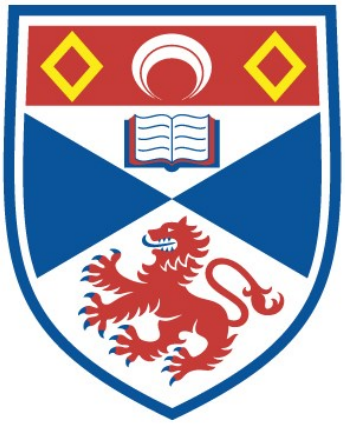
NITROSOTHIOLS AS NO-DONOR DRUGS: SYNTHESIS, MECHANISTIC STUDIES, CHEMICAL STABILITY, PHARMACOLOGICAL AND PHYSIOLOGICAL ACTIVITY

Haitham H. Al-Sa'doni

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Ninewells Hospital & Medical School-Dundee

NITROSOTHIOLS AS NO-DONOR DRUGS

[Synthesis, Mechanistic Studies, Chemical Stability, Pharmacological and Physiological Activity]

HAITHAM H. AL-SA'DONI

(B.Sc., M.Sc.)

Submitted for the degree of

Doctor of Philosophy

April 1996

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Dedicated

To

My Family, My Friends

&

St. Andrews

With

Love & Respect

You Can Be Whatever You Want to Be

There is inside you
all of the potential to be whatever
you want to be ~
all of the energy to do whatever
you want to do.
Imagine yourself as you would like to be,
doing what you want to do,
and each day, take one step
towards your dream.
And thought at times it may seem too
difficult to continue,
hold on to your dream.
One morning you will awake to find
that you are the person
you dreamed of ~
doing what you wanted to do ~
simply because you had to courage
to believe in your potential
and to hold on to your dream.

~ Donna Levine

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ACKNOWLEDGEMENTS

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Finally I would like to express my thanks to my Ma' and Da' for their encouragement and assistance during this work.

LECTURE COURSES

LECTURE COURSES

The following is a statement of the courses attended during the period of research: Organic Research Seminars (3 years attendance); School Colloquia (3 years); Computer Course (Macintosh Computer), Mr J. Bews (5 lectures); Introduction to Instrumentation , Dr R. K. Mackie (3 lectures); NMR, Dr R. K. Mackie (1 lectures); Advanced NMR , Dr F. G. Riddell (5 lectures); Case Studies of Reaction Mechanisms, Dr A. R. Butler (5 lectures); Molecular Modelling in the Design of New Drugs, Dr C. Thomson (5 lectures); Carbohydrates Dr R. Field (5 lectures); Ligand Design Prof. R. W. Hay (5 lectures); Molecular Rearrangements, Dr J. C. Walton (5 lectures).

TABLE OF CONTENTS

TABLE OF CONTENTS

ABSTRACT

ABSTRACT

S-Nitrosothiols (RSNO) are an important class of NO-donor drugs. They have been used clinically and occur naturally where they may have a role in several biological and physiological processes in the human body. The medical importance of *S*-nitrosothiols has been highlighted recently by several reports which describe the clinical use of GSNO (**13**) to inhibit platelet aggregation during coronary angioplasty and also to treat a form of pre-eclampsia , a high blood pressure condition suffered by some pregnant women.

We set out to extend the range of compounds of this type by synthesising a novel series of biologically active *S*-nitrosothiols (**1-13**) and to look for a correlation between structure, chemical stability, and physiological activity. The situation has been complicated by the recent discovery that the main route for the release of NO from *S*-nitrosothiols is a copper-catalysed process. A detailed kinetic study of copper ions, and thiols on the stability of the compounds we synthesised has shown that the dominant pathway for the decomposition of *S*-nitrosothiols in most circumstances is one catalysed by Cu^{+} ions. We suggest that Cu^{+} ions are formed by the reaction of Cu^{2+} ions with thiol, present in the *S*-nitrosothiols as an impurity. The implications of this discovery for an understanding of the biological action of *S*-nitrosothiols is suggested.

All the new *S*-nitrosated dipeptides (**2-12**) examined show less susceptibility to copper (I)-catalysed release of NO than SNAP (**1**) but are more reactive than GSNO (**13**). We found that *S*-nitrosated dipeptides are potent vasodilators and suitable inhibitors of platelet aggregation but are chemically very stable in the absence of copper ions. All thirteen compounds combine the favoured property of chemical stability with a high level of biological activity.

We found that copper(I)-chelation induced reduction of the biological activity of *S*-nitrosothiols in smooth muscle relaxation. The results show that responses to both SNAP and GSNO are reversibly inhibited by neocuproine. We conclude that relaxation of vasodilator smooth muscle by SNAP and GSNO is caused in part by NO released into solution *via* a Cu^{+}-dependent catalytic reaction, and provide evidence that endogenous Cu^{+} ions may also contribute to the maintenance of vasodilator 'store' *in vivo* by catalysing the decomposition of naturally-occurring *S*-nitrosothiols. A particularly interesting finding recently by Gordge *et al.* (1995) shows that the inhibition of platelet aggregation activity shown by GSNO is much reduced in the presence of neocuproine and the closely related bathocuproine, both specific Cu^{+}-chelating agent. However, it was shown very recently (Schrammel *et al.*, 1996) that copper ions inhibit basal and NO-stimulated recombinant soluble guanylate cyclase activity and that Cu^{+} is more effective than Cu^{2+} in this regard.

L-Ascorbic acid (vitamin C) could play a role in the *in vivo* release of NO from naturally occurring and exogenous *S*-nitrosothiols and so play a part in smooth muscle relaxation and in inhibition of platelet aggregation.

All thirteen compounds examined show the ability to release NO *in vitro*. The inhibitory effect of Hb, a recognised NO scavenger, was investigated. In smooth muscle, responses to intermediate doses of *S*-nitrosothiols were significantly inhibited by Hb, though not abolished entirely. In platelet, we found that the inhibitory activity of these *S*-nitrosothiols was reversed by haemoglobin, indicating the involvement of NO in the process.

We found that the solution stability of the *S*-nitrosothiols did not correlate with relaxation of vascular smooth muscle or inhibition of platelet aggregation, again suggesting that the tissue specificity is a function of the R- group.

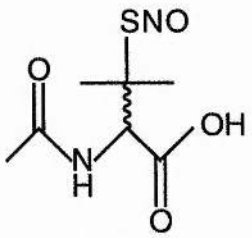
We conclude that the biological activity of *S*-nitrosothiols depends upon the release of NO in a process catalysed by Cu(I), and that the decomposition may occur inside or outside the cell, depending upon the structure of RSNO.

NO-DONOR DRUGS USED IN THIS RESEARCH

NO-DONOR DRUGS USED IN THIS RESEARCH

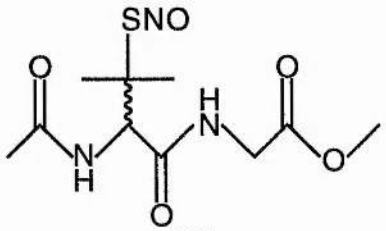
The following list of the NO-donor drugs used in this research is given for ease of reference.

I. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteine (SNAP; **1**):



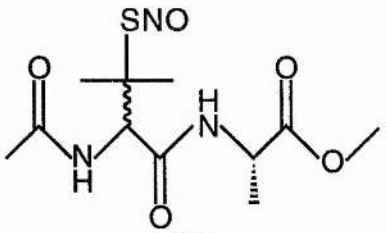
(SNAP; **1**)

II. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester (**2**):



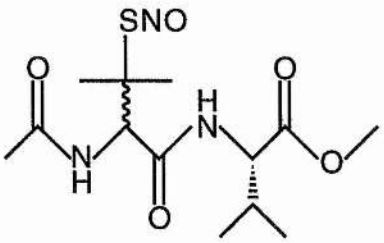
(**2**)

III. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-alanine methyl ester (**3**):



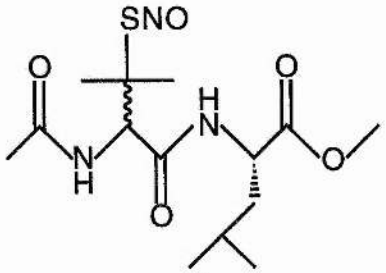
(**3**)

IV. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-valine methyl ester (**4**):



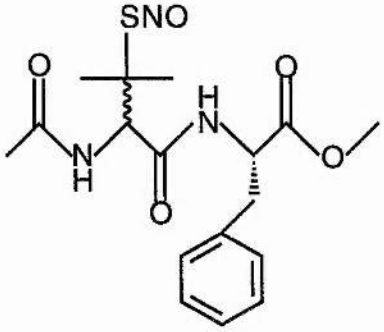
(**4**)

V. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-leucine methyl ester (**5**):



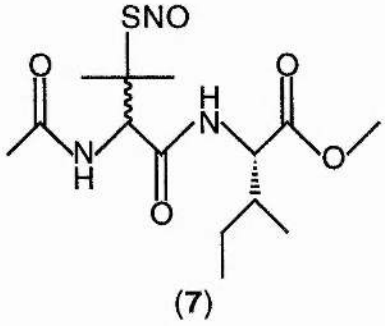
(**5**)

VI. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-phenylalanine methyl ester (**6**):



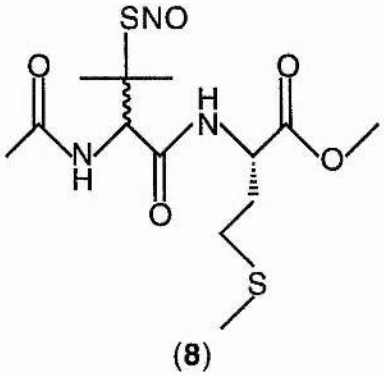
(**6**)

VII. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-isoleucine methyl ester (**7**):



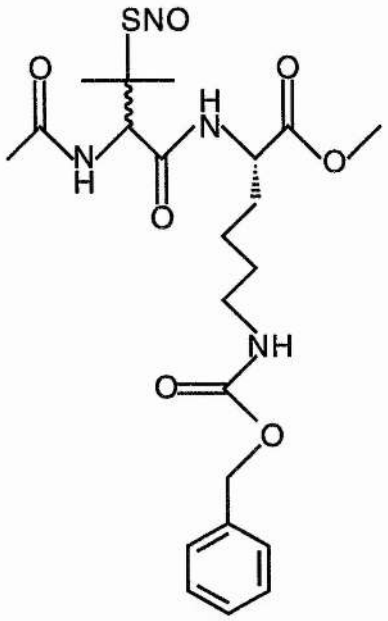
(**7**)

VIII. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-methionine methyl ester (**8**):



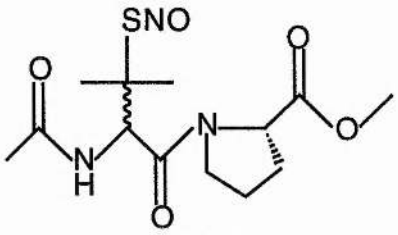
(**8**)

IX. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-N_ε-CBZ-L-lysine methyl ester (**9**):



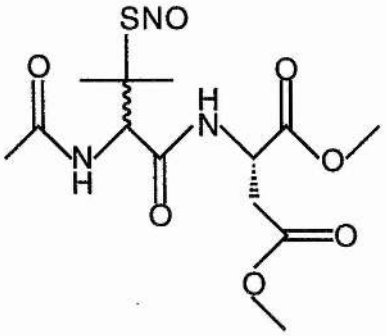
(**9**)

X. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-proline methyl ester (**10**):



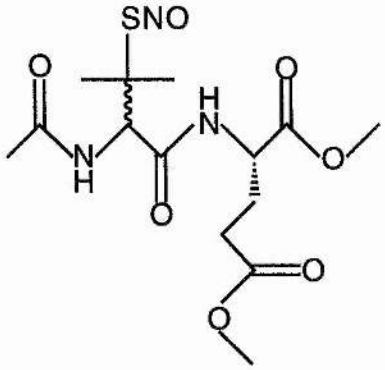
(**10**)

XI. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-aspartic acid dimethyl ester (**11**):



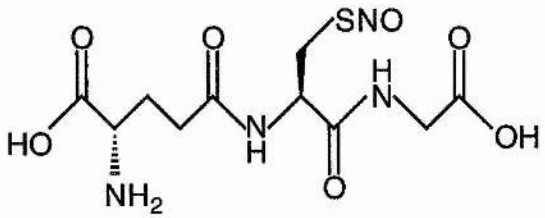
(**11**)

XII. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-glutamic acid dimethyl ester (**12**):



(**12**)

XIII. *S*-nitroso-L-glutathione (GSNO; **13**):



(GSNO; **13**)

LIST OF FIGURES

LIST OF FIGURES

LIST OF TABLES

LIST OF TABLES

LIST OF SCHEMES

LIST OF SCHEMES

LIST OF STRUCTURES

LIST OF STRUCTURES

I. INTRODUCTION

In this chapter we will briefly discuss the following subjects: S-nitrosothiols as NO-donor drugs, the biological activity of S-nitrosothiols, Endothelium-Derived Relaxation Factor (EDRF), physiological function of nitric oxide (NO), and the purpose of the present work.

INTRODUCTION

Twenty-six years ago, it was claimed that (Vasu, 1967): "from an industrial point of view, nitric oxide is probably the most important oxide of nitrogen". After that, nitric oxide was largely ignored and simply considered as a toxic molecule, one of a number of environmental pollutants.

However, during recent years, certain discoveries have shown that NO is involved in several important biological events. Its biological roles are so extensive that in 1992 it became the molecule of the year (Culotta and Koshland, 1992): "a startlingly simple molecule unites neuroscience, physiology, immunology, and revises scientists' understanding of how cell communicate".

NO seems to be implicated in a wide range of biological processes: neurotransmission (Garthwaite, 1991); immune regulation (Hibbs, 1991; Marletta et al., 1988; Stuehr et al., 1989; Moncada et al., 1991); smooth muscle relaxation (Ignarro, 1989a&b; Moncada et al., 1986a&b); and platelet inhibition (Azuma et al., 1986; Furlong et al., 1987; Radomski et al., 1987a,b,c&d). Several reviews on the biology of NO have recently been published (Butler and Williams, 1993; Feldman et al., 1993; Knowles and Moncada, 1992; Nathan, 1992; Stamler, 1992a,b,c,d&e; Galla, 1993).

This contribution attempts to bring together the following aspects of S-nitrosothiol chemistry: (a) Synthesis, chemical stability, and physiological activity (vasodilator activity and inhibition of platelet aggregation). (b) Reactions of S-nitrosothiols with particular emphasis on two recently discovered decomposition pathways in aqueous solution leading to nitric oxide formation, the first brought about by catalytic quantities of metal ions most notably Cu^{2+} the second involving L-ascorbic acid (vitamin C). (c) Detection of NO release from the S-nitrosothiols.

1. 1. *S*-Nitrosothiols as NO-Donor Drugs

S-Nitrosothiols (RSNO) are the sulphur analogues of the alkyl nitrites RONO. They are not as well-known as the alkyl nitrites, mainly because many of them are unstable in the pure state at room temperature (Williams, 1988).

S-Nitrosothiols have come to prominence in recent years as part of the nitric oxide story, since they are believed to decompose non-enzymatically to give nitric oxide, and could, in principle, be used therapeutically as NO-producing drugs. They might replace the much-used glyceryl trinitrate, which is subject to a tolerance problem in some patients (Butler, 1995). Additionally, *S*-nitrosothiols have been detected *in vivo* and many believe that they play a part in the mechanisms of some well-documented physiological processes which have been attributed to nitric oxide (Stamler and Loscalzo, 1991). Indeed, there have been suggestions that *S*-nitrosocysteine, rather than nitric oxide itself, constitute the so-called Endothelium Derived Relaxing Factor (EDRF), but this is not the current majority view (Myers *et al.*, 1990; Rubanyi *et al.*, 1991a&b). Because of these possibilities much more attention has been paid to the chemistry (and pharmacology) of *S*-nitrosothiols in recent years. Studies have concentrated on their synthesis, reactions which lead to nitric oxide formation and reactions whereby the NO group can be tranferred to other thiols.

1. 1. 1. Synthesis of *S*-Nitrosothiols

S-Nitrosothiols are red or green compounds which can be easily obtained during treatment of thiols with a variety of nitrosating agents (Oae and Shinhama, 1983; Williams, 1985). The most common synthetic reactions are:

(a) Thiol and any electrophilic nitrosating agent XNO (NOCl, RONO, N_2O_4, NO_2, N_2O_3, HNO_2 etc.) which can act as a reagent capable of delivering "NO^+" as shown in the following equation:

$$RSH + XNO \longrightarrow RSNO + X^- + H^+$$

The most commonly used reagent is an aqueous solution of nitrous acid generated from sodium nitrite and a mineral acid and the nitrosothiol is separated by filtration or by solvent extraction.

(b)Thiol and alkyl nitrites in aqueous acid or alkaline solution and also in a number of non-aqueous solvents such as acetone and chloroform:

$$RSH \xrightarrow{t\text{-butyl nitrite}} RSNO$$

The nitrosation by alkyl nitrites in water may occur by two mechanisms depending on the pH (Fontecave and Pierre, 1994):

(a) acidic medium:

$$R'ONO + H^+ \longrightarrow R'OH + NO^+$$

$$RSH + NO^+ \longrightarrow RSNO + H^+$$

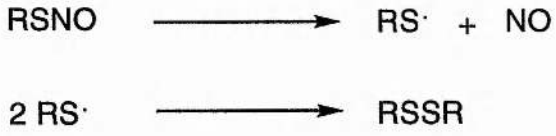
(b) basic medium:

$$RSH + OH^- \longrightarrow RS^- + H_2O$$

$$RS^- + R'ONO \longrightarrow RSNO + R'O^-$$

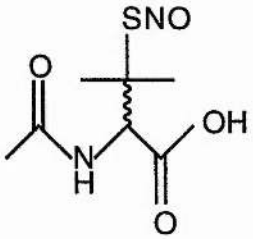
The reaction is the sulphur analogue of the reaction whereby alkyl nitrites are generated from alcohols and a source of "NO^+". One difference between the two reactions is that whilst alkyl nitrite formation is a significantly reversible process (the reaction driven to completion by the removal of the alkyl nitrite by distillation), the reaction of thiols is essentially irreversible which makes separation of the product easier. The difference can readily be explained in terms of the difference in nucleophilicities and basicities of the sulphur and oxygen atoms in these systems.

S-Nitrosothiols are usually quite unstable in water at physiological pH. They appear to release NO spontaneously without redox or photochemical activation, even though light greatly accelerates the reaction. Moreover, the decomposition is strongly catalyzed by Cu^{2+} or Fe^{3+}, even as contaminants of aqueous buffered solutions, and is thus inhibited by chelators (McAninly *et al.,* 1993). The reaction proceeds through homolytic cleavage of the S-N bond generating a thiyl radical and NO:



These NO generators are also very interesting since it is possible to get a wide range of rates of production of NO by simple chemical modification of the R group of *S*-nitrosothiols (RSNO; Mathews and Kerr, 1993). The highest stabilities are obtained with tertiary *S*-nitrosothiols (Williams, 1988).

S-Nitroso-*N*-acetyl-D,L-penicillamine (SNAP) is indefinitely stable as a powder, allowing determination of its crystal structure by X-ray diffraction (Field *et al.,* 1978):

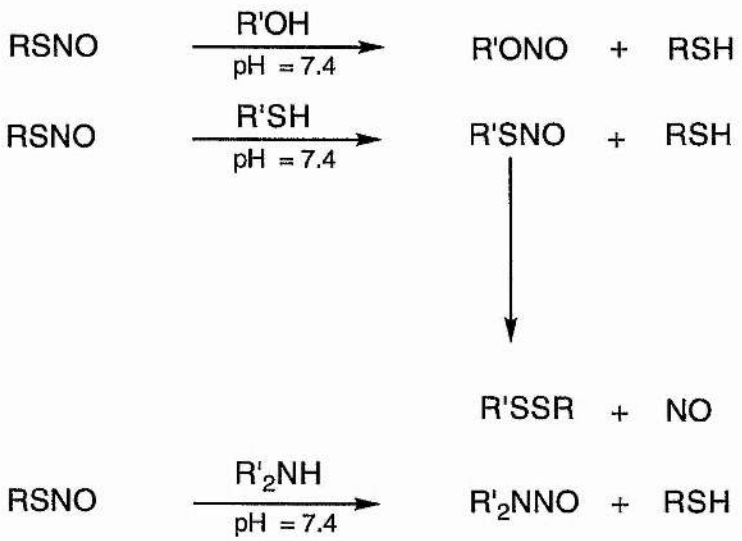


S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP)

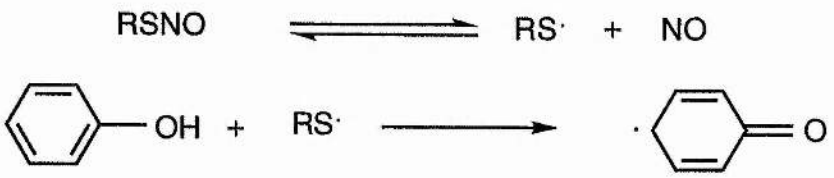
SNAP is slow generator of NO in water at pH =7.0, and can be used as a vasodilator. *S*-Nitrosothiols are also good inhibitors of ribonucleotide reductase and all cell growth. It has been recently shown that nitrosation of cysteine in proteins may occur during exposure to NO (Stamler *et al.*, 1992a,b,c,d&e; McDonald *et al.*, 1993; Girard *et al.*, 1993). However, the reaction can not be a simple one between NO and cysteine. The stability of such protein-bound nitrosothiols is remarkable, especially considering the

extreme lability of the free *S*-nitrosocysteine. Whether this process is involved in the regulation of vascular tone or in cytotoxicity remains to be established. Moreover, endogenous *S*-nitroso-L-glutathione has been found to be present at nano- to micromolar concentrations in the human airways (Gaston *et al.*, 1993).

While alkyl nitrites are essentially NO^+ donors and thus useful nitrosating agents towards nucleophiles, in water *S*-nitrosothiols may behave as either NO or NO^+ donors. This will depend on the substrate and the medium, but also in the stability of the *S*-nitrosothiols. One may suggest that unstable *S*-nitrosothiols will be NO donors while stable ones will be NO^+ donors, converting alcohols, thiols and secondary amines into alkylnitrites, disulphides, and nitrosoamines, respectively (Fontecave and Pierre, 1994). Nitrosation of proteins by *S*-nitrosothiols is likely to occur, as suggested in the cases of the NMDA receptor (Lipton *et al.*, 1993) and ribonucleotide reductase:

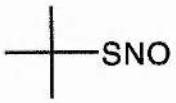


On the other hand, the nitrosation of phenols by nitrosothiol (*e.g.* SNAP) involves a radical mechanism (Oh and Williams, 1991):

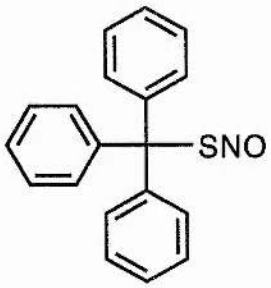


1. 1. 2. The Chemical Stability of *S*-Nitrosothiols

In general *S*-nitrosothiols are not as stable in the pure state as are the alkyl nitrites. A number however have been successfully purified and characterised; some have been known for a number of years (Rheinboldt, 1959). These include the *S*-nitrosothiols derived from *t*-butyl thiol and triphenyl methyl thiol:

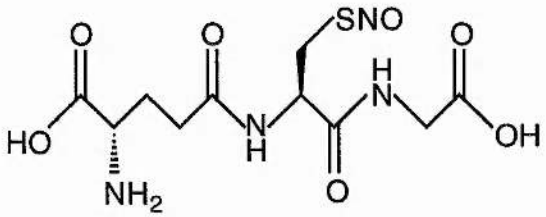


S-nitroso-*t*-butane thiol



S-nitrosotriphenylmethane thiol

More recently it has been shown that the *S*-nitrosothiols from *N*-acetyl-D,L-penicillamine (Field *et al.*, 1978) and L-glutathione (Hart, 1985) can be isolated and kept as solids at room temperature indefinitely:



S-nitroso- L-glutathione (GSNO)

A detailed X-ray crystal structre determination has been carried out for the former (Field *et al.*, 1978). Similarly some *S*-nitrosothiols from proteins containing the -SH group are quite stable (Stamler *et al.*, 1992a,b,c,d&e) as is *S*-nitrosocysteine within a polypeptide chain (Myers *et al.*, 1990) (even though *S*-nitrosocysteine itself is quite unstable) and also the di-nitroso derivative of a penicillamine dipeptide (Moynihan and Roberts, 1994). A range of *S*-nitrosothiols based on cysteamine and its derivatives has recently been described (Roy *et al.*, 1994). Most of the successful syntheses and isolation of stable products were carried out with nitrous acid or *t*-butyl nitrite. Quite often attempted syntheses have resulted in decomposition at the last moment with the evolution of brown fumes of nitrogen dioxide (Williams, 1988).

1. 1. 3. The Physical Properties of *S*-Nitrosothiols

The stable *S*-nitrosothiols are green or red solids or liquids. Generally tertiary structures (such as SNAP) are green and primery ones (such as GSNO) pink or red (Field *et al.*, 1978; Hart, 1985). There are characteristic infrared frequencies at 1480-1530 cm^{-1} (N=O stretch) and 600-730 cm^{-1} (C-S stretch). In the uv-visible region there are two absorption bands in the 330-350 nm and 550-600 nm regions. The former has the larger extinction coefficient (typically 10^3 $dm^3mol^{-1}cm^{-1}$) and is the one usually used to monitor the disappearance of *S*-nitrosothiols in quantitative studies. Both ^1H and ^{13}C nmr spectroscopy have been used to characterise *S*-nitrosothiols. The shift due to both α protons and α carbons on *S*-nitrosothiols is quite diagnostic (Roy *et al.*, 1994)

1. 1. 4. The Decomposition of *S*-Nitrosothiols

S-Nitrosothiols can decompose to yield the corresponding disulphide and nitric oxide as in the following equation:

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

by different pathways:

(a) Photochemical decomposition

S-Nitrosothiols readily decompose photochemically to give disulphide and nitric oxide (Barrett *et al.*, 1965).

(b) Thermal decomposition

Also, *S*-nitrosothiols readily decompose thermally to give disulphide and nitric oxide (the same products obtained photochemically; Field *et al.*, 1978; Rheinbolt and Mott, 1932).

(c) Copper ions catalysed the decomposition of S-nitrosothiols:

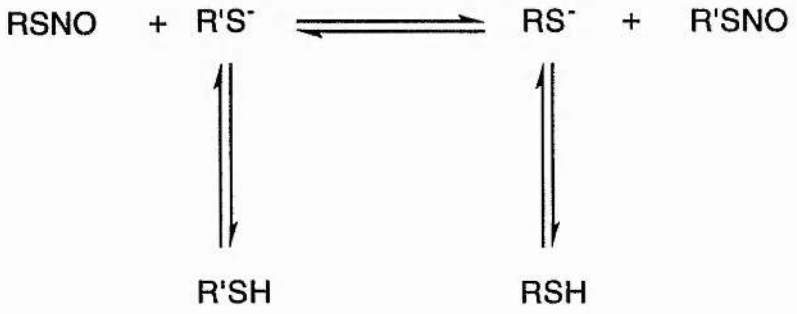
In solution at room temperature in the absence of light, decomposition also occurs and the reaction has been much studied in recent years, because of the intense interest the development of NO-releasing compounds. The quantitative results reported in the literature are however extremely erratic, and there are major difference in the rate form (*i.e.* in the kinetic order of reactions) and in the reactivities (*e.g.* as measured in half-lives) between the measurements made on the same or similar compounds in different laboratories. Consequently, until 1993 when the importance of the presence of catalytic quantities of Cu^{2+} was realised (McAninly *et al.*, 1993), not even a qualitative mechanistic picture was available for these reactions. The current position will be discussed in the next chapter (Results & Discussion; Dicks *et al.*, 1996).

(d) Nitroso group is transferred to another molecule

There are a number of literature reports referring to transfer to amines and thiols (McAninly *et al.*, 1993). In principle such a reaction could occur directly, where the *S*-nitrosothiols act as carriers of NO^{+} as do alkyl nitrites, or indirectly by initial NO loss, which is then oxidised to a higher oxidation state of nitrogen which will allow nitrosation of amines *etc.* to occur. If this oxidation is not possible (e.g. if the reaction is carried out

anaerobically) then reaction will not occur, as nitric oxide itself will not act as a nitrosating agent.

The NO group exchange between a *S*-nitrosothiols and a thiol has been demonstrared (Park, 1988), although under certain circumstances the *S*-nitrosothiols will also decompose leading to mixtures of disulphides. It has been shown (Barnett *et al.*, 1994) that the reaction actually involves the attack of the thiolate anion, since the pH-rate profile follows the thiol ionisation. Reaction occurs quite generally (the following equation) for a wide range of R and R' structures:



The reaction rate is much faster than that of the decomposition of RSNO and occurs quite readily at the physiological pH, depending on the pKa of the thiol. Rate (Barnett *et al.*, 1994) and equilibrium (Meyer *et al.*, 1994) constants have been reported.

(e) L-Ascorbic acid

We very recently discovered that addition of L-ascorbic acid (Vitamin C) increases the rate of *S*-nitrosothiols decomposition. Thus, L-ascorbic acid could play a role in the *in vivo* release of NO from naturally occuring *S*-nitrosothiols (Dasgupta *et al.*, 1996). The role of L-ascorbic acid in the breakdown of *S*-nitrosothiols will be discussed in the next chapter (Results & Discussion).

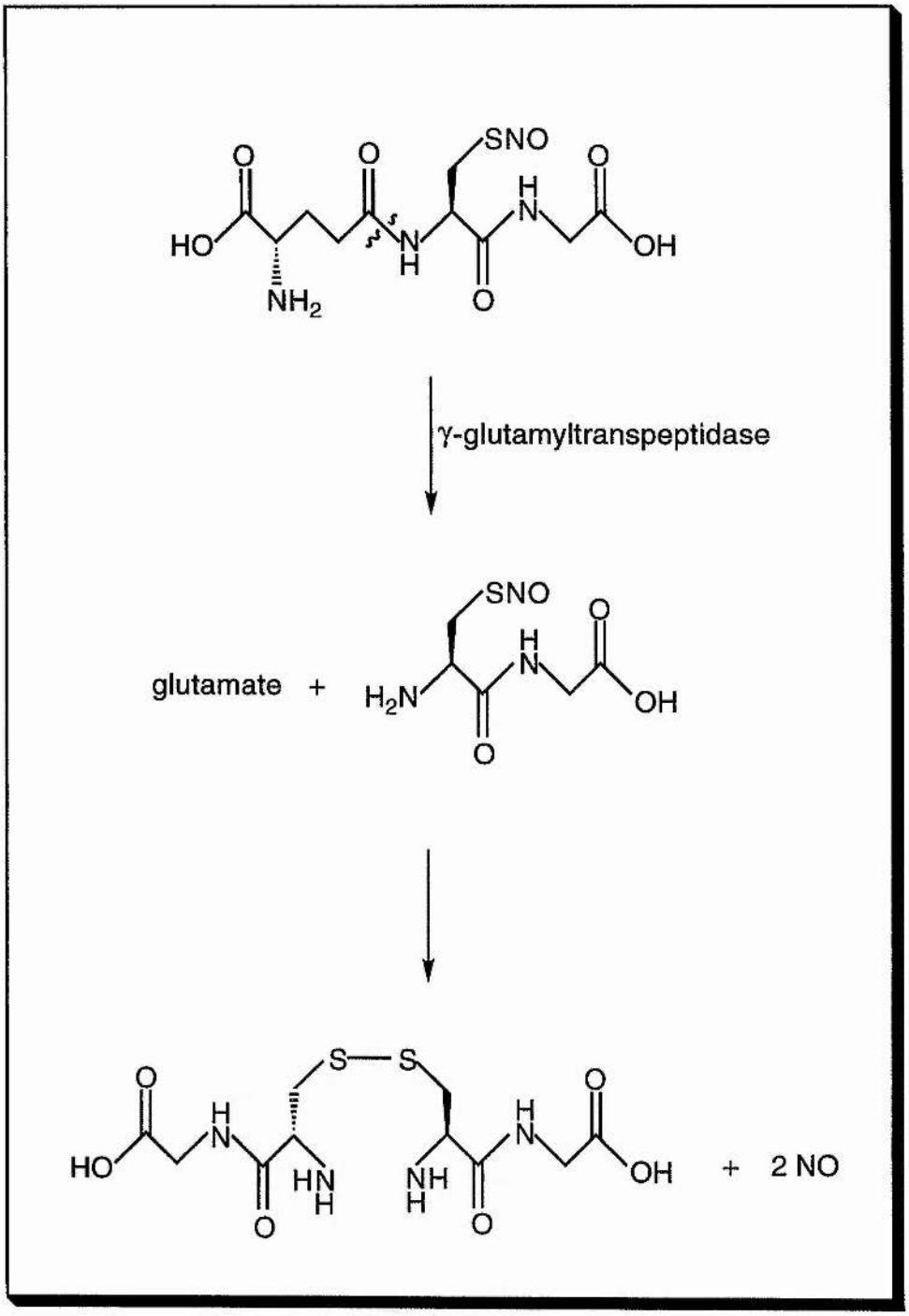
(f) Enzymatic decomposition

S-Nitroso-L-glutathione (GSNO) decomposes to give NO and other products. *In vivo* the first step is enzymatic cleavage of the glutamyl-cysteinyl peptide bond by the enzyme γ-glutamyl transpeptidase (γ-GT; Askew *et al.*, 1994). The resulting *S*-nitrosothiol, *S*-nitrosocysteinylglycine, would be expected to be more susceptible to release of NO by metal (copper) ion catalysis.

The proposed mechanism of action of γ-glutamyl transpeptidase is show in Scheme 1. 1.

(g) Reaction with other metals

The discovery of copper catalysis of *S*-nitrosothiol decomposition was made during a systematic investigation of the effect of metals. Addition of EDTA greatly reduced the rate of decomposition but the rate increased when copper salts were added at concentrations greater than that of EDTA. Thus copper ion catalysis came to light. The exact nature of the catalytic copper will be described in next chapter (Results and Discussion). No catalysis by added Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+} or Fe^{3+} was detected. Williams *et al.* found also catalysis by Fe^{2+} generated by reduction of Fe^{3+} under anaerobic conditions, but this reaction is still under investigation.



Scheme 1. 1: The proposed mechanism of action of γ-glutamyl transpeptidase (adapted from Askew, 1994).

1. 1. 5. The Purity of *S*-Nitrosothiols

The fact that *S*-nitrosothiol are less stable than the corresponding oxygen analogues, such as alkyl nitrites, made them difficult to isolate and purify. Only a few however have been isolated and characterised. A possible reason why pure *S*-nitrosothiols are so difficult to isolate in the pure solid state could be due to spontaneous decomposition (Park, 1988). It has been proposed, although no evidence was presented, that they decompose by second order kinetics and therefore evaporation of the solvent would lead to increase concentration and more chance of rapid decomposition to their disulphide (Park, 1988). Also, it is expected that there would be thiol present even in the pure *S*-nitrosothiol (McAninly *et al.*, 1993).

1. 1. 6. *S*-Nitrosothiols *in vivo*

S-Nitrosothiols have been detected and quantified *in vivo*. Most occur naturally in human plasma as the *S*-nitroso albumin (Stamler *et al.*, 1992a,b,c,d&e). Further, *S*-nitroso-L-glutathione (GSNO) has been measured at micromolar concentrations in human bronchial fluid (Gaston *et al.*, 1993). A number of *S*-nitrosothiols including GSNO have been shown to possess vasodilator activity (Mellion *et al.*, 1983) and also have the ability to inhibit platelet aggregation (Ignarro *et al.*, 1981a&b). They are also implicated in a number of other biological processes. An earlier suggestion (Myers *et al.*, 1990) that the EDRF is not free nitric oxide but a *S*-nitrosocysteine is now not a view which is widely held, but *S*-nitrosothiols may well play a role in nitric oxide storage within the body, given the ready pathway for NO group transfer to thiolate ion. Already *S*-nitrosothiols, specifically GSNO are being used therapeutically (a) to inhibit platelet aggregation after coronary angioplasty (Langford *et al.*, 1994) (b) to treat a form of pre-eclampsia in pregnant women (Belder *et al.*, 1995). Its activity is believed to be associated with its ability to inhibit platelet aggregation at dose levels which do not lower blood pressure, in contrast to the other available NO donors.

1. 2. The Biological Activity of *S*-Nitrosothiols

The first demonstration that these compounds are biologically active was the description of the antibacterial effects of *S*-nitrosocysteine (CysNO; Incze *et al.*, 1974). More recently, attention has focused on the effects of *S*-nitrosothiols on vascular smooth muscle. *S*-Nitrosothiols such as *S*-nitroso-*N*-acetylpenicillamine (SNAP), CysNO, *S*-nitroso-*N*-acetylcysteine (NACysNO) and *S*-nitrososocaptopril (SNOCAP) have been shown to be potent smooth muscle relaxations in a variety of vascular smooth muscle preparations (Ignarro *et al.*, 1989a&b; Henry *et al.*, 1989,Cooke *et al.*, 1989), and *S*-nitrosoglutathione (GSNO), CysNO, *S*-nitrosohomocysteine (HCysNO), NACysNO, SNAP and SNOCAP have been shown to relax tracheal smooth muscle (Jansen *et al.*, 1992). Smooth muscle relaxation is believed to be mediated by elevated cGMP levels, and the ability to stimulate soluble guanylate cyclase (GC) has been described for several *S*-nitrosothiols, including SNAP, NACysNO, SNOCAP, *S*-nitrosomercaptoethylamine, *S*-nitroso-3-mercaptopropanoic acid and *S*-nitroso-β-D-thioglucose (Ignarro *et al.*, 1981a&b; Mellion *et al.*, 1983; Loscalzo *et al.*, 1989). Inhibition of platelet aggregation by *S*-nitrosothiols has been described for several compounds, including SNAP, CysNO, NACysNO, SNOCAP and *S*-nitrosothioglucose (Mellion *et al.*, 1983; Loscalzo *et al.*, 1989; Mendelsohn *et al.*, 1990).

In addition to their pharmacological properties, *S*-nitrosothiols may be important physiologically. *S*-Nitrosothiols have been suggested to be the active metabolites responsible for the smooth muscle relaxing activity of nitroglycerine and related nitrovasodilators (Ignarro *et al.*,1981a&b). According to this proposal, *S*-nitrosothiols such as CysNO are formed from endogenous thiols *in vivo* during metabolism of nitrovasodilators. Similarly , the antithrombotic activity of the nitrovasodilators has been proposed to be due to the formation of *S*-nitrosothiols in platelets (Stamler and Loscalzo, 1991). Recently, the biological activities of two potent endogenous vasodilators, the

bovine retractor penis inhibitory factor (Kerr *et al.*, 1992) and endothelium-derived relaxing factor (EDRF, Myers *et al.*, 1990; Rubanyi *et al.*, 1991a&b) have been suggested to be due to *S*-nitrosothiols. A number of groups have provided evidence that EDRF is identical to nitric oxide (NO) (Furchgott, 1988; Palamer *et al.*, 1987; Ignarro *et al.*, 1987), whereas other dispute that claim (Shikano *et al.*, 1987; Vedernikov *et al.*, 1988; Myers *et al.*, 1990; Sata *et al.*, 1990: Rosenblum, 1992). If EDRF is not nitric oxide, it is probably a closely related compound such as a *S*-nitrosothiols (Myers *et al.*, 1990; Rubanyi *et al.*, 1990). Protein *S*-nitrosothiols, predominantly the *S*-nitroso derivative of albumin, have been found in human plasma and may represent storage forms of nitric oxide (Stamler *et al.*, 1992a,b,c,d&e).

The fact that NO, a decomposition product of *S*-nitrosothiols, can directly relax vascular smooth muscle (Gruetter *et al.*, 1979), inhibit platelet aggregation (Mellion *et al.*, 1983) and stimulate soluble GC (Arnold *et al.*, 1977) has led to the suggestion that the biological activities of *S*-nitrosothiols are a direct consequence of decomposition and NO production. All of the known *S*-nitrosothiols are unstable in solution, and although the decompostion of most of them has not been characterized in detail, it probably occurs by homolytic cleavage of the S-N bond, resulting in the production of NO and the corresponding disulphide (Williams, 1983). Alternatively, NO could be produced *via* a one-electron reduction of *S*-nitrosothiols (Rubanyi *et al.*, 1991a&b). Feelisch and Noack (1991) compared the stability and the biological activity of various *S*-nitrosothiols and concluded that stimulation of soluble GC is mediated by NO released upon decomposition. The ability of CysNO to stimulate soluble GC has likewise been attributed to NO (Graven and DeRubertis, 1983). On the other hand, other researchers, including Kowaluk and Fung (1990a&b), Rubanyi *et al.* (1991a&b) and Myers *et al.* (1990), provide evidence that the smooth muscle relaxing activety of *S*-nitrosothiols is not due to spontaneous libration.

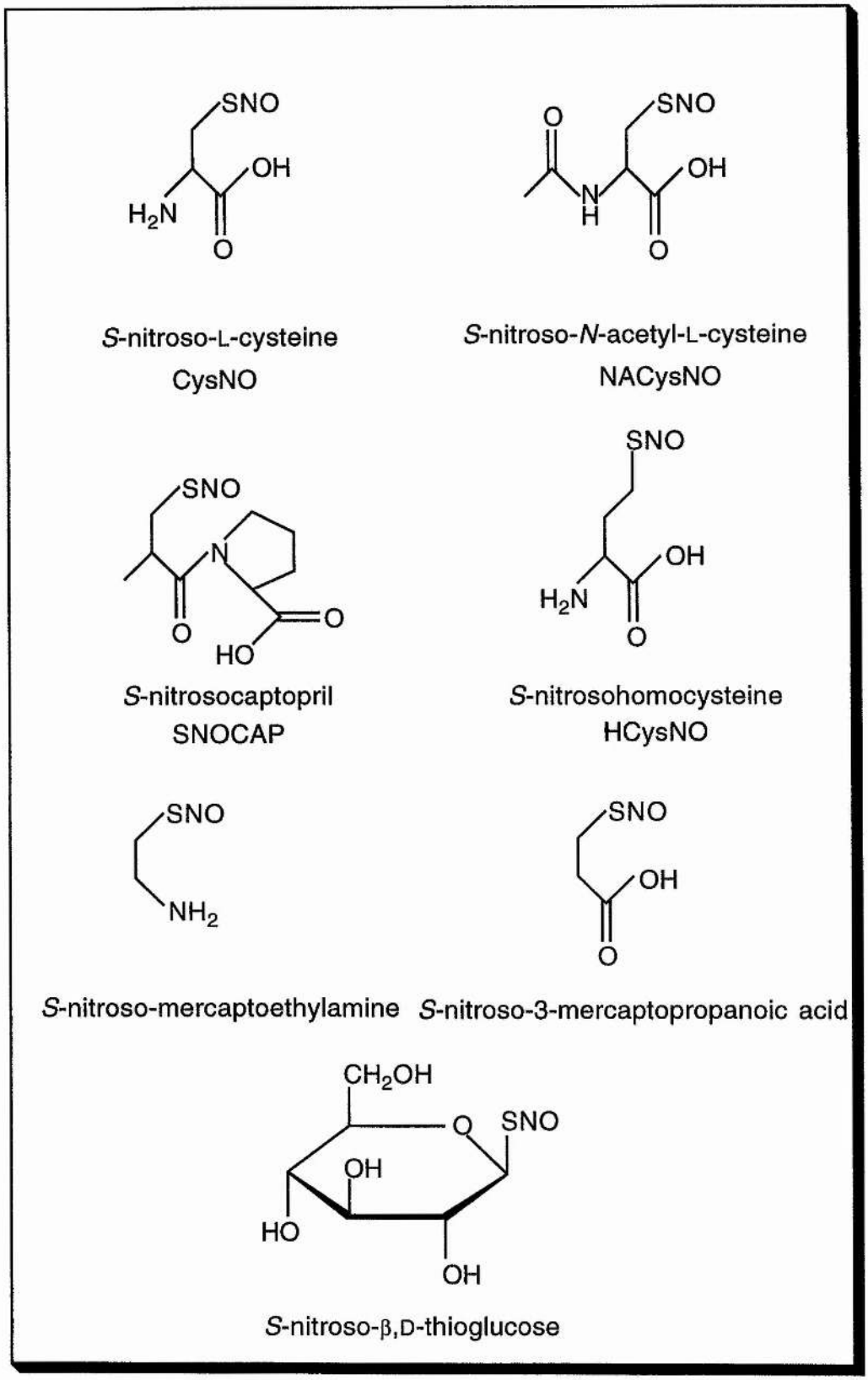
The trouble with all previous studies of the *in vitro* release of NO from *S*-nitrosothiols which do not take into account the effect of copper ions from adventitious copper present in the buffer and thiol contaminating *S*-nitrosothiols need reinterpretation. This will be discussed in the next chapter (Results & Discussion).

Some synthetic and naturally occurring *S*-nitrosothiols are shown in Scheme 1. 2.

1. 3. Endothelium-Derived Relaxation Factor (EDRF)

Early suggestion 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 enzyme (Pinto *et al.*, 1985; Macdonald *et al.*, 1986) or was a compound with a carbonyl group near its active site (Griffith *et al.*, 1984) did not lead to the identification of its chemical structure. Based on the similarities in the pharmacological behaviour of EDRF and NO generated from acidified NO_2^-, Furchgott suggested in 1986 that EDRF may be NO (Furchgott, 1988). At the same time, Ignarro *et al.* also speculated that it may be NO or a closely related species (Ignarro *et al.*, 1988).

The first evidence for the formation of NO by mammalian cells came from experiments in which EDRF released from vascular endothelial cells was identified as NO by chemical means. NO may be measured directly as the chemiluminescent product of its reaction with ozone (Downes *et al.*, 1976). It was shown using this method that the concentrations of bradykinin that induced the release of EDRF from procine aortic endothelial cells in culture also caused a concentration-dependent release of NO. Moreover, the amount of NO released by the cells were sufficient to account for the relaxation of vascular strips (Palmer *et al.*, 1987). Furthermore, the levels of NO released by these cells also accounted for the inhibition of platelet aggregation and adhesion induced by EDRF (Radomski *et al.*, 1987a,b,c&d). A key element in all of these studies was the correlation the amounts of NO measured by bioassay and those detected by chemiluminescence.



Scheme 1. 2: Some synthetic and naturally occurring *S*-nitrosothiols.

A detailed comparison of the biological actions of EDRF and NO on vascular strips (Palmer *et al.*, 1987; Hutchinson *et al.*, 1987) and on platelets (Radomski *et al.*, 1987a,b,c&d) also showed that the two compounds were indistinguishable (Moncada *et al.*, 1988). Both EDRF and NO caused a relaxation of the vascular strips that declined at the same rate during passage down the bioassay cascade (Palmer *et al.*, 1987). Furthermore, the rate of the decay during transit in polypropylene tubes was slower but similar for both compounds, indicating that they have identical chemical stability even under these artificial conditions. Both EDRF and NO also inhibited platelet aggregation (Radomski *et al.*, 1987a,b,c&d), induced the disaggregation of aggregated platelets (Radomski *et al.*, 1987a,b,c&d), and inhibited adhesion (Radomski *et al.*, 1987a,b,c&d). Moreover, their biological half-lives as inhibitors of platelet aggregation were similar (Radomski *et al.*, 1987a,b,c&d).

The actions of EDRF and NO on vascular strips and on platelet were similarly potentiated by SOD (superoxide dismutase) and cytochrome c and inhibited by Fe^{2+} and some redox compounds (Palmer *et al.*, 1987; Hutchinson *et al.*, 1987; Radomski *et al.*, 1987a,b,c&d). Furthermore, the potency of redox compounds as inhibitors of EDRF-induced and NO-induced vascular relaxation was attenuated by SOD (superoxide dismutase) to a similar extent. In addition, the inhibitory action of Hb on EDRF can be explained by the fact that this substance binds avidly to NO (Hermann, 1965; Gibson and Roughton, 1957; Martin *et al.*, 1986). Finally, both EDRF and NO act on vascular smooth muscle (Kukovetz *et al.*, 1979; Rapoport and Murad, 1983a,b&c) and platelets (Mellion *et al.*, 1981) through the stimulation of soluble guanylate cyclase and elevation of cyclic GMP.

NO release from vascular endothelial cells from the other species and from a number of vascular preparations, in amounts sufficent to account for the biological actions of EDRF, has also been demonstrated. It was also shown, using a chemical assay based on the diazotization of sulphamic acid and subsequent coupling with *N*-(1-naphthyl)-ethylene diamine, that NO or a labile nitroso species was released from perfused bovine

pulmonary artery. Furthermore, perfusion of segments of pulmonary artery or pulmonary vein with ACh, caused relaxant responses and elevation of vascular cyclic GMP level in the bioassay tissues that could be matched by NO (Ignarro *et al.*, 1987). Similar, results were obtained in prefused rabbit aorta stimulated with ACh, and substance P (Khan and Furchgott, 1987; Chen *et al.*, 1989). Later, the use of a spectrophotometric assay, based on the reaction between NO and Hb, also demonstrated the release of NO from vascular endothelial cells in culture (Kelm *et al.*, 1988a&b). Furthermore, the release of NO from isolated perfused rabbit (Amezcua *et al.*, 1988) or guinea pig (Kelm and Schrader, 1988b) hearts has been shown to account for the vasodilator actions of ACh and bradykinin in these preparation. All of this evidence strongly supported the proposal that EDRF is NO.

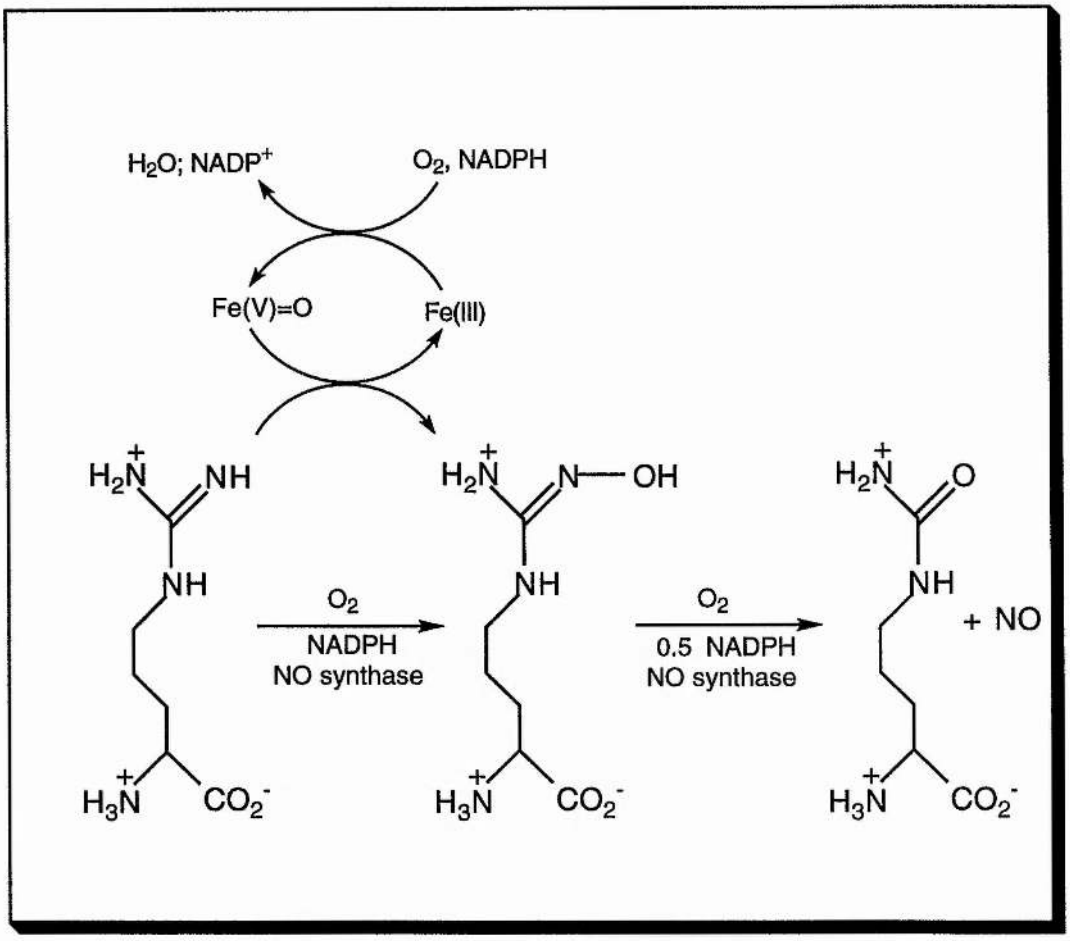
1. 3. 1. Generation of NO

In general, nitric oxide can be generated *in vivo* by two main pathways. Firstly, from enzymatic sources; secondly , from chemical sources. Here we will briefly review the two main pathways.

1. 3. 1. 1. Enzymatic Sources

In mammalian cells, NO is synthesized by NO synthases, which catalyze the oxidation of L-arginine by dioxygen. There are two types of NO synthases that have been recently described in great detail in several excellent reviews (Feldman *et al.*, 1993; Marletta, 1993). One group (macrophages, hepatocytes, tumor cells) is made of inducible NO synthases, the other (cerebellar, endothelial cell, platelets) of constitutive enzymes. In the latter, the enzyme activity is dependent on Ca^{2+} and calmodulin.

These enzymes have strong homologies with both cytochrome P450 reductase and cytochrome P450 with FMN, FAD and haem as prosthetic groups. Moreover, they require tetrahydrobiopterin and NADPH as cofactors. A simplified reaction scheme is shown in Scheme 1. 3.



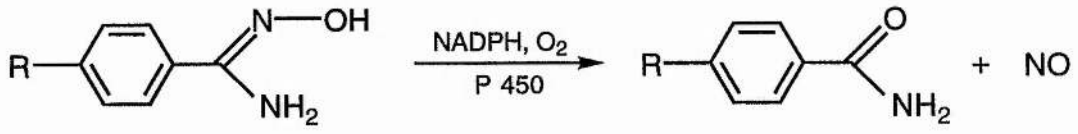
Scheme 1. 3: The proposed NO synthase reaction (adapted from Knowles and Moncada, 1994; Marletta, 1993).

The haem moiety serves to carry out a reductive activation of O_2, leading to a high-valent ferryl complex which transfers its oxygen atom to L-arginine. The resulting *N*-hydroxyarginine (NOHA) is further oxidized to NO and citrulline. The source of oxygen in both NO and citrulline is molecular oxygen. The nitrogen atom of NO is the guanidino nitrogen atom of arginine. Several arginine derivatives (*N*-methylarginine) are powerful inhibitiors of NO synthases.

Other haemoproteins have recently been found to generate NO from *N*-hydroxyarginine. With cytochrome P450 from rat liver microsomes, the oxidation can be performed with

NADPH and O_2 as well as with H_2O_2 or hydroperoxides (Boucher *et el.*, 1992a&b). On the other hand, horseradish peroxidase, hemoglobin and catalase could also catalyze the oxidative cleavage of a C-N bond of NOHA by H_2O_2 or alkyl hydroperoxides (Boucher *et al.*, 1992a&b).

The reaction has been further extended to a large family of molecules, the aromatic amidoximes (Andronik *et al.*, 1992):

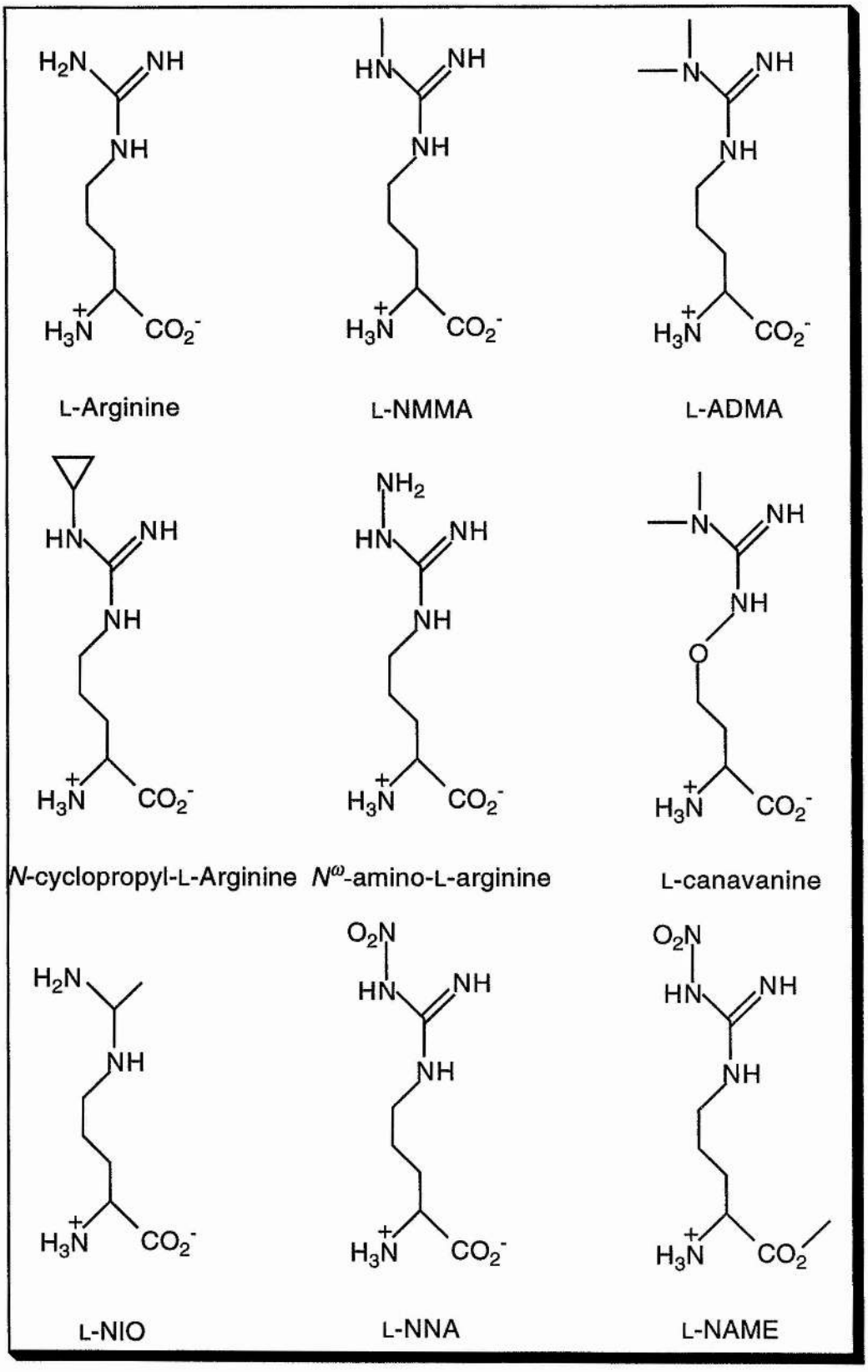


In all these reactions, the oxidizing conditions rapidly convert NO into NO_2^- or other nitrogen oxides.

Some compounds related to arginine are effective inhibitors of NO-synthase and their use has proved invaluable in many investigations on the biology of NO.

The structure of L-arginine and of the L-arginine analogues most frequently used as inhibitors of NO synthases are displayed in Scheme 1. 4 (adapted from Knowles *et al.*, 1994; Butler and Williams, 1993).

NO-synthase in endothelial cells is a constitutive enzyme. This means it is present all the time and responds rapidly to activation. On the other hand, the enzyme in the macrophages is inducible, that is, it is not present until the macrophages have been activated by a cytokine. There is a delay of some hours between activation and the appearance of nitrite and nitrate, indicative of macrophage activity. The differerance between the two can be understood in terms of their different physiological roles. NO is cytotoxic only when large quantities are produced and this explains why NO used to activate guanylate cyclase in muscle cells has no adverse effects. Macrophage activity must, necessarily, be long-lasting to rid the body of infection but, in order to respond to the rapidly changing needs of the body, the effect of NO in muscle cells must be short-term (Butler and Williams, 1993).



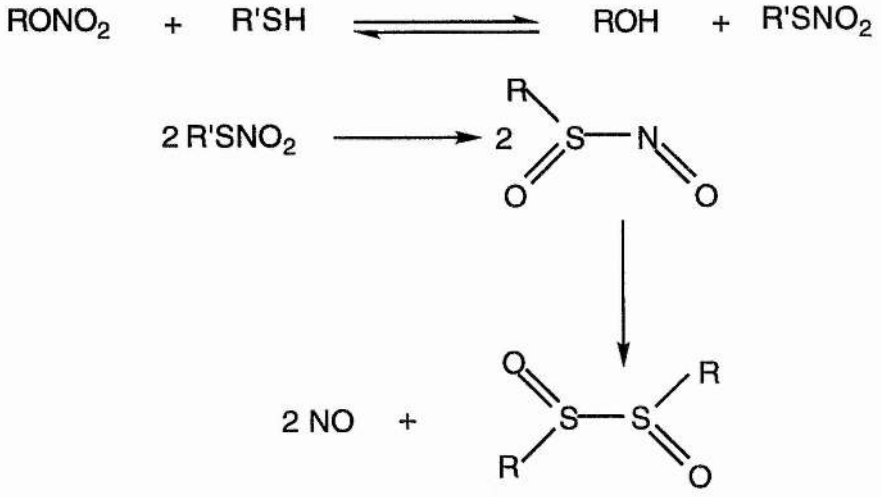
Scheme 1. 4: The structure of L-arginine and of the L-arginine analogues most frequently used as inhibitors of NO synthases.

1. 3. 1. 2. Chemical Sources

Nitric oxide can be generated from several chemical sources, such as organic nitrates, iron-nitrosyl complexes, sydnonimines, *C*-nitroso compounds, secondary amine/NO complex ions and *S*-nitrosothiols. All these sources act as NO-donor drugs in direct and indirect ways. Here we will discuses each source briefly to provide simple background information about these drugs.

(a) Organic Nitrates

Nitroglycerin has been used as a vasodilator (a substance which enlarges blood vessels) for the treatment of angina pectoris. Its vasodilatory effects are releated to its metabolization to NO within the artery.

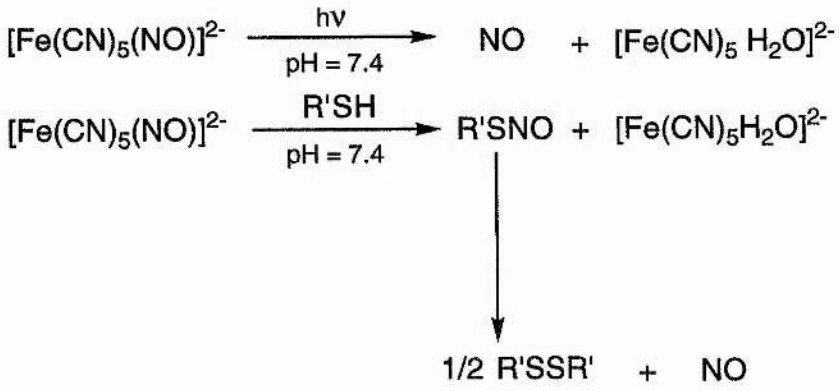


However, the release of NO from organic nitrates is not spontaneous and requires activation through enzymatic pathways, that have not been unambiguously identified. Thiols might be involved in such an activation (Fontecave and Pierre, 1994).

(b) Iron-Nitrosyl Complexes

Sodium nitroprusside (SNP) has also been used as a vasodilator. However, SNP does not spontanously liberate NO *in vitro*. It requires either reductive (for example, with a thiol)

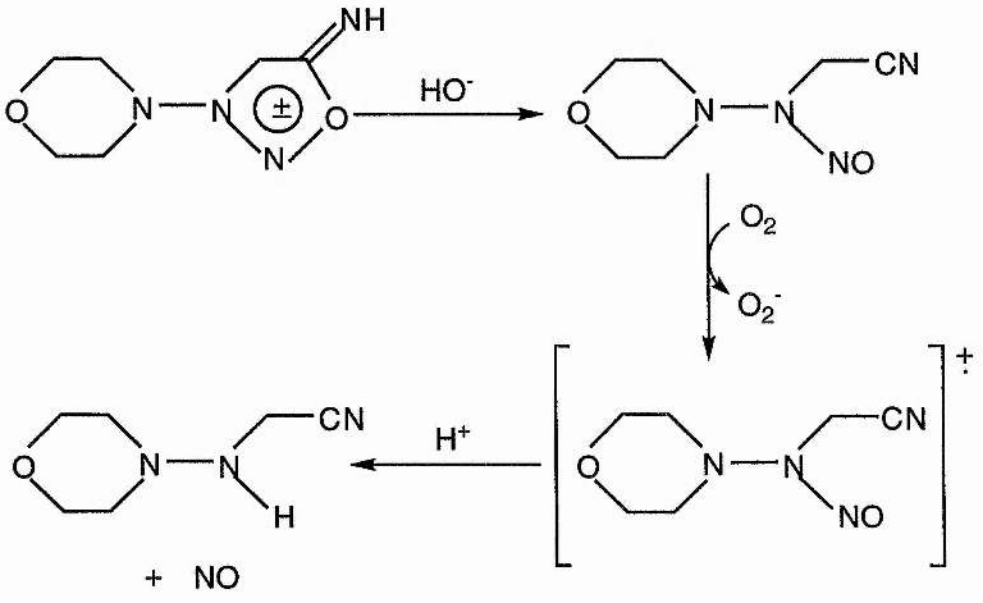
or light activation. This is very often insufficiently appreciated in experiments using SNP as a source of NO (Butler, 1994; unpublished observation).



SNP is actually more a source of nitrosonium NO^+, and thus behaves as a nitrosating electrophilic species. It has been shown to convert amines into nitrosamines, ketones into oximes but *S*-nitrosation has not been unambiguously detected.

(c) Sydnonimines

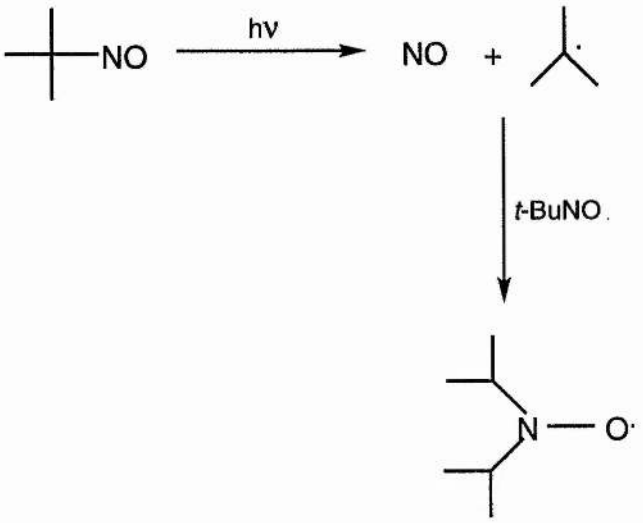
These molecules are heterocyclic compounds derived from morpholine. The liberation of NO requires both alkaline pH and the presence of oxygen:



Superoxide is generated at the same time and may combine with NO to generate peroxynitrite (Fontecave and Pierre, 1994).

(d) C-Nitroso Compounds

Upon exposure to light, *C*-nitroso compounds may undergo a homolytic cleavage of the C-NO bond and generate NO (Chamulitrat *et al.*, 1993):



(e) Secondary Amine/NO Complex Ions

Compounds of formula $R_2N[N(O)NO]$ (so called amine NONOate) (Maragos *et al.*, 1991; Hrabie *et al.*, 1993) have the following interesting properties:

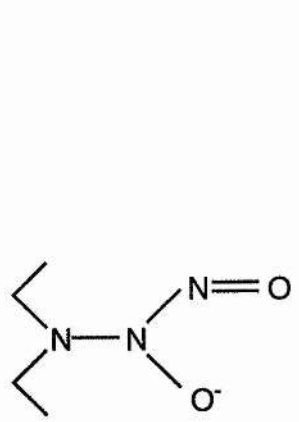
(a) They stabilize NO during storage in a solid form.

(b) They are highly soluble in water.

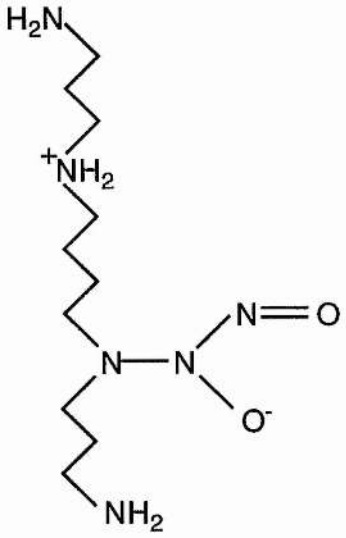
(c) They can release NO at rates that can be reliably adjusted over a wide range with judicious choice of the carrier nucleophile R_2NH.

(d) They release of NO is spontaneous and does not require redox or light activation.

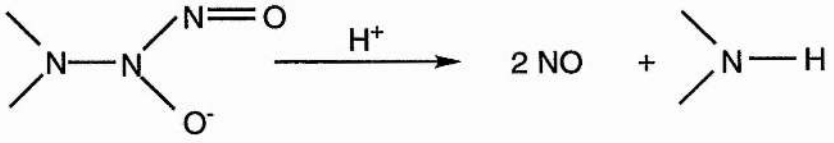
Examples:



diethylamine NONOate



spermine NONOate



These compounds have both vasorelaxant and antiproliferative (inhibition of DNA synthesis in tumor cells) activities (Maragos *et al.*, 1993).

(f) S-Nitrosothiols

S-Nitrosothiols are an important class of NO-donor drugs. They decompose by several pathways giving NO and the corresponding disulphide (Section 1.1).

1. 4. Physiological Functions of Nitric Oxide

Nitric oxide or EDRF seems to be implicated in a wide range of biological processes (Moncada *et al.*, 1991):

(a) Production of nitric oxide acts as a transduction mechanism for the activation of soluble guanylate cyclase (in many cells and tissuse).

(b) Nitric oxide is an effector molecule in immunological reactions.

