Chemical and Biological Properties of *S*-Nitrosothiols

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by

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Since its discovery, nitric oxide (NO) has been identified to influence a large number of physiological processes. This project examines *S*-nitrosothiols (RSNO) as pro-drugs of NO.

The overall aim of this project was to improve our present understanding of the chemical and biological properties of RSNOs. This project has demonstrated that under physiological conditions the stability of RSNOs varies with structure. Results have shown that *S*-nitrosocysteine and *S*-nitroso-L-cysteinylglycine were the least stable of the RSNOs investigated, whereas *S*-nitroso-*N*-acetyl-L-cysteine, *S*-nitroso-3-mercaptopropionic acid and *S*-nitroso-2-mercapto-ethane sulphonic acid were the most stable. The decomposition of certain RSNOs is catalysed by trace amounts of copper. This phenomenon was particularly evident with the RSNOs, *S*-nitrosocysteine and *S*-nitroso-L-cysteinylglycine. Copper catalysed decomposition appears to occur more readily with RSNOs that allow the formation of a stable ring structure, in which Cu^{+} is bound to the nitrogen of the NO group and another electron-rich atom such as the nitrogen of an amino group. Copper catalysed the decomposition of *S*-nitrosoglutathione and *S*-nitroso-L-γ-glutamyl-L-cysteine, but to a lesser extent. Investigations have shown that the decomposition of a stable RSNO is more rapid in the presence of a thiol which gives rise to an unstable RSNO *via* a transnitrosation reaction. In contrast, decomposition of an unstable RSNO is slower in the presence of a thiol which leads to the formation of a stable RSNO.

All RSNOs studied inhibited platelet aggregation and relaxed vascular smooth muscle in a dose dependent manner. In addition all the RSNOs exhibited a dose-dependent inhibition of growth of A549 cells.

Generally no real correlation between the chemical and biological properties of RSNOs was observed.

It is clear that there are many factors controlling the release of NO from RSNOs which may have implications regarding the biological activity of these compounds.

For my Mom and Dad

With much love and thanks

Acknowledgements

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Chapter 1

Introduction

In 1980 Furchgott and Zawadzki made the discovery that the vascular endothelium contains a vasorelaxing substance, endothelium-derived relaxing factor (EDRF), that is released upon stimulation with acetylcholine and other mediators (Furchgott and Zawadzki, 1980). It was established using a variety of pharmacological preparations that EDRF was a diffusible substance with a half-life of several seconds (Griffith *et al*, 1984). The effects of EDRF were shown to be inhibited by haem-containing proteins (Martin *et al*, 1985) and mediated by stimulation of soluble guanylyl cyclase (Rapaport and Murad, 1983). Early investigators in this field suggested that EDRF might be a product of the arachidonic acid lipoxygenase (Singer and Peach, 1983; Forstermann and Neufang, 1984) or of the cytochrome P_{450} system (Pinto *et al*, 1986). It was not until the mid 1980's that EDRF was suggested to be nitric oxide (NO, Furchgott, 1988; Ignarro *et al*, 1987). The first evidence for the formation of NO by mammalian cells came from experiments in which EDRF was measured as the chemiluminescent product of its reaction with ozone (Palmer *et al*, 1987).

Since its discovery, NO has been implicated in a wide variety of physiological pathways. Nitric oxide is responsible for endothelium-dependent vascular relaxation (Rees *et al*, 1989a; Rees *et al*, 1989b) and inhibition of platelet aggregation (Radomski *et al*, 1990a; Radomski *et al*, 1990b). It is also involved in central and peripheral neurotransmission (Garthwaite *et al*, 1988; Knowles *et al*, 1989; Duarte *et al*, 1990), and in the defence mechanisms of the body utilised by macrophages (Hibbs *et al*, 1988; Stuehr *et al*, 1989) and many other cells (Moncada *et al*, 1991a). In addition NO is involved in the pathophysiology of certain diseases such as septic shock (Wright *et al*, 1992) and hypertension (Panza *et al*, 1990).

Nitric oxide, the tenth smallest known molecule and one of the simplest odd-electron species, is a colourless gas at room temperature. For its physiological activity NO is always present as an aqueous solution where its solubility is unchanged over the pH range 2-13. In view of the diverse physiological roles of NO it has been suggested that NO exists as the nitrosonium ion, NO^+, or the nitroxide ion, NO^- (Stamler *et al*, 1992a; Lipton *et al*, 1993). Chemically it is possible that NO^+ could be formed by the oxidation of NO at pH 7, but in aqueous solution it will immediately form nitrous acid (equation 1.1, Butler *et al*, 1995).

Equation 1.1 $NO^+ + H_2O \rightarrow HNO_2 + H^+$

The possibility that nitroxide ions have a physiological role is quite feasible. Nitroxide ions result from the ionisation of nitroxyl (equation 1.2). The pK_a of HNO is 4.7 and hence ionisation will occur at physiological pH but to date little has been published on the solution chemistry of NO^- and so it is difficult to comment on its role as a cell mediator (Butler *et al*, 1995). For the duration of this thesis NO is assumed to be the radical, $NO^{\cdot}$.

Equation 1.2 $HNO \rightleftharpoons H^+ + NO^-$

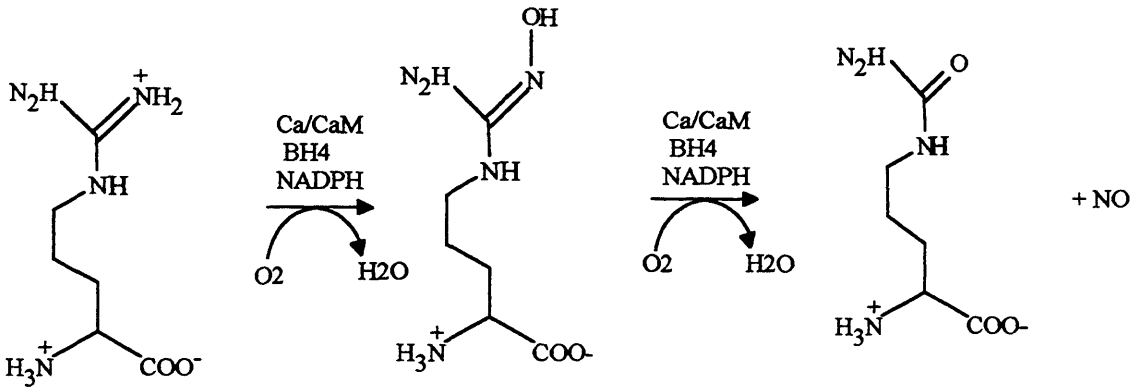
Although the evidence for EDRF being NO is conclusive there has been some controversy regarding this judgement. It has been suggested that EDRF may be an *S*-nitrosothiol (RSNO) (Myers, *et al*, 1990; Rubanyi *et al*, 1991), nitroxyl (Fukuto *et al*,1992), dinitrosyl-iron-cysteine complex (Vanin, 1991) or hydroxylamine (Thomas and Ramwell, 1989). Investigations have been carried out by Feelisch *et al* (1994) comparing the pharmacology of these species with NO and EDRF. The results suggested that all of the aforementioned candidates for EDRF could be eliminated as they are more stable than EDRF itself and less susceptible to inhibition by oxyhaemoglobin. It has also been suggested that low molecular weight RSNOs, such as *S*-nitrosoglutathione (GSNO) and *S*-nitrosocysteine (CysNO) may represent a mechanism for the storage or transport of NO (Mellion *et al*, 1983, Myers *et al*, 1990; Girard and Potier, 1993). This thesis is concerned with the chemical, pharmacological and toxicological properties of RSNOs as NO precursors. I will review briefly the roles of NO in the cardiovascular system and as a cytotoxic agent, and examine other classes of NO donors.

1.1 Biosynthesis of Nitric Oxide

Nitric oxide is synthesised from the amino acid L-arginine to yield L-citrulline and NO (equation 1.3). This reaction is catalysed by the group of enzymes, the nitric oxide

synthases (NOS). There are currently known to be three distinct mammalian isoforms of NOS, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Knowles and Moncada, 1994). These enzymes have been broadly classified as either constitutive (eNOS and nNOS) or inducible isoforms (iNOS). One of the primary differences between the constitutive and inducible forms is in the regulation of activity. It is generally thought that constitutive isoforms are Ca^{2+} and calmodulin (CaM) dependent, whereas the regulation of the inducible isoform is Ca^{2+} independent. The inducible isoform does contain CaM but it is so tightly bound that it is considered to be a subunit and therefore not subject to any regulation by Ca^{2+}(Cho *et al*, 1992).

Nitric oxide synthases generate NO by catalysing the oxygen and NADPH-dependent oxidation of a guanidino nitrogen on L-arginine. The overall process is a five electron oxidation of nitrogen.



L-Arginine *N*-Hydroxy-L-arginine L-Citrulline

Equation 1.3 Biosynthesis of nitric oxide.

Nitric oxide synthases are members of the cytochrome P_{450} enzyme family, requiring many different cofactors, namely NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH_4) and molecular oxygen. Flavin adenine dinucleotide, FMN and BH_4 all play roles in moving electrons from NADPH to the haem complex at the enzyme active site. Molecular oxygen is incorporated both into NO and L-citrulline (Leone *et al*, 1991). Reduced thiol also plays an as yet, unknown role in the activity of the enzyme.

The synthesis of NO from L-arginine can be inhibited by certain analogues of L-arginine, including N^G-monomethyl-L-arginine (L-NMMA), *N*-iminomethyl-L-orthinine and N^G-nitro-L-arginine methyl ester, which compete with L-arginine and act as stereospecific inhibitors of NOS (Rees *et al*, 1990a). These inhibitors have been used extensively *in vitro* and *in vivo* to characterise the part played by NO in its various roles. Methylated arginine analogues including L-NMMA and N^G, N^G-dimethylarginine are also found endogenously and might modify NO synthesis *in vivo* (Vallance *et al*, 1992).

1.2 Reactivity of Nitric Oxide

Nitric oxide reacts in biological systems with molecular oxygen, superoxide (O_2^-), other radicals including lipid protein radicals and transition metals. The products of these reactions, higher nitrogen oxides, peroxynitrite ($OONO^-$) and metal-nitrosyl complexes, have been implicated in the biological and toxicological activity of NO.

The best known reaction of NO is its conversion to nitrogen dioxide ($NO_2^{\bullet}$) which could occur *in vivo* (equation 1.4). In the gas phase this reaction is third order, second order in [NO]. The aqueous-phase reaction of NO with molecular oxygen is slow at concentrations that exist in cells suggesting that the short physiological half-life of NO (6-30 sec) cannot be due to oxidation by O_2 (Wink *et al*, 1993; Butler *et al*, 1995).

Equation 1.4 $$2NO^{\bullet} + O_2 \rightarrow 2NO_2^{\bullet}$$

Nitrogen dioxide reacts with water to give a mixture of nitrate (NO_3^-) and nitrite (NO_2^-) ions (equation 1.7). It was thought that this was the fate of NO in oxygenated water but there is now evidence to suggest that the only product obtained is NO_2^- (Wink *et al*, 1993). Nitric oxide is assumed to react rapidly with NO_2 as it is formed to give N_2O_3, the anhydride of nitrous acid (equation 1.5), which then hydrolyses to nitrite (equation 1.6).

Equation 1.5 $NO_2 + NO \rightarrow N_2O_3$

Equation 1.6 $N_2O_3 + H_2O \rightarrow 2HNO_2 \rightleftharpoons 2H^+ + 2NO_2^-$

Equation 1.7 $2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$

The reactions of NO with oxygen species have been associated with its cytotoxicity. These are well described in the central nervous system, gut and airway where $OONO^-$ and $NO_2^{\cdot}$ are frequently implicated (Gaston *et al*, 1993; Lipton *et al*, 1993). The possibility of the formation of $ONOO^-$ under normal physiological conditions suggests that certain biological functions of the molecule are not excluded. Recent reports have shown $ONOO^-$ to induce thiol-dependent relaxations of vascular smooth muscle (Wu *et al*, 1994) and to be both proaggregatory and antiaggregatory in human platelets (Moro *et al*, 1994).

Reactions with transition metal centres are vital to the biological activities of NO. Many of the biological effects of NO proceed *via* a soluble guanylyl cyclase-dependent pathway to increase intracellular levels of guanosine 3',5'-mono-phosphate (cGMP). Activation of soluble guanylyl cyclase is thought to result from conformational changes within the porphyrin ring brought about by NO binding, leading to the conversion of magnesium guanosine 5'-triphosphate to cGMP. There are a number of types of guanylyl cyclase, these falling into the categories of particulate or membrane-receptor and soluble or cytosolic guanylyl cyclase. By contrast particulate guanylyl cyclase is activated by peptide ligands which bind to cell membrane receptors (Schmidt *et al*, 1993). Nitric oxide induced activation of the haem centre of soluble guanylyl cyclase is inhibited by addition of haemoglobin (Ignarro, 1989). Likewise, the addition of haemoglobin limits the formation of intracellular iron-nitrosyl adducts in cells producing NO (Stadler *et al*, 1993).

There are now known to exist a number of actions of NO that are independent of cGMP. Generally these reactions involve metallo-proteins or thiol-containing proteins as regulators (Stamler and Feelisch, 1996). For example the interaction of NO with metals has been suggested to account for the cytotoxicity of activated macrophages (Hibbs *et al*, 1988) and growth arrest of tumour cells (Hibbs *et al*, 1990). Examples of

iron-sulphur-cluster enzymes which are inhibited by NO include aconitase, NADPH: ubiquinone oxidoreductase (mitochondrial complex I), succinate: ubiquinone oxidoreductase (mitochondrial complex II), and ribonucleotide reductase (Drapier and Hibbs, 1986; Hibbs *et al*, 1990; Lepoivre *et al*, 1992; Henry *et al*, 1993).

1.3 Nitric Oxide in the Cardiovascular System

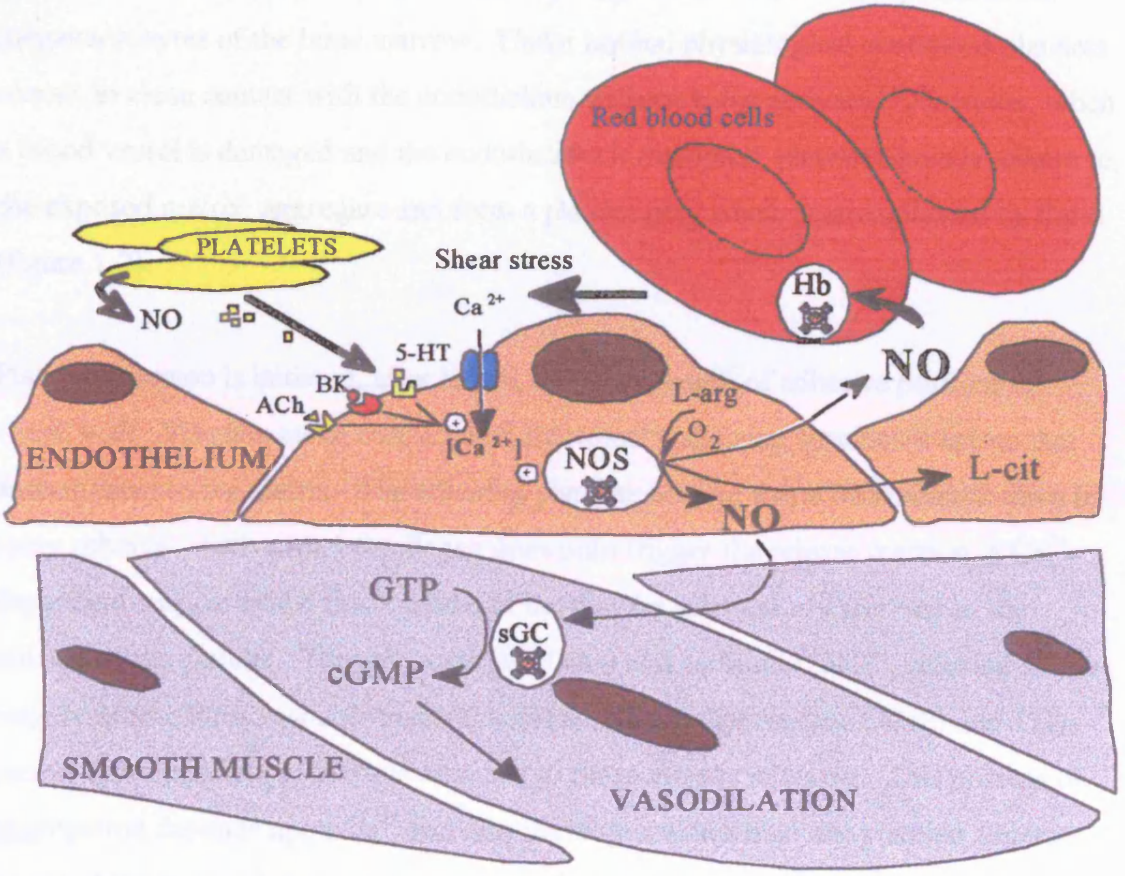


Figure 1.1 Roles of NO in the cardiovascular system.

The discovery of endothelial derived NO highlighted the importance of this cellular monolayer for vascular homeostasis. Stress-mediated release of NO (Rubanyi *et al*, 1986) regulates arterial blood flow and pressure by mediating a vasodilator tone (Rees *et al*, 1989a).

Nitric oxide controls haemostasis by inhibiting cell-cell contact (adhesion and aggregation) at the sensitive interphase between platelets and endothelial cells (Radomski and Moncada, 1993). In addition NO appears to regulate leukocyte

adhesion and activation of endothelial cells (Kubes *et al*, 1991; Moilanen *et al*, 1993; Salas *et al*, 1994).

1.3.1 Anti-Platelet Activity of Nitric Oxide

Platelets are small anucleate cells formed by fragmentation of the cytoplasm of the megakaryocytes of the bone marrow. Under normal physiological conditions platelets remain in close contact with the endothelium without being activated. However, when a blood vessel is damaged and the endothelium is disrupted, platelets quickly adhere to the exposed matrix, aggregate and form a platelet plug which is strengthened by fibrin (figure 1.2).

Platelet adhesion is initiated, after lesion, by the exposure of adhesive portions of vessel wall. Platelets make contact with the vessel wall using specific receptors that anchor them to the matrix. The adhering platelets change shape from smooth discs to spiny spheres. Activation by collagen goes onto trigger the release reaction, a Ca^{2+}-dependent step, in which the contents of the platelet granules are secreted to the outside of the platelet. Thromboxane A_2 (TxA_2) and serotonin (5HT), released in this way, reinforce local vasoconstriction, while adenosine diphosphate (ADP) and TxA_2 causes more platelets to become attached to those already adhering. This process of aggregation depends upon Ca^{2+} and also fibrinogen which links the platelets together by attaching to the glycoprotein receptors IIb and IIIa. Thrombin is generated and this causes the formation of fibrin from fibrinogen. Finally, platelet activation facilitates the formation of platelet-leukocyte aggregates by allowing the α-granule protein P-selectin to be translocated to the platelet surface membrane and bind to the leukocyte ligand (Radomski, 1996).

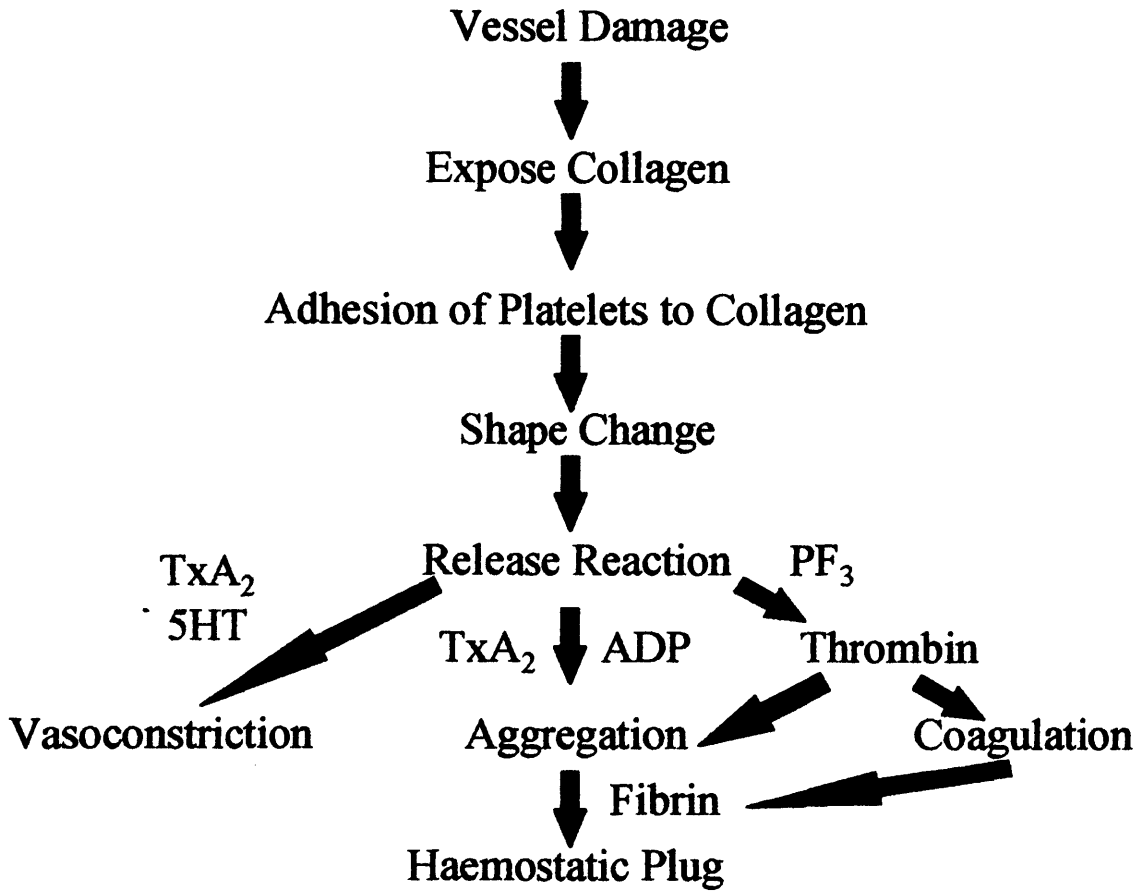


Figure 1.2 Haemostatic reactions of platelets. ADP, adenosine diphosphate; 5HT, 5-hydroxytryptamine (serotonin); PF_3, platelet factor; TxA_2, thromboxane A_2.

Two principal endothelial products that account for the inhibition of activation, adhesion and aggregation of platelets are NO (Azuma *et al*, 1986; Radomski *et al*, 1987a) and prostacyclin. These products act synergistically to inhibit platelet aggregation (Radomski *et al*, 1987b). The finding that inhibition of NOS is accompanied by thrombosis is strong evidence for the inhibition of platelet function *in vivo* by NO (Shultz and Raji, 1992; Herbaczynska-Cedro *et al*, 1991; Freedman *et al*, 1994).

Platelets themselves have the ability to synthesise NO (Radomski *et al*, 1990a; Radomski *et al*, 1990b), with both a constitutive and inducible form of NOS identified in human megakaryoblasts (Lelchuk *et al*, 1992).

Inhibition of platelet function by NO can be potentiated by thiols (Stamler, 1989), this effect may be a consequence of RSNO formation (Loscalzo 1985; Ignarro *et al*, 1981). *S*-Nitrosothiols have been detected *in vivo* and may be responsible for stabilising NO and for many of the biological effects attributed to NO in the vasculature (Stamler *et al*, 1992a; Simon *et al*, 1993; Keaney *et al*, 1993; Mellion *et al*, 1983).

The mechanism(s) by which NO inhibits platelet function is complex. Nitric oxide binds to the soluble guanylyl cyclase in the platelet which leads to a rise in cGMP. This increase in cGMP is accompanied by inhibition of platelet aggregation (Mellion *et al*, 1981). Elevated concentrations of cGMP activates cGMP dependent protein kinase and the phosphorylation of various target proteins including 46/50 kDa vasodilator-stimulated phosphoprotein (Radomski, 1996). Activation of cGMP-dependent protein kinase may also lead to phosphorylation of calcium transporters which reduces intracellular calcium concentration (Ignarro *et al*, 1985). A reduction in intracellular calcium is associated with a change in the conformation of glycoprotein IIb/ IIIa to a state that does not bind fibrinogen (Mendelsohn *et al*, 1990). In addition cGMP inhibits the translocation of P-selectin from platelet granules to the plasma membrane decreasing formation of platelet-leukocyte aggregates (Salas *et al*, 1994).

1.3.2 Vasodilatory Action of Endothelial Derived Nitric Oxide

Endothelium-dependent relaxation has been demonstrated in arteries, arterioles, veins, microvasculature and lymph vessels (Calver *et al*, 1993). Inhibition of NOS with L-NMMA induces endothelium dependent constriction of isolated arteries and arterioles from many species, indicating that there is a continuous release of NO from the endothelium of these preparations (Calver *et al*, 1993). The hypertensive effects of L-NMMA is likely to be due to inhibition of NO synthesis.

Nitric oxide causes relaxation of vascular smooth muscle. The sensitivity of different types of blood vessel varies. Generally it appears that NO or an NO-donor is most potent in veins (Collier *et al*, 1978), less potent in large arteries and least potent in small arterioles (Calver *et al*, 1993). It appears that venous tissue (Seidel and La

Rochelle, 1986) and the venous circulation (Vallance *et al*, 1989) have a lower basal release of NO as well as the increased sensitivity to NO donors. This increase in sensitivity is either the result of soluble guanylyl cyclase being more sensitive to exogenous NO or that venous smooth muscle contains more soluble guanylyl cyclase (Moncada *et al*, 1991a).

The vasodilator actions of NO are mediated by the activation of soluble guanylate cyclase. The pathway involved is thought to be the same as that operative in the inhibition of platelet aggregation. The contractile apparatus of smooth muscle cells consists of actin filaments (thin filaments) and myosin molecules (thick filaments). As the muscle changes in length, the thick and thin filaments slide over each other. Contraction is initiated by the release of calcium into the sarcoplasm from binding sites on the smooth muscle membrane. Calcium then activates an enzyme on the myosin cross-bridge (myosin light-chain kinase), which phosphorylates the myosin bridge so that it can react with actin. Hence the decrease in calcium associated with the activation of cGMP-dependent protein kinase translates to relaxation of smooth muscle.

The endothelium acts as a signal transducer, detecting chemical and physical stimuli and altering NO synthesis accordingly. Under normal physiological conditions the vasculature is regulated by NO synthesised from constitutive NOS present in vascular endothelium (eNOS). Nitric oxide-dependent vasodilator tone is controlled by the physical action of pulsatile flow and shear stress of the circulating blood on endothelial cells. However, in certain pathological conditions e.g. septic shock, NO is also synthesised within endothelial cells and smooth muscle by activation of iNOS. Exposure of blood vessels to bacterial lipopolysaccharide (endotoxin) leads to expression of iNOS in smooth muscle (Knowles *et al*, 1990; Rees *et al*, 1990b) and endothelium (Radomski *et al*, 1990c). The overproduction of NO produces vascular relaxation and hyporesponsiveness to vasoconstrictors (Rees *et al*, 1990b) and even tissue damage (Palmer *et al*, 1992).

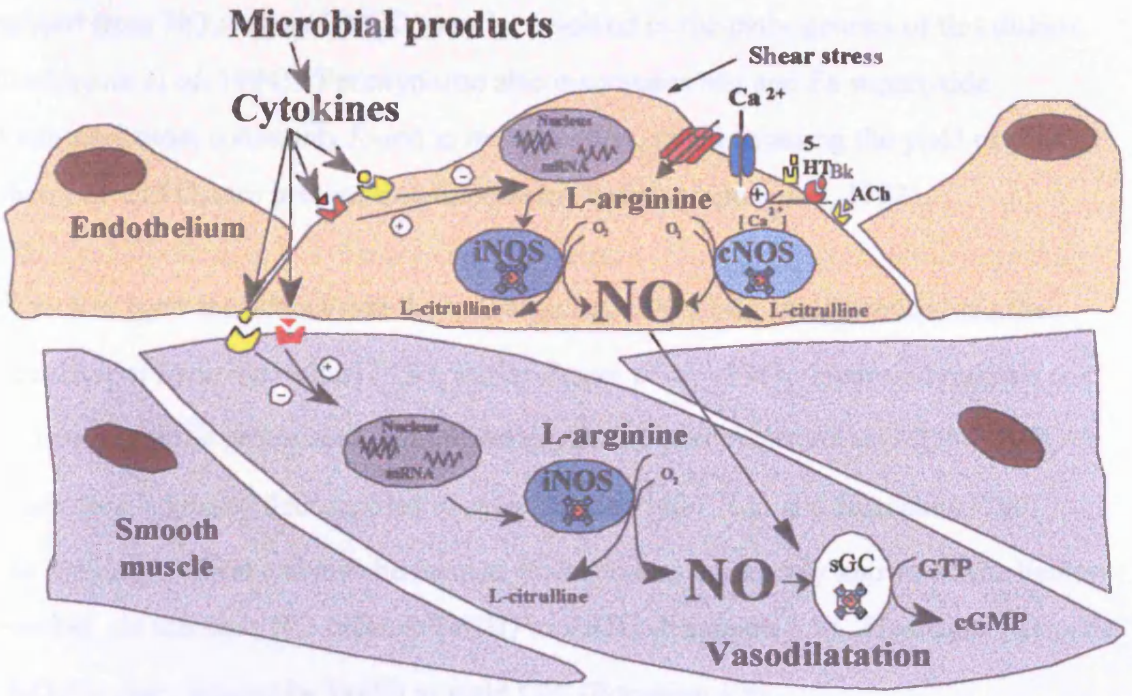


Figure 1.3 L-Arginine-NO pathway in the vasculature.

1.4 Toxicity of Nitric Oxide

In order to fulfil its physiological roles, low concentrations of NO are generally sufficient to effect these functions. However, during infection (as described earlier), and inflammation, *in vivo* formation of NO, measured as excreted levels of nitrate, is elevated demonstrating that NO concentrations in infected tissues are far above those found in normal physiological function. This release of NO may bring about some untoward effects such as formation of *N*-nitroso compounds (Liu *et al*, 1991), deamination of DNA bases and mutagenesis (Wink *et al*, 1991; Nguyen *et al*, 1992), oxidation of DNA bases and tissue injury caused by potent oxidative agents (Beckman *et al*, 1990; Tannenbaum *et al*, 1994), hypotension and shock (Kilbourn *et al*, 1990).

Macrophages and neutrophils can produce NO simultaneously with O_2^- (Ischiropoulos *et al*, 1992) and hence produce $OONO^-$. Peroxynitrite is stable in alkaline solutions but not at physiological pH, with a half-life of under 1 sec. Peroxynitrite has strong oxidising properties towards biomolecules including thiols (Radi *et al*, 1991), deoxyribose and membrane phospholipids (Liu and Hotchkiss, 1995). Nitration of protein tyrosines in human atherosclerosis has been detected indicating that oxidants

derived from NO such as $ONOO^-$ may be involved in the pathogenesis of this disease (Beckmann *et al*, 1994). Peroxynitrite also inactivates Mn and Fe superoxide dismutases most commonly found in mitochondria, thus increasing the yield of $ONOO^-$ when NO and O_2^- are present simultaneously (Ischiropoulos *et al*, 1992).

It has also been speculated that the cytotoxic actions of NO could proceed *via* the formation of hydroxyl radical ($^{\cdot}OH$, Farias-Eisner *et al*, 1996). Hydroxyl radicals can be formed *via* the protonated form of peroxynitrite, peroxynitrous acid (ONOOH), which spontaneously decomposes to give $^{\cdot}OH$ and $NO_2^{\cdot}$ (Liu and Hotchkiss, 1995). The hydroxyl radical can also be formed *via* a process commonly known as the Fenton reaction. In this case NO reduces Fe(III) to Fe(II) (Equation 1.8). Hydrogen peroxide (H_2O_2) is then reduced by Fe(II) to yield $OH^{\cdot}$ (Equation 1.9).

Equation 1.8

$$NO + Fe(III) \rightarrow [Fe(III)\text{-}NO \rightleftharpoons Fe(II)\text{-}^{+}NO]$$

$$[Fe(III)\text{-}NO \rightleftharpoons Fe(II)\text{-}^{+}NO] + H_2O \rightarrow Fe(II) + NO_2^- + 2H^+$$

Equation 1.9

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^- + {}^{\cdot}OH$$

Overall

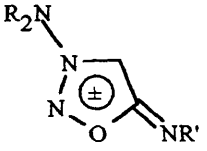
$$NO + H_2O_2 \rightarrow NO_2^- + {}^{\cdot}OH + H^+$$

The synthesis of NO by cytotoxic-activated macrophages is a prerequisite for cellular dysfunctions including growth arrest of target cells and inhibition of mitochondrial and cytosolic enzymes (section 1.2) both in target cells and macrophages themselves (Drapier and Hibbs, 1986; Hibbs *et al*, 1990). Peroxynitrite can also cause damage to a wide range of cellular constituents, within the mitochondrial chain this includes irreversible inhibition of complexes I-III, which is prevented in the presence of GSH or glucose (Lizasoain *et al*, 1996).

1.5 Nitric Oxide Donors

Since the interest in NO has escalated there has been an increased interest in using compounds that are capable of generating NO *in situ*. Nitric oxide donors, or nitrovasodilators, are pro-drugs of NO and can be divided into different groups that include organic nitrates ($RONO_2$), organic nitrites (RONO) and RSNOs (table 1.1).

Table 1.1 Chemical structures of common NO donors (Bauer and Fung, 1995).

Agent	General Structure
Organic nitrates e.g. Glyceryl trinitrate	$R_3C\text{-}O\text{-}NO_2$
Organic nitrites e.g. Isoamyl nitrite	$R_3C\text{-}O\text{-}N{=}O$
Iron Nitrosyls e.g. Sodium nitroprusside	$[(CN)_5Fe^{+}]\text{-}N{=}O$
Sydnonimines e.g. Molsidomine	
S-Nitrosothiols e.g. *S*-Nitrosoglutathione *S*-Nitroso-*N*-Acetyl penicillamine	$R_3C\text{-}S\text{-}N{=}O$
Nucleophile/NO adducts (NONOates) e.g. Diethylamine/NO Spermine/NO	$R_2N\text{-}[N(O)NO]^{-}$

Although a chemically heterogeneous group of compounds the nitrovasodilators have one major property in common in that they exert their pharmacodynamic effects *via* the release of NO. All the compounds differ in their requirement of specific cofactors required for their biotransformation and the rate and extent of formation of metabolites other than NO.

In addition to the main groups quoted here there are other less often used groups of NO donors which includes *N*-nitrosoamines, hydroxylamine, Roussin's Black salt and oxadiazoles.

1.5.1 Organic Nitrates

Organic nitrates are nitric acid esters of mono- and polyhydric aliphatic and aromatic alcohols. Several aliphatic nitrates are used as propellants and explosives. Glyceryl trinitrate (GTN), one of the more widely used $RONO_2$, was first introduced as a treatment for angina pectoris more than a century ago. (Murrell, 1879).

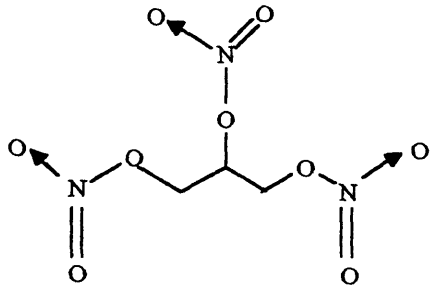


Figure 1.4 Structure of glyceryl trinitrate.

Organic nitrates are metabolised to NO by a combination of enzymatic and non-enzymatic processes. The activity of glutathione-*S*-transferase (Lau *et al*, 1992; Hill *et al*, 1992) and NADPH-dependent cytochrome P_{450} related enzymes is thought to be involved (Servent *et al*, 1989; McDonald and Bennett, 1990), however the contribution of these pathways has been questioned. (Sakanashi *et al*, 1991; Liu *et al*, 1993; Haefeli *et al*, 1993).

Thiols present in the cytosol are likely to account for a major part of non-enzymatic nitrate metabolism. Sulphydryl-containing compounds, such as cysteine, have been shown to react with $RONO_2$ to form inorganic nitrite ions and NO (Feelisch *et al*, 1988). In the course of this reaction thiols are oxidised to their respective disulphides, and nitrite, but under physiological conditions only a few (e.g. cysteine, *N*-acetylcysteine, but not glutathione) produce NO concomitantly (Feelisch and Noack, 1987; Feelisch *et al*, 1988; Chong and Fung, 1991). One of most likely mechanisms for the generation of NO from $RONO_2$ is the formation of a thionitrate arising from the transesterification between the organic nitrate and a thiol compound (equations 1.10 - 1.14) (Feelisch, 1993; Feelisch and Stamler, 1996).

Equation 1.10. $RONO_2 + R'S^- \rightarrow R'SNO_2 + RO^-$

Equation 1.11. $RO^- + H_2O \rightleftharpoons ROH + OH^-$

Equation 1.12. $R'SNO_2 + RS^- \rightarrow R'SSR + NO_2^-$

Equation 1.13. $R'SNO_2 \rightleftharpoons RSONO \rightarrow RSO^{\cdot} + NO^{\cdot}$

Equation 1.14. $RSONO + R'S^- \rightarrow R'SNO + RSO^-$

The main drawback of the clinical use of $RONO_2$ is the impairment of bioactivity on long-term administration *in vivo* (nitrate tolerance) which may be due to depletion of intracellular thiol concentration or down-regulation of the enzymes involved (Feelisch, 1993).

Although NO itself is a potent inhibitor of platelet aggregation $RONO_2$ do not inhibit platelet activation *in vitro*, for platelets do not appear to be able to metabolise these compounds to produce NO. Vascular smooth muscle cells will carry out this transformation and a proportion of the NO will be transferred to the platelets (Benjamin *et al*, 1991) hence these drugs may exhibit a small degree of anti-platelet activity *in vivo*.

1.5.2 Organic Nitrites

Organic nitrites are highly volatile colourless or yellow liquids. The antianginal effects of amyl nitrite were first discovered more than a century ago (Brunton, 1867).

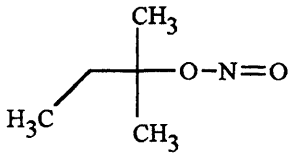


Figure 1.5 Structure of amyl nitrite.

Organic nitrites typically display a rapid onset and short duration of action. They are susceptible to alkaline hydrolysis but in aqueous buffers at pH 7.4, hydrolysis is relatively slow, yielding inorganic nitrite and the corresponding alcohol (equation 1.15).

Equation 1.15 $$RONO + OH^- \rightarrow ROH + NO_2^-$$

Under these conditions release of NO requires reaction with sulfhydryl groups yielding RSNOs, which are active intermediates in this process. The rates of NO release are a function of the rate of formation and metabolism of the RSNO involved (Feelisch, 1991; Ignarro *et al*, 1981; Feelisch and Stamler, 1996). In contrast with organic nitrates, RONO's lack requirement for specific thiols as coactivators. *S*-Nitrosothiols have not been detected *in vivo*, but this may reflect rapid metabolism of RSNOs or alternative pathways of RONO metabolism which are not known (Feelisch and Stamler, 1996).

1.5.3 Iron Nitrosyls

The most widely studied of the iron nitrosyls is sodium nitroprusside (SNP) an inorganic complex where the iron is in the ferrous state and NO is formally bound as NO^+.

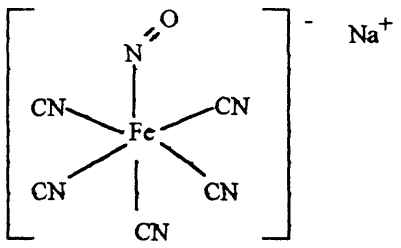
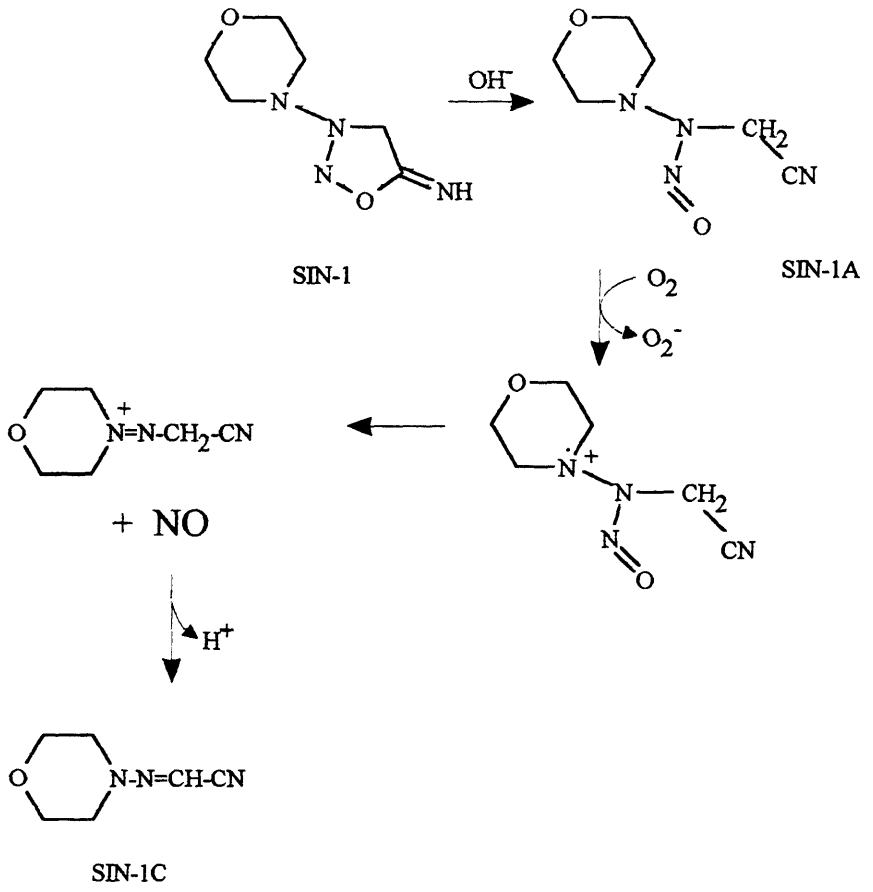


Figure 1.6 Structure of sodium nitroprusside.

