Mechanistic studies of nitric oxide formation from s-nitrosothiols

Barnett, D.Johnathon

MECHANISTIC STUDIES OF NITRIC OXIDE FORMATION FROM S-NITROSOTHIOLS

by

D. Jonathan Barnett, B.Sc. (Dunelm)

(Collingwood College)

A thesis submitted for the degree of Doctor of Philosophy in the
Department of Chemistry, University of Durham
October 1994

MECHANISTIC STUDIES OF NITRIC OXIDE FORMATION FROM S-NITROSOTHIOLS

by D. Jonathan Barnett

A thesis submitted for the degree of Doctor of Philosophy in the Department of Chemistry, University of Durham, October 1994

ABSTRACT

A study of the reactions of S-nitrosothiols in solution was undertaken. S-Nitrosothiols were known to produce NO, a physiologically important regulatory chemical, in solution but the mechanism of the reaction was unknown. Kinetic measurement of the S-nitrosothiol decomposition spectroscopically was erratic and irreproducible. The reaction was found to be mediated by adventitious Cu^{2+} in the water supplies used. The reaction of Cu^{2+} with S-nitrosothiols was studied. The rate equation was established and the reaction was found to be first order in S-nitrosothiol and the added Cu^{2+}. Second order rate constants for the reaction were determined so that the effect of structure could be studied. S-Nitrosothiols containing no other functional groups did not react with Cu^{2+}. S-Nitrosothiols containing a β-amino group were found to be the most reactive. The S-nitrosothiols were thought to be bidentately bound about the Cu^{2+}, which leads to rate limiting S-N bond breaking producing the thiyl radical and nitric oxide. A similar reaction was found to occur between S-nitrosothiols and Cu^{+} and Fe^{2+} ions, but no other transition metals appeared to catalyse nitric oxide formation from S-nitrosothiols.

The reaction of S-nitrosothiols with other thiols was also studied. The transnitrosation reaction was rapid and the variation of the observed rate constant with pH over the range 6-13 produced a sigmoidal curve which was indicative of a reaction involving attack by thiolate anion. It is believed that this reaction could be important *in vivo* as a mechanism for storing NO in the form of a stable S-nitrosothiol and then transferring the NO group to a more reactive S-nitrosothiol for rapid NO release. The effect of the thiol group alone was also investigated and depending on the concentration of thiol used the reaction was either catalysed or inhibited. Other conditions such as changing pH, adding metal chelators and the oxygen dependency of the reaction were studied.

Methods of measuring NO levels in solution were tested, the best being the use of a NO specific electrode. The reactions of NO with thiols were also studied to see if a mechanism for *in vivo* formation of S-nitrosothiols could be determined.

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor, Prof. D. Lyn H. Williams, for his time, guidance and advice throughout this research. I am also grateful to my fellow workers Jav, John, Tim, Rachel, Ling, Alex, Simon, Ana, Helen, Gaynor and Sahab for their friendship and advice. Special thanks to Mr. C. Greenhalgh for his computer wizardry and for keeping all the machines running.

I would also like to thank Prof. Harold F. Hodson, for advice on synthetic methods and his help, and Heather, Keith, Dave, Richard, Don, Phil and everyone else at the Department of Medicinal Chemistry, Wellcome Research Laboratories, Beckenham for making my time there thoroughly enjoyable and worthwhile.

My thanks also go to Stuart Askew and Dr. A.R. Butler, at the University of St. Andrews, for many fruitful discussions. I must also thank the Earls House boys for putting up with me for the past few weeks.

Finally, I'd like to thank EPSRC and the Wellcome Foundation Ltd. for funding this research.

DECLARATION

The material in this thesis is the result of research carried out in the Department of Chemistry, University of Durham, between October 1991 and September 1994. It has not been submitted for any other degree and is the authors own work, except where acknowledged by reference.

STATEMENT OF COPYRIGHT

"What we anticipate rarely happens and what we expect seldom occurs."

Benjamin Disraeli

To my family

CONTENTS

CHAPTER 1

Introduction

1.1 Nitric Oxide

Within the last six years our view of nitric oxide (NO), the simplest paramagnetic molecule, has undergone a radical change. It is no longer only a toxic pollutant and ozone depleter, but has been recently found to have a myriad of physiological roles (section 1.4).

1.1.1 Preparation

Nitric oxide is produced on a large scale industrially by oxidation of ammonia with a Pt-Rh catalyst at 800-900°C[1] (equation 1.1).

$$4\,NH_3 + 5\,O_2 \xrightarrow{\text{Pt-Rh}} 4\,NO + 6\,H_2O \quad \text{eqn. 1.1}$$

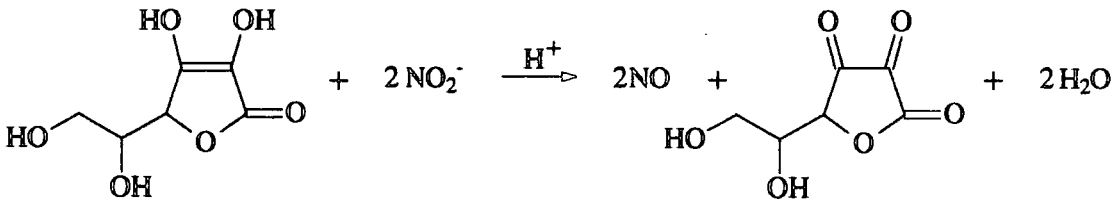
The nitric oxide produced is then oxidised to nitrogen dioxide and reacted with water to give nitric acid.

Formation by combination of the elements, nitrogen and oxygen, can be achieved industrially but only at temperatures in excess of 2000°C or in an electric arc. Both methods are very expensive in terms of energy, and the yields are low. Nitric oxide is formed in lightning and in the internal combustion engine. Without catalytic converters, which reduce NO to N_2, the NO is partially oxidised and gives rise to air pollution by NO_2 and nitrous and nitric acid. The build up of such pollution can give rise to smogs as in Los Angeles, Mexico City and Tokyo. Depletion of the ozone layer by NO reactions is a concern especially with plans for the next generation of supersonic commercial aircraft.

In the laboratory nitric oxide can be prepared by dropping nitric acid onto copper turnings (equation 1.2).

$$8\,HNO_3 + 3\,Cu \longrightarrow 2\,NO + 3\,Cu(NO_3)_2 + 4\,H_2O \quad \text{eqn. 1.2}$$

An easier method is by reduction of sodium nitrite with ascorbic acid (vitamin C) (equation 1.3).

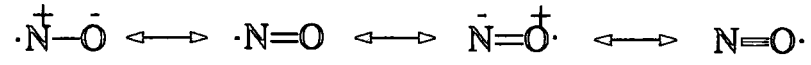


eqn. 1.3

The gas can be purified by passing through a 1M sodium hydroxide solution to remove the higher oxides of nitrogen, and a drying tower if necessary.

1.1.2 Physical Properties

Nitric oxide is a colourless gas at room temperature with a melting point of -163.6°C and a boiling point of -151.8°C[1]. Both the solid and liquid are colourless. Its solubility in aqueous solution is constant in the pH range 2-13[2], and at standard temperature and pressure is 1.8×10^{-3} mol dm^{-3}. Nitric oxide is one of the simplest paramagnetic molecules known. Its structure, however, has been the subject of much speculation as to where the lone electron lies on the molecule.



These canonical forms are the best representation in terms of valence bond theory. The lone electron reduces the bond order to ~2.5 (it is close to 3 in NO^+). The delocalization also goes some way to explaining why there is no appreciable dimerisation of NO.

1.1.3 Reactions of NO

The most important reaction, industrially, of nitric oxide is its oxidation to nitrogen dioxide (equation 1.4). This is part of the route to the production of nitric acid. It has been studied kinetically as it is a classical example of a third order gas phase reaction.

$$2NO + O_2 \longrightarrow 2NO_2 \quad \text{eqn. 1.4}$$

$$\text{Rate} = k[O_2][NO]^2 \quad \text{eqn. 1.5}$$

The reaction has been shown to be second order in nitric oxide and first order in oxygen (equation 1.5). There are two possible mechanisms for the reaction; firstly an equilibrium involving initial dimerisation of NO followed by reaction with oxygen (equation 1.6); or alternatively, another equilibrium between NO and O_2 giving the peroxynitrite radical can be envisaged, which then reacts with a further nitric oxide molecule to give the product (equation 1.7).

$$2NO \rightleftharpoons N_2O_2 \xrightarrow{O_2} NO_2 \quad \text{eqn. 1.6}$$

$$NO + O_2 \rightleftharpoons NO_3 \xrightarrow{NO} NO_2 \quad \text{eqn. 1.7}$$

Increasing the reaction temperature lowers the observed rate constant. The rationale for this is that the dimerisation of NO to N_2O_2 has a negative ΔH^o value[3], and it is this mechanism that is generally accepted.

The oxidation of NO has also been studied in aqueous solution. The rate equation has been established and is found to be the same as for the gaseous reaction. The predicted products of this reaction would be those of the hydrolysis of NO_2 giving nitrite and nitrate (equation 1.8).

$$2NO_2 + H_2O \rightleftharpoons NO_2^- + NO_3^- + 2H^+ \quad \text{eqn. 1.8}$$

Rather surprisingly the product of aqueous oxidation is nitrite (or nitrous acid, depending on pH) alone[3]. The mechanism proposed (scheme 1.1) is that NO is oxidised to NO_2 as the rate limiting step, which then reacts with NO to give N_2O_3. Hydrolysis of N_2O_3 gives nitrous acid, which will dissociate at pH >4.

$$NO + {}^{1}/_{2}\, O_2 \longrightarrow NO_2$$

$$NO + NO_2 \rightleftharpoons N_2O_3$$

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

Scheme 1.1

The radical nitric oxide will react with atoms, radicals and other paramagnetic species. Determination of atomic oxygen can be carried out with NO (equation 1.9). The reaction is chemiluminescent.

$$NO + O \longrightarrow NO_2 + h\nu \qquad \text{eqn. 1.9}$$

There is an analogous reaction with halogen atoms (scheme 1.2).

$$NO + X \longrightarrow NOX^*$$

$$NOX^* \longrightarrow NOX + h\nu$$

$$X = Cl, Br \text{ or } I$$

Scheme 1.2

The reaction with halogens is dealt with later (section 1.2.5). Nitric oxide will undergo a reaction with ozone (scheme 1.3). The reaction is also chemiluminescent and is the basis for a very sensitive nitric oxide assay (detection $\geq 10^{-9}$M).

$$NO + O_3 \longrightarrow NO_2^* + O_2$$

$$NO_2^* \longrightarrow NO_2 + h\nu$$

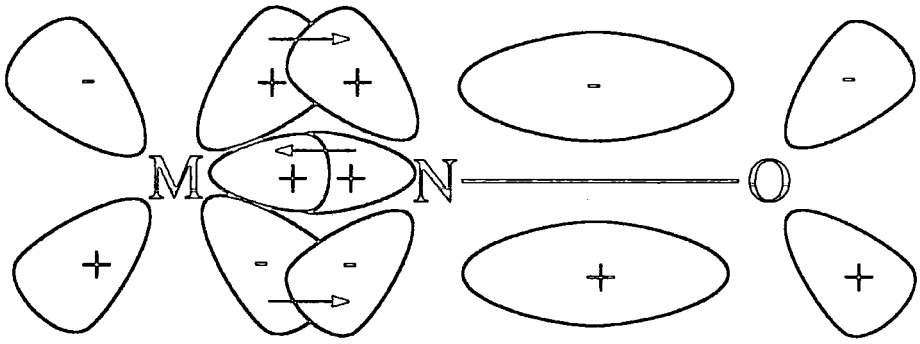
Scheme 1.3

Other methods of nitric oxide detection will be discussed later.

1.1.4 Reaction of NO with Metal Centres

Nitric oxide will act as a ligand to a number of metal centres including Fe, Mn, Co, Rh, Ni, Ru, Pt and Mo. Metal nitrosyls have been known for a long time and recent interest lay in their potential as homogenous catalysts. One of the most well known complexes is $[Fe(H_2O)_5NO]^{2+}$, which is produced in the brown-ring test for nitrates. Recently a book has been published [4] containing a comprehensive review of metal nitrosyl chemistry.

The bonding between metal centres and nitric oxide is best considered in terms of molecular orbital theory. The nitric oxide molecule donates electron density in the form of a σ-bond to the metal. There is also back donation of electron density from the occupied d-orbitals on the metal to the empty π* anti-bonding orbital on NO. This process is synergic, with the excess charge on the metal centre being off loaded back onto the ligand (figure 1.1).



σ-bond and dπ-π* overlap in M-NO bonding

Figure 1.1

The bonding is analogous to that of metal carbonyls. However, nitric oxide is more electro-negative than CO[5] and a better π acceptor and weaker σ donor.

Nitric oxide can donate one or three electrons to a metal giving, respectively, a bent or linear structure (figure 1.2).

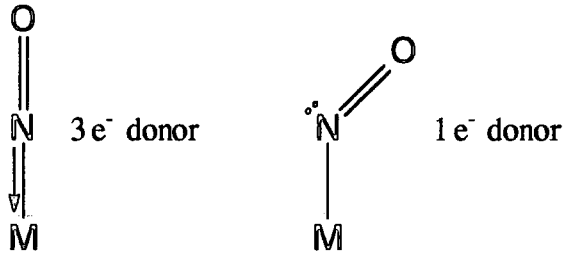


Figure 1.2

1.1.5 Synthesis and Reactions

Nitrosyl metal complexes are usually synthesised by reaction with the metal and nitric oxide, or nitrosonium salt. A simple adduct may be formed reversibly or irreversibly (equation 1.10). Nitric oxide can also displace other ligands (equation 1.11). In the latter case each NO molecule displaces the equivalent of a 3-electron ligand[6].

$$Fe(edta) + 2NO \rightleftharpoons Fe(NO)_2(edta) \qquad \text{eqn. 1.10}$$

$$Cr(CO)_6 + 4NO \longrightarrow Cr(NO)_4 + 6CO \qquad \text{eqn. 1.11}$$

The nitrosonium ion is a 2-electron donor and will readily displace CO in co-ordinatively saturated complexes[7] (equation 1.12).

$$CpMn(CO)_3 + NO^+ \longrightarrow [CpMn(CO)_2(NO)]^+ + CO \qquad \text{eqn. 1.12}$$

Metal nitrosyls will react with nucleophiles already co-ordinated to the metal. Ligands such as ammonia[8], amines[9] and alkyl substituted aromatics [10] will undergo nitrosation reactions with metal nitrosyls.

Recent work on metal nitrosyl complexes has centred on the interaction of nitric oxide with haem compounds. It has been known for a long time [11] in the food industry that sodium nitrite added as a preservative forms a nitric oxide haemoprotein complex. This iron nitrosyl gives cured meats their distinctive pink colouring. In addition to its colouring properties the added nitrite prevents bacterial growth such as *Clostridium botulinum* and hence food poisoning. It follows that NO will also bind to haem units in haemoglobin (see fig. 1.3) and myoglobin.

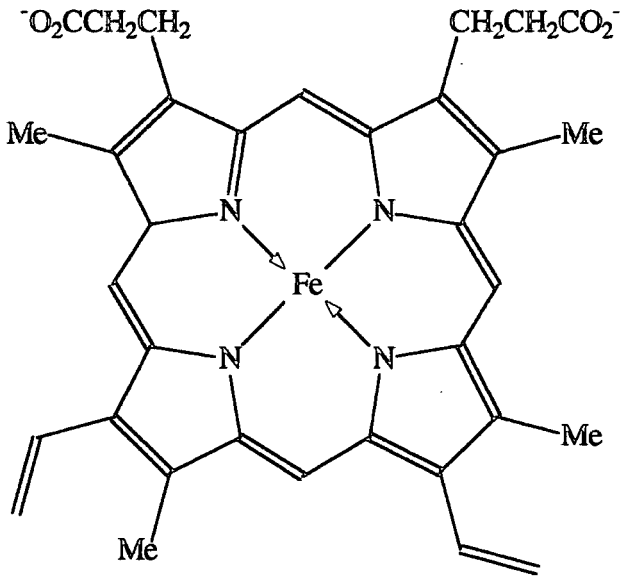


Figure 1.3

When NO binds to Fe^{II} in a haem unit it still has an unpaired electron, which can be detected by electron spin resonance spectroscopy. This technique, along with X-ray studies has been used to probe NO-haem interactions and structures. Nitric oxide will bind to haem more strongly than oxygen (about 1300 times more) [12] and this electron spin label has been used to probe haem reactions such as the eventual reduction of oxygen *in vivo* to water by cytochromes. The NO-haem interaction is also an important physiological process itself and will be discussed in section 1.4.

1.2 Nitrosation

1.2.1 Reaction and Reagents

The nitrosation reaction is an electrophilic addition of "NO^{+}" to a substrate. It is an important reaction both industrially and preparatively. Important products of nitrosation are azo-dyes, ε-caprolactam (for nylon 6) and alkyl nitrites (as nitrovasodilators). Nitrosamines, formed by the nitrosation of secondary amines, have been found to be carcinogenic and show a more hazardous side to this chemistry.

1.2.2 Nitrous Acid HNO_2

There are many ways of adding NO^+ to a substrate. The most commonly used nitrosating reagent is derived from nitrous acid. Nitrous acid is a weak acid, with a pK_a value of 3.1[13]. It is prepared by acidifying nitrite salts at less than room temperature as it is unstable to decomposition (equation 1.13).

$$3\,HNO_2 \rightleftharpoons 2\,NO + HNO_3 + H_2O \quad \text{eqn. 1.13}$$

The structure of nitrous acid has been established by infra-red studies[1]. Both the cis and trans isomers are present (figure 1.4), however, the trans isomer is the

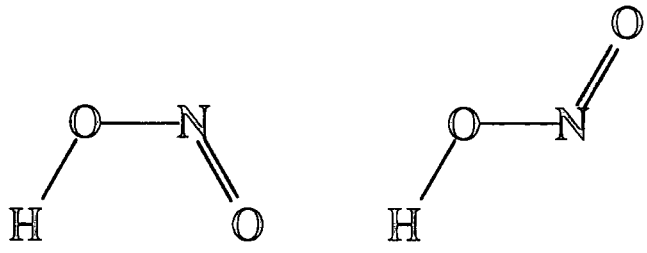


Figure 1.4

more stable of the two and is prevalent. The bond lengths are N=O 1.20Å, O-N 1.46Å and H-O 0.98Å.

1.2.3 Dinitrogen Trioxide N_2O_3

Nitrous acid exists in equilibrium with another nitrosating agent, dinitrogen trioxide (equation 1.14), and this is favoured at quite high concentration of nitrous

$$2\,HNO_2 \rightleftharpoons N_2O_3 + H_2O \quad \text{eqn. 1.14}$$

acid and in low acidic solution. Typically at ~0.1M nitrous acid the equilibrium lies well over to the right. The blue coloured N_2O_3 is visible to the eye. Dropping

concentrated sulphuric acid onto solid sodium nitrite will produce a 1:1 mixture of NO and NO_2 which will condense out as N_2O_3 below 0°C.

The reaction of N_2O_3 with substrates has been studied[14]. This involves attack on substrate, S, by N_2O_3 (scheme 1.4). The rate equation predicts second order dependence on nitrous acid and first order on substrate.

$$2\,HNO_2 \underset{}{\overset{K}{\rightleftharpoons}} N_2O_3 + H_2O$$

$$N_2O_3 + S \xrightarrow{k} S^{+}\!-\!NO + NO_2^{-}$$

Scheme 1.4

$$\text{Rate} = k\,[N_2O_3][S] = kK\,[HNO_2]^2[S] \qquad \text{eqn. 1.15}$$

In the case of very reactive substrates[15], such as ascorbic acid, aniline and azide or for substrates at high concentration, then reaction with substrate can be faster than the hydrolysis of N_2O_3 to nitrous acid. The rate determining step then becomes the formation of N_2O_3 (equation 1.16). The rate equation will be second order in nitrous acid and zero order in substrate (equation 1.17).

$$2\,HNO_2 \xrightarrow{k} N_2O_3 + H_2O \qquad \text{eqn. 1.16}$$

$$\text{Rate} = k\,[HNO_2]^2 \qquad \text{eqn. 1.17}$$

The mechanism is confirmed[14] by using $H_2{}^{18}O$ as the solvent, as ^{18}O is exchanged between the solvent and nitrous acid. The exchange is second order in nitrous acid.

1.2.4 Nitrous Acidium Ion $H_2NO_2^+$

At acidities above that normally used for N_2O_3, but with lower overall nitrous acid concentration another nitrosating agent predominates. This can be shown experimentally[16] as the rate equation is now first order in nitrous acid and first order in free proton concentration (equation 1.18). This is generally the case for most

$$\text{Rate} = k\,[HNO_2]\,[H^+]\,[S] \qquad \text{eqn. 1.18}$$

substrates including C, N, O or S-nitrosation.

The acid catalysed nitrosation reaction has two possible mechanisms to explain the rate equation[17]. There is a pre-equilibrium forming a nitrosating species followed by rate limiting attack on the substrate. The first possibility is that the nitrosating agent is the nitrous acidium ion and the other possibility is the nitrosonium ion (scheme 1.5).

$$HNO_2 + H^+ \rightleftharpoons H_2NO_2^+$$

$$H_2NO_2^+ \rightleftharpoons NO^+ + H_2O$$

Scheme 1.5

The pre-equilibrium process for nitrous acidium ion formation is rapid, whereas, the formation of the nitrosonium ion involves a N-O bond breaking step which in principle is a slower process. If the formation of the nitrosonium ion could be made rate-limiting then this would provide good evidence for that mechanism. This limit has not been successfully achieved in water[18]. There is evidence against NO^+ formation. The rate equation for ^{18}O exchange between water and nitrous acid has been established (equation 1.19).

$$\text{Rate} = k\,[HNO_2]\,[H^+] \qquad \text{eqn. 1.19}$$

The rate constant for the exchange is 230 l mol^{-1} s^{-1} at 0°C[19]. If the nitrosonium ion is involved then the exchange of one oxygen atom in nitrous acid occurs for each NO^+ ion formed, which then rehydrates to the nitrous acidium ion. For equation 1.18 to be correct, then the exchange must be faster than reaction with the substrate. However, for reaction with an anion such as azide (N_3^-) there is a limiting value for k ~ 2500 l mol^{-1} s^{-1}. For the case where $[N_3^-] \geq 0.1M$ the step in equation 1.20 is faster than the formation of NO^+. This would mean there was a zero order dependence on azide which is in disagreement with equation 1.18.

$$NO^+ + N_3^- \longrightarrow N_2O + N_2 \qquad \text{eqn. 1.20}$$

It is reasonable to assume that the nitrosating agent is $H_2NO_2^+$ rather than NO^+. It is not impossible for NO^+ to be formed by dehydration, but the rehydration is likely to be very rapid and possibly with the same water molecule that it was previously bound to, thus not allowing ^{18}O exchange.

1.2.5 Nitrosonium Salts NO^+X^-

At very high acidities (~60% perchloric acid) the nitrosonium ion is quantitatively the nitrosating agent[20] derived from nitrous acid (equation 1.21).

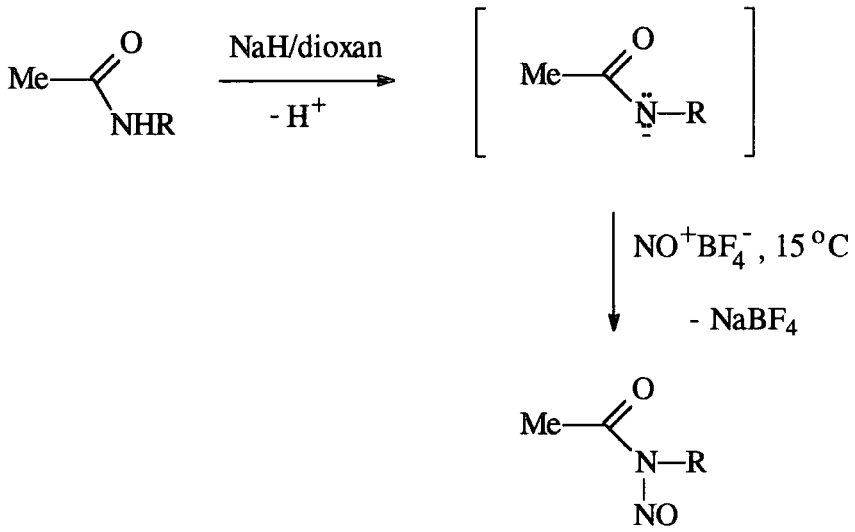
$$H_3O^+ + HNO_2 \rightleftharpoons NO^+ + 2\,H_2O \qquad \text{eqn. 1.21}$$

The nitrosonium ion can also be prepared as a stable salt for use in non-aqueous media. They are prepared by adding N_2O_4, N_2O_3 or NOX to a source of the anion (equation 1.22). The most common nitrosonium salts are tetrafluoroborate, hydrogen sulphate and perchlorate (although the latter is explosive).

$$N_2O_4 + H_2SO_4 \rightleftharpoons NO^+HSO_4^- + HNO_3 \qquad \text{eqn. 1.22}$$

Nitrosonium salts are very reactive nitrosating agents and are able to react with species that are unreactive to other nitrosating agents. Using nitrosonium salts primary amides can be hydrolysed to the corresponding acid[21] (equation 1.23). Under basic conditions acetamides can be nitrosated[22] by deprotonation of the amide with sodium hydride followed by reaction with a nitrosonium salt in dioxan under reflux (scheme 1.6).

$$ArCONH_2 + NO^+BF_4^- \longrightarrow ArCO_2H + N_2 + HF + BF_3 \quad \text{eqn. 1.23}$$



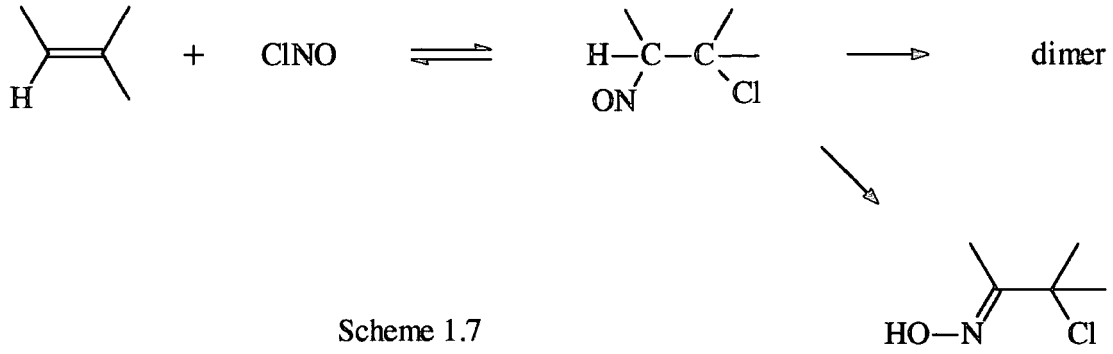
Scheme 1.6

1.2.6 Nitrosyl Halides

Nitrosyl halides are gases of the form XNO and are widely used nitrosating agents as they dissolve readily in organic solvents. This is particularly useful for species that have a low solubility in aqueous media. The most commonly used is nitrosyl chloride. Nitrosyl halides are readily formed[23] by the direct reaction between nitric oxide and the halogen (equation 1.24).

$$2NO + X_2 \rightleftharpoons 2XNO \quad \text{eqn. 1.24}$$

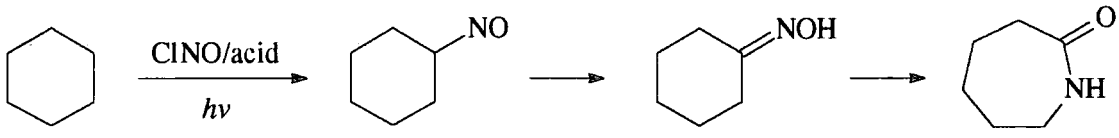
Nitrosyl chloride will react with a range of substrates including alkenes (scheme 1.7), carbonyls and alcohols (equation 1.25).



Scheme 1.7

$$ROH + ClNO \rightleftharpoons RONO + HCl \qquad \text{eqn. 1.25}$$

Free radical reactions with nitrosyl chloride in the presence of uv-radiation are possible, giving rise to C-nitroso compounds. Cyclohexane will react under such conditions in the presence of an acid to give the caprolactam (scheme 1.8) [24] . This is one industrial process for caprolactam production which gives nylon 6 on ring opening polymerisation.



Scheme 1.8

Nitrosyl halides can also be generated in aqueous solution using nitrous acid in the presence of the halide anion (equation 1.26). These conditions give rise to equilibria for nitrosyl halide formation, which then react with any substrate present. The equilibrium constants for ClNO and BrNO have been determined[25] (table 1.1).

Table 1.1

XNO	K_{XNO} (25°C) l^2 mol^{-2}
Nitrosyl chloride	1.1×10^{-3}
Nitrosyl bromide	5.1×10^{-2}

As bromide ion is more polarisable than chloride, and hence a stronger nucleophile we would expect the equilibrium constant for BrNO formation to be larger than that for ClNO, as is found.

$$HNO_2 + X^- + H^+ \overset{K_{XNO}}{\rightleftharpoons} XNO + H_2O \qquad \text{eqn. 1.26}$$

Addition of the halide makes the N of the nitrosating agent more electrophilic and acts as a catalyst for the reaction (equation 1.27) by making more nitrosating agent available for reaction.

$$XNO + S \rightleftharpoons S^{+}\!\!-\!\!NO + X^- \qquad \text{eqn. 1.27}$$

There has been no reported nitrosyl fluoride catalysis.

The rate equation for halide catalysis has been established for diazotization of aniline derivatives (equation 1.28).

$$\text{Rate} = k_2 K_{XNO} K_a [X^-] [HNO_2]_T [ArNH_2]_T \qquad \text{eqn. 1.28}$$

The mechanism is interpreted as rate limiting attack by the nitrosyl halide on the unprotonated amine. K_a is the acid dissociation constant of $ArNH_3^+$, and $[HNO_2]_T$ and $[ArNH_2]_T$ are the total stoichiometric concentrations of nitrous acid and amine. Thus if K_{XNO} and K_a are known, then the value of k_2 the second order rate constant

for nitrosyl halide attack on the substrate can be obtained. The value of K_{INO} is not known, so rate constants for attack by nitrosyl iodide cannot be obtained.

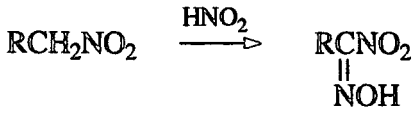
Since chlorine is more electronegative than bromine, the nitrosyl chloride is expected to be the more reactive electrophile of the two. However, bromide catalysis is greater than chloride catalysis because of the difference in the K_{XNO} values, which is due to the nucleophilicity of the halide.

Thiocyanate ion is also a good nucleophilic catalyst. At high concentration it can be seen as a red species. The nitroso group is bound to the S-atom as predicted by HASB theory, as the S (a soft base) is more nucleophilic than N (a hard base). Comparing ONSCN with the nitrosyl halides, it is seen that catalysis is more pronounced than with the halides. This is due to the value for the equilibrium constant K_{ONSCN} of formation of nitrosyl thiocyanate, which is 30 l^2 mol^{-2} at 298K[26]. This value is almost three orders of magnitude larger than the values for the halides. The reactivity of the nitrosyl thiocyanate is much less than that of the nitrosyl halides, as the thiocyanate is not as electronegative as the halides. It is the equilibrium values of XNO formation rather than intrinsic reactivity that is the most important factor in establishing catalytic activities.

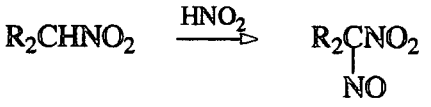
1.2.7 C-Nitrosation

The products of C-nitrosation can exists as monomers, dimers or oximes. The reaction consists of the replacement of a hydrogen by a nitroso group, which may then undergo a rearrangement. Aliphatic C-nitrosation requires the presence of an electron withdrawing group next to the carbon to be nitrosated. Such activators are carbonyl, carboxyl, nitro and cyano groups but they will activate compounds towards nitrosation to different extents.

The history of C-nitrosation goes back to the 1870's[27],when Victor Meyer discovered that careful acidification of an alkaline solution of nitroparaffin and an alkyl nitrite procuced a nitrolic acid (equation 1.29) and a secondary nitroparaffin gave a pseudonitrole (equation 1.30).

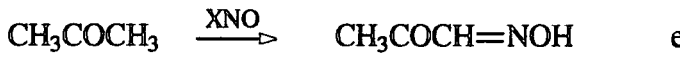


eqn. 1.29

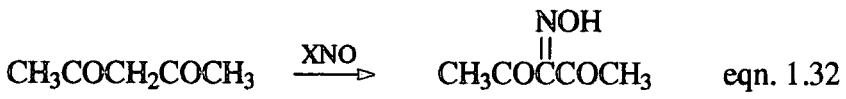


eqn. 1.30

The reaction can be extended to ketones (equation 1.31), β-diketones (equation 1.32), β-ketoacids and β-ketoesters.

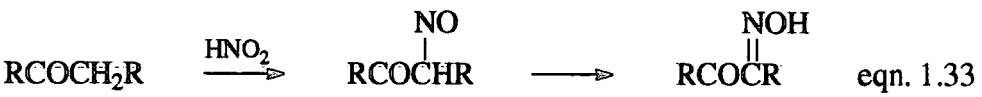


eqn. 1.31



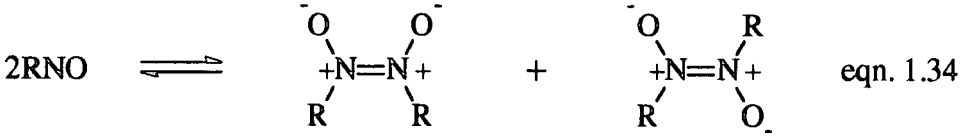
eqn. 1.32

Where a methylene or methyl group is nitrosated there is usually a rearrangement to the oxime (equation 1.33).



eqn. 1.33

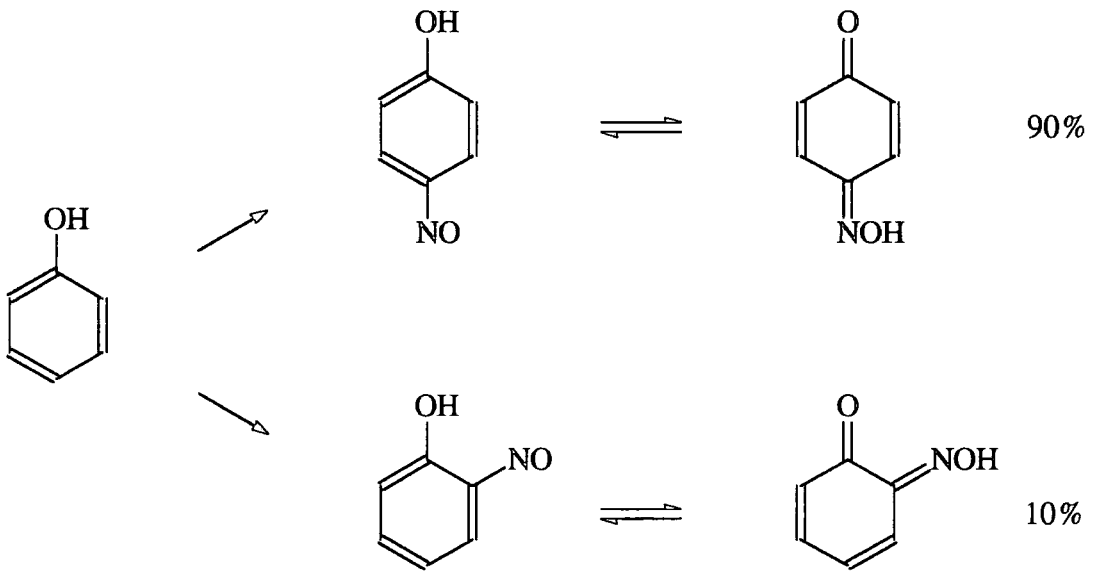
Not all C-nitrosations give the oxime as product[28]. When the C-nitroso product is stable it is seen as a blue or green colour, absorbing between 630-790nm with a low extinction coefficient (ε = 1-60 mol^{-1} l cm^{-1}). The dimer of the C-nitroso compound can also be formed (equation 1.34) and its structure has been established.



eqn. 1.34

Both the Z and E isomers are formed, with the E isomer being the more stable. The dimers are colourless solids and on heating melt to give the coloured monomers. Tertiary nitroso compounds exist as the monomer due the steric strain of dimerisation, and electron withdrawing groups also stabilise the monomer.

Aromatic C-nitrosation is dependent on substantial activation by electron donating groups. The most familiar reactions are with phenols and naphthols (scheme 1.8).



Scheme 1.8

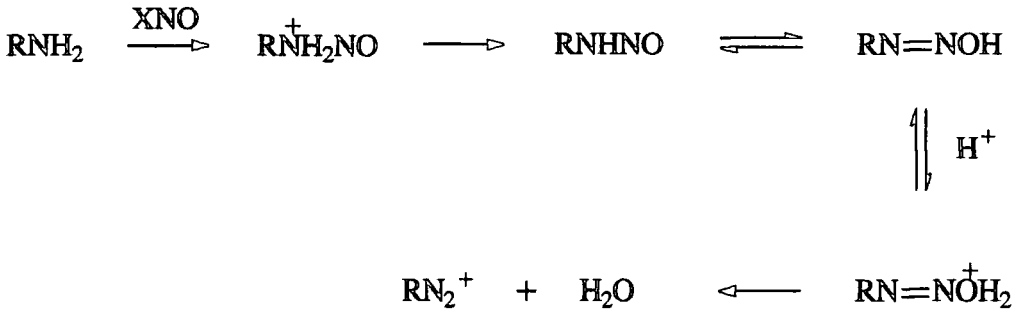
In solution the products exist mainly as the benzoquinones, and the main product is the 4-nitrosophenol[29].

1.2.8 N-Nitrosation

One of the most important industrial applications of nitrosation reactions is the formation of diazo compounds from aromatic amines, which are then used to produce azo-dyes. There is also a great interest in the study of nitrosamines since the discovery that they are powerful carcinogens.

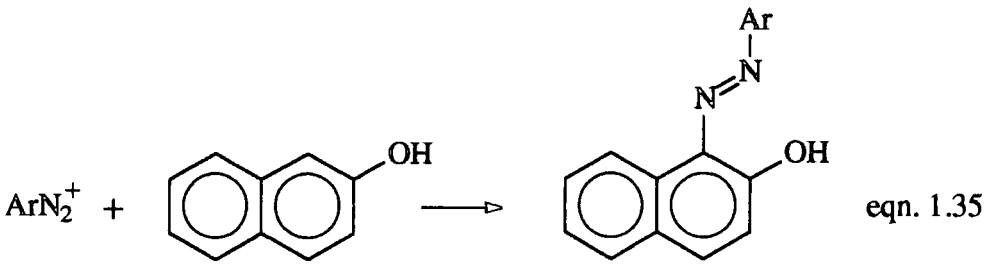
Aliphatic and aromatic primary amines undergo deamination reactions upon nitrosation. The products from such a reaction include alcohols, alkenes and phenols.

The initial nitrosation step of primary amines is rate limiting, which is followed by rapid proton transfer and water loss to give a diazonium ion (scheme 1.9).

