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# The Chemistry of Nitric Oxide-Induced Deamination and Cross-Linking of DNA

by

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B.S. Chemistry
University of Richmond, 1991

SUBMITTED TO THE DEPARTMENT OF CHEMISTRY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY
September 1997



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## Abstract

Nitric oxide has long been studied as an environmental pollutant but only recently was discovered to play a major role in mammalian physiology. It is produced by numerous cell types and serves as a neurotransmitter, vasodilator, and cytostatic/cytotoxic agent in the immune system. Along with the necessary roles of NO· *in vivo*, it can be involved in reactions that may produce cell damage. The autoxidation of nitric oxide results in formation of the nitrosating agent $N_2O_3$ which can damage DNA directly by nitrosation of primary amines on DNA bases leading to deamination. This work aims to better understand NO·-induced mutations by determining relative rates of formation of the deamination products of guanine and cytosine (xanthine and uracil) in NO·-treated deoxynucleosides, single and double stranded DNA, and a G-quartet oligonucleotide. A silastic membrane delivery system was used to deliver NO· at a rate of ~10 nmol/ml/min for 1 hour resulting in a final ~600 uM $NO_2^-$ concentration. Treated samples include: 1) the deoxynucleosides 2'-deoxycytidine & 2'-deoxyguanosine, 2) the single stranded oligomer AACCCCAA & 2'-deoxyguanosine, 3) the single stranded oligomer TGTGTGTG & 2'-deoxycytidine, 4) the G-quartet oligomer TTGGGGTT & 2'-deoxycytidine, and 5) the double stranded oligomer CGCGCGCGCGCG & 2'-deoxycytidine. Using GC/MS, xanthine formation was found to be more than twice the amount of uracil formation which may have important consequences for mechanisms of NO·-induced mutations. Interestingly, single stranded oligomers were significantly more reactive toward $N_2O_3$ than deoxynucleosides. However, the double stranded oligomer was less reactive suggesting that Watson-Crick base pair formation may protect DNA from deamination in agreement with previous reports. Therefore, genome integrity may be best preserved in double stranded DNA. The reactivity of a G-quartet oligonucleotide was also decreased presumably due to hydrogen bonding.

Interstrand cross-linking induced by nitric oxide was investigated using an ATATCGATCGATAT oligomer labeled with a 5'-fluorescein. After treatment with nitric oxide, a late eluting peak in the region where double stranded oligomers elute was observed using an HPLC-laser induced fluorescence (HPLC-LIF) system. Quantification demonstrated that cross-link formation is dose dependent and is formed at ~6% of the amount of the deamination product xanthine. The identity of the nucleus of the interstrand cross-link has not been established but most likely matches the dG-dG interstrand cross-link previously observed upon nitrous acid treatment.

During the course of DNA deamination studies, it was discovered that *N*-nitrosation was inhibited by $NaHCO_3$. This inhibition was previously shown to occur with phosphate and chloride due to anion scavenging of $N_2O_3$ by formation of nitrosyl compounds which are rapidly hydrolyzed to nitrite. An analogous reaction of bicarbonate with $N_2O_3$ is significant due to the substantial concentrations of bicarbonate in physiological fluids (up to 40 mM $NaHCO_3$). In order to determine the rate constant for reaction of bicarbonate with $N_2O_3$, a reactor designed for the study of nitrosation kinetics was implemented. The rate constant for the bicarbonate/$N_2O_3$ reaction relative to that for $N_2O_3$ hydrolysis was found to be $9.3 \times 10^2$ $M^{-1}$ at pH 8.9 and 25°C suggesting a strong scavenging ability of bicarbonate. The knowledge of the bicarbonate/$N_2O_3$ rate constant will contribute to kinetic modeling of the fate of $N_2O_3$ specifically in the calculation of the amount of $N_2O_3$ scavenged by bicarbonate in particular systems.

Thesis Supervisor: Steven R. Tannenbaum
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# Acknowledgments

I would first like to thank my thesis advisor Professor Steven Tannenbaum for his patience and understanding throughout my graduate work. I feel privileged to have been in this laboratory and have had the opportunity to acquire even a small fraction of his knowledge. In addition, enormous debts of gratitude go out to the numerous members of the Tannenbaum group who have shared my time here. In no particular order, special thanks to Samar Burney, Teresa Wright, Can Ozbal, Olga Parkin, Jacquin Niles, Vicky Singh, Laura Kennedy, Shawn Harriman, Snait Tamir, Tim Day, Pete Wishnok, Paul Skipper, Sara Stillwell, LaCreis Kidd, Carlton SooHoo, Koli Taghizadeh, Gerald Cozier, Teresa deRojas-Walker, Don Brunson, Ching-Hung (Gene) Hsu, Jim and Tiffany Parkin for making the past several years a great experience. Extra thanks to my lunch buddies Samar Burney and Teresa Wright for critically reading this thesis. You are all great friends and I will miss you.

I have had the opportunity to work with many terrific scientists through several of my projects. The bicarbonate chapter would not have been possible without the help of Vicky Singh and Professor Bill Deen. I want to acknowledge Vicky for her development of the kinetic reactor. Bill Deen was instrumental in the analysis of bicarbonate kinetics. The deamination work required numerous hours of GC/MS analysis. Pete Wishnok was always available for questions regarding the GC/MS and LC/MS. Those questions were quite frequent. Thank you, Pete, for taking the time to teach me to clean the source and for the patience to make me learn on my own. Technical thanks should be given to Joe Glogowski for the analysis of numerous nitrite/nitrate samples. Finally, the chapter of this thesis dealing with cross-linking of DNA could never have been completed without the OZ001 laser system and the expert, Can Ozbal. Thank you for all of the laughs.

I must thank all of my friends in Boston and beyond for providing me with much needed diversions. Special thanks are given to my husband, Scott, who has survived this journey with me from the start. He always (well, almost always) listened to all of my frustrations and I am forever grateful. Thank you for being so patient. The members of my family - dad, Louise, Nick, Jeff, Dominic, and Socks - have always been a tremendous support. I would never have stuck with this if it weren't for my dad, Dick Caulfield. At this point, I am grateful that you pushed me even harder than I was willing to push myself. Thank you for all of your support!

Finally, this thesis is dedicated to my mother, Sharon Caulfield. I wish more than anything that she could be here to share this with me. I never would be at this point if it weren't for her and the rest of my family. I love you all.

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## Abbreviations

| | |
|---|---|
| NO· | Nitric Oxide |
| NOS | Nitric Oxide Synthase |
| NMA | $N^G$-Methyl-L-arginine |
| EDRF | Endothelium Derived Relaxation Factor |
| SOD | Superoxide Dismutase |
| Fe-S | Iron-Sulfur Center |
| GC | Guanylate Cyclase |
| cGMP | Cyclic Guanosine-3',5'-Monophosphate |
| LPS | Lipopolysaccharide |
| γ-INF | Interferon-γ |
| PARS | Poly(ADP-Ribose)Synthetase |
| $HNO_2$ | Nitrous Acid |
| $O_2^{-\cdot}$ | Superoxide Anion |
| OH· | Hydroxyl Radical |
| $H_2O_2$ | Hydrogen Peroxide |
| $^1O_2$ | Singlet Oxygen |
| $N_2O_3$ | Nitrous Anhydride |
| $ONOO^-$ | Peroxynitrite Anion |
| $ONOOCO_2^-$ | Nitrosoperoxycarbonate Anion |
| NMor | *N*-nitrosomorpholine |
| ROS | Reactive Oxygen Species |
| GC/MS | Gas Chromatography/Mass Spectrometry |

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# 1. Background and Literature Review

# Nitric Oxide

## Biological Importance

Nitric oxide ($NO^{\cdot}$) is a relatively unstable molecule that is potentially toxic due to the high reactivity of its unpaired electron. $NO^{\cdot}$ has long been studied as an environmental pollutant because it is produced by the internal combustion engine and contributes to the formation of photochemical smog, acid rain and is involved in destruction of the ozone layer (1,2). Due to its short lifetime in air (~5-10 seconds), it was surprising when this small, highly reactive gaseous molecule was found to play an extremely important role in mammalian physiology.

Nitric oxide is relevant biologically due to its production by numerous different cell types and its role in many physiological processes (2,3). In fact, virtually every type of mammalian cell is under the influence of $NO^{\cdot}$ (4). It is the lowest molecular weight of any known bioactive mammalian cell secretory product (5). Unlike other compounds whose actions depend on molecular shape, the actions of $NO^{\cdot}$ *in vivo* depend on its chemical properties including reactivity, small size, and diffusibility. Nitric oxide facilitates intercellular signalling due to its electrical neutrality and small size which allow it to be able to diffuse freely through cell membranes (6). It has been demonstrated that the lipid bilayer portion of the biological membrane is not a barrier to $NO^{\cdot}$ movement (6); therefore, nitric oxide can travel significant distances from its point of origin.

There are an astounding number of functions of $NO^{\cdot}$ *in vivo* and continuous new discoveries demonstrate $NO^{\cdot}$'s importance in additional processes. A number of molecular targets of $NO^{\cdot}$ are listed in Table 1. It would be impossible to cover all of the biologically essential roles of $NO^{\cdot}$ in this review; however, three important roles of nitric oxide - vasodilation, neurotransmission and host defense - will be briefly discussed here.

*Vasodilation - Endothelium Derived Relaxation Factor (EDRF)*

Nitric oxide was first determined to be important biologically when its identity was confirmed as the endothelium derived relaxing factor, EDRF (7). Production of NO· by blood vessel endothelial cells regulates the relaxation of the surrounding smooth muscle cells causing vasodilation (2,3). A number of observations led up to the identification of NO· as EDRF including the fact that NO· caused arterial relaxation, and that endothelial cells were essential for vascular smooth muscle relaxation (8,9). Eventually, the proof was presented that endothelial cells are capable of NO· synthesis and that NO· accounts for the biological properties of endothelium derived relaxation factor thus confirming NO·'s role in vasodilation (7).

The mechanism of vasodilation involves a blood-borne signal such as acetylcholine, histamine, or a hormone acting on the endothelial cells lining the blood vessel's inner wall. These signals open $Ca^{2+}$ channels allowing a calcium influx. Calcium then binds to calmodulin allowing it to associate with endothelial nitric oxide synthase (eNOS). NOS is thus activated, so that, in the presence of oxygen and nicotinamide-adenine dinucleotide phosphate (NADPH), it converts L-arginine to L-citrulline and NO·. This entire process occurs in milliseconds because the enzyme is constitutively present and available for activation (2). In turn, the endothelial cells release nitric oxide (EDRF) causing extension of smooth muscle cells and blood vessel dilation (1). It seems that maintaining normal blood pressure requires constant synthesis of NO· by vascular endothelial cells. In addition to its role in vasodilation, NO· is important in the circulatory system through another pathway involving inhibition of blood clotting by preventing platelet aggregation (10). Nitric oxide decreases affinity of platelets for each other and their affinity for vascular endothelial cells thereby decreasing platelet aggregation and platelet adhesion. The mechanism is believed to involve guanylyl cyclase and cGMP as many other functions of NO· are regulated through its interactions with guanylyl cyclase.

*Neurotransmission*

Nitric oxide is important in intercellular communication and cell signalling as demonstrated by its role in neurotransmission discussed briefly here. NO· has been shown to be essential for neurotransmission in the central nervous system due to its important functions in the brain (2,3,10-12). NO· is also involved in stimulation of noncholinergic/nonadrenergic neurons in the peripheral nervous system (2,5). In addition, it is postulated that NO· may be involved in learning and memory due to its role in long-term synaptic depression and long-term potentiation of synaptic transmission.

Nitric oxide helps to mediate neuronal responses to the excitatory amino acid glutamate released from a stimulated neuron. Upon glutamate binding to an *N*-methyl-D-aspartate receptor, calcium ion channels open in the receptor allowing $Ca^{2+}$ influx (2,10). Calcium then binds to calmodulin which activates neuronal nitric oxide synthase (nNOS) to produce NO·. Again, response is rapid due to the fact that this enzyme is constitutively present and available for activation (2).

Nitric oxide is atypical when compared to all other neurotransmitters which are usually stable chemicals stored in synaptic vesicles in nerve terminals (10). Nitric oxide, on the other hand, is not stored in vesicles and is synthesized on demand in a neuron (10). Its release apparently involves simple diffusion from the nerve ending. It does not act at a membrane receptor protein but instead diffuses into the adjacent neuron. The receptor target for nitric oxide is primarily the iron in the active center of guanylyl cyclase (GC) where it initiates a three-dimensional change in the enzyme thereby increasing its activity and, consequently, the production of cyclic GMP. This cascade of events initiated by NO· is a unique method of action for a neurotransmitter (10).

*Host Defense - Macrophages*

A particularly interesting role of nitric oxide is its involvement in host defense. NO· is produced by macrophages as a cytotoxic agent in the immune or inflammatory response as demonstrated by Marletta and independently by Hibbs (2,3,13-15). A number of cytotoxic and cytostatic actions of the nitric oxide released by macrophages are listed in Table 2. Nitric oxide is released in combination with numerous other reactive species including hydrogen peroxide, tumor necrosis factor, and inflammatory cytokines; however, nitric oxide is believed to be the key mediator of macrophage-induced cytostasis because nitric oxide scavengers block the cytostatic effect of macrophages (16-18). In regions of inflammation, particularly chronic inflammation, local nitric oxide production can be quite high resulting in larger amounts of nitric oxide available for further reaction with oxygen or superoxide generating additional highly reactive species as discussed in later sections.

Infection and resulting tissue inflammation from gastritis, hepatitis and colitis are recognized risk factors for a variety of human cancers and it has been proposed that reactive oxygen species formed during the inflammation response may play a role in DNA damage and cellular injury (19). Nitric oxide may also be intimately involved in carcinogenesis resulting from inflammation because chronic inflammation involving constant activation of macrophages can continue for several months and sometimes years (19,20). Nitric oxide or its derivatives have also been shown to induce apoptosis in tumor cells as well as in generator cells (21,22).

Therefore, based on the essential biological roles of NO· such as vasodilation and neurotransmission discussed above and the fact that nitric oxide has been shown to be mutagenic and genotoxic by inducing DNA damage, endogenous nitric oxide has been classified as a double-edged sword with beneficial as well as detrimental roles *in vivo*. The remainder of this thesis will be focused on the detrimental roles of NO· with particular focus on the DNA-damaging effects of NO·.

# Table 1
# Molecular Targets of Nitric Oxide

| Type of Molecule & | Result |
|---|---|
| Heme proteins | Soluble guanylyl cyclase activity increased<br>Hemoglobin, myoglobin activity decreased |
| Fe-S proteins | NADH:ubiquinone oxidoreductase activity decreased<br>NADH: succinate oxidoreductase activity decreased<br>cis-Aconitase activity decreased |
| Non-heme Fe proteins | Ferritin activity decreased<br>Ribonucleotide reductase activity decreased |
| Tyrosyl radical protein | Ribonucleotide reductase activity decreased |
| Protein thiols | Tissue-type plasminogen activator activity increased<br>Dehydrogenases activity decreased |
| DNA | Gain of function mutations<br>Loss of function mutations |
| Superoxide Anion | Generation of reactive oxidants |

# Table 2

## Cytostatic/Cytotoxic Actions of Nitric Oxide

### Target Molecules & Consequent Effects

| Molecular Target | Mechanism of Action | Consequences |
|---|---|---|
| Fe-S Proteins | Enzyme inactivation<br>-Aconitase<br>-Succinate Ubiquinone Reductase | Inhibition of citric acid cycle & mitochondrial respiratory chain |
| Tyrosyl Radical Proteins | Ribonucleotide Reductase Inactivation | Inhibition of DNA Synthesis |
| Sulfhydryls & Protein Thiols | S-Nitrosylation of SH-Dependent Dehydrogenases | Inactivation of Dehydrogenases |
| Non-Heme Fe | Ferritin Activity Decreased | Iron Release |
| DNA | Deamination | DNA Damage & Mutations |
| RNA | Deamination | Inhibition of Protein Synthesis |

## Nitric Oxide Biosynthesis - Nitric Oxide Synthase

Nitric oxide is produced *in vivo* by the dimeric enzyme nitric oxide synthase (NOS) which catalyzes the conversion of L-arginine to L-citrulline releasing nitric oxide as shown in Figure 1 (13,14,23). D-arginine is not a substrate for the enzyme (24). The first step of NO· synthesis involves a two electron oxidation of arginine resulting in formation of $N^G$-hydroxy-L-arginine. Overall, the guanidino nitrogen of L-arginine undergoes a five electron oxidation. A complete mechanism of NOS catalysis has been proposed by Marletta and there are many reviews of NOS structure and function (2,13,14,23). Briefly, NOS contains four prosthetic groups essential for enzymatic activity including 5,6,7,8-tetrahydrobiopterin ($BH_4$), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and the iron protoporphyrin IX group of heme. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is the cofactor that provides reducing equivalents (23); therefore, NADPH and molecular oxygen are cosubstrates for the enzyme.

There are now known to be several NO· synthases (NOS; EC 1.14.23) including nNOS (neuronal NOS), eNOS (endothelial NOS), and iNOS (inducible NOS). The three forms display approximately 50% identity in amino acid sequence. All three NOS isozymes demonstrate significant sequence homology to only one other mammalian enzyme - cytochrome P450 reductase. Both nNOS and eNOS are constitutive enzymes of approximately 150 kD which are subject to allosteric regulation. Activation of eNOS and nNOS is rapid; however, the output of these enzymes is transient and low - on the nanomolar level. NO· at this level has a relatively long half-life and is mainly involved in homeostatic processes such as neurotransmission, peristalsis and blood pressure regulation. Neuronal NOS (nNOS) has been localized to the cytosol and exists as a dimer under native conditions. On the other hand, eNOS is membrane bound through myristoylation and palmitoylation. It is not known whether this form is active as a monomer or a dimer.

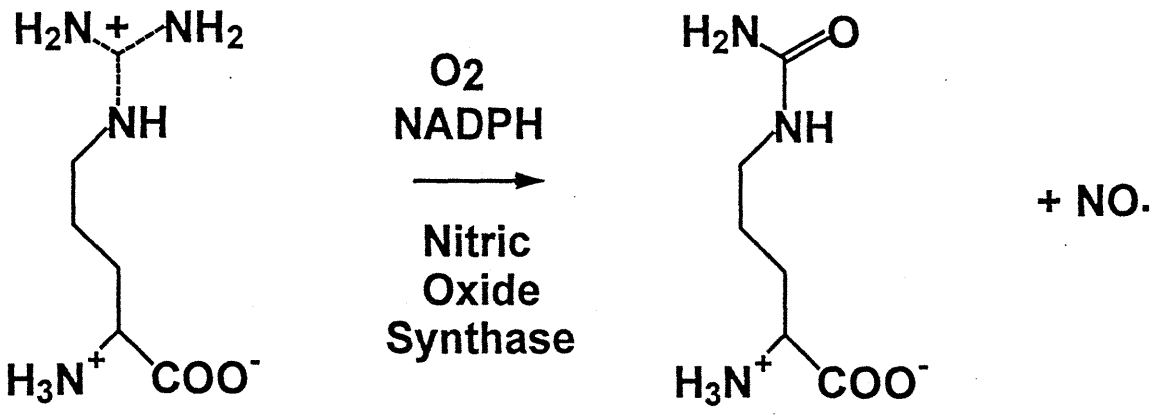
In contrast to the constitutive enzymes, iNOS is the inducible form of the enzyme (approximately 125-135 kD) and is not allosterically regulated. Inducible NOS is found

primarily in macrophages where it exists as a homodimer under native conditions. Dimerization requires tetrahydrobiopterin and is necessary for enzymatic activity. This enzyme releases NO· continuously over days or weeks. In stimulated macrophages, iNOS may produce nitric oxide at $5 \times 10^3$ to $5 \times 10^4$ molecules/cell-sec (25). In cases where inflammation continues over months or even years, neighboring cells may be exposed to significant quantities of highly reactive chemical species including nitric oxide and reactive oxygen species.

Nitric oxide is free to diffuse in all directions from its site of origin thus exhibiting very little specificity of interaction. Therefore, control of nitric oxide synthesis is the key to regulating its activity (2). Calcium$^{2+}$/calmodulin is required for the activation of the constitutive forms of the enzyme; however, calcium$^{2+}$/calmodulin has been shown to be tightly bound to the inducible enzyme making the enzyme insensitive to $Ca^{2+}$ concentration (2). Expression of iNOS is therefore not regulated by calcium/calmodulin but requires gene transcription and *de novo* protein synthesis (5,26).

There are numerous known inhibitors of NOS including N-iminoethyl-L-ornithine and $N^G$-nitro-L-arginine (27,28). The prototype inhibitor is $N^G$-methyl-L-arginine (NMA) which is an analog substituted on one or both of the guanidino nitrogens. This agent is a competitive inhibitor of NOS which is able to block the cytotoxicity and production of nitrite from macrophages stimulated to produce NOS and NO·. The addition of NMA to activated macrophages does indeed inhibit NO· production to a large extent (29).

# Nitric Oxide Synthase




L-Arginine

L-Citrulline

Figure 1. Biosynthesis of Nitric Oxide ($NO^{\cdot}$) by Nitric Oxide Synthase (NOS). The conversion of L-arginine to L-citrulline produces $NO^{\cdot}$.

## Reactive Oxygen Species (ROS)

Due to nitric oxide's many reactions with reactive oxygen species (ROS), a brief summary of the formation, detoxification, and potential reactions leading to cellular damage by reactive oxygen species is included here. ROS arise in normal cellular processes such as metabolism and inflammation and are generated during exposure to environmental chemicals, ionizing radiation, and transition metals (30-33). Substantial evidence exists demonstrating the genotoxic and carcinogenic activities of ROS (30,34). The ability of several ROS to cause cellular damage has been evaluated using cell-free generating systems and scavengers specific for particular species. Overall, ROS have been shown to produce a number of types of DNA damage including large deletions, small deletions, single strand breaks, double strand breaks, DNA-protein crosslinks, point mutations, and sister chromatid exchanges (35).

Molecular oxygen is a triplet in its ground state making it relatively inert. However, oxygen present in its singlet state, $^1O_2$, would readily react with and oxidize all biological material (36). Singlet oxygen is a strong electrophile capable of causing oxidative DNA damage (37). Also, the reduction of molecular oxygen by acceptance of 1, 2, or 3 electrons leads to the formation of additional reactive species including superoxide ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH^{\cdot}$), respectively, as shown here:

$$\underset{\text{oxygen}}{O_2} \xrightarrow{e^-} \underset{\text{superoxide anion}}{O_2^{\cdot-}} \xrightarrow{e^-} \underset{\text{hydrogen peroxide}}{H_2O_2} \xrightarrow{e^-} \underset{\text{hydroxyl radical}}{OH^{\cdot}} \xrightarrow{e^-} \underset{\text{water}}{H_2O}$$

Several of these products are capable of causing cellular damage; therefore, the cell has developed enzymatic systems for protection against these reactive species including superoxide dismutase, catalase, and glutathione peroxidase. These enzymes in combination with antioxidants (vitamin E, glutathione, ascorbate) protect cells against oxidative damage (36).

Superoxide can dismutate spontaneously or through catalysis by one of the several types of superoxide dismutase (SOD) including copper-zinc SOD (CuZn-SOD), manganese SOD (Mn-SOD), or iron SOD (Fe-SOD). The dismutation reaction is as shown below:

$$2O_2^{\cdot-} + 2H^+ \longrightarrow O_2 + H_2O_2$$

The SOD-catalyzed dismutation reaction has a rate constant of $1.9 \times 10^9\ M^{-1}s^{-1}$.

The hydrogen peroxide produced by SOD can then be detoxified by catalase which catalyzes the following reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

The reactivity of $H_2O_2$ is actually relatively low compared to the other reactive oxygen products, principally hydroxyl radical. $H_2O_2$ is able to pass intact through cell membranes and complex biological fluids and can therefore act on distal targets beyond the reach of more reactive oxygen species.

Hydroxyl radical, the most reactive ROS, can be formed through Fenton chemistry where ferrous iron is oxidized to ferric iron generating both hydroxyl radical and hydroxide ion. Ferric iron is reduced back to ferrous iron by $O_2^{\cdot-}$ as shown below:

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^{\cdot}$$
$$O_2^{\cdot-} + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$

Due to the high reactivity of hydroxyl radical, there is no detoxification process *in vivo*.

Substantial evidence exists demonstrating the genotoxic and carcinogenic activities of ROS (38-40). The fact that oxidative stress can lead to strand breaks and oxidized base formation in DNA (40) and that these lesions are mutagenic if not properly repaired (41,42) strongly suggests that oxidative stress is an important risk factor in carcinogenesis.

This overview of reactive oxygen species provides a background for the numerous reactions of ROS with nitric oxide that will be important in the following sections. The number of species involved clearly underscores the complexity of nitric oxide chemistry *in vivo*.

## Nitric Oxide Chemistry

The effect of $NO^{\cdot}$ on cells ultimately depends on many complex conditions such as the rate of $NO^{\cdot}$ production and its rate of diffusion, the concentration of potential reactants such as superoxide and oxygen, the levels of enzymes such as catalase and superoxide dismutase, the levels of antioxidants such as glutathione, and the distances between generator cells and target cells (43). However, the reactions of $NO^{\cdot}$ can be broadly discussed with reference to three main processes which control the fate of $NO^{\cdot}$ in biological systems: 1) diffusion, 2) autooxidation to form nitrous anhydride, $N_2O_3$, and 3) reaction with superoxide to form peroxynitrite, $ONOO^-$ (44).