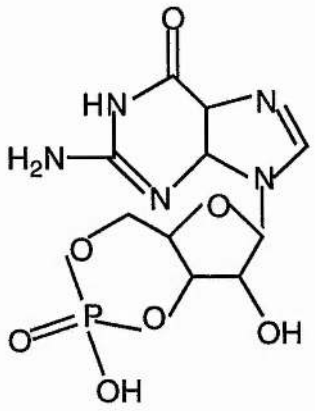
In this research we have studied the biological activity of a range of *S*-nitrosothiols in smooth muscle relaxation, and inhibition of platelet aggregation.

1. 4. 1. Activation of Guanylate Cyclase

Guanylate cyclase, the enzyme responsible for vascular muscle relaxation, is found in most cells and throughout the animal kingdom. It is exist in two forms: a soluble enzyme inside the cell and membrane-associated (particulate). The relative amount of each within the cell varies with the cell type and its physiological state.

Normally purified soluble guanylate cyclase can be activated *in vitro* by NO-donating compounds. It has been shown to be the target enzyme for NO (whether endothelium derived or from an exogenous source). However, NO has its main site of action on the cytoplasmic isoenzyme (Waldman and Murad, 1987). The presence of multiple isoenzyme forms of guanylate cyclase has made the determination of the exact constitution of soluble guanylate cyclase difficult, but it is thought to be a heterodimer with Mr 82,000 and 70,000 subunits (Waldman and Murad 1987). In bovine lung the enzyme contains haem and perhaps copper and when purified, enzyme extracts exhibit 3 absorption maxima at 433, 550 and 565 nm which are shifted on exposure to either CO or NO (Gerzer *et al.*, 1981). Although NO-donor drugs activate soluable guanylate cyclase (Katsuki *et al.*, 1977a,b&c), haem-deficient guanylate cyclase showed modest or little activation by these agents (Ignarro *et al.*, 1982a&b). The involvement of the haem group in the stimulation of the enzyme was clarified by the inhibitory effect of adding ferro metallo-proteins (but not ferric forms) such as haemoglobin or myoglobin, to highly purified extracts of the enzyme. Application of NO-donor drugs to the purified enzyme in the presence of Fe^{2+} metallo-proteins was no longer capable of activating cyclic guanosine monophosphate (cGMP) production (Mittal *et al.*, 1978; Murad *et al.*, 1978).

This was thought to be due to the efficient scavenging ability of NO by the Fe^{+2} metallo-proteins:



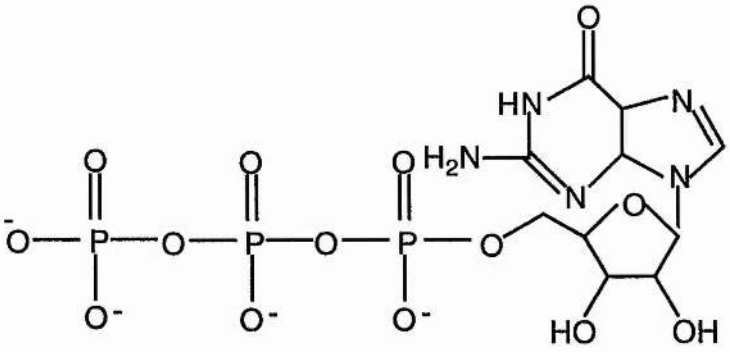
cGMP

It was found that oxidising agents such as hydrogen peroxide, methylene blue, superoxide and ferrocyanide inhibited the activity of the enzyme, whilst reducing agents (ascorbate, cysteine, glutathione, dithiothreitol) promoted the activation of the enzyme by NO-donor drugs (Braughler, 1983; Graven *et al.*, 1978). The inhibitory effect of oxidising agents is likely to be due to a number of reasons; NO may be converted to higher oxides of nitrogen (NO_2) before it reaches the active site of the enzyme; the iron of the recipient haem group at the active site may be oxidised to its ferric form; or, an as yet unexplained 'overoxidation' of key regulatory thiol groups can cause irreversible loss of both basal and nitro-activated cGMP production by the enzyme (Waldman and Murad, 1987). Consequently, reducing agents may potentiate activation by preventing these oxidations.

Partial purification of hepatic soluable guanylate cyclase results in loss of responsiveness to NO-donor drugs, as well as to nitric oxide (Craven *et al.*, 1978). Responses were partially restored by the addition of free haematin, haemoglobin and other haemoproteins, but the activity was potentiated by the addition of reducing agents into the incubations, which facilitate the generation of nitrosyl haem by maintaining the haem iron in the ferrous form (Katsuki *et al.*,, 1977a,b&c; Craven *et al.*, 1978). Preformed nitrosyl-haemoglobin was found to be 10-fold more effective in activating guanylate cylase than

certain nitrovasodilators (Craven *et al.*, 1978). Activation of partially purified guanylate cyclase by preformed nitrosyl-haemoglobin was not potentiated by reducing agents, supporting the suggestion that reducing agents affect the conversion of the parent nitrovasodilator to nitrosyl-haem complexes (Craven *et al.*, 1978). These data imply that activation of guanylate cyclase by nitrovasodilators occurs through reductant-dependent formation of nitrosyl-haem prophyrin complexes.

Thiols such as cysteine, glutatione, and dithiothreitol are capable of releasing NO from a range of nitrovasodilators, although intermediates in the process, such as *S*-nitrosothiol derivatives, have been found to be 100-fold more effective than certain nitrovasodilators in activating guanylate cyclase (Ignarro *et al.*, 1980a,b&c). As thiol groups can undergo oxidation/reduction reactions and thiol disulphide conversions have been demonstrated to regulate protein structure and function, it is reasonable to suggest that they may also be involved in the regulation of guanylate cyclase. Early studies using alkylating agents which modify free thiol groups (eg. ethacrynic acid) were shown to alter basal and nitro-activated cGMP production (Katsuki *et al.*, 1977a,b&c). Similarly, mixed disulphide formation on addition of cysteine or cystamine also inhibited enzyme activity (Ignarro *et al.*, 1981a&b; Waldman *et al.*, 1983). Studies with [^{35}S] cystamine resulted in incorporation of the radioactive label into the enzyme along a time course which paralleled inhibition of the enzyme. This effect was reversed by dithiothreitl which reforms the thiols (Brandwein *et al.*, 1981). It has been proposed that guanylate cyclase possesses multiple thiol sites, one of which is responsible for regulation of basal cGMP production and another for regulation of nitro-activated cGMP production (Braughler *et al.*, 1983). At least one, but probably two, thiol groups are located at or near the catalytic site of the enzyme, as preincubation with the activating agent (nitrovasodilators) or excess substrate (Mg^{2+}-GTP) protected the enzyme against inactivation (Ignarro *et al.*, 1981a&b).



GTP

Although the mechanism by which the nitrosyl-haem complex activates guanylate cyclase remains unknown, a model has been presented which could explain this phenomenon (Figure 1.1).

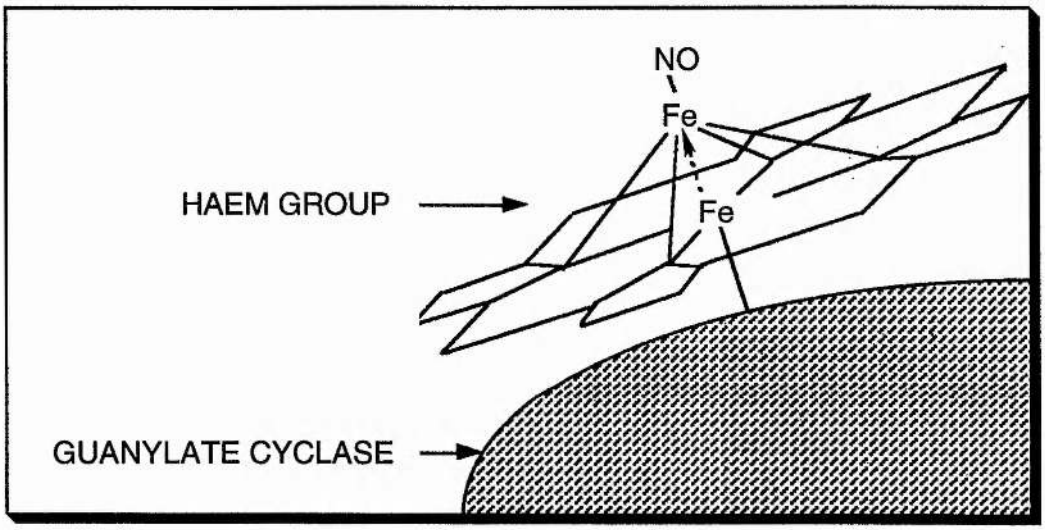


Figure 1.1: A simplified diagram showing the out-of-plane movement of the porphyrin iron centre of guanylate cyclase when activated by NO.

Conformational change in an enzyme is widely belived to be a regulatory phenomenon in regulating its action as envisioned by Koshland in his *induced-fit model* of enzyme

action (Stryer, 1988). If these changes occur at, or near, the active site the conformational change may influence function. Based on the findings that protoporphyrin IX gave the same activation effect as NO-haem, and that haem inhibits, not activates, the enzyme, Ignarro proposed that a haem structural change is caused by NO binding (Ignarro *et al.*, 1982a&b). He suggested that once bound the haem-NO complex elicits an out-of-plane movement of the central iron to produce a haem core size similar to that of a free porphyrin (Ignarro, 1992).

Traylor *et al.* (1993) support this theory with work on model haem systems. Based on the finding that NO binds preferentially to the haem lacking a proximal ligand, they proposed that when NO binds haem, there is a tendency to expel the basic ligand on the proximal side of the haem. This mechanism liberates a free base to catalyse the hydrolysis of the phosphate diester bond of guanosine triphosphate (GTP). This theory was tested using NO to release 1-methylimidazole from a 5-coordinated model haem. This promoted hydrolysis of *p*-nitrophenolate in the aqueous medium used. However there are important aspects of the proposed mechanism which remain to be explained. It is not known whether haem is present in the ferric form in the native enzyme, because reductants such as dithiothreitol are used during enzyme purification. Furthermore, the strong binding of NO to ferrous haem and possible dissociation of haem from proteins due to this binding would tend to severely limit the number of turnovers of the enzyme and make for a very inefficient biocatalyst.

1. 4. 2. Endothelium-Dependent and Independent Smooth Muscle Relaxation

The production of cGMP from activation of guanylate cyclase was shown to correspond to a relaxation of certain smooth muscles and this activation was markedly enhanced by free radicals such as NO and hydroxyl radical (Murad *et al.*, 1979).

Murad and his colleagues proposed that many potent vasodilators, such as sodium nitroprusside (SNP), organic nitrates and inorganic nitrites, activated guanylate cyclase indirectly *via* NO, which may be released as a reaction product (Murad *et al.*, 1979). Speculation that EDRF would stimulate an increase in intracellular cGMP in arterial smooth muscle cells was confirmed by experiments carried out by Rapoport and Murad (1983a,b&c) and Furchgott and Jothianandan (1983) on the rat and rabbit aorta. Stimulation of the tissues with intact endothelium produced elevated levels of cGMP in the smooth muscle which was not present if the endothelium had been removed. This led to speculation that EDRF was a free radical. It has since been shown that the production of EDRF from NO synthase requires intracellular calcium to inititate its release (Palmer *et al.*, 1988). This calcium appears to be released from intracellular stores as there is no evidence of calcium channels cells (Zheng *et al.*, 1994). Endothelium dependent vasodilators such as ACh stimulate membrane-bound muscarinic receptors on endothelial cells initiating the release of calcium. This is required by the constitutive NO synthase for NO production, and leads to stimulation of guanylate cyclase in arterial smooth muscle in a similar way to nitrovasodilators.

Rapoport and co-workers (1983a,b&c) have shown that increase in cGMP in rat aorta due to this process is accompained by a change in the pattern of phosphorylated proteins. This finding was particularly interesting as this change of pattern of phosphorylated proteins was identical to that previously found after exposure to the endothelium independent vasodilator SNP fuelling the hypothesis that the EDRF and the activating agent released by the nitrovasodilators (NO) were the same. They suggested that this processes was mediated through cyclic GMP production. In addition, the entry of calcium into the smooth muscle cells, which is necessary for contraction, diminished. Figure 1. 2 summarises the endothelium-dependent and endothelium-independent mechanisms of vascular smooth muscle relaxtion which bring about vasodilation.

Recently work by Megson, (1993) and Venturini *et al.*, (1993) has shown that vascular smooth muscle cells contain a finite store of a photosensitive molecule(s) which is

capable of releasing NO when irradiated with visible or UV light. This store can be exhausted rapidly by exposing vessels to laser light but subsequently regenerates in the dark with an absolute dependence upon endothelium-derived NO (Megson, 1993). The repriming of the store is prevented by L-NMMA or haemoglobin and accelerated by donor drugs. Venturini *et al.* proposed that the 'store' is a *S*-nitrosothiol, a nitrosoprotein or an iron-sulphur nitrosyl complex. The work of Megson provides evidence that the 'store' is, in fact, an *S*-nitrosothiol or nitrosoprotein, since it is known that ethacrynic acid, a thiol alkylating agent, also prevents repriming of the 'store'.

1. 4. 3. Inhibition of Platelet Aggregation

The discovery of a role for NO in platelet function occurred in 1981, when it was found that NO and other nitrovasodilators inhibited ADP-induced platelet aggregation by a cGMP-dependent mechanism (Mellion *et al.*, 1981). It was not until 1986 that a link was identified between the actions of NO on platelet aggregation and those of EDRF on vascular smooth muscle (Azuma *et al*, 1986). It was shown by these and other workers that EDRF itself was capable of inhibiting platelet aggregation *in vitro* (Furlong *et al.*1987; Radomski *et al.*, 1987a,b,c&d) and *in vivo*. Radomski *et al.* showed that NO and prostacyclin were both capable of enhancing the disaggregating effects of the other on platelets (Radomski *et al.*, 1987a,b,c&d), although the effects of prostacyclin are mediated by cAMP (Radomski *et al.*, 1987a,b,c&d), and that both have cytoprotective properties (Radomski *et al.*, 1988). The cytoprotective effects of NO are now thought to be due to its reacting with the potentially harmful oxygen free radicals (O_2^- in particular) (Rubanyi *et al.*, 1991a&b). It has also been reported that NO inhibited platelet adhesion to collagen, endothelial cell matrix and cultured endothelial cell monolayers by a cGMP-dependent mechanism (Radomski *et al.*, 1987a,b,c&d). This, however, is in contrast to prostacyclin, which only has a weak, cAMP-mediated inhibitory effect on platelet adhesion (Higgs *et al.*, 1987c) and does not synergise with NO in this case.

It has been shown that nitrovasodilators which release NO spontaneously (3-morpholino-sydnonomine) are capable of inhibiting platelet aggregation by a cGMP-dependent mechanism. However, some organic nitrates (e.g. GTN) which can elicit vasodilation in vascular smooth muscle are not able to inhibit platelet aggregation (Gerzer *et al.*, 1988). The inference is that platelets lack the necessary enzymes for biodegradation, leading to release of NO.

It is now known that NO is generated in platelet and that it acts as a negative feedback system to modulate aggregation (Radomski *et al.*, 1990 a,b). Generation of platelet-derived NO is NADPH-dependent, inhibited by LNMMA and dependent on free intracellular Ca^{2+}. Addition of L-arginine to the medium does not alter basal NO production, but enhances the increase in NO production when aggregation is initiated using collagen, or ADP or arachidonic acid. The inference from this is that nitric oxide synthase exists in platelets and is stimulated to produce NO synthesis from L-arginine once aggregation is initiated (due to an increase in intracellular Ca^{2+}: Ware *et al.*, 1986). The increase in intracellular NO has been shown to be accompanied by an increase in cGMP but not cAMP. The mechanism by which cGMP inhibits aggregation is not fully understood, but it may involve sequestration of free intraplatelet Ca^{2+} (Busse *et al.*, 1987).

NO, therefore, may play an important antithrombotic role *in vivo* by inhibiting both platelet aggregation and adhesion to vessel walls, particularly as its actions are enhanced by the synergistic effect of prostacyclin.

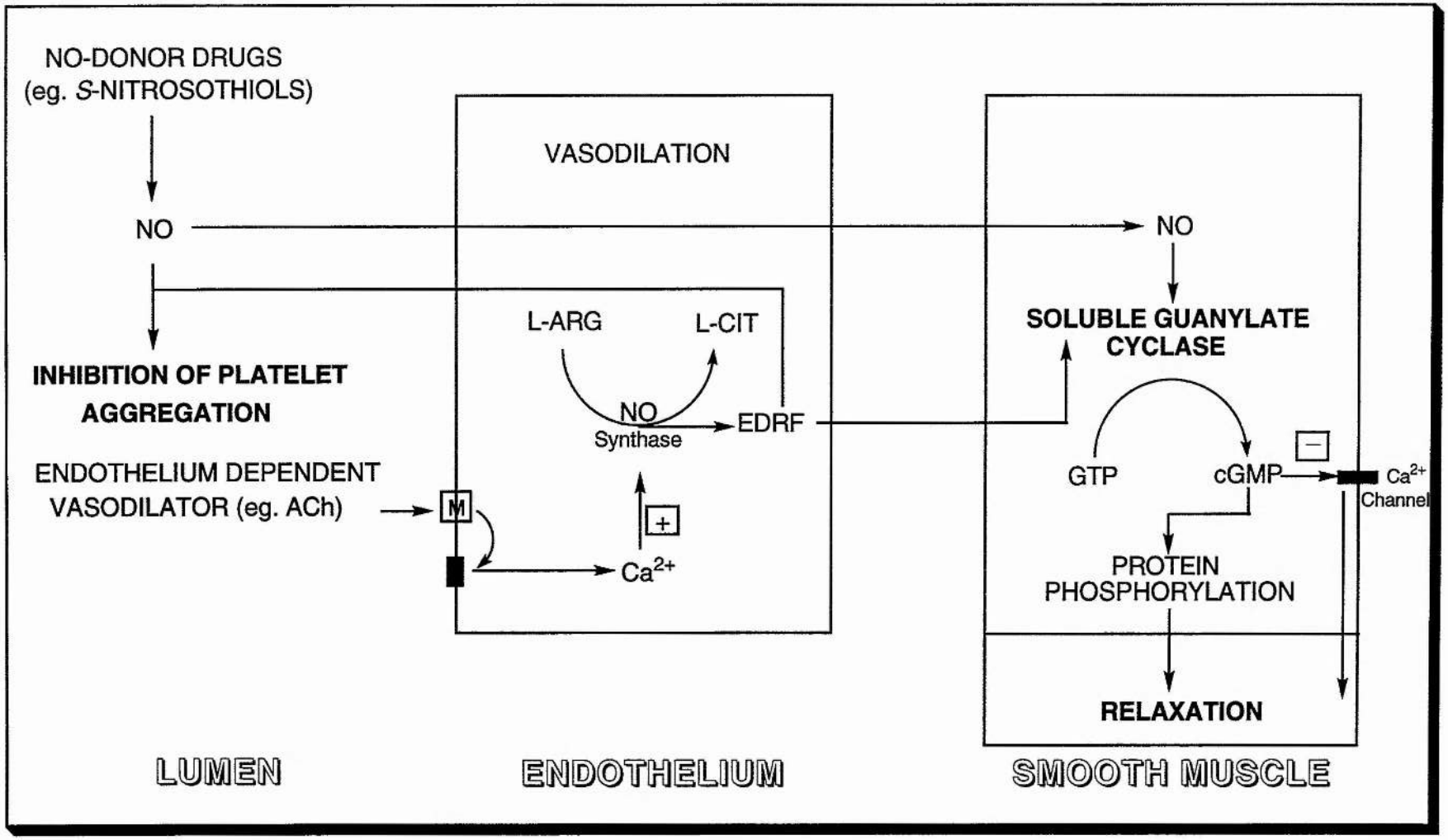


Figure 1. 2: The mechanisms underlying vascular smooth muscle relaxation from endothelium dependent and endothelium independent NO production and the effect of endothelial NO on blood platelets.

1. 5. Purpose of the Present Work

The aims of this research are:

I. The design and synthesis of a new series of biologically active *S*-nitrosothiols as NO-donor drugs with the following properties:

(a) Stable drugs.

(b) Solid or liquid but preferably solid because it will be easier to handle.

(c) Lipophilic drugs in order to increase the ability of these drugs to enter the cell.

(e) With high purity, but we know from literature sources that this will be difficult to achieve due to contamination by thiol and the corresponding disulphide, and the lack of a suitable method of purification due to the nature of *S*-nitrosothiols.

(e) More stable than SNAP but less stable than GSNO.

(f)They can release NO *in vivo*.

(f) They do not release NO during storage.

II. The kinetics and the mechanism of decomposition of *S*-nitrosothiols catalysed by copper ions *in vitro* has not been thoroughly investigated. One aim of this study is to explore the decomposition of SNAP, the new *S*-nitrosothiols, and GSNO in pH = 7.4 in the hope that this will shed light on the mode of action of the copper ions.

III. The role of copper(I) ions in the breakdown of *S*-nitrosothiols *in vivo* is as yet unexplored. Here we investigate the effect of the specific copper(I) chelator, neocuproine (NCu), on vasodilator responses to bolus injections of SNAP or GSNO in the isolated rat tail artery.

IV. Study the chemical stability of the *S*-nitrosothiols.

V. Study the pharmacological and physiological properties of the *S*-nitrosothiols by:

(i) smooth muscle relaxation.

(ii) inhibition of platelet aggregation.

VI. Correlate the structure, chemical stability and biological activity of the *S*-nitrosothiols.

VII. The role of L-ascorbic acid (Vitamin C) in the breakdown of *S*-nitrosothiols *in vivo* is as yet unexplored. Here we investigate the effect of L-ascorbic acid on the decomposition of GSNO *in vitro* and *ex vivo* at pH = 7.4 in the hope that this will shed light on the mode of action of the L-ascorbic acid.

VIII. Detecting of NO by using the sensor IOS-NO to show that *S*-nitrosothiols are NO-donor drugs.

IX. From this study we hope to answer the following question:

Do *S*-nitrosothiols decompose before or after entering the cell?

X. Also we hope to learn something about the tissue selectivity of the drugs. Which can act as good drugs to reduce blood pressure but do not affect the platelet, or *vice versa?*

II. RESULTS AND DISCUSSION

In this chapter, we report the synthesis of (a) an S-nitrosated amino-acid; (b) a series of new S-nitrosated dipeptides; and (c) an S-nitrosated tripeptide. They were prepared in order to study and correlate structure, pharmacological and physiological activity (vasodilator activity and inhibition of platelet aggregation), and chemical stability . Moreover, the mechanism of breakdown of S-nitrosothiols in vitro and in vivo is not fully understood and a study of this matter is reported, to address the questions governing their actual role in the human body, the reason for them being recycled in the body, and to determine if the decomposition is an exo- or endo-cellular event. After thus elucidating the role of S-nitrosothiols as NO-donor drugs attempts will be made to design and synthesise a tissue selective NO-donor drug for further study. Then, detection of NO by three methods to show that S-nitrosothiols are NO-donor drugs.

RESULTS AND DISCUSSION

2. 1. The Design and Synthesis of A New Series of Biologically Active *S*-Nitrosothiols as NO-Donor Drugs

The aim of the work described in Section 2.1 was the design and synthesis of a number of dipeptides where one amino acid contains an -SH group. These compounds were readily converted into the corresponding *S*-nitrosothiols. A number of other syntheses are also described.

Although the role of nitric oxide (NO) in effecting the relaxation of vascular smooth muscle, and thus controlling blood pressure (Butler and Williams, 1993), is firmly established, the part played by *S*-nitrosothiols is far less clear. *S*-Nitrosocysteine (SNC), rather than NO, was proposed (Myers *et al.*, 1990) as the true endothelium derived relaxation factor (EDRF) but a recent study (Feelisch *et al.*, 1994), in which the properties of the EDRF were compared with those of SNC and NO, has shown that this is most unlikely. On the other hand, *S*-nitrosothiols do occur naturally in the body (Stamler *et al.*, 1992a,b,c,d&e) but so far no role has been ascribed to them, although it has been suggested that they provide a pool of nitric oxide for use in metabolic processes.

Exogenous *S*-nitrosothiols are potent vasodilators. *S*-Nitroso-*N*-acetylpenicillamine (SNAP) and *S*-nitrosocaptopril (SNOCAP), *inter alia*, effect relaxation of endothelial smooth muscle (Ignarro *et al.*, 1981a&b). Also, *S*-nitrosoglutathione (GSNO), has been shown to relax bronchial smooth muscle (Gaston *et al.*, 1994). *S*-Nitrosothiols are not particularly stable compounds and breakdown thermally, photochemically (Williams, 1988)

and in a metal ion catalysed process (McAninly *et al.*, 1993; Dicks *et al.*, 1996), to give a disulphide and nitric oxide.

It is possible that *S*-nitrosothiols are vasodilators because they release nitric oxide while in solution before reaching the muscle cells and are merely convenient compounds for the delivery of nitric oxide. However, Kowaluk and Fung (1990a&b) claim that for a number of *S*-nitrosothiols no correlation exists between vasodilator effects and spontaneous liberation of nitric oxide. They conclude that NO production from *S*-nitrosothiols may be catalysed by an external vascular membrane. Mathews and Kerr examined eight *S*-nitrosothiols and found that there was no correlation between solution stability and biological activity. The shortcomings of these two studies are that the changes in chemical structure between *S*-nitrosothiols are rather great and the role of metal ions in NO release was not considered. Metal ions can bring about NO release from *S*-nitrosothiols even if the metals are present as complexed ions (Dicks *et al.*, 1996). However, the two studies mentioned above established the general principle that the vasodilator action of *S*-nitrosothiols is dependent upon structure in a way that does not reflect the ease of NO release.

Moynihan and Roberts (1994) have shown that the nitrosated dipeptide, *S*-nitroso-*N*-acetyl-β,β-dimethylcysteinyl-*S*-nitroso-*N*-acetyl-β,β-dimethylcysteine is an effective vasodilator only 10 times less potent than glyceryl trinitrate.

In view of the importance of *S*-nitrosothiols, peptides containing an -SH group and the complex problems associated with their synthesis, *N*-cyclohexyl-*N'*-2-[*N*-morpholino]ethylcarbodiimide metho-*p*-toluenesulphonate was used to form the peptide bond (Bodanszky and Bodanszky, 1984; Sheehan and Hess, 1955; Sheehan *et al.*, 1956; Bondanszky and Ondetti, 1966; and Sheehan and Hlauka, 1956) and *t*-butyl nitrite was used for nitrosation of the thiol group (Doyle *et al.*, 1983).

S-Nitrosothiols are very easily generated in solution from thiols by electrophilic nitrosation. The current literature surrounding the use of *S*-nitrosothiols has involved the authors

generating them *in situ* because of the difficulties in isolation. Many *S*-nitrosothiols are too unstable in their pure form to be isolated and this has contributed to the lack of knowledge of their chemistry relative to that of alkyl nitrites until comparatively recently. Those with primary alkyl groups are unstable. The presence of *t*-alkyl groups confers some stability (Williams, 1988).

We set out to extend the range of the *S*-nitrosothiols in order to learn more about them. We knew from the literature that they are unstable for several reasons and we will discuss those later in this chapter. The main problem with these drugs is the lack of a suitable procedure for their purification due to their nature. They are usually contaminated by small amounts of the corresponding thiols (McAninly *et al.*, 1993) and, in some cases, by the corresponding disulphides (Park, 1988).

From previous work, we knew that the *S*-nitrosated amino acid SNAP is quite stable (Field *et al.*, 1978). We thought that if we built up a series of *S*-nitrosated dipeptides they may be more stable than SNAP and less stable than the *S*-nitrosated tripeptide GSNO; (Hart, 1985), and that was one aim of our work.

We followed a systematic design for the series by keeping the parent unit for all of them fixed and changing only one part of the drug in order to make it more lipophilic. We thought it might be of value to prepare a series of *S*-nitrosated dipeptides in which one component is kept constant as *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteine and the other is varied to give changes in structure without altering the immediate chemical vicinity of the -SNO group. In this section we report the synthesis of this series of compounds and compare their chemical stability, in the presence and absence of copper ions, with that of SNAP and GSNO. Full details of the way in which copper ions bring about NO release from *S*-nitrosothiols are given in the later sections.

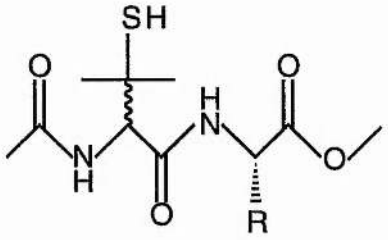
In the present work we describe the preparation and the characterisation of new *S*-nitrosothiols. We have studied their stabilities in aqueous solutions and shown that they spontaneously generate nitric oxide at physiological pH in the presence of copper ions.

The compounds described here might be convenient and useful as drugs for spontaneous generation of nitric oxide in biological systems, at rates that can be finely tuned and controlled over a wide range.

2. 1. 1. Design and Synthesis of a Number of Dipeptides Where One Amino Acid Contains an -SH Group

The basic reaction involved here is the linking of an amino group in one unit with the carboxyl group of another forming an amide (or peptide) (Jones, 1991).

The -SH containing amino acid used in these studies was *N*-acetyl-D,L-β,β-dimethylcysteine (*N*-acetyl-D,L-penicillamine) and this remained unchanged. The other amino acid was an ester to make the product more lipophilic and to simplify the coupling procedures. The general formula of product is as follows:



We thought that the structure of the R group of the dipeptide would play a significant role in determining the biological activity and the chemical stability of the corresponding *S*-nitrosothiol. Once this dependence has been delineated, it should be possible to predict the drug action of other nitrosated dipeptides.

Carbodiimides have been the most important reagent for activating carboxy groups in peptide synthesis ever since Sheehan and Hess reported their results in 1955 (Sheehan and Hess, 1955).

There are two types of carbodiimide: (a) water insoluble carbodiimides; and (b) water soluble carbodiimides (Mackie *et al.*, 1992).

The advantages of the carbodiimide method are (see Scheme 2.2):

(i) good yields in a short reaction time, and

(ii) low racemisation when Z = $(CH_3)_3COCO$ or $PhCH_2OCO$.

Disadvantages include the following (see Scheme 2. 2):

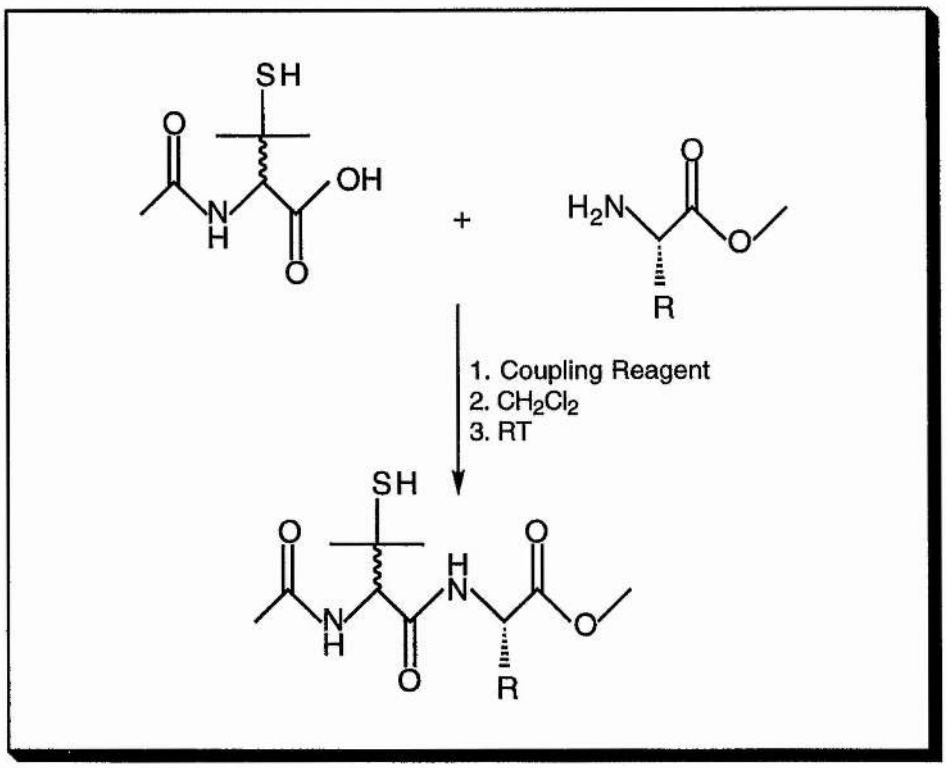
(i) racemisation if Z is an amino acid residue,

(ii) contamination of the product with the carbodiimide (water insoluble carbodiimide) which is difficult to remove, and

(iii) reaction of activated ester with the *N*-protected amino acid to give an anhydride which may be difficult to separate from the peptide.

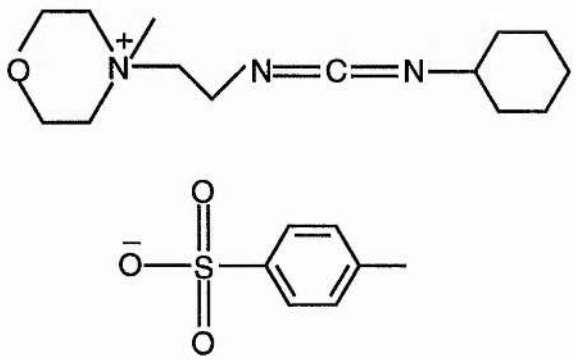
This difficulty can be circumvented by the application of water soluble carbodiimides since they give rise to water soluble urea derivatives.

With the use of *N*-cyclohexyl-*N'*-2-[*N*-morpholino]ethylcarbodiimide metho-*p*-toluenesulphonate (water soluble carbodiimide), *N*-acetyl-D,L-β,β-dimethylcysteine was coupled with glycine methyl ester hydrochloride, L-alanine methyl ester hydrochloride, L-valine methyl ester hydrochloride, L-leucine methyl ester hydrochloride, L-phenylalanine methyl ester hydrochloride, L-isoleucine methyl ester hydrochloride, L-methionine methyl ester hydrochloride, N_ε-CBZ-L-lysine methyl ester hydrochloride, L-proline methyl ester hydrochloride, L-aspartic acid dimethyl ester or L-glutamic acid dimethyl ester in one step. The solvent used was purified methylene chloride at room temperature (Scheme 2. 1).



Scheme 2. 1: The coupling reaction between protected amino acid and amino acid ester to form the corresponding dipeptide.

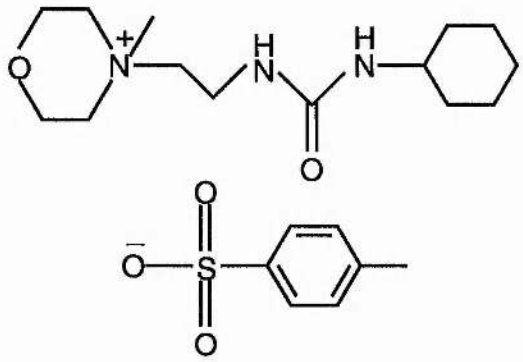
We found it was not necessary to protect the thiol group due presumably to the steric effect of the two β-methyl groups. Since the coupling reagent and its reaction product are both neutral, the synthesis can take place in the presence of acid- and base- sensitive groups in the reactants. The structure of the coupling reagent is:



N-cyclohexyl-*N'*-2-[*N*-morpholino]ethylcarbodiimide metho-*p*-toluenesulphonate

Activation of the carboxyl group occurs through its addition to the N=C double bond in the carbodiimide. The mechanism of the reaction is shown in Scheme 2. 2 .

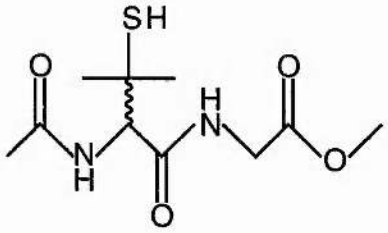
The other product of the coupling reaction is a urea derivative which is insoluble in most organic solvents and is readily removed from the reaction mixtures by filtration. Unreacted carbodiimide was extracted with water (Bodamszky, 1993):



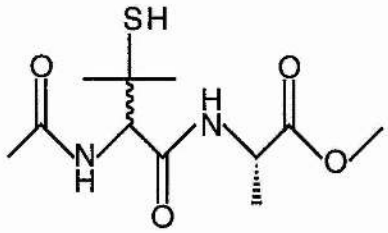
N-cyclohexyl-*N'*-2-[*N*-morpholino]ethylurea metho-*p*-toluenesulphonate

The desired dipeptides were formed in moderate yields (~ 25%).

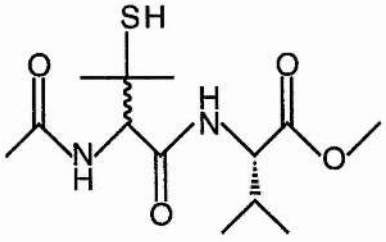
I. *N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester:



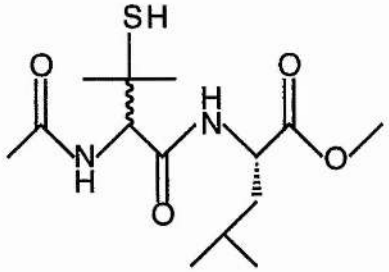
II. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-alanine methyl ester:



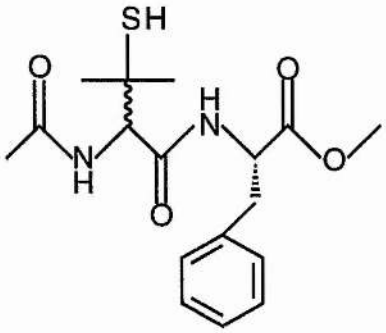
III. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-valine methyl ester:



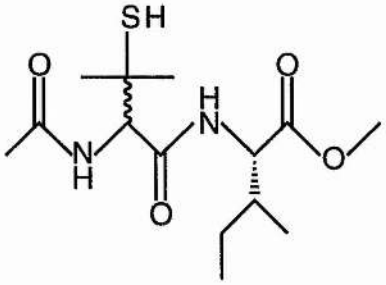
IV. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-leucine methyl ester:

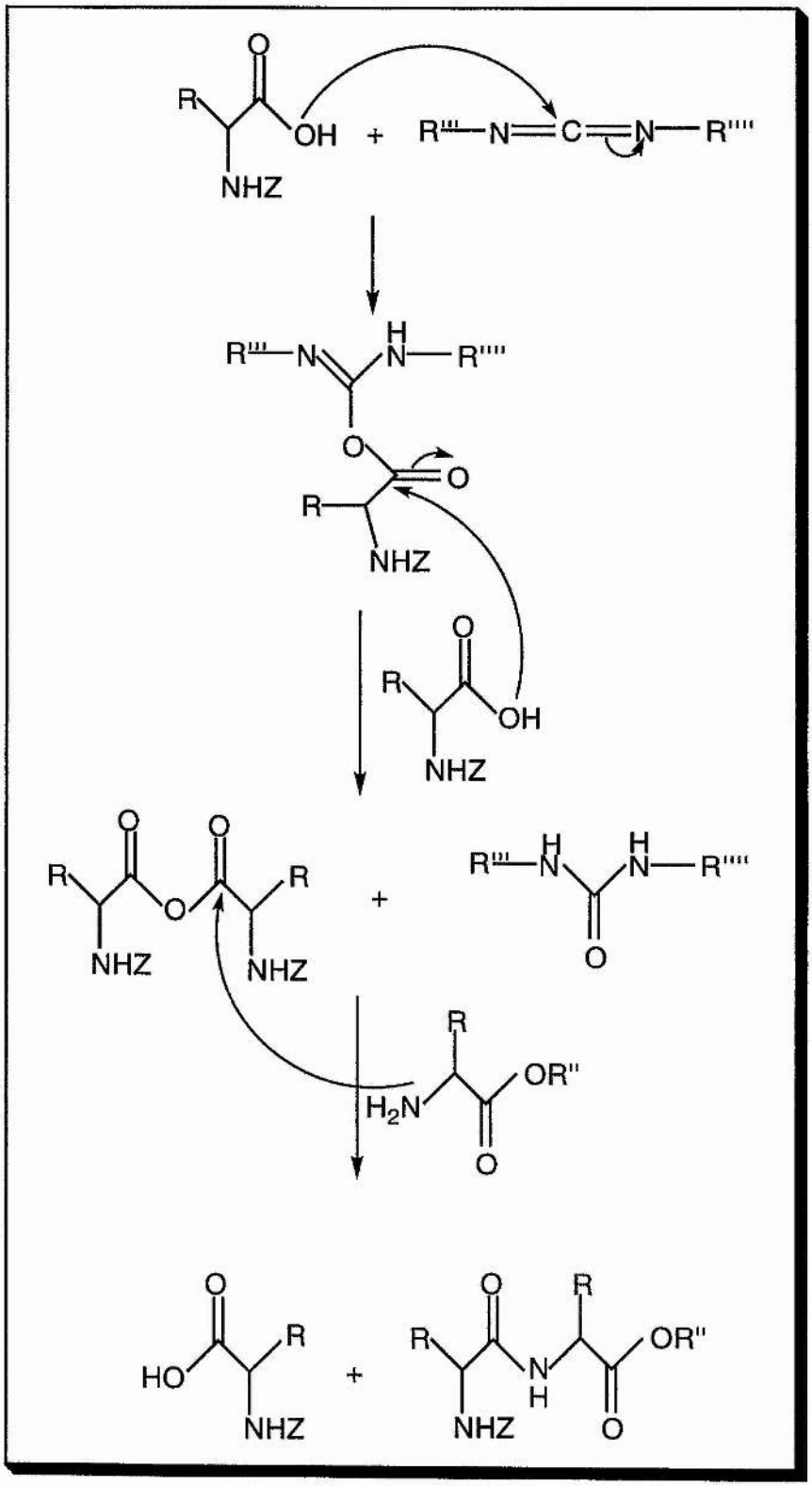


V. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-phenylalanine methyl ester:



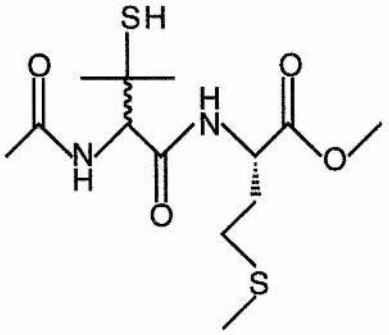
VI. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-isoleucine methyl ester:



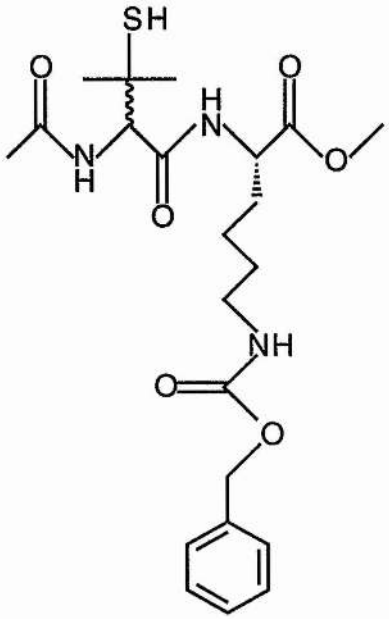


Scheme 2. 2: The mechanism of peptide bond formation using a coupling agent.

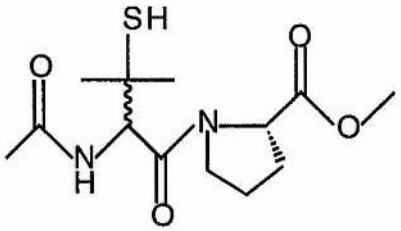
VII. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-methionine methyl ester:



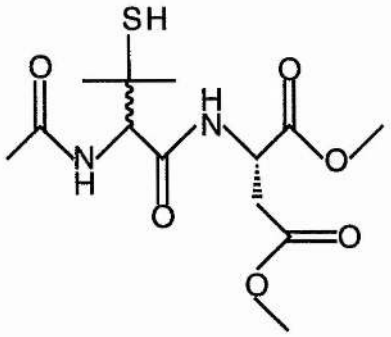
VIII. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-N_ε-CBZ-L-lysine methyl ester:



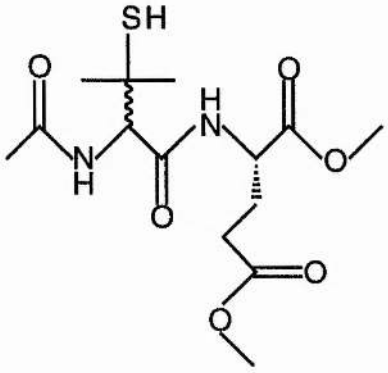
IX. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-proline methyl ester:



X. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-aspartic acid dimethyl ester:



XI. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-glutamic acid dimethyl ester:



The dipeptides were characterised by a variety of criteria, including elemental analysis, IR, ^{1}H-, ^{13}C, DEPT 90° and DEPT 135°-NMR spectroscopy. Accurate mass determinations of the molecular ion peaks were obtained only by the use of high resolution FAB-MS but not by other techniques. Data are displayed in the Experimental Section (next chapter).

2. 1. 2. Synthesis of *S*-Nitrosothiols from the Corresponding Dipeptides

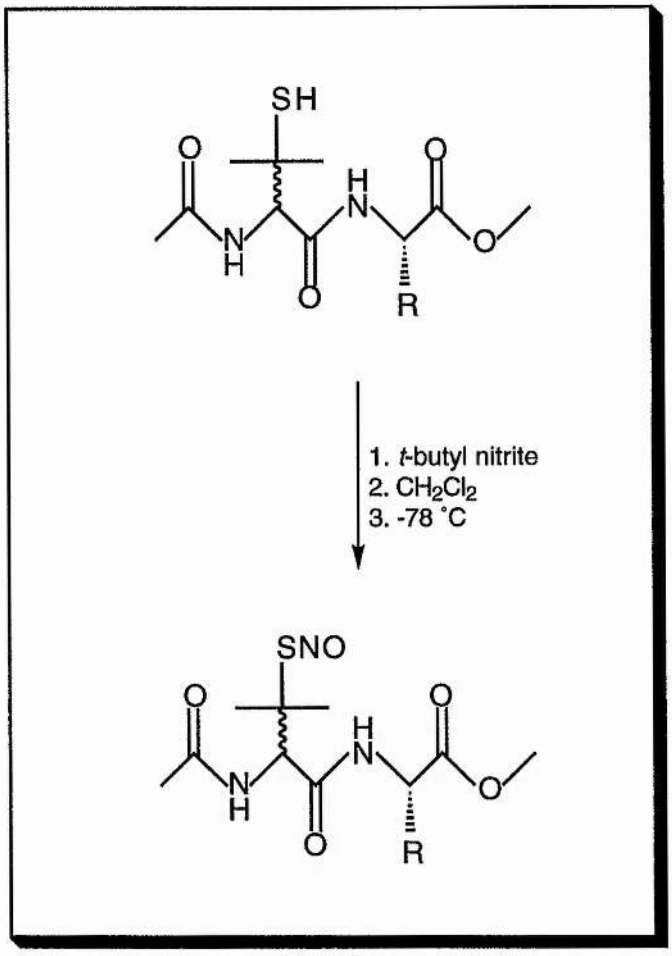
S-Nitrosothiols are now recognised as an important class of nitric oxide (NO) donor drugs as they decompose to give NO and the corresponding disulphide only:

$$RSNO \longrightarrow 1/2\ RSSR + NO$$

If the RS- moiety is a thiol-containing amino acid, *S*-nitrosothiols should have therapeutic potential as the disulphide produced on NO release should have no harmful side effects.

The major requirements for a good NO generator are the following: first, the compound has to be easy to prepare in a stable and pure form, preferably as a solid; second, it has to release NO in aqueous solution, preferably without the need for redox activation; third, it has to generate NO quantitatively at a predictable rate; and fourth, the by-products should be nontoxic. Such compounds are rare.

S-Nitrosothiols are prepared by nitrosation of thiols and no purification procedure is possible because of the ease of decomposition. *S*-Nitrosothiols have been previously prepared by several methods, including reaction of thiols with nitrous acid under acidic conditions, and with dinitrogen tetroxide in carbon tetrachloride or ether (Williams, 1988). The nitrous acid method has limited applicability, and the use of dinitrogen tetroxide is complicated by the requirement for controlled stoichiometry and low reaction temperatures (Williams, 1988). Use of *t*-butyl nitrite was expected to be advantageous for the synthesis of a broad range of *S*-nitrosothiols without the restrictions normally encountered with the use of dinitrogen tetroxide (Doyle *et al.*, 1983). *t*-Butyl nitrite has the requisite chemical and physical properties of a useful synthetic reagent. It has a relatively low boiling point, and the corresponding alcohol can be easily removed from higher boiling or water-insoluble products (Doyle *et al.*, 1983). Doyle *et al.* report that *S*-nitrosothiols were conveniently formed by rapid nitrosyl exchange reactions with *t*-butyl nitrite (Doyle *et al.*, 1983). In our work, *t*-butyl nitrite was used to form *S*-nitrosothiols from the corresponding dipeptides according to the following Scheme (Scheme 2. 3):



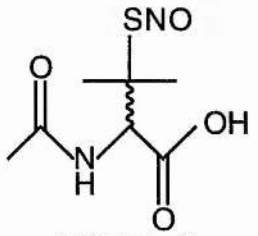
Scheme 2. 3: The nitrosation reaction of the dipeptides to the corresponding *S*-nitrosated dipeptides.

The dipeptides were converted into the corresponding *S*-nitrosothiols (**2-12**) in high yields (more than 80%) by this procedure described in detail in the Experimental Section to give solids or sticky solid (Butler *et al.*, 1996). Compound **1**, **2**, **4**, **7**, **10**, **11**, and **13** were obtained in a pure state but the others (**3**, **5**, **6**, **8**, **9**, and **12**) were contaminated by small amounts of the unreacted thiol and/or by small amounts of the disulphide formed during nitrosation (see element analysis; Experimental Section).

For comparison purposes we also prepared *S*-nitrosated *N*-acetyl-D,L-β,β-dimethylcysteine (SNAP) and glutathione (GSNO).

(a) *S*-Nitrosated Amino-acid

S-Nitroso-*N*-acetyl-D,L-β,β-dimethylcysteine (SNAP; **1**) was synthesised, characterised and its X-ray crystal structure obtained by Field *et al.* in 1978. In the crystalline state, it is the most stable *S*-nitrosothiol known to date, although in solution it decomposes to release NO and form its disulphide. It has been shown to be a potent relaxation agent for vascular smooth muscle and consequently a fast acting vasodilator (Ignarro *et al.*, 1981a&b). Along with other *S*-nitrosothiols, it possesses anticoagulant properties, as it inhibits the aggregation of blood platelets and their adhesion to the sub-endothelium (Radomski *et al.*, 1992). In this work SNAP was synthesised as described by Doyle *et al.* in 1983:

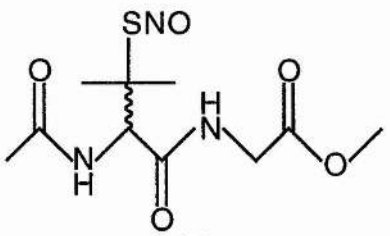


(SNAP; **1**)

(b) *S*-Nitrosated Dipeptides

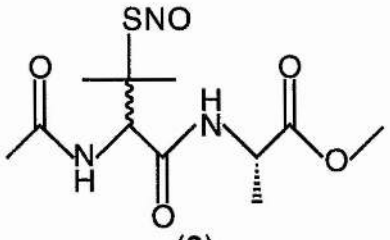
The following *S*-nitrosated dipeptides were prepared by reaction of the thiol with *t*-butyl nitrite:

I. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester (**2**):



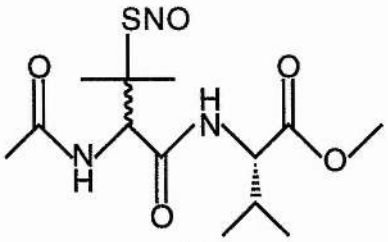
(**2**)

II. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-alanine methyl ester (**3**):



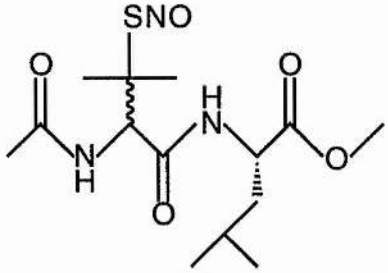
(**3**)

III. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-valine methyl ester (**4**):



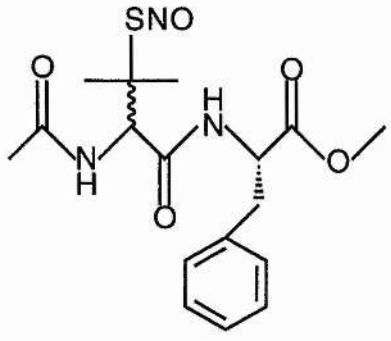
(**4**)

IV. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-leucine methyl ester (**5**):



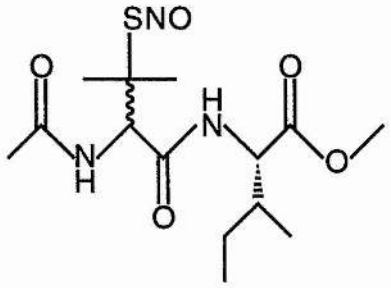
(**5**)

V. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-phenylalanine methyl ester (**6**):



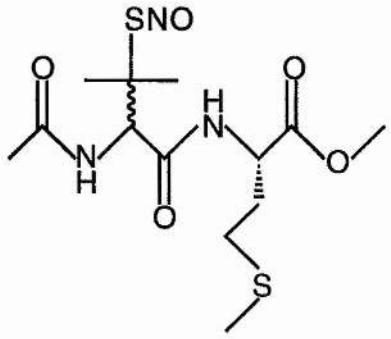
(**6**)

VI. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-isoleucine methyl ester (**7**):



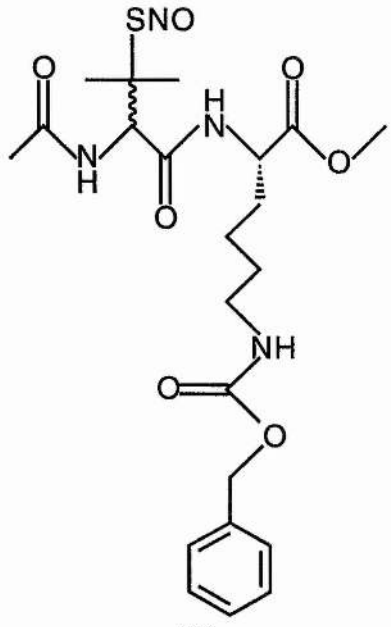
(**7**)

VII. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-methionine methyl ester (**8**):



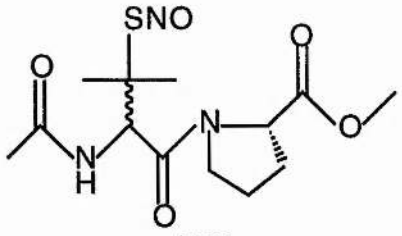
(**8**)

VIII. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-N_{ε}-CBZ-L-lysine methyl ester (**9**):



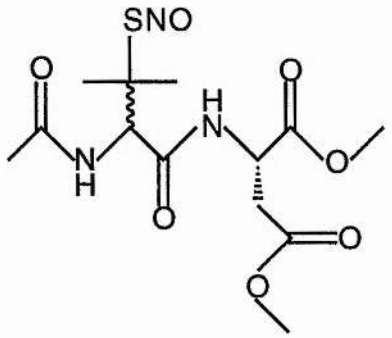
(**9**)

IX. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-proline methyl ester (**10**):



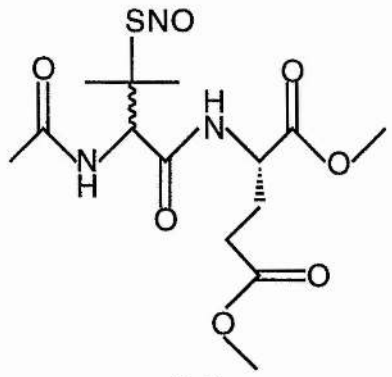
(**10**)

X. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-aspartic acid dimethyl ester (**11**):



(**11**)

XI. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-glutamic acid dimethyl ester (**12**):



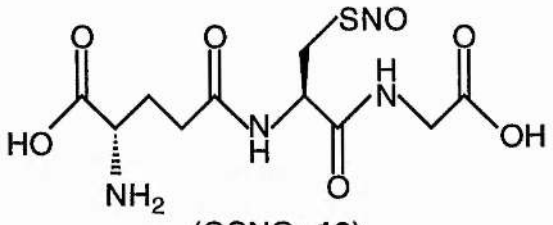
(**12**)

(c) *S*-Nitrosated Tripeptide

S-Nitroso-L-glutathione (GSNO; **13**) was first synthesised and characterised in 1985 by Hart using a modification of a method designed by Saville (1958). GSNO is a derivative of the tripeptide L-glutathione (L-γ-glutamyl-L-cysteinylglycine), which is found in numerous cellular systems and is considered an essential constituent of all living cells. L-Glutathione is generally the most abundant intracellular non-protein thiol (Meister and Anderson, 1983), with a concentration approaching 2-3 mM in erythrocytes. Consequently, the discovery of EDRF and the suggestion that it is an *S*-nitrosothiol, together with evidence that the physiological half-life of NO can be stabilised by thiols (Ignarro *et al.*, 1980a,b&c; Stamler *et al.*, 1992a,b,c,d&e; Feelisch *et al.*, 1994), raises the possibility that *S*-nitrosoglutathione could be the most important *S*-nitrosothiol synthesised *in vivo* and involved in physiological processes.

Since 1985, GSNO has been synthesised by other researchers using different methods (Park and Means, 1989; Clancy and Abramson, 1992).

GSNO has been shown to be a hypotensive drug as effective as sodium nitroprusside (Means and Park, US patent 1990) and a potent inhibitor of platelet aggregation (Radomski *et al.*, 1992). Along with SNAP, it is one of the few stable *S*-nitrosothiols in the crystalline state, and GSNO kept at 4 °C or below is stable over a number of months (Park and Means, 1989; Butler *et al.*, 1996). In this work GSNO was prepared as described in the literature by Hart in 1985.



(GSNO; **13**)

S-Nitrosothiols **1**, **2**, **4**, **7**, **11**, and **13** were obtained as stable green solids in the pure form. *S*-Nitrosothiols **3**, **5**, **6**, and **12** were also green solids and were obtained together with a small amount of the starting material (the corresponding thiols) and/or the corresponding disulphide. *S*-Nitrosothiol **10** was obtained as a green sticky solid in the pure form. *S*-Nitrosothiols **8** and **9** were obtained as green sticky solids, and were contaminated with a small amount of the starting material (the corresponding thiols) and/or the corresponding disulphide.

Dipeptides and their derivatives are sometimes difficult to characterise by elemental analysis as small amounts of the disulphide or thiol are present and we were unable to remove these impurities. In most instances FAB-MS was used to obtain a molecular ion peak and this gave the required characterisation, which was confirmed by a close examination of the ^{13}C-NMR spectrum to ensure that it contained no additional, unassigned peaks.

In order to see how *S*-nitrosothiols might bind to metal ions, which is the main factor affecting their stability and perhaps their biological activity, a representation of the three-dimensional structure was produced using the computer programme Chem 3D and is shown in Figure 2. 1.

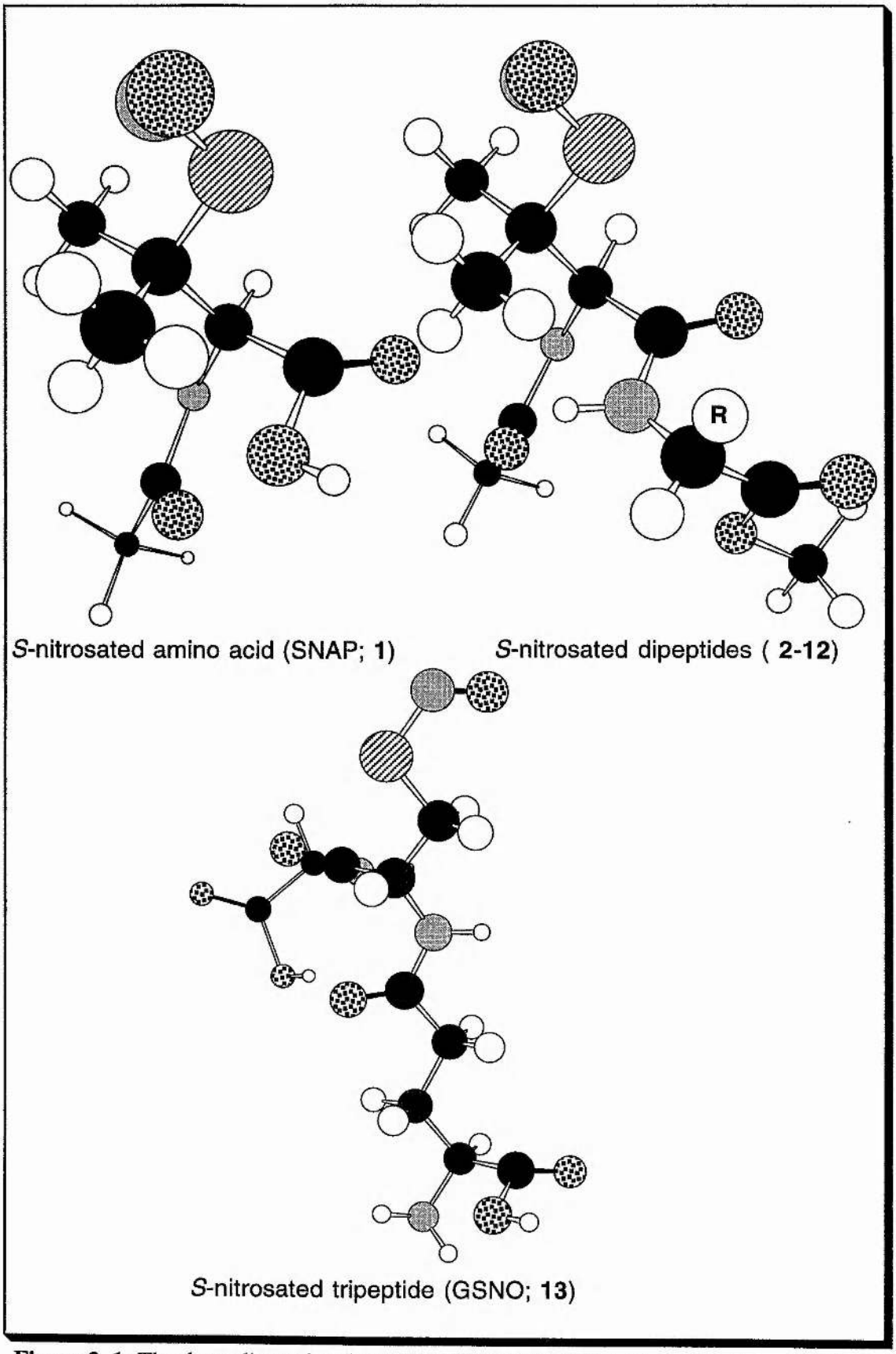


Figure 2. 1: The three-dimensional nature of SNAP (**1**), *S*-nitrosated dipeptides (**2-12**), and GSNO (**13**).

2. 1. 3. Characterisation of *S*-Nitrosothiols

The presence of the -SNO group in a molecule can be readily determined from both infrared and UV-visible spectroscopy.

The broad and strong IR band at 1480-1530 cm^{-1} has been assigned to the stretching vibration of the N=O bond of the *S*-nitrosothiol. N=O vibrations of tertiary *S*-nitrosothiols have been found at frequencies lower than those of primary *S*-nitrosothiols. A second absorption band at 600-730 cm^{-1} is characteristic of the vibration of the C-S bond (see Figure 2. 2).

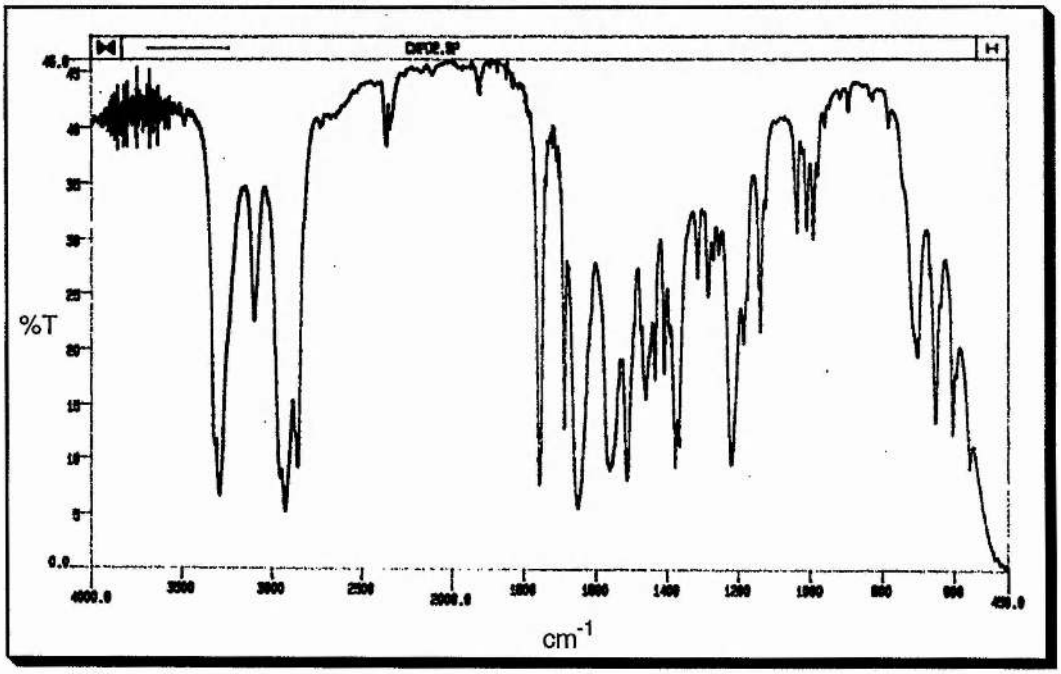


Figure 2. 2: Infrared Fourier Transform spectrum of compound **2**.

The UV-visible spectra of the *S*-nitrosothiols display two bands at around 330-340 nm and 550-600 nm, which are responsible for their red or green colour in solution. These bands are shifted to lower energies by substitution at the α carbon atom. Tertiary *S*-nitrosothiols are usually green compounds, whereas primary and secondary *S*-nitrosothiols are red (see Figure 2. 3).

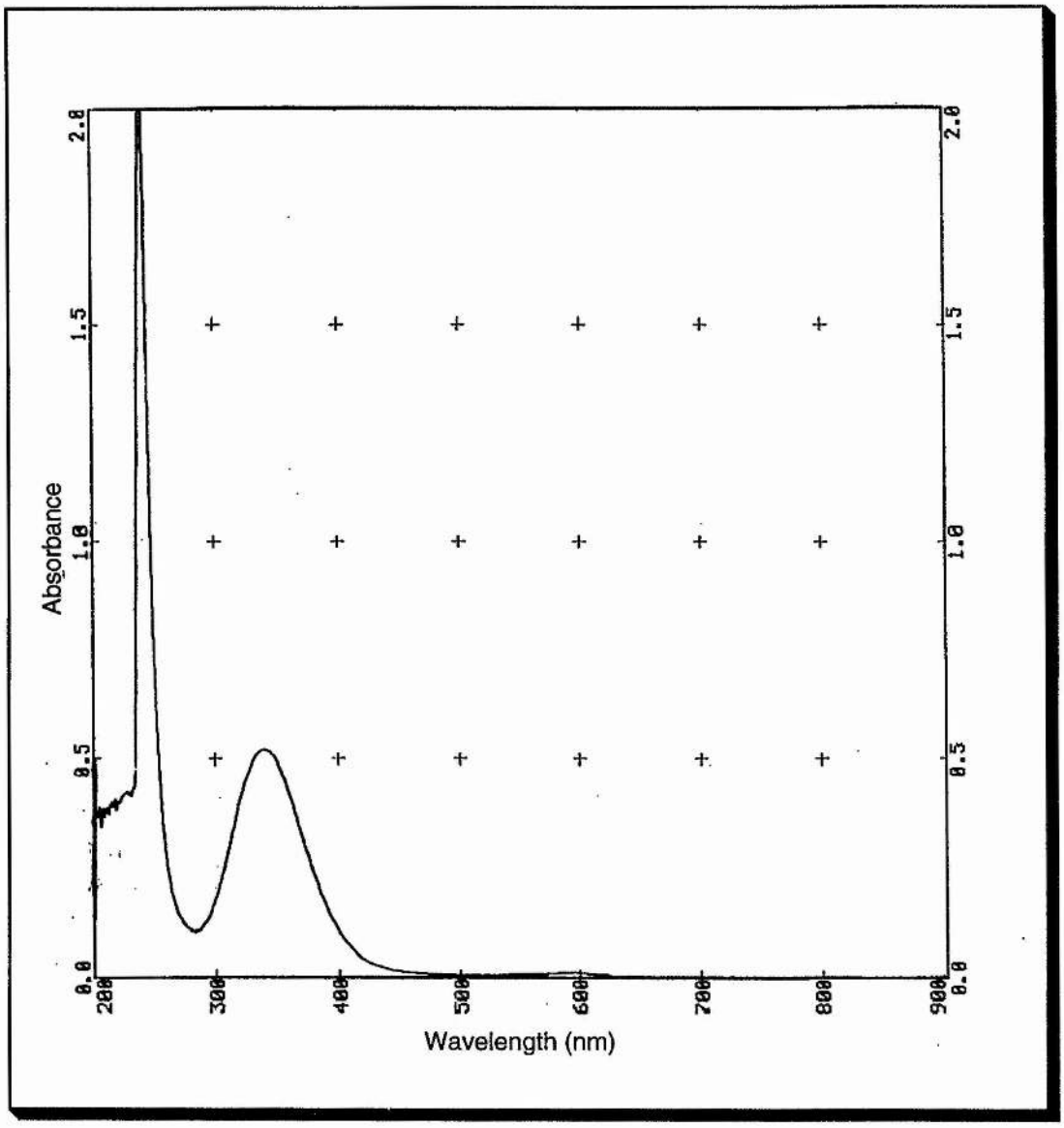


Figure 2. 3: UV-visible spectrum of compound **2** (5 x 10^{-4} mol dm^{-3}).

