Studies of the reactions of S-nitroso-N-acetylcysteine

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by

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Abstract

The discovery of the many important roles of nitric oxide *in vivo* has prompted a large research effort, in recent times, into the chemistry of nitric oxide and of nitric oxide donors. The chemical and biological aspects of NO and S-nitrosothiols (thionitrites RSNO) are reviewed in Chapter 1. It is known that RSNO decomposition is Cu^{+} catalysed, while formation by S-nitrosation of thiols ensures the presence of some thiolate ion in solutions of RSNO generally. The extent of free thiolate ion in solutions of S-nitroso-N-acetylcysteine (SNAC) has been determined by the Ellman procedure. The effects of added Cu^{2+}, thiolate ion and ascorbic acid on the reactivity of SNAC have been determined experimentally, and conditions established where the thiolate ion acts either primarily as a reducing agent or a complexing agent for Cu^{2+}. UV spectral evidence is produced for the intermediacy of Cu^{+}, by the observation of its complex with neocuproine, a specific chelator for Cu^{+}. Finally the results of the reactions of SNAC with both ascorbate and thiolate ion at significantly higher added concentrations have confirmed the existence of other reaction pathways, yielding ammonia from thiolate ion and nitric oxide from ascorbate in reactions which do not involve copper ions. Reaction pathways consistent with the results are presented.

Acknowledgements

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Finally, I would like to indicate my gratitude towards all of my family and to my husband Zhihui who have always been there for me.

Declaration

The substance of this thesis is the result of research carried out in the Department of Chemistry, University of Durham, during October 1996 and October 1998. It has not been submitted for other degree and is the author's own work, except where acknowledged by reference.

Statement of copyright

To my husband Zhihui and my daughter Sijia

3. Chapter three. The reaction of SNAC with N-acetylcysteine (NAC) and ascorbate at high concentration

1. Background and literature survey

1.1. Background

Until the late 1980s nitric oxide was primarily known for its contribution to environmental pollution. But since then, NO has been found to occur naturally in many cell types in various parts of the body. In 1992, NO was named *Molecule of the Year* by *Science* magazine in recognition of hundreds of research papers that reported on NO activities in the brain, arteries, the immune system, liver, pancreas, peripheral nerves and lungs.

Nitric oxide has multifarious functions in the body and it also has some therapeutic possibilities. NO has been used by itself recently to treat persistent pulmonary hypertension of the new-born and inhaled NO has been used successfully in the treatment of severe adult respiratory distress syndrome (ARDS). The role played by NO in preventing platelet aggregation offers the possibility of drugs, which may be useful in the prevention of embola formation and hence reduce strokes.

Nitric oxide is an extremely important and versatile messenger in biological systems. At first, it was recognised as an endothelium-derived relaxing factor in the vascular system[1]. It has also been identified as a neuronal system[2] and a cytotoxic factor in the immune system[3,4]. Also, it is believed to relate to some tissue damage such as schema/repercussion tissue[5,6,7] damage and excitatory neuronal death[8, 9,10].

The discovery of the several roles of nitric oxide has lead to many studies of its chemistry and some of its little-studied derivatives, for example, S-nitrosothiols (thionitrites, RSNO). RSNO species, where R can be any one of a large range of chemical entities, are the sulfur analogues of the better known alkyl nitrites (RONO). One reason for the great interest in S-nitrosothiols has been their possible involvement in the transfer and storage of the NO (nitric oxide) molecule within the human body. Only in recent years with the discovery of the immense biological role of nitric oxide and the possible *in vivo* link with S-nitrosothiols, has intense interest been shown in this class of compounds[11].

The emergence of NO as a cell signalling agent is one of the most important and exciting developments in human physiology in this decade[12]. Long before NO was identified as the EDRF a number of NO donating compounds were recognised as vasodilators (substances which enlarge blood vessels) and used in the treatment of diseased conditions where increased blood flow relieve the symptoms.

Vasodilators such as GTN (nitroglycerin) and other organic nitrates ($RONO_2$) are the most well-known, and by far the most widely used anti-angina drugs[13]. However, it has been observed that they lose their effectiveness in many patients on continual use. Tolerance to these types of drugs is believed to develop when the vascular smooth-muscle receptor is depleted of free thiol groups on its surface[14]. Sulfhydryl groups (SH) are oxidised during the denitration process and with continuous nitrate exposure, decreased nitrate metabolism within the vascular smooth muscle cell occurs as a direct result of the depletion of reduced SH groups. Thus less NO is formed with a subsequent

decrease or absence of vasodilatation.

Exogenous SH donors, particularly N-acetyl-cysteine(NAC), have been employed to provide intracellar thiols in efforts to prevent or reverse nitrate tolerance. S-Nitrosothiols have been considered to be potential alternative anti-angina drugs, and may also be involved in the storage and transport of NO *in vivo*. One of the reasons for lack of knowledge for this class of compounds is due to their instability compared to alkyl nitrites. Many S-nitrosothiols have been detected in solution, but attempts to purify them have not always been successful as a result of their instability in the pure state[15]. However, at least two S-nitrosothiols have been prepared as stable solids and have been characterised: S-nitroso-acetylpenicillamine (SNAP) and S-nitroso-glutathione (GSNO).

1.2. Nitric Oxide

1.2.1. NO chemistry

Nitric Oxide Preparation

Nitric oxide can be prepared in the laboratory via the reduction of nitric acid using copper metal as a reducing agent. (Equation 1-1)

$$8HNO_3 + Cu \longrightarrow 3Cu(NO_3)_2 + 4H_2O + 2NO$$

Equation 1-1

The extremely efficient commercial route to NO is by means of the catalytic oxidation of ammonia (Equation 1-2)

oxidation of ammonia (Equation 1-2)

$$4NH_3 + 5O_2 \xrightarrow{\text{Pt-Rh catalyst}} 4NO + 6H_2O$$

Equation 1-2

Aerial oxidation of the product gases leads to brown NO_2 being formed (Equation 1-3) The reaction leads to air pollution and build up of photochemical smog.

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

Equation 1-3

The properties of nitric oxide

Nitric oxide is a colourless gas at room temperature with bp -151.8^0C and mp -163.6^0C. The liquid and solid are also colourless if the material is pure. The solubility in water at 25^0C and 1 atmosphere pressure is 1.8×10^{-3} mol dm^{-3} which is unchanged within the pH range 2-13. For its physiological action nitric oxide is always is always present as an aqueous solution. It is a relatively stable free-radical, whose structure is best represented by the canonical forms shown below (*Equation 1-4*).

$$.N^+—O^- \longleftrightarrow .N{=}O \longleftrightarrow N^-{=}O^+. \longleftrightarrow N{\equiv}O.$$

Equation 1-4

reaction 3, so simple aqueous NO solutions give nitrite anion as the final product.

$$NO + 1/2O_2 \longrightarrow NO_2 \qquad 1$$

$$2NO_2 \rightleftharpoons N_2O_4 \qquad 2$$

$$N_2O_4 + H_2O \rightleftharpoons NO_2^- + NO_3^- + 2H^+ \qquad 3$$

$$NO + NO_2 \rightleftharpoons N_2O_3 \qquad 4$$

$$N_2O_3 + H_2O \longrightarrow 2NO_2^- + 2H^+ \qquad 5$$

Equation 1-5

The most well-known gas-phase reaction of nitric oxide is also its oxidation to nitrogen dioxide (NO_2) which occurs rapidly at reasonably high concentrations and results in the formation of its characteristic brown fumes. Since the reaction is third order, the aqueous-phase reaction of NO with molecular oxygen is slow with a half-life of several hours at nitric oxide concentrations that exist in cells. In aqueous solution NO reacts rapidly with NO_2 to give N_2O_3 , the anhydride of nitrous acid. Hence a colorimetric assay for NO_2^- (such as the Griess Test) can be used as a measure of NO produced.

1.2.2. NO *in vivo*

More important categories of NO chemistry relative to biological action are the following three forms, NO, NO^+, NO^-, as proposed by Stamler et

al[16]. NO has one electron in the 2p-π antibonding orbital. If this electron is lost through oxidation, NO changes to the nitrosonium cation (NO^+), and if another electron is added in the orbital through reduction, the nitroxyl anion (NO^-) is made.

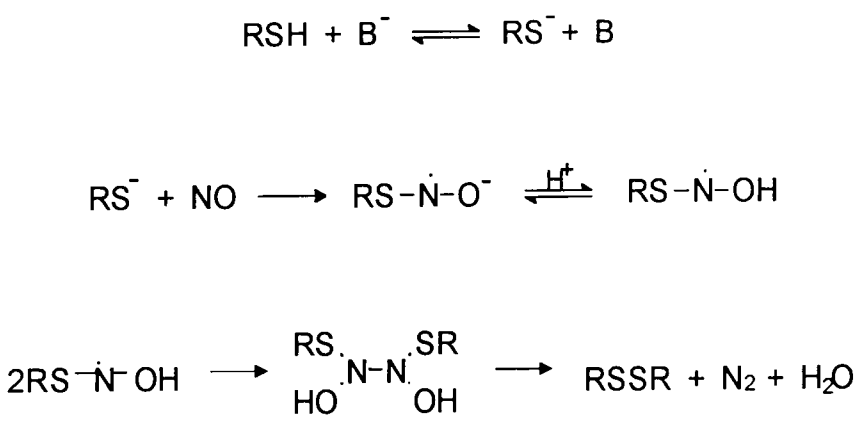
NO, nitric oxide

One of the main reactions of NO, is its complexation with some transition metal cations. For example, NO binds to the ferrous ion (Fe^{2+}) in deoxyhemoglobin to make a stable complex. NO also reacts with other metalloproteins[17]. Various iron-nitrosyl compounds are also formed in activated macrophages[18]. One of the characteristic binding properties of NO is the ability to bind with Fe^{3+}-heme, which is different from O_2 or CO. The complex produced, however, is unstable and tends to release an NO^+ equivalent with an attack by nucleophiles (thiol, amine etc.).

NO^+, nitrosyl cation

Free NO^+ can be present in aqueous media at high acidity but a major form of NO^+ equivalent in biological environments, can be a variety of nitroso compounds which can deliver NO^+, i.e. they are electrophilic nitrosating agents. Nitroso compounds can be made chemically by reaction with sodium nitrite in the presence of acid, in this context the most important form is $H_2NO_2^+$. Amines can also form N-nitroso compounds. However, simple amines are usually protonated in physiological pH to prohibit N-nitrosation. It was believed that S-nitrosothiols can not be made by direct reaction of NO and thiols, but at high

base (B^-) concentration it has been suggested that the thiol is simply oxidised by NO to form the disulphide[19] (Equation 1-6).



Equation 1-6

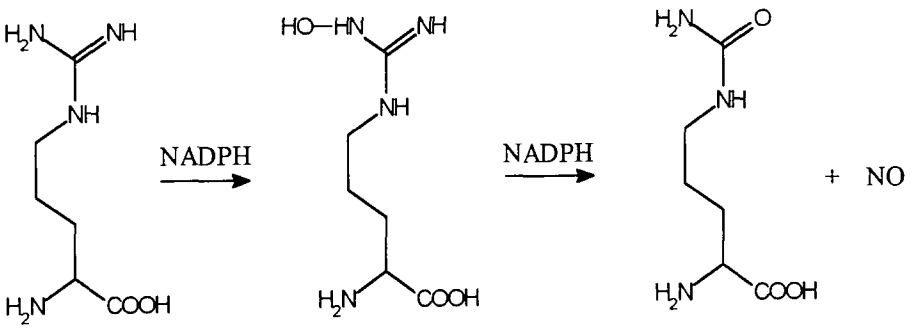
NO^-, nitroxyl anion

NO can be reduced to the anion form, NO^-. NO^- is believed to be formed biologically by the 4-electron oxidation of guanidino nitrogen in arginine by NO synthase. The physiological meaning of NO^- has not been clarified. Murphy et al. have suggested that NO^- might act as a stabilised form of NO to prolong its lifetime with superoxide dismutase(SOD)[20] .

NO synthesis in vivo

Intense studies of the pathways for the formation of nitric oxide within the body has led to the discovery of two or possibly three distinct enzymes which can effect NO formation. The substrate for the NO-synthases is L-arginine and the products of the reaction are NO and L-citrulline (Equation 1-7). The nature of the enzyme depends on the tissue within which it is found. So far these

distinct forms have been found within the brain, endothelial cells, and macrophages.



L-arginine N-hydroxy-L-arginine L-citrulline

NADPH is reduced nicotinamide adenine dinucleotide phosphate.

Equation 1-7

1.2.3. Physiological role of nitric oxide

NO in the vascular system: Smooth muscle relaxation and regulation of blood pressure

An endothelium derived relaxing factor (EDRF), was discovered by Furchgott[21], and Moncada et al. suggested that it was simply nitric oxide. Relaxation of smooth muscle may be the result of one or more biological mechanisms. There appears to be receptors on smooth-muscle membranes that are activated by β-adrenergic agonists (e.g., isoproterenol); these, in turn, activate adenylate cyclase to increase the levels of cyclic adenosine monophosphate (cAMP) in the cell. The increased level of cAMP is associated with smooth-muscle relaxation. Drugs such as papaverine and theophylline also function to relax smooth muscle through a cAMP-mediated mechanism. These

drugs inhibit phosphodiesterase and reduce the rate of conversion of cAMP to 5'-AMP in the cell.

Organic nitrates, nitrites, nitroso compounds, and a variety of other nitrogen-containing substances such as sodium nitroprusside, cause relaxation of vascular smooth muscle by a mechanism that does not include cAMP. These compounds have the common property of generating or releasing the unstable and lipophilic free radical nitric oxide *in situ*. The nitric oxide containing vasodilators are believed to first react with thiols in the cell to form unstable S-nitrosothiols, which break down to yield nitric oxide. The liberated nitric oxide activates guanylate cyclase and increases the cellular level of gnosine 3'5'-monophosphate (cGMP). Cyclic-GMP activates a cGMP-dependent protein kinase, which alters the phosphorylation state of several proteins. Included in this change is the dephosphorylation of the light chain of myosin. The altered state of the light chain of myosin now cannot play a normal role in the contractile process of smooth muscle and result in relaxation. The scheme in Figure 1-1 summarises these events.[14.]

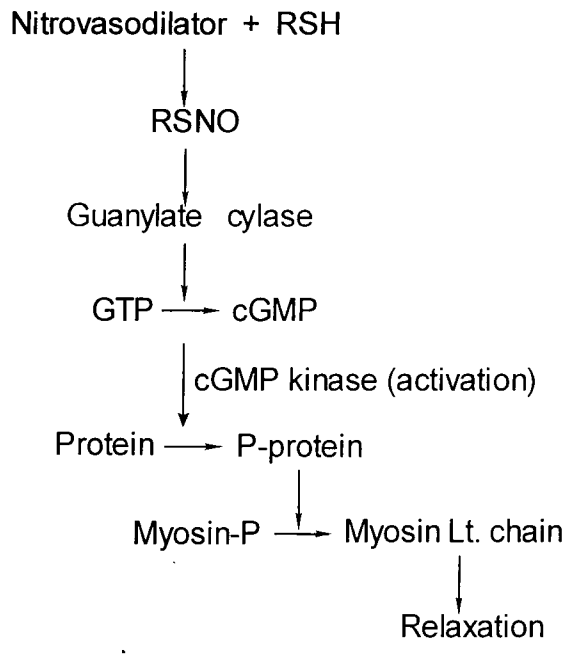


Figure 1-1. Mechanism of nitrovasodilator relaxation of smooth muscle.

Muscle relaxation results in the dilation of the vessel with a corresponding lowering in the blood pressure. Maintenance of a normal blood pressure relies on the constant synthesis of NO within the body, as smooth muscle relaxation is a positive process rather than just the absence of contraction.

Inhibition of platelet aggregation

Nitric oxide has also been found to inhibit both platelet aggregation and adhesion, so breaking up and limiting formation of any harmful clotting within the body. This could result in a heart attack if such a clot formed within a coronary vessel. The nitric oxide responsible for these effects is derived from two sources, the contact between the platelets and endothelial cells, via an enzymatic system within the actual platelets, which release nitric oxide from L-

arginine.

Neuronal NO: Role of nitric oxide as a neurotransmitter

The NO-synthase present within neurotransmitters is found in the postsynaptic neurone and the grial cell in central or peripheral nerve systems[22]. NO made in the central nervous system has attracted the attention of many researchers because it may be closely related to neuronal plasticity[23]. In the peripheral nerve system, the main function of NO is vasoregulation[24]. The renal blood flow is suggested to be regulated indirectly with NO through the attenuation of sympathetic neuronal activity in addition to the direct action[25,26] .

NO also is believed to act as a retrograde messenger diffusing back to the presynaptic neuron where it activates another enzyme guanylyl cyclase into sythesising cyclic guanosine triphosphate. This step has been proposed as a possible mechanism in long term memory. The full physiological roles of NO within the brain are as yet still poorly understood though it appears to be involved in a variety of functions.

A problem with the formation of NO within the brain is when the neurones are deprived of blood flow as in the case of a stroke. The Ca^{2+} intake cannot be controlled, resulting in too much Ca^{2+} being admitted into the cell, so when oxygen supply is restored, NO production reaches toxic levels with resultant brain damage occurring.

NO in the immune system: Cytotoxic activity of nitric oxide

The activity of activated macrophages in the non-specific immune response to a foreign body has been found to release substantial amounts of nitrite and nitrate, the formation of which is believed to be a by-product of oxidation of nitric oxide, which was originally produced in the cytotoxicity mechanism. Further studies have also observed that the cytotoxicity of the macrophages depends upon the presence of L-arginine with nitrite and L-citrulline being formed.

Immune cells, including activated macrophage, monocyte, and kuppfer cells can release a greater amount of NO than endothelium or nerve cells. Macrophages contain the third type of NO-synthase which is inducible rather than constitutive as in the other two cases. The main difference between this and the endothelial or brain NO-Synthase is that it is not Ca^{2+}dependent because the enzyme always contains a tightly bound cadmodulin unit which allows the active enzyme to work at low levels of Ca^{2+} ions. So once the enzyme has been synthesised, the production of NO will continue rapidly until it becomes deactivated or until all the required substrate is consumed.

The formation of this enzyme is stimulated by the presence of a mixture of cytokines. Once formed, it immediately starts to catalyse the conversion of L-arginine to NO and L-citrulline. One of the cytotoxic effects of the macrophage is believed to be the diffusion of NO formed in the macrophage into the adjacent tumour/bacteria cell, which attacks the iron-sulfur centres of many very important enzymes involved, e.g. those involved in DNA synthesis and cell division.

The amounts of NO released by activated macrophages can in some cases become life threatening. If the body is exposed to a large infection a condition known as septic shock can occur where vast quantities of NO are formed which result in widespread relaxation of vascular smooth muscle and hypotension[27]. Understanding the mechanism behind this condition has lead to a number of improved methods of treating this condition with drugs, which inhibit the NO-Synthase[28].

