SOME STUDIES OF BIOLOGICALLY ACTIVE S-NITROSOTHIOLS

Stuart Clive Askew

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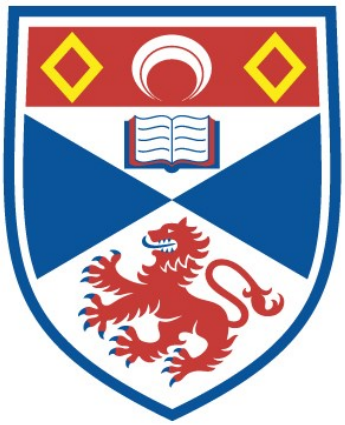

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SOME STUDIES OF BIOLOGICALLY ACTIVE S-NITROSOTHIOLS

By Stuart Clive Askew

for the degree of
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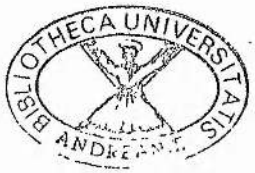

DECLARATION

I, Stuart Clive Askew, hereby certify that this thesis has been composed by me, that it is an accurate representation of the work undertaken by me in the University of St. Andrews since my admission as a Research Student in 1st October 1991, and that it has not been accepted in any previous application for any Higher Degree or professional qualification.

October 1994 Signed.....

I hereby certify that Stuart Clive Askew has fulfilled the Regulations appropriate to the Degree of Doctor of Philosophy.

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I dedicate this thesis to;

My parents, for their continuous support throughout the past seven years, and without whom I would not have been able to come to university, let alone study for a PhD!

To Jill for everything, but particularly for putting up with me being a student for a further 3 years!

and to the university and town of St Andrews, which have provided me with countless friends and opportunities I would otherwise not have had, and a perfect home.

"I think it is a tremendous moment, something we have worked so hard for finally comes to fruition"

Edward Heath, 1973, on joining the EEC.

The Golden Hour, Simon Mayo, Radio One.

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THE INSPIRATION

This view from the laboratory window encompasses the essential ingredients that have carried me through the last 3 years. In reverse order; the beautiful coast line, the 'one & only' golf course and *the 'hallowed' (?!) turf* of Madras Rugby Pitches. However, some of my more 'socially-influenced' colleagues may say that I should replace this picture with one of Berts Bar, but thats another story.....

THE SUPERVISORS

The biggest thankyou must go to my supervisors Dr Anthony Butler and Dr Eric Flitney. For their support, supervision and friendship I am greatly indebted. I wish them both well for the future in this exciting field of research. I would also like to thank the British Heart Foundation for their generous sponsorship over the past 3 years, hopefully the money raised from the 'sweat and toil' of running the London Marathon will go some way towards repayment.

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ABSTRACT

S-nitrosothiols are effective NO-donating drugs which can elicit vasodilation of vascular tissue and disaggregate or inhibit the aggregation of platelets in blood. The chemistries of two S-nitrosothiols, S-nitroso-N-acetyl-DL-penicillamine (SNAP) and S-nitrosoglutathione (GSNO) have been investigated in an attempt to identify the chemical and physiological mechanisms which underlie their biological actions as vasodilators and modulators of platelet behaviour. Although SNAP and GSNO have been found to be susceptible to decomposition by similar chemical mechanisms, such as by thermal and photochemical means, evidence is presented to suggest that they are both capable of NO transfer to other thiol containing compounds such as cysteine. This produces a very unstable S-nitrosothiol, S-nitrosocysteine, which readily produces NO. However, they can both be decomposed by different, distinctive mechanisms.

Metal ion catalysis by copper is shown to greatly accelerate the decomposition of SNAP, but has little effect on GSNO. Instead, NO release from GSNO is effected by enzymatic cleavage of the glutamyl-cysteinyl peptide bond by the enzyme γ-glutamyl transpeptidase (γ-GT). The resulting S-nitrosothiol, S-nitrosocysteinylglycine, would be expected to be more susceptible to release of NO by metal (copper) ion catalysis.

It is concluded that transnitrosation (NO-transfer) between thiol groups, or enzymatic cleavage are obligatory steps in the mechanism of NO release from GSNO, whereas SNAP requires only the presence of trace amounts of metal ions like copper to effect this process. The different modes of NO production may go some way towards explaining the different physiological effectiveness of these S-nitrosothiols as vasodilators and inhibitors of platelet aggregation.

CONTENTS

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ABBREVIATIONS

The following abbreviations are used in the text and are defined on initial use. The following list is for ease of reference.

Oxides of Nitrogen

NO	Nitric oxide
NO^+	Nitrosonium ion
NO^-	Nitroxide ion
NO_2^-	Nitrite
NO_3^-	Nitrate
NO_2	Nitrogen dioxide
N_2O_3	Dinitrogen trioxide

S-Nitrosothiols and Other Drugs Used or Referenced

SNAP	S-Nitroso-N-acetyl-DL-penicillamine
NAP	N-acetyl-DL-penicillamine
$(NAP\text{-})_2$	N-acetyl-DL-penicillamine disulphide
SNFP	S-Nitroso-N-formyl-DL-penicillamine
SNpen	S-Nitroso-DL-penicillamine
GSNO	S-Nitrosoglutathione
SNAG	S-Nitroso-N-acetylglutathione
SNFG	S-Nitroso-N-formylglutathione
SNC	S-Nitroso-DL-cysteine
SNAC	S-Nitroso-N-acetyl-DL-cysteine
SNBC	S-Nitroso-N-Boc cysteine
SNP	Sodium nitroprusside
ISMN	Isosorbide-5-mononitrate
RBS	Roussins Black Salt
GTN	Glycerol trinitrate
PE	Phenylephrine
oxyHb	Oxyhaemoglobin
metHb	Methaemoglobin
Hb	Ferro-haemoglobin
SOD	Superoxide Dismutase
Cys	Cysteine
EDTA	Ethylenediaminetetraacetic acid, disodium salt dihydrate
EA	Ethacrynic Acid