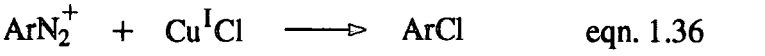


Scheme 1.9

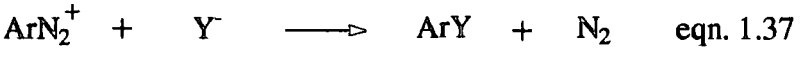
Diazonium ions are very reactive species, which will undergo loss of nitrogen giving the deamination products in the case of aliphatic primary amines. Aromatic diazonium salts can be isolated and can react with other aromatic compounds to produce azo-dyes (equation 1.35), or they will undergo substitution reactions (equations 1.36 and 1.37). The aromatic diazonium ions are more stable than the aliphatic counterparts because the positive charge on the N can be delocalised through the aromatic system.



eqn. 1.35



eqn. 1.36



eqn. 1.37

The electrophilic aromatic substitution reaction by the aryl diazonium ion produces the well-known azo-dyes. Adding substituents to both the aromatic substrate

and the diazonium ion generates new compounds with different colouring properties. The other main reaction of aromatic diazonium ions is the Sandmeyer reaction. Using the copper(I) salts a range of substituted aromatic compounds can be produced for example ArBr, ArI, ArSCN, etc.

Secondary amines undergo the same initial rate limiting nitrosation as primary amines. However, there is no α-hydrogen to allow formation of the diazonium ion. Instead, the reaction stops at the formation of a nitrosamine (equation 1.38).

$$R_2NH + XNO \longrightarrow R_2NNO + HX \qquad \text{eqn. 1.38}$$

The revelation that nitrosamines are powerful carcinogens has led to increased concern about *in vivo* formation from naturally occuring secondary amines, sources of nitrite which is used in food preservation and nitrate in water supplies from overuse of fertilizers.

Nitrosamines can also be formed from tertiary amines along with the corresponding carbonyl compound and nitrous oxide. Kinetic and stoichiometric work led to the proposed mechanism (scheme 1.10)[30].

$$R_2N{-}CHR'_2 \underset{\text{fast}}{\overset{HNO_2/H^+}{\rightleftharpoons}} R_2\overset{+}{N}\begin{matrix}CHR'_2\\ \diagdown\\ NO\end{matrix} \xrightarrow[\text{slow}]{} R_2\overset{+}{N}{=}CR'_2 + HNO$$

$$R_2\overset{+}{N}{=}CR'_2 \xrightarrow{H_2O} R'_2C{=}O + R_2NH$$

$$R_2NH + HNO_2 \xrightarrow{H^+} R_2NNO + H_2O$$

$$2\,HNO \longrightarrow N_2O + H_2O$$

Scheme 1.10

The α-C-H bond breaking is the rate limiting step as there is a primary isotope effect $k_H/k_D = 3.8$ for the reactions of $R_2NCHR'_2$ and $R_2NCDR'_2$.

1.2.9 O-Nitrosation

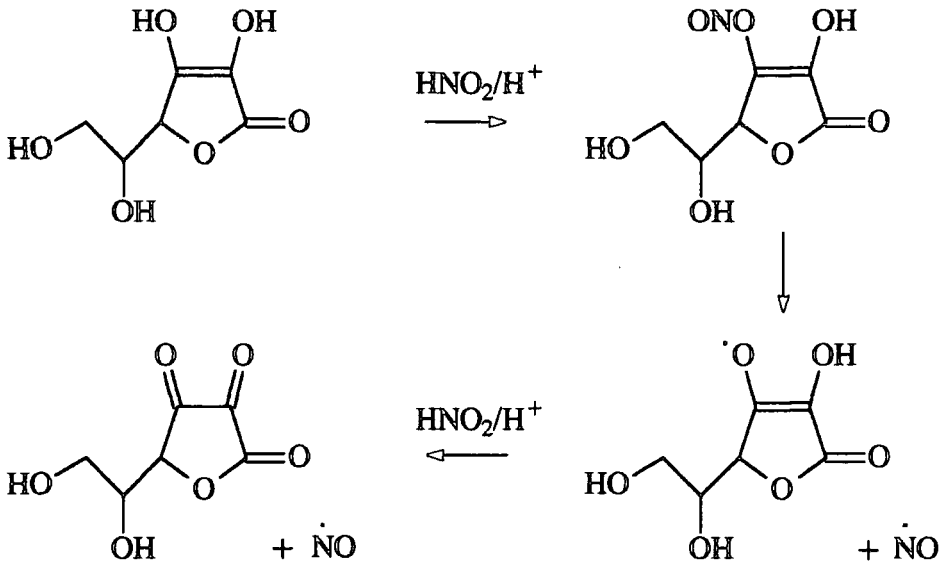
The most common example of O-nitrosation is the formation of alkyl nitrites from alcohols and nitrous acid (equation 1.39). The reaction is reversible and since the boiling point of alkyl nitrites is much less than those of the corresponding alcohol, the product can be removed by distillation. Any nitrosating agent will effect the reaction.

$$ROH + XNO \rightleftharpoons RONO + HX \qquad \text{eqn. 1.39}$$

where X =Cl, HO or R'O

The reaction has been shown to be O-nitrosation[31] rather than nucleophilic attack by nitrite ion. Optically active alcohols retain their configuration. Hydrolysis of the alkyl nitrite similarly retains the configuration. If the hydrolysis is carried out with $H_2{}^{18}O$ then there is no uptake of ^{18}O into the alcohol. Comparison of the equilibrium constants for alkyl nitrite formation[32] shows K decreasing along the series $R = CH_3 > C_2H_5 > i\text{-}C_3H_7 > t\text{-}C_4H_9$. The values for K do not vary much for mono-, di- or tri-alcohols, and even some carbohydrates are in the same area. This implies that steric, rather than electronic effects are more important in O-nitrosation.

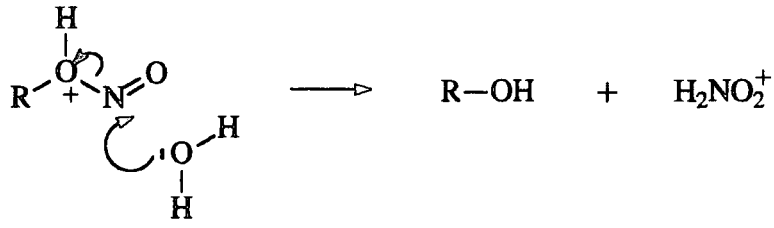
A noteworthy reaction (scheme 1.11) is that of the nitrosation of ascorbic acid (vitamin C). The products of reaction are dehydroascorbic acid and two equivalents of nitric oxide. The nitrite ester initially formed can undergo homolytic fission to give nitric oxide and a resonance stabilised oxygen radical. This is rapidly oxidised by a second molecule of nitrous acid to the products. Rate measurements on the acid catalysed reaction show the reaction of the ascorbate ion (ascorbic acid pK_a ~4) to be at the diffusion controlled limit, as has been found for most other ions[33].



Scheme 1.11

As the O-nitrosation of ascorbic acid is irreversible it has been used as an inhibitor of other nitrosation reactions for example of amines. This competitive reaction is important for *in vivo* inhibition of nitrosamine formation. It appears that as well as the important anti-oxidant properties of vitamin C, its dietary inclusion may also help reduce production of carcinogenic nitrosamines and hence gastric cancers. Vitamin C fits all the criteria required of an inhibitor; high reactivity, non-toxicity and reactivity over a wide pH range.

Alkyl nitrites formed by O-nitrosation are widely used as nitrosating agents themselves. In acid conditions alkyl nitrites are rapidly hydrolysed[31] to the corresponding alcohol and nitrous acid (scheme 1.12).

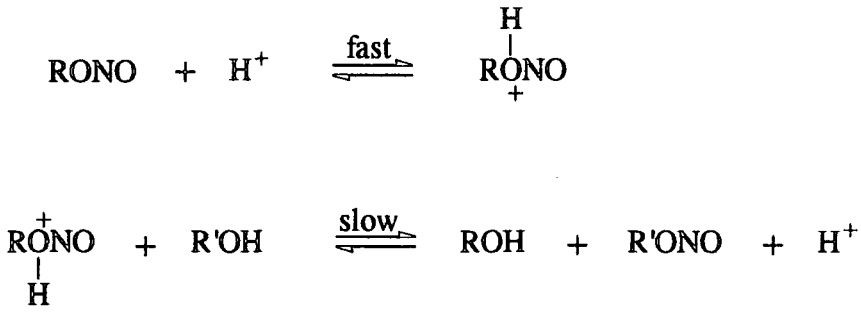


Scheme 1.12

The nitrous acidium ion formed may then nitrosate any substrate present.

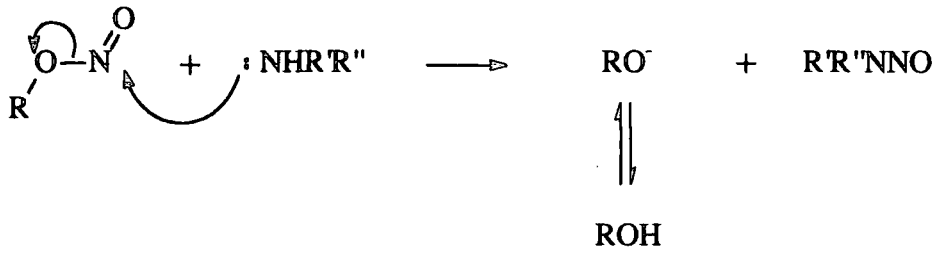
Acid hydrolysis followed by reaction is a waste of alkyl nitrite as inorganic nitrite will achieve the same result. Alkyl nitrites can transfer the nitroso group directly to other substrates. Such reactions have previously been mentioned; aromatic diazonium ions, nitrosamines, alkyl nitrites and nitrosothiols can all be formed by nitrosation by alkyl nitrites.

Transnitrosation between alkyl nitries and alcohols is a further example of O nitrosation. It has been shown[34] that the reaction is reversible and first order in both [RONO] and [H^+] with the alcohol as the solvent (scheme1.13).



Scheme 1.13

Here the reactive species is the protonated alkyl nitrite. Nitrosation will also occur under basic conditions. Both amines and thiols can easily be nitrosated this way (scheme 1.14).

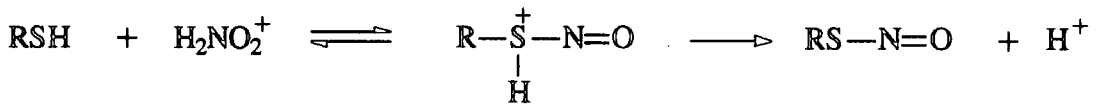


Scheme 1.14

Nucleophilic attack on the alkyl nitrite nitrogen by the amine (or thiolate anion) will give the corresponding nitrosation product. In this case, a nitrosamine for a secondary amine, or various hydrocarbon products for a primary amine.

1.2.10 S-Nitrosation

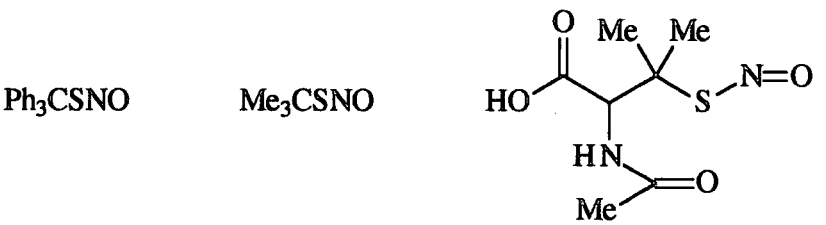
S-Nitrosation is analogous to O-nitrosation, except that it is essentially irreversible (scheme 1.15) due to the difficulty of protonating the product S-nitrosothiol. S-Nitrosation will be discussed in the next section.



Scheme 1.15

1.3 S-Nitrosothiols

S-Nitrosothiols (or thionitrites) are the sulphur analogues of the organic nitrites, and are of the general formula RSNO. Both primary and secondary nitrosothiols are red whilst tertiary nitrosothiols are green. Generally, nitrosothiols are relatively unstable to decomposition, however, several have been isolated and characterised. Trityl nitrosothiol[35] (1.1) is a fairly stable green solid, as is t-butyl nitrosothiol[36] (1.2), although over a period of weeks they will decompose to the corresponding disulphide. The nitrosothiol of N-acetylpenicillamine[37] (1.3) is indefinitely stable as a green solid. Most nitrosothiols have a u.v. absorption at 330-350nm with ε values of the order of 10^3 mol^{-1} dm^3 cm^{-1}.



(1.1) (1.2) (1.3)

For primary and secondary compounds there is an absorption at about 540nm and for tertiary compounds at around 590nm. Both absorption bands have small extinction values of 10-20 mol^{-1} dm^3 cm^{-1}. These absorptions have had electronic transitions

assigned[38], so that the absorption at 540/590nm is due to $n_N \rightarrow \pi^*$ transition and the absorption at around 340nm is due to $n_O \rightarrow \pi^*$.

The molecular structure of S-nitroso-N-acetylpenicillamine (SNAP) was determined by X-ray studies[37] (figure1.4).

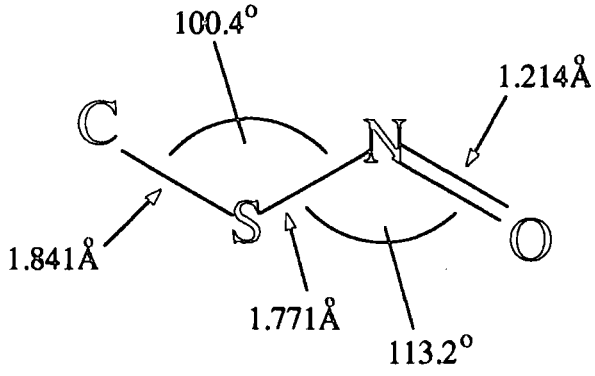


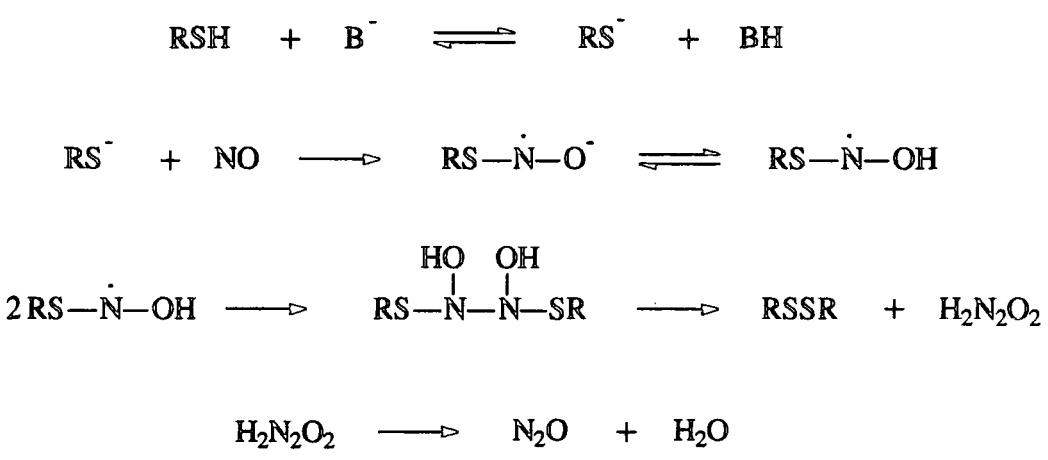
Figure 1.4

1.3.1 Nitrosothiol Formation

These compounds are generally prepared by nitrosation of the parent thiol. Most nitrosating agents will effect the transformation; NOCl, RONO, N_2O_3, N_2O_4, $H_2NO_2^+$, etc. (equation 1.40).

$$RSH + XNO \longrightarrow RSNO + HX \qquad \text{equation 1.40}$$

Interestingly, it has often been reported[39] that nitric oxide itself will nitrosate thiols in the absence of oxygen. It has been shown[40] (see Chapter 2) that nitric oxide alone will not nitrosate thiols. Under basic conditions, however, the thiolate anion will react[41] with nitric oxide to give the disulphide and nitrous oxide (scheme 1.15).



Scheme 1.15

The reaction can only proceed if pH > 4. If oxygen is not totally excluded it will react with NO to produce an electrophilic nitrosating agent N_2O_3 or N_2O_4, which will produce the nitrosothiol and ultimately the disulphide.

Photolysis of a disulphide in the vapour phase, in the presence of nitric oxide[42] will give the desired product (equation 1.41).

$$RSSR \xrightarrow{h\nu} 2\,[RS^{\cdot}] \xrightarrow{NO} 2RSNO \qquad \text{eqn. 1.41}$$

1.3.2 Kinetics of S-Nitrosation

Kinetic studies have been carried out on the nitrosation of thiols[16] and the rate equation established (equation 1.42). For the aqueous reaction with acidified nitrite, the reaction was shown to be first order with respect to nitrous acid concentration, under the pseudo first order condition of [RSH] >> $[HNO_2]$. It was also first order in thiol and acid concentration.

$$\text{Rate} = k\,[HNO_2]\,[RSH]\,[H^+] \qquad \text{eqn. 1.42}$$

The rate equation is consistent with the rate-limiting step involving the nitrous acidium ion ($H_2NO_2^+$) (or the nitrosonium ion NO^+) attacking the thiol. This reaction is rapid and data have to be obtained from stopped flow spectrophotometry.

For the most reactive thiols the values of the third order rate constants[16] approach 7000 $dm^6mol^{-2}s^{-1}$, which is believed to be the diffusion controlled limit.

The reaction is catalysed by nucleophiles, such as halide anions and the thiocyanate anion (equation 1.43). A nitrosyl halide or thiocyanate is produced, which then reacts with the thiol.

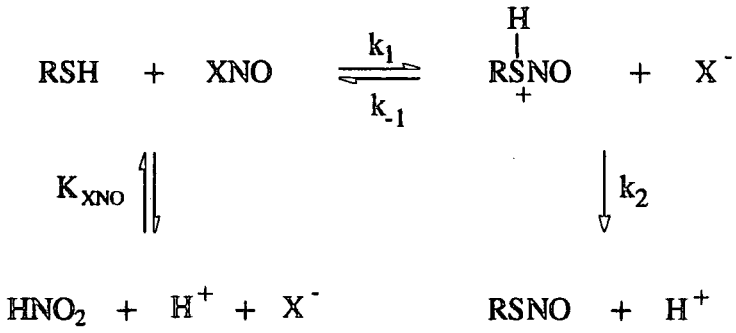
$$XNO + RSH \longrightarrow RS\overset{H}{\underset{+}{N}}O + X^- \qquad \text{eqn. 1.43}$$

where X = Cl, Br, I and SCN.

The observed rate equation is consistent with this being the rate limiting step (equation 1.44).

$$\text{Rate} = k\,[RSH]\,[HNO_2]\,[H^+]\,[X^-] \qquad \text{eqn. 1.44}$$

The catalysis increases with increasing nucleophilicity, such that $I^- > SCN^- > Br^- > Cl^-$. When studying observed rate dependence on nucleophile concentration, both chloride and bromide show linear dependence. For iodide and thiocyanate, however, plots of k_{obs} versus [nucleophile] level off at high concentration. These observations are explained if the initial nitrosation of the thiol is reversible (scheme 1.16). Thus nucleophilic attack by X^- on the nitrogen is competing with loss of H^+ in the final step.



Scheme 1.16

Under pseudo-first order conditions a rate equation (equation 1.45) is found with two terms, one for the uncatalysed reaction and one for nucleophilic catalysis.

$$\text{Rate} = k_o [HNO_2] \qquad \text{eqn. 1.45}$$

$$\text{where } k_o = \frac{k_1 k_2 K_{NOX}[RSH][H^+][X^-]}{k_{-1}[X^-] + k_2} + k_{HNO_2}[RSH][H^+] \qquad \text{eqn. 1.46}$$

The rate constant k_{HNO2} is for the acid catalysed reaction only and K_{XNO} is the equilibrium constant of formation of XNO.

For the weak nucleophiles Cl^- and Br^-, $k_{-1}[X^-] << k_2$ and equation 1.45 can be reduced to a form where k_1, the rate limiting step, can be calculated (equation 1.47).

$$k_o = k_1 K_{XNO} [RSH][H^+][X^-] + k_{HNO_2} [RSH][H^+] \qquad \text{eqn. 1.47}$$

The rate limiting constant k_1 can be obtained for SCN^- by manipulation of equation 1.46. At high nucleophile concentration $k_{-1}[X^-] >> k_2$ and the rate determining step becomes loss of a proton. At such nucleophilic concentrations the primary kinetic isotope effect, using deuterated compounds and solvents, should be observable.

The main difference between O and S nitrosation is that the former is reversible and the latter is essentially irreversible. Nitrosothiols can only be denitrosated at high acidity in the presence of a nitrous acid trap[43]. In comparing reactivities the relative nucleophilicities are important. Sulphur is more nucleophilic than oxygen. This is borne out in nitrosation studies, where N-acetylpenicillamine (as a model for t-BuSH) is much more reactive than tertiary butyl alcohol[16].

Oxygen is significantly more basic in organic molecules than sulphur (ΔpKa ~5), so the rate of the reverse reaction is greater for the alkyl nitrite. The forward reaction is governed by nucleophilicity (S > O) and the reverse reaction governed by basicity (O > S).

1.3.3 Reactions of Nitrosothiols

All nitrosothiols will decompose thermally and photolytically to give the disulphide (equation 1.48).

$$2RSNO \xrightarrow{h\nu/\Delta} RSSR + 2NO \qquad \text{eqn. 1.48}$$

This is due to the ease with which the S-N bond is homolytically broken. Indeed, nitrosation of thiols has been used as a synthetic method for making disulphides. The nitrosating species effectively oxidising the thiol.

Thionitrates can be prepared by oxidation of the nitrosothiol with fuming nitric acid[44] (equation1.49), although they will readily decompose to give the disulphide and thiosulphonate.

$$RSNO \xrightarrow{HNO_3} RSNO_2 \longrightarrow RSSR + R\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}SR \qquad \text{eqn. 1.49}$$

Reduction of nitrosothiols gives the thiol (equation 1.50). This is achieved with mercuric ion and a trap to remove the nitrous acid formed[45]. Usually sulphamic acid or sodium azide are used as traps.

$$RSNO \xrightarrow{Hg^{2+}} RSHg^{+} + HNO_2 \qquad \text{eqn. 1.50}$$

HNO_2 $\downarrow$ removed by trap

Similarly, nitrosothiols can be hydrolysed in strongly acidic solutions (1-4M H^+) with a nitrous acid trap. Once more this is catalysed by nucleophiles, in the same sequence $SCN^- > Br^- > Cl^-$ (equation 1.51).

$$RS\overset{H}{\underset{+}{N}}O + X^- \longrightarrow RSH + XNO \qquad \text{eqn. 1.51}$$

XNO $\downarrow$ removed by trap

The reaction between nitrosothiols and thiols is an efficient synthesis of mixed disulphides[46]. This reaction is very clean, producing the desired product and N_2O (equation 1.52).

$$RSNO \xrightarrow{R'SH} RSSR' \qquad \text{eqn. 1.52}$$

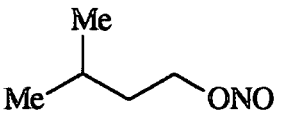
1.4 Physiological Roles of Nitric Oxide

1.4.1 Introduction

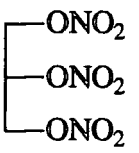
When nitric oxide was demonstrated to be physiologically important in the late 1980's a new area of biochemical and physiological research was opened. Nitric oxide has stimulated new research in chemistry, biochemistry, physiology and pharmacology. Its many essential biological roles have been discovered in the last six years. These include smooth muscle vasodilation, macrophage cytotoxicity neuro-transmission and platelet anti-aggregation. At present over 200 papers are published each month on some aspect of physiology or biochemistry of nitric oxide. A number of reviews have been published and amongst the most recent and significant are those by Moncada, Palmer and Higgs[47] and by Butler and Williams[3].

1.4.2 A History of NO

Organic nitrates and nitrites have been used in medicine for over 100 years, yet it was not understood how they worked until recently. In 1844 amyl nitrite (1.4) was synthesised and Guthrie[48] noted that inhalation of the vapours caused flushing of the face, throbbing of the carotid arteries and acceleration of the pulse rate. It was first used to treat angina in 1867[49]. Nitroglycerin (GTN) (1.5) was first synthesised around the same time as amyl nitrite, but it was not used to treat angina until 1879[50]. Organic nitrates are still widely used to treat conditions including angina, heart failure, hypertension and coronary angioplasty. However, by the end of the 19th century it was noted that munitions workers were becoming tolerant to the vasodilatory effects of nitroglycerin on prolonged exposure[51].



(1.4)

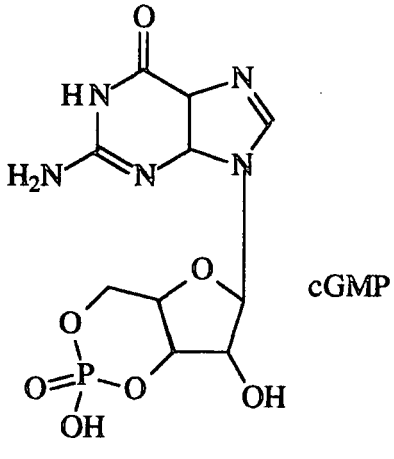


(1.5)

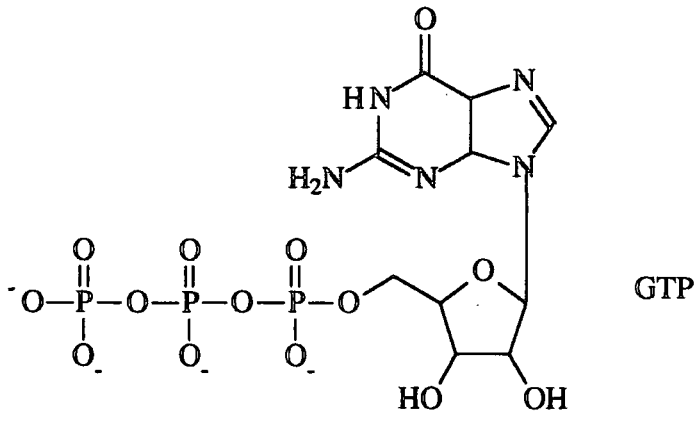
1.4.3 Vasodilation

It was believed that the vasodilator action of GTN was due to its conversion to nitrite, which has some vasodilator action. In the 1940's though, it was demonstrated that quantitative conversion of GTN to nitrite did not yield enough nitrite to account for all the vasodilation measured[52]. The conclusion drawn from this was that the vasodilatory action was due to the whole GTN molecule.

In 1980 Furchgott and Zawadzki[53] reported that aortic relaxation was dependent on the presence of the endothelial cells that line the artery walls. Relaxation can be triggered by a number of substances including acetylcholine and bradykinin, but they are dependent on the endothelial cells for their action. The substances must be acting on the endothelium layer, which then produces another substance that will act on the smooth muscle around the artery, causing relaxation. This second substance was termed the endothelium derived relaxing factor (EDRF). The EDRF passes into the sooth muscle and activates the soluble guanylate cyclase enzyme which increases intracellular levels of cyclic guanosine monophosphate[54] (cGMP) (1.6). The cGMP is derived from guanosine triphosphate (GTP) (1.7). The cGMP induces a sequence of protein phosphorylations associated with smooth muscle relaxation[47].



(1.6)



GTP

(1.7)

Work then concentrated on determining what the EDRF was, using various organ bath arrangements[55,56,58]. It was established that the EDRF was a short lived species with a half life of a few seconds[54] in oxygenated physiological buffer solution. It was also found that the physiological activity of EDRF was inhibited by haemoglobin, methylene blue[58] and hydroquinone[57]. However, the identity of EDRF remained unknown.

In parallel to this, work done on the mechanism of GTN induced vasodilation showed it to be independent of the endothelium[55]. The work on GTN and EDRF was converging. In 1986 both Furchgott[59] and Ignarro[60] suggested that EDRF may be nitric oxide (NO) or a closely related species. Subsequently this was shown to be the case by Palmer, Ferrige and Moncada in England and by Ignarro et.al. in the U.S. in 1987[61]. Endothelial cells were stimulated to produce EDRF and this was put through a chemiluminescent assay for NO. The assay is the reaction of NO with ozone[62] (scheme 1.3). It was shown[61] that the vascular relaxation and half lives of EDRF and NO were indistinguishable.

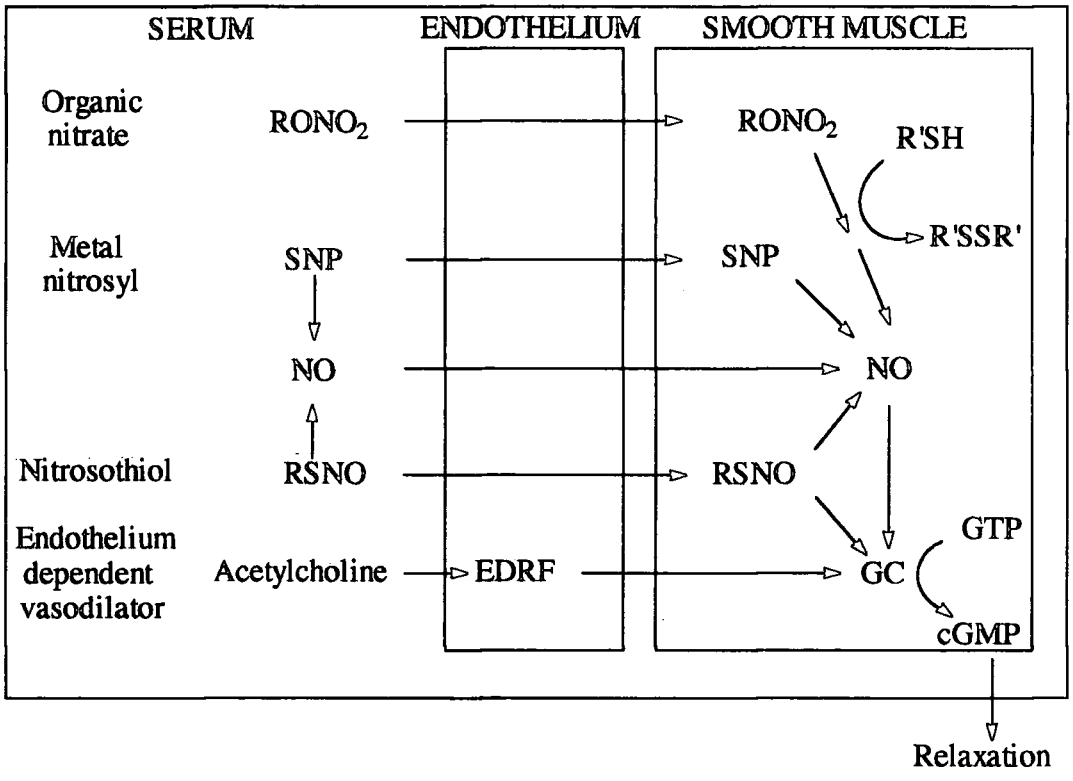
The identification of NO as EDRF explained why the vasodilatory action was inhibited by haemoglobin[58], and why it was prolonged by super oxide dismutase (SOD)[57]. Nitric oxide is known to have a large affinity for haemoglobin, whereas SOD removes O_2^- which reacts with NO (equation 1.53). Nitric oxide was also known to activate guanylate cyclase by binding to its haem site[63].

$$\dot{N}O + O_2^{\cdot} \longrightarrow ONOO^{-} \xrightarrow{H^+} \dot{N}O_2 + HO^{\cdot} \quad \text{eqn. 1.53}$$

Nitrovasodilators, which are endothelium independent, must be metabolised *in vivo* to give NO[64]. Organic nitrate metabolism requires a thiol, and intracellular thiol depletion is observed on treatment with GTN[64]. Nitrosothiols may be an intermediate in this reaction (figure 1.4). This may account for the nitrate tolerance. Vasodilators that do not require a thiol for activation, such as sodium nitroprusside (SNP), are found not to give rise to tolerance[65].

Figure 1.4

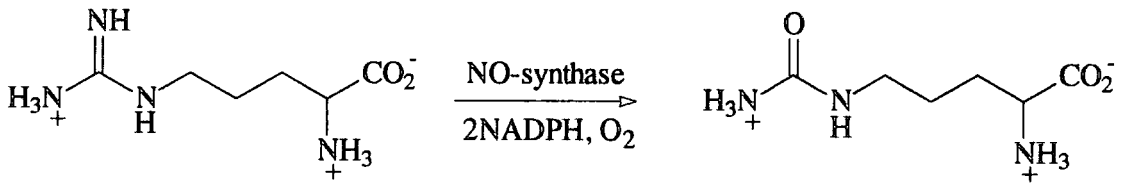
Mode of Action of Nitrovasodilators.



1.4.4 Biosynthesis of Nitric Oxide

In 1988 L-arginine was shown to be the precursor for NO in cell cultures[66]. The production of NO is substrate specific, D-arginine and L-homoarginine do not effect NO formation. The reaction is enzymatic and the product along with NO is L-citrulline (equation 1.54). The enzyme is called NO-synthase. Experiments with

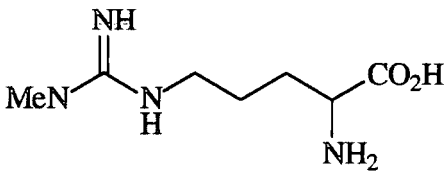
$^{18}O_2$ have shown that the enzyme incorporates molecular oxygen into both NO and L-citrulline[67].



eqn. 1.54 + NO

At least two NO-synthase enzymes have been isolated[68]. Both convert L-arginine to NO and L-citrulline. The NO-synthase found in endothelial cells is a constitutive enzyme, which means it is present all the time and is rapidly activated. It appears that the NO is being produced continuously preventing constriction of the artery[3]. It is important to say that the constitutive enzyme only produces picomoles of nitric oxide[47]. The other main characteristics of this enzyme[47] are that it is dependent on NADPH, Ca^{2+} and calmodulin, a protein that binds calcium.

The enzyme can be inhibited by using derivatives of L-arginine such as N-monomethyl-L-arginine (L-NMMA) (1.8)[69].



(L-NMMA)

(1.8)

Another NO-synthase is present in macrophages and will be discussed along with macrophage cytotoxicity (section 1.4.6).

1.4.5 Neurotransmission

Nerve signals are sent as electrical impulses along nerve cells. The gaps between the nerve cells are known as synapses and the chemical messenger that

crosses the synapse is a neurotransmitter. The major neurotransmitters are glutamate and aspartate and they are associated with increased levels of cGMP[70], paralleling smooth muscle vasodilation. Indeed, cultured brain cells produced NO[71] when stimulated and a NO-synthase was isolated[72].

The nitric oxide produced in the post synaptic nerve cell diffuses out of the cell and into the presynaptic and surrounding nerve cells. This strengthens the connection between nerve cells[73] and nitric oxide is one of the candidates for the retrograde messenger. The retrograde messenger passes back from the post synaptic nerve cell to the presynaptic nerve cell, acting as a feed-back loop. It has been suggested that over production of nitric oxide in the brain during a stroke may be the cause of brain damage as it can generate hydroxyl radicals[74]. NO also seems to have a role in the peripheral nervous system with the NANC nerve cells (non-adrenergic non-cholinergic). It is believed that NO is the neurotransmitter for these nerve cells[75].

1.4.6 Macrophage Cytotoxicity

Macrophages are cells which non-selectively protect the body against foreign substances by engulfing or killing the invader. The macrophages are found throughout the body. Before it was shown that nitric oxide was involved with macrophage activity there was a known correlation between urinary nitrate levels and the activity of the immune system. Cultured macrophages do indeed produce nitrite and nitrate[76] and the precursor common to both is NO. Macrophages can engulf foreign matter (phagocytosis) and also inject cytotoxic substances. Invading microbes can also be killed without contact with macrophages. It is the killing process which involves NO.

The enzyme responsible for macrophage production of NO is different to the NO-synthase found in endothelial cells. The macrophage NO-synthase[47] is inducible, that is it is not present until the macrophage is activated. There is a delay between macrophage activation and NO production. The enzyme is similar to the constitutive enzyme in that it is dependent on NADPH and is inhibited by L-NMMA and other

analogues[69]. However, the inducible enzyme is Ca^{2+}-calmodulin independent and it produces much more NO (nanomoles) for longer than the constitutive NO-synthase. Over production of NO by macrophages can lead to endotoxic shock[3], whereby blood pressure can fall to dangerously low levels, often this condition is fatal. Selective inhibition of the inducible enzyme by L-NMMA type compounds could be therapeutically used to counter the drop in blood pressure.

The toxicity of NO may be due to its radical character enabling it to destroy lipid membranes. Oxidation of NO by superoxide (a by-product of macrophage activity) leads to production of hydroxyl radicals (equation 1.53) which can destroy lipid membranes and DNA. Alternatively, NO may bind to iron-sulphur clusters in enzymes essential to metabolism. Such iron nitrosyls have been detected[77] by EPR techniques in tumor cells cultured with macrophages. This question of toxicity remains to be fully resolved.

NO is toxic towards bacteria as part of the bodies immune system and also to non-bacterial parasites. NO produced by macrophages will kill the protozoa *Leishmania major*[78].

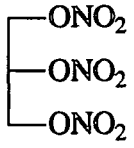
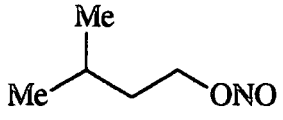
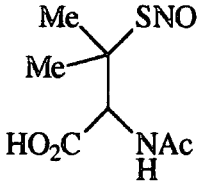
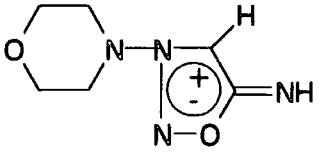
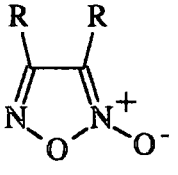
1.4.7 Platelet Aggregation

Blood plasma contains, among other things, cell fragment containing granules known as platelets. When bleeding occurs prostacyclin production leads to the blood platelets adhering together and to the vessel walls to plug the cut. Further aggregation leads to formation of a clot which prevents bleeding after the injury. NO inhibits platelet aggregation and vessel wall adhesion[79]. A heart attack may be caused by over clotting of platelets in a coronary vessel coated with a plaque of cholesterol. The blockage of the coronary blood vessel restricts the oxygen supply to the heart resulting in a heart attack. Prostacyclin and NO act in a synergy to inhibit platelet aggregation and adhesion[79]. However, there is no synergy in initial platelet adhesion. NO acts as a feedback to aggregation after injury. The NO is produced by endothelial cells which come into contact with platelets and also by an arginine reaction with an enzyme in the platelets.

1.4.8 Nitrovasodilators

Nitrovasodilators are compounds which can spontaneously produce NO, or compounds which release NO after activation or reaction with another compound. They bypass the endothelium delivering NO to the smooth muscle. The different types of nitrovasodilators and the chemicals required for activation[40] are listed below (table 1.2).