1.2.4. NO donors and a NO scavenger

NO donors

Figure 1-2 shows some classical NO donors that release NO, in biological environments. These donors can be classified into the following categories: organic nitrates, organic nitrites, furoxane derivatives, iron-nitrosyls, S-nitrosothiols and sydnonimines.

Organic nitrates have the general formula, $RONO_2$. They are esters of alcohols and nitric acid. The chemical reactions involved in NO release require special thiols such as cysteine and N-acetylcysteine. Organic nitrites have the general structure RONO, which represents the ester of an alcohol and nitrous acid. This type of reagent can release NO with thiolate leading to S-nitrosothiols. Furoxanes can also react with thiols to release NO. The above three types of NO donor all require thiols as the cofactor for generating NO. In these cases, the NO moiety is transferred to thiol and then NO is released from the S-nitrosothiol or S-nitrososulfoxide. The real NO-releasing agent is a thiol derivative. Thus, these

types of reagents consume endogenous thiols in biological samples to release NO. If the thiol is depleted, further dosages of such reagents have no more activity, this condition is called nitrate tolerance[29]. Co-administration of N-acetylcysteine suppresses the tolerance in GTN[30]. Iron-nitrosyl and sydnonimine can also release NO spontaneously. As NO can undergo various reactions, the rate, amount and period or profile of NO release are very important factors affecting NO bioactivity.

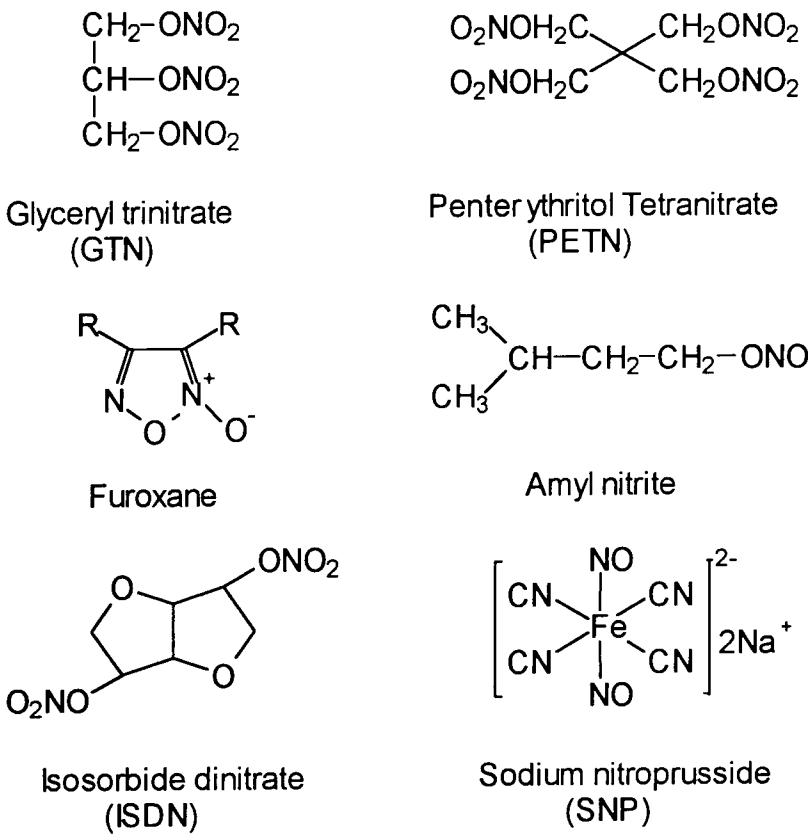
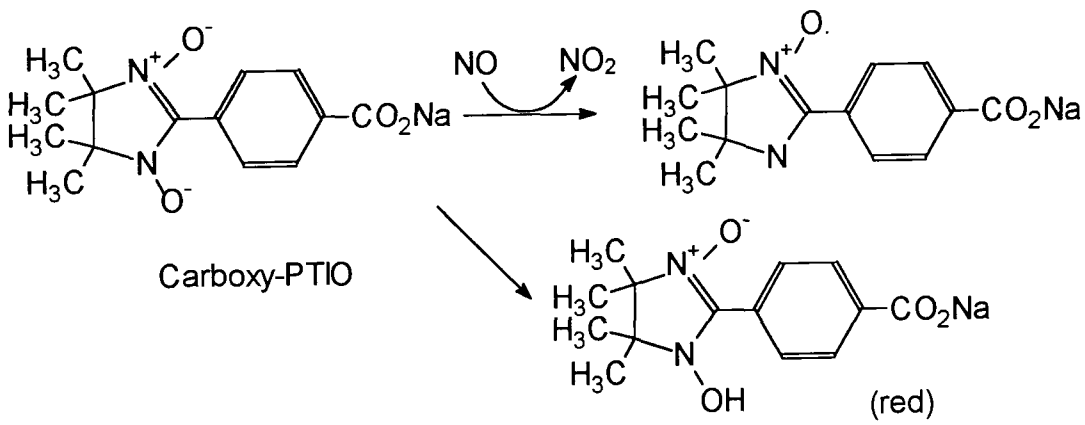


Figure 1-2 Some examples of classical NO donors

A NO scavenger

The NO scavenger, carboxy-PTIO reacts directly with nitric oxide to oxidise it to the NO_2 radical. For scavenging endogenous NO, NOS inhibitors

that are arginine derivitives and hemoglobin have been used as NO traps (Equation 1-8)



Equation 1-8

An inhibitory effect of Carboxy-PTIO on vasodilatation by endogenous NO and exogenous NO donors has been found and there are also some reports that it augmented the antiviral activity of NO[31].

1.3. S-Nitrosation

Nitrosation of thiols is an easy process to achieve due to the reactivity of the sulfur atom of the thiol to electrophilic attack. It may be achieved using a range of reagents such as alkyl nitrites, nitrosyl chloride and nitrous acid. The most widely used reagent in nitrosation is nitrous acid, which is readily produced from sodium nitrite (or any other nitrite salt) and aqueous mineral acid. Nitrous acid itself is only commonly known in solution, in which it decomposes fairly quickly. Such solutions are therefore used immediately after preparation and quantitative work has to take account of the decomposition. However, many

nitrosation reactions are sufficiently rapid at 25^0C, to allow the decomposition reaction to be ignored. Unless oxygen is very rigorously removed, nitrosothiols will be formed when NO is bubbled in a thiol solution, almost certainly after oxidation of NO to NO_2 and consequent formation of N_2O_3. If the dissolved oxygen level is very low then there is, as expected, no reaction between NO and thiols.

$$RSH + XNO \rightarrow RSNO + HX$$

$$RSH + HNO_2 \rightarrow RSNO + H_2O$$

$$3HNO_2 \rightarrow 2NO + HNO_3 + H_2O$$

Equation 1-9

S-nitrosation describes the electrophilic addition of 'NO^+' to a sulfur atom. It is a reaction that has been much less studied than the analogous O-nitrosation, which consequently has led to a smaller literature referring to S-nitroso compounds. This is also partially due to the greater susceptibility of the S-N bond to homolytic fission, making such compounds unstable. The current interest in nitric oxide donor drugs has promoted much research activity related to the mechanism of S-nitrosation in vivo and subsequent decomposition of the relevant species formed.

1.3.1. Reagents involved in nitrosation

Nitrous Acid

It is possible to add NO^+ to a sulfur-containing substrate in many

ways. The most popular reagent utilised being nitrous acid. HNO_2 will be formed on the acidification of sodium nitrite, with solutions being used immediately due to the decomposition reaction occurring. Nitrous acid is a weak acid (pKa value of 3.1)[32]. Its structure has been determined in the gas phase by infrared studies[33] and it is known to exist in both *cis* and *trans* forms (Equation 1-10), with the trans form prevalent in solution.

Equation 1-10

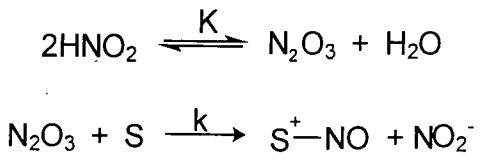
Reaction via dinitrogen trioxide (N_2O_3)

Another common reagent used to effect nitrosation is dinitrogen trioxide, N_2O_3. It is formed from high concentrations of nitrous acid due to the existence of an equilibrium (Equation 1-11).

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

Equation 1-11

Reaction of N_2O_3 with substrates has been observed generally to involve rate-limiting attack on the substrate (S) by dinitrogen trioxide (Equation 1-12). The rate equation (Equation 1-13) shows a second order dependence on nitrous acid and first order dependence on substrate[15].



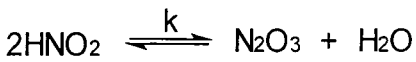
Equation 1-12

$$Rate = k[N_2O_3][S]$$

$$Rate = kK[HNO_2]^2[S]$$

Equation 1-13

For very reactive substrates (or substrates at high concentration) the reaction with dinitrogen trioxide can be faster than the hydrolysis of dinitrogen trioxide to nitrous acid, and so the rate-limiting step becomes the formation dinitrogen trioxide (Equation 1-14).



Equation 1-14

The rate equation is then second order with respect to HNO_2 but zero order in substrate (Equation 1-15).

$$Rate = k[HNO_2]^2$$

Equation 1-15

S-nitrosation of thiols, however, is generally carried out at lower concentrations of nitrous acid. Under these conditions another nitrosati agent prevails. The rate equation now becomes first order in substrate and HNO_2 (Equation 1-16), and the reaction shows acid catalysis.

$$Rate = k\,[HNO_2][S][H^+]$$

Equation 1-16

Two possible mechanisms can be proposed to explain the rate equation, both of which involve a pre-equilibrium forming a nitrosating species followed by attack on the substrate. Two different equilibrium reactions have been suggested, one is involving the formation of the nitrous acidium ion (Equation 1-17), the other involving the nitrosonium ion (Equation 1-18). In aqueous solution it is very difficult to determine which species is the nitrosating agent[15].

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

Equation 1-17

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

Equation 1-18

Nitrosyl halides (XNO), nitrosyl thiocyanate (ONSCN), nitrosyl acetate (CH_3COONO), nitrosonium salts (NO^+X^-), nitrosothiouronium ions and nitrogen oxides have also been much used as electrophilic nitrosating agents, often

generated and used *in situ.* Other less well-known nitrosating agents include alkyl nitrites, nitrosothiols, nitrosamines and metal nitrosyl complexes.

Alkyl nitrites RONO as nitrosating agents:

Alkyl nitrites can react directly with the thiolate ion to yield the nitrosothiol in solution (Equation 1-19). The rate equation was established and the second-order rate constants k_2 were obtained as a function of the pH of the solution[34]. These reactions are of some interest in connection with the well-known vasodilatory properties of alkyl nitrites, since it has been suggested that alkyl nitrites may act in this way by first effecting (*in vivo*) S-nitrosation of tissue-bound thiol groups.

$$RSH + R'ONO \longrightarrow RSNO + R'O$$

$$Rate = k_2 [RSH][R'ONO]$$

Equation 1-19

When describing the kinetics of nitrosothiol formation, a comparison of alcohol and thiol nitrosation is instructive. O-nitrosation is the most commonly used method of preparation of alkyl nitrites[35]. Equilibrium constants for alkyl nitrite formation have been measured for a number of simple alcohols by different procedures. The results show that values of K decrease along the series R = CH_3 > C_2H_5 > *i*-C_3H_7 > t-C_4H_9, implying that steric effects rather than electronic effects are more important in O-nitrosation of alcohols.

Alkyl nitrite formation (RONO) from nitrous acid and the corresponding alcohol is very rapid but not quantitative. Aldred et al[36] measured the observed first order rate constant for the reaction of ROH with HNO_2 ([ROH]>>[HNO_2]) at various [ROH]. A plot of k_{obs} against [ROH] was linear (confirming a first order dependence of ROH on the rate equation) with a positive slope and intercept, which gives the rate constant for the forward reaction and reverse reaction(alkyl nitrite hydrolysis). This is characteristic of a reversible reaction; first order in both directions where the observed first order rate constant, k_0 is the sum of those for the forward and reverse reactions.

1.4. S-Nitrosothiols

1.4.1. Introduction

S- nitrosothiols (or thionitrites) are of the general formula RSNO and are the sulfur analogues of the much more widely studied alkyl nitrites (RONO). A comprehensive study of S-nitrosothiols was reviewed by Oae and Shinhama[37], their physical properties are comparable with that of RONO compounds but are influenced by the smaller electronegativity associated with sulfur than oxygen.

It has been suggested that the EDRF has two types of components, short-lived and long-acting, one of the strong candidates for the long-acting component is S-nitrosothiols. The bioaction of S-nitrosothiols was reported to be similar to that of NO in many cases. However, there are some differences in their physiological activities and there may be another messenger in addition to the NO carrier to intracellular space.

The development of tolerance while on organic nitrate therapy is a common clinical problem. Although the mechanisms involved have not yet been pinned down, some aspects are thiol-related. Provision of both a thiol and NO in some form (possibly a nitrosothiol) is attractive as a means to circumvent this problem but no progress has yet been reported. Relative potency and arterio-venous selectivity of various NO donors have been explored *in vivo* in humans[38]. In the search for tissue-selective NO-donor drugs, S-nitrosothiols have an important place.

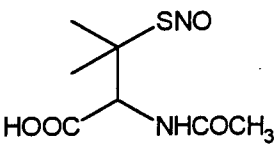
Platelet activation is a fundamental clinical problem in many vascular disorders, which is associated with malfunction of endothelial cell NO production. Most NO donors have profound vasodilator actions at concentrations where there is little antiplatelet effect. The search for NO donors with platelet-weighted effects has thus been energetic. A host of RSNOs inhibit adhesion of human platelets to fibrillar collagen and human endothelial cells *in vitro* and also reduces ADP-induced platelet aggregation *in vivo* in the rat[39]. These include S-nitrosoglutathione, S-nitrosocysteine, S-nitroso-N-acetylcysteine, S-nitroso-N-acetylpenicillamine and S-nitroso-β-D-thioglucose, as well as high-molecular-weight species such as S-nitrosoalbumin[40,41].

Only a few nitrosothiols have so far been isolated and characterised. These include such as S-nitroso-N-acetylpenicillamine (SNAP)[42], which forms stable green crystals and S-nitrosoglutathione (GSNO) which forms stable pink crystals and which has been detected *in vivo*[43]. Other RSNO have been detected in solution, but less is known about S-nitroso compounds compared with that of

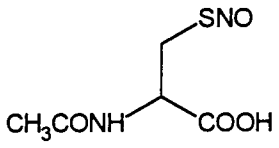
O-nitroso species. This is thought to be due to the apparent relative ease of homolytic cleavage of the RS-NO bond within these compounds, whereas alkyl nitrites generally exhibit far greater stability to homolytic cleavage of the RO-NO bond and hence are found to be more stable. Another factor is probably the greater reactivity of sulfur compounds in nitrosation, which usually requires special fast reaction techniques for the measurement of rate constants.

1.4.2. Physical Properties

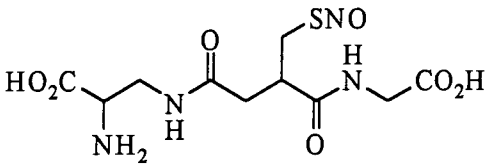
S-Nitrosothiols, in common with many S-nitroso species have a broad absorption band in the uv-visible region. Visible absorption occurs at around 540 or 590 nm with a low extinction coefficient (10-20 $dm^3mol^{-1}cm^{-1}$) for the $n_N \rightarrow \pi^*$ transition and there is an UV absorption centred at around 340nm ($n_0 \rightarrow \pi^*$) with extinction coefficient of ca 10^3 $M^{-1}cm^{-1}$. This property of nitrosothiols has been used analytically in the identification of thiols in solution[44], and in kinetic studies to follow the disappearance of RSNO. In addition, primary and secondary compounds exhibit a characteristic visible absorbance at 540nm whereas tertiary nitrosothiols absorb at around 590nm. These bands result in the compounds being coloured. The electronic transitions responsible for ultraviolet/visible absorption have been assigned by Barrett et al[45]. In the IR there is a N=O stretching vibration in the region 1480-1530cm^{-1} and another C-S vibration at 600-730 cm^{-1}.



SNAP



SNAC



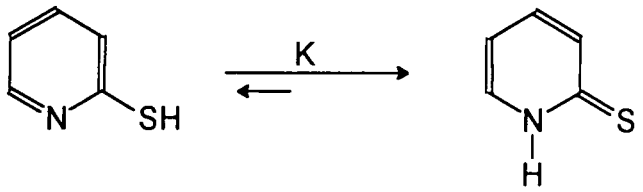
GSNO

S-nitrosothiols (RSNO) are typically red or green compounds, that are light and heat sensitive to various degrees. The R-SNO group is responsible for the coloured nature of the compounds but the structure of the rest of the molecule may have some effect. Primary and secondary nitrosothiols, such as S-nitrosoglutathione (GSNO) and S-nitroso-N-acetylcysteine (SNAC), are reddish whereas more sterically hindered tertiary nitrosothiols, such as S-nitroso-N-acetyl penicillamine (SNAP), are green.

RSNO derived from heterocyclic and aromatic thiols

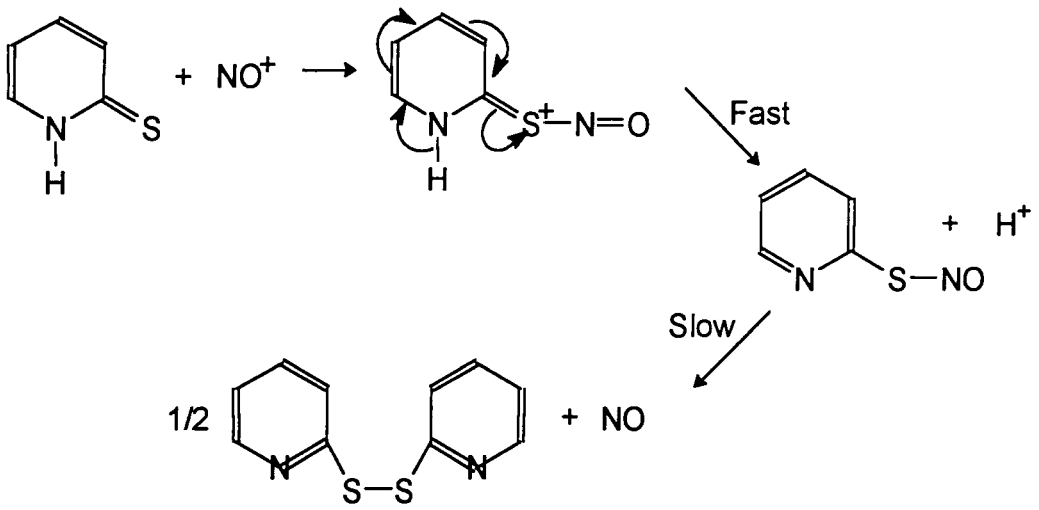
The nitrosocompounds derived from heterocyclic and aromatic thiols eg. 2-mercaptoimidazole, 2-mercaptopyridine, 2-mercaptopyrimidine, and 2-mercaptophenol, do not necessarily exhibit the characteristic absorbance maximum at around 340 nm. The existence of thiol/thione tautomerism in some of these compound means that it is more relevant to term the nitrosated species 'S-nitrosated thiols' rather than 'S-nitrosothiols'. Such S-nitrosated thiols have little use therapeutically as nitric oxide donor compounds but are mechanistically

of great interest. For 2-mercaptopyridine, the predominant form is the thione (Equation 1-20). From extensive ultraviolet studies Albert and Barlin[46] quoted value of the equilibrium constant K as 49,000.