ACh	Acetylcholine
DMGA	Dimethylglutaric acid
CD	Cyclodextrin

Biologically-Derived Compounds

EDRF	Endothelium-derived relaxing factor
AA	Arachidonic acid
PGI_2	Prostacyclin
t-PA	Tissue-type plasminogen activator
O_2^-	Superoxide anions
H_2O_2	Hydrogen peroxide
OH·	Hydroxyl radical
$ONOO^-$	Peroxynitrite anion
ACE	Angiotensin converting enzyme
NANC	Non-adrenergic, non-cholinergic
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
BH_4	Tetrahydrobiopterin
cGMP	Cyclic guanosine monophosphate
cAMP	Cyclic adenosine monophosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
GTP	Guanosine triphosphate

NO-Synthase Inhibitors

L-NMMA	N^{ω} monomethyl-L-arginine
L-NNA	N^{ω} nitro-L-arginine
L-NAME	N^{ω} nitro-L-arginine methyl ester
L-NIO	N^{ω} iminoethyl-L-ornithine
L-ADMA	N^{ω} N^{ω} dimethyl-L-arginine

NO-Synthases

iNOS	Inducible NO synthase
eNOS	Endothelial NO synthase
nNOS	Neuronal NO synthase

Analytical Techniques

HPLC	High Performance Liquid Chromatography

GCMS	Gas chromatography mass spectrometry
GCIRMS	Gas chromatography combustion isotope mass spec.
CZE	Capillary zone electrophoresis
ESR	Electron spin resonance

Miscellaneous

PRP	Platelet rich plasma
WP	Washed platelet suspensions
LL	Luciferin-Luciferase
PNS	Peripheral nervous system
ED_{50}	Equivalent dose for 50% response
IC_{50}	Concentration required to inhibit by 50%

Chemical Structure Diagrams

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

The demonstration in 1987 (Palmer *et al.*) that formation of nitric oxide (NO) from vascular endothelial cells accounted for the physiological effect of the endothelial derived relaxing factor (EDRF), as described by Furchgott and Zawadzki in 1980, has resulted in a dramatic escalation of research into the biological roles of this 'gaseous' messenger molecule. It has become a rapidly growing area of biological research, and one in which the established modes of action of biological messengers are challenged. Its trafficking is independent of specific transporters or channels used by other chemical messengers. Instead, NO appears to diffuse freely in all directions from its site of origin (Moncada & Leone *et al.*, 1994, unpublished pictures), making control of its synthesis the key to regulating its activity. Due to its small size, its radical nature and its diffusibility, NO more than any other biological messenger depends on its chemical properties, rather than its molecular shape to govern its biological action.

This inorganic gas is synthesised by a wide variety of animals ranging from barnacles, fruit flies and blood sucking insects, to horse shoe crabs, chickens, trout and humans (Feldman *et al.*, 1993a, Moncada *et al.*, 1993).

The discovery of NO as a biological mediator resulted from years of intense research on the functional significance of the endothelium, a monolayer of cells which lines the lumen wall of blood vessels throughout the vascular network. These unique cells play a crucial role in the control of local blood pressure and blood platelet adhesion and aggregation. An important breakthrough came with the discovery that prostacyclin (PGI_2), a prostaglandin synthesised from arachidonic acid (AA), was released by endothelial cells and acted as a vasodilator of certain tissues and an anti-aggregating agent (Moncada *et al.*, 1976). It became clear that this was one of a plethora of biologically active substances that are synthesised in, and released from, vascular endothelial cells. Prostacyclin, a peptide (tissue-type plasminogen activator (t-PA),

Collen *et al.*, 1986) and more importantly EDRF constitute the most eminent triad of these.

1.2 THE DISCOVERY OF ENDOTHELIUM DERIVED RELAXING FACTOR

In 1980 Furchgott and his co-workers investigating the effect of acetylcholine (ACh) on rabbit aorta showed that different responses were obtained when the endothelial cell layer was present or removed. They showed that precontracted aortic rings relaxed on exposure to ACh when the endothelium was intact, but failed to relax (or even contracted) when it was removed. They concluded that there was an 'endothelium derived relaxing factor' produced by ACh which was then termed an endothelium dependent vasodilator. Many other endothelium dependent vasodilators have since been identified including bradykinin, histamine, calcium ionophore A23187, substance P, thrombin and adenine nucleotides (for review see Furchgott, 1984).

Endothelium dependent relaxations have also been found to be elicited by changes in the physical nature of the blood vessels such as hypoxia, electrical stimulation and changes in 'shear stress' resulting from increased luminal flow (Furchgott, 1984; Moncada *et al.*, 1986a; Busse *et al.*, 1985).

1.3 PROPERTIES OF EDRF

The humoral nature of EDRF was first demonstrated using a variety of pharmacological preparations based on a donor-detector bioassay in which the biologically active substance is transferred. One such arrangement designed by Furchgott used an aortic strip with an intact endothelium as the donor 'sandwiched' against a similar strip without its endothelium acting as the detector (intimal surface facing intimal surface) (Furchgott & Zawadski, 1980). Other workers used a similar principle in their

systems, one of which involved perfusing the lumen of an isolated intact rabbit aorta and using the effluent to superfuse endothelium denuded vascular rings (Griffith *et al.*, 1984; Rubanyi *et al.*, 1985). Stimulation of the donor aorta with ACh caused relaxation of the detector tissue.

It was later shown that endothelial cells cultured on microcarriers packed in chromatography columns were capable of bradykinin-stimulated relaxation of detector tissues when superfused with the column effluent (Cocks *et al.*, 1985; Gryglewski *et al.*, 1986a).