From the literature sources, only few a *S*-nitrosothiols have been previously characterised by ^{1}H or ^{13}C-NMR spectroscopy. Both methods allowed a very convenient and reliable proof for nitrosation of thiol compounds. While thiols and disulphides had very similar ^{1}H-NMR spectra, the α protons in the *S*-nitrosothiols were strongly shifted downfield (about 1ppm) (Roy *et al.*, 1994). The β protons were also affected by the *S*-nitrosation, to an extent that was dependent on the substitution at the α carbon. Almost no shift was observed for the primary *S*-nitrosothiols, about 0.1 ppm for the secondary *S*-nitrosothiols and about 0.5-0.8 ppm for the tertiary *S*-nitrosothiols (Roy *et al.*, 1994).

The ^{13}C-NMR spectra were also changed by nitrosation of thiols. Resonances of α carbon atoms were shifted downfield, with stronger shifts observed for tertiary *S*-nitrosothiols and resonances of β-carbon atoms were shifted upfield. The ^{13}C-NMR chemical shifts for *S*-nitrosothiols were in general found to be intermediate between those of the corresponding thiols and those of the disulphide (Roy *et al.*, 1994).

As the *N*-acetyl-D,L-β,β-dimethylcysteine used was a racemic mixture the products were formed as a mixture of two diastereomers: e.g. *N*-acetyl-D-β,β-dimethylcysteinyl-L-amino acid methyl ester, and *N*-acetyl-L-β,β-dimethylcysteinyl-L-amino acid methyl ester. Due to this the compounds have complicated ^{1}H-NMR spectra in most cases, but we were able to identify the -S<u>H</u>, CH3CON<u>H</u>, and the proton of the peptide bond (-CON<u>H</u>-) which indicate that we have the correct dipeptide. After the nitrosation of the dipeptides, there are still two diastereomers: *S*-nitroso-*N*-acetyl-D-β,β-dimethylcysteinyl-L-amino acid methyl ester, and *S*-nitroso-*N*-acetyl-L-β,β-dimethylcysteinyl-L-amino acid methyl ester, and it was possible to identify the CH3CON<u>H</u>- and -CON<u>H</u>- signals and the disappearance of -S<u>H</u> which indicates that nitrosation has occurred. Some decomposition occurred while the spectra were being run.

In ^{13}C-NMR for both the thiols and the *S*-nitrosothiols there are two lines for each carbon due to the presence of the two diastereomers and usually DEPT 90° and DEPT 135° enabled <u>C</u>H3-, -<u>C</u>H2-, and -<u>C</u>H- to be distinguished.

In the case of compound **2** the two isomers formed are enantiomers rather than diastereomers. Accordingly only one set of lines is seen in the ^{13}C-NMR and the proton spectrum is also simpler.

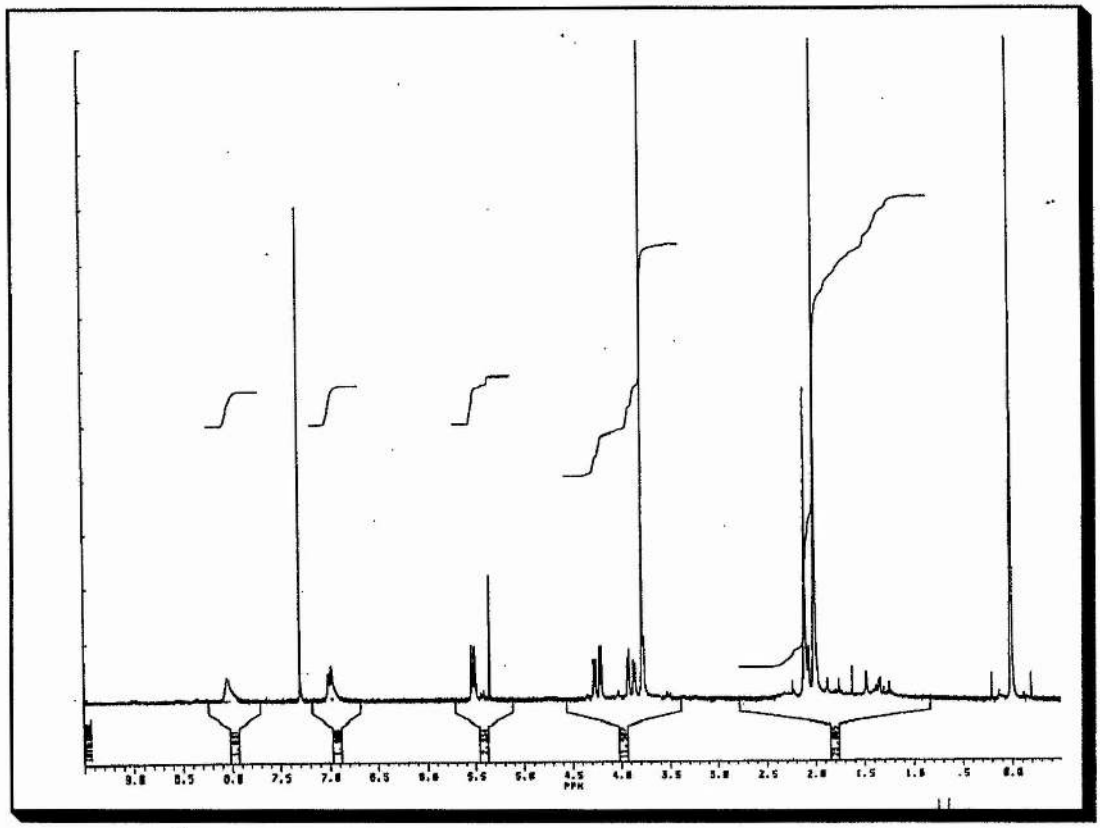


Figure 2. 4: ^{1}H-NMR spectrum of compound **2**.

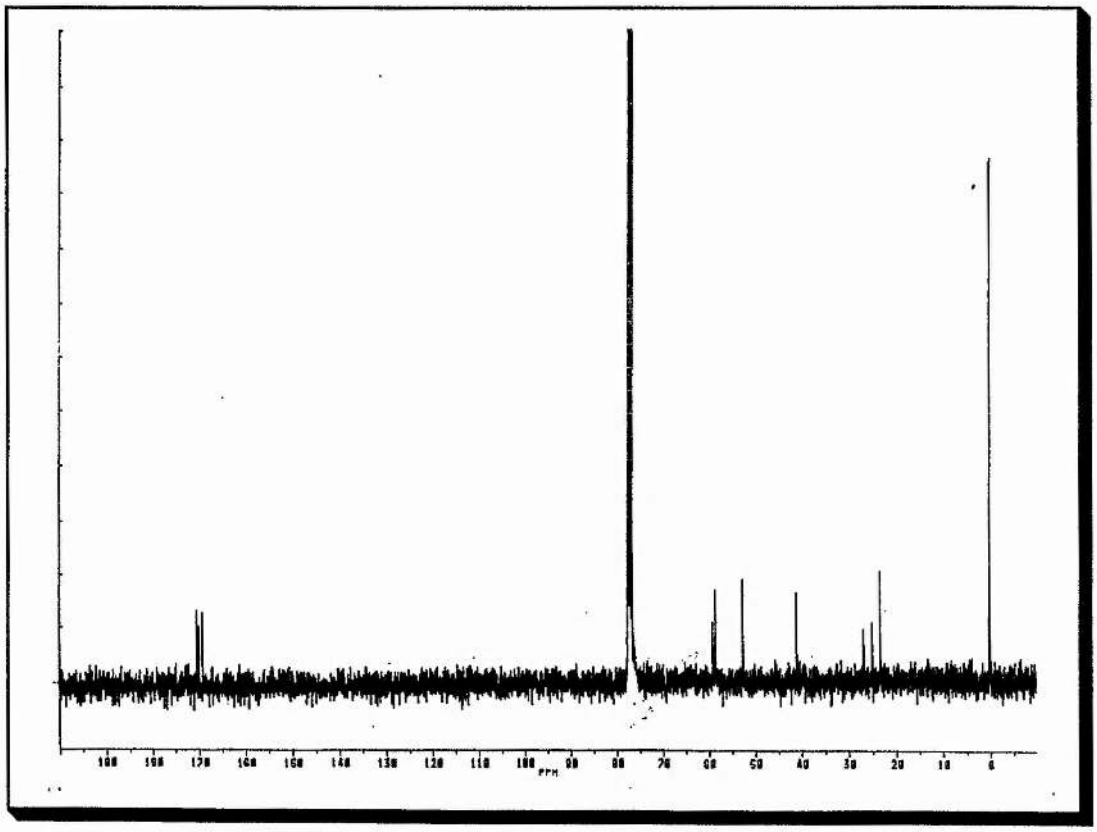


Figure 2. 5: ^{13}C-NMR spectrum of compound **2**.

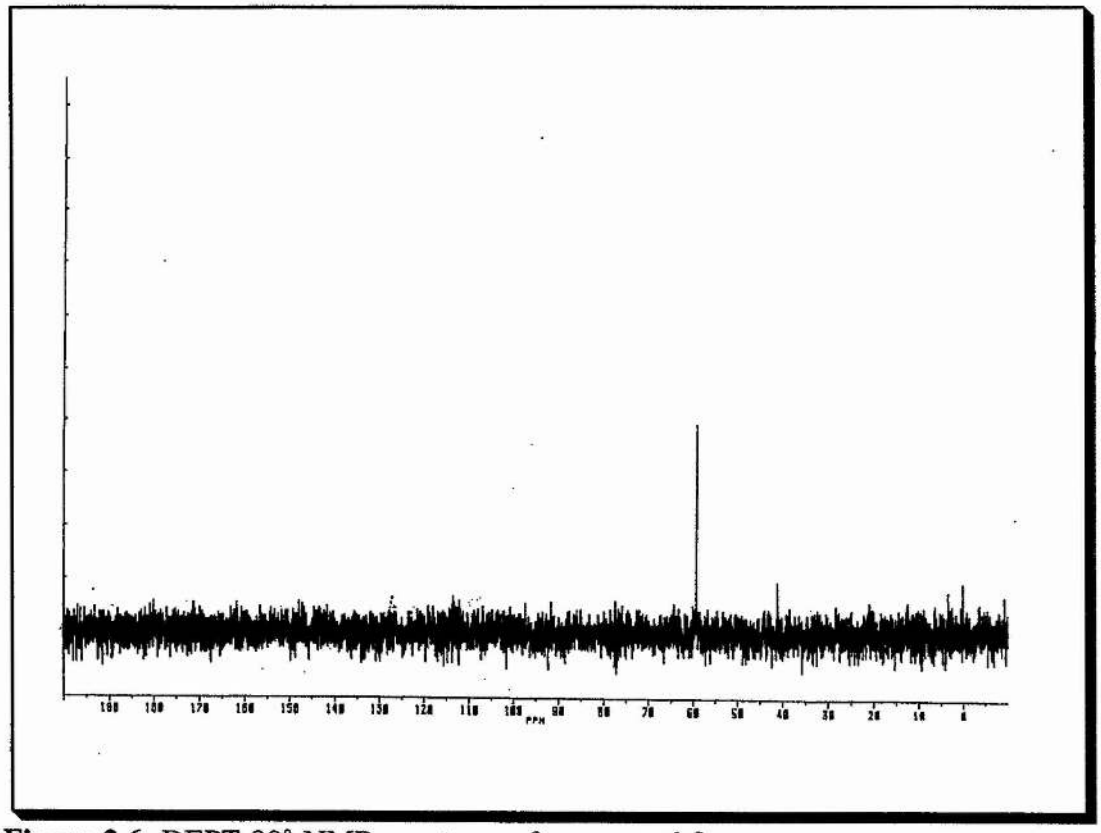


Figure 2.6: DEPT 90°-NMR spectrum of compound **2.**

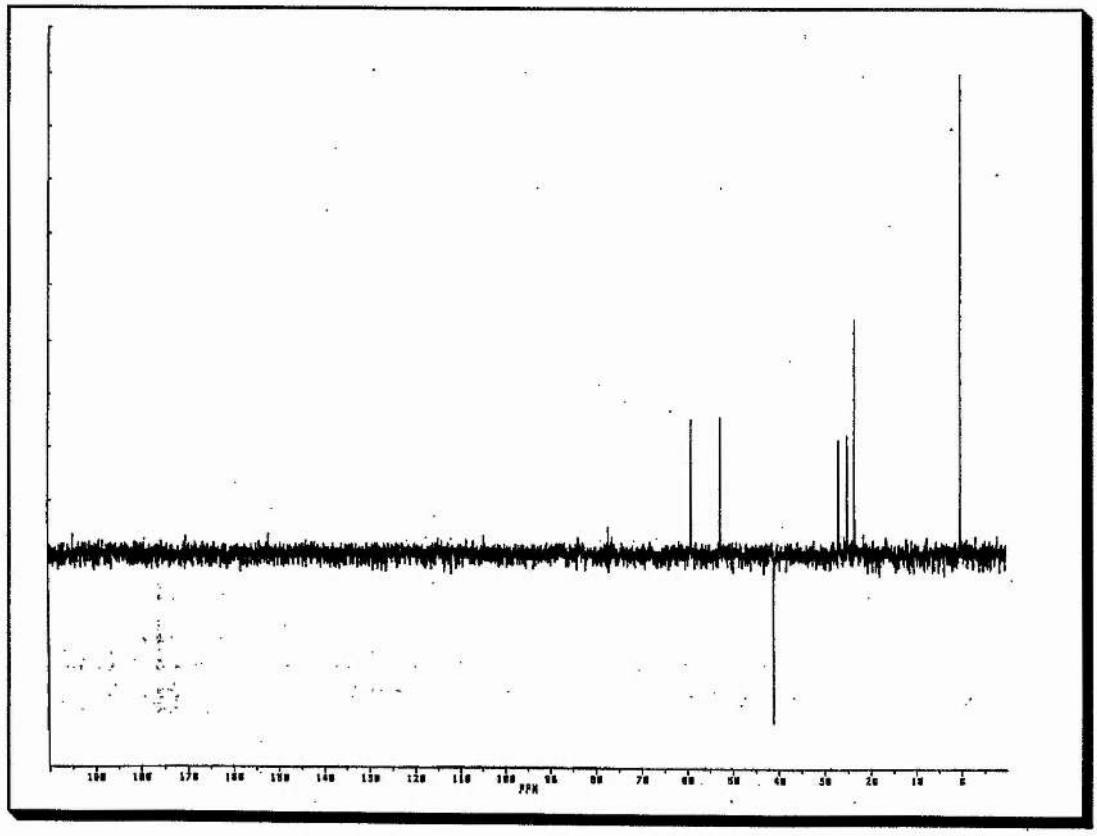


Figure 2. 7: DEPT 135°-NMR spectrum of compound **2.**

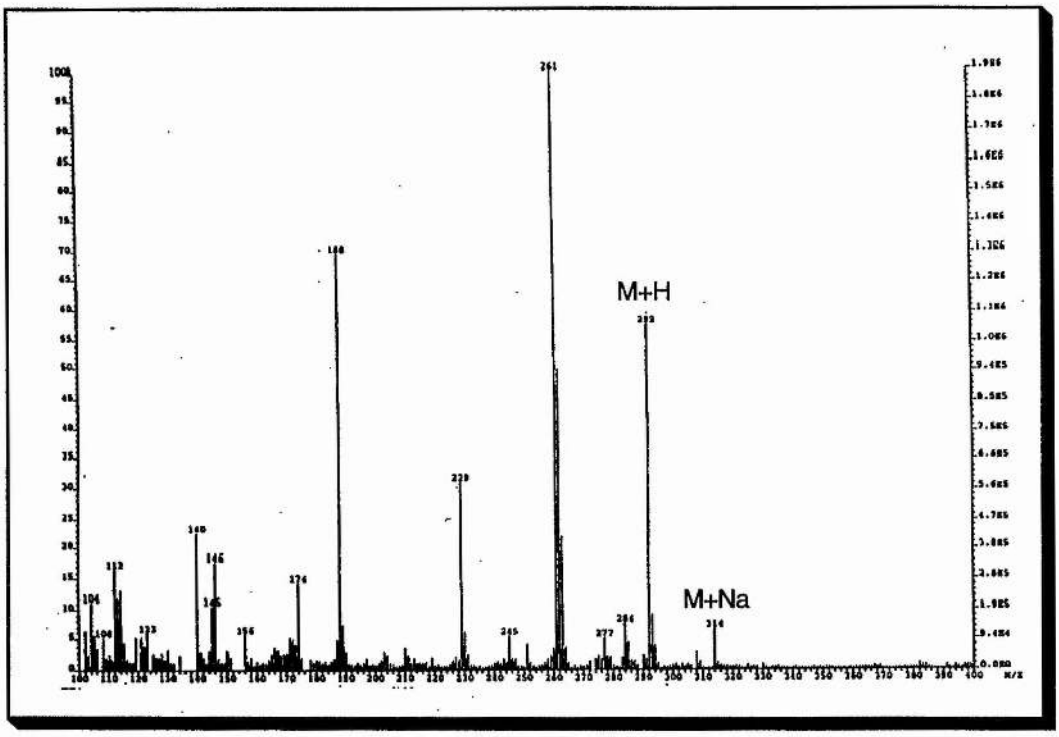


Figure 2. 8: FAB-MS spectrum of compound **2.**

Compound **2** is pure but the others are contaminated by small amounts of the corresponding thiol and/or disulphide. This may be due to steric hindrance by the R group in the other compounds interfering with the nitrosation reaction.

Microanalysis of the green solid or green sticky solid obtained from some *S*-nitrosothiols showed that some disulphide had formed in its isolation as indicated by the high carbon and low nitrogen values (see elemental analyses; Experimental).

Samples of *S*-nitrosothiols were characterised by a variety of criteria, including elemental analysis, IR (e.g. Figure 2. 2), UV-visible (e.g. Figure 2. 3), ^{1}H-(e.g. Figure 2. 4), ^{13}C-(e.g. Figure 2. 5), DEPT 90° (e.g. Figure 2. 6), and DEPT 135°-NMR spectroscopy (e.g. Figure 2. 7). Accurate mass determinations of the molecular ion peaks were obtained only by the use of high resolution FAB-MS but not by other techniques (e.g. Figure 2. 8). Data are displayed in the Experimental Section (next chapter).

A chemical and physiological comparison of the action of (a) *S*-nitrosated amino acid (SNAP; **1**) (b) *S*-nitrosated dipeptides (**2-12**) and (c) *S*-nitrosated tripeptide (GSNO; **13**) is the subject of a large proportion of this thesis. Data have been compiled which give an insight into why these three types of *S*-nitrosothiols (**1-13**) act differently and how *S*-nitrosothiols might be developed in the future to improve their pharmacokinetic profiles.

2. 2. Mechanistic Study of the Decomposition of *S*-Nitrosothiols

S-Nitrosothiols are endothelium-independent vasodilators which are susceptible to decomposition by a number of mechanisms. The rates of decomposition of *S*-nitrosothiols *in vitro* have been shown to be influenced by:

I. Metal ions,

II. Transnitrosation (NO transfer from *S*-nitrosothiols to other thiols),

III. Enzymatic decomposition of *S*-nitrosothiols,

IV. Photochemical decomposition, and

V. Thermal decomposition.

The extent to which each of these different mechanisms causes decomposition of *S*-nitrosothiols *in vivo* is difficult to ascertain quantitatively. In this thesis we are concerned, almost exclusively, with the effect of metal ions, and mention only briefly the other four mechanisms.

2. 2. 1. Metal Ions Catalysed Release of NO from *S*-Nitrosothiols

S-Nitrosothiols are decomposed by metal ions to the corresponding disulphide and nitric oxide. A survey of commonly occurring-metals showed that copper is the most effective catalyst. It enhanced the rate of decomposition even at concentrations as low as 10^{-6} mol dm^{-3}.

2. 2. 1. 1. The Role of Copper Ions in the *in vitro* and *in vivo* Release of NO from *S*-Nitrosothiols

The work (Section 2. 2. 1. 1) was carried out in close collaboration with a group at the University of Durham (Prof. D. L. H. Williams, A. P. Dicks and H. R. Swift). Some of the results reported are from the Durham group as they are key observations in the elucidation of this rather complex mechanism. The computer simulations were carried out by Dr B. G. Cox of Zeneca Fine Chemicals Manufacturing Organisation in Huddersfield, to whom we extend grateful thanks.

Decomposition of *S*-nitrosothiols in aqueous solution at pH 7.4 is brought about by copper ions, either present as an impurity or specifically added. The primary products are nitric oxide and the disulphide.

2. 2. 1. 1. 1. The First Report of the Role of Copper Ions in the Release of NO from *S*-Nitrosothiols

At first quantitative rate measurements of RSNO decomposition reactions in aqueous solution yielded erratic results, with reported half-lives of reaction varying considerably using the same substrate, usually SNAP. Further, the rate-form reported varied widely, zero-, first-, second- and various intermediate orders having been reported at some stage. This picture was resolved when it was realised that reaction occurred by a Cu^{2+}-catalysed reaction pathway (Figures 2. 9, 2. 10, and 2. 11), and that for some reactants there can be enough Cu^{2+} in the distilled water/buffer components used, to bring about reaction. The $[Cu^{2+}]$ varied from source to source and often daily within the same source which goes some way to explaining the erratic nature of the reported results.

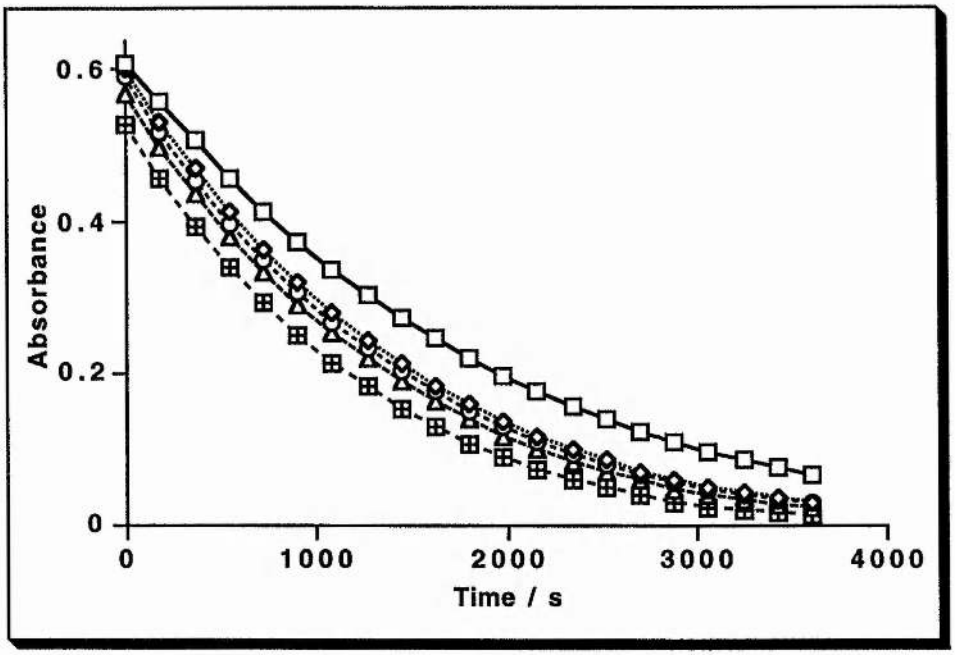


Figure 2. 9: Absorbance time plots for the decomposition of SNAP (**1**) (5 x 10^{-4} mol dm^{-3}) (a) $[Cu^{2+}]$ 2.7 x 10^{-6} (square) (b) $[Cu^{2+}]$ 5.4 x 10^{-6} (diamond) (c) $[Cu^{2+}]$ 8.1 x 10^{-6} (circle) (d) $[Cu^{2+}]$ 10.8 x 10^{-6} (triangle) (e) $[Cu^{2+}]$ 13.5 x 10^{-6} mol dm^{-3} (segment square) at pH = 7.4, 340 nm and 30 °C.

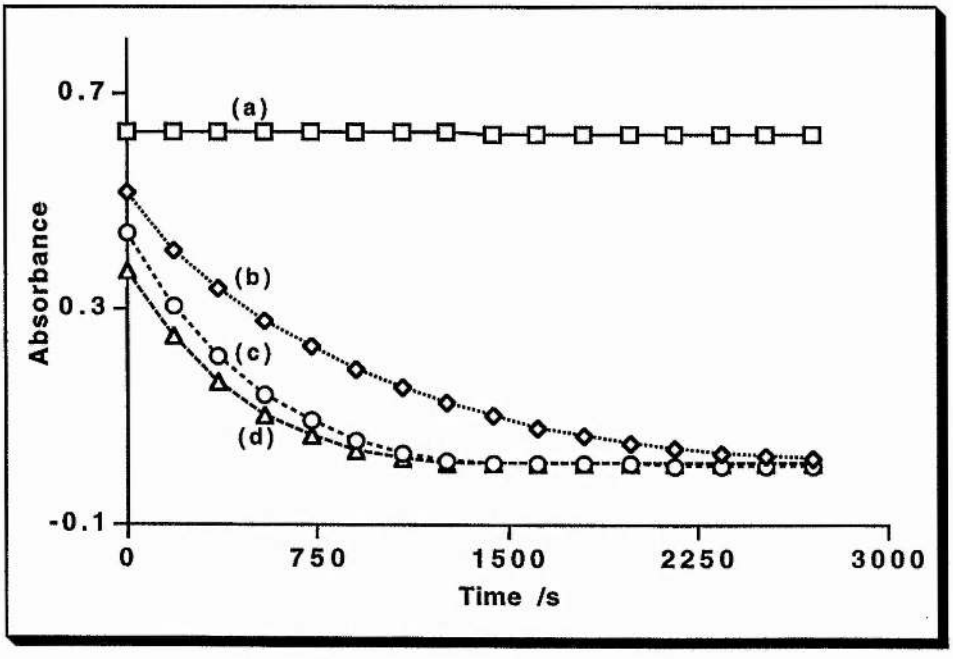


Figure 2. 10: Absorbance time plots for the decomposition of SNAP (**1**) (5 x 10^{-4} mol dm^{-3}) (a) no added Cu^{2+} (b) $[Cu^{2+}]$ 1 x 10^{-5} (c) $[Cu^{2+}]$ 2 x 10^{-5} (d) $[Cu^{2+}]$ 3 x 10^{-5} mol dm^{-3} at pH = 7.4, 340 nm and 30 °C.

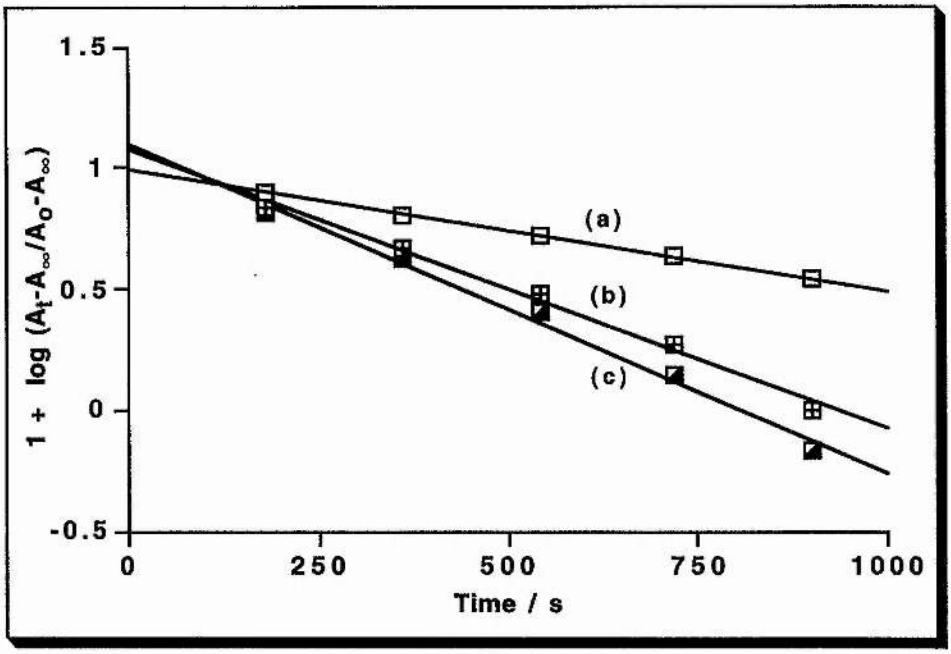


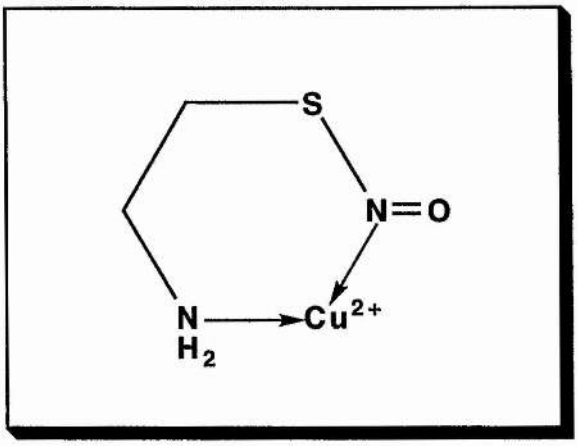
Figure 2. 11: Plots of 1 + log (A_t - A_∞ / A_0 - A_∞) versus time for the decomposition of SNAP (**1**) (a) when [Cu^{2+}] = [EDTA] (b) when 1/2 [Cu^{2+}] = [EDTA] (c) when 1/3 [Cu^{2+}] = [EDTA] at pH = 7.4, 340 nm and 30 °C (r = 1.0, 0.996, and 0.995 respectively).

When Cu^{2+} is removed by complexation with EDTA, virtually no reaction takes place (Scheme 2. 4). There was no catalysis for a range of other metal ions investigated, including Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+}, and Fe^{3+}.

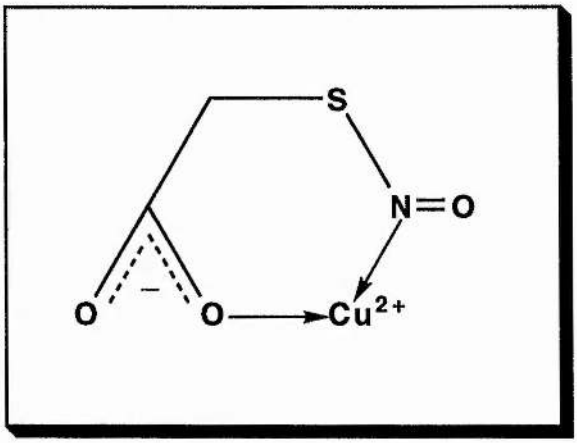
In the earlier work Askew *et al.* reported (1995a&b) that for many RSNO compounds over a given [Cu^{2+}] range (which varied with the substrate), the following equation:

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

applied, often with a small autocatalytic component, which was ignored. The copper is fully regenerated and so is truly catalytic.



Structure 2. 1: The proposed six-membered ring intermediate formed when copper binds to an amine group of the *S*-nitrosothiols.



Structure 2. 2: The proposed six-membered ring intermediate formed when copper binds to a carboxylate group of the *S*-nitrosothiols.

These two binding sites are shown as the nitroso-nitrogen atom in each case and either an amine or carboxylate group. In the absence of either of these features reaction was very slow indeed.

Although a qualitative structure-reactivity pattern was established it was clear that a full mechanistic picture of this reaction had not been described, since outside a given [Cu^{2+}] (which differed for different substrates) other kinetic patterns emerged. In particular, at low [Cu^{2+}] there was an increasing tendency for autocatalysis to be observed and at high [Cu^{2+}] there was a move towards a zero-order dependence upon [RSNO] and also upon [Cu^{2+}].

These rate forms together with a whole range of intermediate situations clearly led to quite a complex set of data. A further unusual feature occurred in the reaction of *N*-acetyl-*S*-nitrosocysteine in that there was a very long induction period (many hours) before reaction set in, with approximately a first-order dependance. This thesis describes in more detail the more unusual kinetics and proposes a mechanism which is consistent with all of the experimental results.

2. 2. 1. 1. 2. Experimental Observations Which Indicate that the True Catalyst is not Cu^{2+}

The complex kinetic behaviour of *S*-nitrosothiols during decomposition has four characteristics: (a) there is no reaction in the absence of copper, (b) in the presence of moderate amounts of copper the reaction may be zero or first order, dependent upon the nature of RSNO, (c) at low copper concentrations the reaction appear to be autocatalytic, and (d) at high copper concentrations the rate becomes independent of the copper concentration. How can these effects be explained?

Ealier work in our laboratories (Askew *et al.*, 1995a&b) concentrated upon kinetic analysis of rate forms, which gave first order dependencies upon both RSNO and Cu^{2+}, in order to establish structure-reactivity factors. We now report the results of a more systematic kinetic study in which other rate forms appeared. In all cases we followed the decrease in absorbance at *ca.* 340 nm due to the RSNO reactant, usually at an initial concentration of around $5x10^{-4}$ mol dm^{-3}. We have been able to observe four limiting absorbance-time patterns: (a) first order reaction with an induction period, (b) first order reaction with no induction period, (c) zero order reaction with induction period, (d) zero order reaction with no induction period.

In addition we observed many forms which could be regarded as intermediate between any two of the four limiting forms. Example of each are given in Figures 2. 12, 2. 13, 2. 14, 2. 15, and 2. 16.

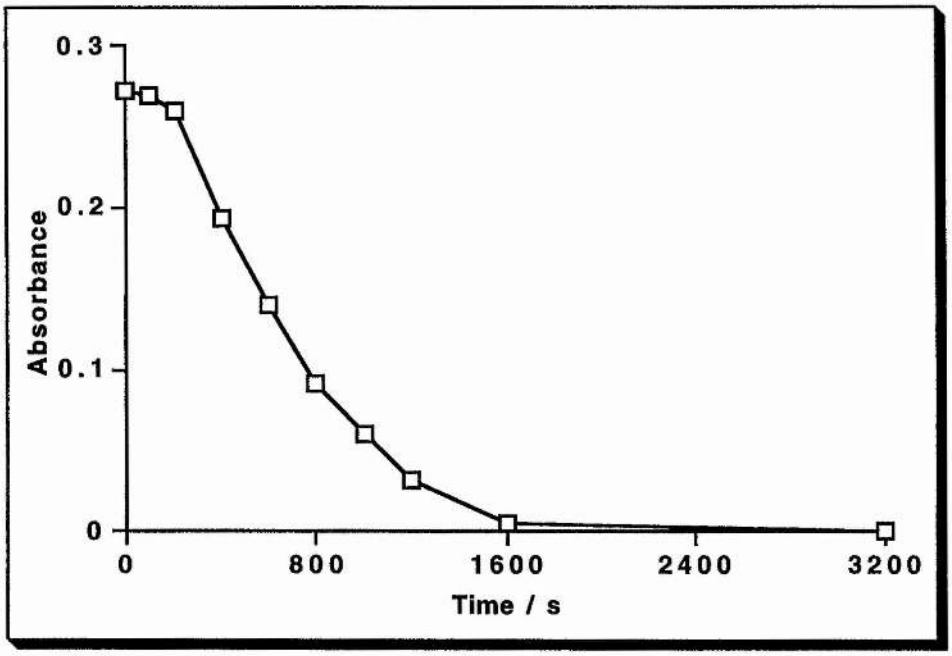


Figure 2. 12: Absorbance-time plot for the reaction of *S*-nitroso-2-*N*,*N*-dimethylaminoethanethiol (5×10^{-4} mol dm^{-3}) in the presence of added Cu^{2+} (1×10^{-6} mol dm^{-3}).

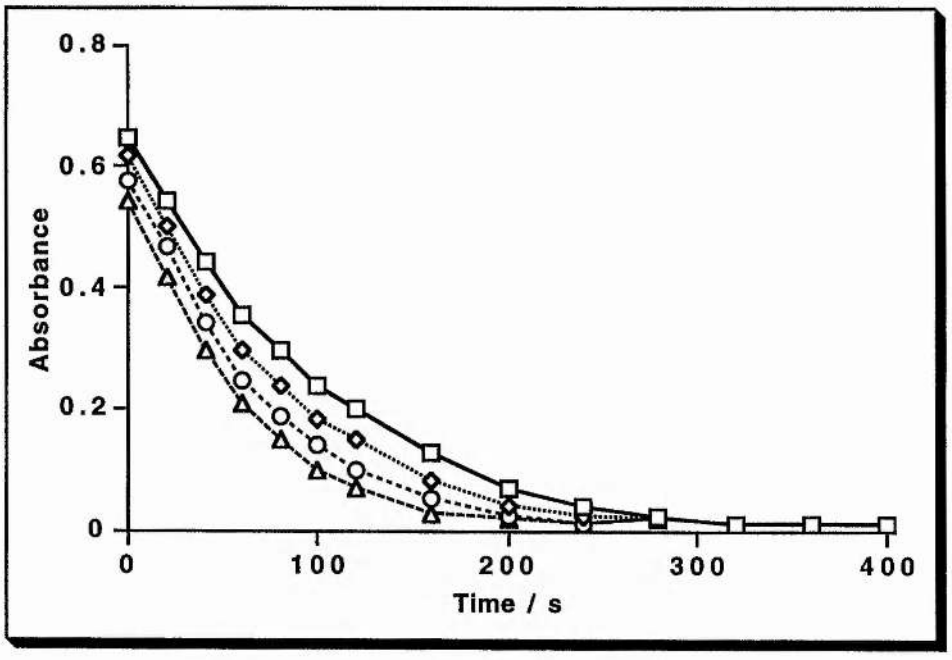


Figure 2. 13: Absorbance-time plots for the reaction of SNAP (1×10^{-3} mol dm^{-3}) in the presence of Cu^{2+}(1×10^{-5} mol dm^{-3}) and varying concentrations of added *N*-acetyl-D,L-penicillamine (NAP) (a) 4×10^{-6} mol dm^{-3} NAP (b) 6×10^{-6} mol dm^{-3} NAP (c) 8×10^{-6} mol dm^{-3} NAP (d) 1×10^{-5} mol dm^{-3} NAP.

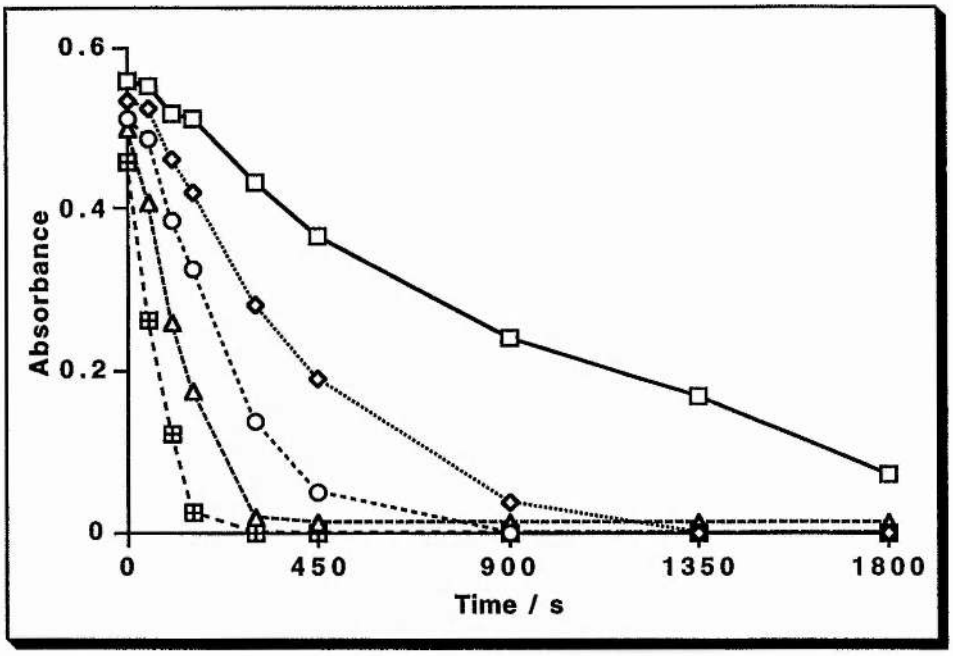


Figure 2. 14: Absorbance-time plots for the reaction of *S*-nitroso-2-*N*,*N*-dimethylaminoethanethiol (1 x 10^{-3} mol dm^{-3}) in the presence of added Cu^{2+} (a) 5 x 10^{-6} mol dm^{-3} Cu^{2+} (b) 7.5 x 10^{-6} mol dm^{-3} Cu^{2+} (c) 1 x 10^{-5} mol dm^{-3} Cu^{2+} (d) 3 x 10^{-5} mol dm^{-3} Cu^{2+} (e) 6 x 10^{-5} mol dm^{-3} Cu^{2+}.

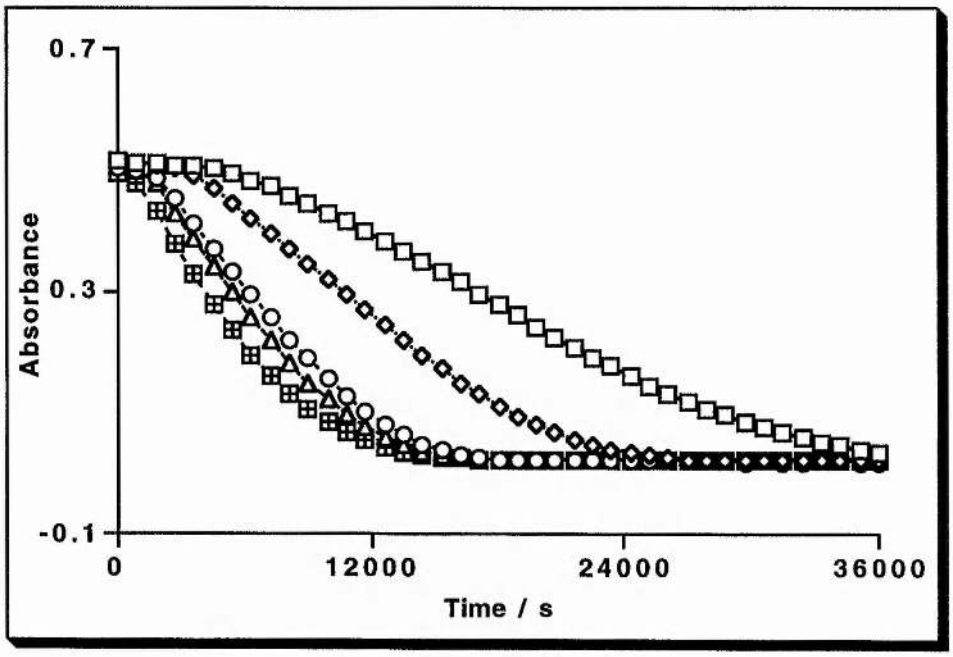


Figure 2. 15: Absorbance-time plots for the reaction of **2** (1 x 10^{-3} mol dm^{-3}) in the presence of added Cu^{2+} (a) 2.7 x 10^{-6} mol dm^{-3} Cu^{2+}, (b) 5.4 x 10^{-6} mol dm^{-3} Cu^{2+}, (c) 8.1 x 10^{-6} mol dm^{-3} Cu^{2+}, (d) 1.1 x 10^{-5} mol dm^{-3} Cu^{2+}, and (e) 1.4 x 10^{-5} mol dm^{-3} Cu^{2+}.

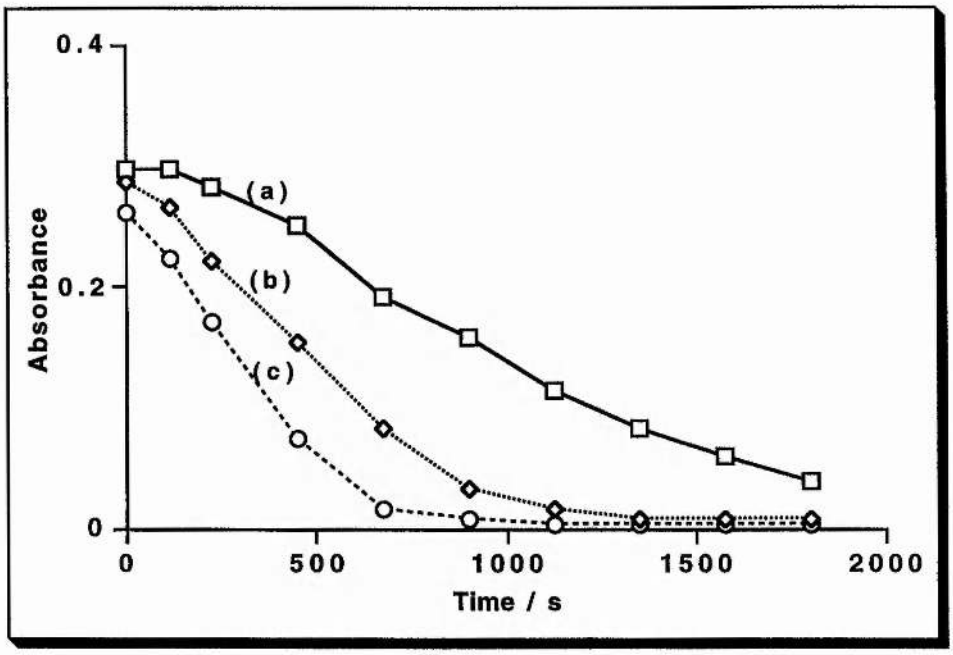
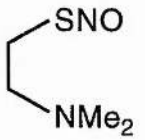


Figure 2. 16: Absorbance-time plots for the reaction of *S*-nitroso-2-*N*,*N*-dimethylaminoethanethiol (2 x 10^{-4} mol dm^{-3}) in the presence of added Cu^{2+} (1 x 10^{-6} mol dm^{-3}) as a function of added 2-*N*,*N*-dimethylaminoethanethiol (a) no added thiol (b) added thiol (1 x 10^{-6} mol dm^{-3}) (c) added thiol (3 x1 0^{-6} mol dm^{-3}).

Figure 2. 12 is an absorbance/time plot for the reaction of *S*-nitroso-2-*N*,*N*-dimethylaminoethanethiol (SNDMA), at low [Cu^{2+}] with no added thiol, Figure 2. 13 shows the reaction of SNAP at 1x10^{-5} mol dm^{-3} Cu^{2+} in the presence of various [NAP], Figure 2. 14 shows the reaction of *S*-nitroso-2-*N*,*N*-dimethylaminoethanethiol with various concentrations of added Cu^{2+}, Figure 2. 15 shows a similar pattern for the reaction of *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester (**2**) with different [Cu^{2+}], and Figure 2. 16 shows the results for SNDMA at low [Cu^{2+}] varying the concentrations of added thiol.



S-nitroso-2-*N*,*N*-dimethylaminoethanethiol (SNDMA)

The induction period is quite clear in Figures 2. 12, 2. 14, and 2. 15 and is completely absent in Figures 2. 13, and 2. 16 at high [RSH]. Equally clear is the first order pattern in Figure 2. 12, and 2. 13 and zero order dependence in 2. 14, 2. 15, and 2. 16.

2. 2. 1. 1. 3. Explanation of the Experimental Data if Cu^{+}, rather than Cu^{2+}, is the True Catalyst

The question then arises: if Cu^{+} rather than Cu^{2+} effects catalysis of *S*-nitrosothiol decomposition, why does addition of a Cu^{2+} salt result in decomposition? *S*-Nitrosothiols are prepared by nitrosation of thiols and no purification procedure is possible because of the ease of decomposition. Contamination of the *S*-nitrosothiol sample by thiol is not only possible but probable and this leads immediately to the reduction of the Cu^{2+} ions present in the buffer. The concentration of copper ions required for catalysis is so low that only *ca.* 1% contamination of *S*-nitrosothiol by thiol would be needed for complete conversion to Cu^{+}.

However, there is one, initially puzzling, observation which provides further confirmation of the formulation and catalytic role of Cu^{+} ions. The catalytic effect of added copper nitrate on the decomposition of *S*-nitroso-*N*-acetyl-β,β-dimethylcysteinylglycine methyl ester (**2**) is shown in Figure 2. 15. Although at low concentrations of added copper ions the catalytic effect is concentration dependent, at high copper levels the rate becomes independent of the amount of copper salt added. The concentration of the catalytic Cu^{+} ions can never be greater than the concentration of thiol present as an impurity in the *S*-nitrosothiol. When the concentration of added copper ions exceeds the concentration of contaminating thiol no further Cu^{+} ions are formed and no increased catalytic effect is produced.

2. 2. 1. 1. 3. 1. Reduction of Cu^{2+} to Cu^{+} by Thiol

For this study we use SNAP with the addition of the corresponding thiol, *N*-acetyl-D,L-penicillamine (NAP). Use of a different thiol would complicate the situation by rapid NO group transfer from RSNO to R'SH leading to R'SNO formation (Barnett *et al.*, 1995a&b). With [SNAP] 1×10^{-3} mol dm^{-3}, added [Cu^{2+}] 1×10^{-5} mol dm^{-3} and [NAP] in the range 1×10^{-6}-1×10^{-3} mol dm^{-3}, reactions are kinetically first order. The results are shown more dramatically in Figures 2. 17, 2. 18, and 2. 19.

As expected at low added NAP there is a very sharp, linear increase in the rate constant until [NAP] ~ 1 x 10^{-5} mol dm^{-3}. The reduction of Cu^{2+} by thiolate is a well-known process (Davis *et al.*, 1983; Scrivens *et al.*, 1995; Reid, 1958) and has been studied mechanistically as the Cu^{2+} catalysed oxidation of thiols to give disulphide:

$$2Cu^{2+} + 2RSH \rightleftharpoons 2Cu^{+} + 2H^{+} + RSSR$$

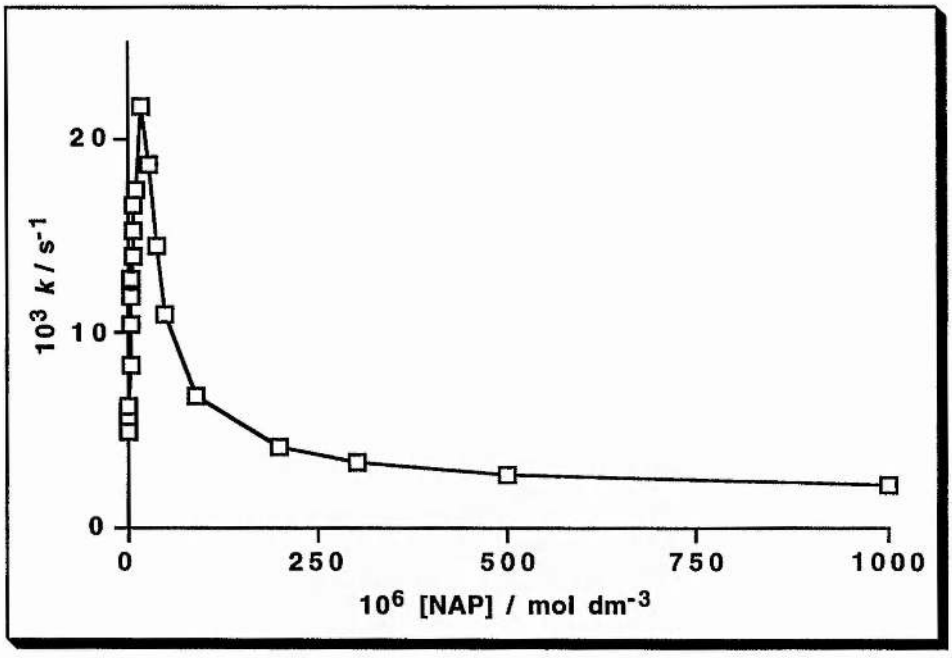
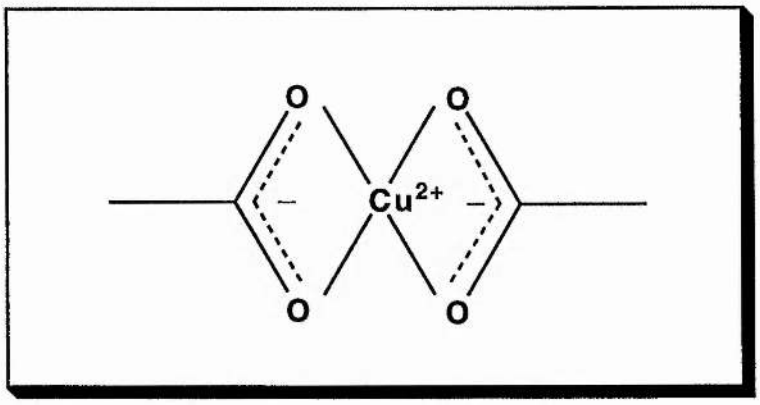


Figure 2. 17: First order rate constants (k) for the reaction of SNAP (1 x 10^{-3} mol dm^{-3}) as a function of added *N*-acetyl-D,L-penicillamine (NAP).

At higher [NAP] there is initially a sharp drop in k followed by a gradual decrease towards zero. We can explain this pattern in terms of the complexing of Cu^{2+} by NAP, thus effectively removing it from solution. These results explain the apparently contradictory reports in the literature (Feelisch *et al.*, 1994) some of which report catalysis of RSNO decomposition by added thiols, whilst other find a reduction in rate upon thiol addition. It is now clear at low added [RSH] there will be catalysis, as this favours the reduction of Cu^{2+} whereas complexation of Cu^{2+}, probably by the carboxylate group, takes over at higher added [RSH] resulting in inhibition of nitric oxide formation. Such copper-carboxylates are well-known (Structure 2. 3) and some have been isolated and examined structurally (Doedens, 1976).



Structure 2. 3: The general binuclear structure of copper binding to carboxylate groups.

We have previously noted (Askew *et al.*, 1995a&b) a reduction in reactivity with increasing buffer concentration when the buffer contains a carboxylic acid, an effect we attributed to competitive complexation with the carboxylic acid.

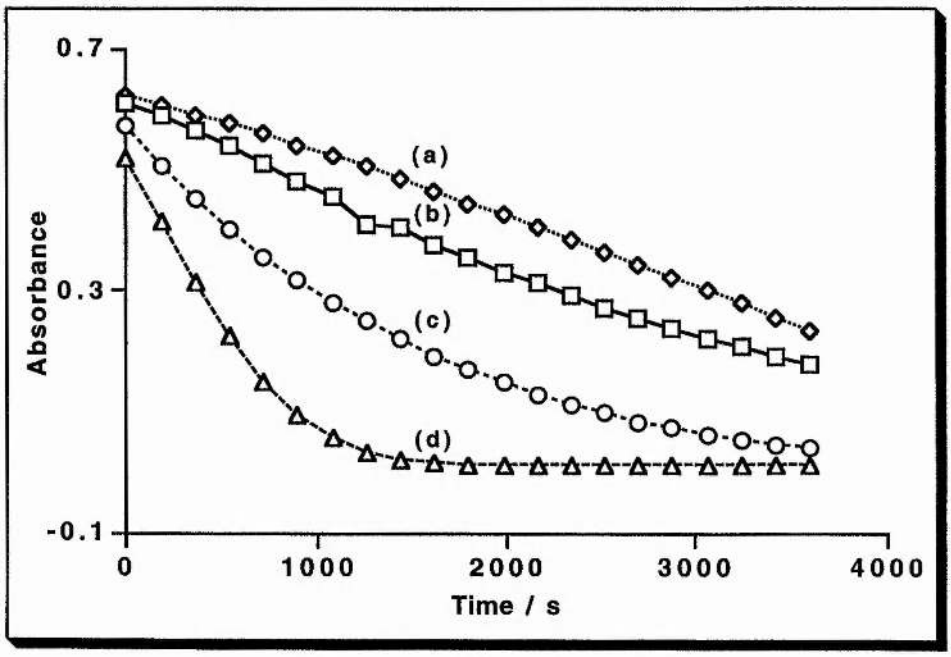


Figure 2. 18: Absorbance-time plots for the decomposition of SNAP (**1**) (5 x 10^{-4} mol dm^{-3}) (a) [NAP] 2.5 x 10^{-4} and [Cu^{2+}] 2.7 x 10^{-6} (b) no added NAP and [Cu^{2+}] 2.7 x 10^{-6} (c) no added NAP and [Cu^{2+}] 8.1 x 10^{-6} (d) [NAP] 2.5 x 10^{-4} and [Cu^{2+}] 8.1 x 10^{-6} mol dm^{-3} at pH = 7.4, 340 nm and 30 °C.

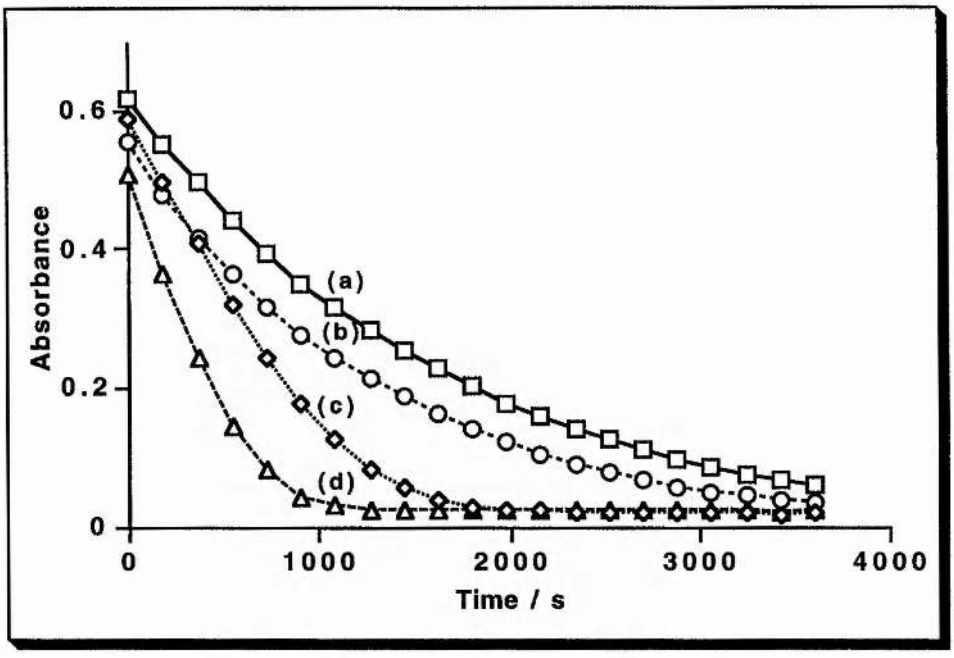
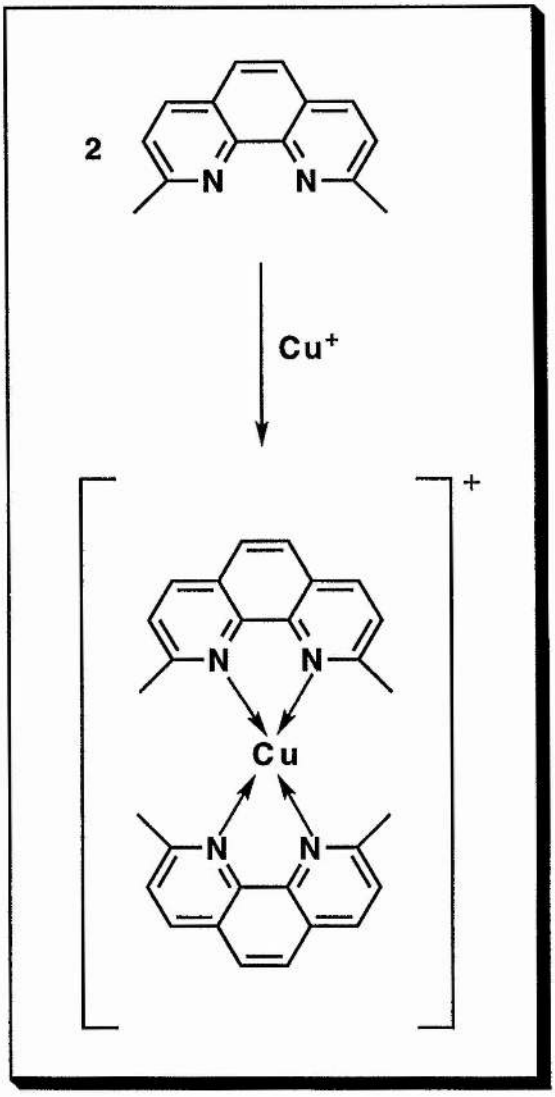


Figure 2. 19: Absorbance-time plots for the decomposition of SNAP (**1**) (5 x 10^{-4} mol dm^{-3}) (a) no added NAP and [Cu^{2+}] 8.1 x 10^{-6} (b) [NAP] 2.5 x 10^{-4} and [Cu^{2+}] 8.1 x 10^{-6} (c) no added NAP and [Cu^{2+}] 13.5 x 10^{-6} (d) [NAP] 2.5 x 10^{-4} and [Cu^{2+}] 13.5 x 10^{-6} mol dm^{-3} at pH = 7.4, 340 nm and 30 °C.

2. 2. 1. 1. 3. 2. Direct Proof that the True Catalyst of the Decomposition of *S*-Nitrosothiols is Cu^{+} by use of Neocuproine

The unusual kinetic forms found for a number of RSNO species under different conditions of [Cu^{2+}], particularly the tendency in some cases towards zero-order behaviour, led us to consider the possibility that the effective reagent in these reactions is in fact Cu^{+} and not Cu^{2+}. Zero-order dependence upon [RSNO] might then be interpreted in terms of a rate-limiting $Cu^{2+} \longrightarrow Cu^{+}$ reduction. Earlier Askew *et al.* considered this possibility and rejected it on the basis that there was no evidance for it from preliminary esr experiments with SNAP. In an alternative approach, we have now made use of the specific Cu^{+}-chelating agent neocuproine (Smith and McCurdy, 1952; Yoshida *et al.*, 1994; James and Williams, 1961) shown in the complexed form in Scheme 2. 5. In aqueous solution the stability constant of Cu^{+}-neocuproine complex is ~ $1x10^{19}$ $mol^{-2}dm^{6}$. When a Cu^{2+} solution ($2x10^{-5}$ mol dm^{-3}) was added to one of neocuproine hydrochloride ($4x10^{-5}$ mol dm^{-3}) there was no detectable change in the uv/visible spectrum.



Scheme 2. 5: The general binuclear structure of Cu^{+} binding to neocuproine.

However, upon addition of sodium dithionite (a well-known reducing agent for Cu^{2+}----------> Cu^{+}) an immediate yellow colour was noted and an absorbance maximum at 453 nm was found, as reported in the literature (Smith and McCurdy, 1952; James and Williams, 1961) for the spectrum of Cu^{+}-neocuproine complex. We then examined the decomposition of SNAP (1 x 10^{-3} mol dm^{-3}) at pH 7.4 containing added Cu^{2+} (2 x 10^{-5} mol dm^{-3}) in the presence of increasing concentrations of neocuproine in the range 4 x 10^{-5} - 1x10^{-3} mol dm^{-3}. The resulting absorbance-time plots taken at 340 nm (the absorbance maximum for SNAP and **2**) are shown in Figures 2. 20, and 2. 21.

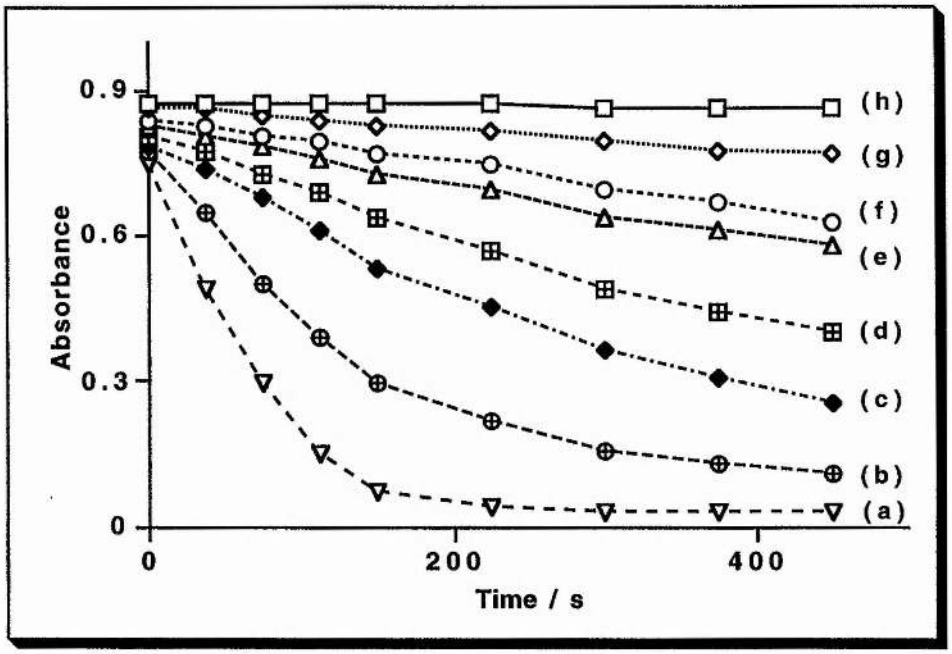


Figure 2. 20: Reaction of SNAP (1 x 10^{-3} mol dm^{-3}) in the presence of Cu^{2+} (2 x 10^{-5} mol dm^{-3}) and varying concentrations of neocuproine (a) no added neocuproine (b) 4 x 10^{-5} mol dm^{-3} neocuproine (c) 5 x 10^{-5} mol dm^{-3} neocuproine (d) 6 x 10^{-5} mol dm^{-3} neocuproine (e) 8 x 10^{-5} mol dm^{-3} neocuproine (f) 1 x 10^{-4} mol dm^{-3} neocuproine (g) 2 x 10^{-4} mol dm^{-3} neocuproine (h) 1 x 10^{-3} mol dm^{-3} neocuproine.

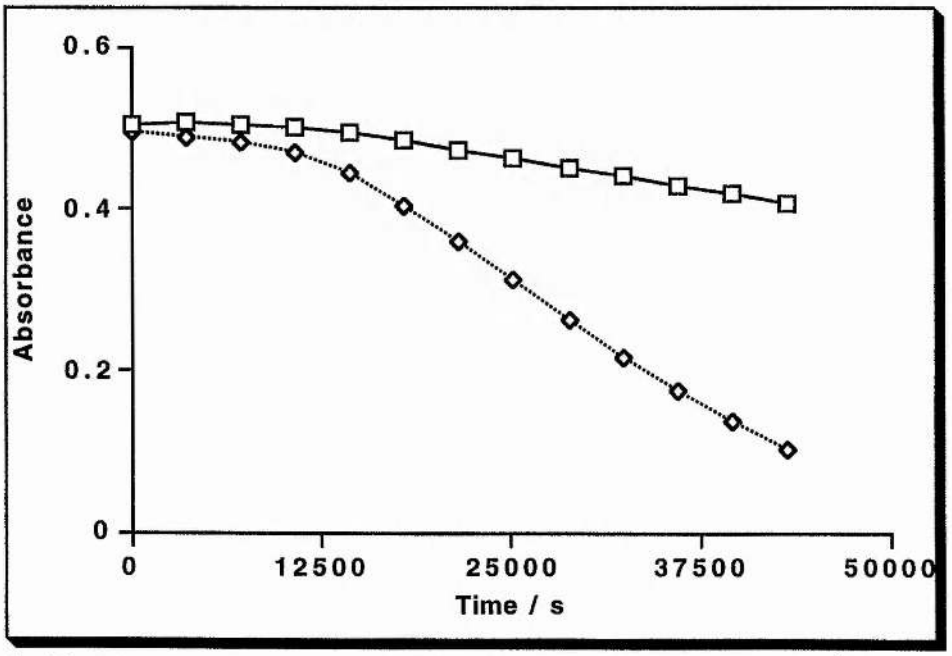


Figure 2. 21: Absorbance time plots for the decomposition of **2** (5 x 10^{-4} mol dm^{-3}) (a) neocuproine 5 x 10^{-4} mol dm^{-3} (square) (b) no added neocuproine at pH = 7.4, 340 nm and 30 °C (diamond).

It is immediately clear that the presence of neocuproine reduces the reaction rate progressively and at 1 x 10^{-3} mol dm^{-3} neocuproine the reaction is completely suppressed. The full spectra showed the increasing absorbance at 453 nm as expected for the formation of Cu^{+}-neocuproine complex. Similar experiments over a slightly smaller range of [added neocuproine] yielded reasonably good first-order plots and the data are given in Figure 2. 22 showing clearly the inhibiting effect of neocuproine.

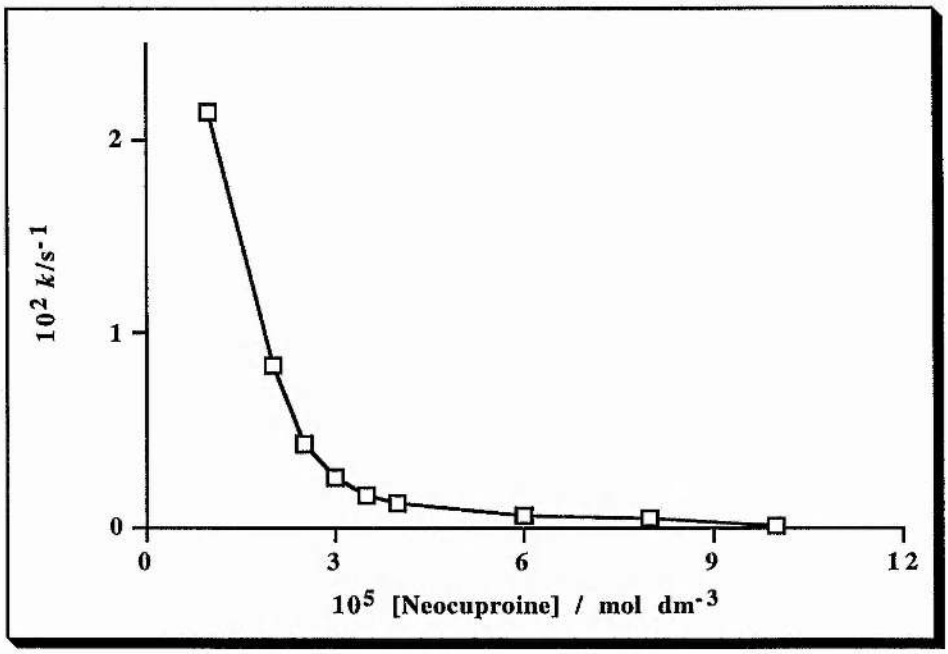


Figure 2. 22: First order rate constants (k) for the reaction of SNAP (1 x 10^{-3} mol dm^{-3}) in the presence of Cu^{2+} (2 x 10^{-5} mol dm^{-3}) as a function of [neocuproine].