Table 1.2

Nitrovasodilator type	Example	Co-factors for activation
Organic nitrates	 GTN	Cysteine/ or enzyme
Organic nitrites	 Iso-amyl nitrite	Any thiol
Nitrosothiols	 SNAP	*hν*/Δ (also see chapter 4)
Sydnonimines	 SIN-1	HO^-, O_2
Furoxans		Any thiol
Metal nitrosyls	$Na_2[Fe(CN)_5NO]$ SNP	*hν*

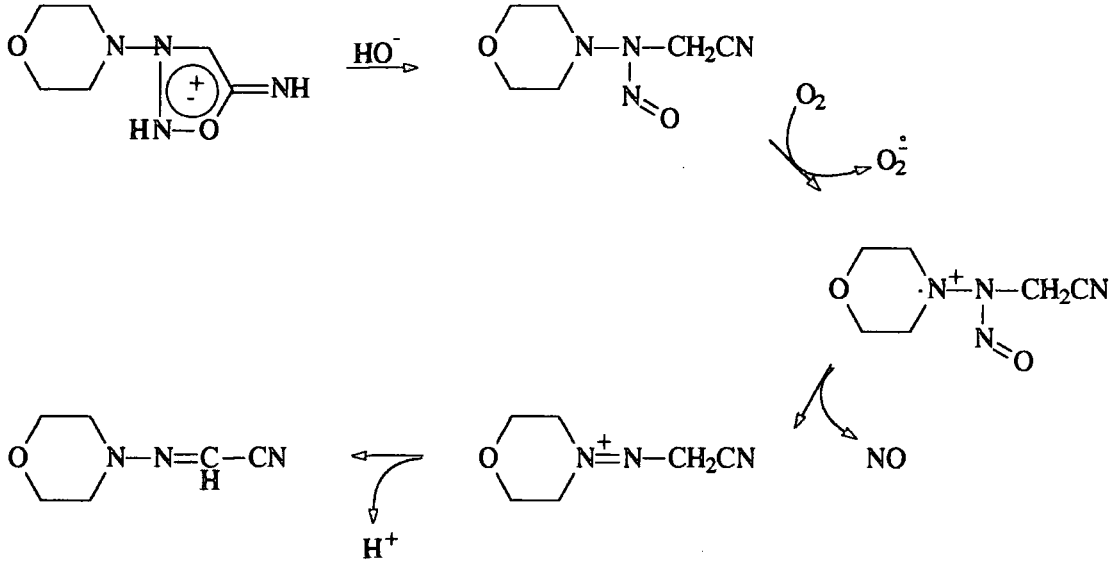
Organic nitrates have to react with thiols in order to release NO. It is suggested[80] that there are enzymatic and non-enzymatic processes and one mirrors the other. Thiols such as cysteine and N-acetylcysteine decompose organic nitrates to generate NO, but most others generate nitrite only[40]. Organic nitrites like amyl nitrite require any thiol as a co-activator. The transnitrosation reaction (equation 1.55) produces a nitrosothiol which decomposes to NO (equation 1.56).

$$RONO + R'SH \rightleftharpoons ROH + R'SNO \qquad \text{eqn. 1.55}$$

$$2R'SNO \longrightarrow R'SSR' + 2NO \qquad \text{eqn. 1.56}$$

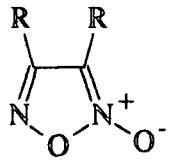
S-Nitrosothiols decompose to give NO. These compounds have anti-bacterial and anti-oxidant properties in cured meats[40]. They are also known to be potent vasodilators[81] and inhibit platelet activation[82]. The mechanism of nitrosothiol decomposition in solution was not understood and will be discussed later.

Sydnonimines undergo attack by hydroxide to give an N-nitrosamine followed by aerial oxidation which releases NO[40] (scheme 1.16).



Scheme 1.16

Furoxans are another class of heterocyclic compounds that will react to produce a thiol[40]. A thiol is required for reaction to give a nitrosothiol which will then release NO on decomposition (equation 1.57).



+ R'SH ⟶ R'SNO ⟶ R'S· + ·NO eqn. 1.57

There are a number of metal nitrosyls which will donate NO. Those that have been studied are sodium nitroprusside (1.9) and Roussin's Black Salt (RBS) (1.10). Sodium nitroprusside is known to release NO on photolysis[83], however the non-photochemical release is not understood. Roussin's Black Salt decomposes chemically and photolytically[3] to release NO, it also has a high lipid solubility. The lipid solubility makes RBS more potent as it is not removed by fluid flow.

$Na_2[Fe(CN)_5NO]$ (1.9)

$Na[Fe_4S_3(NO)_7]$ (1.10)

1.5 Conclusion

NO has a long history in physiology, it is known to be present in the limulus crab which has existed for over 300 million years[84]. Recently it has found the attention of the quality press, finding a place in The Independent as "Molecule of the Month". The discovery and work on NO *in vivo* has given us a new insight into how our bodies work and possible therapeutic compounds for a range of conditions including, angina, hypertension, septic shock and bacterial infection. Understanding how nitrovasodilators produce NO is extremely important if we are to tailor compounds for selective use in the body.

References

1. K. Jones in Comprehensive Inorganic Chemistry, Vol. 2, Eds. J.C. Bailar, H.R.Emeleus, R. Nyholm & A.F. Trotman-Dickenson ,Pergamon Press ,New York , 1973, 329.
2. J.N. Armor, *J. Chem. Eng. Data*, 1974, Vol. 19, No. 1, 82.
3. A.R. Butler & D.L.H. Williams, *Chem. Soc. Rev.*, 1993, 233.
4. G.B. Richter-Addo & P. Legzins, 'Metal Nitrosyls', Oxford University Press, NY, 1992.
5. G.D. Stucky, R.R. Rye, D.R. Jennison & J.A. Kelber, *J. Am. Chem. Soc.*, 1982, 104, 5951.
6. I.H. Sabherwal, A.B. Burg, *J. Chem. Soc., Chem. Commun.*, 1970, 1001.
7. N.G. Connelly, F.F. Dahl, *J. Chem. Soc., Chem. Commun.*, 1970, 880.
8. S.D. Pell & J.N. Armor, *J. Am. Chem. Soc.*, 1973, 95, 7625.
9. W.A. Freeman, *J. Am. Chem. Soc.*, 1983, 105, 2725.
10. E.H. Bartlett & M.D. Johnson, *J. Chem. Soc. A*, 1970, 523.
11. J. Haldane, *J. Hyg.*, 1901, 1, 115.
12. Q.H. Gibson & F.J.W. Roughton, *J. Physiol. London*, 1957, 136, 507.
13. J. Tummavuori & P. Lumme, *Acta Chem. Scand.*, 1968, 22, 2003; P. Lumme, P. Lahermo & J. Tummavuori, *ibid.*, 1965, 19, 9; P. Lumme & J. Tummavuori, *ibid.*, 1965, 19, 617.
14. J.H. Ridd, *Quart. Rev.*, 1961, 15, 418.
15. D.L.H. Williams, Nitrosation, CUP, Cambridge, 1988, 3.
16. S.E. Aldred & D.L.H. Williams, *J. Chem. Soc. Perkin Trans. II*, 1982, 777.
17. E.D. Hughes, C.K. Ingold & J.H. Ridd, *J. Chem. Soc.*, 1958, 88.
18. L.R. Dix & D.L.H. Williams, *J. Chem. Res. (S)*, 1982, 190.
19. C.A. Bunton & G. Stedman, *J. Chem. Soc.*, 1959, 3466.
20. Reference 15, p.2.
21. G.A. Olah & J.A. Olah in Friedel Crafts and Related Reactions, Vol. 3, part II., Ed. G.A. Olah, Interscience, New York, 1964, 1267.
22. J.M. Simpson, D.C. Kapp & T.M. Chapman, *Synthesis*, 1979, 100.

23. L.J. Beckmann, W.A. Fessler & M.A. Kise, *Chem. Rev.*, (1951), 48, 319.

24. Reference 15, 54.

25. Reference 15, 13.

26. G. Stedman & P.A.E. Whincup, *J. Chem. Soc.*, 1963, 5796.

27. O. Touster in Organic Reactions, Ed. R. Adams, Wiley, New York, 1966, Vol. 2, 328.

28. Reference 15, 36.

29. S. Veibel, *Ber.*, 1930, 63, 1577.

30. P.A.S. Smith & R.N. Loeppky, *J. Am. Chem. Soc.*, 1967, 89, 1147.

31. A.D. Allen, *J. Chem. Soc.*, 1954, 1968.

32. Reference 15, 152.

33. J.H. Ridd, *Adv. Phys. Org. Chem.*, 1978, 16, 22.

34. A.D. Allen & G.R. Schonbaum, *Can. J. Chem.*, 1961, 39, 940.

35. H. Rheinboldt, *Ber.*, 1926, 59, 1311.

36. G. Kresze & U. Uhlich, *Chem. Ber.*, 1959, 92, 1048.

37. L. Field, R.V. Dilts, R. Ravichandran, P.G. Lenhert & G.E. Carnahan, *J. Chem. Soc., Chem. Commun.*, 1978, 249.

38. J. Barrett, D.F. Debenham & J. Glauser, *Chem. Comm.*, 1965, 12, 248.

39. L.J. Ignarro, B.K. Barry, D.Y. Gruetter, J.C. Edwards, E.H. Ohlstein, C.A. Gruetter & W.H. Barricos, *Biochem. Biophys. Res. Comm.*, 1980, Vol. 94, No. 1, 93.

40. M. Feelisch, *J. Cardiovasc. Pharmacol.*, 1991, 17(Suppl.3), 25.

41. W.A. Pryor, D.F. Church, C.K. Govindan & G. Crank, *J. Org. Chem.*, 1982, 47, 156.

42. P.M. Rao, J.A. Copeck & A.R. Knight, *Can, J. Chem.*, 1967, 45, 1369.

43. S.S. Al-Kaabi, D.L.H. Williams, R. Bonnett & S.L. Ooi, *J. Chem. Soc., Perkin Trans. 2*, 1982, 227.

44. H. Rheinboldt & F. Mott, *Ber.*, 1932, 65, 1223.

45. B. Saville, *Analyst*, 1958, 83, 670.

46. S. Oae, Y.H. Kim, D. Fukushima & T. Takata, *Chem. Lett.*, 1977, 893.

47. S. Moncada, R.M.J. Palmer & E.A. Higgs, *Pharmacol. Rev.*, 1991, 43, 109.

48. F. Guthrie, *Q. J. Chem. Soc.*, 1859, 11,245.

49. T.L. Brunton, *Lancet*, 1867, 2, 97.

50. W. Murrell, *Lancet*, 1879, 1, 80.

51. G.C. Laws, *J.A.M.A.*, 1898. 31, 793.

52. J.C. Krantz Jr.,C.J. Carr, S.E. Forman & N. Cone, *J. Pharmacol. Exp. Ther.*, 1940, 70, 323.

53. R.F. Furchgott & J.V. Zawadzki, *Nature*, 1980, 288, 373.

54. F. Murad, C.K. Mittal, W.P. Arnold, S. Katsuki & H. Kimura, *Adv. Cyclic Nucleotide Res.*, 1978, 9, 145.

55. R.F. Furchgott, *Ann. Rev. Pharmacol. Toxicol.*, 1984, 24, 175.

56. T.M. Griffith, D.H. Edwards, M.J. Lewis, A.C. Newby & A.H. Henderson, *Nature*, 1984, 308, 645.

57. R.J. Gryglewski, R.M.J. Palmer & S. Moncada, *Nature*, 1986, 320, 454.

58. W. Martin, G.M. Villani, D. Jothianandem and R.F. Furchgott, *J. Pharmacol. Exp. Therap.*, 1985, 232, No.3, 708.

59. R.F. Furchgott in Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium, Ed. P.M. Vanhoutte, Raven Press, New York, 1988, pp 401-414.

60. L.J. Ignarro, R.E. Byrns & K.S.Wood, ibid., pp 427-436.

61. R.M.J. Palmer, A.G. Ferrige & S. Moncada, *Nature*, 1987, 327, 524; L.J. Ignarro, G.M. Buga, K.S. Wood & G. Chaudhuri, *Proc. Natl. Acad. Sci., USA*, 1987, 84, 9265.

62. M.J. Downes, M.W. Edwards, T.S. Elsey & C.L. Walters, *Analyst*, 1976, 101, 742.

63. C.A. Gruetter, D.Y. Gruetter, J.E. Lyon, P.J. Kadowitz & L.J. Ignarro, *J. Pharmacol. Exp. Ther.*, 1981, 219, 181.

64. J. Abrams, *The American Journal of Medicine*, 1990, 91(Suppl.3C), 106.

65. S. Moncada, R.M.J. Palmer & E.A. Higgs, *Hypertension*, 1988, Vol.12, No.4, 365.

66. R.M.J. Palmer, D.S. Ashton & S. Moncada, *Nature*, 1988, 10.

67. A.M. Leone, R.M.J. Palmer, R.G. Knowles, P.L. Francis, D.S. Ashton & S. Moncada, *J. Biol. Chem.*, 1991, 266, No.35, 23790.

68. D.S. Bredt & S.H. Snyder, *Proc. Natl. Acad. Sci., USA*, 1990, 87, 682.

69. S. Moncada, E.A. Higgs, H.F. Hodson, R.G. Knowles, P. Lopez-Jaranillo, T. McCall, M.W. Radomski & D.D. Rees, *J. Cardiovasc. Pharmacol.*, 1991, 17(Suppl. 3), 1.

70. J. Garthwaite, *TINS*, 1991, Vol.14, No.2, 60.

71. J. Garthwaite, S.L. Charles & R. Chess-Williams, *Nature*, 336, 385.

72. R.G. Knowles, M. Palacios, R.M.J. Palmer & S. Moncada, *Proc. Natl. Acad. Sci., USA*, 1989, 86, 5159.

73. M. Baringa, *Science*, 1991, 254, 1296.

74. J. Kanner, S. Harel & R. Granit, *Arch. Biochem. Biophys.*, 1991, 289, 130.

75. H. Bult, G.E. Boeckxstaens, P.A. Pelckmans, F.H. Jordaens, Y.M. Van Maercks & A.G. Herman, *Nature*, 1990, 345, 346.

76. D.J. Steuhr & M.A. Marletta, *Cancer Res.*, 1987, 138, 550.

77. A.R. Butler, C. Glidewell, A.R. Hyde & J.C. Walton, *Polyhedron*, 1985, 4, 303.

78. F.Y. Liew, S. Millott, C. Parkinson, R.M.J. Palmer & S. Moncada, *J. Immunol.*, 1990, 144, 4794.

79. R.M.J. Palmer, S. Moncada & M.W. Radomski, *Brit. J. Pharmacol.*, 1987, 92, 181.

80. M. Feelisch & E. Noack, *Eur. J. Pharmacol.*, 1987, 139,19.

81. W.R. Mathews & S.W. Kerr, *J. Pharmacol. Exp. Ther.*, 1993, 267, No.3, 1529.

82. M.W. Radomski, D.D. Rees, A. Dutra & S. Moncada, *Br. J. Pharmacol.*, 1992, 107, No. 3, 745.

83. F.W. Flitney & G. Kennovin, *J. Physiol.*, 1987, 392, 43P.

84. T. McCall & P. Vallance, *TIPS*, 1992, 13, 1.

CHAPTER 2

Detection of Nitric Oxide

2 Detection of Nitric Oxide

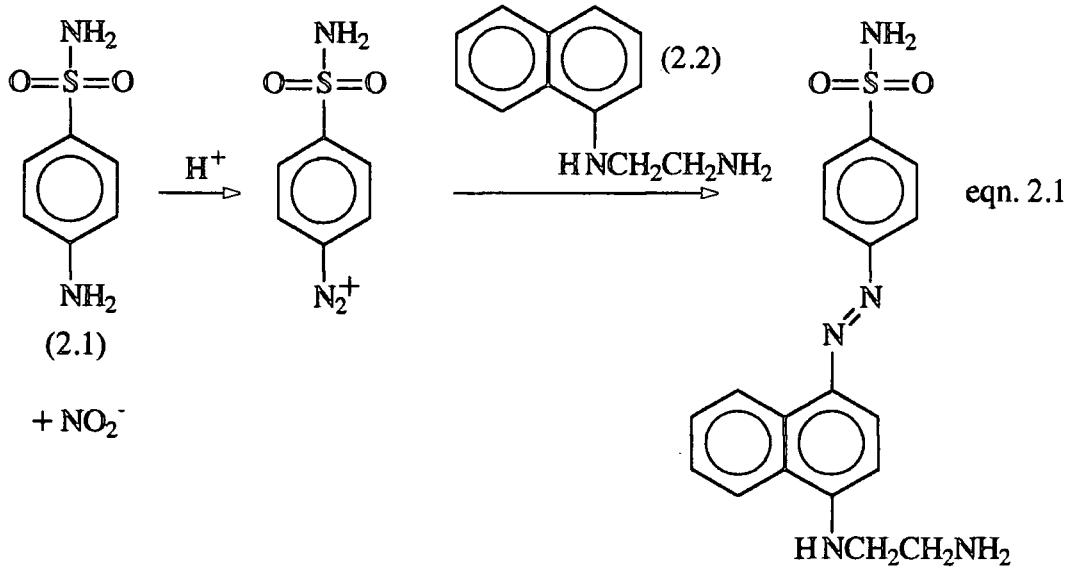
If we are to study the chemistry of NO donors, in particular nitrosothiols, and the chemistry of NO itself we need to find methods of detecting NO reliably and accurately. Recently a review on methods of NO detection has been published[1] describing methods to be used in conjunction with biological studies of NO. Any such method for measuring NO has to be calibrated with authentic NO. NO is commercially available in lecture bottles, but the gas still has to be bubbled through NaOH to ensure removal of all higher oxides. An easier and more economical method is reduction of nitrite by ascorbic acid (section 1.2.8) followed by the same higher oxide removal. The main advantage of this method is that the total amount of NO produced can be controlled.

2.1 Methods of Detection

Most methods of NO detection involve an indirect measurement of NO. A reaction is carried out and the products are used as the measure of NO present. A very sensitive assay of NO is the measurement of chemiluminescence produced by reaction with ozone[2]. The reaction produces NO_2 in an excited state which on relaxation emits light. Obviously the light produced is proportional to the concentration of NO. This method requires the NO to be in the gas phase, so NO in solution has to be expelled by bubbling an inert gas into the solution. The ozone is generated by electrical discharge and is mixed with the NO/inert gas mixture in front of a photo-multiplier tube. Using this method down to 20 picomoles of NO can be detected. The assay is specific for NO but if the sample is refluxed in either acid or reducing conditions then NO can be produced from nitrite, nitrosothiols and other NO generating compounds.

It is known that nitrosothiols will decompose to disulphide and nitric oxide and that the ultimate fate of the nitric oxide in solution is nitrite[3]. The standard test for inorganic nitrites may therefore be carried out to determine the original NO content. The analysis[4] involves diazotization of sulphanilamide (2.1) by the acidified nitrite,

followed by coupling to N-(1-naphthyl)-ethylenediamine (2.2) (equation 2.1). The product is an azo-dye which absorbs strongly in the visible region at around 550nm.



eqn. 2.1

The detection limits of this assay are 1μM-400μM. Contamination by nitrite will give an artificially high reading and care should be taken to ensure that all glass ware is thoroughly cleaned.

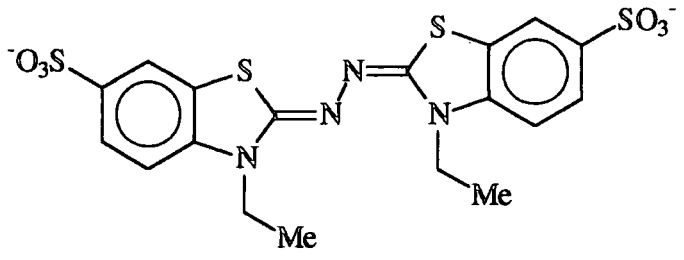
Paramagnetic NO can form stable complexes which are also paramagnetic and can be detected using electron paramagnetic spin resonance spectroscopy (EPR)[5]. Haemoglobin can be used as a spin trap because nitrosyl-haemoglobin is readily measured by EPR. Haemoglobin is commercially available and easily reduced to Fe^{II} haemoglobin using dithionite. The detection limit on this assay is 1 nanomolar.

The discussed methods all have disadvantages to being used to determine NO produced by nitrosothiols, they are either non-specific or very expensive to set up. Alternative methods for NO detection, using uv/vis spectrophotometry where possible were investigated.

The NO donor compounds under study are nitrosothiols which are known to decompose in solution. However, the decomposition is erratic[3] so direct measurement of RSNO decomposition is not helpful. Detection of the breakdown products may elucidate the reasons for this.

2.2 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (2.3) will undergo oxidation to give a species which absorbs strongly in the visible region of the spectrum (fig 2.1).



(2.3)

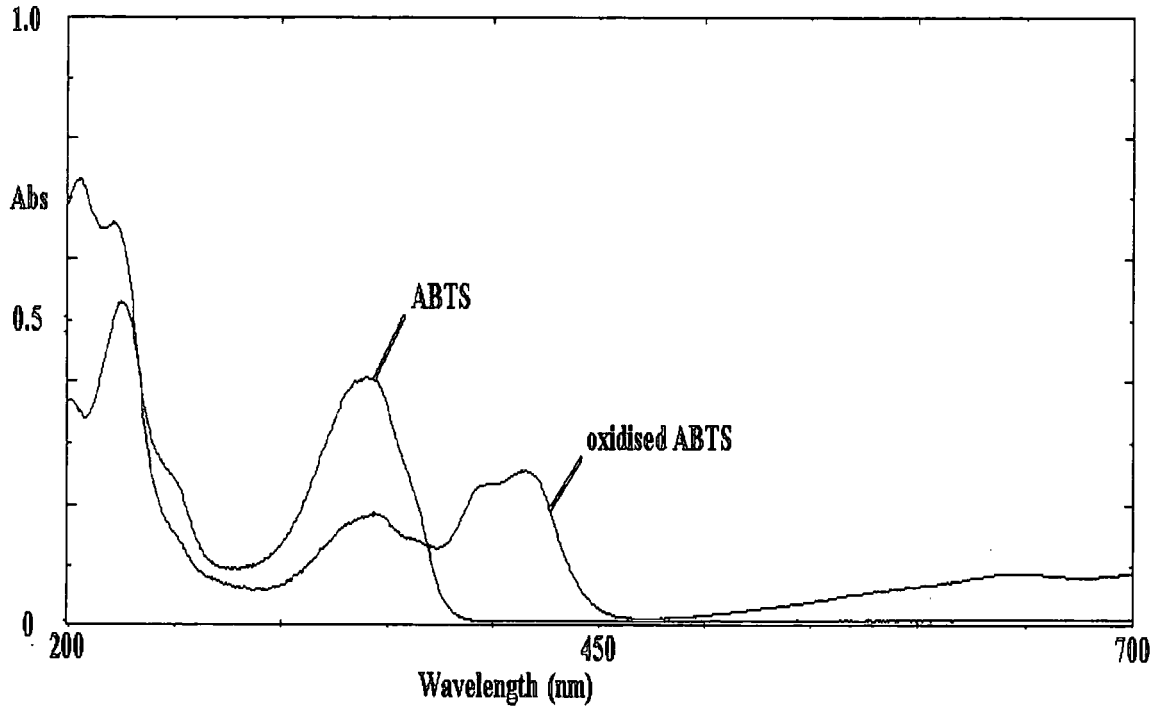
The assay was designed for analysing nitrite[6] in an acidic medium to determine nitrite levels in rainwater.

2.2.1 Reaction of ABTS with NO

ABTS was reacted with NO generated in unbuffered solution by nitrite and ascorbic acid. The ascorbic acid was in excess over the nitrite. No change in the absorbance spectrum was observed. The reaction was repeated by generating NO gas and bubbling this into an aerobic unbuffered solution of ABTS. Oxidation of the ABTS occurred rapidly and the solution turned a dark green colour. New absorbances appeared at 420nm and 645nm with ε values of 24800 and 8800 mol^{-1} dm^3 cm^{-1} respectively (fig 2.1).

Figure 2.1

Spectra showing the different absorbances ofABTS and oxidised ABTS.



If the NO was added to an anaerobic solution of ABTS then no reaction occurred. Addition of ascorbic acid to the oxidised ABTS decolourised the solution because it is a reducing agent. No oxidation was observed in the first experiments because the excess ascorbic acid was reducing any product formed.

2.2.2 Reaction of ABTS with Nitrosothiols

ABTS was reacted with an excess of S-nitroso-N-acetylpenicillamine (SNAP) in unbuffered solution and the reaction monitored at 645nm. There was little reaction because at this pH (~3.1) the SNAP is very stable. The reaction was repeated at pH 7.5 in dimethylglutaric acid (DGA) buffer. This buffer was chosen because it has a low absorbance across the spectrum. The ABTS was oxidised upon the breakdown of the SNAP. A calibration curve was constructed by reacting different ABTS concentrations (unbuffered pH ~5) with an excess of NO generated separately. As the object of the assay was to detect the breakdown of nitrosothiols the ABTS was put in excess. Varying SNAP concentrations gave little variation in k_{obs} (table 2.1).

Table 2.1

First order rate constants for the oxidation of ABTS (1mM) by SNAP at pH 7.4

10^5[SNAP] (M)	$10^4 k_{obs}$ (s^{-1})
2.0	3.4
1.5	3.1
1.25	2.6
1.0	3.8

Repeating the reaction in anaerobic conditions gave no reaction. If NO will not react with ABTS then the reaction must be with a higher oxide of nitrogen (scheme 2.1).

$$2RSNO \longrightarrow RSSR + 2NO$$

$$NO \xrightarrow{O_2} NO_x$$

$$NO_x + ABTS \longrightarrow ABTS^+ + NO_x^-$$

Scheme 2.1

Using the calibration curve for ABTS oxidation it is possible to calculate the yield from the reaction with SNAP. The yields varied between 60% and 90%, the latter figure being almost quantitative.

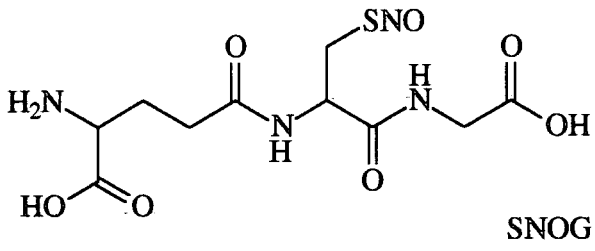
The reaction was repeated with a different water supply containing virtually no Cu^{2+} and no reaction was observed. Addition of Cu^{2+}, which catalyses the breakdown of nitrosothiols (see chapter 4), promoted the reaction with ABTS. Varying the concentrations of the SNAP and ABTS did not affect the observed rate constant (table 2.2). The rate determining step is either the breakdown of SNAP or the oxidation of NO. The kinetic traces produced were not very good first order curves so ABTS is not a satisfactory method of NO measurement.

Table 2.2

First order rate constants for the oxidation of ABTS by SNAP decomposition in pH 7.0 dimethyl glutaric acid buffer with 2 x 10^{-5}M Cu(II).

[SNAP] (mmol dm^{-3})	[ABTS] (mmol dm^{-3})	10^3 k_{obs} (s^{-1})
0.5	10	10.8
0.25	10	10.0
0.125	10	9.4
0.125	5	10.2
0.125	2.5	10.3

Another nitrosothiol, S-nitrosoglutathione (SNOG) (2.4), was reacted under the same conditions with ABTS. This compound was known to be very stable to decomposition in solution. A control solution containing no SNOG was also run in parallel. As expected the reaction was extremely slow progressing over a period of days.



SNOG

(2.4)

2.2.3 Reaction of ABTS with Nitrous Acid

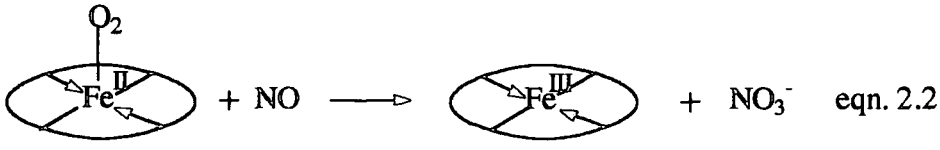
The reaction of ABTS with nitrite in pH 7 buffered solution was followed at 645nm. There was no appreciable reaction over a period of hours. At neutral pH the assay was effectively nitrite blind.

ABTS was then reacted with nitrous acid as an alternative calibration to the NO reaction and to try to determine the identity of "NO_x". The nitrous acid oxidised

the ABTS to a much greater extent than the NO reaction. The oxidising reagent in the ABTS/SNAP reaction was not like nitrous acid (at pH 7 there would only be nitrite present anyway).

2.3 NO Detection Using Haemoglobin

Oxy-haemoglobin will react rapidly with nitric oxide to give met-haemoglobin and nitrate (equation 2.2).



eqn. 2.2

The reaction is monitored spectrophotometrically by observing the Soret absorbance peak move from 416nm to 405nm. The haemoglobin was obtained from a sample of blood[3] generously donated by Prof. Lyn Williams. The haemoglobin was diluted in an anaerobic pH 7.4 phosphate buffer and NO was passed through. The reaction was rapid with λ_{max} moving from 416nm to 405nm. SNAP was reacted with a kinetic excess of haemoglobin under the same conditions and was followed at 400nm. The reaction was extremely slow taking in excess of 12 hours. A haemoglobin solution was also run as a control experiment. Diffusion of oxygen into the cells oxidised the haemoglobin at a similar rate but to a lesser extent to that of reaction with SNAP.

An excess of SNAP was reacted with haemoglobin and observed at 400nm. The reaction was zero order in haemoglobin but some scatter of values was found on changing SNAP concentrations (table 2.3).

Table 2.3

Zero order rate constants for the oxidation of oxy haemoglobin (1 x 10^{-5}M) by SNAP at pH 7.4 in deaerated buffer solution.

10^4[SNAP] (mol dm^{-3})	$10^4 k_{obs}$ (mol dm^{-3} s^{-1})
7.4	4.8
7.3	10.4
4.2	0.9
3.4	4.7
1.4	0.3

The scattering was seen because the experiments were difficult to repeat as aerial oxidation interfered with the reaction. However, the reactions were always zero order in haemoglobin. The breakdown of the SNAP must be rate limiting followed by rapid reaction of the NO with oxy-haemoglobin (scheme 2.2).

$$RSNO \xrightarrow{slow} \tfrac{1}{2} RSSR + NO$$

$$NO + HbO_2 \xrightarrow{fast} MetHb + NO_3^-$$

Scheme 2.2

It appeared that the SNAP was more stable in the presence of oxy-haemoglobin than SNAP alone in buffer. It has subsequently been shown that nitrosothiol decomposition is mediated by Cu^{2+} (see chapter 4). Any Cu^{2+} present in solution would be complexed by the haemoglobin protein drastically reducing the decomposition of the nitrosothiol. The reaction with oxy-haemoglobin is dependent on the decomposition of the nitrosothiol. A direct reaction between a nitrosothiol and haemoglobin would be useful to study since this may give an indication of the *in vivo*

role of nitrosothiols in activating the enzyme guanylate cyclase as part of the mechanism of vasodilation.

Kinetic methods of measuring the nitrosyl-haemoglobin complex have been published[7]. The amount of haemoglobin (Fe^{II}) is measured optically and then reacted with NO; the amount of met-haemoglobin (Fe^{III}) present at the end of the experiment is obtained and the difference is taken to be due to nitrosyl-haemoglobin.

Measurement of the disappearance of haemoglobin (Fe^{II}) can thus be correlated to nitrosyl haemoglobin production. A kinetic excess of SNAP was reacted with haemoglobin in an anaerobic pH 7.4 phosphate buffer solution and measured at 574nm as a decrease of the met-haemoglobin absorbance. The haemoglobin was prepared by passing air through the haemoglobin solution to oxidise all the HbO_2 to MetHb and then reducing to Hb with an equivalent of dithionite. First order rate constants for the reaction were obtained (table 2.4). Once again the results were repeatable within one set of runs, but on repeating with a new solution the results varied. Thus it was not possible to have exactly the same conditions each time.

Table 2.4

Pseudo-first order rate constants for the reaction of SNAP with reduced haemoglobin at pH 7.4

10^4[SNAP] (mol dm^{-3})	$10^4 k_{obs}$ (s^{-1})
4.5[a]	2.05
4.5[a]	1.86
3.0[a]	1.11
3.0[a]	1.08
1.5[a]	0.65
0[a]	0
3.0[b]	1.94
3.0[b]	1.90
3.0[b]	1.97
3.0[b]	2.57
3.0[b]	1.97

[Hb] = 1.4×10^{-5} mol dm^{-3}, measurements taken at 574nm and 298K.

a. one set of runs from same solution of freshly reduced Hb

b. second set of runs with new solution of reduced Hb

If we take the results from the first experiment and plot k_{obs} against [SNAP] we can obtain a value for the second order rate constant for the reaction between Hb and SNAP (equation 2.3).

$$\text{Rate} = k_2 [\text{Hb}][\text{SNAP}] \qquad \text{eqn. 2.3}$$

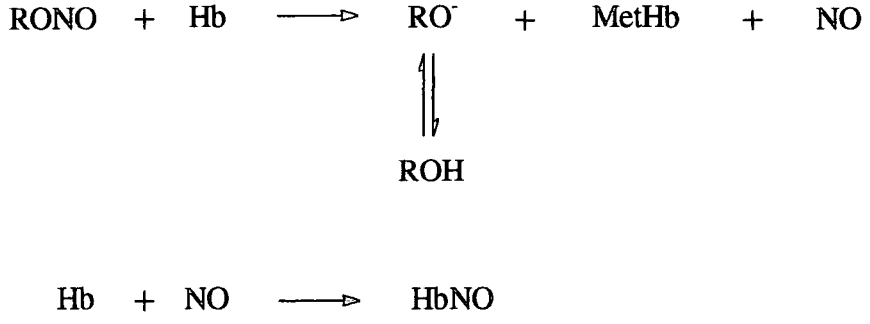
The second order rate constants for reaction between Hb and nitrite and ethyl nitrite are known[7] and can be compared with the value obtained for SNAP (table 2.5).

Table 2.5

Second order rate constants for the reaction of nitrosating agents with Hb

Nitrosating agent	k_2 (mol^{-1} dm^3 s^{-1})	pH (temp °C)
HNO_2	2.69	7.0 (25)
EtONO	1120	7.0 (10)
SNAP (RSNO)	0.42	7.4 (25)

The alkyl nitrite is more reactive than the nitrous acid at neutral pH as expected, there will only be a small amount of free nitrous acid present as its pK_a is 3.1[8]. The nitrosothiol is the least reactive. The reaction between Hb and RONO is thought to involve reduction of the alkyl nitrite[7] to NO (scheme 2.3), followed by reaction with Hb.



Scheme 2.3

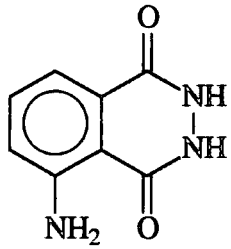
A similar reaction can be envisaged for the nitrosothiol, so the difference in k_2 must be due to a difference in the reactivity with Fe^{II}haem.

This work was a preliminary study into whether or not the reaction with haemoglobin was a good method of observing nitrosothiol decomposition. Further investigation of the reactions with haemoglobin needs to be carried out before any

conclusions can be drawn. However, this lies outside the scope of this study which is concerned with nitrosothiols and nitric oxide production.

2.4 NO Detection Using Luminol

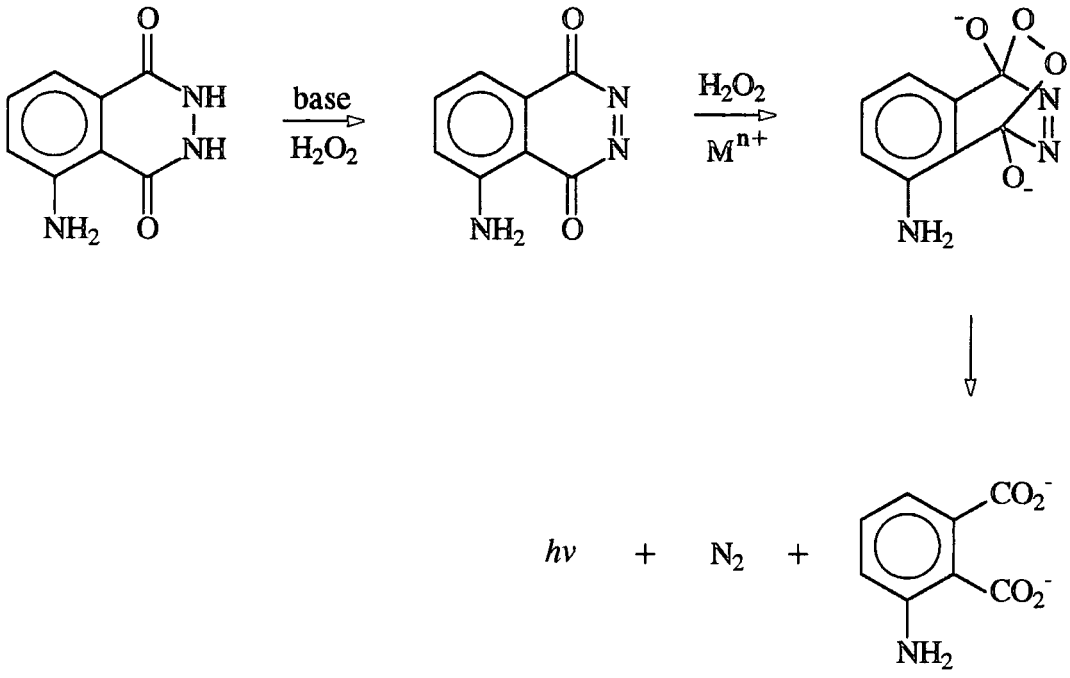
In 1928 Albrecht reported the emission of light from a reaction of luminol[9] (5-amino-2,3-dihydrophthalazine-1,4-dione) (2.5).



(2.5)

Under basic conditions luminol will undergo oxidation in the presence of hydrogen peroxide and transition metal ions to form an excited intermediate which liberates N_2 (scheme 2.4). It is believed that the metal ions and hydrogen peroxide react to give oxygen and either the hydroperoxy or hydroxyl radical[10].

Nitrogen dioxide can be substituted for the metal ions and the chemiluminescence used to measure the amount of NO_2 present[11]. This assay was developed to measure NO_2 levels from air samples taken from high up in the atmosphere. However, NO can also react with the H_2O_2 and luminol giving rise to luminescence[12]. This method of detection can only be as sensitive as the chemiluminescence detector used.



Scheme 2.4

Nitric oxide solutions were prepared in deaerated distilled water produced by passing N_2 gas through and then saturating with NO generated by reduction of nitrite with ascorbic acid. Solutions of NO were then prepared by diluting the saturated solution with more dearated distilled water. A 15mM stock luminol solution was prepared along with a 0.19M H_2O_2 solution. The reaction was carried out in buffer solutions and the chemiluminescence at 420nm detected by a Perkin Elmer LS50 spectrofluorimeter.

Initially the reaction was carried out at pH 7.4 in DGA buffer over the NO range 10-50μM. Taking the peak height as the measure of NO, the change with concentration was not linear. The readings were not repeatable either, the NO solution being oxidised over a period of hours. It took 2 minutes to reach a maximum reading and then over 15 minutes for the chemiluminescence to drop back to the background level. Increasing the pH increased the intensity of chemiluminescence and decreased the time to reach its maximum. However, at pH 10 the H_2O_2 was unstable in solution. The NO standard solutions still gave inconsistent results and even within one sample there was no linear relationship between NO and intensity of chemiluminescence. Even though the method remained uncalibrated it was used to

attempt to observe NO production from the decomposition of SNAP. Aliquots of a SNAP solution were taken at known times and added to a pH 9.0 buffer solution which effectively prevented further decomposition and then reacted with luminol and H_2O_2. Due to the length of time required to measure each sample and the susceptibility of NO to oxidation only a small number of readings were taken. The result was a very rough curve showing the increase of NO with time, but it was not possible to analyse the curve. Again this method proved not to be useful in the measurement of nitrosothiol decomposition.