Bioassay systems such as these allowed the chemical and biological characteristics of the EDRF to be investigated. The short half life of EDRF in physiological solutions was the first property to be noticed and subsequently investigated. Cocks *et al.* (1985) and Griffith *et al.* (1984) claimed that the half-life of EDRF was a matter of seconds, and Griffith along with other researchers showed that it could be released under basal conditions as well as after stimulation by ACh (Griffith *et al.*, 1984; Rubanyi *et al.*, 1985; Martin *et al.*, ,1985). Other properties of EDRF were shown to include inhibition of its biological effects by haemoglobin and methylene blue (Martin *et al.*, 1985), as well as dithiothreitol and hydroquinone (Griffith *et al.*, 1984). It was also demonstrated that the effect of EDRF was mediated by stimulation of soluble guanylate cyclase in vascular smooth muscle, resulting in the elevation of intracellular cyclic GMP (but not cAMP) levels (Rapoport & Murad, 1983a). Superoxide anions (O_2^-) were shown to have a dramatic effect on the biological half life of EDRF, as superoxide dismutase (SOD), the endogenous enzyme which scavenges O_2^- ions, converting them to H_2O_2, substantially stabilised EDRF (Rubanyi & Vanhoutte, 1986). Subsequent work by Moncada *et al.* (1986b) showed that certain inhibitors of EDRF acted by generating O_2^- in solution as a result of their redox properties and more recent work has shown that haemoglobin and methylene blue inhibit EDRF, at least in part, by this process (Steele *et al.*, 1991; Marczin *et al.*, 1992). However, the main cause of EDRF

inhibition by haemoglobin is attributed to its ability to bind EDRF (Martin *et al* ., 1985; Hutchinson *et al.*, 1987).

Armed with these observations regarding the properties of EDRF, Furchgott (1988) and Ignarro (1987) both proposed at the same conference, that it could be NO or a closely related compound. It was only a few months later that Palmer, Ferrige and Moncada (1987) provided crucial evidence strongly supporting the theory that EDRF was indeed NO, using the reaction between NO and ozone (O_3) to yield a chemiluminescent product (Downes *et al.*, 1976). They showed that bradykinin was capable of generating enough NO from cultured endothelial cells to account for the physiological effects of EDRF and in comparative experiments using a cascade system, demonstrated that NO and EDRF had very similar biological properties. In subsequent experiments, NO was also shown to be susceptible to the same inhibitors as EDRF (Fe^{2+}, methylene blue, other redox reagents and haemoglobin) and its half-life was prolonged by agents such as SOD and cytochrome C (which oxidises O_2^- to O_2). Like EDRF, NO also elevated intracellular cGMP levels mediated by stimulation of guanylate cyclase.

In addition, an intensive study by Radomski and co-workers showed that EDRF and NO inhibited blood platelet aggregation (1987a), caused disaggregation of aggregated platelets (1987b) and also inhibited platelet adhesion to endothelial monolayers (1987c&d). Radomski also showed that there was synergistic activity between EDRF and prostacyclin (PGI_2) in the inhibition of platelet aggregation (Radomski *et al.*,1987a&b).

1.4 THE CHEMICAL IDENTITY OF EDRF

The evidence for EDRF being NO is compelling, but not conclusive and this has been an issue of intense speculation and controversy. The main argument surrounds the

wide variation in half-life of EDRF (from 3 to 50s, Palmer *et al*, 1987; Griffith *et al.*, 1984; Forstermann *et al.*, 1984; Cocks *et al.*, 1985; Rubanyi *et al.*, 1985; Gryglewski *et al.*, 1986b; Ignarro *et al.*, 1987), although this discrepancy is thought to be due to differing experimental conditions with regard to relative O_2^- and O_2 concentrations. Other points of contention are: differential binding of EDRF and NO to anion exchange columns; differential activity of EDRF and NO on smooth muscle preparations and poor correlation between biological activity and amounts of NO detectable by chemical methods (Cocks *et al.*, 1985; Myers *et al.*, 1990; Long *et al.*, 1987). Most of these factors can be explained by the different experimental methodologies implemented and side reactions of NO in the 'artificial' surroundings that these methodologies employ. For instance, in experiments using anion exchange columns (Cocks *et al.*, 1985; Long *et al.*, 1987;), EDRF has been reported to bind to columns, whereas NO (which is not anionic) binds to a lesser extent (Khan *et al.*, 1987). It is possible that EDRF undergoes a reaction with amines when passing down the column as NO is known to be a nitrosating agent under certain conditions (Williams *et al.*, 1985). Also, the response of the bioassay tissues to bolus administration of an agonist differs quantitatively from that to an infusion, therefore the comparison between NO released from cells or tissues and bolus injections of NO may be misleading.

Evidence from electron paramagnetic resonance (EPR) studies also raises a question mark concerning the exact identity of EDRF, since NO, but not EDRF, can form paramagnetic nitrosyl-haem (Greenberg & Rubanyi *et al.*, 1990). It has been proposed that an S-nitrosothiol, such as S-nitrosocysteine, more closely mimicks EDRF than NO (Rubanyi *et al.*, 1991; Myers *et al.*, 1990). Further experiments showed that like EDRF, S-nitrosocysteine did not produce an EPR signal of nitrosyl-haem when a solution of the nitrosothiol is passed down a reduced haemoglobin-agarose column (Rubanyi *et al.*, 1991). Furthermore, in concentrations necessary to produce vascular relaxation, S-nitrosocysteine did not spontaneously release NO (Rubanyi *et al.*, 1991). Further (indirect) evidence supporting the theory that an S-nitrosothiol could be EDRF

is provided by bradykinin-induced ^{35}S efflux studies using endothelial cells previously loaded with cysteine containing the isotope. This experiment showed a similar time course of EDRF release and suggested that EDRF carried the ^{35}S label. More recent work by Fukuto *et al.*. (1992b) has proposed a possible role of nitroxyl (HNO) as having similar biological activity to EDRF and NO. Hydroxyguanidines, one of which, N^{ω}-hydroxyarginine, is a biosynthetic intermediate in the endogenous enzymatic production of NO (see section 1.3), have been shown to generate NO or nitroxyl (Fukuto *et al.*, 1992a).