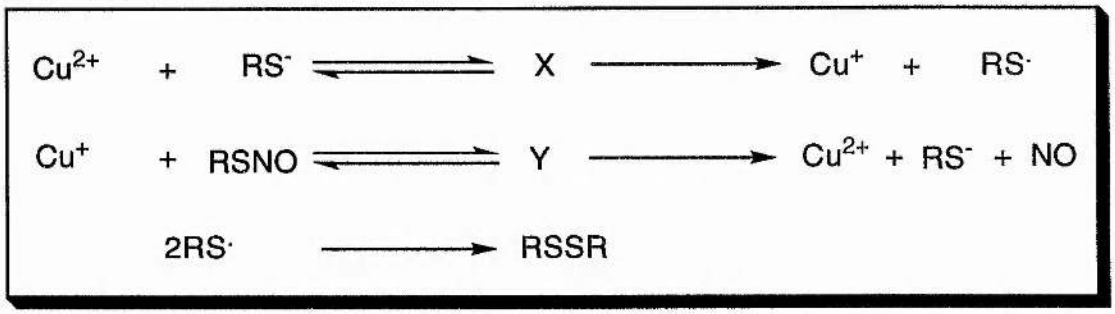
Clearly Cu^{+} is being generated and the question arises as to the nature of the reducing agent. Previously we have suggested that the reduction could be achieved by thiolate ion present from a small quantity of thiol impurity in the *S*-nitrosothiol sample. However in some experiments we were able to avoid any thiol impurity by carrying out reactions on solution samples of RSNO generated from thiols and nitrous acid with the nitrous acid present in a slight excess, and reaction still occurs. An alternative suggestion is that thiolate is generated by hydrolysis of the *S*-nitrosothiols:

$$RSNO + H_2O \longrightarrow RS^- + NO_2^- + 2H^+$$

This is not expected to be a rapid process given earlier studies of the hydrolysis in acid solution (Al-Kaabi *et al.*, 1982), but even a few percent reaction could be enough to initiate the reduction. When the corresponding thiol is added to the reaction mixtures a rapid increase in the rate constant is found.

2. 2. 1. 1. 4. Proposed Mechanism of the Copper Ion Catalysed Decomposition of *S*-Nitrosothiols

On the basis of these and earlier results we propose the following outline mechanism (Scheme 2. 6):



Scheme 2. 6: The proposed mechanism of copper ion catalysed decomposition of *S*-nitrosothiols.

Cu^{2+} is reduced by RS^- (generated from RSNO or added as RSH) *via* intermediate X to give Cu^+ and RS·. Intermediate X is probably $RSCu^+$. Reaction then occurs between Cu^+ and RSNO *via* intermediate Y releasing Cu^{2+}, RS^-, and NO. This is essentially the mechanism we suggested earlier (McAninly *et al.*, 1993) as a possibility, but without at that time sufficient evidence to support it.

Qualitatively we can account for the induction period by the time required for the generation of RS^- and Cu^+. A first-order dependence occurs if the reaction of RSNO with Cu^+ is rate-limiting and a zero order dependence occurs (for the more reactive RSNO species) when Cu^+ formation is rate limiting. The length of the induction period can be reduced by the addition of either RSH or Cu^{2+} which will increase the rate of formation of Cu^+.

Alternatively, if only RSNO and Cu^{2+} are present initially, the step for the generation of Cu^{+} must be included in the simulation. This results in an absorbance-time profile given in Figure 2. 23, with an autocatalytic feature which reproduces that observed experimentally in Figure 2. 12.

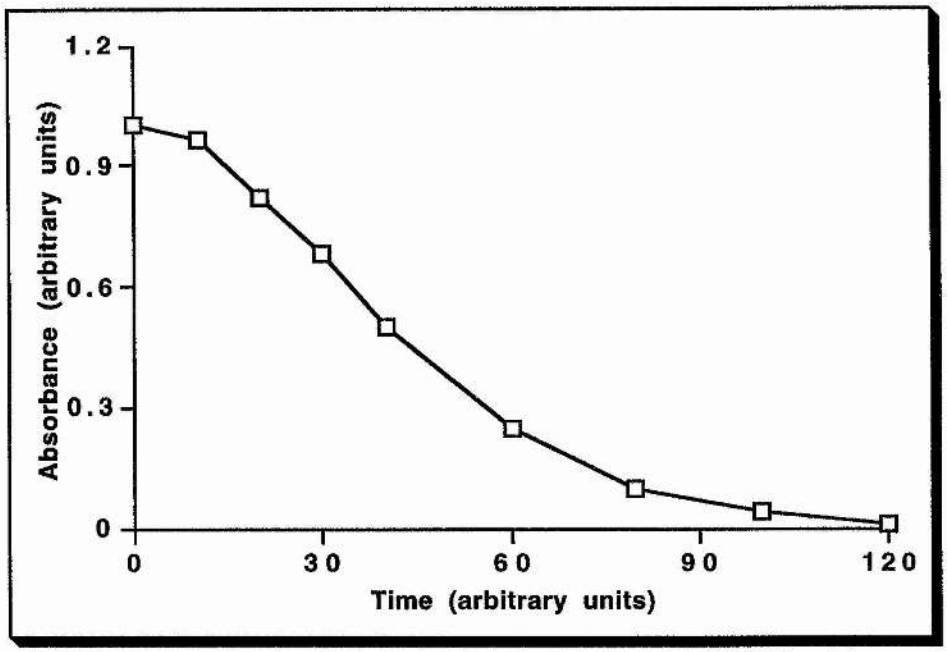


Figure 2. 23: Computed absorbance-time plot with no added RSH with rate-limiting RSNO reaction with Cu^{+}.

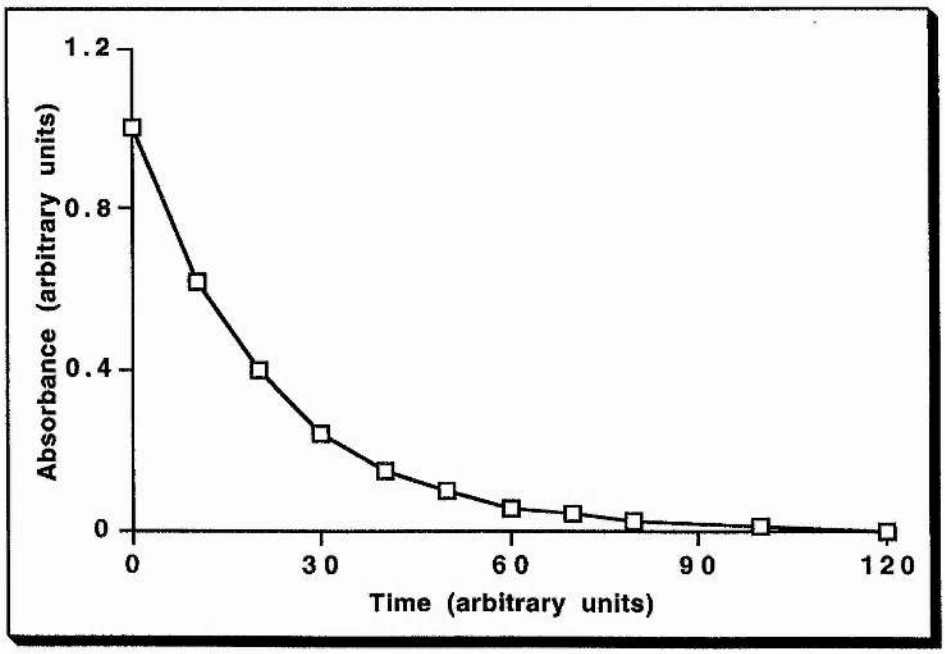


Figure 2. 24: Computed absorbance-time plot with added RSH with rate-limiting RSNO reaction with Cu^{+}.

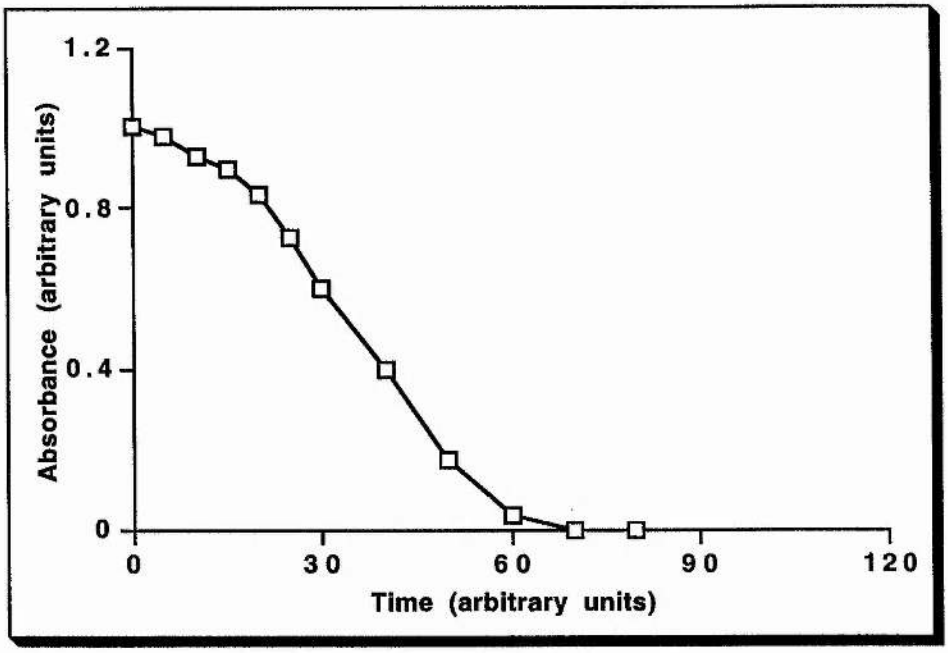


Figure 2. 25: Computed absorbance-time plot with no added RSH with rate-limiting Cu^{+} formation.

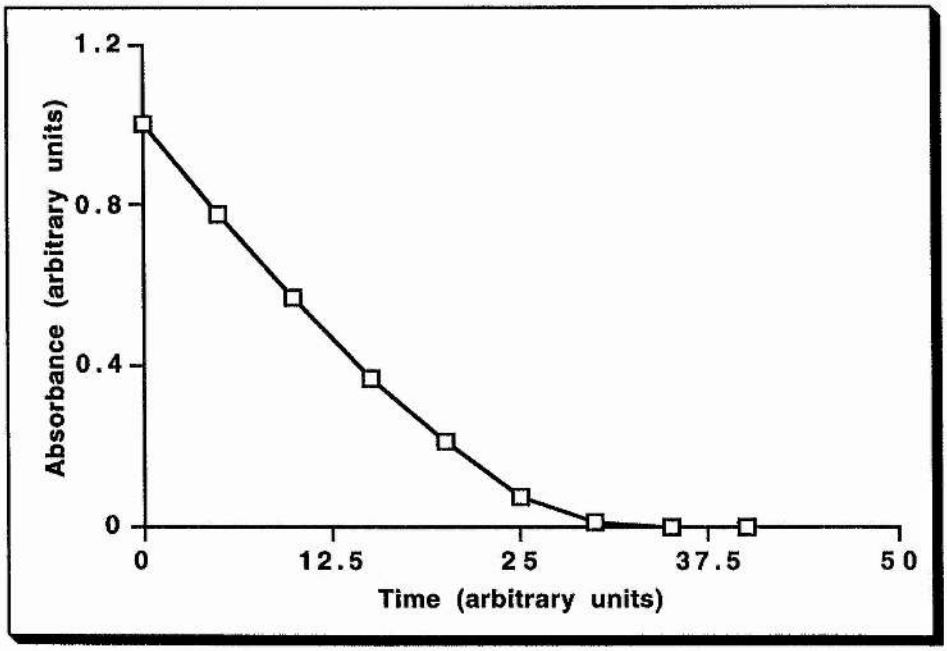
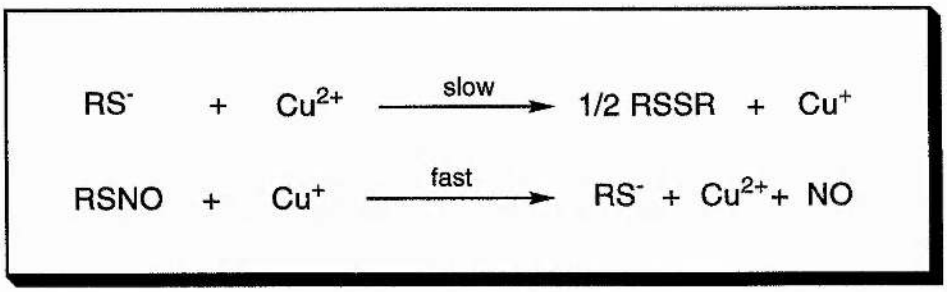


Figure 2. 26: Computed absorbance-time plot with added RSH with rate-limiting Cu^{+} formation.

(2) Rate-limiting Cu^+ formation

Under these circumstances Scheme 2. 6 effectively reduces to the following equations:



Scheme 2. 8: The proposed mechanism of copper ion catalysed decomposition of *S*-nitrosothiols in the case of rate-limiting Cu^+ formation.

If RSNO, Cu^+, and RS^- are all present initially then we get a truly zero-order reaction (simulation in Figure 2. 26) with RSNO scavenging Cu^+ as it is formed and immediately regenerating RS^- and Cu^{2+}. However, if only RSNO and Cu^{2+} are present initially, then RS^- has to be generated as before and this results in an induction period followed by a zero-order reaction (Figure 2. 25). Experimental examples of both types of behaviour have been observed as in Figure 2. 16 as $[RS^-]$ is increased, 2. 14 and 2. 15. Figures 2. 14 and 2. 15 clearly show a saturation effect in Cu^{2+} at high $[Cu^{2+}]$ which requires that in Cu^+ formation there is an equilibrium involving intermediate X.

All of these experiments were conducted at pH 7.4 in normal aerated solvent. When oxygen was rigorously excluded then we found no significant difference in behaviour for any of the limiting rate forms. This implies that any oxidation reaction such as of Cu^+ by dissolved oxygen is not a significant reaction under these conditions. However, for very slowly reacting RSNO compounds, such as *N*-acetyl-*S*-nitrosocysteine (SNAC), we found a very long induction period, typically 3 hours, as shown in Figure 2. 27. This result was obtained using a rather impure solid sample of SNAC which was known to contain some of the thiol and was very difficult to purify. Repetition of this experiment with SNAC generated *in situ* in solution gave no reaction whatsoever over a 16 hour period (Figure 2. 28), so it is likely that the thiol is playing a part here in Cu^+ generation as expected.

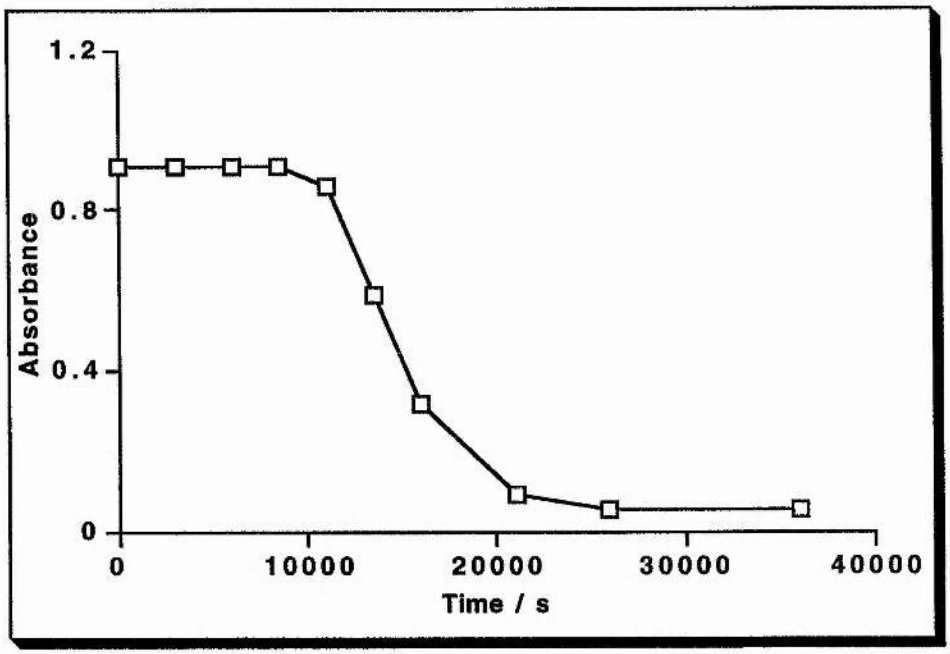


Figure 2. 27: Absorbance-time plot for the reaction of *N*-acetyl-*S*-nitrosocysteine (SNAC; crude sample).

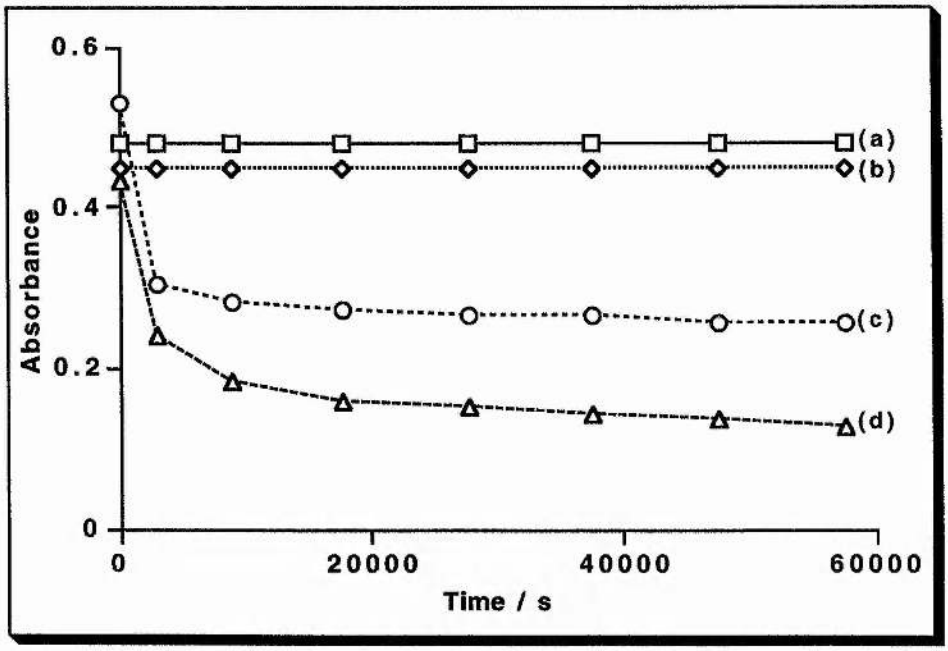


Figure 2. 28: Absorbance-time plots for the reactions of GSNO and *N*-acetyl-*S*-nitrosocysteine (SNAC) showing the effect of the presence of oxygen (a) GSNO under aerobic conditions (b) SNAC under aerobic conditions (c) GSNO under anaerobic conditions (d) SNAC under anaerobic conditions.

However, when the same reaction was carried out in the absence of oxygen, reaction occurred immediately with no induction period. Exactly the same pattern was found with *S*-

nitroso-L-glutathione (GSNO), although in both cases it is not clear why complete reaction does not occur. There is, however, a very dramatic effect due to the presence of oxygen here, which implies that oxidation of Cu^+ by oxygen is a competing reaction with that of Cu^+ with RSNO.

$$Cu^+ + O_2 \longrightarrow Cu^{2+} + O_2^-$$

When the oxygen is removed, decomposition can occur. For both of these substrates reaction can be induced by the addition of the corresponding thiol. An induction period occurs which is reduced as the concentration of added thiol is increased until we get a good first order plot at very high [thiol] (~ 0.15 mol dm^{-3}). In the presence of thiol, the effect of increasing the $[Cu^{2+}]$ is also to reduce the induction period and, as before, the removal of oxygen also removes the induction period completely. For these slower reacting substrates it does seem that re-oxidation of Cu^+ by dissolved oxygen is an important pathway in their decomposition reactions.

2. 2. 1. 2. Copper Ions *in vivo*

Copper is present in blood plasma (*ca.* 1.0 x 10^{-6} mol dm^{-3}), approximately 5% of which is bound to albumin protein and is known to be in rapid equilibrium with copper in tissues (Bearn and Kunkel, 1954). Later studies (Neumann and Sass-Kortsak, 1967; Sarkar and Kruck, 1966) established the presence of an equilibrium between this fraction and copper bound to amino acids in blood plasma as shown by the set of equilibria:

$$Cu^{2+} + \text{Albumin} \rightleftharpoons [Cu^{2+}\ (\text{Albumin})]$$

$$[Cu^{2+}\ (\text{Albumin})] + AA \rightleftharpoons [Cu^{2+}\ (AA)] + \text{Albumin}$$

$$Cu^{2+} + AA \rightleftharpoons [Cu^{2+}\ (AA)]$$

The amino acid complexes are thought to mediate the transport of copper through the biological membranes by virtue of their small molecular size.

Although earlier studies (Sarkar and Kruck, 1966) established histidine as the major ligand for Cu in the amino acid fraction, the low concentration of the metal ion and the complex equilibria have defied detailed experimental study to date. Instead, computer simulation models have been developed (Hallman *et al.*, 1971; May *et al.*, 1977a&b) to delineate the speciation in this low molecular weight plasma fraction and have been put to use to determine the influence of chelating drugs on the low molecular weight plasma fraction equilibria (May *et al.*, 1977a&b).

It is noteworthy that cysteine is present in blood plasma (*ca.* 23 x 10^{-6} mol dm^{-3}) (Brigham *et al.*, 1960) and cysteine is known to reduce rapidly Cu^{2+} according to the stoichiometry:

$$2Cu^{2+} + 2CysH \rightleftharpoons 2Cu^{+} + 2H^{+} + CysSSCys$$

However, there is an even larger excess of other amino acids in the low molecular weight plasma fraction which, by chelation, could stabilise the Cu^{2+} state by lowering the Cu^{2+}/Cu^{+} redox potential.

2. 2. 1. 3. Is *in vitro* Release of NO from *S*-Nitrosothiols due to Copper Ions?

We believe that we have shown that Cu^{+} is the effective reagent in bringing about decomposition of *S*-nitrosothiols to yield nitric oxide. Both the experiments with neocuproine and all the kinetic evidence support the outline mechanism given in Scheme 2. 6. Some details remain as yet unresolved, *e.g.* the detailed mechanism for the breakdown of intermediate Y, for which at present we have no experimental evidence.

These findings could well have implications for the reactions of *S*-nitrosothiols *in vivo*. It is known that they have specific biological activity, notably vasodilation (Ignarro *et al.*, 1981a&b), the inhibition of platelet aggregation (Radomski *et al.*, 1992), and the inhibition of neutrophil functions (Clancey and Abramson, 1992). In the body, copper is present not as free Cu^{2+}, but in a bound form with amino acids and proteins. Possibly this is reducible to Cu^{+} by thiolate. Experiments to test this possibility are described in later following sections.

The reactivity pattern of GSNO is particularly interesting. In aerobic solution it is particularly stable, with only a negligibly small amount of decomposition occurring over many hours. However, in the presence of L-glutathione (GSH), decomposition occurs much more rapidly, but possibly with an induction period (Figure 2. 29), the length of which is dependent on [RSH] and $[Cu^{2+}]$.

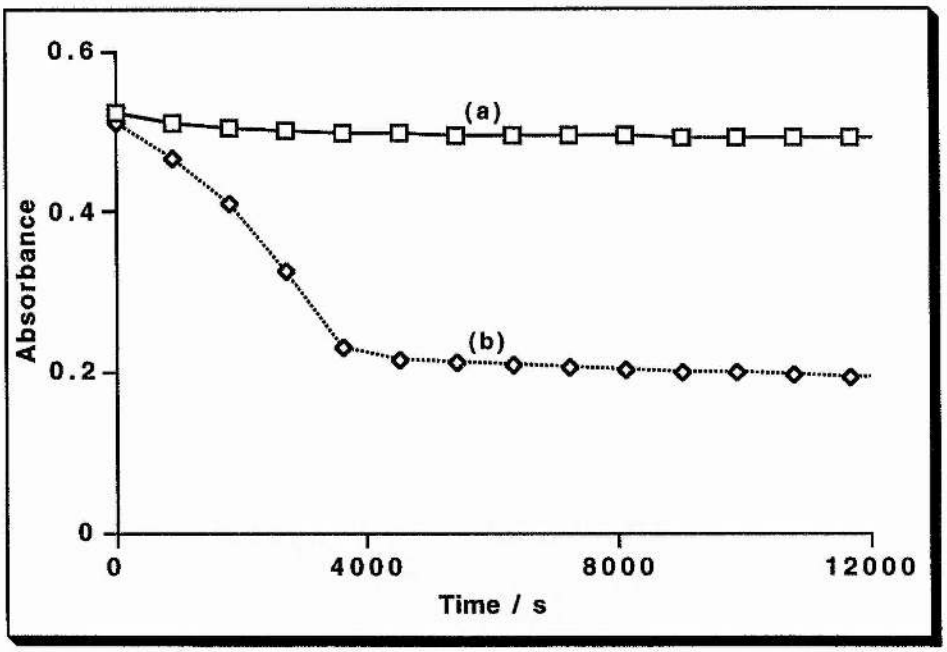


Figure 2. 29: Effect of added L-glutathione (GSH) on the copper-catalysed decomposition of GSNO (5×10^{-5} mol dm^{-3}), $[Cu^{2+}]$ = (a) 1.4×10^{-5} and (b) 1.4×10^{-5} mol dm^{-3}, and [GSH] = 2.5×10^{-5} mol dm^{-3}.

Although in the presence of L-cysteine (CysH), decomposition also occurs much more rapidly, but possibly with an induction period (Figure 2. 30) the length of which is dependent on [CysSH] and [Cu^{2+}].

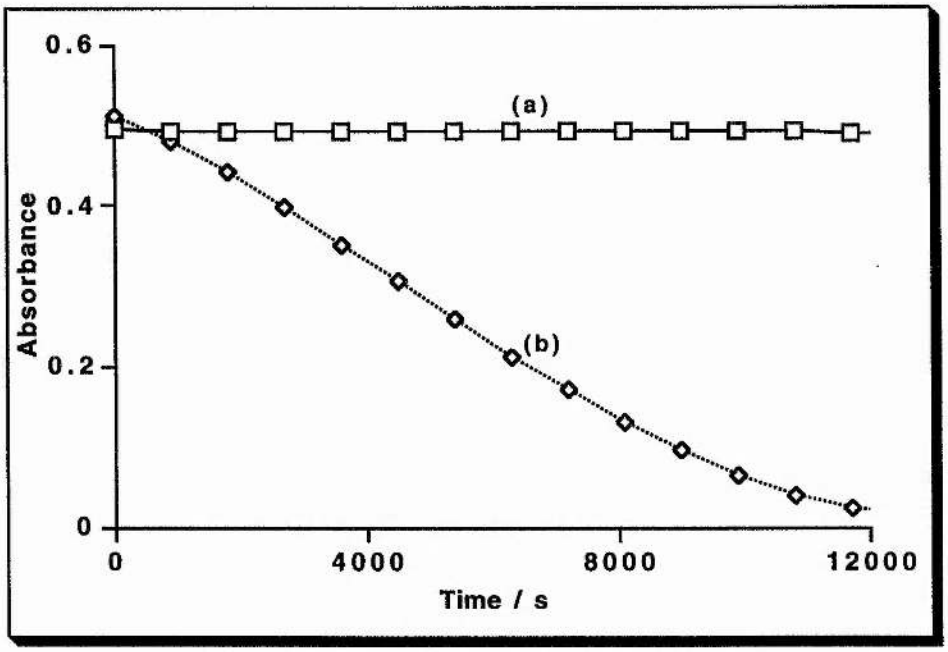


Figure 2. 30: Effect of added L-cysteine (CysH) on the copper-catalysed decomposition of GSNO (5 x 10^{-5} mol dm^{-3}), (a) no added cysteine and (b) added cysteine (12.5 x 10^{-5} mol dm^{-3}).

Further, in the absence of oxygen and without added GSH then reaction occurs quite readily without an induction period. Finally, the presence of another oxidising agent (*e.g.* hydrogen peroxide) has the effect of stabilising GSNO, again over a period of many hours. In our experiments no perceptible decomposition occurred overnight. Again, the re-oxidation of Cu^{+} to Cu^{2+} does not allow Cu^{+} to have a catalytic role.

2. 2. 2. Transnitrosation (NO Transfer from *S*-Nitrosothiols to other Thiols)

When the *in vivo* release of NO from *S*-nitrosothiols is considered, the effect of metal ions is supplemented by possible NO transfer to one or more of the numerous sulphydryl containing molecules present. Clearly, the process of transnitrosation can also affect the nature and stability of *S*-nitrosothiols. The sulphydryl groups present *in vivo* are contained in molecules which are either membrane bound, or free, either in the intracellular matrix (e.g. cysteine and glutathione) or in the plasma (albumin). These sulphydryl containing molecules, together with the tissue-type plasminogen activator and cathepsin B, are susceptible to nitrosation and NO transfer from *S*-nitrosothiols under physiological conditions (Stamler *et al.*, 1992a,b,c,d&e). NO transfer to these proteins or amino acids, and the extent to which it happens, are likely to depend on a number of factors. These include the relative pK_a's of sulphydryl moieties, the concentration at which they are present, and the steric hindrance likely to be caused by the movement of the NO group to the acceptor thiol. It has recently been suggested that bioactive NO equivalents in plasma are bound predominantly to thiol groups of proteins and that this reservoir has a role in modulating vasodilator tone (Stamler *et al.*, 1992a,b,b,c&d).

If transnitrosation occurs from relatively stable *S*-nitrosothiols, such as GSNO or SNAP, to a thiol such as cysteine, which is a relatively abundant thiol *in vivo* (Jocelyn, 1972), the formation of the unstable *S*-nitrosothiols, such as *S*-nitrosocysteine, could facilitate the release of NO and account in part for the biological action of GSNO.

Addition of cysteine appears to enhance copper catalysis in the decomposition of SNAP. Figures 2. 31 and 2. 32 show the effect of added cysteine. It is clear that without cysteine

Figure 2. 33 shows the effect on the decomposition rate of **2**. It is clear that without cysteine the decomposition rate is very small, but in the presence of cysteine it is rapid. Similar results for compounds **3-13** were found.

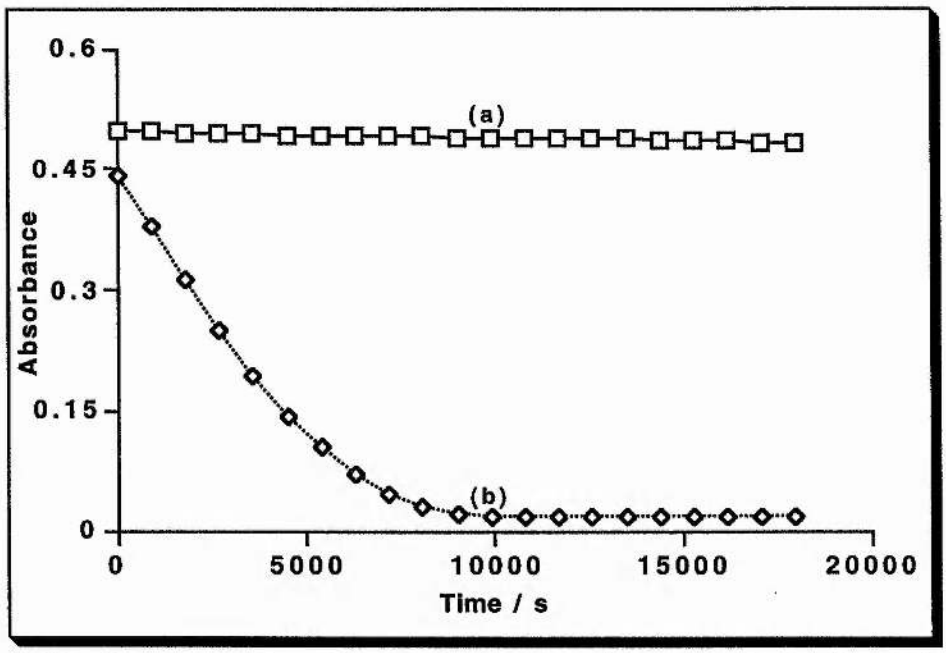


Figure 2. 33: Absorbance-time plots for the decomposition of **2** (5 x 10^{-4} mol dm^{-3}) (a) no added cysteine (b) cysteine 12.5 x 10^{-4} mol dm^{-3}.

Although cysteine addition has a dramatic effect on the release of NO from *S*-nitrosothiols, it appears that copper ions are still required. The results displayed in Figures 2. 34 and 2. 35 show the effects on the decomposition rate of **2** of adding cysteine, and of adding cysteine and 2,9-dimethyl-1,10-phenanthroline (k = 1.2 x 10^{-4} and 3.0 x 10^{-5} s^{-1} respectively). Even when *S*-nitrosocysteine is formed, its decomposition rate is very slow in the absence of copper ions.

From these results we suggest a mechanism whose details are reproduced in Scheme 2. 9

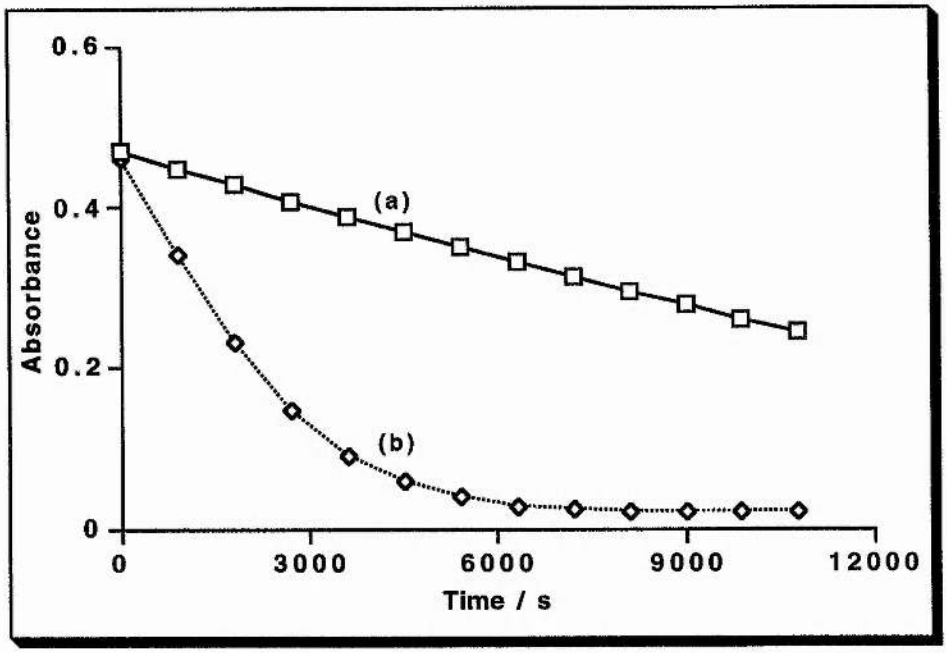


Figure 2. 34: Absorbance-time plots for the decomposition of **2** (5 x 10^{-4} mol dm^{-3}) (a) Cysteine 12.5 x 10^{-4} mol dm^{-3} and 2,9-dimethyl-1,10-phenanthroline 5 x 10^{-4} mol dm^{-3} (b) Cysteine 12.5 x 10^{-4} mol dm^{-3}.

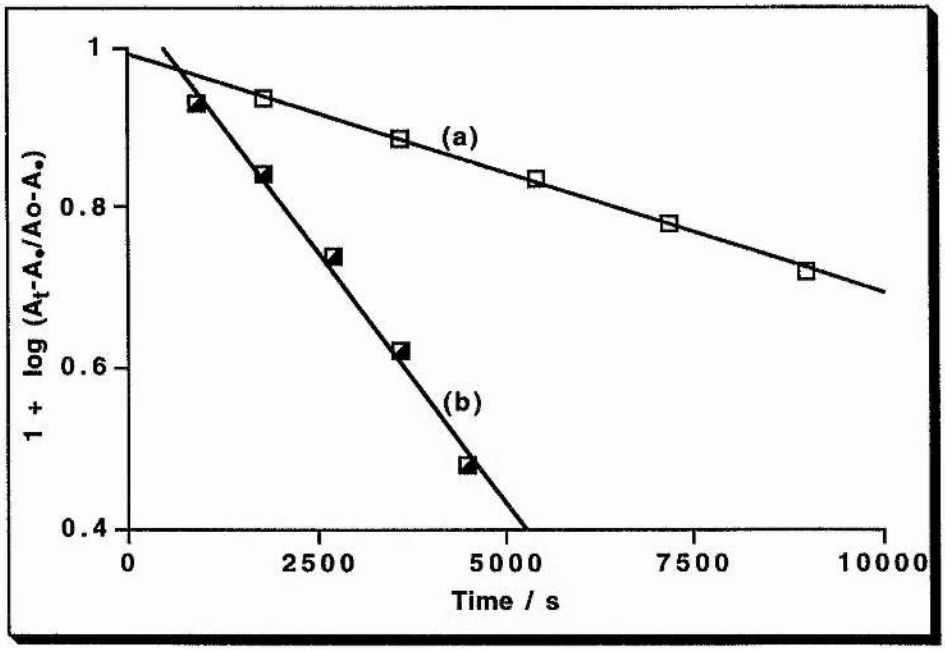
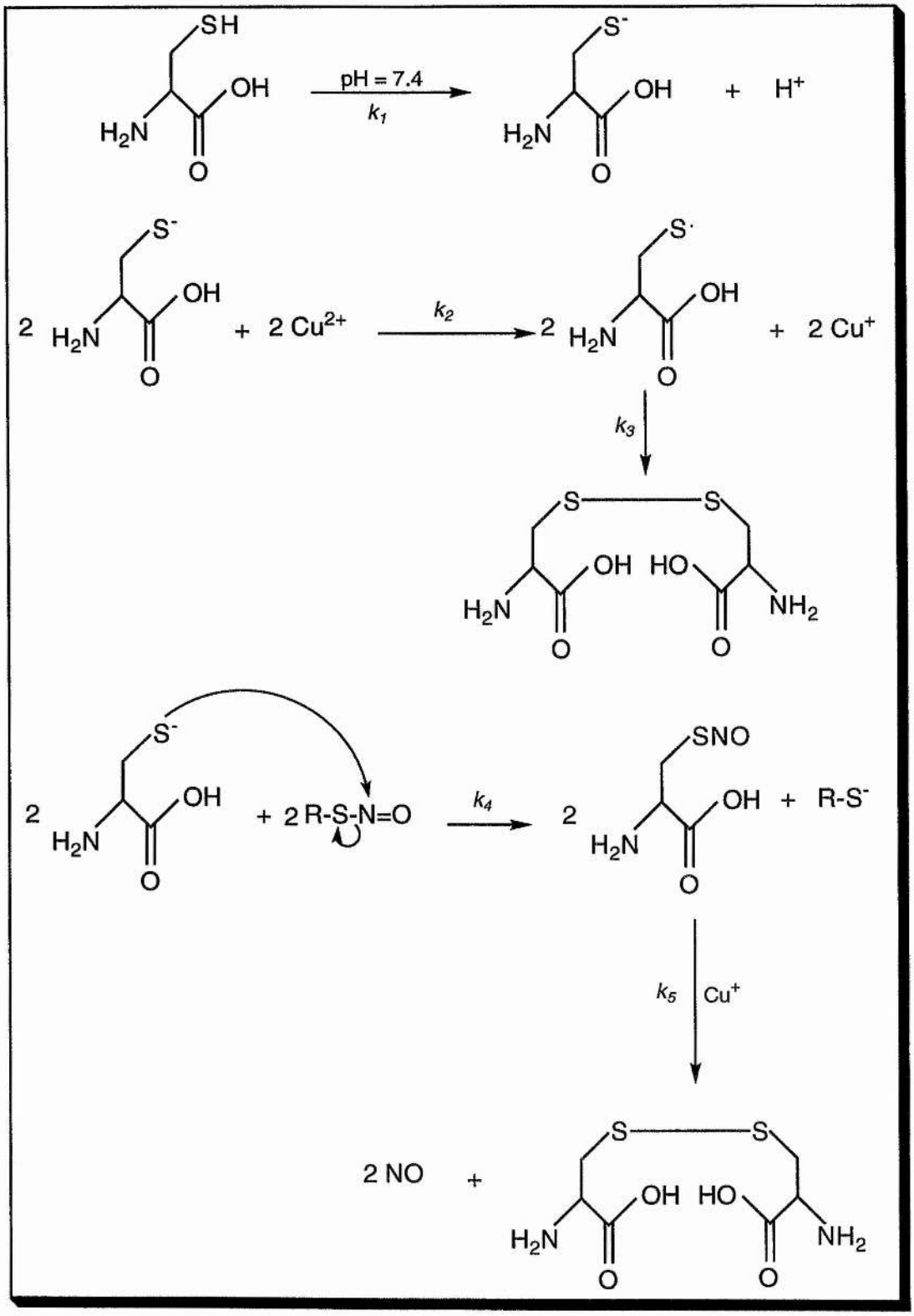


Figure 2. 35: Plots of 1 + log (A_t - A_∞ / A_0 - A_∞) versus time for the decomposition of (a) **2**, Cysteine 12.5 x 10^{-4} mol dm^{-3}, and neocuproine 5 x 10^{-4} mol dm^{-3} (b) **2** and Cysteine 12.5 x 10^{-4} mol dm^{-3} (r = 0.999, 0.996 respectively).



Scheme 2. 9: The proposed mechanism of transnitrosation.

According to this mechanism (Scheme 2. 9), at pH = 7.4, the cysteine (Cys-H) will be converted into the corresponding thiolate (Cys^-); (a) the thiolate (Cys^-) will react with Cu^{2+} to give the corresponding thiyl radical (Cys·), which is converted directly into the corresponding disulphide (Cys-Cys), and Cu^+; (b) the thiolate (Cys^-) will react with the *S*-nitrosothiols (RSNO) to give *S*-nitrosocysteine and the corresponding thiolate (RS^-), then *S*-nitrosocysteine, unstable in the presence of Cu^+, will decompose into cystine and nitric oxide.

If step 4 is the rate determining step, therefore:

rate = k_4 [RSNO] [Cys^-]

Because [RSNO] << [CysH], therefore, the reaction is pseudo-first order with respect to *S*-nitrosothiols.

rate = k_{obs} [RSNO]

where $k_{obs} = k_4$ [Cys^-]

The rate constants for *S*-nitrosothiol decomposition in the presence of added cysteine are listed (Table 2. 1). In the absence of cysteine decomposition is very slow for **2-13** but the rate of spontaneous decomposition for SNAP is quite high.

Our results indicate that the transnitrosation mechanism could be important in the endogenous decomposition of *S*-nitrosothiols.

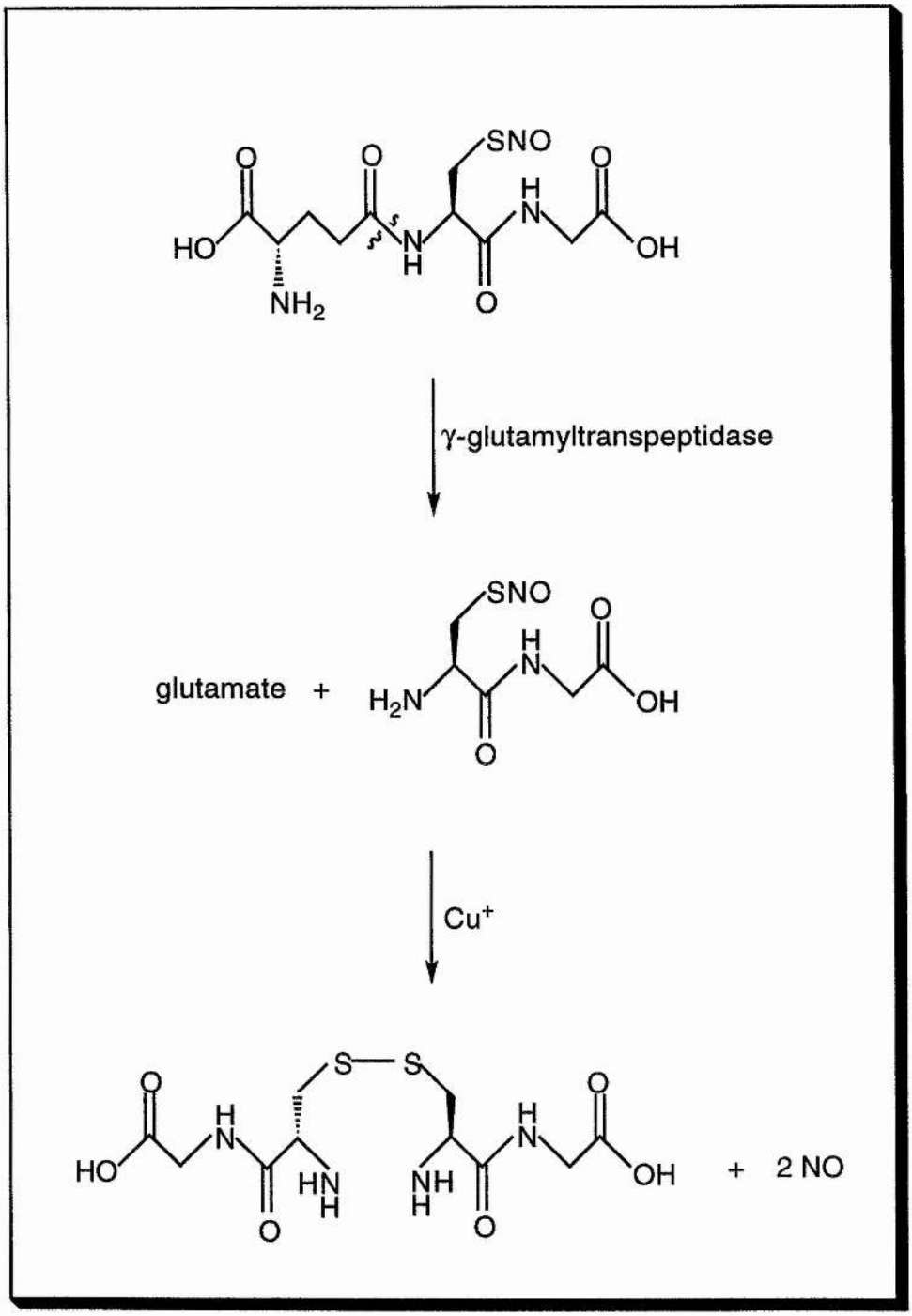
2. 2. 3. Enzymatic Decomposition of *S*-Nitrosothiols

GSNO, a derivative of the endogenous peptide glutathione, has been detected *in vivo* at concentrations of approximately 0.25 μM in venous plasma and up to 1.2 μM in arterial plasma (Stamler *et al.*, 1992a,b,c,d&e; Meyer *et al.*, 1994). Therefore it is more likely that this *S*-nitrosothiol could be acted upon enzymatically to produce NO and vasodilation.

Previous work in our laboratories (Askew *et al.*, 1995a&b) has shown that γ-glutamyl transpeptidase (γ-GT) could bring about decomposition of GSNO, by γ-glutamyl cleavage as a first step. This would form an *S*-nitrosothiol which is likely to have greatly reduced stability compared with GSNO and which could readily release NO.

We have also shown (Section 2. 2. 1) that in general *S*-nitrosothiols do not breakdown to release NO except in the presence of Cu^{+}. Probably GSNO, a naturally occurring *S*-nitrosothiol, undergoes enzymatic metabolism to give *S*-nitrosocysteinylglycine and this will be decomposed again by Cu^{+} catalysis to give the corresponding disulphide and NO. In view of the previous results, a modified mechanism of action of γ-glutamyl transpeptidase is shown in Scheme 2. 10.

Evidence that this mechanism is possible may imply that this enzyme, or a closely related enzyme, or a number of other members of this gene family (Morris *et al.*, 1993), could be responsible, in part, for the biological activity of GSNO.



Scheme 2. 10: The modified mechanism of action of γ-glutamyl transpeptidase.

2. 2. 4. The Photochemical Decomposition of *S*-Nitrosothiols

S-Nitrosothiols are decomposed photochemically to the corresponding disulphide and nitric oxide (Josephy *et al.*, 1984):

$$RSNO \xrightarrow{h\nu} 1/2\ RSSR + NO$$

Barrett *et al.* (1966) reported that the absorption of 365 nm-radiation by *S*-nitrosotoluene resulted in excitation to the extent of 79 kcal mol^{-1}, which was sufficient to cause fission of the S-N bond. The reaction mechanism was formulated as follows (Scheme 2. 11):

$$RSNO \xrightarrow{h\nu} RSNO^* \longrightarrow RS^{\cdot} + NO$$

$$RS^{\cdot} + RSNO \longrightarrow RSSR + NO$$

Scheme 2. 11: The proposed mechanism of photochemical decomposition of *S*-nitrosothiols (Mechanism A).

Josephy *et al.* (1984) and Mile *et al.* (1995) suggested another mechanism. The initial step in *S*-nitrosothiol decomposition is believed to be homolytic cleavage of the S-N bond to give NO and an alkyl thiyl radical. In the final step the thiyl radical will react by combination with another thiyl radical to give the corresponding disulphide (Scheme 2. 12):

$$2\ RSNO \xrightarrow{h\nu} 2\ RS^{\cdot} + 2\ NO$$

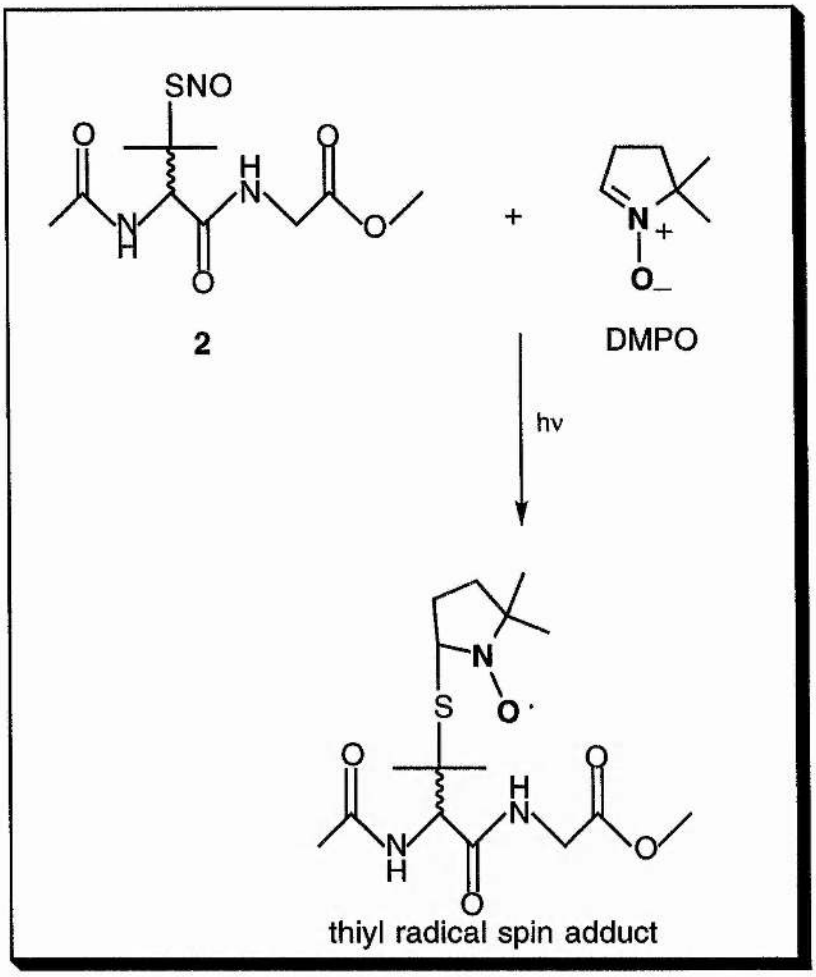
$$2\ RS^{\cdot} \longrightarrow RSSR$$

Scheme 2. 12: The suggested mechanism of photochemical decomposition of *S*-nitrosothiols (Mechanism B).

Several studies of spin trapping of thiyl radicals have been reported using 2-methyl-2-nitrosopropane (MNP; Wargon and Williams, 1975), α-phenyl-*N*-*t*-butylnitrone (PBN; Graceffa, 1983; Janzen *et al.*, 1978), and 5,5-dimethyl-3,4-dihydropyrrole-*N*-oxide (DMPO; Josephy *et al.*, 1984). The DMPO spin adducts gave at least one hundred times more intense signals than the MNP or PBN spin adducts, and in many cases the latter spin traps did not give detectable signals. Probably, DMPO is more reactive towards RS· than are MNP or PBN. DMPO adducts show considerable dependence of hyperfine splitting constants on the structure of the thiyl radical (Josephy *et al.*, 1984).

The intermediates in the photochemical decomposition of tertiary *S*-nitrosothiol **2** in acetonitrile solution were investigated by ESR spectroscopy. When **2** in acetonitrile solution was photolysed in the presence of excess DMPO (Scheme 2. 13) in the cavity of an ESR spectrometer the spectrum shown in Figure 2. 36a was obtained. The spectrum of the major radical was short-lived, and was recorded within a few minutes of initiation of the reaction at 298 K. Inspection of Figure 2. 36a indicates the presence of one major radical showing a triplet hyperfine splitting by one nitrogen and a doublet splitting by one hydrogen atom. In addition, a minor radical is also present which probably has similar basic interactions, but slightly different hyperfine splitting. The spectrum was simulated using a software package due originally to Heinzer (1972; Figure 2.36b) and the best fit hyperfine splitting as given in Table 2. 2. The ESR parameters of the major radical are consistent with the thiyl radical spin adduct. The identity of the minor radical is uncertain, but might be due to a diastereomer of the thiyl radical spin adduct.

From our results, we suggest that the initial step in *S*-nitrosothiol photolytic decomposition might be homolytic cleavage of the S-N bond to give NO and an alkyl thiyl radical. In the final step the thiyl radical will react by combination with another thiyl radical to give the corresponding disulphide. More work should be carried out in this area in order to determine the actual mechanism.



Scheme 2. 13: The alkyl thiyl radical produced by the homolytic decomposition of **2** was detected by ESR spin trapping using (DMPO).

Photochemical decomposition may be important due to the fact that, the endogenous *S*-nitrosothiols decompoosed to the corresponding disulphide and NO by light. Therefore, using laser light may be another way of supplying NO to the cells in the human body instead of using exogenous drugs.

Recently the photochemistry of GSNO has been examined in more detail and the quantum yield and a first-order rate constant have been determined (Sexton *et al.*, 1994). In this reaction it is likely that homolysis of the S-N bond is the primary process.

TABLE 2. 2

Hyperfine interactions in DMPO spin adducts of thiyl radical

Radical	a_N	$a_{\beta-H}$	T / K
Thiyl radical spin adduct [a]	1.48	1.76	298
Minor radical [a, b]	1.40	1.56	298

Footnotes:
(a) in acetonitrile
(b) obtained by computer simulation of spectra, in mT.

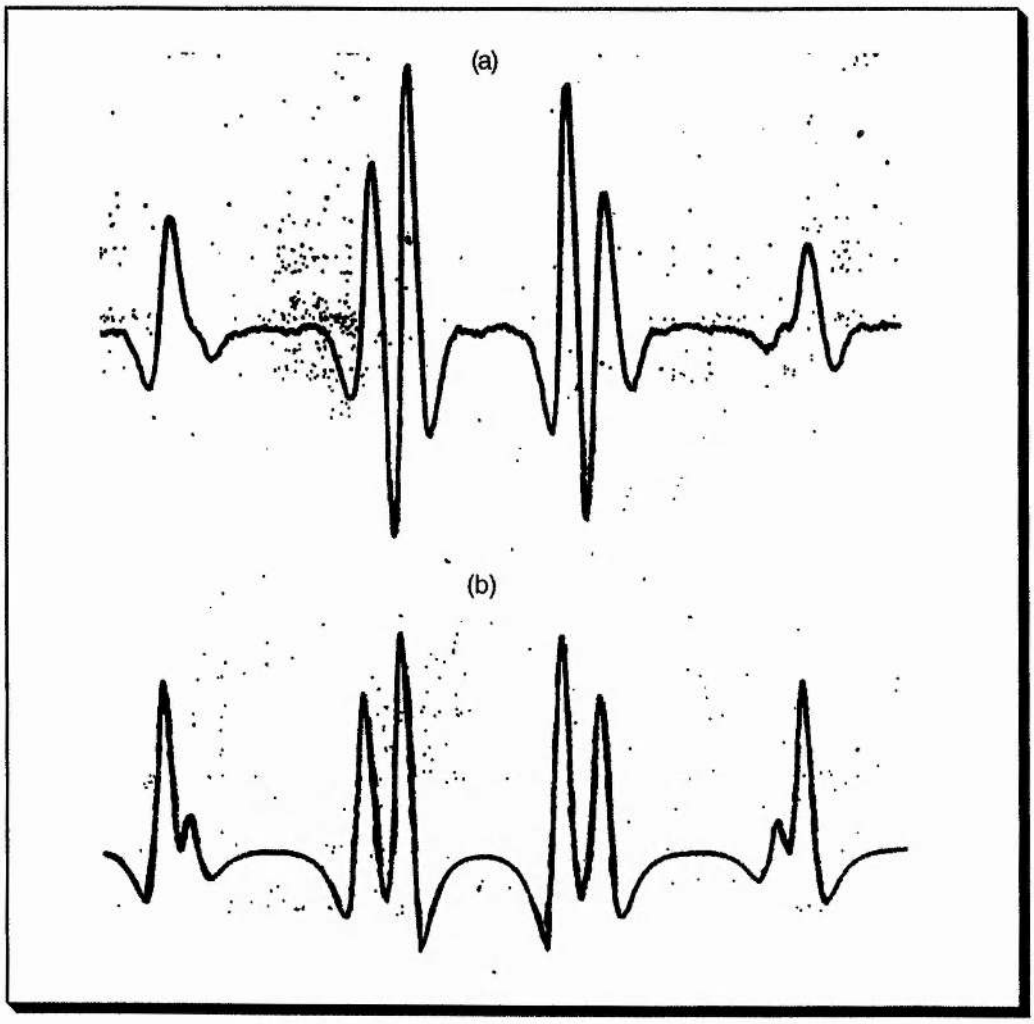


Figure 2. 36: Experimental and simulated 9.3 GHz ESR spectra of spin adducts from DMPO and *S*-nitrosothiol **2** in acetonitrile: (a) experimental spectrum at 298 K (b) simulation of spectra using the parameters given in the table. ESR spectra were recorded on a BRUKER ER 200 D spectrometer.

2. 2. 5. The Thermal Decomposition of *S*-Nitrosothiols

S-Nitrosothiols have also been shown to decompose thermally to give the corresponding disulphide and NO (Josephy *et al.*, 1984):

$$RSNO \xrightarrow{\Delta} 1/2\ RSSR + NO$$

This is similar to photochemical decomposition and probably occurs by homolytic cleavage of the S-N bond to give NO and an alkyl thiyl radical. Then the thiyl radical will react with another thiyl radical to give the corresponding disulphide (Scheme 2. 14):

$$2\ RSNO \xrightarrow{\Delta} 2\ RS^{\cdot} + 2\ NO$$

$$2\ RS^{\cdot} \longrightarrow RSSR$$

Scheme 2. 14: The proposed mechanism of thermal decomposition of *S*-nitrosothiols (Mechanism A).

Another mechanism, may be by homolytic cleavage of the S-N bond to give NO and an alkyl thiyl radical, followed by reaction of the thiyl radical with another *S*-nitrosothiol molecule to give the corresponding disulphide and NO (Scheme 2. 15):

$$RSNO \xrightarrow{\Delta} RS^{\cdot} + NO$$

$$RS^{\cdot} + RSNO \longrightarrow RSSR + NO$$

Scheme 2. 15: The suggested mechanism of thermal decomposition of *S*-nitrosothiols (Mechanism B).

Most *S*-nitrosothiols are unstable at room temperature although some, such as tertiary *S*-nitrosothiols, are quite stable (e.g. SNAP; **1**) (Field *et al.*, 1978).

Previous work in our laboratories has shown that SNAP (**1**), and also GSNO (**13**) decompose thermally to the corresponding disulphide and NO. More work should be carried out in this area in order to determine the actual mechanism.

2. 2. 6. Which Mechanism is Predominant *in vivo*?

Interest in *S*-nitrosothiols was heightened when it was discovered that they occur naturally, principally as the *S*-nitrosothiol of human serum albumin (7 μM) (Stamler *et al.*, 1992a,b,c,d&e) , but fairly high levels of GSNO also occur in human blood plasma (2μM) (Leone and Rhodes, 1996) and human bronchial lavage fluid (0.3 μM) (Gaston *et al.*, 1993). It has been generally assumed that naturally occurring *S*-nitrosothiols act as stores of NO which can be released when required (Stamler *el. al.*, 1992a,b,c,d&e). However, it has not been established how endogenous *S*-nitrosothiols are formed (reaction of NO with a thiol does not produce an *S*-nitrosothiol) nor how NO is released on demand. With GSNO enzymatic cleavage of one of the peptide bonds does result in much more rapid decomposition of the *S*-nitrosothiol moiety (Askew *et al.*, 1995a&b) but this does not address the problem of what causes rupture of the S–NO bond.

As we have seen, *S*-nitrosothiols are susceptible to decomposition by a number of mechanisms. The extent to which each of these different mechanisms causes decomposition of *S*-nitrosothiols *in vivo* is difficult to ascertain quantitatively.

Copper ions are present in the human body in a suitable concentration, and thiols are present in high concentration compared with copper ions:

$$Cu^{2+} + RS^{-} \longrightarrow Cu^{+} + RS^{\cdot}$$

Therefore, copper could be present as Cu^+ ions in the body. We proved that Cu^+ is the true catalyst for the decomposition of *S*-nitrosothiols and it is possible that endogenous *S*-nitrosothiols are decomposed by Cu^+ to release NO on demand.

S-Nitrosothiols *in vivo* probably also undergo transnitrosation with cysteine and *S*-nitrosocysteine requires Cu^+ for NO release.

GSNO, a naturally occurring *S*-nitrosothiol, will probably undergo enzymatic metabolism to give *S*-nitrosocysteinylglycine and this will be decomposed again by copper ion catalysis to give the corresponding disulphide and NO. Therefore, all the previous mechanisms need copper ions (i.e. Cu^+) in order to release NO.

Under other circumstances the photochemical mechanism may be important. The vascular smooth muscle cells contain a finite store of a photosensitive molecule(s) which is capable of releasing NO when irradiated with UV or visible light. This store can be exhausted rapidly by exposing vessels to laser light but subsequently regenerates in the dark with an absolute dependence upon endothelium-derived NO (Megson, 1993). Work by Megson *et al.* provides evidence that the photosensitive 'store' is an *S*-nitrosothiol or *S*-nitrosoprotein, since it is known that ethacrynic acid, a thiol alkylating agent, also prevents repriming of the 'store'. As, in some cases, it may not be possible to give a patient *S*-nitrosothiol as an NO-donor it might be possible to rely on endogenous *S*-nitrosothiol which is decomposed by a laser beam to target cancer cells. The laser beam will increase the endogenous decomposition of *S*-nitrosothiols to give a suitable dose of NO and the dose may be regulated by controlling the intensity and the width of the ray. There is no harmful effect from the process by using it for short periods to solve limited problems.

In the human body, some decomposition of the endogenous *S*-nitrosothiols (e.g. CysNO) may be due to thermal decomposition.

In our opinion, the predominant mechanism will be Cu^+ catalysis for the decomposition of *S*-nitrosothiols *in vivo*. If, therefore, naturally occurring *S*-nitrosothiols are a store of NO which can be released on demand at a suitable rate, dose, and time we need Cu^+ in the cell. Both cysteine and copper are present in the cell at reasonable concentrations and it has been proposed that copper is, indeed, present as Cu^+. This is a completely new biological role for Cu^+.

In the next sections we will describe several experiments which prove that Cu^+ is required for endogenous *S*-nitrosothiol decomposition.

2. 3. The Chemical Stability of *S*-Nitrosothiols

The chemical stability of *S*-nitrosothiols in solution depends upon two main factors: a) the presence of metal ions in the buffer; b) the presence of the corresponding thiols as an impurities. Here we report the chemical stability of *S*-nitrosothiols in the presence of chelating agent such as EDTA and neocuproine and in the presence of the corresponding thiol.

2. 3. 1. The Effect of Metal Ions on Decomposition of the *S*-Nitrosothiols

In this section new *S*-nitrosothiols **2-11** will be considered and their decomposition rates compared with those of SNAP (**1**) and GSNO (**13**). Full details of the way in which copper ions bring about NO release from *S*-nitrosothiols are given in the previous section (Section 2. 2). However, there is spontaneous decomposition, as well as a reaction catalysed by Cu^{2+} ions, which leads to the release of NO. In both pathways the reactivities of **2-11** are considerably less than that of SNAP and more than that of GSNO.

2. 3. 1. 1. The Effect of EDTA on the Decomposition of *S*-Nitrosothiols

Workers in the past have discussed the stability of *S*-nitrosothiols but have not taken into account the crucial role of metal ions, at concentrations to be found even in distilled water, in the release of NO. When we say that SNAP is more stable than SNC we mean simply that it is possible to prepare and isolate SNAP but SNC has to be prepared and used *in situ*. It appears that the two β-methyl groups in SNAP are responsible for this difference and this is why we have made dipeptides where the thiol-containing component is β,β-dimethylcysteine rather than cysteine. To give the term stability a more precise meaning we must compare the rate of NO-release from *S*-nitrosothiols in solution in the absence of light and in the presence and absence of metal ions. Askew *et al.* have done this for a number of *S*-nitrosothiols and their results in are given in previous work (Askew *et al.*, 1995a&b). We carried out a similar study with **2-11** and can compare their stabilities with those of SNAP (**1**) and GSNO (**13**). Unfortunately, **2-11** are not readily soluble in an aqueous buffer and addition of 10% DMSO is necessary for solution. To make the comparison precise we used the same DMSO-buffer mixture to study NO-release from SNAP (**1**) and GSNO (**13**).

Addition of EDTA to the buffer effectively removes all metal ions and the disappearance of absorbance at 330-340 nm, due to loss of NO, must be a spontaneous, thermal reaction. Over a short time there appeared to be no reaction, but monitoring over a 24 h period indicated some decomposition which may be due to thermal decomposition. The results are displayed in Figure 2. 37 and show that the decomposition of **2** is somewhat slower than that of SNAP and faster than that of GSNO but the difference is not great.

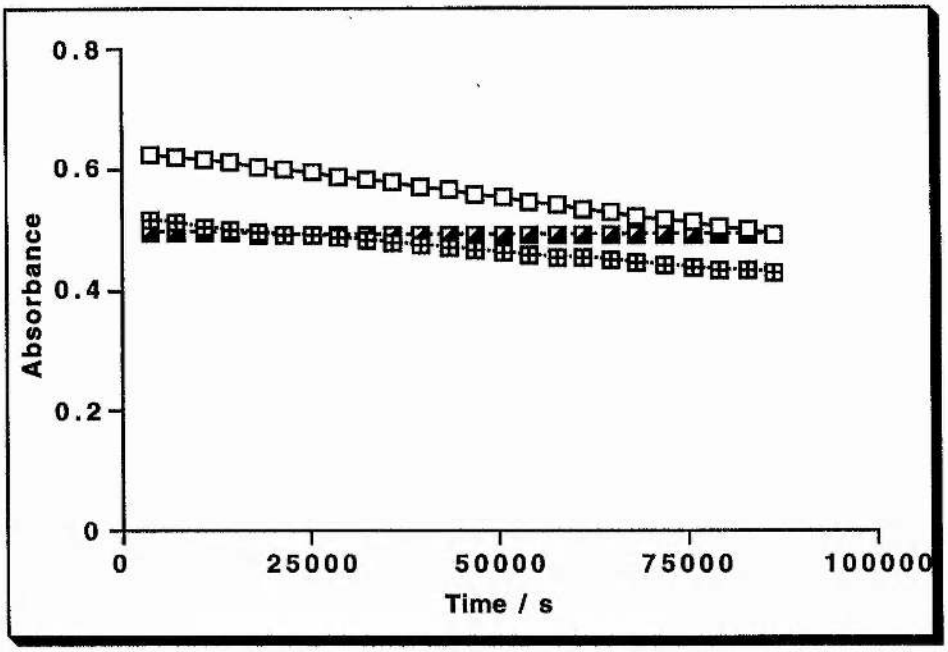


Figure 2. 37: Absorbance-time plots for the decomposition of (a) [SNAP] = 5 x 10^{-4} mol dm^{-3} (square) (b) [**2**] = 5 x 10^{-4} mol dm^{-3} (half filled square) (c) [GSNO] = 5 x 10^{-3} mol dm^{-3} (segmented square), during 24 hours (at pH = 7.4, 340 nm, and 30 °C).

Addition of copper(II) nitrate to the buffer had a dramatic effect on the decomposition of SNAP, **2-11**, and GSNO, the data are displayed in Figure 2. 38 and Table 2. 3. Inspection of the curves shows that, at identical Cu^{2+} ion concentrations, **2** decomposes less rapidly than SNAP (**1**) and more rapidly than GSNO. However, we can give a somewhat more precise measure of this difference.