This compound is used clinically to reduce blood pressure in hypertensive crises and to minimise blood loss during surgery. Sodium nitroprusside spontaneously releases NO by an, as yet, unknown mechanism, but it is clear that SNP requires either irradiation with light or one-electron reduction to release NO (Singh *et al*, 1995; Arnold *et al*, 1984). Loss of cyanide, which appears to be obligatory for the release of NO, limits the dose and duration of administration (Michenfelder and Tinker, 1977).

1.5.4 Sydnonimines

Sydnonimines are a class of heterocyclic NO donor. The most studied representative of this class is molsidomine (*N*-ethoxycarbonyl-3-morpholinosydnonimine), which is in use as an anti-anginal agent. Molsidomine is an *N*-acyl-containing prodrug which is converted by liver esterases to the active metabolite, SIN-1 (3-morpholino-sydnonimine) (Tanayama, *et al*, 1974). At physiological pH SIN-1 undergoes rapid nonenzymatic hydrolysis to the ring-open form SIN-1A. Traces of oxygen promote oxidative conversion to a cation radical intermediate, which, upon release of NO undergoes spontaneous cleavage to the corresponding *N*-morpholino-aminoacetonitrile, SIN-1C (equation 1.16) (Feelisch and Stamler, 1996).



Equation 1.16. Pathway of nitric oxide formation from SIN-1

In the course of this reaction stoichiometric amounts of superoxide anions (O_2^-) are formed as a result of oxygen reduction (Feelisch *et al*, 1989) and hence results in the generation of $ONOO^-$. This may lead to a number of side reactions, for example the simultaneous production of NO and O_2^- from SIN-1 was shown to generate hydroxyl radicals ($^{\cdot}OH$) or a species with hydroxyl radical-like reactivity and to promote lipid peroxidation (Hogg *et al*, 1992; Darley-Usmar *et al*, 1992). Moreover H^+ ions are also generated which may lead to a considerable decrease in pH if solutions are left unbuffered (Feelisch and Stamler, 1996).

Kankaanranta *et al* (1997) have recently shown that SIN-1 inhibits human neutrophil degradation in a cGMP-, $ONOO^-$- and NO-independent manner, suggesting the need to carry out control experiments with NO-deficient metabolites when evaluating sydnonimine NO-donors.

1.5.5 Nucleophile/Nitric Oxide Adducts (NONOates)

Nucleophile/NO adducts are formed by reacting NO gas with strong nucleophiles such as diethylamine, at low temperatures (Maragos *et al*, 1991). The products are generally stable as solids but decompose in solution at a rate that appears to depend on pH, temperature and the identity of the nucleophile (Morley *et al*, 1993a). Nucleophile/NO adducts have been shown to relax vascular smooth muscle in an endothelium-independent manner, to increase tissue cGMP and to inhibit platelet aggregation *in vitro* (Morley *et al*, 1993b; Maragos *et al*, 1993; Mooradian *et al*, 1995), these activities correlating well with the measured rates of NO release. Diethylamine NONOate (DEA/ NO) and spermine NONOate (SPER/NO) have shown a similar vasodilatory potency without any acute toxic effects in an *in vivo* model (Diodati *et al*, 1993).

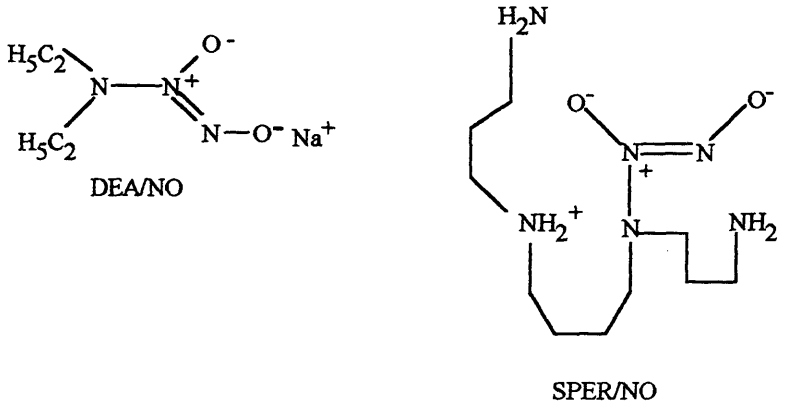


Figure 1.7 Structures of NO/nucleophile adducts.

It is thought that NONOates generate NO spontaneously without requirement for electron transfer, cofactors or metabolic activation (Morley *et al*, 1993b). The rate of NO generation from NONOates in a biological system can be chemically predicted and adjusted by altering the carrier nucleophile.

Nucleophile/NO adducts have proved to be valuable research tools since they predictably and quantifiably release NO both *in vitro* and *in vivo.*

1.5.6 S-Nitrosothiols

It has been suggested that RSNOs are endogenous intermediates of the physiological actions of NO or even the identity of EDRF itself (Myers *et al*, 1990). *S*-Nitrosylation of proteins, in particular *S*-nitrosoalbumin, and low molecular weight thiols such as cysteine has been shown to occur *in vitro* and *in vivo* (Stamler *et al*, 1992b; Stamler *et al*, 1992c; Keaney *et al*, 1993; Gaston *et al*, 1993). More recently Jia *et al* (1996) have described a role for *S*-nitrosohaemoglobin in the transduction of NO-related activities. It has been suggested that NO reacts with the SH groups of the cysteine residues on the β sub unit of haemoglobin. It has been proposed that a dynamic cycle exists in which haemoglobin is *S*-nitrosylated in the lung when red blood cells are oxygenated and NO is released during arterial-venous transit.

S-Nitrosothiols are thermodynamically and photochemically unstable compounds, undergoing photodecomposition by UV and visible light (Sexton *et al*, 1994). For this reason when using RSNOs, solutions always need to be protected from light and stored on ice. Due in part to their chemical instability, RSNOs have been much less studied than their corresponding oxygen analogues, the alkyl nitrites.

Thiols can be easily reacted with nitrous acid (HNO_2), *via* $NaNO_2$ and HCl, to yield the RSNO (Hart, 1985). A number of other methods are available using nitrosating agents such as NOCl, RONO, NO_2, N_2O_3. *S*-Nitrosothiols can also be synthesised by reacting the thiol directly with NO in an oxygen free atmosphere. Another established method is to react the thiol with dinitrogen tetroxide (N_2O_4) in equimolar amounts at -10°C in an inert solvent such as CCl_4 or hexane (Williams, 1988a; Williams, 1985; Oae and Shinhama, 1983). The products obtained are coloured yellow or red and in some cases green/blue i.e. *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP). Only a few RSNOs have been isolated in solid form namely, *S*-nitroso-L-glutathione (GSNO) (Hart, 1985) and SNAP (Field *et al*, 1978).

S-Nitrosothiols decompose, presumably by homolytic fission, to give the disulphide and nitric oxide (equation 1.17).

Equation 1.17. $2RSNO \rightarrow RSSR + 2NO$

It has been shown that homolytic decomposition of RSNOs is catalysed by trace metals, particularly copper ions (McAninly *et al*, 1993; Askew *et al*, 1995a; Dicks *et al*, 1996), and that the biological activity of some but not all RSNOs can be significantly reduced by chelation of the copper (Gordge *et al*, 1995).

In addition to homolytic cleavage RSNOs can also decompose heterolytically with formation of NO^+, or NO^- (Equations 1.18 and 1.19, Arnelle and Stamler, 1995).

Equation 1.18. $RSNO \rightarrow RS^- + NO^+$

Equation 1.19. $RSNO \rightarrow RS^+ + NO^-$

Thus, depending on the reaction (redox) conditions, RSNOs can act as donors of NO, NO^+ or NO^-. Many groups have claimed that the pharmacological activity of RSNOs does not correlate with the lipophilicity, charge, stability in solution or the rate of spontaneous NO release from individual compounds (Gaston *et al*, 1994; Kowaluk and Fung, 1990; Mathews and Kerr, 1993; Salas *et al*, 1994). Moreover, the rank of potency appears to vary between different assays. Therefore when predicating the biological potency of a series of RSNOs both homolytic decomposition and heterolytic mechanisms such as transnitrosation reactions (Equation 1.20) have to be taken into account as well (Arnelle and Stamler, 1995; Barnett *et al*, 1994; Park *et al*, 1993: Park, 1993). The exact mechanism of transnitrosation has not yet been established. It is believed that such reactions may proceed *via* the transfer of NO^+ from the RSNO to a RSH (Singh *et al*, 1996a). Transnitrosation reactions have also been shown to proceed *via* an attack by the thiolate anion (RS^-) of the RSH at the nitrogen atom of the RSNO (Barnett *et al*, 1994; Barnett *et al*, 1995).

Equation 1.20. $RSNO + R'S^- \rightarrow R'SNO + RS^-$

The influence of thiols on the decomposition of RSNOs is complex. It has been shown in certain instances that excess cysteine will increase the half-life of various RSNOs , probably by complexation of transition metals (Feelisch *et al*, 1994). On the other hand excess thiol can dramatically increase the rate of NO formation from a number of structurally different RSNOs (Feelisch and Stamler, 1996). This latter effect may be a consequence of the redox function of the thiol rather than the result of a transnitrosation reaction.

Thus the stability of RSNOs under physiological conditions varies as a function of thiol group, pH, oxygen tension, redox state and the presence or absence of contaminant transition metals.

1.5.7 Therapeutic Uses of Nitric Oxide Donors

Nitrovasodilators have been in clinical use for more than a century for the treatment of angina (Brunton, 1867; Murrell, 1879) and are still used in numerous conditions today. Cardiovascular conditions commonly treated with NO donors include stable and unstable angina , coronary vasospasm, myocardial infarction and congestive heart failure. The beneficial effects of these drugs in angina results from their dilator action on arterial and venous smooth muscle and from their ability to dilate large coronary vessels thus increasing coronary flow (Moncada and Higgs, 1995).

There is presently much interest in using NO donors in the field of obstetrics and gynaecology in the treatment of pre-eclampsia and pre-term labour. Studies have investigated the use of NO donors on the contractility of uterine smooth muscle. A study has been carried out investigating the use of GTN patches on a group of women in preterm labour. The results showed a small prolongation of the pregnancies without any obvious side effects (Lees *et al*, 1994).

Pre-eclampsia is defined as hypertension with proteinurea limited to the duration of pregnancy. There is evidence of an impairment of NO production in women with pre-eclampsia which may be the cause of the hypertension and the increase in platelet activation observed (Seligman *et al*, 1994). An i.v. infusion of GSNO has been given

to a small group of women with severe pre-eclampsia. The finding of a significant reduction in uterine artery resistance and systemic blood pressure could prove to be of great benefit in the future in treating such disorders (Lees *et al*, 1996; Lees, 1996). GSNO has also been given to a woman with HELLP syndrome. HELLP syndrome is a variant of severe pre-eclampsia characterised by haemolysis, elevated liver enzymes and low platelet count. An increase in blood pressure and a concurrent decrease in blood pressure was observed during and after an i.v. infusion of GSNO (de Belder *et al*, 1995).

S-Nitrosoglutathione has also been shown to inhibit the platelet activation that occurs, despite concurrent treatment with aspirin, GTN and heparin, in patients undergoing percutaneous transluminal coronary angioplasty (Langford *et al*, 1994).

1.6 Aims and Objectives

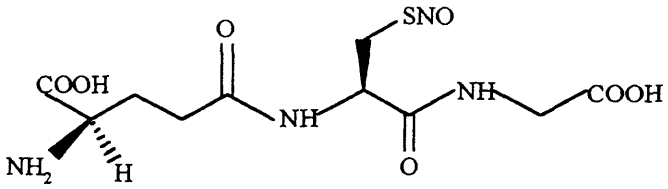
The overall aim of this project was to improve our understanding of the relationship between the chemical and biological properties of RSNOs as nitric oxide donors.

As outlined above there are considerable discrepancies in the relationship between the chemical and biological properties amongst RSNOs. For example *S*-nitrosocysteine (CysNO) in solution is a most unstable RSNO, whilst GSNO is a reasonably stable compound (Mathews and Kerr, 1993). A major aim of this work was to investigate how the structural differences between these two compounds may explain the drastic discrepancy in their stability. To that end molecules intermediate in size to these two RSNOs were synthesised and investigated with particular emphasis on the dipeptides *S*-nitroso-L-cysteinylglycine (CysGlyNO) and *S*-nitroso-L-γ-glutamyl-L-cysteine (GluCysNO) which are related to GSNO. *S*-Nitroso-L-cysteinylglycine and GluCysNO were thought to help determine how the molecular environment of the *S*-NO moiety in GSNO affects the thionitrite stability.

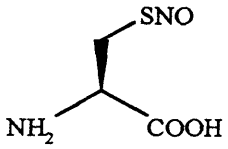
S-Nitrosoglutathione is an avid donor of NO *in vivo* despite its considerable chemical stability. It has been suggested that the difference in stability of GSNO between an

extracellular and intracellular environment might implicate a role for an enzyme in the denitrosation of GSNO, perhaps with a degree of selectivity (Radomski *et al*, 1992; de Belder *et al*, 1994). One such enzyme could be γ-glutamyl transpeptidase (GGT). γ-Glutamyl transpeptidase cleaves the glutamyl group in glutathione (GSH) and also in GSNO (Askew *et al*, 1995b). In the case of GSNO this would generate the *S*-nitrosylated dipeptide, CysGlyNO, which might have a much shorter half-life than GSNO itself (Askew *et al*, 1995b), hence release NO more readily. As GGT has a natural specificity for L-amino acids then by synthesising the D-isomer of GSNO (D-GSNO), it should be possible to investigate the role of GGT in the breakdown of GSNO. Decreased release of NO from D-GSNO, as compared to L-GSNO, would support a role for GGT in the de-nitrosation of GSNO.

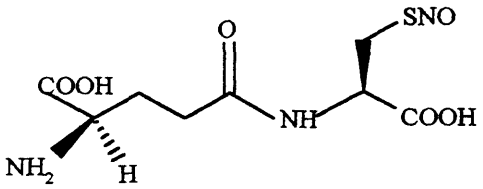
Furthermore, the *S*-nitrosylated forms of a range of amino acid and peptide thiols related to glutathione (GSH) and cysteine (CysH) were synthesised (figure 1.8), and their stabilities and biological activities were studied. The differences in stability were compared to the differences in the pharmacological and toxicological activity and attempts were made to establish a correlation between such properties.



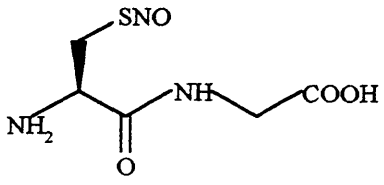
S-Nitroso-L-glutathione
(GSNO)



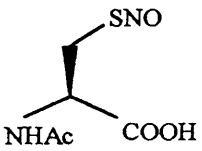
S-Nitroso-L-cysteine
(CysNO)



S-Nitroso-L-γ-glutamyl-L-cysteine
(GluCysNO)



S-Nitroso-L-cysteinylglycine
(CysGlyNO)



S-Nitroso-*N*-acetyl-L-cysteine
(SNAC)

$S(NO)CH_2CH_2COOH$

S-Nitroso-3-mercaptopropionic acid
(SNPROPA)

$S(NO)CH_2CH_2SO_3^-Na^+$

S-Nitroso-2-mercaptoethanesulphonic acid
(MESNO)

Figure 1.8 Structure of *S*-nitrosothiols investigated.

Two derivatives, *S*-nitroso-3-mercaptopropionic acid (SNPROPA) and *S*-nitroso-2-mercapto-ethanesulphonic acid (MESNO) lack the amino acid function. *S*-Nitroso-2-mercapto-ethanesulphonic acid also lacks a carboxylic acid function. The reason for preparing these compounds was to study the mechanism by which trace amounts of copper catalyse the decomposition of certain RSNOs (Askew *et al*, 1995a; Dicks *et al*, 1996). It has been proposed that copper catalysed decomposition of RSNOs proceeds *via* the formation of Cu^+ from Cu^{2+} which then co-ordinates with the RSNO between the N of the NO group and another electron-rich atom such as the N of an amino group forming a stable ring structure which may allow expulsion of NO from

the molecule (Dicks *et al*, 1996). This hypothesis was tested by investigating MESNO and SNPROPA, which lack the ability to form a stable ring structure with Cu^{+}. The *N*-acetylated form of CysNO, *S*-nitroso-*N*-acetyl-L-cysteine (SNAC) was prepared to determine the effect of copper on CysNO when the amino group is substituted.

In addition endogenous thiols have been implicated as playing a key role in the biological activity of RSNOs *via* transnitrosation reactions (Singh *et al*, 1996a). It has been suggested that transnitrosation reactions can stimulate RSNO decomposition if the resulting RSNO is more susceptible to metal-ion catalysed decomposition than the parent RSNO. This hypothesis was investigated by studying the decomposition of several RSNOs in the presence of GSH or CysH.

Chapter 2

Synthesis of Peptides

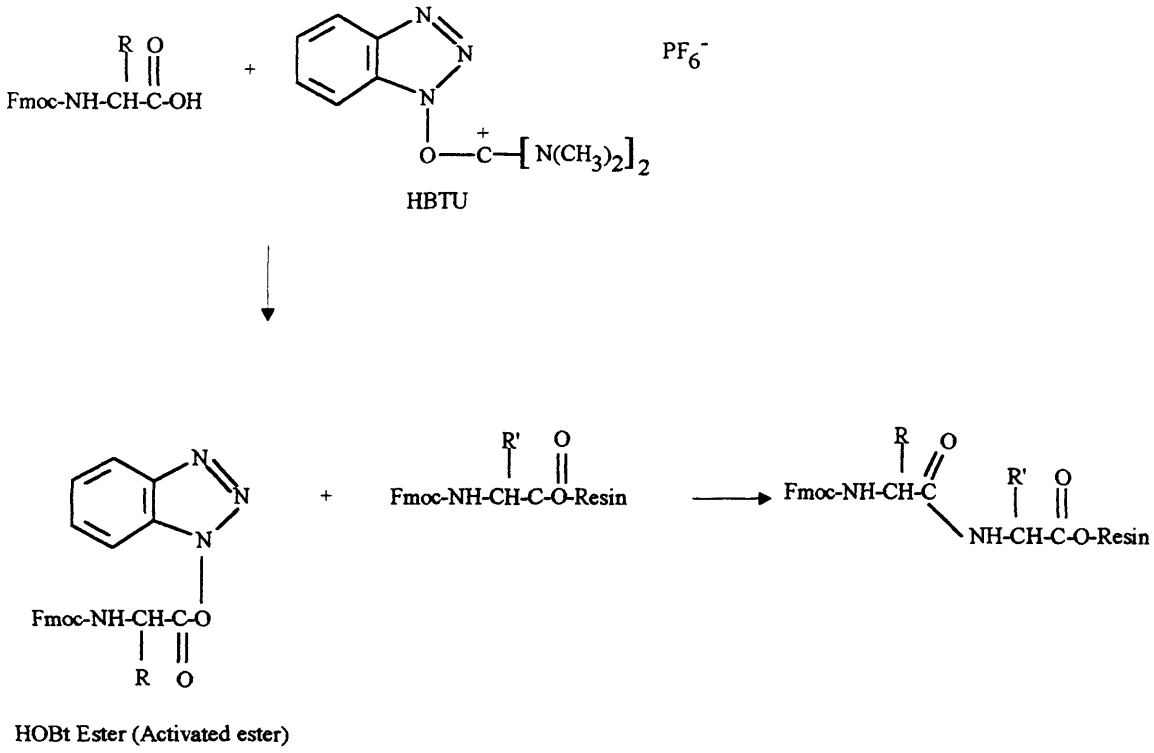
2.1 Introduction

One major aim of this project was to determine how the chemical and biological properties of the *S*-nitrosylated forms of the two glutathione related dipeptides, CysGlyNO and γ-GluCysNO, compared with GSNO and CysNO. In order to accomplish this aim the two glutathione dipeptides CysGly and γ-GluCys were synthesised as detailed (section 2.3).

An additional aim of this thesis was to investigate the role of GGT in the de-nitrosation of GSNO (Radomski *et al*, 1992; de Belder *et al*, 1994). This investigation was to be carried out by synthesising the D-isomer of GSNO. D-Glutathione was synthesised as detailed (section 2.4).

The synthetic routes for all the peptides in question made use of solid phase methods. The basic strategy of solid phase peptide synthesis involves the attachment of the C-terminal amino acid to a "solid" support and the addition of subsequent amino acids in a stepwise manner (Merrifield, 1963). All unreacted soluble reagents can be removed by simple filtration and washing techniques. As the peptide chain "grows" from the C-terminal protection of the amino group needs to be temporary, that is removable after formation of each peptide bond. The use of the acid-labile Boc group and the Fmoc group are popular choices. In addition the Fmoc group is readily removed *via* β-elimination initiated by a secondary organic base such as piperidine which allows the use of acid-sensitive protecting groups for side chains (Carpino and Han, 1972) as demonstrated in this project.

In general for the amide bond to occur the carboxyl entity being coupled with the growing peptide chain needs to be activated. All the synthetic procedures made use of 1-hydroxybenzotriazole /2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HOBt / HBTU) coupling techniques which form an active ester of the incoming amino acid (equation 2.1). Generally HBTU can be used alone but an equivalent of HOBt is added to improve coupling efficiencies and rates (Novabiochem, 1997).



Equation 2.1. Coupling of amino acids *via* HBTU activation.

Once the required chain has been accomplished the crude peptide is cleaved from the solid support, followed by purification and extensive characterisation steps to ensure the correct sequence has been achieved (Barany *et al*, 1987).

2.2 General Methods

2.2.1 Spectroscopic Measurements

NMR spectra were carried out on Bruker 250 and 400 MHz instruments (^{1}H at 250 MHz, ^{13}C at 63 MHz and ^{19}F at 400 MHz). Chemical shifts are shown in ppm (integral, multiplicity and assignment).

Mass spectra were recorded on a VG70 SEQ mass spectrometer. All experiments were carried out in positive fast atom bombardment (FAB) mode.

2.2.2 Preparative HPLC Purification

All HPLC experiments were carried out on a Beckmann System Gold HPLC.

HPLC separation of γ-GluCys and CysGly from other synthetic intermediates was accomplished using a Spherisorb ODS2 preparative HPLC column (25cm x 21.2mm) with a mobile phase of 10% v/v methanol/ 90% v/v 0.1%v/v TFA at a flow rate of 6 ml min^{-1}. UV detection at 210nm was used. Fractions were collected and submitted for positive ion FAB mass spectral analysis and ^1H NMR analysis.

HPLC separation of D-GSH from other synthetic intermediates was accomplished using a Spherisorb ODS1 preparative HPLC column (25cm x 21.2mm) with a mobile phase of 0.1%v/v heptafluorobutyric acid at a flow of 9ml min^{-1}. UV detection at 210nm was used. Fractions were collected and submitted for positive ion FAB mass spectral analysis and ^1H NMR analysis.

2.2.3 HPLC Analysis of Peptides

HPLC analysis of CysGly was accomplished using a Lichrosphere 100RP-18 analytical HPLC column (25cm x 5mm) with a mobile phase of 0.1%v/v TFA at a flow rate of 1 ml min^{-1}. HPLC analysis of γ-GluCys was accomplished using a Lichrosphere 100RP-18 analytical column (25cm x 5mm) with a mobile phase of 90 % 0.1% v/v HFBA/10% methanol. UV detection at 210nm was used.

HPLC analysis of D-GSH was accomplished using a Lichrosphere 100RP-18 analytical HPLC column (25cm x 5mm) with a mobile phase of 0.1%v/v HFBA at a flow rate of 1 ml min^{-1}. UV detection at 210nm was used.

2.2.4 Measurement of Optical Rotation

The optical rotation was measured on a Perkin Elmer 341 polarimeter at 589nm using a Na/HCl lamp. The temperature at which the measurements were made are specified

in the results. Concentration and solvent used for each individual sample are also indicated in the results.

2.3 Synthesis of Cysteinylglycine and γ-Glutamylcysteine

L-Cysteinylglycine and GluCys were both synthesised using the Fmoc-solid phase strategy as shown (figures 2.1 and 2.2 respectively).

2.3.1 Experimental

Fmoc-AA1-Wang resin (AA1:Fmoc-Cys(Trt)-Wang resin for γ-GluCys and Fmoc-Gly-Wang resin for CysGly; weight equivalent to 0.25mmole AA1) was suspended in 20%v/v piperidine/N-methyl pyrrolidone (NMP, 25ml) in order to remove the Fmoc groups. The reaction mixture was left stirring at room temperature for 1hr. The resin was then filtered through Whatman Number 1 filter paper and washed, firstly with NMP (10x5ml) followed by of dichloromethane (CH_2Cl_2, 5x5ml), and left to dry at room temperature.

Fmoc-AA2 (1mmole) was dissolved in NMP (2.1ml) (AA2: Fmoc-Glu-O^{t}Bu for γ-GluCys, Fmoc-Cys(Trt)OPfp for CysGly). HOBt/HBTU mixture (2.2ml, 0.45M of each in DMF) was added to the mixture and left stirring at room temperature for 10 min. AA1-Wang resin and di-isopropylethylamine (DIEA, 0.34ml) were added to the mixture and left stirring at room temperature for 1hr. This yielded the fully protected dipeptide. The protected dipeptide-resin was then filtered and washed with NMP (10x5ml) followed by CH_2Cl_2 (5x5ml), and left to dry at room temperature.

The dipeptide resin was suspended in 20%v/v piperidine/ NMP, (25ml) to remove N-terminal Fmoc groups. The reaction mixture was left stirring at room temperature for 1hr. The resin was then filtered through Whatman Number 1 filter paper and washed, firstly with NMP (10x5ml) followed by dichloromethane (CH_2Cl_2, 5x5ml), and left to dry at room temperature.

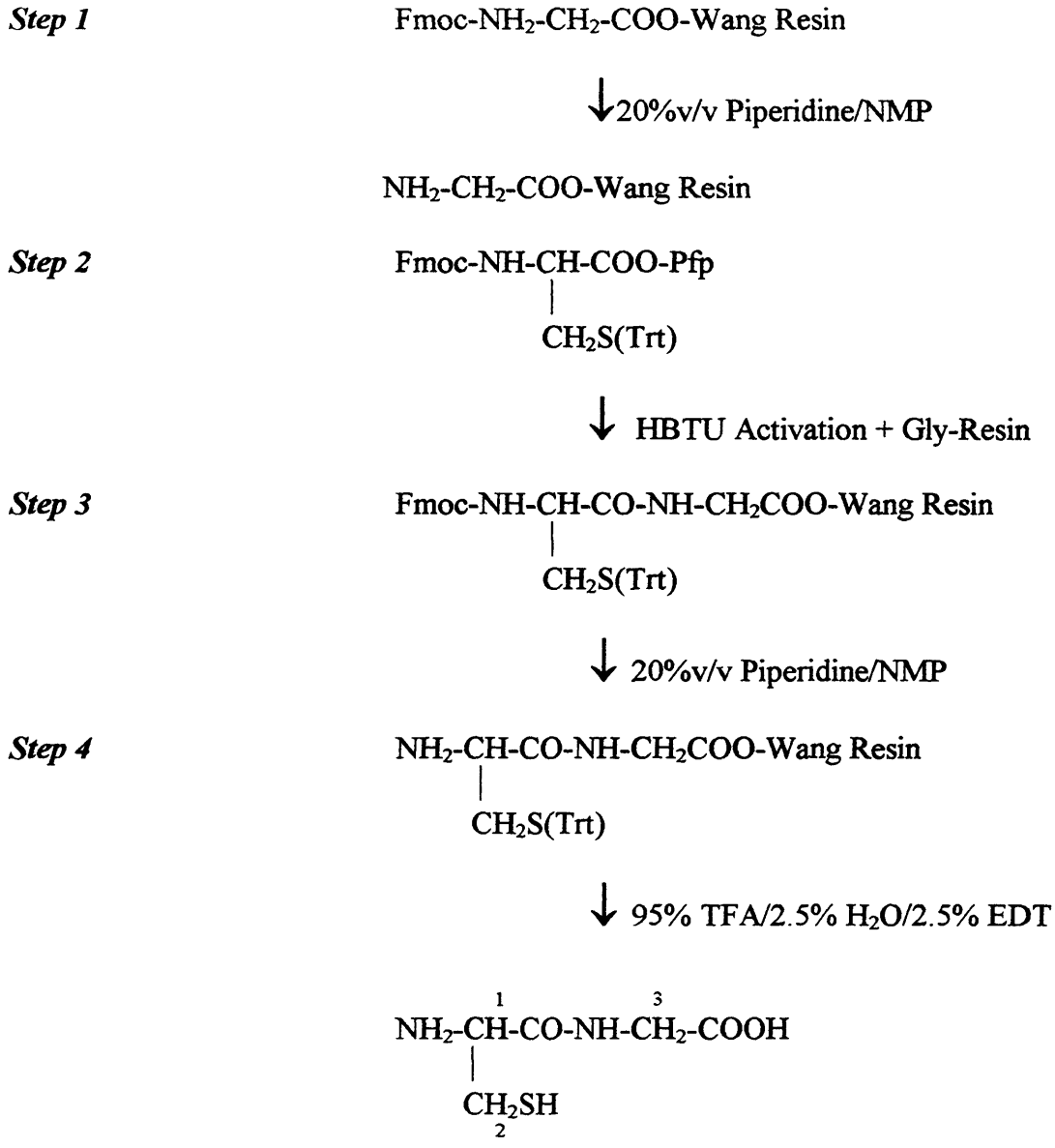


Figure 2.1 Reaction scheme followed in the synthesis of the dipeptide CysGly.

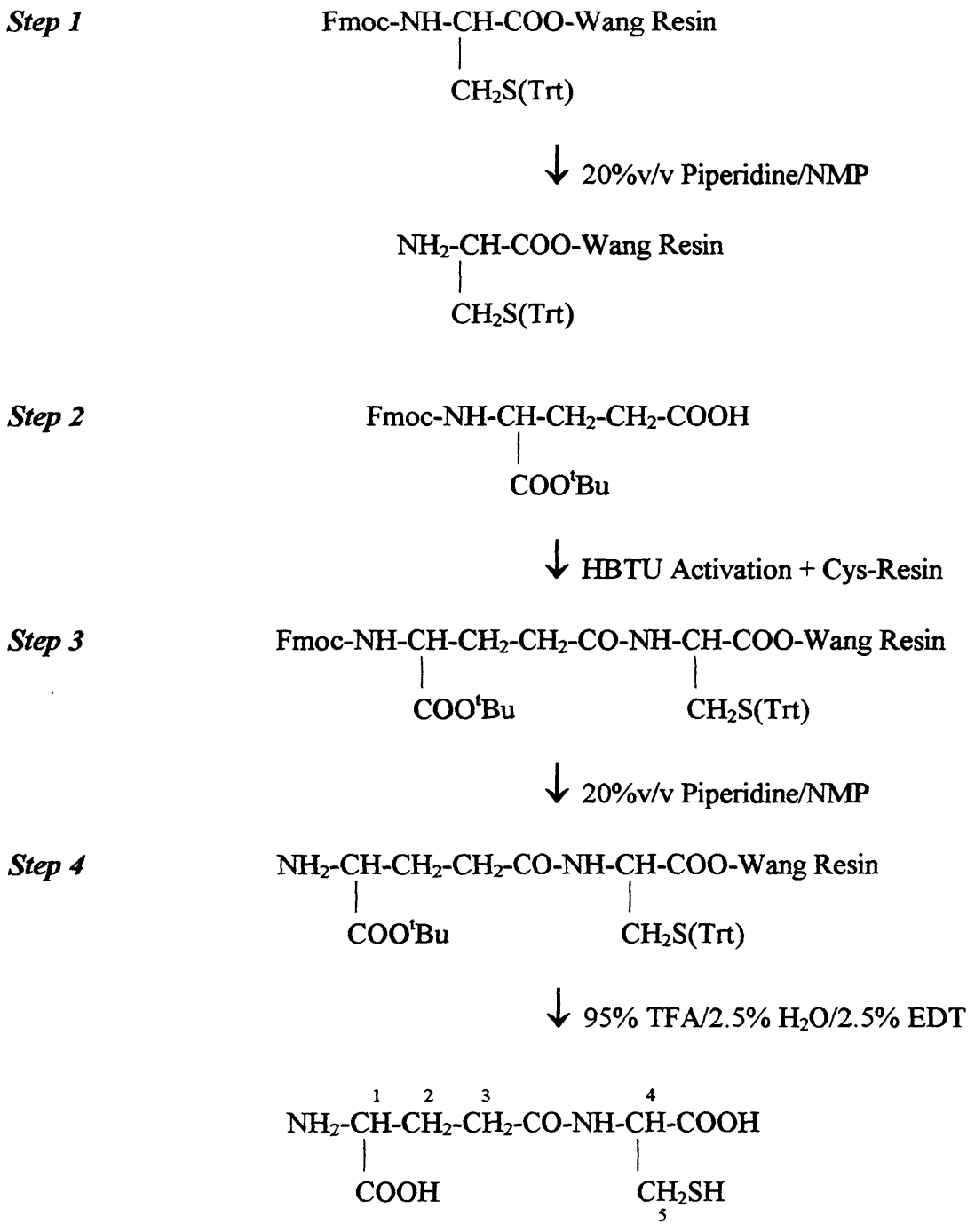


Figure 2.2 Reaction scheme followed in the synthesis of the dipeptide γ-GluCys.

The dipeptide-resin was then suspended in a mixture consisting of 95%v/v trifluoroacetic acid (TFA, 4.75ml), 2.5%v/v water (125µl), 2.5%v/v ethanedithiol (EDT, 125µl). The mixture was left stirring at room temperature for 3 hr. This step removed Trt and ^{t}Bu groups and also cleaved the peptide from the resin.

The dipeptide was filtered through glass wool. The filtered resin was washed with TFA to ensure all the dipeptide had been removed.

An ether extraction was carried out on the supernatant. Water (15 ml) and diethyl ether (15ml) were added to the cleavage mixture in a separating funnel. The two layers were allowed to separate and the lower layer (water) was decanted off into a clean flask. A further portion of ether was added to the water layer and the process was repeated until the aqueous layer no longer had an immediate odour of ethanedithiol present. After the final wash the aqueous layer was evaporated down to dryness overnight in a freeze-drier to yield a pale yellow gum. The yellow coloration was due to the presence of trace amounts of trityl groups. Each dipeptide was cleaned up using preparative HPLC as detailed (section 2.2.2). The final products were submitted for NMR and mass spectral analysis.

CysGly

δ_H (D_2O)

2.95 (2H, doublet, H-2), 3.85 (2H, singlet, H-3), 4.15 (1H, triplet, H-1)

(See figure 2.1 for proton numbering)

δ_C (D_2O)

C: 168.6, 174.3

CH: 54.7

CH_2: 25.2, 42.2

FAB *m/z* 179 (MH^+, 100%), 76

HPLC analysis of CysGly was carried out as detailed (section 2.2.3). The results showed the main peak at 8.68min. (90.2% by peak area).

γ-GluCys

δ_H (D_2O)

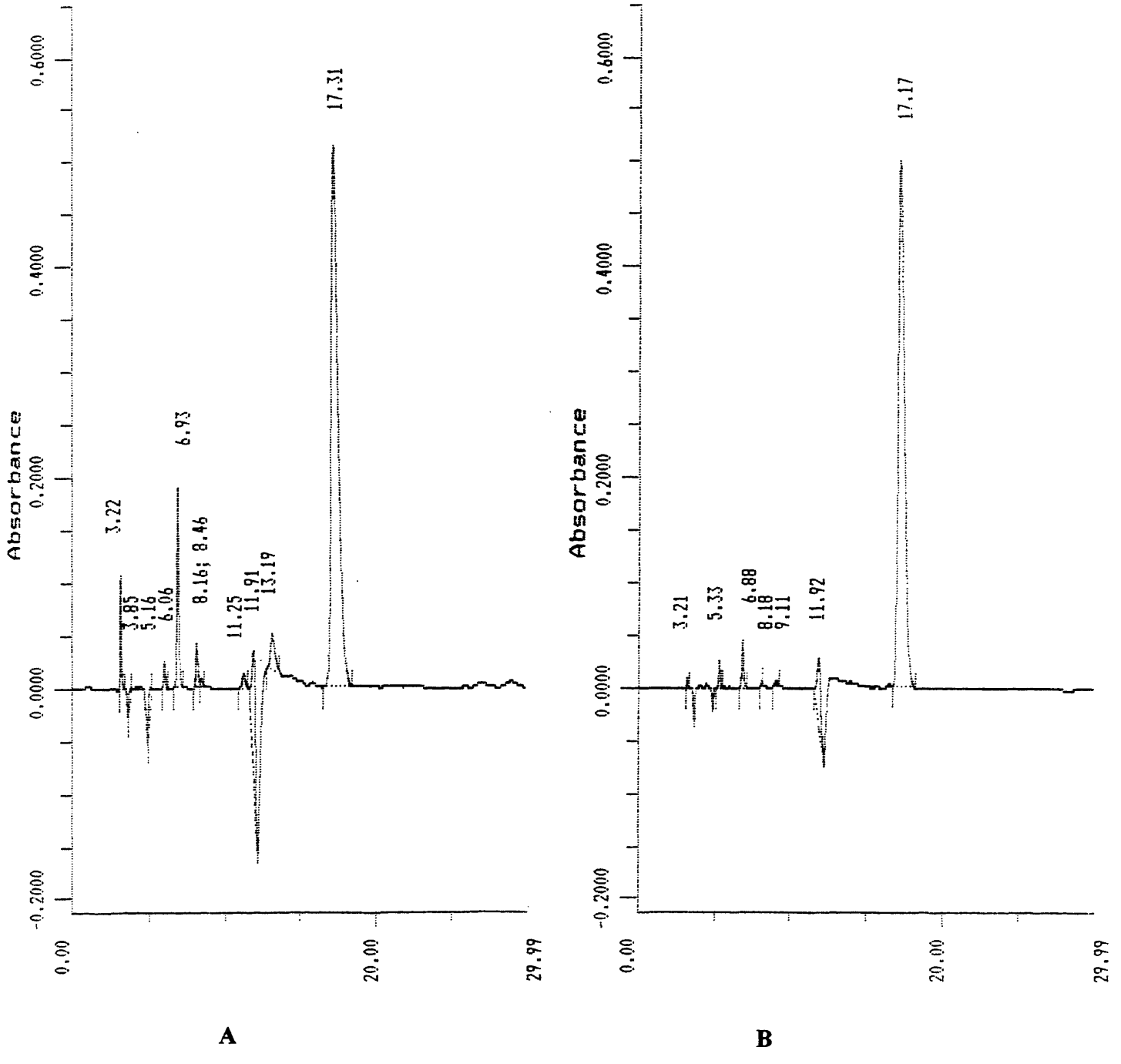
2.1 (2H, multiplet, H-2), 2.5 (2H, multiplet, H-3), 2.9 (2H, doublet, H-5) 3.95 (1H, triplet, H-1) 4.5,(1H, triplet, H-4).

A COSY spectrum establishes the coupling between nuclei and gives evidence of through-bond interactions. A COSY spectrum confirmed the assignments made. (See figure 2.2.for proton numbering)

FAB *m/z* 251 (MH^+, 100%), 86, 115, 131, 223, 245, 337.
The majority of the additional signals showed the presence of potassium and sodium.

HPLC analysis of γ-GluCys as detailed (section 2.2.3) showed the main peak at 17.3min (79.2% by peak area). Many problems were experienced in obtaining a suitable preparative HPLC system for the purification of γ-GluCys. In order to ensure an acceptable clean-up procedure had been obtained a sample of γ-GluCys *ex* Sigma was also analysed and as shown (figure 2.3) displayed the same chromatographic profile as the sample synthesised in-house (R_T =17.2min, 87.3% by peak area).

Figure 2.3 Chromatographic profile of γ-GluCys (A) Synthetic sample (B) *ex* Sigma.



2.4 Synthesis of D-Glutathione

D-Glutathione was synthesised using the Boc-solid phase strategy (figure 2.4).

2.4.1 Experimental

Boc-Gly-MBHA resin (weight equivalent to 0.25mmole of amino acid) was suspended in 50%v/v TFA/CH_2Cl_2 (10ml) in order to remove the Boc groups. The mixture was left stirring at room temperature for 4 hr.