It has also been postulated that there is more than one EDRF (De Mey *et al.*, 1982) and the existence of mechanisms other than NO synthesis which play a role in endothelium-dependent relaxations cannot be excluded. For example, stimulation of the endothelium of some arteries by ACh or histamine results in hyperpolarisation of the adjacent smooth muscle cells which contributes to their relaxation and this has been attributed to the release of a factor, termed endothelium derived hyperpolarising factor. (Feletou *et al.*, 1988; Chen *et al.*, 1989; Taylor *et al.*, 1988). The existence of other endothelium-dependent vasodilator mechanisms would not be suprising, because mechanisms subserving a biological function are usually multiple. It is important to stress however, that endothelium-dependent relaxations susceptible to inhibition by haemoglobin and methylene blue, which indicate the involvement of NO and cyclic GMP, have been demonstrated in many different vascular preparations, whereas evidence for other mechanisms is scant. Furthermore, a recent work by Feelisch and Moncada sides heavily with the view that NO is the EDRF (Feelisch & Moncada et al., 1994). Using a bioassay cascade, comprising three precontracted de-endothelialised rabbit aortic strips, and infusing cysteine over the detector tissues they found that only NO and EDRF had analogous vasodilator profiles unlike all the other possible EDRF candidates examined.

The underlying problem when trying to identify the nature of EDRF as NO, which is evident from most of the studies conducted, is the determination of what happens to it

between its production and release from the endothelium and its stimulation of guanylate cyclase. This could involve a number of chemical reactions to allow its transport to the active site of guanylate cyclase, or this whole process could be conducted by simple diffusion of NO. Whatever the exact chemical nature of EDRF and the way by which it is transported, it is generally agreed that its actions are mediated ultimately by NO.

1.5 SYNTHESIS OF EDRF

The enzymes responsible for the synthesis of NO from L-arginine in mammalian tissues are known as NO synthases. These enzymes are remarkable for three different reasons: the rapidity with which they have been characterised, purified and cloned (first described in 1989, Palmer *et al.*; first purified in 1990, Bredt *et al.*; and first cloned in 1991, Bredt *et al.*); the complexity and number of reactions carried out by a medium-sized subunit of 125-155 KDa (probably as a homodimer) to produce EDRF; and for the number and range of physiological and pathological roles in which they are involved (Moncada *et al.*, 1991).

The first description of NO synthases demonstrated that the synthesis of EDRF required L-arginine. Mass spectrometry studies showed that ^{15}NO was produced when endothelial cells containing ^{15}N labelled L-arginine (on the terminal guanidino nitrogen atom(s)) were stimulated by bradykinin (Palmer *et al.*, 1988a&b).

Two distinct types of NO synthase have been purified; a constitutive, Ca^{2+}/calmodulin dependent enzyme (Bredt *et al.*, 1990; Mayer *et al.*, 1989) first shown to be present in endothelial and brain cells (Palmer *et al.*, 1989; Garthwaite *et al.*, 1989); and an inducible Ca^{2+} independent enzyme initially found in macrophages (Stuehr *et al.*, 1989). Both enzymes are flavoproteins containing bound flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Mayer *et al.*, 1991; Stuehr *et al.*, 1991;

Yui *et al.*, 1991). Furthermore they are both dependent on an e^- donor, nicotinamide adenine dinucleotide phosphate (NADPH; Moncada and Palmer, 1990; Palmer *et al.*, 1989; Bredt *et al.*, 1990) as a cofactor and tetrahydrobiopterin (BH_4) which enhances the enzyme's activity and may stabilise or help assemble the active enzyme (Mayer *et al.*, 1991; Yui *et al.*, 1991).

Subsequent work on the mechanism of NO production from L-arginine by NO synthases demonstrated that molecular oxygen is also a substrate, being incorporated into both NO and citrulline (Kwon *et al.*, 1990, Leone *et al.*, 1991). This discovery has shed some light on the mechanism, which appears to involve 2 separate mono-oxygenation steps with N^{ω}-hydroxyarginine as an intermediate species formed by a reaction requiring one O_2 and one NADPH molecule (Leone *et al.*, 1991) and the presence of tetrahydrobiopterin (BH_4). The second step in the mechanism appears to result in the oxidation of N^{ω}-hydroxyarginine to form citrulline and NO (**Figure 1.1**). The detailed mechanism of this reaction remains to be determined.

The NO synthase enzymes have close homology with *Bacillus megaterium* cytochrome $P\text{-}450_{BM\text{-}3}$. The presence of FMN and FAD (see **Figure 1.1**), the striking sequence similarity with cytochrome P-450 reductase (Bredt *et al.*, 1991) and the presence of a haem centre, which exhibits similar spectral properties (Stuehr *et al.*, 1992a; White *et al.*, 1992) infer that NO synthase is the first self-sufficient mammalian P-450 enzyme to be identified.

There are at least 6 isoforms of NO synthase which have been purified (**Table 1.1**) based on 3 isoenzymes: nNOS originally identified as constitutive in neuronal tissue synthesising NO in response to glutamate; eNOS originally identified as constitutive in vascular endothelial cells synthesising NO in response to ACh; and iNOS originally identified as inducible by cytokines in macrophages and hepatocytes.