Equation 1-20

It has been reported that an unstable S-nitrososulfonium ion ($Ar=S^{+}$-NO) is observable at high concentration as a transient orange colour[47]. This species can rapidly undergo the loss of a proton, which leads to formation of an S-nitrosothiol (Ar-S-NO) (Equation 1-21) which is readily oxidised in the air to the disulfide. 2-Mercaptopyrimidine is also known to exist as the thione tautomer, in solution and is nitrosated under similar conditions using acidified sodium nitrite.



Equation 1-21

1.4.3. Chemical properties

Thermolysis/photolysis

Nitrosothiols decompose both thermally and photochemically initially according to the Equation 1-22, although NO will react further in the presence of oxygen. In aerated solution the nitric oxide produced will be converted to nitrite (NO_2^-) by hydrolysis. A photochemical study[48] with GSNO showed that nitric oxide is released when samples are irradiated at either 340 or 545 nm and that the cytotoxic effect of GSNO on leukemia cells is enhanced upon radiation, raising the possibility that GSNO and other nitrosothiols might have a future as photochemotherapeutic agents.

The thermal decomposition is considered to occur via a homolytic mechanism with fission of the S-NO to form a thiyl radical ($RS^{\cdot}$) and nitric oxide. Subsequently two thiyl radicals can combine to form the observed disulphide product. In aerated solution the nitric oxide produced will be converted to nitrite by hydrolysis. Nitrosothiols readily undergo oxidation by a variety of reagents. Reaction with fuming nitric acid will initially form a thionitrate, which will decompose to form the corresponding disulphide and thiosulphonate[49].

$$2RSNO \xrightarrow{\Delta, h\nu} RSSR + 2NO$$

Equation 1-22

Copper ion catalysis decomposition

The decomposition of S-nitrosothiols (releasing NO) in aqueous buffers

is brought out about by copper but not significantly by any other metal ions (Equation 1-23). The rate of reaction is dependent on the concentration of RS^- and Cu^+, which is generated by reduction of Cu^{2+} by thiolate ion[50,51]. Thiolate ion is always present in these RSNO sample solutions as S-nitrosation is a slightly reversibly reaction, whereas Cu^{2+} is present as an impurity in the water or buffer. The copper ion reduction is a very fast reaction, for most thiols Cu^+ is formed within 10 seconds. Reduction of Cu^{2+} sources where the copper is bound to peptides and proteins also occurs, thus allowing the possibility that these reactions could occur *in vivo*[52]. Over a range of $[Cu^{2+}]$, the rate of decomposition can be described by Rate = k $[Cu^{2+}][RSNO]$.

$$RS^- + Cu^{2+} \rightarrow 1/2\,RSSR + Cu^+$$

$$Cu^+ + RSNO \rightarrow RS^- + NO + Cu^{2+}$$

$$2RSNO \rightarrow RSSR + 2NO$$

Equation 1-23

Silver and mercury ion induduced reaction

RSNO can be decomposed by the Hg^{2+} metal ion, and this reaction has been used for analysis of the thiol group by Saville (Equation 1-24), but this involves NO^+ rather than NO release. Ag^+ ions also induce decomposition without any NO release (Equation 1-25). However, decomposition is not observed in the presence of Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Cr^{3+}, or Fe^{3+}.

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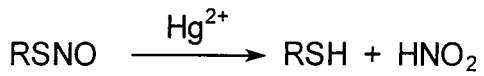
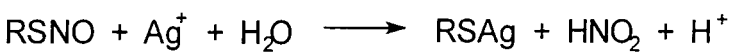


Equation 1-24



Equation 1-25

These Hg^{2+} and Ag^{+} induced reactions are quite different from the recently much studied Cu^{+} catalysed RSNO decomposition, which gives initially nitric oxide and the disulfide rather than nitrous acid and thiol. Another major difference between the Cu^{+} and Hg^{2+} reactions is the lack of structural dependence in the mercury ion case. N-Acetylation of many of the cysteine derivatives causes a major rate reduction in the copper reaction, which does not occur in the reaction with mercury ion.

There is a very wide range of reactivity in the copper catalysed reaction depending on structural differences. The very reactive compounds are those which contain a substituent able to complex with Cu^{+}, i.e. electron donating substituents particularly the $-NH_2$, NR_2, groups or the $-COO^{-}$ group, in addition to co-ordination to the nitroso group, so that the copper can be bidentately bound. If there is no such groups present then the reaction is very slow (as for $(CH_3)_3CSNO$). Similarly when the proposed intermediate would have an

unfavourable seven-membered ring configuration, as in S-nitrosohomocysteine, the reactivity is reduced to almost zero. Esterification of $-COO^-$ and acetylation of $-NH_2$ groups reduces reactivity drastically.

Ferrous ion catalysis decomposition

It has also been claimed that ferrous ion (Fe^{2+}) is capable of acting as a catalyst for the release of nitric oxide from S-nitrosothiols[53]. Due to the instability of ferrous ion with respect to aerial oxidation, any kinetic studies have to be performed under completely anaerobic conditions. An additional problem is that the copper catalysed reaction may interfere with the ferrous ion reaction, due to the naturally occuring Cu^{2+} impurities in the water/buffer components.

Transnitrosation

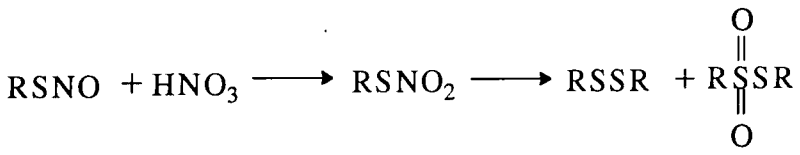
Transnitrosation describes the transfer of the NO group from an S-nitrosothiol (or other nitroso-containing molecule) to a suitable nucleophile. If the nucleophile is thiolate ion then a new S-nitrosothiol is formed (Equation 1-26).

$$RSNO + R'S^- \rightleftharpoons RS^- + R'SNO$$

Equation 1-26

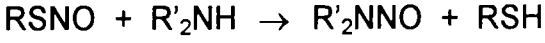
Other reactions

and thiosulphonate (Equation 1-27).[55]



Equation 1-27

Nitrosothiols can also generally nitrosate both secondary amines and aniline derivatives to yields nitrosamines (Equation 1-28) and azo dyes may be formed after coupling with naphthol. In such reactions it is still not clear whether the nitrosation is directly attributable to the S-nitrosothiol, or whether an alternative nitrosating agent is formed from the S-nitrosothiol, e.g. by hydrolysis.



Equation 1-28

1.4.4. Analysis of S-nitrosothiols

Chemiluminescence:

RSNO derivatives occur naturally in plasma and other body fluids as GSNO and as the S-nitrosothiols of sulfur-containing proteins[56,57]. This finding has prompted much research into the detection of low concentrations of S-nitrosothiol. Low-molecular-weight thiols and their nitrosated derivatives are difficult to analyze by direct methods. Some progress has been made and photolytic cleavage of the S-NO bond prior to detection of NO has proved quite

successful.

Chemiluminescence analyzers have been used for over 20 years for the determination of NO, non-volatile nitrosamines, and related nitroso and nitro compounds. Following gas chromatographic and/or HPLC separation, higher N-oxides are first converted to NO by one of several techniques such as pyrolysis, photolysis or reduction by hydroiodic acid (HI). Reaction between nitric oxide and ozone leads to the formation of an excited form of NO_2 (NO_2*) with subsequent release of electromagnetic radiation of characteristic frequency (Equation 1-29).

Detection of near-infrared radiation light produced in this way has been applied to measurement of NO as an atmospheric pollutant and for the analysis of the nitrosamine content of a variety of products. This technique[58] remains one of the most sensitive assays of NO in biological systems, with detection limits of approximately 10^{-13} M.

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

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

Equation 1-29

Direct detection:

High-performance capillary electrophoresis is performed by application of high voltages, between 10 and 30kV, across narrow bore fused silica capillaries. It is an analytical separation technique characterised by high resolving

power, minimal sample preparation, use of aqueous media, rapid analyses, and low sample consumption (1-50nl injected). Capillary electrophoresis is capable of separations of thiols, their disulfides, and their S-nitrosated derivatives, and it allows distinction between cysteine, homocysteine, and glutathione with the rapidity and specificity that is characteristic of capillary electrophoresis[59]. A disadvantage of the assay is the requirement to vary the polarity of the internal power supply and buffer pH in order to analyse the different thiol derivatives. Both these requirements prevent simultaneous analyses. The method lacks the sensitivity that is required for detection of physiological levels of S-nitrosothiols in plasma, and thus cannot be applied to human physiological work.

Mass spectrometry

The presence of NO in breath has been demonstrated indirectly, with nitrosation of thioproline, perhaps after activation by oxidation and analysis of the tert-butyldimethylsilyltrifluoroacetamide derivative of nitrosothioproline by GC-MS. This has been applied to validate chemiluminescence as a method for analysis of NO in breath. The method would therefore have potential for application to S-nitrosothiol analysis, although no examples have been reported so far.

Nitrate and nitrite

Analysis of nitrite and nitrate in biological samples has been performed for many years. NO generated in most biological systems is of limited life (i.e. a matter of seconds, although the exact figure depends on the circumstances). It is

converted under different circumstances into nitrite and nitrate, although additional products may sometimes occur. The use of nitrite measurements to reflect NO concentrations works well in aqueous solution, i.e. in vitro in the presence of oxygen where the conversion of NO to nitrite is quantitative and other sources of nitrite, apart from NO, are eliminated. In vivo both nitrite and nitate are generated from NO and the use of both nitrite and nitrate as indices for *in vivo* NO production is less straight forward than in vitro and so there are special problems with use of nitrite and nitrate concentrations in whole blood.

Conversion of nitrate to nitrite and nitrite to nitric oxide

Reversal of some of the reactions described above is known to occur in vivo in human. For example, nitrate in saliva can be reduced to nitrite by oral bacteria[60,61], the nitrite swallowed could be converted to nitric oxide in the acid environment of the stomach[62]. The most sensitive and specific method available for determination of nitrite and nitrate concentrations is traditionally believed to be GC-MS[63]. The method for nitrate is based on production of a nitroaromatic compound from benzene or related aromatic in the presence of concentrated acid. Nitrite may be derivatized in this way after conversion to nitrate. However, measurement of nitroaromatics by mass spectrometry is not ideal for routine analyses. Other methods other than mass spectrometry are available. These include diazotization (the Griess Reaction), a somewhat laborious colorimetric assay unless it is largely automated.

Probes

Although a number of probe systems are available to detect electrochemical oxidation of NO, they have not been widely used in the quantitative analysis of S-nitrosothiols.

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Chapter 2. The presence of thiols in RSNO samples and their effect on the stability of RSNO

2. The presence of thiols in RSNO and their effect on the stability of RSNO

2.1. Reversibility of S-nitrosation

2.1.1. Background

It is now clear that the decomposition of S-nitrosothiols in an aqueous buffer to give nitric oxide and the disulfide is brought about by Cu^{+}, which is generated by reduction of Cu^{2+} present in the aqueous buffer solution[1,2]. There is a question as to where the thiolate ion comes from. It transpires that the nitrosation of thiols is sufficiently reversible to allow, in many cases, the low thiol concentration present at equilibrium to reduce Cu^{2+} and bring about loss of nitric oxide from S-nitrosothiols.

Nitrosation of alcohols is less commonly encountered than C-nitrosation and N-nitrosation reactions and has been much less studied mechanistically. The obvious and by far the best known example is the formation of an alkyl nitrite from an alcohol and nitrous acid (Equation 2-1). The reaction is significantly reversible and the position of equilibrium is dependent upon the electronic and steric properties of the R group.

$$ROH + HNO_2 \rightleftharpoons RONO + H_2O$$

Equation 2-1

The synthesis of thionitrites (S-nitrosothiols) from thiols is probably the best-known example of S-nitrosation and is the exact sulfur counterpart of

the formation of alkyl nitrites from alcohol. With reactants containing both the thiol group and the amino group (e.g. cysteine), reaction occurs preferentially at sulfur and it is possible to suppress completely nitrosamine formation from a secondary amine in the presence of a sufficient excess of cysteine.

It was believed that the major difference between O- and S-nitrosation is that the former is significantly reversible whilst the latter is effectively quantitative. This is because oxygen is significantly more basic than sulfur, generally in organic molecules ($\Delta pK_a \approx 5$), so that the rate of the reverse reaction is greater for the oxygen case than for the sulfur case. The forward reaction is governed by the nucleophilicity (-SH > -OH), whereas the reverse reaction is governed by the basicity (-OH > -SH), because the first stage of the reverse process is protonation[3] (Equation 2-2).

$$RSH + XNO \rightleftharpoons RS^{+}(NO)H \rightleftharpoons RSNO + H^{+} + X^{-}$$

$$ROH + XNO \rightleftharpoons RO^{+}(NO)H \rightleftharpoons RONO + H^{+} + X^{-}$$

Equation 2-2

Nitrosation of alcohols is a well-known reversible process, and equilibrium constants have been determined for a number of alcohols. Some typical values are 3.5 and 1.2dm^3 mol^{-1} for methanol and ethanol respectively. A kinetic method for demonstrating this reversibility is by the presence of a positive intercept at [ROH] = 0, when the first-order rate constant (k_{obs}) is plotted against [ROH], when reactions are carried out with [ROH] >> [HNO_2]. Equilibrium

constants determined in this way[4] are in reasonable agreement with those obtained from direct measurements[5].

$$ROH + HNO_2 \rightleftharpoons RONO + H_2O$$

$$K = \frac{[RONO]}{[ROH][HNO2]}$$

Equation 2-3

Such plots for the nitrosation of thiols show no measurable intercept[6], so it was generally assumed that those reactions are effectively irreversible. However by working with nitrosothiol solutions generated under mildly acid conditions it was found that some thiol is still present at equilibrium (measured using the Ellman reagent) suggesting that the S-nitrosation is a somewhat reversible process[7]. These results provide an explanation as to why reduction of Cu^{2+} by thiolate ion occurs so readily in RSNO solutions where it would be expected that there would be no thiolate present.

$$RSH + HNO_2 \rightleftharpoons RSNO + H_2O$$

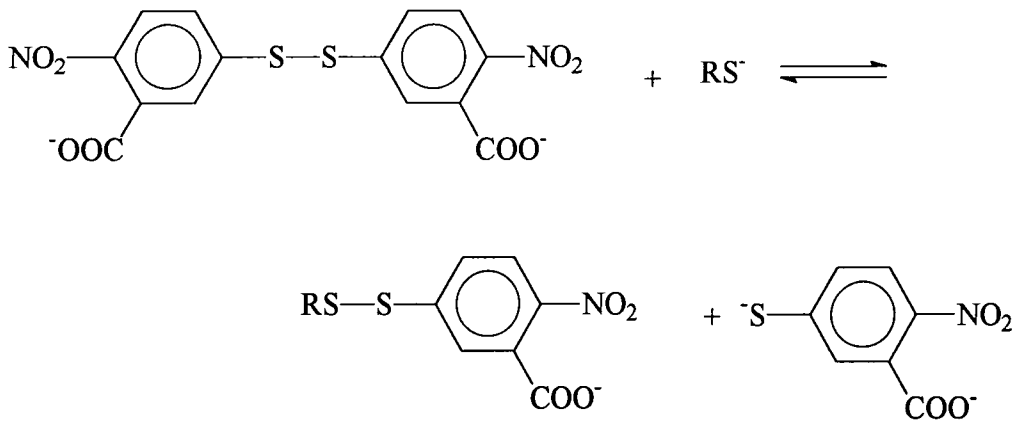
$$K = \frac{[RSNO]}{[RSH][HNO2]}$$

Equation 2-4

2.1.2. Experimental result and discussion

The percentage of RSH impurity was determined using Ellman reagent: 2,2'-Dinitro-5,5'-dithiobenzoic acid (DTNB). Ellman reagent is an aromatic

symmetric disulfide and reacts with a reduced thiolate ion to form a mixed disulfide plus one mole of 2-nitro-5-thiobenzoic acid (TNB^{2-}), which is quantified by its strong visible absorbance. DTNB has little if any absorbance, but when it react with-SH groups under mild alkaline conditions (pH 7-8), the TNB^{2-} gives an intense yellow colour at 412nm (Equation 2-5). There has been some controversy about the most correct extinction coefficient for TNB^{2-}. The original work by Ellman[8,9] cited a value of $13600M^{-1}cm^{-1}$. Later, values ranging from 11400 to 14150 $M^{-1}cm^{-1}$ had been reported for TNB^{2-}.



Equation 2-5

The Ellman procedure is sensitive to temperature, pH and various buffer ions, so the extinction coefficient must be matched to the reaction conditions[10]. Most experiments below were carried out in pH 7.4 phosphate buffer to relate to possible *in vivo* reaction. Calibration experiments revealed that we were able to measure thiol concentrations in the range of 8×10^{-6} to 5×10^{-5} mol dm^{-3} very readily and the extinction coefficient at 412nm we measured was

within 5% of the literature value. The calibration data for the nitrosation of penicillamine are given in Figure 2-1.

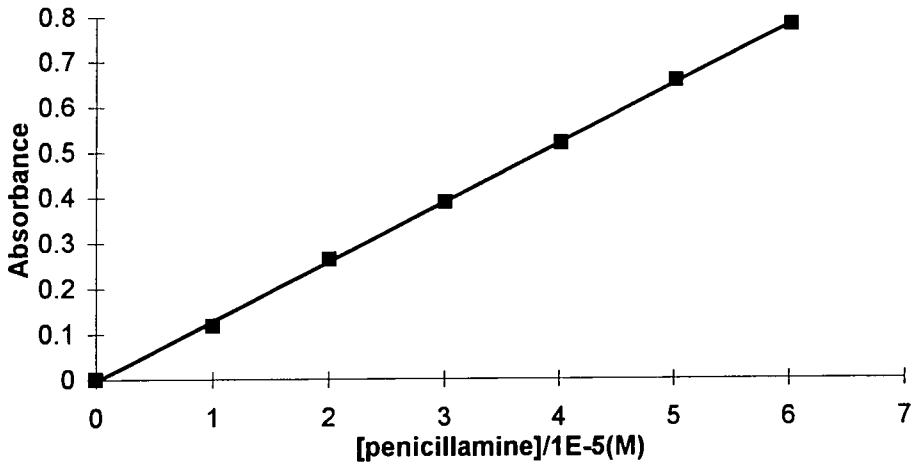


Figure 2-1 Extinction coefficient of TNB^{2-} (ε =13080M^{-1} cm^{-1}) generated from penicillamine at pH 7.4, 25^{0}C

In the study of nitrosation reversibility, all nitrosothiols solutions used were prepared *in situ* with a mildly acidic thiol solution (*ca.* 0.05 mol dm^{-3} H^+) mixed with a sodium nitrite solution. After nitrosation, addition to a pH 7.4 phosphate buffer solution was followed by the addition of the Ellman reagent. Results were obtained for the product of nitrosation of thiols (1.34×10^{-3}mol dm^{-3}), using stoichiometric ratios of [RSH]:[HNO_2] ranging from 1:1 to 1:2. The figures in Table 2-1 and Table 2-2 give approximate equilibrium constant in the range of 10^5-10^6 dm^3 mol^{-1} for a number of thiol nitrosations. Values of K of this magnitude would not lead to a measureable intercept for plots of k_{obs} against [thiolate], so the kinetic method is obviously not sufficiently sensitive to measure equilibrium constants of this magnitude[4]. The low concentration of thiol at

equilibrium makes it difficult to get more precise values of K, the following values are probably reliable to about ±10% of each value.