2.5 Measurement by NO Specific Electrode

Detection of NO by an electrode is the most direct method of measuring NO levels in solution. To date there are two types of electrode. Malinski[13] and Taha have developed a micro electrode. A nickel(II) semi-conducting porphyrin polymer is deposited on carbon fibres and covered with a negatively charged Nafion membrane. The metallo-porphyrin catalyses the oxidation of NO to NO^+ and the Nafion prevents interference by nitrite ion. The current generated is measured and the electrode calibrated. As the carbon fibres are so small (diameter = 0.5μm) this electrode can be used to measure NO release from a single cell. The detection limits for the electrode are 10nM to 300μM.

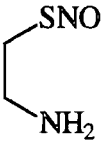
A commercially available NO electrode was purchased from World Precision Instruments. This method of NO detection is based on a platinum electrode with a disposable steel jacket and membrane across the electrode. The platinum electrode also measures the oxidation of NO and the membrane makes the electrode nitrite blind. This electrode has a tip diameter of 2mm and the detection limits are 1nM to 20μM.

2.5.1 Detection of NO from the Catalytic Decomposition of S-Nitrosothiols by Transition Metal Ions

It has subsequently been shown[14] that nitrosothiols are decomposed in the presence of catalytic amounts of copper(II) ions. It had not been unequivocally shown though that NO was the primary product of reaction (equation 2.4).

$$2RSNO \xrightarrow{Cu^{2+}} RSSR + 2NO \qquad \text{eqn. 2.4}$$

The nitrosothiol SNAP (20μM) was reacted in pH 7.4 phosphate buffer with Cu^{2+} (20μM) in the presence of the NO electrode. The reaction was slow, proceeding over 10 minutes until a maximum reading was observed. The amount of NO detected was 15% of the amount of nitrosothiol reacted. This was a low yield, but as the reaction was slow the NO formed initially would be oxidised by the end of the reaction as it is not possible to remove all traces of oxygen from the system. A more reactive nitrosothiol was then reacted with Cu^{2+}, the nitrosothiol of cysteamine (2.6) was reacted under the same conditions. This reaction was rapid and up to 75% of the NO expected was detected. As the NO was released more rapidly in this case, less NO was lost through oxidation.



(2.6)

The catalytic decomposition of nitrosothiols by Cu^{2+} gives a virtually quantitative yield of NO. If we use a fairly unreactive nitrosothiol (e.g. SNAP) we can probe the reactivities of the metal ions that are known to decompose nitrosothiols. The results are summarised in table 2.6.

Table 2.6

Amount of NO detected from the reaction between SNAP (20μM) and metal ions in pH 7.4 phosphate buffer.

Metal Ion	% NO detected
Cu^{2+}	15
Cu^{+}	45
Hg^{2+}	0

Copper(I) is more reactive than copper(II) towards nitrosothiol decomposition, however, Hg^{2+} gives no NO. Mercury(II) salts are known to decompose nitrosothiols[15]. The product from this reaction is NO^{+} which is immediately hydrolysed to nitrite at pH 7.4.

2.5.2 NO Detection in Photolysis Experiments

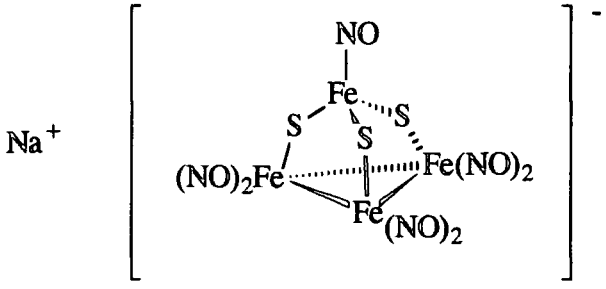
Photolysis of nitrosothiols leads to their decomposition (equation 2.5). Previous work[16] had shown the formation of the disulphide as evidence of the homolytic breaking of the S-N bond. Recently SNOG has been photolysed[17] and the NO_x products of decomposition detected. Detection of NO directly would confirm the other product of decomposition.

$$2RSNO \xrightarrow{h\nu} RSSR + 2NO \quad \text{eqn. 2.5}$$

The nitrosothiol of glutathione (SNOG) was chosen for photolysis as its reaction with Cu^{2+} is very slow and can be ignored for this experiment. An excess of EDTA was also added to complex any Cu^{2+} in solution and thus prevent any slow reaction with SNOG. A tuneable laser was used to photolyse the SNOG at 340nm. As very low concentrations of nitrosothiol were used with the electrode there was little absorbance at 340nm. A small amount of NO (~5%) was detected but it was not quantitative. Photolysis of a nitrosothiol does produce NO but the reaction is

extremely slow at low concentrations making quantitative NO measurement impractical.

Better results were obtained by using the electrode as part of a flow system and photolysing Roussin's Black Salt (RBS) (2.7).



(2.7)

Roussin's Black Salt is known to undergo decomposition when photolysed, indeed adding RBS to biological assays with photolysis gives physiological responses typical of NO[18]. Similar photolysis experiments were repeated, but instead of the organ bath a NO electrode was added to the flow system. The NO could then be detected rather than inferred. A peristaltic pump was used to push the solution through a system of tubes that connected a glass tube containing a quartz window and the NO electrode.

The reaction was carried out in unbuffered aerobic solution. The background level of NO was measured and then the laser was used to photolyse the 1μM RBS solution as it passed through the flow system. NO was detected and the signal obtained rose to a maximum value and remained there. Switching the laser off caused the signal to fall back to the background level. The amount of NO detected depended on the intensity of the laser (table 2.7).

Table 2.7

Variation of NO detected from photolysis of RBS (1μM) by a NO electrode with intensity of laser light used

457nm		514nm	
Intensity (mW)	10^6 [NO] (mol dm^{-3})	Intensity (mW)	10^6 [NO] (mol dm^{-3})
0	0.30	20	0.61
10	0.84	50	1.01
20	1.24	75	1.14
30	1.65	100	1.36

There seems to be a linear relationship between the intensity of laser light and the amount of NO produced. However, there is a large intercept if they are plotted out. It may be that at lower intensities the system is not saturated with light and that the relationship is not linear. It is clear, though, that RBS was producing more than one equivalent of NO, however, the experiment had its limitations as the amount of NO detected was not near the expected seven equivalents. The solutions were not anaerobic so the NO produced would react with O_2 producing nitrite[19], thus the amount detected would always be less than 100%. Also the window that allows the laser light into the experiment must be perpendicular to the beam otherwise reflections would reduce the amount of light entering. Repeating the experiment in an oxygen saturated Krebs buffer solution (95% O_2, 5% CO_2, K^+, Ca^{2+}, Mg^{2+}, Cl^-, CO_3^{2-}, HCO_3^- and sucrose), in which the bioassays were carried out[18], reduced the amount of NO detected by 5-6 fold (table 2.8). The difference must be due to an increased amount of oxidation of NO by O_2.

Table 2.8

Variation of [NO] detected from photolysis of RBS (1μM) with laser intensity at 514nm in oxygen saturated buffer.

Intensity (mW)	10^6 [NO] (mol dm^{-3})
10	0.053
20	0.105
50	0.165
100	0.204

Here, then, we have a method for on-line measurement of NO from photolysis experiments.

2.6 Conclusion

Measurement of NO from the decomposition of nitrosothiols did not elucidate the reaction until the advent of the Cu^{2+} catalysed reaction. Even then the electrode was the only satisfactory method of measuring NO. The ABTS and both haemoglobin methods inhibited the Cu^{2+} reaction with nitrosothiols. The use of the electrode in NO measurement is restricted by the low upper limit on detection (20μM) and on the fact that the moiety being measured is very susceptible to oxidation. It is not practically possible to make kinetic readings with the electrode, this is best left to uv/vis measurements of nitrosothiols.

References

1. S. Archer, *FASEB*, 1993, 7, 349.
2. M.J. Downes, M.W. Edwards, T.S. Elsey & C.L. Walters, *Analyst*, 1976, 101, 742.
3. J. McAninly, Thesis, to be published.
4. Vogel's Textbook of Quantitative Inorganic Analysis, Fourth Edition, Longman, New York, 1978, 755.
5. J.C.W. Chien, *J. Am. Chem. Soc.*, 1969, 91, 2166.
6. T. Okutani, A. Sakuragawa, S. Kamikura, M. Shimura & S. Azuchi, *Analytical Sciences*, 1991, 7, 793.
7. M.P. Doyle, R.A. Pickering, T.M. DeWeert, J.W. Hoeckstra & D. Pater, *J.Biol. Chem.*, 1981, 256, No. 23, 12393.
8. J. Tummavuori & P. Lumme, *Acta Chem. Scand.*, 1968, 22, 2003.
9. G.B. Schuster & S.P. Schmidt, *Adv. Phys. Org. Chem.*, 1982, 18, 229.
10. E.H. White, E.G. Nash, D.R. Roberts & O.C. Zafiriou, *J. Am. Chem. Soc.*, 1968, 90, 5932.
11. G.J. Wendel, D.H. Stedman & C.A. Cantrell, *Anal. Chem.*, 1983, 55, 937.
12. K. Kikuchi, H. Hayakawa, T. Nagano, Y. Hirata, T. Sugimoto & M. Hirobe, *Chem. Pharm. Bull.*, 1992, 40, 2233.
13. T. Malinski & Z. Taha, *Nature*, 1992, 358, 676.
14. J. McAninly, D.L.H. Williams, S.C. Askew, A.R. Butler & C. Russell, *J.Chem. Soc., Chem. Commun.*, 1993, 1758.
15. B. Saville, *Analyst*, 1958, 83, 670.
16. J. Barrett, L.J. Fitygibbones, J. Glauser, R.H. Stille & P.N.W. Young, *Nature*, 1966, 211, 848.
17. D.J. Sexton, A. Muruganandam, D.J. McKenney & B. Mutus, *Photochemistry and Photobiology*, 1994, 59, No. 4, 463.
18. F.W. Flitney, I.L. Megson, D.E. Flitney & A.R. Butler, *Brit. J. Pharmacol.*, 1992, 107, 842.
19. A.R. Butler & D.L.H. Williams, *Chem. Soc. Rev.*, 1993, 233.

CHAPTER 3

Kinetic Studies on the S-Nitrosation of Thiols

3 Kinetic Studies on the S-Nitrosation of Thiols

3.1 Kinetic Measurement of SNAP Formation

Much work has been carried out on the nitrosation of a range of thiols[1]. The rate equation has been established (equation 3.1) with the rate limiting step being electrophilic attack on the thiol by the nitrous acidium ion (section 1.3.2). Kinetic measurements had been carried out on N-acetylpenicillamine at 31°C[2]. The work needed to be repeated at 25°C.

$$\text{Rate} = k\,[HNO_2]\,[RSH]\,[H^+] + k'[HNO_2]\,[RSH] \quad \text{eqn. 3.1}$$

The acid catalysed nitrosation of N-acetylpenicillamine was carried out under pseudo first order conditions with the thiol in excess. S-Nitrosation is generally a rapid process so the reaction was measured by stopped flow spectrophotometry, observing the increase in absorption at 340nm due to nitrosothiol production. The reaction has already been shown to be first order in thiol, acid and nitrous acid[2]. The acid concentration was varied so that a value for the third order rate constant for nitrosation could be obtained. All the results used to calculate the third order rate constant were obtained from at least five runs so that the average had an error ±5% (table 3.1).

The third order rate constant was determined to be 165 ± 8 $mol^{-2}\ dm^6\ s^{-1}$. The value for the rate constant[2] at 31°C was 840 $mol^{-2}\ dm^6\ s^{-1}$. These values are not compatible as they give an activation energy for the reaction of 180 kJ mol^{-1} which is taken to be too large for the reaction. We can compare these results with those previously obtained for other thiols (table 3.2[3]).

Table 3.1

Rate data for nitrosation of N-acetylpenicillamine

$[H^+]$ (mol dm^{-3})	10^3 k_{obs} (s^{-1})
0.005	4.8
0.010	6.6
0.020	11.1
0.030	14.6
0.040	18.4
0.050	22.0

[RSH] = 2.5 x 10^{-3} mol dm^{-3}, [$NaNO_2$] = 1.25 x 10^{-4} mol dm^{-3}, T = 298K.

Table 3.2

Values of k (equation 3.1) for acid catalysed nitrosation in water at 25°C.

Thiol	k (mol^{-2} dm^6 s^{-1})
t-Butyl thiol (3°)	47[a]
Cysteine methyl ester (1°)	213
Cysteine (1°)	443
Glutathione (1°)	1080
Mercaptosuccinic acid (2°)	1330
N-Acetyl cysteine (1°)	1590
Thioglycolic acid (1°)	2630
Mercaptopropionic acid (1°)	4760

[a] In 50% dioxan-water

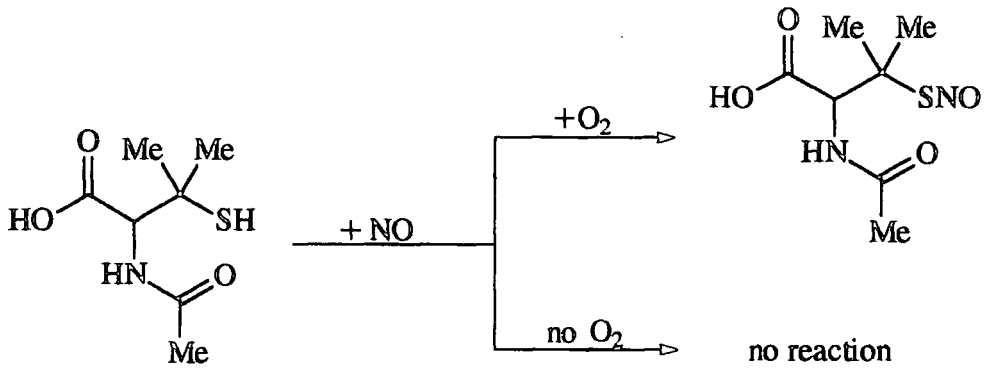
The most reactive substrates are the primary thiols and the least reactive the tertiary thiols. This trend is similar to that found in alkyl nitrites where steric

considerations are more important than electronic ones. The value obtained for N-acetylpenicillamine at 25°C fits in with the data in table 3.2 showing that tertiary thiols are not as reactive as secondary and primary.

3.2 Reaction Between Nitric Oxide and N-Acetylpenicillamine.

There are many reports that gaseous nitric oxide will react with solutions of amines to produce N-nitrosamines. A detailed study[4], however, has shown that under anaerobic conditions, provided by a vacuum line, secondary amines were nitrosated extremely slowly, with half lives of eight days. The rate appeared to be zero order with respect to amine concentration. This suggests that nitrosation is due to a higher oxide of nitrogen and that the rate determining step is oxidation of NO via oxygen entering the system. Addition of oxygen to the system allowed the reaction to proceed in five minutes. However, it has been reported that nitric oxide could nitrosate thiols under anaerobic conditions[5]. Again this must be due to oxygen entering the system. The confusion has arisen because of the difficulty of achieving fully de-oxygenated systems.

In order to study the reaction, addition of nitric oxide to a thiol solution must be carried out under strictly anaerobic conditions. Solutions of N-acetylpenicillamine were degassed in a sonic bath under reduced pressure and then oxygen-free nitrogen was passed through the solutions. Nitric oxide gas, generated by the reduction of sodium nitrite by ascorbic acid, was then passed through the thiol solution. There was no colour change observable on passage of the nitric oxide. Immediate scanning on a uv/vis spectrophotometer showed no absorbance at 340nm, which is where authentic S-nitroso-N-acetylpenicillamine (SNAP) absorbs. If nitric oxide was passed through an aerated 10mM N-acetylpenicillamine solution then an immediate green solution was produced with large absorbance peaks at 340nm and 590nm identical to those of authentic SNAP. The reaction of NO with thiols needs oxygen to proceed (scheme 3.1).



Scheme 3.1

The reaction was repeated using anaerobic buffered solutions. The anaerobic samples initially gave no absorbance at 340nm but upon standing the absorbance increased as oxygen gradually diffused into the cells. The increase in absorbance could be measured and was followed by a much slower decrease in absorbance. The decrease was due to the SNAP decomposing in solution. However, at the end of the decomposition there was still a noticeable absorbance ($A > 0.1$) at 355nm. The buffer used for these experiment was based on maleic acid and the double bond was undergoing some nitrosation reaction. Passing NO through the aerated buffer solution alone gave the same absorbance at 355nm so the buffer solutions were discarded and the thiol containing solutions had their pH adjusted by addition of acid or base. Again, prior to measurement the thiol did not react with the NO. The reaction only proceeds when the NO encounters air (figure 3.1). Measurements taken (table 3.3) will be dependent on the amount of oxygen allowed into the cells rather than the pH of the solution.

Figure 3.1

Kinetic trace for SNAP formation at 340nm from N-acetylpenicillamine and NO in anaerobic solution.

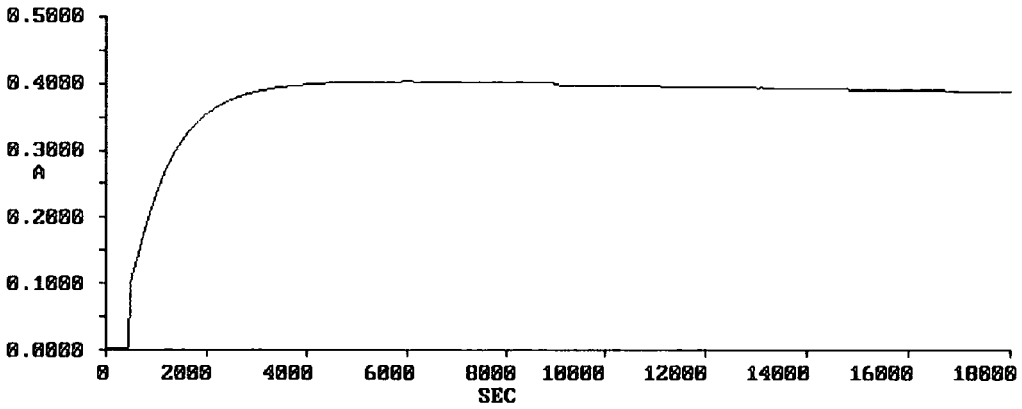


Table 3.3

Rate data for reaction of N-acetylpenicillamine with NO and oxygen.

pH	$10^3 k_{formation}$ (s^{-1})	$10^5 k_{decomposition}$ (s^{-1})
1.4	not measured	9 ± 4
2.4	4.2 ± 2.1	3 ± 2
3.4	0.8 ± 0.1	1 ± 0.5
4.4	6.8 ± 3.2	7 ± 3
5.4	24 ± 13	8 ± 4

3.3 Reaction of Thiols with NO in the Presence of Cu(II) Ions.

There has been a great deal of discussion about the formation of S-nitrosothiols *in vivo*[6,7]. However, no conclusive evidence has been shown as to how free thiols react with NO, rather than usual nitrosating agents such as N_2O_3. Enzymatic mechanisms have been proposed to account for the nitrosylation of thiols by nitric oxide. S-Nitrosothiols are known to circulate in the blood plasma[7] and are thought to be a store for nitric oxide *in vivo*.

It has been shown that in non-aqueous media nitrosation reactions can be carried out with nitric oxide in the presence of copper (II) salts[8,9]. Nitric oxide dissolved in acetonitrile will form complexes with Cu(II) salts (scheme 3.2) which can then nitrosate substrates, such as amines[8,9].

$$CuCl_2 + NO \rightleftharpoons Cu^{II}NOcomplex$$

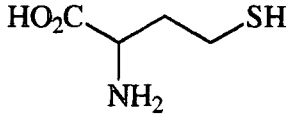
$$Cu^{II}NOcomplex + R_2NH \longrightarrow Cu^{I}complex + R_2NNO + H^+$$

Scheme 3.2

Many enzymes have copper ions in their active site, for example superoxide dismutase and mono- and di- oxygenases[10]. Other copper containing substituents in the body include serum albumin, which also contains a free thiol group. Oxidation of NO bound to the copper site of serum albumin could lead to nitrosation of the thiol group. Smaller thiols found in the blood plasma such as cysteine and glutathione could then undergo transnitrosation to produce nitrosothiols that could form a stable store of nitric oxide. To test this hypothesis NO was reacted with Cu(II) ions in the presence of thiols.

The reaction in acetonitrile is thought to be extremely complex, however, the product in water should be NO^+ which under acidic conditions will effect nitrosation. The whole system needs to be anaerobic and this was achieved by bubbling oxygen-

free nitrogen through all the solutions being used. The Cu(II) and thiol solutions were placed in modified glassware which had a scintered glass disc for the incoming gas and a septum for removal of samples with a gas tight syringe. All flasks were placed in series in the following order; a 5mM Cu(II) solution; a plant for NO production; a 0.2M sodium hydroxide solution as a higher N-oxide scrubber; a 0.5mM control thiol solution; a 0.5mM thiol solution for the experiment and finally aerated water for the waste NO. The thiol solutions were prepared in 0.1M $HClO_4$. The nitrogen was passed through for 2 hours to remove most of the oxygen and then the NO was passed through the experiment. An aliquot of the Cu(II) solution was syringed into the thiol solution. Samples were then removed from both the experiment and the control and the initial absorbance at 340nm measured. Homocysteine (3.1) was initially used as the thiol.

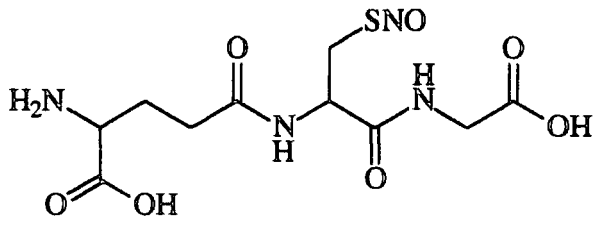


(3.1)

The 0.1mM Cu(II)/ 0.5mM thiol solution gave 60% yield of the nitrosothiol, whereas the thiol solution without Cu(II) gave 7% yield of the nitrosothiol. The nitrosothiol in the control experiment probably resulted because of the presence of residual oxygen in the system. Scans of the product of the reaction were identical with an authentic sample of the S-nitrosothiol of homocysteine. Addition of the Cu(II) solution to the thiol could have admitted oxygen into the system, so an equal amount of deaerated water needed to be added to the control experiment on repeating.

The experiment was repeated in pH 7.4 phosphate buffer to ascertain whether the thiol was reacting with a copper-nitrosyl complex or with the nitrous acidium ion. The scans of the products of this reaction were not identical with the nitrosothiol. The products had absorbance peaks at ~213nm which is typical for disulphide absorption. As the reaction was carried out in slightly basic conditions this was not unexpected. The presence of the Cu(II) was also causing any nitrosothiol formed to

decompose (see chapter 4), so the thiol glutathione was used as the corresponding nitrosothiol, SNOG (3.2), has a negligible reaction with Cu(II).



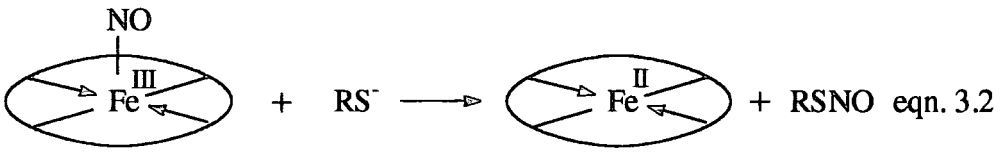
(3.2)

The reaction was carried out as above in pH 1 solution (0.1M $HClO_4$) of glutathione. Addition of Cu(II) to acidic thiol solutions produced no appreciable change in the absorption spectrum so the Cu(II) was added to the thiol before deaerating with nitrogen. Two experiments were carried out simultaneously; one flask contained glutathione (0.02M) and Cu(II) (4mM); and the other a dilute solution of glutathione (0.5mM) and Cu(II) (0.1mM). If there is a nitrosation reaction then it should have been visible in the concentrated solution. The more dilute solution was used for spectral measurements. After passing oxygen-free nitrogen through the solutions the nitric oxide was generated and allowed through the system. No colour change was observed. Samples taken from the second solution by gas tight syringe gave absorbance spectra identical with authentic SNOG. Measurement of these samples at 340nm showed that the absorbance was still rising 30 seconds after taking the sample. The NO in solution was thus reacting with oxygen and nitrosating the thiol after the sample was taken. The thiol, Cu(II) and NO had been present together for at least 10 minutes before the sample was taken and any nitrosation reaction should have taken place within this time. The reaction that was subsequently measured was solely due to oxygen. Nitrogen was then passed through the system for a further 30 minutes to remove any NO dissolved in the solutions. Samples taken subsequently showed only a small reaction on standing. There was no reaction between NO and Cu(II) leading to nitrosation.

3.4 Conclusion

There must be little doubt that NO will not react with thiols under strictly anaerobic conditions contrary to previous reports[5,6,7]. Reaction with O_2 must be a prerequisite for any reaction with NO in aqueous solution. Neither is NO oxidised by Cu^{2+} in aqueous solution. These results do not preclude an enzymatic reaction between thiols and NO leading to *in vivo* production of nitrosothiols, but it does show that Cu^{2+} ions in aqeuous solution do not undergo redox reactions with NO (see section 4.2.2).

No reasonable mechanism for formation of nitrosothiols *in vivo* has been proposed other than some unknown enzymatic process. Metal nitrosyls such as nitrosyl haems are known[11] to act as sources of NO^+. It may be a possibility that nitrosyl methaems[12] undergo transnitrosation reactions with thiols to give nitrosothiols *in vivo* (eqn. 3.2).



eqn. 3.2

It would be extremely difficult, practically, to verify this mechanism due to the small extinction coefficient associated with nitrosothiols compared to haems. However, the idea remains attractive due to its simplicity.

References

1. P.A. Morris & D.L.H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1988, 513; L.R. Dix & D.L.H. Williams, *ibid.*, 1984, 109.
2. S.E. Aldred, D.L.H. Williams & M. Garley, *J. Chem. Soc., Perkin Trans. 2*, 1982, 777.
3. D.L.H. Williams, Nitrosation, CUP, Cambridge, 1988, 176.
4. B.C. Challis & S.A. Kyrtopoulos, *J. Chem. Soc., Perkin Trans. 1*, 1979, 299.
5. L.J. Ignarro, B.K. Barry, D.Y. Gruetter, J.C. Edwards, E.H. Ohlstein, C.A. Gruetter & W.H. Barricos, *Biochem. Biophys. Res. Commun.*, 1980, 94, 93.
6. J.S. Stamler, D.I. Simon, J.A. Osbourne, M.E. Mullins, O. Jaraki, T. Michel, D.J. Singel & J. Loscalzo, *Proc. Natl. Acad. Sci. USA*, 1992, 89, 444.
7 J.S. Stamler, O. Jakari, J. Osbourne, D.I. Simon, J. Keaney, J. Vita, D.J. Singel, C.R. Valeri & J. Loscalzo, *Proc. Natl. Acad. Sci. USA*, 1992, 89, 7674.
8. W. Brackman & P.J. Smit, *Rec. Trav. Chim. Pays-Bas*, 1965, 84, 357.
9. A.G. McKenna & J.C. Fanning, *Abs. Papers Am. Chem. Soc.*, 1990, 200, 374.
10. K.D. Karlin & Z. Tyeklár, Eds., Bioinorganic Chemistry of Copper, 1993, Chapman & Hall, New York.
11. D.L.H. Williams, Nitrosation, Cambridge University Press, Cambridge, 1988, 209.
12. J.C.W. Chien, *J. Am. Chem. Soc.*, 1969, 91, 2166.

CHAPTER 4

Cu(II) Catalysed Nitric Oxide Formation From S-Nitrosothiols

4.1 Introduction

With the current enormous expansion in NO research, much interest has been generated in compounds that produce NO. S-Nitrosothiols are known to produce NO both photochemically[1] and thermally[2] (equation 4.1).

$$RSNO \xrightarrow{\Delta / h\nu} RS\cdot + \cdot NO \qquad \text{eqn. 4.1}$$

Much work has been carried out on the mechanism of nitrosothiol decomposition[3,4] with regard to their possible therapeutic and pharmacological uses. Kinetic measurement were always erratic and irreproducible. The data have on occasions been seen to fit anything from second order, first order or even half order kinetics. Recently it was shown[5] that the decomposition of nitrosothiols was mediated by trace quantities of copper(II) ions found in the water supplies and buffer solutions used. Decomposition of S-nitroso-N-acetylpenicillamine (SNAP) in aqueous solution gave almost quantitative production of nitrite[5] as detected by the diazotization and azo coupling method (section 2.1). It is known[6] that NO in oxygenated aqueous solution is quantitatively oxidised to nitrite with no nitrate formation (scheme 4.1).

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

$$NO_2 + NO \xrightarrow{\text{fast}} N_2O_3$$

$$N_2O_3 + H_2O \xrightarrow{\text{fast}} 2\,NO_2^- + 2H^+$$

$$2\,NO_2 + H_2O \xrightarrow{\text{slow}} NO_2^- + NO_3^- + 2\,H^+$$

Scheme 4.1

The rate equation for nitrosothiol decomposition had not been established and no satisfactory mechanism proposed. The reaction between nitrosothiols and Cu^{2+} needed to be measured quantitatively and the mechanism elucidated as far as possible.

4.2 Reaction of Cu(II) with S-Nitrosothiols

A large range of thiols were available for nitrosation and the spectral data for those used is summarised in table 4.1.

Table 4.1

Spectral data obtained for various nitrosothiols in pH 7.4 phosphate buffer.

S-Nitrosothiol	ε_{340nm} (mol^{-1} dm^3 cm^{-1})	λ_{max} (nm)
S-Nitroso-N-acetylpenicillamine	956 ± 20	338, 592
S-Nitrosopenicillamine	858 ± 26	339, 594
S-Nitrosocysteamine	553 ± 7	332, 548
S-Nitrosocysteine	855 ± 47	336, 542
S-Nitrosocysteine ethyl ester	772 ± 21	336, 541
S-Nitroso t-butylthiol	690 ± 40	333, 591
S-Nitrosohomocysteine	840 ± 25	331, 543
S-Nitroso-N-acetylcysteine	920 ± 29	336, 543
S-Nitroso-N-acetylcysteamine	816 ± 27	332, 549
S-Nitrosoglutathione	895 ± 7	337, 546
S-Nitrosocaptopril	1108 ± 24	332, 546
S-Nitrosomercaptoacetic acid	1026 ± 33	326, 546
S-Nitroso mercaptomethylacetate	880 ± 35	329, 542
S-Nitroso mercatopropionic acid	1053 ± 21	328, 548
S-Nitroso mercaptomethylpropionate	1046 ± 28	329, 544
S-Nitroso thiolactic acid	1118 ± 21	331, 551
S-Nitroso thiomalic acid	1061 ± 59	335, 548
S-Nitroso-2-hydroxyethanthiol	1055 ± 33	333, 546

Many of the thiols used were based on cysteine, which is an amino acid found at levels of 30-100 nano-moles per gram[7] in mammalian tissue. Therapeutic use of such nitrosothiols should potentially have minimal adverse side-effects as the decomposition products (disulphides) are also found *in vivo*. All primary and secondary nitrosothiols are red and absorb between 540nm and 550nm, whereas all tertiary nitrosothiols are green and absorb around 590nm.

Only a few nitrosothiols can be synthesised as solids and characterised, they are SNAP, SNOG, SNOCAP and SNAC. The synthesis is based on the method by Field et. al.[8]. The remaining thiols were nitrosated in acid solution, using sodium nitrite, and then diluted with pH 7.4 buffer to the required concentration.

4.2.1 Detection of Products

S-Nitrosothiols can be monitored spectrophotometrically by observation of the characteristic absorbance at ~340nm. As mentioned in section 2.5.1, a nitric oxide specific electrode shows almost quantitative production of NO for the reaction with Cu^{2+} (equation 4.2). The other product of the reaction is the corresponding disulphide. Concentrated solutions (0.01M) of cysteine nitrosothiol will, upon decomposition, precipitate the disulphide which can be identified by its melting point.

$$2RSNO \xrightarrow{Cu^{2+}} RSSR + 2NO \qquad \text{eqn. 4.2}$$

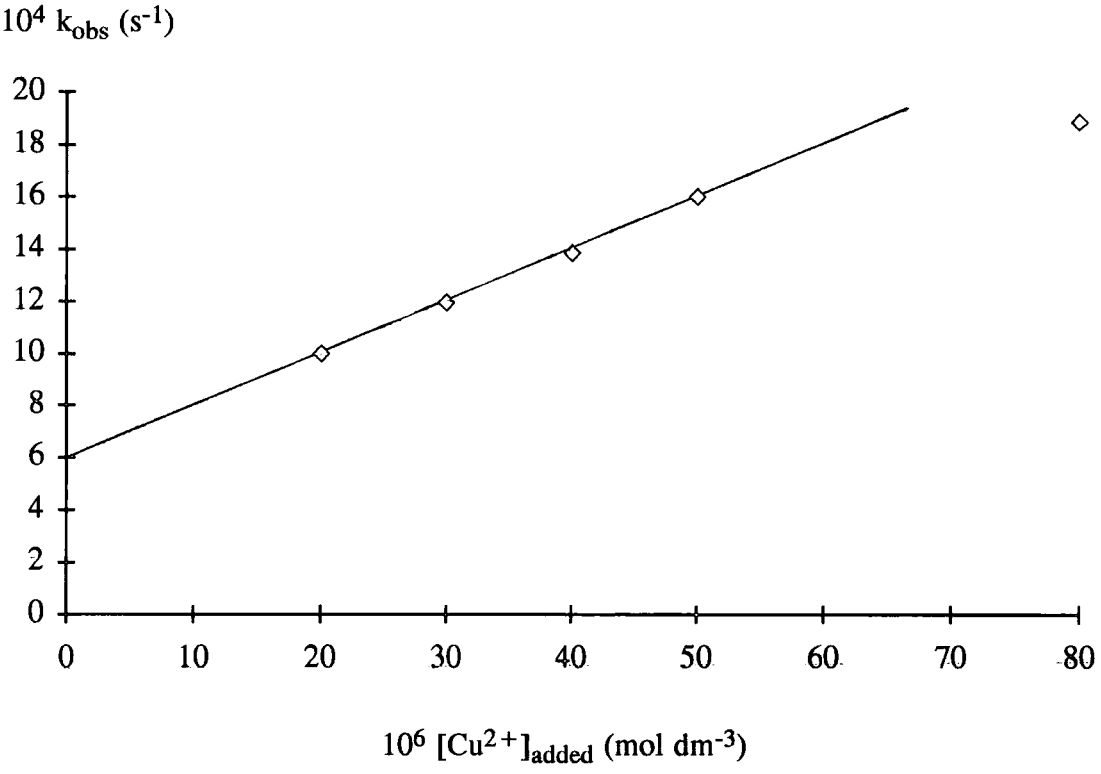
4.2.2 Kinetics of the Reaction with Cu^{2+}

The reaction between Cu^{2+} ions and nitrosothiols was observed at 340nm in pH 7.4 phosphate buffer (0.12M) and at 298K. The decrease in absorbance at 340nm was measured as a function of time and generally good first order kinetics were obtained. The first nitrosothiol considered was that derived from N-acetylpenicillamine. This was synthesised, according to the method by Field et. al.[8], as a stable solid and then made up in solution before reaction. The reaction was studied for a range of Cu^{2+} concentrations (table 4.2) and a plot of k_{obs} against $[Cu^{2+}]$ was linear (figure 4.1) over the range 20 - 50 μM Cu^{2+}. The rate equation

was established (equation 4.3) and the reaction was found to be first order in S-nitroso-N-acetylpenicillamine (SNAP). The reaction was also found to be first order with respect to the amount of Cu^{2+} added to the system. There is a significant intercept on the plots of k_{obs} against $[Cu^{2+}]$ which is probably due to the residual amount of Cu^{2+} already present in the phosphate buffer solution (determined by atomic absorption spectroscopy to be typically 1 x 10^{-6}M).

Figure 4.1

Plot of k_{obs} against $[Cu^{2+}]_{added}$ for the catalytic formation of NO from SNAP (5 x 10^{-4} M) in pH 7.4 buffer



$$\text{Rate} = k_2 [\text{RSNO}] [Cu^{2+}]_{added} + c \qquad \text{eqn. 4.3}$$

Cu^{2+} is catalytic, so $[Cu^{2+}]_{added}$ is constant.

$$k_{obs} = k_2 [Cu^{2+}]_{added} + \text{constant} \qquad \text{eqn. 4.4}$$

Table 4.2

Rate data for the reaction of S-nitroso-N-acetylpenicillamine (5×10^{-4}M)with Cu^{2+} at pH 7.4

10^6 $[Cu^{2+}]_{added}$ (mol dm^{-3})	10^4 k_{obs} (s^{-1})
20	10.0 ± 0.1
30	12.0 ± 0.1
40	13.9 ± 0.05
50	16.0 ± 0.1
80	18.9 ± 0.1

Using equation 4.4 a value for k_2, the second order rate constant, can be obtained. For the reaction with SNAP the value of k_2 is 20 ± 1 mol^{-1} dm^3 s^{-1}. The rate equation holds for a range of reactive nitrosothiols and k_2 values can be obtained for these compounds. Comparison of the k_2 values should enable us to comment on the mechanism of the reaction. The rate data for the nitrosothiols reacted are shown below (tables 4.3 - 4.10) and all the k_2 values summarised in table 4.11.