The resin was filtered through Whatman Number 1 filter paper and washed with CH_2Cl_2 (5 x 5ml), methanol (5x5ml) and finally CH_2Cl_2 (5x 5ml). The resin was left to dry at room temperature in a desiccator.

Fmoc-D-Cys(Trt)-OH (1mmole) was dissolved in NMP (2.1ml). HOBt/HBTU mixture (2.2ml, 0.45M of each in DMF) was added and the mixture was left stirring at room temperature for 10 minutes. Gly-MBHA resin and DIEA (0.34ml) were added to the mixture and left stirring at room temperature for 3 hr. The dipeptide was then filtered through number 1 filter paper and washed with NMP (5x5ml) and CH_2Cl_2 (5x 5ml). The CysGly-resin was then left to dry at room temperature.

Fmoc-D-Cys(Trt)-Gly-MBHA resin was suspended in 20% v/v piperidine in NMP (25ml) to remove terminal Fmoc groups. The resin was then filtered as detailed in section 2.3.1 and left to dry at room temperature.

Boc-D-Glu-OBzl (1mmole) was coupled to the amino terminal of D-Cys(Trt)-Gly-MBHA resin *via* HBTU activation as previously described. This yielded the fully protected tripeptide, Boc-D-Glu-OBzl-D-Cys(Trt)-Gly-MBHA resin.

Step 1 BOC-NH-CH_2-COO-MBHA Resin

↓50%v/v TFA/CH_2Cl_2

NH_2-CH_2-COO-MBHA Resin

Step 2 Fmoc-NH-CH-COOH
|
CH_2S(Trt)

↓ HBTU Activation + Gly-Resin

Step 3 Fmoc-NH-CH-CO-NH-CH_2-COO-MBHA Resin
|
CH_2S(Trt)

↓ 20%v/v Piperidine/NMP

Step 4 NH_2-CH-CO-NH-CH_2-COO-MBHA Resin
|
CH_2S(Trt)

↓ HBTU Activation
+ BOC-NH-CH-CH_2-CH_2-COOH
|
COOBzl

Step 5 BOC-NH-CH-CH_2-CH_2-CO-NH-CH-CO-NH-CH_2-COO-MBHA Resin
| |
COOBzl CH_2S(Trt)

↓50% v/v TFA/CH_2Cl_2 (EDT)

Step 6 NH_2-CH-CH_2-CH_2-CO-NH-CH-CO-NH-CH_2-COO-MBHA Resin
| |
COOBzl CH_2S(Trt)

plus some trityl de-protected material

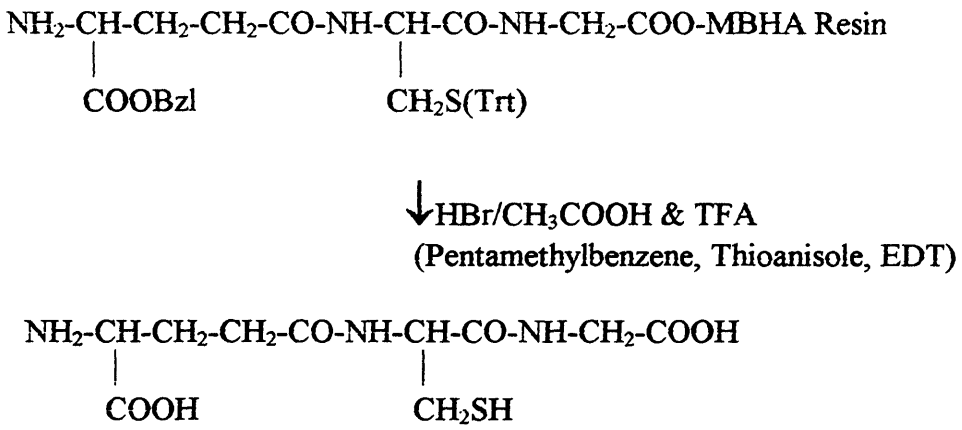


Figure 2.4 Reaction scheme followed in the synthesis of D-GSH.

The final product was in the form of the HFBA salt (figure 2.5) following HPLC purification using HFBA in the mobile phase. The equivalent L-GSH salt was obtained by reacting equimolar amounts of GSH in water and HFBA and freeze drying down overnight. This compound was used for comparison with the D-GSH obtained

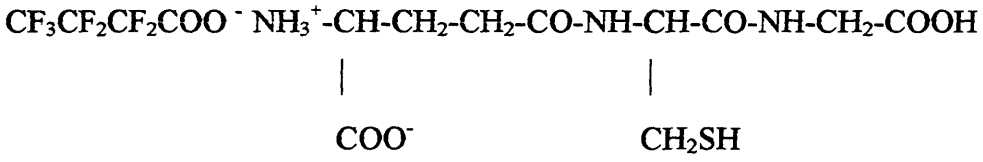


Figure 2.5 Expected structure of the D-GSH-HFBA salt formed.

Boc-D-Glu-OBzl-D-Cys(Trt)-Gly-MBHA resin was suspended in a solution of 50% v/v TFA/ CH_2Cl_2 (10ml) plus 200µl of EDT to remove the Boc/Trt groups. The mixture was left stirring at room temperature for 2 hr. The resin was filtered through Whatman number 1 filter paper and washed with CH_2Cl_2 (5x5ml), methanol (5x5ml) and finally CH_2Cl_2 (5x5ml). The resin was left to dry at room temperature.

The protected D-GSH-resin was suspended in a mixture consisting of TFA (10ml), hydrobromic acid in acetic acid (1ml), pentamethylbenzene (0.46g), thioanisole (600µl) and EDT (200µl) in order to remove all remaining protective groups and to cleave the tripeptide from the resin. The mixture was left stirring unstoppered overnight.

The D-GSH mixture was then filtered through glass wool. The filtered resin was washed with TFA to ensure all the tripeptide had been removed.

An ether extraction was carried out on the supernatant (section 2.3.1).
The resulting D-GSH was cleaned up using preparative HPLC (section 2.2.2) and submitted for NMR and mass spectral analysis.

δ_H (D_20) (figure 2.6)
2.15 (2H, triplet, H-2), 2.55 (2H, triplet, H-3), 2.9 (2H, doublet, H-5), 3.85 (2H, singlet, H-6), 3.95 (1H, triplet, H-1), 4.45 (1H, triplet, H-4)

The COSY spectrum shown (figure 2.7) confirmed the assignments made.

δ_C (D_20) (figure 2.8)
C: 172.5, 173.1, 174.3, 175
CH: 53.3, 56.3
CH_2: 25.5, 26.0, 31.3, 42.5

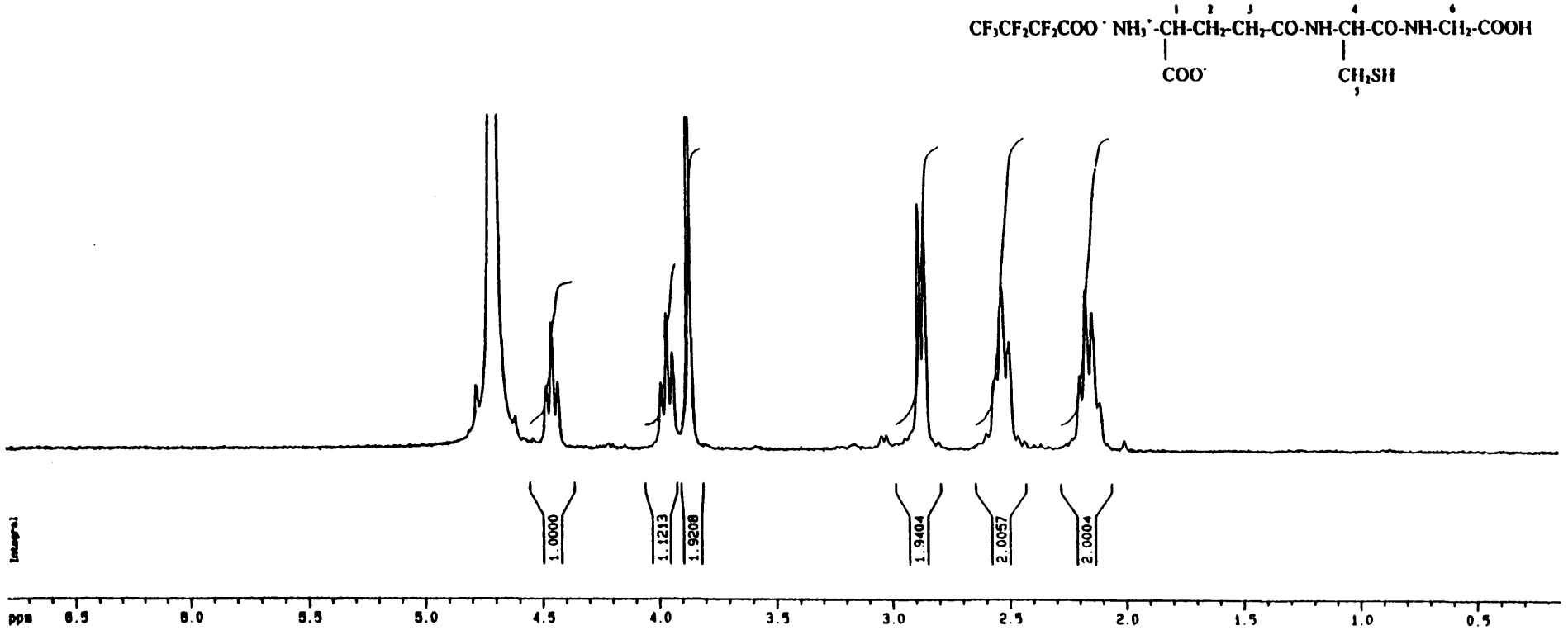
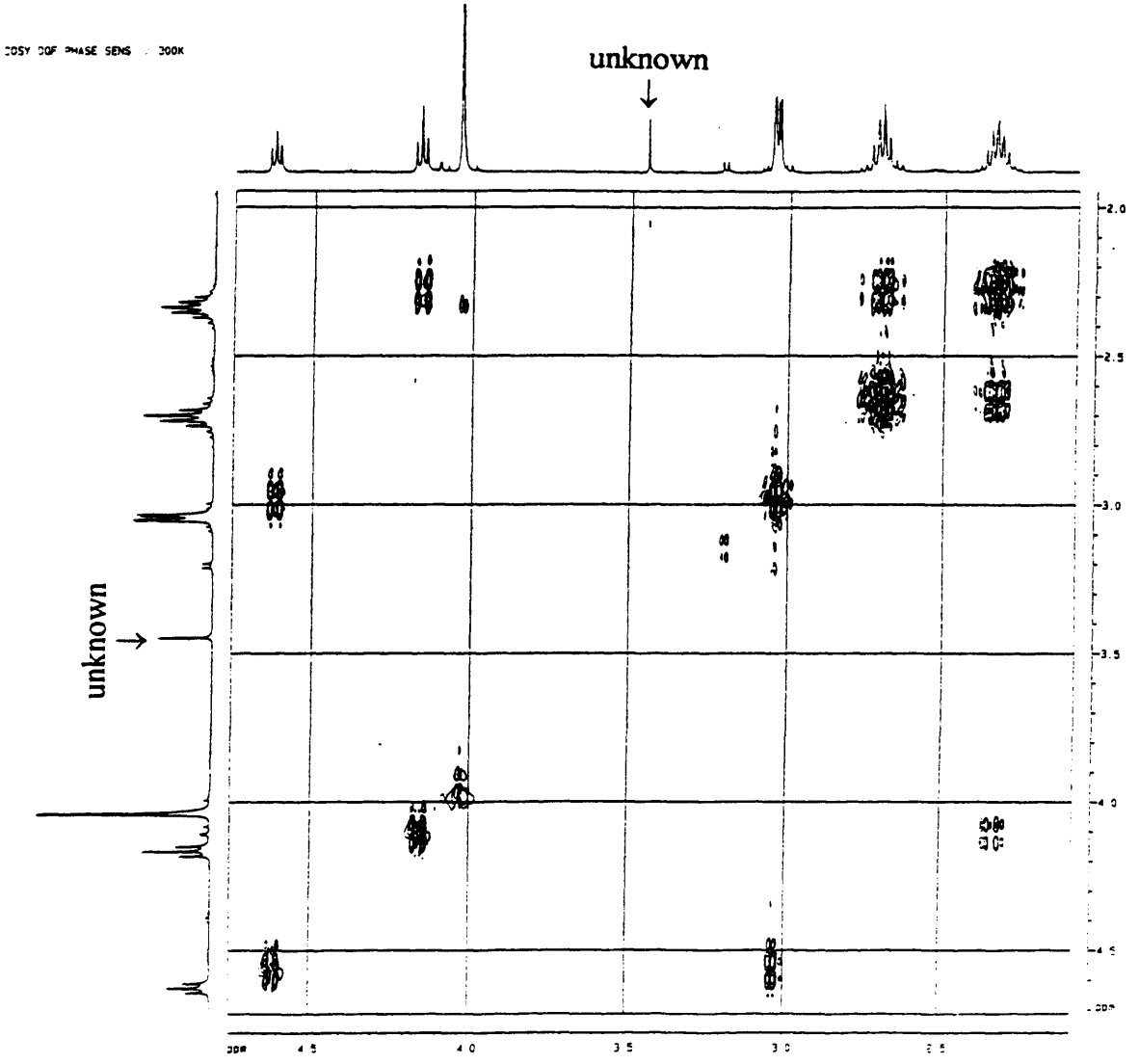


Figure 2.6 ^{1}H-NMR Spectrum of D-GSH.

Figure 2.7 COSY-NMR Spectrum of D-GSH



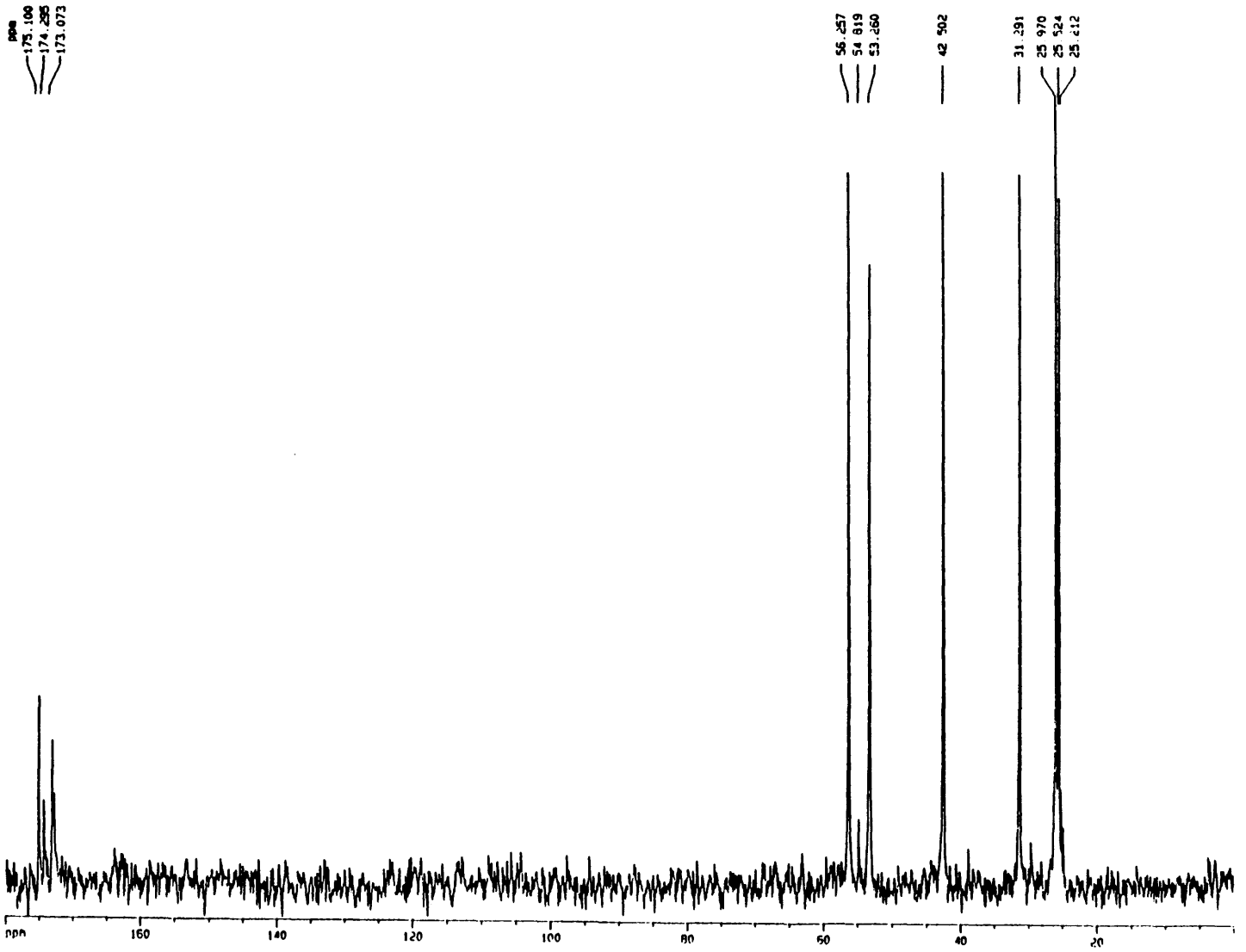


Figure 2.8 ^{13}C-NMR Spectrum of D-GSH.

δ_F (D_2O) (figure 2.9)

-128 (2F, singlet, F-3), -119 (2F, quartet, F-2), -82 (3F, triplet, F-1)

The spectrum shown confirmed that the final product was in the form of the heptafluorobutyrate salt.

FAB *m/z* 307 (M^+, 6%), 345 (80%), 131, 151, 223, 242, 437

The majority of the additional signals showed the presence of potassium and sodium

i.e. 115 = glycerol + Na

131 = glycerol + K

223 = (2 x glycerol) + K

The optical rotation of D-GSH was determined as detailed (section 2.2.4).

L-GSH $[\alpha]_D^{18}$ -17.21° (c=2, H_2O)

D-GSH $[\alpha]_D^{19}$ +5.21° (c=2, H_2O)

These results indicate that the D-GSH synthesised may not be the D,D-isomer as expected.

An Ellman's test for free thiols was carried out on batches of D-GSH synthesised. 5,5' Dithio-bis(2-nitrobenzoic) acid (DTNB, 4mg ml^{-1}) was prepared in Ellman's buffer. A standard solution of L-GSH (1mg ml^{-1}) was prepared in water. This was further diluted in water to give concentrations of 0.5, 0.25 and 0.1mg ml^{-1}. A standard solution of D-GSH (0.5mg ml^{-1}) was also prepared in water.

A 50µl aliquot of each standard and DTNB solution was added to Ellman's buffer (1ml). The resulting solution was left to incubate at room temperature for 15min. The UV absorbance of each mixture was read at 412nm.

A calibration curve for L-GSH was constructed by plotting absorbance against concentration on Microcal Origin v 2.8. The concentration of thiol in D-GSH was determined from the calibration line.

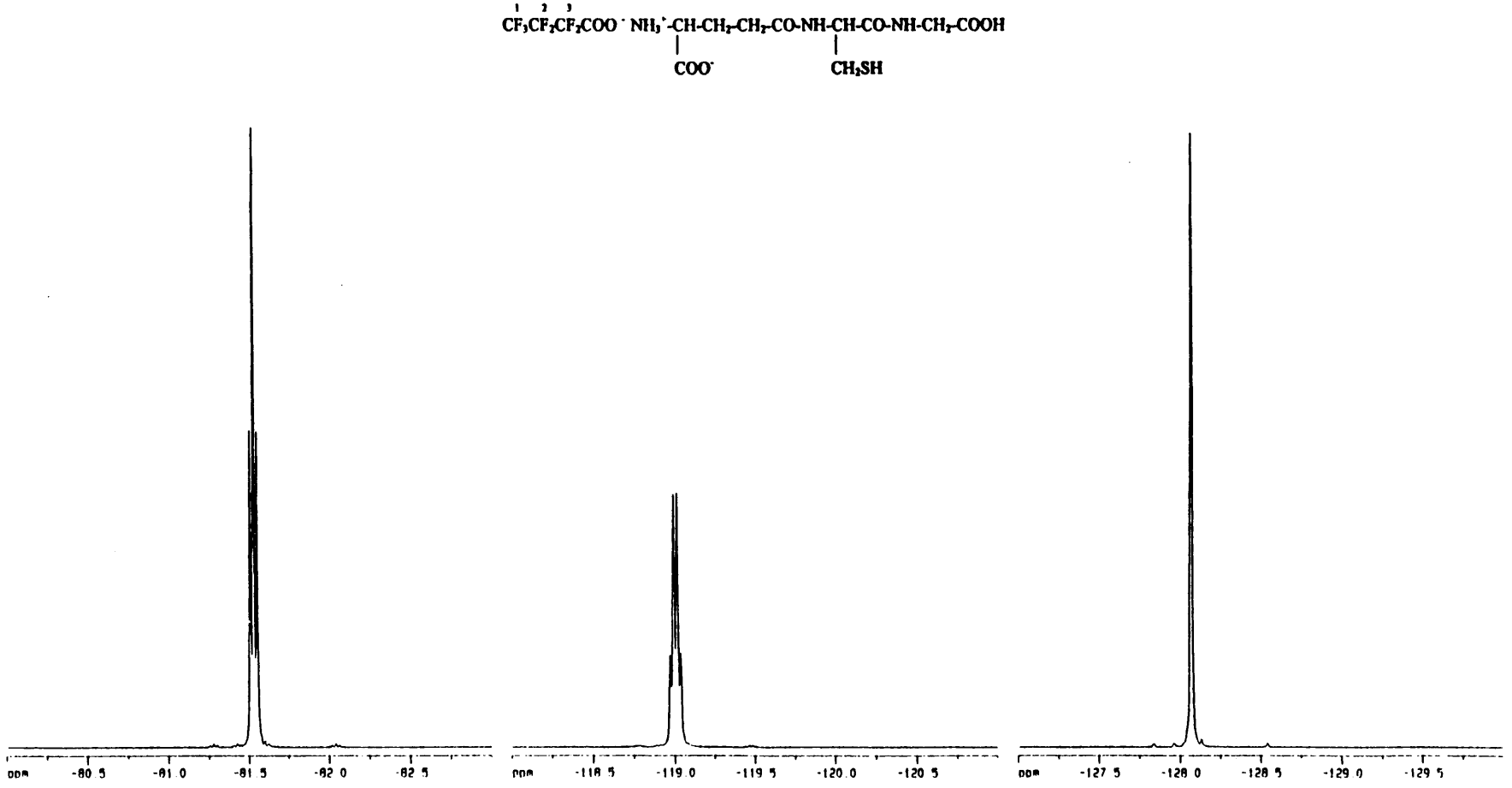


Figure 2.9 ^{19}F-NMR Spectrum of D-GSH-HFBA salt.

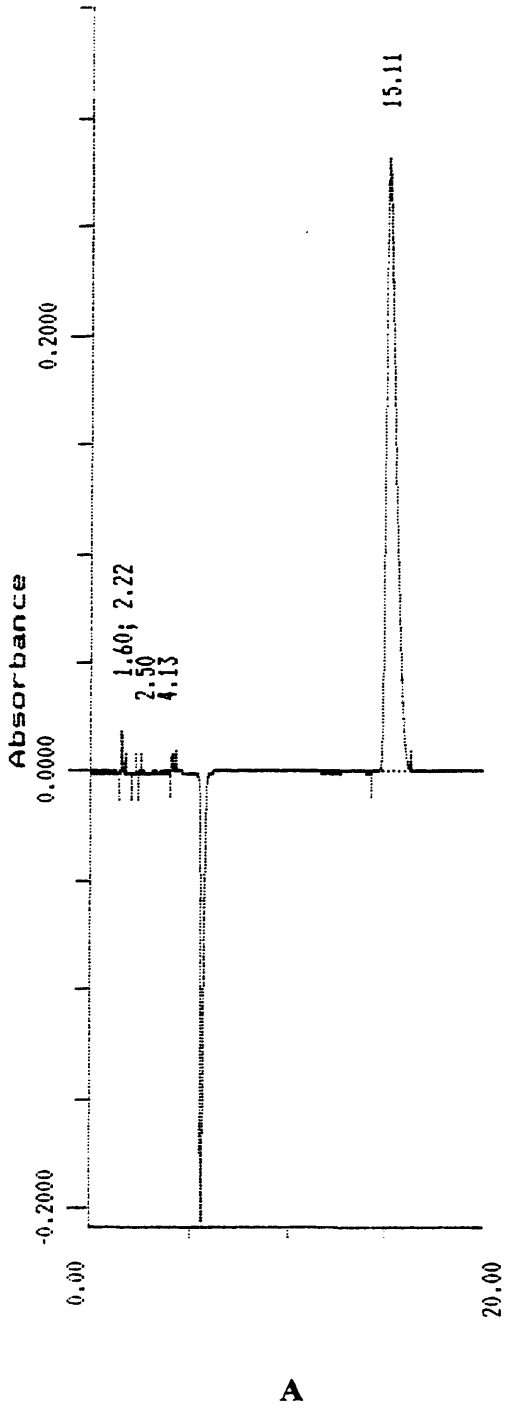
A positive yellow colour was observed for each batch of D-GSH tested indicating the presence of free thiol groups. The results showed a >90% yield of thiol groups as compared to the expected value. These results confirmed that the D-GSH synthesised was not oxidised. The final product was also nitrosated *via* acidified nitrite (section 3.2) to give the corresponding RSNO. UV spectral analysis gave the expected $\lambda_{max.}$ of an RSNO. This result suggests that the thiol group in the D-GSH was reduced.

HPLC analysis of D-GSH and L-GSH was carried out (section 2.2.3).
Sample chromatograms for the above compounds are shown (figure 2.10).

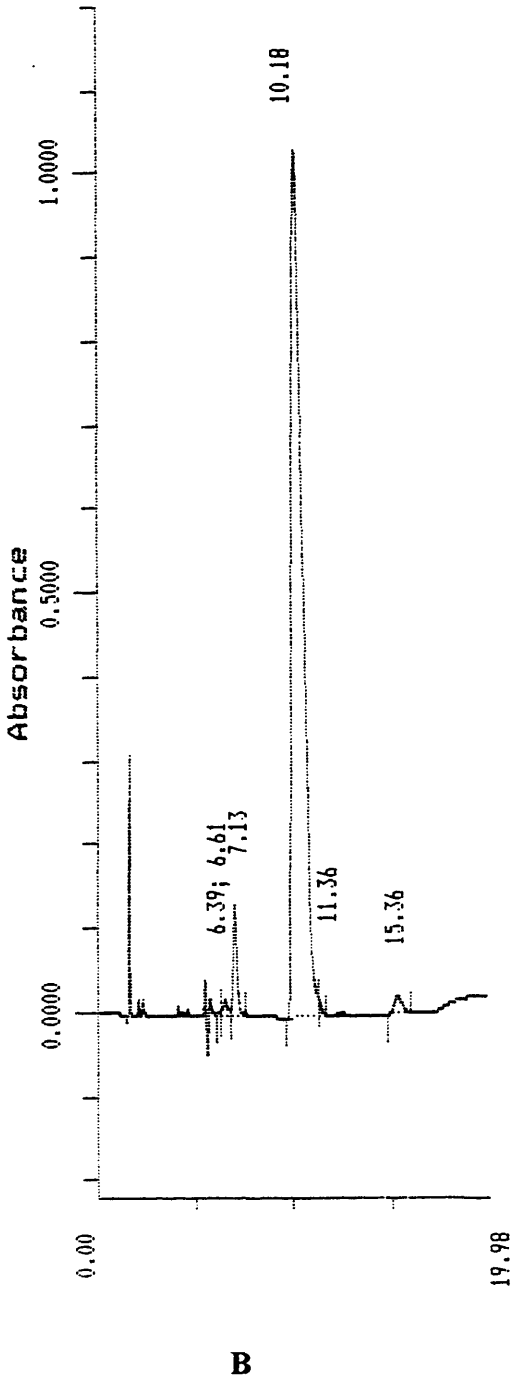
D-GSH R_T of main peak = 10.2 min (93.5% by peak area)
L-GSH R_T of main peak = 15.1 min (99% by peak area)

This is one of the main pieces of evidence that suggests that the compound synthesised was not D-GSH.

Figure 2.10 Chromatographic profile of (A) L-GSH (B) D-GSH.



A



B

2.5 Investigation into the Identity of D-GSH

As is apparent from the results already quoted the D-GSH synthesised did not appear to be the correct material. There are a number of possibilities has to where the synthetic route may have gone wrong. One of the most obvious explanations is that one or both of the chiral centres may have undergone racemization during synthesis. Another possibility is that the wrong starting materials may have been provided.
In order to investigate this problem further the following experiments were performed:

The optical rotation of the following starting materials was determined (section 2.2.4) in order to ensure the compounds supplied were of the correct chirality.

Boc-Glu-OBzl	$[\alpha]_D^{26}$ -31.43° (c=1, MeOH)
Boc-D-Glu-OBzl	$[\alpha]_D^{27}$ +30.19° (c=1, MeOH)
Fmoc-Cys(Trt)-OH	$[\alpha]_D^{25}$ +17.51° (c=1, DMF)
Fmoc-D-Cys(Trt)-OH	$[\alpha]_D^{26}$ -21.79° (c=1, DMF)

In addition there is also the possibility that the γ-protected glutamic acid had been supplied which would result in α-GluCysGly being synthesised instead of glutathione which is γ-GluCysGly. In order to investigate this problem the NMR spectrum of the two dipeptides, γ-GluVal and α-GluVal were compared to each other and to the D-GSH synthesised to determine if there was any change in shift of any of the signals (figure 2.11)

γ-GluVal

δ_H (D_2O) (figure 2.11)

0.85 (6H, doublet-doublet, H-6, H-7), 2.1 (2H + 1H, multiplet, H-2, H-5), 2.5 (2H, multiplet, H-3), 4.0 (1H, triplet, H-1) and 4.1ppm (1H, doublet, H-4).

α-GluVal

δ_H (D_2O) (figure 2.11)

0.85 (6H, doublet-doublet, H-6, H-7), 2.1 (1H+2H, multiplet, H-2, H-5), 2.5 (2H, multiplet, H-3), 4.1 (1H, triplet, H-1) and 4.2ppm (3H, doublet, H-4).

All of the signals where in the same position for both compounds except for the two signals at 4 and 4.1ppm in γ-GluVal have been shifted slightly down-field in α-GluVal.

In addition a second batch of Boc-D-Glu-OBzl was obtained from a second supplier and the ^{1}H-NMR spectra was compared to that obtained from the original supplier. The spectra shown (figure 2.12) are identical suggesting that the original batch obtained was correct.

L-Glutathione and L-GSSG were also submitted for ^{1}H-NMR analysis (section 2.2.1). These spectrum shown were used to compare with spectra obtained from D-GSH (figures 2.13 and 2.14).

L-GSH

δ_H (D_2O) (figure 2.13)

2.15 (2H, quartet, H-2), 2.6 (2H, multiplet, H-3), 2.95 (2H, multiplet, H-5), 3.85 (1H, triplet, H-1) and 4.0ppm (2H, singlet, H-6). There is thought to be an additional signal under the D_2O signal responsible for 1H (H-4)

L-GSSG

δ_H (D_2O) (figure 2.14)

2.45 (2H, quartet, H-2), 2.8 (2H, multiplet, H-3,), 3.3 (1H, quartet/doublet-doublet, H-5), 3.5 (1H, doublet-doublet, H-5), 4.1 (1H, triplet, H-1), 4.25 (2H, singlet, H-6) and 5.0ppm (1H, quartet, H-4).

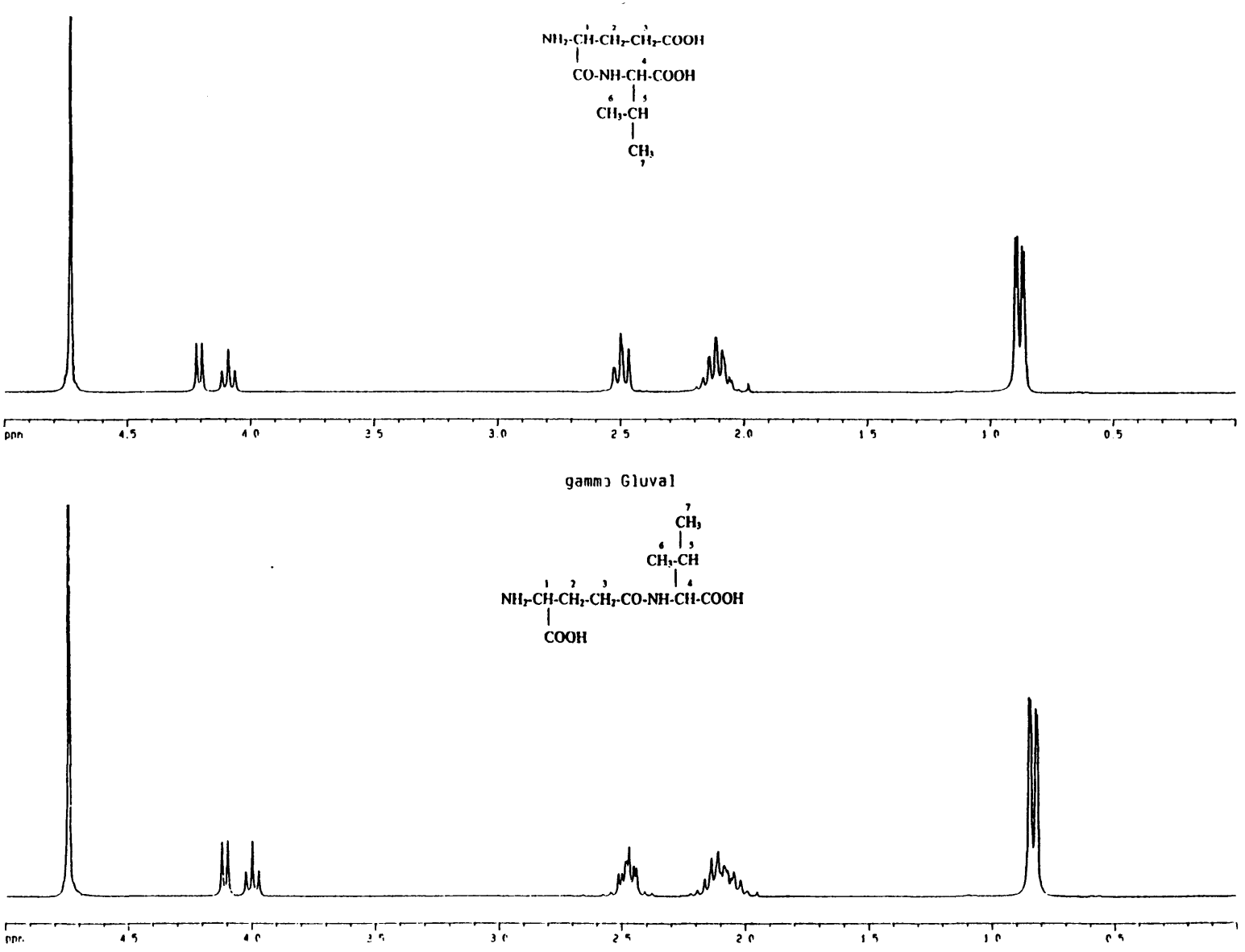


Figure 2.11 ^{1}H-NMR Spectrum of α and γ-GluVal.

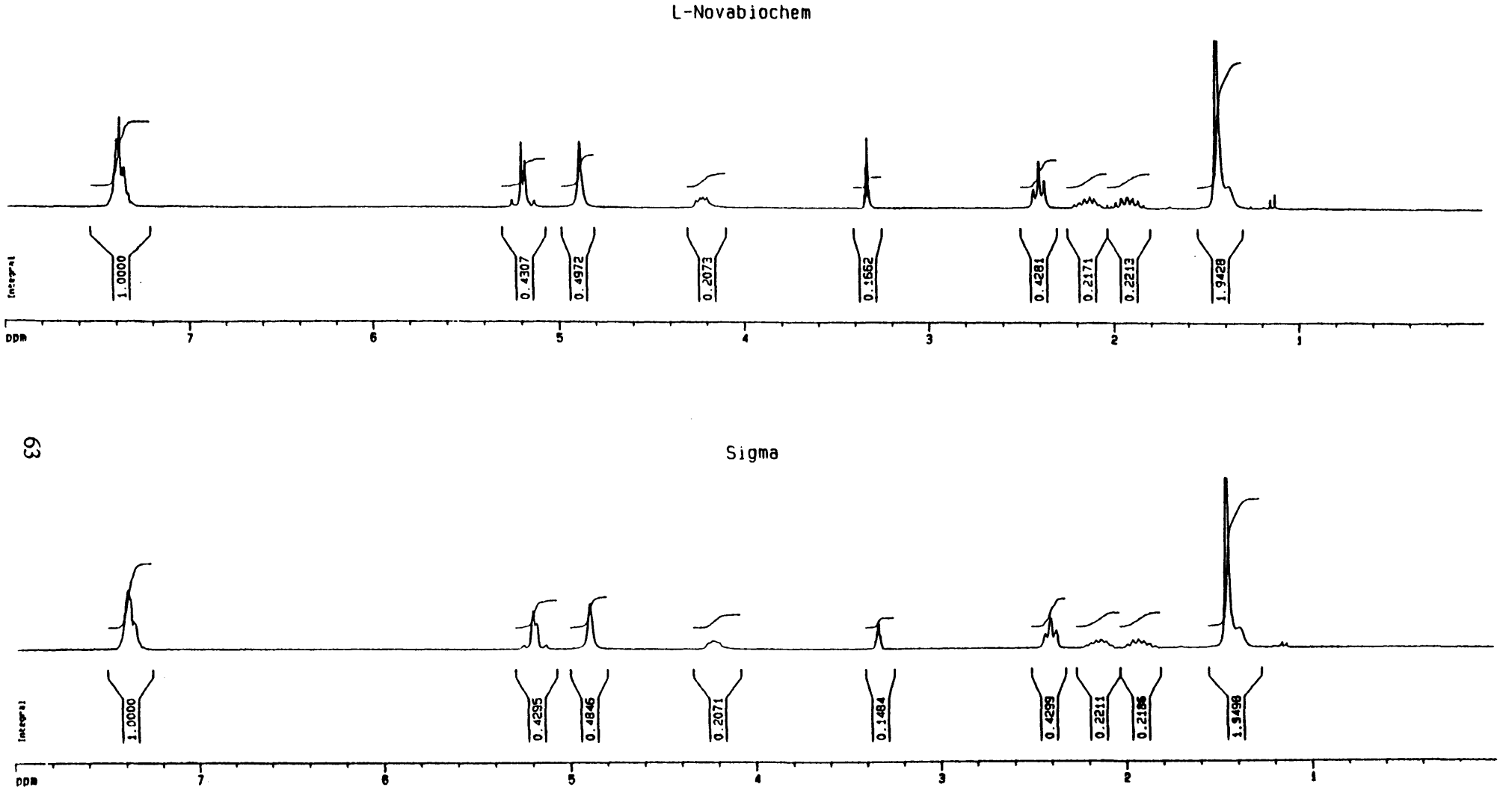


Figure 2.12 Comparison of ^{1}H-NMR spectrum of separate batches of Boc-D-Glu-OBzl.