This section focuses on the formation of these reactive species which will be important in later sections detailing DNA damage by the derivatives of $NO^{\cdot}$. Briefly, the types of DNA damage that may arise from $NO^{\cdot}$ through the species $ONOO^-$ and $N_2O_3$ are mentioned here. Peroxynitrite, $ONOO^-$, can oxidize and nitrate DNA and may potentially cause single strand breaks through attack on the sugar-phosphate backbone. Nitrous anhydride, $N_2O_3$, can nitrosate secondary amines forming *N*-nitrosamines which after metabolic activation can alkylate DNA through an indirect mechanism. $N_2O_3$ can also directly nitrosate the primary amine functionalities of DNA bases leading to deamination. Both DNA-DNA cross-links and DNA-protein cross-links can also be formed through $NO^{\cdot}$-derived reactive species. Figure 2 schematically demonstrates the potential for deamination, oxidation and nitration to target cell DNA upon exposure to the $NO^{\cdot}$-derived reactive species $N_2O_3$ and $ONOO^-$ arising from macrophage activation. Due to the importance of these types of damage for this thesis work, the DNA-damaging effects of $NO^{\cdot}$ through the reactive species $N_2O_3$ and $ONOO^-$ are discussed extensively in the following sections.

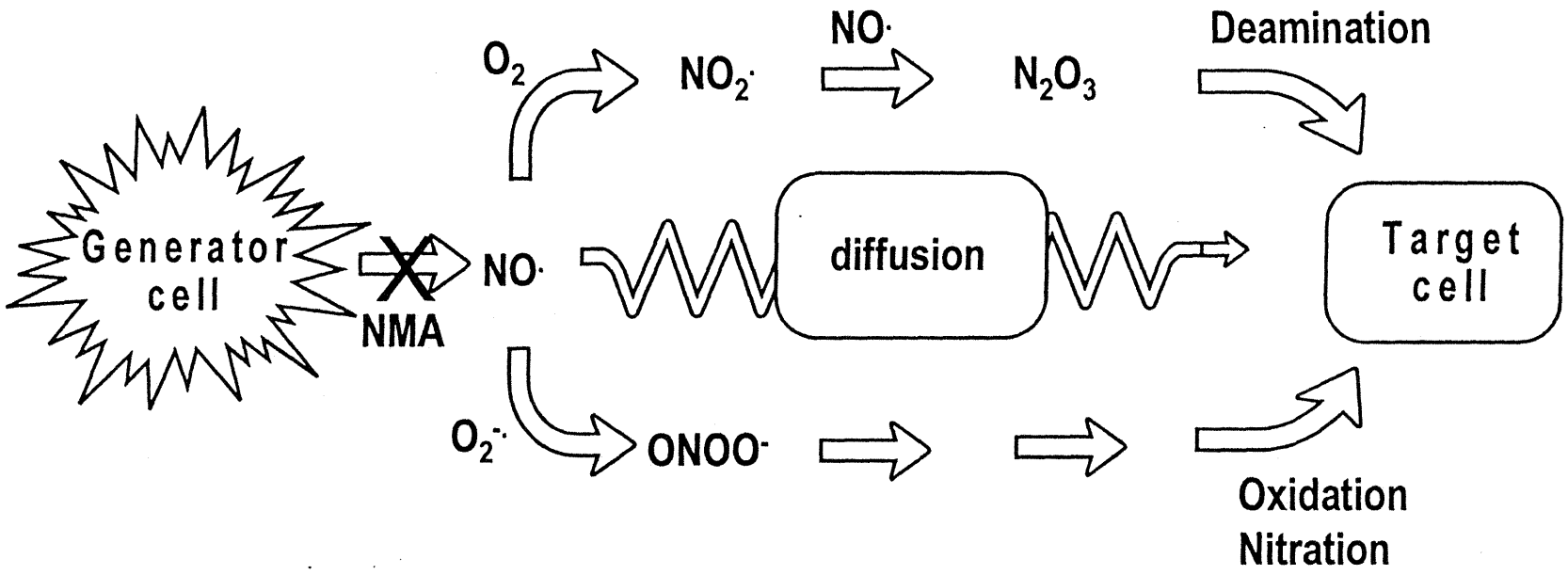



**Figure 2. Three main fates of nitric oxide discussed in this thesis - diffusion, autooxidation to form $N_2O_3$, and reaction with superoxide forming $ONOO^-$. The types of DNA damage that can result from these species is shown above.**

*Diffusion of Free Nitric Oxide*

The chemistry of nitric oxide in oxygenated biological systems is extremely complex due to the significant number of chemical species formed and the numerous parallel reactions to consider. The first pathway involves direct reaction of NO· with cellular targets after simple diffusion of NO·. Once produced, nitric oxide itself may directly react with myoglobin or hemoglobin in the extracellular space (5,45,46). Inside the cell, free NO· can exert a number of diverse effects. Nitric oxide may react with the non-heme iron or quench the tyrosyl radical in ribonucleotide reductase leading to enzyme inhibition and inhibition of DNA synthesis (47,48). Nitric oxide also inactivates aconitase, a citric acid cycle enzyme with a catalytically active iron-sulfur cluster. In addition, NO· is known to regulate the enzymatic activity of guanylate cyclase, glyceraldehyde-3-phosphate dehydrogenase, cyclooxygenase, and cytochrome P450 mixed function oxygenases by its interaction with Fe-S centers and tyrosyl radical groups (2,5,49,50).

A critical role of nitric oxide *in vivo* is the activation of soluble guanylate cyclase. The neurotransmission and vasodilation actions of NO· are largely mediated by guanylate cyclase after binding of NO· to the sixth coordination position of the enzyme's iron protoporphyrin IX group forming a nitrosyl-heme (51-53). Stimulation of GC leads to the synthesis of the biologically important second messenger, cyclic GMP or cyclic guanosine 3',5'-monophosphate, and subsequent activation of cGMP-dependent kinases in responder cells. Nanomolar concentrations of nitric oxide are capable of activating guanylate cyclase and increasing cyclic GMP levels (52,53).

Free nitric oxide can also bind to sulfhydryls and protein thiols such as glutathione, serum albumin, tissue plasminogen activator, and hemoglobin resulting in *S*-nitrosylation and possibly the inactivation of SH-dependent dehydrogenases (2,5,54-59). Nitric oxide may also be involved in the release of non-heme iron from iron storage proteins such as ferritin which could be a possible cause of tumor cell cytotoxicity induced by activated macrophages (60).

*Nitric Oxide Reaction with Superoxide Forming Peroxynitrite, $ONOO^-$*

Nitric oxide also undergoes reactions forming additional reactive species which can participate in other types of chemistry. One major fate of nitric oxide is reaction with superoxide anion ($O_2^{\cdot-}$) forming peroxynitrite, $ONOO^-$, which is an extremely fast reaction due to the fact that both species are radicals. The rate of nitric oxide/superoxide reaction is near the diffusion limit with a rate constant of $6.7 \times 10^9$ $M^{-1}$ $s^{-1}$ (61-63) . This rate constant is approximately 3.5 times faster than that for the superoxide dismutase (SOD)-catalyzed decomposition of $O_2^{\cdot-}$ indicating that the nitric oxide/superoxide reaction may predominate over the superoxide/SOD reaction. However, nitric oxide does not consume all of the available superoxide due to the observation that peroxynitrite formation is attenuated to some extent by the presence of SOD. The formation of both nitric oxide and superoxide does indeed occur simultaneously in cells such as macrophages, neutrophils, Kupffer cells, and endothelial cells (64). In the vicinity of these cells, peroxynitrite may be formed at high concentrations. However, the mechanism and extent of $ONOO^-$ formation are strongly influenced by the relative fluxes of $O_2^{\cdot-}$ and $NO^{\cdot}$ (65,66).

Peroxynitrite has a $pK_a$ of 6.8; therefore, it is protonated in acidic solution to form peroxynitrous acid which then decays rapidly to the predominant product nitrate. Peroxynitrite is a potent one electron and two electron oxidant and is therefore capable of oxidizing protein and non-protein sulfhydryls (54,67,68). In addition, peroxynitrite is a nitrating agent which is supported by the evidence that both nitrotyrosine and 8-nitroguanine are found in cells exposed to peroxynitrite (63,69,70). *In vitro* and *in vivo*, the most important modulator of peroxynitrite chemistry is carbon dioxide (71). The rate constant for reaction of peroxynitrite anion with carbon dioxide has been determined to be $3 \times 10^4$ $M^{-1}s^{-1}$ which indicates that $CO_2$ greatly accelerates the decomposition of $ONOO^-$ (68). This rate is one of the fastest reactions known for peroxynitrite. Given the high concentrations of carbon dioxide/bicarbonate in biological fluids (>25 mM), it is believed that the reaction of peroxynitrite with carbon dioxide will be the predominant

pathway for decay of peroxynitrite *in vivo* (68). The species formed is postulated to be the nitrosoperoxycarbonate ion O=N-OOCOO$^-$ as shown here:

$$CO_2 + O{=}NOO^- \longrightarrow O{=}N{-}OOCO_2^-$$

which may rearrange to the nitrocarbonate ion, $O_2N$-$OCO_2^-$. This species is capable of carrying out the types of chemistry associated with peroxynitrite including 1 and 2 electron oxidations and nitrations (68,71-73). In addition, the hydrolysis of this species will result in nitrate which is the observed product of peroxynitrite breakdown. However, no direct evidence for this species exists.

The balance between oxidation products and nitration products is influenced by a number of factors including the presence of carbon dioxide. Lymar and Hurst have studied the relative formation of oxidation and nitration products by examining the yields of 3-nitrotyrosine and 3,3'-dityrosine formed upon tyrosine/peroxynitrite reaction (73). Nitrosoperoxycarbonate is postulated to be the first intermediate involved in the oxidation and nitration chemistry. In the absence of other reactants, this species decomposes to give bicarbonate and nitrate (73). The mechanism of tyrosine oxidation and nitration is postulated to be a one-electron oxidation of tyrosine by O=NOOCO$_2^-$ which generates tyrosyl and $NO_2$ radicals as intermediary species. The formation of dityrosine eliminates the idea that nitrotyrosine formation occurs through direct attack of a nitronium ion on tyrosine. This provides evidence for one electron chemistry. The 3-nitrotyrosine/3,3'-dityrosine product ratio depends upon the pH, tyrosine concentration, and absolute reaction rate.

Peroxynitrite may be extremely important when considering NO·-induced DNA damage due to the types of DNA damage that can occur from ONOO$^-$ - oxidized bases, nitrated bases, and single strand breaks. The reactions of ONOO$^-$ with DNA are discussed in more detail in a later section.

*Nitric Oxide Reaction with Oxygen Forming Nitrous Anhydride, $N_2O_3$*

The most important reaction of nitric oxide for this thesis work is the autooxidation of nitric oxide forming $N_2O_3$, a powerful nitrosating agent (74). There are a number of other nitrosating agents that may be important under certain conditions including the nitrosonium ion ($NO^+$), nitrous acidium ion ($NO\text{-}OH_2^+$), NOX, and $N_2O_4$ (74,75). For example, the formation of $NO^+$ is favored by high acidity due to the reaction:

$$HONO + H^+ \longrightarrow NO^+ + H_2O$$

However, these species are primarily important in the study of acid-catalyzed nitrosation. At physiological pH, $N_2O_3$ formation from nitric oxide has been demonstrated to be most important (76). The chemistry of $N_2O_3$ formation is nearly identical to that from nitrous acid, i.e. nitrite at an acidic pH because dinitrogen trioxide is the anhydride of $HNO_2$. The same nitrosating agent, $N_2O_3$, is formed in both cases (76).

$$2HNO_2 \longrightarrow N_2O_3 + H_2O$$

The formation of the nitrosating agents $N_2O_3$ and $N_2O_4$ from nitric oxide at physiological pH is shown below and also in Figure 3. In theory, $N_2O_3$ and $N_2O_4$ can both be formed. These nitrosating agents can be hydrolyzed by water resulting in nitrite and nitrate as shown below:

$$2NO\cdot + O_2 \longrightarrow 2NO_2\cdot$$

$$NO\cdot + NO_2\cdot \longrightarrow N_2O_3$$

$$NO_2\cdot + NO_2\cdot \longrightarrow N_2O_4$$

$$N_2O_3 + H_2O \longrightarrow 2HNO_2 \longrightarrow 2NO_2^- + 2H^+$$

$$N_2O_4 + H_2O \longrightarrow NO_3^- + NO_2^- + 2H^+$$

Hydrolysis of $N_2O_3$ produces only nitrite whereas hydrolysis of $N_2O_4$ produces equimolar nitrate and nitrate. *In vitro*, nitric oxide delivery into buffer or water results in only nitrite formation indicating that $N_2O_3$ formation predominates over $N_2O_4$ production. In addition, $N_2O_4$ is a poor nitrosating agent for primary amines and therefore may not be important for the deamination chemistry in this work (74).

The rate of reaction of NO· with $O_2$ to form $N_2O_3$ is second order in nitric oxide concentration and first order in oxygen concentration. The rate equation is

$$\text{Rate} = k[NO]^2[O_2] \qquad (59,77,78).$$

where k= $3.5 \times 10^6$ $M^{-2}$ $s^{-1}$ at 37 °C and $6 \times 10^6$ $M^{-2}$ $s^{-1}$ at 22 °C (78). Based on this equation, it can be seen that the biological half life of nitric oxide is dependent on the initial nitric oxide concentration (77). The second order dependency of autooxidation dictates that $t_{1/2}$ of NO· be inversely proportional to its concentration (78). The maximal concentration of nitric oxide in the cellular microenvironment has been shown to be 0.45-10 mM which corresponds to a $t_{1/2}$ of 1-500 seconds for NO· in air-saturated aqueous solution (78).

Numerous studies have been performed on the chemistry of reactions of $N_2O_3$ generated from nitrous acid and the rate constants for reaction of many compounds with $N_2O_3$ is known. $N_2O_3$ can react with thiols resulting in an *S*-nitrosation event. For example, glutathione is known to react in this way and therefore may be an effective scavenger of $N_2O_3$ *in vivo* (59,79-81). The reactions of $N_2O_3$ with secondary and primary amines and its relation to DNA damage will be discussed at length in later sections. The interaction of DNA with nitrous acid, i.e. nitrite at an acidic pH, has been extensively studied - usually pH 4.2 or below due to the fact that the $pK_a$ for nitrous acid is 3.36.

There are also a few studies of the effects of NO· on DNA that will be summarized in following sections.

# Autooxidation of Nitric Oxide

Formation of nitrosating agents:

$$NO^{\cdot} + O_2 \longleftrightarrow O_2NO^{\cdot}$$

$$O_2NO^{\cdot} + NO^{\cdot} \longleftrightarrow 2NO_2^{\cdot}$$

$$2NO_2^{\cdot} \longleftrightarrow N_2O_4$$

$$NO^{\cdot} + NO_2^{\cdot} \longleftrightarrow N_2O_3$$

Hydrolysis of nitrosating agents:

$$N_2O_3 + H_2O \longleftrightarrow 2H^+ + 2NO_2^-$$

$$N_2O_4 + H_2O \longleftrightarrow 2H^+ + NO_2^- + NO_3^-$$

Figure 3. The autooxidation of nitric oxide forms the nitrosating agents $N_2O_3$ and $N_2O_4$. Hydrolysis of these species results in nitrite and nitrate. $N_2O_3$ is the predominant nitrosating agent arising from $NO^{\cdot}$ under physiological conditions which is proven by the fact that nitrite is the exclusive product of hydrolysis.

*Reaction of Nitrous Anhydride with Biological Anions*

The effect of anions on nitrous acid chemistry has long been known. Several anions are known to enhance nitrosation reactions at acidic pH with the following order of effect: $SCN^- >>> Br^- >> Cl^- > SO_4^{2-} = ClO_4^- = H_2PO_4^- = RCO_2^-$ (82,83). Thiocyanate is an extremely strong promoter of nitrosation through the formation of nitrosyl thiocyanate ON-NCS. The effectiveness of the various anions appears to be approximately related to their relative nucleophilicity; however, nucleophilicity alone cannot explain their catalytic effectiveness (83).

Lewis *et al.* have described the opposite effect of these anions on nitrosation at neutral pH - phosphate and chloride were demonstrated to be inhibitors of nitrosation at physiological pH (76). Other anions including nitrate, thiocyanate, and perchlorate were shown to have little or no effect on nitrosation at physiological pH (76). The explanation for the reverse reactivity of anions with $N_2O_3$ at acidic and neutral pH results from the following reaction demonstrating the formation of a nitrosyl halide:

$$X^- + N_2O_3 \longrightarrow XNO + NO_2^-$$

This reaction is reversible at acidic pH because the product nitrite can be protonated and form additional XNO and $N_2O_3$ which are both nitrosating agents. Nitrosation by $N_2O_3$, ON-NCS, and NOX are all similar reactions. In fact, thiocyanate- and halide-catalyzed nitrosation of amines will compete favorably with nitrosation by $N_2O_3$ at acidic pH (82). For this reason, there is continuous production of nitrosating agents and nitrosation chemistry is enhanced by the presence of these anions. However, at neutral pH, the above reaction is irreversible so the reaction of an anion with $N_2O_3$ forming $NO_2^-$ will serve as a sink for nitrogen at physiological pH as opposed to a source of nitrogen at acidic pH (76).

Following the equations below, the nitrosyl anion species can act as a nitrosating agent or be hydrolyzed to nitrite:

$$XNO + R_2NH \longrightarrow R_2NNO + H^+ + X^-$$

$$XNO + H_2O \longrightarrow HNO_2 + H^+ + X^- \longrightarrow NO_2^- + 2H^+ + X^-$$

The first reaction dominates at acidic pH which is observed as an enhancement of nitrosation by anions. However, at physiological pH, the hydrolysis seems to be favored thus irreversibly destroying the nitrosating agent XNO resulting in decreased nitrosation in the presence of these anions.

Lewis *et al.* performed important studies to demonstrate the inhibitory effect of phosphate and chloride on nitrosation chemistry at neutral pH. Morpholine nitrosation was inhibited by the presence of these anions and the rate constants for $N_2O_3$/anion reactions were calculated and are discussed at length in Chapter 2 of this thesis (76). In additional studies, Lewis *et al.* performed experiments with macrophages to determine the fate of nitrogen in a more biological experiment. A mass balance indicated that there was significantly more nitrite formed than predicted even after the effects of phosphate and chloride were taken into account (25). This is most likely due in part to the effect of anion scavenging of $N_2O_3$ forming nitrite; however, although the effects of phosphate and chloride do contribute to the hydrolysis, there are still other components in the system contributing to $N_2O_3$ hydrolysis that are as yet unknown giving rise to the increased nitrite formation (25). It is likely that a key component is another biologically important anion.

## Direct and Indirect DNA Damage by Nitric Oxide

The previous sections have described the formation of reactive species from nitric oxide - primarily nitrous anhydride, $N_2O_3$, and peroxynitrite, $ONOO^-$. Here, the interaction of these species with DNA will be discussed in detail in order to outline the numerous types of DNA damage that can ultimately result from nitric oxide exposure. Several of these products have been the subjects of the experiments in later chapters of this thesis.

DNA damage is caused by at least two major pathways: one arising from reaction of nitric oxide with oxygen forming $N_2O_3$, and the other from reaction of nitric oxide with superoxide forming $ONOO^-$. The formation of $N_2O_3$ can cause either direct or indirect DNA damage. Direct damage results from the nitrosation of primary amines on DNA bases ultimately leading to deamination. Indirect damage results from the nitrosation of secondary amines forming carcinogenic or mutagenic *N*-nitrosamines. Alternatively, reaction of nitric oxide with superoxide gives the peroxynitrite anion that decomposes via reactive intermediates as described in previous sections which can result in DNA oxidation, nitration, and single strand breaks. The following discussion describes in detail the different types of DNA damage that may result from nitric oxide exposure including:

1) Oxidation and Strand Breaks by Peroxynitrite, $ONOO^-$
2) Endogenous Formation of N-Nitroso Compounds by $N_2O_3$
3) Deamination of DNA Bases by $N_2O_3$
4) Cross-Link Formation by $N_2O_3$
    - -DNA-Protein Cross-Link Formation
    - -DNA Intrastrand Cross-Link Formation
    - -DNA Interstrand Cross-Link Formation

## 1. *Oxidation and Strand Breaks by Peroxynitrite, $ONOO^-$*

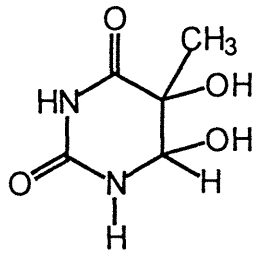
Peroxynitrite is capable of carrying out three types of chemistry which will be discussed here - oxidation, nitration, and isomerization to nitrate. Peroxynitrite has been demonstrated to oxidize numerous macromolecules including lipids and thiols such as sulfhydryl groups of proteins (54,84,85). Within DNA, a number of oxidized bases shown in Figure 4 can be formed in cells exposed to oxidants such as peroxynitrite. Three oxidized bases including FAPY-guanine, 8-oxoguanine, and 5-hydroxymethyluracil were shown to form in the DNA of murine macrophages activated for $NO^{\cdot}$ production presumably through peroxynitrite formation (29). The presence of 8-oxoguanine in DNA was first shown in 1984 by Kasai *et al.* and others soon followed (86-88). The development of a specific and extremely sensitive electrochemical detection system by Floyd (36,89) has made 8-oxoguanine an attractive marker for monitoring DNA damage in studies with various oxidizing agents, including peroxynitrite (90,91). Using a sensitive immunoaffinity/HPLC-ECD methodology, the levels of 8-oxoguanine detected in plasmid DNA treated with increasing concentrations of peroxynitrite demonstrated a dose-dependent increase (91). It has recently been determined that it is also possible for 8-oxoguanine to undergo further oxidation by peroxynitrite which explains why 8-oxoguanine is not observed in studies using high peroxynitrite concentrations (92). Previously, the DNA damaging species arising from peroxynitrite was believed to be the hydroxyl radical (61); however, the use of hydroxyl radical scavengers such as DMSO and mannitol did not decrease yields of peroxynitrite-induced oxidation. Therefore, the current idea is that the DNA damaging species may be a high-energy form of peroxynitrous acid (85). The high energy intermediate causing the oxidation is believed to be less reactive and more selective than the hydroxyl radical (85,93). As discussed in previous sections, the species formed through reaction of peroxynitrite with carbon dioxide may be the ultimate oxidant and nitrating species.

The nitration product 8-nitroguanine has also been observed upon $ONOO^-$ treatment of DNA (94). The formation of 8-nitroguanine may be important because it has been shown to depurinate with a half life of approximately 4 hours at 20 °C (94) and

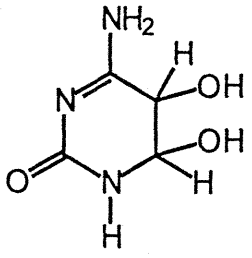
therefore may lead to abasic sites and potentially single strand breaks. In addition, peroxynitrite chemistry may lead directly to hydrogen atom abstraction from a sugar in the DNA backbone ultimately resulting in a single strand break which may be lethal to the cell (93). Single strand breaks have been observed in rodent cells treated with $ONOO^-$ and plasmid DNA treated with $ONOO^-$ (44). The cytotoxicity observed in cells exposed to $ONOO^-$ may be mediated by DNA strand breakage and the subsequent activation of the DNA repair enzyme poly-(ADP-ribose)-synthetase (PARS) leading to depletion of the cellular $NAD^+$ and ATP pool (95,96).

The mutagenicity of $ONOO^-$ has been studied by Juedes *et al.* using a plasmid containing the supF gene as a target (97) . The plasmid was treated with $ONOO^-$ and subsequently replicated both in bacterial and in human cells. In bacterial cells, the majority of mutations were G:C → T:A. In human cells, the spectrum was more complex but again most point mutations were G:C → T:A mutations. Large deletions, insertions, tandem and multiple mutations were also found. The spectrum was similar to the mutational spectrum for singlet oxygen ($^1O_2$) but very different from that of nitric oxide.