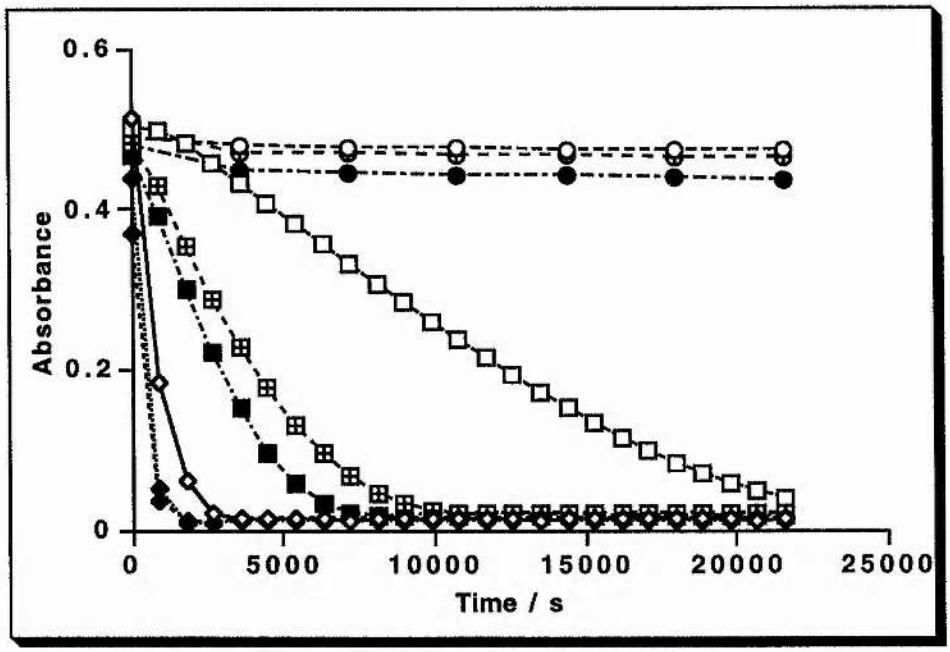


Figure 2. 38: Absorbance-time plots for the decomposition of (a) [SNAP] = 5 x10^{-4} mol dm^{-3}, [EDTA] = 1x 10^{-5} mol dm^{-3}, and [Cu^{2+}] 1x 10^{-5}, 2 x10^{-5}, 3 x10^{-5} mol dm^{-3} respectively (circle) (b) [**2**] = 5 x10^{-4} mol dm^{-3}, [EDTA] = 1x 10^{-5} mol dm^{-3}, and [Cu^{2+}] 1x 10^{-5}, 2 x10^{-5}, 3 x10^{-5} mol dm^{-3} respectively (square) (c) [GSNO] = 5 x10^{-4} mol dm^{-3}, [EDTA] = 1x 10^{-5} mol dm^{-3}, and [Cu^{2+}] 1x 10^{-5}, 2 x10^{-5}, 3 x10^{-5} mol dm^{-3} respectively (diamond).

As noted from previous work by Askew *et al.* (1994) Cu^{2+} catalysed SNAP decomposition obeys reasonably good first-order kinetics over a restricted range of Cu^{2+} concentrations. We found this also to be the case using a DMSO-buffer mixture and the data are displayed in Table 2. 3.

Inspection of Table 2. 3 shows that, if we take the value of k for SNAP, and the values of k for **2-11**, the ratio of the two k values is ~ 10, indicating that the Cu^+ catalysed decompositions of **2-11** occur less readily than that of SNAP. So, for both the spontaneous and the Cu^+ catalysed decomposition of **2-11** are less reactive than SNAP. For the latter reaction it could be that, because of the additional amino acid moiety, Cu^+ binds less readily to **2-11** than to SNAP.

Also, if we take the values of k for **2-11**, and the value of k for GSNO, the ratio of the two k values is ~ 100, indicating that the Cu^+ catalysed decomposition of GSNO occurs less readily than that of **2-11**. So, for both the spontaneous and the Cu^+ catalysed reactions GSNO is less reactive than **2-11**. For the latter reaction it could be that, because of the additional amino acid moiety, Cu^+ binds less readily to GSNO than to **2-11**.

Moreover, if we take the value of k for SNAP, and the value of k for GSNO, the ratio of the two k values is ~ 1000, indicating that the Cu^+ catalysed decomposition of GSNO occurs less readily than that of SNAP. So, for both the spontaneous and the Cu^+ catalysed decomposition, GSNO is less reactive than SNAP. For the latter reaction it could be that, because of the additional two amino acid moieties, Cu^+ binds less readily to GSNO than to SNAP.

TABLE 2. 3

Values of *k* for the decomposition of the *S*-nitrosothiols (**1-13**; 5.0 x 10^{-4} mol dm^{-3}) in the presence of added EDTA (1.0 x 10^{-5} mol dm^{-3}) and various Cu^{2+} ions concentrations

Compound Number	Rate constant (k/s^{-1})		
	Added Cu^{2+} (1.0x10^{-5} mol dm^{-3})*	Added Cu^{2+} (2.0x10^{-5} mol dm^{-3})**	Added Cu^{2+} (3.0x10^{-5} mol dm^{-3})***
SNAP (**1**)	5.0 x 10^{-4}	11.5 x 10^{-4}	13.7 x 10^{-4}
2	3.3 x 10^{-5}	13.3 x 10^{-5}	18.3 x 10^{-5}
3	1.6 x 10^{-5}	11.6 x 10^{-5}	23.0 x 10^{-5}
4	1.6 x 10^{-5}	8.3 x 10^{-5}	18.3 x 10^{-5}
5	1.6 x 10^{-5}	10.0 x 10^{-5}	15.0 x 10^{-5}
6	1.7 x 10^{-5}	8.3 x 10^{-5}	16.7 x 10^{-5}
7	6.2 x 10^{-5}	7.0 x 10^{-5}	10.8 x 10^{-5}
8	1.7 x 10^{-5}	9.3 x 10^{-5}	9.3 x 10^{-5}
9	insoluble in the buffer	insoluble in the buffer	insoluble in the buffer
10	2.5 x 10^{-5}	7.8 x 10^{-5}	10.5 x 10^{-5}
11	2.8 x 10^{-5}	8.9 x 10^{-5}	12.5 x 10^{-5}
12	4.3 x 10^{-5}	7.8 x 10^{-5}	12.3 x 10^{-5}
GSNO (**13**)	2.5 x 10^{-7}	3.7 x 10^{-7}	4.3 x 10^{-7}

(* r = 1.000, 0.993, 0.997, 0.994, 0.998, 0.999, 0.997, 0.999, insoluble in the buffer, 0.995, 0.975, 1.000, and 0.998 respectively).
(** r = 0.996, 0.997, 0.999, 1.000, 0.999, 1.000, 0.0937, 1.000, insoluble in the buffer, 0.999, 1.000, 0.999, and 1.000 respectively).
(*** r = 0.995, 0.992, 0.997, 1.000, 1.000, 1.000, 0.980, 1.000, insoluble in the buffer, 1.000, 0.998, 0.997, and 1.000 respectively).

2. 3. 1. 2. The Effect of Neocuproine on *S*-Nitrosothiols Decomposition

Neocuproine is a complexing agent specific for Cu^+ (Smith and McCurdy, 1952). Addition of neocuproine to give a 5 x 10^{-4} M solution in phosphate buffer at pH 7.4 completely suppresses the decomposition of *S*-nitrosothiols (**1-13**) which normally occurs in about 30 minutes to 7 days, depending upon the structure of *S*-nitrosothiols, in a clean first-order reaction (Figure 2. 39). The effect is less pronounced at lower concentrations of neocuproine (Figure2. 22). As has been reported previously (Askew, 1994), EDTA has the same effect but it is a non discriminating complexing agent. It is the specificity of neocuproine for Cu^+ which allows us to propose that it is the interaction of Cu^+ with the *S*-nitrosothiol which results in NO release (Section 2. 2). *S*-Nitrosothiols other than (**1-13**) have been examined in the same way and the stabilising effect of neocuproine is general.

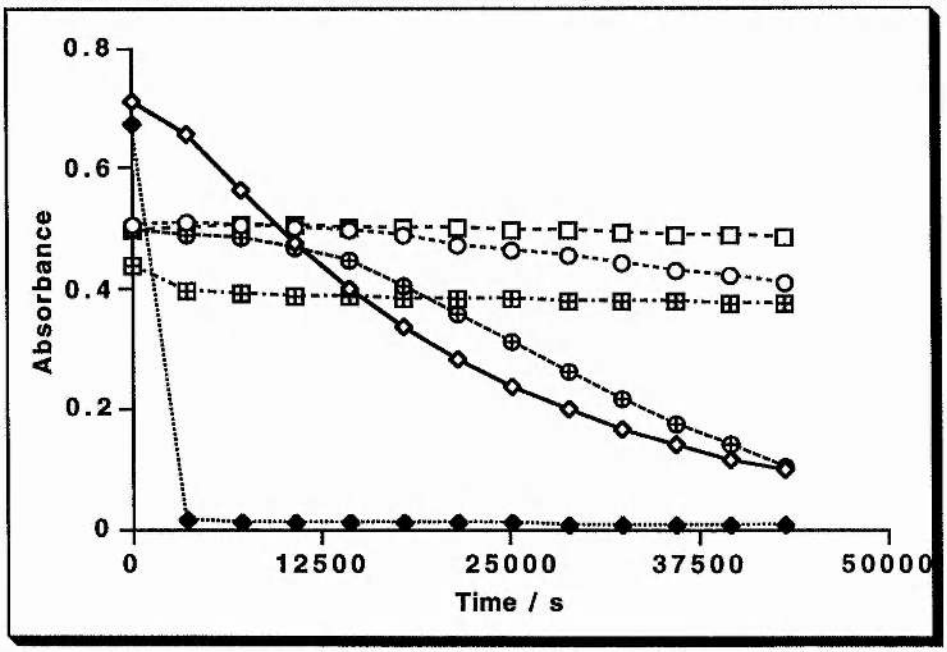


Figure 2. 39: Effect of neocuproine on the rate of decomposition of SNAP (5 x 10^{-4} mol dm^{-3}) in the presence of added copper (1.8 x 10^{-5} mol dm^{-3}); [neocuproine] = 5 x 10^{-4}, 0 mol dm^{-3} respectively (circles), **2** (5 x 10^{-4} mol dm^{-3}) in the presence of added copper (1.8 x 10^{-5} mol dm^{-3}); [neocuproine] = 5 x 10^{-4}, 0 mol dm^{-3} respectively (squares), GSNO (5 x 10^{-4} mol dm^{-3}) in the presence of added copper (1.8 x 10^{-5} mol dm^{-3}); [neocuproine] = 5 x 10^{-4}, 0 mol dm^{-3} respectively (diamonds).

Inspection of Table 2. 4 shows that in the case of neocuproine we found similar behaviour to that of EDTA. In view of this similarity we can conclude that the only catalytic effect occurring in buffered solution is due to the presence of Cu^+ ions.

TABLE 2. 4

Values of *k* for the decomposition of the *S*-nitrosothiols (**1-13**; 5.0 x 10^{-4} mol dm^{-3}) in the presence and absence of added neocuproine (5.0 x 10^{-4} mol dm^{-3}) and in the presence of added Cu^{2+} (1.8 x 10^{-5} mol dm^{-3})

Compound Number	Rate Constant (k/s^{-1})	
	Added Neocuproine	No Added Neocuproine
SNAP (**1**)	no decomposition	4.4 x 10^{-4}
2	no decomposition	1.5 x 10^{-5}
3	no decomposition	1.5 x 10^{-5}
4	no decomposition	1.3 x 10^{-5}
5	no decomposition	0.6 x 10^{-5}
6	no decomposition	1.3 x 10^{-5}
7	no decomposition	1.7 x 10^{-5}
8	no decomposition	8.5 x 10^{-5}
9	insoluble in the buffer	insoluble in the buffer
10	no decomposition	2.2 x 10^{-5}
11	no decomposition	0.7 x 10^{-5}
12	no decomposition	8.7 x 10^{-5}
GSNO (**13**)	no decomposition	4.9 x 10^{-7}

(r = 1.000, 0.970, 1.000, 1.000, 1.000, 0.989, 0.999, 0.986, the compound is insoluble in the buffer, 1.000, 0.998, 0.999, and 0.994 respectively).

2. 3. 2. The Effect of Thiol on the Decomposition of *S*-Nitrosothiols

There is direct evidence for the involvement of Cu^{+} in nitrosothiol decomposition from a study of the effect of added thiol on the decomposition of *S*-nitrosothiols. The results of a typical experiment are shown in Figure 2. 40. The observation is consistent with the catalytic effect of Cu^{+}, rather than Cu^{2+}, as it is known that cysteine (Stricks and Kolthoff, 1951) and penicillamine (Laurie and Prime, 1979) reduce Cu^{2+} to Cu^{+} (Section 2. 2):

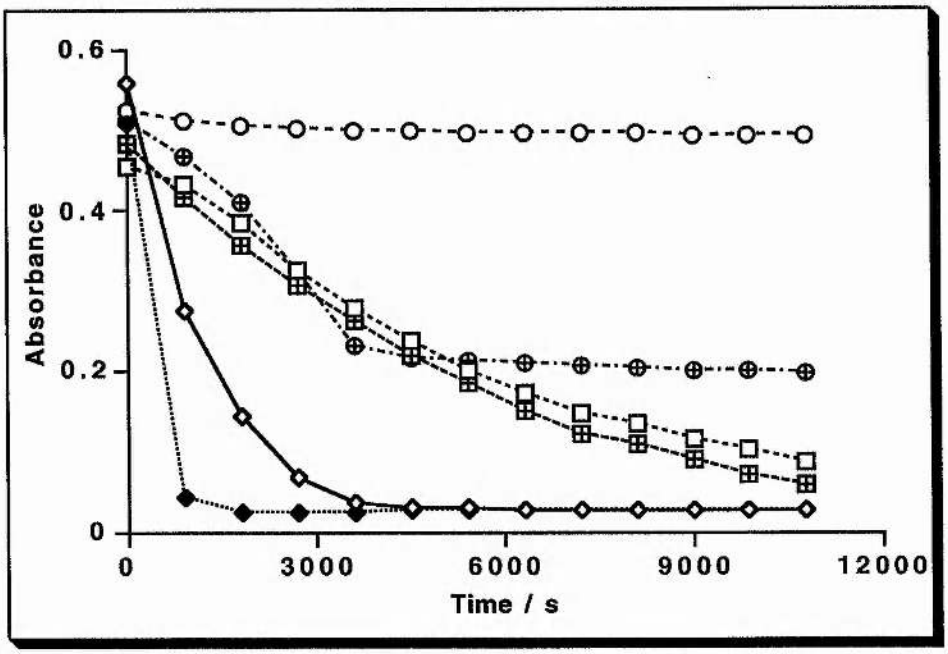


Figure 2. 40: Effect of added thiol on the copper-catalysed decomposition of SNAP (5 x 10^{-4} mol dm^{-3}) (a) [added Cu^{2+}] = 1.4 x 10^{-5} mol dm^{-3}; [NAP] = 0 (diamond)(b) [added Cu^{2+}] = 1.4 x 10^{-5} mol dm^{-3}; [NAP] = 2.5 x 10^{-4} mol dm^{-3} (segmented diamond), **2** (5 x 10^{-4} mol dm^{-3}) (a) [added Cu^{2+}] = 1.4 x 10^{-5} mol dm^{-3}; [NAP-Gly-OMe] = 0 (square) (b) [added Cu^{2+}] = 1.4 x 10^{-5} mol dm^{-3}; [NAP-Gly-OMe] = 2.5 x 10^{-4} mol dm^{-3} (segmented square), GSNO (5 x 10^{-4} mol dm^{-3}) (a) [added Cu^{2+}] = 1.4 x 10^{-5} mol dm^{-3}; [GSH] = 0 (circle) (b) [added Cu^{2+}] = 1.4 x 10^{-5} mol dm^{-3}; [GSH] = 2.5 x 10^{-4} mol dm^{-3} (segmented circle).

TABLE 2. 5

Values of *k* for the decomposition of the *S*-nitrosothiols (**1-13**; 5.0 x 10^{-4} mol dm^{-3}) in the absence and presence of added the corresponding thiol (2.5 x 10^{-4} mol dm^{-3}) and in the presence of added Cu^{2+} (1.8 x 10^{-5} mol dm^{-3})

Compound Number	Rate Constant (k/s^{-1})	
	No Added Thiol*	Added Thiol**
SNAP (**1**)	3.6 x 10^{-4}	18.1 x 10^{-4}
2	8.0 x 10^{-5}	8.0 x 10^{-5}
3	2.0 x 10^{-5}	7.5 x 10^{-5}
4	0.8 x 10^{-5}	6.7 x 10^{-5}
5	2.2 x 10^{-5}	9.2 x 10^{-5}
6	3.0 x 10^{-5}	10.3 x 10^{-5}
7	3.3 x 10^{-5}	2.5 x 10^{-5}
8	6.3 x 10^{-5}	6.3 x 10^{-5}
9	insoluble in the buffer	insoluble in the buffer
10	3.8 x10^{-5}	4.7 x 10^{-5}
11	0.6 x 10^{-5}	6.2 x 10^{-5}
12	34.2 x 10^{-5}	5.0 x 10^{-5}
GSNO (**13**)	3.7x 10^{-7}	23.3 x 10^{-5}

(* r = 1.000, 0.995, 0.988, 0.999, 0.999, 0.999, 0.995, 1.000, the compound is insoluble in the buffer, 0.999, 0.999, 0.937, and 0.999 respectively).

(** r = 0.981, 0.995, 0.998, 0.970, 1.000, 0.996, 0.979, 1.000, the compound is insoluble in the buffer, 0.997, 0.999, 0.999, and 0.974 respectively).

The results in Table 2. 5 show clearly that addition of thiol in the presence of added Cu^{2+} increases the rate of NO release. This result is consistent with the mechanism of reaction set out earlier. The effect is greatest with SNAP and GSNO, and least with **2-12**. Also, these results explain the apparently contradictory ones in the literature (Feelisch *et al.*, 1994) some of which report catalysis of RSNO decomposition by added thiols whilst other find a reduction in rate upon thiol addition (Section 2. 2. 1. 1. 3. 1).

The reason for the great catalytic effect of Cu^{+} ions is not, at the moment, understood. Probably Cu^{+} ions bind to *S*-nitrosothiols but, as the latter have at least four binding sites, the structure of the complex is far from certain. This matter has been discussed briefly in previous work (Askew *et al.*, 1995a&b). Cu^{+} ions are rapidly oxidised to Cu^{2+} by dioxygen but we found that for most *S*-nitrosothiols purging the solutions with argon had little effect on the kinetics. This would be the case if binding of Cu^{+} to the *S*-nitrosothiol is faster than oxidation.

It is clear from what has been reported in this thesis that all previous studies of the *in vitro* release of NO from *S*-nitrosothiols which do not take into account the production of Cu^{+} ions from adventitious copper present in the buffer and thiol contaminating the *S*-nitrosothiol need reinterpretation. This need for reinterpretation is true even for *S*-nitrosocysteine which, until now, has been regarded as thermally very unstable. Its formation had been seen as a mechanism whereby more stable *S*-nitrosothiols (such as GSNO) could release NO *in vivo* as GSNO readily transfers NO^{+} to cysteine with formation of *S*-nitrosocysteine (Barnett *et al.*, 1994; Meyer *et al.*, 1994; Arnelle and Stamler, 1995), which could then undergo thermal homolytic fission to release NO. Transnitrosation reactions are very fast (Barnett *et al.*, 1994). However, addition of EDTA to a buffered solution of *S*-nitrosocysteine, prepared *in situ*, renders the compound relatively stable. It is clear that the metal ion catalysed pathway for the decomposition of *S*-nitrosothiols is not a minor one. In the presence of even micromolar quantities of Cu^{+} it is thermal fission which is of little importance.

2. 4. Study of the Pharmacological and Physiological Properties of *S*-Nitrosothiols

In this section, the pharmacological and physiological properties (smooth muscle relaxation and inhibition of platelet aggregation) of compounds **2-12** are compared with those of SNAP and GSNO in an effort to understand the relationship of structure to chemical stability and biological activity. Also, there is a report of an investigation of the effect of neocuproine on vasodilator responses to bolus injections of SNAP and GSNO in an *ex vivo* model, using a rat tail artery preparation. As well, there is a report of a direct proof from the literature that Cu^{+} is involved in the biological activity of GSNO on platelet aggregation and soluble guanylate cyclase.

2. 4. 1. Smooth Muscle Relaxation

SNAP and GSNO are NO-donor drugs whose biological activities in water are well documented (Megson, 1993; Askew, 1994). Here we compare the biological activities of SNAP (**1**) and GSNO (**13**) with those of *S*-nitrosated dipeptides (**2-12**) in 10% aqueous DMSO.

Male Wistar rats (300 - 400 g) were killed by cervical dislocation and their tails removed. Lengths of tail artery (8 - 11 mm) were dissected free, cannulated and perfused internally with Krebs solution (37 °C) at a constant flow rate (2 cm^3 min^{-1}). Temperature was maintained by superfusion with pre-warmed Krebs solution. Vessels were precontracted with phenylephrine hydrochloride (PE; 2 - 8 μM) in the internal perfusate, generating perfusion pressures of 100 - 120 mm Hg, measured upstream of the artery by a differential pressure transducer (SenSym; SCX15DNC). Microinjections (10 μl) of SNAP or GSNO

were made through a resealable rubber septum located immediately upstream of the cannula (transit time *ca.* 4 sec). Increasing control doses (10^{-7} - 10^{-3} M) of *S*-nitrosothiol injected into the internal perfusate caused relaxations which were allowed to recover fully before subsequent doses were administered. Doses were then repeated in the presence of ferrohaemoglobin, a recognised NO 'scavenger' (Hb; 15 μM; prepared by the method described by Martin *et al.* 1985; Figures 2. 41 - 2. 54 respectively) for comparison with controls. Results are expressed as the peak amplitude of relaxation as a % of PE-induced pressure. Results are mean + S.E.M. of 5-7 separate experiments. They were compared by unpaired students' *t*-test and $p < 0.05$ was considered as statistically significant (details of the rat tail artery preparation are reported elsewhere ; Flitney *et al.*, 1992).

For statistical comparison a student's paired/unpaired *t*-test was carried out individual data points. All the plots obtained by use of a cricket graph program.

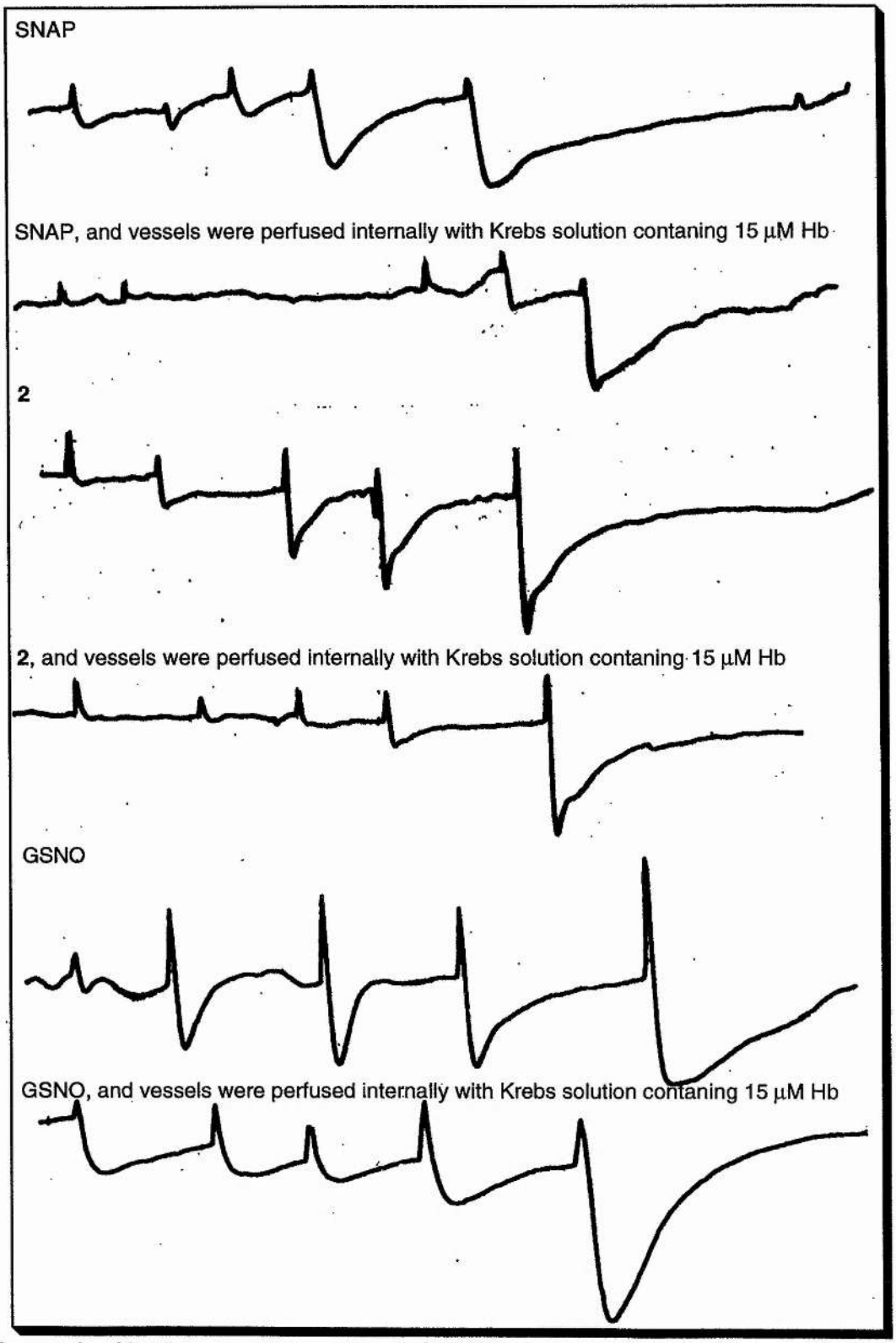


Figure 2. 41: Pressure recordings showing the vasodilator effects of 10 μl microinjections of SNAP, **2**, and GSNO in the absence and presence of 15 μM Hb.

S-Nitrosothiols (**1-13**) are fast acting and potent vasodilators. As can be seen in Figure 2. 41, the responses are sharp, appearing soon after injection, and there is full recovery, in a matter of minutes, to the original precontracted tone of the artery.

When traces like those in Figure 2. 41 are translated into log dose-response curves like those shown in Figures 2. 42 - 2. 54 it is evident that all *S*-nitrosothiols (**1-13**) are effective vasodilators, and are excellent NO-donor drugs.

The results show that bolus injections of all compounds into the internal perfusate elicit transient responses which recover rapidly. Hb significantly inhibits, but does not abolish, the responses to all drugs, with SNAP being less sensitive than the others.

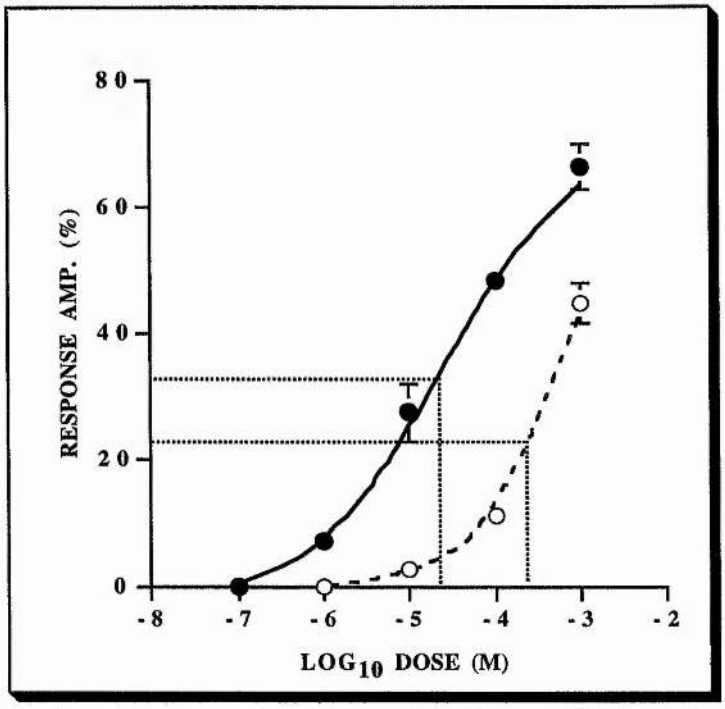


Figure 2. 42: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of SNAP; **1**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols shows responses in the presence of oxyhaemoglobin (15 μM).

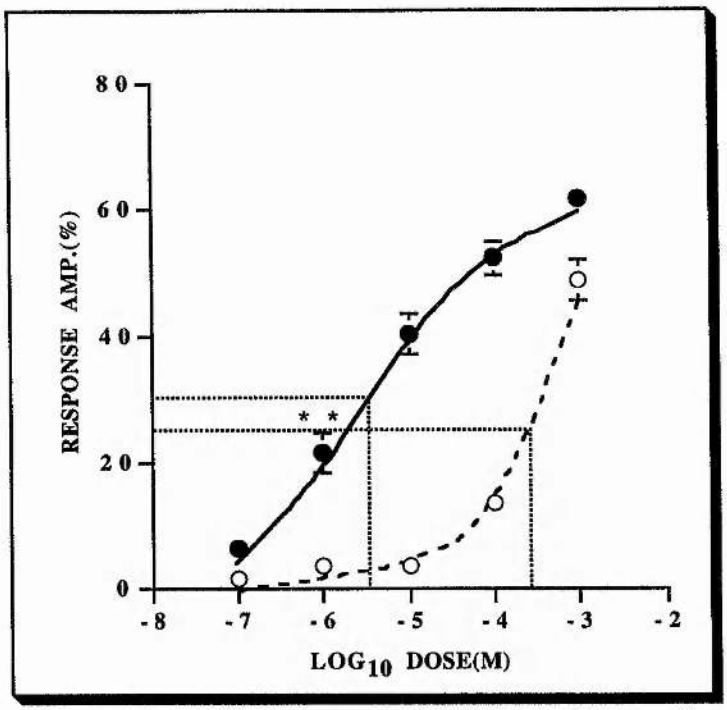


Figure 2. 43: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of **2**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbol shows responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine the statistical significance of responses to **2** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

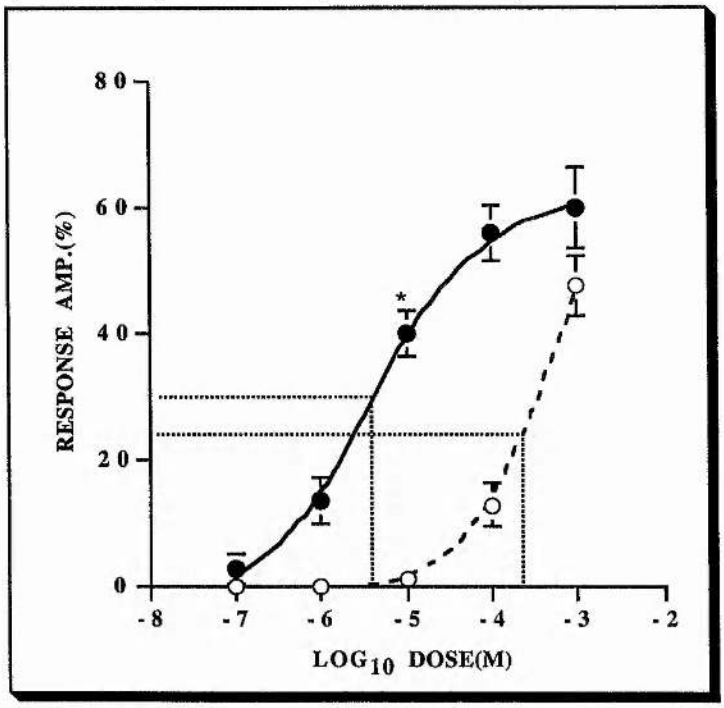


Figure 2. 44: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of **3**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols show responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine the statistical significance of responses to **3** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

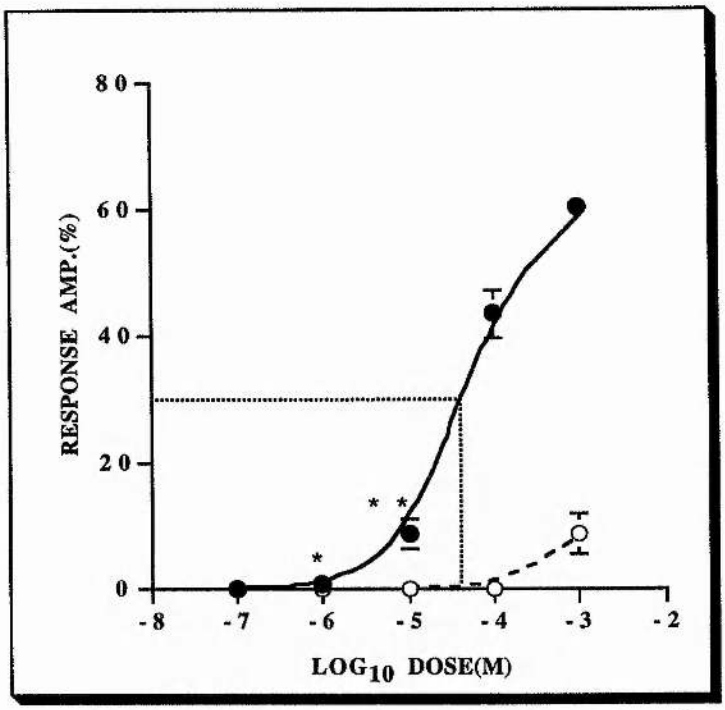


Figure 2. 47: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of **6**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols show responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine the statistical significance of responses to **6** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

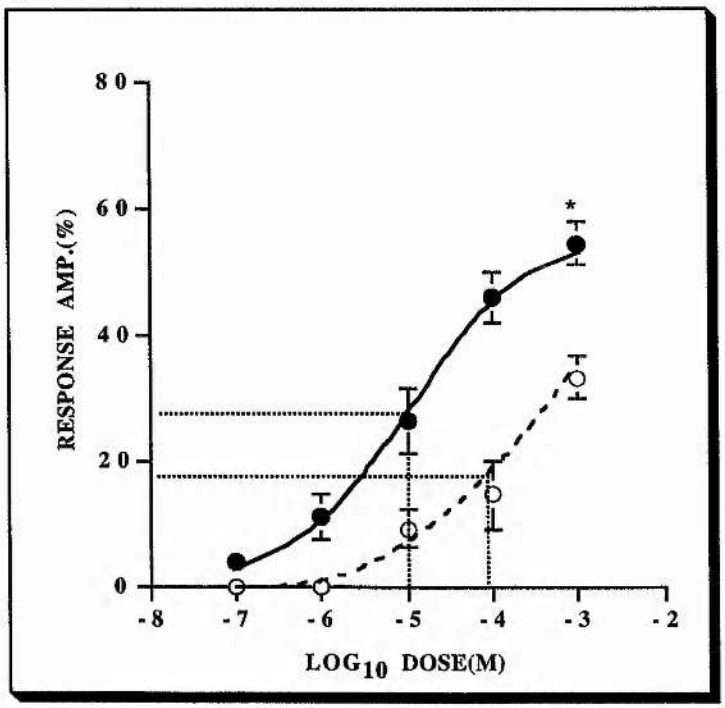


Figure 2. 48: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of **7**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols show responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine the statistical significance of responses to **7** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

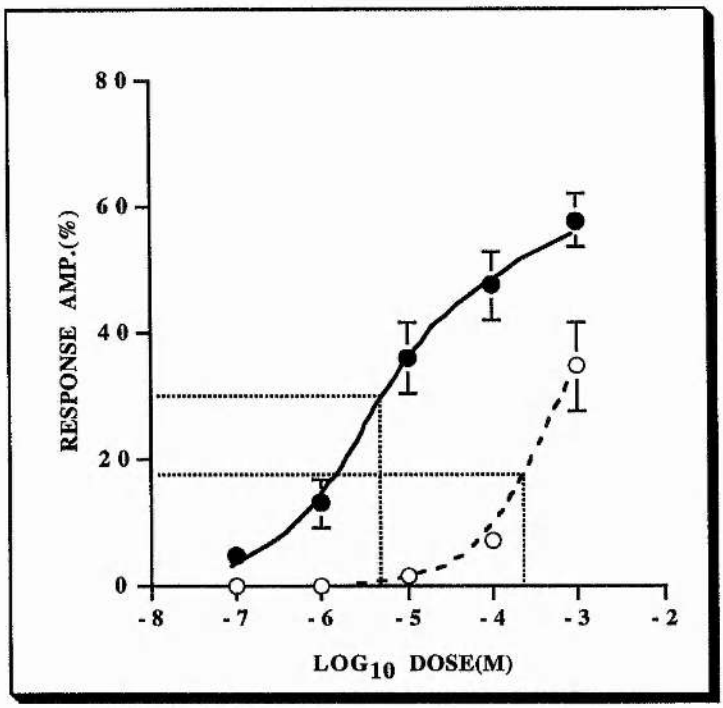


Figure 2. 49: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of **8**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols show responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine the statistical significance of responses to **8** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

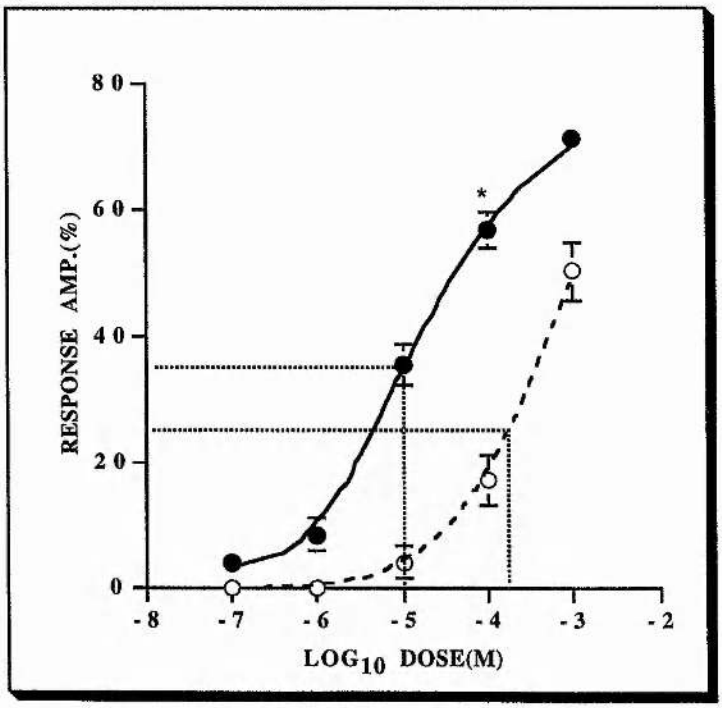


Figure 2. 50: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of **9**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols show responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine statistical the significance of responses to **9** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

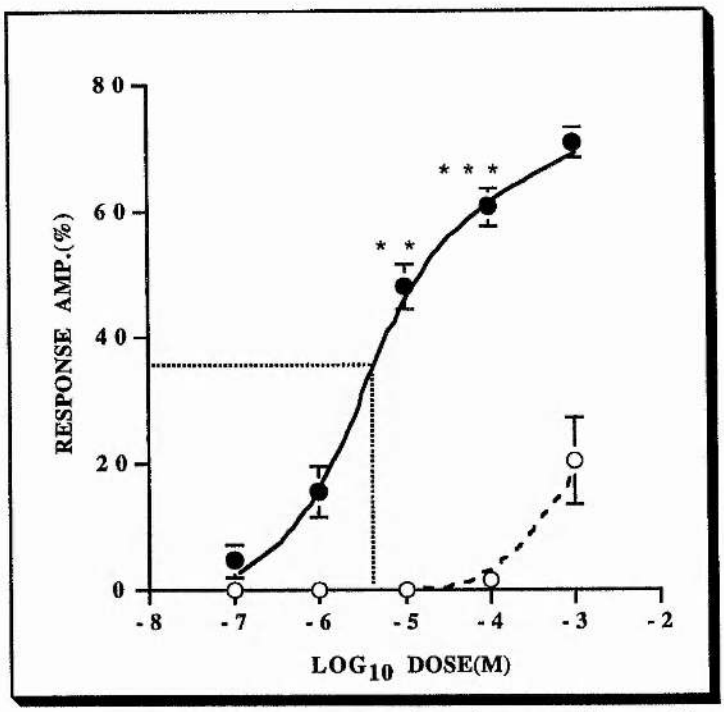


Figure 2. 51: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of **10**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols show responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine the statistical significance of responses to **10** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

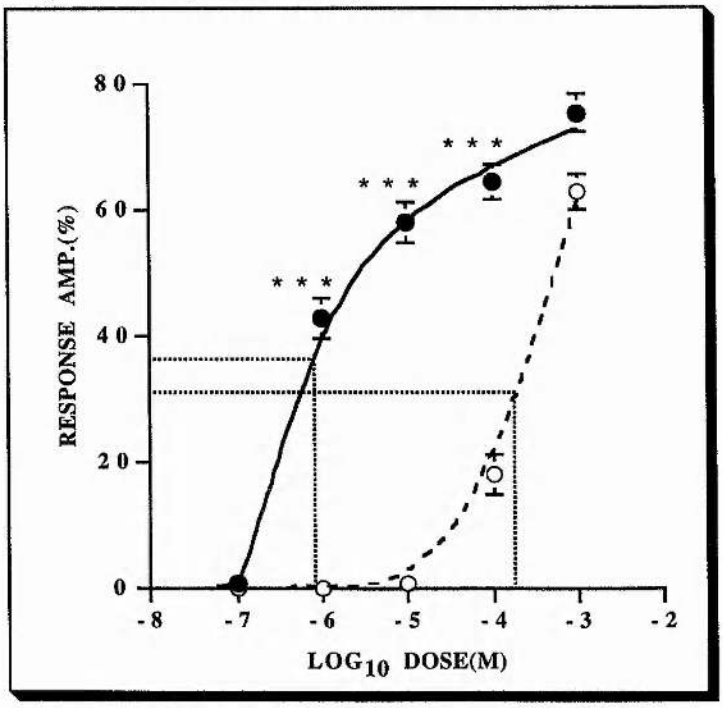


Figure 2. 52: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of **11**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols shows responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine the statistical significance of responses to **11** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

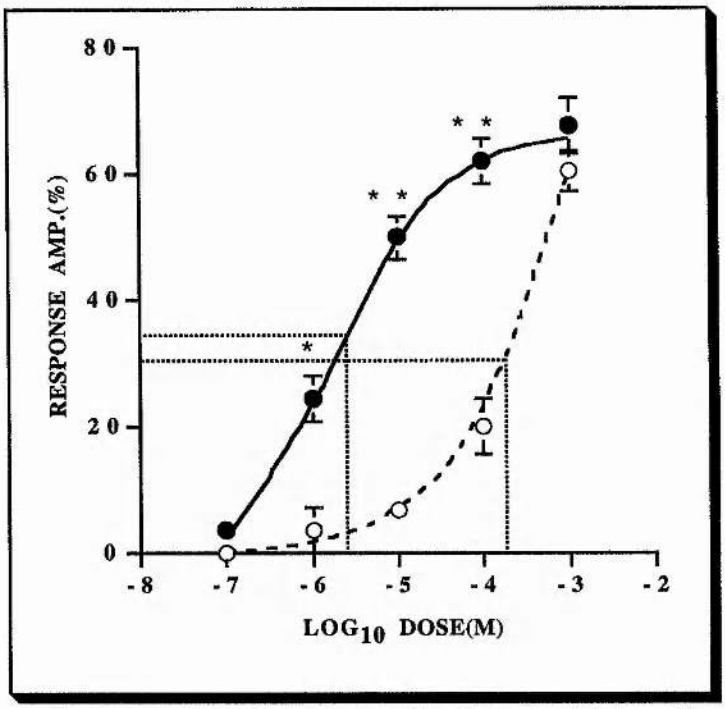


Figure 2. 53: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of **12**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols show responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine the statistical significance of responses to **12** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

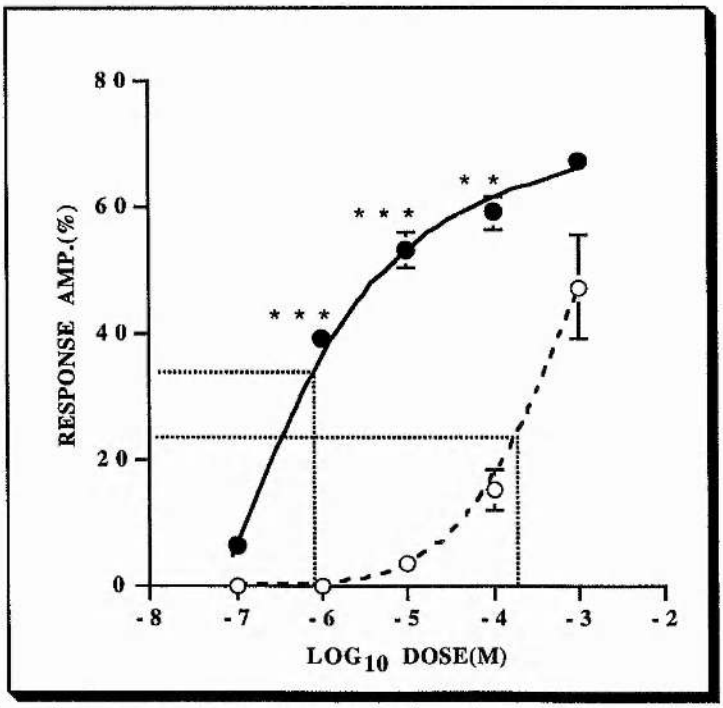


Figure 2. 54: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of GSNO;**13**. Filled symbols show responses in the absence of oxyhaemoglobin and open symbols show responses in the presence of oxyhaemoglobin (15 μM). Unpaired *t*-tests were carried out to determine the statistical significance of responses to **13** compared to those of SNAP. (*= P<0.05, **= P<0.01, ***= P<0.001).

Figures 2. 42 - 2. 54 show log dose-response curves (closed circles) derived from experiments involving microinjections of (a) SNAP, (b) **2-12** and (c) GSNO. In all cases, ED_{50} values have been determined using the response to 10^{-3} M injections as the maximum. 10^{-2} M injections could not be carried out since DMSO in concentrations greater than 10% produced a significant relaxation response which would preclude the effect of the drug alone. 'ED_{50}' values of 0.8 - 45 μM have been estimated for SNAP , **2-12** , and GSNO respectively. The addition of 15 μM Hb to the internal perfusate caused a significant reduction in potency for all compounds (open circles). Hb is a well recognised NO scavenger and these results indicate that NO is the effector agent in vasodilation by all thirteen compounds. Figures 2. 41 -2. 54 show the effect of Hb on vasodilator responses to all compounds. There is no significant difference between the response amplitudes at any concentration. Therefore, responses to **2-12** and GSNO were inhibited to a greater extent than those to SNAP. ($P<0.01$ for all doses of compounds **1-13** when compared to control values using an unpaired Student's *t*-test).

These results show that all *S*-nitrosothiols (**1-13**) were capable of causing vasodilation in pre-contracted lengths of isolated rat tail artery. **11** was significantly more effective at causing vasodilation (Table 2. 6) than the others. This result was contrary to that expected from the chemical stability (Section 2. 3).

ED_{50} : equivalent dose for 50% response.

TABLE 2. 6

A comparison of the vasodilator effectiveness of SNAP, **2-12**, and GSNO on rat tail artery

Compound Number	ED_{50} (μM) * 10^{-5} M
SNAP (**1**)	20 .....
2	4 ↑
3	3.5 ↑*
4	3 ↑
5	1.5 ↑***
6	45 ↓**
7	10 ↑
8	4.5 ↑
9	9.5 ↑
10	5 ↑**
11	0.8 ↑***
12	2 ↑**
GSNO (**13**)	0.9 ↑***

↑: the drug was significantly more effective at causing vasodilation than SNAP.

↓: the drug was significantly less effective at causing vasodilation than SNAP.

Statistical analysis of data in the table:
statistical analysis on the data was carried out using an unpaired students *t*-test.
* indicates data is significantly different at the P = 0.05 confidence level.
** indicates data is significantly different at the P = 0.01 confidence level.
*** indicates data is significantly different at the P = 0.001 confidence level.

A very close scrutiny of the results leads to the following conclusions:

(a) We have examined the biological activity of these compounds on smooth muscle relaxation and compared it with that of SNAP(**1**) and GSNO(**13**). We have found that *S*-nitrosated dipeptides are potent vasodilators but are chemically very stable in the absence of copper ions (SNAP<*S*-nitrosated dipeptides<GSNO).

(b) Differences in spontaneous (thermal) decomposition do not account for differences in NO release from these *S*-nitrosothiols *in vivo*.

(c) Since *S*-nitrosothiols are thermally unstable there is likely to be release of some NO spontaneously in the lumen where it would be susceptible to Hb inactivation. Recent experiments (Section 2. 2. 2) have shown that *S*-nitrosothiols undergo a process known as transnitrosation in the presence of -SH-containing compounds, such as cysteine. It is possible, therefore, that NO could be 'stripped' from *S*-nitrosothiols to form a compound whose action may not be susceptible to Hb inhibition. Potentially, therefore, *S*-nitrosothiols can release NO by several mechanisms, only some of which are susceptible to Hb inhibition.

(d) A number of mechanisms are involved in the decomposition of *S*-nitrosothiols *in vivo*. The dominant mechanism in the decomposition of *S*-nitrosated dipeptides may be different to that operative with SNAP and/or GSNO.

(e) **2**, **3**, **4**, **5**, **7**, **8**, **9**, **10**, **11**, **12**, and GSNO (**13**) are more effective than SNAP (**1**) . Compound (**6**) was found to be less effective than SNAP but that may be because **6** was an impure compound. The similarity is surprising as in **1-12** the local chemical environment of the -SNO groups are almost identical (i.e. **1-12** (3° carbon) but different in GSNO (1° carbon)). This finding is contrary to what might be expected on the basis of their chemical stabilities *in vitro* and may indicate that the *S*-nitrosated dipeptides and GSNO interact with tissue components or show greater permeability than SNAP.

(f) In *ex vivo* and *in vivo* situations, many factors may influence NO release from *S*-nitrosothiols.

2. 4. 2. Copper Chelation-Induced Reduction of the Biological Activity of *S*-Nitrosothiols in Smooth Muscle Relaxation

In vitro kinetics studies have shown that *S*-nitrosothiols spontaneously decompose in solution (pH = 7.4) to release NO, a reaction which is accompanied by formation of the corresponding disulphide only. In section 2. 2. 1. 1, we reported that this process is dependent upon catalysis by adventitious metal ions (Dicks *et al.*, 1996; Butler and Al-Sa'doni, 1996; Al-Sa'doni *et al.*, 1996). The release of NO by *S*-nitrosothiols is inhibited by the non-specific metal ion chelating agent EDTA and accelerated by addition of Cu^{2+} ions. Most importantly, a selective Cu^{+}-chelating agent (Smith and McCurdy, 1952), neocuproine, also prevents their decomposition and is equally as effective as EDTA in this regard.

The experiments to be reported here employed an *ex vivo* model, the isolated, internally perfused rat tail artery preparation (Flitney *et al.*, 1992), to ascertain whether Cu^{+} catalysis plays a role in the vasodilatation actions of SNAP and GSNO.

The experiments were performed in a dark laboratory since SNAP, GSNO, and NP are all photosensitive and release NO when exposed to light. The following protocol was used throughout. First, phenylephrine (1-7 μM) was added to the Krebs solution (in P1; Figure 3. 1) to precontract the artery, generating a perfusion pressure of 100-120 mm Hg. Bolus microinjections (10 μM) of SNAP or GSNO (10^{-7}-10^{-3} M; prepared by serial dilution of the stock solutions) were then delivered into the internal perfusate. Stock solutions of SNAP and GSNO (10^{-3} M) were prepared in 10% aqueous dimethyl sulphoxide (DMSO). These produced transient vasodilator responses which were allowed to recover fully between successive injections. The injection sequence was then repeated with SNAP and GSNO after pre-mixing with neocuproine (10^{-4} M) for 30 min. Finally, vessels were perfused internally with Krebs solution containing neocuproine (10^{-5}M, added to P1; Figure 3. 1) and solutions of SNAP or GSNO alone were injected into the artery. Stock solution of neocuproine (10^{-2} M) was prepared in 25% ethanol.

Control experiments showed that the highest working concentrations of DMSO (1%) and ethanol (2.5%) alone did not cause vasodilation of the artery. Control experiments were performed with neocuproine previously complexed to Cu^{+}.

Vasodilator responses to standard bolus injections of sodium nitroprusside (NP; 10^{-5} M) were recorded periodically (Figure 2. 55). Like SNAP and GSNO, NP relaxes vascular smooth muscle by increasing cyclic GMP levels, but it does so by reacting with rather than by spontaneous release of NO in solution (Flitney *et al.*, 1992).

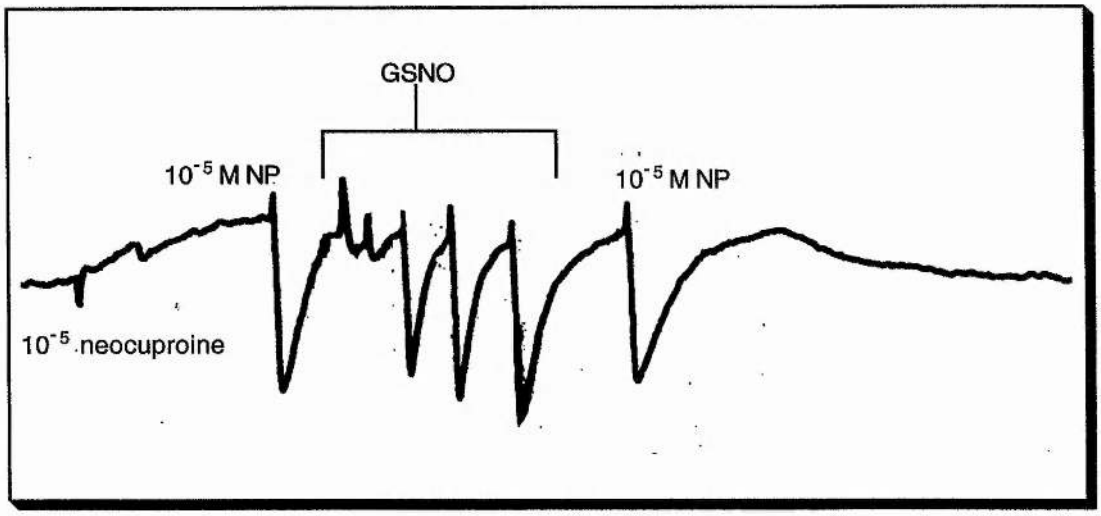


Figure 2. 55: Pressure recording showing the vasodilator effect of 10 µl microinjection of sodium nitroprusside (NP; 10^{-5} M), GSNO, and sodium nitroprusside (NP; 10^{-5} M) respectively.

The concentration of contaminating copper in Krebs solution (*ca.* 10^{-6} M; in distilled water) was determined by atomic absorption spectroscopy.

Figure 2. 56 shows vasodilator responses obtained by injecting drug concentrations of:

(a) SNAP or GSNO alone in pre-contracted arteries.

(b) SNAP or GSNO pre-mixed with neocuproine (10^{-4} M) in pre-contracted arteries.

(c) SNAP or GSNO alone, and vessels were perfused internally with Krebs solution containing neocuproine (10^{-5} M) in pre-contracted arteries.

The vasodilator effect of both compounds was severely attenuated in the presence of neocuproine. Log dose-response curves (data from 5-7 experiments) are presented in Figures 2. 57 and 2. 58 (solid symbols). Neocuproine caused a marked rightward shift in the dose-response curve: the ED_{50} values for both compounds were increased approximately 100 fold.

The addition of neocuproine (10^{-5} M) alone to reservoir P1, containing the internal perfusate (= Krebs + neocuproine), elicited a prompt vasoconstrictor response (mean +/- SEM = 17.9 +/- 3.1%; n = 7 vessels) which was fully reversible on washout (Figure 2. 55). The magnitude of the effect was significantly less than that produced by including either haemoglobin (5 μM; 56.5 +/- 4.9%; n =8) or by the nitric oxide synthase inhibitor L-monomethyl-L-arginine (100 μM: 57.1 +/- 6.1%; n = 10) in the internal perfusate. In contrast, control experiments showed that the pre-prepared stoichiometric complex of neocuproine and Cu^{+} had no effect on phenylephrine-induced tone.

Vasodilator responses to bolus injections of SNAP or GSNO (empty symbols) administered while vessels were being continuously perfused with Krebs and neocuproine were also impaired, and under these conditions the maximum vasodilator response was depressed too (Figures 2. 57 and 2. 58). The effect was greater for SNAP than GSNO. Again, the neocuproine-Cu^{+} complex had no effect on vasodilator responses to SNAP (GSNO was not tested).

Finally, vasodilator responses to NP (10^{-5} M) were unaffected by the presence of neocuproine in the internal perfusate: the amplitudes were 44.3 +/- 3.7% (n = 5-7 separate experiments) in normal Krebs solution and 43.2 +/- 5.3% (n = 5-7 separate experiments) in Krebs solution containing neocuproine.

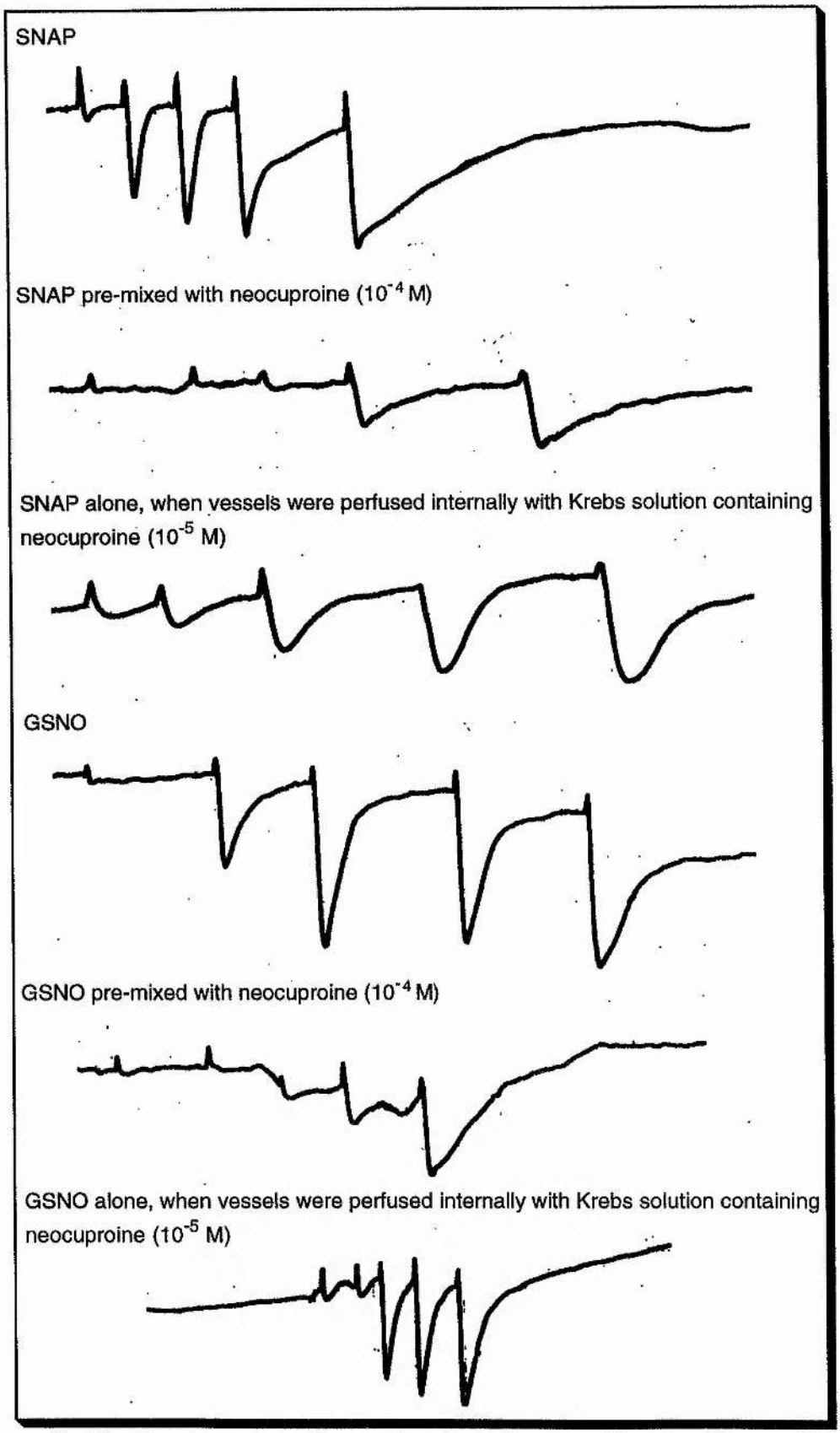


Figure 2. 56: Pressure recordings showing the vasodilator effects of 10 µl microinjections of SNAP, and GSNO in the absence and presence neocuproine.

Neocuproine is a selective chelating agent of Cu^+ ions. The results presented here show that it strongly inhibits vasodilator responses of isolated rat tail artery to bolus injections of SNAP or GSNO, demonstrating that Cu^+-catalysed release of NO from *S*-nitrosothiols is important in a functionally-intact *ex vivo* physiological system.

The nature of the inhibitory effect was found to depend upon the experimental protocol, specifically on the manner in which SNAP and GSNO were administered: that is, whether (a) pre-mixed with neocuproine prior to injection or (b) injected without pre-mixing into vessels that were continually perfused with Krebs solution and neocuproine. The dose-response curves to bolus injection of SNAP or GSNO pre-mixed with neocuproine demonstrate a competitive inhibition: that is, a marked rightward shift of the curve but with similar maximum responses at higher doses (Figures 2. 57 and 2. 58). On the other hand, the form of the dose-response curves to SNAP or GSNO alone when injected into arteries perfused with Krebs solution and neocuproine is suggestive of a non-competitive inhibition (Figures 2. 57 and 2. 58). Here, maximum responses to both compounds are significantly diminished. Importantly, responses to bolus injections of NP were not impaired under these conditions, demonstrating that prolonged exposure of vessels to neocuproine *per se* does not impair vascular smooth muscle relaxation directly.

The period of time that vessels are exposed to neocuproine appears to be the important factor which determines the form of the dose-response curve. This was negligible (*ca.* 300 ms) in the 'pre-mix' experiments, but considerable (1-12 s) in those experiments which involved continuous infusions of Krebs and neocuproine.

The results of the kinds of the experiment can be interpreted if we postulate a dual mechanism of action for SNAP and GSNO. First, a 'direct' relaxant effect, due to Cu^+-catalysed release of free NO into solution; and second, an 'indirect' relaxant effect, due to a process involving Cu^+-catalysed release of NO within the tissue.

There can be little doubt that the concentration of neocuproine present (10^{-4} M) in the 'pre-mixed' solution was sufficient to chelate the trace amounts of Cu^+ likely to be present.

Atomic absorption spectroscopy showed that this was in the region of 10^{-6} M. The ability to give near maximal responses at the highest doses of SNAP or GSNO under these conditions (Figures 2. 57 - 2. 58) can therefore be attributed to trace copper ions and contaminating thiols present in the internal perfusate, to which each compound was momentarily exposed following injection, while *en route* to, and within, the artery.

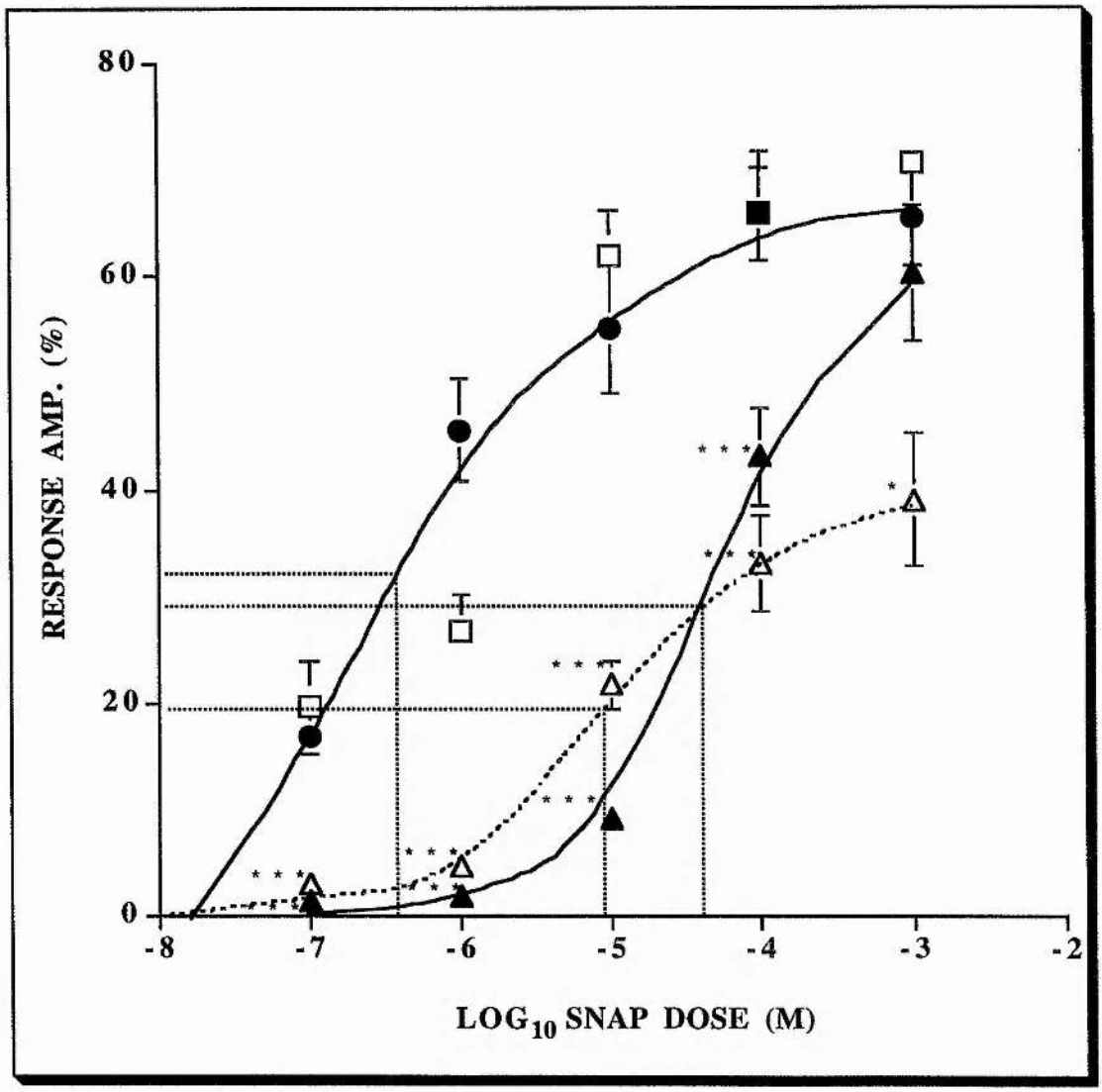


Figure 2. 57: Log dose-response curves comparing the vasodilator effect of 10 µl bolus injections of SNAP. Filled circles show responses of SNAP alone, closed triangles show SNAP pre-mixed with neocuproine (10^{-4} M), open triangles show SNAP alone when vessels were perfused internally with Krebs solution containing neocuproine (10^{-5} M), and open squares show SNAP alone when vessels were perfused internally with Krebs solution containing neocuproine (10^{-5} M), copper(II) acetate (0.5×10^{-5} M), and L-glutathione (0.5×10^{-5} M).

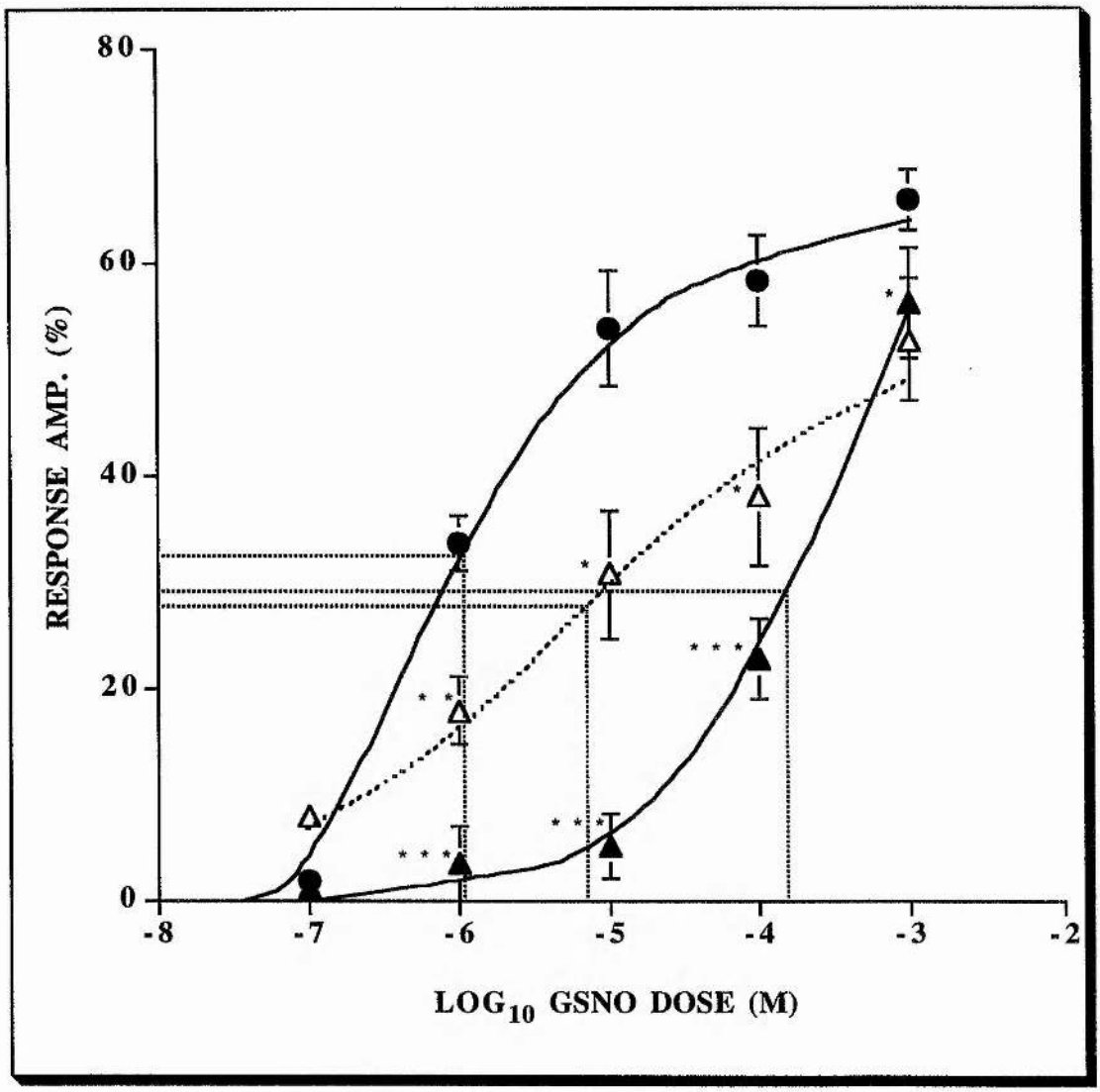


Figure 2. 58: Log dose-response curves comparing the vasodilator effect of 10 µl bolus injections of GSNO. Filled circles show responses of GSNO alone, closed triangles show GSNO pre-mixed with neocuproine (10^{-4} M), and open triangles show GSNO alone when vessels were prefused internally with Krebs solution containing neocuproine (10^{-5} M).