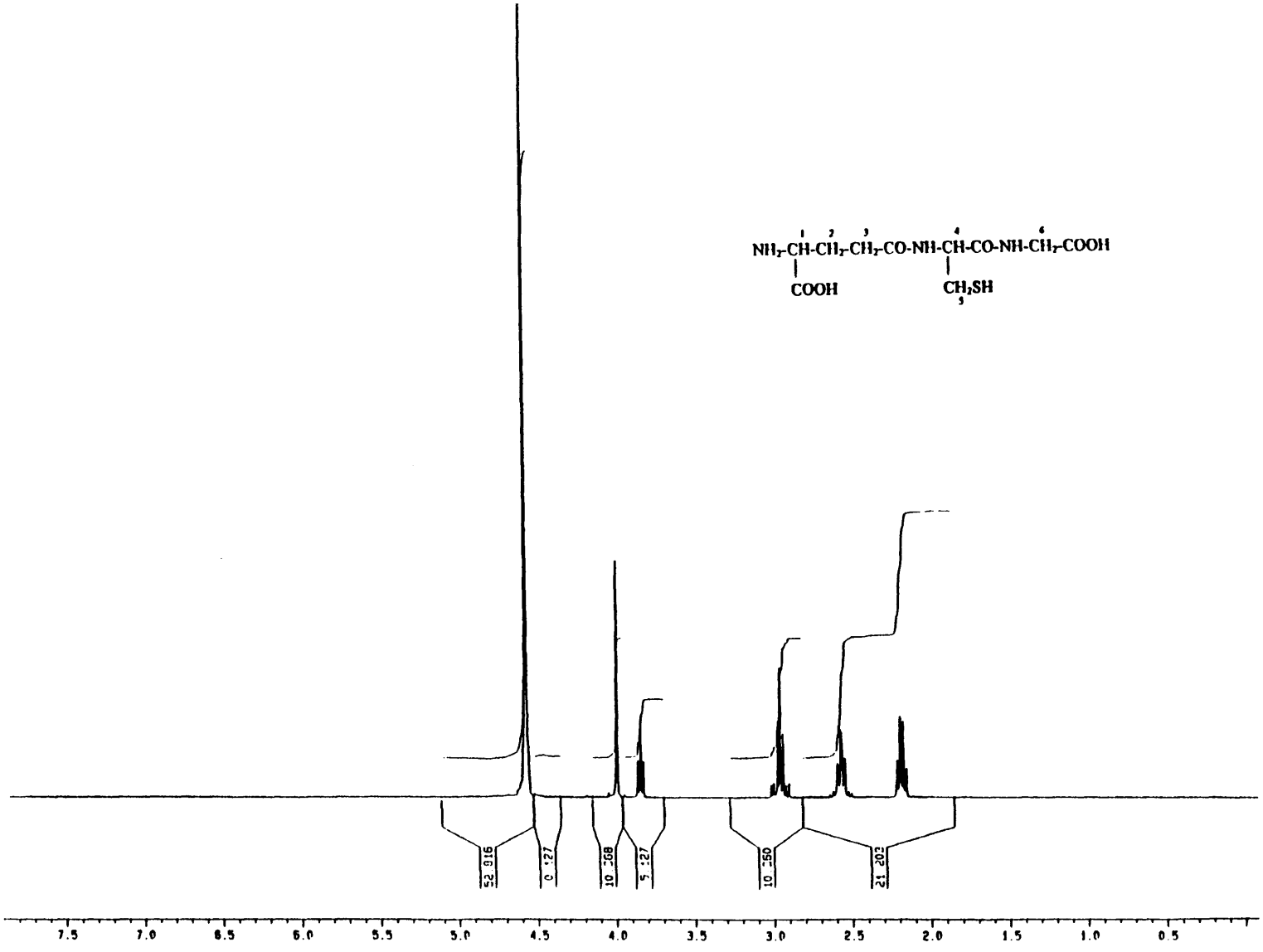


Figure 2.13 ^{1}H-NMR Spectrum of L-GSH.

SPECIAL NOTE

THIS ITEM IS BOUND IN SUCH A MANNER AND WHILE EVERY EFFORT HAS BEEN MADE TO REPRODUCE THE CENTRES, FORCE WOULD RESULT IN DAMAGE

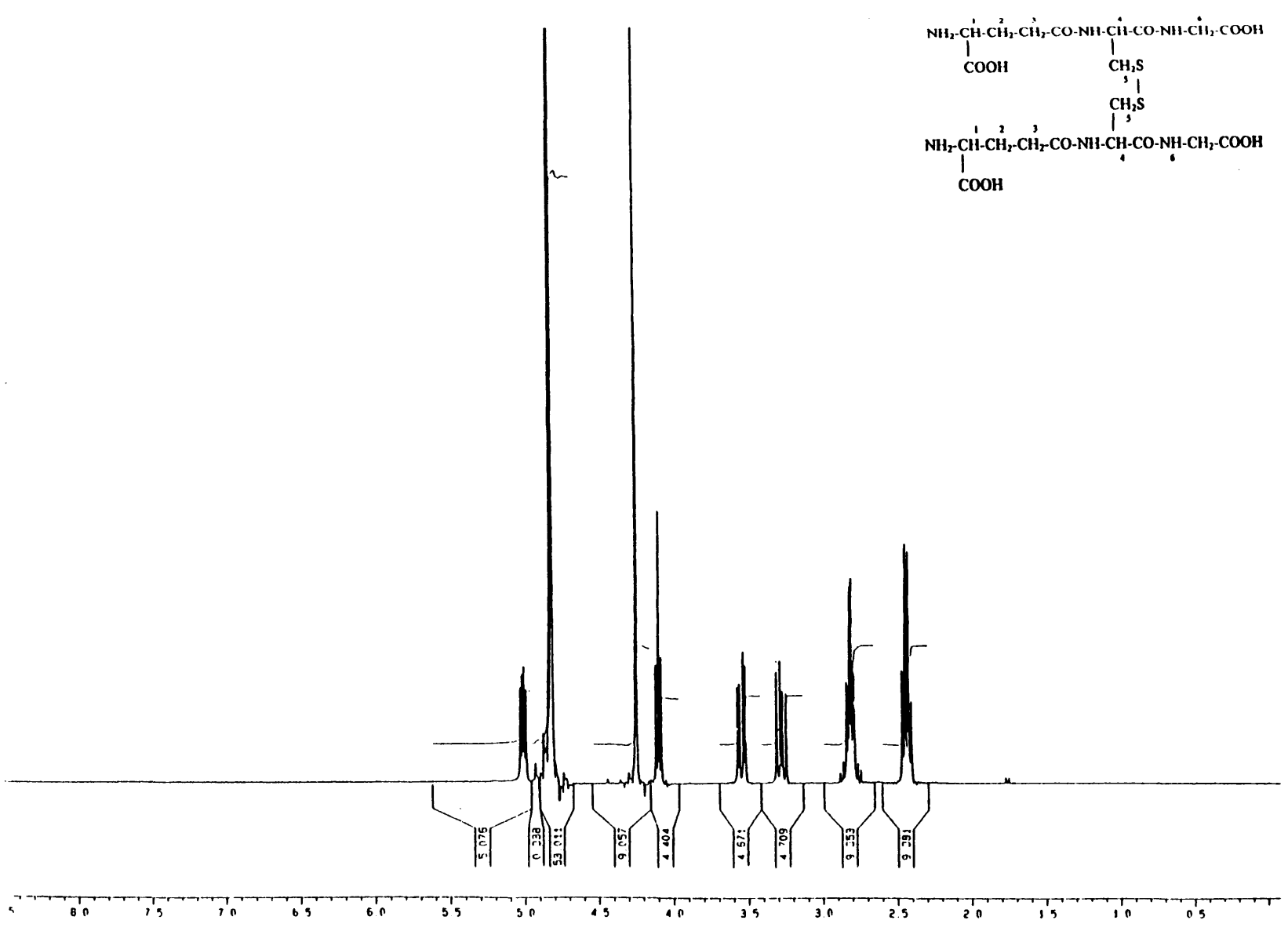


Figure 2.14 ^{1}H-NMR Spectrum of L-GSSG.

In order to confirm that D-GSH and L-GSH should give the same NMR spectrum a ^{1}H-NMR analysis (section 2.2.1) of the two dipeptides D and L-γ-Glu-Gly was obtained. Both spectrum shown were identical as expected (figure 2.15).

D and L-γ-Glu-Gly

δ_H (D_2O) (figure 2.15)

2.15 (2H, multiplet, H-2), 2.45 (2H, triplet, H-3), 3.9 (2H, singlet, H-4) and 4.05ppm (1H, triplet, H-1).

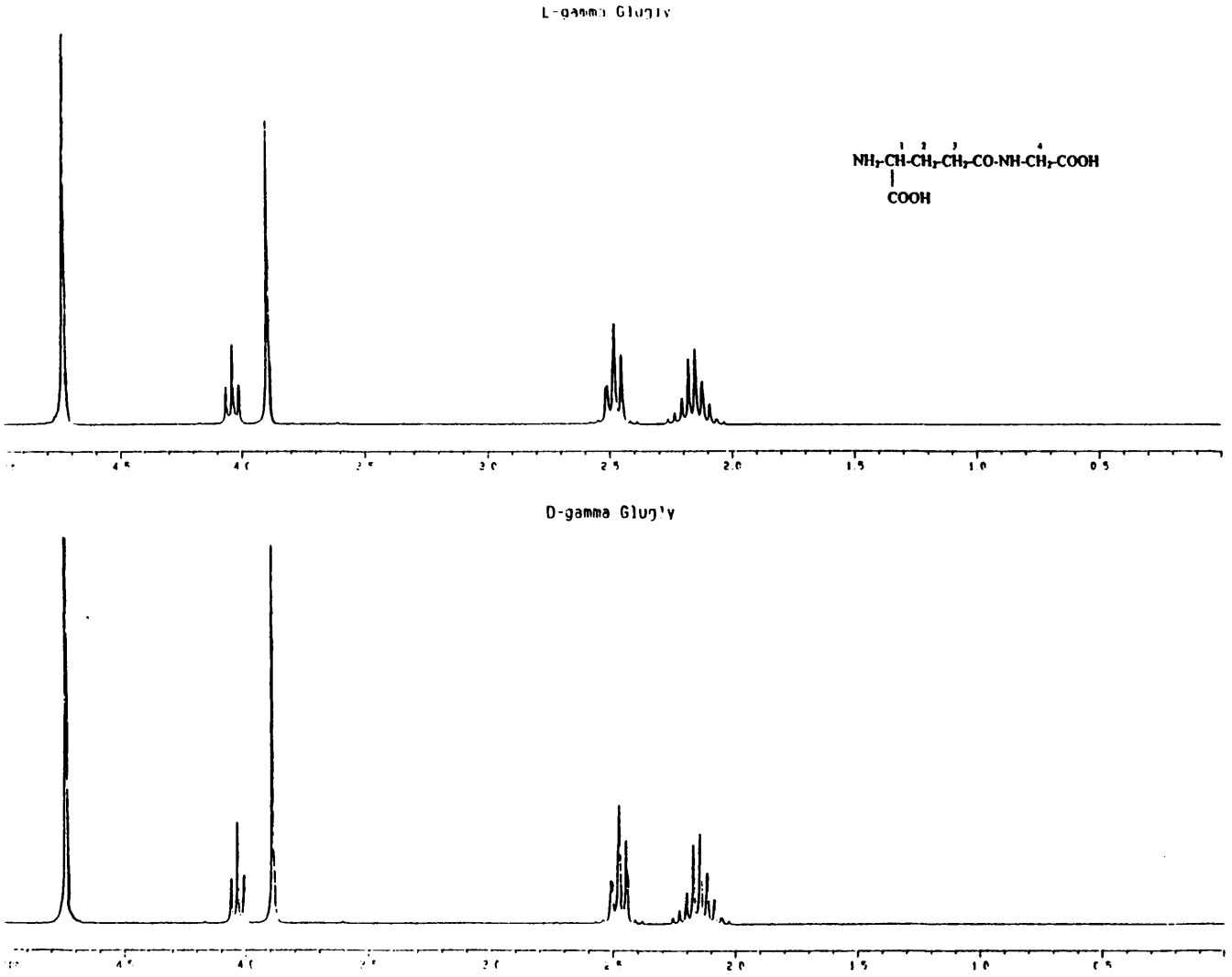


Figure 2.15 ^{1}H-NMR Spectrum of L and D-GluGly.

2.6 Discussion

The two dipeptides CysGly and γ-GluCys were synthesised in order to investigate how the structural differences between the two RSNOs, GSNO and CysNO, influence their chemical and biological properties. Since starting this project the two dipeptides CysGly and γ-GluCys are now available commercially. The synthetic CysGly was not used in any further testing and γ-GluCys was used for some initial stability experiments. The main outcome of this exercise was that the steps required in synthesising these two compounds were utilised in the synthesis of D-GSH.

All the synthetic routes followed made use of solid phase peptide synthesis. This type of peptide synthesis has fundamental chemical and physical advantages over the solution approach. In particular the attachment of a protected peptide to a resin support overcomes the problem of poor solubility of protected peptide intermediates (Kent, 1988). From a practical point of view a major advantage in using a solid phase strategy is that essentially no complicated clean-up procedures are required throughout the synthesis.

The analytical results obtained for D-GSH appear to suggest that the final compound was not actually the D-isomer of glutathione. ^{1}H-NMR results gave virtually the same spectra for D-GSH as L-GSH except for the signals for H-1 and H-6 have swapped around between the two spectra. There is the chance that this may be a pH effect as the α-hydrogen of glutamic acid is very pH-sensitive (Corazza *et al*, 1996). An ^{1}H-NMR spectrum of L-GSH in the presence of sodium carbonate was obtained. The spectrum was identical for all protons except for H-1 and H-6 where both signals had shifted up-field to 3.7ppm (results not shown). This spectra clearly demonstrates the sensitivity of these protons to pH.

The mass spectral data gave an *m/z* of 307 instead of 308 as is expected for D-GSH. The hypothesis originally put forward was that the final product was in the form of the disulphide which may be cleaved at the S-S bond ,pick up a proton and hence give an

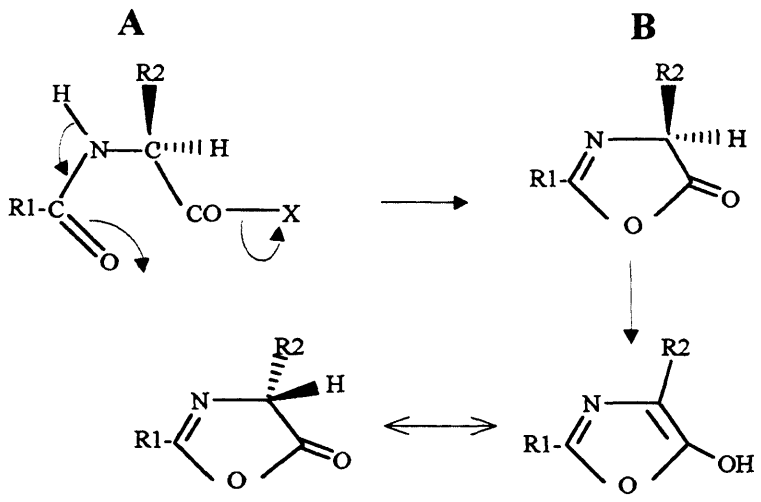
m/z of 307, but from the Ellmans data and the nitrosation reaction this was shown not to be the case. It is unclear at this stage why the *m/z* is one less than expected.

The ^{19}F-NMR spectrum clearly demonstrated that the final D-GSH was in the form of the heptafluorobutyrate salt. The splitting pattern observed for the salt was not as expected, with the signal for F-3 being a singlet rather than a triplet. The signal for F-3 was a singlet due to the coupling between F-2 and F-3 being reduced or even abolished. This de-coupling effect is thought to be caused by the COOH group in the salt. Although the final D-GSH was in the form of the heptafluorobutyrate salt, the salt was not observed in the ^{13}C-NMR experiment. This anomaly is due there being no nuclear Overhauser enhancements, caused by the long relaxation times of the COOH of the salt. Nuclear Overhauser enhancement is the ability of nuclei to interact through space and enhance the signal observed.

There are a number of possible explanations as to why the synthesis of D-GSH did not give the expected results. The most plausible explanation is that one or both of the chiral centres in D-GSH may have undergone racemization. D and L-glutathione are a pair of enantiomers, that is they are mirror images of each other and should have identical physical and chemical properties except for the direction of rotation of the plane of polarised light. If one of the chiral centres had undergone racemization this would give rise to the formation of diastereoisomers. Diastereoisomers are stereoisomers that are not mirror images of each other and do not have the same physical properties and have only similar chemical properties. The possibility that racemization had occurred is partially supported by the optical rotation values and the HPLC data. In the case of the $[\alpha]_D$ values, D-GSH gave a lower value but in the expected direction which suggests that only one of the chiral centres has undergone racemization. With the HPLC data the retention time of both isomers of GSH should have been the same. The results observed different retention times for both L and D-GSH suggesting the formation of diastereoisomers. Alternatively if racemization had occurred there should have been at least two products detected by HPLC. Also it is expected that the ^{1}H-NMR spectrum would be more "complicated" than observed (P Cullis and G Roberts, personal communications).

Racemization during the formation of a peptide bond has long been an area of concern. Nineteen of the twenty common amino acids are optically active. All of these amino acids have hydrogen atoms as one of the substituents at their asymmetric α-carbons. Removal and reattachment of this hydrogen atom constitutes a potentially easy mechanism for racemization. The enhanced reactivity of the adjacent carboxyl group when activated for a coupling reaction provides a simple mechanism where the α-hydrogen may be removed. There is therefore a risk of racemization by direct ionisation of the α-hydrogen during activation and coupling procedures (Atherton and Sheppard, 1989).

Generally it seems that direct ionisation of this α-hydrogen is not usually a major source of racemization during peptide synthesis. A second mechanism is the result of the formation of oxazolone intermediates (equation 2.2). In this process the activated carbonyl group effectively acylates the acylamino group (peptide bond) preceding it in the peptide chain (**A**). The resulting oxazolone intermediate (**B**) may racemize more readily. Oxazolones of type (**A**) are reactive species generally lowering the yield and purity of synthetic peptides. When the amide group in (**A**) is part of a urethane structure, as in Boc- and Fmoc-amino acids the tendency to oxozalone formation is apparently reduced. In addition the oxazolone, if formed, is more resistant to racemization. Thus solid-phase peptide synthesis from a carboxy terminus (particularly glycine which is not prone to racemization) using urethane-protected amino acids is relatively free from racemization (Atherton and Sheppard, 1989; Davies, 1992).



Equation 2.2 Oxazolone formation and deprotonation during activation of peptides

Thus from the information available racemization is unlikely to occur during this synthetic procedure firstly because glycine is the carboxy terminus and secondly all amino acids used urethane protecting groups.

A second explanation is that the protected glutamic acid used was the N-α-t-Boc-D-glutamic acid γ-benzyl ester which would give α-GluCysGly and not γ -GluCysGly as required. The ^{1}H-NMR spectra of α-GluVal and γ-GluVal were compared and showed the signal for H-1 and H-4 have shifted slightly (0.1ppm) up-field in γ-GluVal. As previously mentioned the signals for the same glutamic acid proton H-1 have moved up-field (0.1ppm) in D-GSH compared to L-GSH. This result suggested that the compound synthesised is actually α-GluCysGly. This will have occurred if the wrong starting material had been provided. In order to ascertain if this was the case a second batch of the same glutamic acid derivative was obtained from a different source and compared with the original compound. The results showed (figure 2.12) an identical ^{1}H-NMR spectrum which does seem to infer that this is not the case.

Another possibility is that one of the starting materials used was of the wrong optical activity. The optical rotation of the D- and L-isomers of the starting materials were compared and results obtained suggested that this was not the case (section 2.5).

There are several other experiments which can be carried out to probe further into this problem. One logical experiment would be to de-protect the actual starting compounds used and obtain the optical rotation of the de-protected materials to ensure the amino acids were of the correct chirality. The main drawback of this experiment is that the resultant amino acids will have little UV activity which makes purification procedures difficult to perform.

There is also the possibility that the final material could have been in the oxidised form but together with the results from the Ellmans test and the nitrosation reaction it does appear that this is not the case. In addition the ^{1}H-NMR spectrum for L-GSSG showed the signal for H-5 had split in comparison with L-GSH. This splitting pattern was not observed with D-GSH, supporting further that the final product was not oxidised.

As can be seen no firm conclusions have been made has to where the problems in the synthesis of D-GSH occur. One plausible explanation is that one or both of the chiral centres in D-GSH may have undergone racemization. This possibility was supported by the different $[\alpha]_D$ values for L and D-GSH and by the different HPLC retention times for the two enantiomers. In contrast it does seem unlikely that the final product is a diastereoisomer of GSH due to the fact that only one product was detected by HPLC and the ^{1}H-NMR spectrum is expected to be more 'complicated' than observed.

With the data available the most probable explanation is that the final product is α-GluCysGly, this would occur if the wrong starting material had been provided. A comparison of two different batches of starting material by ^{1}H-NMR gave identical results suggesting that the two batches were identical but it is not improbable that both batches were the γ-protected material obtained from the same manufacturer. In addition this hypothesis was supported with the ^{1}H-NMR data where the signal for H-1 had shifted up-field. Further work is required to confirm this hypothesis which may include peptide mapping to determine the identity of each individual amino acid.

Chapter 3

Chemical Properties of

S-*Nitrosothiols*

3.1 Introduction

S-Nitrosothiols are thermodynamically and photolytically unstable compounds (Feelisch, 1991). The decomposition of RSNOs has been shown to be catalysed by trace metal ions such as copper (Askew *et al*, 1995a; Dicks *et al*, 1996). A major aim of this work was compare the stability of each RSNO under investigation and to study the effect of copper on the decomposition of these compounds.

Another important reaction of RSNOs is the transfer of the NO group to a second thiol (Park, 1988; Barnett *et al*, 1994) which could lead to the formation of a less stable RSNO resulting in the release of NO more readily. These reactions may have implications for the mechanism of action of RSNOs in biological assays and *in vivo* studies. Experiments were carried out to investigate this phenomenon further with the RSNOs in question.

All RSNOs were synthesised *in situ* due to the low stability of these compounds. This method of synthesising fresh RSNO solutions is generally accepted in the field of RSNO research (Feelisch and Stamler, 1996). The concentration of RSNOs in all of the assays in this chapter was assessed by conventional spectrophotometric methods, taking advantage of the characteristic absorption maxima of RSNOs at around 330-335nm.

3.2 Determination of Extinction Coefficients (ε)

S-Nitrosothiols were prepared using the method of Hart (1985), reacting equimolar amounts of thiol, $NaNO_2$ and HCl.

A 50mM stock of RSNO was prepared as follows: Each thiol (50μmoles) was dissolved in water (980μl). $NaNO_2$ (50μmoles) was added to this solution along with 2.5M HCl (20μl). This immediately yielded a red solution of RSNO. This RSNO solution was then diluted serially in water to give concentrations of 2.5, 1.0, 0.5, 0.25,

0.05mM and the absorbance of the solutions at the $\lambda_{max.}$ *ca* 330nm was measured. The top standard was first scanned between 200 and 600nm to determine the λ_{max} values.

3.2.1 Treatment of Results

Plots of absorbance *vs* concentration were drawn, linear regression was carried out on the lines obtained and the ε value was determined from the gradient (Microcal Origin v 2.8). Experiments were carried out in triplicate for each compound.

3.2.2 Results

Table 3.1 Molar absorption coefficients for *S*-nitrosothiols, $\lambda_{max.}$ *ca* 330nm.

RSNO	ε ± SD ($M^{-1}cm^{-1}$)	λ_{max} (nm)
GSNO	795 ± 15	334
CysNO	666 ± 57	336
CysGlyNO	411 ± 69	337
γ-GlucysNO	918 ± 27	336
SNAC	872 ± 22	335
MESNO	980 ± 49	330
SNPROPA	978 ± 95	330

Mean ± SD for 3 experiments

3.3 Determination of Stability of S-Nitrosothiols by UV Spectroscopy

3.3.1 Experimental

An RSNO solution (50mM) was prepared by dissolving the equivalent RSH (125μmoles) in ultra-pure water (2ml). $NaNO_2$ (125μmoles) was added to this solution along with 2.5M HCl (500μl). This immediately yielded a red solution of RSNO. A suitable dilution of the RSNO in water was then scanned in a UV spectrophometer (Perkin Elmer Lambda 2S) between 200 and 600nm against water as a blank. This spectrum determined the λ_{max} value at *ca* 330nm.

1M NaOH (100µl) and 1M phosphate buffer pH7.4 (890µl) were mixed in 1.5ml matched quartz cuvettes. Each RSNO (10µl) was added to the cuvettes within 10-15 min of preparation. The pH of this solution was determined intermittently to ensure it was kept at 7.4. The UV absorption of the resulting solutions was measured at 37°C at the previously determined λ_{max} against a blank which contained a dilution of the $NaNO_2$ and HCl in the previously described buffer. Data were collected immediately, at 5 min intervals for the less stable compounds i.e. CysNO and CysGlyNO and at 1 hr intervals for the stable compounds. The data were collected for 3 half-lives for CysNO and CysGlyNO and for 24 hr for the remaining RSNOs. Plots of absorbance against time were prepared using Microcal Origin v2.8. The half-lives and rate constant for the decomposition of each RSNO were calculated (section 3.3.2).

Experiments were also carried out in the presence of the copper chelator, diethylenetriaminepentaacetic acid (DTPA). 1M NaOH (100µl), 1mM DTPA (100µl) and 1M phosphate buffer pH7.4 (790µl) were placed in 1.5ml matched quartz cuvettes. Each RSNO (10 µl) was added to the cuvettes within 10-15min of preparation. Data collected as previously described.

3.3.2 Treatment of Results

Data were obtained as a series of absorbance values for a given time course. The half-life and rate constant for the less stable compounds without DTPA was calculated assuming first-order kinetics i.e. A plot of ln RSNO concentration against time for up to 3 half-lives gave a straight line with a gradient equal to the rate constant k, from which the half-life was calculated (equation 3.1).

Equation 3.1

$$t_{\frac{1}{2}} = \frac{\ln 2}{k}$$

In the presence of DTPA the kinetics of the decomposition of the less stable RSNO appeared to be zero order. A plot of concentration against time gave a straight line with a gradient equal to the rate constant k from which the half life was calculated

(equation 3.2). The half-life value obtained in this case is the time for half of the RSNO to disappear and not the true half-life of the compound. The half-lives obtained for the more stable compounds can only be regarded as an approximation as data were not collected for at least one half-life.

Equation 3.2 $$t_{\frac{1}{2}} = \frac{C_0}{2k}$$

C_0 = concentration at t = 0

Concentration of RSNO was calculated from absorbance values using the Beer-Lambert Law (equation 3.3).

Equation 3.3 $$A = \varepsilon C l$$

A= absorption, ε = molar absorption coefficient (M^{-1} cm^{-1}), C = concentration (M), l = path length (cm)

Individual ε values for each RSNO were determined as detailed (section 3.2).

3.3.3 Results

Rate constants and half-life values of the RSNOs of interest both in the presence and absence of adventitious amounts of copper are shown (table 3.2).

Table 3.2 Stability of *S*-nitrosothiols under physiological conditions.

	Without DTPA		With DTPA	
RSNO	k (hr^{-1})	$t_{\frac{1}{2}}$ (hr)	k ($mmol.dm^{-3}hr^{-1}$)	$t_{\frac{1}{2}}$ (hr)
CysNO	1.35 ± 0.294	0.542 ± 0.151	0.0278 ± 0.003	13.1 ± 1.55
CysGlyNO	2.22 ± 1.02	0.309 ± 0.103	0.0526 ± 0.004	5.67 ± 0.645
GSNO	0.0178 ± 0.005	41.6 ± 13.7	0.00306 ± 0.00162	115 ± 62.1
γ-GluCysNO	0.0165 ± 0.0008	42.2 ± 1.98	0.00274 ± 0.00405	411 ± 329
SNAC	ND	Stable*	ND	Stable
MESNO	ND	Stable	ND	Stable
SNPROPA	ND	Stable	ND	Stable

Mean ± SD for 3 experiments, * $t_{\frac{1}{2}}$ >500hr

Concentration-time profiles for all RSNOs under investigation were obtained (figure 3.1). The results show that CysNO and CysGlyNO were the least stable irrespective of the presence of DTPA. Diethylenetriaminepentaacetic acid increased the half-lives of GSNO, γ-GluCysNO, CysNO and CysGlyNO significantly (figure 3.2) indicating the catalytic effect of trace metals, particularly copper, on the decomposition of certain RSNOs. *S*-Nitroso-*N*-acetyl-L-cysteine, MESNO and SNPROPA appear to be the most stable of the compounds tested under these experimental conditions.

Figure 3.1 Stability of *S*-nitrosothiols under physiological conditions.

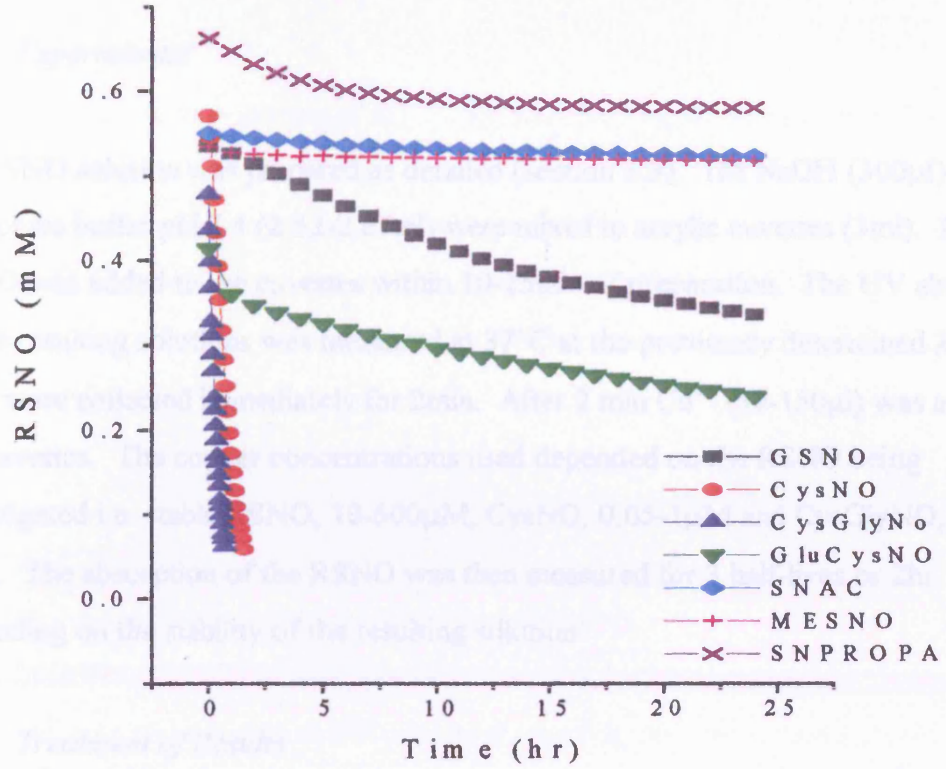
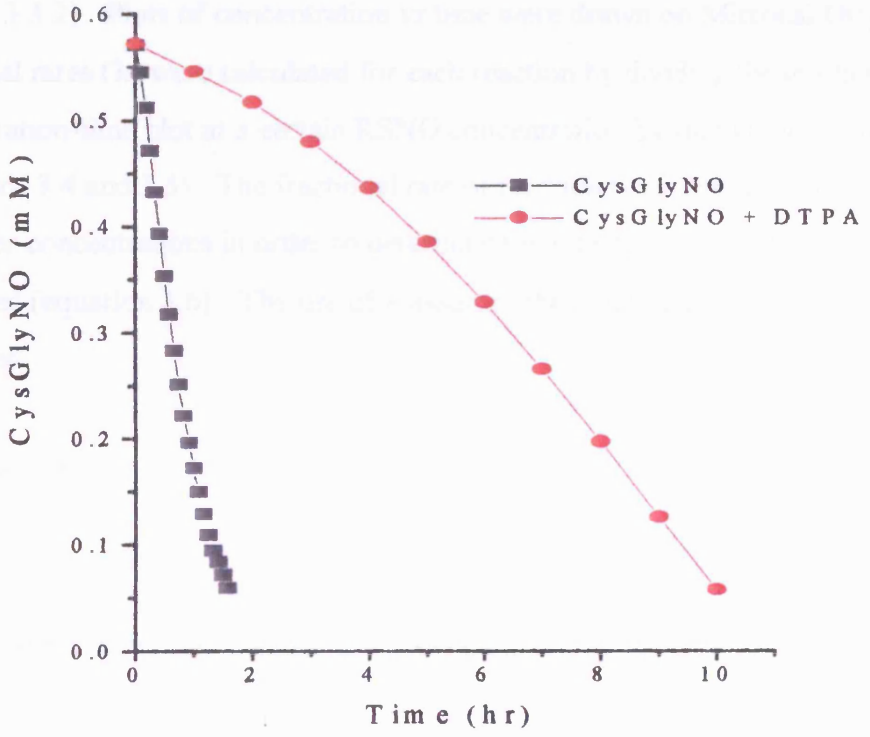


Figure 3.2 Stability of CysGlyNO in the presence of DTPA.



3.4 Effect of Copper on the Stability of S-*Nitrosothiols*

3.4.1 Experimental

An RSNO solution was prepared as detailed (section 3.3). 1M NaOH (300μl) and 1M phosphate buffer pH 7.4 (2.52-2.67ml) were mixed in acrylic cuvettes (3ml). Each RSNO was added to the cuvettes within 10-15min of preparation. The UV absorption of the resulting solutions was measured at 37°C at the previously determined λ_{max}. Data were collected immediately for 2min. After 2 min Cu^{2+} (30-150μl) was added to the cuvettes. The copper concentrations used depended on the RSNO being investigated i.e. stable RSNO, 10-500μM, CysNO, 0.05-1μM and CysGlyNO, 0.5-5μM. The absorption of the RSNO was then measured for 3 half-lives or 2hr depending on the stability of the resulting solution.

3.4.2 Treatment of Results

Data were obtained as a series of absorbance values for a given time course. Concentration of RSNO was calculated from absorbance values as previously detailed (section 3.3.2). Plots of concentration *vs* time were drawn on Microcal Origin v2.8. Fractional rates (λ) were calculated for each reaction by dividing the gradient of the concentration-time plot at a certain RSNO concentration by that known concentration (equations 3.4 and 3.5). The fractional rate of reaction was plotted against the range of copper concentrations in order to determine the rate constant, k_1 for these reactions as defined (equation 3.6). The use of λ assumes the reaction is first order with respect to copper.

Equation 3.4 $$\frac{C_0}{C_t} = e^{-\lambda t}$$

C_0 = Initial RSNO concentration, C_t = Concentration at time t, λ=Fractional rate.

Equation 3.5 $$\frac{dC/dt}{C} = \lambda$$

The fractional rate was measured at $C_0/2$ for the faster reactions or after a fixed time period for slower reactions. The C_0 used was not always the first data point collected because it appears with some of the reactions that there may have been some impurities present which were causing a rapid initial decay of the RSNO followed by a slower phase of decomposition. In these cases C_0 was taken as the initial concentration of the second slower phase.

Equation 3.6 $$\frac{d[RSNO]_0}{dt} = k_1[RSNO]_0[Cu^{2+}]$$

$[RSNO]_0$ represents the initial concentration of RSNO.

Representative concentration-time profiles for each RSNO are shown (figures 3.3 - 3.7) from which fractional rates for the reaction of GSNO, CysNO, CysGlyNO and GluCysNO were calculated (tables 3.3 -3.6).

3.4.3 Results

Figure 3.3A Reaction of 0.5mM GSNO and 10-500μm Cu^{2+}.

Figure 3.3B Plot of fractional rate *vs.* [added Cu^{2+}] for the reaction between GSNO and Cu^{2+}.

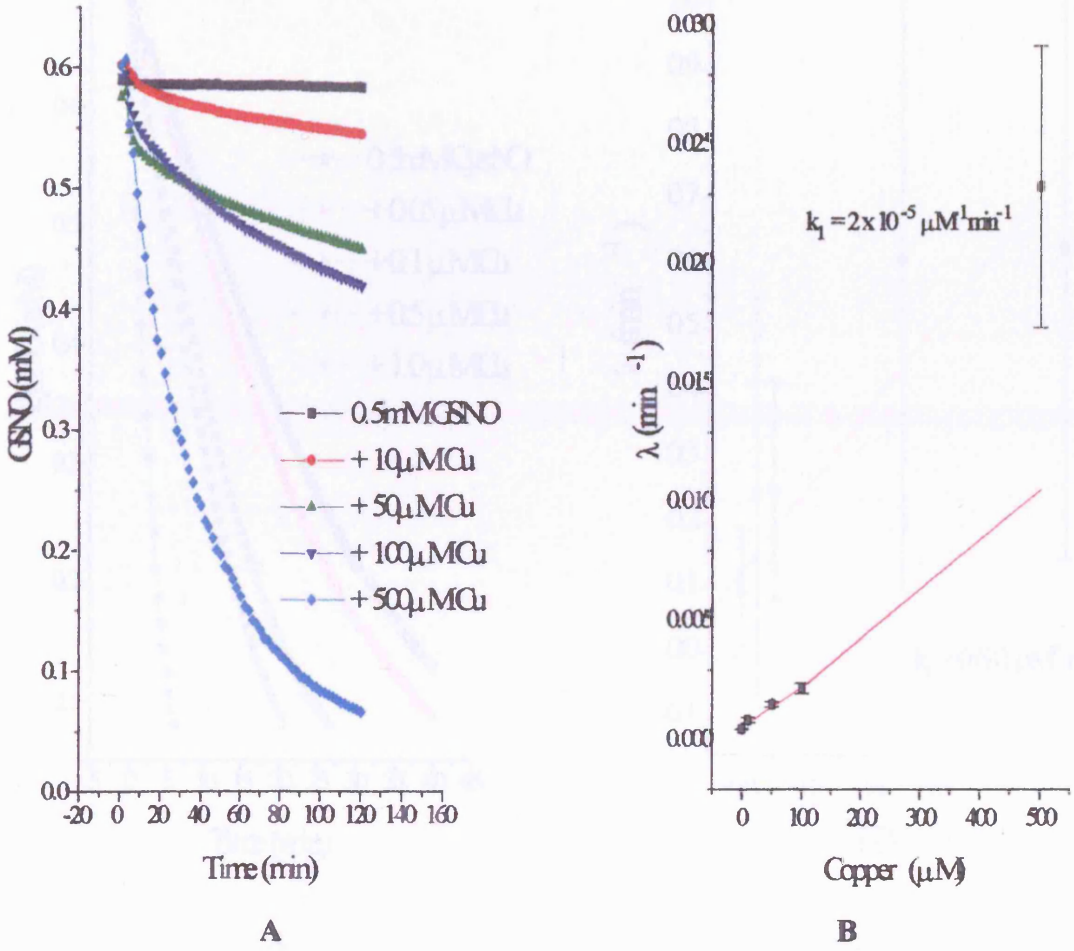


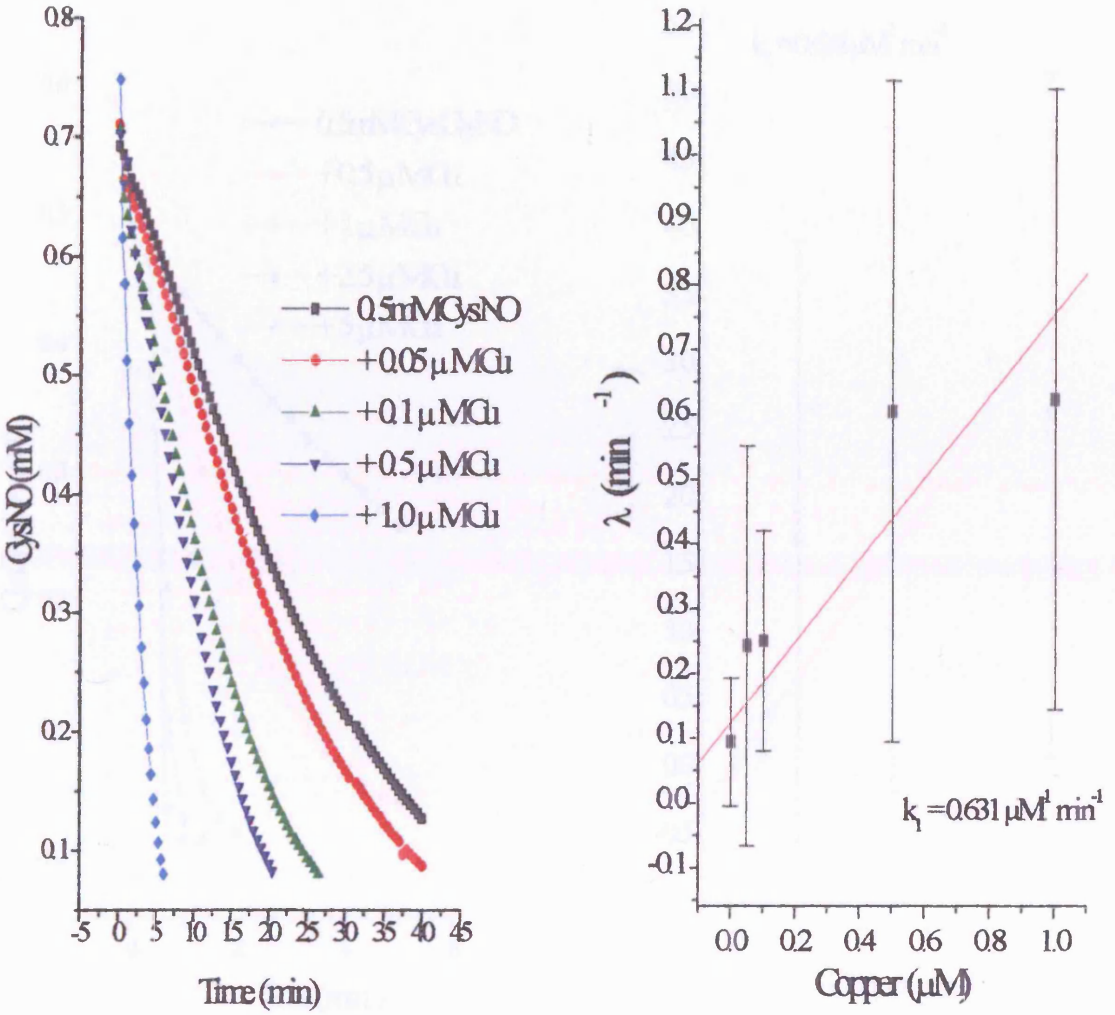
Table 3.3 Fractional rates for the reaction between 0.5mM GSNO and 10-500μM Cu^{2+}.