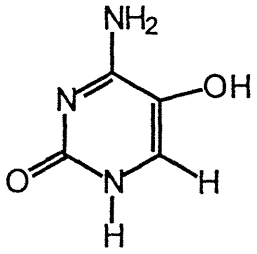
# Oxidized DNA Bases



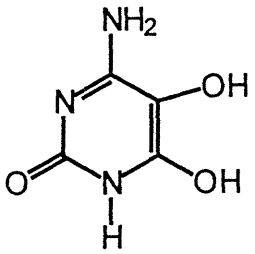
Thymine Glycol
(cis- and trans-)



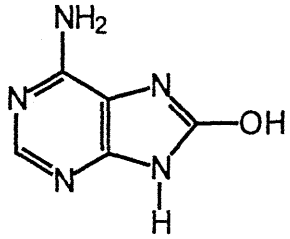
Cytosine Glycol



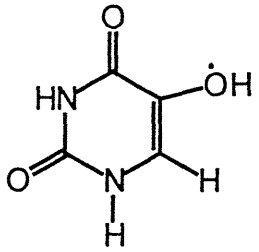
5-Hydroxycytosine



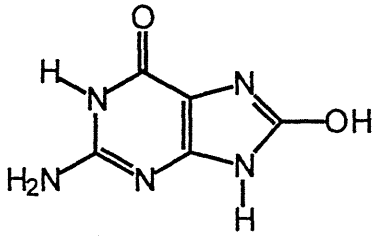
5,6-Dihydroxycytosine



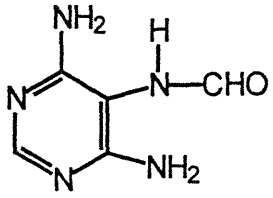
8-Hydroxyadenine



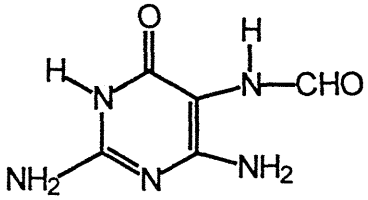
5-Hydroxyuracil



8-Hydroxyguanine



4,6-Diamino-
5-formamidopyrimidine



2,6-Diamino-4-hydroxy-
5-formamidopyrimidine

Figure 4. Oxidized DNA Bases. These modified bases can presumably be formed by the action of reactive oxygen species on cells. $ONOO^-$ is also believed to induce these types of oxidative damage in DNA.

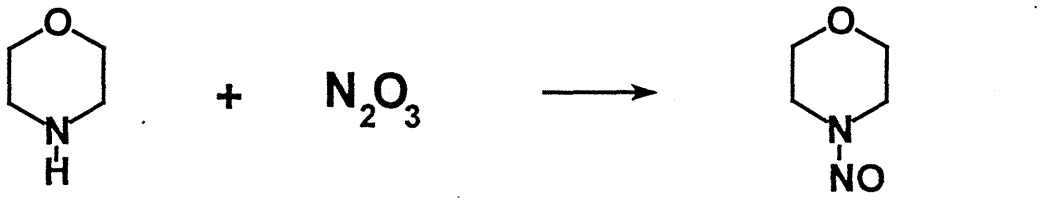
## 2. *Endogenous Formation of N-Nitroso Compounds by $N_2O_3$*

*N*-nitroso compounds are a group of carcinogens occurring in tobacco products, nitrite-cured meat and other foods, drugs, and certain industrial settings (98). The *N*-nitrosamines are both mutagenic and carcinogenic (99). Exposure to *N*-nitroso compounds has been implicated as an etiological factor for cancer of the stomach, esophagus, nasopharynx, urinary bladder, and colon (82,100-102). The chemistry of nitrosation has been reviewed extensively by Ridd (103), Mirvish (82), and Challis (104). Originally, the focus was on acid-catalyzed nitrosation in which the nitrite ion exists in equilibrium with several nitrosating agents including nitrous anhydride, $N_2O_3$, and nitrosonium ion, $NO^+$ (105).

*N*-nitroso compounds have also been shown to be formed endogenously. Nitric oxide-associated formation of *N*-nitrosamines has been demonstrated in aqueous solution, in macrophages activated for NO· production, and in infected patients (19,25,106,107). In one experiment, nitrosamines formed from activated macrophage cytosol upon addition of the nitrosatable amine morpholine without added nitrite or nitrate (108). This nitrosation was indeed mediated by NOS due to its dependence on L-arginine, oxygen, NADPH and its inhibition by $N^G$-monomethyl-L-arginine (NMA). Ohshima *et al.* present a three-step mechanism for macrophage-induced nitrosamine formation: 1) NO· generation from L-arginine by NOS, 2) NO· oxidation by molecular oxygen to form $NO_2$·, and 3) formation of the nitrosating agents $N_2O_3$ and $N_2O_4$ which react with amines forming *N*-nitrosamines (108-110). Figure 5 shows the nitrosation of morpholine by $N_2O_3$ giving rise to *N*-nitrosomorpholine.

After metabolic activation by cytochrome P450 enzymes, *N*-nitrosamines form powerful alkylating electrophiles which can attack DNA at several nucleophilic sites. Figure 6 shows the metabolic activation and subsequent chemistry that give rise to the powerful electrophiles which are capable of damaging DNA. The formation of *N*-nitrosamines and subsequent alkylation of DNA is an indirect method of DNA damage caused by $N_2O_3$. Repair of DNA alkylation is mediated by nucleotide excision repair mechanisms (111,112).

# Nitrosation of Secondary Amines - Morpholine Nitrosation




Morpholine

N-Nitrosomorpholine

Figure 5. An example of nitrosation of a secondary amine forming an N-nitrosamine. Here, morpholine is nitrosated by $N_2O_3$ forming N-nitrosomorpholine.

# Methylation of DNA by *N*-nitrosamines
# Example: *N*-nitrosodimethylamine

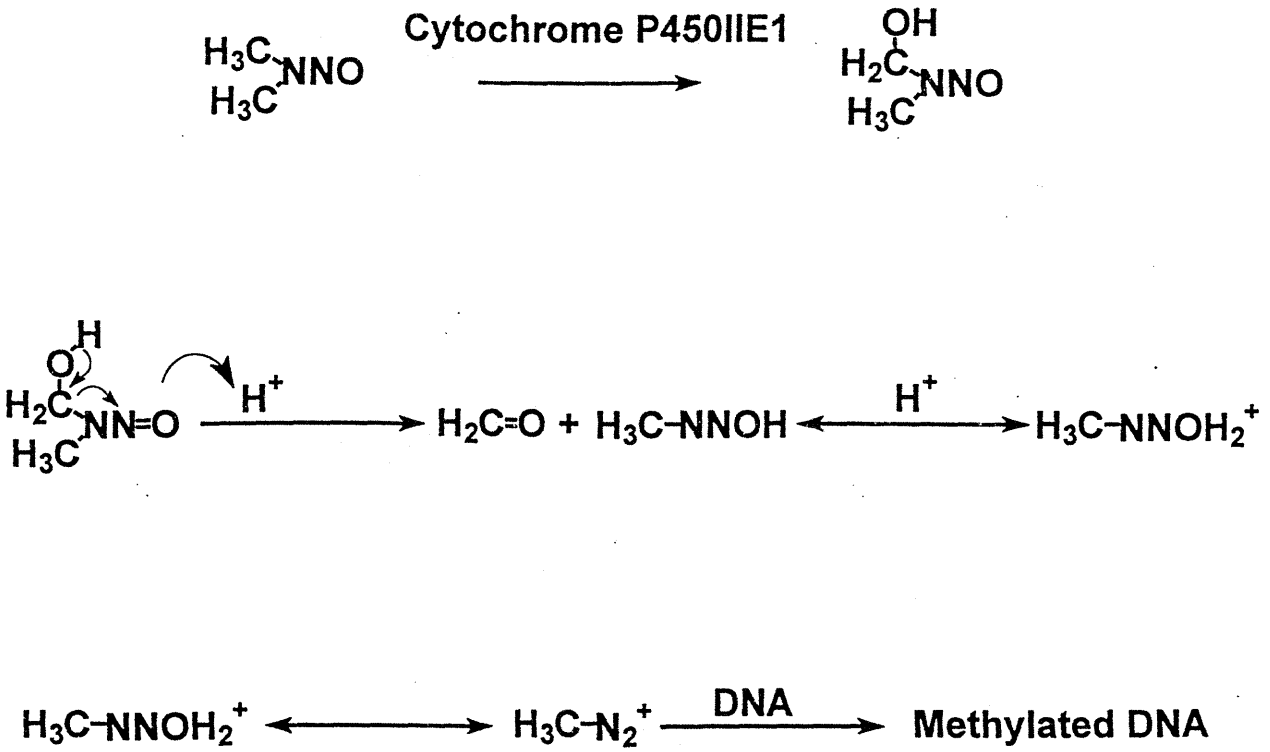



**Figure 6. Mechanism of DNA alkylation via the formation of N-nitrosamines and subsequent metabolic activation by cytochrome P450 enzymes.**

### 3. *Deamination of DNA Bases by $N_2O_3$*

DNA damage can also result from the direct attack of $N_2O_3$ on the DNA bases. Any DNA base containing an exocyclic amino group can undergo deamination upon reaction with $N_2O_3$. Therefore, cytosine, 5-methylcytosine, guanine, and adenine can all be deaminated forming uracil, thymine, xanthine, and hypoxanthine, respectively. The chemistry involves attack of the exocyclic amino group on $N_2O_3$ forming a primary nitrosamine which breaks down to a diazonium ion. Diazotization is rate-controlled by the formation of a primary nitrosamine - subsequent transformations of the primary nitrosation product do not affect the kinetics of diazotization. The end result is formation of a diazonium ion which is stabilized by conjugation with the aromatic ring. There is a marked correlation of the rate of diazotization with base strength of the amine. This chemistry is shown in Figure 7. Hydrolysis of the diazonium ion completes the deamination. The end result of this process is the net replacement of an amino group by a hydroxyl group as demonstrated for guanine deamination in Figure 8. The formation of the electrophilic nitrosating agent $N_2O_3$ may ultimately result in DNA deamination events which can lead to mutagenesis as described in later sections (29,113).

Shapiro *et al.* have performed a number of studies on the kinetics of reaction of $HNO_2$ with DNA components leading to deamination (114-116). Guanine has been shown to be the most reactive base as shown by its higher deamination rate than adenine and cytosine - guanine is approximately two times as reactive as cytosine in native DNA (114,117-119). It has also been observed in numerous studies that heat-denatured DNA deaminates much faster than native DNA (117,118,120,121). It is clear from these earlier studies that relative rates of $HNO_2$-induced deamination depend on both the individual base and the nucleic acid structure. Also, in previous studies on spontaneous cytosine deamination, Frederico *et al.* have observed a greater than 100 fold increase in spontaneous deamination for cytosine in single stranded DNA over double stranded DNA (122). This indicates that the formation of secondary structure protects DNA from deamination. Within double stranded DNA, cytosine may deaminate through a single stranded intermediate (122). Cytosine in mispairs also is 1-2 orders of magnitude more

prone to deamination than properly paired double stranded DNA again providing evidence for the importance of Watson-Crick base pairs in maintaining genetic integrity (123). The overall conclusion is that secondary structure is a major determinant of the extent of deamination.

Deamination is an important type of modification because it changes the base pairing abilities and may result in mutations as shown in Figure 9. The specific types of mutations that can arise from each deamination are discussed here. The deamination product of 5-methylcytosine is thymine which is a "perfect" mutation because it forms a normal DNA base. The end result of this deamination is a G:C → A:T transition mutation (124,125). Deamination of cytosine forms uracil which will also give rise to a G:C → A:T mutation through mispairing. However, most cell types have significant quantities of the enzyme uracil glycosylase which is known to excise uracil from single and double stranded DNA with preference for the former (126). Therefore, the deamination of cytosine to uracil may not be critical because the resulting uracil will be repaired before causing a mutation. The deamination of adenine to hypoxanthine may result in an A:T → G:C mutation due to pairing of hypoxanthine with cytosine. The deamination of guanine to xanthine will lead to a G:C → A:T transition upon pairing; however, this deamination is also extremely important because the product xanthine is believed to be unstable in DNA and could depurinate leaving an abasic site (114,127). The cell may replicate past the abasic site following the "A rule" involving insertion of an adenine opposite the abasic site resulting in a G:C → T:A transversion mutation (128). The abasic site may also be cleaved by base catalysis or an endonuclease resulting in a single strand break which may be toxic to the cell (129). Indeed, strand breaks were observed in intact DNA treated with NO· gas and in rodent cells treated with NO· gas (44,120). Single strand breaks may arise from the formation of abasic sites or other indirect mechanisms (44). In support of the notion that strand breaks occur as a result of nitrosative deamination, it was shown that single strand breaks could be caused by $NO_2$· but not NO· in the absence of $O_2$. Cytotoxicity in cells exposed to NO· may be mediated by DNA strand breakage and the subsequent activation of the DNA repair enzyme

poly(ADPribose) synthetase (PARS) leading to the depletion of the cellular $NAD^+$ and ATP pool as suggested for $ONOO^-$ (95,96).

*Additional Reaction Products*

In the study of nitrous acid-treated DNA, it was determined that not all of the guanine modifications were observed as xanthine but there must have been some guanine consumed in an unknown reaction. One report describes recovery of only 35-55% of missing guanine as xanthine whereas 90-98% of missing adenine is recovered as hypoxanthine (118). Therefore, there is an additional reaction taking place. In experiments using high concentrations of nitrous acid, the product 2-nitroinosine has been observed (114,130). This is presumably due to nucleophilic displacement of the diazonium ion by nitrite ion resulting in a nitro group at the N2 position. This mechanism is deduced because the formation of 2-nitroinosine was favored by increasing the nitrite concentration from 1 to 8 N (114). Another fate for guanine was proposed to be cross-link formation.

In recent studies, Suzuki *et al.* have isolated an additional product which may explain the disappearance of guanine not accounted for by 2-nitroinosine and cross-link formation. The product they have isolated is 2-deoxyoxanosine (131). Oxanosine, the ribonucleoside of this compound, had previously been isolated as a novel antibiotic in 1981 from a bacterial culture. The compound 2'-deoxyoxanosine was synthesized from that and exhibited a stronger antineoplastic activity than oxanosine. The structures of the additional reaction products 2-nitroinosine and oxanine are shown in Figure 10. The product 2'-deoxyoxanosine has been identified by its IR spectrum and NMR spectrum and is a major product under those reaction conditions. The yield appears to be approximately one half that of xanthine. In these experiments, however, the amount of nitrite used is extremely high (100 mM $NaNO_2$) and the pH is low (pH 3.7); therefore, it is not certain whether this product would appear in NO·-treated DNA at physiological pH. At the acidic pH used and the high nitrite concentration, there may be different chemistry occuring. The nitrosating agent may be different - for example, at a lower pH, there

could be more $NO^+$ available for reaction. Oxanine formation was also demonstrated after nitric oxide treatment; however, in that treatment, the pH dropped to 2.9. This low pH indicates that the chemistry is actually that of nitrous acid.

In subsequent work, Suzuki *et al.* examined the deglycosylation susceptibility and base pairing stability of 2'-deoxyoxanosine in oligodeoxynucleotides (132). Oligodeoxynucleotides containing oxanine, xanthine, and guanine were compared for the stability of the N-glycosidic bond. Suzuki's results indicate that oxanine is as stable as guanine but is hydrolyzed significantly slower than xanthine. The structure of 2'-deoxyoxanosine can exist in a ring-opened and ring-closed configuration due to the fact that it contains a lactone structure. The $pK_a$ has been reported to be 9.4 for the ring-opening and ring-closing equilibrium implying that dOxo is in the ring-closed form at physiological pH.

Since dOxo does not appear to hydrolyze spontaneously, it is of interest to determine which base dOxo is likely to pair with upon replication. The $T_m$ values for the duplexes decrease in the order of C > T > A > G as counter bases (132); however, oxanine does not seem to be able to hydrogen bond with one specific counter base causing very low $T_m$ values for all counter bases. Also, it has been determined that pairing of dOxo with another base does not induce global structural change to DNA (132).

# Nitrosation of Primary Amines -Diazonium Ion Formation

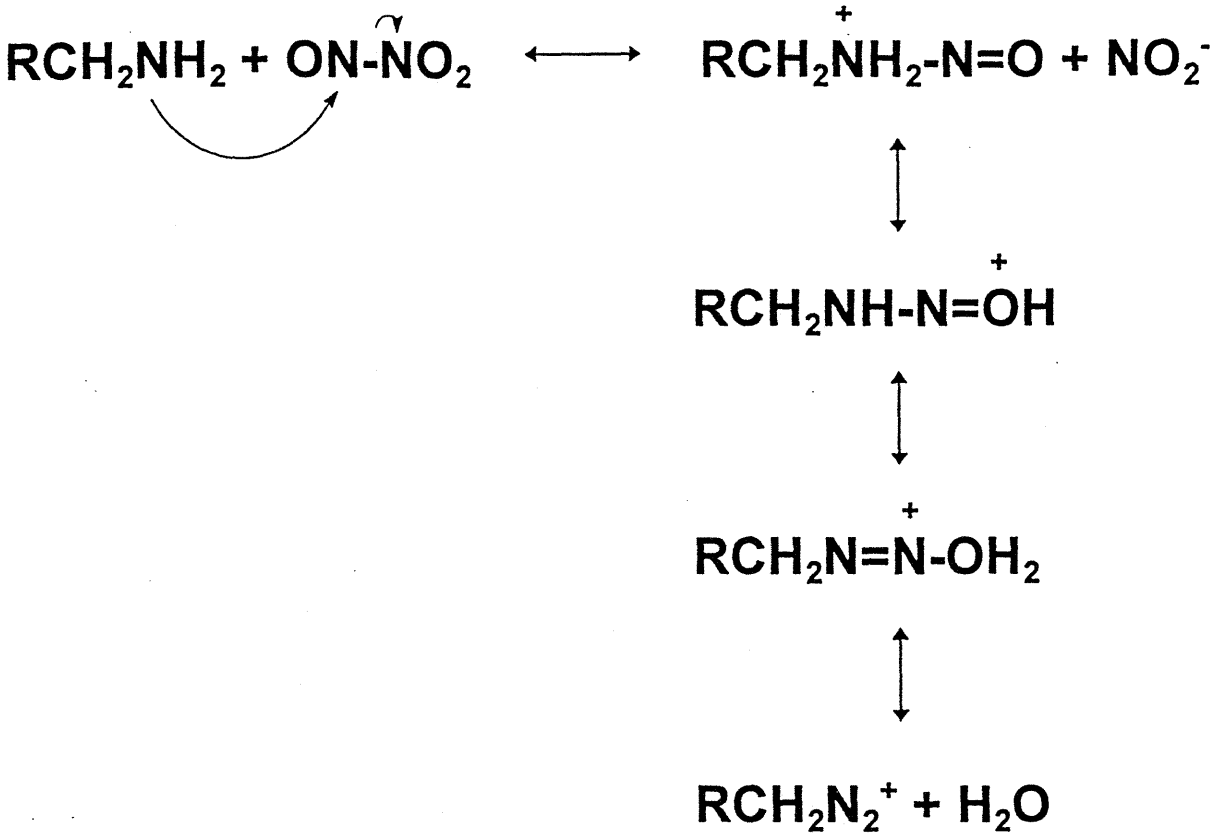



Figure 7. An example of nitrosation of a primary amine by $N_2O_3$ leading to diazonium ion formation.

# DNA Base Deamination

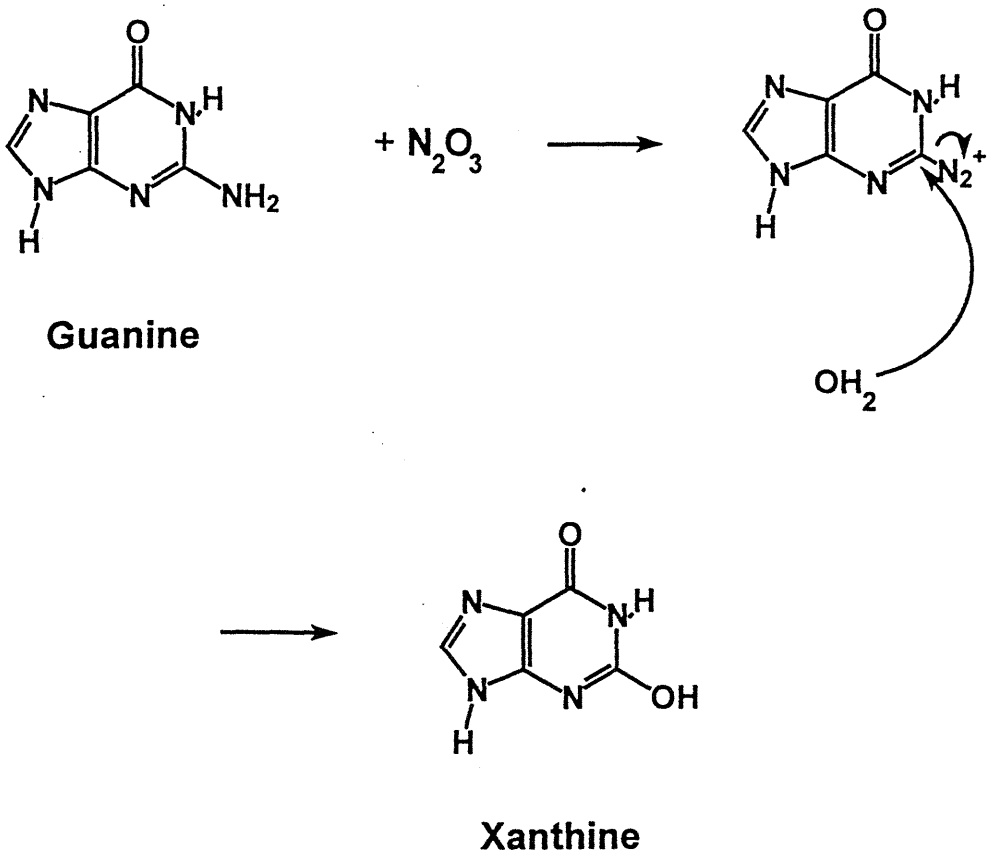



**Figure 8. An example of DNA base deamination - hydrolysis of the diazonium ion leads to deamination of guanine forming xanthine.**

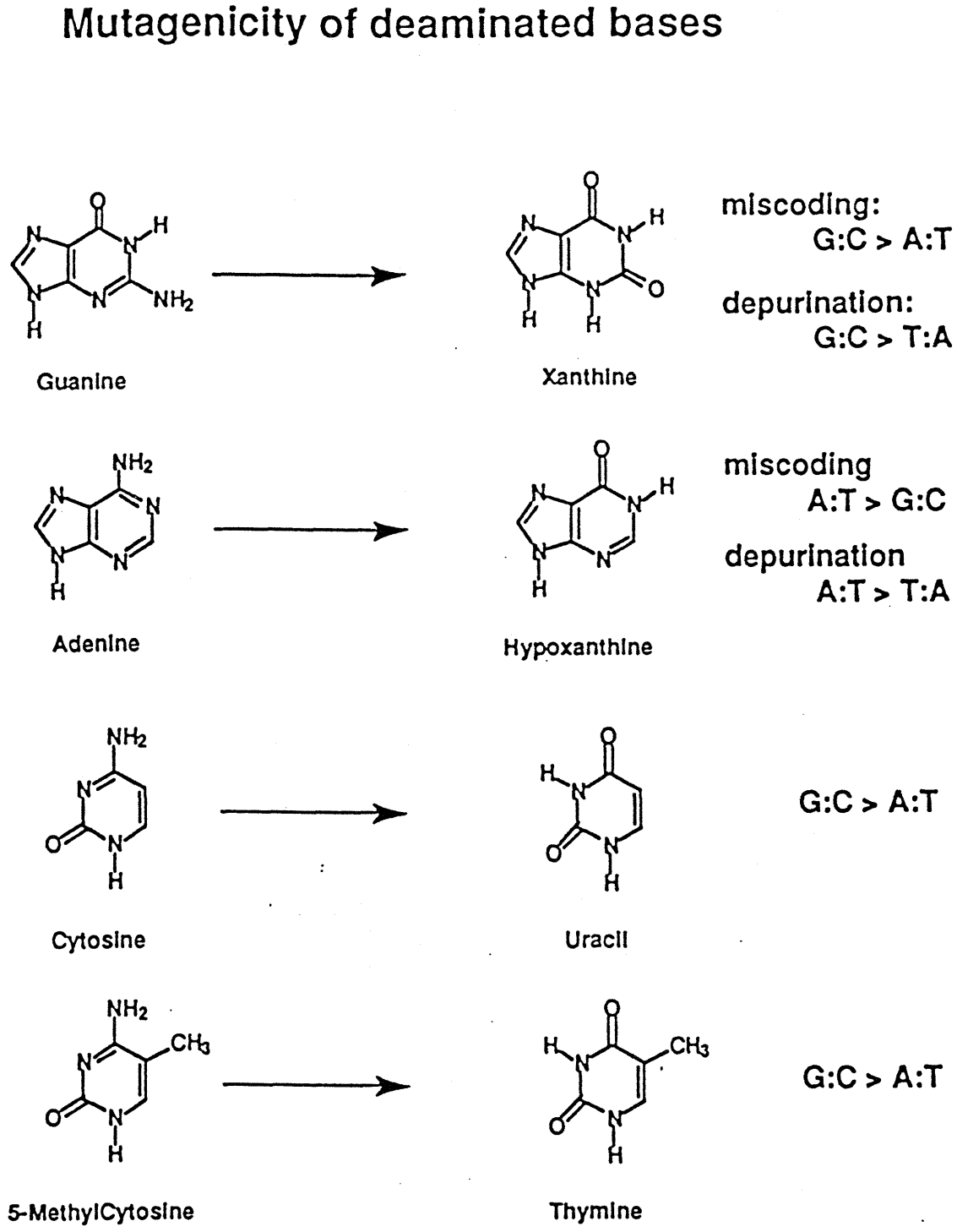
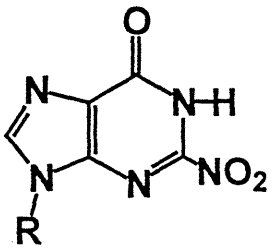
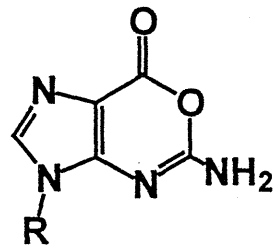



**Figure 9. Deamination products of DNA bases and their potential mutagenicity.**

# Additional Reaction Products




2-Nitroinosine




Oxanine

**Figure 10. The structures of oxanine and 2-nitroinosine - additional products that have been demonstrated to arise from nitrous acid treatment and may also be generated from NO· exposure.**

## 4. *Cross-Link Formation by $N_2O_3$*

There are three important types of cross-links that have been demonstrated to form upon nitrous acid treatment including DNA-protein cross-links, DNA intrastrand cross-links, and DNA interstrand cross-links which will all be discussed here. There have been no reports of formation of these cross-links upon nitric oxide treatment.