	[RSH]:[HNO_2]				
	1:1	1:1.1	1:1.2	1:1.5	1:2
Nitroso-acetylcysteine	5.1	4.4	4.2	3.8	3.2
Nitroso-glutathione	3.2	2.0	1.5	1.0	0.9
Nitroso-thiomalic acid	1.6	1.1	0.8	0.6	0.4
Nitroso-thioglycerol	2.2	1.7	1.4	1.2	1.1
nitroso-thioglycolic acid	3.4	2.8	2.2	1.8	1.2
Nitroso-penicillamine	1.4	1.0	0.6	0.4	0.2
Nitroso-cysteine	1.8	1.4	1.1	0.9	0.7

Table 2-1 The percentage of free thiol remaining at equilibrium in the S-nitrosation of thiol (1.34×10^{-3}M), with different [RSH]:[HNO_2] ratios, at 25^0C.

S-nitrosothiol	Free thiol (%)	K(dm^3mol^{-1})
Nitrosoacetylcysteine	5.1	2.7×10^5
Nitrosocysteine	1.8	2.4×10^5
Nitrosoglutathione	3.2	7.0×10^5
Nitrosothioglycerol	2.2	1.5×10^6
Nitrosothioglycolic acid	3.4	6.1×10^5
Nitrosothiomalic acid	1.6	3.0×10^6
Nitrosopenicillamine	1.4	3.8×10^6

Table 2-2. The percentage of free thiol present at equilibrium and values of equilibrium constants of the nitrosation reaction. ([RSNO] = 1.34×10^{-3} M, at 25 ^{0}C, 1:1 ratio of reactants is used)

All of these S-nitrosations are very rapid processes and low thiol concentration was found in all of these solutions. Generally the thiol concentration is quite significant for the 1:1 experiments, dropping as expected as we move to a 1:2 mixture. But the solutions of S-nitrosothiols generated in solution will always contain some free thiols. This will also apply to solutions of

nitrosothiols made up from the pure RSNO form in any mildly acidic solution, but not presumably to solutions made up in basic solution since the equilibrium process is acid catalysed. The result shown above provide an explanation as to why Cu^{2+} is reduced by thiolate ion so readily in solutions of a nitrosothiol where it would be expected that no thiolate existed. We also noticed that the thiol concentrations are higher for some 'stable' RSNO samples than the 'reactive' RSNO.

Discussion

The UV spectrum of DTNB alone was recorded, displaying a maximum absorption band at 320nm. The addition of RSH led to the formation of TNB^{2-}, with absorbance maximum at 412nm, which is comparatively stable. But when using Ellman reagent to determine the thiolate concentration in the RSNO samples, the yellow colour appears slowly and this is followed by colour bleaching (loss of absorbance at 412nm). So all the absorbance measurement made during the evaluation of the SH level in RSNO were recorded as a time drive course at 412nm and the highest absorbance reading was used to calculate the thiol concentration.

During the determination of the extinction coefficients of the thiols, the colour of TNB^{2-} fades also slowly due to autoxidation. This is delayed by the inclusion of metal chelator in the medium so EDTA was used in the thiol assay method. Thiols generally are not stable in neutral pH, but will be slowly oxidized to corresponding disulfides RSSR[11]; the reaction is catalysed by traces of metal

ions. Oxidation rates usually increase continuously with pH and temperature; EDTA removes traces of metal ions responsible for RSH oxidation.

Oxidation of the SH group by NO and a decrease of thiol concentration after S-nitrosation have been measured by Ellman reagent[12,13,14,15,16]. The Ellman reagent has also been used for the determination of thiol oxidation by peroxynitrite, a potent oxidant produced from the reaction of NO with superoxide[17]. Zhang and Means recently described a method[18] for the spectrophotometric determination of serum albumin nitrosation by assaying formation of S-nitrosothiols using the Ellman reagent.

Since TNB^{2-}, produced during the Ellman assay, is a thiolate anion, it was of interest to evaluate the possible interaction of NO with TNB^{2-} and establish whether this would influence the determination of thiols by the Ellman assay. The exact mechanism of interaction of TNB with NO is not clear. We suggest that a possible mechanism for the loss of absorbance of TNB at 412nm reflects the interaction of TNB with NO (presumably NO_x), resulting in the formation of an unstable intermediate followed by dimerization to reform the corresponding disulfide, DTNB.

The Ellman reagent is widely used as a sensitive assay for the quantification of thiol groups in tissues and proteins[19,20]. However the presence of NO donors during the determination of SH groups by the Ellman reagent can bleach the yellow colour of TNB[21]. The kinetics of NO interaction with TNB depends on the ability of the NO donors to release NO. The donors with a short half-life bleached the yellow colour of TNB faster than relatively stable RSNO,

which slowly releases NO. This raises the possibility that evaluation of SH groups by the Ellman reagent after S-nitrosation may be underestimated due to the bleaching of TNB. This can give an answer to why the low thiol concentration at equilibrium could not be determined more precisely.

2.1.3. Conclusion

The discovery of the existence of a certain amount of thiol in RSNO samples proved the hypothesis that S-nitrosation is a reversible process. It also helps us to understand the fact that reduction of Cu (II) ions happens readily in the RSNO solution. It is extremely difficult to separate a thiol from its S-nitrosated derivative due to the reversibility of S-nitrosation, and the effect of thiols on the decomposition of RSNO cannot be ignored. The existence of thiol in RSNO sample is crucial especially for the amino group containing thiols (sulfur containing amino acids), which have dual ability of reducing and complexing.

2.2. SNAC decomposition

2.2.1. Introduction

The decomposition of GSNO (nitrosoglutathione) has been widely studied because GSH (glutathione) is the most abundant thiol in the body, and plays many essential roles *in vivo*. The structure of N-acetylcysteine is similar to the central part of glutathione and acetylcysteine is used to produce intracellular glutathione *in vivo*[22]. Thus as a contrast to GSNO, decomposition of SNAC has been studied.

S-nitroso-N-acetylcysteine (SNAC) is easily made in solution by electrophilic nitrosation of N-acetylcysteine (NAC) in acid conditions. It is one of the most stable nitrosothiols in solution. SNAC made *in situ* is stable in pH7.4 phosphate buffer for hours and there is a very long induction time before the decomposition starts. For such a stable RSNO the oxygen dissolved in the solution makes the induction time even longer. In fact in the kinetic study carried in pH 7.4 phosphate buffer at 25^0C, the reaction time is observed to be increased in the absence of oxygen[23], and an explanation in terms of an O_2-promoted release of Cu^+ from a GSH-Cu^+ complex was proposed.

Most RSNO compounds are not stable, and will readily release nitric oxide in pH 7.4 phosphate buffer and result in disulfide (RSSR) formation. Decomposition of S-nitrosothiols in aqueous solution at pH 7.4 is brought about by Cu^+, which is in turn generated by reduction of Cu^{2+} by thiolate ion, or in principle by any other reducing agent[24] (Equation 2-6).

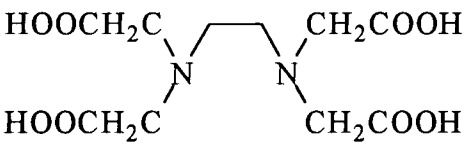
$$RS^- + Cu^{2+} \rightarrow 1/2\,RSSR + Cu^+$$

$$Cu^+ + RSNO \rightarrow RS^- + NO + Cu^{2+}$$

Equation 2-6

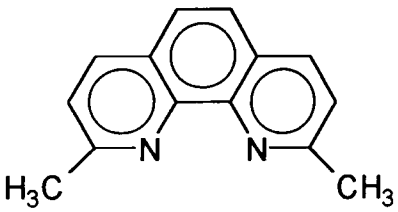
There is very large range of reactivity, which depends on structural differences. Acetylation of $-NH_2$ and esterification of $-COO^-$ groups reduces reactivity drastically[25]. For the very reactive RSNO compounds, Cu^{2+} reduction to Cu^+ can be the rate limiting step. This results in the observation of zero order kinetics i.e. linear absorbance-time plots[25].

EDTA (ethylenediaminetetra-acetic acid) can inhibit or stop RSNO decomposition, which is metal ion promoted. The equilibrium constant of EDTA for formation of the copper complex is well known (*ca.* 5×10^{18}). It has been often used to retard metal-ion catalysed pathways in the oxidation of thiols and has also been used to study the metal ion induced RSNO decomposition.

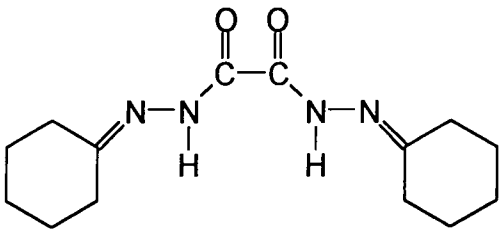


EDTA

A specific Cu^{+} chelator neocuproine (2,9-dimethyl-1,10-phenanthroline) was used to establish that Cu^{+} was responsible for the RSNO decomposition[26]. The RSNO decomposition is stopped in the presence of small amounts of neocuproine, whereas RSNO decomposition can not be stopped by biscyclohexanoneoxalyldihydrazone (cuprizone), a specific Cu^{2+} chelator added after the start of the reaction[26].



Neocuproine



Cuprizone

The mechanism of RSNO decomposition has been studied with reference to the possible therapeutic and pharmaceutical applications[26]. In most

cases addition of Cu^{2+} ions and reducing agents (thiolate ion) at very low concentration can accelerate RSNO decomposition whereas at high thiol concentration the RSNO decomposition can be slowed down. The addition of NAC to the copper ion induced SNAC solution will act similarly with a significant stablising effect at higher concentration showing strong complexing ability. N-Acetylcysteine (NAC) has received some attention as an antioxidant for components of cigarette smoke and for treatment of various respiratory diseases and there have been several reports suggesting that it is a cardioprotective agent[27,28]. These effects are thought to be due to its copper and other metal ion complexing properties, so the possible stabilising effect of NAC could be attributed to complexing of copper ions, e.g. NAC-Cu^{2+}(Cu^{+}).

2.2.2. Experimental Results and Discussion

The measurements on the decomposition of SNAC(1×10^{-3}M) were carried out in aqueous pH 7.4 buffer solutions at 25^{0}C, by monitoring the disappearance of the absorbance at 333nm. In the absence of added reducing agents and copper ion, SNAC is very stable and very little decomposition occurred even after many hours. When the [Cu^{2+}] was increased reaction did occur, but via an induction period which decreased with increasing [Cu^{2+}], a shown in Figure 2-1. When the experiments were repeated in the absence of oxygen, no induction periods were observed and decomposition followed a first-order process which was much more sensitive to the [Cu^{2+}], see Figure 2-3, when the added Cu^{2+} was varied from $1\text{-}4\times10^{-6}$M. It is likely here that oxygen reoxidises Cu^{+} to Cu^{2+} in a process that competes with the reaction of Cu^{+} with the nitrosothiol to generate NO. In the absence of oxygen, the measured first-

order rate constants (k_0) increase linearly with $[Cu^{2+}]$, (Figure 2-4) following Equation 2-7 the small positive intercept at $[Cu^{2+}] = 0$ is possibly due to the catalytic effect of Cu^{2+} impurity present in the water-buffer components.

$$\text{Rate} = k_o\,[\text{RSNO}]$$

$$k_o = k\,[Cu^{2+}]$$

Equation 2-7

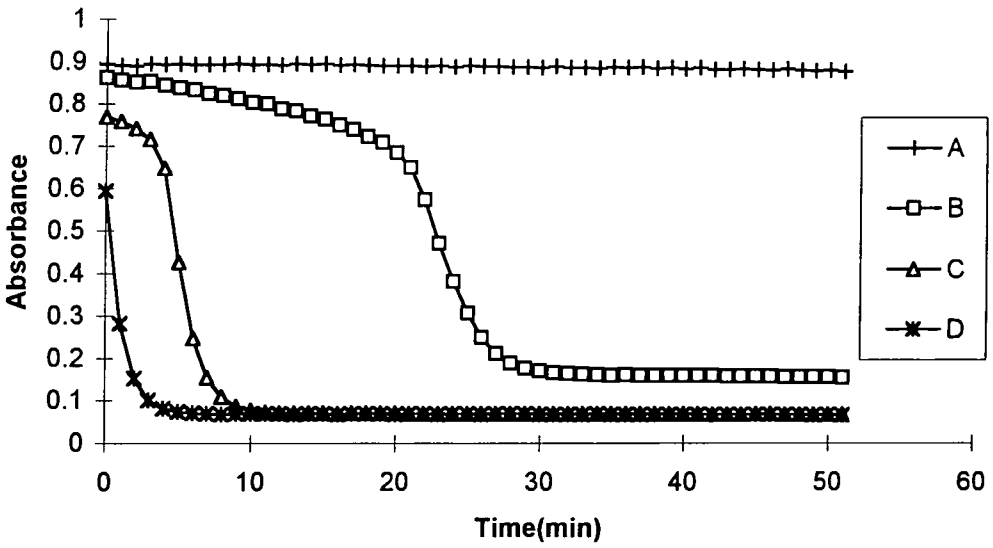


Figure 2-2 Effect of added Cu^{2+} on the decomposition of SNAC, in the presence of oxygen, [SNAC]=1×10^{-3}M.
A) $[Cu^{2+}] = 0$ B) $[Cu^{2+}] = 5\times10^{-6}$ M C) $[Cu^{2+}] = 1\times10^{-5}$ M
D) $[Cu^{2+}] = 1.5\times10^{-5}$ M

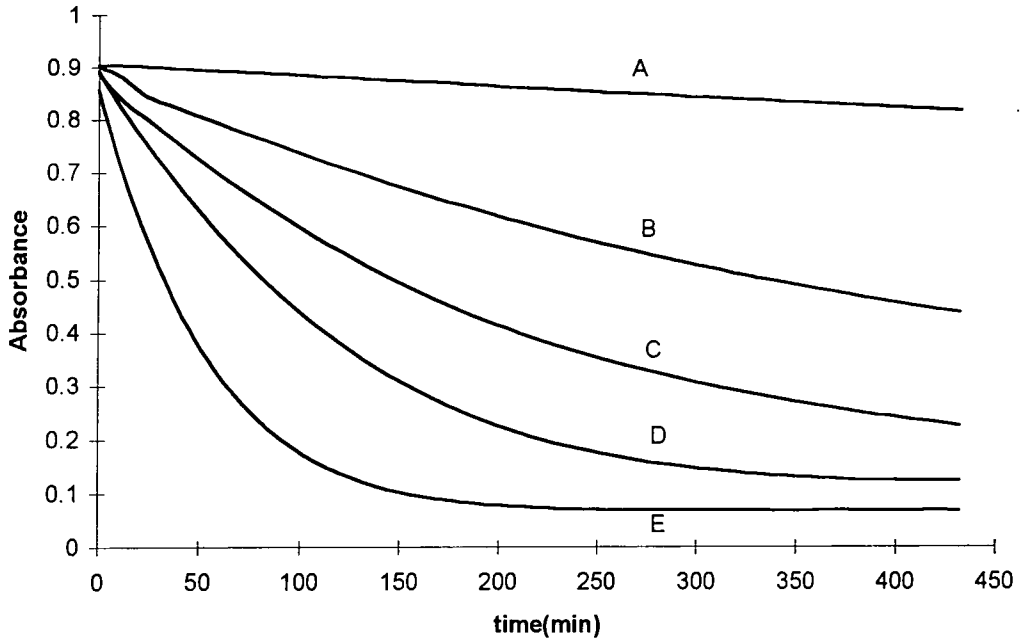


Figure 2-3 Effect of added Cu^{2+} on the decomposition of SNAC when oxygen has been carefully removed from the solution.
A) $[Cu^{2+}] = 0$ B) $[Cu^{2+}] = 1\times 10^{-6} M$ C) $[Cu^{2+}] = 2\times 10^{-6} M$
D) $[Cu^{2+}] = 3\times 10^{-6} M$ E) $[Cu^{2+}] = 4\times 10^{-6} M$

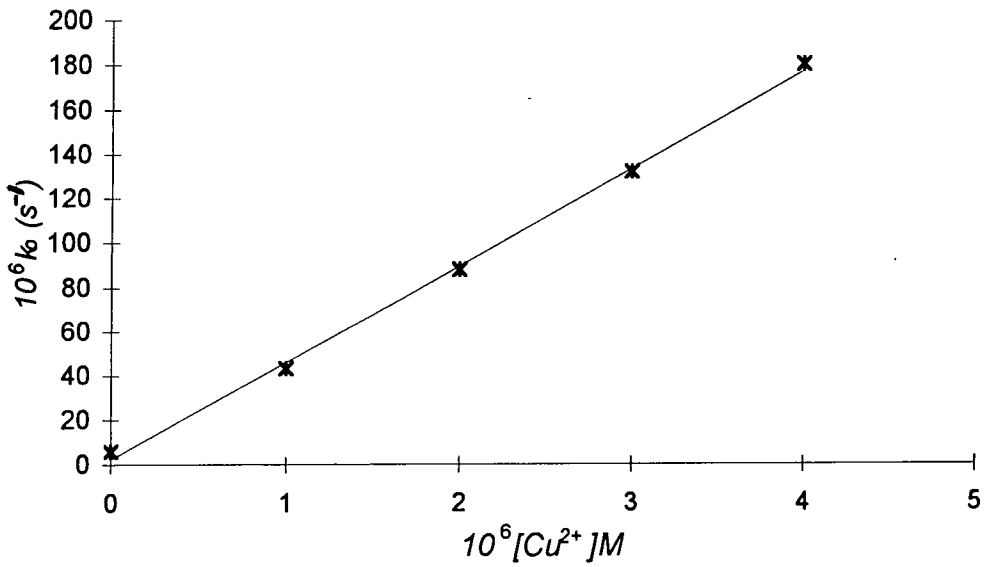


Figure 2-4 Values of k_0 for the reaction of SNAC in pH7.4 buffer at 25^{0}C

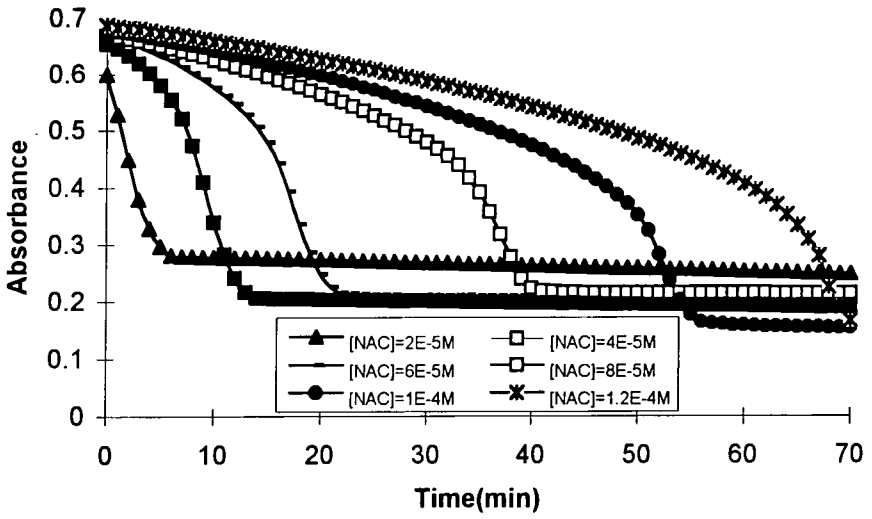


Figure 2-5. Decomposition of SNAC in the presence of added Cu^{2+} ($1 \times 10^{-5}M$) and NAC, [SNAC]=$8 \times 10^{-4}M$.