Cu^{2+}(μM)	λ (min^{-1})
0	0.00003 ± 0.00002
10	0.00042 ± 0.00009
50	0.00108 ± 0.00011
100	0.00175 ± 0.00023
500	0.0228 ± 0.00592

Mean ± SD for 3 experiments

Figure 3.4A Reaction of 0.5mM CysNO and 0.05-1μm Cu^{2+}.

Figure 3.4B Plot of fractional rate *vs.* [added Cu^{2+}] for the reaction between CysNO and Cu^{2+}.



A B

Table 3.4 Fractional rates for the reaction between 0.5mM CysNO and 0.05-1μM Cu^{2+}.

Cu^{2+}(μM)	λ (min^{-1})
0	0.0946 ± 0.0989
0.05	0.249 ± 0.308
0.1	0.251 ± 0.169
0.5	0.605 ± 0.51
1	0.624 ± 0.479

Mean ± SD for 3-4 experiments

Figure 3.5A Reaction of 0.5mM CysGlyNO and 0.5-5μm Cu^{2+}.

Figure 3.5B Plot of fractional rate *vs.* [added Cu^{2+}] for the reaction between CysGlyNO and Cu^{2+}.

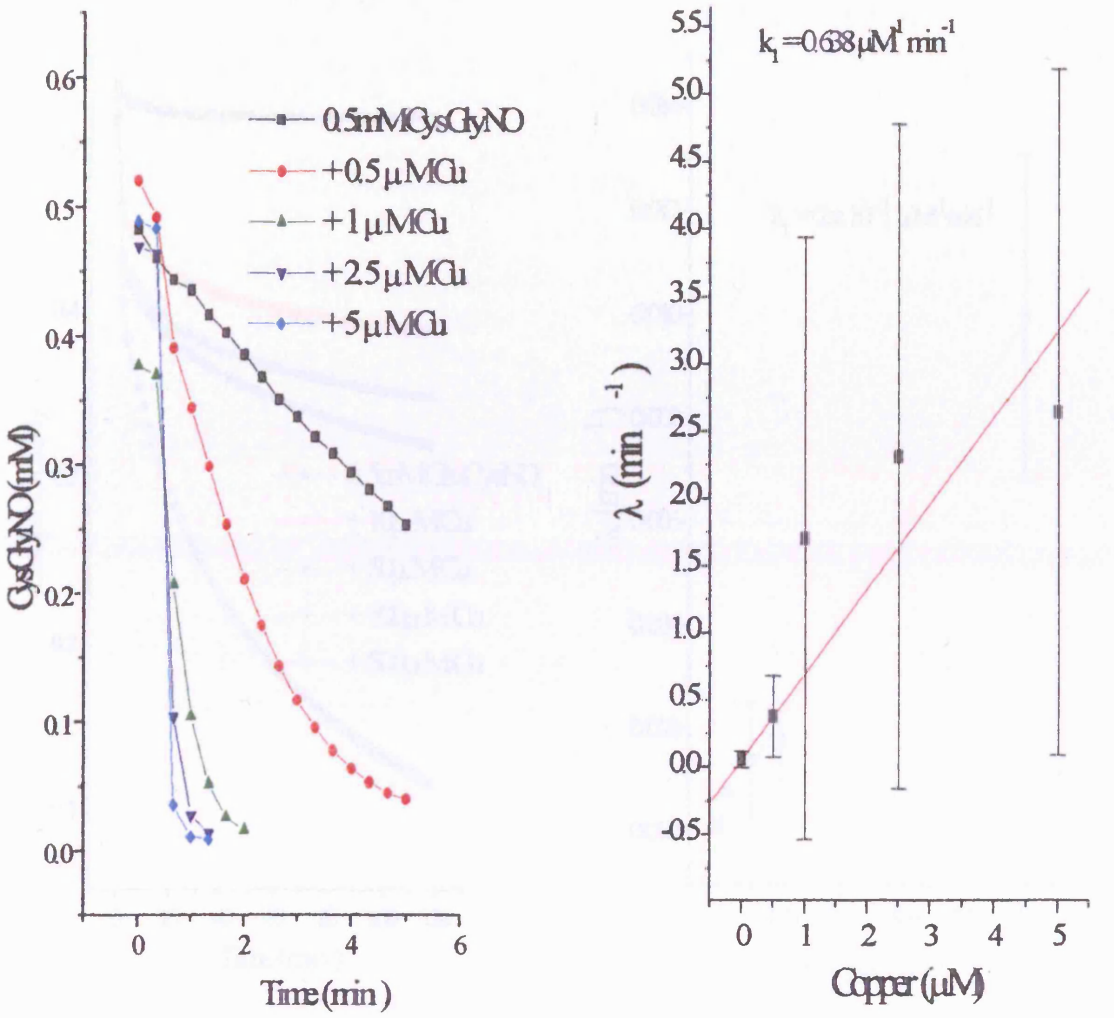


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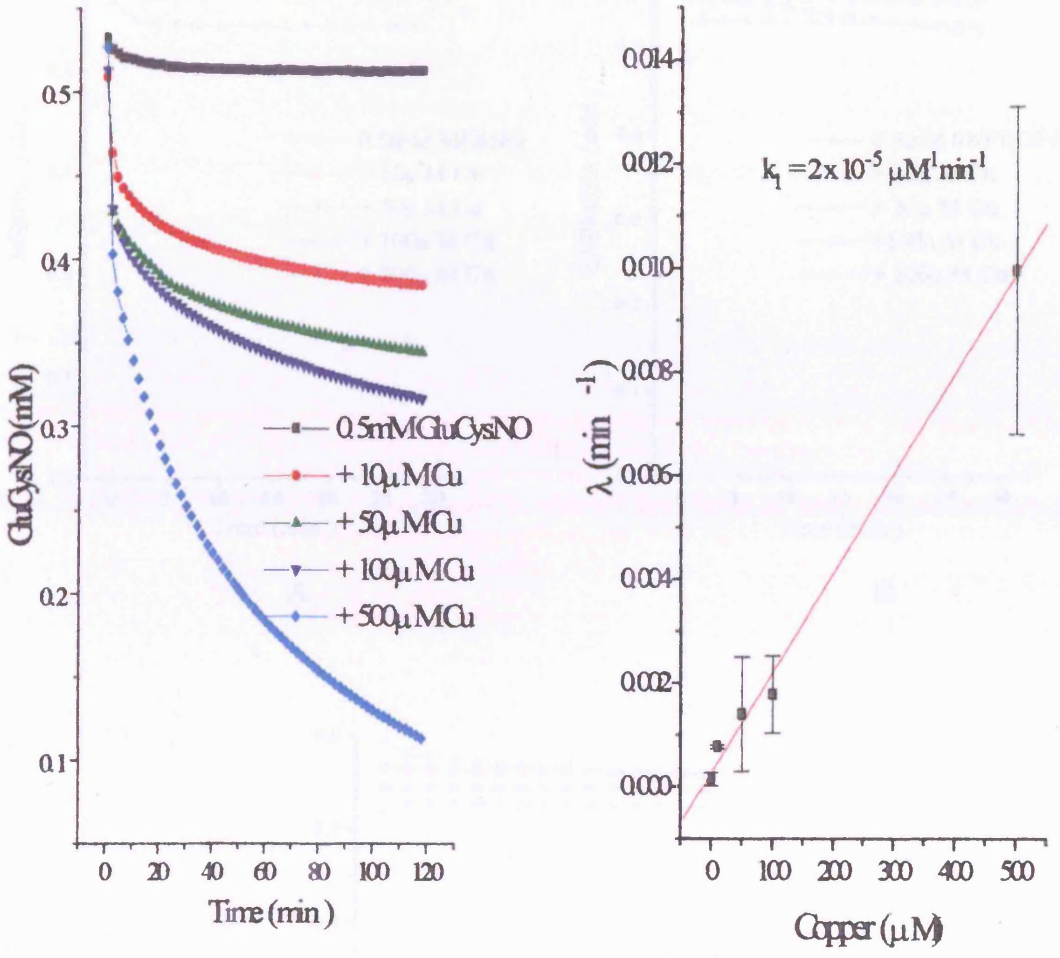
Table 3.5 Fractional rates for the reaction between 0.5mM CysGlyNO and 0.5-5μM Cu^{2+}.

Cu^{2+}(μM)	λ (min^{-1})
0	0.0573 ±0.0610
0.5	0.377 ± 0.301
1	1.70 ± 2.24
2.5	2.31 ± 2.47
5	2.64 ± 2.55

Mean ± SD for 3-4 experiments.

Figure 3.6A Reaction of 0.5mM GluCysNO and 10-500μm Cu^{2+}.

Figure 3.6B Plot of fractional rate *vs.* [added Cu^{2+}] for the reaction between GluCysNO and Cu^{2+}.



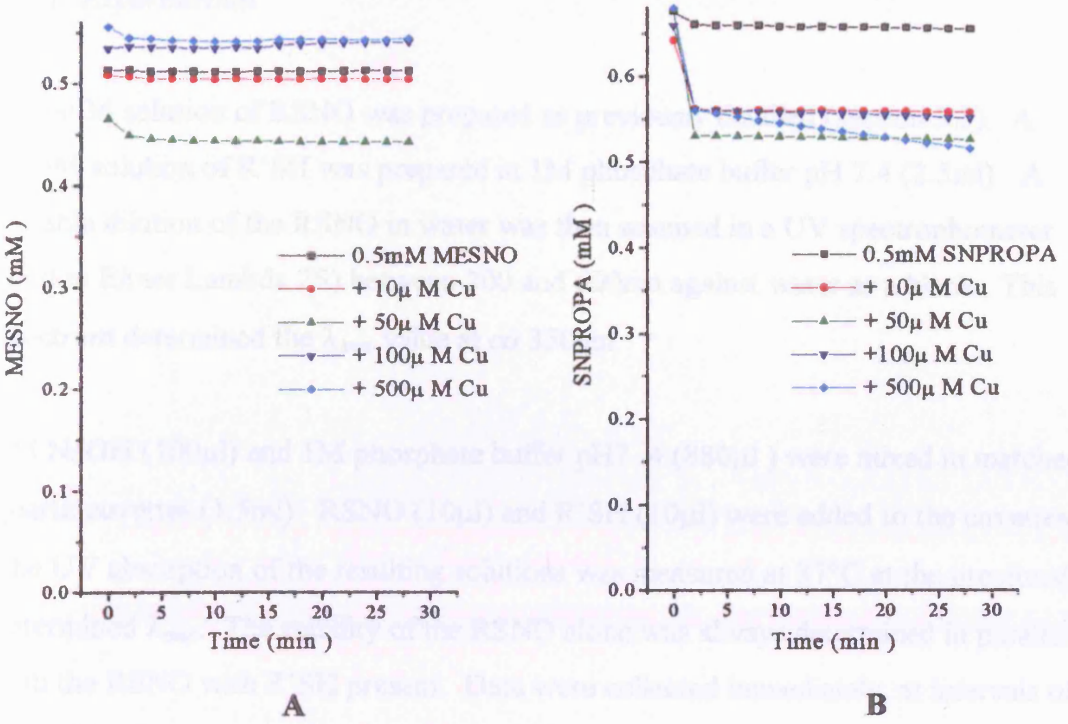
A B

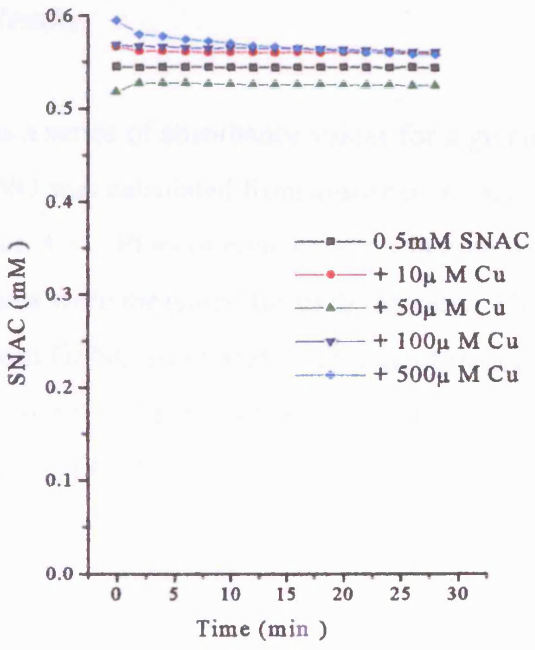
Table 3.6 Fractional rates for the reaction between 0.5mM GluCysNO and 10-500μM Cu^{2+}.

Cu^{2+}(μM)	λ (min^{-1})
0	0.00015 ± 0.00013
10	0.00078 ± 0.00004
50	0.0014 ± 0.0011
100	0.00179 ± 0.00074
500	0.00997 ± 0.00316

Mean ± SD for 3 experiments

Figure 3.7 Reaction of 0.5mM (A) MESNO, (B) SNPROPA and (C) SNAC with 10-500μm Cu^{2+}.





3.5 Stability of S-Nitrosothiols in the Presence of Added Thiols

3.5.1 Experimental

A 50mM solution of RSNO was prepared as previously detailed (section 3.3). A 50mM solution of R'SH was prepared in 1M phosphate buffer pH 7.4 (2.5ml). A suitable dilution of the RSNO in water was then scanned in a UV spectrophometer (Perkin Elmer Lambda 2S) between 200 and 600nm against water as a blank. This spectrum determined the λ_{max} value at *ca* 330nm.

1M NaOH (100µl) and 1M phosphate buffer pH7 .4 (880µl) were mixed in matched quartz cuvettes (1.5ml). RSNO (10µl) and R'SH (10µl) were added to the cuvettes. The UV absorption of the resulting solutions was measured at 37°C at the previously determined λ_{max}. The stability of the RSNO alone was always determined in parallel with the RSNO with R'SH present. Data were collected immediately, at intervals of every 30 min.

3.5.2 Treatment of Results

Data were obtained as a series of absorbance values for a given time course. Concentration of RSNO was calculated from absorbance values using the Beer-Lambert Law (equation 3.3). Plots of concentration *vs* time were drawn on Microcal Origin v2.8. Initial rates were measured for each reaction. The initial rate of the transnitrosation between GSNO and CysH at a range of thiol concentrations was used to measure the rate constant (k_1) for this reaction (equation 3.7), where $d[GSNO]_0/dt$ is the initial rate of decay of GSNO.

Equation 3.7 $$\frac{d[GSNO]_0}{dt} = k_1[GSNO]_0[CysH]_0$$

$[GSNO]_0$ and $[CysH]_0$ represent the initial concentrations of GSNO or CysH. Representative concentration-time profiles for each transnitrosation reaction are shown (figures 3.8, 3.10 - 3.11).

3.5.3 Results

Figure 3.8 Stability of 0.5mM GSNO in the presence of 0.05-5mM CysH.

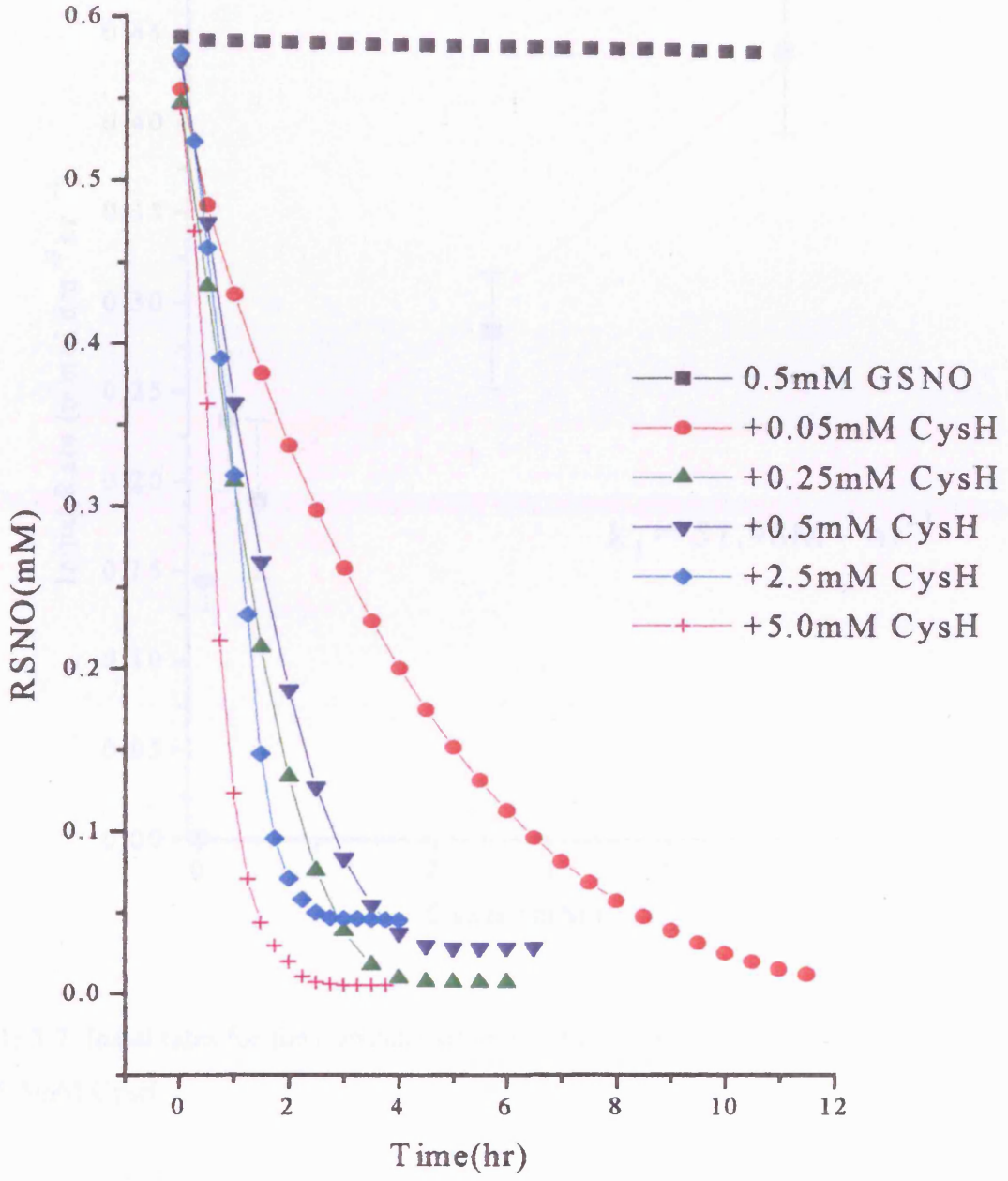


Figure 3.9 Plot of initial rate *vs.* [added CysH] for the reaction between GSNO and CysH.

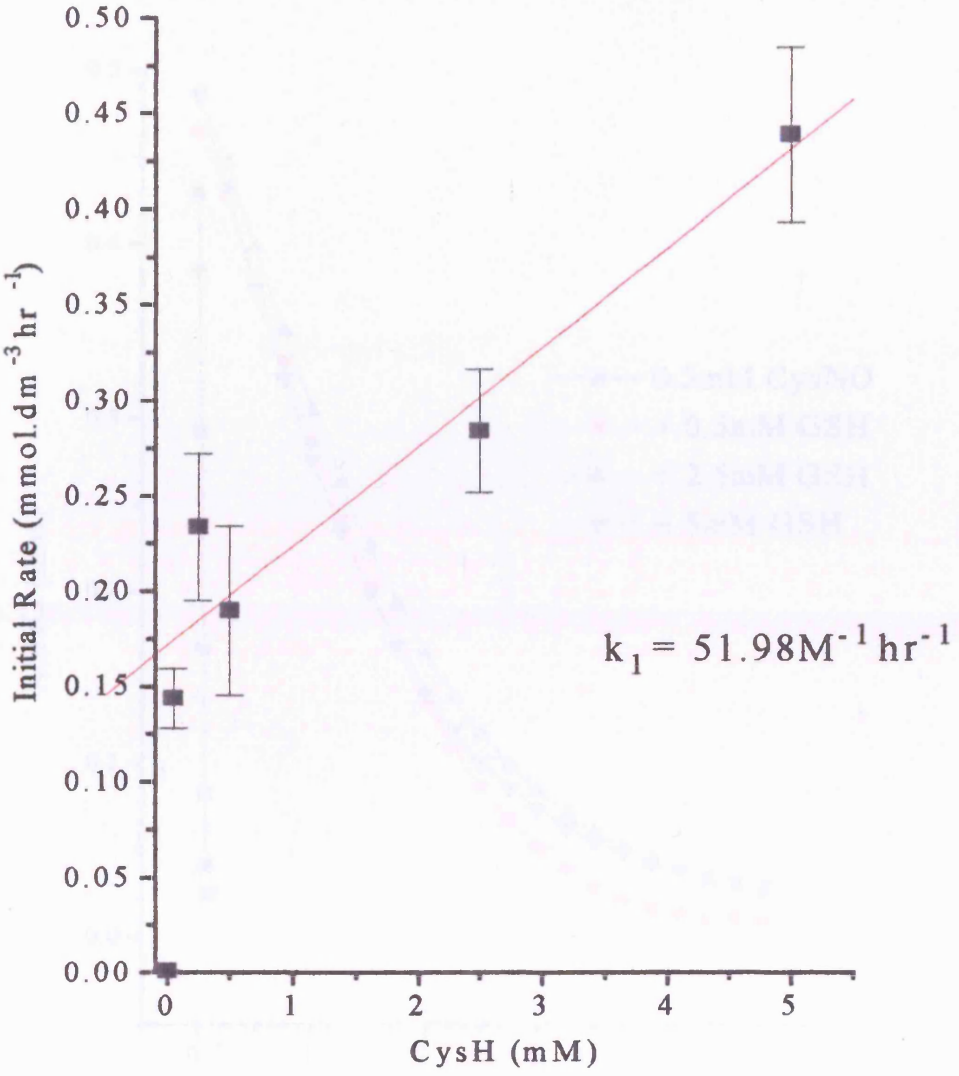


Table 3.7 Initial rates for the transnitrosation reaction between 0.5mM GSNO and 0.05-5mM CysH.

CysH (mM)	Rate ($mmol.dm^{-3}hr^{-1}$)
0	0.000872 ± 0.00018
0.05	0.144 ± 0.016
0.25	0.234 ± 0.038
0.5	0.190 ± 0.044
2.5	0.284 ± 0.034
5.0	0.439 ± 0.043

Mean ± SD for 3-5 experiments.

Figure 3.10 Stability of 0.5mM CysNO in the presence of 0.5-5mM GSH.

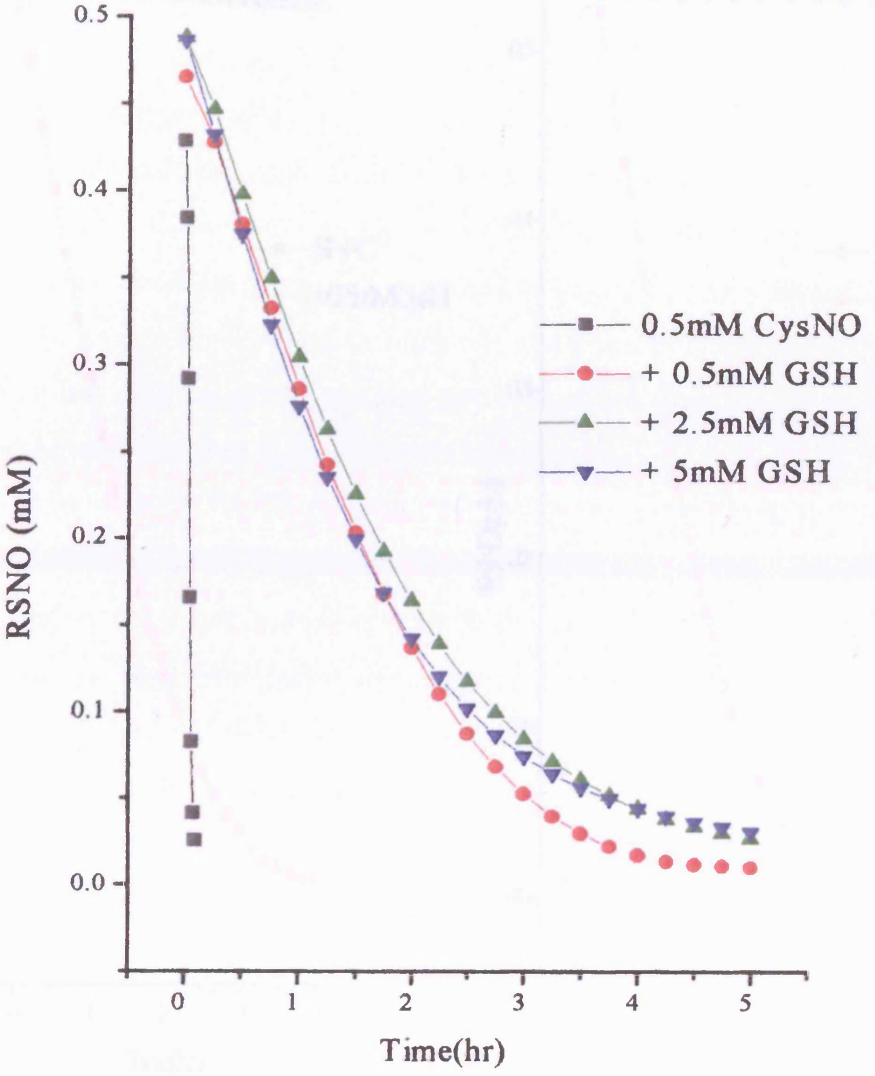


Table 3.8 Initial rates for the transnitrosation reaction between 0.5mM CysNO and 0.5-5mM GSH.

Experiment	Initial Rate ($mmol.dm^{-3}$ hr^{-1})
0.5mM CysNO	3.29 ± 0.716
0.5mM CysNO + 0.5mM GSH	0.270 ± 0.004
0.5mm CysNO + 2.5mM GSH	0.269 ± 0.003
0.5mM CysNO + 5mM GSH	0.289 ± 0.003

Mean ± SD for 3 experiments

Figure 3.11 Stability of 0.5mM SNAC and MESNO in the presence of 0.5mM CysH.

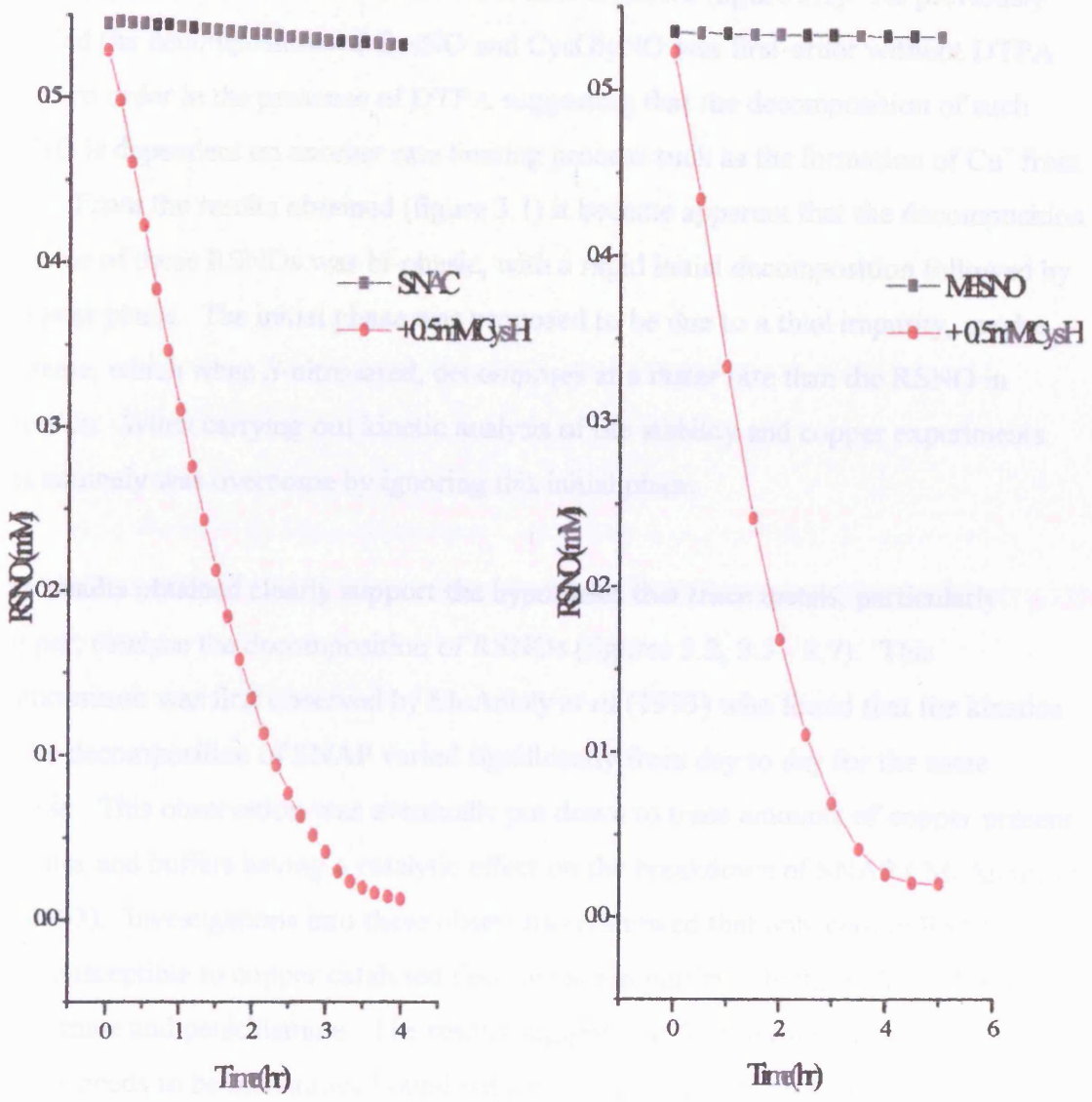


Table 3.9 Initial rates for the transnitrosation reaction Between 0.5mM MESNO and SNAC and 0.5mM CysH.

Experiment	Initial Rate (mmol. $dm^{-3}hr^{-1}$)
0.5mM MESNO	0.00257 ± 0.0015
0.5mM MESNO + 0.5mM CysH	0.181 ± 0.004
0.5mM SNAC	0.00268 ± 0.00088
0.5mM SNAC + 0.5mM CysH	0.237 ± 0.069

Mean ± SD for 3-4 experiments

3.6 Discussion

The decay of each RSNO as a function of time is shown (figure 3.1). As previously detailed the decomposition of CysNO and CysGlyNO was first-order without DTPA but zero order in the presence of DTPA suggesting that the decomposition of such RSNO is dependent on another rate limiting process such as the formation of Cu^{+} from Cu^{2+}. From the results obtained (figure 3.1) it became apparent that the decomposition of some of these RSNOs was bi-phasic, with a rapid initial decomposition followed by a slower phase. The initial phase was proposed to be due to a thiol impurity, maybe cysteine, which when *S*-nitrosated, decomposes at a faster rate than the RSNO in question. When carrying out kinetic analysis of the stability and copper experiments this anomaly was overcome by ignoring this initial phase.

The results obtained clearly support the hypothesis that trace metals, particularly copper, catalyse the decomposition of RSNOs (figures 3.2, 3.3 - 3.7). This phenomenon was first observed by McAninly *et al* (1993) who found that the kinetics of the decomposition of SNAP varied significantly from day to day for the same sample. This observation was eventually put down to trace amounts of copper present in water and buffers having a catalytic effect on the breakdown of SNAP (McAninly *et al*, 1993). Investigations into these observations showed that only certain RSNOs were susceptible to copper catalysed decomposition particularly those derived from cysteamine and penicillamine. The results suggest that for the catalysis to occur the copper needs to be bidentately bound within an intermediate. This co-ordination would probably occur between the nitrogen atoms of the nitroso group and the free amine group or another electron rich atom such as the oxygen in the carboxylate group yielding a stable ring structure (Askew *et al*, 1995b). Further investigations by Dicks *et al* (1996) have identified Cu^{+} and not Cu^{2+} as the effective catalyst. A mechanism has been proposed, as outlined in equations 3.8 - 3.10, in which Cu^{2+} is reduced by RS^{-} (generated from RSNO or RSH) *via* intermediate X to give Cu^{+} and $RS^{\cdot}$. Intermediate X is probably $RSCu^{+}$. Reaction then occurs between Cu^{+} and RSNO *via* intermediate Y releasing Cu^{2+}, RS^{-} and NO. Intermediate Y is thought to be a stable ring structure of the form shown (figure 3.12). Gorren *et al* (1996) have also recently demonstrated that the copper catalysed decomposition of GSNO is brought about by Cu^{+}.

Equation 3.8 $Cu^{2+} + RS^{-} \rightleftharpoons X \rightarrow Cu^{+} + RS^{\cdot}$

Equation 3.9 $RSNO + Cu^{+} \rightleftharpoons Y \rightarrow Cu^{2+} + RS^{-} + NO$

Equation 3.10 $2RS^{\cdot} \rightarrow RSSR$

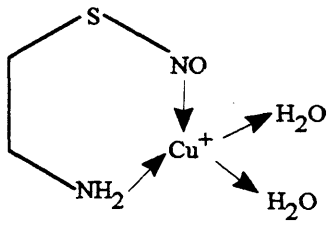


Figure 3.12 Structure of the expected ring formed in the copper catalysed decomposition of RSNOs.

Looking at the RSNOs in question results showed a high reactivity of copper with CysNO and CysGlyNO (sections 3.2 and 3.3). With these two compounds the intermediate Y would be a six membered ring (figure 3.12). *N*-Acetylation of the amine group, as in the case of SNAC, reduced the effect of copper by making the nitrogen atom less nucleophilic and hence reduce co-ordination with Cu^{+}. With MESNO there are no available sites (except for NO) for the co-ordination to occur. In the case of SNPROPA, an initial reaction with copper was observed, in this case Y would be a seven membered ring (figure 3.13), and hence energetically unfavourable.

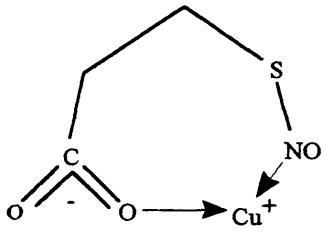


Figure 3.13 Structure of seven membered ring formed in the copper catalysed decomposition of SNPROPA.

Higher concentrations of copper (10-500 μM) did have a catalytic effect on the decomposition of GSNO (section 3.3). In addition the presence of DTPA increased the half-life of this RSNO. Following the ring formation theory the reactivity of this

the half-life of this RSNO. Following the ring formation theory the reactivity of this compound with copper is expected to be very low due the fact that the two sites of co-ordination would require the formation of either a ten or eleven membered ring which are obviously both energetically unfavourable (figure 3.14). There is also the possibility that co-ordination can occur intra-molecularly i.e. between two different molecules of GSNO which could account for the reaction of copper with GSNO at higher concentrations. The same applies to GluCysNO but in this case for co-ordination to occur a seven or eleven-membered ring would have to be formed (figure 3.15).

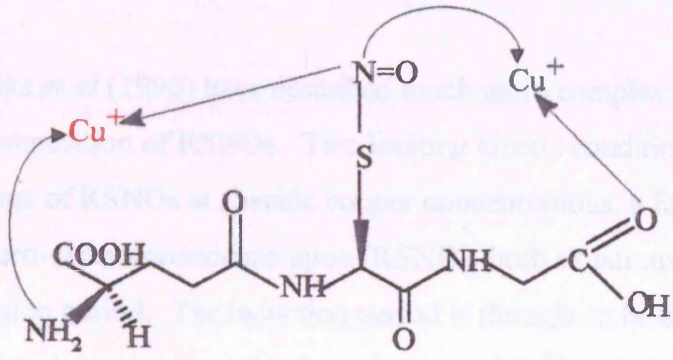


Figure 3.14 Points of co-ordination in the copper catalysed decomposition of GSNO.

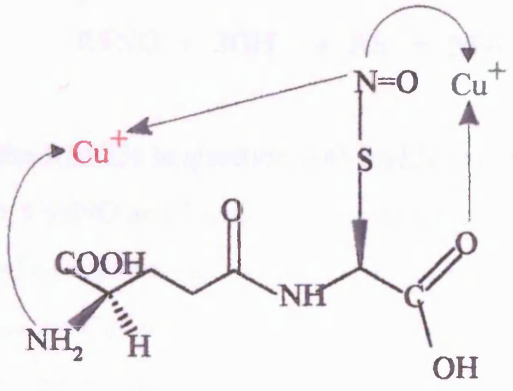


Figure 3.15 Points of co-ordination in the copper catalysed decomposition of GluCysNO.

copper, and RSNO concentration. At higher copper concentration plots of rate *vs.* $[Cu^{2+}]$ lost linearity and showed erratic kinetic behaviour which was explained by the formation of a complex between Cu^{2+} and the phosphate component of the buffer system used. The rate equation below was proposed.

Equation 3.11 $$\text{Rate} = k[RSNO][Cu^{2+}] + k'$$

The k' term was thought to represent the component of the reaction catalysed by residual copper present in the reaction solution together with any thermal or photolytic decomposition.

More recently Dicks *et al* (1996) have described much more complex kinetics for the Cu-catalysed decomposition of RSNOs. Two limiting kinetic conditions have been identified for a range of RSNOs at specific copper concentrations, a first-order dependence and zero-order dependence upon [RSNO], both situations sometimes involving an induction period. The induction period is thought to be due to the generation of thiolate ions necessary for the reduction of Cu^{2+} to Cu^{+} for the catalysis to occur as previously described. The thiolate ion may be present as a thiol impurity or generated by partial hydrolysis of the RSNO (equation 3.12).

Equation 3.12 $$RSNO + 2OH^- \rightarrow RS^- + NO_2^- + H_2O$$

The effect of copper on the RSNOs in question was highly variable (section 3.4). With the less stable RSNOs i.e. CysNO and CysGlyNO lower concentrations of copper (0.05-5μM) were required to activate the Cu-catalysis and hence any small changes in concentrations of adventitious Cu^{2+}/Cu^{+} ions will effect the results considerably. The kinetic order for the reactions of each RSNO with Cu^{2+} was not clear. Reasonable first-order fits were obtained for the copper catalysed decomposition of CysNO and CysGlyNO (figure 3.16). This observation is in agreement with Dicks *et al* (1996). Although a linear trend was observed with the plots of fractional rate *vs* $[Cu^{2+}]$ for

Figure 3.16 First order reaction between 0.5mM CysNO and 1μM Cu^{2+}.

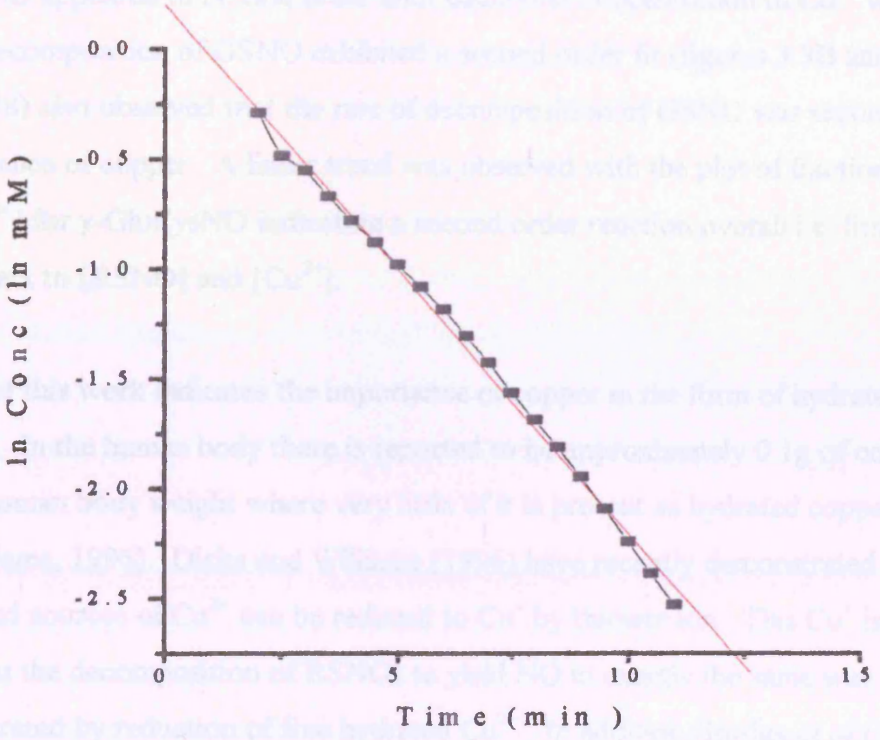
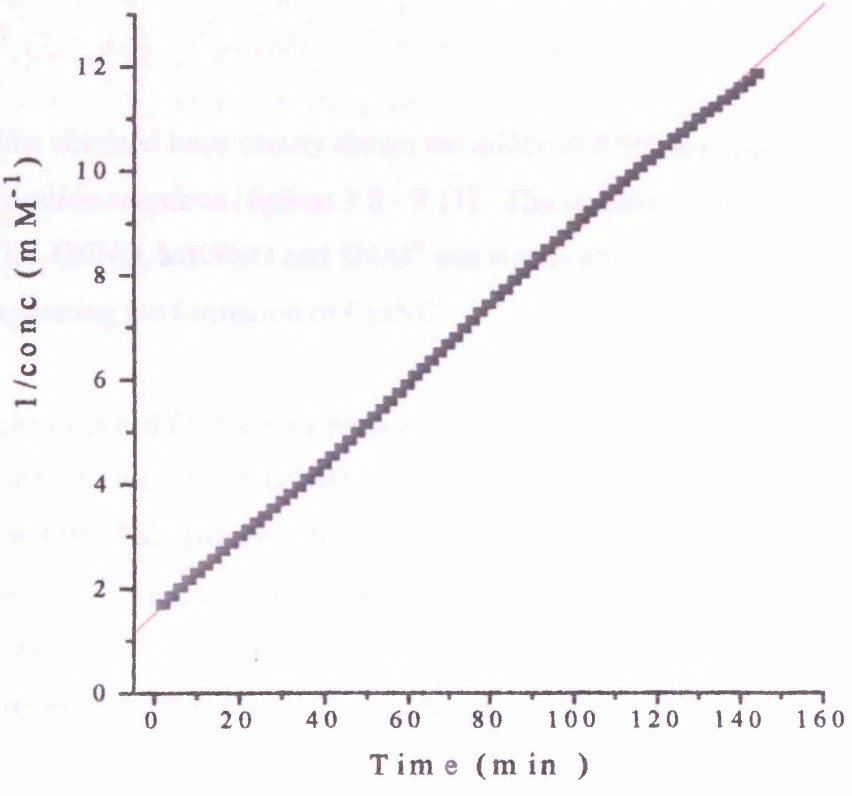


Figure 3.17 Second order reaction between 0.5mM GSNO and 0.5mM Cu^{2+}.



these two RSNOs, the fractional rates were highly variable. The reaction of Cu^{2+} with GSNO appeared to be first order until equimolar concentration of Cu^{2+} where the rate of decomposition of GSNO exhibited a second order fit (figures 3.3B and 3.17). Park (1988) also observed that the rate of decomposition of GSNO was second order in the presence of copper. A linear trend was observed with the plot of fractional rate *vs* $[Cu^{2+}]$ for γ-GluCysNO indicating a second order reaction overall i.e. first-order with respect to [RSNO] and $[Cu^{2+}]$.