Table 4.3

Rate data for the reaction of S-nitrosopenicillamine (5×10^{-4}M) with Cu^{2+} at pH 7.4

10^6 $[Cu^{2+}]_{added}$ (mol dm^{-3})	k_{obs} (s^{-1})
0.1	0.1729 ± 0.0111
0.2	0.1853 ± 0.0045
0.5	0.2316 ± 0.014
0.8	0.2230 ± 0.015
2.5	0.3350 ± 0.013

$$k_2 = 67000 \pm 2000 \text{ mol}^{-1} \text{ dm}^3 \text{ s}^{-1}$$

Table 4.4

Rate data for the reaction of S-nitrosocysteamine (5×10^{-4}M) with Cu^{2+} at pH 7.4

10^6 $[Cu^{2+}]_{added}$ (mol dm^{-3})	k_{obs} (s^{-1})
0.04	0.0200 ± 0.0003
0.08	0.0218 ± 0.0013
0.10	0.0283 ± 0.0034
0.25	0.0383 ± 0.0030
0.40	0.0451 ± 0.0038
0.60	0.0572 ± 0.0050
0.80	0.0639 ± 0.0048
1.00	0.0693 ± 0.0083
2.50	0.1056 ± 0.0067

$k_2 = 65000 \pm 1300$ mol^{-1} dm^3 s^{-1}

Table 4.5

Rate data for the reaction of S-nitrosocysteine (5×10^{-4}M) with Cu^{2+} at pH 7.4

10^6 $[Cu^{2+}]_{added}$ (mol dm^{-3})	k_{obs} (s^{-1})
0.5	0.0891 ± 0.0096
1	0.0984 ± 0.0031
2	0.1392 ± 0.0120
3	0.1497 ± 0.0068
4	0.1749 ± 0.0120

$k_2 = 24700 \pm 500$ mol^{-1} dm^3 s^{-1}

Table 4.6

Rate data for the reaction of S-nitrosomercapto acetic acid (5 x 10^{-4}M) with Cu^{2+} at pH 7.4

10^6 $[Cu^{2+}]_{added}$ (mol dm^{-3})	10^3 k_{obs} (s^{-1})
10	2.85 ± 0.2
15	4.41 ± 0.3
17.5	4.95 ± 0.2
20	6.13 ± 0.3
25	6.71 ± 0.5
30	8.52 ± 0.3
40	12.11 ± 2.1

$k_2 = 300 \pm 5$ mol^{-1} dm^3 s^{-1}

Table 4.7

Rate data for the reaction of S-nitrosomercapto propionic acid (5 x 10^{-4}M) with Cu^{2+} at pH 7.4

10^6 $[Cu^{2+}]_{added}$ (mol dm^{-3})	10^4 k_{obs} (s^{-1})
50	4.7 ± 0.2
100	12.3 ± 0.6
125	17.3 ± 0.7
200	21.5 ± 1.0
300	22.1 ± 0.9

$k_2 = 16.4 \pm 0.3$ mol^{-1} dm^3 s^{-1}

Table 4.8

Rate data for the reaction of S-nitrosothiolactic acid (5×10^{-4}M) with Cu^{2+} at pH 7.4

10^6 $[Cu^{2+}]_{added}$ (mol dm^{-3})	10^3 k_{obs} (s^{-1})
0.5	1.32 ± 0.09
1.0	2.45 ± 0.02
1.5	2.48 ± 0.01
2.0	2.94 ± 0.01
3	3.81 ± 0.01
4	4.56 ± 0.01
5	5.77 ± 0.06

$k_2 = 900 \pm 60$ mol^{-1} dm^3 s^{-1}

Table 4.9

Rate data for the reaction of S-nitrosothiomalic acid (5×10^{-4}M)with Cu^{2+} at pH 7.4

10^6 $[Cu^{2+}]_{added}$ (mol dm^{-3})	10^3 k_{obs} (s^{-1})
2	1.46 ± 0.07
4	1.89 ± 0.11
6	3.58 ± 0.16
8	5.71 ± 0.27
10	6.35 ± 0.37

$k_2 = 1100 \pm 60$ mol^{-1} dm^3 s^{-1}

The k_2 values are determined from the linear relationship between k_{obs} and $[Cu^{2+}]_{added}$. However, above the linear regions shown above the relationship broke down. The results became erratic and this may be due to complex formation with the phosphate buffer.

The k_2 values show a large substrate dependence (table 4.11). There were some nitrosothiols that were very unreactive towards Cu^{2+}, these include; tertiary butylnitrosothiol; S-nitroso-N-acetylcysteamine; S-nitrosocaptopril; S-nitrosoglutathione and S-nitrosomethylmercaptopropionate. None of these compounds showed any significant reaction with Cu^{2+} even after 20 hours. Three other compounds had an intermediate reactivity with Cu^{2+}. The S-nitrosothiol of methylmercaptoacetate decomposed in the presence of Cu^{2+}, but variation of Cu^{2+} levels over the range 1-30 μM produced no change in k_{obs} (table 4.10). This reaction also produced a new absorbance peak at 285nm the identity of which was not ascertained.

Table 4.10

Rate data for the reaction of S-nitrosomethylmercaptoacetate (5×10^{-4}M) with Cu^{2+} at pH 7.4

$10^6 [Cu^{2+}]_{added}$ (mol dm^{-3})	$10^3 k_{obs}$ (s^{-1})
1	3.22 ± 0.10
2	3.27 ± 0.03
4	3.29 ± 0.08
8	3.48 ± 0.12
15	3.23 ± 0.05
30	3.35 ± 0.15

S-Nitroso-N-acetylcysteine (SNAC) decomposed very slowly over 20 hours but would not completely react. A similar reaction occurred with the nitrosothiol of 2-mercaptoethanol, of which only 50% decayed over 4 hours followed by no further

change over 10 hours. The species remaining had the same absorbance characteristics as the nitrosothiol.

Table 4.11

Values of k_2 (equation 4.3) for Cu^{2+} catalysed nitrosothiol decomposition in pH 7.4 buffer at 298K

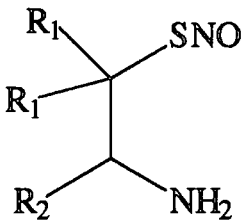
Nitrosothiol	k_2 (mol^{-1} dm^3 s^{-1})
S-Nitroso-N-acetylpenicillamine (SNAP)	20 ± 1
S-Nitrosopenicillamine	67 000 ± 2000
S-Nitrosocysteamine	65 000 ± 1300
S-Nitrosocysteine	24 700 ± 500
S-Nitrosocysteine ethyl ester[a]	270 000 ± 11000
t-Butyl-S-nitrosothiol	0
S-Nitrosohomocysteine[a]	16 ± 0.5
S-Nitroso-N-acetylcysteine (SNAC)	0
S-Nitroso-N-acetylcysteamine	0
S-Nitrosoglutathione (SNOG)	0
S-Nitrosocaptopril (SNOCAP)	0
S-Nitrosomercaptoacetic acid	300 ± 5
S-Nitrosomethylmercaptoacetate	0
S-Nitrosomercaptopropionic acid	16 ± 0.3
S-Nitrosomethylmercaptopropionate[a]	0
S-Nitrosothiolactic acid	900 ± 60
S-Nitrosothiomalic acid	1100 ± 60
S-Nitroso-2-hydroxyethanthiol	0

[a] Results from work carried out by S. Ayris, 3H project student.

When there is only one functional group on the nitrosothiol, as in tertiary butylnitrosothiol, there is no reaction with Cu^{2+}. This is possibly due to the Cu^{2+} not forming a stable complex with the S-nitroso group alone. Other functional groups on the nitrosothiol are needed in order for the nitrosothiol to react with Cu^{2+} and produce NO. It seems likely that the Cu^{2+} may be bidentately bound for the reaction to occur.

The most reactive nitrosothiols (mainly based on cysteine) are those containing a free β-amine group. However, at pH 7.4 the amines will be present mostly (> 90%) in the protonated form (as $-NH_3^+$) and will not coordinate Cu^{2+}. The simplest amino-nitrosothiol (see figure 4.2) is based on cysteamine (R_1 = R_2 = H) which has a k_2 value of 65000 mol^{-1} dm^3 s^{-1} and we can compare other similar compounds to it.

Figure 4.2

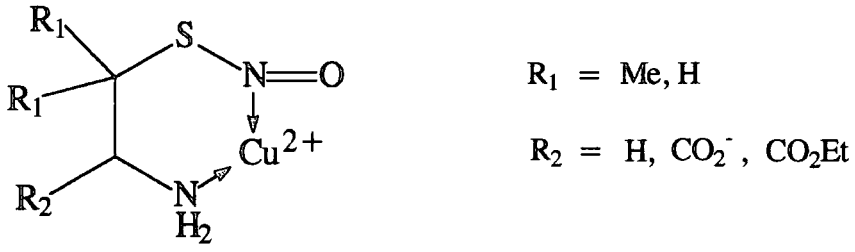


S-Nitrosocysteine has an additional carboxylic acid group (R_1 = H, R_2 = CO_2^-) which causes competitive complex formation with the Cu^{2+}. Complexation of Cu^{2+} by carboxylic acids is well known and a number of complexes have been isolated and examined structurally[9]. This is why the value of k_2 for S-nitrosocysteine, k_2 = 24500 mol^{-1} dm^3 s^{-1}, is almost three times less than that of S-nitrosocysteamine. S-Nitrosopenicillamine (R_1 = Me, R_2 = CO_2^-) is as reactive, k_2 = 67000 mol^{-1} dm^3 s^{-1}, as S-nitrosocysteamine and this could be due to a gem-dimethyl effect[10] holding the reactive groups in close proximity and preventing rotational isomers with a configuration unfavourable to reaction. This effect is seen to overcome the effect of unfavourable complexation via the acid group. The ethyl ester

of S-nitrosocysteine (R_1 = H, R_2 = CO_2Et), k_2 = 270000 mol^{-1} dm^3 s^{-1}, is four times as reactive as S-nitrosocysteamine. The ester prevents much of the competitive complexation seen in cysteineSNO as the negative charge has been removed. However, the reason for the extra reactivity must be that the electron-withdrawing effect of the ester lowers the pK_a value for the amine[11] making more unprotonated amine available for co-ordination to Cu^{2+}.

An explanation for their reactivity is that the nitrosothiol binds the Cu^{2+} in a bidentate manner through the N-atom on the nitroso group and the N-atom of the free amine (figure 4.3). The intermediate thus formed is a six-membered ring which will rapidly decompose the nitrosothiol to give nitric oxide and the thiyl radical.

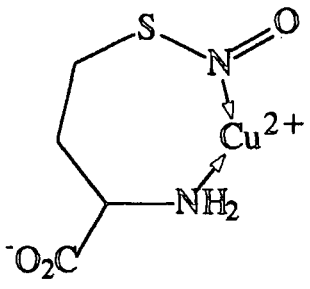
Figure 4.3



The co-ordination chemistry of Cu^{2+} is known[12] to be dominated by binding through N and O groups because Cu^{2+} is an intermediate Lewis acid. Where there is no such group available for binding, as in tertiary butylnitrosothiol, there is no reaction with Cu^{2+}.

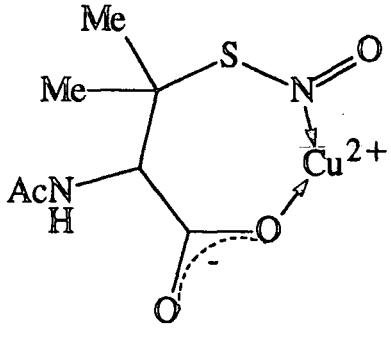
Addition of an extra methylene group, as in S-nitrosohomocysteine, in between the nitroso and amine groups drastically reduces the reactivity of the nitrosothiol, k_2 = 16 mol^{-1} dm^3 s^{-1}. The intermediate complex with Cu^{2+} must now be a less favourable 7-membered ring (figure 4.4).

Figure 4.4



The reactivity can be reduced to almost immeasurable levels by acetylation of the amino group, as in the S-nitroso-N-acetyl derivatives of cysteine and cysteamine. The lone pair on the N is now delocalised into the carbonyl group and unavailable for co-ordination. N-Acetylation of penicillamine (giving SNAP) does not completely prevent reaction though. The Cu^{2+} can still be co-ordinated by the nitroso N and an O-atom on the carboxylic acid group (figure 4.5). This produces a 7-membered intermediate but the reaction still occurs.

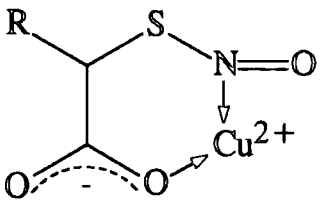
Figure 4.5



S-Nitrosothiol carboxylic acids are not as reactive as compounds containing a free amino group but they still react with Cu^{2+} to produce NO. S-Nitrosomercapto-acetic acid (R = H) is the simplest example and will react with Cu^{2+} with a second order rate constant of 300 mol^{-1} dm^3 s^{-1}. The proposed reaction goes via a 6-membered intermediate (figure 4.6) similar to that proposed for the amino-

nitrosothiols. Esterification of the acid does not completely stop the reaction but it is certainly slowed and in the case of S-nitroso methylmercaptoacetate, there appears to be a zero order dependence on Cu^{2+}.

Figure 4.6

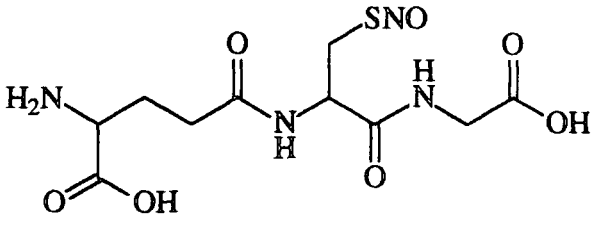


Again if we increase the separation of the nitroso and acid groups by one methylene group (S-nitrosomercaptopropionic acid) then the reactivity drops to a level similar to that for SNAP. These two compounds would be expected to have a similar reactivity as the proposed intermediate with Cu^{2+} is a 7-membered ring co-ordinating through the nitroso N-atom and an O-atom from the acid group. Esterification of this compound leads to suppression of the reaction so that the reaction cannot be accurately measured. The S-nitrosothiols of thiolactic acid (R = Me) and thiomalic acid (R = $CH_2CO_2^-$) must both go through the 6-membered intermediate (figure 4.6). These compounds are more reactive than the basic S-nitrosomercaptoacetic acid but the difference is not great and is probably due to a small steric effect favouring the formation of the ring.

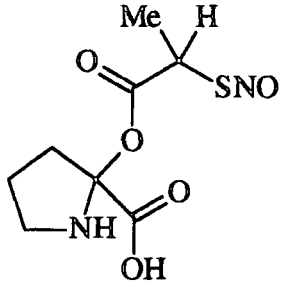
The absence of a complete reaction between Cu^{2+} and S-nitroso-2-hydroxyethanthiol was somewhat surprising. There is obviously some co-ordination as ~ 50% of the reaction takes place, but since an appreciable amount of S-nitroso compound remains the Cu^{2+} must be preferentially complexed by the disulphide product. The electron density on the alcohol O does not seem to be sufficient for the formation of a reactive intermediate when there is a competitive Cu^{2+} chelator (in this case the disulphide). The overall reaction between Cu^{2+} and the nitrosothiol is very slow anyway, suggesting that the co-ordination is not very favourable.

The interaction of thiols with Cu^{2+} has been studied[13] by esr. β-Aminothiols and β-mercapto acids will coordinate to Cu^{2+} forming stable bis complexes. These complexes will slowly decay to give Cu^{+} and RS·. Thiols such as benzenethiol, 2-hydroxyethanethiol and β-mercaptoesters will not form complexes with Cu^{2+} to any measureable degree and rapidly form Cu^{+} and RS·. The electron withdrawing -NO group will affect the coordination slightly, but should show similar trends. This helps explain possible reasons why β-amino and acid thiols are reactive and the esters and alcohol are unreactive to Cu^{2+} catalysed decomposition.

Using these observations it is clear why SNOG (4.1) and SNOCAP (4.2) are such stable compounds. There are no free amino or acid groups in the vicinity of the nitroso group in SNOG as both are involved in amide linkages. SNOCAP also has no local available binding sites for Cu^{2+} which could lead to decomposition.

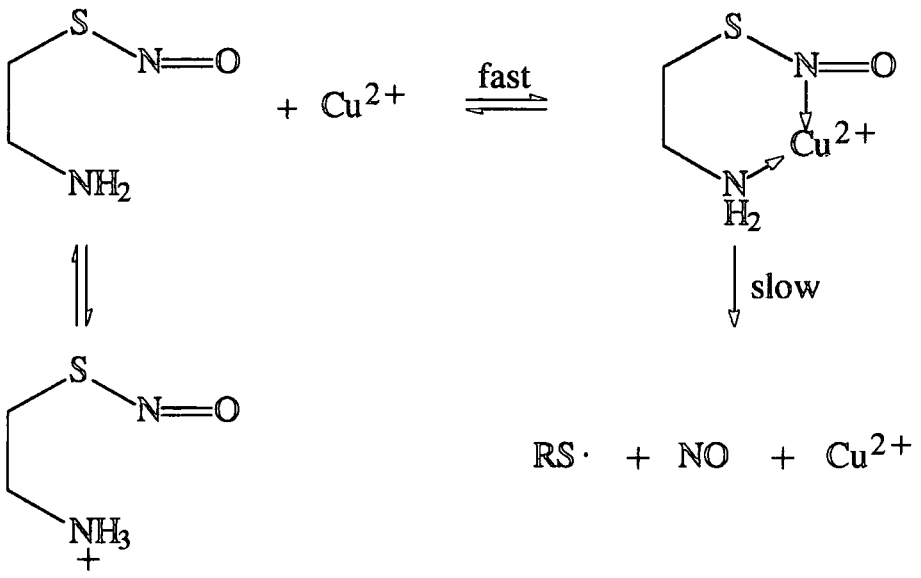


(4.1)



(4.2)

The structural requirements for reaction with Cu^{2+} have been established. The mechanism for NO release is not fully understood. It is proposed that upon rapid formation of the intermediate the complex slowly reacts causing homolytic fission of the weak S-N bond and then breaks apart to give NO, Cu^{2+} and RS· (scheme 4.2).



Scheme 4.2

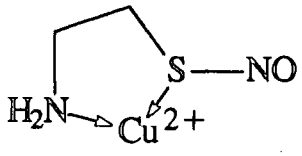
The reactive intermediate has been written as a 2-co-ordinate complex. The complex is likely to be 4- or 6-co-ordinate in solution with the other co-ordination sites being taken by water molecules or even hydroxide ions (at pH 7.4). Given the first order dependency of the reaction on nitrosothiol it is unlikely that two nitrosothiols will complex one Cu^{2+}. There is a possibility though that the reaction involves a redox process between Cu^{2+} and Cu^{+}. The biochemistry of Cu^{2+} is dominated by the inter conversion between the two oxidation states[14] for one electron transfers. The esr spectrum of Cu^{2+} was observed during the reaction with SNAP in static and flow cells. No change in the spectrum due to removal of Cu^{2+} by reduction was seen. Quantitative change of the Cu^{2+} to Cu^{+} can therefore be ruled out of the mechanism.

It was shown (section 3.3) that no redox reaction occurs between Cu^{2+} and NO in aqueous solution. This would appear to preclude the reduction of Cu^{2+} to Cu^{+} by NO upon the dissociation of the nitrosothiol/Cu^{2+} complex after reaction. The Cu^{2+} seems to be undergoing no reduction during the reaction.

An alternative to the 6-membered reactive intermediate would be a 5-membered intermediate with the Cu^{2+} co-ordinated through the S-atom and a N- or O-atom of the neighbouring group (figure 4.7). It is known[15] that amino acids such

as cysteine and penicillamine bind Cu^{2+} bidentately through the S- and N-atoms. In the case of D-penicillamine this binding is so strong that it is used in the treatment of Wilson's disease[16], which is caused by elevated levels of Cu^{2+} in the body. Indeed, recently it has been shown[17] that penicillamine will form a bis complex with Cu^{2+} even when the Cu^{2+} is already complexed by EDTA.

Figure 4.7



Two denitrosation reactions of nitrosothiols are known to involve the S-atom. Acid catalysed denitrosation involves protonation of the S-atom and the Hg^{2+} catalysed reaction proceeds via S-Hg^{2+} co-ordination[18]. Both reactions lead to NO^+ being released rather than NO. As the Cu^{2+} catalysed reaction gives virtually quantitative NO it seems unlikely that the mechanism follows the 5-membered intermediate.

4.3 Reaction of Cu(I) with S-Nitrosothiols

S-Nitrosothiols were also reacted with Cu^+ in pH 7.4 buffer to see whether a change in the oxidation state of the copper would affect the reaction. In normal aerated water Cu^+ is rapidly oxidised to Cu^{2+}, so the reaction with nitrosothiols needs to be carried out in deaerated buffer solution. Initially the reaction was observed using a NO specific electrode. The reaction with SNAP gave a much larger yield of NO (40%) than with Cu^{2+} (15%), which suggests that the reaction is appreciably faster than with Cu^{2+}. Spectrophotometric measurements were then conducted to establish the rate equation and obtain a second order rate constant, k'_2, (equation 4.5) for the reaction of Cu^+ with SNAP. Solutions of $CuSO_4$ were

reduced[19] with dithionite to give Cu^+ salts. When this was carried out in anaerobic solution the results gave bad first order kinetics. However, the reduction in aerobic solution produced a red/pink solution that was probably Cu_2O. Reaction of this solution with SNAP in anaerobic buffer solution gave good first order kinetics (table 4.12). The reduced copper solution was used immediately, as Cu^+ will disproportionate[19] to Cu^0 and Cu^{2+}.

Table 4.12

Rate data for the decomposition of SNAP by Cu^+ at pH 7.4

10^6 [Cu^+] (mol dm^{-3})	10^2 k_{obs} (s^{-1})
5	1.42 ± 0.07
10	1.77 ± 0.17
15	2.39 ± 0.30
19	2.99 ± 0.04
25	3.61 ± 0.15
30	3.91 ± 0.21

The rate equation has been established (equation 4.5) and the reaction is first order in both nitrosothiol and Cu^+. A plot of k_{obs} against [Cu^+] is linear over 5-30 μM Cu^+ (figure 4.7) and there is an intercept, probably due to the residual Cu^{2+} in the buffer or, less likely, the thermal decomposition of the nitrosothiol. The value determined for the second order rate constant, k'_2, for the reaction of Cu^+ with SNAP is 1100 ± 100 mol^{-1} dm^3 s^{-1}. The corresponding reaction with Cu^{2+} gives a second order rate constant k_2 = 20 ± 1 mol^{-1} dm^3 s^{-1}.

$$\text{Rate} = k'_2 [\text{RSNO}] [Cu^+] + c \quad \text{eqn. 4.5}$$

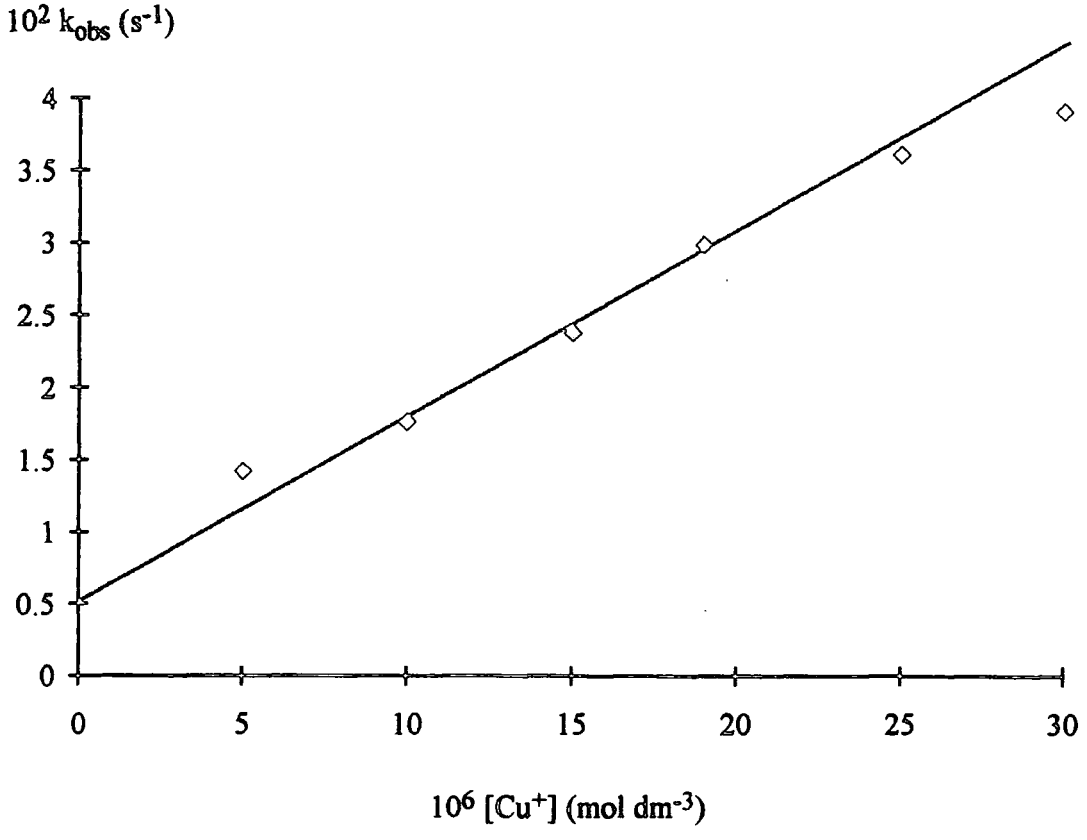
$$\text{Where } c = k_2 [\text{RSNO}] [Cu^{2+}]$$

and k_2 is the second order rate constant for the reaction of SNAP and Cu^{2+}

$$k_{obs} = k'_2 [Cu^+] + k_2 [Cu^{2+}]_{residual} \quad \text{eqn. 4.6}$$

Figure 4.7

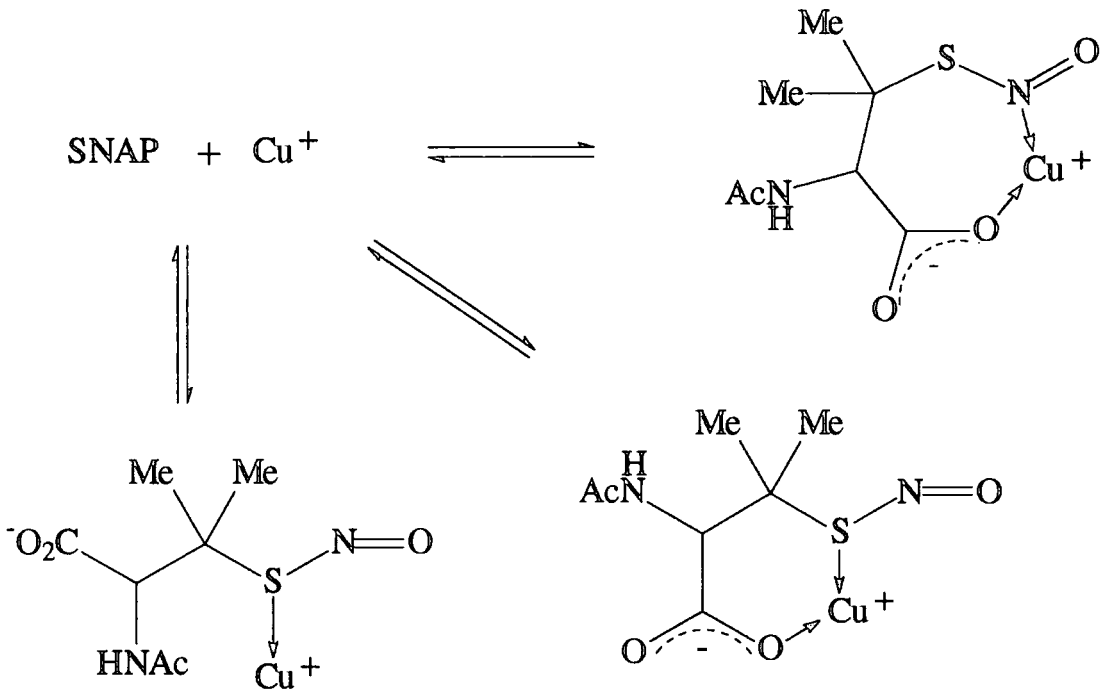
Graph of k_{obs} against [Cu+] for the decomposition of SNAP (5×10^{-4} M) at pH 7.4



In anaerobic solution the reaction is catalytic with respect to the Cu^+. The reaction cannot involve a redox reaction giving Cu^{2+} because their is no way of regenerating Cu^+ under these conditions. Comparison of the second order rate constants for reaction with SNAP shows that Cu^+ is some fifty times more reactive towards NO production than Cu^{2+}. Cu^+ is a soft Lewis acid and its co-ordination chemistry[12] is dominated by S-atoms, which are generally soft Lewis bases. The reaction may therefore be expected to proceed via Cu^+ co-ordination to the S-atom of the S-nitroso group, instead of the N-atom, and a N-atom or O-atom of a neighbouring group depending on the substrate. This would give 6- or 7-membered ring intermediate (figure 4.9) in a similar manner to the Cu^{2+} reaction or even coordination to the S-atom alone.

neighbouring group depending on the substrate. This would give 6- or 7-membered ring intermediate (figure 4.9) in a similar manner to the Cu^{2+} reaction or even coordination to the S-atom alone.

Figure 4.9



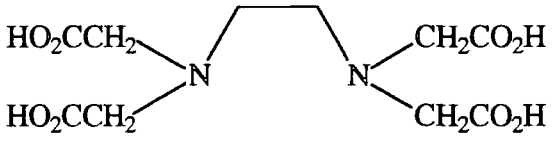
The reaction with SNOG has also been investigated using the NO specific electrode. Reaction with Cu^{2+} usually gives < 2% yield of NO but yields of 15% were obtained with Cu^{+}. This suggests that Cu^{+} may not need to be bidentately complexed by the nitrosothiol in order to react (figure 4.8). A range of nitrosothiol substrates would need to be examined to show this, for example t-butylnitrosothiol only has the S-nitroso group capable of binding to a metal centre. When more reactive nitrosothiols are reacted there is only a slight increase in the observed rate constant as compared with the Cu^{2+} reaction. S-Nitrosocysteamine when reacted with Cu^{+} (1×10^{-6} M) reacts twice as fast as with Cu^{2+} at the same concentration.

As there is seemingly little variation in reactivity between nitrosothiols when reacting with Cu^+ it is possible that co-ordination to the S-nitroso group alone occurs. In this case the formation of the Cu^+/S-nitroso intermediate must lead to fission of the S-N bond producing NO and the thiyl radical. The major drawback to this mechanism is that co-ordination to the S-atom in S-nitrosothiols usually leads to NO^+ formation. Thus far there is no evidence to support any of these mechanisms, and more work needs to be done with the Cu^+ catalysed reaction.

4.4 Effect on Reaction of Known Metal Chelators

4.4.1 Ethylenediaminetetraacetic Acid (EDTA)

Transition metals were first shown to mediate S-nitrosothiol decomposition to RS· and NO by the fact that addition of excess EDTA (4.3), a known metal chelator, could prevent the reaction.



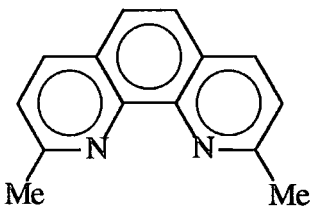
(4.3)

Addition of EDTA to a solution of SNAP will slow the reaction with Cu^{2+}. The reaction of SNAP with 50 μM Cu^{2+} has an observed rate constant of 18.4 x 10^{-4} s^{-1}, but on addition of 50 μM EDTA the rate constant decreases to 4 x 10^{-4} s^{-1}. Increasing the EDTA concentration slows the reaction further until the reaction is completely inhibited at > 70 μM EDTA. The addition of EDTA also gives increasingly poorer first order kinetics. The reason for the inhibition of the reaction is the competitive complexation of the Cu^{2+}. The same result is obtained if S-nitroso cysteine is used instead of SNAP. However, a larger concentration (~1mM) is

required to prevent reaction as this nitrosothiol is better Cu^{2+} chelator as is shown by its greater reactivity.

4.4.2 Neocuproine

Neocuproine (4.4) (2,9-dimethyl-1,10-phenanthroline) is a Cu^{+} chelator[20]. This chelator was added to the reaction of S-nitrosothiolactic acid with Cu^{2+} to investigate whether any Cu^{+} was formed. If Cu^{+} was involved in the process then complexing it should slow or even stop the reaction with the S-nitrosothiol.



(4.4)

Due to the large absorption of neocuproine at < 400nm it was not possible to observe the decomposition of nitrosothiol directly. The complex between Cu^{+} and neocuproine had a maximum absorbance at 456nm. Reaction of Cu^{2+} with S-nitrosothiolactic acid at pH 7.4 produced no significant absorbance increase at 456nm. The reaction was then repeated using the nitric oxide specific electrode. The results (table 4.13) showed that the neocuproine was also complexing Cu^{2+}, as in this case the Cu^{2+} was in excess of the nitrosothiol. If the neocuproine complexed Cu^{+} only then under these conditions we would expect the reaction to progress as if there were no chelator present.

Table 4.13

Yields of NO for reaction of Cu^{2+} with S-nitrosothiolactic acid (20μM) at pH 7.4 in the presence of neocuproine

% NO detected	10^6 [neocuproine] (mol dm^{-3})	10^6 [Cu^{2+}] (mol dm^{-3})
55	-	40
53	-	40
3.5	120	40
4.1	120	40

Neocuproine will complex Cu^{+} and Cu^{2+}, but also makes observation of the reaction more difficult. The use of neocuproine did not elucidate any further the mechanism of the metal catalysed decomposition of nitrosothiols.

4.5 Conclusion

Using the ideas of bidentate complexation around Cu^{2+} and 6- or 7-membered intermediates it should be possible to make a good estimate of how reactive nitrosothiols are towards Cu^{2+} catalysed NO formation. Compounds containing amine groups α or β to the nitroso group should be very reactive; whereas compounds that have no adjacent functional groups, or groups that have had their electron density delocalised (e.g. by acetylation), should be very stable to reaction with Cu^{2+}.

It would be nice to report that these trends were followed when S-nitrosothiols are used *in vivo* or in organ baths, but this is not the case. A recent paper[3] on the biological activity of S-nitrosothiols shows that there appears to be no trend between "test-tube" reactivity and biological activity. A well known example of this is SNOG which is very unreactive to metal catalysed decomposition, but is a potent inhibitor of platelet aggregation[21]. Of course there is no free Cu^{2+} *in vivo* as it is bound in proteins or in enzyme centres, but there may be reactions with enzyme centres or with haem groups to effect reaction. Enzymic reactions within vessels and at cell surfaces

have been put forward[3] as the modes of action of nitrosothiols in biological systems, rather than "spontaneous" breakdown to give NO, but the explanations and the evidence to support them are not as yet totally satisfactory. S-Nitrosothiols will produce NO on reaction with metal centres such as Cu^{2+}, Cu^{+} and Fe^{2+} (equation 4.7); and via a one electron reduction with dithionite or ascorbic acid (equation 4.8).

$$RSNO + M^{n+} \longrightarrow RS\cdot + NO + M^{n+} \qquad \text{eqn. 4.7}$$

Where $M^{n+} = Cu^{2+}$, Cu^{+} or Fe^{2+}.

$$RSNO + e^{-} \longrightarrow RS^{-} + NO \qquad \text{eqn. 4.8}$$

One or any combination of the above reactions may be responsible for the reaction of nitrosothiols in biological systems. However, as biological systems are complex and contain many variables it is not yet possible to explain the biological behaviour of nitrosothiols and hence predict their putative therapeutic properties.

References

1. J. Barrett, D.F. Debenham & J. Glauser, *Chem. Comm.*, 1965, 12, 248.
2. S. Oae & K. Shinhama, *Organic Preparations and Procedures Int.*, 1983, 15, 165.
3. W.R. Mathews & S.W. Kerr, *J. Pharm. Exp. Ther.*, 1993, 267, 1529.
4. J.W. Park, *Biochem. Biophys. Res. Comm.*, 1988, 152, 916.
5. J. McAninly, D.L.H. Williams, S.C. Askew, A.R. Butler & C. Russell, *J. Chem. Soc., Chem. Commun.*, 1993, 1758.
6. D.A. Wink, J.F. Darbyshire, R.W. Nims, J.E. Saavedra & P.C. Ford, *Chem. Res. Toxicol.*, 1993, 6, 23.
7. P.C. Jocelyn, Biochemistry of the SH Group, Academic Press, London, 1972, 11.
8. L.Field, R.V. Dilts, R. Ravichandran, P.G. Lenhert & G.E. Carnhan, *J. Chem. Soc., Chem. Comm.*, 1978, 249.
9. R.J. Doedens, *Prog. Inorg. Chem.*, 1976, 21, 209.
10. W.P. Jencks, Catalysis in Chemistry and Enzymology, Dover Publications Inc., New York, 1987, 12.
11. H.M.S. Patel & D.L.H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1990, 37.
12. B.J. Hathaway, in Comprehensive Co-ordination Chemistry, Pergamon, 1987, Vol. 7, 594.
13. F.J. Davis, B.C. Gilbert & R.O.C. Norman, *J. Chem. Soc., Perkin Trans. 2*, 1983, 1763.
14. R.W. Hay, Bio-inorganic Chemistry, Wiley, Chichester, 1984, 128.
15. S.H. Laurie, in Comprehensive Coordination Chemistry, Pergamon, Oxford, 1987, Vol. 2, 748.
16. H.E. Howard-Lock & C.J.L. Lock, in Comprehensive Coordination Chemistry, Pergamon, Oxford, 1987, Vol. 6, 769.
17. A. Hanaki & H. Sago, *Chem. Lett.*, 1994, 109.
18. B. Saville, *Analyst*, 1958, **83**, 670.

19. A.G. Sharpe, Inorganic Chemistry, Longman, New York, 1986, 611.

20. Y. Yoshida, J, Tsuchiya, & E. Niki, *Biochim. Biophys. Acta*, 1994, 1200, 85.

21. M.W. Radomski, D.D. Rees, A. Dutra & S. Moncada, *Br. J. Pharmacol.*, 1992, 107, 745.

CHAPTER 5

Other Factors Affecting NO Formation from S-Nitrosothiols

5 Other Factors Affecting NO Formation from S-Nitrosothiols

5.1 Introduction

The *in vivo* reactions of S-nitrosothiols are expected to be complex, involving more than just reactions at metal centres. The action of thiols, sulphides, disulphides and other factors were investigated to observe how the reaction of S-nitrosothiols with Cu^{2+} was modified.

5.2 Transnitrosation

Transnitrosation is the transfer of the NO^+ moiety from one substrate to another by nucleophilic attack on the nitroso compound. This reaction is known to occur between alkyl nitrites and alcohols[1] and between alkyl nitrites and amines[2] (equation 5.1).

$$\mathrm{RONO + R'XH \longrightarrow R'XNO + RO^- + H^+} \qquad \text{eqn. 5.1}$$

$\mathrm{RO^-} \rightleftharpoons \mathrm{ROH}$

where X = O or NR"

The analogous reaction with thiols (X = S) has also been studied[3] and the pH-rate constant profile has all the characteristics of nucleophilic attack by the thiolate anion (RS^-) on the alkyl nitrite. It seems reasonable then to expect transnitrosation to occur between nitrosothiols and thiols (equation 5.2).

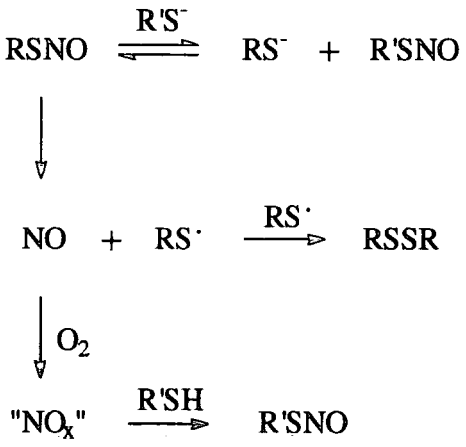
$$\mathrm{RSNO + R'S^- \rightleftharpoons RS^- + R'SNO} \qquad \text{eqn. 5.2}$$

$\mathrm{R'S^-} \rightleftharpoons \mathrm{R'SH}$ $\qquad$ $\mathrm{RS^-} \rightleftharpoons \mathrm{RSH}$

This reaction had not been studied kinetically, however, it was known that addition of thiols to nitrosothiols in organic solvents produced the mixed disulphide[4] (equation 5.3). This reaction may proceed via a transnitrosation step.

$$2RSNO + 2R'SH \longrightarrow 2RSSR' + N_2O + H_2O \quad \text{eqn. 5.3}$$

It has also been shown[5] that transnitrosation occurs between SNOG and cysteine in pH 7.4 buffer solution producing the mixed disulphide. There was a possibility that transnitrosation was occurring via the loss of NO from the nitrosothiol followed by some nitrosation process to give the new nitrosothiol. Work was carried out to determine which mechanism fitted the reaction (scheme 5.1).