### *DNA-Protein Cross-Links*

The reversible interactions between proteins and nucleic acids are important for processes such as replication, transcription and translation. Disruption of these interactions can have serious genetic consequences because it can interfere with gene expression. A large variety of agents have been found to form DNA-protein cross-links including formaldehyde, cisplatin, psoralen, chromium, and UV light (133-135). The presence of these DNA-protein cross-links may be an eventual biomarker of exposure to cross-linking agents because these types of adducts are persistent due to poor repair capacity.

The formation of DNA-protein cross-links is a possibility by nitrous acid and nitric oxide exposure if the nucleophile attacking the diazonium ion were from an amino acid side chain instead of water or the guanine N2 amino group as in deamination or DNA interstrand cross-linking, respectively. However, the formation of DNA-protein cross-links induced by nitrous acid or nitric oxide has not been extensively studied. Nitrous acid was once reported to cause DNA-protein cross-links. In 1971, Potti *et al.* demonstrated that upon treatment of calf thymus nucleohistone with nitrous acid, protein could not be completely removed from nucleic acid after 5 extractions with phenol followed by isoamyl alcohol/$CHCl_3$ treatment (136). Upon hydrolysis, the product showed both amino acids and nucleic acid bases. No further study was performed to determine the actual identity of the true components. Their conclusion was that a covalent cross-link formed between histone and nucleic acid; however, they present no

information to prove that the protein involved in the cross-link is actually a histone protein.

*DNA Intrastrand Cross-Links*

The formation of intrastrand cross-links upon nitrous acid treatment has been observed by Dubleman and Shapiro and independently by Murphey-Corb *et al.* (137,138). There has been no further characterization with respect to the structure, the kinetics of formation, or possible site-specificity of cross-link formation. Hartman *et al.* postulate that this type of cross-link would be important due to the possible distortion of the duplex which may facilitate deamination of residues in that region (139). Also, the presence of this cross-link could block repair enzymes (139).

*DNA Interstrand Cross-Links*

Despite the fact that cross-links are a minor component of the DNA, many types of DNA interstrand cross-links have been shown to occur (140). The list of compounds that form these cross-links is quite varied including carcinogens such as polycyclic aromatic hydrocarbons (PAHs) and therapeutically active drugs including antibiotics and chemotherapeutic drugs (137). Other agents that form DNA interstrand cross-links are psoralens, formaldehyde, spermine, and ultraviolet radiation (141-143).

DNA interstrand cross-link formation in nitrous acid-treated DNA was discovered in 1961 when Geidushek observed reversible denaturation of nitrous acid-treated DNA (144). This suggested that covalent bonds had formed linking the complementary strands so the strands are held in close proximity upon denaturation which allows for fast renaturation kinetics (144,145). A low number of cross-links is effective to yield reversible DNA (146). However, Geidushek did not investigate the actual structure of the cross-link.

In 1977, Shapiro *et al.* were the first to isolate candidate structures for the cross-link and tentatively determine the structures using nuclear magnetic resonance

spectroscopy (NMR) and mass spectroscopy (MS) (147). They actually found two different cross-links present - a dG-dG cross-link and a dG-dA cross-link. Later work has not shown the dG-dA structure to be of much importance (148). This dG-dA cross-link is not expected to be formed without significant disruption of the helix because the reactive centers are in opposite grooves in the DNA. It may have been formed in denatured regions of calf thymus DNA in Shapiro's study. Focus will therefore be only on the dG-dG interstrand cross-link.

The structure of the dG-dG interstrand cross-link is shown in Figure 11 (147). Further studies by Kirchner *et al.* showed the formation of this DNA interstrand cross-link upon nitrous acid treatment of oligomers (148,149). After enzymatic digestion of the oligomers, the resulting nucleoside was structurally characterized by NMR and Electrospray-MS (148). The nucleus of the cross-link was shown to be a G-G interstrand cross-link with a connection through the N2 amino groups. This structure is not in agreement with the mechanism previously proposed by Shapiro in which an aldehyde-β-phosphodiester group resulting from depurination cross-links to an amino group on the opposite strand (147,150). The currently proposed mechanism of cross-link formation is diazotization of a dG residue followed by nucleophilic attack on C2 of that guanine by the N2 exocyclic amino group of a neighboring guanine on the opposite strand and loss of a molecule of $N_2$ gas (147,149). In this case, the dG amino group acting as the nucleophile in the cross-link formation reaction can be viewed as analogous to the role of water in the deamination reactions. An alternative mechanism involves the formation of a triazine structure that ultimately breaks down releasing a molecule of nitrogen gas and forming the G-G interstrand cross-link. The proposed triazine mechanism may be more likely because the diazonium ion is closer to the N2 than the C2 of the guanine. Therefore, reaction with the N2 position may be more likely to occur than reaction with C2 as proposed in the previous mechanism.

This interstrand cross-linking is possible due to the close van der Waals contact between the N2 amino groups of neighboring guanines in the double helix. In the sequence 5'-CG, the N2 amino groups are approximately 3.2 angstroms apart as determined by X-ray crystallography of the Dickerson dodecamer (151). In 1992,

Kirchner *et al.* demonstrated that nitrous acid-induced cross-link formation is indeed sequence specific with 5'CG being preferred over 5'GC (148). More distortion is needed for cross-link formation in the sequence 5'GC because the distance between N2 atoms is not easily bridgeable in this sequence and requires a 3 angstrom sliding of the base pairs to bring the reactive centers into contact. Based on molecular mechanics calculations, Kirchner postulates that cross-linked DNA shows no significant bending of the helix axis and also that there will not be much difference between cross-linked and normal DNA other than a severe propeller twisting of the two linked dG residues (149). To date, no studies have been performed to determine the effect of this cross-link on cell viability. It may interfere with DNA synthesis or translation due to the inability of the strands to dissociate. The role of these lesions in NO·-associated cytotoxicity and mutagenesis has yet to be established (152); however, cross-links are considered to be toxic if not lethal to the cell (148).

# DNA Interstrand Cross-Linking via Nitrosative Deamination

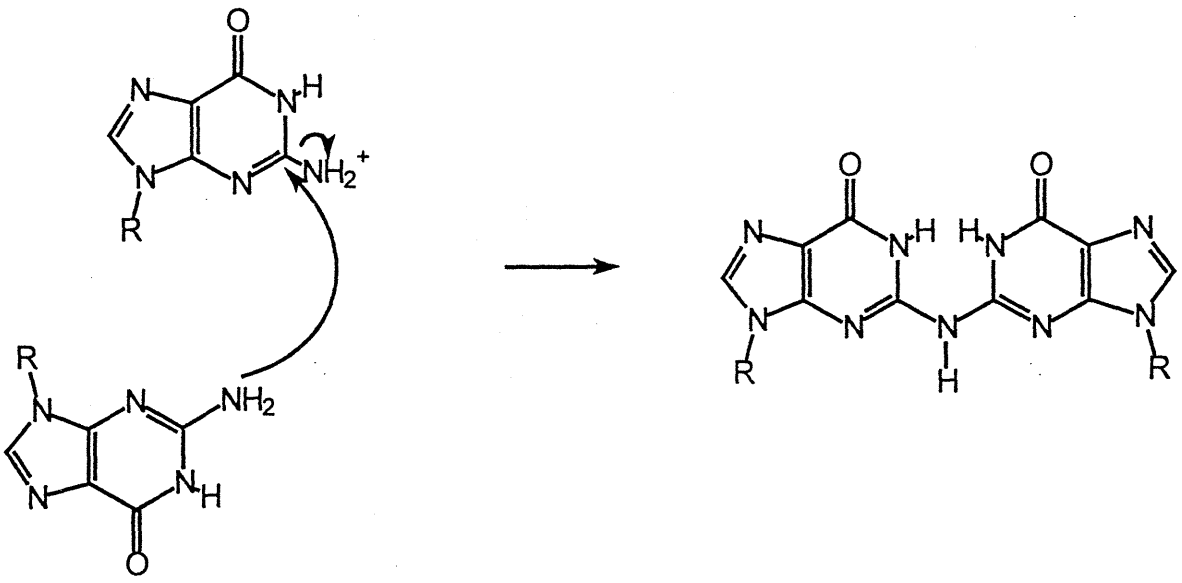


**Figure 11. Structure and possible mechanism for formation of a dG-dG interstrand cross-link through nitrosative deamination. This cross-link has been reported to form upon nitrous acid treatment and may also be generated from NO· exposure.**

## Nitric Oxide-Induced Mutagenicity and Cytotoxicity

The numerous types of DNA damage outlined above could result in several types of mutations which are known to be important in human cancer genes. In a number of human cancers including colon, liver, breast and lung, G:C → A:T transitions have been found in the p53 tumor suppressor gene (153,154). This type of mutation could arise through the deamination of cytosine, guanine, or 5-methylcytosine which demonstrates the potential importance of deamination chemistry as previously shown in Figure 9. In particular, the relative reactivity of guanine and cytosine is of interest to determine which of the bases in the original base pair is modified leading to the observed G:C → A:T mutation. The possible mutagenic effects of cytosine, adenine and guanine deamination forming uracil, hypoxanthine, and xanthine, respectively, are discussed below.

Deamination of cytosine will lead to a C:G → T:A transition mutation upon replication due to pairing of uracil with adenine (155,156). However, the uracil may be removed by the enzyme DNA uracil glycosylase before replication proceeds (157). Uracil glycosylase excises uracil from both single and double-stranded DNA with preference for single stranded DNA (126). The importance of cytosine deamination is emphasized by the fact that organisms which lack this enzyme have an increased spontaneous mutation rate and specifically more C:G → T:A transitions (158).

Adenine can deaminate to hypoxanthine which can base pair with cytosine, leading to an A:T → G:C transition. Deamination of guanine to xanthine will lead to a G:C → A:T transition mutation upon replication because xanthine is believed to base pair with thymine. There is no known repair mechanism for xanthine. Deoxyxanthosine has been noted to have high acid lability of its N-glycoside bond (114,127); therefore, one possible fate of xanthine is depurination resulting in an abasic site. Many organisms will replicate past such a lesion by invoking the "A rule" which involves insertion of an adenine opposite the abasic site (128). The ultimate result of such an event would be a G:C → T:A transversion. The sugar phosphate backbone of DNA may also be cleaved at the abasic site by an endonuclease or by base catalysis to yield a single strand break

which could be toxic to the cell (129). Indeed, the formation of xanthine and single strand breaks have been observed in NO·-treated cells (29,120).

Nitric oxide is indeed mutagenic as observed by many including Nguyen *et al.* who determined the mutagenicity of NO· at both the HPRT and TK gene loci of TK6 human lymphoblastoid cells (120). The deaminated bases xanthine and hypoxanthine were observed in the DNA of these treated cells which presents a possible mechanism for nitric oxide mutagenicity. In addition, *Salmonella typhimurium* TA1535 was treated with SPER/NO and the observed reversion mutations were 99% G:C → A:T indicating that cytosine or guanine deamination may be involved (159). Also, mutations induced by saturated aqueous nitric oxide in the forward mutation system utilizing the supF gene of the pSP189 shuttle vector in human and *E. coli* cells found a majority of A:T → G:C transitions followed by G:C → A:T mutations both of which could result from deamination. Bubbling nitric oxide gas leads to predominantly A:T → G:C transitions which may arise from adenine deamination to hypoxanthine (160). When the same shuttle vector was treated with millimolar concentrations of the NO·-donor drugs DEA/NO and SPER/NO, the most predominant mutation observed was G:C → A:T, which could result from deamination of either guanine or cytosine (160,161). Further support of nitric oxide's mutagenicity being caused by a deamination mechanism is provided by the mutational spectra of tetranitromethane. Tetranitromethane, a known nitrosating agent which can cause deamination, has been shown to yield transformed K-*ras* genes with predominantly G:C → A:T transitions in lung tumors of treated rats and mice. This difference in mutational spectra resulting from the two types of NO· exposure could arise from different reactive intermediates formed, i.e. by bubbling NO·, there could be significant $NO_2$ formation leading to higher levels of $N_2O_4$ which may have different specificity in its reaction with DNA than $N_2O_3$. This is discussed further in the next section regarding nitric oxide delivery.

The G:C → A:T transition has frequently been observed as the primary type of mutation upon NO· treatment which suggests a role for NO·-induced deamination in the initiation of particular cancers (160,161). Approximately 35% of point mutations within

coding regions of genes causing human genetic diseases including cancer have occured within CpG dinucleotides and over 90% are G:C → A:T transition mutations which demonstrates the importance of this type of mutation in human disease (153). Nitric oxide-induced deamination could therefore play a role in causing G:C → A:T mutations.

*Effect of Nitric Oxide Delivery on Cytotoxicity and Mutagenicity*

A number of studies have been performed on the cytotoxic effects of NO· using NO· donors or by directly bubbling NO· gas into aerobic solutions (50,162-166). Significant deamination of cytosine in calf thymus DNA was observed in previous experiments with high doses of NO· - accumulated amounts of 0.1-1 mole of NO· per liter delivered by syringe (159). Bubbling of NO· into solution is an extremely inefficient method of delivering nitric oxide due to significant loss of NO· into the gas phase so it will not be available to form $N_2O_3$ in solution. The major advantage of the silastic membrane delivery system used here and shown in Figure 12 is slow, steady delivery of low levels of nitric oxide which approaches the delivery rates of stimulated cells such as macrophages. Rates of delivery using this system are ~10-20 nmol/ml/min resulting in a final concentration of ~600-1200 uM $NO_2^-$. Tamir *et al.* (43) observed increased cytotoxicity to TK6 and *S. typhimurium* cells using this system as compared to bubbling NO· into the solution due to the fact that NO· is not released into the gas phase when delivered at such low levels thereby increasing the effective nitric oxide concentration in solution. Due to the fact that $N_2O_3$ formation is dependent on the square of the nitric oxide concentration, the dose rate is critical because it determines the local nitric oxide concentration. Therefore, even a small change in NO· dose rate will significantly alter the amount of $N_2O_3$ formed.

Experiments performed using NO· donors may also result in a variable rate of NO· generation. In addition, NO·-releasing drugs give rise to side products that may be toxic or bind directly to DNA; therefore, data using this delivery system can be difficult to interpret. For example, sodium nitroprusside leads to the formation of cyanide which is toxic. Also, *S*-nitroso-N-acetyl-D,L-penicillamine(SNAP) produces *N*-acetyl-D,L-penicillamine which is a metal chelator. In addition, the NO· donor SPER/NO decomposes to nitrite and spermine which may be problematic because nitrite-polyamine mixtures are mutagenic by an unknown mechanism (138,139).

The silastic membrane delivery system used in this thesis best reflects physiological conditions because it maintains a constant steady state of NO· over time

whereas NO·-releasing drugs and bubbling lead to a bolus or a variable NO· concentration. A steady rate of NO· delivery is important because dose rate can indirectly influence the underlying chemistry.

The complexity of cytotoxicity of nitric oxide recapitulates the complexity of nitric oxide chemistry. The many reactive compounds generated by nitric oxide can damage many crucial cellular processes. Toxicity may occur through a number of pathways including DNA synthesis inhibition, mitochondrial inactivation, cell membrane lysis, cell cycle arrest, DNA strand break formation, and apoptosis (167). The environment and dose of nitric oxide will change its effect on the cell as will the cell type. Comparison of the response of two cell types, the human TK6 cell line and the Chinese Hamster Ovary (CHO) cell line, shows that specific cell types have differential sensitivity to nitric oxide (167). The details of different mechanisms of nitric oxide-induced cytotoxicity are beyond the scope of this thesis; however, a detailed discussion of the parameters of nitric oxide cytotoxicity can be found in Burney *et al.* (167).

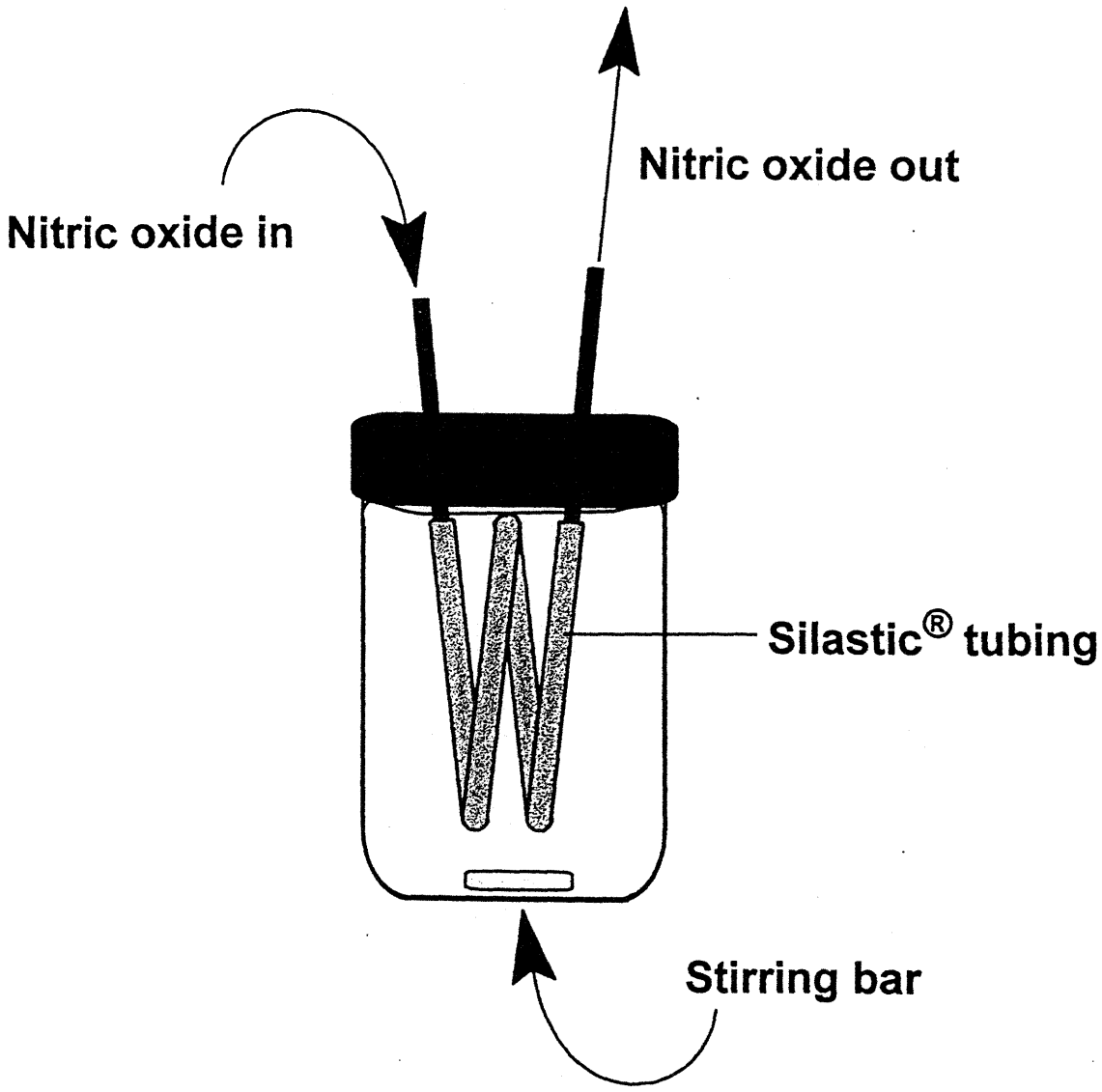



**Figure 12. Silastic membrane system for delivery of nitric oxide. A 10% NO· in argon mixture is passed through the gas permeable Silastic tubing for delivery.**

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# 2. Bicarbonate Inhibits *N*-Nitrosation in Oxygenated Nitric Oxide Solutions

## Abstract:

N-nitrosation in oxygenated nitric oxide ($NO^{\cdot}$) solutions was previously shown to be significantly inhibited by phosphate and chloride presumably by anion scavenging of the nitrosating agent nitrous anhydride, $N_2O_3$ (1). Here, bicarbonate is shown to exhibit this same inhibitory effect. Rate constants for reaction of morpholine, phosphate, and bicarbonate with $N_2O_3$ relative to $N_2O_3$ hydrolysis at pH 8.9 were determined to be $(3.7 \pm 0.2) \times 10^4\ M^{-1}$, $(4.0 \pm 0.9) \times 10^2\ M^{-1}$ and $(9.3 \pm 1.5) \times 10^2\ M^{-1}$, respectively. The morpholine and phosphate rate constants at pH 8.9 are similar to those reported at pH 7.4 assuring that these results are relevant to physiological conditions. The rate constant for this previously unrecognized reaction of bicarbonate with $N_2O_3$ suggests the strong scavenging ability of bicarbonate; accordingly, bicarbonate may contribute to reducing deleterious effects of $N_2O_3$. This is biologically important due to substantial bicarbonate concentrations *in vivo* - approximately 30 mM. Bicarbonate was previously shown to alter peroxynitrite reactivity; however, carbon dioxide is the probable reactive species (2-7). Bicarbonate is therefore potentially important in determining the fate of two reactive species generated from nitric oxide, $N_2O_3$ and $ONOO^-$, and may thus act as a regulator of $NO^{\cdot}$-induced toxicity.

## Introduction:

Nitric oxide ($NO^{\cdot}$) is an important physiological messenger that is produced by several different cell types and is involved in many processes *in vivo* including inhibition of platelet aggregation, blood vessel relaxation, and neurotransmission (8). An alternative to these physiologically important pathways is the formation of reactive species that may ultimately result in cytotoxic or mutagenic events by a number of possible mechanisms. Mutagenic effects may arise from the reaction of nitric oxide with superoxide ($O_2^{\cdot-}$) forming peroxynitrite ($ONOO^-$) that can in turn oxidize many types of molecules including DNA. Alternatively, reaction of $NO^{\cdot}$ with molecular oxygen results in the formation of nitrous anhydride ($N_2O_3$) which can cause cytotoxic effects through the nitrosation of both primary and secondary amines. DNA bases containing primary amine functionalities undergo nitrosative deamination upon treatment with $NO^{\cdot}$ resulting in a modified base (9-10). $N_2O_3$ can also nitrosate secondary amines forming carcinogenic *N*-nitrosamines that can damage DNA following metabolic activation. $N_2O_3$ can modify other cell constituents including protein sulfhydryl groups and low molecular weight thiols such as glutathione resulting in S-nitrosothiols.

The kinetics of morpholine N-nitrosation by nitric oxide at physiological pH have recently been studied by Lewis *et al.* using a novel reactor that allows continuous and simultaneous measurements of $NO^{\cdot}$, nitrite ($NO_2^-$), and N-nitrosomorpholine (NMor) concentrations. In this system, $N_2O_3$ was identified as the key nitrosating agent (1). The measured rate constant for the reaction of morpholine with $N_2O_3$ relative to $N_2O_3$ hydrolysis was $4.0 \times 10^4\ M^{-1}$. A key finding was the inhibitory effect of phosphate and chloride on morpholine nitrosation; the rate constants for reaction of these anions with $N_2O_3$ relative to $N_2O_3$ hydrolysis were $4.0 \times 10^2\ M^{-1}$ and $9.0 \times 10^1\ M^{-1}$, respectively. Participating anions react with $N_2O_3$ forming nitrosyl compounds (XNO) that can in turn react with amines or be hydrolyzed to $HNO_2$ and ultimately nitrite. At physiological pH, hydrolysis of XNO is much faster than nitrosation of amines by XNO; therefore, the anions will scavenge $N_2O_3$ and lower the rate of N-nitrosation (1).

Other anions including nitrate, nitrite, thiocyanate, and perchlorate have little or no effect on nitrosation.

During the course of DNA deamination studies (J.L. Caulfield, unpublished results), it was found that NO·-related deamination of calf thymus DNA at physiological pH was inhibited by sodium bicarbonate, $NaHCO_3$. Bicarbonate therefore seems to protect biomolecules from the nitrosative effects of NO· presumably due to an ability to scavenge $N_2O_3$ and consequently inhibit nitrosation at pH 7.4. The initial evidence for bicarbonate's inhibitory effect at pH 7.4 prompted the use of a modified reactor similar to that developed by Lewis *et al.* (11) to determine the rate constant for reaction of bicarbonate with $N_2O_3$. However, bicarbonate cannot be reliably studied at pH 7.4 in this system due to extensive argon degassing resulting in a shift in the equilibrium between bicarbonate and carbon dioxide and a consequent pH increase. The rate constants were therefore determined at pH 8.9.