The 'indirect' process would be significantly impaired if there was sufficient time available for neocuproine to chelate all contaminating Cu^{+} in the Krebs solution and also any cellular Cu^{+}, either by entering vascular smooth muscle cells and/or by acting as an external 'sink'. The sustained vasoconstrictor response observed when vessels were continuously perfused with neocuproine alone (Figure 2. 55) provides some support for this hypothesis and

raises the possibility that vascular smooth muscle tone may be regulated, at least in part, by Cu^{+}-catalysed release of NO from the endogenous *S*-nitrosothiols. It will be recalled that the magnitude of the effect was considerably less than that produced by continuous infusions of either haemoglobin (5 μM) or LNMMA (100 μM), so this source of NO may be less important than endothelium-derived NO in regulating vessel tone. However, it was shown recently (Schrammel *et al.*, 1996) that copper ions inhibit basal and NO-stimulated recombinant soluble guanylate cyclase activity and that Cu^{+} is more effective than Cu^{2+} in this regard (Section 2. 4. 5). Neocuproine may therefore exert opposing effects on vessel tone: inhibition of NO release from endogenous *S*-nitrosothiols, causing a vasoconstrictor action, and a simultaneous vasodilation, due to removal of the inhibitory effect of Cu^{+} on guanylate cyclase activity. Clearly, the amplitude of the net response will depend upon the relative contributions from the two processes, making it difficult at present to decide whether endothelial- or *S*-nitrosothiols-derived NO plays the more important role in controlling smooth muscle contractility.

The amplitude of the vasodilator response to SNAP or GSNO is given as the maximum drop in pressure expressed as a percentage of that existing immediately prior to injecting the vessel. Results presented are mean (+/- SEMSs) for 5-7 experiments. Statistical significances were calculated using a paired Student's *t*-test, comparing responses obtained at each dose of SNAP/GSNO in the presence of neocuproine (pre-mixed or added to the internal perfusate) with control responses recorded in Krebs solution only. A p value of 0.05 or less was taken to indicate a statistically significant difference between mean values.

2. 4. 3. Inhibition of Platelet Aggregation

It has recently been shown that *S*-nitrosoproteins such as *S*-nitroso albumin and *S*-nitroso-tissue type plasminogen activator, like the low-molecular-weight *S*-nitrosothiols such as GSNO (Radomski *et al.*, 1992), have strong anti-platelet effects with IC_{50}'s in the range of 1.5 μM (Simon *et al.*, 1993). Furthermore, it has been postulated that these stable adducts release and deliver NO by transfer to low-molecular-weight thiols such as cysteine and glutathione (Simon *et al.*, 1993).

It is known that NO is generated in platelets and that it acts as a negative feedback system to modulate aggregation (Radomski *et al.*, 1990 a&b). Generation of platelet-derived NO is NADPH-dependent, inhibited by LNMMA and dependent on free intracellular Ca^{2+}. Addition of L-arginine to the medium does not alter basal NO production, but enhances the increase in NO production when aggregation is initiated using collagen, ADP or arachidonic acid. The interference from this is that nitric oxide synthase exists in platelets and is stimulated to enhance NO synthesis from L-arginine once aggregation is initiated (due to an increase in intracellular Ca^{2+}: Ware *et al.*, 1986). The increase in intracellular NO has been shown to be accompanied by an increase in cGMP but not cAMP. The mechanism by which cGMP inhibits aggregation is not fully understood, but it may involve sequestration of free intraplatelet Ca^{2+} (Busse *et al.*, 1987). Nitric oxide, therefore, may play an important antithrombotic role *in vivo* by inhibiting both platelet aggregation and adhesion to vessel walls, particularly as its actions are enhanced by the synergistic effect of prostacyclin. This evidence suggests that *S*-nitrosothiols have a possible role as thromboregulators controlling platelet aggregation. Although blood platelets play a central role in maintaining haemostasis and in the pathogenesis of thrombosis, the precise mechanisms regulating platelet production, release and ageing, are still not fully understood.

In a normal human, it is estimated that approximately 35 x 10^9 platelets are produced per litre of blood every day (Harker and Finch, 1969). This production rate is under relatively strict control and finely regulated as evidenced by very narrow limits within the blood platelet count variations from day to day, or month to month (Brecher and Cronkite, 1950).

In the last decade knowledge of some of the regulating mechanisms of megakaryocyte proliferation and maturation has become much more certain, but many unanswered questions remain. The final stage of the liberation of platelets from these precursors is also poorly understood. Our lack of insight is highlighted by the controversy concerning whether platelets are released from the megakaryocytes in the bone marrow or in the lungs. It is also not clear why platelet volume is so heterogeneous and what determines the ultimate size of the platelet.

Platelets in the circulation change with ageing (Hirsh *et al.*, 1968), but little is known of the characteristics of newly formed platelets that distinguish them, or could make it possible to isolate senescent platelets from those of average age. In respect of human platelets at least, the claims that young platelets can be identified by their characteristic buoyant density, volume, and biochemical features (Karpatkin, 1969a&b, 1970; Corash *et al.*, 1977, 1978), must now be regarded as mistaken (Boneu *et al.*, 1980; Martin *et al.*, 1983).

The major cause of the difficulties relating age with features such as size, has been the confusion between 'stress platelets' and normal young platelets. Stress platelets are produced under conditions of stimulated platelet production (Karpatkin 1984; Penington 1984), and they differ from normal young platelets in respect of volume and functional characteristics (McDonald *et al.*, 1964; Ginsberg and Aster, 1972; Harker and Slichter, 1972). The presence of megathrombocytes in the blood reflects increased platelet turnover. This characteristic has become clinically important with the advent of automated measurement of platelet volume (Levin and Bessman, 1983). Although the presence of megathrombocytes on the peripheral blood film may be used as an index of megakaryocyte number (Garg *et al.*, 1971), it should be recognised that in a steady state the distribution of

platelet volume is log-normal (Paulus, 1975). The peripheral blood will therefore always have some large platelets. There is now conclusive evidence that the volume of platelets does not decrease with ageing (Thompson *et al.*, 1983). An increase of stimulated platelet production and turnover, should therefore be interpreted simply to reflect a shift to the right of the log-normal distribution curve describing platelet size (Penington and Streatfield, 1975).

There are many important variables in platelet aggregation:

(a) Type of anticoagulant:

Citrate is satisfactory for most studies. It should be noted that an increase in the concentration of citrate results in reduced platelet aggregation.

(b) Methods of preparation of platelet-rich plasma:

This should be carefully standardised. Some platelets will not be recovered in platelet-rich plasma prepared by centrifugation and it is possible that, in some circumstances, a population of platelets of higher density may exhibit aggregation responses different from those of the remainder of the platelet population. Such platelets may not be represented in the platelet-rich plasma.

The study of platelet aggregation by light transmission methods necessitates the separation of platelets from other blood cells. This is clearly an entirely artificial situation as platelet response *in vivo* may be influenced by these other cellular elements. Thus, for example, red cells are a potent source of ADP.

(c) pH:

Maximum responses occur in a pH range of 7.4 to 8.0 and steps should be taken to ensure that the pH is standardised and within this range (Mustard and Packham, 1970; Rogers,

1972). pH is largely dependent on CO_2 content and this should be controlled.

(d) Temperature:

Platelet aggregation tests should be performed at 37 °C (Mustard and Packham, 1970; Han and Ardlie, 1974). Aggregation is sensitive to temperature variations and secondary responses to ADP are not seen at temperatures below 30 °C (Valdorf-Hansen and Zucker, 1971).

(e) Stirring :

The number of collisions between platelets is governed by the rate of stirring and the physical characteristics of the stirrer bar. These should be standardised (Born, 1962; Coller and Gralnick, 1976).

(f) Platelet count in platelet-rich plasma:

For meaningful comparisons of responses between samples, the platelet counts must be matched. Responses are difficult to interpret with a platelet count in platelet-rich plasma of less than 100 x 10^9/l. A method has been described for the analysis of samples with counts as low as 50 x 10^9/l (Levine, 1976).

(g) Difference in light transmission:

It should be noted that the difference in light transmission between platelet-poor and platelet-rich plasma in lipaemic samples is narrowed and analysis of aggregation responses may not be possible.

The responses to adrenaline are variable, a proportion of normal subjects failing the respond to this agonist at any concentration. This is also a characteristic of neonatal platelets (Corby and Schulman, 1971).

We have examined the pharmacological effects of the previous *S*-nitrosothiols (**2-12**) on human platelets *ex vivo* and compared them with the pharmacological properties of SNAP and GSNO.

Human blood was collected from healthy volunteers who had not ingested drugs known to affect platelet function for two weeks prior to the study (details of inhibition of platelet aggregation procedure are described elsewhere; Experimental Section). Briefly, the light beam from the spectrophotometer (Platelet Aggregation Profiler) passed through the sample cuvette containing a stirred solution of platelet rich plasma (PRP) at 37 °C and the absorbance noted. Collagen added to PRP was used to aggregate the platelets. This caused a dramatic decrease in the absorbance due to reduced light scattering by the aggregating platelets. Example traces are shown in Figure 2. 59. Various concentrations of the *S*-nitrosothiols to be tested were injected into the cuvette prior to the addition of collagen.

Incubation of the drugs with platelet rich plasma (PRP) resulted in a concentration-dependent inhibition of the collagen-induced aggregation (Figure 2. 60 and Table 2. 7, results are the mean of at least two separate experiments). The inhibitory activity of these *S*-nitrosothiols was reversed by haemoglobin, indicating the involvement of NO in the process.

2, **3**, **4**, **9**, **10**, **11**, **12**, and GSNO were significantly more potent than SNAP as inhibitors of platelet aggregation, and, **5**, **6**, **7**, and **8** were significantly less potent than SNAP. No significent difference were found in the inhibition of collagen release from platelets caused by the drugs (Table 2. 7).

It is clear that *S*-nitrosothiols, as a class of NO donor drugs, are very effective at inhibiting platelet aggregation. Whether endogenously or exogenously produced, they are far more potent than other commercially available NO-donor drugs and, with two exceptions, they were found to be more potent than NO itself.

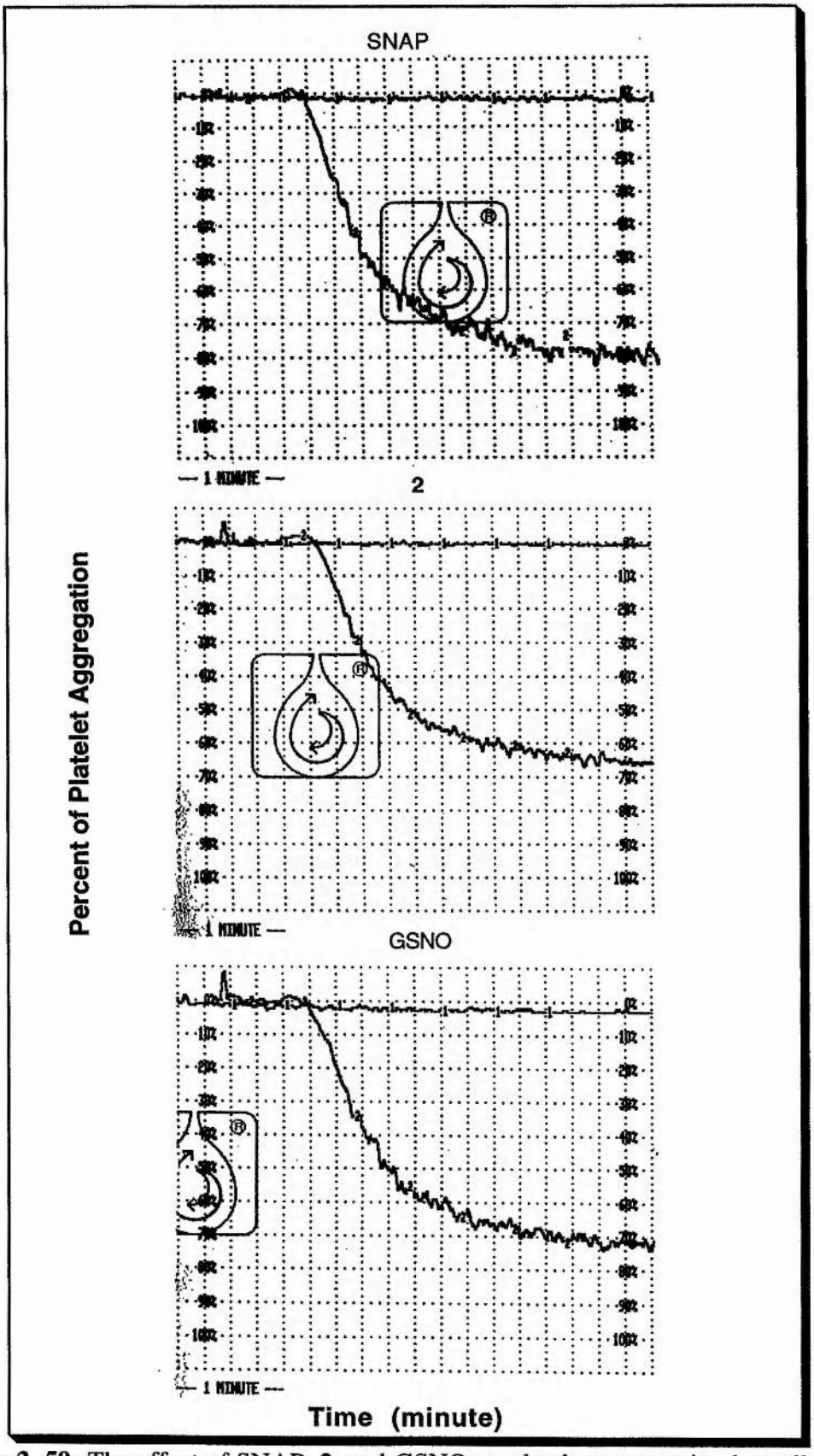


Figure 2. 59: The effect of SNAP, **2**, and GSNO on platelet aggregation by collagen in platelet rich plasma (1. added drug, and 2. no added drug).

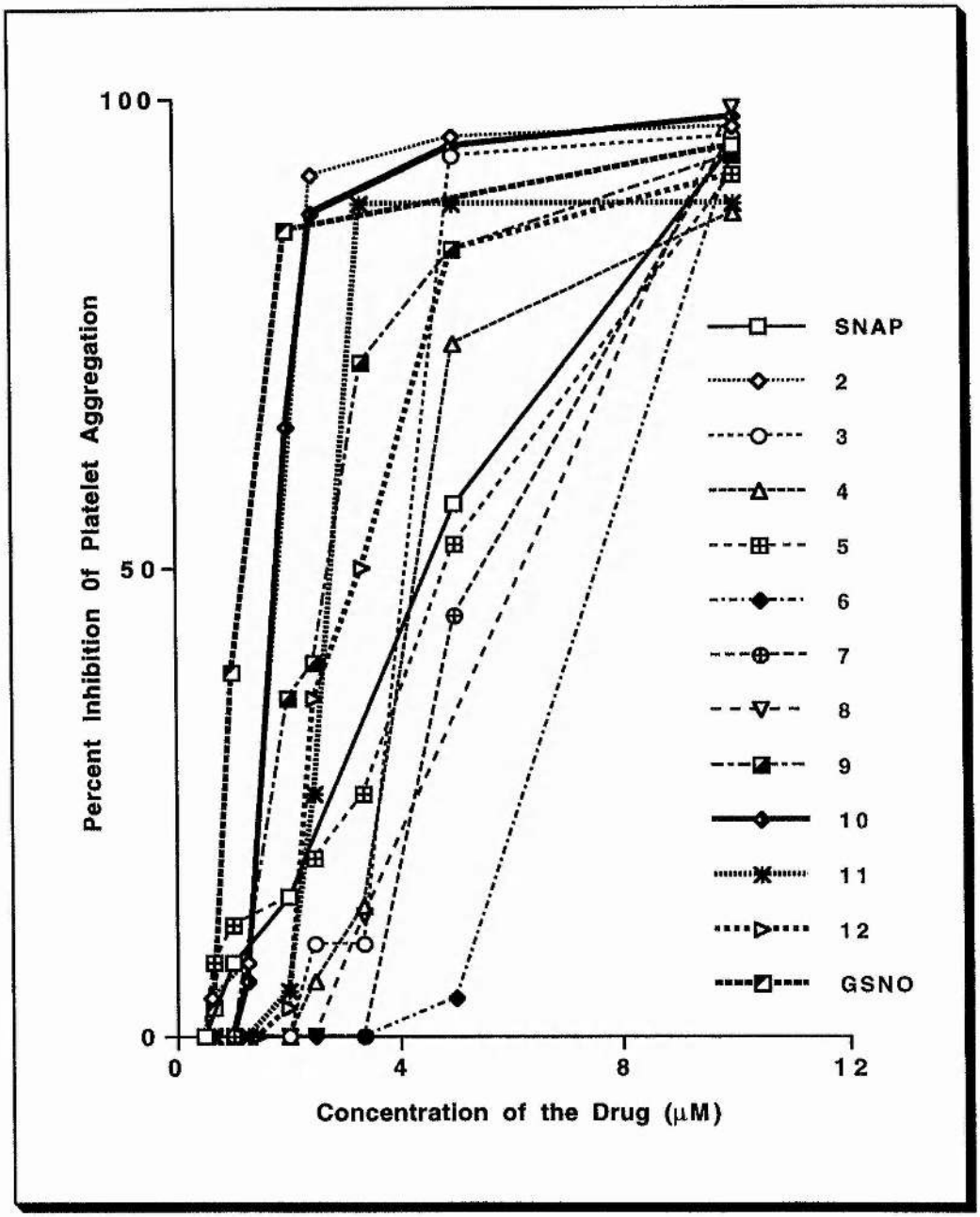


Figure 2. 60: Inhibition of collagen-induced platelet aggregation by *S*-nitrosothiols (**1-13**).

TABLE 2. 7

A comparison of the relative effectiveness at inhibiting platelet aggregation of the *S*-nitrosothiols (**1-13**) by collagen-induced aggregation

Compound Number	IC_{50} (μM)*
SNAP (**1**)	4.4
2	1.8
3	4.1
4	4.1
5	4.6
6	7.1
7	5.3
8	6.2
9	2.7**
10	1.9
11	2.8
12	3.2
GSNO (**13**)	1.3

* IC_{50}: concentration required to inhibit by 50%.
** The stock solution was the drug dissolved in DMSO, because the drug is insoluble in the buffer.

2. 4. 4. Copper Chelation-Induced Reduction of the Biological Activity of *S*-Nitrosothiols in Inhibition of Platelet Aggregation

A particularly interesting finding recently (Gordge *et al.*, 1995) shows that the inhibition of platelet aggregation activity by GSNO is much reduced in the presence of neocuproine and the closely related bathocuproine, both specific Cu^{+}-chelating agent. In addition, bathocuproine sulphonate reduces the stimulation of platelet guanylate cyclase by both *S*-nitroso-L-cysteine and GSNO.

2. 4. 5. The Effect of Copper Ions on Soluble Guanylate Cyclase

Very recently, Schrammel *et al.* (1996) found that recombinant bovine lung soluble guanylate cyclase (sGC) purified from a baculovirus overexpression system (Weld *et al.*, 1994) was inhibited by low concentrations of $CuSO_4$. Their data are shown in Figure 2. 61. $CuSO_4$ inhibited cGMP formation by the enzyme stimulated with 1 μM 2,2-diethyl-1-nitroso-oxyhydrazine (DEA/NO) with an IC_{50} of ~ 2 μM; complete inhibition was observed with 10 μM $CuSO_4$. GSH protected sGC from copper-induced inhibition, as revealed by a more than 10-fold reduced potency of $CuSO_4$ in the presence of 1 mM of the thiol (Figure 2. 61). Haemoglobin exhibits a high-affinity binding site for Cu(I) ions, which may be involved in copper-induced, thiol-sensitive methaemoglobin formation (Rifkind, 1981; Smith *et al.*, 1993). Since sGC was reported to contain stoichiometric amounts of copper (Gerzer *et al.*, 1981) it is conceivable that copper-induced enzyme inhibition may be due to oxidation of haem-iron, resulting in reduced affinity for NO and thus deactivation of the NO-stimulated cyclase. However, their data indicate that the inhibitory effect of $CuSO_4$ is not due to interference with stimulation of the enzyme by NO, because (a) $CuSO_4$ inhibited basal and NO-stimulated enzyme activities with similar potency, (b) higher concentrations of DEA/NO did not prevent inhibition, and (c) reaction of $CuSO_4$ with NO was negligible under their experimental conditions.

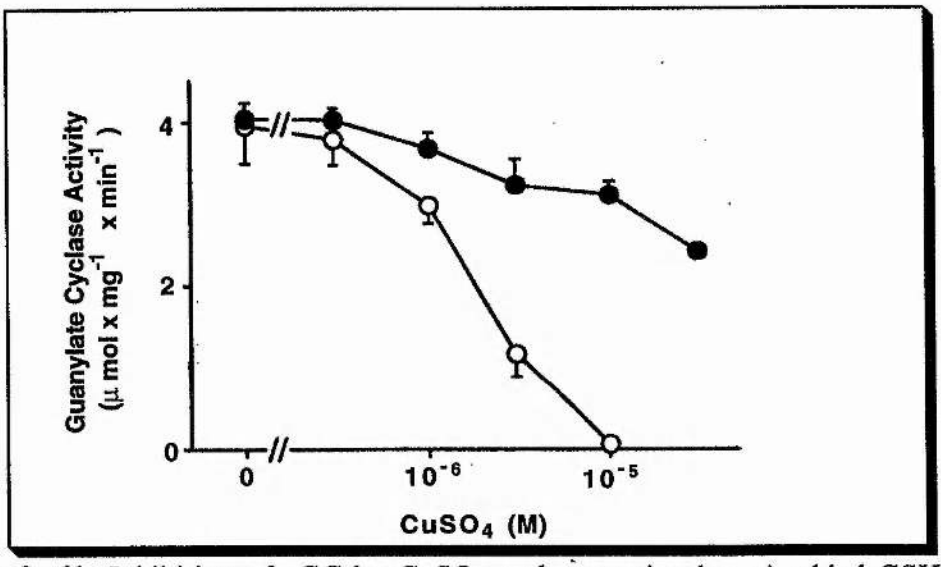


Figure 2. 61: Inhibition of sGC by $CuSO_4$ and protection by a) added GSH (filled circles) b) no added GSH (empty circles); (adapted from Schrammel *et el.*, 1996).

Inhibition of sGC due to the oxidative properties of copper was further excluded in experiments performed with selective Cu^{+} and Cu^{2+} chelating agents (Gordge *et al.*, 1995). Figure 2. 62 shows that the inhibitory effect of 3 μM $CuSO_4$ was almost completely antagonized by the Cu^{+}-specific antagonist neocuproine, whereas cuprizone, a compound with Cu^{2+} selectivity, was much less effective.

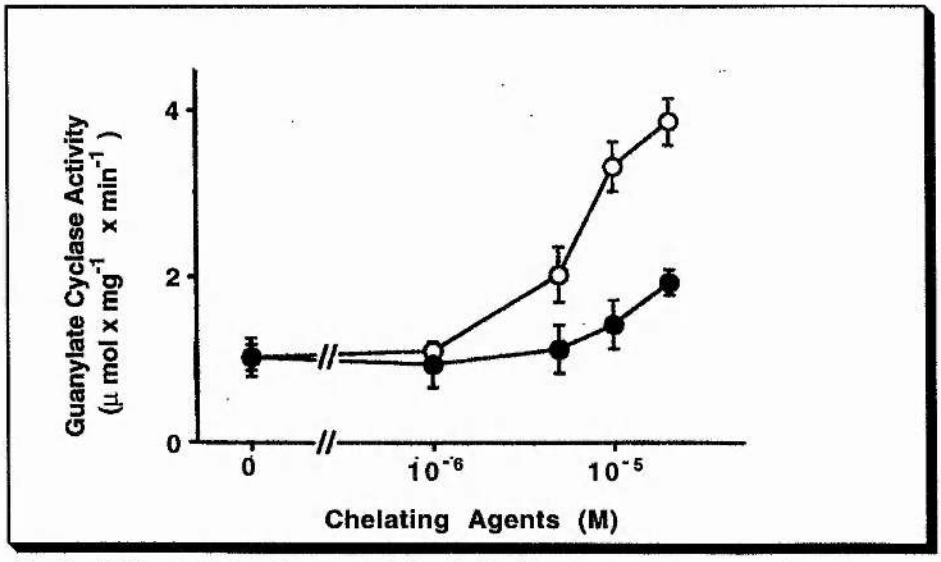


Figure 2. 62: Effect of the Cu^{+}-specific chelating agents, neocuproine (empty circles) and cuprizone (filled circles) on sGC activity in the presence of 3 μM $CuSO_4$ (adapted from Schrammel *et. al.*, 1996).

GSH is known to form highly stable complexes with Cu^+ ions (Miller *et al.*, 1990), but the protective role of the thiol was probably not due to chelation of copper, as several other thiols were also effective.

The present data show that copper ions induce a pronounced inhibition of cGMP formation by sGC. The inhibitory effect of $CuSO_4$ may be due to the binding of Cu^+ ions to one or more sulphhydryl groups critically involved in the catalytic function of sGC, since the enzyme was protected by a Cu^+-selective chelating agent and several thiols. Intracellular GSH levels are in the millimolar range and copper occurs primarily in chelated, redox-inactive forms, suggesting that Cu^+-induced inhibition of sGC is insignificant under physiological conditions. However, oxidative stress may cause depletion of tissue GSH (Bray and Taylor, 1993), and redox-active copper was reported to be mobilized in the course of myocardial ischemia (Chevion, 1993) or may be released from caeurloplasmin by peroxynitrite (Swain, 1994). Thus, impaired accumulation of cGMP induced by free copper may contribute to the pathophysiology of certain diseases.

2. 4. 6. Cu^+ Catalysed NO Release from Endogenous *S*-Nitrosothiols

All the previous results are a direct proof that Cu^+ causes NO release from endogenous *S*-nitrosothiols. This strongly suggests that nitric oxide is generated from *S*-nitrosothiols by a process which involves Cu^+. Copper is therefore required for the full activity of these *S*-nitrosothiol compounds. However, another recent article (Bannenberg *et al.*, 1995) claims that bronchodilation induced by GSNO does not require the formation of NO. The medical importance of *S*-nitrosothiols has been highlighted recently by two reports (Longford *et al.*, 1994; de Belder *et al.*, 1995) which describe the clinical use of GSNO to inhibit platelet aggregation during coronary angioplasty and also to treat a form of pre-eclampsia, a high blood pressure condition suffered by some pregnant women.

2. 5. The Correlation between Structure, Chemical Stability, and Physiological Activity

As described earlier, the chemical stability of the *S*-nitrosothiols was monitored spectrophotometrically in the presence of different copper concentrations or in the presence of the corresponding thiols. The synthetic *S*-nitrosothiols (**1-13**) were tested for biological activity in two assay systems, an isolated smooth muscle preparation and a platelet aggregation assay. All the *S*-nitrosothiols tested were active in each assay. They relaxed smooth muscle preparations (rat tail artery), and inhibited collagen-induced platelet aggregation. Although the structures and chemical properties of the synthetic *S*-nitrosothiols varied considerably, all the compounds were active in both bioassay systems. Coupled with the fact that the starting thiols and the product disulphide were inactive, this indicates that the -S-N=O functional group is responsible for the biological activity of these compounds. The R- group, however, significantly influences the biological activity. We have found that *S*-nitrosated dipeptides are potent vasodilators and suitable inhibitors for platelet aggregation but are chemically very stable in the absence of copper ions (SNAP < *S*-nitrosated dipeptides < GSNO; Table 2.8). The potency of the *S*-nitrosothiols varied as a function of the structure by as much as 0.8 - 45 μM orders of magnitude in the isolated smooth muscle preparation, and 1.3 - 7.1 μM in the platelet aggregation assay. It is clear that the platelet aggregation assay is more sensitive to *S*-nitrosothiols than the isolated smooth muscle preparation. The effect of the R- group on biological activity was different in each assay; this is reflected by the different rank orders of activity observed in the systems. We found that the solution stability did not correlate with inhibition of platelet aggregation or relaxation of vascular smooth muscle. The effect of the structure on activity is pronounced; similar structures had different activity profiles (Table 2. 8).

A close inspection of Table 2.8 indicates that **2-12** (except **6** which was impure) are substantially more potent than SNAP in the smooth muscle relaxation bioassay, although the chemical environments of the -SNO groups are almost identical. There is an inverse correlation between chemical reactivity and biological activity only in the smooth muscle relaxation bioassay, a result which provides further evidence that extracellular decomposition of *S*-nitrosothiols (**2-12**) to give NO cannot account for all their vasodilator effects. It suggests, rather, that the *S*-nitrosothiol enters the cell intact, a process controlled partly by the lipophilicity of the complete molecule, before decomposition occurs or *S*-nitrosated dipeptides interact with tissue components. Also the biological activity of the *S*-nitrosothiols (**1-13**) in the platelet aggregation assay does not correlate with their chemical stability.

Unlike acidified nitrite, where biological activity is due to NO (Furchgott, 1988), the activity of *S*-nitrosothiols is not due to the generation of NO in solution. These results are consistent with previous reports suggesting that *S*-nitrosothiols do not act by releasing NO in solution. For example, the relaxation of smooth muscle by *S*-nitrosothiols was shown to be enhanced by cysteine (Askew *et al.*, 1995a&b) and superoxide dismutase and inhibited by *N*-acetyl-D,L-penicillamine, agents that had the opposite effect on *S*-nitrosothiol degradation (Kowaluk and Fung, 1990a&b), indicating that spontaneous liberation of NO was not responsible for the vascular activity of these compounds.

TABLE 2. 8

The correlation between structure, chemical stability, and physiological activity

Compound Number	Relative Rate of Decomposition ($k_{S\text{-Nitrosothiol}}/k_{GSNO}$)*	Smooth Muscle Relaxation ED_{50} (μ M)	Inhibition of Platelet Aggregation IC_{50} (μ M)
SNAP (**1**)	2000	20	4.4
2	132	4	1.8
3	64	3.5	4.1
4	64	3	4.1
5	64	1.5	4.6
6	68	45	7.1
7	248	10	5.3
8	68	4.5	6.2
9	insoluble in the buffer	9.5	2.7**
10	100	5	1.9
11	112	0.8	2.8
12	172	2	3.2
GSNO (**13**)	1	0.9	1.3

* When [EDTA] = [added Cu]
[Cu] = [added Cu] + [Cu present in the buffer]
** The stock solution was the drug dissolved in DMSO, because the drug is insoluble in the buffer.

Relaxation of vascular smooth muscle by a variety of agents, including *S*-nitrosothiols, is believed to be due to stimulation of soluble GC (Ignarro and Kodowitz, 1995). Elevated cGMP levels may also regulate tone in nonvascular trached smooth muscle (Katsuki and Murad, 1977b) and may be involved in platelet aggregation (Mellion *et al.*, 1981). Inhibition of GC using methylene blue (Ignarro *et al.*, 1981a&b) and *N*-methylhydroxylamine (Gibson *et al.*, 1992) blocks the activity of *S*-nitrosothiols. Thus, all of the actions of *S*-nitrosothiols (**1**-**13**) that we have reported here, could be explained by stimulation of soluble GC. However, the biological activity of the *S*-nitrosothiols in the smooth muscle relaxation assays and in the platelet aggregation assay does not correlate with the ability to stimulate platelet soluble GC. This suggests that additional factors are important in determining activity in these systems.

The stimulation of soluble GC in these systems could occur either directly or indirectly *via* NO release. Direct stimulation would require that the intact *S*-nitrosothiols enter the cell and activate GC, perhaps by transnitrosation of the active site haem moiety to form catalytically active NO haem (Ignarro, 1990). Alternatively, once inside the cell, the *S*-nitrosothiols could liberate NO to stimulate GC. Although the ability of *S*-nitrosothiols to cross membranes is not known, some of the *S*-nitrosothiols examined here, such as SNAP (**1**) and GSNO (**13**), would not be expected to be able to enter cells easily. From our results we suggest that the ability of *S*-nitrosothiols to cross membranes and enter cells is required for activity.

2. 6. The Role of L-Ascorbic Acid (Vitamin C) in the Breakdown of *S*-Nitrosothiols *in vitro* and *in vivo*

L-Ascorbic acid (vitamin C) is a water-soluble vitamin, although it does not function as a coenzyme in the manner of the B vitamins. Like vitamin E, the role of vitamin C in nutrition is still a subject of uncertainty. Whereas vitamin E protects the lipid portion of cells, vitamin C (a highly polar compound) serves as an antioxidant in the aqueous regions. Vitamin C participates in several biological oxidation reactions, such as the hydroxylation of proline and lysine moieties in collagen. It reacts with oxygen and/or oxidising agents to form dehydroascorbic acid (Scheme 2. 17; Baum, 1987).

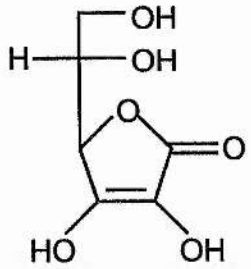
In 1747, James Lind discovered that citrus fruit was effective in treating sailors suffering from scurvy, a weakening of the collagenous tissues. The symptoms are swollen gums, loose teeth, sore joints, bleeding under the skin, and slow healing of wounds. It was not until 1932, however, that the vitamin was isolated from citrus fruit. Vitamin C was the first dietary component to be recognised as essential for preventing a human disease (Baum, 1987).

The controversy surrounding vitamin C received extra impetus in 1970, when Linus C. Pauling published his best-selling book, Vitamin C and the Common Cold. He stated that vitamin C in doses ranging from 1 to 5 g a day could prevent colds and that as much as 15 g a day could cure a cold. Scientists investigating Pauling's claims have obtained conflicting results and the RDA of vitamin C for adults is 60 mg (Baum, 1987).

Some scientists report that taking 1.5 g of vitamin C every half hour over a period of 2 hours can cure a cold. Others concede that this may be true for a small group of the population. However, as one researcher puts it " For the remainder of the population, vitamin C is relatively or completely ineffective in curing the common cold, since large-scale controlled studies have been quite unimpressive" (Baum, 1987).

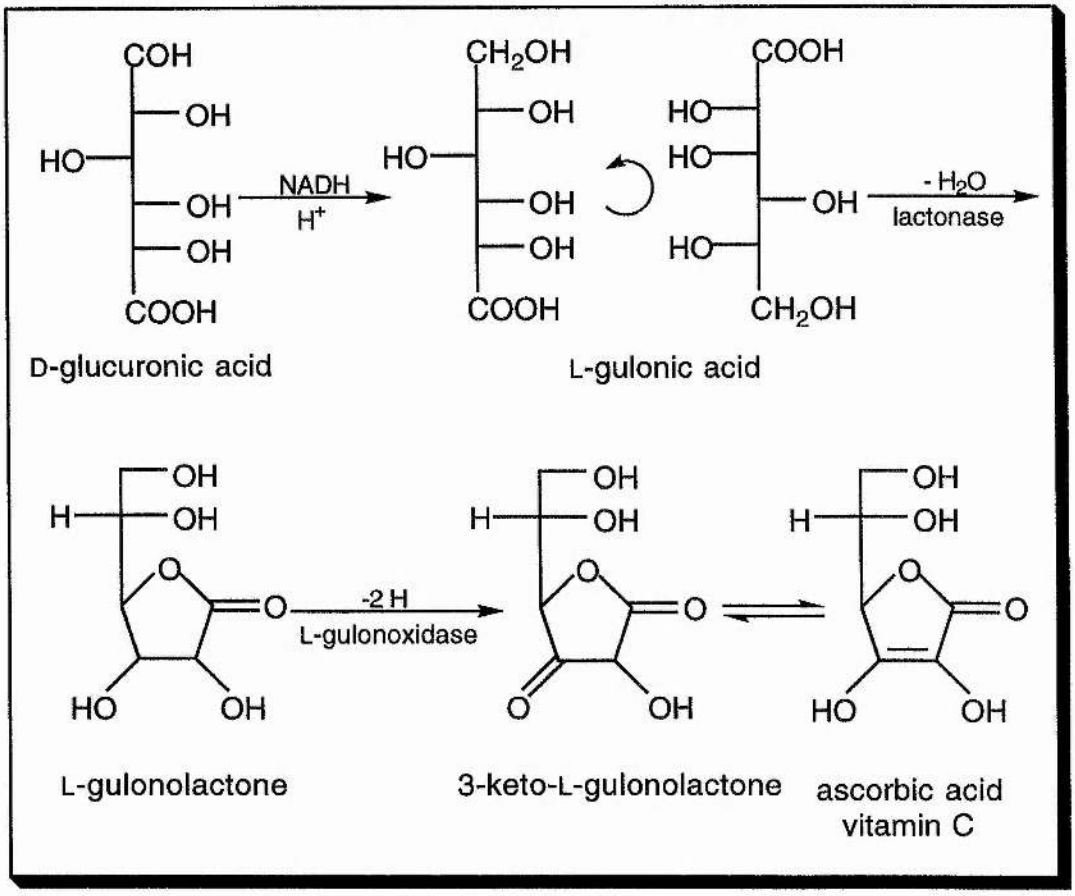
There have also been claims that vitamin C can prevent cancer and that it is useful in the therapy and management of cancer patients. Its role in cancer/cold prevention is related to its supposed stimulation of the immune system and its activity as an antioxidant in suppressing the damage caused by free radicals and in blocking the formation of nitrosamines. There is as yet no concrete evidence to substantiate or to invalidate these claims (Baum, 1987).

Since vitamin C is water soluble, excess is excreted rather than being stored in the body. However, some scientists point to the possible dangers of taking massive doses of this vitamin. Such doses, for example, may raise the uric acid level in body fluids and thus cause gout in people predisposed to this disease. Also, it is known that the ingestion of 5 g of ascorbic acid by the normal adult human will cause diarrhoea (Baum, 1987).



ascorbic acid
vitamin C

Plants and animals, except guinea pigs and primates (including man), can synthesise ascorbic acid from D-glucose. The enzyme which is missing in the species that are unable to produce the ascorbic acid is L-gulonoxidase, which converts L-gulonolactone to 3-keto-L-gulonolactone (Conn and Stumpf, 1976):



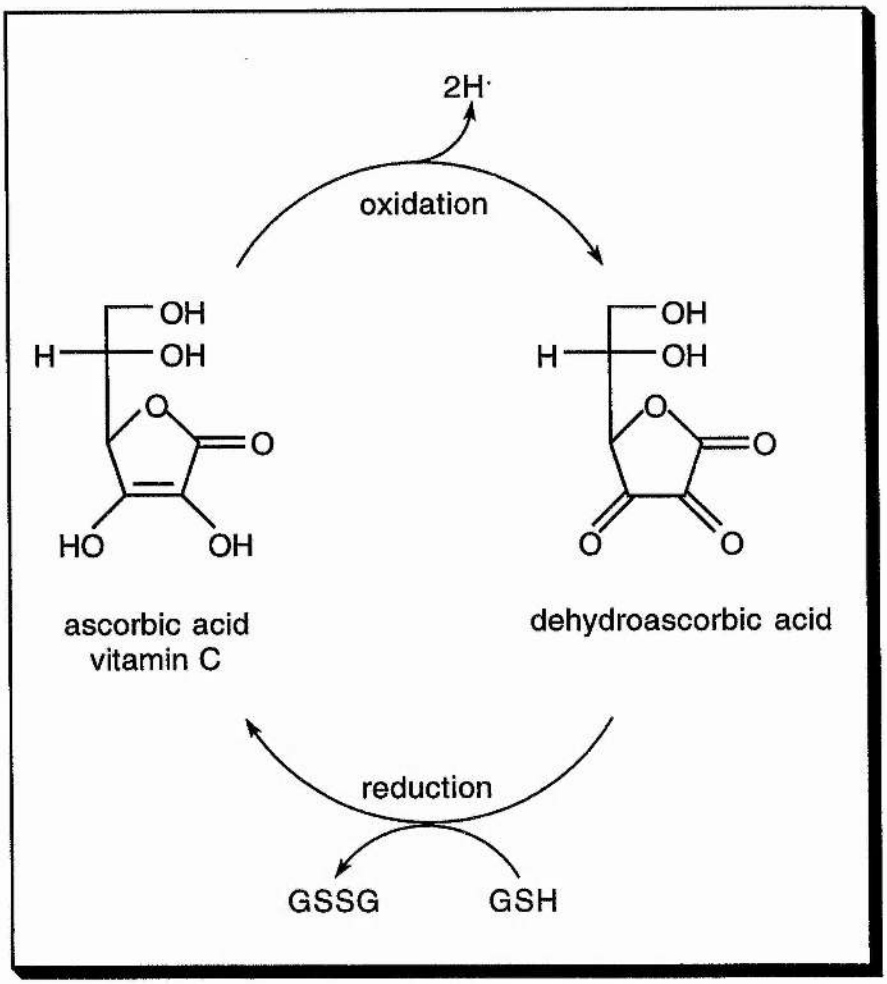
Scheme 2. 16: Synthesis of ascorbic acid from D-glucose.

The absence of ascorbic acid in the human diet gives rise to scurvy, a disease characterised by oedema, subcutaneous hemorrhages, anemia, and pathological changes in the teeth and gums. The disease was known to the ancients, especially among sailors, who often travelled for extended periods of time away from sources of fresh fruits and vegetables that were known to prevent scurvy (Conn and Stumpf, 1976).

A primary characteristic of scurvy is a change in connective tissue. In ascorbic acid deficiency, the mucopolysaccharides of the cell ground substance are abnormal in character, and there are significant changes in the nature of the collagen fibrils that are formed. The presence of ascorbic acid is required for the formation of normal collagen in experimental animals. At the enzyme level, there is an induction that ascorbic acid is involved in the conversion of proline to hydroxyproline, an amino acid found in relatively high concentrations in collagen (Conn and Stumpf, 1976).

The biochemical role which ascorbic acid plays is undoubtedly related to its being a good reducing agent. Its oxidized form, dehydroascorbic acid, is capable of being reduced again by various reductants including glutathione (GSH), and the two forms of ascorbate constitute a reversible oxidation-reduction system (Scheme 2. 17; Conn and Stumpf, 1976).

In the case of collagen formation, ascorbic acid can function as the external reductant that is required in the conversion of proline to hydroxyproline. Ascorbic acid can function as an external reductant in the hydroxylation of *p*-hydroxyphenylpyruvic acid to homogentisic acid in the liver and in the conversion of dopamine to norepinephrine that occurs in the adrenals. Moreover, guinea pigs that are maintained on an ascorbic-acid-deficient diet will excrete *p*-hydroxyphenylpyruvic acid in their urine.



Scheme 2. 17: The biochemical role of ascorbic acid.

Thus, it appears that the biochemical role of ascorbic acid is related to its involvement in hydroxylation reactions in the cell. It is interesting in this connection that the highest concentrations of ascorbate in animal tissues are found in the adrenals (Conn and Stumpf, 1976).

The aim of the work described in Section 2.6 (2. 6. 1 & 2. 6. 2) was to elucidate the role of L-ascorbic acid (vitamin C) in the breakdown of *S*-nitrosothiols *in vitro* and *in vivo*.

2. 6. 1. The Effect of L-Ascorbic Acid on the Decomposition of *S*-Nitrosothiols *in vitro*

Addition of L-ascorbic acid enhanced the decomposition of *S*-nitrosothiols. Figures 2. 63 and 2. 64 show the effect on the decomposition rate of adding L-ascorbic acid (Dasgupta *et al.*, 1996).

Figure 2. 63 shows the following behaviour: (a) at low L-ascorbic acid concentrations the rate becomes dependent on the L-ascorbic acid concentration, and (b) at high L-ascorbic acid concentrations the rate becomes independent of the L-ascorbic acid concentration. Therefore, addition of more L-ascorbic acid will not increase the rate of the GSNO decomposition. This behaviour is similar to the copper ion catalysed *S*-nitrosothiol decomposition.

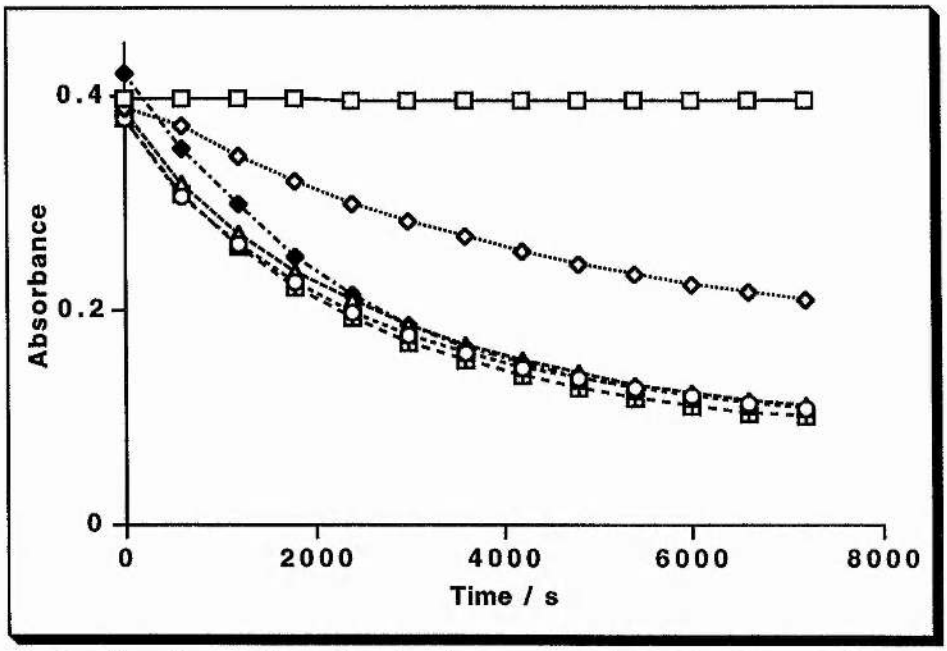


Figure 2. 63: Absorbance-time plots for the reaction of GSNO (6.0×10^{-5} mol dm^{-3}) in the presence of added ascorbic acid at pH = 7.4, 340 nm, and 30 °C.
(a) no added ascorbic acid (square), (b) 6.0×10^{-5} mol dm^{-3} ascorbic acid (diamond), (c) 3.0×10^{-4} mol dm^{-3} ascorbic acid (segmented diamond), (d) 6.0×10^{-4} mol dm^{-3} ascorbic acid (triangle), (e) 9.0×10^{-4} mol dm^{-3} ascorbic acid (circle), (f) 1.5×10^{-3} mol dm^{-3} ascorbic acid (segmented square).

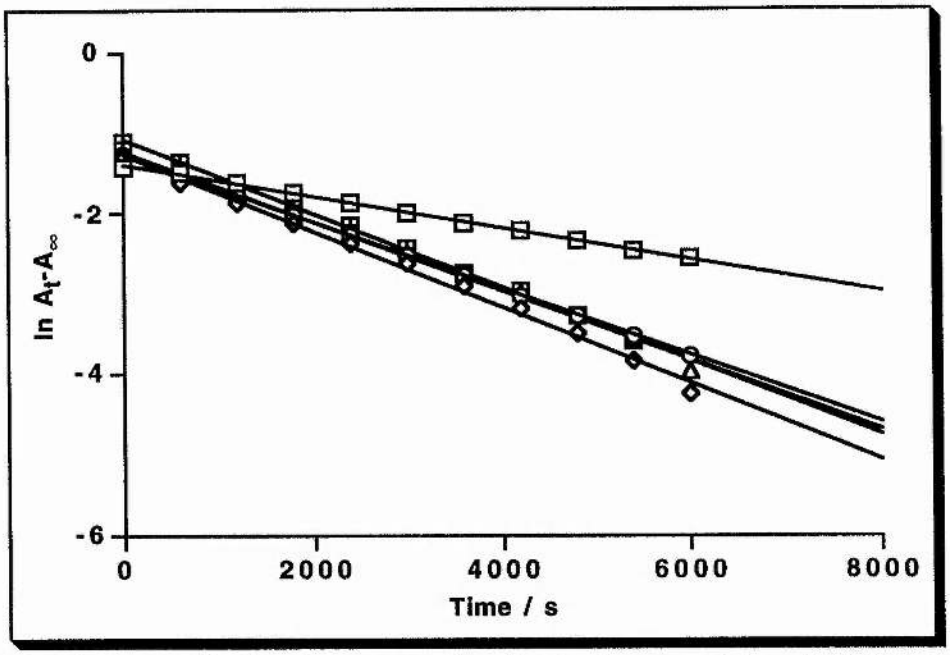


Figure 2. 64: Plots of ln (A_t-A_∞) versus time for the decomposition of GSNO (6.0 x10^{-5} mol dm^{-3}) in the presence of added ascorbic acid at pH = 7.4, 340 nm, and 30 °C. (a) 6.0 x 10^{-5} mol dm^{-3} ascorbic acid (square), (b) 3.0 x 10^{-4} mol dm^{-3} ascorbic acid (diamond), (c) 6.0 x 10^{-4} mol dm^{-3} ascorbic acid (circle), and (d) 9.0 x 10^{-4} mol dm^{-3} ascorbic acid (triangle), (e) 1.5 x 10^{-3} mol dm^{-3} ascorbic acid (segment square). (r = 1.000, 0.998, 1.000, 0.999, and 0.999 respectively).

On the other hand, addition of L-ascorbic acid and EDTA reduces the decomposition of *S*-nitrosothiols compared with that caused by L-ascorbic acid only. Figures 2. 65 and 2. 66 show the effect on the decomposition of adding L-ascorbic acid and EDTA. The rate of GSNO decomposition in the presence of L-ascorbic acid and EDTA decreases by a factor of two compared with the GSNO decomposition in the presence of L-ascorbic acid only (Figure 2. 67 and Table 2. 9). This means that the EDTA must remove most of the metal ions in the buffer. From the previous Figure it is seem that, at low L-ascorbic acid concentration the reaction was first order with respect to L-ascorbic acid, at high L-ascorbic acid concentration the reaction was zero order with respect to L-ascorbic acid.

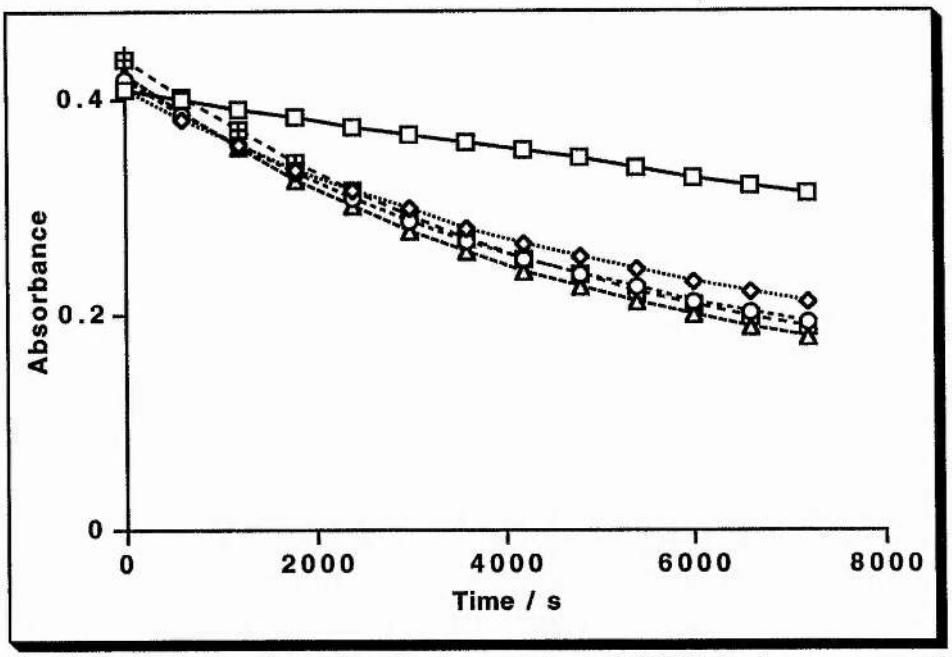


Figure 2. 65: Absorbance-time plots for the reaction of GSNO in the presence of EDTA ([GSNO] = [EDTA]; 6.0 x 10^{-5} mol dm^{-3}) and varying concentrations of added ascorbic acid at pH = 7.4, 340 nm, and 30 °C. (a) 6.0 x 10^{-5} mol dm^{-3} ascorbic acid (square), (b) 3.0 x 10^{-4} mol dm^{-3} ascorbic acid (diamond), (c) 6.0 x 10^{-4} mol dm^{-3} ascorbic acid (circle) , (d) 9.0 x 10^{-4} mol dm^{-3} ascorbic acid (segmented square), (e) 1.5 x 10^{-3} mol dm^{-3} ascorbic acid (triangle).

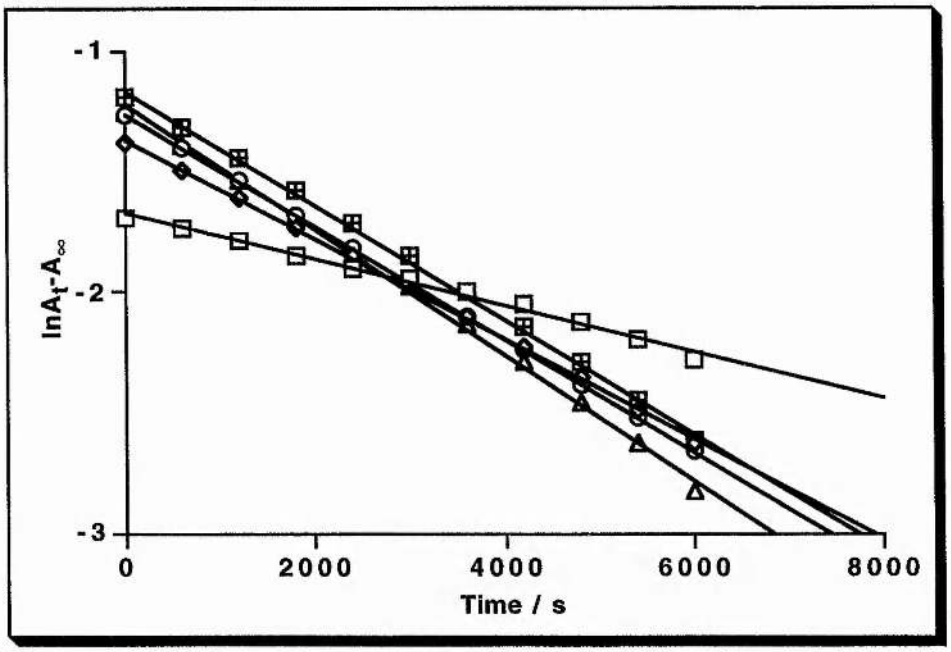


Figure 2. 66: Plots of ln (A_t-A_∞) versus time for the decomposition of GSNO in the presence of EDTA ([GSNO] = [EDTA]; 6.0 x 10^{-5} mol dm^{-3}) and varying concentrations of added ascorbic acid at pH = 7.4, 340 nm, and 30 °C. (a) 6.0 x 10^{-5} mol dm^{-3} ascorbic acid (square), (b) 3.0 x 10^{-4} mol dm^{-3} ascorbic acid (diamond), (c) 6.0 x 10^{-4} mol dm^{-3} ascorbic acid (circle), (d) 9.0 x 10^{-4} mol dm^{-3} ascorbic acid (triangle), and (e) 1.5 x 10^{-3} mol dm^{-3} ascorbic acid (segment square). (r = 0.997, 1.000, 0.999, 0.999, and 0.999 respectively).

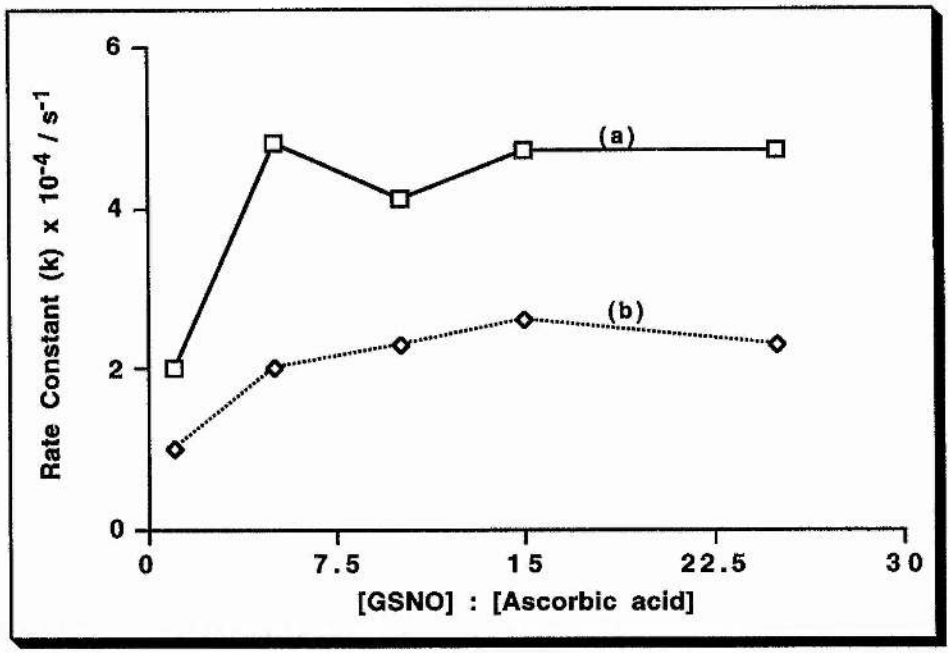


Figure 2. 67: First order rate constants (k) for the reaction of GSNO (6.0 x 10^{-5} mol dm^{-3}) at pH = 7.4, 340 nm, and 30 °C (a) No added EDTA , and (b) Added EDTA (6.0 x 10^{-5} mol dm^{-3}) as a function of added ascorbic acid.

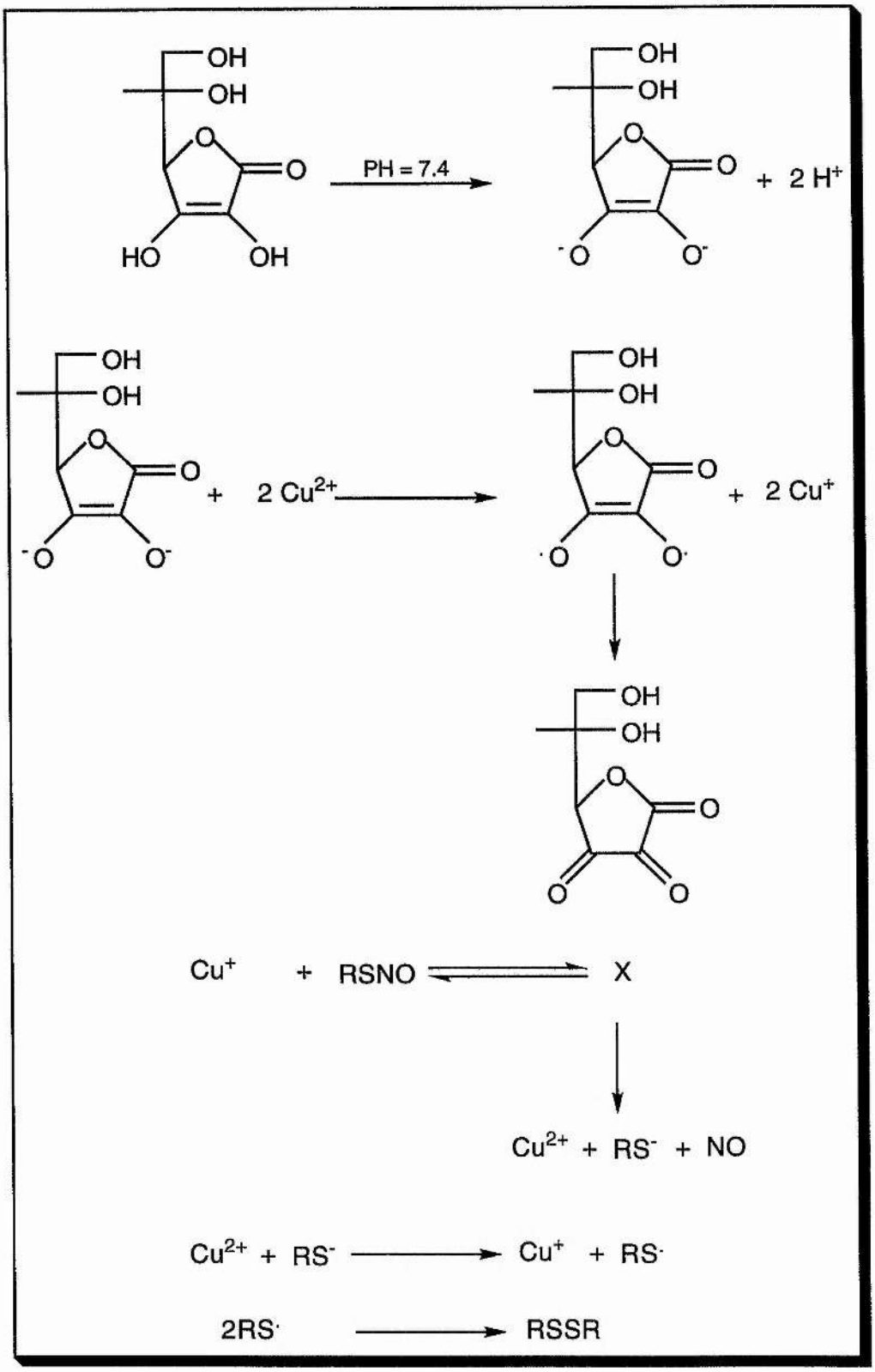
From the primary previous results, we suggest two possible mechanisms of *S*-nitrosothiol decomposition by L-ascorbic acid. The first one involves the reduction of Cu^{2+} by L-ascorbic acid to Cu^{+}. Details are reproduced below (Scheme 2. 18). The second one involves electron-transfer from L-ascorbic acid to RSNO to give dehydroascorbic acid and *S*-nitrosothiol radical anion $[RSNO]^{\cdot}$. Details are also reproduced below (Scheme 2. 19).

At this stage it is difficult to decide if either of these mechanisms functions *in vivo*. In the human body many chemicals and enzymes function and it is very difficult to decide which one is favoured.

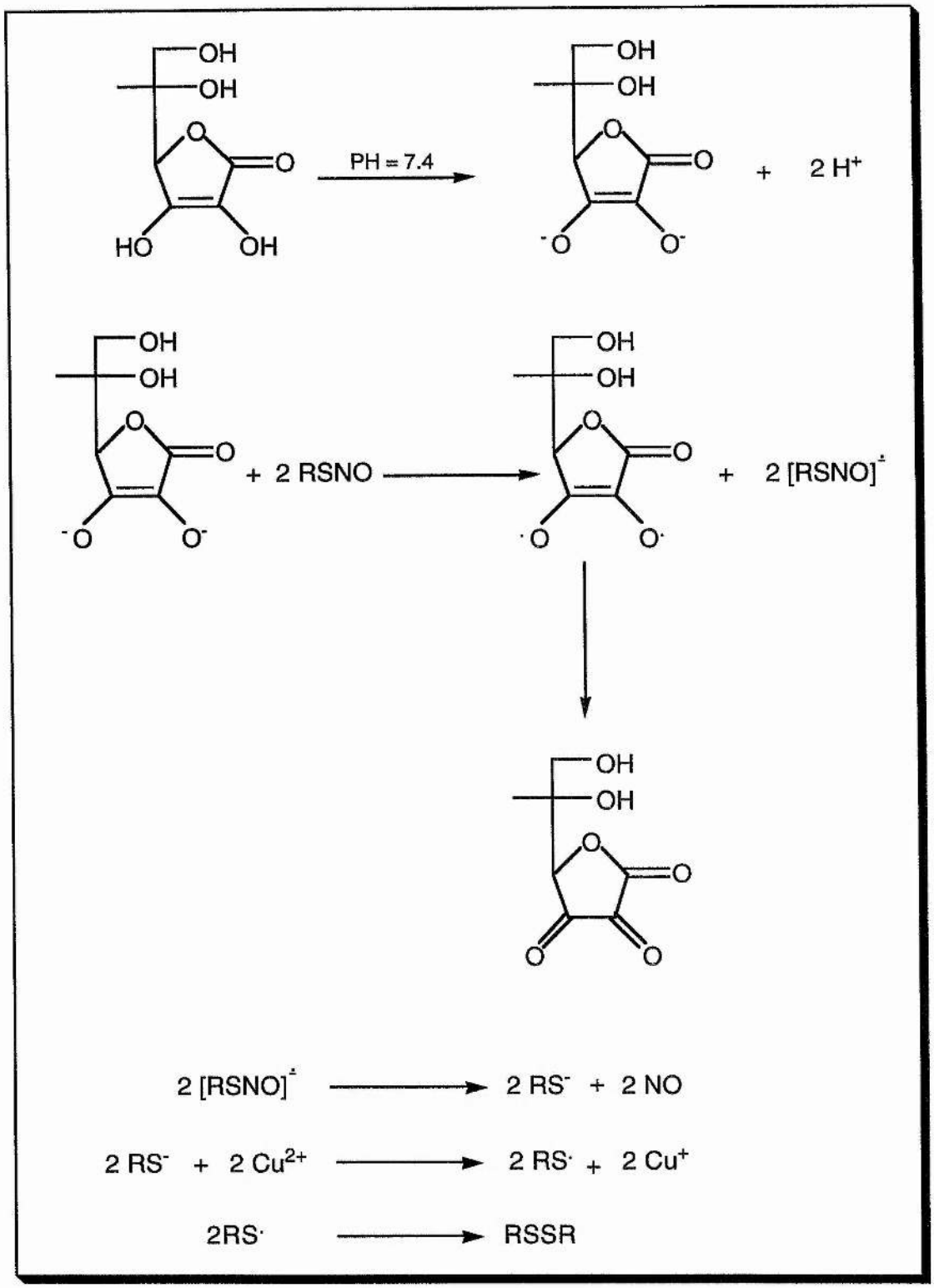
TABLE 2. 9

First order rate constants (k) for the decomposition of GSNO (6.0×10^{-5} mol dm^{-3}) in the absence and in the presence of EDTA (6.0×10^{-5} mol dm^{-3}) and various concentrations of added ascorbic acid at pH = 7.4, 340 nm, and 30 °C

[GSNO] : [ascorbic acid]	Rate Constant x 10^{-4} /s^{-1} No Added EDTA	Rate Constant x 10^{-4}/s^{-1} Added EDTA
1 : 1	2.0	1.0
1 : 5	4.8	2.0
1 : 10	4.1	2.3
1 : 15	4.7	2.6
1 : 25	4.7	2.3



Scheme 2. 18: The proposed mechanism of *S*-nitrosothiol decomposition by L-ascorbic acid (vitamin C; mechanism A).



Scheme 2. 19: The proposed mechanism of *S*-nitrosothiol decomposition by L-ascorbic acid (vitamin C; mechanism B).

According to the first mechanism (mechanism A; Scheme 2. 18) at pH 7.4 L-ascorbic acid will be converted into the corresponding ascorbate which will react with Cu^{2+} (present in the buffer) to give the ascorbate diradical (dehydroascorbic acid) and Cu^{+}. Cu^{+} will then react with the *S*-nitrosothiols as described previously.

According to the second mechanism (mechanism B; Scheme 2. 19) at pH 7.4 L-ascorbic acid will converted into the corresponding ascorbate which will react with *S*-nitrosothiol by an electron-transfer mechanism to give the ascorbate diradical and *S*-nitrosothiol radical anion $[RSNO]^{\cdot}$.The *S*-nitrosothiol radical anion $[RSNO]^{\cdot}$ will be converted directly into the corresponding thiolate (RS^{-}) and nitric oxide.

Due to the effect of EDTA on the ascorbic acid catalysed reaction, the experimental evidence is consistent with the first mechanism. Thus, L-ascorbic acid could play a role in the *in vivo* release of NO from naturally occurring *S*-nitrosothiols and could be responsible, in part, for the biological activity of the endogenous *S*-nitrosothiols. We suggest that the role of L-ascorbic acid in the decomposition of *S*-nitrosothiols is as a reducing agent for Cu^{2+} which is present *in vivo*.

2. 6. 2. The Effect of L-Ascorbic Acid on the Decomposition of *S*-Nitrosothiols *ex vivo*

Here we will study the effect of L-ascorbic acid on smooth muscle relaxation and platelet aggregation.

2. 6. 2. 1. The Effect of L-Ascorbic Acid on Smooth Muscle Relaxation

The effect of L-ascorbic acid on the activity of GSNO (**13**) *ex vivo* was investigated using L-ascorbic acid and GSNO with rat tail artery as target cells. *In vitro*, GSNO decomposes rapidly to the corresponding disulphide and NO in the presence of L-ascorbic acid. So, we were not able to study the *ex vivo* action of bolus injections of the drug and L-ascorbic acid premixed before injection.

Therefore, the *ex vivo* vasodilation action of bolus injections of GSNO was studied with L-ascorbic acid in the internally perfused Krebs buffer supply (Figure 2. 68). The effect of L-ascorbic acid on the action of GSNO was not great (Figure 2. 69).