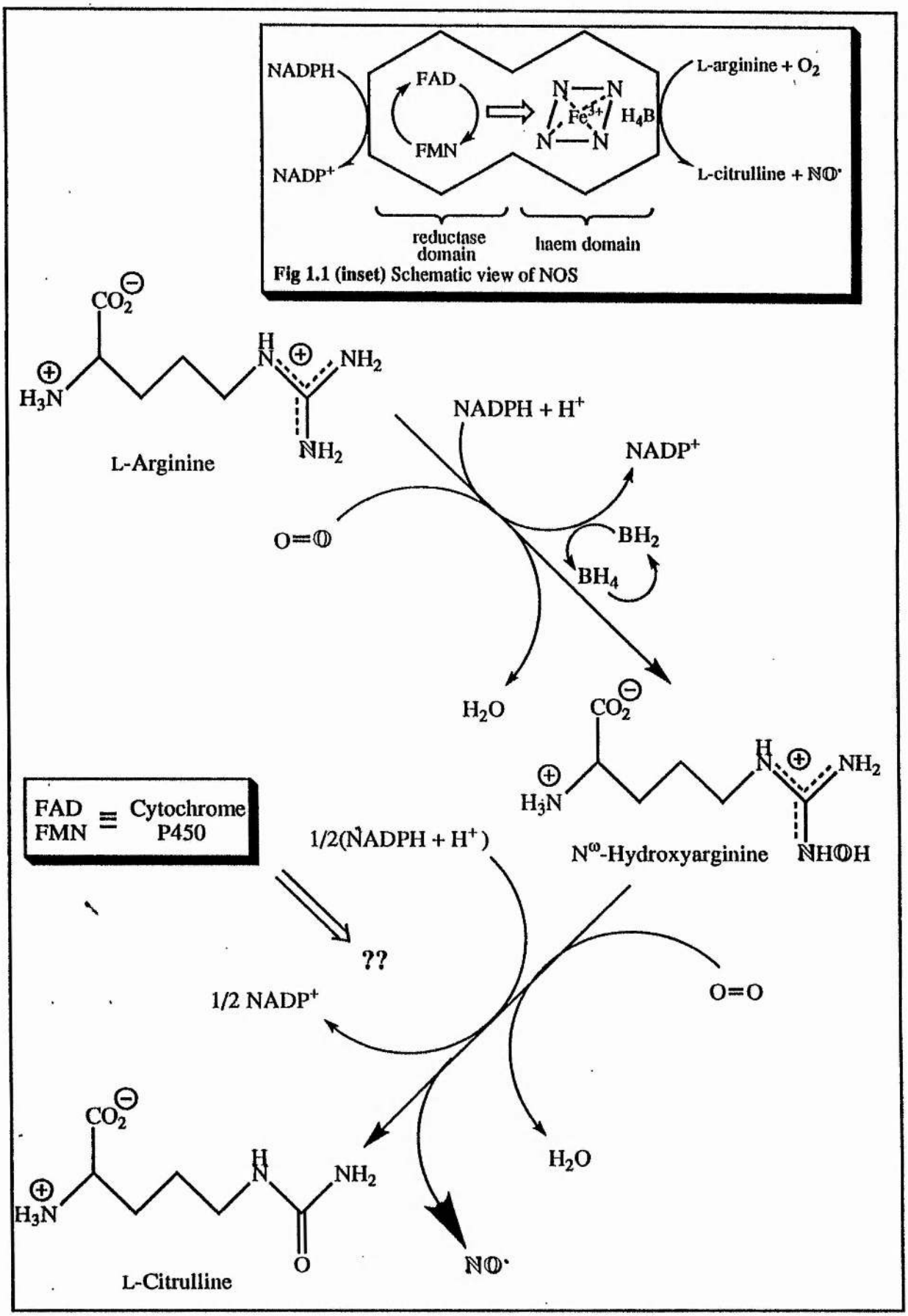


Figure 1.1 *The proposed NO synthase reaction (adapted from Knowles & Moncada, 1994; Marletta, 1993). In brain NO synthase the flavin coenzymes have been shown to be involved in the production of NO & citrulline from hydroxyarginine with analogy to cytochrome P450 reductase enzymes.*

Type/source	Type isoenzyme	Cell type	Ca^{2+}-dependent	CaM-dependent	Constitutive	Cytosolic/particulate
Constit. Vascular Endothelial	eNOS	Platelets, Endothelium	Yes	Yes	Yes	Both
Constit. neuronal	nNOS	cerebellum, NANC nerves,	Yes	Yes	Yes	Cytosolic
Macrophage	iNOS	immune cell. VSM, endothelial, Kupfer,	No	No	No	Cytosolic
Liver	iNOS	hepatocytes	No	Yes/No	No	Cytosolic
Rabbit chondrocyte	iNOS		Yes	No	No	Cytosolic
Human chondrocyte	iNOS		No	No	No	Cytosolic

Table 1.1 *Isoforms of NO synthase (adapted from Knowles & Moncada, 1994). All isoforms use L-arginine as a substrate and are inhibited by LNMMA and LNIO (see section 1.6). CaM-dependent = calmodulin dependent.*

1.6 INHIBITORS OF NO SYNTHESIS

The study of NO synthesis in mammalian systems has been aided by the early identification of competitive inhibitors of NO synthase. Palmer and coworkers (1988a) discovered that N^{ω} monomethyl-L-arginine (L-NMMA) was a substrate-competitive inhibitor, and it has since been shown that it inhibits all NO synthases so far examined with a Ki of ~1mM. This inhibition is enantiomer-specific. In the vasculature, the diminished blood flow resulting from administration of L-NMMA shown by infusion into the human brachial artery, (Vallance *et al.*, 1989) confirmed the proposal that NO is the endogenous vasodilator responsible for maintaining a 'dilator tone' of vessels (Moncada *et al.*, 1991).

L-NMMA and asymmetric N^{ω}-N^{ω}-dimethyl-L-arginine (L-ADMA), another NO synthase inhibitor, are both naturally occuring compounds (Vallance *et al.*, 1992) that can be derived from methylated protein arginine residues during protein turnover.

However, symmetrical N^{ω}-N^{ω}-dimethylarginine, which is present in the same concentration as L-ADMA, is not an inhibitor of NO-synthase. Normally the concentrations of these compounds are low (1mM), but with patients suffering from renal failure L-NMMA and L-ADMA accumulate in sufficient quantities to inhibit NO synthesis (Vallance *et al.*, 1992). It is possible that under certain conditions these two inhibitors can be metabolised to L-citrulline and/or L-arginine in some cells and tissues (Hecker *et al.*, 1990) and so reduce their inhibitory effects, possibly leading to a paradoxical stimulation of NO synthesis.