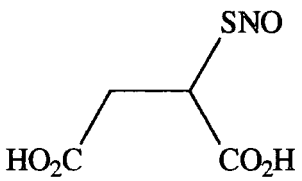
Scheme 5.1

Transnitrosation can be observed spectrophotometrically by simply mixing a thiol and a nitrosothiol in a basic solution. This is the case when SNOG (5×10^{-4}M) is added to N-acetylpenicillamine (5×10^{-3}M) in a pH 7.4 phosphate buffer. There is a rapid transnitrosation reaction. Scanning the solution shortly after mixing shows the presence of both the nitrosothiols SNOG and SNAP due to their characteristic absorbances at 542nm and 592nm respectively. The transnitrosation reaction is rapid so stopped flow techniques are needed to measure the observed rate constants.

When a large excess of thiol is added the equilibrium (equation 5.2) lies over to the side of formation of the new nitrosothiol. The reaction of SNAP with thioglycolic acid (mercaptoacetic acid) had been studied[6] using the difference in the absorption spectra at 600nm. Since SNAP is a green compound it absorbs at 600nm, whereas the S-nitrosothioglycolic acid is a red compound absorbing at 540nm. The extinction values at these wavelengths are small, typically 20 mol^{-1} dm^3 cm^{-1}, so high concentrations of reactants are required. Problems arose at high pH values due to the poor solubility of SNAP.

As has already been shown (table 4.1) nitrosothiols have a range of extinction coefficients at 340nm (700-1100 mol^{-1} dm^3 cm^{-1}). Exploiting the difference in ε_{340} values made it possible to measure rate constants for transnitrosation.

S-Nitrosocysteine (ε_{340} = 850 mol^{-1} dm^3 cm^{-1}) was reacted with thiomalic acid (RSNO ε_{340} = 1060 mol^{-1} dm^3 cm^{-1}) over the pH range 6.5-12.7 observing the increase in absorbance due to S-nitrosothiomalic acid (5.1) formation. The thiomalic acid was in large excess over S-nitrosocysteine and good first order behaviour was observed throughout.



(5.1)

The S-nitrosocysteine was prepared by the quantitative nitrosation of cysteine by sodium nitrite in 0.1M $HClO_4$, and was then diluted for use. The thiomalic acid solutions were prepared in buffered solutions using a range of buffer systems (table 5.1). The thiol solutions were made up and then used immediately, since oxidation of thiols to disulphides is mediated by oxygen and transition metal ions in basic solution[7]. Table 5.1 shows the variation of k_{obs}, the observed first order rate constant, with pH.

Table 5.1

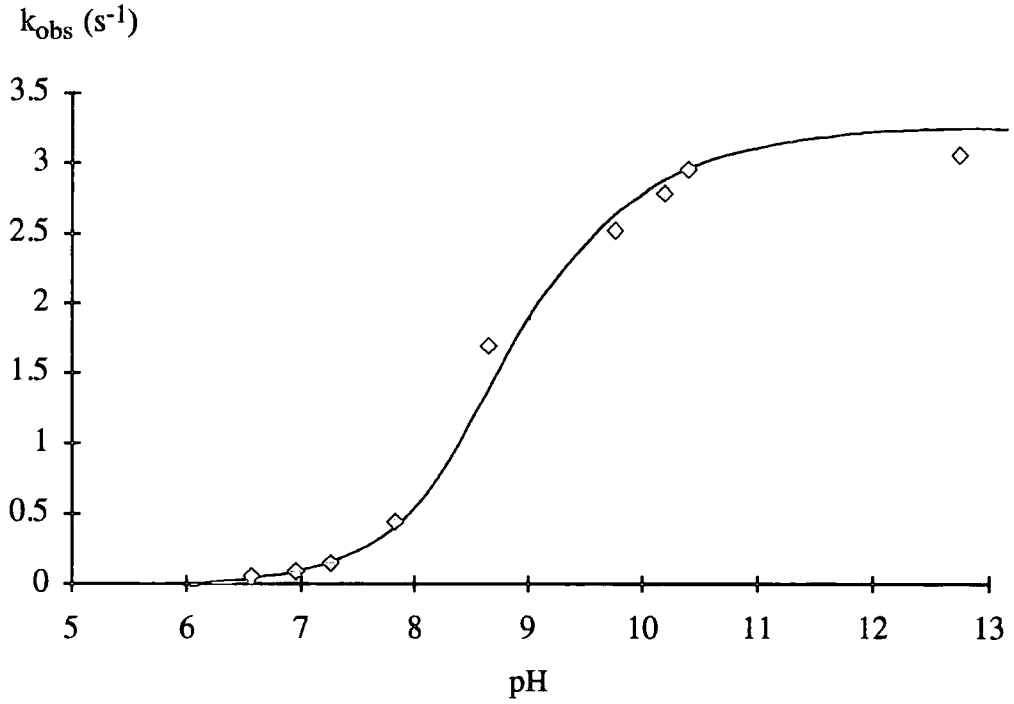
Variation of k_{obs} with pH for the reaction of S-nitrosocysteine (5×10^{-4} mol dm^{-3}) and thiomalic acid (0.02 mol dm^{-3})

pH	k_{obs} (s^{-1})	Buffer systems
6.56	0.0558 ± 0.001	KH_2PO_4/NaOH
6.95	0.0895 ± 0.008	KH_2PO_4/NaOH
7.25	0.1480 ± 0.003	KH_2PO_4/NaOH
7.82	0.4450 ± 0.030	KH_2PO_4/NaOH
8.65	1.696 ± 0.065	$Na_2B_4O_7$/HCl
9.75	2.524 ± 0.114	$Na_2B_4O_7$/NaOH
10.18	2.787 ± 0.140	$Na_2B_4O_7$/NaOH
10.40	2.961 ± 0.138	$Na_2B_4O_7$/NaOH
12.74	3.062 ± 0.121	KCl/NaOH

A plot of k_{obs} against pH gives a sigmoidal curve (figure 5.1) which is characteristic of reaction via the thiolate anion. The observed rate constant, k_{obs}, increases with increasing pH to an upper limit where all the thiol has been deprotonated. The point of inflexion should correspond to the pK_a of the thiol. The alternative mechanism, involving NO formation followed by nitrosation of the thiol, can be discounted. The observed rate constants of the transnitrosation reaction are orders of magnitude greater than the Cu^{2+} catalysed formation of NO. The Cu^{2+} levels in the buffer solutions should not have been large enough to allow the NO-producing reaction to compete with the transnitrosation reaction. As a further check runs were repeated with EDTA added as a Cu^{2+} chelator. The observed rate constants remained unchanged.

Figure 5.1

Plot of the observed rate constants against pH for the reaction of S-nitrosocysteine (5×10^{-4} M) with thiomalic acid (0.02M)



Variation of the thiomalic acid concentration at the same pH value (pH 7.75) showed a first order dependency on the total thiol concentration (table 5.2) and hence the thiolate anion concentration. A plot of k_{obs} against the total thiol concentration (figure 5.2) was linear with a slope of 26.1 ± 0.3 mol^{-1} dm^3 s^{-1} and so the rate equation was established (equation 5.4).

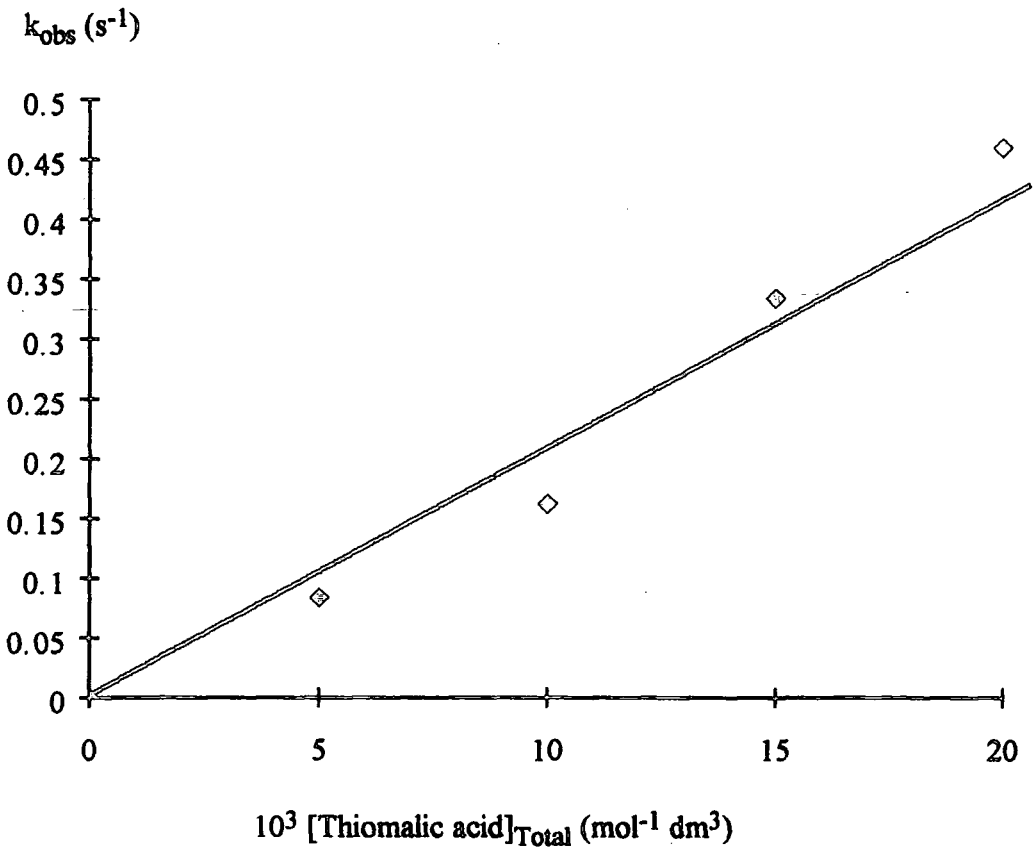
Table 5.2

Variation of k_{obs} with [thiomalic acid] in the transnitrosation reaction of S-nitrosocysteine (5×10^{-4} mol dm^{-3}) at pH 7.75

10^3 [Thiomalic acid] (mol dm^{-3})	k_{obs} (s^{-1})
5.0	0.084
10	0.162
15	0.334
20	0.460

Figure 5.2

Plot of the observed rate constants against total thiol concentration for the reaction of S-nitrosocysteine (5×10^{-4} mol^{-1} dm^3) with thiomalic acid at pH 7.75



$$\text{Rate} = k_2 [\text{RSNO}] [\text{R'S}^-] \qquad \text{eqn. 5.4}$$

$$\text{Rate} = k_{obs} [\text{RSNO}] \qquad \text{eqn. 5.5}$$

$$\text{where } k_{obs} = \frac{k_2 K'_a [\text{R'SH}]_{Total}}{K'_a + [H^+]}$$

$$\text{at pH 7.75 } K_a << [H+]$$

$$\text{thus } k_{obs} = \frac{k_2 K'_a [\text{R'SH}]_{Total}}{[H^+]} \qquad \text{eqn. 5.6}$$

$$\text{slope} = \frac{k_2 K'_a}{[H^+]} = 26.1 \text{ mol}^{-1} \text{ dm}^3$$

In order to obtain a value for k_2, the second order rate constant for transnitrosation, a value for the thiol dissociation constant is needed. Literature values[8] for the pK_a of the thiol group of thiomalic acid range from 9.7 to 10.5 which give values for k_2 in the range 2600 - 17400 mol^{-1} dm^3 s^{-1}. Clearly an accurate pK_a value is vital for obtaining a meaningful result. The sigmoidal curve produced by plotting k_{obs} against pH (figure 5.2) should show where the pK_a of thiomalic acid lies. If the curve is symmetrical then the point of inflexion will lie where k_{obs} is half the maximum value. The plot gives the pK_a value to be 8.55 and this produces a value for k_2 of 184 ± 20 mol^{-1} dm^3 s^{-1}. Alternatively, using the k_{obs} value obtained for the reaction at pH 12.74 we can calculate a value for k_2 since $[R'S^-] = [RSH]_{Total}$. The value for the second order rate constant is thus calculated to be 165 ± 6 mol^{-1} dm^3 s^{-1}, which is in quite good agreement with the earlier value obtained.

Further transnitrosation reactions between nitrosothiols and thiols have been carried out[9] in order to compare the reactivity of nitrosothiols with that of alkyl nitrites[3]. These reactions were carried out in 0.1M NaOH so that all the thiols were deprotonated. This makes determination of the k_2 values possible without the need

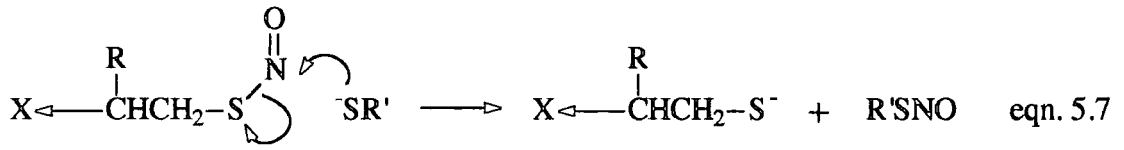
for an accurate pK_a value for the thiol. The reaction was carried out in the same manner as previously described. Table 5.3 summarises the results for the reaction of various thiols with S-nitroso-2-hydroxyethanethiol.

Table 5.3

Second order rate constants for the transnitrosation reaction between various thiols and S-nitroso-2-hydroxyethanethiol in 0.1M NaOH

Thiol	k_2 (mol^{-1} dm^3 s^{-1})
Methylthioglycolate	710 ± 40
Cysteine	445 ± 7
N-Acetylcysteine	432 ± 6
Cysteine ethyl ester	416 ± 10
Cysteamine	300 ± 7
Glutathione	257 ± 4
Thiomalic acid	251 ± 5
N-Acetylpenicillamine	56 ± 2
Penicillamine	23 ± 0.5

Comparing the result from the reaction between S-nitrosocysteine and thiomalic acid and the results in table 5.3 we can see that a value of 8.5 is more reasonable for the pK_a of thiomalic acid than 10.5. The above results show similar trends to those obtained when reacting thiols with alkyl nitrites[3]. The reactivity sequence primary > secondary > tertiary for the attacking nucleophile must be due to steric hindrance. Steric considerations are more important than the electron releasing α-substituents here. Comparing the k_2 values for the reaction of thiomalic acid with the two nitrosothiols we can see that the presence of an β-electron withdrawing group increases k_2 since it stabilises the thiolate leaving group in the transition state (equation 5.7).

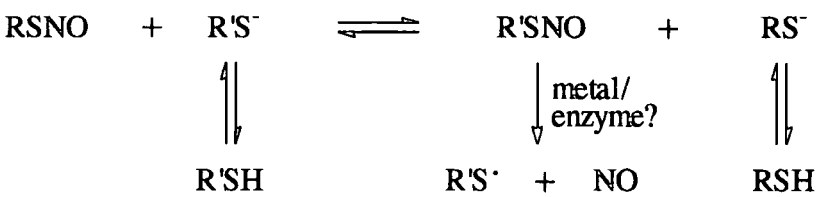


eqn. 5.7

Alkyl nitrites[3] are about ten times less reactive than the nitrosothiols. We might expect this as thiolate groups are better leaving groups than alkoxides. Thiols are stronger nucleophiles than alcohols because the thiolate group is more polarizable than the alkoxide. The S-N bond is also known[10] to be weak (218 kJ mol^{-1}). The transnitrosation process is written as a synchronous process. It is postulated[11] that transnitrosation involving alkyl nitrites is a synchronous process. However, there is no compelling evidence for or against the existence of an intermediate in the transnitrosation reaction[3].

5.3 Transnitrosation at pH 7.4

If nitrosothiols are to be used *in vivo* as sources of NO it is important to know what possible side reactions may also be occurring. Thiols, such as cysteine and glutathione, are known to be present *in vivo* at milli-molar levels[7]. Transnitrosation to a new nitrosothiol followed by NO formation (scheme 5.2) may be a significant process in the body.



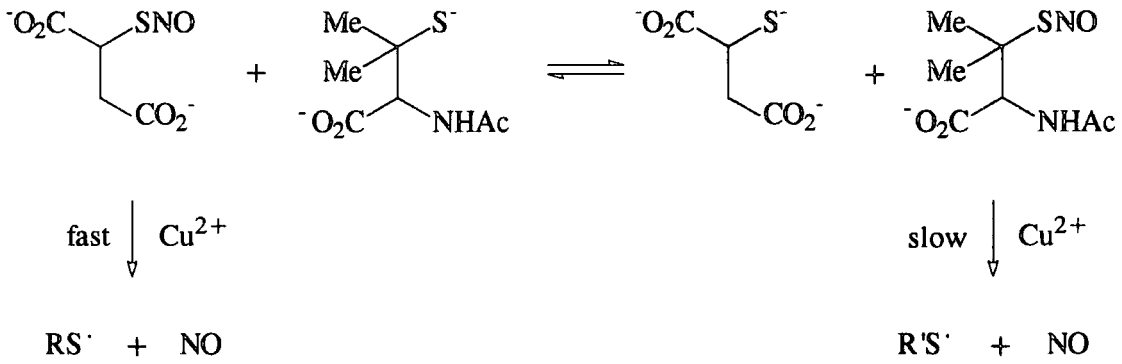
Scheme 5.2

S-Nitrosothiols that reacted very slowly, or even negligibly, with Cu^{2+} were chosen to react with various thiols. SNOG (S-nitrosoglutathione) is essentially unreactive towards Cu^{2+} and so any observed reaction upon addition of a thiol should

be due to the reaction of a different nitrosothiol. Over three hours the absorbance of a solution of SNOG (5 x 10^{-4} M) at pH 7.4 remained constant. Addition of Cu^{2+} (1 x 10^{-5} M) did not produce any observable decrease in absorption. An equivalent amount of N-acetylpenicillamine when added to the SNOG solution produced a 30% decrease in absorption at 340nm in three hours. When Cu^{2+} (1 x 10^{-5} M) was added to a solution of SNOG and N-acetylpenicillamine all the absorbance due to the nitrosothiol was removed within three hours. Scanning this solution after ten minutes showed the small but characteristic absorbance peaks at 542nm and 592nm for SNOG and SNAP respectively. It appears that there is a fast transnitrosation step followed by a much slower reaction with Cu^{2+}. Only SNAP will react with Cu^{2+} in this system. The observed rate constant for the reaction with Cu^{2+} is 3.4 x 10^{-4} s^{-1} which is less than half the value for the reaction of SNAP (5 x 10^{-4} M) with Cu^{2+} (1 x 10^{-5} M) alone at pH 7.4. There must be a competitive complexation between glutathione and Cu^{2+} giving effectively a lower concentration of free Cu^{2+} for reaction.

Since both SNOG and SNAP can be observed after mixing equivalents of SNOG and N-acetylpenicillamine, the reaction must be reversible (scheme 5.2) and rapidly reach equilibrium. The Cu^{2+} reacts with the SNAP pulling the equilibrium completely over to the right hand side so that all the nitrosothiol is reacted. Addition of EDTA (1 x 10^{-4} M) prevents the reaction with Cu^{2+} so only the transnitrosation reaction occurs and no decomposition is observed.

Alternatively, a reactive nitrosothiol, such as that derived from thiomalic acid, can have its reaction with Cu^{2+} slowed by the addition of N-acetylpenicillamine. A ten-fold excess of N-acetylpenicillamine drives the equilibrium over to the side of SNAP formation thus slowing the overall reaction (scheme 5.3).

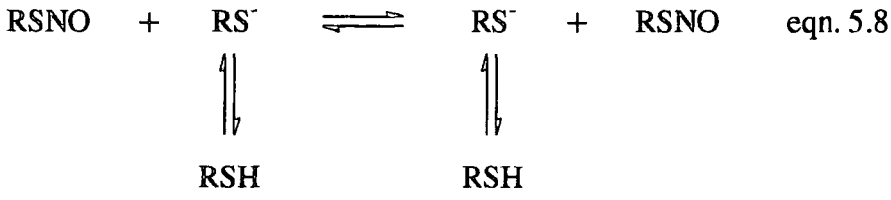


Scheme 5.3

The most common thiols found *in vivo* are cysteine and glutathione. We have shown (section 4.2.2) that their nitrosothiols have vastly different reactivities towards Cu^{2+}. SNOG is virtually unreactive towards NO formation by Cu^{2+}, whereas S-nitrosocysteine is very reactive. When cysteine (5 x 10^{-3}M) is added to SNOG (5 x 10^{-4}M) in pH 7.4 buffer there is a rapid transnitrosation as the equilibrium between the two nitrosothiols is reached and then a slower reaction with Cu^{2+}. EDTA can prevent the reaction of S-nitrosocysteine with Cu^{2+}, but if the same amount of EDTA is added to the SNOG/cysteine mixture the nitrosothiol decomposition still proceeds. The system is dynamic and the Cu^{2+} will be complexed to different chelators with respect to time. This allows the some of the S-nitrosocysteine to complex the Cu^{2+} and react. As expected, increasing [Cu^{2+}] increases the observed rate constant and increasing [EDTA] has the opposite effect.

5.4 Effect of Adding Thiols to S-Nitrosothiols

The addition of thiols to solutions of nitrosothiols derived from different thiols leads to transnitrosation and formation of a pair or one new nitrosothiol (equation 5.2). The new nitrosothiol may have a different reactivity towards Cu^{2+} and such reactions were considered in section 5.3. However, it would also be interesting to see what the effect would be of the thiol group alone on the reaction. This is achieved by adding the same thiol that the nitrosothiol is based on. Transnitrosation will still occur but the product is the same as the starting material (equation 5.8)

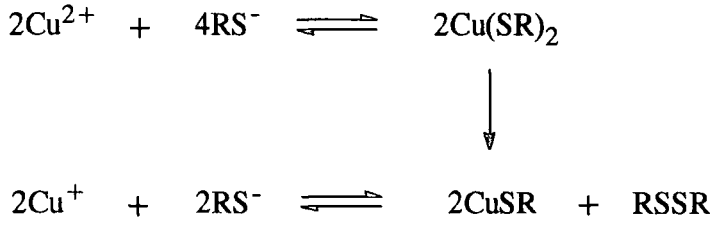


eqn. 5.8

It is thus possible to observe what effect the thiol group has on the Cu^{2+} catalysed reaction with nitrosothiols. There are three possibilities for the reaction:

1. there will be no effect,
2. the thiol will competitively complex Cu^{2+} thus slowing the reaction,
3. or, the thiol will react with Cu^{2+} to produce Cu^{+} which is more reactive towards nitrosothiols.

It is well known[12] that thiols will reduce Cu^{2+} to Cu^{+} in basic solution. The reaction is believed to proceed by formation of a copper(II) dimercaptide followed by a redox step to form Cu^{+} and the disulphide (scheme 5.4).



Scheme 5.4

The effect of adding cysteine to S-nitrosocysteine was considered firstly. The nitrosothiol was reacted with Cu^{2+} alone in pH 7.4 phosphate buffer. Cysteine was added over the range 5μM - 1mM and the results are summarised in table 5.4.

Table 5.4

Kinetic data for the Cu^{2+} (5 x 10^{-5}M) catalysed reaction of S-nitrosocysteine (5 x 10^{-4}M) with added cysteine at pH 7.4

10^6 [Cysteine] (mol dm^{-3})	k_o (s^{-1})[a]
0	0.254
5	0.251
10	0.366
25	0.470
50	0.551
75	0.294
100	0.191
250	0.057
500	0.028
750	0.018
1000	0.014

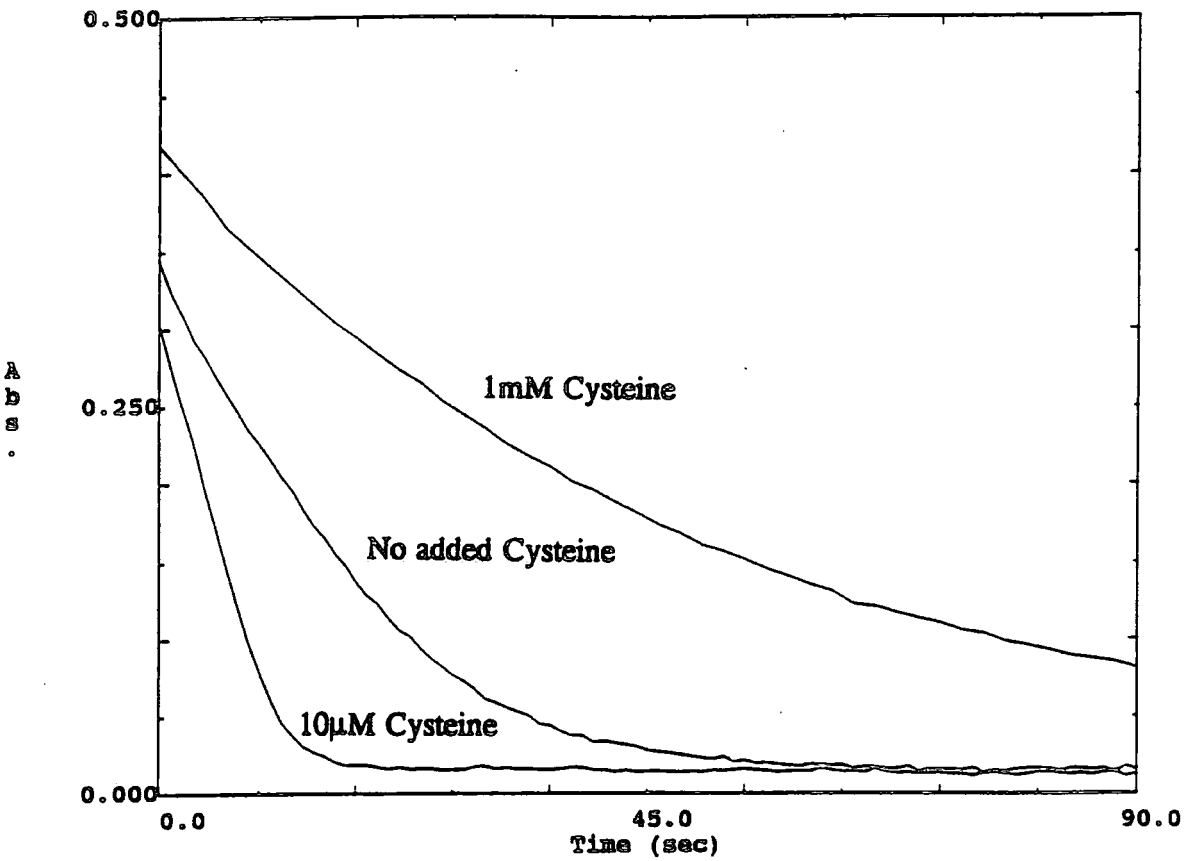
[a] First order kinetics obtained had correlation coefficients of < 0.90 associated with them, so were not purely first order reactions.

Adding the thiol has two effects on the reaction of S-nitrosocysteine. When greater than 50μM cysteine is added the reaction is inhibited and the observed rate constant lies below that obtained for the reaction without added thiol. This must be due to the cysteine complexing the Cu^{2+}. At concentrations of 50μM cysteine or less the reaction is catalysed. Low concentrations of cysteine will not act as competitive chelators since the nitrosothiol is in excess over the thiol. However, any Cu^{+} formed will now complex with the nitrosothiol rather than the thiol, thus increasing the rate of the reaction. The kinetics in these reactions were not good and contained elements of zero and first order kinetics. When cysteine acts as a competitive chelator the first

order kinetics are not as good as without the added cysteine. This is similar to the effect of adding EDTA (section 4.4.1). The kinetics of the cysteine catalysed reaction involve a rapid reaction followed by a slower one. The initial fast reaction is probably with Cu^{+}, but this will be oxidised by dissolved oxygen and can only be generated as long as there is thiol available. The thiol is quickly used up and then the slower Cu^{2+} reaction will dominate (figure 5.4).

Figure 5.4

Traces Showing the Decomposition of S-Nitrosocysteine (5×10^{-4}M) with Cu^{2+} (1×10^{-5}M) at pH 7.4 in the Presence of Cysteine



A similar result is obtained by reacting N-acetylpenicillamine with SNAP. However, in this case addition of the thiol always catalyses the reaction. The results are summarised in table 5.5. The catalysis must be due to Cu^{+} formation by N-

acetylpenicillamine from Cu^{2+}. The Cu^{+} produced will also be complexed by the N-acetylpenicillamine which is why k_o increases with decreasing thiol concentration. The results were not very reproducible, but the trend always remained the same.

Table 5.5

Kinetic results for the reaction of SNAP (5×10^{-4}M) with Cu^{2+} (1×10^{-5}M) catalysed by N-acetylpenicillamine (NAP) at pH 7.4

10^5 [NAP] (mol dm^{-3})	10^3 k_o (s^{-1})
0	0.9
60	3.4
40	3.9
20	6.0
10	10.2
5	16.5
2	30.6

Repeating the reaction under seemingly identical conditions gave absorbance/time traces that were definitely split into two parts: a fast reaction followed by a much slower one. First order rate constants could not be obtained. In this reaction the thiol seemed to have been used up before the reaction had finished.

Two other reactions of the nitrosothiol/thiol systems were briefly investigated. Glutathione (5×10^{-3}M) added to SNOG (5×10^{-4}M) and Cu^{2+} (1×10^{-5}M) increased the rate of decomposition. However, this process was still very slow with only 30% reacting in 60 minutes. No kinetics were measured but the reaction was probably due to Cu^{+} formation. Conversely, adding thiomalic acid (0.01M) to S-nitrosothiomalic acid (5×10^{-4}M) and Cu^{2+} (1×10^{-5}M) stopped the reaction. In this case, the Cu^{2+} is complexed by the carboxylic acid groups which prevents reaction with the nitrosothiol and with the thiol group. Hence, there is no measurable reaction ($< 3\%$ in 90 minutes) with Cu^{2+}.

5.5 Effect of Adding Disulphide to a S-Nitrosothiol

Although there appeared to be no auto catalysis in the Cu^{2+}/nitrosothiol reaction it seemed worthwhile investigating whether the products of nitrosothiol decomposition, the disulphide, had any effect on the reaction. The addition of an equimolar amount of N-acetylpenicillamine disulphide to a solution of SNAP (5×10^{-4}M) and Cu^{2+} (1×10^{-5}M) caused decomposition far more rapidly than without the added disulphide. The disulphide concentration was varied to observe the effect on the observed rate constant (table 5.6).

Table 5.6

Kinetic data for the effect on the Cu^{2+} (1×10^{-5}M) catalysed decomposition of SNAP (5×10^{-4}M) by N-acetylpenicillamine disulphide at pH 7.4

10^5 [Disulphide] (mol dm^{-3})	10^4 k_{obs} (s^{-1})
0	9.0 ± 0.2
4	8.5 ± 0.4
6	7.0 ± 0.3
10	11.3 ± 3.7
20	16.6 ± 4.2
30	28.4 ± 4.3
40	81.3 ± 2.1
50	80.7 ± 1.3
60	117.1 ± 3.2
70	127.5 ± 0.7
80	150.0 ± 20.4
100	216.8 ± 11.7

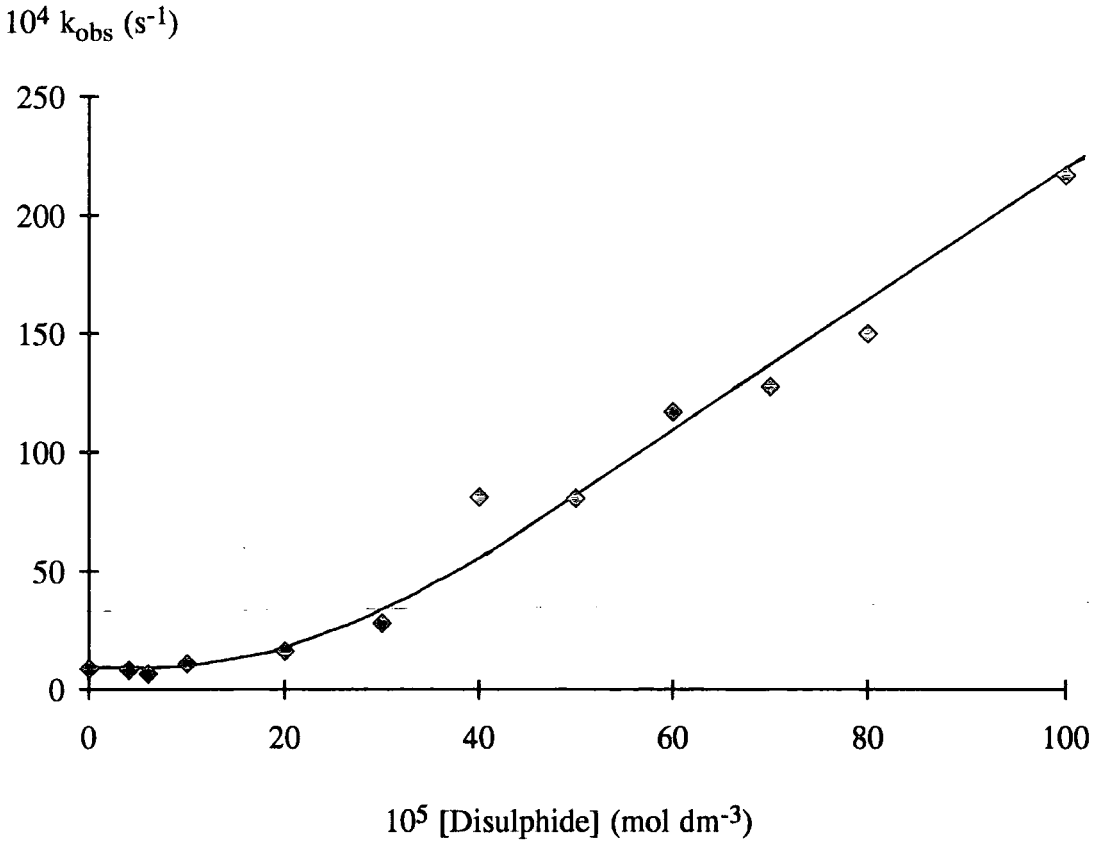
The disulphide has no great effect on the reaction until the concentration reaches 0.4mM and above (figure 5.4). The disulphide may be forming Cu^{+} in a

reaction with Cu^{2+}, however, there is no precedent for this in the literature. Disulphides are known to bind heavy metal ions through O and N atoms rather than the disulphide bridge[13] and first row transition metals bind either weakly or not at all.

Since a large disulphide concentration is required before catalysis is observed this process is probably not an important process either *in vivo* or during the reaction between Cu^{2+} and SNAP.

Figure 5.4

Plot of k_{obs} against added disulphide concentration for the catalysis of NO formation from SNAP (5×10^{-4}M) and Cu^{2+} (1×10^{-5}M) at pH 7.4

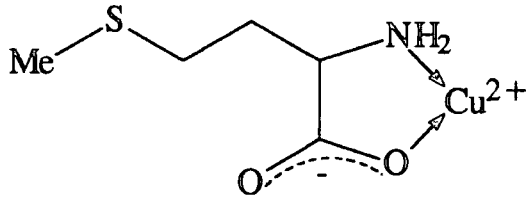


5.6 Other Factors Affecting S-Nitrosothiol Reaction

5.6.1 Addition of a Sulphide

The observation of catalysis of the nitrosothiol/Cu^{2+} reaction by disulphide lead to the investigation of the effects of sulphides. The amino acid methionine (S-methylhomocysteine) is a sulphide and an equimolar amount was added to SNAP (5 x 10^{-4}M) and Cu^{2+} (1 x 10^{-5}M). The Cu^{2+} is complexed by the methionine (figure 5.5) so the concentration of free Cu^{2+} is drastically reduced. There is no evidence for methionine complexing Cu^{2+} through the S atom and it is believed to bind through the O and N atoms[13].

Figure 5.5

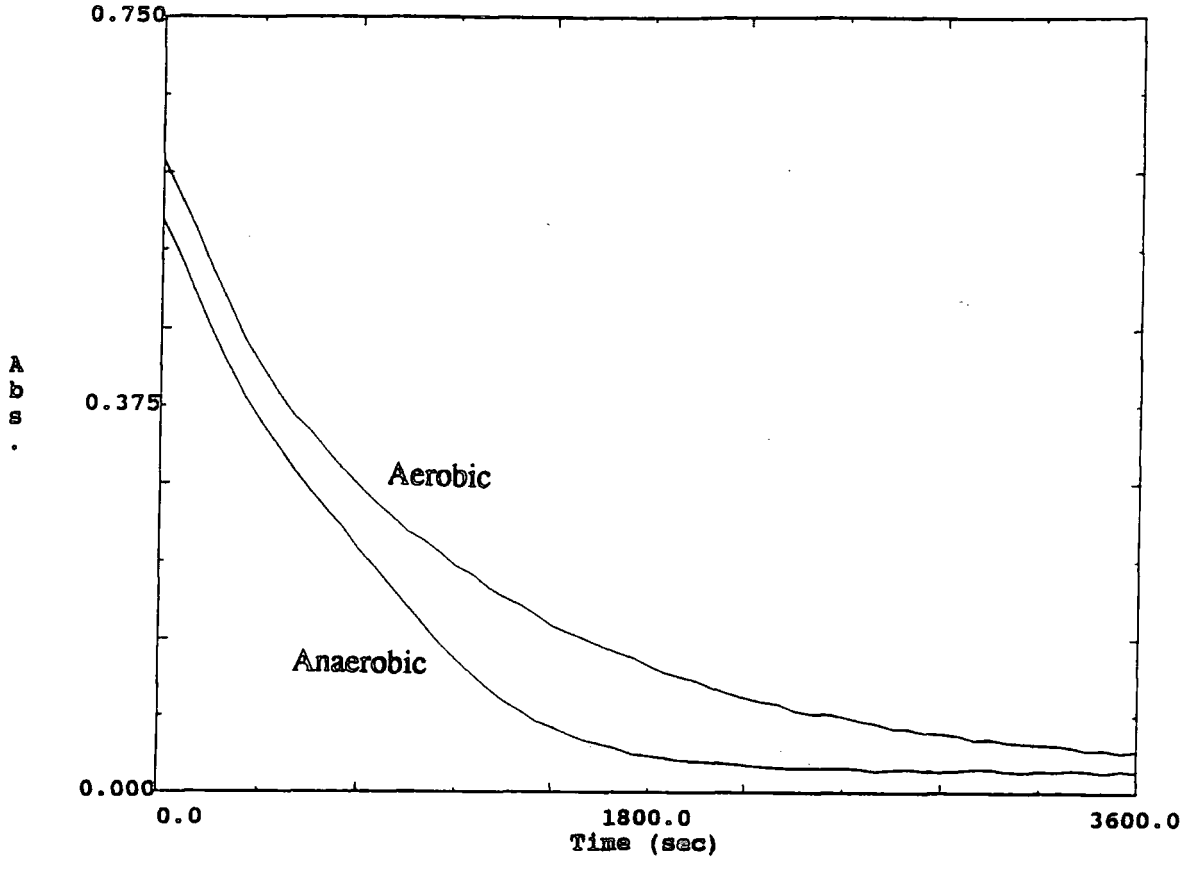


5.6.2 Oxygen Dependency

The major problem involving the possibility of formation of Cu^{+} *in situ* in the nitrosothiol/Cu^{2+} reaction was that oxidation by dissolved oxygen would drastically reduce the life-time of any Cu^{+} present. To this end, SNAP (5 x 10^{-4}M) was reacted with Cu^{2+} (1 x 10^{-5}M) in anaerobic solution at pH 7.4. If Cu^{+} was involved in the reaction the rate of reaction of the nitrosothiol should be significantly enhanced. It has been shown (section 4.3) that Cu^{+} is fifty times more reactive towards SNAP than Cu^{2+}. The observed rate constant for the anaerobic reaction was only twice that for the aerobic reaction (figure 5.6).