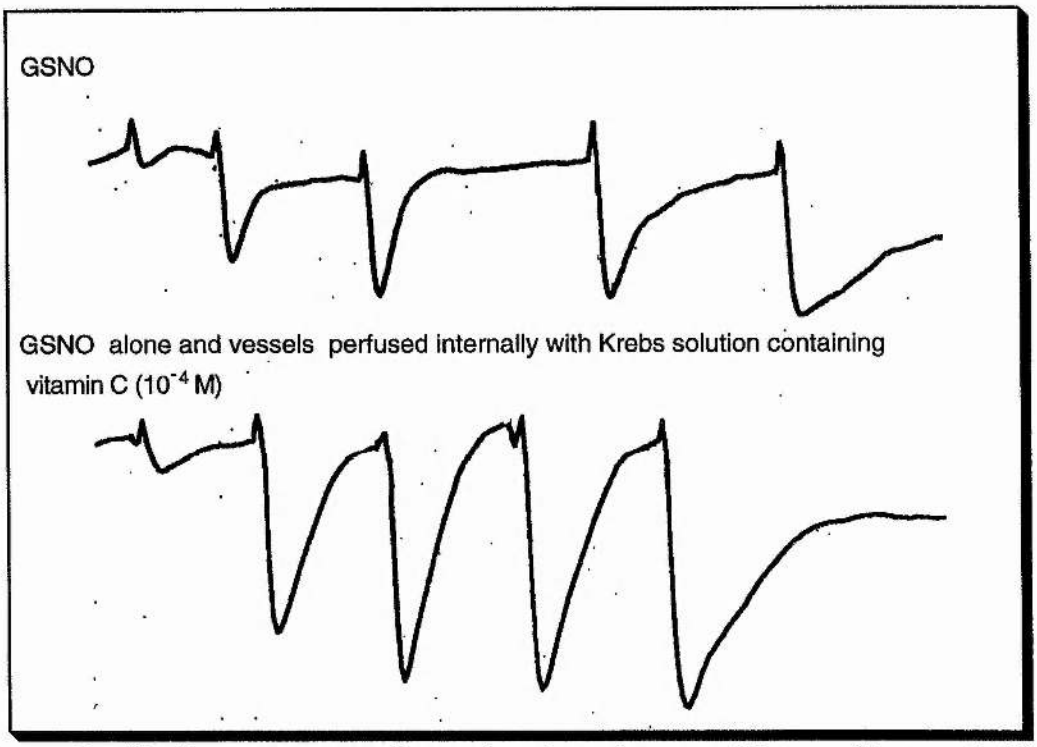


Figure 2. 68: Pressure recordings showing the vasodilator effects of 10 µl microinjections of GSNO (10^{-3} - 10^{-7} M) in the absence and presence of L-ascorbic acid (vitamin C).

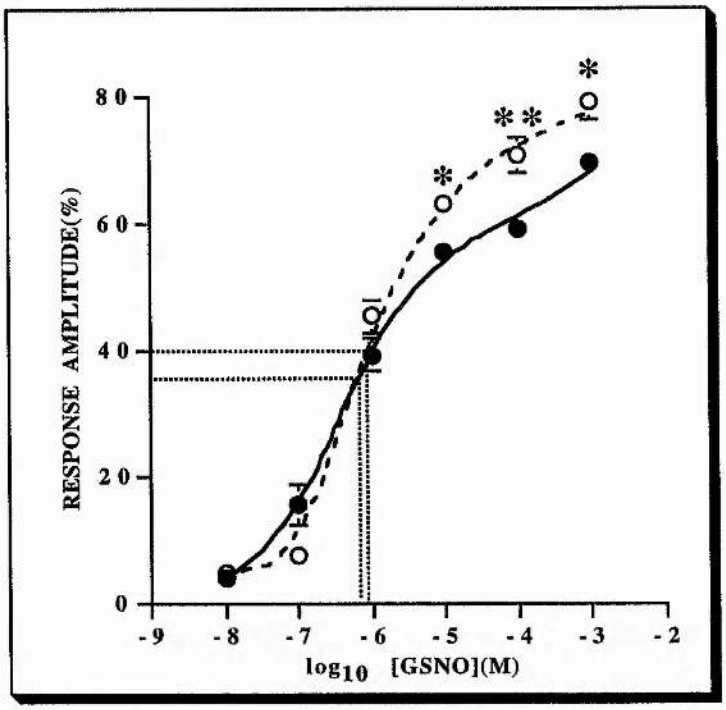


Figure 2. 69: Log dose-response curves comparing the vasodilator effects of 10 μl bolus injections of GSNO; **13**. Filled symbols show responses in the absence of L-ascorbic acid and open symbols show responses in the presence of L-ascorbic acid (100 μM). Paired *t*-tests were carried out to determine the statistical significance of responses to **13** in the presence of L-ascorbic acid compared to those of GSNO. (*= $P<0.05$, **= $P<0.01$, ***= $P<0.001$).

The ED_{50} value for microinjection of GSNO was found to be approximately the same in the absence and in the presence of 10^{-4} M L-ascorbic acid (Figure 2. 69). As GSNO has 3-5 seconds to react with L-ascorbic acid before reaching the artery, it is reasonable to suppose that it decomposes to the corresponding disulphide and NO.

From our results, we conclude that L-ascorbic acid has no significant role in vasodilation.

2. 6. 2. 2. The effect of L-ascorbic acid on platelet aggregation

The effect of L-ascorbic acid on the activity of GSNO (**13**) *ex vivo* was investigated using human platelets as target cells.

We found that L-ascorbic acid had no significant effect or only a very small effect on the anti-aggregation action of GSNO. The IC_{50} value for microinjection of GSNO was found to be approximately the same in the absence and in the presence of 10^{-4} M L-ascorbic acid (IC_{50} = 2.4 μM (no added vitamin C), and IC_{50} = 2.1 μM (added vitamin C); Figure 2. 70).

This, may be due to the following reasons:

a) The concentration of L-ascorbic acid in the human platelet is high already. Therefore, addition of 2 μl L-ascorbic acid (10^{-4} M) will not increase the concentration of L-ascorbic acid in the human platelet that much.

b) From the kinetics results, we found that at high concentration of L-ascorbic acid the rate becomes independent of the L-ascorbic acid concentration, which means addition of more L-ascorbic acid will not increase the rate of the decomposition.

From our results, we conclude that L-ascorbic acid has no significant role in inhibition of platelet aggregation.

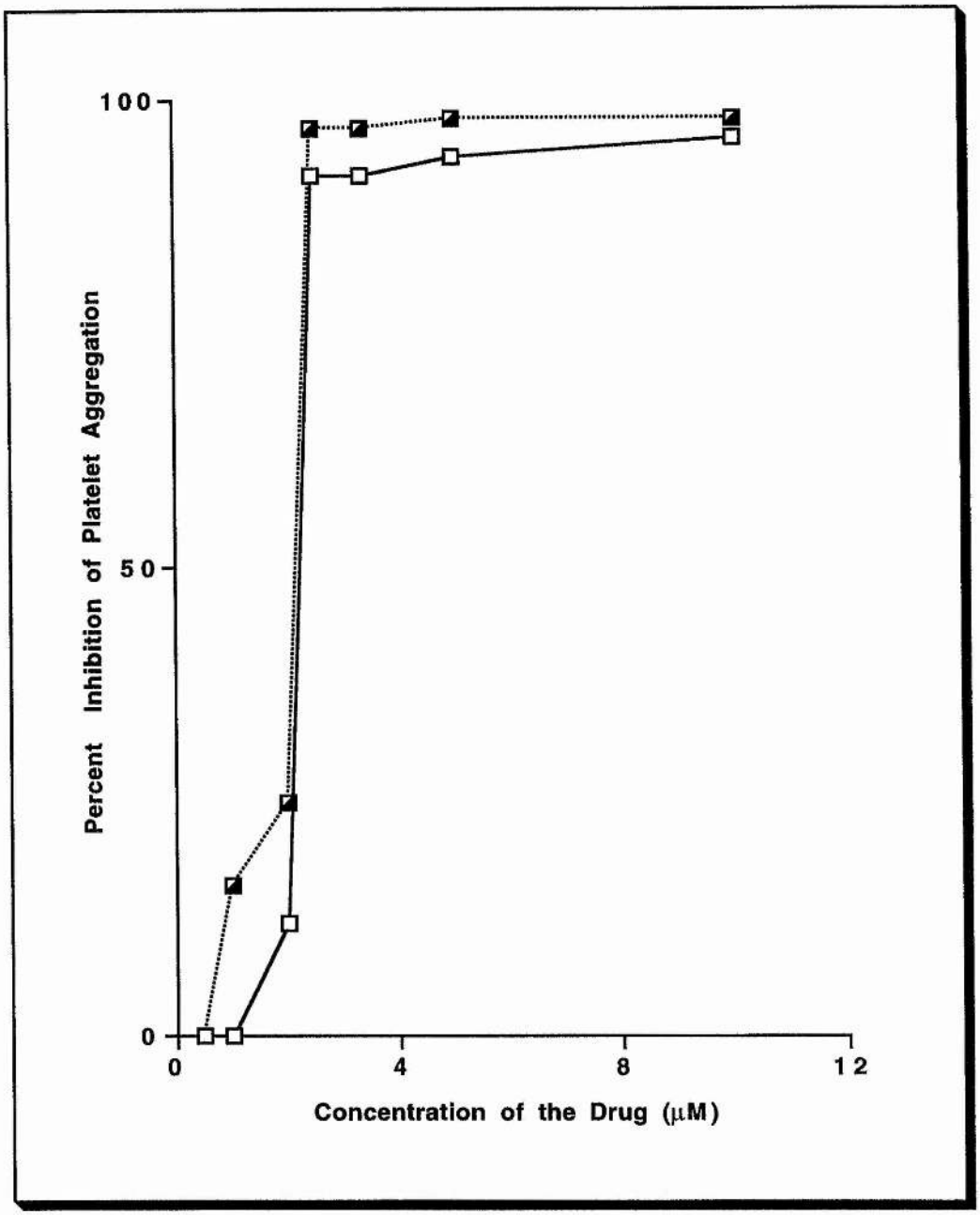


Figure 2. 70: Inhibition of collagen-induced platelet aggregation by GSNO.
(a) added vitamin C (10^{-4} M; half filled square).
(b) no added vitamin C (square).

The work in this section is preliminary. Therefore, more experiments *in vitro* and *in vivo* should be carried out to give a clear picture about the mode of action of L-ascorbic acid *in vivo*.

2. 7. Detection of NO to Show that *S*-Nitrosothiols are NO-Donor Drugs

Here we describe efforts to detect NO by three methods to show that *S*-nitrosothiols are NO-donor drugs. The first method is the use of an ISO-NO sensor, the second method is addition of haemoglobin (Hb) to the internal perfusate of smooth muscle, and the third method is addition of Hb to the platelet suspension.

a) Evidence from NO-Probe

We detected nitric oxide from the decomposition of *S*-nitrosothiols (**1-13**) using the NO-probe electrode system when the reaction was carried out anaerobically. Yields of more than 70% have been noted for the more reactive *S*-nitrosothiols (e.g. SNAP) but the yield was always far from quantitative. However, as yet we have not taken steps to minimise the loss of NO to the headspace and by oxidation and hydrolysis to nitrite (Wink *et al.*, 1993), so that for the slower reacting compounds (**2-13**) this loss is a major competitor. Figure 2. 71 shows the response of an ISO-NO sensor (2 mm) to NO released from GSNO, one of the less reactive *S*-nitrosothiols in our series (0.5×10^{-3} mol dm^{-3}) catalysed by L-ascorbic acid (0.5×10^{-3} mol dm^{-3}) under aerobic conditions at pH = 7.4 and at RT. In the presence of oxygen, however, the final product is nitrite ion. Separate experiments using only nitric oxide solutions under aerobic conditions have confirmed (Wink *et al.*, 1993) that nitrite ion is the only product. The rationalisation of this is given by the following equations:

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

$$NO_2 + NO \rightleftharpoons N_2O_3$$

$$N_2O_3 + 2OH^- \rightleftharpoons 2NO_2^- + H_2O$$

Nitric oxide is oxidised to nitrogen dioxide which, in turn, reacts with more nitric oxide to give dinitrogen trioxide. In aqueous solution at pH = 7.4 this is converted into nitrite anion.

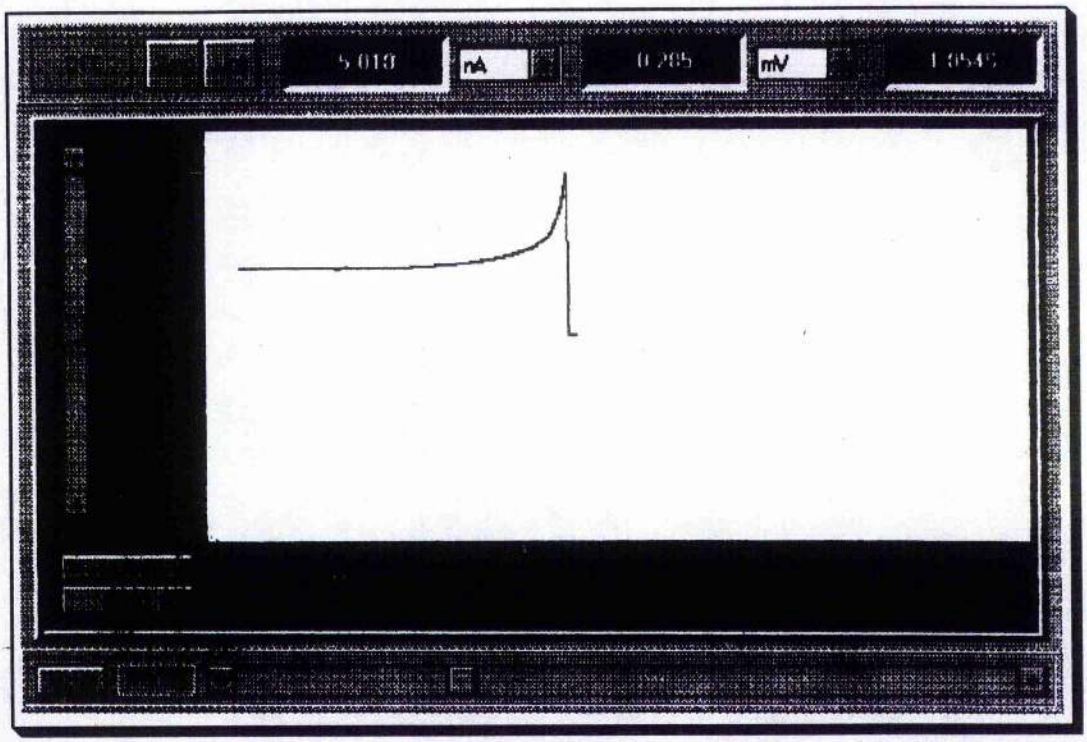


Figure 2. 71: The response of an ISO-NO sensor (2 mm) to NO released from GSNO (0.5×10^{-3} mol dm^{-3}) by L-ascorbic acid (0.5×10^{-3} mol dm^{-3}) in aerobic conditions at pH = 7.4 and at RT.

b) Evidence from Smooth Muscle Relaxation

The ability of NO to bind to free coordination sites of iron complexes has important implications in biology. The formation of such nitrosyl complexes may result to activation / inhibition of enzymatic systems containing iron centers.

One well-known example is deoxyhaemoglobin which binds NO with a much greater affinity than O_2 and CO (Traylor and Sharma, 1992):

$$HbFe(II) + NO \rightleftharpoons HbFe(II)(NO)$$

$$HbFe(II)(NO) + O_2 \rightleftharpoons HbFe(III)$$

Nitrosylhaemoglobin is paramagnetic and can be easily detected by EPR spectroscopy at 77 K. This offers a very convenient method for the spin trapping of NO (Henry *et al.*, 1991).

The iron centre is oxidized in the presence of oxygen. Methaemoglobin is formed together with nitrite and nitrate. It should be noted that, unlike O_2, NO binds to both iron(II) and iron(III) porphyrin. The great reactivity of NO towards haemoglobin suggests that in red blood cells NO will be rapidly converted into NO_3^- and eliminated.

In this research, the inhibitory effect of Hb, a recognised NO scavenger, was investigated. Addition of Hb to the internal perfusate produced a rapid rise in pressure. Injections of drugs in the presence of Hb are still capable of producing substantial vasodilator responses. Responses to intermediate doses of *S*-nitrosothiols were significantly inhibited by Hb, though not abolished entirely. It is possible, therefore, that only part of *S*-nitrosothiols-induced vasodilation is due to NO released in solution in the lumen. The Hb-resistant element of vasodilator responses to *S*-nitrosothiols may be due either to an NO-independent mechanism, or to 'cross-nitrosation' of *S*-nitrosothiols with membrane-bound or intracellular thiols. In the latter case, free NO would not exist as a single entity outside the tissue and would be resistant to Hb inactivation.

Exogenous Hb, which binds NO extracellularly, has been used as a classic probe of NO generation in the extracellular space (Martin *et al.*, 1985; Ignarro, 1989a&b). Typical traces showing the effect of perfusing ferro-haemoglobin (Hb, 15 μM) through the internal circuit, on SNAP (**1**), (**2**), and GSNO(**13**) vasodilation responses are shown in Figure 2. 41. Ferro-haemoglobin reduced the ED_{50} value for SNAP (**1**) by a factor of 10 only, by different factors of 70 to 200 for **2-12** whereas that for GSNO (**13**) is reduced by a factor of 200 (Table 2. 10, and Figures 2. 42 - 2. 54 respectively). These results are consistent with the finding that SNAP is less stable than GSNO in solution and suggest that SNAP does not require tissue to release NO, decomposing, at least in part, in the lumen. The vasodilator actions of SNAP (**1**), **2-12**, and GSNO (**13**) are only partially inhibited by 15 μM Hb. This suggests that a significant percentage of each drug reaches the vascular smooth muscle cells before releasing NO, probably at the membrane surface (Kowaluk and Fung, 1990a&b).

TABLE 2. 10

A comparison of the vasodilator effectiveness of SNAP, **2-12**, and GSNO on rat tail artery in the absence and presence of 15 μM Hb
(the vasodilator actions of *S*-nitrosothiols are only partially inhibited by 15 μM Hb)

Compound Number	ED_{50} (μM) no added Hb	ED_{50} (μM) 15 μM added Hb
SNAP (**1**)	20	223
2	4	272
3	3.5	223
4	3	223
5	1.5	202
6	45	332
7	10	90
8	4.5	223
9	9.5	165
10	5	406
11	0.8	165
12	2	182
GSNO (**13**)	0.9	182

c) Evidence from Inhibition of Platelet Aggregation

From our results, we found that the inhibitory activity of these *S*-nitrosothiols was reversed by haemoglobin, indicating the involvement of NO in the process.

2. 8. Do *S*-Nitrosothiols Decompose Inside or Outside the Cell?

From our *in vitro* experiments, we found that the *S*-nitrosated amino acid (**1**) was less stable than *S*-nitrosated dipeptides (**2-12**), and the latter were less stable than the *S*-nitrosated tripeptide (**13**). This means that, by increasing the molecular size, the chemical stability is increased.

From a chemical point of view, SNAP (**1**), and GSNO (**13**) were less lipophilic than *S*-nitrosated dipeptides. On the other hand, GSNO is said to be more lipophilic than SNAP. GSNO is more bulky, more polar, and more ionic than SNAP, which suggests that SNAP may enter the cell more easily than GSNO. If *S*-nitrosothiols decompose before entering the cell, we would expect that SNAP will be more effective in biological assays than *S*-nitrosated dipeptides, and the latter will be more effective than GSNO, but this not observed. Moreover, if *S*-nitrosothiols decompose after entering the cell we expect that *S*-nitrosated dipeptides would be more effective than SNAP and the latter more effective or similar to GSNO.

From our results, we found that the latter expectation is more likely. Furthermore, the polar, and probably the ionised nature of SNAP and GSNO at physiological pH, makes it unlikely that they would traverse cell membranes readily, which supports this hypothesis.

So **2-12** are substantially more potent than SNAP although the chemical environment of the -SNO groups are almost identical. Thus, there is an inverse correlation between chemical reactivity and biological activity, a result which provides further evidence that extracellular decomposition of *S*-nitrosothiols to give NO cannot account for its vasodilator effect. It suggests, rather, that the *S*-nitrosothiol enters the cell intact, a process controlled partly by the lipophilicity of the complete molecule, before decomposition occurs.

There are three possibilities for the decomposition of *S*-nitrosothiols inside or outside the cell:

(a) the differences in effectiveness are possibly due to the rate of NO generation at the cell surface, due to differences in the ability of different tissues to catalyse RSNO decomposition,

(b) the target cell may have a transport system that could allow entry of a specific RSNO into the cell making it more effective on the tissue than on another tissue in which it is not transported, and

(c) *S*-nitrosothiols enter the cell first, then *S*-nitrosothiols decompose by one of the previous mechanisms (Section 2. 2 and 2.6), and this possibility depends upon the lipophilicity of the RSNO.

SNAP can spontaneously release NO without the presence of tissue. *S*-Nitrosated dipeptides (**2-12**) may release NO in the presence of tissue, because they show that they are more effective than SNAP and also they are more stable (Section 2. 4. 1).

The response of *S*-nitrosated dipeptides and GSNO is not completely abolished by ferro-haemoglobin and a significant percent of the drug, or 'bound NO' from interaction of SNAP with tissue such as the endothelium, reaches the vascular smooth muscle cells.

From our results, we suggest that *S*-nitrosothiols which are polar, ionic, and can spontaneously release NO may decompose before entering the cell or at the cell surface. *S*-Nitrosothiols which are less polar, not ionic, lipophilic, and cannot spontaneously release NO may decompose inside the cell, or may use a transport system that could allow entry of a specific RSNO into the cell. GSNO is more lipophilic than SNAP and it is more likely that GSNO decomposes inside the cell by one of the previous mechanisms.

We are forced to conclude that *S*-nitrosothiols decompose inside and outside the cell, depending upon the structure of RSNO.

2. 9. A Tissue Selective NO-Donor Drug

Smooth muscle and platelet respond differently to different *S*-nitrosothiols. These differences are not likely to be related to any intrinsic activity of the R- group of the RSNOs, because the starting thiols were inactive in each of the assays. The difference could be related to factors such as the agent used to contract the tissue (Rapoport *et al.*, 1983a,b&c) or the sensitivity of the different soluble GCs to activation by NO. The differences could also be due to variations in the rate of NO generation at the cell surface. The difference could even be due to variations in the rate of NO generation inside the cell, depending upon the structure of the *S*-nitrosothiol. In addition to displaying differences in overall sensitivity to *S*-nitrosothiols, the various tissues revealed different rank orders of activity, indicating that these tissues can somehow distinguish among the various *S*-nitrosothiols. One possibility is that the cell surfaces in the various tissues differ in their ability to catalyse *S*-nitrosothiol decomposition. Alternatively, NO release at the cell surface is not correct in all cases (Section 2. 8). For example, in some cases the target cell might have a transport system that could allow entry of a specific *S*-nitrosothiol into the cell, and this compound could be very potent in the cell. The same *S*-nitrosothiol might be much less potent at other target cells which do not have this transport system.

The profile of biological activity of the *S*-nitrosothiols that is described here is similar to that seen with clinically used vasodilators. This is not surprising, because *S*-nitrosothiols have been suggested to be the active metabolites of the nitrovasodilators in the development of tolerance. Several reports in the literature indicate that *S*-nitrosothiols may not develop tolerance (Henry *et al.*, 1989; Shaffer *et al.*, 1992; Bauer and Fung, 1991; Kowaluk and Fung, 1990a&b). Our findings that the activity of *S*-nitrosothiols is not related to their chemical stability and that the tissue specificity is a function of the R- group suggest that a stable, active *S*-nitrosothiol targeted to a specific site may be found. Further research in this area may yield useful therapeutic agents.

2. 10. Conclusion

S-Nitrosothiols are an important class of NO-donor drugs. These compounds are of interest because of their potent pharmacological properties and possible physiological role in smooth muscle relaxation and inhibition of platelet aggregation.

A series of eleven new *S*-nitrosothiols (**2-12**) derived from dipeptides has been prepared, and their chemical properties and physiological effects studied and compared with those of SNAP (**1**), derived from an amino acid, and GSNO (**13**), derived from a tripeptide. In order to look for a correlation between structure, chemical stability and physiological activity. Among the thirteen *S*-nitrosothiols (**1-13**) whose structure are depicted in the previous Section (Section 2. 1) only SNAP (**1**) and GSNO (**13**) have been prepared and characterised previously.

The situation has been complicated by the recent discovery that the main route for the release of NO from *S*-nitrosothiols is a copper (I)-catalysed process. Therefore, we examined the effect of copper ions on the stability of the compounds we synthesised.

A detailed kinetic study has shown that the dominant pathway for the decomposition of *S*-nitrosothiols in most circumstances is one catalysed by Cu^{+} ions. We suggest that the thiolate ion necessary to bring about Cu^{2+} reduction is either present as a thiol impurity or is generated in small quantities by partial hydrolysis of *S*-nitrosothiols, which results in an induction period. The implications of this discovery for an understanding of the biological action of *S*-nitrosothiols are discussed.

In the absence of Cu(I) the *S*-nitrosothiols are much more stable. All the new compounds examined show less susceptibility to copper (I)-catalysed release of NO than SNAP but are more reactive than GSNO. We have found that *S*-nitrosated dipeptides are potent vasodilators and suitable inhibitor for platelet aggregation but are chemically very stable in the absence of copper ions.

All thirteen compounds (**1-13**) combine the favoured property of chemical stability with a high level of biological activity.

However, no correlation was found between the solution stability of the compounds and their biological activity, suggesting that other factors may be important. For example, due to their thermal instability the compounds are likely to release some NO spontaneously in the lumen where it would be susceptible to Hb inactivation. On the other hand, *S*-nitrosothiols undergoes a process known as 'cross nitrosation' or transnitrosation in the presence of -SH-containing compounds, such as cysteine. It is possible, therefore, that NO could be 'stripped' from *S*-nitrosothiols and then transported into a cell where it may not be susceptible to Hb inhibition. Potentially, therefore, *S*-nitrosothiols can release NO by several mechanisms, only some of which is susceptible to Hb inhibition.

The effect of L-ascorbic acid (vitamin C) on the activity of the *S*-nitrosothiols was also examined, but was found to be negligible. This may be due to the two factors. Firstly, the concentration of L-ascorbic acid in human platelet is already high, and so may not be changed very much by the addition of a small amount, and, secondly, the chemical studies showed that at high concentrations the effect of the decomposition of the *S*-nitrosothiols become independent of the L-ascorbic acid concentration, so that an increase in concentration will have no effect on the rate.

All thirteen compounds examined show ability to release NO *in vitro*. The inhibitory effect of Hb, a recognised NO scavenger, was investigated. Responses to intermediate doses of *S*-nitrosothiols were significantly inhibited by Hb, though not abolished entirely. We found that the inhibitory activity of these *S*-nitrosothiols was reversed by haemoglobin, indicating the involvement of NO in the process.

The potency of the compounds studied was found to be very markedly as a function of structure, by up to 5 order of magnitude in the platelet aggregation studies, and up to 55 order of magnitude in the smooth muscle relaxation studies, even though the difference in the group R- were relatively small. This suggests that it may be possible to produce a stable, active *S*-nitrosothiols targeted to a specific site by a suitable choice of the group R.

Overall, then, we conclude that the biological activity of *S*-nitrosothiols depends upon the release of NO in a process catalysed by Cu(I), and that the decomposition may occur inside or outside the cell, depending upon the structure of RSNO.

We found that copper(I) chelation induced reduction of the biological activity of *S*-nitrosothiols in smooth muscle relaxation. The results show that responses to both SNAP and GSNO are reversibly inhibited by neocuproine. We conclude that relaxation of vasodilator smooth muscle by SNAP and GSNO is caused in part by NO released into solution *via* a Cu^{+}-dependent catalytic reaction. One may speculate that a similar process is involved in the maintenance of a vasodilator 'store' *in vivo*, in which endogenous Cu(I) ions catalyse the decomposition of naturally occurring *S*-nitrosothiols.

It is interesting to compare the results given here with those recently reported by Gordge *et al.* (1996), who showed that the inhibition of platelet aggregation activity shown by GSNO is much reduced in the presence of neocuproine and the closely related bathocuproine, both specific Cu(I) chelating agents, and those of Schrammel *et al.* (1996), who showed that copper ions inhibit basal and NO-stimulated recombinant soluble guanylate cyclase activity, and that Cu(I) is more effective than Cu(II) in this regard.

2. 11. Scope of Future Work

Due to the importance of NO-donor drugs there are many areas of work which can be further researched:

1. The design and synthesis of a new generation of *S*-nitrosothiols with the following properties:

(a) Stable drugs.

(b) Solid drugs.

(c) Lipophilic drugs.

(d) They can release NO *in vivo*.

(e) They do not release NO during storage.

(f) With high purity.

2. Initial results in this thesis show that vitamin C has a dramatic effect on the stability of GSNO and presumably other *S*-nitrosothiols. As vitamin C is present in larger concentrations than copper *in vivo*, a full study of the effect of vitamin C on *S*-nitrosothiols is needed, in order to understand the role of vitamin C *in vitro* and *in vivo*, and to suggest the mechanism of action.

3. More work should be carried out using the NO electrode in order to gain more quantitative results.

4. Synthesis of *S*-nitrosothiols which have alternative metal ion binding sites away from the -SNO moiety.

5. More studies should be carried out on human blood plasma. This would give a better insight into how robust this class of drugs are likely to be in the *in vivo* situation, and whether they are still susceptible to copper catalysis in this more complex media.

6. Initial results presented in this thesis show that the photochemical decomposition of *S*-nitrosothiols occurs. As laser light could be used to accelerate the endogenous decomposition of *S*-nitrosothiols.

7. More research into the synthesis of tissue selective drugs to release NO in specific tissues.

8. Despite the fact that *S*-nitrosothiols are potent mediators of NO signalling, the mechanism(s) by which they donate NO and alter cellular function are poorly understood. More experiments *in vivo* should be carried out in order to increase our knowledge of these exogenous *S*-nitrosothiols.

III. EXPERIMENTAL

In this chapter, we describe in detail the various methods employed in our work. These include instrumental, synthetic, pharmacological and physiological procedures.

EXPERIMENTAL

3. 1. Chemicals

The following is a list of chemicals and materials used throughout this work: *N*-cyclohexyl-*N'*-2-[*N*-morpholino]ethylcarbodiimide metho-*p*-toluenesulphonate (Aldrich), *t*-butyl nitrite (Aldrich), *N*-acetyl-D,L-β,β-dimethylcysteine (Sigma), glycine methyl ester hydrochloride (Sigma), L-alanine methyl ester hydrochloride (Sigma), L-valine methyl ester hydrochloride (Sigma), L-leucine methyl ester hydrochloride (Sigma), L-phenylalanine methyl ester hydrochloride (Sigma), L-isoleucine methyl ester hydrochloride (Sigma), L-methionine methyl ester hydrochloride (Sigma), N_{ε}-CBZ-L-lysine methyl ester hydrochloride (Sigma), L-proline methyl ester hydrochloride (Sigma), L-aspartic acid dimethyl ester (Sigma), L-glutamic acid dimethyl ester (Sigma), and L-glutathione (Sigma). They were used without purification. Dichloromethane was distilled from CaH_2 prior to use (Vogel, 1989). Dry diethyl ether was distilled before use (Vogel, 1989). Dimethyl sulfoxide (DMSO; Sigma-Aldrich Chemicals) was HPLC grade (99.9%). Neocuproine, sodium nitroprusside, phenylephrine and haemoglobin were obtained from Sigma Ltd. Krebs and Henks solution components were obtained from BDH and were Aristar grade. LNMMA was a gift from Dr Harold Hodson of the Wellcome Research Laboratories, Beckenham, Kent. Compounds were dried *in vacuo* over self-indicating silica gel. All the buffer components for the kinetics and chemical stability studies were Aristar grade.

3. 2. Instrumentation

Melting points were determined on a capillary apparatus (Gallenkamb). Elemental analyses were carried out using an Elemental Analyzer Mod. 1106 (CARLO ERBA STRUMENTAZIONE). IR spectra were recorded on a Perkin-Elmer 1710 (Infrared Fourier Transform Spectrometer). UV-Visible spectra were recorded on a Phillips PU8700 UV-Visible scanning spectrophotometer. ^{1}H-NMR spectra were recorded at 300 MHz on a Bruker AM300. ^{13}C-, DEPT 90°-, and DEPT 135°-NMR spectra were recorded at 75 MHz on a Bruker AM300. All chemical shifts (δ) are reported in parts per million (ppm) reference to TMS and coupling constants (J) are quoted in Hz. FAB-Mass spectra were recorded on a VG Auto Spec. (FISONS Instruments) run in 3-nitrobenzyl alcohol. The a apparatus used for perfusing isolated segments of rat tail artery is described in Section 3. 3. 4. 1. All platelet aggregation studies were performed in a Platelet Aggregation Profiler, Blood Platelet Counter (COULTER T-540), and IEC Centra-3C Centrifuge. The NO-sensitive electrode was obtained from World Precision Instruments. Measurements of pH were measured using a Digital pH Meter (Griffin).

3. 3. Experimental Procedures

In this section, we will describe the following procedures: synthesis of the dipeptides, synthesis of the *S*-nitrosothiols, chemical stability of the *S*-nitrosothiols, pharmacological and physiological activity, the effect of L-ascorbic acid, trapping the thiyl radical and detection of NO from *S*-nitrosothiols.

3. 3. 1. Synthesis of the Dipeptides

(i). Procedure A (Bodanszky and Bodanszky, 1984):

A solution of the amino acid methyl ester hydrochloride (50 mmol) in water (40 cm^3) was treated with a solution of potassium carbonate (72 mmol) in water (20 cm^3) and the mixture was extracted with methylene chloride (3x50 cm^3). The methylene chloride extracts were combined, dried over magnesium sulphate and the solvent removed under moderately

reduced pressure. The residue (about 40 mmol) was added to a suspension of *N*-acetyl-D,L-β,β-dimethylcysteine (20 mmol) in purified dichloromethane (100 cm^3) followed by the addition of *N*-cyclohexyl-*N'*-2-[*N*-morpholino]ethylcarbodiimide metho-*p*-toluenesulphonate (20 mmol). A precipitate of the urea derivative started to separate almost immediately and its amount gradually increased. After one day at room temperature the urea derivative was removed by filtration and washed with dichloromethane (40 cm^3). The combined filtrate and washing were extracted with a saturated solution of citric acid (60 cm^3), a saturated solution of potassium hydrogen carbonate (60 cm^3), and water (60 cm^3), dried over magnesium sulphate and evaporated to dryness *in vacuo*. The residue was dissolved in dry diethyl ether; scratching of the walls with a glass rod induced crystallization of the dipeptide. The crystalline solid was collected by filtration, washed with cold dry diethyl ether and dried. The desired dipeptides were formed in moderate yields (~ 25%; data are shown in Table 3.1). The dipeptide was used for the next step without further purification.

(ii). Procedure B (Bodanszky and Bodanszky, 1984):

A solution suspension of *N*-acetyl-D,L-β,β-dimethylcysteine (20 mmol) in purified dichloromethane (50 cm^3) was cooled in an ice-water bath and treated with *N*-cyclohexyl-*N'*-2-[*N*-morpholino]ethylcarbodiimide metho-*p*-toluenesulphonate (20 mmol) and then with a solution of the amino acid ester hydrochloride (21 mmol) and triethylamine (20 mmol) in purified dichloromethane (50 cm^3) . Stirring was continued at 0°C for about an hour and then at room temperature for one day. A precipitate of the urea derivative started to separate almost immediately and its amount gradually increased. After one day at room temperature the urea derivative was removed by filtration and washed with dichloromethane (40 cm^3). The combined filtrate and washing were washed with a saturated solution of citric acid (60 cm^3), a saturated solution of potassium hydrogen carbonate (60 cm^3), and water (60 cm^3), dried over magnesium sulphate and evaporated to dryness *in vacuo*. The residue was dissolved in dry diethyl ether; scratching of the walls with a glass rod induced crystallization of the dipeptide. The crystalline solid was collected by filtration, washed

with cold solvent and dried. The desired dipeptides were formed in moderate yields (~ 25%; data are shown in Table 3.1). The dipeptide was used for the next step without further purification.

The dipeptides were characterised by IR, ^{1}H-, and ^{13}C, DEPT 90° and DEPT 135°-NMR spectroscopy. Accurate mass determinations of the molecular ion peaks were obtained only by the use of high resolution FAB-MS but not by other techniques. Melting points, elemental analyses, and other data are shown in Table 3. 1.

Notes:

(i) Preferably freshly distilled solvents were used.

(ii) The free amino acid ester was not stored: it is gradually transformed into the diketopiperazine.

(iii) Use of a water soluble carbodiimide ensures that the product is not contaminated with urea.

3. 3. 2. Synthesis of the *S*-Nitrosothiols (Nitrosation Reaction)

In this work, *S*-nitrosothiols were prepared by three different methods depending upon the structure of the thiol.

(a) *S*-nitrosated amino-acid

S-Nitroso-*N*-acetyl-D,L-β,β-dimethylcysteine (SNAP; **1**) was prepared as a pure solid by a modified literature procedure (Royle *et al.*, 1983) in high yield (81%). A solution of *N*-acetyl-D,L-β,β-dimethylcysteine (383 mg, 2.00 mmol) in 15 cm^3 of acetone and 1 cm^3 of water was treated with 2.0 cm^3 of *t*-butyl nitrite. After 30 min., the red solution was evaporated to remove the 15 cm^3 acetone, then additon of water, and the green solid was separated off, filtered, washed well with water and then dried under vacuum (355 mg , 1.61 mmol, 81%).

(b) *S*-nitrosated dipeptides

S-Nitrosothiols were synthesised by treatment of the corresponding dipeptide (0.125 mmol) in methylene chloride (2 cm^3) with *t*-butyl nitrite (1 cm^3) at -78 °C. After 30 min., a green solution was obtained and evaporated to dryness *in vacuo*. The residue was suspended in dry diethyl ether a suitable and the green solid *S*-nitrosothiol filtered off and then dried *in vacuo*. In case where the product was green sticky solid, the residue was suspended in a dry diethyl ether (Table 3. 2), then decanted and the rest of the solvent removed by evaporation to dryness *in vacuo*. The *S*-nitrosothiols (**2-12**) were obtained in high yields (greater than 80%) and data are shown in Table 3. 2.

(c) *S*-nitrosated tripeptides

S-Nitroso-L-glutathione(GSNO) was synthesised according to Hart's procedure (1985). To a stirred ice-cold solution of L-glutathione (1.53 g, 5 mmol) in water (8 cm^3) containing 2N HCl (2.5 cm^3) was added in one portion sodium nitrite (0.345 g, 5 mmol). After 40 minutes at 5°C the red solution was treated with acetone (10 cm^3) and stirred for a further 10 minutes. The resulting fine pale red precipitate was filtered off and then washed successively with ice-cold water (5 x 1 cm^3), acetone (3 x 10 cm^3) and ether (3 x 10 cm^3) to afford *S*-nitroso-L-glutathione (1.29 g, 3.8 mmol , 76%).

Samples of *S*-nitrosothiols were characterised by IR (e.g. Figure 2. 2), UV-Visible (e.g. Figure 2. 3), ^1H (e.g. Figure 2. 4), and ^{13}C (e.g. Figure 2. 5), DEPT 90° (e.g. Figure 2. 6), DEPT 135°-NMR spectroscopy (e.g. Figure 2. 7). Accurate mass measurements on the molecular ion peaks were obtained using high resolution FAB-MS.

TABLE 3. 1

Characterisation of the dipeptides by m.p., NMR, FAB-MS, and elemental analysis

Depeptides are sometimes difficult to characterise by elemental analyses as small amounts of the disulphide are present which are difficult to remove. Similar compounds were authenticated by Moynihan and Roberts (1994) without elemental analyses.

I. *N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester[*#]

m.p. 142 °C (dec.). δ_C (300 MHz; $CDCl_3$) 23.24, 28.64, 30.74, 52.31 (CH_3), 41.12 (CH_2), 60.23 (CH), 45.99 (C), 169.87, 170.23, and 170.39 (CO). (Found: m/z (FAB) 263.1061 (MH^+) $C_{10}H_{19}N_2O_4S$ requires 263.1066 (MH^+)). [Found: C, 44.9; H, 6.9; N, 10.6 Calc. for $C_{10}H_{18}N_2O_4S$: C, 45.8; H, 6.9; N, 10.7%].

II. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-alanine methyl ester[*#†‡]

m.p. 150 °C (dec.). δ_C (300 MHz; $CDCl_3$) 17.58, 17.97, 23.22, 23.27, 28.58, 28.63, 30.50, 31.07, 52.36, and 52.44 (CH_3), 48.20, 48.23, 60.01, and 60.27 (CH), 46.03, and 46.28 (C), 169.38, 169.45, 170.20, 170.32, 172.76, and 172.91 (CO). (Found: m/z (FAB) 277.1229 (MH^+) $C_{11}H_{21}N_2O_4S$ requires 277.1222 (MH^+)). [Found: C, 48.7; H, 6.7; N, 10.2 Calc. for $C_{11}H_{20}N_2O_4S$: C, 47.8; H, 7.3; N, 10.1%].

III. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-valine methyl ester[#†‡]

m.p. 174 °C (dec.). δ_C (300 MHz; $CDCl_3$) 17.76, 18.08, 19.00, 19.10, 23.22, 23.22, 28.47, 28.57, 31.27, 31.27, 52.07, and 52.11 (CH_3), 30.64, 30.93, 57.61, 57.64, 60.18, and 60.18 (CH), 45.88, 46.15 (C), 169.93, 170.03, 170.21, 170.28, 171.68, and 171.89 (CO). (Found: m/z (FAB) 305.1565 (MH^+) $C_{13}H_{25}N_2O_4S$ requires 305.1535 (MH^+)). [Found: C, 51.6; H, 8.0; N, 9.2 Calc. for $C_{13}H_{24}N_2O_4S$: C, 51.3; H, 7.9; N, 9.2%].

continued

IV. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-leucine methyl ester[*#†‡]

m.p. 124 °C (dec.). δ_C (300 MHz; $CDCl_3$) 21.61, 21.79, 22.74, 22.81, 23.21, 23.26, 28.50, 28.64, 30.41, 31.06, 52.19, and 52.22 (CH_3), 40.75, and 41.02 (CH_2), 24.80, 24.86, 50.98, 51.09, 60.02, and 60.11 (CH), 46.02, and 46.35 (C), 169.81, 169.85, 170.16, 170.30, 172.80, and 172.93 (CO). (Found: m/z (FAB) 319.1705 (MH^+) $C_{14}H_{27}N_2O_4S$ requires 319.1691 (MH^+)). [Found: C, 53.5; H, 8.8; N, 8.8 Calc. for $C_{14}H_{26}N_2O_4S$: C, 52.8; H, 8.2; N, 8.8%].

V. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-phenylalanine methyl ester[*#†‡]

m.p. 116 °C (dec.). δ_C (300 MHz; $CDCl_3$) 23.19, 13.19, 28.42, 28.49, 30.64, 30.81, 52.34, and 52.40 (CH_3), 37.71, and 37.86 (CH_2), 53.33, 53.43, 60.30, and 60.30 (CH), 45.73, and 45.89 (C), 126.99, 127.21, 128.43, 128.54, 128.66, 128.93, 129.20, 129.32, 135.60, and 135.99 (aromatic carbons), 169.47, 169.53, 170.07, 170.18, 171.55, and 171.62 (CO). (Found: m/z (FAB) 353.1545 (MH^+) $C_{17}H_{25}N_2O_4S$ requires 353.1535 (MH^+)). [Found: C, 59.5; H, 7.3; N, 8.1 Calc. for $C_{17}H_{24}N_2O_4S$: C, 57.9; H, 6.9; N, 7.9%].

VI. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-isoleucine methyl ester[*#†‡]

m.p. 136 °C (dec.). δ_C (300 MHz; $CDCl_3$) 11.50, 11.59, 15.60, 15.63, 23.23, 23.23, 28.45, 28.52, 30.62, 31.23, 52.03, and 52.07 (CH_3), 25.14, and 25.35 (CH_2), 37.31, 37.54, 56.79, 56.88, 60.14, and 60.24 (CH), 45.90, and 46.16 (C), 169.78, 169.88, 170.21, 170.30, 171.69, and 171.84 (CO). (Found: m/z (FAB) 319.1690 (MH^+) $C_{14}H_{27}N_2O_4S$ requires 319.1691 (MH^+)). [Found: C, 52.0; H, 7.5; N, 8.7 Calc. for $C_{14}H_{26}N_2O_4S$: C, 52.8; H, 8.2; N, 8.8%].

continued

VII. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-methionine methyl ester[*#†‡]

m.p. 128 °C (dec.). δ_C (300 MHz; $CDCl_3$) 15.40, 15.40, 23.24, 23.29, 28.44, 28.65, 31.12, 31.20, 52.46, and 52.48 (CH_3), 30.00, 30.09, 30.59, and 31.04 (CH_2), 51.64, 51.64, 60.17, and 60.32 (CH), 45.92, and 46.15 (C), 169.79, 169.79, 170.27, 170.37, 171.78, and 171.92 (CO). (Found: m/z (FAB) 337.1250 (MH^+) $C_{13}H_{25}N_2O_4S_2$ requires 337.1255 (MH^+)). [Found: C, 47.7; H, 7.5; N, 8.6 Calc. for $C_{13}H_{24}N_2O_4S_2$: C, 46.4; H, 7.2; N, 8.3%].

VIII. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-N_ε-CBZ-L-lysine methyl ester[*¤#†‡]

m.p. 96 °C (dec.). δ_C (300 MHz; $CDCl_3$) 23.12, 23.16, 28.52, 28.73, 30.59, 30.95, 52.34, and 52.38 (CH_3), 22.40, 22.48, 29.26, 29.33, 31.19, 31.38, 40.30, 40.48, 66.67, and 66.67 (CH_2), 52.05, 52.21, 60.28, 60.44 (CH), 45.89, and 46.01 (C), 128.08, 128.08, 128.08, 128.08, 128.50, 128.50, 128.50, 128.50, 136.64, and 136.64 (aromatic carbons), and 156.59, 156.76, 169.76, 169.92, 170.40, 170.52, 172.20, 172.30 (CO). (Found: m/z (FAB) 468.2153 (MH^+) $C_{22}H_{34}N_3O_6S$ requires 468.2168 (MH^+)). [Found: C, 57.7; H, 7.7; N, 9.3 Calc. for $C_{22}H_{33}N_3O_6S$: C, 56.5; H, 7.1 N, 9.0%].

IX. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-proline methyl ester[*¤#†‡]

m.p. syrup. δ_C (300 MHz; $CDCl_3$) 23.05, 23.16, 28.34, 28.84, 30.52, 30.63, 52.06, and 52.22 (CH_3), 24.63, 24.93, 29.17, 29.20, 48.06, and 48.16 (CH_2), 56.79, 56.90, 58.84, 59.04 (CH), 46.45, and 46.59 (C), 169.41, 169.79, 170.03, 172.04, 172.30, and 172.73 (CO). (Found: m/z (FAB) 303.1371 (MH^+) $C_{13}H_{23}N_2O_4S$ requires 303.1378 (MH^+)). [Found: C, 52.0; H, 7.7; N, 9.2 Calc. for $C_{13}H_{22}N_2O_4S$: C, 51.6; H, 7.3; N, 9.3%].

continued

X. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-aspartic acid dimethyl ester[#†‡]

m.p. 120 °C (dec.). δ_C (300 MHz; $CDCl_3$) 23.20, 23.26, 28.49, 28.49, 30.63, 31.09, 52.16, 52.24, 52.86, and 52.92 (CH_3), 35.76, and 35.91 (CH_2), 48.41, 48.77, 60.47, and 60.70 (CH), 45.79, 46.08 (C), 169.40, 169.50, 170.20, 170.31, 170.55, 170.64, 171.12, and 171.28 (CO). (Found: m/z (FAB) 335.1273 (MH^+) $C_{13}H_{23}N_2O_6S$ requires 335.1277 (MH^+)). [Found: C, 46.8; H, 6.4; N, 8.3 Calc. for $C_{13}H_{22}N_2O_6S$: C, 46.7; H, 6.6; N, 8.4%].

XI. *N*-acetyl-D,L-β,β-dimethylcysteinyl-L-glutamic acid dimethyl ester[*¤#†‡]

m.p.134 °C (dec.). δ_C (300 MHz; $CDCl_3$) 23.23, 23.29, 28.54, 28.54, 30.72, 31.31, 51.96, 51.96, 52.54, and 52.54 (CH_3), 26.61, 26.74, 29.99, and 30.12 (CH_2), 51.93, 51.93, 60.32, and 60.42 (CH), 45.74, and 46.02 (C), 169.80, 169.80, 170.22, 170.37, 171.63, 171.73, 173.21, and 173.21 (CO). (Found: m/z (FAB) 349.1450 (MH^+) $C_{14}H_{25}N_2O_6S$ requires 349.1433 (MH^+)). [Found: C, 48.7; H,7.1; N, 8.1 Calc. for $C_{14}H_{24}N_2O_6S$: C, 48.3 H, 6.9; N, 8.0%].

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* In this instance the results of the elemental analysis differ slightly from the values required to confirm the identity of the compound. However, the molecular ion peak in the FAB-MS spectrum has the required agreement and the identity of the compound is confirmed by the ^{13}C-NMR spectrum which contains no additional peaks.

¤ In procedure A and/or B (Section 3. 3. 1) the residue was washed with cold dry ether to give solid product.

$\nu_{max.}$ (nujol)/cm^{-1} ~ 2500w (SH), ~1600 (CONH), ~ 1700 (CONH; peptide bond), and ~ 1750 (CO; aliphatic ester).

† As the *N*-acetyl-D,L-β,β-dimethylcysteine used was a racemic mixture the products (the dipeptides) were two diastereomers. Due to this the compound has a complicated ^{1}H-NMR spectrum, but we were able to identify the -SH, CH_3CONH, and the proton of the peptide bond (-CONH-) which indicate that we have the correct dipeptide.

‡ In the ^{13}C-NMR spectrum there are two lines for each carbon due to the presence of the two diastereomers and usually DEPT 90° and DEPT 135° enabled CH_3-, -CH_2-, and -CH- to be distinguished. In the case of *N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester the two isomers formed are enantiomers rather than diastereomers. Accordingly only one set of lines is seen in the ^{13}C-NMR and the proton spectrum is also simpler {δ_H (300 MHz; $CDCl_3$) 1.34 (s, 3H, CH_3) 1.53 (s, 3H, CH_3) 2.07 (s, 3H, CH_3) 2.63 (s, 1H, SH) 3.75 (s, 3H, CH_3) 4.02 (dd, 2H, *J* 18, 6, CH_2) 4.64 (d, 1H, *J* 9, CH) 6.77 (d, 1H, *J* 9, NH), and 7.42 (br s, 1H, NH)}.

TABLE 3. 2

Characterisation of *S*-nitrosothiols by NMR, UV-visible, FAB-MS, and elemental analysis

Satisfactory elemental analyses for compounds **3**, **5**, **6**, **8**, **9** and **12** could not be obtained since the samples are contaminated with unreacted thiol (and possibly disulphide) and we were unable to find any satisfactory purification procedure. Other workers (Moynihan and Roberts, 1994) must have had similar difficulties as their compounds are authenticated without elemental analyses.

I. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteine (SNAP; 1)

SNAP was prepared as a pure solid by a modified literature procedure (Royle *et al.*, 1983). $\lambda_{max.}$ = 340 nm in 10 % DMSO. (Found: m/z (FAB) 221.0582 (MH^+) $C_7H_{13}N_2O_4S$ requires 221.0596 (MH^+)). [Found: C, 38.5; H, 5.7; N, 12.9 Calc. for $C_7H_{12}N_2O_4S$: C, 38.2; H, 5.5; N, 12.2%].

II. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester (2)#§†

δ_C (300 MHz; $CDCl_3$) 23.31, 24.94, 26.77, 52.62 (CH_3), 41.02 (CH_2), 58.54 (CH), 59.11 (C), 169.13, 169.90 and 170.40 (CO). $\lambda_{max.}$ = 336 nm. (Found: m/z (FAB) 292.0976 (MH^+) $C_{10}H_{18}N_3O_5S$ requires 292.0967 (MH^+)). [Found: C, 41.0; H, 6.2; N, 14.2 Calc. for $C_{10}H_{17}N_3O_5S$: C, 41.2; H, 5.9; N, 14.4%].

III. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-alanine methyl ester (3)*¤#§†‡

δ_C (300 MHz; $CDCl_3$) 17.16, 17.75, 23.16, 23.16, 24.90, 25.54, 26.60, 26.82, 52.57, 52.73 (CH_3), 48.11, 48.18, 58.65, 58.87 (CH), 59.09, 59.09 (C), 168.27, 168.48, 170.39, 170.52, 172.78, and 172.91 (CO). $\lambda_{max.}$ = 339 nm. (Found: m/z (FAB) 306.1133 (MH^+) $C_{11}H_{20}N_3O_5S$ requires 306.1124 (MH^+)). [Found: C, 42.6; H, 5.9; N, 12.7 Calc. for $C_{11}H_{19}N_3O_5S$: C, 43.3 ; H, 6.3; N, 13.8%].

continued

IV. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-valine methyl ester (4)#§†‡

δ_C (300 MHz; $CDCl_3$) 17.55, 17.95, 18.96, 19.09, 23.23, 23.23, 25.25, 25.78, 26.64, 26.83, 52.34, 52.41 (CH_3), 30.36, 30.74, 57.56, 57.56, 58.58, 58.89 (CH), 58.97, 59.12 (C), 168.98, 169.12, 170.26, 170.31, 171.68, and 171.85 (CO). $\lambda_{max.}$ = 340 nm. (Found: m/z (FAB) 334.1090 (MH^+) $C_{13}H_{24}N_3O_5S$ requires 334.1436 (MH^+)). [Found: C, 46.4; H, 6.6; N, 12.0 Calc. for $C_{13}H_{23}N_3O_5S$: C, 46.8; H, 7.0; N, 12.6%].

V. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-leucine methyl ester (5)*¤#§†‡

δ_C (300 MHz; $CDCl_3$) 21.11, 21.28, 21.42, 21.52, 22.76, 22.87, 24.54, 24.95, 25.49, 26.60, 52.48, 52.91 (CH_3), 40.04, 40.21 (CH_2), 23.16, 24.47, 50.86, 50.97, 58.60, 58.88 (CH), 59.08, 59.08 (C), 168.88, 168.93, 170.27, 170.54, 172.85, and 172.90 (CO). $\lambda_{max.}$ = 336 nm. (Found: m/z (FAB) 348.1587 (MH^+) $C_{14}H_{26}N_3O_5S$ requires 348.1593 (MH^+)). [Found: C, 47.9; H, 6.3; N, 11.5 Calc. for $C_{14}H_{25}N_3O_5S$: C, 48.4; H, 7.2; N, 12.1%].

VI. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-phenylalanine methyl ester (6)*¤#§†‡

δ_C (300 MHz; $CDCl_3$) 22.98, 22.98, 25.12, 26.55, 28.90, 30.97, 52.42, 52.60 (CH_3) 37.37, 37.37 (CH_2), 53.67, 53.67, 58.71, 58.79 (CH), 59.10, 59.10 (C), 168.72, 168.85, 170.50, 170.50, 171.54, 171.72 (CO), 127.08, 127.08, 128.41, 128.56, 128.71, 129.03, 129.08, 129.08, 129.22, 129.22 (CH; aromatic carbons), 135.57, and 136.01 (C; aromatic carbons). $\lambda_{max.}$ = 339 nm. (Found: m/z (FAB) 382.1326 (MH^+) $C_{17}H_{24}N_3O_5S$ requires 382.1437 (MH^+)). [Found: C, 55.1; H, 6.3; N, 11.7 Calc. for $C_{17}H_{23}N_3O_5S$: C, 53.5; H, 6.1; N, 11.0%].

continued

VII. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-isoleucine methyl ester (7)#§†‡

δ_C (300 MHz; $CDCl_3$) 11.48, 11.71, 15.40, 15.40, 23.20, 23.20, 25.18, 25.73, 26.62, 26.80, 52.27, 52.36 (CH_3), 24.77, 24.98 (CH_2), 36.98, 37.22, 56.76, 56.89, 58.65, 58.65 (CH), 58.95, 58.95 (C), 168.81, 168.97, 170.18, 170.28, 171.57, and 171.73 (CO). $\lambda_{max.}$ = 339 nm. (Found: m/z (FAB) 348.1596 (MH^+) $C_{14}H_{26}N_3O_5S$ requires 348.1593 (MH^+)). [Found: C, 48.9; H, 7.4; N, 11.7 Calc. for $C_{14}H_{25}N_3O_5S$: C, 48.4; H, 7.3; N, 12.1%].

VIII. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-methionine methyl ester (8)*¤#§†‡

δ_C (300 MHz; $CDCl_3$) 15.07, 15.14, 23.01, 23.43, 26.58, 26.58, 30.93, 31.26, 52.66, 52.76 (CH_3), 28.92, 29.65, 30.07, 30.68 (CH_2), 52.39, 52.58, 58.67, 58.82 (CH), 58.99, 59.10 (C), 169.04, 169.87, 170.58, 170.79, 171.89, 171.97 (CO). $\lambda_{max.}$ = 339 nm. (Found: m/z (FAB) 366.1146 (MH^+) $C_{13}H_{24}N_3O_5S_2$ requires 366.1157 (MH^+)). [Found: C, 41.7; H, 6.2; N, 9.6 Calc. for $C_{13}H_{23}N_3O_5S_2$: C, 42.7; H, 6.3; N, 11.5%].

IX. *S*-nitroso- *N* -acetyl- D,L -β,β-dimethylcysteinyl- L -N_ε-CBZ- L -lysine methyl ester (9)*¤#§†‡

δ_C (300 MHz; $CDCl_3$) 22.98, 23.05, 25.37, 25.39, 26.51, 26.90, 52.46, 52.76 (CH_3), 22.18, 22.47, 28.53, 28.91, 30.80, 30.97, 40.16, 40.38, 66.39, 66.56 (CH_2) 52.25, 52.50, 58.86, 58.93 (CH), 59.05, 59.05 (C), 156.56, 156.81, 168.93, 169.16, 170.60, 170.68, 172.10, 172.26 (CO), 127.65, 128.13, 128.13, 128.33, 128.44, 128.44, 128.49, 128.73, 128.73, 128.94 (CH; aromatic carbons), 135.97, 136.09 (C; aromatic carbon). $\lambda_{max.}$ = 340 nm in DMSO (insoluble in the buffer). (Found: m/z (FAB) 497.1342 (MH^+) $C_{22}H_{33}N_4O_7S$ requires 497.2070 (MH^+)). [Found: C, 53.4; H, 6.8; N, 10.3 Calc. for $C_{22}H_{32}N_4O_7S$: C, 53.2 H, 6.5; N, 11.3%]. *continued*

X. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-proline methyl ester (10)#§†‡

δ_C (300 MHz; $CDCl_3$) 22.58, 22.62, 25.82, 26.51, 30.88, 31.08, 52.43, 52.50 (CH_3), 24.38, 24.46, 28.90, 29.01, 48.07, 48.26 (CH_2), 56.24, 56.87, 58.65, 58.72 (CH), 59.10, 59.27 (C), 168.29, 168.41, 170.60, 170.97, 172.00, and 172.16 (CO). $\lambda_{max.}$ = 338 nm. (Found: m/z (FAB) 332.1268 (MH^+) $C_{13}H_{22}N_3O_5S$ requires 332.1280 (MH^+)). [Found: C, 43.4; H, 5.8; N, 11.3 Calc. for $C_{13}H_{21}N_3O_5S$: C, 43.0; H, 5.8; N, 11.7%].

XI. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-aspartic acid dimethyl ester (11):#§†‡

δ_C (300 MHz; $CDCl_3$) 23.17, 23.17, 25.16, 25.87, 26.00, 26.80, 52.38, 52.43, 53.02, 53.09 (CH_3), 35.40, 35.48 (CH_2), 47.97, 48.50, 58.29, 58.74 (CH), 59.01, 59.21 (C), 168.35, 168.54, 170.50, 170.56, 170.65, 170.65, 171.26, and 171.55 (CO). $\lambda_{max.}$ = 339 nm. (Found: m/z (FAB) 364.1119 (MH^+) $C_{13}H_{22}N_3O_7S$ requires 364.1178 (MH^+)). [Found: C, 44.8; H, 6.0; N, 11.3 Calc. for $C_{13}H_{21}N_3O_7S$: C, 44.6; H, 6.1; N, 11.1%].

XII. *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-glutamic acid dimethyl ester (12)*¤#§†‡

δ_C (300 MHz; $CDCl_3$) 23.21, 23.24, 25.04, 25.54, 26.07, 26.33, 51.16, 51.16, 52.66, 52.70 (CH_3), 26.59, 26.78, 29.76, 29.84 (CH_2), 52.72, 52.72, 58.56, 58.95 (CH), 59.11, 59.11 (C), 168.80, 168.88, 170.25, 170.45, 171.49, 171.70, 173.19, and 173.27 (CO). $\lambda_{max.}$ = 339 nm. (Found: m/z (FAB) 378.1185 (MH^+) $C_{14}H_{24}N_3O_7S$ requires 378.1335 (MH^+)). [Found: C, 44.0; H, 5.6; N, 10.7 Calc. for $C_{14}H_{23}N_3O_7S$: C, 44.6; H, 6.1; N, 11.1%].

continued

XIII. *S*-nitroso-L-glutathione (GSNO; 13)

$\lambda_{max.}$ = 336 nm. (Found: m/z (FAB) 337.0856 (MH^+) $C_{10}H_{17}N_4O_7S$ requires 337.0818 (MH^+)). [Found: C, 35.2; H, 4.9; N, 16.4 Calc. for $C_{10}H_{16}N_4O_7S$: C, 35.2; H, 4.7; N, 16.3%].

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* In this instance the results of the elemental analysis differ slightly from the values required to confirm the identity of the compound. However, the molecular ion peak in the FAB-MS spectrum has the required agreement and the identity of the compound is confirmed by the ^{13}C-NMR spectrum which contains no additional peaks.

¤ The drug was contaminated by small amounts of the unreacted thiol and/or by small amounts of the disulphide formed during nitrosation.

In the nitrosation of the dipeptide (Section 2. 3. 2.b) the residue was washed with cold dry ether to give green solid product (except in the case of compounds 9 and 10 which were green sticky solids).

§ $\nu_{max.}$ (nujol)/cm^{-1} ~ 1900w (SNO), ~ 600 (SN), ~1500 (NO), ~ 1600 (CONH), ~1700 (CONH; peptide bond), and ~ 1750 (CO; alipatic ester).

† It was possible to identify the CH_3CONH- and -CONH- signals and the disappearance of -SH which indicates that the nitrosation has occurred. Moreover, the resonances of the α carbon atoms were shifted downfield (~ 15 ppm), and resonance of β-carbon atoms were shifted upfield which also indicates that the nitrosation has occurred. Some decomposition occurred while the spectra were being run.

‡ In the ^{13}C-NMR spectrum there are two lines for each carbon due to the presence of the two diastereomers and usually DEPT 90° and DEPT 135° enabled CH_3-, -CH_2-, and -CH- to be distinguished. In the case of compound **2** the two isomers formed are enantiomers rather than diastereomers. Accordingly only one set of lines is seen in the ^{13}C-NMR and the proton spectrum is also simpler {δ_H (300 MHz; $CDCl_3$) 2.00 (s, 6H, 2 x CH_3) 2.10 (s, 3H, CH_3) 3.78 (s, 3H, CH_3) 4.06 (dd, 2H, *J* 18, 6, CH_2) 5.52 (d, 1H, *J* 10, CH) 6.99 (d, 1H, *J* 11, NH), and 8.04 (br s, 1H, NH).

3. 3. 3. Chemical Stability of the *S*-Nitrosothiols

In this section, we will report the effect of EDTA, the effect of copper ions, the effect of thiol, the effect of neocuproine, and the effect of L-cysteine on the decomposition of *S*-nitrosothiols. All *S*-nitrosothiols gave a characteristic broad absorption band centred around 340 nm. Aliquots of these drugs were used after pH adjustment, taking care to minimise exposure to light.

3. 3. 3. 1. The Effect of EDTA on the Decomposition of *S*-Nitrosothiols

The decomposition of each of the test drugs was followed spectrophotometrically by determining the absorbance of test drug as a function of time.

A stock solution (2.5 mM) of copper(II) nitrate trihydrate was made up (0.302 g in 500 cm^3 water), and diluted by a factor of 10 to form a 2.5 x 10^{-1} mM solution. 0.1 cm^3 Of this solution was added to phosphate buffer (0.1 M KH_2PO_4/0.1 M NaOH; 1.90 cm^3) at pH = 7.4. 0.15 cm^3 Of DMSO and 0.25 cm^3 of EDTA [1.0 x 10^{-4} M] were added to the resulting solution. The solution in the cell was left for one hour at 30 °C to equilibrate in the spectrophotometer. When 0.1 cm^3 of the drug solution (12.5 mM; 12.5 x 10^{-6} mol in 1.0 cm^3 DMSO) was added to this solution in the spectrophotometer cuvette at 30°C, the final concentrations of the drug and copper(II) ions in the cuvette were 5 x 10^{-1} mM and 10 μM respectively. The other concentrations of copper(II) ions were made up in a similar way from further dilution of the copper/distilled water stock solutions, always adding a final volume of 2.5 cm^3 to the cuvette and 0.1 cm^3 of the drug stock solution. Immediately after addition of the 0.1 cm^3 of the drug (which had been kept in ice and in the absence of light prior to addition), the experiment was started and readings taken at timed intervals against a reference cell containing phosphate buffer. For the experiment run in the presence of EDTA, the EDTA (100 μM) was made up in water (250 cm^3) from a stock solution (0.9306 g in 250 cm^3 buffer) which, after addition of the stock drug solution (0.1 cm^3), resulted in a final concentration of EDTA of 10 μM.

3. 3. 3. 2. The Effect of Copper Ions on the Decomposition of *S*-Nitrosothiols

A stock solution (2.5×10^{-1} mM) of copper(II) nitrate trihydrate was made up (0.0060 g in 100 cm^3 buffer). An aliquot of this solution (0.12 cm^3, 0.24 cm^3, 0.36 cm^3, 0.48 cm^3 and 0.60 cm^3) was diluted to 10 cm^3 with phosphate buffer (0.1 M KH_2PO_4/0.1 M NaOH) to form 0.3×10^{-5}, 0.6×10^{-5}, 0.9×10^{-5}, 1.2×10^{-5} and 1.5×10^{-5} mM solutions. 0.15 cm^3 Of DMSO was added to phosphate buffer containing copper ions pH = 7.4 at (2.25 cm^3) and 30 °C in the spectrophotometer cuvette. The solution in the cell was left for one hour at 30 °C to equilibrate in the spectrophotometer. 0.1 cm^3 Of the drug solution (12.5×10^{-6} mol in 1 cm^3 DMSO, 12.5 mM) was added to the cuvette. Immediately after addition of 0.1 cm^3 of drug (which had kept in ice and in the absence of light prior to addition), the experiment was started and readings taken at timed intervals against a reference cell containing phosphate buffer and DMSO.

3. 3. 3. 3. The Effect of Thiol on the Decomposition of *S*-Nitrosothiols

0.1 cm^3 Of 6.25 mM thiol (6.25 μmol in 1 cm^3 DMSO) was added to phosphate buffer containing copper ions (1.5×10^{-2} mM) at pH = 7.4 (2.25 cm^3) and 30 °C in a spectrophotometer cuvette. 0.05 cm^3 Of DMSO was added to the previous solution. The solution in the cell was left for one hour at 30 °C to equilibrate in the spectrophotometer. 0.1 cm^3 Of the drug (12.5 μmol in 1 cm^3 DMSO) was added to the first cuvette and the absorbance decrease at $\lambda_{max.}$ was measured as a function of time for the 2.5×10^{-1} mM thiol / 5.0×10^{-1} mM drug solution.

0.15 cm^3 Of DMSO was added to phosphate buffer (2.25 cm^3) containing copper ions (1.5×10^{-2} mM) at pH = 7.4 and 30 °C was placed in a spectrophotometer cuvette. The solution in the cell was left for one hour at 30 °C to equilibrate in the spectrophotometer. 0.1 cm^3 Of the drug (12.5 mM in 1 cm^3 DMSO) was added to the second cuvette and the absorbance decrease at $\lambda_{max.}$ was measured as a function of time.

The absorbance decrease at $\lambda_{max.}$ was measured as a function of time for the first and second cuvettes versus a standard cuvette containing all but the *S*-nitrosothiol at the same concentrations.

3. 3. 3. 4. The Effect of Neocuproine on the Decomposition of *S*-Nitrosothiols

0.1 cm^3 Of 12.5 mM neocuproine (0.0026 g in 1 cm^3 DMSO) was added to phosphate buffer containing copper ions (2.0 x 10^{-2} mM; 2.25 cm^3) at pH = 7.4 and 30 °C in a spectrophotometer cuvette. 0.05 cm^3 Of DMSO was added to the resulting solution. The solution in the cell was left for one hour at 30 °C to equilibrate in the spectrophotometer. 0.1 cm^3 Of the drug (12.5 mM in 1 cm^3 DMSO) was added to the first cuvette.

The solution in the cell was left for one hour at 30 °C to equilibrate in the spectrophotometer. 0.1 cm^3 Of the drug (12.5 mM in 1 cm^3 DMSO) was added to phosphate buffer containing copper ions (2.0 x 10^{-2} mM; 2.25 cm^3) at pH = 7.4 and 0.15 cm^3 DMSO at 30°C in a second spectrophotometer cuvette.

The absorbance decrease at $\lambda_{max.}$ was measured as a function of time for the 5.0 x 10^{-1} mM drug solution, versus a standard cuvette containing all but the *S*-nitrosothiol at the same concentrations.