The effect of added N-acetylcysteine (NAC) varied considerably with the concentration. Addition of NAC at low concentration (2×10^{-5}M) speeds up decomposition significantly (see Figure 2-5), but increasing [NAC] further slows the reaction down and results in the appearance of long induction effects. A likely explanation is that when NAC is added at low concentration, it acts as a reducing agent, promoting the formation of Cu^{+} and allowing decomposition of SNAC to proceed. As [NAC] is increased however it appear that NAC acts as a complexing agent for Cu^{2+} (or Cu^{+}), with a different stoichiometry from that required for reduction of Cu^{2+}. When a reducing agent, without complexing ability, eg ascorbic acid is added, then, as shown in Figure 2-6 increasing the [ascorbic acid] promotes reaction (although there is some evidence of an induction period at low [ascorbic acid].)

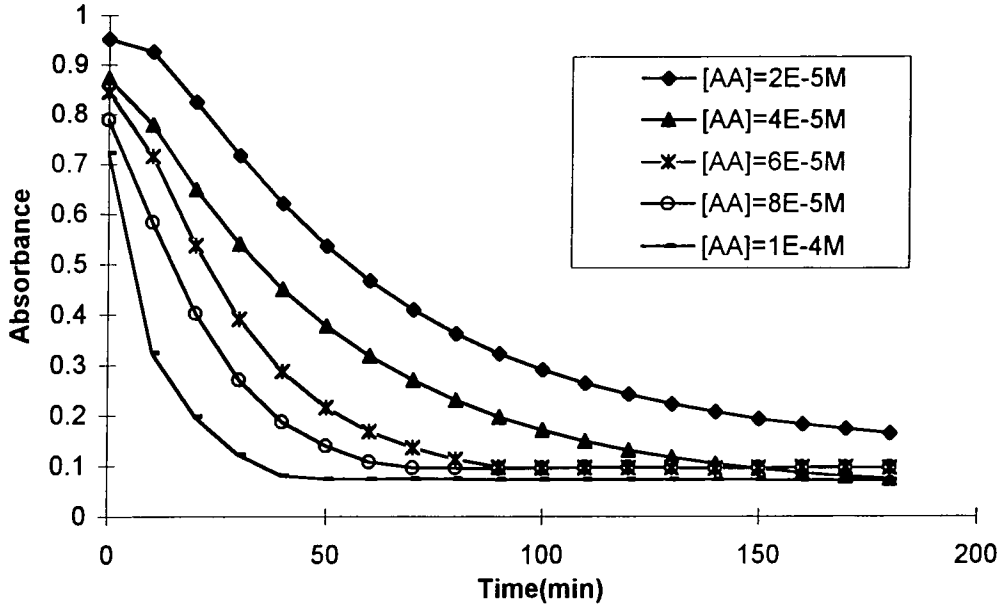


Figure 2-6 SNAC(1×10^{-3}M) decomposition in the presence of ascorbic acid and small amount of Cu^{2+} (5×10^{-6} mol dm^{-3}). ([AA]=[ascorbic acid])

When a different thiol (e.g. cysteine) is added then this also promotes reaction (see Figure 2-7) probably involving initially a transnitrosation process (Equation 2-8), which has been well documented[29], followed by a copper catalysed decomposition of S-nitrosocysteine. In this case with naturally occurring free Cu^{2+}, at low added thiol there is catalysis by the thiol as this favours the reduction of copper

$$\text{SNAC} + \text{Cysteine} \rightleftharpoons \text{NAC} + \text{S-Nitrosocysteine}$$

Equation 2-8

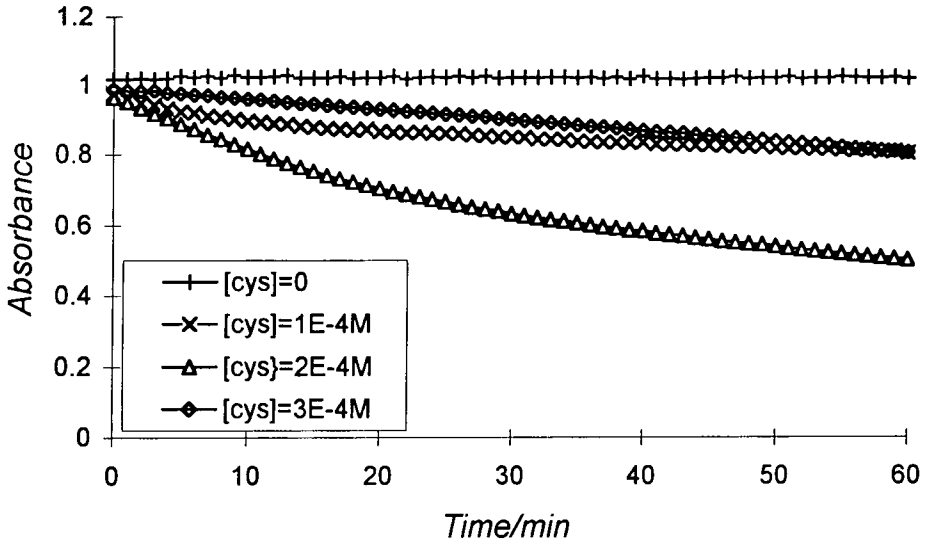


Figure 2-7 The effect of cysteine on the decomposition rate of SNAC, pH 7.4, 25^{0}C, no added copper ion.

The whole area is a very complicated one where added thiols can act as reducing agents and also as complexing agents for Cu^{2+} or Cu^{+}. We do have some spectral evidence, which shows the complexing properties of some thiols (in the next section). A further reaction occurs with thiols (e.g. NAC) and nitrosothiols (e.g. SNAC) at much higher [NAC], which is independent of added Cu^{2+}, added EDTA or added neocuproine, which will be discussed in more detail in Chapter 3.

2.2.3. Some studies on the thiol-copper ion complex

There is a large literature regarding the structure of thiol-copper ion (Cu^{+} and Cu^{2+}) complexes[30,31,32,33]. The reduction of Cu^{2+} by thiolate ion has been examined, using EPR techniques and UV measurements, by a number of worker, notably Cavallini and co-workers[34], and more recently Gilbert and co-workers[35]. Evidence was presented in favour of a 2:1 thiol:Cu^{2+} complex for the complex

with penicillamine. Similar structures were proposed for corresponding complexes with cysteine and mercaptoethanoic acid. The structure of a simple thiol copper complex has been suggested to be of a 2:1 thiol:Cu^{2+} complex and chelated through N and S donation as in the following structure (Figure 2-8).

Figure 2-8

Beloso et al.[36] had suggested that the dithiolate complex is formed reversibly (Equation 2-9), and that Cu^{+} is formed in a parallel reaction of Cu^{2+} with one thiolate ion (Equation 2-10). If this is true then the reaction profile will depend on [RS^{-}]; at high [RS^{-}], complexation will be more dominant, and the rate of Cu^{+} formation will be much reduced, whereas at low [RS^{-}], formation of Cu^{+} will be more favoured since complexation of Cu^{2+} will be less extensive. This then explains at least qualitatively the experimental absorbance-time profiles, which can not be explained in terms of the complex being an intermediate in the reduction of Cu^{2+} to Cu^{+}.

$$Cu^{2+} + 2RS^{-} \rightleftharpoons \text{Dithiolate complex}$$

Equation 2-9

$$Cu^{2+} + RS^{-} \rightarrow Cu^{+} + 1/2RSSR$$

Equation 2-10

We have demonstrated the complexing of copper ion and the formation of a Cu^{+}-NAC by a series of experiments. The UV spectrum from the mixtures of NAC and Cu^{2+} has a maximum absorbance at around 300 nm, which is believed to be a Cu^{2+} or Cu^{+} complex. This complex is not stable and decomposes slowly (Figure 2-9) and the maximum absorbance wavelength is not in the region of 333 nm, which is different from the penicillamine and other RSH-Cu^{2+} complexes[36]. The existence of a Cu^{+}-NAC complex can also be demonstrated by mixing the thiol solution with a quite large amount of cuprous chloride (Cu_2Cl_2) in a ratio of [NAC]:[Cu_2Cl_2] = 2:1 at pH 7.4. Cuprous chloride is very easily oxidised in the air, and special care must be taken to avoid the oxidation. Since Cu_2Cl_2 has a very low solubility in H_2O (0.0062g/100ml)[37] and no precipitate was present in the tube an assumption can be made that all the Cu^{+} present is bound to NAC. Cu_2Cl_2 can also be dissolved in solutions of glutathione, acetylpenicillamine and captopril under same conditions suggesting similar cuprous complexing. The structure of this complex could be determined by methods such as ESR or NMR, but we did not carry out any further structural studies.

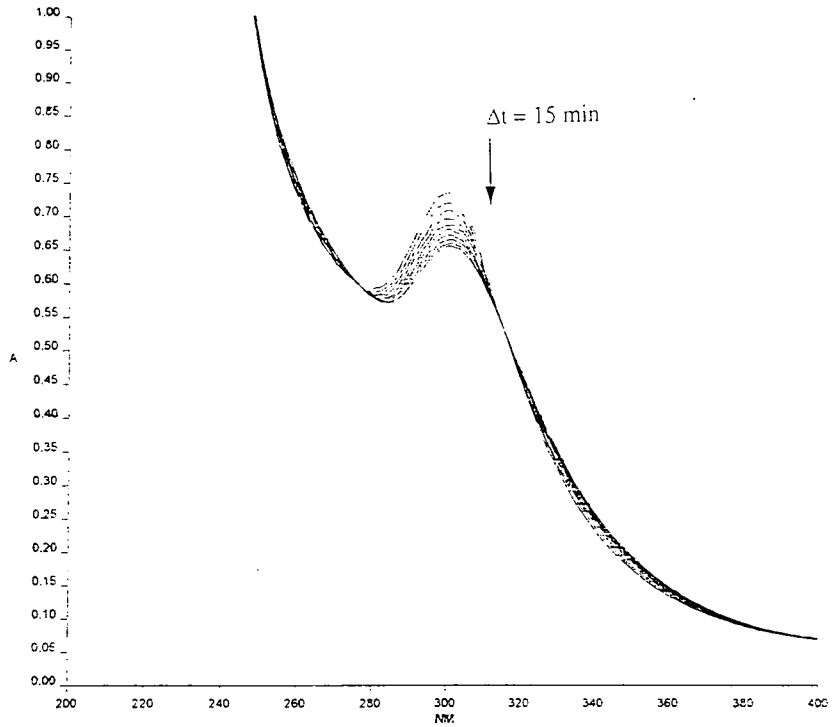


Figure 2-9 The UV spectra of the NAC-Cu^{2+}/Cu^{+}, absorbance at 300 nm

The formation of Cu^{+} from the Cu^{2+}-NAC dithiolate complex was demonstrated by the addition of the specific Cu^{+} chelator neocuproine to a solution of the complex at pH 7.4. A fairly rapid build-up of the absorbance at 453 nm occors, characteristic of the Cu^{+} complex with neocuproine; the stopped-flow technique was used to detect the formation process.

Neocuproine (NC) is a specific Cu^{+} chelator and has been used to determine trace amount of copper ion[38]. Its specific chelating effect is the result of combined use of reducing conditions the strong binding of Cu^{+} to nitrogen donors plus the demand for tetrahedral geometry by the Cu^{+} ion[39] (Figure 2-10). This is achieved by steric effects of the 2,9 dimethyl substituents, which force the complex out of a planar geometry, making it a stronger oxidant than the 1,10-phenanthroline analogue[40,41,42].

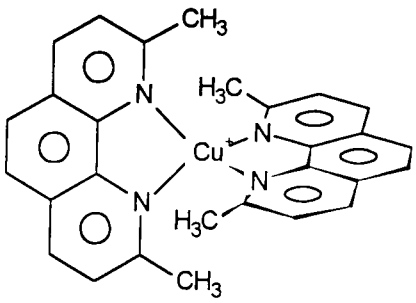


Figure 2-10 structure of the Cu^+-Neucuproine complex

NC has been used to detect Cu^+ produced by thiols and other reducing agents[43,44]. The NC-Cu^+ complex has UV-Visible maximum absorbance at 456 nm, the extinction coefficient[45] is about 7.5×10^3 mol dm^{-3} cm^{-1}. Mixing Cu^{2+} and neocuproine gives no yellow colour, but after adding thiol an intense yellow colour appears almost immediately due to the reduction of Cu^{2+} to Cu^+ and the fast formation of the Cu^+-NC complex. The reduction of copper ion and the formation of this Cu^+-NC complex are both very fast processes and can be followed by the stopped-flow spectrophotometry method; the rate constants are shown in Table 2-3.

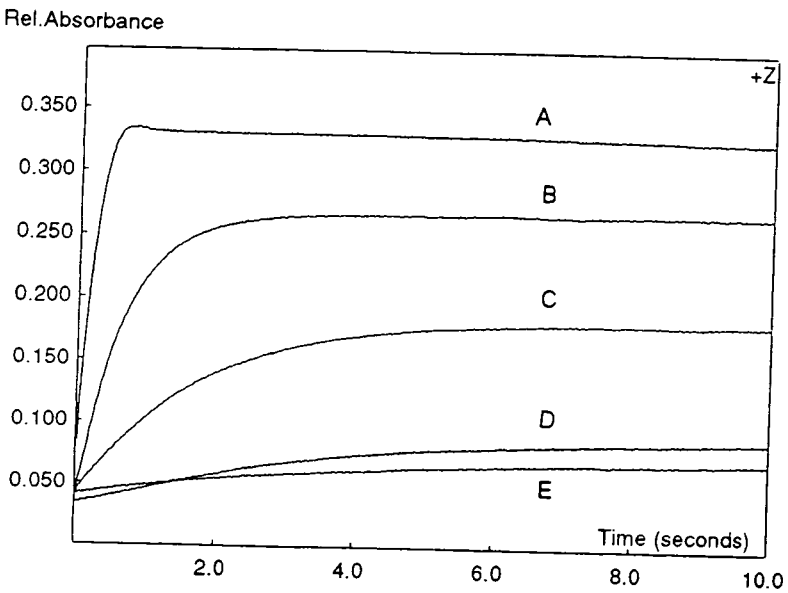


Figure 2-11. Cu^+ formation observed by neocuproine , [RSH]: $[Cu^{2+}]$=100:1 $[RSH]=5\times10^{-3}M$, $[Cu^{2+}]=5\times10^{-4}M$, $[NC]=1\times10^{-4}M$
A) ascorbic acid B) penicillamine C) cysteine D) GSH E) NAC

Reductant	K_{obs} (s^{-1})
Glutathione	0.316
Cysteine	0.915
Penicillamine	1.560
Acetyl-cysteine	0.221
Ascorbic acid	1.780

Table 2-3 The rate constant for the formation of Cu^{+}

The extinction coefficient of the NC-Cu^{+} complex generated by different reducing agents was measured by using an excess of the methanol solution of NC. The absorbances for the eight different reducing agents were measured within the concentration range in which Beer's law is obeyed (Table 2-4). The average values of the molar extinction coefficient and the standard deviations were obtained, giving an overall mean value of $(7.7\pm0.3)\times10^{3}$ dm^{3} mol^{-1} cm^{-1}. Under these condition when neocuproine is in excess and concentration of Cu^{2+} and recucing agent are equal the extinction coefficient for the eight different thiols are similar and didn't show any significant difference. However when the thiol is in excess of the Cu^{2+} centration, the absorbance values are decreased to a greater or lesser extent.