As shown here, bicarbonate is important in determining the fate of $N_2O_3$, the product of nitric oxide oxidation; in addition, bicarbonate has been reported to alter the rate of reactions of peroxynitrite, the product of nitric oxide reaction with superoxide (2,3,12). However, it has now been demonstrated that $CO_2$ is the actual species that reacts with peroxynitrite (5-7). The reactivity of bicarbonate/$CO_2$ with both $N_2O_3$ and $ONOO^-$ and the relatively high concentrations of bicarbonate in interstitial and intracellular fluids (up to 30 mM) suggest that bicarbonate is a key determinant of the fate of the reactive species generated from nitric oxide and that bicarbonate may be protective of NO·-induced toxicity. Consequently, the presence of bicarbonate must be taken into account in all experiments with nitric oxide both in the presence and absence of reactive oxygen species.

## Materials and Methods:

**Reagents.** Morpholine (Aldrich Chemical Co., Milwaukee, WI) was used for the N-nitrosation studies. Phosphate buffer (0.01 M) at pH 7.4 was prepared with $K_2HPO_4$ and $KH_2PO_4$ using double-distilled water. Solutions containing 0.04 M bicarbonate were prepared fresh by adding sodium bicarbonate to the 0.01 M phosphate buffer. Nitric oxide (Matheson, Gloucester, MA) was passed through a column of 4-8 mesh soda lime to remove $NO_x$ impurities. Argon (Ar), after passage through an oxygen trap, was mixed with NO· using electronically-controlled gas flow meters (Porter Instrument Co., Hatfield, PA) to obtain the desired NO· gas concentration.

**Reactor.** The reactor was an ultrafiltration cell modified as previously described and shown schematically in Figure 1. For a detailed description, see reference 1. The only difference is the absence of the chemiluminescence detector to monitor NO·. Briefly, the reactor was a modified 200 mL stirred ultrafiltration cell (Amicon, Danvers, MA, Model 8200) to which gas inlet and outlet ports, two ports for a flow loop connected to a spectrophotometer (for $NO_2^-$ and NMor measurements) and a thermometer were added. A needle was inserted into the gas outlet port for argon/NO· delivery and removed during the reaction. The reactor was at ambient temperature.

**N-Nitrosation of Morpholine.** Morpholine at 50-1500 uM was added to 150 mL of buffer in the reactor and the pH was measured. Stirring was initiated at 40 rpm and circulation through the flow loop was started. The solution was bubbled with Ar for 45 minutes and a mixture of NO·/Ar was then introduced for 30 minutes at 350 sccm to obtain the desired aqueous NO· concentration. Bubbling of the NO·/Ar mixture was terminated and residual NO· in the head space was removed by introducing Ar via the gas inlet for 1.5 minutes. A 21:79 mixture of $O_2$ and $N_2$ was then introduced through the gas inlet at the same flow rate (350 sccm). Diffusion of $O_2$ into the aqueous phase initiated the oxidation of NO· which at this time was approximately 25 uM. The reaction was allowed to proceed for 30 minutes during which $NO_2^-$ and NMor

concentrations were monitored. Upon completion of the reaction, the pH was measured.

**Nitrite and N-Nitrosomorpholine Analysis.** The aqueous solution was continuously circulated at 45 mL/min through the 1/8 in. diameter flow loop (volume ~ 10 mL) and into a 10 mm spectrophotometer flow cell (Hewlett Packard, Model HP8452A UV) using a pulseless pump (Cole Parmer, Chicago, IL). The absorbance was measured at intervals of one minute. Absorbances at 250 nm were linearly proportional to the NMor concentration ($\varepsilon = 5500\ M^{-1}\ cm^{-1}$) with no interference from $NO_2^-$. The nitrite ion concentration was proportional to the absorbance at 210 nm ($\varepsilon = 5200\ M^{-1}\ cm^{-1}$) although absorption of NMor at 210 nm necessitated a correction of -0.27 uM $NO_2^-$/uM NMor. Concentrations of NMor and $NO_2^-$ were calculated at each cycle point for the 30 minute reaction.

**Kinetic Model and Reaction Scheme.** In previous experiments with this reactor, Lewis *et al.* showed that the principal nitrosating agent in the NO· oxidation pathway at physiological pH is $N_2O_3$ which leads primarily to $NO_2^-$ as summarized in reactions 1-3 (1).

$$2NO + O_2 \xrightarrow{k_1} 2\,NO_2 \qquad (1)$$

$$NO + NO_2 \underset{k_3}{\overset{k_2}{\rightleftharpoons}} N_2O_3 \qquad (2)$$

$$N_2O_3 + H_2O \underset{k_5}{\overset{k_4}{\rightleftharpoons}} 2HNO_2 \rightleftharpoons 2\,NO_2^- + 2\,H^+ \qquad (3)$$

Morpholine nitrosation by $N_2O_3$ and enhanced hydrolysis of $N_2O_3$ by various anions ($X^-$) are summarized in equations 4 and 5.

$$N_2O_3 + Mor \xrightarrow{k_6} NMor + NO_2^- + H^+ \quad (4)$$

$$N_2O_3 + X^- \xrightarrow{k_{10}} XNO + NO_2^- \quad (5)$$

Specifically, phosphate and chloride react with $N_2O_3$ as shown below (1):

$$N_2O_3 + P_i^- \xrightarrow{k_{10}^{Pi}} P_iNO + NO_2^- \quad (6)$$

$$N_2O_3 + Cl^- \xrightarrow{k_{10}^{Cl}} ClNO + NO_2^- \quad (7)$$

Any anion that behaves in this manner will scavenge some of the $N_2O_3$ thereby decreasing the rate of N-nitrosation at neutral pH.

In the above reaction scheme, all reaction rate constants are known. The overall nitrogen balance performed by Lewis (1) confirms that pseudo-steady-state approximations for $NO_2$, $N_2O_3$, and XNO are valid. A detailed analysis of the conservation equations at physiological pH is given in reference 1. The overall reaction kinetics where $N_2O_3$ is the only significant nitrosating agent are summarized by the following equations:

$$\frac{\Delta[R_2NNO]}{\Delta[NO_2^-] - \Delta[R_2NNO]} = k^*[R_2NH] \quad (8)$$

and,

$$k^* = \frac{k_6}{2\left(k_4 + \sum k_{10}[X^-]\right)} \quad (9)$$

where the summation in equation 9 is over all participating anions. For those anions that react with $N_2O_3$, the lumped "constant" k* will depend inversely on the anion concentration. By measuring k* in the presence of several concentrations of participating anions, the rate constants were determined for the reaction of phosphate and chloride with $N_2O_3$ (1). In the presence of phosphate and one additional anion, rearrangement of equation 9 yields:

$$k^* = \frac{1}{2}\left(\frac{\left(\frac{k_6}{k_4}\right)}{1+\left(\frac{k_{10}^{P}}{k_4}\right)[P_i]+\left(\frac{k_{10}^{X-}}{k_4}\right)[X^-]}\right) \qquad (10)$$

Further rearrangement gives:

$$\frac{1}{2k^*[P_i]} = \frac{k_{10}^{Pi}}{k_6} + \frac{k_4}{k_6}\left(\frac{1}{[P_i]}\right)+\frac{k_{10}^{X-}}{k_6}\left(\frac{[X^-]}{[P_i]}\right) \qquad (11)$$

Using the data for phosphate alone, linear regression of $1/(2k^*[P_i])$ vs $1/[P_i]$ yields $k_{10}^{Pi}/k_6$ and $k_4/k_6$ as the intercept and slope. The values of $k_6/k_4$ for morpholine and $k_{10}^{Pi}/k_4$ were found in this way (1). In this work, $k_6/k_4$ for morpholine and $k_{10}^{Pi}/k_4$ were determined as described above. In addition, the data for solutions containing bicarbonate and phosphate were used to calculate the rate constant for the reaction of bicarbonate with $N_2O_3$ at pH 8.9 ($k_{10}^{HCO3-}/k_4$).

## Results:

**Morpholine Concentration and pH.** The unprotonated form of morpholine is the substrate for nitrosation and is thus the most important form of morpholine for these experiments. Denoting total morpholine as Mor and the unprotonated form as Mor °, the respective concentrations are related by:

$$[\text{Mor}^{0}] = \frac{[\text{Mor}]}{1 + 10^{\text{pK}-\text{pH}}} \qquad (12)$$

where the pK at 25 °C is 8.5 for morpholine. The amount of morpholine available for nitrosation is 7.4% and 71.5% of the total morpholine concentration at pH 7.4 and 8.9, respectively. In phosphate buffer, the pH was nearly constant during a given experiment. In the bicarbonate reactions, the pH of the buffer rose during degassing to approximately 8.9. However, the pH during the reaction itself (i.e. after $O_2$ addition) remained virtually constant. The concentration of unprotonated morpholine therefore did not change significantly during the reaction. In all experiments, some NMor and $NO_2^-$ were present in the solution prior to introduction of $O_2$ due to a small air leak in the flow loop that could not be eliminated.

**Effect of Hydroxide Ion Concentration on Nitrosation Kinetics.** It has been reported that $OH^-$ enhances the rate of hydrolysis of $N_2O_3$ (13). Using flash photolysis of $NO_2^-$ ions in the presence of NO· in the pH range 9-10, a factor for total $N_2O_3$ hydrolysis was reported to be 2000 $s^{-1}$ + $10^8$ $[OH^-]$ $M^{-1}$ $s^{-1}$ representing terms for both water and hydroxide-induced hydrolysis of $N_2O_3$ (13). In order to determine the rate constant for reaction of hydroxide with $N_2O_3$ under the present conditions, morpholine nitrosation reactions were performed at several pH values in the range pH 7.4 - 8.9. A decrease in morpholine nitrosation at higher pH values was indeed observed indicating an increase in $N_2O_3$ hydrolysis mediated by hydroxide. The k* values obtained by linear regression analysis at pH 7.4, 8.0, 8.5 and 8.9 were 2600 ± 200 $M^{-1}$, 2300 ± 90 $M^{-1}$, 1900 ± 200 $M^-$

$^{1}$, and 1500 $\pm$ 300 $M^{-1}$, respectively. The rate constant for hydroxide reaction with $N_2O_3$ was calculated using a rearrangement of equation 10 by considering hydroxide to be an inhibitor of morpholine nitrosation analogous to the treatment of anions in previous work (1). Using this equation,

$$\frac{1}{2k^*[OH^-]} = \frac{k_{10}^{OH-}}{k_6} + \frac{1}{[OH^-]}\left(\frac{k_4 + k_{10}^{Pi}[P_i]}{k_6}\right) \qquad (13)$$

the intercept of 1 / 2k*[$OH^-$] vs. 1 / [$OH^-$] gives the value of $k_{10}^{OH-}/k_6$ to be (2.3 $\pm$ 0.4) $\times$ $10^1$. The published value for $k_6/k_4$ (4.0 X $10^4$ $M^{-1}$) implies that $k_{10}^{OH-}/k_4 = 9.4 \times 10^5$ $M^{-1}$.

There is some ambiguity in the meaning of $k_4$ depending on whether the hydroxide contribution is included. However, the hydroxide term does not significantly affect the $k_4$ value at pH 7.4 due to the extremely small concentration of hydroxide at this pH. For example, if a value for $k_4$ is assumed to be 1600 $s^{-1}$ at pH 7.4 (1), the incremental increase for the hydroxide contribution is 150 $s^{-1}$. Given the wide range of reported $k_4$ values as discussed by Lewis *et al.* (1), this 10% difference does not seem to be very important. The rate constants here are expressed as ratios to $k_4$.

**Effect of Phosphate on N-Nitrosation at pH 8.9.** To assess the validity of these experiments at pH 8.9, the rate constants for reaction of morpholine and phosphate with $N_2O_3$ relative to $N_2O_3$ hydrolysis ($k_6/k_4$ and $k_{10}^{Pi}/k_4$) were measured and compared to the published results. Various morpholine concentrations (50 uM to 150 uM) were used in buffers of three different phosphate concentrations, 0.01 M, 0.025 M, and 0.05 M.

As seen in Figure 2, the marked decrease in slope of $\Delta$[NMor]/$\Delta[NO_2^-]$-$\Delta$[NMor] vs. [$Mor^0$] with increasing phosphate concentration indicates that phosphate inhibits nitrosamine formation. When the slope was calculated by linear regression using the average data between 3 and 30 minutes, values of k* at pH 8.9 for 0.01 M, 0.025 M and 0.05 M phosphate were 1500 $\pm$ 300 $M^{-1}$, 1100 $\pm$ 100 $M^{-1}$, and 600 $\pm$ 100 $M^{-1}$, respectively. The differences between these values and those previously published are due to the effect of hydroxide at pH 8.9. The rate constants for the reaction of $N_2O_3$

with phosphate and morpholine at pH 8.9 relative to $N_2O_3$ hydrolysis ($k_{10}^{Pi}/k_4$ and $k_6/k_4$) were calculated from a plot of the equation below:

$$\frac{1}{2k^*[P_i]} = \frac{k_{10}^{Pi}}{k_6} + \frac{k_4}{k_6}\left(\frac{1+\left(\frac{k_{10}^{OH}}{k_4}\right)[OH^-]}{[P_i]}\right) \qquad (14)$$

The intercept ($k_{10}^{Pi}/k_6$) was found to be $(1.1 \pm 0.2) \times 10^{-2}$ which agrees nicely with the previously reported value of $1.0 \times 10^{-2}$ (1). The value of the rate constant for the phosphate/$N_2O_3$ reaction relative to $N_2O_3$ hydrolysis ($k_{10}^{Pi}/k_4$) was then calculated to be $4.0 \times 10^2$ $M^{-1}$. The rate constant for the morpholine/$N_2O_3$ reaction relative to $N_2O_3$ hydrolysis ($k_6/k_4$) was determined from the slope to be $(3.7 \pm 0.2) \times 10^4$ $M^{-1}$ which agrees nicely with the literature value of $4.0 \times 10^4$ $M^{-1}$ (1).

**Effect of Bicarbonate on N-Nitrosation at pH 8.9.** Various morpholine concentrations (50 uM to 150 uM) were used in a 0.01 M phosphate buffer solution containing 0.04 M sodium bicarbonate. As seen in Figure 2, there is a significant decrease in the slope of $\Delta[NMor]/\Delta[NO_2^-]$-$\Delta[NMor]$ vs. $[Mor^0]$ with the addition of 0.04 M sodium bicarbonate. Using linear regression, the value of k* using the average data between 3 and 30 minutes for reactions containing 0.01M phosphate and 0.04 M bicarbonate at 25 °C and pH 8.9 was $400 \pm 60$ $M^{-1}$. The rate constant for the bicarbonate/$N_2O_3$ reaction relative to $N_2O_3$ hydrolysis ($k_{10}^{HCO3-}/k_4$) was calculated from equation 10. The resulting $k_{10}^{HCO3-}/k_4$ value is $(9.3 \pm 1.5) \times 10^2$ $M^{-1}$. The rate constants from this study are summarized in Table 1 and their importance is discussed below.

## Discussion:

The finding that N-nitrosation of morpholine is inhibited by bicarbonate provides an additional pathway that affects the fate of $N_2O_3$ *in vitro* and *in vivo*. The rate constant for the bicarbonate/$N_2O_3$ reaction relative to $N_2O_3$ hydrolysis ($k_{10}^{HCO3-}/k_4$) in oxygenated nitric oxide solutions is $(9.3 \pm 1.5) \times 10^2$ $M^{-1}$ which is greater than the rate constant for the phosphate/$N_2O_3$ reaction relative to $N_2O_3$ hydrolysis ($k_{10}^{Pi}/k_4$) found to be $(4.0 \pm 0.9) \times 10^2$ $M^{-1}$. Inhibition by bicarbonate will be significant due to the higher rate constant for bicarbonate and higher extracellular concentrations of bicarbonate relative to phosphate. As summarized in Table 1, the agreement between the published rate constants for the morpholine and phosphate reactions at pH 7.4 and the experimentally determined rate constants at pH 8.9 demonstrates the validity of performing these experiments at pH 8.9 and assures the applicability of these rate constants at pH 7.4. Further work is necessary to determine an exact rate constant for the bicarbonate/$N_2O_3$ reaction at pH 7.4 to compare with that found here at pH 8.9. Nevertheless, it is certain that bicarbonate can efficiently scavenge the nitrosating agent $N_2O_3$ in competition with nitrosation of morpholine.

The importance of including this previously unrecognized reaction of bicarbonate in nitrosation experiments can be demonstrated by analyzing recent work by Lewis *et al.* (14). In an effort to determine whether the rate constants of pertinent reactions measured in simple cell-free systems could account for the rates at which the products are formed in the presence of NO·-generating cells in complex media, they studied the formation of NMor by activated macrophages. The importance of buffer anions is emphasized by the fact that the predicted values for NMor formation were 28 times larger than measured levels of NMor without the inclusion of chloride and phosphate contributions to $N_2O_3$ hydrolysis. The difference between observed and predicted values decreased to only 7-fold when these terms were included. This suggests that while chloride and phosphate are important, the cell culture system contains additional unrecognized compounds that can compete for $N_2O_3$. Bicarbonate is a prime candidate to account for at least a portion of the $N_2O_3$ scavenged. In this case,

$N_2O_3$ hydrolysis by bicarbonate will be more significant than phosphate due to the much higher concentrations of bicarbonate (30 mM $NaHCO_3$ vs 0.9 mM $P_i$). Calculations show that the inclusion of a term for bicarbonate reduces the difference between observed and predicted values for NMor by an additional factor of three.

The contribution to $N_2O_3$ hydrolysis by the different anions can be roughly calculated given the rate constants and the media concentrations. In Lewis' kinetic analysis, a lumped "constant" k* is calculated from the measured concentrations of NMor and $NO_2^-$ using equation 8. This k* value is related to the rate constants for morpholine nitrosation ($k_6$) and several terms for $N_2O_3$ hydrolysis ($k_4$ and $k_{10}^{X-}$) shown below which can also be expressed relative to $N_2O_3$ hydrolysis ($k_4$) as follows:

$$k^* = \frac{1}{2}\left(\frac{k_6}{k_4 + \Sigma k_{10}^{X-}[X^-]}\right)$$

or

$$k^* = \frac{1}{2}\left(\frac{\left(\frac{k_6}{k_4}\right)}{1 + \Sigma\left(\frac{k_{10}^{X-}}{k_4}\right)[X^-]}\right)$$

The anion contribution to $N_2O_3$ hydrolysis is therefore the summation over all anions, $X^-$, which can be expanded to include terms for phosphate, chloride, bicarbonate and remaining unidentified anions as follows:

$$\Sigma\,(k_{10}^{X-}/k_4)[X^-] = (k_{10}^{Pi}/k_4)[P_i] + (k_{10}^{Cl-}/k_4)[Cl^-] + (k_{10}^{HCO3-}/k_4)[HCO_3^-] + (k_{10}^{X-}/k_4)[X^-]$$

The contribution for the anions currently known to scavenge $N_2O_3$ using the media concentrations for these experiments is shown here as a hydrolysis enhancement ratio representing the additional hydrolysis with the anion present:

| | | |
|---|---|---|
| Phosphate: | $(4.0 \times 10^2\ M^{-1})$ ( 0.9 mM) = | 0.4 |
| Chloride: | $(9.0 \times 10^1\ M^{-1})$ ( 110 mM) = | 9.9 |
| Bicarbonate: | $(9.3 \times 10^2\ M^{-1})$ ( 30 mM) = | 27.9 |

Bicarbonate is extremely significant in this case and in all biological situations because it is present at relatively high concentrations. Physiological concentrations of phosphate, chloride, and bicarbonate in interstitial fluid are approximately 5 mM, 110 mM, and 30 mM, respectively, and the concentrations of these species in intracellular fluid are approximately 80 mM, 5 mM, and 12 mM, respectively (15). Predictions for contributions of these participating anions to $N_2O_3$ hydrolysis *in vivo* were calculated using these concentrations for both the intracellular and extracellular environments and are shown below as hydrolysis enhancement ratios:

INTRACELLULAR -

| | | |
|---|---|---|
| Phosphate: | $(4.0 \times 10^2\ M^{-1})$ ( 80 mM) = | 32.0 |
| Chloride: | $(9.0 \times 10^1\ M^{-1})$ ( 5 mM) = | 0.5 |
| Bicarbonate; | $(9.3 \times 10^2\ M^{-1})$ ( 12 mM) = | 11.2 |

EXTRACELLULAR -

| | | |
|---|---|---|
| Phosphate: | $(4.0 \times 10^2\ M^{-1})$ ( 5 mM) = | 2.0 |
| Chloride: | $(9.0 \times 10^1\ M^{-1})$ (110 mM) = | 9.9 |
| Bicarbonate: | $(9.3 \times 10^2\ M^{-1})$ ( 30 mM) = | 27.9 |

Bicarbonate's significant contribution to $N_2O_3$ hydrolysis in the extracellular milieu demonstrated by the large hydrolysis enhancement ratio may be important in protecting cells from $N_2O_3$ formed near NO·-producing cells such as macrophages during an inflammatory response. In this situation, $N_2O_3$ would be scavenged in the extracellular fluid before encountering neighboring cells.

Therefore, bicarbonate which is present at high concentration *in vivo* will be a key determinant of the fate of NO·-derived reactive species due to its reaction with $N_2O_3$ reported here and the previously reported reaction with $ONOO^-$. Carbonate has long been known to alter the activity of several types of oxygen radicals including superoxide anion, hydroxyl radical, and singlet oxygen (4). In addition, peroxynitrite has been known to be unstable in carbonate buffers for many years (12). Recently, it has been demonstrated that bicarbonate does indeed affect peroxynitrite reactivity (2-3). Bicarbonate inhibits the toxicity of peroxynitrite to *E. coli* (2) which may be a direct result of the enhanced isomerization to nitrate leading to $ONOO^-$ decomposition before it can encounter the bacteria. Extremely low levels of bicarbonate (far below physiological concentrations) are required as demonstrated by the fact that 95% protection from toxicity is observed at 5 mM bicarbonate (2). The probable mechanism involves reaction of carbon dioxide with peroxynitrite forming the nitrosoperoxycarbonate anion, $O=N-OOCO_2^-$ (3,5,6). Carbon dioxide increases the rate of peroxynitrite isomerization to nitrate presumably through this species (5). The result is $ONOO^-$ scavenging which will be important in defining amounts of tissue injury including both oxidation and nitration products resulting from peroxynitrite (16-18).

Elucidation of the exact reaction pathways and rate constants for the reaction of bicarbonate/$CO_2$ with peroxynitrite will provide additional information about the fate of NO· in specific systems. Then, reactions of NO· with oxygen and superoxide can be modeled in detail. The newly determined rate constant for the bicarbonate/$N_2O_3$ reaction will contribute to kinetic modeling ultimately enhancing the understanding of the numerous biological roles of NO· both as a messenger and as a cytotoxic or mutagenic agent.

**Acknowledgments:**

This work was supported by grants from the National Institutes of Health (CA09112, CA26731, ES04675, ES07020).