All of this work indicates the importance of copper in the form of hydrated Cu^{2+}/Cu^{+} ions. In the human body there is reported to be approximately 0.1g of copper per 75 kg human body weight where very little of it is present as hydrated copper (Dicks and Williams, 1996). Dicks and Williams (1996) have recently demonstrated that protein-bound sources of Cu^{2+} can be reduced to Cu^{+} by thiolate ion. This Cu^{+} is able to bring about the decomposition of RSNOs to yield NO in exactly the same way as Cu^{+} generated by reduction of free hydrated Cu^{2+}. In addition Gordge *et al* (1995) demonstrated that chelation of copper with the copper chelator bathocuproine sulphonate reduced the anti-aggregatory effect of GSNO in an *in vitro* assay. These results do show the potential importance of copper on the biological activity of RSNO *in vivo.*

The results obtained have clearly shown the ability of RSNOs to participate in transnitrosation reactions (figures 3.8 - 3.11). The stability of some of the more stable RSNOs i.e. GSNO, MESNO and SNAC was significantly reduced in the presence of CysH suggesting the formation of CysNO.

The results obtained for the transnitrosation reaction between GSNO and CysH indicate that the reaction of GSNO was first-order with respect to [CysH] (table 3.6 and figure 3.9). This gives rise to a pseudo first-order rate equation as shown (equation 3.13). Taking the zero CysH point into account demonstrates that there are two phases involved in this reaction, the first of which is a high affinity process which was not investigated any further during the course of this project.

Equation 3.13 $$\text{Rate} = k[\text{GSNO}][\text{CysH}]$$

[GSNO] is constant therefore the equation becomes

$$\text{Rate} = k'[\text{CysH}]$$

where $k' = k[\text{GSNO}]$

The reaction of CysNO with GSH was zero-order with respect to [GSH]. These results suggest that the reaction of CysNO with GSH is so rapid that the equilibrium in this reaction (equation 3.14) favours GSNO formation.

Equation 3.14 $$\text{CysNO} + \text{GSH} \rightleftharpoons \text{GSNO} + \text{CysH}$$

The differences in the order of the reactions in these two systems can be explained by the difference in the stability of the two starting RSNOs. In the case of the GSNO/CysH reaction GSNO is relatively stable, therefore GSNO will be strongly favoured in the equilibrium (equation 3.15). With the CysNO/GSH system the formation of GSNO is favoured due to the high reactivity of CysNO which will drive the equilibrium to the right and hence to the formation of the more stable RSNO (equation 3.14). It should be noted that all experiments were carried out in the presence of oxygen and adventitious metal ions therefore any CysNO present will be subject to copper catalysed decomposition.

Equation 3.15 $$\text{GSNO} + \text{CysH} \rightleftharpoons \text{CysNO} + \text{GSH}$$

The products of the reaction between RSNOs and RSH's is currently a subject of much debate. It is well established that RSNOs can act as nitrosation reagents with R'SH to give a corresponding R'SNO (Barnett *et al*, 1994; Meyer *et al*, 1994). More recently Singh *et al*, (1996b) have proposed the reaction scheme shown (figure 3.18). They have hypothesised that GSH reacts with GSNO to form the GSH conjugate *N*-hydroxylsulfenamide, GS-N(OH)-SG which can react *via* a number of pathways. Subsequent reactions with GSH result in the formation of NH_3.

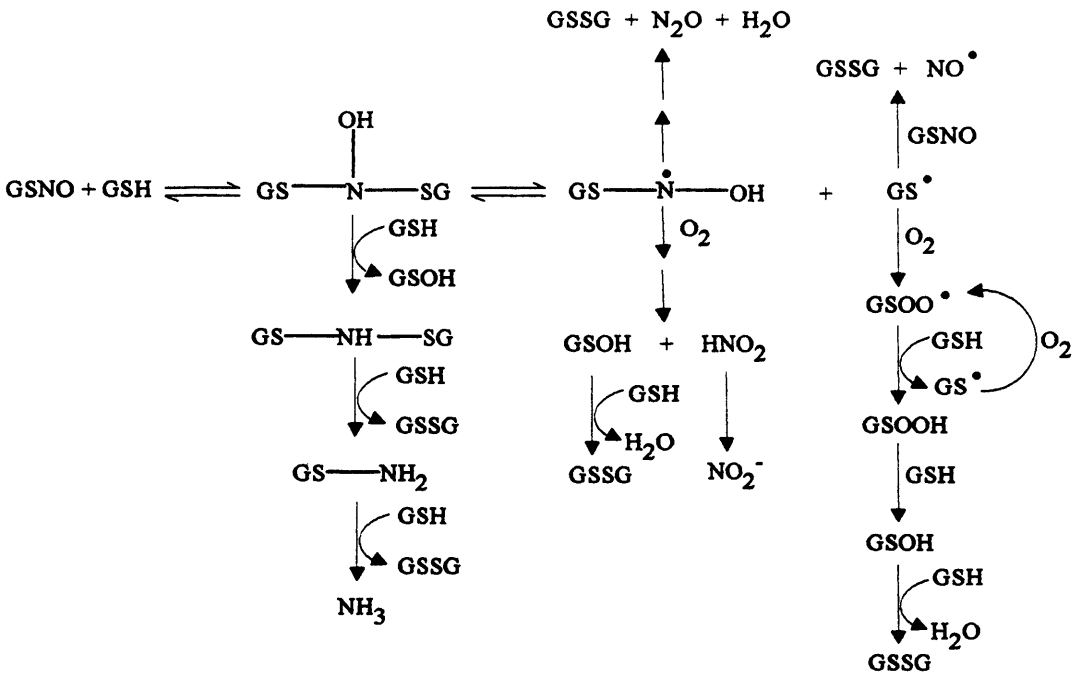
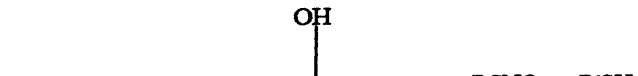


Figure 3.18 Reactions of GSNO with GSH.

From this scheme it has been proposed that transnitrosation occurs through the *N*-hydroxylsulfenamide that arises from nucleophilic attack of the thiol on the R'SNO nitrogen as shown in equation 3.16. The proposed hypothesis suggests that NO^+ is not an intermediate in any of these reactions.

Equation 3.16



The reaction of GS^- with GSNO to form GSSG and NO could account for the faster rate of NO production from GSNO in the presence of GSH. The major end product from this reaction scheme is GSSG, the amount formed depending on availability of oxygen.

There is also the possibility that the added thiols are reducing any Cu^{2+} present to Cu^+ which then catalyses the decomposition of the RSNO (equations 3.8-3.10). If this hypothesis is true then the differences in the results for the GSNO/CysH and CysNO/GSH systems may reflect the differing abilities of each RSH for reducing Cu^{2+}

In comparison Singh *et al* (1996a) have proposed that transnitrosation reactions proceed *via* transfer of NO^+ from an RSNO to a RSH, but in physiological conditions NO^+ would be very short lived, reacting with water to give nitrous acid as (equation 1.1) (Butler *et al*, 1995).

Barnett *et al* (1994) have suggested a direct reaction between the NO group of RSNO and the thiolate anion of R'SH (equation 1.20). This reaction can be readily achieved with alkyl nitrites in a reaction which has all of the characteristics of nucleophilic attack by the thiolate anion (Barnett *et al*, 1994; Barnett *et al* 1995; Williams, 1988b).

The formation of low molecular weight RSNOs *in vivo* such as GSNO and CysNO has been suggested as a mechanism for storage or transport of NO (Girard and Potier, 1993; Myers *et al*, 1990). In such cases it is assumed that these compounds are transported or diffuse to the site of action where the RSNO decomposes to release NO. Little is known about the reaction of GSH with NO *in vivo* but a direct reaction of these two yields GSSG and the nitroxyl anion (NO^-) (Hogg *et al*, 1996). However, in the presence of oxygen it as been demonstrated that GSNO is formed *via* $NO_2^{\cdot}$ and N_2O_3 (Wink *et al*, 1994; Goldstein and Czapski, 1996) but as the rate of formation of these reactive nitrogen/oxygen species is second order in NO and first-order in O_2 the concentrations of these NO/O_2 intermediates are expected to be low *in vivo* (Hogg *et al*, 1996; Goldstein and Czapski, 1996). In an *in vitro* situation the nitrosation of RSH *via* N_2O_3 has been suggested to be affected by phosphate (Keshive *et al*, 1996; Lewis *et al* 1995). The *N*-nitrosation of morpholine was shown to be inhibited in the presence of phosphate buffer. This inhibition was explained by phosphate reacting with N_2O_3 to form nitrosyl compounds, which are then hydrolysed to NO_2^-.

It is clear that there are many factors controlling the release of NO from RSNOs both *in vitro* and *in vivo*, which may have implications regarding the biological activity of these compounds as shown in chapters 4 and 5.

Chapter 4

Pharmacological Properties of S-Nitrosothiols

4.1 Introduction

Endothelial-derived NO plays a pivotal role in the control of blood flow and haemostasis. The aim of this work was to determine the ability of the RSNOs under investigation to inhibit aggregation of human washed platelets (HWP) and induce the relaxation of vascular smooth muscle. These results were compared to the differences in stability of the RSNOs to determine if any correlation occurred between such properties.

Platelet aggregation was monitored using the turbidimetric method of Born and Cross, (1963) in which aliquots of HWP were incubated at 37°C and stirred in an aggregometer (figure 4.1). This method is based upon the spectrophotometric measurement of changes in light transmission during aggregation.

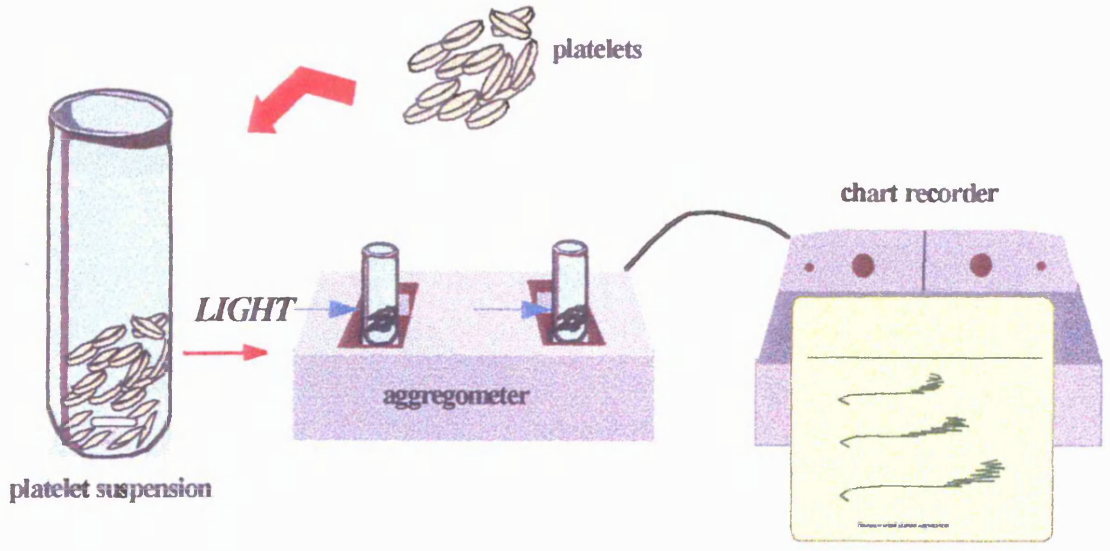


Figure 4.1 Aggregometer set up for platelet studies.

Due to the use of light this assay is only suitable for measuring aggregation in platelet-rich plasma (PRP) or washed platelets (WP) but not whole blood. The aggregometer is adjusted so that unstimulated platelets show 0-10% light transmission (no aggregation) and platelet-poor plasma shows 90-100% transmission (complete aggregation). Aggregation is quantified by measuring the extent of change of light transmittance.

The preparation of human washed platelets was based on the method of Radomski and Moncada (1983) which uses prostacyclin (PGI_2) in order to protect the platelets from activation brought about by preparative manipulations. Prostacyclin appears to be a better choice for washing than other platelet-inhibitory prostaglandins or prostacyclin analogues mainly because of its short half-life. The more stable reagents may cause the recovery time of platelets to be prolonged.

The vasorelaxation effect of the RSNOs was measured using the organ chamber bioassay. Each arterial ring preparation is mounted on two L-shaped stirrups in a chamber in which it is bathed with a physiological salt solution (Krebs buffer) and attached to a signal transducer through which a continuous record of its contractile tension is recorded (figure 4.2).

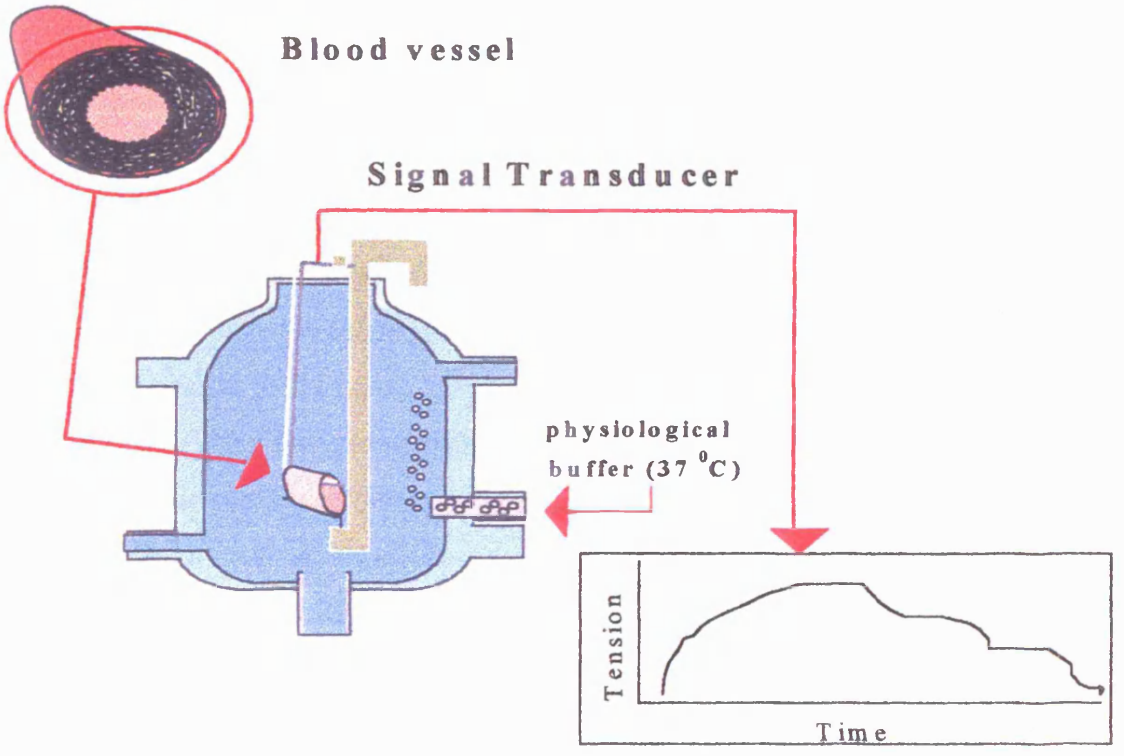


Figure 4.2 Organ chamber apparatus used for the measurement of vasorelaxation.

Concentration-response data to NO/NO donor are obtained by making cumulative additions of the agent to the sub-maximal pre-contracted rings. Cumulative additions

are generally begun at a concentration below or close to threshold and are continued until maximum relaxation to the agent has been achieved.

For these experiments, rings of rat aorta were prepared with the endothelium intact and denuded. Endothelium-free arterial rings avoid any influence of basally released NO on the responses of the added agents.

4.2 Synthesis of S-Nitrosothiols for use in Biological Assays

An alternative method of synthesising the RSNO in solution was devised for use in biological systems. This method was developed mainly to overcome the problems experienced with the resulting low pH of solutions prepared using the Hart method (1985). In addition this preparation of RSNOs is quick which is an added advantage when preparing solutions of unstable compounds such as CysNO.

4.2.1 Experimental

The following method prepares a 1mM solution of RSNO. A solution of thiol (20mM) was prepared in 1mM citrate buffer pH 2.0 (solution A). A solution of $NaNO_2$ (20mM) was prepared in saline (solution B). Solutions A and B (250µl) were added to 1mM citrate buffer pH2 (4.5ml). This reaction yielded a coloured solution of the appropriate RSNO. The concentration of RSNO was assessed by obtaining the UV spectrum of the compound diluted in ultra-pure water. The RSNO was scanned between 200 and 600nm. The absorbance at the λ_{max} at approximately 330nm was determined and the concentration of RSNO was calculated using the Beer-Lambert law and the appropriate ε value (section 3.2).

All RSNO solutions were protected from heat and light by wrapping the containing vessel in aluminium foil and storing on ice. Solutions were always prepared fresh just prior to use.

In the above method a 1 in 20 dilution was made of both the thiol and $NaNO_2$ in citrate buffer. When preparing different concentrations of RSNO this volume was adjusted accordingly but the dilution of $NaNO_2$ was always kept to maximum hence the amount of saline was kept to a minimum to ensure the pH remained at 2.

The RSNO stock prepared was at least 5 times more concentrated than the upper dose required. This allowed for a dilution to be made in the appropriate buffer e.g. Tyrodes solution etc., to increase the pH before addition to the biological solution.

4.3 Inhibition of Platelet Aggregation

4.3.1 Experimental

4.3.1.1 Preparation of Human Washed Platelets (HWP)

Blood was collected from human volunteers in the ratio of 1 part 3.15% w/v trisodium citrate to 9 parts blood. The blood was then centrifuged at room temperature at 1100rpm (220g) for 20 min. The supernatant obtained is termed as PRP. The PRP was transferred into clean centrifuge tubes and PGI_2 was added at a final concentration of $0.3\mu gml^{-1}$, this was then centrifuged at 2300rpm (700g) for 10 min. The resultant pellet contained platelets and some erythrocytes. The supernatant, platelet poor plasma (PPP), was removed. Tyrodes solution (20ml) containing PGI_2 ($0.3\mu gml^{-1}$) was prepared. A 5ml aliquot of this solution was poured onto the platelet pellet. The platelet pellet was then re-suspended. Care was taken to ensure that the red cells remained at the bottom of the tube. The re-suspended platelet solution was transferred to the remaining 15ml of the Tyrodes solution and gently mixed. The resulting solution was then centrifuged at 2100 rpm (680g) for 10 min again at room temperature. The supernatant was removed and the surface of the platelets was washed with 3x1ml of Tyrodes solution without PGI_2. The platelets were then finally re-suspended in 5ml of Tyrodes solution and numbers counted on a Coulter Counter. The final solution was diluted with PGI_2-free Tyrode solution to give a final platelet count of $200\text{-}250x10^9$ /l.

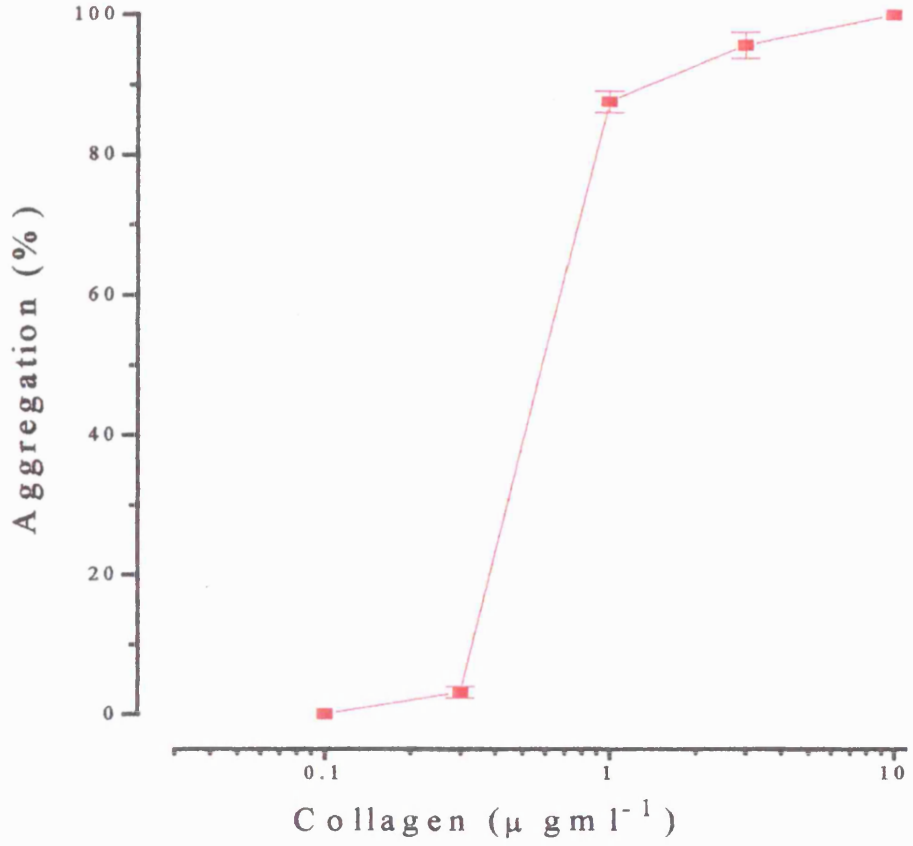
Suspensions of HWP were always left for up to 2 hr at room temperature before use in order to ensure that the anti-aggregatory effects of PGI_2 had decayed. Platelet suspensions were then stored on ice until required.

4.3.1.2 Collagen Induced Platelet Aggregation

The platelet suspension (0.5ml) was placed into a dual-channel aggregometer (Chronolog) at 37°C (figure 4.1). The platelet suspension was stirred continuously at 900rpm throughout the assay. Measurements were made against Tyrodes solution as a blank.

A recording of the aggregation was initiated once the platelet suspension had reached an equilibrium, which was usually after 1.5 min. After 2 min collagen (1-10μgml^{-1}) was added and aggregation monitored for 4 min. A dose response curve for collagen was obtained (figure 4.3) and from this curve the effect of NO donors was assessed using a sub-maximal concentration of collagen. This was usually an EC_{90} and is described as the concentration that gives 90% response of the maximum aggregation induced by collagen. Care was always taken to ensure that the EC_{90} concentration chosen was submaximal. If the EC_{90} used was too high this resulted in maximum aggregation which made it difficult to observe any anti-aggregatory effects.

Figure 4.3 Typical concentration response curve for the induction of aggregation of HWP by collagen.



4.3.1.3 Determination of Potency (IC_{50}) of Individual RSNO

The RSNO stock was prepared as detailed in section 4.2.1. This RSNO stock was diluted serially using Tyrodes solution as the diluent. Again, the platelet suspension (0.5ml) was placed into a dual-channel aggregometer (Chronolog) at 37°C. The platelet suspension was stirred continuously at 900rpm throughout the assay. A recording of the aggregation was started once the platelet suspension had reached an equilibrium, which was usually after 1.5min. After 2 min the RSNO was added (0.003-30μM) and incubated with the platelets for 1 min. After 3 min collagen was added at the EC_{90} previously determined, aggregation was then monitored for a further 3 min.

4.3.1.4 Treatment of Results

Inhibition of aggregation was expressed as the percentage of the maximal aggregation induced by collagen. Plots of % inhibition of aggregation *vs* concentration were obtained using Microcal Origin 2.8 (figure 4.4). Potency values (IC_{50}) were determined for each compound (table 4.1). Each dose response curve was carried out 5 times. Tyrodes solution (10μl) instead of the RSNO, at the beginning and end of each concentration response curve was used to standardise the experiment.

The presentation of results shows standard errors (SE) not standard deviations (SD) as in preceding chapters, this reflects the convention in the use of animal tissues for pharmacological responses which tends to exhibit high variability.

One way analysis of variance was used to determine the significance of differences between means and $P < 0.001$ was taken as statistically significant. The Tukey test was used for multiple comparisons between the group and $P < 0.05$ was taken as statistically significant. These analyses were carried out on SigmaStat for Windows, v2.0.

4.3.2 Results

The RSNOs investigated exhibited concentration-dependent inhibition of aggregation of HWP. All RSNOs except SNAC demonstrated potency values within the range 0.1-1.1 μM (table 4.1). *S*-Nitroso-*N*-acetyl-L-cysteine gave an IC_{50} value of 3.3 μM (Tukey test $P < 0.05$, n = 5) and also exhibited a different profile (figure 4.4).

Figure 4.4 Inhibition of aggregation of human washed platelets by *S*-nitrosothiols.

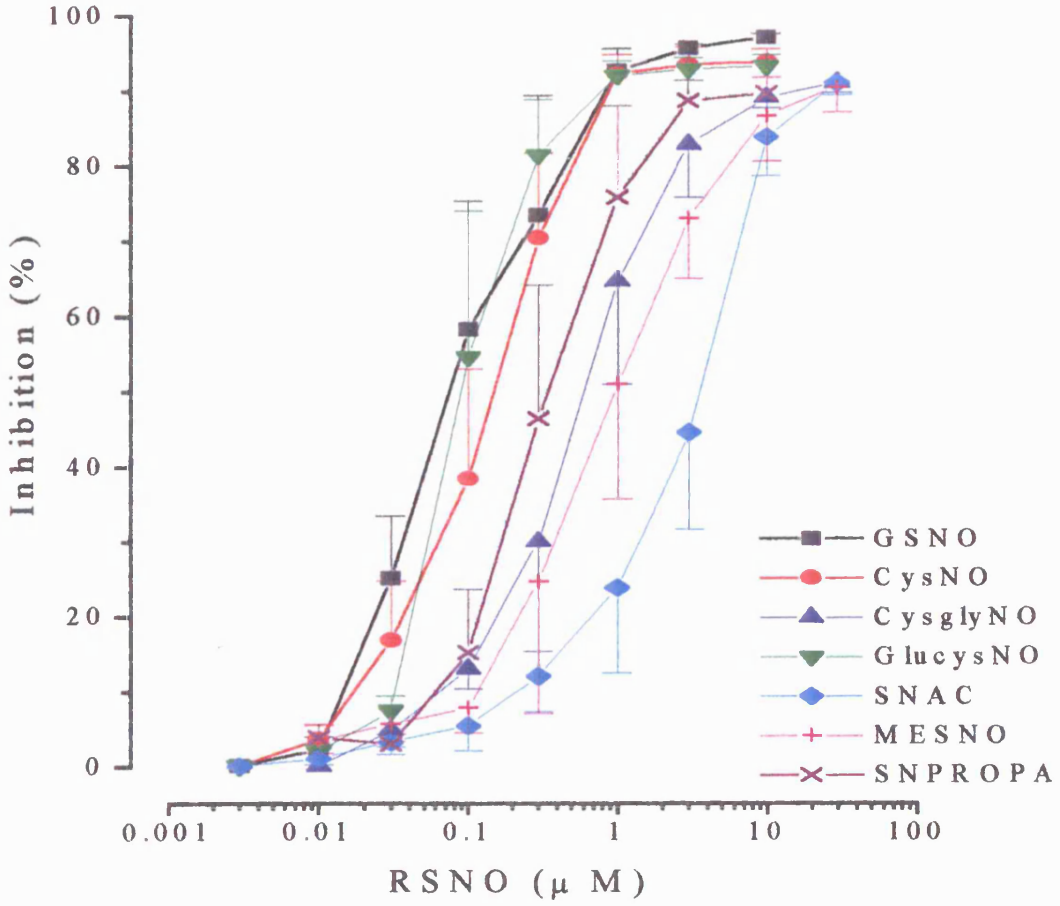


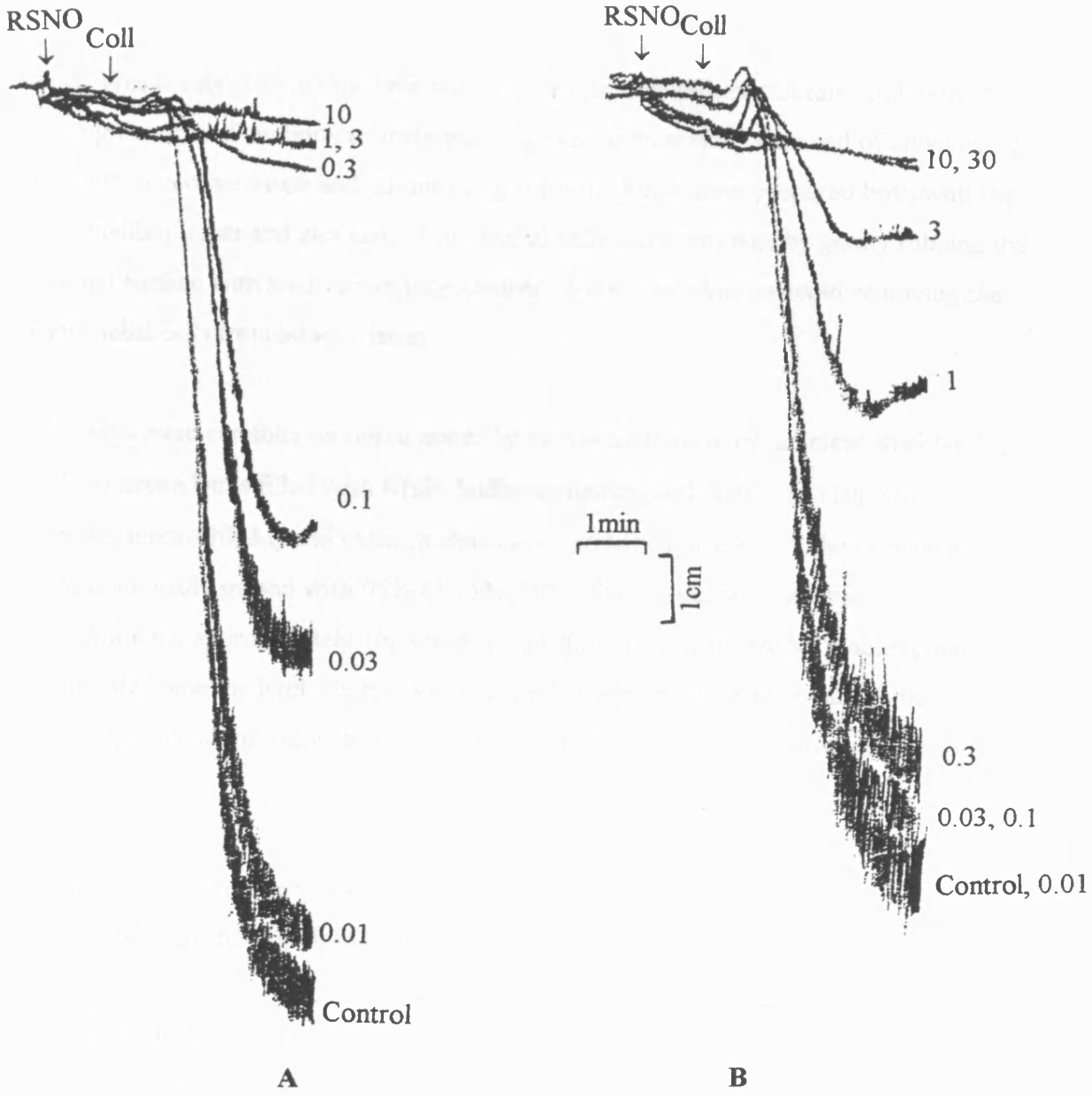
Table 4.1 Potency of *S*-nitrosothiols as inhibitors of aggregation of human washed platelets.

RSNO	IC_{50} ± SE (μM)
GSNO	0.16 ± 0.1
CysNO	0.17 ± 0.07
CysGlyNO	0.79 ± 0.29
γ-GluCysNO	0.11 ± 0.03
SNAC	3.29 ± 0.92*
MESNO	1.07 ± 0.39
SNPROPA	0.45 ± 0.23

Mean ± SE for 5 experiments

$P < 0.001$ by ANOVA, * $P < 0.05$ by Tukey Test

Figure 4.5 (A) Inhibition of aggregation of HWP by CysNO (0.01-10μM) and (B) MESNO (0.01-10μM).



4.4 Relaxation of Vascular Smooth Muscle

4.4.1 Experimental

Male Wistar rats (250-300g) were anaesthetised briefly with isofluorane and killed by exsanguination. The thoracic aorta was removed and carefully trimmed of all adhering fat and connective tissue and cut into rings (4mm). Rings were prepared both with the endothelium-intact and denuded. Endothelial cells were removed by gently rubbing the internal surface with a cut down pipe-cleaner. Care was taken to avoid removing the endothelial cells from intact tissues.

The rings were carefully mounted under 1g of resting tension on stainless steel hooks, in 25ml organ baths filled with Krebs buffer containing indomethacin (10μM), cycloheximide (10μM) and calcium chloride (2.5mM) (figure 4.2). The organ baths were continually gassed with 95% O_2/ 5% CO_2. The tissues were allowed to equilibrate for approximately 1hr which allowed the tissues to reach a stable baseline. During this time the Krebs buffer was changed at regular intervals to ensure the complete removal of vasoactive mediators released from the tissue after removal from the animal.

A concentration-response curve to phenylephrine (1-1000nM) was carried out and the EC_{90} to be used for the relaxation studies was determined (figure 4.8).

Initially the tissues were pre-contracted with the EC_{90} dose of phenylephrine previously determined. Relaxation of the tissues was induced by addition of acetylcholine (0.1-10μM). Failure of the tissue to relax was taken as an indication that the endothelium had been completely removed (figure 4.9). Rings showing less than 80% relaxation were discarded.

The organ baths were then flushed again with Krebs buffer at regular intervals in order to remove acetylcholine and phenylephrine and allowed to equilibrate for a further 60 min.

S-Nitrosothiol stocks were prepared as previously detailed (section 4.2). All standards were diluted serially with Krebs buffer. All solutions were protected from light and stored on ice. The tissues were again pre-contracted by addition of phenylephrine EC_{90} previously determined and cumulative relaxation curves to the RSNOs were obtained (100pM-100μM).

4.4.1.1 Treatment of Results

Concentration-response curves were constructed for each compound (Microcal Origin v 2.8, figures 4.6 and 4.7). EC_{50} values for each compound were determined from the individual concentration-response curves (table 4.2 and 4.3). The EC_{50} is described as the concentration of RSNO required to relax the tissue to 50% of the maximum contraction induced by phenylephrine. The experiments were always carried out on paired rings, with and without endothelium.

One way analysis of variance was used to determine the significance of differences between means and $P < 0.001$ was taken as statistically significant. The Tukey test was used for multiple comparisons between the group and $P < 0.05$ was taken as statistically significant. These analyses were carried out on SigmaStat for Windows, v2.0.

4.4.2 Results

All the RSNOs tested exhibited concentration-dependent relaxation of vascular smooth muscle. All the compounds except SNAC and CysNO exhibited potency values within the range of 300-800nM for endothelium-intact rings (table 4.2). *S*-Nitroso-*N*-acetyl-L-cysteine and CysNO were less potent with EC_{50} values of 1400nM and 2220nM respectively (Tukey test $P < 0.05$). All the RSNOs tested relaxed endothelium-denuded rings with EC_{50} within the range of 60-200nM except for CysNO which exhibited an EC_{50} of 600nM (Tukey test $P < 0.05$) (table 4.3).

Figure 4.6 Relaxation of endothelium-intact rat aortic rings by *S*-nitrosothiols.

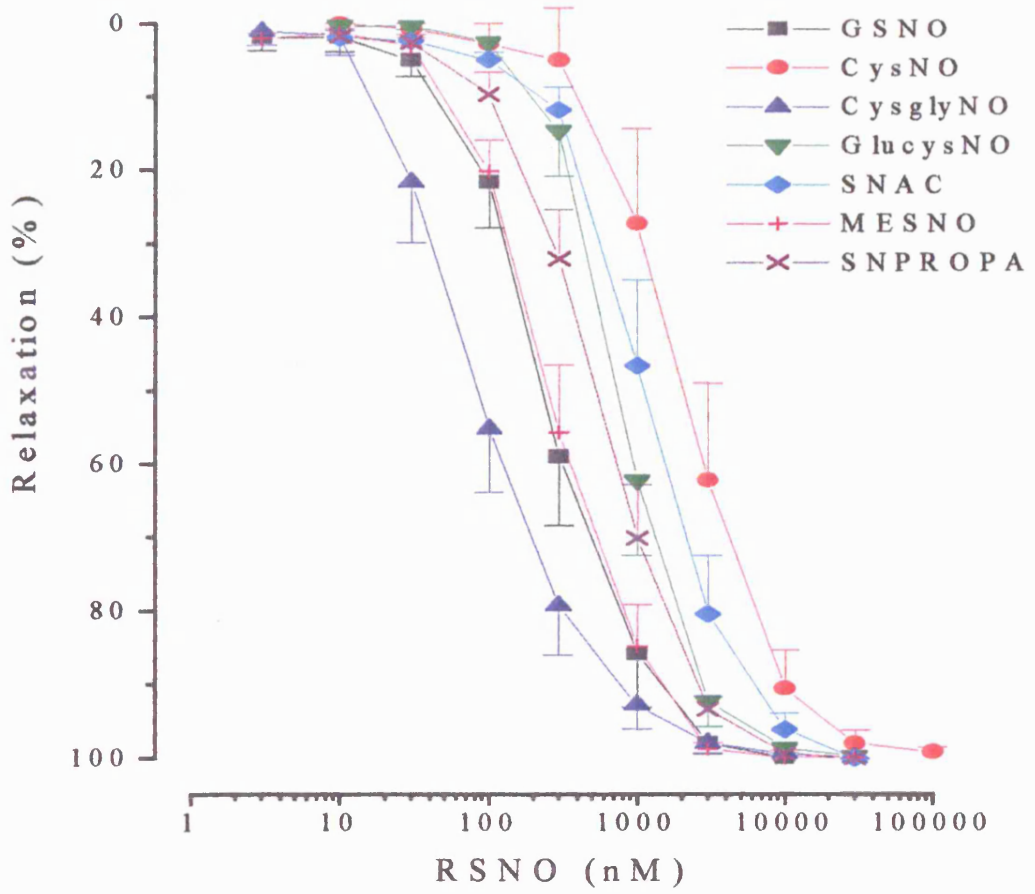


Table 4.2 Potency of *S*-nitrosothiols as vasorelaxation agents in endothelium-intact aortic rings.

RSNO	EC_{50} ± SE (nM)
GSNO**	286.9 ± 48.0
CysNO*	2220 ± 606‡
CysGlyNO*	92 ± 30
γ-GluCysNO*	825 ± 200
SNAC*	1364.6 ± 381.8‡
MESNO*	301.5 ± 82.7
SNPROPA§	344.5 ± 140.6

Mean ± SE for *n=4, § n=5, **n=8

$P < 0.001$ by ANOVA, ‡$P < 0.05$ by Tukey Test

Figure 4.7 Relaxation of endothelium-denuded rat aortic rings by *S*-nitrosothiols.