Reductant	ε/ dm^{3} mol^{-1} cm^{-1}
ascorbic Acid	$(7.80\pm0.35)\times10^{3}$
Penicillamine	$(7.55\pm0.29)\times10^{3}$
Cysteine	$(7.56\pm0.18)\times10^{3}$
acetyl-cysteine	$(7.73\pm0.34)\times10^{3}$
Glutathione	$(7.82\pm0.41)\times10^{3}$
Cysteamine	$(7.69\pm0.23)\times10^{3}$
Captopril	$(7.61\pm0.27)\times10^{3}$
Acetyl-penicillamine	$(7.65\pm0.36)\times10^{3}$

Table 2-4. Extinction coefficient of Cu^{+}-NC produced by different thiols

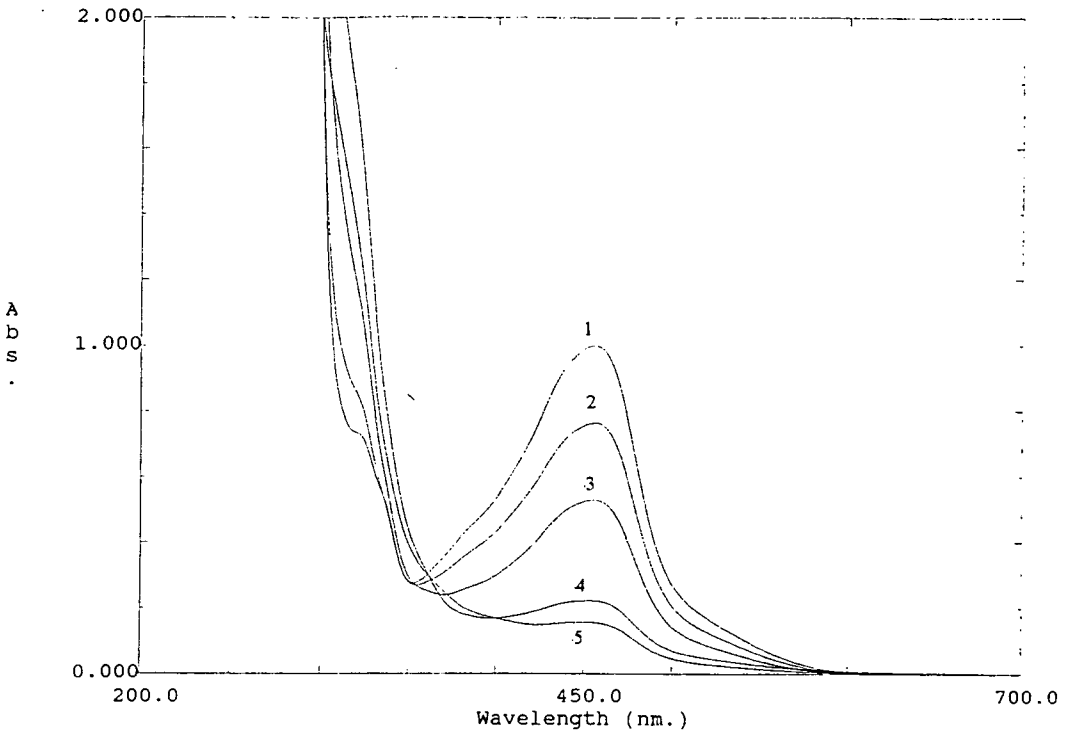


Figure 2-12. UV spectra for NC-Cu^{2+} at 456 nm with different thiols, when the thiol is in excess (thiol:Cu^{2+}=100:1) [RSH]=1×10^{-3}M, [Cu^{2+}]=1×10^{-4}M,
1) ascorbic acid 2) penicillamine 3) cysteine
4) GSH 5) NAC

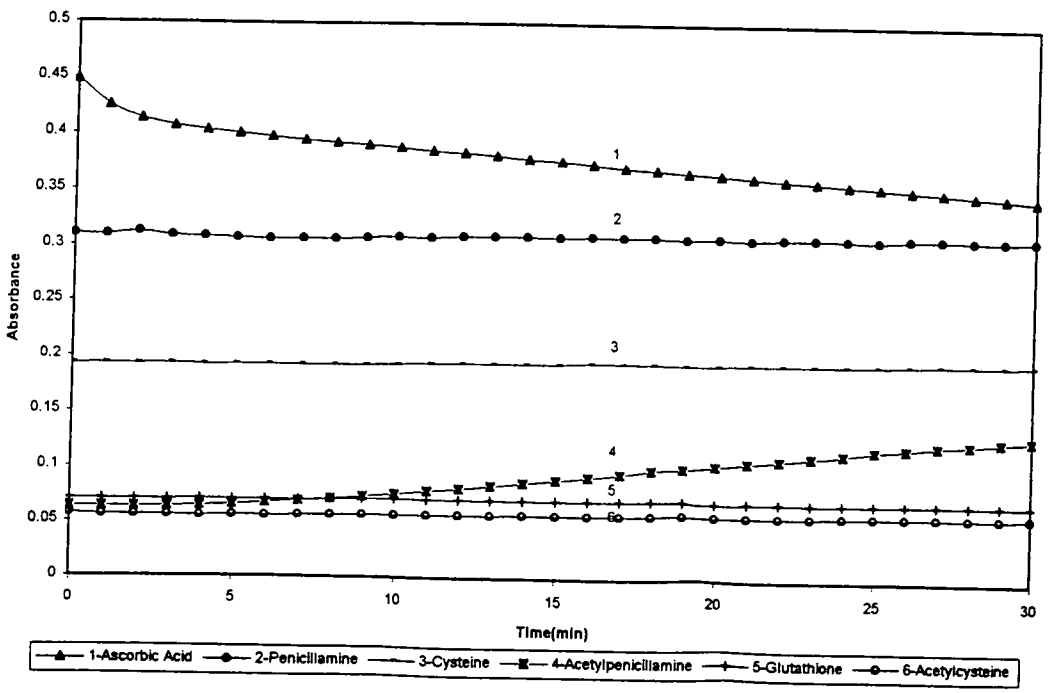


Figure 2-13. The stability of NC-Cu^{+} complex produced by different thiols. [RSH]=5×10^{-4}M, [Cu^{2+}]=5×10^{-5}M.

Clearly Cu^+ is not generated quantitatively in each case and this shows that the thiol is complexing Cu^{2+} making it less available for reduction and formation of the NC-complex at 456 nm. With equal concentration of Cu^{2+} and thiol, the Cu^+ concentrations generated, as measured at 456 nm, are similar and are comparable with that developed from ascorbic acid. When the ratio of RSH:Cu^{2+} is 100 the Cu^+ concentration produced by different thiols are not the same. We found that penicillamine produces quite high [Cu^+]. Even at this concentration GSH and NAC produce very little free Cu^+ initially, but gradually release more and acetylpenicillamine(NAP)-Cu^+ is less stable and releases Cu^+ slowly.

The experimental result have confirmed the hypothesis suggested by Beloso et al[36], which was shown in the Equation 2-9 and Equation 2-10. The decomposition of RSNO in the presence of copper and the way in which several compounds affect the reaction are summarised in Figure 2-14 Cu^+ induces the effective homolytic cleavage of RSNO. At low concentration of thiol, it acts as a reducing agent and stimulates the reaction leading to RSNO decomposition. At higher concentrations, thiols inhibit the overall reaction by formation of the Cu^+ complex. The oxygen effect is clearly important with RSNO for capture of Cu^+ Figure 2-14 was suggested by Gorren et al[14], and includes the additional possibility of regeneration of Cu^+ from a Cu^+-thiol complex.

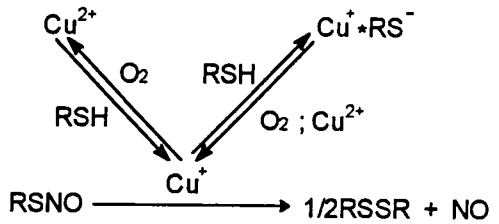


Figure 2-14. Summary of the effects of Cu^{2+} ions, thiolate and oxygen on the decomposition of RSNO.

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Chapter 3. The reaction of SNAC with NAC and ascorbate at high concentration

3. The reaction of SNAC with NAC and ascorbate at high concentration

3.1. The reaction of SNAC with NAC

3.1.1. Background

The release of NO from RSNO is catalysed by cuprous ion. However copper induced reactions in normal plasma appear to be improbable, because the availability of free transition metals is virtually zero[1], although Cu^{2+} bound to protein is accessible for reduction by RS^-. S-Nitroso-albumin is present in micromolar concentrations in the plasma of normal subjects[2] and S-nitroso-glutathione is present in the alveolar lining fluid at 0.2-0.5 micromolar[3]. Since RSNO concentration is less than micromolar, it follows that the concentration of RSNO will always be small. It has been found that low molecular weight thiols such as L-cysteine can strongly enhance the vascular sensitivity of RSNO. These facts suggest that a thiol may control S-nitrosothiol metabolism *in vivo*, possibly through a transnitrosation reaction and resultant decomposition of nitrosocysteine.

Transnitrosation from RSNO to thiols has been reported on a number of occasions[4,5], where the final isolated products are the products of decomposition of nitrosothiols, the disulfide. In view of the potential importance of NO storage and transfer *in vivo*, some study has been carried on to include a large range of thiol and nitrosothiol species[6,7]. The overall reaction is shown in Equation 3-1, with R'SH in large excess over RSNO the reaction is effectively driven in one direction. The reactive species is $R'S^-$ which attacks the nitroso nitrogen-atom.

$$RSNO + R'S^- \rightarrow RSSR + RSSR' + R'SSR' + NO$$

Equation 3-1

However, more recently research has shown that different products have been found at high thiol concentration, indicating a different reaction pathway [11]. The reaction between SNAC and NAC at higher concentration has been studied to understand the pathway of NAC induced SNAC decomposition. Under these conditions the reaction is quite different, as a new product, ammonia, has been found. These reactions do not involve copper ion catalysis, because under the conditions, where the thiol concentration is quite high, these metal ions will be fully complexed. This is shown to be true by working with the chelating agent EDTA, which hardly has any effect on the reaction rate.

3.1.2. Experimental Results

The reaction of SNAC (freshly made in solution via the nitrosation of NAC by acidic sodium nitrite) and NAC was observed in pH 7.4 phosphate buffer at 25^0C by recording the absorbance at 333 nm as a function of time. When incubation of SNAC (1 mM) with NAC was carried out at various concentrations (10-20 mM) in pH 7.4 phosphate buffer, good first order behaviour was observed (Figure 3-1) and the rate constants depended on the concentration of NAC when [RSH]>>[RSNO]$_0$. A plot of the first-order rate constant (k_0) *vs* [NAC] was an excellent straight line (Figure 3-2) and from the slope of the line, value of the second-order rate constant k_2 (defined by Equation 3-2) was obtained. The value for k_2 at pH 7.4 is 8.0×10^{-3} mol^{-1} dm^3 s^{-1}. The

reaction is quite general[8] for a range of other RSNO and the rate constants are independent of both added Cu^{2+} and EDTA. The contrast with the effect of metal ions when the reaction is carried out at low thiol concentration is very marked.

$$RATE = k_2 [SNAC] [NAC]$$

$$k_0 = k_2 [NAC]$$

Equation 3-2

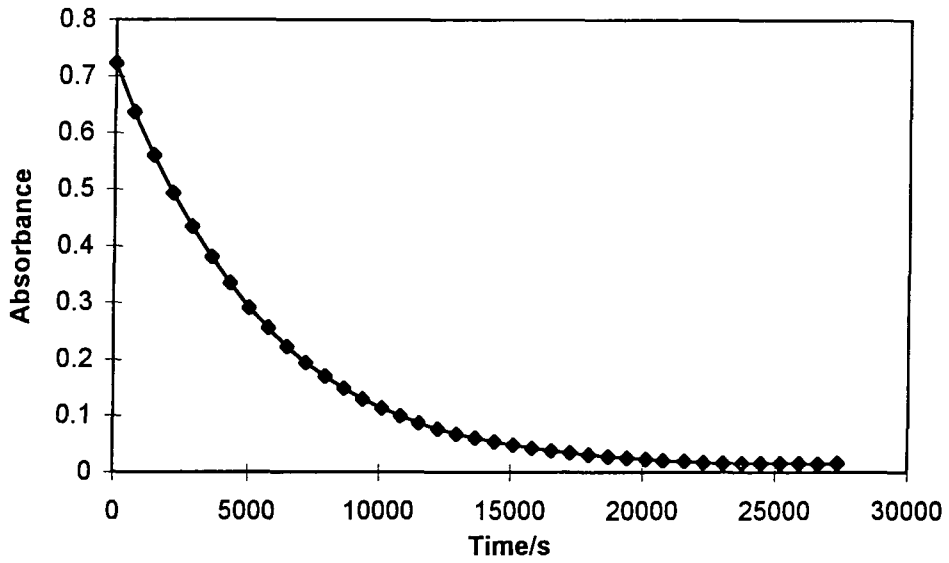


Figure 3-1 .The reaction of SNAC (1 mM) with NAC (20 mM) in pH 7.4 phosphate buffer solution, at 25^{0}C

[NAC]/mM	$10^4 k_{obs}$ (s^{-1})
12.5	1.04
15	1.26
17.5	1.49
20	1.65
22.5	1.88
25	2.03

Table 3-1 Rate data for the reaction of SNAC (1 mM) with NAC in pH 7.4 phosphate buffer solution, 25 ^{0}C

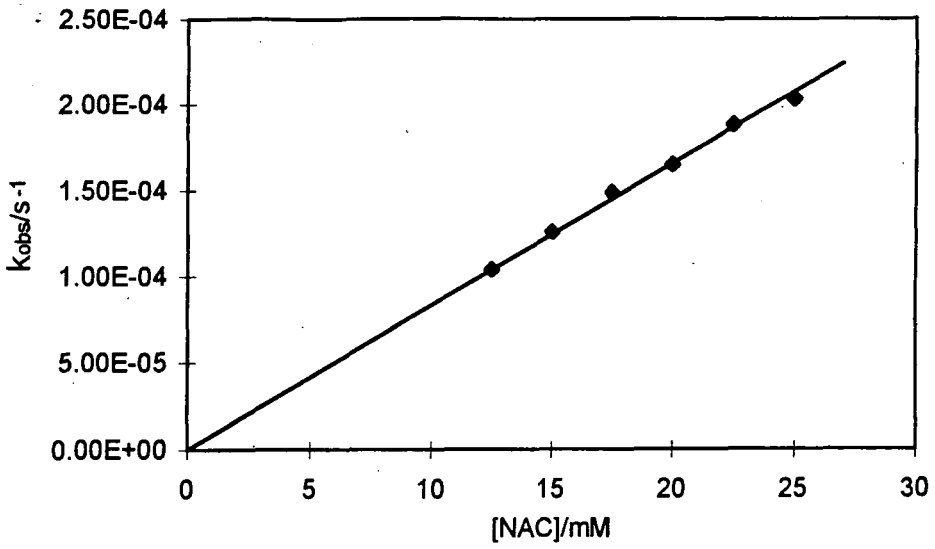


Figure 3-2 k_{obs} for the reaction of SNAC (1 mM) with NAC (12.5 mM-25 mM) in pH 7.4 phosphate buffer at 25^{0}C.

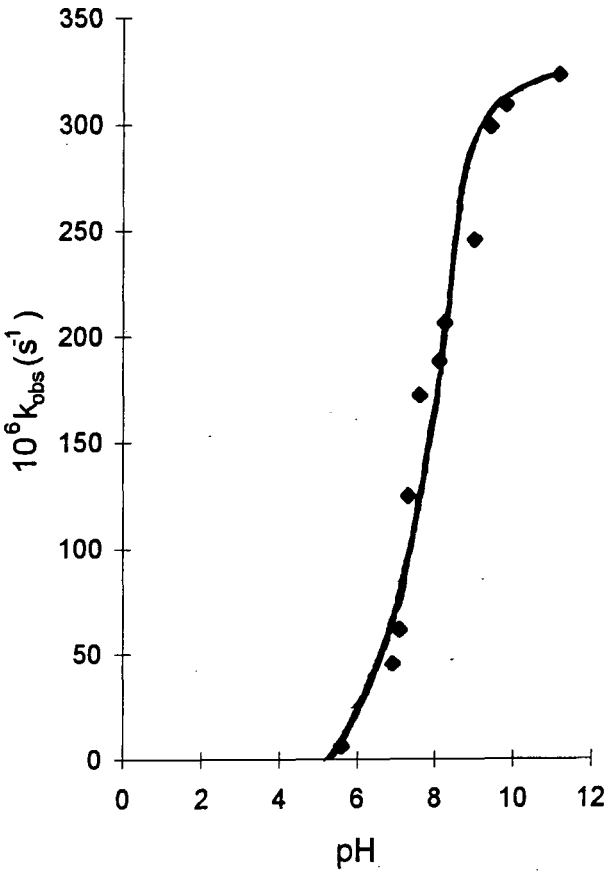


Figure 3-3 The plot of k_{obs} vs pH for the reaction of NAC and SNAC ([SNAC]=1 mM, [NAC]=25 mM)

The reaction rates are markedly dependent upon the pH of the reaction medium. The pK_a of NAC is about 9.76[9], the Figure 3-3 show the pH dependence of the reaction. A plot of k_{obs} *vs* pH is the usual sigmoid curve which indication that the thiolate anion is the reactive species.

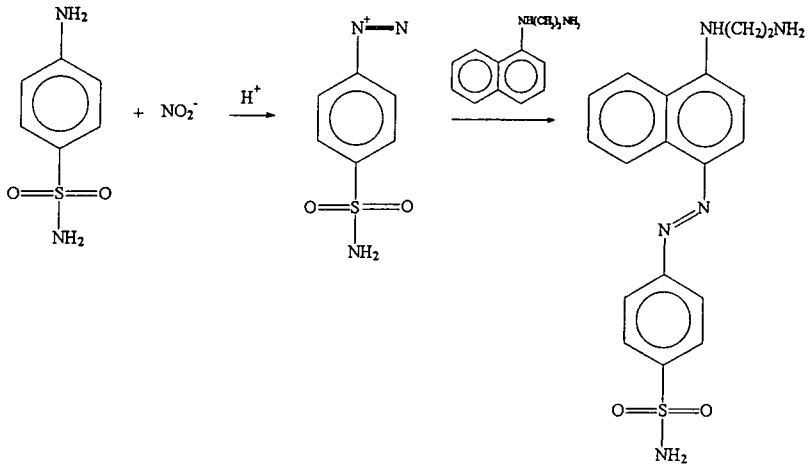
Product Analysis

Incubation of SNAC with NAC at various concentrations in phosphate buffer (pH 7.4) gives nitrite and ammonia as the main products. All experiments were carried out in the presence of EDTA (1×10^{-4}mol dm^3). But literature work with GSNO and GSH has shown that small amounts of N_2O are also formed, together with the disulfide GSSG[11]. Nitrite anion released from the reaction of SNAC and NAC initially as NO has been quantitatively detected using a diazotisation and azo coupling method known as the Griess test. Nitrite was determined as a function of added NAC. When copper ion is used to catalyze SNAC decomposition (in the absence of RSH) the nitrite yield is almost quantitative. However, as the concentration of NAC is increased, the nitrite yield drops sharply. Following the finding by Singh et al [11] that ammonia was a major product in the reaction of GSNO with GSH, we analyzed the reaction products for ammonia. The ammonia is formed at the expense of nitrite ion and the reaction leading to ammonia is dominant when the concentration of NAC is greater than 22.5 mM.

Griess Test (Nitrite)

Sulfanilamide is diazotised via the nitrite ion in an acidic medium (0.4 M HCl) and coupled to N-(1-naphthyl)-ethylenediamine to form a purple dye

which has an absorption maximum at 540 nm (Equation 3-3). Using authentic $NaNO_2$ the extinction coefficient at this wavelength was found to be 45900 ± 400 $mol^{-1} dm^3 cm^{-1}$.