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## Table 1:

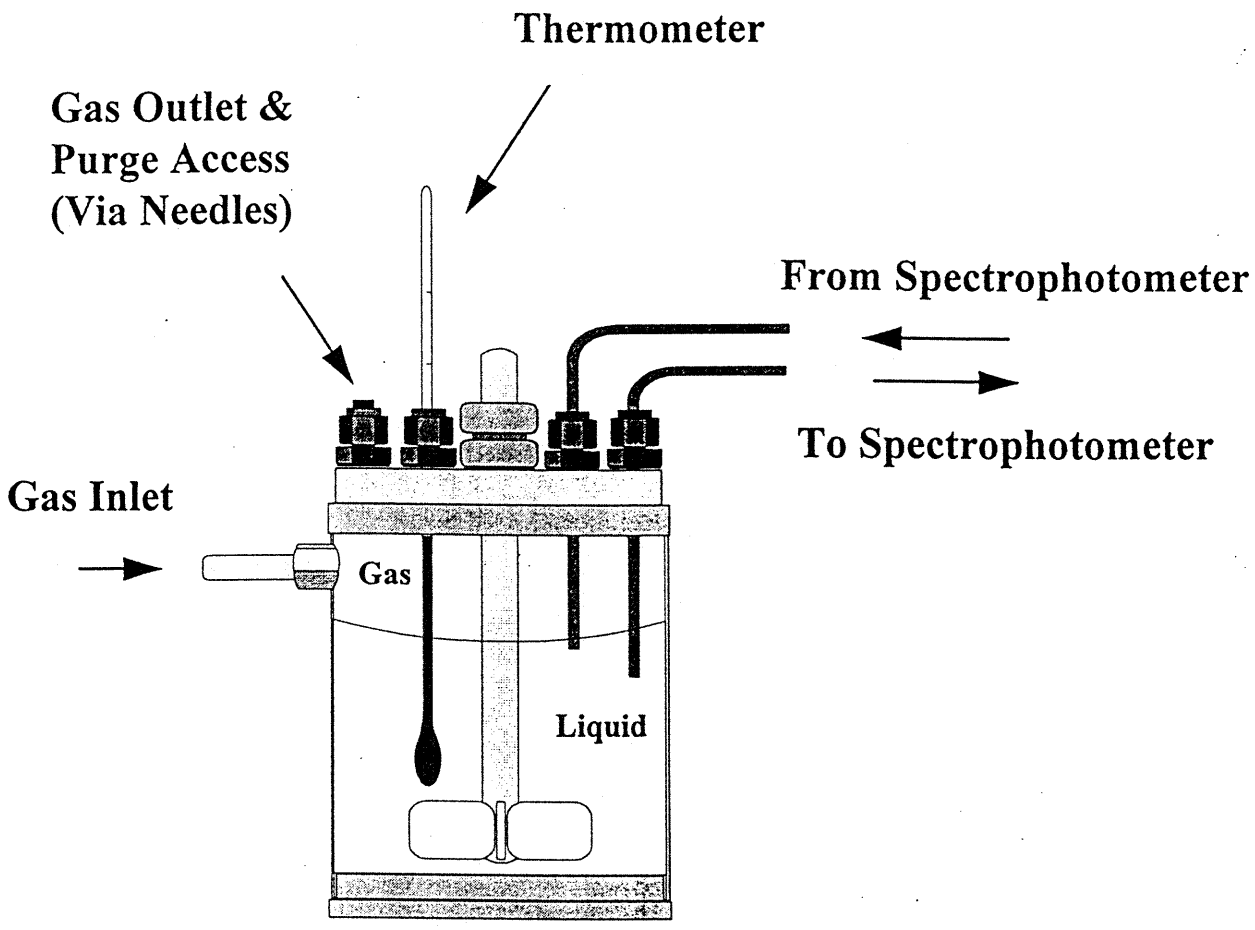
Comparison of the Rate Constants Relative to $N_2O_3$ Hydrolysis with Literature Values

| Rate Constant / Reaction | This Work | Literature Value (1) |
|---|---|---|
| $\mathbf{k_6/k_4}$ | $3.7 \times 10^4\ M^{-1}$ | $4.0 \times 10^4\ M^{-1}$ |
| $Mor + N_2O_3 \longrightarrow NMor + NO_2^- + H^+$ | | |
| $\mathbf{k_{10}^{Pi}/k_4}$ | $4.0 \times 10^2\ M^{-1}$ | $4.0 \times 10^2\ M^{-1}$ |
| $P_i^- + N_2O_3 \longrightarrow P_iNO + NO_2^-$ | | |
| $\mathbf{k_{10}^{HCO3-}/k_4}$ | $9.3 \times 10^2\ M^{-1}$ | Not Previously Reported |
| $HCO_3^- + N_2O_3 \longrightarrow HCO_3NO + NO_2^-$ | | |

## Figure Legends:

**Figure 1.** Schematic of the apparatus used in the morpholine N-nitrosation reactions. The reactor was a modified 200 ml stirred ultrafiltration cell with a flow loop connected to a spectrophotometer for continuous monitoring of N-nitrosomorpholine (NMor) and nitrite ($NO_2^-$) concentrations.

**Figure 2.** Effect of added 0.015 M phosphate (□), 0.04 M phosphate (■) and 0.04 M bicarbonate (×) on N-nitrosomorpholine (NMor) formation in the presence of 0.01 M phosphate (●). The unprotonated morpholine concentration ([Mor $^0$]) remained nearly constant during each reaction. The mean value is shown for reaction times ranging from 3 to 30 minutes.


Thermometer
Gas Outlet &
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(Via Needles)
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To Spectrophotometer
Gas Inlet
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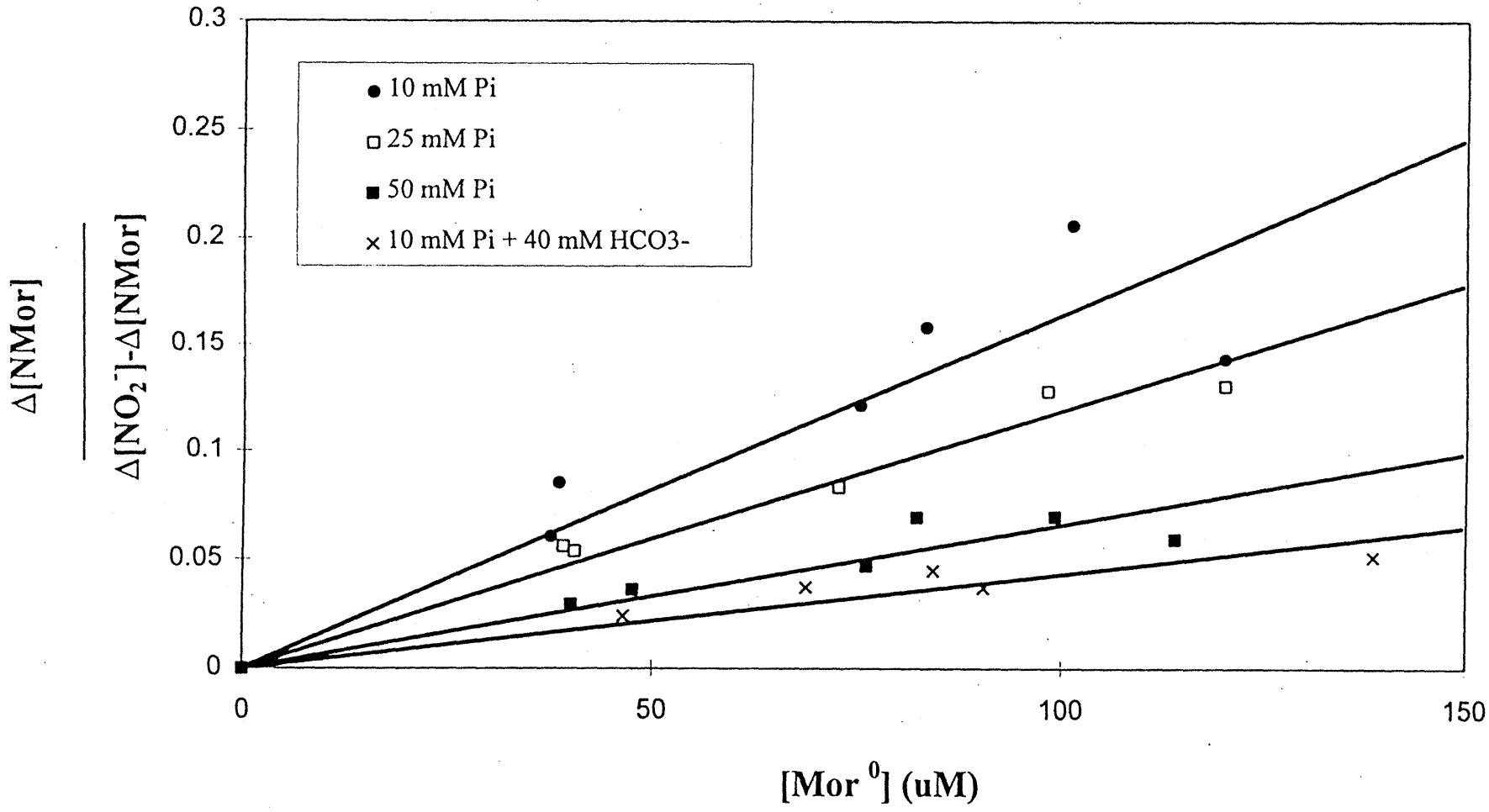
0.3
0.25
0.2
0.15
0.1
0.05
0
0
50
100
150
10 mM Pi
25 mM Pi
50 mM Pi
10 mM Pi + 40 mM HCO3-
Δ[NMor] / Δ[NO2-]-Δ[NMor]
[Mor 0] (uM)

# 3. Nitric Oxide-Induced Deamination of Cytosine & Guanine in Deoxynucleosides & Oligonucleotides

## Abstract:

The autoxidation of nitric oxide ($NO^{\cdot}$) forms the nitrosating agent $N_2O_3$ which can directly damage DNA by nitrosation of primary amines on DNA bases leading to deamination. Within the G:C base pair, deamination results in xanthine and uracil formation, respectively. To determine the effect of DNA structure on guanine and cytosine deamination, the $NO^{\cdot}$-induced deamination rate constants for deoxynucleosides, single and double stranded oligonucleotides and a G-quartet oligonucleotide were investigated. First, a reactor was used to deliver $NO^{\cdot}$ to a 2'-deoxyguanosine/morpholine solution for determination of the 2'-deoxyguanosine deamination rate constant by comparison to the known morpholine nitrosation rate constant. Next, the other deamination rate constants were determined using a silastic membrane to deliver $NO^{\cdot}$ at a rate of ~10-20 nmol/ml/min for 60 minutes yielding a final concentration of ~600-1200 μM $NO_2^-$. Treated samples included: 1) 2'-deoxycytidine & 2'-deoxyguanosine, 2) the single stranded oligomer AACCCCAA & 2'-deoxyguanosine, 3) the single stranded oligomer TGTGTGTG & 2'-deoxycytidine, 4) the G-quartet oligomer TTGGGGTT & 2'-deoxycytidine, and 5) the double stranded oligomer CGCGCGCGCGCG & 2'-deoxycytidine. GC/MS analysis revealed formation of nanomolar levels of deamination products from millimolar concentrations of deoxynucleoside and oligomer. As a result, deamination rate constants for cytosine and guanine in all types of DNA were lower than the morpholine nitrosation rate constant by a factor of ~$10^3$-$10^4$. Xanthine formation was twice that of uracil formation which may have important consequences for mechanisms of $NO^{\cdot}$-induced mutations. Single stranded oligomers were five times more reactive toward $N_2O_3$ than deoxynucleosides. Interestingly, the double stranded oligomer was ten fold less reactive than single stranded oligomers suggesting that Watson-Crick base pair formation protects DNA from deamination. The G-quartet structure also protects against $N_2O_3$ presumably due to hydrogen bonding. Therefore, DNA structure is an important consideration in determining the reactivity of DNA bases with $NO^{\cdot}$-derived species.

## Introduction:

Nitric oxide ($NO^{\cdot}$) is an important physiological messenger that is involved in many processes *in vivo* including inhibition of platelet aggregation, blood vessel relaxation, and neurotransmission (1). Along with the many essential roles of $NO^{\cdot}$ *in vivo*, it can be involved in reactions that may result in cytotoxic or mutagenic events by a number of possible mechanisms. The autoxidation of nitric oxide forms the nitrosating agent nitrous anhydride, $N_2O_3$, which can react with amines, thiols and other available nucleophiles. $N_2O_3$ can damage DNA directly by nitrosation of the primary amine functionalities on DNA bases leading to deamination. Cytosine, adenine, guanine, and 5-methylcytosine can be deaminated resulting in uracil, hypoxanthine, xanthine, and thymine, respectively.

DNA base deamination can occur spontaneously or through reactive nitrogen oxide species such as $N_2O_3$ formed from both nitrous acid and nitric oxide. Pyrimidine bases in DNA are more susceptible to spontaneous hydrolysis than purine constituents (2,3); however, purine constituents were found to be more easily deaminated by nitrous acid (4). Deamination of DNA bases can lead to mutagenesis through misincorporation by DNA polymerase, misrepair or no repair of the resulting deamination products. The types of mutations that potentially arise from deamination of DNA bases are summarized in Table 1. Given the reports of nitric oxide-induced toxicity (5) and the observation of deamination products in $NO^{\cdot}$-treated cells and DNA (5,6), it is believed that deamination may play a key role in nitric oxide-induced mutagenesis. The G:C → A:T mutation is the predominant mutation observed upon $NO^{\cdot}$ treatment (6,7) and is important because it has been observed in numerous human diseases including hemophilia, retinoblastoma, familial Alzheimer's disease and colon cancer (8-11). This mutation could arise from either the deamination of guanine or cytosine; therefore, our main interest was to determine the rate constants for guanine and cytosine deamination. The deaminations of adenine and 5-methylcytosine were not included in this study. At this point, the rates of nitric oxide-induced deamination of DNA bases in different environments is not known.

Therefore, in order to reveal the effects of base pairing and helix structure on nitrosation chemistry, this work has investigated the rates of guanine and cytosine deamination when present as components of 2'-deoxynucleosides, single and double stranded oligonucleotides and a G-quartet oligonucleotide. G-quartet structures, a tetraplex of four parallel strands wherein each guanine donates and accepts two hydrogen bonds, have been demonstrated to form among the tandem repeats of G-rich sequences in telomeric DNA (12). This structure provides an additional polymer containing hydrogen bonding for the study of the reactivity of $N_2O_3$ with different DNA structures.

Two different systems were used for nitric oxide delivery including a reactor and a silastic membrane system. The silastic membrane delivery system was used for most of the experiments to maintain a steady state of NO· and achieve a very low dose rate (~10-20 nmol/ml/min). This system works ideally for compounds whose nitrosation rate constants are close to one another such as the deoxynucleosides and oligomers. However, in order to determine the actual rate constant for the 2'-deoxyguanosine/$N_2O_3$ reaction, a reactor developed by Lewis *et al.* was used to deliver nitric oxide to a deoxygenated solution of 2'-deoxyguanosine and morpholine. Subsequent oxygen introduction results in $N_2O_3$ formation. In this reactor, lower amounts of NO· can be delivered resulting in a very steady pH and therefore constant free morpholine concentrations. The silastic system is not amenable to compounds such as morpholine because the higher amounts of NO· result in a slight pH drop and subsequent unsteady free morpholine concentrations. It is not possible to increase the buffer concentrations to maintain the pH due to $N_2O_3$ scavenging effects demonstrated by several buffer anions (13,14). For these reasons, the reactor was used for the morpholine/2'-dGuo reaction. The rate constant for the morpholine/$N_2O_3$ reaction relative to $N_2O_3$ hydrolysis is known ($4.0 \times 10^4$ $M^{-1}$); therefore, the rate constant for the 2'-deoxyguanosine/$N_2O_3$ reaction relative to $N_2O_3$ hydrolysis can be found by comparing the levels of its nitrosation product (xanthine) and *N*-nitrosomorpholine formed upon exposure of a mixture of the two compounds to NO· using the competitive kinetics approach developed by Keshive *et al.* (15). Using a value of $k_4 = 1600$ $s^{-1}$ (14), the rate constant for the 2'-dGuo/$N_2O_3$ reaction ($k_7^G$) can then be calculated.

The rate constants for reaction of $N_2O_3$ with 2'-deoxycytidine, a G-quartet oligonucleotide, and single & double stranded oligonucleotides containing guanine and cytosine were based on the earlier 2'-deoxyguanosine rate constant using treatment mixtures where the rate constant for $N_2O_3$ reaction with one of the components was known. Using these rate constants, it is possible to determine whether guanine or cytosine is deaminated faster within a G:C base pair. In addition, conclusions can be made regarding the effects of base pairing on deamination chemistry which will contribute to an understanding of NO·-induced modifications to DNA.

## Materials and Methods:

**Materials.** 2'-Deoxycytidine and 2'-deoxyguanosine were purchased from Sigma Chemical Co. (St. Louis, MO) and morpholine was purchased from Aldrich Chemical Co. (Milwaukee, WI). The synthetic oligonucleotides AACCCCAA, TTGGGGTT, TGTGTGTG, and CGCGCGCGCGCG were obtained from the MIT Biopolymers Lab. Phosphate buffer (0.01 M) at pH 7.4 was prepared with $K_2HPO_4$ and $KH_2PO_4$ using double-distilled water. 100% nitric oxide and 10% nitric oxide in argon (Matheson, Gloucester, MA) were passed through a column of 4-8 mesh soda lime to remove $NO_x$ impurities. Silastic tubing (0.025 in ID × 0.047 in OD) was purchased from Dow Corning Corp. (Midland, MI). Sep-Pak tC18 cartridges were obtained from Waters (Bedford, MA). [1,3-$^{15}N_2$]-Xanthine and [1,3-$^{15}N_2$]-uracil were obtained from Cambridge Isotope Laboratories (Cambridge, MA). Silylation grade acetonitrile, pyridine, and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MT-BSTFA) were purchased from Pierce Chemical Co. (Rockford, IL).

**Reactor for NO· Treatment of the 2'-Deoxyguanosine and Morpholine Solution.** A 200 mL reactor was used as previously described (15) with a few modifications. First, the total amount of NO· delivered was ~80 μM in these experiments as opposed to ~30 μM in previous work. Second, the flow loop to the spectrophotometer was disconnected because UV detection cannot be used to simultaneously monitor xanthine and *N*-nitrosomorpholine product concentrations. Because UV detection could not be used, samples were withdrawn from the reactor immediately before $O_2$ addition and 30 minutes after $O_2$ addition (i.e. pre-reaction and post-reaction samples). Using a gas tight syringe previously evacuated with argon, 600 μL of the reaction mixture was withdrawn and added to 400 μL of a 0.15 M azide solution to quench any $N_2O_3$ present. Due to the fact that there is dissolved nitric oxide in the pre-reaction sample, high concentrations of azide are needed to quench all of the $N_2O_3$ so it does not react with morpholine or 2'-deoxyguanosine. Complete $N_2O_3$ scavenging was confirmed by omitting morpholine

from the reaction mixture but including it in the azide solution and determining that there was no *N*-nitrosomorpholine formation. Approximately 80 μM NO· was delivered to a solution of 3 mM 2'-deoxyguanosine and 0.5 - 1.5 mM morpholine in 10 mM potassium phosphate buffer at pH 7.4. The resulting levels of xanthine and *N*-nitrosomorpholine were quantitated by GC/MS as described below.

**Sample Preparation and GC/MS - *N*-Nitrosomorpholine.** A portion (200 μL) of the pre-reaction and post-reaction samples was withdrawn and 50 μL of a 48.4 μM nitrobenzene solution was added for use as an internal standard. The aqueous sample was extracted with methylene chloride (300 μL) and the organic layer analyzed for *N*-nitrosomorpholine and nitrobenzene using a Supelcowax column obtained from Supelco (Bellefonte, PA). Analysis was performed on an HP 5989 GC/MS in the electron ionization mode. Quantitation was done using a standard curve.

**Sample Preparation and GC/MS - Xanthine and Uracil.** A portion (100 μL) of the pre-reaction and post-reaction samples was withdrawn and 50 μL of a 100 pg/ μL [1,3-$^{15}N_2$]- xanthine and 50 μL of a 100 pg/μL[1,3-$^{15}N_2$]-uracil were added as internal standards. Acid hydrolysis was performed in Reacti-Vials using 500 μL 60% formic acid at 100°C for one hour. Samples were then dried in a Speed Vac. Sep-Pak tC18 cartridges were used and the methanol eluant was dried completely in a Speed Vac. Samples were derivatized in a Reacti-Vial with 15 μL acetonitrile, 10 μL pyridine, and 25 μL MT-BSTFA at 130°C for 30 minutes and analyzed on a Hewlett Packard HP-5 column.

**Silastic Membrane Delivery System for NO· Treatment of the 2'-Deoxynucleoside and Oligonucleotide Solutions.** The oligonucleotides studied are shown in Table 2. The mixtures treated were: 1) 1mM 2'-dGuo & 1mM 2'-dCyd, 2) 0.25 mM AACCCCAA & 1 mM 2'-dGuo, 3) 0.25 mM TGTGTGTG & 1 mM 2'-dCyd, 4) 0.25 mM TTGGGGTT & 1 mM 2'-dCyd, and 5) 0.18 mM CGCGCGCGCGCG and 1 mM 2'-dCyd. All NO· treatments were performed in 10 mM potassium phosphate buffer at pH 7.4. After passing through a column of 4-8 mesh soda lime, NO· was administered as a mixture of

10% NO· in argon using a Silastic membrane system shown in Figure 1 and as described by Tamir *et al.* (16) with minor modifications. For sample #1, a 7 mL volume was treated with 10 cm Silastic for 1 hour. For samples #2-5, a volume of 1.5 mL was treated with 2 cm Silastic for 1 hour. All solutions were stirred to minimize the boundary layer at the polymer-liquid interface. The total amount of NO· actually delivered was measured at the end of each experiment as total nitrite (17). In all experiments, the nitric oxide delivery rate was ~10-20 nmol/mL/min resulting in a final $NO_2^-$ concentration of ~600-1200 μM. Samples were analyzed for xanthine and uracil levels using GC/MS as described above. An additional step was necessary for the double stranded oligomer involving separation of the oligomer from the deoxynucleoside using Millipore Ultrafree-MC 2,000 NMWL Filter Units. The oligomer is retained by the filter and recovered in the retentate while the deoxynucleoside is found in the filtrate. Separation was confirmed in trial experiments with each component individually.

**Kinetic Model and Reaction Scheme.** In previous experiments, Lewis *et al.* (14) showed that the principal nitrosating agent in the NO· oxidation pathway at physiological pH is $N_2O_3$ which leads primarily to $NO_2^-$ as summarized in Equations 1-3.

$$2NO + O_2 \xrightarrow{k_1} 2NO_2 \qquad \text{(Eq. 1)}$$

$$NO + NO_2 \underset{k_3}{\overset{k_2}{\rightleftharpoons}} N_2O_3 \qquad \text{(Eq. 2)}$$

$$N_2O_3 + H_2O \underset{k_5}{\overset{k_4}{\rightleftharpoons}} 2HNO_2 \rightleftharpoons 2NO_2^- + 2H^+ \qquad \text{(Eq. 3)}$$

$N_2O_3$ can also react with phosphate, chloride, and bicarbonate (13,14) which has the effect of enhancing the hydrolysis of $N_2O_3$ represented otherwise by equation 3. However, using this competitive kinetics approach, these effects do not need to be

included in the kinetic analysis (15). Morpholine nitrosation by $N_2O_3$ is shown in Equation 4.

$$N_2O_3 + Mor \xrightarrow{k_6} NMor + NO_2^- + H^+ \qquad \text{(Eq. 4)}$$

**Determination of the 2'-dGuo/$N_2O_3$ Rate Constant ($k_7^G$).** In the above reaction scheme, all reaction rate constants are known. The first unknown rate constant to be determined is for the 2'-dGuo/$N_2O_3$ reaction shown below:

$$N_2O_3 + G \xrightarrow{k_7^G} X + NO_2^- + H^+ \qquad \text{(Eq. 5)}$$

The complete kinetic analysis for estimating the unknown rate constant $k_7$ can be found in reference 15. Using that analysis, the rates of formation of NMor and dX are

$$\frac{d[NMor]}{dt} = k_6[Mor^0][N_2O_3] \qquad \text{(Eq. 6)}$$

$$\frac{d[X]}{dt} = k_7^G[G][N_2O_3] \qquad \text{(Eq. 7)}$$

Combining equations 6 and 7, we obtain:

$$\frac{d[X]}{d[NMor]} = \frac{k_7^G[G]}{k_6[Mor^0]} \qquad \text{(Eq. 8)}$$

where $[Mor^0]$ is the concentration of unprotonated morpholine available for nitrosation. Integration of equation 8, with the assumption that [G] and $[Mor^0]$ are essentially constant during the reaction, gives

$$k_7^G = k_6 \frac{\Delta[X][Mor^0]}{\Delta[NMor][G]} \qquad \text{(Eq. 9)}$$

where Δ[X] and Δ[NMor] represent the changes in the respective concentrations during the reaction (i.e. post-reaction amounts minus pre-reaction levels). Rearrangement of equation 9 gives

$$\Delta[X][Mor^0] = \frac{k_7^G}{k_6} \Delta[NMor][G] \qquad \text{(Eq. 10).}$$

The rate constant for the 2'-dGuo/$N_2O_3$ reaction ($k_7^G$) was determined from the slope of the plot of $\Delta[X][Mor^0]$ vs. $\Delta[NMor][G]$, and the previously reported value for $k_6$ ($6.4 \times 10^7$ $M^{-1}$ $s^{-1}$) (14).

**Determination of the 2'-dCyd/$N_2O_3$ Rate Constant ($k_7^C$).** The rate constant for the reaction of $N_2O_3$ with 2'-dCyd was determined using the rate constant for the 2'-dGuo/$N_2O_3$ reaction found above ($k_7^G$), the amounts of xanthine and uracil formed during the 2'-dCyd/2'-dGuo treatment, and the following equation which arises from the same theory as Equation 9 above:

$$k_7^C = k_7^G \frac{\Delta[U][G]}{\Delta[X][C]} \qquad \text{(Eq. 11)}$$

**Determination of the Rate Constant for Reaction of $N_2O_3$ with Cytosine and Guanine in Single Stranded Oligonucleotides and a G-Quartet Oligonucleotide ($k_7^{C(oligo)}$ & $k_7^{G(oligo)}$).** The rate constants for the reaction of $N_2O_3$ with single stranded oligomers and the G-quartet oligomer were determined using the previously determined rate constants for the 2'-dGuo/$N_2O_3$ reaction & 2'-dCyd/$N_2O_3$ reaction, the amounts of xanthine and uracil formed during the oligomer/deoxynucleoside reactions, and the following equations:

$$k_7^{C(oligo)} = k_7^{G} \frac{\Delta[U][G]}{\Delta[X][C\ (oligo)]} \qquad \text{(Eq. 12)}$$

and,

$$k_7^{G(oligo)} = k_7^{C} \frac{\Delta[X][C]}{\Delta[U][G\ (oligo)]} \qquad \text{(Eq. 13)}$$

**Determination of the Rate Constants for Reaction of $N_2O_3$ with Cytosine and Guanine in a Double Stranded Oligonucleotide ($k_7^{C(oligo)}$ & $k_7^{G(oligo)}$).** The rate constants for the reaction of $N_2O_3$ with cytosine and guanine in the double stranded oligomer were determined using the amounts of xanthine and uracil formed from the oligomer, the amount of uracil arising from 2'-dCyd upon treatment of the double stranded oligomer/2'-dCyd mixture, and the following equations:

$$k_7^{C(oligo)} = k_7^{C} \frac{\Delta[U\ (oligo)][C]}{\Delta[U][C\ (oligo)]} \qquad \text{(Eq. 14)}$$

and,

$$k_7^{G(oligo)} = k_7^{C} \frac{\Delta[X\ (oligo)][C]}{\Delta[U][G\ (oligo)]} \qquad \text{(Eq. 15)}$$

## Results:

**Morpholine Concentration and pH.** The unprotonated form of morpholine is the substrate for nitrosation and is thus the most important form of morpholine for these experiments. Denoting total morpholine as Mor and the unprotonated form as $Mor^0$, the respective concentrations are related by:

$$[Mor^0] = \frac{[Mor]}{1 + 10^{pK-pH}} \qquad \text{(Eq. 16)}$$

where the pK at 25°C is 8.5 for morpholine. The amount of morpholine available for nitrosation is 7.4% of the total morpholine concentration at pH 7.4. The pH was nearly constant during a given reaction; therefore, the concentration of unprotonated morpholine did not change significantly. In all experiments, some nitrosation products were present in the solution prior to introduction of $O_2$ due to a small air leak that could not be eliminated.