A range of structurally related compounds has been synthesised (**Figure 1.2**) and found to inhibit NO synthase. Like L-NMMA these compounds are competitive inhibitors. However, there are some differences between them. L-NMMA is a non-selective inhibitor of the various NO synthase enzymes and its action is reversible unless incubation with the enzyme is prolonged (Olken *et al.*, 1991; Feldman *et al.*, 1993b). N^{ω}-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME) show progressive and irreversible, or only slowly reversible, inhibition of brain NO synthase, but weaker and reversible inhibition of macrophage NO synthase, following the initial binding (Dwyer *et al.*, 1991). Furthermore, amidine N^{ω}-iminoethyl-L-ornithine (L-NIO), L-canavanine and N^{ω}-amino-L-arginine show some selective behaviour by inhibiting the inducible macrophage NO synthase more potently than the constitutive brain and endothelial NO synthases (Knowles *et al.*, 1989; Stuehr et al 1992b; Rees *et al.*, 1990). Selective behaviour has also been found with other N^{ω} substituted arginine analogues which have been shown to be competitive inhibitors of macrophage NO synthase (Olken *et al.*, 1992). Furthermore, different types of inhibitor having no structural similarity to arginine have been developed, one of which named Ebselen is a nontoxic selenoorganic compound known to break a cysteine thiolate/ferric iron bond of some P-450 enzymes. It is a relatively selective inhibitor of the endothelial isoform of NO synthase (Zembowicz *et al.*, 1993).

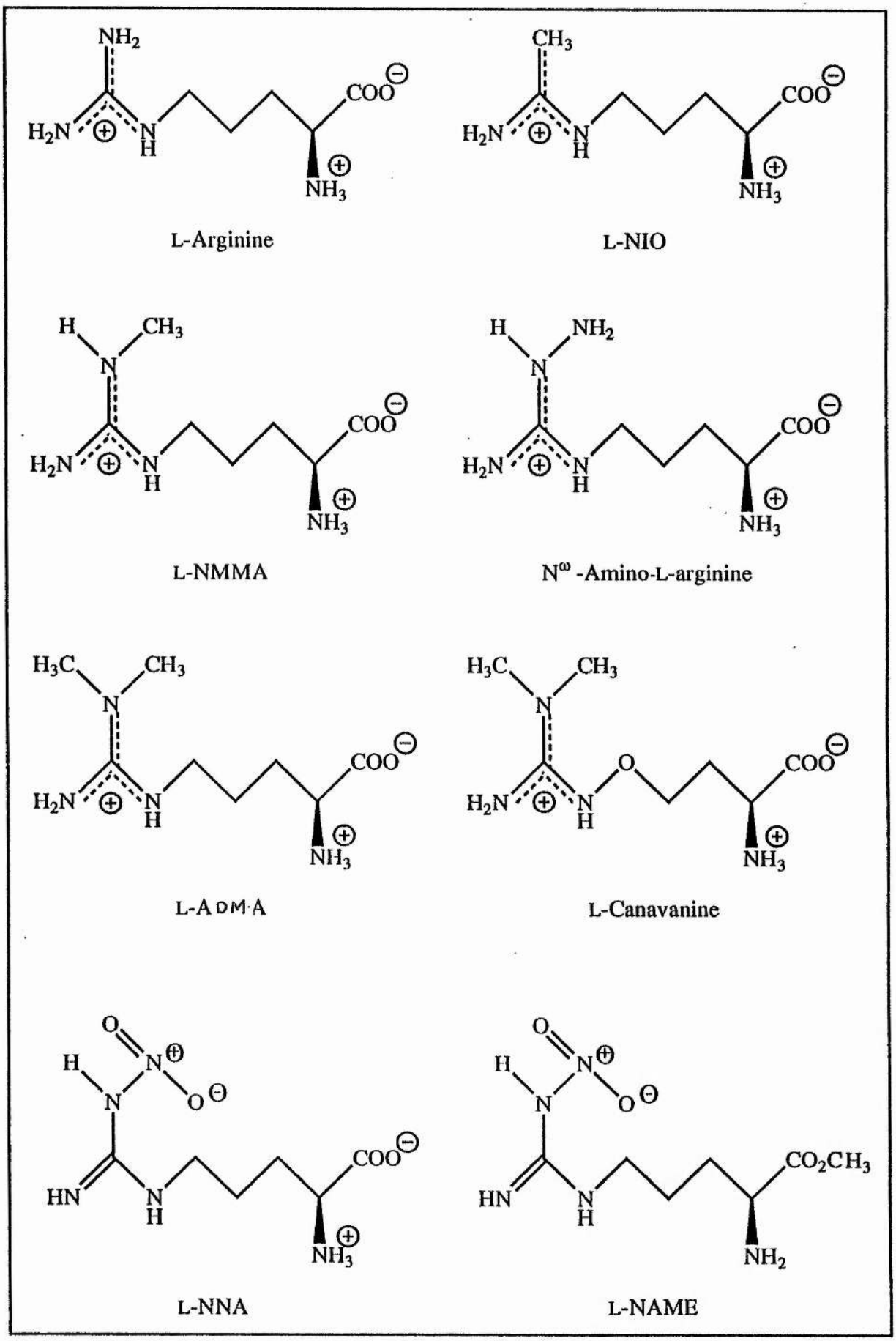


Figure 1.2 *The structure of arginine and of the arginine analogues most frequently used as inhibitors of NO synthases. (adapted from Knowles et al., 1994).*

The charge differences between the arginine analogue inhibitors also plays a part in the differing ways and strengths, in which they exert their effect. L-NNA and L-NAME are neutral at physiological pH (due to their low pK_a~0) and would not be expected to be taken up by the γ+ amino acid transport system, responsible for a high proportion of the cellular uptake of positively charged amino acids, such as L-arginine (White, 1985). L-NMMA, together with other positively charged inhibitors, competes with L-arginine for transport (Bogle *et al.*, 1992). Furthermore, both L-NMMA and L-NAME have been shown to be orally active in rats (Gardiner *et al.*, 1990).