Figure 5.6

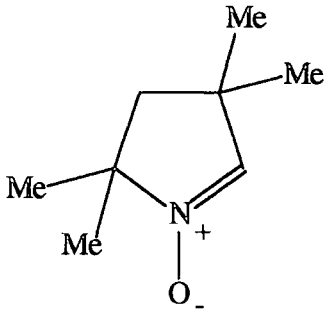
Traces Showing the decomposition of SNAP (5×10^{-4}M) in pH 7.4 buffer by Cu^{2+} (1×10^{-5}M) in Aerobic and Anaerobic Conditions



The data from the anaerobic reaction did not give good first order kinetics. It appears that there may be some conversion of Cu^{2+} to Cu^{+} which accelerates the reaction, but this falls far short of a quantitative redox process. The Cu^{+} may be formed by reaction of the disulphide with the Cu^{2+}, but under aerobic conditions this is rapidly oxidised back to Cu^{2+}. More work needs to be carried out to establish what reaction is occurring under anaerobic conditions.

5.6.2 Spin Trapping

Before disulphides are formed in the breakdown of nitrosothiols, thiyl radicals ($RS^{\cdot}$) are produced. The radicals may affect the Cu^{2+} catalysed reaction of nitrosothiols. 3,3,5,5-Tetramethyl-1-pyrroline-N-oxide (5.1) was used as a radical trap.



(5.1)

This radical trap will donate an electron[14] to a radical producing a very stable radical species which can be detected by esr. The other product of the electron transfer would be the thiolate anion. If the presence of the radical trap were to change the observed rate constant then the presence or absence of the thiyl radical may be important. The radical trap (3×10^{-4}M) was added to SNAP (5×10^{-4}M) and Cu^{2+} (1×10^{-5}M) with and without the N-acetylpenicillamine disulphide (5×10^{-4}M), but no change in k_{obs} was seen. The disulphide was added to see if its reaction with Cu^{2+} involved thiyl radical production. The radical trap was air sensitive and although it was handled under a nitrogen stream it is possible that it was oxidised before it had a chance of reacting with the thiyl radicals. Thus no conclusions can be drawn about the effect of thiyl radicals on the nitrosothiol reaction.

5.6.3 Reaction of S-Nitrosothiols with Other Metal Ions

Other transition metals were added to nitrosothiols to investigate whether this was a general reaction of transition metals or whether Cu^{2+} and Cu^{+} were unique activators of nitrosothiols to NO formation. Both Ni^{2+} and Co^{2+} were added to solutions of S-nitrosothiomalic acid (5×10^{-4}M) in pH 7.4 phosphate buffer. Control experiments were run in parallel which contained no added metal ions. Ni^{2+} and Co^{2+} ions were added over the range 50-500μM. Both these ions have similar sizes and charge densities to Cu^{2+}. The results were variable. Some of the Ni^{2+} and

Co^{2+} experiments gave a slow decomposition measured at 340nm. However, so did some of the control experiments. The reactions were probably caused by contamination by Cu^{2+}, otherwise the results of the controls and the metal experiments were indistinguishable. Both ions showed no catalytic activity towards NO formation from nitrosothiols.

It is known[15] that the Fe^{2+} centre in haemoglobin has a great affinity for NO, so Fe^{2+} was reacted with SNAP. Anaerobic conditions had to be used to prevent aerial oxidation to Fe^{3+} which was found to be unreactive towards nitrosothiols. the Fe^{2+} solution was prepared by reduction of acidified $Fe_2(SO_4)_3$ with sodium dithionite ($Na_2S_2O_4$) in anaerobic solution. The Fe^{2+} was then transferred to a cell containing an anaerobic solution of SNAP (5×10^{-4}M) at pH 7.4. The SNAP was catalytically decomposed. The first order rate constant obtained was half the value (5.2×10^{-4} s^{-1}) of the corresponding aerobic Cu^{2+} reaction. This seems to indicate that Fe^{2+} is not as reactive as Cu^{2+} in the decomposition of nitrosothiols to produce NO. To compare the relative reactivities more work has to be done varying $[Fe^{2+}]$. It has recently been shown[16] that no significant catalysis is exhibited by Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+} or Fe^{3+}.

5.6.4 Effect of pH

Most of this study of nitrosothiols has been carried out at physiological pH 7.4. Changing the pH should affect both the Cu^{2+} environment and the ability of the nitrosothiols to act as ligands. At pH 1 and 12 there is no measurable reaction between any nitrosothiol and Cu^{2+}. Between these two pH values the reaction can be measured and k_2, the second order rate constant, can be evaluated. The results for SNAP decomposition are summarised in table 5.7. There is little change in k_2 over the pH range considered.

Table 5.7

Variation of k_2, the second order rate constant, with pH for the reaction of SNAP with Cu^{2+}

pH	k_2 (mol^{-1} dm^3 s^{-1})
3	13[17]
5.9	16
7.4	20

At pH 1 any nitrosothiol would have all its binding sites except the S-nitroso group fully protonated. The carboxylic acids would be fully protonated, as $-CO_2H$, and the amino groups protonated as ammonium groups, $-NH_3^+$. Co-ordination to Cu^{2+} should now be extremely slow as the concentration of nitrosothiol with unprotonated carboxyl or amino groups would be negligible. At pH 12 all the binding groups would be deprotonated and able to readily co-ordinate about Cu^{2+}. However, at this pH the Cu^{2+} will be co-ordinated by hydroxide groups, which are very strongly bound. The blue copper hydroxide $Cu(OH)_2$ is insoluble, but in strong alkali solution forms ill-defined hydroxo complexes[18]. At intermediate pH values the reactivity seems to be due to a balance between the concentration of nitrosothiol available to bind Cu^{2+} and the concentration of Cu^{2+} available for co-ordination. The reactivity appears to be highest at about neutral pH although there is only a small change overall, however, this has not been rigorously studied.

5.7 Conclusions

Transnitrosation between nitrosothiols and thiols is seen to be a fast process, even at physiological pH. S-Nitrosothiols have been detected *in vivo*[19] at micro-molar concentrations. The majority appears to consist of low molecular weight nitrosothiols with the remainder derived from thiol containing proteins such as serum albumin[19]. *In vivo* nitric oxide is typically found at nano-molar levels. It appears that the

relatively large amounts of nitrosothiols circulating in blood plasma could be a store for nitric oxide. Transnitrosation from the stable S-nitrosoproteins to small thiols could produce reactive nitrosothiols capable of releasing NO rapidly. However, since the endothelial layer is constantly producing NO[20] it is not clear whether this reservoir of NO is a method for storage or a mechanism for removal of excess NO from the body.

Nitrosothiols are known[21] to react rapidly in organ baths and *in vivo* releasing NO which will induce a physiological response. If transnitrosation was a dominant process *in vivo* then there should not be any difference between the biological activity of different nitrosothiols. The nitrosothiols added would undergo transnitrosation with cysteine or glutathione and so the biological activity would always be that of the cysteine and glutathione nitrosothiols. The biological activity of nitrosothiols differs with structure[22] so transnitrosation must not occur significantly within the life-time of the nitrosothiols.

S-Nitrosothiols must be being formed *in vivo* by an unknown process if they can be detected[19]. It may then be that transnitrosation could be an important step in transporting the nitrosothiols from where they are formed (perhaps at some enzyme site or other metallo-protein) to the blood plasma via S-nitrosocysteine or S-nitrosoglutathione.

Although, unlike alkyl nitrates and alkyl nitrites[23], there is no requirement in the case of the nitrosothiols for a free thiol group to be present for NO production, addition of cysteine into bioassays does enhance the biological activity of nitrosothiols[24]. This effect has been ascribed to chelation of metal ions in the buffer solutions used and similar effects were achieved with EDTA. It is still far from clear how nitrosothiols react *in vivo* and what effects the local biochemistry has on reactivity. Elucidation of these mechanisms, as well as broadening our understanding of how our bodies work, may also lead to therapeutic uses for S-nitrosothiols.

References

1. A.D. Allen & G.R. Schonbaum, *Can. J. Chem.*, 1961, 39, 940.
2. D.L.H. Williams, Nitrosation, CUP, Cambridge, 1988, 167.
3. H.M. Patel & D.L.H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1990, 37.
4. S. Oae, D. Fukushima & Y.H. Kim, *J. Chem. Soc., Chem. Commun.*, 1977, 407.
5. J.W. Park, *Biochem. Biophys. Res. Commun.*, 1988, 152, 916.
6. D.J. Barnett, J. McAninly & D.L.H. Williams, *J. Chem. Soc., Perkin Trans. 2*, 1994, 1131.
7. P.C. Jocelyn, Biochemistry of the SH Group, Academic Press, New York, 1972, 94.
8. E.P. Sergeant & B. Dempsey, Ionisation Constants of Organic Acids in Aqueous Solution, Pergamon Press, Oxford, 1979.
9. A.R. Rodriguez, unpublished results.
10. J. Barrett, D.F. Debenham & J. Glauser, *Chem. Comm.*, 1965, 248.
11. S. Oae, N. Asai & K. Fujimori, *J. Chem. Soc., Perkin Trans. 2*, 1978, 1124.
12. E.E. Reid, Organic Chemistry of Bivalent Sulphur, Chemical Publishing, New York, 1958, Vol.1, 145.
13. S.H. Laurie, in Comprehensive Coordination Chemistry, Pergamon, Oxford, 1987, Vol.2, 749.
14. E.G. Janzen, R.V. Shetty & S.M. Kunanec, *Can. J. Chem.*, 1981, **59**, 756.
15. Q.H. Gibson & F.J.W. Roughton, *J. Physiol. London*, 1957, **136**, 507.
16. J. McAninly, D.L.H. Williams, S.C. Askew, A.R. Butler & C. Russell, *J. Chem. Soc., Chem. Comm.*, 1993, 1758.
17. J. McAninly, thesis, to be published.
18. A.G. Sharpe, Inorganic Chemistry, Longman, London, 1986, 613.
19. J.S. Stamler, O. Jaraki, J. Osbourne, D.I. Osbourne, D.I. Simon, J. Keaney, J. Vita, D. Singel, C.R. Valeri & J. Loscalzo, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 7674.

20. A.R. Butler & D.L.H. Williams, *Chem. Soc. Rev.*,1993, 233.

21. M.W. Radomski, D.D. Rees, A. Dutra & S. Moncada, *Br. J. Pharmacol.*, 1992, 107, 745.

22. W.R. Mathews & S.W. Kerr, *J. Pharm. Exp. Therap.*, 1993, 267, 1529.

23. M. Feelisch, *J. Card. Pharm.*, 1991, Vol. 17, Suppl. 13, S25.

24. M. Feelisch, M. te Poel, R. Zamora, A. Deussen & S. Moncada, *Nature*, 1994, 368, 62.

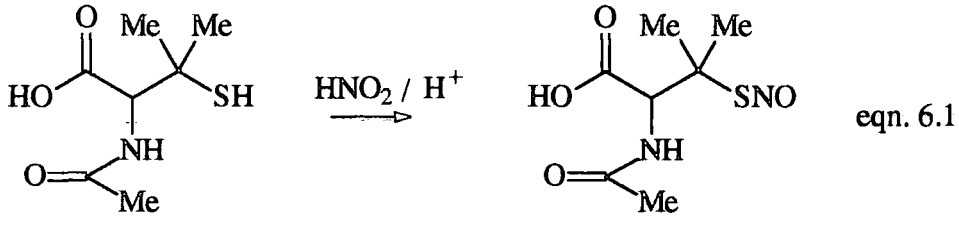
CHAPTER 6

Synthesis of Vasodilatory Compounds

6.1 Synthesis of Stable S-Nitrosothiols

Two S-nitroso compounds were synthesised; S-nitroso-N-acetylpenicillamine (SNAP) (6.1) and S-nitrosoglutathione (SNOG) (6.2). The literature references suggest that both compounds were stable solids.

SNAP was prepared (eqn. 6.1) as described by Field et.al.[1] with some slight modification. The reaction was carried out with N-acetylpenicillamine (4.78g, 25mmol) fully dissolved in a 1:1 mixture (300ml) of methanol and 1M hydrochloric acid with concentrated sulphuric acid (20ml). The reaction vessel was covered with aluminium foil, to minimise formation of the disulphide dimer by photolysis, and cooled to 2°C in an ice bath. A solution of sodium nitrite (3.45g, 50mmol) was added slowly over 10 minutes with stirring. After leaving to warm to room temperature for 15 minutes, the green solid was filtered and dried under vacuum in a desiccator.

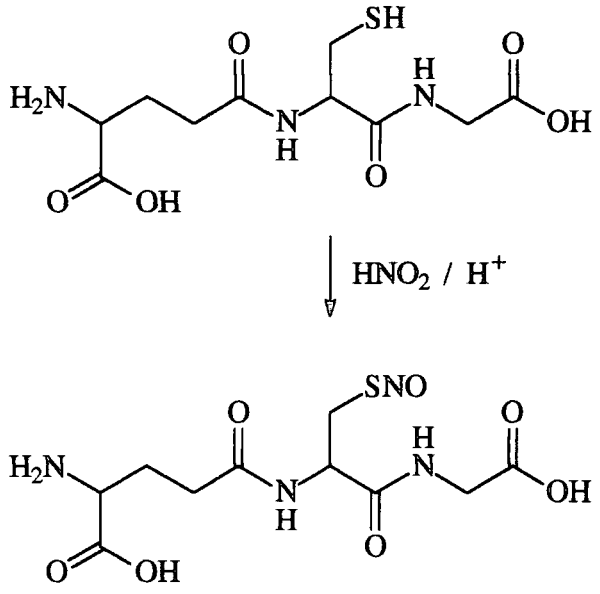


eqn. 6.1

(6.1)

The yield based on weight of product was 74%. Elemental analysis gave a fit for SNAP ($C_7H_{12}N_2O_4S$), requires C=38.17%, H=5.49% and N=12.72%; obtained C=37.49%, H=5.52% and N=12.20%. The difference is probably due to water being present because SNAP is difficult to dry completely. Melting point = 141-143°C decomp. (lit. m.p. = 152°C decomp).

SNOG was prepared (eqn. 6.2) as described by Hart[2], but again the reaction vessel was covered with aluminium foil to minimise photolysis. Glutathione (1.53g, 5mmol) was fairly insoluble in water (~ 10ml), but dissolved rapidly on addition of 2M hydrochloric acid (2.5ml, 5mmol). Addition of the solid sodium nitrite (0.345g, 5mmol) produced a deep red solution. After 1 hour a viscous pink mixture had formed and this was filtered and dried under vacuum in a desiccator giving a pink solid.



eqn. 6.2

(6.2)

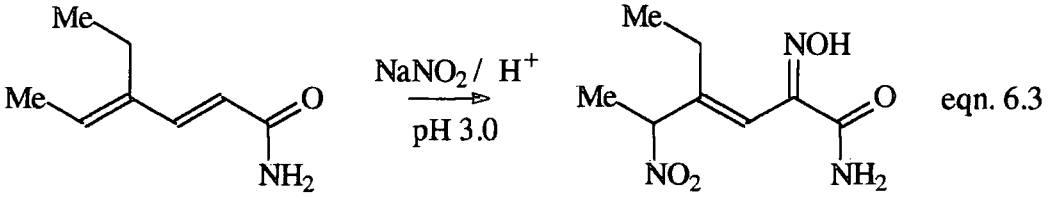
Yield based on product weight = 50%. Elemental analysis gave no clear fit. Required C=35.70%, H=4.80% and N=16.66%, but obtained C=33.25%, H=4.63% and N=14.98%. It may be a mixture of the sodium salt of SNOG with either starting material or disulphide dimer present.

6.2 Synthesis of Nitrovasodilators

6.2.1 Attempted Synthesis of a Known Vasodilator Compound

Work carried out by the Fujisawa Pharmaceutical Company into biologically active compounds from microbial cultures, produced a compound with vasodilator and anti-platelet aggregation activity. A wide range of fermentation broths were tested and the active compound FK409 ((E)-4-ethyl-2-hydroxyimino-5-nitro-3-hexenamide) (6.4) was produced from the acid treated broth of the bacteria *Streptomyces griseosporeus*[3,4,5].

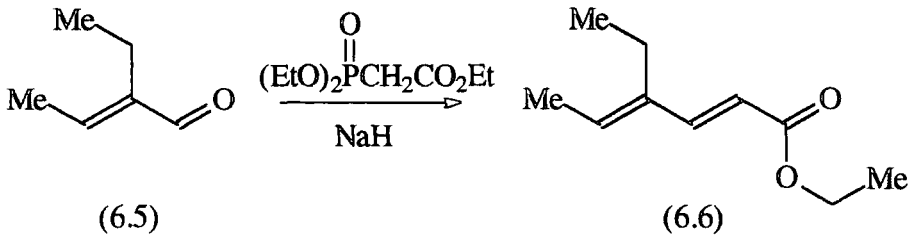
The microbes were cultured with glycerol, glucose, cotton and inorganic salts at pH = 7.4 and 34°C for 4 days. The pH was then adjusted to 3.0 and FK409 extracted with ethyl acetate. In the absence of sodium nitrate, FK409 is not extracted. Instead, a conjugated dienecarboxamide (6.3) is formed. The bacteria reduced the nitrate to nitrite, so when the acid was added, the diene underwent nitrosation to FK409 (eqn. 6.3).



(6.3) (6.4) eqn. 6.3

The synthetic preparation of FK409 was described in a patent[6] and this was followed in an attempt to produce the compound for biological testing.

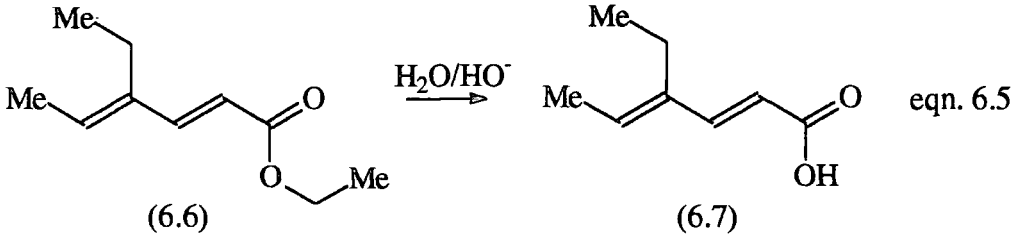
2-Ethyl-2-butenal (6.5) reacts with triethylphosphonoacetate, in the presence of a base (eqn. 6.4) to give a conjugated diene-ester with the correct E-configuration (6.6).



(6.5) (6.6) eqn. 6.4

This is a Wadsworth-Emmons reaction, which is a modified Wittig reaction. A solution of triethyl-phosphonoacetate (10.5ml, 0.053mol) in dry benzene (65ml) was added dropwise, at 0°C under a dry nitrogen atmosphere, to sodium hydride (2.25g, 0.094mol) in dry benzene (50ml). The benzene was dried in a Dean-Stark trap. The mixture was stirred at room temperature for 30 minutes and then 2-ethyl-2-butenal (6.5) (5g, 0.043mol) was added slowly at 0°C, and allowed to stand at room temperature for a further 30 minutes. This reaction mixture was diluted with water (100ml) and extracted with ethyl acetate (2 x 100ml), washed with water and brine and then dried with magnesium sulphate. Evaporation under reduced pressure gave an oil as product. The reaction was then repeated on four times the scale. The total product (29.27g, yield 81%) was distilled and gave the pure ester (20g) with a yield of 69%.

Hydrolysis of ethyl (E,E)-4-ethyl-2,4-hexadienoate (6.6) with aqueous sodium hydroxide produced the corresponding acid (6.7) (eqn. 6.5).

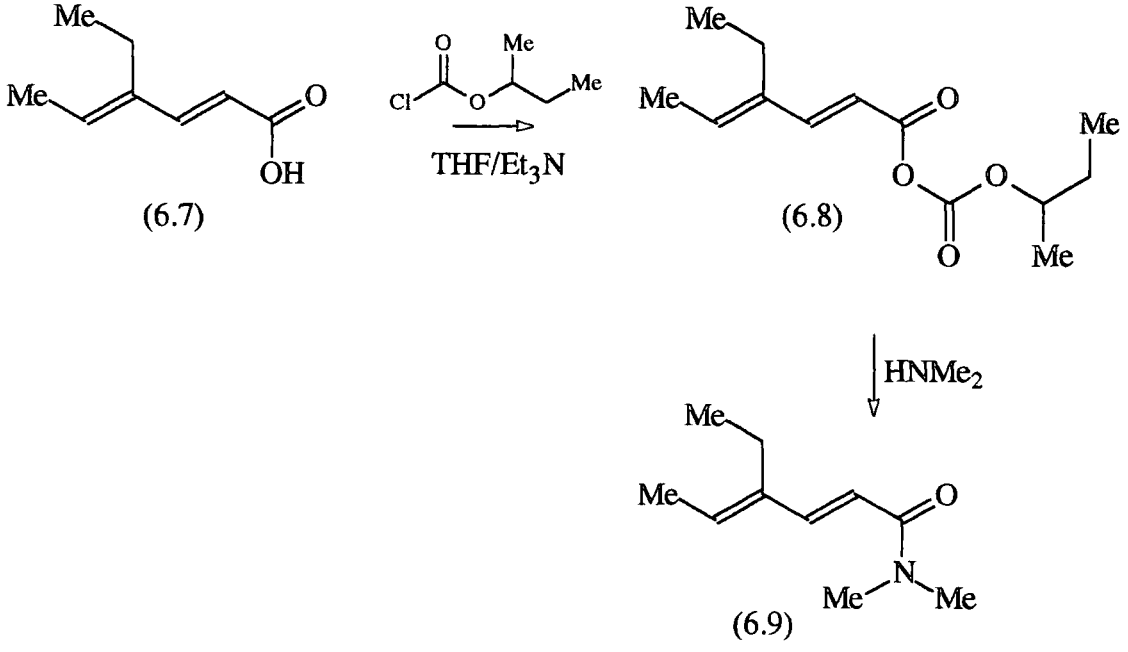


(6.6) (6.7) eqn. 6.5

The ester (20g, 0.119mol) was dissolved in methanol (30ml), and 1M sodium hydroxide (150ml) was added to the solution. This solution was stirred at room temperature for 20 hours. The methanol was removed and the aqueous layer acidified with concentrated hydrochloric acid. Extraction with ethyl acetate gave a solution

which, after washing with water and brine, and then evaporation, gave a white powder. The yield, based on product weight (14.4g) was 86%. Elemental analysis was lower than expected (expected C=68.53%, H=8.63%, actual C=67.26%, H=8.53%), but the melting point was in good agreement with the literature value (m.p. = 78-80°C, lit. m.p. =79-81°C).

(E,E)-N,N-dimethyl-4-ethyl-2,4-hexadienamide (6.9) was prepared by forming a mixed anhydride (6.8), and then the amide (scheme 6.1).

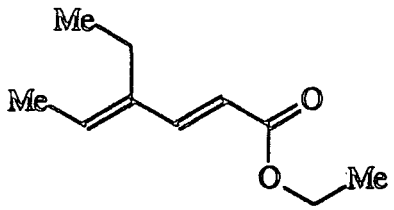


Scheme 6.1

(E,E)-4-Ethyl-2,4-hexadienoic acid (6.7) (5g, 0.035 mol) was dissolved in tetrahydrofuran (40 ml) and triethylamine (10 ml) added. A solution of iso-butyl-chloroformate (4.54 ml, 0.035 mol) in THF (10 ml) was added dropwise at -20°C under nitrogen. The mixture was stirred for 60 minutes and then a 33% solution of dimethylamine in ethanol (15 ml) was added and allowed to stir for two hours at room temperature. After concentration under reduced pressure and dilution with water (100ml), the product was extracted with ethyl acetate. The extracts were washed, dried and evaporated to leave an orange oil. The yield was small and no crystals were obtained. Due to the constraints of time the synthesis was not repeated.

6.2.2 Identification of Products

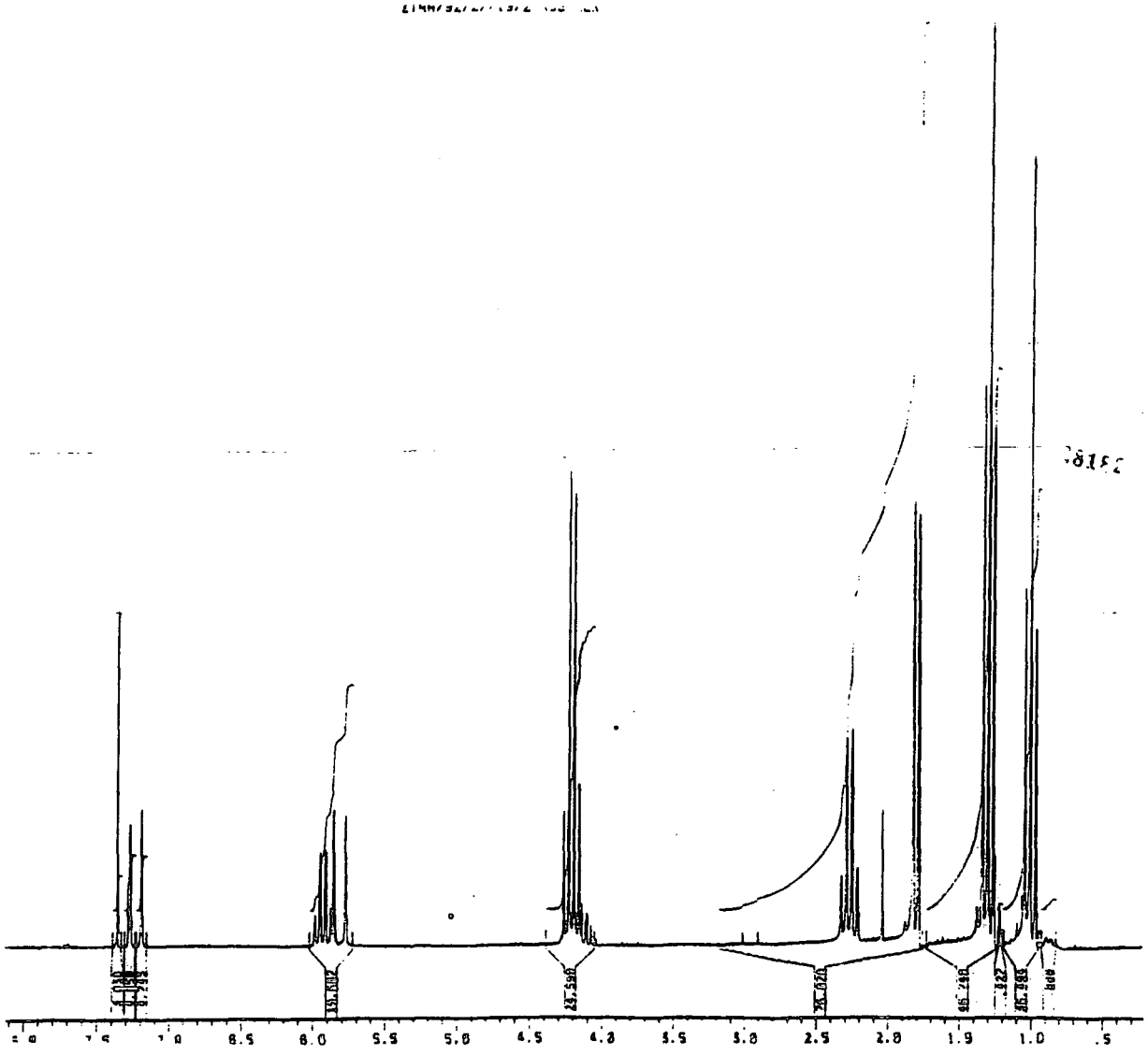
A sample of crude ethyl (E,E)-4-ethyl-2,4-hexadienoate (6.6) before distillation, was characterised by ^1H nmr. The ^1H spectrum in $CDCl_3$ taken on a 200MHz instrument (fig. 6.1).



(6.6)

Figure 6.1

^1H nmr (E,E) 4-ethyl-2,4-hexadienoate / $CDCl_3$.

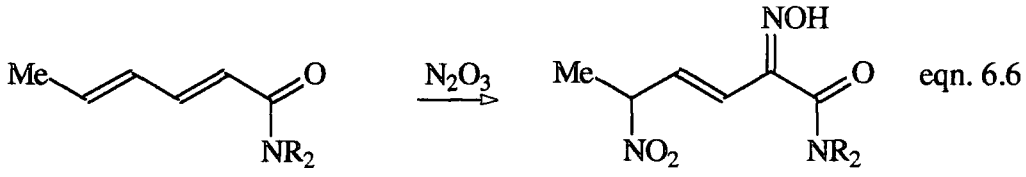


The ethoxy group gave rise to two peaks, a quartet for the methylene at δ 4.2ppm and a triplet for the methyl at δ 1.27ppm. Similarly, the 4-ethyl group gave a quartet for the methylene at δ 2.26ppm and a triplet for the methyl at δ 0.97ppm. The remaining methyl gave a doublet at δ 1.78ppm. The protons on C-3 and C-5 gave a doublet and quartet respectively with adjacent peaks at δ 6.07-5.67ppm. The C-2 proton gave a doublet peak at δ 7.2ppm and a coupling constant J = 16Hz, which is indicative of a trans-coupling.

6.3 Synthesis of Analogous FK409 Compounds.

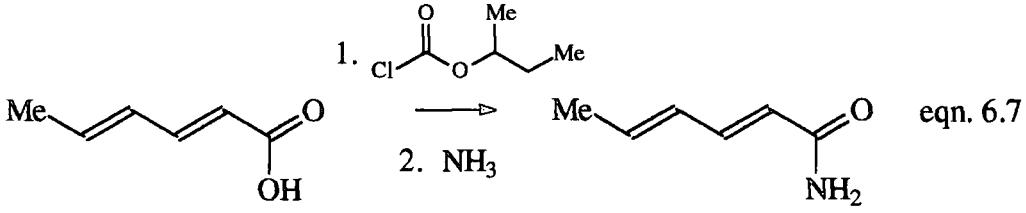
6.3.1 Synthesis of Dienamides

The synthesis of compounds analogous to FK409 was also attempted. Dienamides were prepared which would undergo nitrosation with N_2O_3 (eqn. 6.6).



eqn. 6.6

Sorbamide ((E,E)-2,4-hexadienamide) (6.10)was prepared by reacting ammonia with a mixed anhydride of sorbic acid (6.7) (eqn. 6.7).



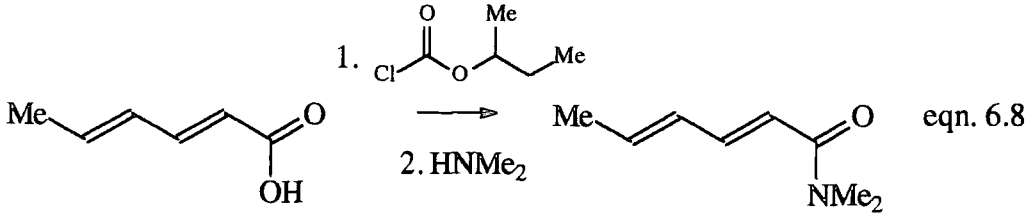
eqn. 6.7

(6.7) (6.10)

Sorbic acid (5.6g, 0.05mol) was added to dichloromethane (40 ml). On addition of triethylamine (5.5 ml, 0.04mol) the sorbic acid rapidly dissolved. Iso-

butyl-chloroformate (3.8 ml, 0.03mol) was added dropwise at -20°C (acetone/CO_2 bath) and stirred for 45 minutes. Ammonia was then passed through the mixture at -20°C for an hour. A white solid formed and the mixture allowed to warm to room temperature. Water was added (200 ml) and two layers were formed. The layers were washed with 1M sodium hydroxide (100 ml) and brine (100 ml), which afforded a white solid that settled on the bottom of the aqueous layer. The solid was collected as product. Elemental analysis fit for sorbamide (C_6H_9NO), which required C=64.84%, H=8.16% and N=12.60%; actual analysis C=63.73%, H=8.26% and N=12.46%. The melting point was 170-172°C (lit. m.p.=171-172°C). The yield was 23% and the infra red spectrum (fig. 6.2) showed absorbances typical of an amide.

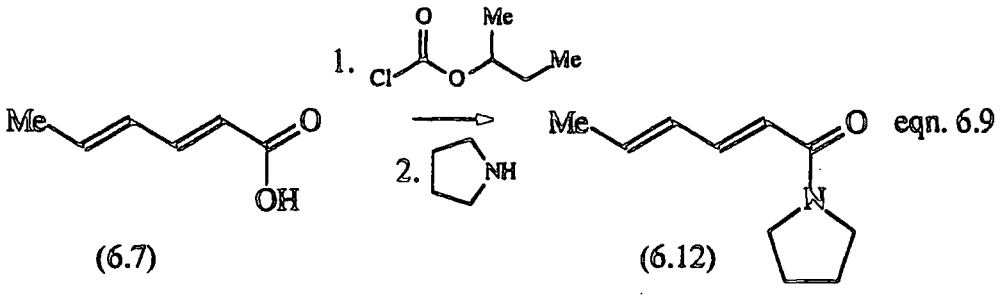
The reaction to produce sorbamide was repeated, but instead of ammonia, dimethylamine was used (eqn. 6.8) to give (E,E)-N,N-2,4-hexadienamide.



(6.7) (6.11) eqn. 6.8

A 33% solution of dimethylamine in ethanol (12 ml) was added to the mixed anhydride and stirred for two hours. All the solvent was removed leaving a white solid. Water was added to remove the triethylamine hydrochloride, and all the solid dissolved. Ethyl acetate (2x200 ml) was used to extract the product (6.11), which was washed with sodium hydrogen carbonate (100 ml) and brine (100 ml). Removal of the ethyl acetate gave a pale yellow solid. Elemental analysis on the product ($C_8H_{13}NO$) required C=69.03%, H=9.41% and N=10.06%; and gave C=68.83%, H=9.39% and N=9.94%. M.p.=59-61°C and the yield was 47%.

Another derivative, N-[(E,E)-2,4-hexadienoyl]-pyrrolidine (6.12), was prepared using pyrrolidine (eqn. 6.9).

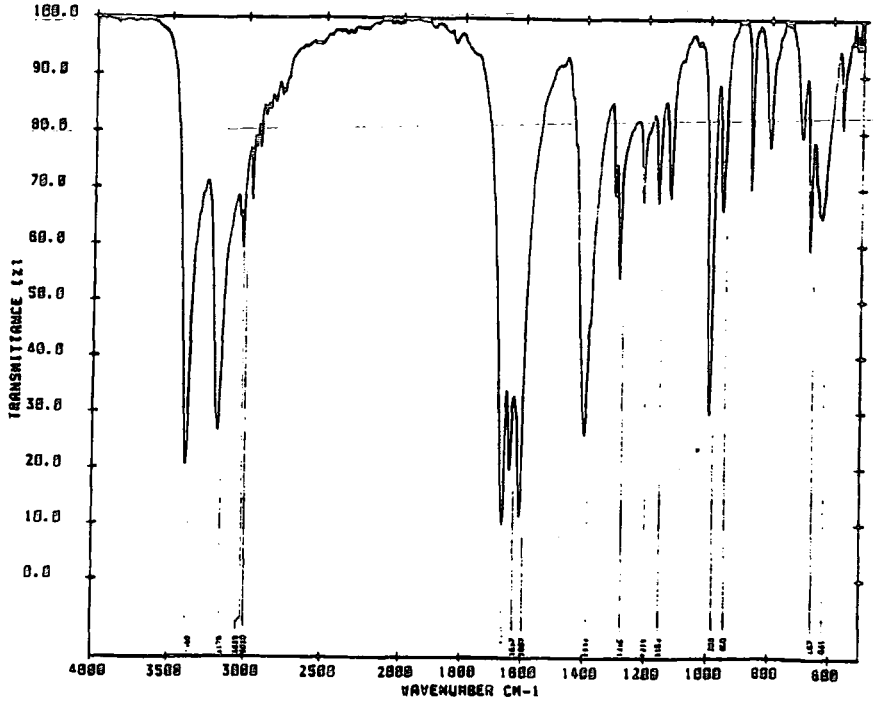


(6.7) (6.12) eqn. 6.9

Pyrrolidine (8.4 ml, 0.1mol) was added and the mixture stirred for two hours. The product was extracted as previously described. The yield, based on weight of product collected, was 41%. The solid was light brown in colour and the melting point was 70-75°C. Elemental analysis fit for product ($C_{10}H_{15}NO$) required C=72.69%, H=9.15% and N=8.48%; and gave C=71.27%, H=9.38% and N=8.24%.

Figure 6.2

IR spectrum of sorbamide.



A ^1H nmr was run on samples of both (E,E)-N,N-dimethyl-2,4-hexadienamide and N-[(E,E)-2,4-hexadienoyl]-pyrrolidine in $CDCl_3$ on a 200MHz instrument (figures 6.3 and 6.4).

Both compounds gave similar spectra. The C-6 methyl group protons gave doublets at 1.8ppm in both spectra with coupling constants of 5.7Hz and 5.3Hz in figures 6.3 and 6.4 respectively. The C-5 proton gave a multiplet for the dimethyl amide at 6.1ppm. This triplett may also be being split by the C-4 proton, but it appears only to be a weak effect. The C-2, C-3 and C-4 protons in both compounds were not assignable, but the areas under the peaks suggest that they were due to the peaks between 6.0ppm and 6.3ppm (insets). The two methyl groups protons in (E,E)-N,N-dimethyl-2,4-hexadienamide gave a singlet at 3.05ppm. The pyrrolidine groups protons in N-[(E,E)-2,4-hexadienamide]-pyrrolidine gave a triplet at 3.45ppm for the four methylene protons adjacent to the N-atom and a very broad peak at 1.92ppm which is probably due to the remaining four protons.