**Determination of the 2'-dGuo/$N_2O_3$ Rate Constant ($k_7^G$).** From the plot of Equation 10, the 2'-dGuo/$N_2O_3$ rate constant is calculated to be $k_7^G = (2.2 \pm 0.2) \times 10^4\ M^{-1} s^{-1}$. Results are summarized in Table 3. In one experiment, morpholine was not present in the reaction mixture but was included in the azide mixture when samples were withdrawn from the reactor to ensure that there was no nitrosation occurring from the nitric oxide present in the pre-reaction sample. No detectable *N*-nitrosomorpholine was observed indicating that all of the $N_2O_3$ present is efficiently scavenged by the azide. Therefore, the levels of xanthine and *N*-nitrosomorpholine reported in the pre-reaction samples are accurate.

**Determination of the 2'-dCyd/$N_2O_3$ Rate Constant ($k_7^C$).** As shown in Table 3, the rate constant for the 2'-dCyd/$N_2O_3$ reaction was determined to be $k_7^C = (1.1 \pm 0.2) \times 10^4\ M^{-1} s^{-1}$ based on the rate constant for the 2'-dGuo/$N_2O_3$ reaction above and the amounts of

xanthine and uracil formed upon treatment of the 2'-dCyd/2'-dGuo mixture using Equation 11.

**Determination of the Rate Constants for Reaction of $N_2O_3$ with Cytosine and Guanine in Single Stranded Oligonucleotides & a G-Quartet Oligonucleotide ($k_7^{C(oligo)}$ & $k_7^{G(oligo)}$).** The rate constant for reaction of $N_2O_3$ with cytosine in the single stranded oligonucleotide AACCCCAA was found to be $k_7^{C(oligo)} = (5.6 \pm 1.1) \times 10^4\ M^{-1}\ s^{-1}$ using Equation 12, the 2'-dGuo/$N_2O_3$ rate constant, and the xanthine and uracil amounts found upon 2'-dGuo/AACCCCAA oligo treatment. The rate constant for reaction of $N_2O_3$ with guanine in the single stranded oligonucleotide TGTGTGTG was found to be $(9.8 \pm 1.3)\ M^{-1}\ s^{-1}$ using Equation 13, the 2'-dCyd/$N_2O_3$ rate constant, and the xanthine and uracil amounts found upon 2'-dCyd/TGTGTGTG oligo treatment. Similarly, the rate constant for the reaction of $N_2O_3$ with guanine in the G-quartet oligonucleotide TTGGGGTT was found to be $k_7^{G(oligo)} = (7.4 \pm 1.1) \times 10^4\ M^{-1}\ s^{-1}$.

**Determination of the Rate Constants for Reaction of $N_2O_3$ with Cytosine and Guanine In A Double Stranded Oligonucleotide ($k_7^{C(oligo)}$ & $k_7^{G(oligo)}$).** As seen in Table 3, the rate constant for the reaction of cytosine in the double stranded oligonucleotide CGCGCGCGCGCG with $N_2O_3$ was found to be $k_7^{C(oligo)} = (5.6 \pm 1.0) \times 10^3\ M^{-1}\ s^{-1}$ using Equation 14, the amounts of uracil arising from the oligo, amounts of uracil from 2'-dCyd and the previously determined 2'-dCyd/$N_2O_3$ rate. Similarly, the rate constant for the reaction of $N_2O_3$ with guanine in the double stranded oligonucleotide was calculated to be $k_7^{G(oligo)} = (1.0 \pm 0.1) \times 10^4\ M^{-1}\ s^{-1}$ using Equation 15.

## Discussion:

Mutations may ultimately result from deamination of any of the bases shown in Table 1. The G:C → A:T transition has frequently been observed as the primary type of mutation upon NO· treatment which could result from the deamination of guanine, cytosine, or 5-methylcytosine (18-20). Focusing on the G:C base pair, this study aims to determine whether guanine or cytosine is deaminated faster to give rise to the G:C → A:T mutation. The possible mutagenic effects of cytosine and guanine deamination forming uracil and xanthine, respectively, are discussed below.

Deamination of cytosine will lead to a G:C → A:T transition mutation upon replication due to pairing of uracil with adenine (18,19). Alternatively, the uracil may be removed by the enzyme DNA uracil glycosylase before replication proceeds (21). Uracil glycosylase excises uracil from both single and double-stranded DNA with preference for single stranded DNA (22). The importance of cytosine deamination is emphasized by the fact that organisms that lack this enzyme have an increased spontaneous mutation rate and more G:C → A:T transitions (23).

Deamination of guanine to xanthine will lead to a G:C → A:T transition mutation upon replication because xanthine is believed to base pair with thymine (20,24). There is no known repair mechanism for xanthine. Deoxyxanthosine has been noted to have high acid lability of its N-glycoside bond (25,26); therefore, one possible fate of xanthine is depurination resulting in an abasic site. Many organisms will replicate past such a lesion by incorporating the "A rule" involving insertion of an adenine opposite the abasic site (27). The ultimate result of such an event would be a G:C → T:A transversion. The abasic site may also be cleaved by an endonuclease or by base catalysis to yield a DNA single strand break which may be toxic to the cell (28).

Indeed, the formation of deamination products and single strand breaks have been observed in *S. typhimurium* and TK6 cells treated with NO· (5,6). Also, upon treatment of calf thymus DNA, significant deamination of cytosine was observed with high doses of NO· - accumulated amounts of 0.1-1 mole of NO· per liter (6) delivered by syringe. This method of bubbling NO· into solution is extremely inefficient due to significant loss

of NO· into the gas phase. The silastic membrane delivery system used in this study results in a slow, steady delivery of low levels of nitric oxide approaching the delivery rates of stimulated cells such as macrophages. Rates of delivery in these experiments are ~10-20 nmol/ml/min resulting in a final concentration of ~600-1200 μM $NO_2^-$. Using this system, Tamir *et al* (16) observed increased cytotoxicity to TK6 and *S. typhimurium* cells as compared to bubbling NO· into the solution due to the fact that the effective nitric oxide concentration in solution is higher because NO· is not released into the gas phase as in syringe delivery.

The rates and products of the reactions of purine and pyrimidine bases, nucleosides and nucleic acids with nitrous acid have been previously established by the elegant studies of Shapiro and Pohl (26). It is clear from these early studies that relative rates of nitrous acid-induced deamination depend on both the individual base and the nucleic acid structure. Due to the many reports of nitric oxide-induced toxicity and the observation of deamination products in the DNA of NO·-treated cells (5,6), it is likely that deamination also plays a key role in mutagenesis induced by nitric oxide (7). This is the motivation behind the current study to determine the rates of reaction of $N_2O_3$ with guanine and cytosine in different environments. In their work on the rates of reaction of DNA with $N_2O_3$, Shapiro and Pohl suggested that in future studies on the chemical modification of nucleic acids, the reaction rates should be discussed with reference to the rates exhibited by suitable model compounds and that rate constants be determined. They also suggest that variables such as pH and the concentration of reactive species be as closely controlled as possible. All of these conditions have been taken into consideration here where the pH and the concentration of reactive species is strictly controlled. In addition, the rates are expressed in relative terms and were determined by comparison with the model compound morpholine. The rate constant for the 2'-deoxyguanosine/$N_2O_3$ reaction was determined to be $(2.2 \pm 0.2) \times 10^4$ $M^{-1}$ $s^{-1}$. This rate constant and all rate constants for cytosine and guanine deamination in oligonucleotides are several orders of magnitude lower than that for morpholine nitrosation ($6.4 \times 10^7$ $M^{-1}$ $s^{-1}$) demonstrating that deoxynucleosides and oligomers react with $N_2O_3$ much slower than a typical amine (14).

The single and double stranded oligonucleotides and the G-quartet oligonucleotide shown in Table 2 were treated with NO· to reveal the effects of base pairing and helix structure on nitrosation chemistry. The rate constants determined here for the reaction of guanine and cytosine present in each type of DNA are shown in Table 3. As seen in Table 3, single stranded oligomers are approximately five times more reactive than deoxynucleosides. Nguyen *et al.* observed a similar effect where the yields of deamination products from single stranded nucleic acids (RNA and heat denatured calf thymus DNA) were higher than those from free bases upon nitric oxide treatment (5). Single stranded oligomers may react differently than monomers or double stranded nucleotides due to folding or stacking interactions. The increased rate for single stranded oligonucleotides may also occur as a result of nearest neighbor effects whereby the nitrosyl group could be passed to neighboring groups within the single stranded oligo.

Results also demonstrate that guanine is more reactive than cytosine in DNA as shown in Table 4. This is in contrast to the thermal hydrolytic deamination reactions in which cytosine>adenine>>guanine (2,3). In several types of RNA and calf thymus DNA, the relative reactivity of guanine to cytosine with nitrous acid has been reported to be approximately 2:1 which is in agreement with the results of this study (26). Therefore, within a G:C base pair, guanine is deaminated faster than cytosine. Table 4 demonstrates that the relative reactivity of guanine and cytosine remains the same regardless of their environment. The only exception is the comparison between the single stranded AACCCCAA oligo and the G-quartet TTGGGGTT oligo.

The reduced reactivity of the G-quartet oligonucleotide TTGGGGTT as compared to the single stranded oligonucleotide TGTGTGTG suggests that the hydrogen bonding upon quadruplex formation decreases the reactivity of the guanine N2 amino group toward $N_2O_3$. The reactivity of the G-quartet oligonucleotide is still significantly greater than the double stranded oligonucleotide perhaps due to the accessibility of the guanine N2 amino group in the quadruplex as opposed to its internal position within the double helix of the double stranded oligonucleotide. The G-quartet structure has been shown to form in telomeric DNA and is perhaps involved in chromosomal stabilization. Tetraplex formation is extremely dependent upon the particular buffer cation. The four strands

form a quadruplex centered around a cation. Potassium ion is the most preferred ion for tetraplex formation and is the buffer cation used in experiments here. Other cations such as sodium promote tetraplex formation to a lesser extent whereas ions such as lithium do not allow for tetraplex formation (12). For a detailed review of G-quartets, see reference 12. The deamination of guanine in G-quartet structures *in vivo* may not be of extreme importance but the study of deamination of this short oligomer provides information regarding the reactivity of $N_2O_3$ with different DNA structures. The important conclusion from the G-quartet oligonucleotide data here is that its reactivity is reduced as compared to the single stranded oligonucleotide of the same composition but different sequence indicating the importance of hydrogen bonding and secondary structure formation in determining the reactivity with $N_2O_3$.

Watson-Crick base pair formation also protects DNA from deamination as demonstrated here by the fact that double stranded oligomers are ten-fold less reactive with $N_2O_3$ than single stranded oligomers. This observed decreased reactivity of double stranded DNA is in agreement with previous reports on the study of spontaneous and heat-induced cytosine deamination (29-32) suggesting that genome integrity may be best preserved in double stranded DNA.

However, spontaneous cytosine deamination was reported to occur over 100 times faster in single than in double stranded DNA (29,31), whereas, in this work, NO·-induced deamination is ~10 times faster in single than in double stranded oligonucleotides. In the absence of other reports of the relative deamination of single and double stranded DNA upon NO· treatment, it is unknown whether single stranded DNA is 10-fold or 100-fold more reactive than double stranded DNA. A possible explanation for the observed difference may be that there is a significant amount of single stranded oligomer in equilibrium with the double stranded oligomer. Due to the fact that the single stranded oligomer is so much more reactive, it could be contributing to the observed reactivity of the double stranded oligomer.

If single stranded DNA is actually 100 times more susceptible to deamination than double stranded DNA, then ~10% single stranded character would significantly alter the observed reactivity of the double stranded oligomer. The result would be an observed

10-fold difference between single and double stranded DNA when it actually is a 100-fold difference. However, based on melting curve data, it is calculated that the fraction of single stranded oligomer in this buffer is <1%; therefore, single stranded oligomer could not be contributing much to the observed rate of reactivity of the double stranded oligomer in these experiments. In fact, 1% single stranded character could affect the observed double stranded reactivity by a factor of at most two.

Steric factors may be a key reason for the reduced reactivity of DNA bases within double stranded oligonucleotides. In addition, protonation of the cytosine N3 position has been reported to be important in deamination of cytosine in solution; however, this nitrogen cannot be protonated in double stranded DNA due to Watson-Crick base pairing (3,33). Therefore, steric factors and lack of protonation of the cytosine N3 position may both play a role in the lower rate of deamination of cytosine in double stranded DNA.

It has been reported that there is a correlation between DNA duplex melting and cytosine deamination indicating that deamination in double-stranded DNA is inversely related to duplex stability (31). Another study demonstrated that spontaneous deamination occurred selectively near AT-rich regions possibly due to the increased single-stranded character of this stretch of DNA. Based on this evidence, it has been proposed that double stranded DNA may deaminate through a single stranded intermediate (29). *In vivo*, DNA is present single-stranded during replication, transcription and breathing (34).

Our results demonstrate that the rate constants for the reaction of guanine and cytosine in any environment are several orders of magnitude lower than that for morpholine nitrosation indicating that the bases react with $N_2O_3$ much slower than a typical amine. In addition, the relative reactivity of guanine and cytosine is the same regardless of the environment - xanthine formation is always approximately double the amount of uracil formation which may have important consequences for mechanisms of NO·-induced mutations. Due to the faster deamination rate of guanine and the high levels of uracil glycosylase in cells, guanine deamination is more likely to be responsible for the observed G:C → A:T mutations. In agreement with previous reports (5,27,35,36), single stranded oligomers are more reactive than double stranded oligomers and

deoxynucleosides. The decreased reactivity of a double stranded oligomer and a G-quartet oligomer toward $N_2O_3$ as compared to single stranded oligomers suggests that hydrogen bonding and Watson-Crick base pair formation may protect DNA from deamination.

The question remains as to the reason for the enhanced reactivity of single stranded oligomers over deoxynucleosides. A possible explanation involves the previously reported proton-rich microenvironment at the surface of single and double stranded DNA molecules (37). Due to this proton field, a ΔpH of ~2 was reported for the pH difference between the DNA surface and the bulk solution (37). This proton field does not form in the presence of nucleosides or nucleotides. Therefore, in the experiments here, the pH at the surface of the single stranded and double stranded oligomer would therefore be ~ pH 5.4 which is nearing the range where $N_2O_3$ will form from nitrous acid. This proton field may therefore significantly enhance the rate of single stranded oligomer reaction due to higher local amounts of $N_2O_3$ at the DNA surface. The rate for double stranded oligomer is not enhanced even though this same proton field is present due to the protective effect of hydrogen bonding. Therefore, it is important to consider the DNA structure when determining its reactivity with nitric oxide-derived species.

**Acknowledgments:**

This work was supported by National Institutes of Health Grants CA09112 and ES04675.

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## Table 1:

Mutations That Potentially Arise From Deamination of DNA Bases:

| Conversion | Type of Mutation | Reference |
|---|---|---|
| 5-Methylcytosine → Thymine | G:C → A:T | (18) |
| Cytosine → Uracil | G:C → A:T | (19) |
| Guanine → Xanthine | G:C → A:T | (20) |
| Adenine → Hypoxanthine | A:T → G:C | (36) |
| Guanine → Xanthine → Apurinic Site | G:C → T:A | (27) |
| Adenine → Hypoxanthine → Apurinic Site | A:T → T:A | (27) |

## Table 2:

Oligonucleotides Used In This Study:

Single Stranded Oligonucleotides:

AACCCCAA

TGTGTGTG

Double Stranded Oligonucleotide:

CGCGCGCGCGCG

G-Quartet Oligonucleotide:

TTGGGGTT

## Table 3:

Rate constants for reaction of $N_2O_3$ with 2'-deoxynucleosides, single and double stranded oligomers & G-Quartet oligomers.

**2'-Deoxynucleosides**:

| | | | |
|---|---|---|---|
| 2'-dCyd | $k_7^C$ | = | $(1.1 \pm 0.2) \times 10^4\ M^{-1} s^{-1}$ |
| 2'-dGuo | $k_7^G$ | = | $(2.2 \pm 0.2) \times 10^4\ M^{-1} s^{-1}$ |

**Single Stranded Oligonucleotides**:

| | | | |
|---|---|---|---|
| AACCCCAA | $k_7^C$ | = | $(5.6 \pm 1.1) \times 10^4\ M^{-1} s^{-1}$ |
| TGTGTGTG | $k_7^G$ | = | $(9.8 \pm 1.3) \times 10^4\ M^{-1} s^{-1}$ |

**Double Stranded Oligonucleotide**:

| | | | |
|---|---|---|---|
| CGCGCGCGCGCG | $k_7^C$ | = | $(5.6 \pm 1.0) \times 10^3\ M^{-1} s^{-1}$ |
| | $k_7^G$ | = | $(1.0 \pm 0.1) \times 10^4\ M^{-1} s^{-1}$ |

**G-Quartet Oligonucleotide:**

| | | | |
|---|---|---|---|
| TTGGGGTT | $k_7^G$ | = | $(7.4 \pm 1.1) \times 10^4\ M^{-1} s^{-1}$ |

Mean ± Standard Deviation

## Table 4:

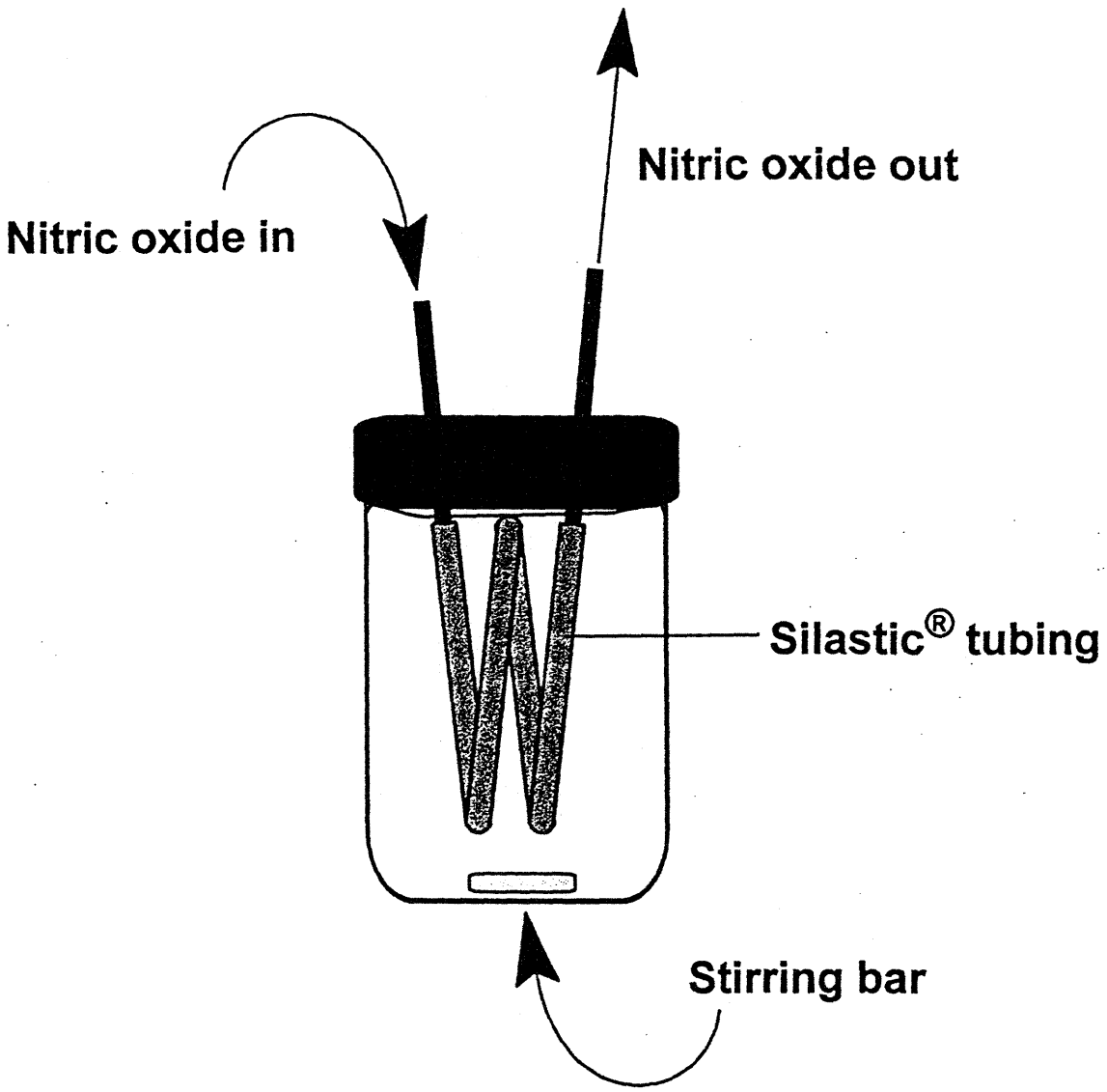
**Relative deamination rates of C & G in different contexts.**

| | Reactivity of G/C |
|---|---|
| Nucleosides:<br>2'-dCyd & 2'-dGuo | 2.0 ± 0.3 |
| Single Stranded Oligonucleotides<br>AACCCCAA & TGTGTGTG | 1.7 ± 0.3 |
| Double Stranded Oligonucleotide<br>CGCGCGCGCGCG | 1.8 ± 0.4 |
| G-Quartet Oligonucleotide &<br>Single Stranded Oligonucleotide<br>AACCCCAA & TTGGGGTT | 1.3 ± 0.3 |

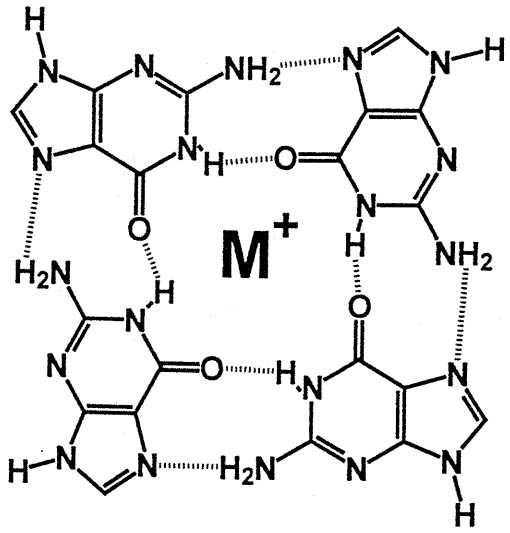
## Figure Legends:

**Figure 1: Nitric Oxide Membrane Delivery System.** Measured lengths of Silastic tubing were threaded onto hypodermic needles that pass through a rubber septum. Solutions were stirred to minimize the boundary layer at the polymer-liquid interface. 10% NO· in argon was then delivered for 1 hour resulting in a final $NO_2^-$ concentration of ~600-1200 μM delivered at ~10-20 nmol/ml/min.

**Figure 2: G-Quartet Structure.** In this schematic, each of the guanine moieties represented is a component of a different strand. Four different strands of DNA containing a series of guanines can form a stable tetraplex structure around a cation as shown here. Potassium ion is most efficient for forming a tetraplex whereas sodium promotes tetraplex formation to a lesser extent. G-quartet structures do not form in the presence of lithium. (Adapted from reference 12).