There are ways in which NO synthases can be inhibited other than substrate-competitive inhibition. Calmodulin antagonists have been shown to inhibit some NO synthase isoenzymes and indirect inhibition of NO synthase by depletion of one of its cofactors BH_4, has proven to limit NO synthesis (Gross *et al.*, 1991). It has recently been demonstrated that NO synthase is subject to feedback inhibition by NO itself. Brain (constitutive) and macrophage (inducible) isoforms, were shown to be inhibited by NO from the enzyme itself, or NO donors, possibly by interaction with the haem of NO synthase (Assreney *et al.*, 1993; Rogers *et al.*, 1992). This claim is substantiated by work showing that CO and methylene blue which interact with haem, also inhibit NO synthase (Stuehr *et al.*, 1992a; White *et al.*, 1992).

1.7 STIMULATION OF GUANYLATE CYCLASE: THE ROLE OF PORPHYRINS AND THE REGULATORY EFFECTS OF THIOLS

Guanylate cyclase has been described in virtually all cell types examined. In most cells the enzyme exists as a polymorphic protein with both cytosolic (soluble) and membrane associated (particulate) forms existing in the same cell. 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 565nm which are shifted on exposure to either CO or NO (Gerzer *et al.*, 1981). This is consistent with binding of these species to the haem moiety of the enzyme.

A range of 'nitrovasodilators' (compounds containing NO in some form) including glycerol trinitrate (GTN), sodium nitroprusside, and amyl nitrite, have been shown to activate soluble guanylate cyclase (Katsuki *et al.*, 1977a). All of these compounds are potential sources of NO under the appropriate conditions (Katsuki *et al.*, 1977a). However haem-deficient guanylate cyclase demonstrated modest or little activation by these agents (Ignarro *et al.*, 1982a). The involvement of the haem group in stimulation of the enzyme was clarified by the inhibitory effect of adding ferro (Fe^{2+}) metallo-proteins (but not ferric (Fe^{3+}) 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 for NO of the Fe^{2+}metallo-proteins.

It was also 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; Craven *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 soluble 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 heme by maintaining the heme iron in the ferrous form (Katsuki *et al.*, 1977b; Craven *et al.*, 1978). Preformed nitrosyl-haemoglobin was found to be 10-fold more effective in activating guanylate cyclase 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 porphyrin complexes.

Thiols such as cysteine, glutathione, and dithiothreitol are capable of releasing NO from a range of nitrovasodilators, although intermediates in this 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 sulphydryl 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). Similarly, mixed disulphide formation on addition of cysteine or cystamine, also inhibited enzyme activity (Ignarro *et al.*, 1981b; 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 dithiothreitol which reforms the thiols (Brandwein *et al.*, 1981). It has been proposed that guanylate cyclase possesses multiple sulphydryl 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, sulphydryl 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).

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

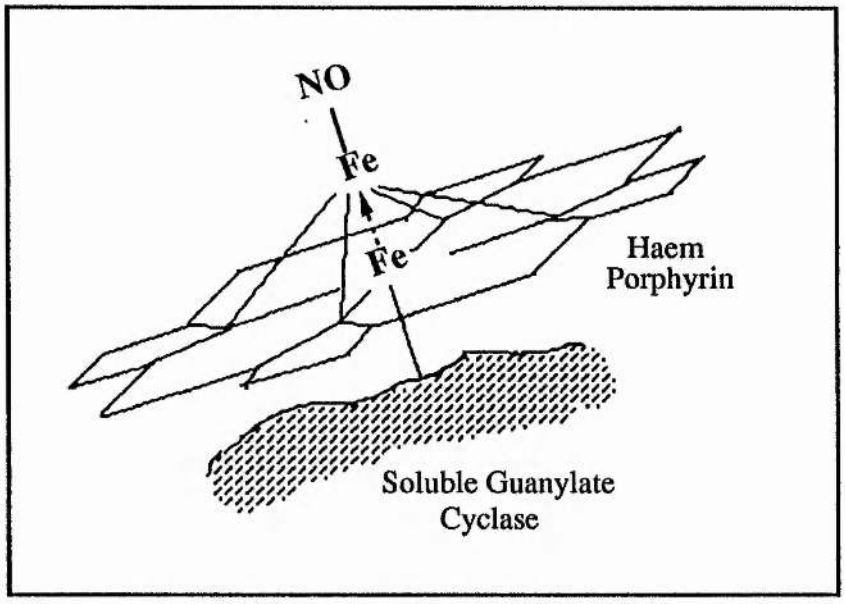


***Figure 1.3** A simplified diagram showing the out-of-plane movement of the porphyrin iron centre of guanylate cyclase when activated by NO.*

Conformational change in enzymes is widely believed to be a regulatory phenomenon in regulating its action as envisioned by D.Koshland Jr. 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 free protoporphyrin IX gave the same activation effect as NO-haem, and that free haem inhibits (not activates) the enzyme (Ignarro *et al.*, 1982b), Ignarro proposed that a haem structural change is caused by NO binding. He suggested that once bound the haem-NO complex elicits an out-of-plane movement of the centre 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.

1.8 THE PHYSIOLOGICAL PROCESSES UNDERLYING ENDOTHELIUM- DEPENDENT AND INDEPENDENT VASCULAR 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 nitrite, activated guanylate cyclase indirectly via NO, which they release 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 & Murad (1983a) and Furchgott & Jothianandan (1983) on the rat and rabbit aorta. Stimulation of the tissues with intact endotheliums 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 initiate its release (Palmer *et al.*, 1988b). This calcium appears to be released from intracellular stores as there is no evidence of calcium channels in endothelial 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 for the constitutive NO synthase for EDRF production (see **Table 1.1**), and leads to stimulation of guanylate cyclase in arterial smooth muscle in a similar way to nitrovasodilators.