Equation 3-3

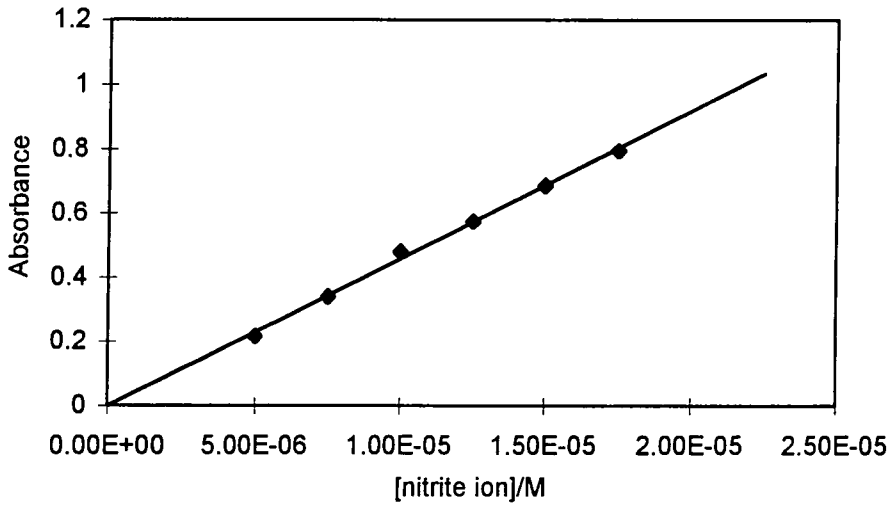


Figure 3-4 Extinction coefficient of Griess Test

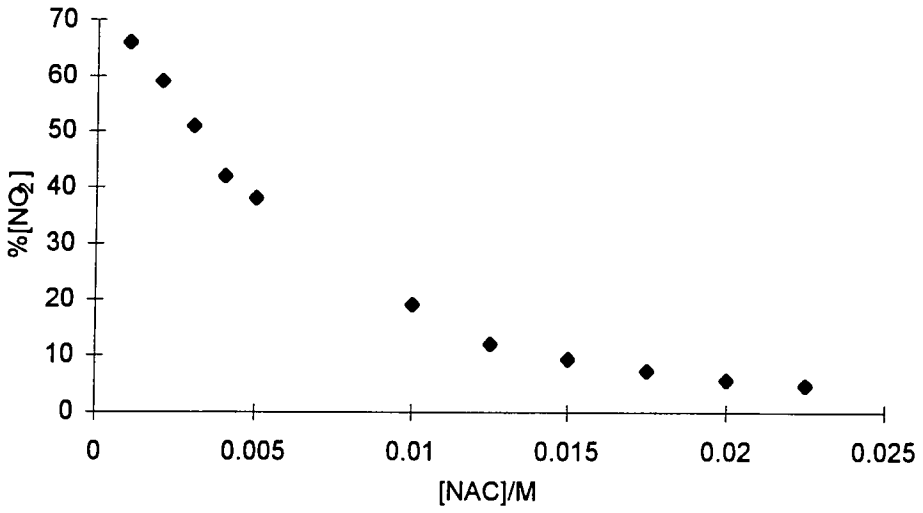


Figure 3-5 Griess test results for the reaction of SNAC (1 mM) and NAC (1-25 mM) in pH 7.4 phosphate buffer at 25^{0}C

Ammonia Test:

The concentrations of NH_3 were measured with a commercial ammonia diagnostic kit (Sigma) using the method of van Anken Shiporst [10]. The assay method (Equation 3-4) is based on reductive amination of 2-oxoglutarate, using glutamate dehydrogenase (GLDH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The decrease in absorbance at 340 nm due to the oxidation of NADPH is proportional to the ammonia concentration.

$$\text{Oxoglutarate} + NH_3 + \text{NADPH} \xrightarrow{GLDH} \text{Glutamate} + \text{NADP}$$

Equation 3-4

The decomposition rate is increased in the presence of added thiol. However the release of nitric oxide measured as nitrite anion in the presence of excess NAC is decreased whereas the release of NH_3 is increased. The major product in the presence of excess NAC is not NO, as had been assumed but instead, NH_3. And it was reported by Singh et al[11] that small yields of N_2O under

anaerobic conditions are inversely dependent on the NAC concentration, a trend similar to that noted for nitrite.

[NAC] /M	$[NH_3]$%
0.01	40.6
0.0125	45.6
0.0150	49.9
0.0175	59.2
0.0200	65.2
0.0225	77.1

Table 3-2 Ammonia test for the reaction of SNAC (1 mM) with NAC (10-22.5 mM)

3.1.3. Discussion

We have confirmed the observation by Singh et al.[11] that at reasonably high thiol concentrations there is a reaction between thiols and nitrosothiols (observed also kinetically by Komiyama and Fujimori[12]), leading to disulfide formation, which is not catalyzed by Cu^+ or any other metal ion. The reactions are first order in each reactant and lead principally to the formation of ammonia and not nitric oxide. This behaviour is in marked contrast with the behaviour at low concentrations of thiol (either added or present in equilibrium concentrations from the preparation of the RSNO from nitrous acid and thiols), when the addition of EDTA or the specific Cu^+ chelator neocuproine completely suppresses reaction. Our experiment results are in reasonable agreement with those found by Singh et al [11].

The ammonia-forming reaction is clearly a thiolate-promoted reaction at the nitroso group, of which there are a number of other known examples and

where the final product is often an amine[13]. The scheme presented in Figure 3-6 summaries the reactions suggested by Singh et al [11] and a reasonable sequence of reactions, based on the above suggestion, which are compatible with our findings, are given. Presumably the rate limiting step is the first reaction of RS^- at the nitrogen atom of the S-nitrosothiol (Equation 3-5). Followed by other reactions of RSH at the sulfur atom and various proton transfers, leading to the formation of hydroxylamine, which would be expected to undergo thiolate reduction to give ammonia (Equation 3-5 to Equation 3-11).

$$RSNO + RSH = RS\text{-}N(OH)\text{-}SR \qquad \textit{Equation 3-5}$$

$$RS(NO^-)SR + H_2O = RS(NOH)SR + OH^- \qquad \textit{Equation 3-6}$$

$$RS(NOH)SR + RS^- = RSSR + RSN^-OH \qquad \textit{Equation 3-7}$$

$$RSN^-OH + H_2O = RSNHOH + OH^- \qquad \textit{Equation 3-8}$$

$$RSNHOH + RS^- = RSSR + {}^-NHOH \qquad \textit{Equation 3-9}$$

$${}^-NHOH + H_2O = NH_2OH + OH^- \qquad \textit{Equation 3-10}$$

$$NH_2OH + RS^- \rightarrow NH_3 \qquad \textit{Equation 3-11}$$

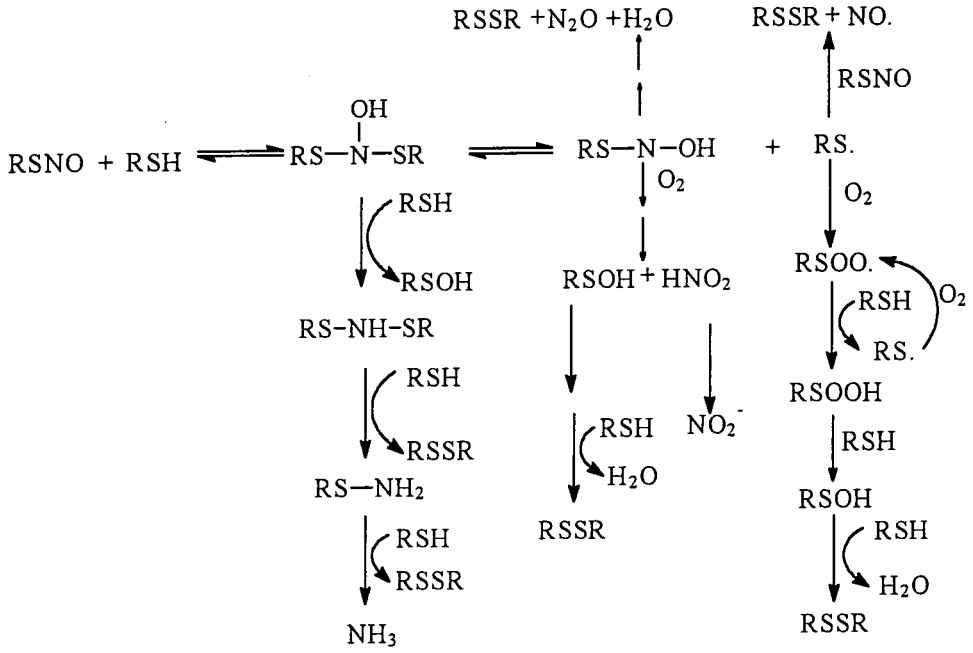


Figure 3-6. The reaction of RSNO with RSH suggested by Singh et al.[11]

According to the reaction scheme suggested by Singh. et al[11] RSH reacts with RSNO to form the RSH conjugate N-hydroxysulfenamide (RS-N(OH)-SR). The N-hydroxysulfenamide can react via different pathways depending on the availability of RSH and oxygen. Reaction of N-hydroxysulfenamide with RSH results in the sulfenamide (RS-NH-SR). Subsequent reductions with RSH result in the formation of NH_3. Homolytic cleavage of the S-N bond of N-hydroxysulfenamide would generate the thiol radical and the N-hydroxyl radical which can dimerize to the unstable dihydroxyhydrazine and subsequently form hyponitrous acid (HO-N=N-OH). The RS· radicals can dimerize to form RSSR.

The RS· radical could in principle also react with RSNO to form NO and RSSR, this would explain the faster rate of formation of NO from RSNO in the presence of RSH. The ultimate fate of NO in aerated phosphate buffer at pH

7.4 is nitrite ion but little or no nitrate is formed, consistent with our observations. The decrease in nitrite formation, and increase in NH_3, with increasing RSH concentrations, reflect the increased reduction of the N-hydroxysulfenamide in the presence of excess RSH leading to increased formation of NH_3.

At low [RSH], the above sequence of reactions is in competition with the copper ion promoted reaction that gives NO, but as [RSH] increases the ammonia reaction soon dominates, and at >25mM NAC, this pathway accounts for ~80% of the total for the reaction of SNAC. The effect of oxygen on the reaction has not been studied in the reactions of SNAC and NAC, but results in the Singh group suggest that a partitioning of reaction pathways occurs after the rate limiting step, and that oxygen plays a part in one of these steps. A detailed mechanism of that part of the reaction awaits further study.

In the scheme proposed by Singh et al.[11], nitrous oxide is believed to derive from the radical RS-N.-OH, while Stamler[14] has suggested that earlier reports of nitrous oxide formation can be explained in terms of a heterolytic mechanism involving attack of RS^- at the sulfur atom of RSNO, releasing NO^-, which would lead to nitrous oxide formation. Nitrous oxide has also been detected in the reaction products from sodium nitroprusside and glutathione[15]. We cannot at this time establish whether either of these suggestions is correct.

3.2. The reaction of SNAC with ascorbate

3.2.1. Background

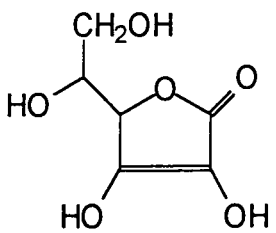
The ascorbate ion concentration in the plasma is higher than that of thiols (the mean levels of L-cysteine and ascorbate are 9±4mM and 50±20mM respectively[16,17]), so ascorbate may play a more important role in the NO release from RSNO *in vivo*. In the copper catalyzed SNAC decomposition ascorbate is used as a reducing agent (an alternative to RS^-), which can speed up the reaction at very low concentration, typically 10^{-5} M. This reaction is stopped when a very small amount of EDTA is added. However the decomposition of SNAC, which is induced by large amounts of ascorbate is not inhibited by EDTA. Surprisingly we find that the main products of this reaction do not include disulfide. Free thiol formed during the ascorbate induced reaction process is detected by the use of Ellman reagent at the end of the reaction. Approximately 80% of RSNO is recovered as thiolate ion and 70% of nitrite is detected at the end of the reactions.

3.2.2. Experimental Results

SNAC again is used as the solution made *in situ*, which is freshly made via the nitrosation of NAC by sodium nitrite under acidic condition. The kinetic reactions of the ascorbate induced SNAC decomposition were carried out in pH 7.4 phosphate buffer at 25^0C. Relatively high concentrations of ascorbate are required for the kinetic studies to speed up the reaction to a measurable value, otherwise other side reactions, such as the oxidation of ascorbate, may interfere with the recording of the reaction data. Special care must be taken to keep the

pH value in the component constant; sodium hydroxide solution is used to neutralize the ascorbic acid stock solution.

For SNAC the absorbance was measured at 400 nm instead of 333 nm to exclude the absorbance interference coming from the further reaction of the product dehydroascorbic acid, which generates a yellow colour as the result of oxidation of ascorbate. At this wavelength the absorbance interference arise from the dehydroascorbic acid can be ignored. For the faster reactions a wavelength of 340 nm was used, where there is a higher extinction coefficient[18], and where the side reaction of the product is less important.



Ascorbic Acid (AsA)

Good first order behaviour was observed and the first order rate constants was measured as a function of the concentration of AsA. A plot of the first-order rate constant (k_0) vs [AsA] was an excellent straight line and a value of the second-order rate constant k_2 (defined by Equation 3-12) was obtained. The value for k_2 at pH 7.4 was found to be 4.0×10^{-3} mol^{-1} dm^3 s^{-1}. Other workers in the group[18] have found values of k_2 (for reaction with ascorbate) of 2.0×10^{-2}, 2.5×10^{-1} and 1.40 mol^{-1} dm^3 s^{-1} for the S-nitroso derivatives of glutathione, cysteine and penicillamine respectively.

$$RATE = k_2 [SNAC] [AsA]$$

$$k_0 = k_2 [AsA]$$

Equation 3-12

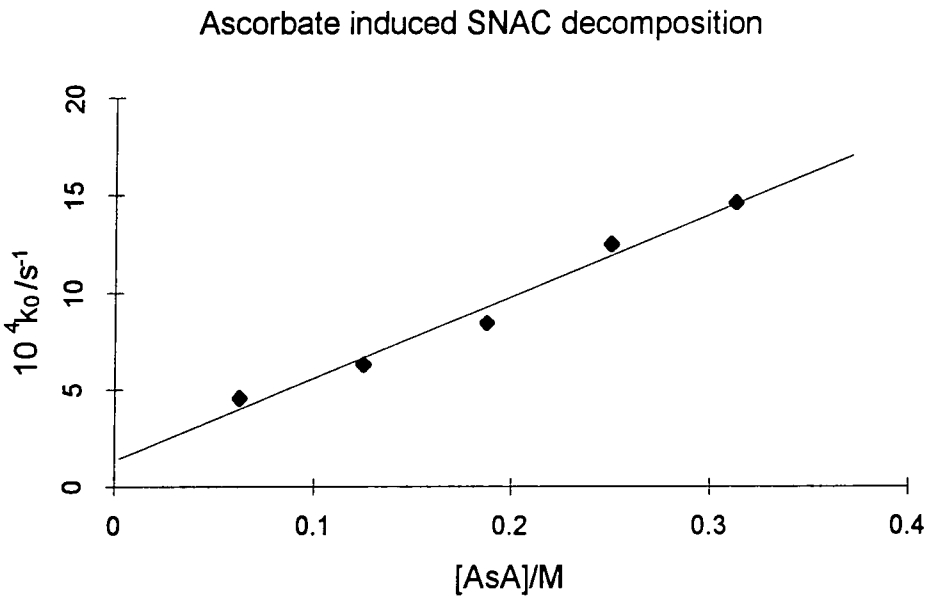


Figure 3-7 Ascorbate induced SNAC decomposition

The end product of those reactions is also the free thiolate and not the disulfide. This was detected by using the Ellman Reagent. Ellman reagent can react with free thiolate and give a intense yellow colour with the extinction coefficient at 412 nm about 14,000 $cm^{-1} M^{-1}$. Free thiolate as low as 10^{-6} M, can be detected by this reagent[19]. The details about this reagent have been discussed in chapter 2. In these reactions at the end of the decomposition, > 80% SNAC was recovered as thiolate ion and >70% nitrite is found as product. Also NO has been detected in high yield by Holmes[18], for the nitrosothiols derived from glutathione, captopril, N-acetylpenicillamine, homocysteine and thioglycerol, using the direct reading NO electrode system, when the reactions were carried out anaerobically.

3.2.3. Discussion

Under these conditions we can rule out a copper ion reaction, since EDTA is present in all the kinetic reactions. Both NAC and ascorbate enhanced nitric oxide release from SNAC, and the reaction rates depend on the concentration of NAC and ascorbate by a first order dependence. In contrast to the thiol-dependent reaction, which forms the disulfide, the ascorbate-dependent release of nitric oxide yields the thiol product.

$$RSNO + \text{ascorbate} \rightarrow RS^{\cdot} + \text{dehydroascorbate} + NO$$

Equation 3-13

The results suggest here that ascorbate is acting as a nucleophile, attacking the nitroso nitrogen atom probably giving the O-nitrosated product (and the thiolate ion) which reacts again (at the other oxygen atom) to give the dinitroso compound which eliminates NO and yields dehydroascorbic acid. There is a well established precedent for this in the literature[20], since nitrous acid reacts readily with ascorbic acid in this way as indeed does an alkyl nitrite[21]; this reaction has been studied mechanistically[20,21].

Earlier the transnitrosation reaction has been discussed[7], which involved the thiolate ion as a nucleophile and more recently[22] kinetic results have been observed for a range of nitrogen nucleophiles, including amines (primary and secondary), hydrazine, hydroxylamine etc. and also the sulfite ion SO_3^{2-}. In each case the RSNO acts as an electrophilic nitrosation reagent. It is therefore not surprising that the ascorbate ion can also be nitrosated in a direct reaction with RSNO. In the ascorbate reaction we have not carried out a rate-pH profile which

would indicate the exact nature of the nucleophile, but at pH 7.4 this is likely to be the ascorbate mono anion, given the two pK_a values of ascorbic acid are 4.3 and 11.8. This contrasts with the work with the alkyl nitrite, which was carried out at pH 12 where it was suggested that the dianion is the reactive species.

Whatever the detailed mechanism of NO release in these reactions with ascorbate, it is clear that there is a reaction pathway for NO formation from RSNO, which is independent of the presence of metal ions, particularly Cu^{2+}. This reaction is dominant at high [ascorbate], whereas at low [ascorbate], where the direct ascorbate reaction is too slow to be significant, the Cu^{+} -promoted reaction takes over.

Finally, for these reactions in the living cells, it is not likely that the ammonia-forming reaction is important. Given that the most prevalent thiol glutathione could be present at 10 mM, and the second order rate constant[18] is $\sim 5\times10^{-3}$ mol^{-1} dm^3 s^{-1} for the GSNO and GSH reaction, the half life at 25^0C is approaching 4 hours. Similarly the ascorbate reaction with glutathione, although more rapid generates NO only relatively slowly. The conclusion is, that in the absence of any enzyme catalysis, it is unlikely that NO or NH_3 forming reactions will occur rapidly *in vivo*, from ascorbate or thiolate.

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Appendix 1 . Experimental Details

Ultraviolet/Visible Spectrophotometry

All ultraviolet/visible spectra were obtained from solutions in quartz cuvettes of 1cm path length at 25°C on either a Perkin-Elmer Lambda 2, Perkin-Elmer Lambda 12 or Shimadzu UV-2101PC spectrophotometer. The same three instruments were generally used to measure reaction kinetics at fixed wavelengths. For more rapid reactions ($t_{1/2} \geq 2$ms) a stopped-flow technique was used.

All kinetic measurements were made under pseudo-first order conditions. The observed rate constants were calculated from the noted absorbance change as a function of time at a specific wavelength (usually 333 nm). The absorbance/time data from each spectrophotometer were transferred to an Epson AX2 personal computer and utilised in a software program designed for rate constant calculation (Enzifitter). This program allowed the calculation of observed rate constants, k_{obs}, based on the following derivation.