Nitric oxide out
Nitric oxide in
Silastic® tubing
Stirring bar

# G-Quartet Structure



# 4. Laser Detection of Nitric Oxide-Induced Interstrand Cross-Links in DNA

## Abstract

Along with its many essential roles *in vivo*, nitric oxide is capable of inducing a number of types of DNA damage. The DNA damaging effects of nitrous acid, an agent that acts through similar chemistry, have been extensively studied and the formation of interstrand cross-links has been observed. The potential for this interstrand cross-linking to occur through a nitric oxide mechanism is investigated here. Using a high performance liquid chromatography - laser induced fluorescence (HPLC-LIF) system, the amount of interstrand cross-link formed upon nitric oxide treatment of the 5'-fluorescein-labeled oligomer ATATCGATCGATAT was determined. This self-complementary sequence contains two 5'-CG sequences which are the preferred site for nitrous acid-induced cross-linking. Nitric oxide was delivered to an 0.5 mM oligomer solution at 15 nmol/ml/min to give a final nitrite concentration of 652 μM. The resulting concentration of xanthine in this sample was found to be (211 ± 39) nM using GC/MS and the amount of interstrand cross-link was (13.0 ± 2.5) nM. Therefore, upon nitric oxide treatment, the cross-link is found at approximately 6% of the amount of the deamination product xanthine. Using this system, detection of the cross-link is also possible for significantly lower doses of nitric oxide as demonstrated by treatment of the same oligomer with NO· at a rate of 18 nmol/mL/min for 7 minutes resulting in a final nitrite concentration of 126 μM. The concentration of interstrand cross-link was determined to be (3.6 ± 0.1) nM in this sample. Therefore, using the same dose rate, when the total nitric oxide concentration delivered drops by a factor of approximately 5, the concentration of cross-link drops by a factor of about 4 indicating a quasi-linear response. Through further method development, it will be possible to use even lower total doses and dose rates of nitric oxide. This method is also applicable to other DNA interstrand cross-links and DNA modifications where the HPLC retention time of the modified oligomer is significantly different from the that of the unmodified oligomer.

## Introduction

There are three important types of cross-links that have been demonstrated to form upon nitrous acid treatment including DNA-protein cross-links, DNA intrastrand cross-links, and DNA interstrand cross-links (1-3); however, there have been no reports of formation of these cross-links upon nitric oxide treatment. The focus of this work is DNA interstrand cross-links. Nitrous acid-treated DNA was discovered to contain interstrand cross-links in 1961 when Geidushek observed reversible denaturation of nitrous acid-treated DNA (4). This suggested that covalent bonds had formed linking the complementary strands so the strands are held in close proximity upon denaturation which allows for fast renaturation kinetics (4,5). A low number of cross-links is effective to yield reversible DNA (6). However, Geidushek did not investigate the actual structure of the cross-link.

In 1977, Shapiro *et al.* were the first to isolate candidate structures for the cross-link and tentatively determine the structures using nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS) (7). They actually found two different cross-links present - a dG-dG cross-link and a dG-dA cross-link. Later work has not shown the dG-dA structure to be of much importance (8). A dG-dA interstrand cross-link is not expected to form without significant disruption of the helix because the reactive centers are in opposite grooves of the DNA.

Further studies by Kirchner *et al.* showed the formation of the dG-dG interstrand cross-link upon nitrous acid treatment of oligomers (8,9). After enzymatic digestion of the oligomers, the resulting nucleoside was structurally characterized by NMR and Electrospray-MS (8). The nucleus of the cross-link was demonstrated to be a G-G interstrand cross-link with a connection through the N2 amino groups. This structure is not in agreement with the mechanism previously proposed by Shapiro in which an aldehyde-β-phosphodiester group resulting from depurination cross-links to an amino group on the opposite strand (7,10). The currently proposed mechanism of cross-link formation is diazotization of a dG residue followed by nucleophilic attack on C2 of that

guanine by the N2 exocyclic amino group of a neighboring guanine on the opposite strand and loss of a molecule of $N_2$ (7,9). In this case, the dG amino group acting as the nucleophile in the cross-link formation reaction can be viewed as analogous to the role of water in the deamination reactions.

This interstrand cross-linking is possible due to the close van der Waals contact between the N2 amino groups of neighboring guanines in the double helix. In the sequence 5'-CG, the two guanine N2 amino groups are approximately 3.2 angstroms apart as demonstrated by X-ray crystallography of the Dickerson dodecamer (11). In 1992, Kirchner *et al.* demonstrated that nitrous acid-induced cross-link formation is indeed sequence specific with 5'CG being preferred over 5'GC (8). More distortion is needed for cross-link formation in the sequence 5'GC because the distance between the N2 atoms of guanine is not easily bridgeable in this sequence and requires a 3 angstrom sliding of the base pairs to bring the reactive centers into contact. Based on molecular mechanics calculations, Kirchner *et al.* postulate that cross-linked DNA shows no significant bending of the helix axis and also that there will not be much difference between cross-linked and normal DNA other than a severe propeller twisting of the two linked dG residues (9). To date, no studies have been performed to determine the effect of this cross-link on cell viability. It may interfere with DNA synthesis or translation due to the inability of the strands to dissociate. The role of these lesions in NO·-associated cytotoxicity and mutagenesis has yet to be established (12); however, cross-links are considered to be toxic if not lethal to the cell (8).

The formation of this interstrand cross-link has been of considerable interest; however, due to the low levels of cross-linked DNA resulting from low doses of NO· treatment, detection and quantification are difficult. Therefore, the extremely sensitive and powerful high performance liquid chromatography - laser-induced fluorescence (HPLC-LIF) method was implemented. The detection limit of the HPLC-LIF system used is approximately one attomole of fluorescein itself and approximately one hundred attomoles of fluorescein-labeled DNA. Due to the ability to detect attomole levels of fluorescein-labeled DNA, a 5'-fluorescein labeled oligomer containing two 5'CG

sequences was used for this study and the resulting cross-linked oligomer was detected using an argon laser.

## Materials and Methods

**Materials.** The synthetic oligonucleotides ATATCGATCGATAT and $T_{22}$ were obtained from the MIT Biopolymers Lab. These oligomers contain a fluorescein label at the 5'-end through a thiourea linkage using the product 5'-fluorescein obtained from Glen Research (Sterling, VA). Purification was performed using reversed phase HPLC with a 100 mM ammonium acetate/acetonitrile gradient. After purification, the oligomers were desalted using a Sep-Pak tC18 cartridge (Waters, Bedford, MA). A mixture of 10% nitric oxide in argon (Matheson, Gloucester, MA) was passed through a column of 4-8 mesh soda lime to remove $NO_x$ impurities. Silastic tubing (0.025 in ID × 0.047 in OD) was purchased from Dow Corning Corp. (Midland, MI). Silylation grade acetonitrile, pyridine, and *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MT-BSTFA) were purchased from Pierce Chemical Co. (Rockford, IL).

**Silastic Membrane Delivery System for NO· Treatment.** The NO· treatment was performed in 10 mM potassium phosphate buffer at pH 7.4. NO· was administered to an 0.5 mM solution of ATATCGATCGATAT oligomer as a mixture of 10% NO· in argon using a Silastic membrane system as described by Tamir *et al.* with minor modifications (13). The higher dose and lower dose samples in a total volume of 1.5 mL were treated with 2 cm Silastic for 45 minutes and 7 minutes, respectively. The solutions were stirred to minimize the boundary layer at the polymer-liquid interface. The total amount of NO· actually delivered was measured at the end of the delivery as total nitrite (14).

**GC/MS for Xanthine Quantification.** A portion (100 μL) of the untreated and treated samples was withdrawn in triplicate and 50 μL of a 100 pg/μL [1,3-$^{15}N_2$]-xanthine was added to each as an internal standard. Acid hydrolysis was performed in Reacti-Vials using 500 μL 60% formic acid at 100°C for one hour. Samples were then dried in a Speed Vac. Sep-Pak tC18 cartridges were used and the methanol eluant was dried completely in a Speed Vac. Samples were derivatized in a Reacti-Vial with 15 μL

acetonitrile, 10 μL pyridine, and 25 μL MT-BSTFA at 130°C for 30 minutes and analyzed using a Hewlett Packard HP-5 column.

**Sample Purification for Cross-Link Analysis.** A preliminary purification is necessary to rid the sample of all single stranded oligomer that has not been covalently cross-linked. Injection of the original level of fluorescein onto the HPLC-LIF system would overwhelm the detector and cause a maximum signal to persist indefinitely. Preliminary purification was therefore performed using an analytical C18 column with an Eppendorf model CH-30 column heater and a model TC-50 controller set at 80 °C. The high temperature ensures that the oligomer will indeed be single stranded unless covalently cross-linked. The water/MeOH gradient used is as follows: 0% B for 10 minutes, 0 to 50% B from 10 to 30 minutes, 50% B to 90% B from 30 to 45 minutes. Early studies demonstrated that the 27.5 - 29.5 minute fraction contained the cross-link; therefore, this fraction was collected for each sample and dried using a concentrator with grade 5 helium and a hydrocarbon trap. The dried fraction is then re-dissolved in 20 μL water for laser analysis of cross-link amounts.

**Instrument.** An HPLC-LIF system shown schematically in Figure 1 was used consisting of a Hewlett Packard 1050 quaternary pump used at 0.4 ml/min equipped with an Accurate flow splitter which is used to deliver very low flow rates to the column - in this case - 40 ul/min. Analysis was performed using 8% MeOH isocratic delivery. The injector is a Rheodyne model 7520 which injects 1 μL of sample. The HPLC column used was a 300 μm ID Vydac C18 column with a column heater to maintain a constant 60°C temperature during runs. The argon laser is an Ion Laser Technology model 5425ASL-00 laser with a model 5400A-115-00 power supply. The laser output wavelength is adjustable from 457 to 514.5 nm; however, the main argon line at 488 nm is the most stable and efficient wavelength and is used here for the fluorescein labeled samples. The fluorescence at the desired wavelength is monitored by a Spex 0.25 meter monochromator using 1.25 mm entrance and exit slits with a Spex model CD2A compudrive controller. The photomultiplier tube (PMT) used in this system is a

Hamamatsu Corporation model R928 side-on PMT which is powered by a Bertan Associates power supply model 230. A lockin amplifier (SciTech Model 500MC) with an analog chopper is used to reduce noise from laser flicker and HPLC pump variations. The fluorescence beam is "chopped" with an optical chopper which has the effect of turning the beam on and off at a given frequency. The amplifier is then phased to only detect signal at this frequency.

The optical system consists of a 2 cm focal length lens to focus the laser beam onto the 250 μm ID fused silica capillary. A window of fused silica in the polyimide coating of the capillary was previously exposed using a flame. The HPLC eluent is passed through this capillary and the laser is focused onto the exposed window. The capillary is set vertically in a stable holder to prevent vibration. A 40X microscope objective is used to collect the photons of fluorescence. The laser beam and the collection optics are placed at right angles to minimize scattered light from the circular capillary. The focusing lens and the collection microscope are set on 3 micrometers allowing minute adjustments in the x, y, and z directions. A third lens with a focal length of 10 cm is used to focus the fluorescence from the microscope onto the entrance slit of the monochromator. This lens can be moved in the x and y directions to focus the image directly onto the monochromator entrance slit.

Filters and dichroic mirrors are used to reduce the effects of laser scatter. Two filters are used including a laser bandpass filter which is in effect a double-prism cube with a grating between the prisms. The grating allows for reflectance at 90 degrees of the 488 line while all other wavelengths are transmitted, absorbed by the coatings, or spatially filtered with an iris just before the focusing lens. This cube has a transmission efficiency of 90% of the 488 line and completely blocks off all other radiation within ~200 wavenumbers on either side. The second filter used is a holographic notch filter which blocks radiation at 488 nm (± 2 nm) which is placed just after the collection optics to prevent capillary Rayleigh scatter from entering the monochromator and interfering with the relatively close fluorescence signal. The signal is collected using a DAQ system and analysis is performed with a Gramms 386 program.

## Results:

**Sample Purification for Cross-Link Analysis.** Preliminary purification using C18 chromatography shows that the unmodified oligomer elutes in a broad peak in the fifteen minute range. The treated oligomer has a very similar peak; however, the peak becomes more broad and appears to have a number of component peaks underlying it presumably representing deaminated oligomers or oligomers that have had a strand break through NO·-chemistry. These peaks can be resolved to a certain extent using an anion exchange HPLC column or using buffers instead of $H_2O$. However, due to the ease of sample preparation when using a C18 column with a $H_2O$/MeOH gradient, buffers were not used so as to avoid the introduction of salt into the sample which would need to be cleaned up.

There is also the appearance of a peak with absorbance only at 488 nm indicating that it contains only fluorescein with no DNA components. The exact identity of this fraction has not been confirmed but it probably results from hydrolysis of a small amount of fluorescein from the 5'-end of the oligomer. The fraction of interest in the chromatogram of the treated oligomer is the region from 27.5 - 29.5 minutes and therefore this region was collected for laser analysis. There is absolutely no UV signal observed in this region of the chromatogram.

**Cross-Link Quantification.** The oligomer $T_{22}$ was used in the preparation of a standard curve. It is necessary to use an oligomer with a fluorescein label to prepare the standard curve due to the fact that fluorescein within a DNA molecule fluoresces on the order of 100 times less than fluorescein itself. This is an understandable occurence and a similar effect has been observed with the fluorescence of benzo[a]pyrene alone or as adducted to a peptide or protein. Known quantities of the $T_{22}$ oligomer were injected and the area of the resultant peaks was used to generate the standard curve. Sample areas were compared with the standard curve for determination of the amount of cross-linked oligomer in the peak present in the NO·-treated sample but absent in the untreated sample.

Laser chromatograms demonstrate the complete absence of a peak in the region for single stranded oligomer indicating that the preliminary purification was adequate for

removing all single stranded oligomer from the sample. Therefore, the only component of the mixture isolated after preliminary purification is covalently cross-linked oligomer. Support for the evidence of this peak's identity as cross-linked oligomer is that the cross-linked peak's retention time matches that of double stranded oligomer which is observed when the HPLC column is not heated.

**Higher Dose Sample - Xanthine, Nitrite, and Cross-Link Quantification.** The results of this treatment are listed in Table 1. Nitric oxide was delivered at a rate of 15 nmol/mL/min for 45 minutes resulting in a final $NO_2^-$ concentration of 652 μM. The amount of xanthine present in the sample was determined to be (211 ± 39) nM. By comparison to the standard curve, the amount of cross-linked oligomer was found to be (13.0 ± 2.5) nM.

**Lower Dose Sample - Nitrite and Cross-Link Quantification.** The results of this treatment are listed in Table 1. In this treatment, the nitric oxide delivery rate was 18 nmol/mL/min for 7 minutes resulting a final $NO_2^-$ concentration of 126 μM. The amount of xanthine formed was not quantitated in this sample. Cross-linked oligomer was found at the level of (3.6 ± 0.1) nM.

## Discussion

Due to its extreme sensitivity, the HPLC-LIF system has enabled the quantification of DNA interstrand cross-links induced by nitric oxide that were not detectable by a number of other methods. Previously, denaturing gel electrophoresis using $P^{32}$ detection was used for the detection of oligomers containing an interstrand cross-link after nitrous acid treatment. Using high concentrations of nitrous acid, the yield of cross-linked oligomer was several percent (8). Denaturing gel electrophoresis with $P^{32}$ detection may be applicable to the lower levels of nitric oxide-induced cross-links; however, the yield of interstrand cross-links in experiments here using NO· is approximately 0.001%. The HPLC-LIF system using fluorescein-labeled oligomers is powerful enough to easily measure material at this level and it avoids the use of the radioactive isotope $P^{32}$.

The investigation of the nitric oxide-induced interstrand cross-link is hindered by the inability to generate a standard for the intact cross-linked oligomer. With other types of damage formed upon nitric oxide treatment (i.e. deamination), adequate amounts of standard can be generated by treating DNA with high levels of nitrous acid because it is not important that the DNA molecule remain intact for the recovery of deamination products. However, in order to prepare a standard for the intact cross-linked molecule, high doses of nitrous acid cannot be used because many other types of damage will occur (i.e. deamination of cytosine, guanine, adenine, and possibly strand breaks) which may destroy the oligomer leaving nothing intact.

Our results demonstrate cross-link levels at approximately 6% of the amount of the deamination product xanthine as seen in Table 1 for the higher dose sample where both xanthine and cross-link were measured. Therefore, hydrolysis of the guanine diazonium ion by water is much more likely than attack by the N2 amino group of a neighboring guanine on the opposite strand even though the N2 group is held in van der Waals contact in the double helix (8). The fact that interstrand cross-links were found at ~6% of the amount of xanthine is important because it provides information regarding the

fate of the guanine diazonium ion; however, the amount of cross-link will be a significantly lower percentage of all deamination events.

The rate equation for $N_2O_3$ formation is as follows:

$$\text{Rate} = k[O_2][NO\cdot]^2$$

where $k = 3.5 \times 10^6\ M^{-2}\ s^{-1}$ at 37 °C and $6 \times 10^6\ M^{-2}\ s^{-1}$ at 22 °C (15). The above equation indicates that $N_2O_3$ formation is first order in $O_2$ concentration and second order in NO· concentration. It is important to note that in the above equation, the nitric oxide concentration can be thought of as the delivery rate(nmol/mL/min) × time (min).

Based on our results of the analysis of two samples treated for different times but using nearly the same dose rate, a near linear response is generated. Given the same dose rate, the amount of $N_2O_3$ (and consequently interstrand cross-link) should give a linear response with the time of treatment as discussed above. The dose rate or the time can be changed to change the amount of NO· delivered. Therefore, this demonstrates that the delivery rate and the time of treatment are the key parameters in determining the amount of $N_2O_3$ formed in oxygenated solutions and will be a major determinant of the amount of DNA damage occuring as a result of nitric oxide.

Future work will be focused on the confirmation of the identity of the cross-link in the peak of interest. The exact structure is unknown at this point; however, it is likely that the structure matches that of the dG-dG interstrand cross-link previously observed upon nitrous acid treatment (7-9,16). The experimental approach for structure confirmation involves a preliminary purification as performed for laser quantification of the cross-link. Subsequent enzymatic digestion or acid hydrolysis of the resulting cross-linked oligomer will yield the nucleoside or base form of the cross-link, respectively. Both of these can be analyzed using electrospray ionization mass spectrometry for structure confirmation. Appropriate standards can be made for the base or nucleoside form of the cross-link through nitrous acid treatment of an oligomer with numerous 5'-CG sequences. Once again, when monitoring the individual base or nucleoside, standards can be made because intact cross-linked DNA is not the eventual goal. Using the

standards for the base and nucleoside form, the best method can be determined based on which form gives a greater response using electrospray-MS. The identity of the cross-link can then be determined using the form that gives the best sensitivity.

In the experiments here, a product from an oligomer treated with low doses of nitric oxide was discovered with a highly sensitive HPLC-LIF method. The preliminary purification at high temperature (80 °C) was effective at removing all non-covalently cross-linked duplex oligomer. The retention time of the additional product matches that of double stranded oligomer which is expected due to the fact that the cross-link has been postulated to only minimally structurally alter the double helix. Kirchner *et al.* state that only local reorganization in the form of propellor twisting of the cross-linked residues will occur and there will be no significant bending of the helix axis (8). This additional product is thus thought to be cross-linked oligomer which is significant due to the fact that interstrand cross-links can be lethal to the cell. Therefore, the HPLC-LIF method has allowed the observation of cross-linked DNA arising from quantities of nitric oxide that approach the amounts synthesized endogenously. Structural confirmation will be additional support for the presence of the interstrand cross-link.

# Table 1:

Resulting $NO_2^-$, Cross-Link, and Xanthine Levels after Two NO·-Treatments of Fluorescein Labeled ATATCGATCGATAT Oligomer

| $[NO_2^-]$ | [Cross-Link] | [Xanthine] |
|---|---|---|
| 652 μM (NO· delivery - 15 nmol/mL/min) | 13.0 nM | 211 nM |
| 126 μM (NO· delivery - 18 nmol/mL/min) | 3.6 nM | --------- |

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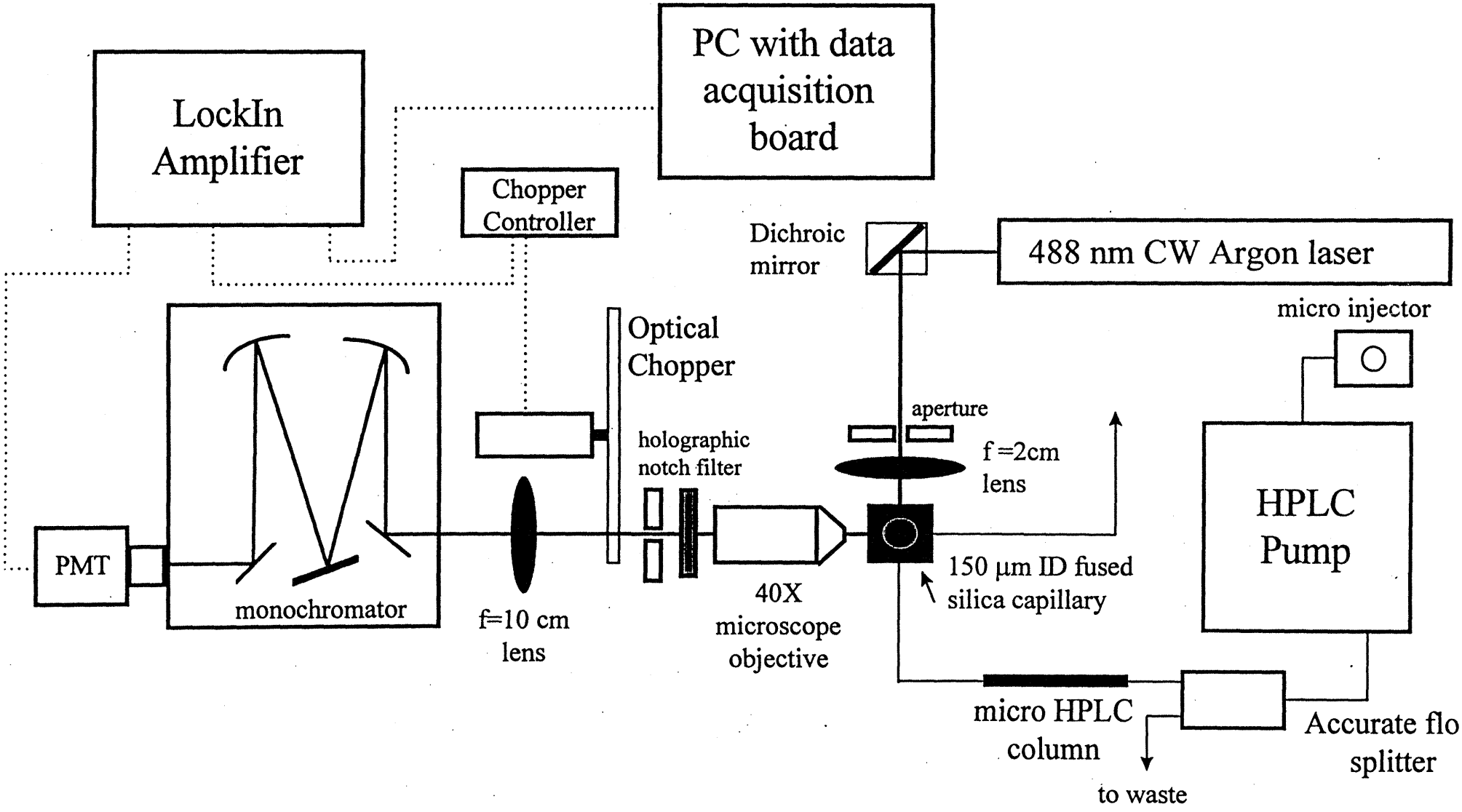
**Figure Legends**:

**Figure 1: HPLC-Laser Induced Fluorescence (HPLC-LIF) System** - Schematic diagram of the system used to quantitate the amount of fluorescence in 5'-fluorescein labeled oligomers. The entire system is described in detail in Materials and Methods.

# HPLC-LIF System



# 5. Conclusions

## Conclusions:

This thesis has focused on the fate of $N_2O_3$ in biological systems including study of the inhibition of *N*-nitrosation by bicarbonate, the deamination of DNA bases by nitric oxide, and the interstrand cross-linking induced by nitric oxide. First, the importance of bicarbonate in mediating nitrosation chemistry by nitric oxide has been demonstrated. Bicarbonate had previously been shown to be important in modulating peroxynitrite chemistry. Second, the relative rates of deamination of guanine and cytosine in nucleosides, single and double stranded oligomers and a G-quartet oligomer were investigated. Guanine was shown to be deaminated two times faster than cytosine which may be important for NO·-induced mutations, particularly if xanthine is unstable within DNA. Due to the fact that a double stranded oligomer and a G-quartet oligomer were less susceptible to attack by $N_2O_3$, it is postulated that hydrogen bonding protects DNA from deamination. Third, the formation of interstrand cross-links upon nitric oxide treatment has been demonstrated. The interstrand cross-link forms at approximately six percent of the amount of xanthine indicating that hydrolysis of the diazonium ion will predominate over cross-link formation. All of the information in this thesis contributes to the knowledge of the fate of $N_2O_3$ in biological systems and the rate constants determined here will contribute to kinetic modeling of the reaction of $N_2O_3$ with cellular components.

## Suggestions for Future Research:

The area of research in this thesis that holds the most potential for future research involves interstrand cross-link formation. First, structure confirmation of the nucleus of the interstrand cross-link using electrospray mass spectrometry will be further support for the conclusions drawn in the cross-link chapter of this thesis. Second, continued method development of the HPLC-LIF system for other applications will be interesting. It will be important to study interstrand cross-link formation using lower total nitric oxide delivery and lower nitric oxide delivery rates. In addition, interstrand cross-linking as a result of treatment with different agents is a possible application of this system. The system may also eventually be applied to the study of other types of modifications that give rise to a modified oligomer with retention time significantly different from the unmodified oligomer. Construction of an additional HPLC-LIF system will allow for application of this system to a number of different projects.