Figure 6.3

^1H nmr of (E,E)-N,N-dimethyl-2,4-hexadienamide in $CDCl_3$

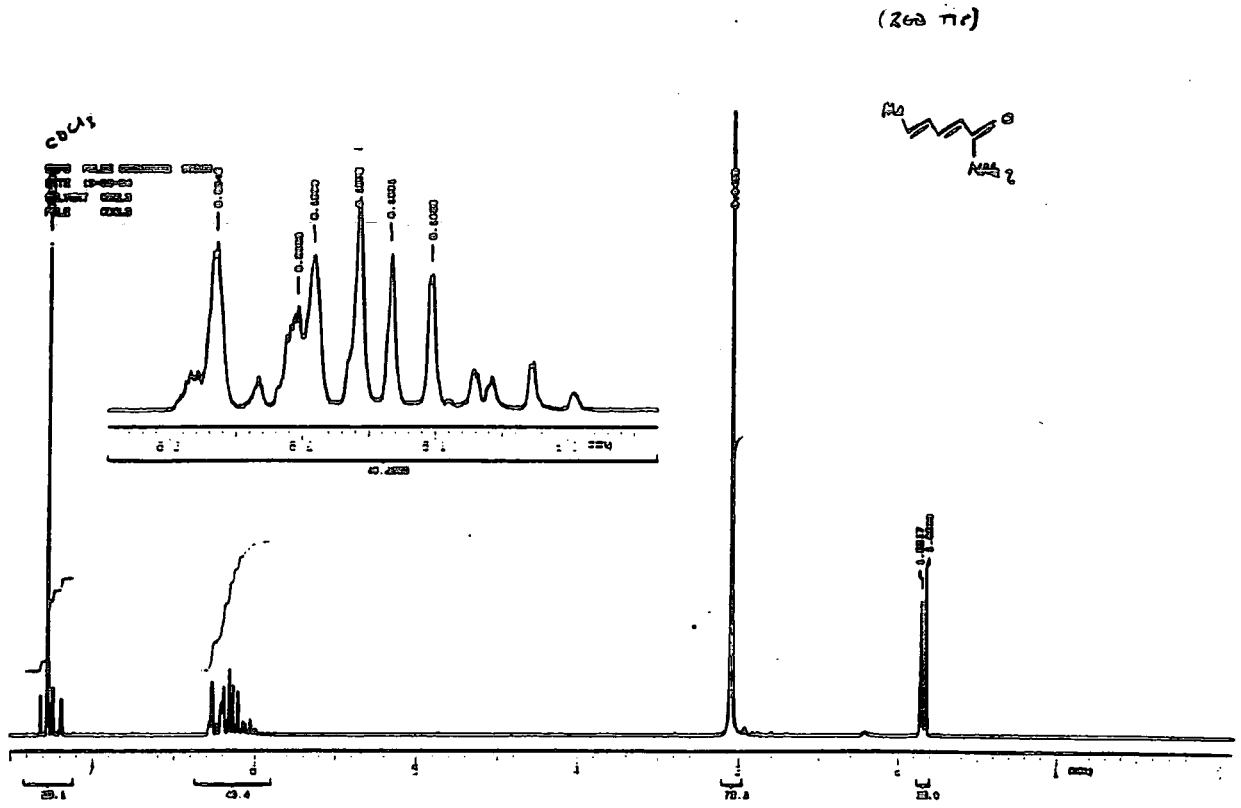
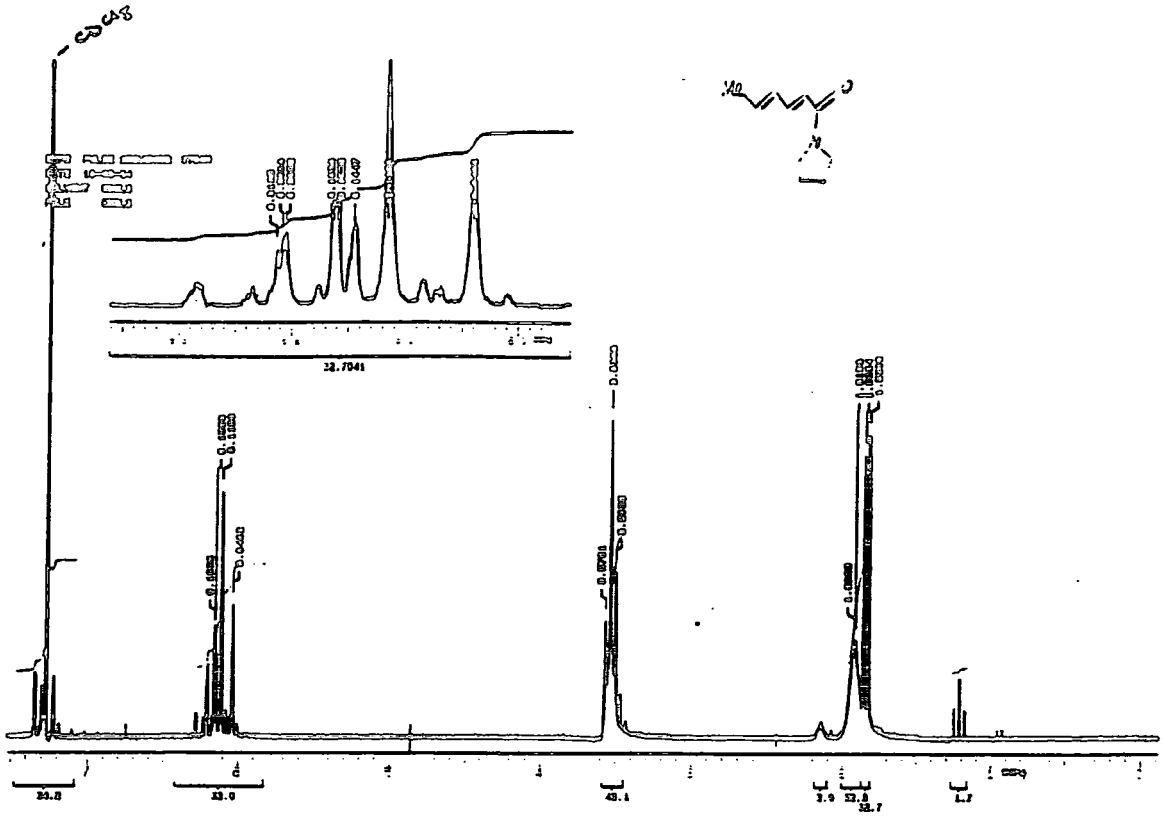


Figure 6.4

^1H nmr of N-[(E,E)-2,4-hexadienamide]-pyrrolidine in $CDCl_3$



6.3.2 Nitrosation of Dienes.

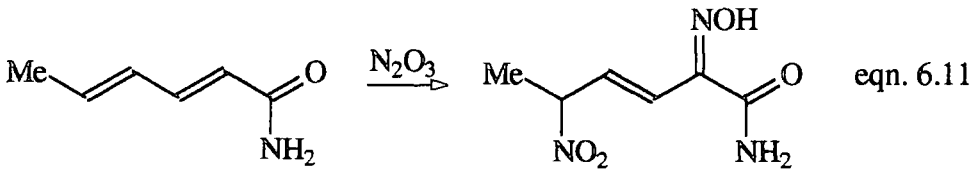
It is known that dinitrogen trioxide will add across a double bond[7]. In a reaction with the conjugated diene a 1,4-addition was required for the desired product.

Gaseous dinitrogen trioxide was produced by dropping concentrated sulphuric acid onto sodium nitrite[8] in a nitrogen atmosphere (eqn. 6.10).

$$H_2SO_4 + NaNO_2 \longrightarrow NO + NO_2 + Na_2SO_4 + H_2O \quad \text{eqn. 6.10}$$

Dinitrogen trioxide can be condensed out from NO and NO_2 at any temperature around or below 0°C.

The first method for the nitrosation/nitration reaction was addition of liquid dinitrogen trioxide to solid sorbamide (eqn. 6.11).

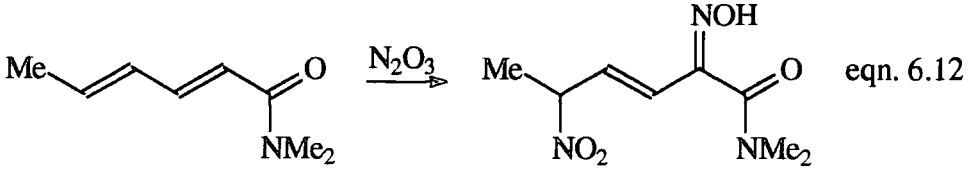


eqn. 6.11

(6.10) (6.13)

The blue liquid dinitrogen trioxide was condensed onto solid sorbamide (0.5g) at -78°C in a test tube in an acetone/CO_2 mixture. The results were mixed. Some reactions gave a highly exothermic response, vapourising the gas and leaving a charred, black residue. Other reactions gave an orange oil, which produced a few crystals, however, there was insufficient product for analysis. The oil was probably the nitroso dimer. Addition of ethyl acetate gave more crystals but the elemental analysis showed this was not the desired product. The expected analysis was C=38.57%, H=4.85% and N=22.45%; but the result was C=20.07%, H=4.33% and N=5.72%. The m.p.=126-128°C. Mass spectrometry gave virtually nothing and ^1H nmr (in D_2O) gave a peak typical of H_2O.

Nitrosation was attempted with the N,N-dimethyl sorbamide (eqn. 6.12) as this would rule out the possibility of any reaction with the amide group, as may have occurred with sorbamide. The reaction was repeated but again the results were unsatisfactory.



eqn. 6.12

(6.10) (6.14)

This method of reacting dinitrogen trioxide with the dienamides was too unreliable so a solvent was introduced. N,N-dimethylamide (0.5g) was dissolved in diethyl ether (60 ml) and cooled to -20°C. Dinitrogen trioxide was passed through the solution. A fine yellow solid rapidly appeared and the addition of more dinitrogen trioxide turned the solution green. On standing, a yellow crystalline solid appeared, along with an orange oil. The solid was collected. The reaction was repeated, but as soon as the first solid formed it was filtered off. Not enough solid was produced to collect off the sintered disc. More dinitrogen trioxide was passed through the remaining solution and it was left to stand overnight. Clumps of crystals appeared and were collected. Both solids analysed the same, but were not the expected product. Elemental analysis expected C=44.65%, H=6.09% and N=19.53%; the actual analysis was C=29.92%, H=3.45% and N=21.50%. The product may be nitrosated across both double bonds, although the analysis does not fit for this either. The structure of this compound has not been elucidated. The melting point was 93-95°C and the mass spectrum gave no information.

The reaction was repeated again and after leaving the solution overnight crystals appeared. These pale yellow crystals gave the correct analysis. The analysis requires C=44.65%,H=6.09% and N=19.53%; and gave C=44.08%, H=6.07% and N=19.45%. A fast atom bombardment (FAB) mass spectra gave peaks for $(M+1)^+=216$ and $(M+Na)^+=238$ (figs. 6.5 and 6.6), which analyse correctly. TLC showed that it was neither the starting material nor the unknown product. The melting point was 142-145°C.

It was observed that the characterised product was not very water soluble, so the reaction was attempted in water. The amide (0.5g) was dissolved in water (50 ml) and the solution cooled to ca. 2°C. Dinitrogen trioxide was passed through the solution briefly, giving a yellow solution. On standing, pale yellow crystals were produced. The analysis was the same as the previous product. This was the best synthesis, as it was reproducible.

Fig. 6.5

FAB mass spectrum of (E) N,N-dimethyl-2-hydroxyimino-5-nitro-3-hexenamide.

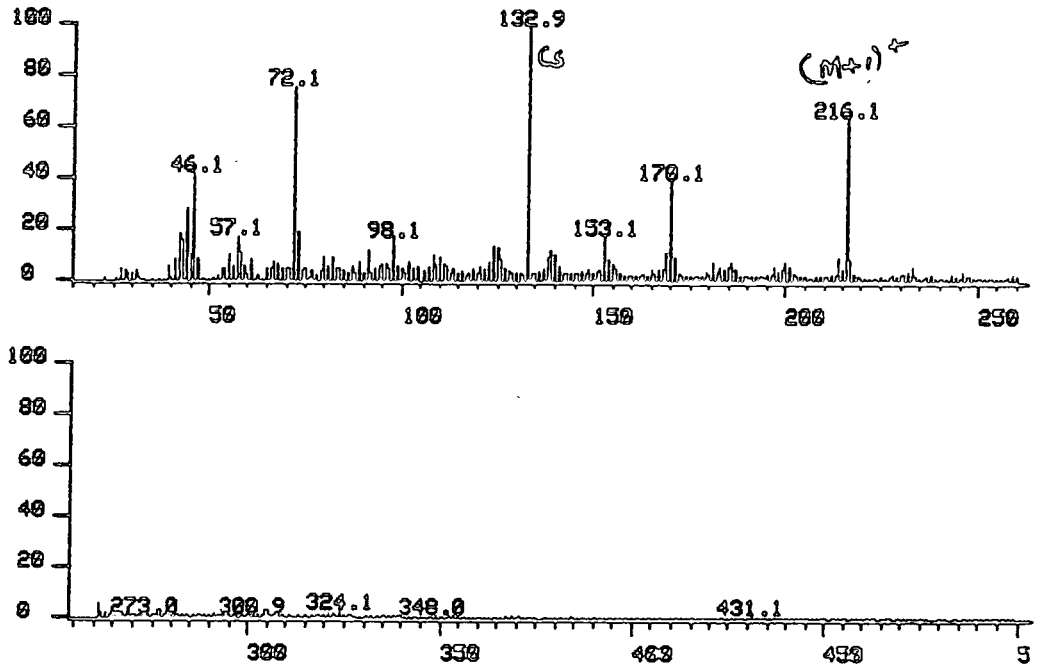
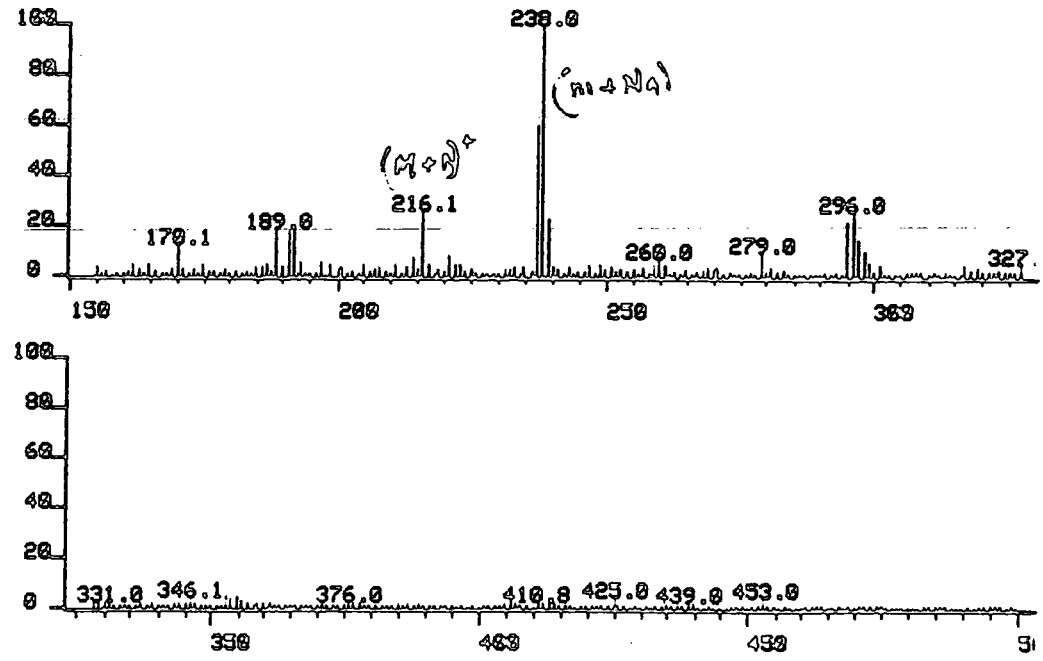


Fig. 6.6

FAB mass spectrum of (E) N,N-dimethyl-2-hydroxyimino-5-nitro-3-hexenamide.



6.4 Phamacological testing.

(E) N,N-dimethyl-2-hydroxyimino-5-nitro-3-hexenamide was submitted for pharmacological testing, to observe its efficacy in anti-platelet aggregation. It was found to be less potent than glyceryl trinitrate. Its biological activity was probably due to the presence of the nitro group.

References

1. L. Field, R. V. Dilts, R. Ravichandran, P.G. Lenhert & G.E. Carnahan, J. *Chem. Soc., Chem. Commun.*, 1978, 249.
2. T.W. Hart, *Tet. Lett.*, 1985, 26, 2013.
3. M. Hino, M. Iwami, M. Okamoto, K. Yoshida, H. Haruta, M. Okuhara, J. Hosoda, M. Kohsaka, H. Aoki & H. Imanaka, *J. Antibiotics*, 1989, 42, 1578.
4. M. Hino, M. Iwami, M. Okamoto, K. Yoshida, H. Haruta, M. Okuhara, J. Hosoda, M. Kohsaka, H. Aoki & H. Imanaka, *J. Antibiotics*, 1989, 42, 1584.
5. M. Hino, M. Iwami, M. Okamoto, K. Yoshida, H. Haruta, M. Okuhara, J. Hosoda, M. Kohsaka, H. Aoki & H. Imanaka, *J. Antibiotics*, 1989, 42, 1589.
6. Guthrie, *J. Chem. Soc.*, 1861, 13, 129.
7. P.I. Protsenko & V.B. Stradomsky, *Zhur. obshchei Khim.*, 1955, 25, 1043.

CHAPTER 7

Experimental

7.1 Experimental Techniques

7.1.1 U.V./Vis Spectrophotometry

All uv/vis spectra were obtained from solutions in 1cm path length quartz cells on either a Perkin-Elmer Lambda 2 or a Shimadzu 2102-PC instrument. The same instruments were used when measuring reactions kinetically. For rapid reactions ($t_{1/2} \geq 2 \times 10^{-3}$ s) a stopped flow technique was used (section 7.1.2).

Kinetic measurements were made under pseudo first order conditions. The observed rate constants were calculated from the change in absorbance with time at a fixed wavelength. The absorbance/time data from the Lambda 2 were used in a program designed for rate constant calculation on an Epsom AX2 personal computer. Similarly for the data from the Shimadzu instrument, a program run on an Opus 386 DX was used to determine rate constants. The programs determined the observed rate constants, k_o, using a calculation based on the derivation of k_o *vide infra.*

For a first order process A $\longrightarrow$ B, the rate of formation of B or the removal of A can be expressed by equation 7.1.

$$-\frac{d[A]}{dt} = \frac{d[B]}{dt} = k[A] \qquad \text{eqn. 7.1}$$

Intergration of equation 7.1 gives an expression for the observed first order rate constant, k_o (equation 7.2).

$$k_o = \frac{1}{t} \ln \frac{[A]_0}{[A]_t} \qquad \text{eqn. 7.2}$$

Where $[A]_0$ and $[A]_t$ are the concentrations of A at times t = 0 and t = t respectively.

Using the Beer-Lambert law (A = εcl, where A is the absorbance, ε is the molar extinction coefficient, c is the concentration and l is the path length) and assuming the path length to be 1cm the expression of the absorbance at t = 0 and t = t can be derived (equations 7.3 and 7.4).

$$A_0 = \varepsilon_A[A]_0 \qquad \text{eqn. 7.3}$$

$$A_t = \varepsilon_A[A]_t + \varepsilon_B[B]_t \qquad \text{eqn. 7.4}$$

Now as $[B]_t = [A]_0 - [A]_t$ substituting for $[B]_t$ into equation 7.4 gives:

$$A_t = \varepsilon_A[A]_t + \varepsilon_B[A]_0 - \varepsilon_B[A]_t \qquad \text{eqn. 7.5}$$

But $A_\infty = \varepsilon_B[A]_0 = \varepsilon_B[B]_\infty$ since $[B]_\infty = [A]_0$

Thus $(A_t - A_\infty) = \varepsilon_A[A]_t - \varepsilon_B[A]_t$

$$[A]_t = \frac{(A_t - A_\infty)}{(\varepsilon_A - \varepsilon_B)} \qquad \text{eqn. 7.6}$$

Similarly $A_o = \varepsilon_A[A]_o$

and $A_\infty = \varepsilon_B[B]_\infty = \varepsilon_B[A]_0$

$$(A_0 - A_\infty) = \varepsilon_A[A]_0 - \varepsilon_B[A]_0$$

$$[A]_0 = \frac{(A_0 - A_\infty)}{(\varepsilon_A - \varepsilon_B)} \qquad \text{eqn. 7.7}$$

Substituting equations 7.6 and 7.7 into equation 7.2 gives:

$$k_o = \frac{1}{t} \ln \frac{(A_0 - A_\infty)}{(A_t - A_\infty)} \qquad \text{eqn. 7.8}$$

Rearranging gives:

$$\ln(A_t - A_\infty) = -k_o t + \ln(A_0 - A_\infty) \qquad \text{eqn. 7.9}$$

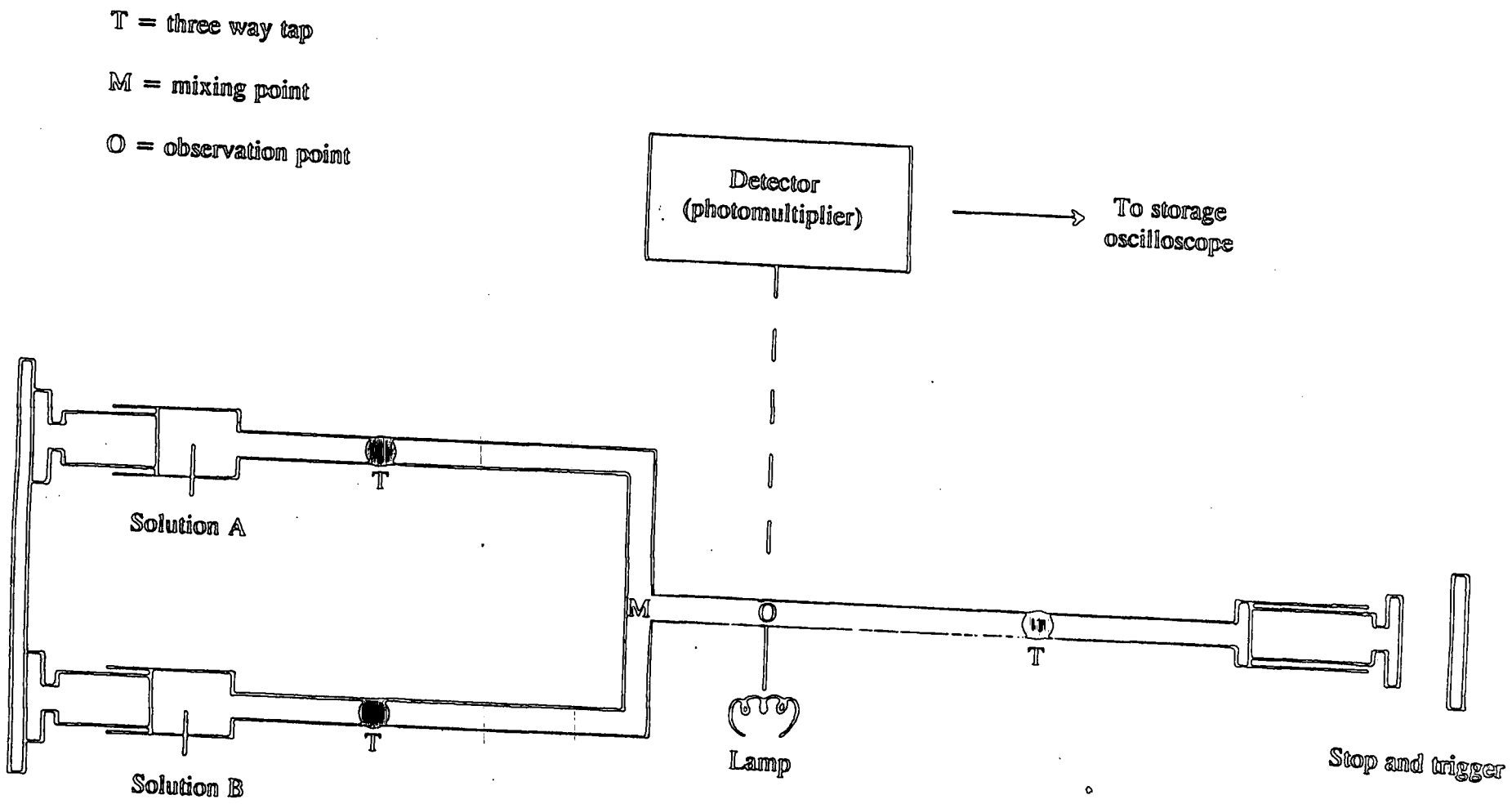
Therefore a plot of $\ln(A_t - A_\infty)$ against t should be linear with a slope of $-k_o$. The infinity values A_∞, were determined after a period of ten half lives and the disappearance of absorbance followed for at least two half lives.

7.1.2 Stopped Flow Spectrophotometry

For measurement of rate constants of reactions too fast to measure by conventional machines a Photophysics Biosequential Stopped Flow ASVD Spectrofluorimeter and a Hi-Tech Scientific SF-3L stopped flow spectrometer were used. This is shown schematically in figure 7.1. The two solutions to be reacted, A and B, are stored in reservoirs and are drawn into two identical syringes so that equal volumes are mixed. The syringes are compressed simultaneously, either manually or using a compressed air supply, and mixing occurs at point M extremely rapidly (< 1ms) The mixture flows into a thermostatted 2mm path length quartz cell at point O. The plunger of the third syringe will hit a stop and the flow of solution will be stopped. Hitting the stop also triggers the acquisition of data from the reaction. The reaction is observed by passing a beam of monochromatic light of the appropriate wavelength through the cell by fibre optic cable. The light is passed through a photomultiplier and the change in voltage measured due to a change in absorbance of the solution is observed. Software on the computers that run the stopped flow machines can transform voltage/time data into absorbance/time data and also calculate the observed rate constants.

Figure 7.1

Schematic Diagram of a Stopped Flow Spectrophotometer



7.2 pH Measurements

All pH measurements were carried out using a PTI-6 Universal digital pH meter (accurate to ± 0.02 pH units). The pH meter was calibrated over the range pH 4.0 to 7.0 or pH 7.0 to 10.0 depending on the solution to be measured.

7.3 Nitric Oxide Electrode Calibration

A World Precision ISO-NO nitric oxide specific electrode was used to measure aqueous NO solutions. Calibration was carried out with ascorbic acid (0.1M) and sodium nitrite (2.5×10^{-3}M) stock solutions. Since the passage of NO across the membrane is temperature dependent, the solutions were thermostatted at 298K. The electrode was zeroed in the ascorbic acid solution with just the tip (10mm) below the surface. Nitrogen was passed through to remove most of the oxygen in solution. When the electrode reading had stabilised the solution was vigorously stirred and the nitrite solution injected. A calibration curve of current (nA) against concentration of NO produced (μM) was constructed. It was assumed that NO was produced quantitatively by the reaction. The electrode was then calibrated on the concentration setting. When the current reached its known maximum, the adjustment screw was turned until the correct value was shown. Readings could then be taken either as a current measurement or by reading the NO concentration directly from the meter.

7.4 Reagents

All reagents of the highest grade were purchased commercially except NO (section 3.2) and N_2O_3 (section 6.2.4) which were prepared as previously mentioned. Nitrosothiols were produced *in situ* (section 4.2) from the commercially availble thiols except SNAP and SNOG (section 6.1) which were synthesised as solids and stored in a freezer. The potassium dihydrogen orthophosphate used for preparing buffer solutions was purchased commercially and used as supplied. the perchloric acid

solutions were prepared by dilution of concentrated perchloric acid which had been standardised by standard sodium hydroxide solution using phenol red as an indicator.

APPENDIX

Research Colloquia, Seminars, Lectures and Conferences

The Board of Studies in Chemistry requires that each postgraduate research thesis contains an appendix listing:

A. all research colloquia, seminars and lectures arranged by the Department of Chemistry and by the Durham University Chemical Society during the period of the author's residence as a postgraduate student;

B. all research conferences attended and papers presented by the author during the period when research for the thesis was carried out;

C. details of the postgradute induction course.

A. Colloquia, Lectures and Seminars from Invited Speakers Organised by the Durham University Chemistry Department, 1991 - 1994

(* denotes lectures attended)

*	17.10.91	Dr. J.A. Salthouse, University of Manchester Son et Lumiere - a Demonstration Lecture
*	31.10.91	Dr. R. Keely, Metropolitan Police Forensic Science Modern Forensic Science
	06.11.91	Prof. B.F.G. Johnson#, Edinburgh University Cluster-Surface Analogies
*	07.11.91	Dr. A.R. Butler, St. Andrews University Traditional Chinese Herbal Drugs: a Different Way of Treating Disease
*	13.11.91	Prof. D. Gani#, St. Andrews University The Chemistry of PLP Dependent Enzymes
*	20.11.91	Dr. R. More O'Ferrall#, University College Dublin Some Acid-Catalysed Rearrangements in Organic Chemistry
	28.11.91	Prof. I.M. Ward, IRC in Polymer Science, University of Leeds The SCI Lecture: The Science and Technology of Orientated Polymers
*	04.12.91	Prof. R. Grigg#, Leeds University Palladium-Catalysed Cyclisation and Ion-Capture Process
*	05.12.91	Prof. A.L. Smith, ex Unilever Soap, Detergents and Black Puddings
*	11.12.91	Dr. W.D. Cooper#, Shell Research Colloid Science: Theory and Practice
*	22.01.92	Dr. K.D.M. Harris#, St. Andrews University Understanding the Properties of Solid Inclusion Compounds
*	29.01.92	Dr. A. Holmes#, Cambridge University Cycloaddition Reactions in the Service of the Synthesis of Piperidine and Indolizidine Natural Products
*	30.01.92	Dr. M. Anderson, Sittingbourne Research Centre, Shell Research Recent Advances in the Safe and Selective Chemical Control of Insect Pests

	12.02.92	Prof. D.E. Fenton#, Sheffield University Polynuclear Complexes of Molecular Clefts as Models for Copper Biosites
	13.02.92	Dr. J. Saunders, Glaxo Group Research Limited Molecular Modelling in Drug Discovery
*	19.02.92	Prof. E.J. Thomas#, Manchester University Applications of Organostannanes to Organic Synthesis
*	20.02.92	Prof. E. Vogel, University of Cologne *The Musgrave Lecture* Porphyrins: Molecules of Interdisciplinary Interest
*	25.02.92	Prof. J.F. Nixon, University of Sussex *The Tilden Lecture* Phosphaalkynes: New Building Blocks in Inorganic and Organometallic Chemistry
	26.02.92	Prof. M.L. Hitchman#, Strathclyde University Chemical Vapour Deposition
	05.03.92	Dr. N.C. Billingham, University of Sussex Degradable Plastics - Myth or Magic
*	11.03.92	Dr. S.E. Thomas#, Imperial College Recent Advances in Organoiron Chemistry
	12.03.92	Dr. R.A. Hann, ICI Imagedata Electronic Photography - an Image of the Future
*	18.03.92	Dr. H. Maskill#, Newcastle University Concerted or Stepwise Fragmentation in a Deamination-Type Reaction
	07.04.92	Prof. D.M. Knight, Philosophy Department, University of Durham Interpreting Experiments: the Beginning of Electrochemistry
	13.05.92	Dr. J-C. Gehret, Ciba Geigy, Basel Some Aspects of Industrial Agrochemical Research
	15.10.92	Dr. M. Glazer & Dr. S. Tarling, Oxford University & Birbeck College, London It Pays to be British - The Chemist's Role as an Expert Witness in Patent Litigation
	20.10.92	Dr. H.E. Brynda, Du Pont Central Research Synthesis, Reactions and Thermochemistry of Metal (Alkyl) Cyanide Complexes and their Impact on Olefin Hydrogenation Catalysis

* 22.10.92 Prof. A. Davies, University College London
The Ingold-Albert Lecture The Behaviour of Hydrogen as a Pseudometal

28.10.92 Dr. J.K. Cockcroft, University of Durham
Recent Developments in Powder Diffraction

* 29.10.92 Dr. J. Emsley, Imperial College, London
The Shocking History of Phosphorous

04.11.92 Dr. T.P. Kee, University of Leeds,
Synthesis and Co-ordination of Silylated Phosphites

* 05.11.92 Dr. C.J. Ludman, University of Durham
Explosions, a demonstration lecture

* 11.11.92 Prof. D. Robins#, Glasgow University
Pyrrolizidine Alkaloids: Biological Activity, Biosynthesis and Benefits

* 12.11.92 Prof. M.R. Truter, University College, London
Luck and Logic in Host-Guest Chemistry

18.11.92 Dr. Nix#, Queen Mary College, London
Characterisation of Heterogenous Catalysts

* 25.11.92 Prof. Y. Vallee, University of Caen
Reactive Thiocarbonyl Chemistry

25.11.92 Prof. L.D. Quin#, University of Massachusetts, Amherst
Fragmentation of Phosphorous Heterocycles as a Route to Phosphoryl Species with Uncommon Bonding

26.11.92 Dr. D. Humber, Glaxo Greenford
AIDS - The Development of a Novel Series of Inhibitors of HIV

* 02.12.92 Prof. A.F. Hegarty, University College, Dublin
Highly Reactive Enols Stabilised by Steric Protection

* 02.12.92 Dr. R.A. Aitken#, University of St. Andrews
The Versatile Cycloaddition Chemistry of $Bu_3P.CS_2$

* 03.12.92 Prof P. Edwards, Birmingham University
The SCI Lecture - What is a Metal?

09.12.92 Dr. A.N. Burgess#, ICI Runcorn
The Structure of Perfluorinated Ionomer Membranes

	20.01.93	Dr. D.C. Clary#, University of Cambridge Energy Flow in Chemical Reactions
*	21.01.93	Prof. L.Hall, Cambridge NMR - Window to the Human Body
*	27.01.93	Dr. W. Kerr, University of Strathclyde Development of the Pauson-Khand Annulation Reaction: Organo-cobalt Mediated Synthesis of Natural and Unnatural Products
*	28.01.93	Prof. J. Mann, University of Reading Murder, Magic and Medicine
	03.02.93	Prof. S.M. Roberts, University of Exeter Enzymes in Organic Synthesis
	10.02.93	Dr. D. Gillies#, University of Surrey NMR and Molecular Motion in Solution
	11.02.93	Prof. S. Knox, Bristol University *The Tilden Lecture* Organic Chemistry at Polynuclear Metal Centres
*	17.02.93	Dr. R.W. Kemmitt#, University of Leicester Oxatrimethylenemethane Metal Complexes
	18.02.93	Dr. I. Fraser, ICI Wilton Reactive Processing of Composite Materials
	22.02.93	Prof. D.M. Grant, University of Utah Single Crystals, Molecular Structure and Chemical-Shift Anisotropy
*	24.02.93	Prof. C.J.M. Stirling#, University of Sheffield Chemistry on the Flat-Reactivity of Ordered Systems
*	10.03.93	Dr. P.K. Baker, University College of North Wales, Bangor Chemistry of Highly Versatile 7-Co-ordinate Complexes
*	11.03.93	Dr. R.A.Y. Jones, University of East Anglia The Chemistry of Wine Making
*	17.03.93	Dr. R.J.K. Taylor#, University of East Anglia Adventures in Natural Product Synthesis
*	24.03.93	Prof. I.O. Sutherland#, University of Liverpool Chromogenic Reagents for Cations

*	13.05.93	Prof. J.A. Pople, Carnegie-Mellon University, Pittsburgh, USA *The Boys-Rahman Lecture* Applications of Molecular Orbital Theory
	21.05.93	Prof. L.Weber, University of Bielefeld Metallo-Phospha Alkenes as Synthons in Organometallic Chemistry
*	01.06.93	Prof. J.P. Konopelski, University of California, Santa Cruz Synthetic Adventures with Enantiomerically Pure Acetals
	02.06.93	Prof. F. Ciardelli, University of Pisa Chiral Discrimination in the Stereospecific Polymerisation of Alpha Olefins
	07. 06.93	Prof. R.S. Stein, University of Massachusetts Scattering Studies of Crystalline and Liquid Crystal Polymers
	16.06.93	Prof. A.K. Covington, University of Newcastle Use of Ion Selective Electrodes in Ion Chromatography
	17.06.93	Prof. O.F. Nielsen, H.C. Ørsted Institute, University of Copenhagen Low Frequency IR and Raman Studies of Hydrogen Bonded Liquids
	13.09.93	Prof. A.D. Schlüter, Freie Universität Berlin, Germany Synthesis and Characterisation of Molecular Rods and Ribbons
	13.09.93	Dr. K.J. Wynne, Office of Naval Research, Washington, USA Polymer Surface Design for Minimal Adhesion
	14.09.93	Prof. J.M. DeSimone, University of North Carolina, Chapel Hill, USA Homogenous and Heterogenous Polymerisations in Environmentally Responsible Carbon Dioxide
	28.09.93	Prof. H. Ila, North Eastern Hill University, India Synthetic Strategies for Cyclopentanoids via Oxoketene Dithioacetals
	04.10.93	Prof. F.J. Feher#, University of California, Irvine, USA Bridging the Gap between Surfaces and Solution with Sessilquioxanes
*	14.10.93	Dr. P. Hubberstey, University of Nottingham Alkali Metals: Alchemist's Nightmare, Biochemist's Puzzle and Technologist's Dream

*	20.10.93	Dr. P. Quayle#, University of Manchester Aspects of Aqueous ROMP Chemistry
*	21.10.93	Prof. R. Adams#, University of South Carolina, USA Chemistry of Metal Carbonyl Cluster Complexes: Development of Cluster Based Alkyne Hydrogenation Catalysts
	27.10.93	Dr. R.A.L. Jones#, Cavendish Laboratory, Cambridge Perambulating Polymers
	10.11.93	Prof. M.N.R. Ashfold#, University of Bristol High Resolution Photofragment Translational Spectroscopy: A New Way to Watch Photodissociation
*	17.11.93	Dr. A. Parker#, Rutherford Appleton Laboratory, Didcot Applications of Time Resolved Resonance Raman Spectroscopy to Chemical and Biochemical Problems
*	24.11.93	Dr. P.G. Bruce#, University of St. Andrews Structure and Properties of Inorganic Solids and Polymers
	25.11.93	Dr. R.P. Wayne, University of Oxford The Origin and Evolution of the Atmosphere
*	01.12.93	Prof. M.A. McKervey#, Queen's University, Belfast Synthesis and Applications of Chemically Modified Calixarenes
*	08.12.93	Prof. O. Meth-Cohen#, University of Sunderland Friedel's Folly Revisited - A Super Way to Fused Pyridines
	26.01.94	Prof. J. Evans#, University of Southampton Shining Light on Catalysts
	02.02.94	Dr. A. Masters#, University of Manchester Modelling Water Without Using Pair Potentials
*	09.02.94	Prof. D. Young#, University of Sussex Chemical and Biological Studies on the Coenzyme Tetrahydrofolic Acid
	16.02.94	Prof. K.H. Theopold, University of Delaware, USA Paramagnetic Chromium Alkyls: Synthesis and Reactivity
	23.02.94	Prof. P.M. Maitlis#, University of Sheffield Across the Border: From Homogenous to Heterogenous Catalysis
*	02.03.94	Dr. C. Hunter#, University of Sheffield Noncovalent Interactions between Aromatic Molecules

09.03.94 Prof. F. Wilkinson, Loughborough University of Technology
Nanosecond and Picosecond Laser Flash Photolysis

10.03.94 Prof. S.V. Ley, University of Cambridge
New Methods for Organic Synthesis

25.03.94 Dr. J. Dilworth, University of Essex
Technetium and Rhenium Compounds with Applications as Imaging Agents

* 28.04.94 Prof. R.J. Gillespie, McMaster University, Canada
The Molecular Structure of some Metal Fluorides and Oxofluorides: Apparent Exceptions to the VSEPR Model

* 12.05.94 Prof. D.A. Humphreys, Mcmaster University, Canada
Bringing Knowledge to Life

Invited specially for the graduate training programme.

B. Conferences Attended

4th European Symposium on Organic Reactivity and 2nd Newcastle Meeting on Molecular Mechanisms in Bioorganic Processes, Newcastle, England, 11-16 July 1993.

3rd International Meeting of the Biology of Nitric Oxide, Cologne, Germany, 3-6 October, 1993.

Poster presented: "Cu(II) Catalysed Nitrosothiol Decomposition"

C. First Year Induction Course, October 1991

The course consists of a series of one hour lectures on the services available in the department.

1. Introduction, research resources and practicalities
2. Safety matters
3. Electrical appliances and hands-on spectroscopic services
4. Departmental computing
5. Chromatography and high pressure operations
6. Elemental Analysis
7. Mass spectrometry
8. Nuclear magnetic resonance spectroscopy
9. Glassblowing techniques