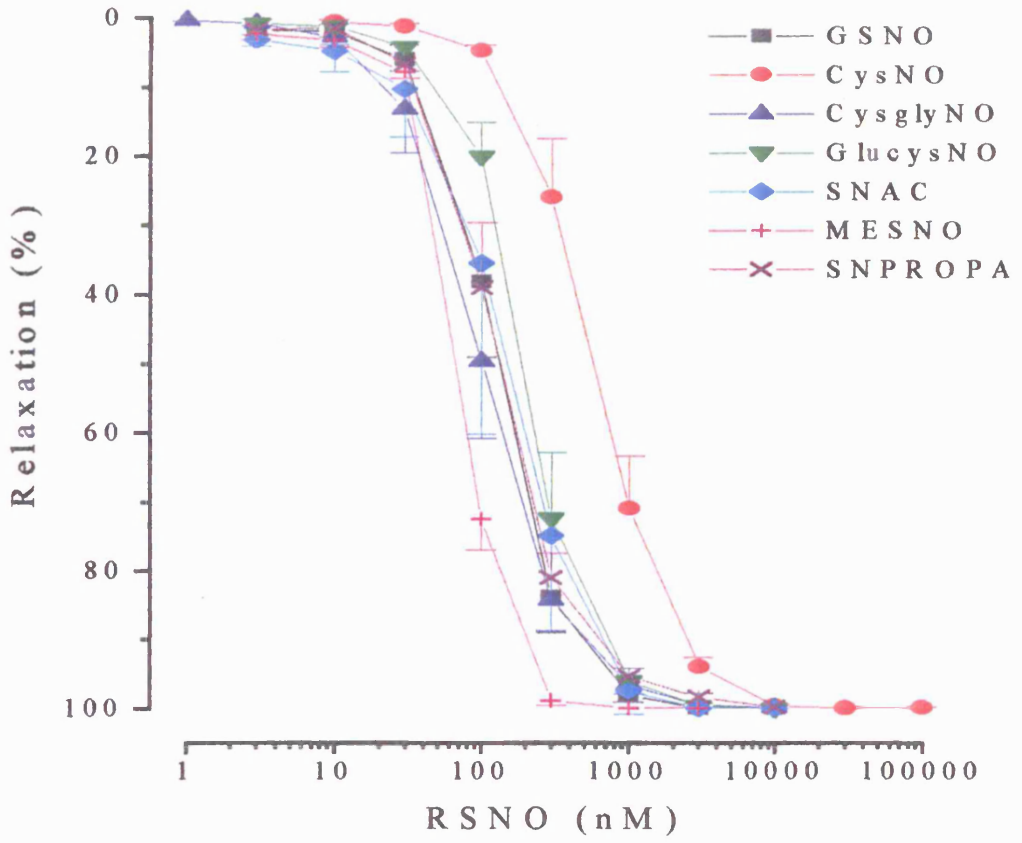


Table 4.3 Potency of *S*-nitrosothiols as vasorelaxation agents in endothelium-denuded aortic rings.

RSNO	EC_{50} ± SE (nM)
GSNO**	148 ± 24.1
CysNO*	617.1 ± 149.9‡
CysGlyNO*	110.5 ± 24.9
γ-GluCysNO†	204.9 ± 40.5
SNAC*	171.9 ± 37.5
MESNO*	69.3 ± 5.7
SNPROPA§	138.4 ± 21.3

Mean ± SE for † n=3, *n=4, § n=6, **n=7

$P < 0.001$ by ANOVA, ‡ $P < 0.05$ by Tukey Test

Figure 4.8 Typical concentration response curve obtained for the contraction of endothelium-intact and denuded vascular smooth muscle by phenylephrine (1-1000nM).

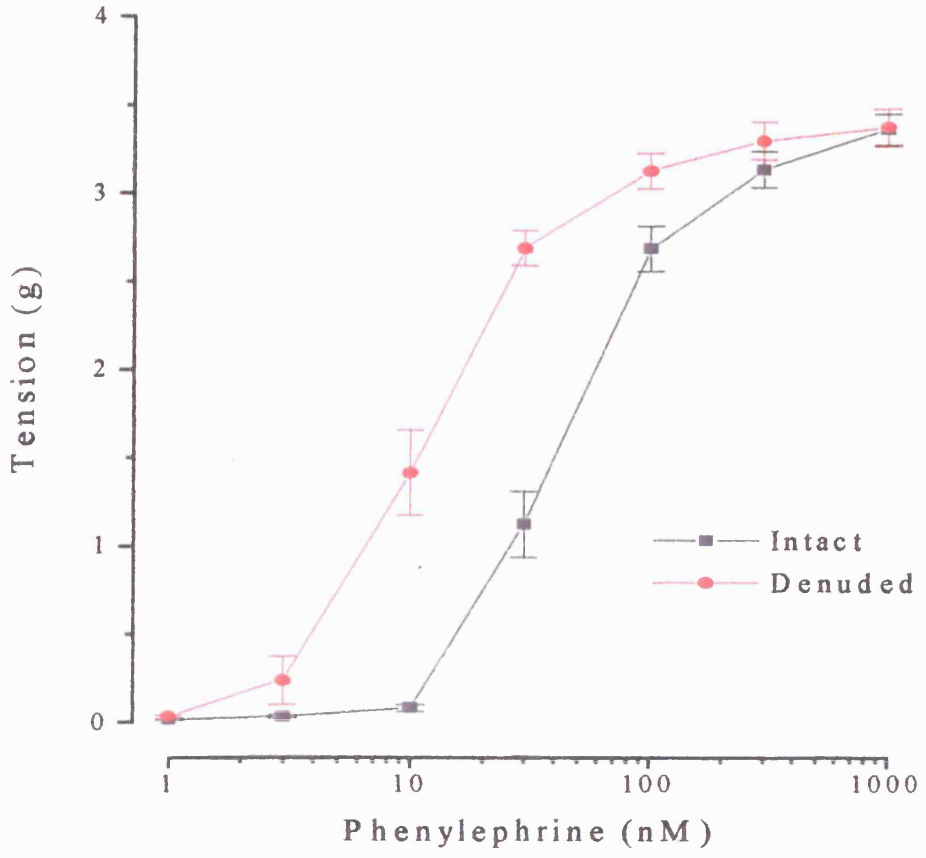
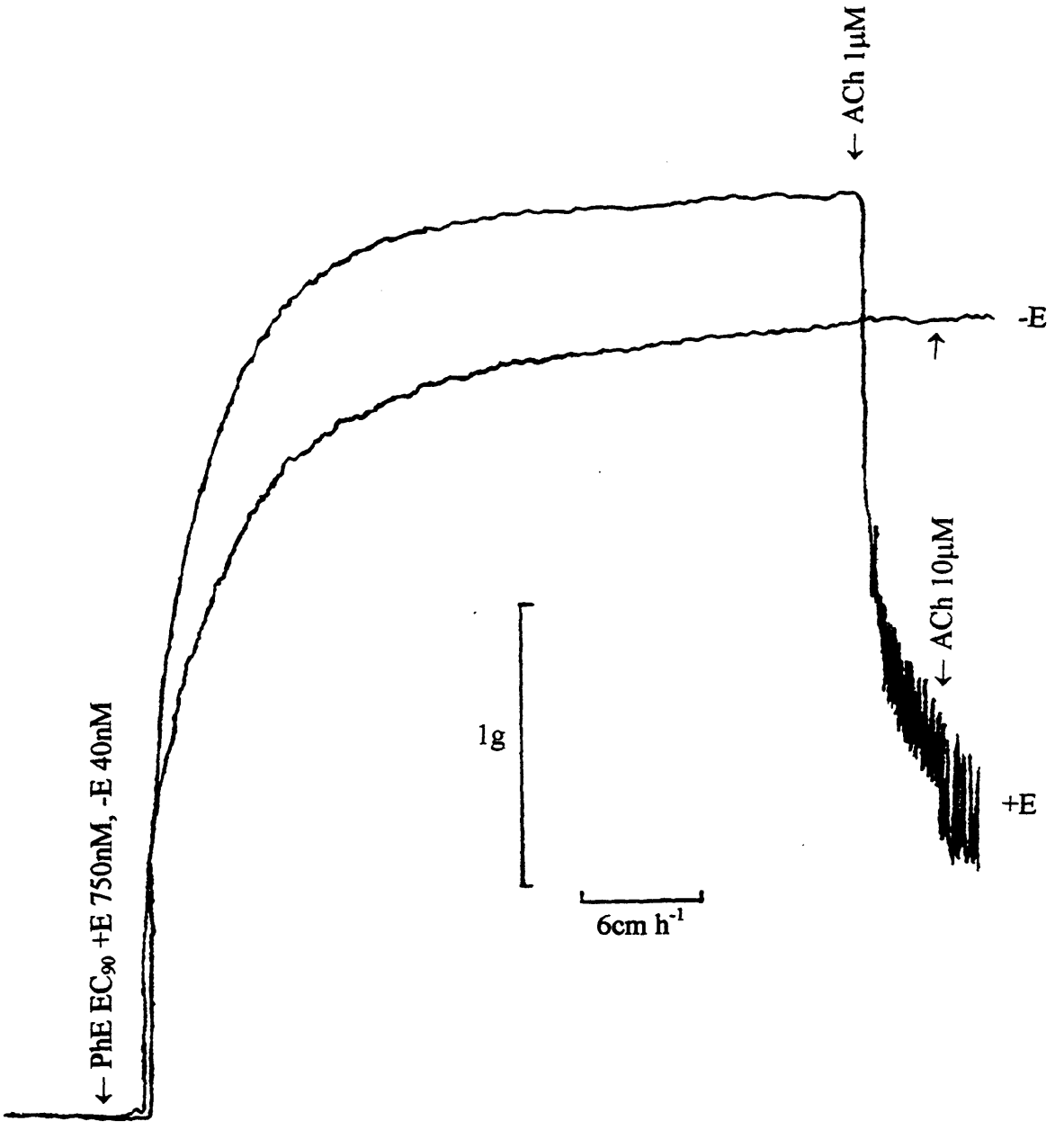


Figure 4.9 Relaxation of endothelium intact and denuded vascular smooth muscle by acetylcholine.



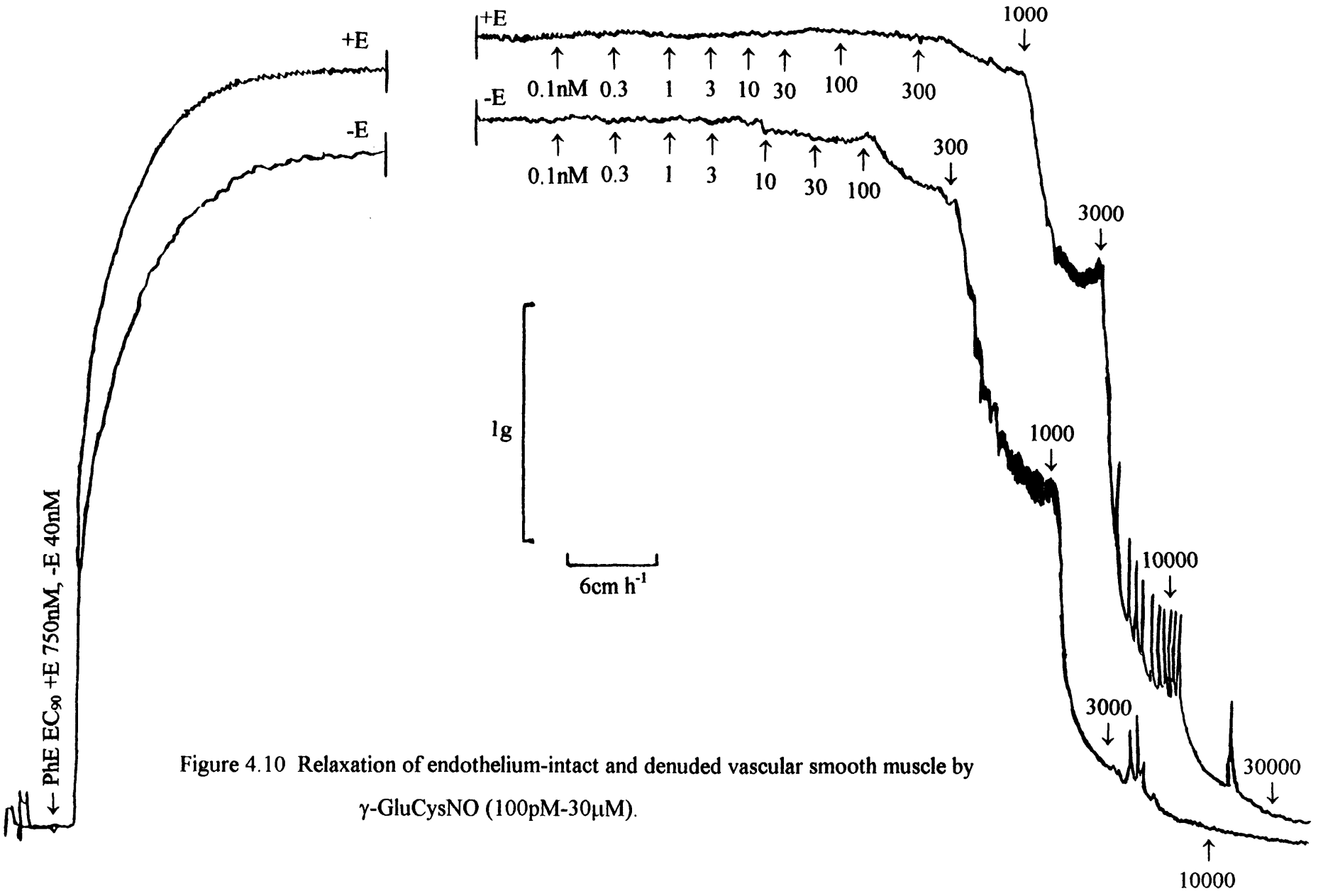


Figure 4.10 Relaxation of endothelium-intact and denuded vascular smooth muscle by γ-GluCysNO (100pM-30μM).

4.5 Discussion

The RSNOs investigated exhibited concentration-dependent inhibition of aggregation of HWP. All RSNOs except SNAC demonstrated potency values within the range 0.1-1.1 μM (table 4.1). *S*-Nitroso-*N*-acetyl-L-cysteine gave a higher IC_{50} value and also exhibited a different profile (figure 4.4).

Again all RSNOs studied exhibited concentration-dependent relaxation of vascular smooth muscle. The more stable RSNOs except CysNO and SNAC exhibited potency values within the range of 90-800nM for endothelium-intact rings. Again CysNO exhibited a higher EC_{50} for denuded rings with the remainder of the compounds exhibiting potency values within the range of 60-200 nM (tables 4.2 and 4.3). Generally RSNOs were more potent as smooth muscle relaxing agents with endothelium-denuded rings. This may be due to there being no interference from endothelial derived NO, differences in O_2^- concentration and other NO scavengers or even due to the presence of an endothelial dependent mechanism of RSNO breakdown. Therefore the results from denuded rings generally give a better estimation of order of potency when comparing each compound. The increased sensitivity of RSNOs in denuded rings may be related to an up-regulation of soluble guanylyl cyclase rather than to the removal of the endothelium which may act as a diffusion barrier to these compounds (Moncada *et al,* 1991b).

S-Nitrosocysteine and CysGlyNO which are both very unstable, exhibited varying activity in both bioassays. *S*-Nitrosocysteine proved to be the least potent RSNO in the organ bath method whereas CysGlyNO was the most potent in the same assay. As inhibitors of platelet aggregation CysNO was equipotent with GSNO, one of the more stable compounds, but CysGlyNO was less potent.

Hence from these results no real correlation between pharmacological activity and ability of the RSNO to release NO has been observed, under the experimental conditions used. This apparent lack of correlation may be due to the instability of RSNOs which could prevent accurate biological measurements being made. In

addition the stability of the RSNOs has not been measured under the same conditions used in each of the biological assays. The stability of several of the RSNOs synthesised using the citrate method were measured as previously detailed (section 3.3), but the half-lives obtained were comparable to the same RSNOs synthesised using the Hart method (results not shown). However, the results obtained suggest that the spontaneous liberation of NO is not solely responsible for the vascular activity of these compounds. The same observations have been reported previously (Mathews and Kerr, 1993; Kowaluk and Fung, 1990; Park *et al*, 1993). The pharmacological activity of RSNOs has been suggested to be due to the intact RSNO (Myers *et al*, 1990) but these compounds are polar and are probably ionised at physiological pH hence this explanation seems unlikely (Kowaluk and Fung, 1990). A more probable hypothesis is that the NO is released from the exogenous RSNO and is transported across cell membranes by a carrier molecule such as another thiol (Girard and Potier, 1993), this would give rise to a second R'SNO *via* a transnitrosation mechanism as previously described (chapter 3). The R'SNO formed is likely to have stabilities and biological properties that differ from the original RSNO.

There is presently a great deal of evidence to support this hypothesis. Park (1988 and 1993) and Park *et al* (1993) have implicated the role of transnitrosation reactions in the biological activity of GSNO, demonstrating the production of protein RSNO *in vitro*. In agreement with this Simon *et al* (1993) have shown that *S*-nitroso proteins such as *S*-nitroso-albumin and *S*-nitroso-tissue type plasminogen activator undergo RSH- RSNO exchange with low molecular weight thiols i.e. GSH, CysH, and that these protein RSNOs are potent inhibitors of platelet aggregation *in vitro*, *ex vivo* and *in vivo*. The formation of low molecular weight RSNOs from *S*-nitroso-albumin has been demonstrated *in vivo* (Scharfstein *et al*, 1994). The relevance of this data is highlighted by the work of Stamler *et al* (1992c) who suggested that naturally produced NO circulates in plasma primarily as an RSNO complex, particularly *S*-nitroso-serum albumin. This RSNO would act to stabilise NO and serve as a reservoir with which levels of NO can be regulated. In addition the production of endogenous RSNOs has been demonstrated *in vivo* and *in vitro*, suggesting this as a method of stabilising NO (Keaney *et al*, 1993; Stamler *et al* 1992b; Gaston *et al* 1993).

Overall in both assays the majority of RSNOs gave potency values within the same region. With the exception of SNAC all the RSNOs inhibited platelet aggregation within one log unit i.e.0.1-1μM. Again with the relaxation of endothelium-denuded vascular smooth muscle all the RSNOs gave potency values within one log unit. This observation again supports the theory that the pharmacological effect of these RSNOs may proceed *via* a common pathway such as transnitrosation.

A major aim of this project was to investigate if the glutathione enzyme, GGT has a role to play in the de-nitrosation of GSNO. This enzyme may enhance the release of NO from GSNO *via* the formation of CysGlyNO. As previously shown (chapter 3) CysGlyNO is considerably less stable than GSNO and hence would release NO more readily and be more potent as a pharmacological agent. This in fact was not the case, as CysGlyNO was less potent than GSNO as an inhibitor of platelet aggregation but was more potent as a vasorelaxation agent. These data suggest that the formation of CysGlyNO is not a major pathway involved in the pharmacological activity of GSNO. Rees *et al* (unpublished data) have also looked at the effect of the GGT inhibitor Acivicin on the vasorelaxant effect of GSNO and observed that the potency was unaffected. Again this implies that GGT is not involved in the de-nitrosation of GSNO but it is feasible that a different enzyme may be involved in this process. These results are in disagreement with Askew *et al* (1995b) who claimed that the release of NO from GSNO was caused by both transnitrosation between GSNO and CysH and the GGT cleavage of GSNO to give CysGlyNO. Several groups have proposed that the selectivity of GSNO as an inhibitor of platelet aggregation is due to the de-nitrosation of GSNO *via* an enzymatic mechanism present in platelets (de Belder *et al*, 1994; Radomski *et al*, 1992).

The evidence suggests that the transnitrosation between the exogenous RSNO and endogenous thiols could contribute to the pharmacological activity of the RSNOs investigated. It appears at this stage that GGT does not have a role to play in the de-nitrosation of GSNO but this cannot be confirmed until the pharmacological properties of D-GSNO have been investigated.

Chapter 5

Toxicological Properties of S-Nitrosothiols

5.1 Introduction

The toxicity and reactivity of NO is modest in comparison to oxidants such as NO_2 (Brunelli *et al*, 1995) and is often overestimated. The aim of this work was to assess in a preliminary fashion, the toxicological potential of RSNOs, as NO donors, and to ascertain if there is any correlation between the chemical stability and toxicological properties of these compounds.

The human lung carcinoma cell line (A549) was chosen as the toxicity model system and the cytotoxicity of the RSNOs against these cells was assessed using the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The MTT assay is based upon the principle that dehydrogenase enzymes within active mitochondria oxidise the yellow MTT salt to a dark blue formazan product by cleaving the tetrazolium ring (Mosmann, 1983; Twentymann and Luscombe, 1987). This conversion only takes place in viable cells hence the amount of formazan present is proportional to the number of cells present. Dead cells do not interfere with the MTT absorbance as cell debris is not stained by formazan (Keepers *et al*, 1991). The formazan product is measured quantitatively using visible absorption.

There are several points which need to be considered when using the MTT assay. Optimal conditions need to be determined for each cell line, both in terms of cell number plated and assay duration. Sufficient time must be allowed for cell death and loss of dehydrogenase activity to occur (Carmichael *et al*, 1987). The extent of formazan production depends upon cell density and amount of MTT added and therefore has to be optimised. A major disadvantage of the MTT assay is the fact that serum interferes with the optical density of formazan in solution. Therefore medium needs to be removed as much as possible and the cells should be rinsed (Twentymann and Luscombe, 1987). DMSO catalyses the production of formazan from MTT therefore plates should be read as soon as possible after DMSO addition (Twentymann and Luscombe, 1987).

5.2 Experimental

5.2.1 Routine Cell Maintenance

All cell culture procedures were carried out in a laminar flow cabinet using aseptic techniques. Cells were maintained in incubators at 37°C with 5% CO_2. A549 cells had been originally obtained from the European Collection of Animal Cell cultures (ECACC), Porton Down, Wilts and were free of mycoplasma contamination. Cells were passaged when approaching confluence which was normally every 3-4 days. Cells were used in experiments between two and six months after resurrection from the cell bank. A549 cells were routinely grown in Ham's F12 medium supplemented with 10% v/v foetal calf serum, penicillin (100iu ml^{-1}), streptomycin (100μg ml^{-1}) and glutamine (2mM). Trypsin solution was used for detachment of monolayers from culture vessels.

5.2.2 MTT Assay

A549 cells were seeded at $1.1x10^3$ cells /well in 96 well plates. After 24 hr the medium was removed and replaced with fresh medium (210μL) containing the RSNO or thiol (0.05-0.5mM) and incubated for 72 hr. All plates were wrapped in aluminium foil before being placed in the incubator to prevent light-catalysed degradation of the RSNO. The RSNO stock was diluted 10-fold with 0.25M Tris buffer pH 7.4 prior to further dilution with media before addition to the cells so that the stock solution maintained physiological pH. Control wells were also set up containing a 1 in 10 dilution of the citrate buffer, which was the solution in which the RSNOs were prepared (section 4.2), in Tris, pH 7.4, followed by dilution with media. Blank wells were prepared containing media alone. The plates were incubated at 37°C for 72hr.

A stock solution of MTT in medium (5mg ml^{-1}) was prepared and passed through a 0.2μm filter. The medium/RSNO was removed and the cells were washed with PBS (200μl). To each well an aliquot (200μl) of the MTT solution was added and the plates were then incubated for a further 4hr. Monolayers were then washed twice with PBS (200μL) and the formazan crystals were then dissolved in buffered DMSO (1 part

glycine buffer to 8 parts DMSO). The plates were agitated using a plate shaker for 20 min. Formazan production was measured quantitatively using visible absorption at 540nm on a Labsystems iEMS plate reader. In each step solution was removed from the wells by aspiration to avoid disruption of the cells.

5.2.3 Treatment of Results

The results obtained were in the form of an absorbance value. Results from triplicate wells were averaged and the mean blank value was subtracted from this average. Results were expressed as a percentage of controls and plotted against concentration to calculate the IC_{50} value. An IC_{50} dose is described as the dose that gives 50% growth of control cells.

5.2.4 Results

Of all the RSNOs tested SNPROPA appeared to be the most toxic exhibiting an IC_{50} of 0.0425μM. *S*-Nitrosoglutathione, GluCysNO, SNAC and MESNO exhibited similar toxicities giving IC_{50} values between 0.107-0.181μM. IC_{50} values for CysNO and CysGlyNO were unobtainable under these experimental conditions. Dose response curves and IC_{50} values for each RSNO are shown (figure 5.1 and table 5.2).

Figure 5.1 Inhibition of growth of A549 cells by *S*-nitrosothiols.

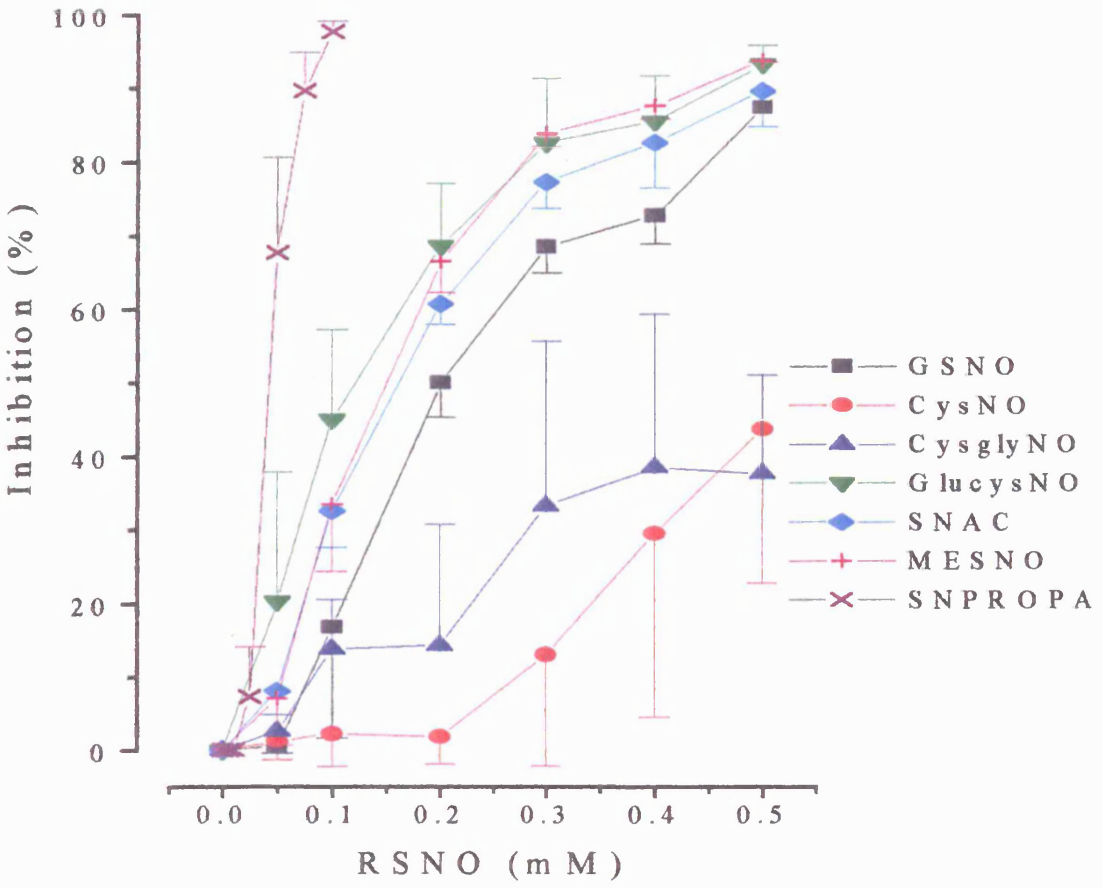


Table 5.1 Inhibition of growth of A549 cells by *S*-nitrosothiols.

RSNO	IC_{50} ± SD (mM)
GSNO	0.181 ± 0.005
CysNO	> 0.5
CysGlyNO	> 0.5
GluCysNO	0.107 ± 0.034
SNAC	0.144 ± 0.008
MESNO	0.138 ± 0.023
SNPROPA	0.0425 ± 0.006

Mean ± SD for 5 experiments

5.2.5 Mechanism of Toxicity

In order to test the hypothesis that the toxicity of RSNOs particularly GSNO and SNPROPA, is caused by the oxidised or reduced parent thiol, cells were treated with GSH (0.05mM-0.5mM), GSSG (0.025-0.25mM) and 3-mercaptopropionic acid (0.005-0.1mM). Cells were exposed to GSNO (0.05-0.5mM) and [1*H*-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one] (ODQ, 10μM), the soluble guanylyl cyclase inhibitor, to test the hypothesis that the toxicity was caused by a cGMP-dependent pathway. Cells were also incubated with GSNO (0.05-0.5mM) and oxyhaemoglobin (OxyHb, 10μM) in order to confirm that the NO released from an RSNO is the cytotoxic agent. The extent of toxicity of these compounds was assessed using the MTT assay as detailed in section 5.2.2.

Students *t* test was used to determine the significance of differences between means and $P < 0.05$ was taken as statistically significant.

5.2.6 Results

Table 5.2. Investigation into the mechanism of toxicity of *S*-nitrosothiols.

Experiment	IC_{50} (mM)
GSNO	0.181 ± 0.005
GSH	> 0.5
GSSG	> 0.25
GSNO + OxyHb	0.310 ± 0.042
GSNO + ODQ	0.186 ± 0.096
SNPROPA	0.0425 ± 0.006
3-Mercaptopropionic Acid	> 0.1

Mean ± SD for 3 experiments

5.3 Discussion

All RSNOs tested exhibited a concentration-dependent inhibition of growth of A549 cells. The inability to obtain IC_{50} values for CysNO and CysGlyNO may be explained by these RSNOs undergoing rapid decomposition once in contact with the media due to the presence of copper. There appears to be some degree of correlation between the chemical stability and the toxicity of these compounds, that is the stable RSNOs with the exception of SNPROPA all exhibited similar IC_{50} values.

The results suggest that the toxicity of GSNO is not due to the formation of GSSG from the decomposition of GSNO (table 5.2). Addition of OxyHb, a known scavenger of NO, significantly increased the IC_{50} of GSNO to 0.31mM ($P = 0.006$) suggesting that the NO released from GSNO is responsible for the inhibition of cell growth. With GSNO and SNPROPA the free thiol did not effect the growth of A549 cells at the concentrations studied.

It is not clear at this stage why SNPROPA is more toxic than the other RSNO investigated. The results indicate (table 5.2) that the free thiol is not as toxic as its *S*-nitrosylated counterpart, suggesting that the toxicity is due to NO or a product of SNPROPA decomposition such as oxidised 3-mercaptopropionic acid. In order to ascertain which of the species is responsible investigations need to be carried out to assess the toxicity of the 3-mercaptopropionic acid disulphide under these experimental conditions and also the toxicity of SNPROPA in the presence of OxyHb to confirm that the toxicity is due to NO.

As previously shown (chapter 1) a large number of the biological effects of NO occur *via* the activation of soluble guanylyl cyclase, which has recently been shown to be inhibited by the compound ODQ (Garthwaite *et al*, 1995; Moro *et al*, 1996). This soluble guanylyl cyclase inhibitor was added to A549 cells in conjunction with GSNO to determine if the mechanism of toxicity involves soluble guanylyl cyclase. The IC_{50} value obtained was not significantly different to GSNO alone ($P = 0.933$), suggesting that at the concentration tested ODQ did not inhibit the toxicity of GSNO which

implies that the soluble guanylyl cyclase is not involved with the inhibition of cell growth by GSNO (table 5.2).

The present literature regarding the toxicity of RSNOs and other NO precursors concentrates on their NO donating properties. *S*-Nitrosoglutathione has been shown to be mutagenic in the Ames test using the *Salmonella typhimurium* strain TA 100 (Carter and Josephy, 1986). This mutagenic effect was explained by the generation of thiol radicals in agreement with Glatt *et al* (1983) who suggested that the mutagenicity of GSH and cysteine was due to radical species. It must be pointed out that this work was carried out before NO was discovered as a biological mediator, and hence if repeated today the results may be interpreted differently.

S-Nitrosoglutathione, CysNO and SNAP (10mM) have been shown to induce strand breaks in pBluescript plasmid DNA in the presence of hydrogen peroxide and DTPA (Park and Kim, 1994). Again this toxicity was suggested to be due to formation of thiol radicals and ultimately the production of $^{\cdot}OH$ (equation 5.1) by reactions other than the Fenton reaction (equations 1.8 and 1.9).

Equation 5.1 $$RS^{\cdot} + H_2O_2 \rightarrow ROH + {}^{\cdot}OH$$

A large number of the toxic reactions of NO have been shown to be due to RSNO intermediates. Niknahad and O'Brien (1995) have implicated GSNO as an active intermediate in the cytotoxicity of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine observed in rat hepatocytes. The mechanism of toxicity is thought to involve NO and further reactions to give $OONO^{-}$, $^{\cdot}OH$ and nitrite. Investigations into the cytotoxic mechanism of butyl nitrite in hepatocytes again suggested GSNO formation (Meloche and O'Brien, 1993).

Laval and Wink (1994) suggested that the DNA repair enzyme O^6-methylguanine-DNA -methyltransferase is inhibited by NO due to the formation of a protein -*S*-nitrosothiol adduct at the enzymes active site. Another important class of DNA interacting proteins containing thiols are those with zinc finger motifs. It has been

shown that under aerobic conditions NO results in degradation of proteins containing such a moiety (Wink and Laval, 1994). Reactive nitrogen-oxygen species formed in the NO/O_2 reaction are thought to attack the sulphur residues resulting in the labilization of the zinc ion and destroying the protein structure (figure 5.2).

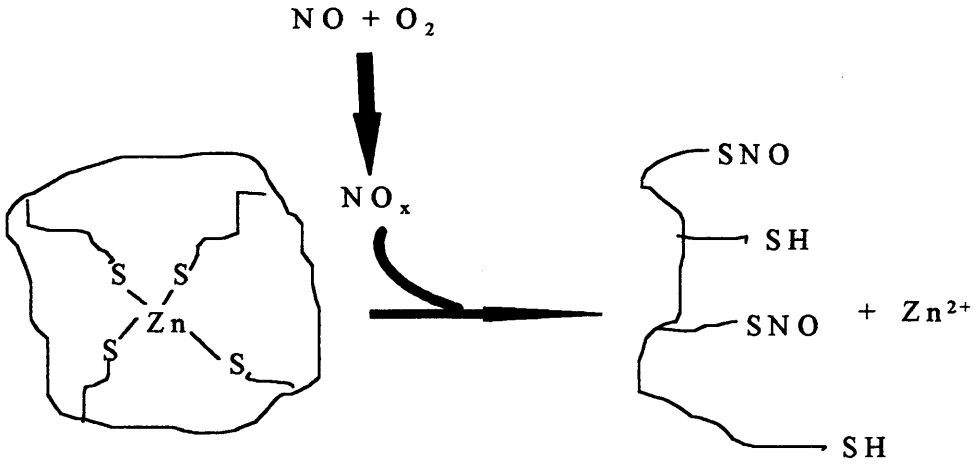


Figure 5.2 Effect of NO on zinc finger-containing enzymes (Wink *et al*, 1996).

In contrast, the toxicity of oxidised low-density lipoprotein in endothelial cells has shown to be inhibited by GSNO (Struck *et al*, 1995). It as been proposed that the NO released from GSNO inhibits the toxicity of lipid hydroperoxide by scavenging peroxyl and alkoxyl free radicals. Walker *et al* (1995) also demonstrated the role of GSH in the prevention of cytotoxicity of NO in HA1 fibroblasts. The formation of an RSNO intermediate is hypothesised as an explanation for the results observed. It was suggested that intracellular GSH pools scavenge reactive species derived from NO directly or act as cofactors in reactions which detoxify oxidised cell components, such as lipids, after damaging reactions mediated by NO-derived species. In agreement with these results it has recently been suggested that thiol groups in metallothionein, the major protein thiol induced in cells exposed to cytokines, are capable of forming *S*-NO adducts with products from the NO/O_2 reaction, and hence reduce cytotoxicity induced by such species (Schwarz *et al*, 1995; Misra *et al*, 1996). Additionally Wink *et al* (1994) have demonstrated that the products from reactions of cellular constituents with the intermediates from the NO/O_2 reaction are *S*-NO adducts. These data suggest that GSNO may represent a physiological scavenger of reactive nitrogen-oxygen

species, and that enzymes containing cysteine residues critical to their function may be inhibited by such species. Together these results indicate the role of RSNOs in the detoxification of NO.

In comparison to the effective doses required for pharmacological activity the RSNOs under investigation are not particularly cytotoxic. As shown previously (chapter 4) the effective doses for the inhibition of platelet aggregation and to induce relaxation of vascular smooth muscle are in the nM-μM range in comparison with μM-mM required for cytotoxicity. However, the toxicity will depend on environmental conditions such as GSH concentration and antioxidant levels.

There is a possibility that the NO released from these compounds maybe interfering with the mitochondrial dehydrogenase enzymes responsible for the oxidation of the MTT salt. Peroxynitrite, formed from NO, is a known inhibitor of succinate dehydrogenase. Therefore, it maybe that the reduction in MTT oxidation observed at the higher concentrations of RSNOs is caused by $ONOO^-$-induced inhibition of the dehydrogenase enzymes rather than a reduction in the number viable cells.

In summary there are a number of ways as to how RSNOs may exert a toxic effect. Obviously the actual cause of inhibition of cell growth by these compounds cannot be detected by the MTT assay. It is also impossible to determine if the RSNOs exert a cytostatic or cytotoxic effect as the MTT assay is unable to distinguish between the two. These results should be considered as an indicator of the toxicological potential of these compounds.

Chapter 6

General Discussion

The field of NO research is a rapidly growing one. The discovery that mammalian cells generate NO (Furchgott and Zawadzki, 1980; Palmer *et al*, 1987) provided important information about many physiological processes some of which have been described previously (chapter 1). Much work is being carried out in discovering methods of manipulating NO-derived pathways, and balancing the physiological and protective actions of NO in order to achieve useful new therapies (Moncada and Higgs, 1995). Current novel therapeutic areas involve NO donors as a therapy for pre-eclampsia (section 1.5.7), development of NOS inhibitors such as L-NMMA for the treatment of septic shock (section 1.3.2) and inhalation of NO gas as a treatment for adult respiratory distress syndrome (Rossaint *et al*, 1993; Gerlach *et al*, 1993).

Table 6.1 Summary of the chemical and biological of *S*-nitrosothiols.

	Half-life (Hr)		Inhibition of Aggregation IC_{50} (μM)	Vasorelaxation EC_{50} (nM)		Toxicity IC_{50} (mM)
RSNO	-DTPA	+DTPA		+E	-E	
CysGlyNO	0.309	5.67	0.79	92	110.5	>0.5
CysNO	0.542	13.1	0.17	2220	617.1	>0.5
GSNO	41.6	115	0.16	286.9	148	0.181
GluCysNO	42.2	411	0.11	825	204.9	0.107
SNAC	Stable	Stable	3.29	1364.6	171.9	0.144
MESNO	Stable	Stable	1.07	301.5	69.3	0.138
SNPROPA	Stable	Stable	0.45	344.5	138.4	0.0425

This thesis is aimed at providing a further insight into the chemical and biological properties of RSNOs in order to aid the development of an RSNO as a novel treatment for certain arterial/cardiovascular disorders. The results obtained in the course of the PhD are summarised in table 6.1. This project has demonstrated the following: (i) Under the experimental conditions the stability of RSNOs varies with structure. Results have shown (section 3.3) that CysNO and CysGlyNO were the least stable of the RSNOs investigated, whereas SNAC, MESNO and SNPROPA were the most

stable under these conditions. (ii) The decomposition of certain RSNOs is catalysed by trace amounts of copper (section 3.4). This phenomenon was particularly evident with the RSNOs, CysNO and CysGlyNO. Copper catalysed decomposition appears to occur more readily with RSNOs that allow the formation of a stable ring structure, in which Cu^{+} is bound to the nitrogen of the NO group and another electron-rich atom such as the nitrogen of an amino group or an oxygen of a carboxylate group. Copper also catalysed the decomposition of GSNO and GluCysNO, but to a lesser extent. (iii) Results have shown (section 3.5) that the decomposition of a stable RSNO e.g. GSNO is more rapid in the presence of a thiol, e.g. CysH, which gives rise to an unstable RSNO *via* a transnitrosation reaction. In contrast, decomposition of an unstable RSNO e.g. CysNO is slower in the presence of a thiol, e.g. GSH, which leads to the formation of a stable RSNO. (iv) RSNOs inhibited platelet aggregation and relaxed vascular smooth muscle in a dose dependent manner (chapter 4). (vi) All RSNOs studied exhibited a dose-dependent inhibition of growth of A549 cells (chapter 5). The use of 'physiological' to describe the stability conditions is debatable. For true physiological conditions, in addition to pH and temperature, other properties such as oxygen tension and ionic strength need to be controlled.