For a first order kinetic process (Equation 1), the rate of formation of B or the removal of A can be expressed by Equation 2.

$$A \xrightarrow{k_{obs}} B \qquad \textit{Equation 1}$$

$$-\frac{d[A]}{dt} = \frac{d[B]}{dt} = k_{obs}[A] \qquad \textit{Equation 2}$$

Integration of the equation gives an expression for the observed first order rate constant, k_{obs}, where $[A]_0$ and $[A]_t$ are the concentrations of species A at times t = 0 and t = t respectively.

$$\ln[A]_0 - \ln[A]_t = k_{obs}t \qquad \textit{Equation 3}$$

Using the Beer-Lambert law ($A = \varepsilon cl$, where A is the absorbance, ε is the molar extinction coefficient, c is the concentration and l the path length), and assuming the latter to be 1cm, the expression for the absorbance at t = 0 and t = t can be derived.

$$A_0 = \varepsilon_A[A]_0 \qquad \textit{Equation 4}$$

$$A_t = \varepsilon_A[A]_t + \varepsilon_B[B]_t \qquad \textit{Equation 5}$$

As $[B]_t = [A]_0 - [A]_t$, substituting for $[B]_t$ into Equation 5 gives-

$$A_t = \varepsilon_A[A]_t + \varepsilon_B[A]_0 - \varepsilon_B[A]_t \qquad \textit{Equation 6}$$

At the end of reaction, $t = \infty$ and $[B]_\infty = [A]_0$, so $A_\infty = \varepsilon_B[A]_0$, thus

$$[A]_t = \frac{(A_t - A_\infty)}{(\varepsilon_A - \varepsilon_B)} \qquad \textit{Equation 7}$$

Similarly, at time t = 0

$$A_0 = \varepsilon_A[A]_0 \qquad \textit{Equation 8}$$

Hence,

$$A_0 - A_\infty = \varepsilon_A[A]_0 - \varepsilon_B[B]_0 \qquad \textit{Equation 9}$$

Then,

$$[A]_0 = \frac{(A_0 - A_\infty)}{(\varepsilon_A - \varepsilon_B)} \qquad \textit{Equation 10}$$

$$k_{obs} = \frac{1}{t}\ln\frac{(A_0 - A_\infty)}{(A_t - A_\infty)} \qquad \textit{Equation 11}$$

Rearranging gives

$$ln\,(A_t - A_\infty) = -k_{obs}\,t + ln\,(A_0 - A_\infty)$$

Therefore, a plot of $ln\,(A_t - A_\infty)$ against t should be linear with a slope of $-k_{obs}$. 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.

Stopped-Flow Spectrophotometry

For the determination of rate constants of reactions too fast to measure by conventional machines a Applied Photophysics Stopped-Flow spectrophotometer or a Hi-Tech Scientific SF-3L Stopped-Flow spectrophotometer were used. The experimental apparatus is shown schematically in figure 1. All reactions were carried out under pseudo-first order conditions. The two solutions to be reacted, A and B, are stored in reservoirs and drawn into two identical syringes so that equal volumes are mixed. The syringes are simultaneously compressed by using a compressed air supply with reactant mixing taking place at point M extremely rapidly (< 1ms). The mixture then flows into a thermostatted 2mm path length quartz cell at point O, which causes the plunger of the third syringe to hit a stop, with a cessation in the solution flow. The acquisition of absorbance/time data from the reaction is triggered by the stop being hit. The observation of reaction is maintained 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 recorded. Software on the computers that run the stopped-flow machines is capable of transforming voltage/time data into absorbance/time data and can also calculate the observed rate

constants.

pH Measurements

All pH measurements were carried out using a Jenway 3020 digital pH meter, which was accurate to ± 0.02 pH units. The pH meter was calibrated over the range pH 4.0 to 7.0 to pH 7.0 to 10.0 depending on the solution to be measured.

GSNO synthesis

To a stirred ice-cold solution of glutathione (1.53g, 5mmol) in water (5ml) containing 2M HCl (2.5ml) was added in one portion sodium nitrite (0.345g, 5mmol). After 20 minutes at 5^0C the red solution was treated with acetone (10ml) and stirred for a further 5 minutes. The resulting fine pale red precipitate was filtered off and then washed successively with ice- cold water (5×1ml), acetone (3×10ml) and ether (3×10ml) to afford S-nitrosoglutathione. The same method was attempted in the synthesis of S-nitroso-N-acetylcysteine but failed to precipitate a solid sample. The ^{1}H NMR spectrum for SNAC in solution gave signals at δ_H/ppm 4.48 (H,t, J=4Hz), 2.84 (2H, d, J=4Hz), 1.93 (3H, m), NH and COOH hydrogen will undergo rapid exchange with the solvent, D_2O, Figure 2.

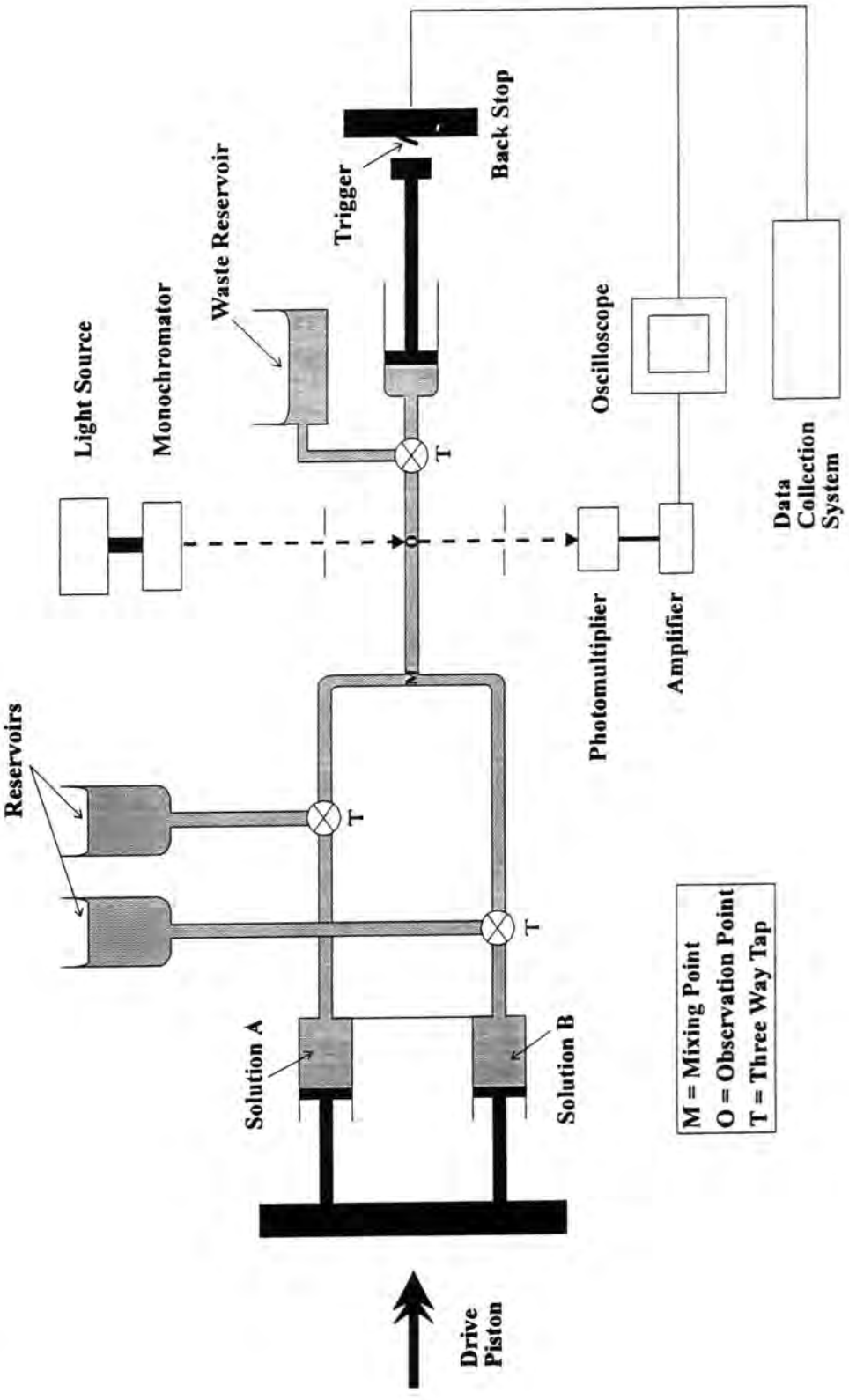

Figure 1. Schematic diagram of a stopped-flow spectrophotometer

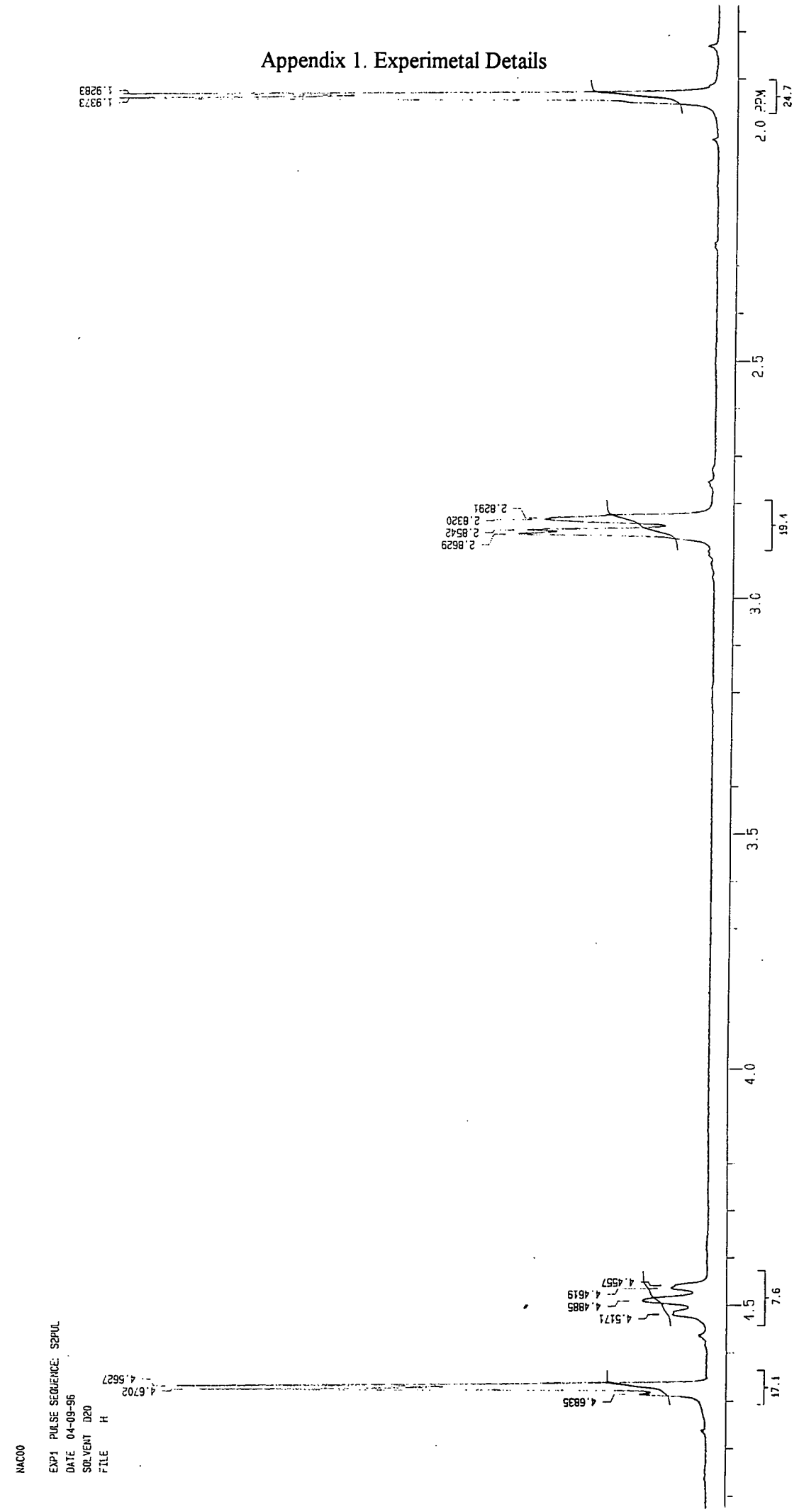

Figure 2 ^1H NMR spectrum of SNAC in D_2O

Reagents

All reagents used for synthetic and kinetic purposes were of the highest grade commercially available. Generally, S-nitrosothiols were produced *in situ* as described using acidified sodium nitrite and a variety of thiols, which were purchased commercially. The potassium dihydrogenphosphate and sodium hydroxide used to prepare pH 7.4 buffer solutions were purchased commercially and used as supplied. The perchloric acid solutions used for nitrosation were prepared by dilution of concentrated perchloric acid, which had been standardised using standard sodium hydroxide solution and phenol red as an indicator.

Appendix 2

The Board of Studies in Chemistry requires that each postgraduate research thesis containing 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 posters presented by the author during the period when research for the thesis was carried out;

C. Details of the postgraduate induction course.

A. Colloquia, lectures and seminars from Invited Speakers Organised by the Durham University Chemistry Department. 1996-1998

(* denotes lectures attended)

1996

October 9	Prof. G. Bowmaker, University of Auckland, New Zealand Coordination and Materials Chemistry of the Group 11 and Group 12 Metals- Some Recent Vibrational and Solid State NMR Studies
*October 16	Prof. Ojima, State University of New York, USA Silylformylation and Silylcarbocyclisations in Organic Synthesis
October 22	Prof. B.J. Tighe, University of Aston Synthetic Polymers for Biomedical Application – Can We Meet Nature's Challenge?
*October 23	Prof. H. Ringsdorf, Johannes Gutenberg-Universitat, Mainz, Germany *Perkin Centenary Lecture* – Function Based on Organisation
*October 29	Prof. D.M. Knight, Department of Philosophy, University of Durham The purpose of Experiment – A Look at Davy and Fraday
October 30	Dr. P. Mountford, University of Nottingham Recent Developments in Group 4 Imido Chemistry
November 12	Prof. R. J. Young, UMIST New Materials – Fact of Fantasy?
*November 13	Dr. G. Resnati, University of Milan, Italy Perfluorinated Oxaziridines – Mild Yet Powerful Oxidising Agents
*November 19	Prof. R. E. Grigg, University of Leeds Assembly of Complexs Molecules by Palladium – Catalysed Queueing Processes
November 20	Prof. J. Earnshaw, Department of Physics, Belfast Surface Light Scattering – Ripples and Relaxation
December 3	Prof. D. Philips, Imperial College, London

	A Little Light Relief
*December 4	Prof. K. Muller-Dethlets, University of York Chemical Applications of Very High Resolution ZEKE Photoelectron Spectroscopy
*December 11	Dr. C.Richards, University of Wales, Cardiff Stereochemical Games with Metallocenes
1997	
January 15	Dr. V.K. Aggarwal, University of Sheffield Sulfur Mediated Asymmetric Synthesis
January 16	Dr. S. Brooker, University of Otago, New Zealand Macrocycles-Exciting yet Controlled Thiolate Coordination Chemistry
January 22	Dr. N. Cooley, BP Chemicals, Sunbury Synthesis and Properties of Alternating Polyketones
*February 5	Dr. A. Haynes, University of Sheffield Mechanism in Homogeneous Catalytic Carbonylation
*February 6	Prof. B. Bartlett, University of Southampton Immobilisation of Enzymes in Electrochemically Polymerised Films
*February 18	Prof. Sir J. Black, Sir James Black Institute My dialogues with Medicinal Chemists
February 19	Prof. B. Hayden, University of Southampton Reaction Dynamics and Fuel Cells
*February 25	Prof. A.G. Sykes, University of Newcastle The structure, Properties and Design of Blue Copper Proteins
*February 26	Dr. A. Ryan, UMIST Making Hairpins from Rings and Chains
*March 4	Prof. C.W. Rees, Imperial College, London Some Very Heterocyclic Chemistry
March 11	Dr. A. D. Taylor, Rutherford Appleton Laboratory, Didcot Neutron Scattering
*March 19	Dr. K. Reid, University of Nottingham Probing Dynamical Processes with Photoelectrons
*October 8	Prof. E. Atkins, Dept. of Physics, University of Bristol

	Advances in the Control of Architecture for Polyamides: From Nylons to Genetically Engineered Silks to Monodisperse Oligoamides.
*October 15	Dr. R. M. Ormerod, Dept of Chemistry, Keele University Studying Catalysts in Action.
October 22	Prof. R.J. Puddephatt, University of Western Ontario Organoplatinum Chemistry and Catalysis.
October 23	Prof. M.R. Bryce, University of Durham, New Tetrathiafulvalene Derivatives in Molecular, Supramolecular and Macromolecular Chemistry: Controlling the Electronic Properties of Organic Solids.
October 29	Prof. B. Peacock, University of Glasgow Probing chirality with Circular dichroism.
November 5	Dr. M. Hii, University of Oxford Studies of the Heck Reaction
*November 12	Dr. J. Frey, University of southampton Spectroscopy of Liquid Interfaces: From Bio – organic Chemistry to Atmospheric Chemistry
November 19	Dr. G. Morris, University of Manchester Pulsed Field Gradient NMR Techniques: Good News for the Lazy and DOSY
*November 20	Dr. L. Spiccia, University of Monash, Melbourne, Australia Polynuclear Metal Complexes
*November 26	Prof. R.W. Richards, University of Durham A Random Walk in Polymer Science
December 3	Prof. A.P. Davis, Trinity College Dublin Steroid – based Frameworks for Supramolecular Chemistry
*December10	Sir Gordon Higginson, former Professor of Engineering in Durham and Retired Vice-Chancellor of University of Southampton 1981 and all that
*December 10	Prof. M. Page, University of Huddersfield The Mechanism and Inhibition of Beta-Lactamases

1998

*January 8	Dr. Ian D. Williams, Hong Kong University of Science and technology Control of Structure and Dimensionality in Mixed Organic-Inorganic Solids
January 14	Prof. D. Andrews, University of East Anglia Energy Transfer and Optical Harmonics in Molecular Systems
January 21	Prof. David Cardin, University of Reading Aspects of metal and carbon cluster chemistry
January 27	Prof. R.Jordan, University of Iowa, USA Cationic Transition Metal and Main Group Metal Alkyl Complexes in Olefin Polymerisation
January 28	Dr. S. Rannard, Courtaulds Coatings (Coventry) The Synthesis of dendrimers using highly selective chemical reactions
February 4	Prof. P.Fowler, University of Exeter Classical and Non-classical Fullerenes
February 18	Prof. G. Hancock, University of Oxford Surprises in the Photochemistry of Tropospheric Ozone
February 25	Dr. C. Jones, University of Swansea Low Coordination Arsenic and Antimony Chemistry
March 11	Prof. M.J. Cook, University of East Anglia How to Make Phthalocyanine films and what to do with them

D. First Year Induction Course, October 1996

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