3. 3. 3. 5. The Effect of L-Cysteine on the Decomposition of *S*-Nitrosothiols

0.1 cm^3 Of 31.25 mM L-cysteine (0.0378 g in 10 cm^3 buffer) was added to phosphate buffer at pH = 7.4 (2.15 cm^3) and 30 °C in a spectrophotometer cuvette. 0.15 cm^3 Of DMSO was added to the resulting solution. The solution in the cell was left for one hour at 30 °C to equilibrate in the spectrophotometer. 0.1 cm^3 Of the drug (12.5 μmol in 1 cm^3 DMSO; 12.5 mM) was added to the first cuvette and the absorbance decrease at $\lambda_{max.}$ was measured as a function of time for the 12.5 mM L-cysteine / $5x10^{-1}$ mM drug solution.

0.15 cm^3 DMSO was added to phosphate buffer (2.25 cm^3) at pH = 7.4 and 30°C in a spectrophotometer cuvette. The solution in the cell was left for one hour at 30 °C to equilibrate in the spectrophotometer. 0.1 cm^3 Of the drug (12.5 mM in 1 cm^3 DMSO) was added to the second cuvette and the absorbance decrease at $\lambda_{max.}$ was measured as a function of time for the 5 x 10^{-1} mM drug solution.

3. 3. 4. Pharmacological and Physiological Activity of the *S*-Nitrosothiols

In this section, we will report the effect of *S*-nitrosothiols on smooth muscle relaxation, and inhibition of platelet aggregation.

3. 3. 4. 1. Smooth Muscle

Methods used to study the effect of *S*-nitrosothiols, ferro-haemoglobin, and neocuproine on smooth muscle relaxation are reported.

3. 3. 4. 1. 1. Materials and Methods

(i). Preparation

Experiments were performed on 8 - 12 mm segments of tail artery taken from normotensive adult Wistar rats (270 - 538 g). Animals were killed by cervical dislocation and the tail removed. The artery was exposed following removal of the overlying skin and connective tissue. Once exposed, the artery was cannulated (Portex cannula), dissected free and transferred to a perspex bath. Care was taken to identify and tie-off all side-branches.

(ii). Apparatus

The apparatus is shown in Figure 3.1. The vessel (V) was perfused internally at a constant flow rate of 2 $cm^3\ min^{-1}$ driven by a peristaltic pump (P1: Gilson minipuls 2). The perfusate was pre-warmed to 37 °C by passage through the heat exchanger (HE). Drugs were introduced into the lumen of the vessel by bolus injection (10 µl) through a resealable

rubber septum (RS). A differential pressure transducer (P: Sensym type SCX 150NC; Farnell Electronic Components, Leeds) detected changes in back pressure determined by arterial tone.

The outer surface of the vessel was superfused continuously (*ca.* 8 cm^3 min^{-1}) with solution driven by a second peristaltic pump (P2: BDH minipump). The solution was pre-warmed to 37 °C by passage through a heat exchanger similar to that used in the internal circuit.

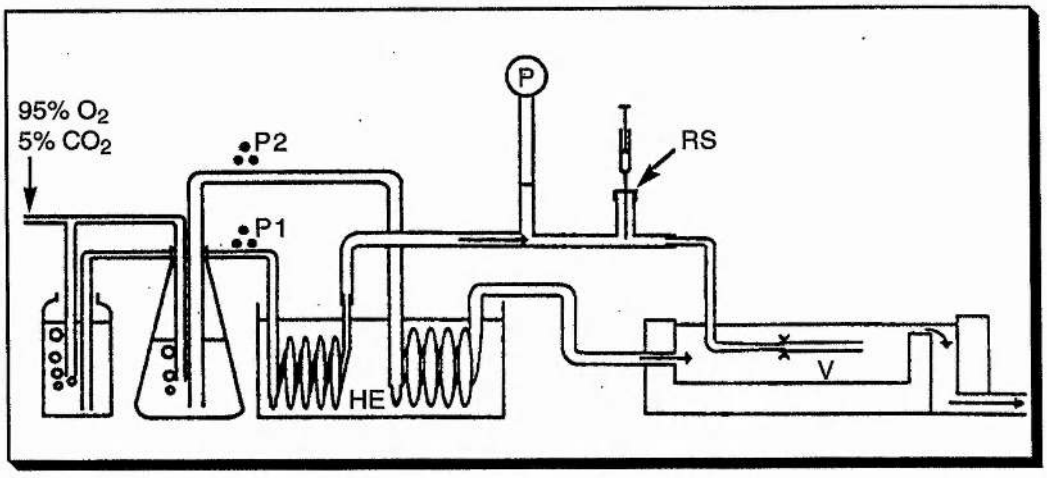

Figure 3. 1: Apparatus used for perfusing isolated segments of rat tail artery. See text for full description and explanation of lettering.

(iii). Experimental Protocol

Arteries were perfused internally and externally with oxygenated Krebs solution (composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, NaH_2PO_4 1.15, $CaCl_2$ 2.5, $MgCl_2$ 1.1, glucose 5.6, gassed with 95% O_2 / 5% CO_2 to maintain pH 7.4). Internal perfusion of the cannulated vessel was initially slow (flow rate *ca.* 0.2 cm^3 min^{-1}). The working flow rate of 2 cm^3 min^{-1} was attained by gradually increasing the pump speed over a period of 10-20 mins. The preparation was then allowed to stabilise for a further 20-30 mins, at which time a 'passive' pressure of 5 - 24 mm Hg was recorded. Precontraction was induced by addition of phenylephrine hydrochloride (PE) to both internal and external perfusates (Figure 3.2).

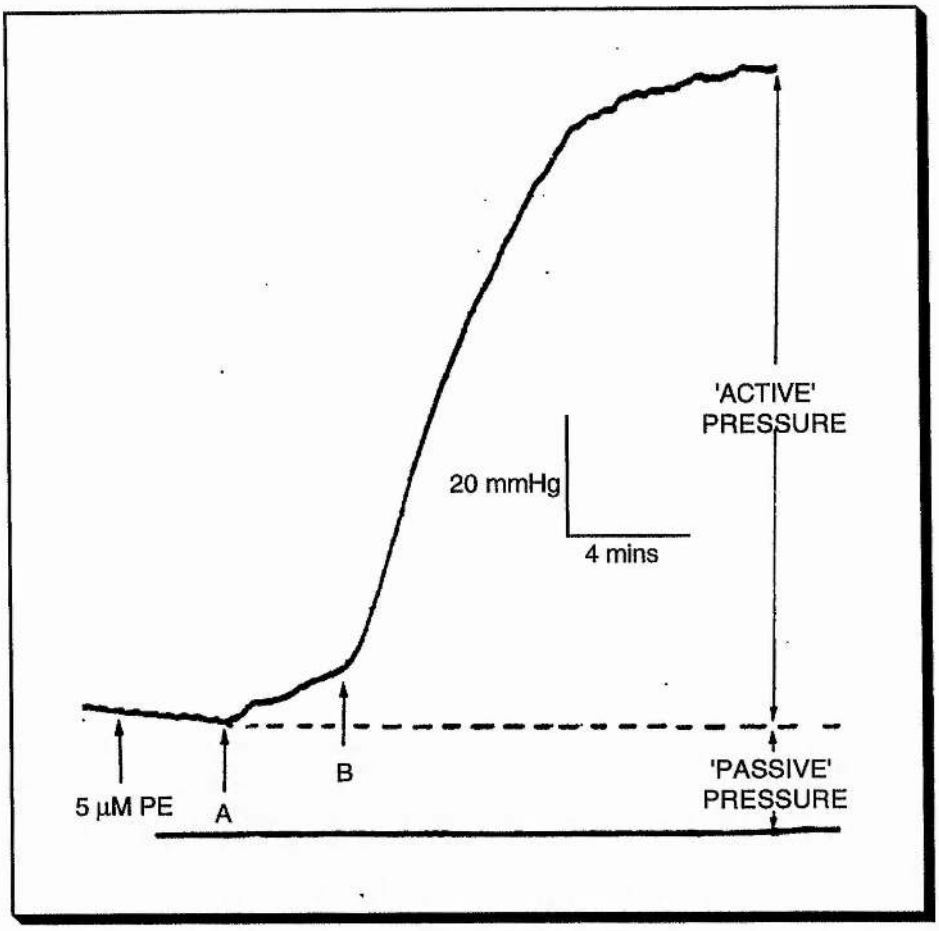

Figure 3. 2: Pressure recording showing precontraction with PE. 5 µM PE was added to both internal and external perfusate reservoirs at the time indicated, reaching the vessel through the external perfusate at A and the internal at B. The pressure maintained by the vessel before PE addition is termed 'passive', whilst agonist-induced pressure is 'active'. Expt. № 920304.

The PE concentration was adjusted to maintain a pressure of 90 - 138 mm Hg above the passive pressure.

At this point in the experimental procedure, the laboratory lights were switched off, leaving a red safelight (60 W) as the only means of illumination. This precaution served to prevent photorelaxation of the vessel and to protect any NO donor drugs in use from photolytic decomposition.

(iv). Drugs

L-phenylephrine hydrochloride :

Appropriate volumes of a 10^{-3} M stock solution were added to both the internal and external perfusates to produce concentrations ranging from 1 - 12 μM.

S-Nitroso-N-acetyl-D,L-β,β-dimethylcysteine:

S-Nitroso-*N*-acetyl-D,L-β,β-dimethylcysteine (SNAP; **1**) was prepared as a pure solid by a modified procedure (Section 3. 3. 2. a). The solid was first dissolved in dimethyl sulphoxide (Sigma-Aldrich Chemicals; HPLC grade; 99.9%) to produce a 10^{-2} M stock solution which was then serially diluted with Krebs solution to produce the desired range of concentrations immediately prior to use. Solutions were kept in ice during experiments.

S-Nitroso-N-acetyl-D,L-β,β-dimethylcysteinyl-L-amino-acid methyl esters:

S-Nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-amino-acid methyl esters (**2-12**) are not soluble in water and cannot be dissolved in Krebs solution. The solid was first dissolved in dimethyl sulphoxide (Sigma-Aldrich Chemicals; HPLC grade; 99.9%) to produce a 10^{-2} M stock solution which was then serially diluted with Krebs solution to produce the desired range of concentrations immediately prior to use. Solutions were kept in ice during experiments.

S-Nitroso-L-glutathione:

The solid was first dissolved in Krebs solution (9 cm^3) followed by addition of 1 cm^3 of dimethyl sulphoxide (Sigma-Aldrich Chemicals; HPLC grade; 99.9%) to produce a 10^{-2} M

stock solution which was then serially diluted with Krebs solution to produce the desired range of concentrations immediately prior to use. Solutions were kept in ice during experiments.

Preparation of Ferro-haemoglobin:

Ferro-haemoglobin (Mr 64500) was prepared by reduction of 100 cm^3 of 1 mM bovine haemoglobin using 10 mM sodium dithionite. The dithionite was dialysed out of solution against 3 x 2 litre volumes of deaereated distilled water at 0 - 5 °C. The stock ferro-haemoglobin was then split into 3 cm^3 aliquots and frozen (-10 °C). Aliquots were added to 200 cm^3 of Krebs buffer used for the internal perfusate of the artery, immediately prior to perfusion, to make up the 15 mM concentration required. Samples were used within 2 weeks.

3. 3. 4. 1. 2. The Effect of Ferro-haemoglobin

The same procedure was carried out as described in Section 3. 3. 4. 1. 1. Bolus injections of *S*-nitrosothiols (1 mM - 10 μM) were administered to the artery *via* the injection port (see Figure 3. 1) to obtain a control dose response curve. Ferro-haemoglobin (15 μM) and superoxide dismutase (150 units $(cm^3)^{-1}$; Sigma Chemical) were then perfused through the artery in the internal Krebs buffer supply and the *S*-nitrosothiol injections were repeated. The mean response amplitudes of experiments were obtained for each concentration and statistical analysis of the results was carried out.

Statistical analysis of data in figures (Section 2. 4. 1):
statistical analysis on the data was carried out using an unpaired students *t*-test.
* indicates data is significantly different at the P = 0.05 confidence level.
** indicates data is significantly different at the P = 0.01 confidence level.
*** indicates data is significantly different at the P = 0.001 confidence level.

3. 3. 4. 1. 3. The Effect of Neocuproine on Smooth Muscle Relaxation

(a) Pre-mixed

The same procedure was carried out as described in the experimental section (Section 3. 3. 4. 1. 1). Bolus injections of SNAP and/or GSNO (1 mM - 10 μM) were administered to the artery *via* the injection Port (Figure 3. 1) to obtain a control dose response curve.

A stock solution (10 cm^3) of neocuproine (10^{-2} M) in 2.5 cm^3 ethanol and 7.5 cm^3 Krebs solution was prepared. The drug was dissolved in Krebs buffer containing dimethyl sulfoxide (10% DMSO; Sigma-Aldrich chemicals; HPLC grade; 99.9%) to produce a 10^{-2} M stock solution which was then serially diluted with Krebs solution containing neocuproine (10^{-4} M; 0.1 cm^3 of neocuproine (10^{-2} M) and 0.8 cm^3 Krebs solution) to produce the desired range of concentrations immediately prior to use. Bolus injections of SNAP and/or GSNO (1 mM - 10 μM) were administered to the artery *via* the injection port (Figure 3. 1) to obtain a dose response curve and the SNAP and/or GSNO injections were repeated. The mean response amplitudes of 6 to 8 experiments were obtained for each concentration and statistical analysis of the results was carried out.

(b) Perfused

The same procedure was carried out as described in the experimental section (Section 3. 3. 4. 1. 1). Bolus injections of SNAP and/or GSNO (1 mM - 10 μM) were administered to the artery *via* the injection port (see Figure 3. 1) to obtain a control dose response curve. A stock solution (10 cm^3) of neocuproine (10^{-2} M) in 2.5 cm^3 ethanol and 7.5 cm^3 Krebs solution was prepared. Neocuproine solution (10^{-4} M; 1 cm^3 from the neocuproine stock and 99 cm^3 Krebs solution) was then perfused through the artery in the internal Kerbs buffer supply and the SNAP and/or GSNO injections were repeated. The mean response amplitudes of 6 to 8 experiments were obtained for each concentration and statistical analysis of the results was carried out.

3. 3. 4. 2. Platelet Aggregation

Human whole blood was collected over 3.2% trisodium citrate at a ratio of 9 to 1 (blood to citrate). Platelet-rich plasma (PRP) was prepared from human whole blood by centrifugation at 900 rpm for 20 min. at 25 °C, a platelet account performed(~ 300 ± 50 x 10^9L) and stored in plastic tubes closed to air. Platelet-poor plasma (PPP) was used as a blank (200 μL) in the aggregometer and was prepared by centrifugation of PRP for 10 min. at 3500 rpm. All platelet aggregations were performed in 196 μl aliquots of PRP in a Platelet Aggregation Profiler at 37 °C with a stirring rate of 900 rpm. The test drug (2 μl) was added to PRP 5 min. before the addition of 2 μl collagen to stimulate aggregation (run for 5 min.).

The percent inhibition of aggregation was determined by dividing (the percent aggregation in the control minus the percent aggregation in the test sample which contains the drug) by that observed in the control, then multiplying by 100%.

3. 3. 5. The Effect of L-Ascorbic Acid

In this section, we will study the effect of L-ascorbic acid on the decomposition of *S*-nitrosothiols, the effect of L-ascorbic acid and EDTA on the decomposition of *S*-nitrosothiols, the effect of L-ascorbic acid on smooth muscle relaxation, and the effect of L-ascorbic acid on platelet aggregation.

3. 3. 5. 1. The Effect of L-Ascorbic Acid on the Decomposition of *S*-Nitrosothiols

The solutions in all the cells were left for one hour at 30 °C to equilibrate in the spectrophotometer before the addition of the drug.

0.1 cm^3 Of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer (2.4 cm^3) at pH = 7.4 and 30 °C in a spectrophotometer cuvette (first cell).

0.1 cm^3 Of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer containing L-ascorbic acid (15 mM; 2.4 cm^3) at pH = 7.4 and 30 °C in a spectrophotometer cuvette (second cell).

0.1 cm^3 of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer containing L-ascorbic acid (75 mM; 2.4 cm^3) at pH = 7.4 and 30 °C in a spectrophotometer cuvette (thired cell).

0.1 cm^3 Of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer containing L-ascorbic acid (150 mM; 2.4 cm^3) at pH = 7.4 and 30 °C in a spectrophotometer cuvette (fourth cell).

0.1 cm^3 Of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer containing L-ascorbic acid (225 mM; 2.4 cm^3) at pH = 7.4 and 30 °C in a spectrophotometer cuvette (fifth cell).

The bsorbance decrease at $\lambda_{max.}$ was measured as a function of time for the 15 mM GSNO, versus a standard cuvette containing all but the *S*-nitrosothiol at the same concentration.

3. 3. 5. 2. The Effect of L-Ascorbic Acid and EDTA on the Decomposition of *S*-Nitrosothiols

The solutions in all the cells were left for one hour at 30 °C to equilibrate in the spectrophotometer before the addition of the drug.

0.1 cm^3 Of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer containing EDTA (15 mM; 2.4 cm^3) at pH = 7.4 and 30 °C in a spectrophotometer cuvette (first cell).

0.1 cm^3 Of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer containing EDTA and L-ascorbic acid ([EDTA] = 15 mM; [L-ascorbic

acid] = 15 mM; 2.4 cm^3) at pH = 7.4 and 30 °C in a spectrophotometer cuvette (second cell).

0.1 cm^3 Of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer containing EDTA and L-ascorbic acid ([EDTA] = 15 mM; [L-ascorbic acid] = 75 mM; 2.4 cm^3) at pH = 7.4 and 30 °C in spectrophotometer cuvette (third cell).

0.1 cm^3 Of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer containing EDTA and L-ascorbic acid ([EDTA] = 15 mM; [L-ascorbic acid] = 150 mM; 2.4 cm^3) at pH = 7.4 and 30 °C in a spectrophotometer cuvette (fourth cell).

0.1 cm^3 of 15 mM GSNO (0.0050 g in 1 cm^3 phosphate buffer; pH = 7.4) was added to phosphate buffer contains EDTA and L-ascorbic acid ([EDTA] = 15 mM; [L-ascorbic acid] = 225 mM; 2.4 cm^3) pH = 7.4 at 30 °C in spectrophotometer cuvette (fifth cell).

The absorbance decrease at $\lambda_{max.}$ was measured as a function of time for the 15 mM GSNO, versus a standard cuvette containing all but the *S*-nitrosothiol at the same concentration.

3. 3. 5. 3. The Effect of L-Ascorbic Acid on Smooth Muscle Relaxation

The same procedure was carried out as described in the experimental section (Section 3. 3. 4. 1. 1). Bolus injections of SNAP and/or GSNO (1 mM-10 μM) were administered to the artery *via* the injection port (Figure 3. 1) to obtain a control dose response curve. L-Ascorbic acid solution (10^{-4} M) was then perfused through the artery in the internal Kerbs buffer supply and the SNAP and/or GSNO injections were repeated. The mean response amplitudes of 6 to 8 experiments were obtained for each concentration and statistical analysis of the results was carried out.

3. 3. 5. 4. The Effect of L-Ascorbic Acid on Platelet Aggregation

The same procedure was carried out as described in the experimental section (Section 3. 3. 4. 2). All platelet aggregations were preformed in 194 μl aliquots of PRP in a Platelet Aggregation Profiler at 37 °C with a stirring rate of 900 rpm. L-Ascorbic acid (2 μl; 10^{-2} M) were added to PRP. The test drug (2 μl) was added to the previous mixture 5 min. before the addition of 2 μl collagen to stimulate aggregation (run for 5 min.). The final concentration of L-ascorbic acid was 10^{-4} M.

The percent inhibition of aggregation was determined by dividing (the percent aggregation in the control minus the percent aggregation in the test sample which contains the drug) by that observed in the control, then multiplying by 100%.

3. 3. 6. Trapping Thiyl Radical

5,5-Dimethyl-3,4-dihydropyrrole-*N*-oxide (DMPO) was used as a spin trap agent. *S*-Nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester (**2**) (0.0029 g, 1 mmol) was added to a solution of 5,5-dimethyl-3,4-dihydropyrrole-*N*-oxide (0.05 cm^3 of DMPO in 0.95 cm^3 of acetonitrite) in an ESR tube. The solution was degassed with nitrogen. Before addition no ESR signal (response) was observed. Upon addition of *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester (**2**) a spectrum was obtained directly after the photolysis .

3. 3. 7. NO Assay

A World Precision ISO-NO sensor was used and standardised with a solution of NO prepared from sodium nitrite and L-ascorbic acid.

Statistical analysis of data in figures (Sections 2. 4. 2 & 2. 6. 2. 1):
statistical analysis on the data was carried out using a paired students *t*-test.
* indicates data is significantly different at the P = 0.05 confidence level.
** indicates data is significantly different at the P = 0.01 confidence level.
*** indicates data is significantly different at the P = 0.001 confidence level.

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PUBLICATIONS

Vasodilator properties of some novel *S*-nitrosated dipeptides: comparative chemical and pharmacological studies.

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Introduction.

S-Nitrosothiols (RS-NO) are relatively unstable compounds and break down thermally, photochemically [1] and in a metal ion catalysed process [2] to give a disulphide and nitric oxide (NO):

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

These compounds are of interest because of their potent pharmacological properties and possible physiological role in smooth muscle relaxation and inhibition of platelet aggregation [3]. Endothelium-derived relaxing factor, or EDRF, has pharmacological properties identical to NO or a closely related compound, such as an *S*-nitrosothiol [4]. We have synthesised *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteine (**1**; SNAP) and three novel *S*-nitrosated dipeptides, *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinylglycine methyl ester (**2**), *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-alanine methyl ester (**3**) and *S*-nitroso-*N*-acetyl-D,L-β,β-dimethylcysteinyl-L-valine methyl ester (**4**). The vasodilator properties of compounds **2-4** were compared with that of SNAP to investigate the relationship of structure to chemical stability and biological activity.

Materials and Methods.

S-Nitrosothiols: SNAP was prepared according to the method of Field *et al* [5]. The synthesis of the *S*-nitrosated dipeptides (**2-4**) is described by Butler & Al-Sa'doni [6]:

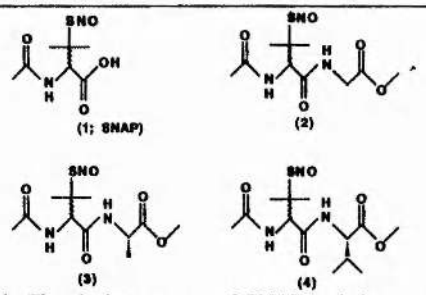

Figure 1: Chemical structures of SNAP and three novel *S*-nitrosated dipeptides

Rat Tail Artery Bioassays: Male Wistar rats (300-400g) were killed by cervical dislocation and their tails removed. The tail artery was dissected free, cannulated, and perfused internally with Krebs solution (37°C) at a constant flow rate (2ml min^{-1}). Vessels were precontracted with phenylephrine hydrochloride (PE; 1-7μM), generating perfusion pressures of 100-120mmHg.

Results and Discussion.

The biological activity of *S*-nitrosothiols depends upon the structure of R [7]. Thus, changing R results in a new compound with different chemical and physiological properties.

The chemical stability of the *S*-nitrosated dipeptides was monitored spectrophotometrically (at λ_{max} for -SNO group; 30°C and pH 7.4). All four compounds decomposed to the corresponding disulphide and NO, but the *S*-nitrosated dipeptides were found to be more stable than SNAP. The addition of cysteine, a transnitrosating agent, accelerated the rate of decomposition of all compounds.

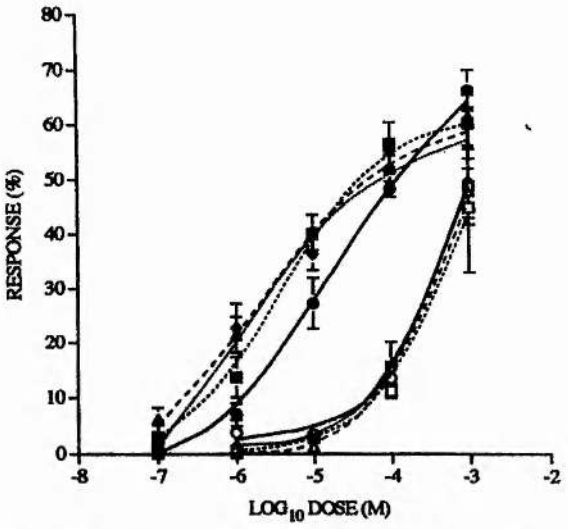

Figure 2: Log dose-response curves comparing the vasodilator effects of SNAP (filled circles), **2** (filled squares), **3** (filled triangles), and **4** (filled diamonds). Open symbols shows responses in presence of oxyhaemoglobin (15μM).

Figure 2 shows log-dose response curves resulting from bolus injections of compounds **1-4** into pre-contracted tail arteries. Oxyhaemoglobin (Hb; 15μM) significantly attenuated responses to all four compounds ($P<0.01$ for all doses of compounds **1-4** when compared to control values using an unpaired Student's t-test). Hb is a well recognised NO scavenger and these results indicate that NO is the effector agent in vasodilation to all four compounds.

S-Nitrosated dipeptides are more effective vasodilators than SNAP (ED_{50}s of 4.1, 2.8, 3.7μM, respectively compared with 17.8μM for SNAP). SNAP responses were statistically significantly different for 10^{-5}M (**3**) and 10^{-6}M (**2, 4**) doses even though the local chemical environment of the -SNO groups are almost identical. This finding is contrary to what might be expected on the basis of their chemical stabilities *in vitro* and may indicate that the *S*-nitrosated dipeptides interact with tissue components or show greater permeability than SNAP.

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Synthesis of a novel series of biologically active *S*-nitrosothiols

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Introduction.

S-Nitrosothiols (RSNO) are an important class of NO-donor drugs. They decompose giving NO and a disulfide:

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

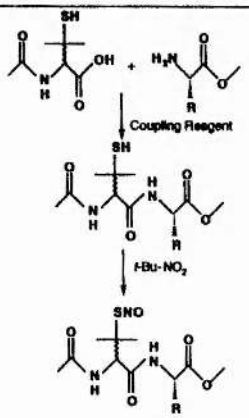
They have been used clinically and occur naturally where they may have a role in smooth muscle relaxation and in the prevention of platelet aggregation. The most extensively examined nitrosothiols are *S*-nitroso-*N*-acetyl-D,L-β,β–dimethylcysteine(SNAP) and *S*-nitroso-L-glutathione(GSNO). We set out to extend the range of compounds of this type and to look for a correlation between chemical structure and biological activity. The situation has been complicated by the recent discovery that the main route for the release of NO from a nitrosothiol is a copper(I)-catalysed process(A. R. Butler, D. L. H. Williams, H. H. Al-Sa'doni, S. C. Askew, A. P. Dick and H. R. Swift; unpublished results). Therefore we examined the effect of copper ions on the stability of the compounds we synthesised.

Materials and Methods.

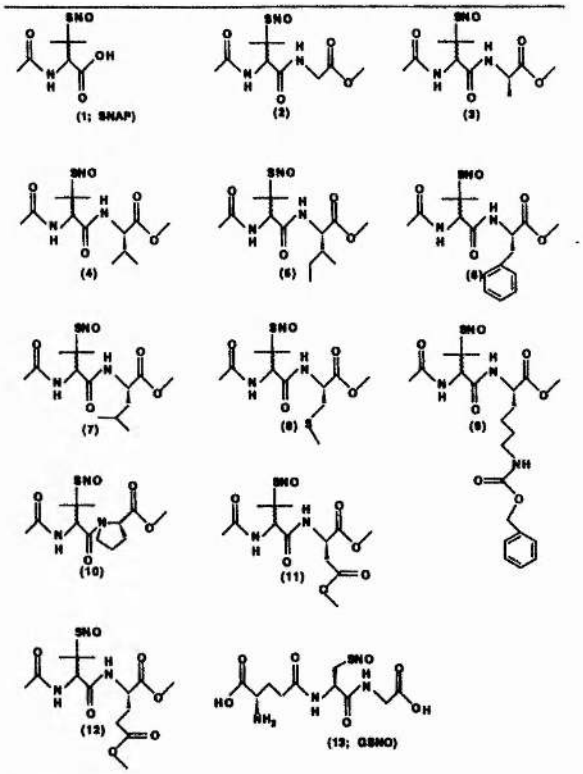
SNAP(**1**) and GSNO(**13**) were prepared by literature methods[1, 2]. The dipeptides were prepared by coupling *N*-acetyl-D,L-β,β–dimethylcysteine with the methyl esters of a number of amino acids. The coupling agent was *N*-cyclohexyl-*N'*-2-[*N*-morpholino]ethylcarbodiimide metho-*p*-toluenesulphonate and we found that using a *N*-acetyl-D,L-β,β–dimethylcysteine derivative, it was unnecessary to protect the thiol group during the reaction. The thiol group was nitrosated by the use of *t*-butyl nitrite. For the kinetic studies NO release was monitored by observing the decrease in absorbance at the λ_{max} (normally 339 nm). The vasodilator effect of nitrosothiols was examined using a length of pre-contracted isolated rat tail artery. The drug was delivered either by bolus injection or as an internal prefusate. The prevention of platelet aggregation was studied using a Platelet Aggregation Profiler. For the detection of NO a WP NO sensor was used.

Results and Discussion.

The *S*-nitrosothiols were prepared according to the following scheme:



The new compounds (**2**-**12**) prepared are shown in the figure below:



The copper catalysed release of NO from nitrosothiols is known((A. R. Butler, D. L. H. Williams, H. H. Al-Sa'doni, S. C. Askew, A. P. Dick and H. R. Swift; unpublished results) to occur by the following mechanism:

$$RS^{\cdot} + Cu^{++} \longrightarrow RS^{\cdot} + Cu^{+} \quad \text{(i)}$$

$$RS^{\cdot} \rightarrow 1/2\ RSSR$$

$$RS^{-} + Cu^{+} \longrightarrow RS^{\cdot} + Cu^{++} \quad \text{(ii)}$$

$$RS^{\cdot} + Cu^{++} \longrightarrow RS^{\cdot} + Cu^{+} \quad \text{(iii)}$$

$$RS^{\cdot} \rightarrow 1/2\ RSSR$$

All the new compound examined show less susceptibility to copper(I)-catalysed release of NO than SNAP but are more reactive than GSNO. However, they are all active as vasodilator[3] and in the prevention of platelet aggregation(A. R. Butler, H. H. Al-Sa'doni, A. J. Bancroft and M. McLaren ; unpublished results).

As a class they combine the favoured property of chemical stability with a high level of biological activity.

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Identification of Cu^+ as the effective reagent in nitric oxide formation from *S*-nitrosothiols (RSNO)

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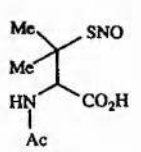
Decomposition of *S*-nitrosothiols (RSNO) in aqueous solution at pH 7.4 is brought about by copper ions, either present as an impurity or specifically added. The primary products are nitric oxide and the disulfide. In the presence of the specific Cu^+ chelator, neocuproine, reaction is progressively inhibited as the [neocuproine] is increased, the reaction eventually stopping completely. The characteristic UV–VIS spectrum of the Cu^+ adduct can be obtained from the reaction solutions. This shows clearly that Cu^+ and not Cu^{2+} is the effective catalyst. Two limiting kinetic conditions can be identified for a range of *S*-nitrosothiols at specific copper ion concentrations (*a*) a first-order dependence and (*b*) a zero-order dependence upon [RSNO]. Normally both situations also have a short induction period. This induction period can be removed by the addition of the corresponding thiol RSH. A mechanism is proposed in which Cu^+ is formed by reduction of Cu^{2+} by thiolate anion *via* an intermediate, possibly $RSCu^+$. Loss of nitric oxide from RSNO is then brought about by Cu^+, probably *via* another intermediate in which Cu^+ is bound to the nitrogen atom of the NO group and another electron-rich atom (such as nitrogen from an amino group, or oxygen from a carboxylate group) involving a six-membered ring. As well as NO this produces both RS^- and Cu^{2+} which then are part of the cycle regenerating Cu^+. Thiolate ion is oxidised to RS' which dimerizes to give the disulfide. Depending on the structure (and hence reactivity) of RSNO either Cu^+ formation or its reaction with RSNO can be rate-limiting. Computer modelling of the reaction scheme allows the generation of absorbance time plots of the same forms as those generated experimentally, *i.e.* first- or zero-order, both with or without induction periods. We suggest that the thiolate ion necessary to bring about Cu^{2+} reduction is either present as a thiol impurity or is generated in small quantities by partial hydrolysis of the nitrosothiol, which results in an induction period. Addition of small quantities of thiol removes the induction period and leads to catalysis but larger quantities bring about a rate reduction by, it is suggested, complexation of the Cu^{2+}. For two very unreactive substrates, *S*-nitrosoglutathione and *S*-nitroso-*N*-acetylcysteine very large induction periods were observed, typically three hours. This results, we suggest, from competitive re-oxidation of Cu^+ to Cu^{2+} by the dissolved oxygen. Experiments carried out anaerobically confirm this, since there is then no induction period. Addition of hydrogen peroxide extends the induction period ever further. The results are discussed in terms of the biological properties of *S*-nitrosothiols which are related to nitric oxide release.

There is currently much interest in the chemistry of nitric oxide following the discoveries that it is involved in a range of human physiological processes.[1–3] Within the area some attention has been focussed on the release of nitric oxide from *S*-nitrosothiols (or thionitrites) RSNO, not only from the point of view of their potential use therapeutically as alternative NO-releasing drugs, but also with regard to their possible involvement *in vivo* as potential NO-storage and transport vehicles. There is a very real case for the generation of alternative drugs given the tolerance problem associated in many cases with the widespread use of glyceryl trinitrate for the treatment of angina and other circulatory problems.

S-Nitrosothiols are very easily generated in solution from thiols by electrophilic nitrosation,[4] *e.g.* in aqueous solution using acidified sodium nitrite. These reactions have been examined mechanistically[4,5] and show all the characteristics of electrophilic nitrosation reactions. Many *S*-nitrosothiols are too unstable in their pure form to be isolated (contrasting with their oxygen counterparts the alkyl nitrites) and this has contributed to the lack of knowledge of their chemistry relative to that of the alkyl nitrites, until comparatively recently. A number of *S*-nitrosothiols however are sufficiently stable to allow a full structural characterization. In particular *N*-acetyl-*S*-nitrosopenicillamine (SNAP) **1** and *S*-nitrosoglutathione (GSNO) **2** appear to be indefinitely stable as solids at room temperature. It has been known for some time[6,7] that RSNO



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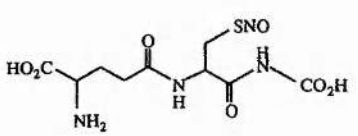

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species decompose photochemically and thermally to give nitric oxide and the corresponding disulfide [eqn. (1)]. Recently the

$$2RSNO \xrightarrow[\text{or } \Delta]{h\nu} RSSR + 2NO \qquad (1)$$

photochemical reaction of GSNO has been examined in more detail and the quantum yield and a first-order rate constant have been determined.[8] In both reactions it is likely that homolysis of the S–N bond is the primary process.

In solution, particularly aqueous solution, and in the absence of heat and light many investigations have reported that the same overall reaction occurs. In the presence of oxygen the final product is nitrite anion, as expected from the known reaction[9] of nitric oxide [eqn. (2)], but in the absence of oxygen, nitric

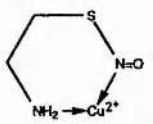
$$4NO + O_2 + 4OH^- = 4NO_2^- + 2H_2O \quad (2)$$

oxide has been detected using an electrode system.[10] At first quantitative rate measurements of RSNO decomposition reactions in aqueous solution have yielded erratic results, with reported half-lives of reaction varying considerably in different reports using the same substrate, usually SNAP. Further, the rate-form reported varied widely, zero-, first-, second- and various intermediate orders having been reported at some stage. This picture was resolved recently when it was realized that reaction occurred by a Cu^{2+}-catalysed reaction pathway, and that for some reactants there can be enough Cu^{2+} in the distilled water/buffer components used, to bring about reaction.[10] The $[Cu^{2+}]$ varied from source to source and often daily within the same source, which goes some way to explaining the erratic nature of the reported results. When Cu^{2+} is removed by complexation with EDTA, virtually no reaction takes place. There was no catalysis for a range of other metal ions investigated, including Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+} and Fe^{3+}.

In the earlier paper[10] we reported that for many RSNO compounds over a given $[Cu^{2+}]$ range (which varied with the substrate) the second-order rate equation, eqn. (3), applied,

$$\text{Rate} = k[\text{RSNO}][Cu^{2+}] \quad (3)$$

often with a small autocatalytic component, which we ignored. The copper is fully regenerated and so is truly catalytic. That work concentrated on the structure–reactivity dependence using nitrosothiol species which were of biological interest, mainly derivatives of cysteine and glutathione. Values of k [eqn. (3)] varied considerably with structure and clearly led to the conclusion that high reactivity was associated with RSNO structures in which the copper could bind with two sites within the molecule *via* six-membered ring intermediates such as those shown in structures **3** and **4**. These two binding sites we wrote as



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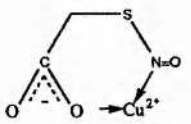

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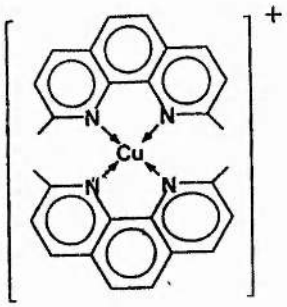
the nitroso–nitrogen atom in each case and either an amine or carboxylate group. In the absence of either of these features reaction was very slow indeed.

Although we did establish a qualitative structure–reactivity pattern it was clear that a full mechanistic picture of this reaction had not been described, since outside a given $[Cu^{2+}]$ (which differed for different substrates) other kinetic patterns emerged, in particular at low $[Cu^{2+}]$ there was an increasing tendency for autocatalysis to be observed and at high $[Cu^{2+}]$ there was a move towards a zero-order dependence upon [RSNO] and also upon $[Cu^{2+}]$. These rate forms together with a whole range of intermediate situations clearly led to quite a complex set of data. A further unusual feature occurred in the reaction of *N*-acetyl-*S*-nitrosocysteine in that there was a very long induction period (many hours) before reaction set in, with approximately a first-order dependence. This paper describes in more detail the more unusual kinetics and proposes a mechanism which is consistent with all of the experimental results.

Results and discussion

The unusual kinetic forms found for a number of RSNO species under different conditions of $[Cu^{2+}]$, particularly the tendency in some cases towards zero-order behaviour, led us to consider the possibility that the effective reagent in these reactions is in fact Cu^+ and not Cu^{2+}. Zero-order dependence upon [RSNO] might then be interpreted in terms of a rate-limiting $Cu^{2+} \longrightarrow Cu^+$ reduction. Earlier[10] we had considered this possibility and had rejected it on the basis that we had failed to find evidence for it from preliminary EPR experiments with SNAP. In an alternative approach, we have now made use of the specific Cu^+-chelator neocuproine[11] shown in the complexed form in **5**. In aqueous solution the stability constant of **5** is



5

about 1×10^{19} dm^6 mol^{-2}. When a Cu^{2+} solution (2×10^{-5} mol dm^{-3}) was added to one of neocuproine hydrochloride (4×10^{-5} mol dm^{-3}) there was no detectable change in the UV–VIS spectrum. However upon addition of sodium dithionite (a well-known reducing agent for $Cu^{2+} \longrightarrow Cu^+$) an immediate yellow colour was noted and an absorbance maximum at 453 nm was found, as reported in the literature[11] for the spectrum of **5**. We then examined the decomposition of SNAP (1×10^{-3} mol dm^{-3}) at pH 7.4 containing added Cu^{2+} (2×10^{-5} mol dm^{-3}) in the presence of increasing concentrations of neocuproine in the range 4×10^{-5}–1×10^{-3} mol dm^{-3}. The resulting absorbance–time plots taken at 340 nm (the absorbance maximum for SNAP) are shown in Fig. 1. It is immediately clear that the presence of neocuproine reduces the reaction rate progressively and at 1×10^{-3} neocuproine the reaction is completely suppressed. The full spectra showed the increasing absorbance at 453 nm as expected for the formation of **5**. Similar experiments over a slightly smaller range of [added neocuproine] yielded reasonably good first-order plots and the data are given in Table 1 and Fig. 2 showing clearly the inhibiting effect of neocuproine.

Clearly Cu^+ is being generated and the question arises as to the nature of the reducing agent. Previously we have suggested that the reduction could be achieved by thiolate ion present from a small quantity of thiol impurity in the *S*-nitrosothiol sample. However we have been careful to avoid any thiol impurity by carrying out reactions on solution samples of RSNO generated from thiols and nitrous acid with the nitrous acid present in a slight excess, and reaction still occurs. An alternative suggestion is that thiolate is generated by hydrolysis of the *S*-nitrosothiol [eqn. (4)]. This is not expected to be a

$$\text{RSNO} + 2OH^- \longrightarrow RS^- + NO_2^- + H_2O \quad (4)$$

rapid process given our earlier studies of the hydrolysis in acid solution,[12] but even a few percent reaction could be enough to initiate the reduction. When the corresponding thiol is added to the reaction mixtures initially a rapid increase in the rate constant is found. We worked with SNAP with the addition of the corresponding thiol *N*-acetylpenicillamine NAP. Use of a different thiol would complicate the situation by rapid NO

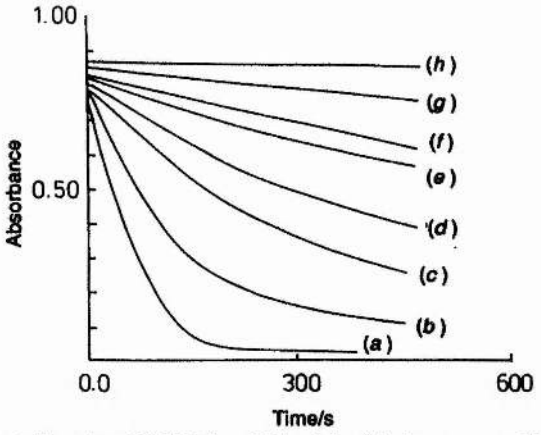


Fig. 1 Reaction of SNAP (1×10^{-3} mol dm^{-3}) in the presence of Cu^{2+} (2×10^{-5} mol dm^{-3}) and varying concentrations of neocuproine: (*a*) no added neocuproine; (*b*) 4×10^{-5} mol dm^{-3} neocuproine; (*c*) 5×10^{-5} mol dm^{-3} neocuproine; (*d*) 6×10^{-5} mol dm^{-3} neocuproine; (*e*) 8×10^{-5} mol dm^{-3} neocuproine; (*f*) 1×10^{-4} mol dm^{-3} neocuproine; (*g*) 2×10^{-4} mol dm^{-3} neocuproine; (*h*) 1×10^{-3} mol dm^{-3} neocuproine

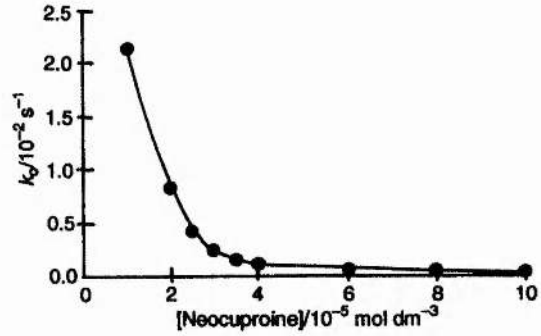


Fig. 2 First-order rate constants (k_o) for the reaction of SNAP (1×10^{-3} mol dm^{-3}) in the presence of Cu^{2+} (2×10^{-5} mol dm^{-3}) as a function of [neocuproine]

Table 1 Values of k_o for the decomposition of SNAP in the presence of added Cu^{2+} (2×10^{-5} mol dm^{-3}) and increasing amounts of neocuproine

Neocuproine/10^{-5} mol dm^{-3}	$k_o/10^{-2}$ s^{-1}
1.0	2.14
2.0	0.83
2.5	0.43
3.0	0.26
3.5	0.16
4.0	0.12
6.0	0.06
8.0	0.04
10.0	0.003

group transfer from RSNO to R'SH leading to R'SNO formation.[13] With [SNAP] of 1×10^{-3} mol dm^{-3}, added [Cu^{2+}] of 1×10^{-5} mol dm^{-3} and [NAP] in the range of 1×10^{-6}–1×10^{-3} mol dm^{-3}, reactions are kinetically first-order. The results are given in Table 2 and are also shown more dramatically in Fig. 3. As expected at low added NAP there is a very sharp linear increase in the rate constant until [NAP] $\approx 1 \times 10^{-5}$ mol dm^{-3}. The reduction of Cu^{2+} by thiolate is a well-known process[14,15] and has been studied mechanistically as the Cu^{2+} catalysed oxidation of thiols to give disulfides [eqn. (5)].

$$2Cu^{2+} + 2RSH = 2Cu^{+} + 2H^{+} + RSSR \qquad (5)$$

At higher [NAP] there is initially a sharp drop in k_o followed

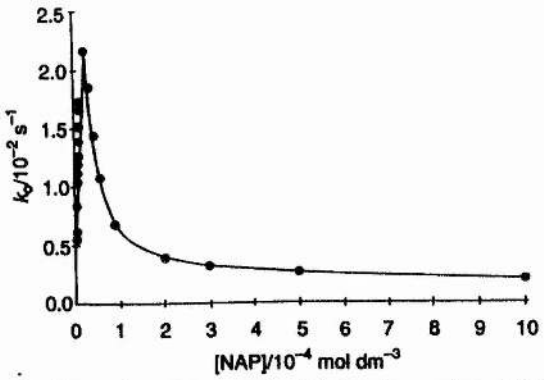
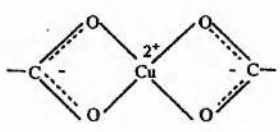


Fig. 3 First-order rate constants (k_o) for the reaction of SNAP (1×10^{-3} mol dm^{-3}) as a function of added *N*-acetylpenicillamine (NAP)

Table 2 Values of k_o for the decomposition of SNAP in the presence of added Cu^{2+} (1×10^{-5} mol dm^{-3}) and increasing amounts of NAP

[NAP]/10^{-6} mol dm^{-3}	$k_o/10^{-3}$ s^{-1}
0	4.97
1.0	5.63
2.0	6.25
3.0	8.35
4.0	10.4
5.0	11.9
6.0	12.8
7.0	14.0
8.0	15.3
9.0	16.6
10	17.4
20	21.7
30	18.7
40	14.5
50	10.9
90	6.79
200	4.14
300	3.37
500	2.67
1000	2.12

by a gradual decrease towards zero. We can explain this pattern in terms of the complexing of Cu^{2+} by NAP, thus effectively removing it from solution. These results explain the apparently contradictory reports in the literature,[16] some of which report catalysis of RSNO decomposition by added thiols whilst others find a reduction in rate upon thiol addition. It is now clear that at low added [RSH] there will be catalysis, as this favours the reduction of Cu^{2+}, whereas complexation of Cu^{2+}, probably by the carboxylate group, takes over at higher added [RSH] resulting in inhibition of nitric oxide formation. Such copper-carboxylates are well-known (see structure **6**) and some have



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been isolated and examined structurally.[17] We have previously noted[10] a reduction in reactivity with increasing buffer concentration when the buffer contains a carboxylic acid, an effect we attributed to competitive complexation with the carboxylic acid.

Earlier[10] we concentrated our kinetic analysis on rate forms which gave first-order dependencies upon both RSNO and Cu^{2+} in order to establish structure–reactivity factors. We now

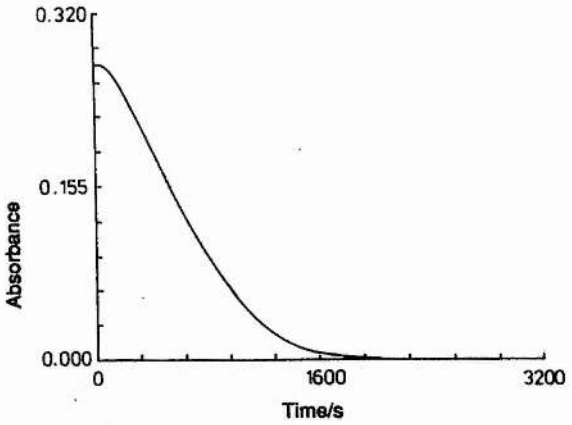


Fig. 4 Absorbance–time plot for the reaction of *S*-nitroso-2-dimethylaminoethanethiol (5×10^{-4} mol dm^{-3}) in the presence of added Cu^{2+} (1×10^{-6} mol dm^{-3})

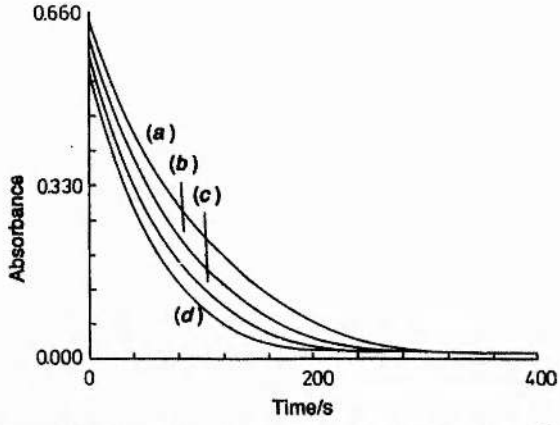


Fig. 5 Absorbance–time plots for the reaction of SNAP (1×10^{-3} mol dm^{-3}) in the presence of Cu^{2+} (1×10^{-5} mol dm^{-3}) and varying concentrations of added *N*-acetylpenicillamine (NAP): (*a*) 4×10^{-6} mol dm^{-3} NAP; (*b*) 6×10^{-6} mol dm^{-3} NAP; (*c*) 8×10^{-6} mol dm^{-3} NAP; (*d*) 1×10^{-5} mol dm^{-3} NAP

report the results of a more systematic kinetic study in which other rate forms appeared. In all cases we have been following the decreasing absorbance at *ca.* 340 nm due to the RSNO reactant, usually at an initial concentration of around 5×10^{-4} mol dm^{-3}. We have been able to isolate four limiting absorbance–time patterns: (*a*) first-order reaction with an induction period, (*b*) first-order reaction with no induction period, (*c*) zero-order reaction with an induction period and (*d*) zero-order reaction with no induction period. In addition we observed many forms which could be regarded as intermediate between any two of the four limiting forms. Examples of each are given in Figs. 4, 5, 6, 7 and 8. Fig. 4 is the reaction of *S*-nitroso-2-dimethylaminoethanethiol (SNDMA) **8**, at low [Cu^{2+}] with no added thiol, Fig. 5 is the reaction of SNAP at 1×10^{-5} mol dm^{-3} Cu^{2+} in the presence of varying [NAP], Fig. 6 is the reaction of *S*-nitroso-2-diethylaminoethanethiol with various concentrations of added Cu^{2+}, Fig. 7 is a similar pattern for the reaction of *N*-acetyl-D,L-2,2-dimethylcysteinyl-glycine methyl ester **7** with different [Cu^{2+}] and Fig. 8 is SNDMA **8** at low [Cu^{2+}] varying the concentration of added thiol. The induction period is quite clear in Figs. 4, 6 and 7 and is completely absent in Figs. 5 and 8 at high [RSH]. Equally clear is the first-order pattern in Figs. 4 and 5 and zero-order dependence in Figs. 6, 7 and 8.

On the basis of these and earlier results we propose the following outline mechanism: Cu^{2+} is reduced by RS^- (generated from RSNO or added as RSH) *via* intermediate X

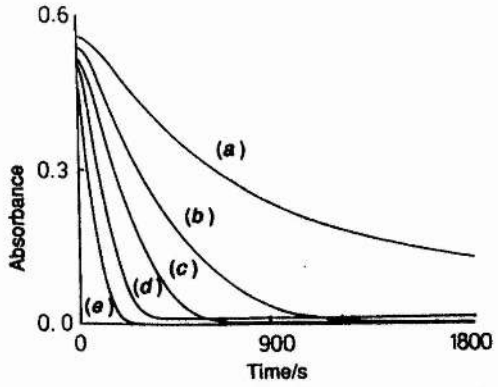


Fig. 6 Absorbance–time plots for the reaction of *S*-nitroso-2-diethylaminoethanethiol (1×10^{-3} mol dm^{-3}) in the presence of added Cu^{2+}: (*a*) 5×10^{-6} mol dm^{-3} Cu^{2+}; (*b*) 7.5×10^{-6} mol dm^{-3} Cu^{2+}; (*c*) 1×10^{-5} mol dm^{-3} Cu^{2+}; (*d*) 3×10^{-5} mol dm^{-3} Cu^{2+}; (*e*) 6×10^{-5} mol dm^{-3} Cu^{2+}

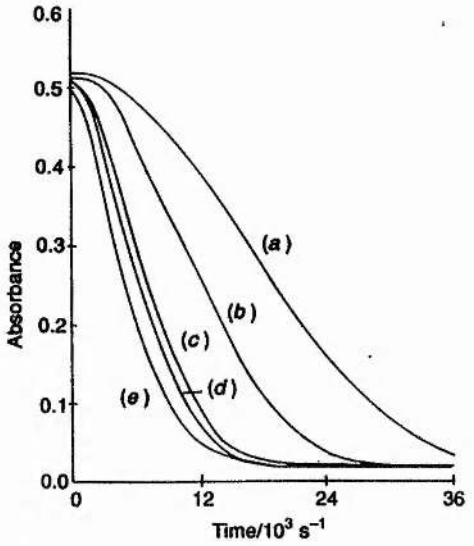


Fig. 7 Absorbance–time plots for the reaction of SNAP–Gly (1×10^{-3} mol dm^{-3}) in the presence of added Cu^{2+}: (*a*) 2.7×10^{-6} mol dm^{-3} Cu^{2+}; (*b*) 5.4×10^{-6} mol dm^{-3} Cu^{2+}; (*c*) 8.1×10^{-6} mol dm^{-3} Cu^{2+}; (*d*) 1.1×10^{-5} mol dm^{-3} Cu^{2+}; (*e*) 1.4×10^{-5} mol dm^{-3} Cu^{2+}

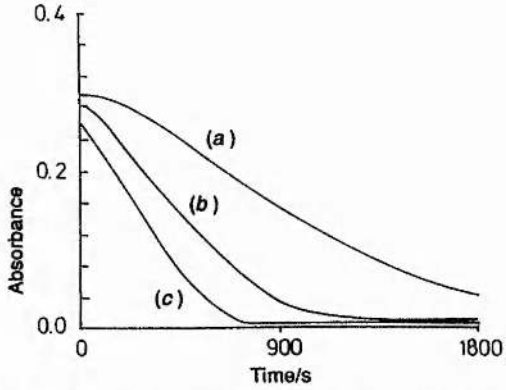


Fig. 8 Absorbance–time plots for the reaction of *S*-nitroso-2-dimethylaminoethanethiol (2×10^{-4} mol dm^{-3}) in the presence of added Cu^{2+} (1×10^{-6} mol dm^{-3}) as a function of added 2-*N*,*N*-dimethylaminoethanethiol: (*a*) No added thiol; (*b*) Added thiol (1×10^{-6} mol dm^{-3}) (*c*) Added thiol (3×10^{-6} mol dm^{-3})

to give Cu^+ and RS$^{\bullet}$. Intermediate X is probably $RSCu^+$. Reaction then occurs between Cu^+ and RSNO *via* intermediate Y releasing Cu^{2+}, RS^- and NO. This is essentially the

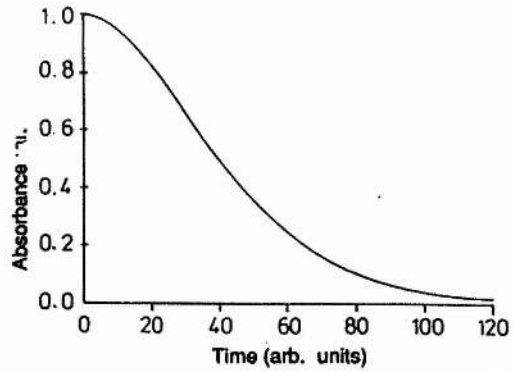


Fig. 9 Computed absorbance–time plot with no added RSH for rate-limiting RSNO reaction with Cu^+

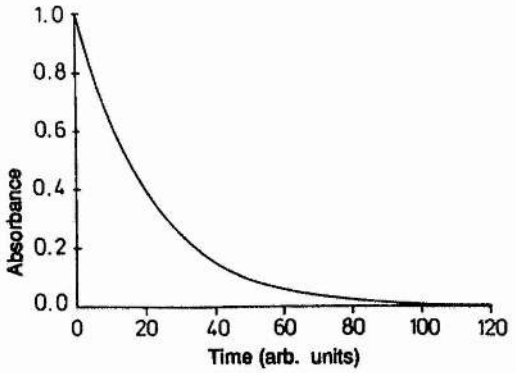


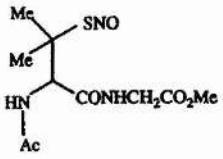
Fig. 10 Computed absorbance–time plot with added RSH for rate-limiting RSNO reaction with Cu^+

$$Cu^{2+} + RS^- \rightleftharpoons X \longrightarrow Cu^+ + RS^\cdot$$

$$Cu^+ + RSNO \rightleftharpoons Y \longrightarrow Cu^{2+} + RS^- + NO$$

$$2RS^\cdot \longrightarrow RSSR$$

Scheme 1



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mechanism we suggested earlier [18] as a possibility, but without at that time sufficient evidence to support it. Intermediates Y we believe are akin to structures **3** and **4**, now with Cu^+ and not Cu^{2+}, probably also co-ordinated to two water molecules.

Qualitatively we can account for the induction period as the time required for the generation of RS^- and Cu^+. A first-order dependence occurs if the reaction of RSNO with Cu^+ is rate-limiting and a zero-order dependence occurs (for the more reactive RSNO species) when Cu^+ formation is rate limiting. The length of the induction period can be reduced by the addition of either RSH or Cu^{2+} which will increase the rate of formation of Cu^+.

We have attempted to explain the various rate patterns encountered experimentally more quantitatively by use of a computer model of Scheme 1, and to extract the limiting forms which this model predicts. We have not aimed at a full treatment optimizing all of the parameters because of the uncertainties of the values of the initial $[Cu^{2+}]$ and $[RS^-]$ particularly when an induction period occurs. Our hope was that we would be able to reproduce qualitatively the absorbance–time experimental data with those predicted from Scheme 1, particularly under four limiting conditions.

(1) Rapid formation and regeneration of Cu^+

Scheme 1 reduces (for kinetic purposes) to eqns. (6) and (7). If RSNO, Cu^{2+} and RS^- are present at the start then the result

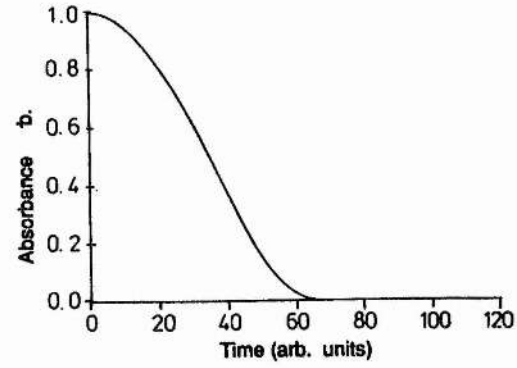

Fig. 11 Computed absorbance–time plot with no added RSH for rate-limiting Cu^+ formation

$$RSNO + Cu^+ \xrightarrow{slow} RS^- + NO + Cu^{2+} \quad (6)$$

$$RS^- + Cu^{2+} \xrightarrow{fast} \tfrac{1}{2}RSSR + Cu^+ \quad (7)$$

will be a simple first order rate equation [eqn. (8)] where the

$$-d[RSNO]/dt = d[NO]/dt = k_o[RSNO] \quad (8)$$

observed rate constant k_o will be given by $k[Cu^{2+}]$ when $[Cu^{2+}] \ll [RS^-]$ (the normal state of affairs with added RS^-) and by $k[RS^-]$ when $[Cu^{2+}] \gg [RS^-]$. The computer simulation is given in Fig. 10. We have observed many examples of such behaviour experimentally (as in Fig. 5 as [NAP] is increased). In effect we now have rapid formation and regeneration of Cu^+, the concentration of which remains constant in any one experiment.

Alternatively, if only RSNO and Cu^{2+} are initially present the step for the generation of Cu^+ [eqn. (4)] must be included in the simulation. This results in an absorbance–time profile given in Fig. 9, with an autocatalytic feature which reproduces that observed experimentally in Fig. 4.

(2) Rate-limiting Cu^+ formation

Under these circumstances Scheme 1 effectively reduces to eqns. (9) and (10). If RSNO, Cu^{2+} and RS^- are all present

$$RS^- + Cu^{2+} \xrightarrow{slow} \tfrac{1}{2}RSSR + Cu^+ \quad (9)$$

$$Cu^+ + RSNO \xrightarrow{fast} RS^- + NO + Cu^{2+} \quad (10)$$

initially then we get a truly zero-order reaction (simulation in Fig. 12) with RSNO scavenging Cu^+ as it is formed immediately regenerating RS^- and Cu^{2+}. However if only RSNO and Cu^{2+} are present initially, then RS^- has to be generated as before and this results in an induction period

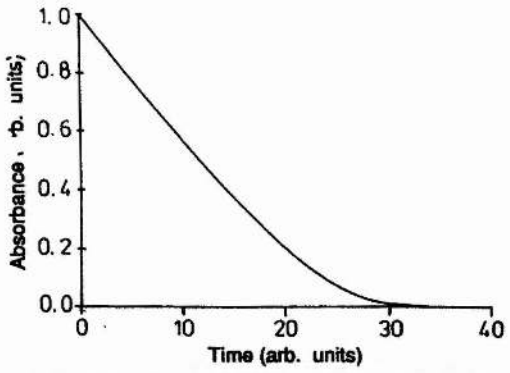

Fig. 12 Computed absorbance–time plot with added RSH for rate-limiting Cu^+ formation

followed by a zero-order reaction (Fig. 11). Experimental examples of both types of behaviour have been observed as in Fig. 8, as $[RS^-]$ is increased, Figs. 6 and 7. Figs. 6 and 7 clearly show a saturation effect in Cu^{2+} at high $[Cu^{2+}]$ which requires that in Cu^+ formation we have an equilibrium involving intermediate X.

All of these experiments were conducted at pH 7.4 in normal aerated solvent. When oxygen was rigorously excluded then we found no significant difference in behaviour for any of the limiting rate forms. This implies that any oxidation reaction, such as of Cu^+ by dissolved oxygen, is not a significant reaction under these conditions. However for very slow reacting RSNO compounds, such as *N*-acetyl-*S*-nitrosocysteine (SNAC) we found a very long induction period typically 3 hours as shown in Fig. 13. This result was obtained using a rather impure solid sample of SNAC which was known to contain some of the thiol and was very difficult to purify. Repetition of this experiment with SNAC generated *in situ* in solution gave no reaction whatsoever over a 16 hour period (Fig. 14), so it is likely that the thiol is playing a part here in Cu^+ generation as expected. However when the same reaction was carried out in the absence of oxygen, reaction occurred immediately, with no induction period. Exactly the same pattern was found with *S*-nitrosoglutathione (GSNO), although in both cases it is not clear why complete reaction does not occur. There is however a very dramatic effect due to the presence of oxygen here, which implies that oxidation of Cu^+ by oxygen [eqn. (11)] is a competing reaction with the Cu^+ reaction

$$Cu^+ + O_2 = Cu^{2+} + O_2^- \qquad (11)$$

with RSNO.

When the oxygen is removed decomposition can occur. For both of these substrates reaction can be induced by the addition of the corresponding thiol. An induction period occurs which is reduced as the concentration of added thiol is increased until we get a good first-order plot at very high [thiol] ≈ 0.15 mol dm^{-3}. In the presence of thiol, the effect of increasing the $[Cu^{2+}]$ is also to reduce the induction period, and as before the removal of oxygen also removes the induction period completely. For these slower reacting substrates it does seem that re-oxidation of Cu^+ by dissolved oxygen is an important pathway in their decomposition reactions.

We believe that we have shown that Cu^+ is the effective reagent in bringing about decomposition of *S*-nitrosothiols to yield nitric oxide. Both the experiments with neocuproine and all the kinetic evidence support the outline mechanism given in Scheme 1. Some details remain as yet unresolved, *e.g.* the detailed mechanism for the breakdown of intermediate Y, for which at present we have no experimental evidence.

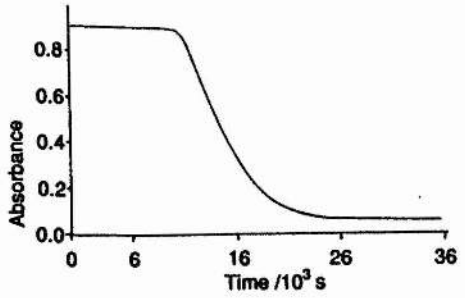

Fig. 13 Absorbance–time plot for the reaction of *N*-acetyl-*S* nitrosocysteine SNAC (crude sample)

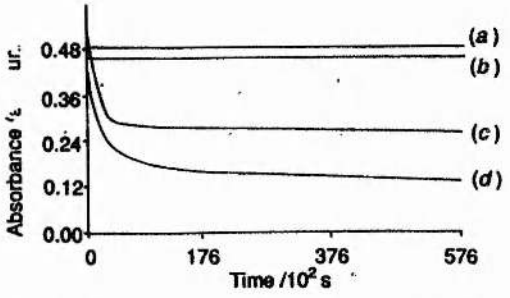

Fig. 14 Absorbance–time plots for the reactions of GSNO and *N*-acetyl-*S*-nitrosocysteine (SNAC) showing the effect of the presence of oxygen: (*a*) GSNO under aerobic conditions; (*b*) SNAC under aerobic conditions; (*c*) GSNO under anaerobic conditions; (*d*) SNAC under anaerobic conditions

These findings could well have implications for the reactions of *S*-nitrosothiols *in vivo*. It is known that they have specific biological activity notably, vasodilation,[19] the inhibition of platelet aggregation[20] and the inhibition of neutrophil functions.[21] In the body copper is present not as free Cu^{2+} but in a bound form with amino acids and proteins. Possibly this is reducible to Cu^+ by thiolate. Experiments to test this possibility are in hand. A recent particularly interesting finding[22] shows that the inhibition of platelet aggregation activity shown by GSNO is much reduced in the presence of neocuproine and the closely related bathocuproine, both specific Cu^+-chelators. This strongly suggests (*a*) that nitric oxide is required to effect the activity and (*b*) that it is generated from *S*-nitrosothiols by a process which involves Cu^+. However another recent article[23] claims that bronchodilation induced by GSNO does not require the formation of NO. The medical importance of *S*-nitrosothiols has been highlighted recently by two reports,[24,25] which describe the clinical use of GSNO to inhibit platelet aggregation during coronary angioplasty and also to treat a form of pre-eclampsia, a high blood pressure condition suffered by some pregnant women.

The reactivity pattern of GSNO is particularly interesting. In aerobic solution it is particularly stable, with only a negligibly small amount of decomposition occuring over many hours. However, in the presence of glutathione GSH decomposition occurs much more rapidly but possibly with an induction period, the length of which is dependent on [GSH] and $[Cu^{2+}]$. Further, in the absence of oxygen and without added GSH, reaction occurs quite readily without an induction period. Finally, the presence of another oxidizing agent (*e.g.* hydrogen peroxide) has the effect of stabilizing GSNO, again over a period of many hours, in our experiments no perceptible decomposition occurred overnight. Again the re-oxidation of $Cu^+ \longrightarrow Cu^{2+}$ does not allow the Cu^+ + RSNO to occur.

Experimental

All thiols, buffer components, *etc.* were commercial samples of the highest purity grade available. The *S*-nitrosothiols were mostly synthesized in solution, and not isolated, by nitrosation of the thiols with an equivalent of nitrous acid under mildly acid conditions. All gave the characteristic broad absorption band centred around 340 nm. Aliquots of these freshly prepared solutions were used after pH adjustment, taking care to minimize exposure to light. In the case of SNAP **1** and GSNO **2**, the solid derivatives were prepared as described in the literature.[26,27] An impure sample of *N*-acetyl-*S*-nitrosocysteine was prepared by a modification of the method used for GSNO. The *S*-nitroso dipeptide **7** was a new compound and was prepared as follows.

A solution of glycine methyl ester hydrochloride (6.28 g, 50 mmol) in water (40 cm^3) was treated with a solution of potassium carbonate (72 mmol) in water (20 cm^3) and the mixture extracted with dichloromethane. After drying ($MgSO_4$), the solvent was removed by evaporation. The residue was added to a suspension of *N*-acetyl-D,L-penicillamine (3.28 g, 20 mmol) in purified dichloromethane (100 cm^3) followed by 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (8.47 g, 20 mmol). The urea derivative started to precipitate immediately and after 2 days was removed by filtration and washed with dichloromethane. The combined filtrate and washings were extracted with a saturated solution of citric acid (60 cm^3), a saturated solution of potassium hydrogen carbonate (60 cm^3) and water (60 cm^3), dried ($MgSO_4$) and the solvent removed by evaporation. The residue was washed with cold ether to give white, flaky crystals of product, mp 142 °C (decomp.). It was used without purification for the next stage [Found: *m/z* (FAB) 263.1061 (MH^+). $C_{10}H_{19}O_4N_2S$ requires 263.1066 (MH^+)]. The *S*-nitroso derivative was prepared by dissolving the dipeptide (0.26 g, 1 mmol) in dichloromethane (8 cm^3) and adding *tert*-butyl nitrite (1 cm^3). After 1 h the solvent from the green solution was removed by evaporation to give a green solid. After washing with water the solid was dried *in vacuo* (0.24 g, 82%), *m/z* (FAB) 292.0967 (M^+, 292.0976) [Found: C, 41.0; H, 6.2; N, 14.2. $C_{10}H_{17}O_5N_3S$ requires C, 41.2; H, 5.9; N, 14.4%].

Products

The disulfide products, nitrite anion and nitric oxide (in the absence of oxygen) were determined as reported earlier.[10]

Kinetics

These were carried out also as reported earlier [10] by monitoring the decreasing absorbance at *ca.* 340 nm.

Acknowledgements

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