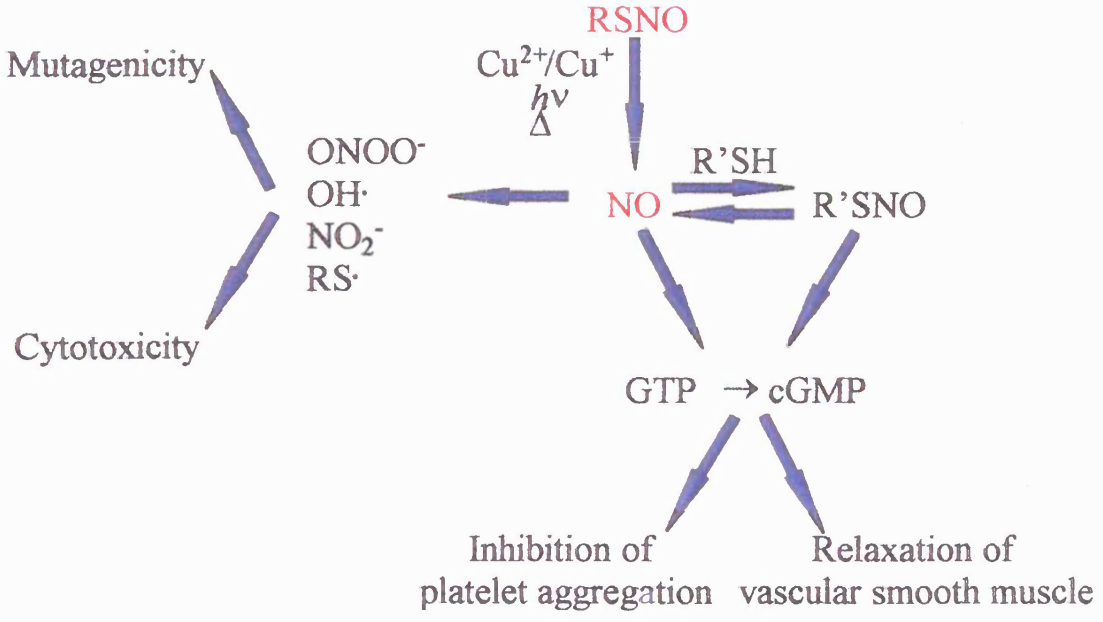


Figure 6.1 Chemical and biological properties of *S*-nitrosothiols.

A major aim of this project was to ascertain if a correlation exists between chemical stability and biological activity of RSNOs. Generally such a correlation was not observed. It was expected that the less stable RSNOs, i.e. CysNO and CysGlyNO, would be pharmacologically more potent than the stable RSNOs, on the basis of the assumption that NO release is a pivotal determinant of pharmacological action, but this was not the case. CysNO and CysGlyNO exhibited varying activity in both assays (chapter 4). The inhibition of A549 cell growth by the RSNOs did show a certain degree of correlation with stability, except for SNPROPA, which was considerably more potent as an inhibitor of cell growth. Analogous observations have been made by several other groups (Mathews and Kerr, 1993; Gaston *et al*, 1994; Kowaluk and Fung, 1990), who concluded that spontaneous liberation of NO from RSNOs is not solely responsible for the biological activity of these compounds. The pharmacological results obtained in this thesis suggest that the biological activity of RSNOs may involve transnitrosation reactions with an endogenous thiol(s), and as shown (chapter 3), these reactions are feasible under physiological conditions. The same may hold true for the cytotoxic properties of RSNOs. As previously outlined (section 5.3), many of the toxic reactions of NO may involve RSNO intermediates which could be formed by transnitrosation reactions between the added RSNO and endogenous thiols (Niknahad and O'Brien, 1995; Laval and Wink, 1994).

The cytotoxicity results (chapter 5) indicate that RSNOs are toxic but at much higher concentrations than those required for inhibition of platelet aggregation and relaxation of vascular smooth muscle. These results are similar to those obtained with authentic NO where studies have shown NO to be toxic in the mM range (Nguyen *et al*, 1992; Walker *et al*, 1995), but able to exert pharmacological activity within the nM range (Palmer *et al*, 1987; Radomski *et al*, 1987c). The cytotoxicity results might only be considered as an indicator of the toxicological potential of these compounds.

An agent of major importance for the elucidation of the role of GGT into the de-nitrosation of GSNO would have been D-GSH. As shown (chapter 2) the compound synthesised did not appear to be D-GSH but may be a diastereoisomer i.e. D,L-GSH or L,D-GSH. The results obtained do suggest that racemization may have occurred during synthesis but it is not known how or to what extent. Firstly, the synthesis was

carried out using urethane-protected amino acids which are expected to discourage racemization (Atherton and Sheppard, 1989). In addition only one product was detected by HPLC instead of two which would have been expected. Racemization is thought to be a non-selective process but according to these results only one chiral centre has undergone racemization. The most important experiment to be carried out is to identify the compound obtained. This may be achievable by carrying out circular dichroism analysis or by NMR experiments using chiral shift reagents.

Due to the unavailability of D-GSH no firm conclusions can be made regarding the involvement of GGT in the de-nitrosation of GSNO. Results obtained regarding the pharmacological activity of GSNO/CysGlyNO (chapter 5) suggest that GGT is not involved in the biological decomposition of GSNO. Further work is required in this area particularly with D-GSNO. In addition the role of transnitrosation reactions may be investigated further by alkylating free thiol groups on platelets using reagents such as iodoacetate or another thiol. This would hopefully lead to marked differences in the potency of a range of RSNOs. An analogous experiment would be to pre-incubate vascular smooth muscle in the organ bath experiment with a free thiol such as GSH. This again would probably lead to greater differences in potency of the RSNOs investigated due to a 'blocking' effect of free thiols in the vascular tissue. In addition GSNO may be generated which may be more stable than the RSNO under investigation.

In addition to measuring the chemical stability of RSNOs it would be useful to measure the amounts and rate of NO release from these compounds. Attempts were made in the duration of this project to measure the rate of NO release from the RSNOs at pH 7.4, 37°C using an NO-sensitive electrode. The aim was to determine if the rate of NO release correlated with the biological properties of these RSNOs. However these experiments were not very successful due to the extreme temperature sensitivity of the electrode.

Further investigations need to be made into the theory proposed by Dicks *et al* (1996) explaining the copper catalysed decomposition of RSNOs. The formation of a stable

ring structure involving Cu^{+} holds well for CysNO and CysGlyNO but does not explain why GSNO and GluCysNO decomposed in the presence of higher concentrations of copper, whilst the stability of other stable RSNOs i.e. SNAC, MESNO and SNPROPA was unaffected. There is the possibility that the catalysis occurs by the co-ordination of Cu^{+} between two molecules of RSNO but this still does not explain why only GSNO and GluCysNO were effected by the higher concentrations of Cu^{2+}.

The toxicity of the RSNOs in question needs to be investigated further, in particular why SNPROPA was more potent as a cytotoxic agent. In addition it would be interesting to determine whether these compounds are cytotoxic or cytostatic. Further insights would be gained by LDH assays for acute cytotoxicity and observation of cell number and clonogenicity to determine more subtle effects on growth. In addition further studies into the mechanism of toxicity of RSNOs in general may be carried out by investigating the toxic effect of these compounds in the presence of superoxide dismutase (SOD). Superoxide dismutase reduces the levels of O_2^{-}, and in turn would reduce the levels of $ONOO^{-}$. The failure of SOD to diminish the toxicity of RSNO would suggest that the toxicity of RSNOs may be due to the NO released and not due to the formation of $ONOO^{-}$ (Delaney *et al*, 1993).

Generally there are still many questions to be answered with regards to how the structure of RSNOs effects their stability and in turn the biological activity. Further investigations into structure-activity relationships of additional GSNO and CysNO analogues might lead to a greater understanding of the factors controlling the release of NO from RSNOs which would ultimately lead to the design of 'optimally' active NO donors.

Chapter 7

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Chapter 8

Appendices

8.1 Abbreviations

AA	Amino acid
ACh	Acetylcholine
ADP	Adenosine Diphosphate
BH_4	Tetrahydrobiopterin
Bk	Bradykinin
BOC	tert. Butoxycarbonyl
^tBu	Tertiary Butyl
cGMP	Cyclic Guanosine Monophosphate
CaM	Calmodulin
Coll	Collagen
CysH	Cysteine
CysNO	S-Nitroso-L-cysteine
CysGlyNO	S-Nitroso-L-cysteinylglycine
D-GSNO	S-Nitroso-D-glutathione
DEA/NO	Diethylamine NONOate
DIEA	Diisopropylethylamine
DMF	N,N'-dimethylformamide
DMSO	Dimethyl sulphoxide
+E	Endothelium Intact
-E	Endothelium Denuded
EDRF	Endothelium Derived Relaxation Factor
EDT	Ethanedithiol
FAB	Fast Atom Bombardment
FAD	Flavine Adenine Dinucleotide
FMN	Flavine Mononucleotide
Fmoc	9-Fluorenylmethoxycarbonyl
GC	Guanylyl Cyclase
Glu	Glutamic acid
GluCysNO	S-Nitroso-L-γ-glutamyl-L-cysteine
Gly	Glycine

GGT	γ-Glutamyltranspeptidase
GSH	Glutathione
GSSG	Oxidised Glutathione
GSNO	S-Nitroso-L-glutathione
GTN	Glyceryl Trinitrate
HBTU	2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOBt	1-Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
hr	Hour
5HT	Serotonin
HWP	Human Washed Platelets
MBHA	Methylbenzhydrylamine
MeOH	Methanol
MESNO	S-Nitroso-2-mercaptoethanesulphonic acid
min	Minutes
MTT	3-4,5 Dimethylthiazol-2,5 diphenyl tetrazolium bromide
ND	Not Determined
L-NMMA	N^G-Monomethyl-L-arginine
NMP	N-Methylpyrrolidone
NMR	Nuclear Magnetic Resonance Spectroscopy
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
ODQ	1*H*-[1,2,4]Oxadiazolo[4,3-*a*]quinoxalin-1-one
OPfp	Pentafluorophenyl ester
OxyHb	Oxyhaemoglobin
PGI_2	Prostacyclin
PhE	Phenylephrine
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
$RONO_2$	Organic nitrate
RONO	Organic nitrite

RSH	Thiol
RSNO	*S*-Nitrosothiol
RSSR	Oxidised Thiol
R_T	Retention Time
SNAP	*S*-Nitroso-*N*-acetyl D,L penicillamine
SNAC	*S*-Nitroso-*N*-acetyl-L-cysteine
SNP	Sodium Nitroprusside
SNPROPA	*S*-Nitroso-3-mercaptopropionic acid
SOD	Superoxide Dismutase
SPER/NO	Spermine NONOate
TFA	Trifluoroacetic acid
Trt	Trityl/ Triphenylmethyl
TxA_2	Thromboxane A_2
UV	Ultra Violet

8.2 Chemicals and Reagents

Materials were obtained from the sources as indicated below:

Aldrich Chemical Co., The Old Brickyard, New Rd, Gillingham, Dorset, SP8 4XT:
Hydrogen bromide in acetic acid, Hydrogen chloride in acetic acid, 3-Mercaptopropionic acid

Applied Biosystems, Kelvin Close, Birchwood Science Park, Warrington, Cheshire, WA3 7PB:
(All of the following solvents were of peptide synthesis grade)
Piperidine, Diethylpropylethylamine, Dichloromethane, Methanol, *N*-methyl-pyrrolidone, Trifluoroacetic acid

Fisher Scientific UK, Bishop Meadow Rd, Loughborough, Leics, LE11 0RG:
Dichloromethane, Diethylenetriaminepentaacetic acid, HPLC Grade Acetonitrile HPLC Grade Methanol, Trifluoroacetic acid, Glacial acetic acid, Diethyl ether Acetone

Fluka Chemicals, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL:
Pentamethylbenzene, Thioanisole

Fresenius Health Care, Basingstoke, England:
0.9%w/v Sodium Chloride (Saline)

Gibco, Life Technologies Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF:
L-Glutamine, Hams F12 Media, Penicillin Streptomycin, Tyrodes salt

Goss Scientific, 100 Vicarage Lane, Great Baddow, Essex, CM2 8JB:
D_2O

Novabiochem, Calbiochem-Novabiochem (UK) Ltd, Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR

Boc-Gly-MBHA Resin , Boc-D-Glu-OBzl , Fmoc-D-Cys(Trt)-OPfp, Fmoc-D-Glu(O^{t}Bu)-OH, Fmoc-Cys(Trt)-Wang Resin, Fmoc-Gly-Wang Resin, Fmoc-Cys(Trt)-OH, Fmoc-Glu-O^{t}Bu, Fmoc-D-Cys(Trt)-OH, HBTU, HOBT

Nycomed Arzneimittel GmbH, Freisinger Landstraβe 74, D-800 Munchen 45:

Collagen

Phenomenex, Melville House, Queens Avenue, Hurdsfield Industrial Estate, Macclesfield, Cheshire, SK10 2BN.

HPLC Columns

Sigma Aldrich Company Ltd, Fancy Rd, Poole, Dorset, BH12 4QH:

N-Acetyl-L-cysteine, Cycloheximide, Cysteine, CysGly

Citric acid monohydrate, 5,5' Dithio-bis(2-nitrobenzoic) acid (Ellmans Reagent).

Ethanedithiol, γ-GluCys, Glutathione, Heptafluorobutyric acid, Indomethacin

2-Mercaptoethane sulphonic acid (MESNA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Phenylephrine, Sodium Chloride, Sodium Hydrogen Carbonate, Sodium Nitrite

Wellcome

Prostacyclin (Eprostenol sodium), Oxyhaemoglobin

8.3 Solutions and Buffers

Unless otherwise stated all water used in the preparation of solutions was of ultra-pure grade (Milli-Q).

Peptide Synthesis

0.45M HOBt/HBTU:

N-Hydroxybenzotriazole (HOBt) (0.3378g), 2-(1H-Benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) (0.9475g), 5.5ml DMF

Preparative HPLC:

(Solvents used of HPLC grade)

0.1% v/v Trifluoroacetic acid (1.0ml/l water), 0.1% v/v Heptafluoroacetic Acid (1.0ml/l water)

All mobile phases filtered through HAWP filters (0.45μM) ex Millipore and degassed with He for 5-10 min prior to use.

Ellmans Buffer:

$Na_2HPO_4.2H_2O$ (3.37g/ 100ml water), $NaH_2PO_4.H_2O$ (0.15g/100ml water)

Stability of RSNO

2.5M HCl:

10M HCl (250ml/l water)

1M Phosphate Buffer:

KH_2PO_4 (43.5g/ 250ml water), K_2HPO_4 (34.0/250ml water)

Add KH_2PO_4 to K_2HPO_4 until pH 7.4

1M Sodium Hydroxide

NaOH (4.0g/100ml water)

1mM DTPA

Diethylenetriamine pentaacetic acid (39.75mg/100ml water)

10mM $CuSO_4$

$CuSO_4.5H_2O$ (24.97mg/10ml water)

Stock serially diluted with water (1mM-1μM)

Synthesis of RSNO for use in Biological Assays

Citrate Buffer pH 2.0

The concentration of citrate buffer used was always the same as the concentration of RSNO being synthesised in order to maintain sufficient buffer capacity. Generally it was prepared by dissolving citric acid (monhydrate) in deionised water and adjusting to pH 2.0 with conc. HCl

Thiol solution

For a 1mM solution of RSNO a 20mM solution of the corresponding thiol was prepared in 1mM citrate buffer pH2.0.

Saline Solution (0.9 w/v sodium chloride)

Sodium Chloride (9.0g/l water)

Sodium Nitrite:

For a 1mM solution of RSNO a 20 mM solution of $NaNO_2$ was prepared in saline e.g. (1.38mg/ml saline)

Inhibition of Platelet Aggregation

3.15%w/v Trisodium Citrate:

Trisodium citrate (3.15g/l water)

Solution filter-sterilised and stored at 4°C

Tyrodes Solution:

Tyrodes Salt (9.5g/l water), Sodium Bicarbonate (1.0g/l water)

Solution filter-sterilised and stored at 4°C

Tyrodes solution contains (g/l):

$CaCl_2$ (anhyd.) (0.2), KCl (0.2), $MgCl_2$ (anhyd.) (0.0469), NaCl (8.0), $NaH_2PO_4.H_2O$ (0.05) and D-glucose (1.0)

Tyrodes Salt (9.5g/l water), Sodium Bicarbonate (1.0g/l water)

Solution filter-sterilised and stored at 4°C

1M Tris Buffer:

Trizma HCL.(52.8g/l water), Trizma base (80.6g/l water)

1mgml^{-1} Prostacyclin Solution:

Eprostenol sodium (1.5g/1.5ml Tris buffer)

Solutions stored at -80°C

Collagen Solutions:

1mgml^{-1} solution provided by manufacturer and serially diluted down using manufacturers buffer to concentrations of 100 and 10μgml^{-1}.

Relaxation of Vascular Smooth Muscle

10mM Cycloheximide:

Cycloheximide (14.07g/5ml saline)

50mM Indomethacin

Indomethacin (8.9mg/ml $NaHCO_3$)

Krebs Ringer Solution:

10 x Krebs (500ml), Cycloheximide (5ml), Indomethacin (1ml)

1 M Calcium Chloride (12.5ml)

Krebs ringer solution contains (mM):

NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.17), $CaCl_2$ (2.5), $NaHCO_3$ (25) and glucose (8.4).

Volume made up to 5 litres with distilled water and continually gassed with 95% O_2/ 5% CO_2

25 mM Phenylephrine:

Phenylephrine (5.09mg/ml saline)

25mM Acetylcholine:

Acetylcholine (4.54mg/ml saline)

Cell Culture

Hams F12 Medium:

Hams F12 Media (500ml), Pen/Strep. (5ml), Glutamine (5ml), Foetal Calf Serum (50ml)

Trypsin:

10 x Trypsin EDTA (10ml), PBS (90ml)

MTT Assay

10M Sodium Hydroxide:

Sodium Hydroxide (400g/l water)

0.25M Tris Buffer:

Tris HCl (7.56g/250ml water).

Adjust to pH 7.4 with conc. HCl.

Glycine Buffer:

Glycine (375.4mg/50ml water), NaCl (292.2mg/50ml water)

Adjust to pH 10.5 with 10M NaOH

5mgml^{-1} MTT Solution:

MTT Salt (5mg/ml media)

Buffered DMSO:

DMSO (24ml), Glycine buffer (3ml)

8.4 Communications

A. Abstract submitted for a poster presentation at the 'The Biology of Nitric Oxide', Amelia Island, 1995.

STABILITY OF S-NITROSOTHIOLS RELATED TO GLUTATHIONE

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S-Nitrosothiols are being widely studied as pro-drugs of NO. In general S-nitrosothiols are considered to be very unstable. S-Nitrosocysteine (CysNO) is short lived whereas S-nitrosoglutathione (GSNO) is stable (W R Mathews and S W Kerr, J. Pharmacol. Exp. Ther. 267(3): 1529, 1993). The reason for this difference was studied by examining the stability of the S-nitroso analogues of the glutathione-related dipeptides, L-cysteinylglycine (CysglyNO) and L-γ-glutamyl-L-cysteine. Stability of the S-nitrosothiol in solution (pH7.4) at 37°C was determined by monitoring its UV absorbance at 330nm. The results indicate that CysNO and CysglyNO were the least stable agents with half-lives of 32.4min and 18.9 min, respectively. There appear to be discrepancies between half-lives quoted in the current literature. It has been proposed that these differences may be due to Cu^{2+} catalysed decomposition of S-nitrosothiols (J McAninly *et al*, J. Chem. Soc., Chem. Commun. 1758, 1993). The metal ion is thought to co-ordinate between the S-NO group and the NH_2 group forming a ring structure, the stability of which determines if denitrosation occurs. The hypothesis was tested that the removal of Cu^{2+} and other transition metals would prolong the half-lives of the S-nitrosothiols. In the presence of the chelator diethylenetriaminepentaacetic acid the half-lives of CysNO and CysglyNO were increased between 10-20 fold, but for GSNO there was little difference. The results suggest that Cu^{2+} affects the stability of thionitrites such as CysNO and CysglyNO in which co-ordination leads to a six membered ring

B. Abstract submitted for a poster presentation at the 'The Biochemistry and Molecular Biology of Nitric Oxide', Los Angeles, 1996.

PHARMACOLOGICAL PROPERTIES OF S-NITROSOTHIOLS RELATED TO GLUTATHIONE

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S-Nitrosothiols (RSNO) are under investigation as pro-drugs of NO. In general RSNOs are considered to be very unstable, however they are both potent vasodilators and inhibitors of platelet aggregation *in vivo* and *in vitro*, each property being mediated by NO. The aim of this study was to examine the relationship between pharmacological activity and chemical stability of S-nitroso analogues of glutathione (GSNO), cysteine (CysNO), and the glutathione related dipeptides L-cysteinylglycine (CysglyNO) and L-γ-glutamylcysteine (GlucysNO). Stability of the RSNO in solution (pH 7.4) at 37°C was determined by monitoring the UV absorbance at 330nm. CysNO and CysglyNO were the least stable agents with half-lives of 32.4±8.9 min and 18.9±6.4 min, respectively (mean±SD). It has been proposed that the decomposition of RSNOs is catalysed by trace metals such as Cu^{2+}. The hypothesis was tested that the removal of $Cu^{2+}$and other transition metals would prolong the half-lives of the RSNO. In the presence of the chelator diethylenetriaminepentaacetic acid the half-lives of CysNO and CysglyNO were increased between 10-20 fold but for GSNO and GlucysNO there was little difference. Each RSNO displayed a dose-dependent inhibition of collagen induced aggregation of human washed platelets . IC_{50}values showed that GSNO, CysNO and GlucysNO were of similar potencies (IC_{50} = 0.15±0.09, 0.17±0.07 and 0.12±0.04μM, respectively (mean±SE)), but CysglyNO was less active with an $IC_{50}$of 0.78μM. All RSNOs relaxed rat aortic smooth muscle . Cumulative dose-response curves were generated for each RSNO. EC_{50} values for the relaxation of intact rat aortic rings ranged from 92±30 nM for CysglyNO to 2.2±0.6 μM for CysNO. GSNO and GlucysNO exhibited similar potencies of 512±198 μM

and 825±200μM respectively (mean±SE). The results indicate that GSNO and GlucysNO, which are of similar stability, exhibit similar biological activity. CysNO and CysglyNO are both very unstable, and exhibited varying activities. These differences might reflect their susceptibility towards factors such as the presence of Cu^{2+}.

8.5 Publications

Manuscript submitted for publication in 'Nitric Oxide Protocols', Methods in Molecular Biology Series, Humana Press, New Jersey. In press.

Use of NO Donors in Biological Systems

Jayne M Tullett and Daryl D Rees

1.Introduction

The endogenous production of nitric oxide (NO) plays a key role in many bioregulatory systems including the control of vascular tone, inhibition of platelet aggregation, neurotransmission and macrophage toxicity, many of these pathways being activated by the stimulation of soluble guanylyl cyclase (1). Due to the inconvenient handling of solutions of NO there is an increased interest in compounds capable of generating NO *in situ*. These compounds can be divided into different groups that include organic nitrates (e.g. glyceryl trinitrate, GTN), organic nitrites (e.g. iso amyl nitrite), inorganic nitroso compounds (e.g. sodium nitroprusside, SNP), sydnonimines (e.g. molsidomine, SIN-1) and S-nitrosothiols (RSNO) (e.g. S-nitrosoglutathione, GSNO). All these compounds differ in their need for specific cofactors required to release NO(2).

Organic nitrates are metabolised by enzymatic and non-enzymatic pathways. The activity of glutathione-S-transferase and cytochrome P450 related enzymes are thought to be involved. Thiols present in the cytosol are likely to account for the non-enzymatic pathway and in both cases an unstable thionitrate may be the common intermediate in the production of NO. The main drawback of using these compounds clinically is the occurrence of tolerance to the drug. This is thought to be due to a down-regulation of the enzymes involved or a depletion in the amount of available thiol groups (3,4). Organic nitrates have been shown to be less potent in the inhibition of platelet aggregation *in vitro* than the relaxation of vascular smooth muscle presumably due to the absence of the metabolising enzymes in platelets (5). Inorganic nitrites are thought to react with available thiol groups forming RSNOs which decompose to give NO. The extent of NO generation will depend on the rate of formation of the RSNO and its rate of decomposition, which will vary with temperature and pH. SNP spontaneously liberates NO by as yet an unknown mechanism. This NO donor is very potent and has a very short biological half-life (approximately 2 minutes in humans). The main drawback of SNP treatment is the concomitant production of cyanide which appears to be obligatory for the release of NO (6). The sydnonimine, molsidomine requires metabolism by liver esterases to give the active metabolite SIN-1. This then undergoes hydrolysis to yield the open-ring form SIN-1A which releases NO *via* a radical process involving molecular oxygen. In addition to the release of NO there is a stoichiometric formation of superoxide anions which may give rise to oxidative side reactions due to hydrogen peroxide and hydroxyl radical generation. The sydnonimines are

highly susceptible to light and oxygen and should therefore be prepared just before use and kept protected from light and heat. RSNOs liberate NO spontaneously by homolytic fission of the S-NO bond (4,6) (See table 1).

The biological effects of the majority of these NO donors has already been extensively covered except for RSNOs which are still very much an experimental class of compound mainly due to their attributed instability. This varies depending on the pH, oxygen tension, redox state, transition metal contamination and the presence of thiol groups. RSNOs decompose both thermally and photochemically yielding NO and the corresponding disulphide. The decomposition of certain RSNOs has been shown to be catalysed by trace amounts of copper (7). RSNOs are usually red or green in colour thus their stability can be assessed by monitoring the UV absorbance (320-360nm) of the compound at one of the $\lambda_{max.}$ normally *ca*335 nm with a detection limit of approximately 0.1-10mM.

RSNOs can be synthesised by acid catalysed nitrosation of the corresponding thiol with nitrite in mineral acid (8). With certain RSNOs it is often difficult to obtain the compound in a pure form due to it's instability and is often easier to use the original reaction solution. For biological assays, using an RSNO reaction solution generated from mineral acid is generally not suitable due to the resulting low pH. An alternative method as been outlined below in section 3.1.

Two of the major properties of NO and hence NO donors is their ability to inhibit platelet aggregation and relax vascular smooth muscle both *in vitro* and *in vivo*. This chapter outlines the methods used in assessing these properties *in vitro*.

NO has often been cited as being a toxic molecule (9), thus NO donors may also fall into this category. When investigating a new NO donor it may be desirable to assess it's toxicological potential. A method has been outlined in section 3.4. It should be pointed out that this assay does not distinguish between cytotoxicity or cytostasis.(10)

2.Materials

2.1 Preparation of NO Donors

1) Citrate buffer pH 2. The concentration of citrate buffer used will be the same as the concentration of RSNO being synthesised in order to ensure sufficient buffer capacity. Generally it is prepared by dissolving citric acid (monohydrate) in deionised water and adjusting to pH2 with conc. HCl.
2) 0.9 %w/v Saline solution: 9g of NaCl in 1 litre of deionised water.
3) For the more stable NO donors i.e. GTN, these are available commercially and should be made up according to the manufacturers recommendation.

2.2 Inhibition of Platelet Aggregation

1. 3.15% w/v Trisodium citrate. Filter sterilise and store at 4°C.
2. At least 400ml of blood collected from healthy volunteers who have not taken any drugs (in particular aspirin based products which prevent aggregation) in the previous 10 days.
3. 1M TRIS buffer: 52.8g Trizma HCl and 80.6g Trizma base to 1 litre of water.
4. 1mg/ml Prostacyclin (PGI_2) in 1M TRIS buffer. Solutions stored at -80°C.

TABLE 1. Properties of Common NO Donors

CLASS	COMPOUND	STABILITY OF SOLID	STABILITY OF SOLUTION	NOTES
Organic Nitrates	Glyceryl trinitrate (GTN)	NA	Stable stock solutions prepared in ethanol or DMSO. Protect from light.	Metabolised by enzymatic and non-enzymatic pathways. Thionitrate common intermediate in the release of NO concomitantly with nitrite. Less potent inhibitor of platelet aggregation.
	Isosorbide dinitrate(ISDN)	NA	As above	As above
Iron Nitrosyls	Sodium Nitroprusside	Store desiccated in dark at RT.	Prepare aqueous solutions fresh and protect from light.	Enzymatic and photochemical release of NO. Produces concomitant amounts of cyanide.
	Roussin's Black Salt (heptanitrosyltri-μ3-thioxotetraferrate)	Store at -20°C under argon.	Water soluble. Prepare in assay buffer immediately before use. Protect from light.	Highly photosensitive. NO formation depends on intensity of illumination.

Organic Nitrites	Amyl nitrite	NA	Store in sealed glass containers at 4°C.	Release of NO requires presence of thiol groups. RSNO are active intermediates, and rates of NO release are a function of rate of formation and metabolism of RSNO involved.
Sydnonimines	3-Morpholinosydnonimine (SIN-1)	HCl salts stable as solids. Store desiccated at 4°C. Protect from light.	Water soluble. Stable in acidic solution, pH 5.0. Keep cool and protected from light.	SIN-1 undergoes hydrolysis to yield the open-ring form, SIN-1A. NO released from SIN-1A via a radical process. Forms stoichiometric amount of superoxide ions. NO release enhanced by superoxide dismutase. Peroxynitrite and hydroxyl radicals may be formed.
	Molsidomine	Stable solid. Store at RT and protected from light.	Prepare stock solutions in DMSO.	Inactive in vitro. Converted to active metabolite SIN-1 by liver esterases.

S-Nitrosothiols	S-Nitrosoglutathione (GSNO)	Store desiccated at -20°C. Protect from light.	Prepare fresh using citrate/HCl buffer, pH 2.0 (See section 2.1), or 0.5-1M HCl. Store at 4°C and protected from light.	Rapid decomposition to yield the disulphide and NO. Thiol radicals may form. Decomposition catalysed by trace amounts of Cu^{2+}.
	S-Nitroso-N-acetyl-D,L-penicillamine (SNAP)	Store desiccated at RT. Protect from light.	Prepare fresh. Store at 4°C.	As above.
NONOates	Diethylamine/NO (DEA/NO)	Store under argon or nitrogen at -80°C.	Water soluble. Prepare fresh in dilute NaOH. Store on ice and under argon.	Generate NO spontaneously, independent of tissue. High concentrations of thiols decrease release. Release pH-dependent, stable at alkaline pH, rapid decomposition at pH ,5.0. Predictable NO release (see Chapter x).
	Spermine/NO (SPER/NO)	As above.	As above	Produces spermine which may have biological activity.

Data obtained form (3)

5. Tyrodes solution containing 0.1% w/v sodium bicarbonate. 9.5g of Tyrodes salt (Gibco) and 1g of sodium bicarbonate to 1 litre of water. Filter sterilise and store at 4°C.
6. 1mg/ml collagen diluted to 100 and 10µg/ml in buffer specified by manufacturer. Store solutions at 4°C.

2.3 Relaxation of Vascular Smooth Muscle

1. Krebs ringer solution 10x diluted 1 in 10 with deionised water containing the following:
   a) 10 µM Cycloheximide, to inhibit the induction of inducible nitric oxide synthase (iNOS).
   b) 10 µM Indomethacin, to inhibit prostanoids e.g. PGI_2
   c) 2.5 µM Calcium chloride
2. 25 mM phenylephrine in saline.
3. 25 mM acetylcholine in saline.
4. 0.9% w/v saline solution (see section **2.1** item 2).

2.4 MTT Toxicity Assay

1. Glycine buffer: 375.4mg glycine and 292.2mg of NaCl to 50 ml of water. Adjust pH to 10.5 with 10M NaOH. Store at 4°C.
2. Dimethyl sulphoxide (DMSO).
3. DMSO/Glycine Buffer. 3ml Glycine buffer pH10.5 added to 24ml DMSO.
4. Phosphate buffered saline.
5. MTT solution: 100mg MTT to 20ml of media.
6. Hams F12 media supplemented with 10% foetal calf serum, penicillin 100iu/ml, streptomycin 100µg/ml and glutamine (2mM).

3. Methods

3.1 Preparation of NO Donors

The following method prepares a 1mM solution of RSNO, all concentrations will have to be altered according to the concentration of RSNO required (See notes 2-5).

1. Prepare a solution of thiol (20mM) in 1mM citrate buffer pH2.0 (Solution A)
2. Prepare a solution of $NaNO_2$ (20mM) in saline (Solution B)
3. Take 250µL of solution A and B and add to 4.5ml of citrate buffer pH2.0. This should yield a coloured solution of the appropriate RSNO.
4. Assess the concentration of RSNO by obtaining a UV spectrum of the compound. It is usual to dilute the RSNO in deionised water and scan between 200 and 600nm. Determine the absorbance at the $\lambda_{max.}$ at approx. 330nm and calculate the concentration of RSNO using the Beer-Lambert law and the appropriate ε value. (e.g. ε values for the following RSNOs in water GSNO 0.79mMcm^{-1}, S-nitroso-L-cysteine 0.67mMcm^{-1} and S-nitroso-N-acetyl-L-cysteine 0.87mMcm^{-1}, see (11) for further values)

3.2. Inhibition of Platelet Aggregation

3.2.1 Preparation of Human Washed Platelets (HWP)

1. Collect blood using 3.15% w/v trisodium citrate (as the anti-coagulant), in the ratio 1 part citrate to 9 parts blood.
2. Centrifuge the blood at 220g for 20 minutes at room temperature. The supernatant is termed as platelet rich plasma (PRP).
3. Transfer the PRP into clean centrifuge tubes and add PGI_2 to a final concentration of 0.3µg/ml. Care is needed so as not to foam the PRP or disturb the collected cells from the pellet. PRP should be dispensed into the tube by running the PRP down the wall of the tube , this avoids turbulence which could activate the cells
4. Centrifuge the mixture at 700g for 10 minutes at room temperature, no brake must be used to ensure that the platelet pellet is not disrupted.
5. The resultant pellet contains platelets and some erythrocytes. The supernatant, platelet poor plasma (PPP), should be removed using a 5000µL pipette and finished off with a 1000µL pipette. Take care so as not to disturb the pellet.
6. Prepare 20ml of Tyrode solution and add PGI_2 to a final concentration of 0.3 µg/ml . Carefully pour 5ml of this solution onto the pellet.
7. Re-suspend the pellet using a 1000µL pipette, do this gently and try to leave the red cells at the bottom of the tube.
8. Transfer the re-suspended platelet solution into the remaining 15 ml of Tyrode solution and swirl gently.
9. Centrifuge at 680g for 10 minutes at room temperature.
10. Remove the supernatant carefully. Wash the surface of the platelets with 3 x 1ml of Tyrode solution without PGI_2 . Take care as not to disturb the pellet during the washes. Remove any traces of PGI_2 from the walls of the tube with tissue paper.
11. Add 5ml of Tyrode solution and re-suspend the pellet gently again discarding any region that contains erythrocytes.
12. Obtain a platelet count and adjust the final volume with Tyrode solution (PGI_2 free) in order to have a final platelet count of 200-250 x 10^{-9} /L. (See Note 6)

3.2.2 Platelet Aggregation Assay.

Platelet aggregation is measured in an aggregometer by the method of Born and Cross (12).

3.2.2.1 Determination of Collagen EC_{90}

1. Place 0.5ml of the platelet suspension into an aggregometer at 37°C and incubate for 1.5 min. The platelet suspension should be stirred continuously at a rate of 900rpm.
2. Start recording aggregation once the platelet suspension has reached an equilibrium which is normally after 1.5 min.
3. After 2 min. add collagen (1-10µg/ml) and monitor aggregation for 4 min.
4. From the dose-response curve obtained for collagen the effect of the NO donors can then be assessed using a sub-maximal concentration of collagen. This is normally an EC_{90} and is described as the dose that gives a 90% response of the maximum aggregation induced by collagen. (See Note 8)

3.2.2.2 Determination of Potency (IC_{50}) of Individual NO Donors

1. Prepare NO donor stock as detailed in section 2.1. Dilute down accordingly in Tyrodes solution prior to use. Store all solutions on ice. With the RSNO solutions ensure the compound is also protected from light.
2. Repeat steps 1 and 2 from section 3.2.2.1.
3. At 2 min add the NO donor (0.003-30μM) and incubate for 1 min. Care should be taken so as not to suck any platelets back up into the pipette.
4. Add collagen (EC_{90} previously determined) at 3 min and monitor aggregation for a further 3 minutes.
5. Inhibition of aggregation is expressed as a percentage of the maximal aggregation induced by collagen. Determine potency (IC_{50}) values for each compound.

   (See Notes 9-11)

3.3. Relaxation of Vascular Smooth Muscle

The following method is a general principle used for smooth muscle from Male Wistar rats but can be adapted for other vessels.

1. Male Wistar rats (250-300g) are anaesthetised briefly with isofluorane and killed by exsanguination.
2. Remove the thoracic aorta and carefully trim off all adhering fat and connective tissue and cut into 4mm rings . Prepare rings of aorta both with the endothelium intact and denuded since the endothelium generates NO which can interfere with exogenous NO donors. Endothelial cells are removed by gently rubbing the internal surface with a cut down pipe-cleaner. Care must be taken to avoid removing the endothelial cells from the intact tissues.
3. Mount rings under 1g of resting tension (other vessels will require the optimal tension for that tissue), on stainless steel hooks, in organ baths filled with Krebs buffer containing indomethacin, cycloheximide and gassed with 95% O_2 / 5%CO_2 at 37°C. (See Note 12-13)
4. Allow the tissues to equilibrate for approximately 1 hour which will allow the tissues to reach a stable baseline (this will differ depending on the tissue used). During this time the Krebs buffer should be changed at regular intervals in order to remove vasoactive mediators released from the tissue after removal from the animal.
5. Carry out a dose response curve to phenylephrine (1-10000 nM), or other contractile agents, and determine the EC_{90} dose to be used for the relaxation studies. The EC_{90} will be less for endothelium-denuded tissues than intact tissues due to the removal of the continuous dilatory actions of NO.
6. Pre-contract tissues by addition of the EC_{90} dose of phenylephrine. Induce relaxation of the tissues by addition of acetylcholine or other endothelium-dependent relaxant (0.1-10μM). Failure of the tissue to relax is taken as an indication of endothelium removal. Rings showing less than 80% relaxation at this stage should be discarded.
7. Flush the organ bath with Krebs solution at regular intervals to remove acetylcholine and phenylephrine and allow to equilibrate for a further 60 minutes.
8. Prepare NO donor stock as detailed in section 2.1. Dilute down accordingly in Krebs buffer prior to use. Store all solutions on ice.

9. Again pre-contract tissues by addition of phenylephrine EC_{90} and obtain cumulative relaxation curves for the NO donors in question (0.1-30000nM).
10. Determine EC_{50} values for each compound. The EC_{50} is described as the concentration of NO donor required to relax the tissue to 50% of the maximum contraction induced by phenylephrine.

3.4. MTT Assay

The following assay described below uses the human lung carcinoma cell line A549 but can be adapted for other cells.

1. Seed A549 cells at $1.1x10^3$ cells/well in a 96 well plate. Incubate for 24 hours.
2. Remove medium, (See Note 15) being careful not to dislodge any cells. Replace with medium (200-250µL) containing the NO donor (0.05-0.5mM) (For preparation see section 2.1). Incubate for 72 hours.
3. Remove medium after 72 hours and wash cells with 200 µL PBS.
4. Add 200 µL of the MTT solution to each well and incubate for a further 4 hours at 37°C.
5. Remove the MTT solution and wash cells twice with PBS.
6. Add 200 µL of the DMSO/Glycine buffer to dissolve formazan product.
7. Shake the plate for 20 minutes to ensure complete dispersal of the blue product.
8. Read the absorbance of each well at 540nm.
9. The absorbance obtained is proportional to the number of viable cells since it is only living cells that can convert the yellow tetrazolium salt to the blue formazan product. Results expressed as % inhibition of growth as compared to control cells.

4. Notes

1. Unless otherwise stated water used in all of these experiments should be of the ultra-pure grade.
2. RSNO solutions should be protected from light and heat by wrapping the containing vessel in aluminium foil and storing on ice (4°C). Solutions should always be made up fresh on the day of the study, just prior to use.
3. The lifetime of RSNO can be prolonged by adding a copper chelator such as DTPA (approx. 100µM) to the reaction solution.
4. In the method outlined in section 2.1 for the preparation of RSNO a dilution of 1 in 20 is made of both the thiol and $NaNO_2$ in the citrate buffer. This can be adjusted accordingly but it is always advisable to keep the dilution of $NaNO_2$ to a maximum. This will keep the amount of saline added to a minimum in order to ensure that the pH remains at 2.
5. It is advisable to make the RSNO stock at least 5 times more concentrated than the upper dose required. This will allow for a dilution to be made in the appropriate buffer which will increase the pH before addition to the tissue/platelets.
6. When preparing HWP always leave platelet suspensions for approx. 2 hours before using in order for the anti-aggregatory effects of PGI_2 to wear off. Although PGI_2 is unstable its anti-aggregatory effects, once initiated, last longer.
7. Platelets and aortic rings must be used on day of preparation.

8. Care must be taken when choosing the EC_{90} of collagen in the platelet aggregation assay and phenylephrine with the aortic rings to ensure that the dose is submaximal. If the EC_{90} used is too high this will result in maximum aggregation and contraction which makes it difficult to observe any anti-aggregatory or relaxation effects.
9. Tyrodes solution to be used as a blank in the aggregometer with HWP.
10. PRP can be used instead of HWP, but PPP must be used as a blank.
11. With the platelet aggregation assay, run a control at the beginning and the end of the dose response curve.
12. Aortic rings must be kept in oxygenated Krebs buffer at all times.
13. When mounting the aortic rings care must be taken not to over-stretch the vessels as this can result in damage.
14. Unless otherwise stated all solutions used in the MTT assay must be pre-warmed to 37°C before use. All solutions must also be sterilised prior to use.
15. Remove media, PBS etc. from a 96 well plate by aspiration, using a hypodermic needle attached to a vacuum line.

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