Rapoport and co workers (1983) have shown that increases in cGMP in rat aorta due to this process are accompanied 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 hypothesised 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 1.4** summarises the endothelium-dependent and endothelium independent mechanisms of vascular smooth muscle relaxation which bring about vasodilation.

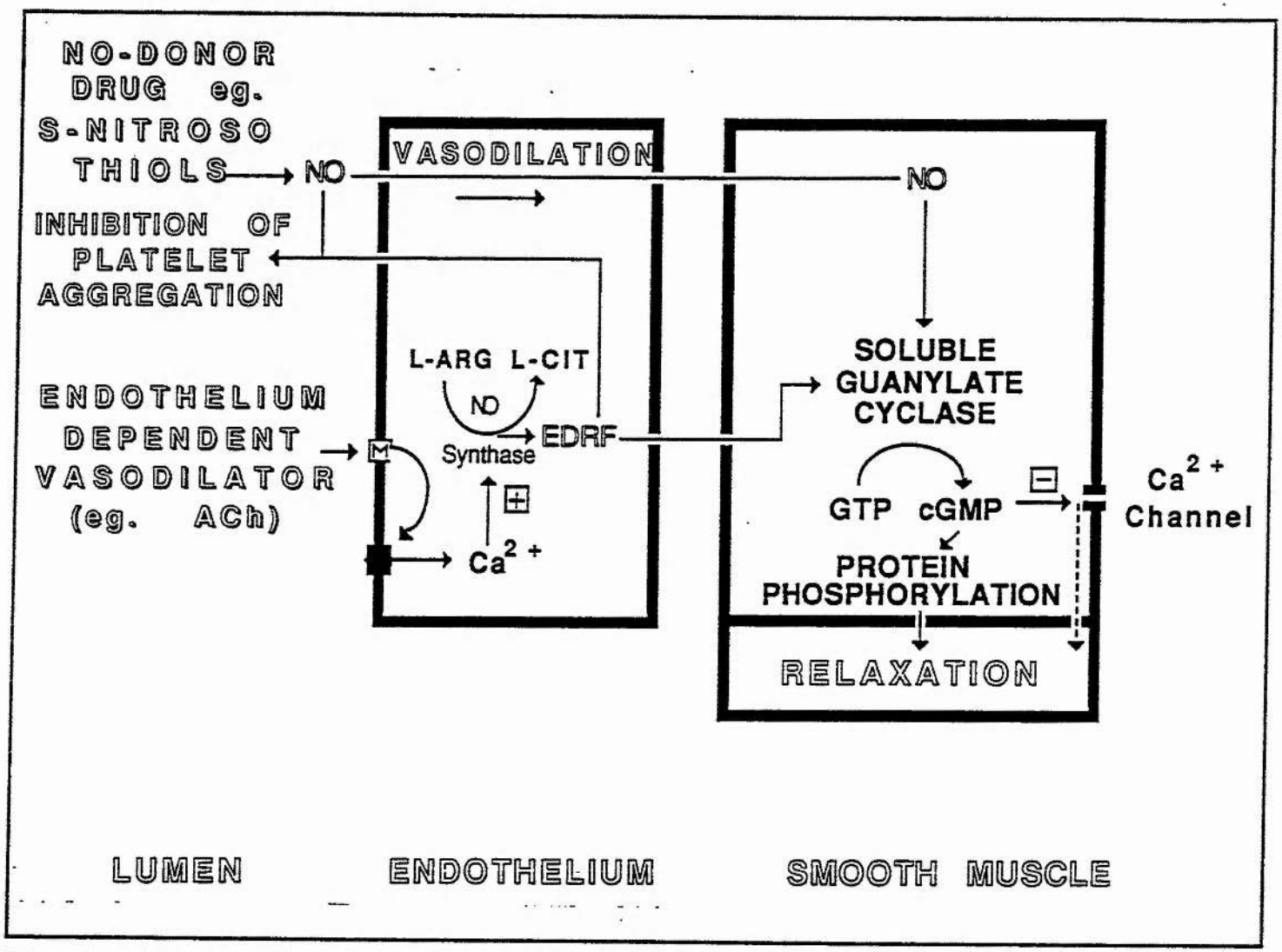


Figure 1.4 *The mechanisms underlying vascular smooth muscle relaxation from endothelium dependent and endothelium independent NO production & the effect of endothelial NO on blood platelets (see Chapter 1, Section 1.12)*

1.9 THE PROPERTIES OF NITRIC OXIDE AND ITS REDOX FORMS: THE NITROSONIUM ION (NO^+) AND THE NITROXIDE ION (NO^-)

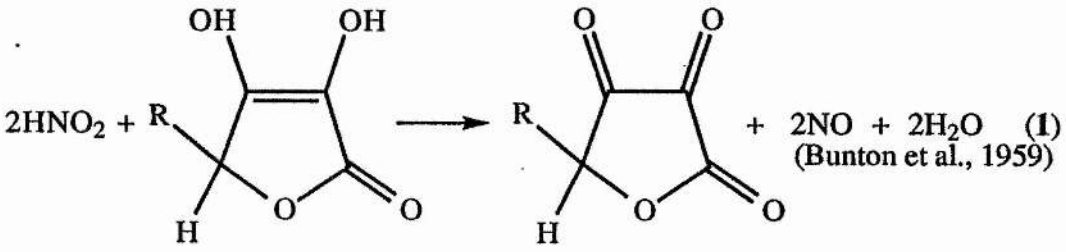
i) NO

NO is one of the 10 smallest molecules known. It is a colourless gas at room temperature with Bp. -151.8°C and Mp. -163.6°C. It is also colourless in the solid and liquid states. At 25°C and 1 atmosphere pressure it has a solubility of 1.8mM (Feelisch *et al.*, 1991a) which remains unchanged within the pH range 2-13. The small amount of NO required for its physiological action means that it is always soluble in aqueous solution and therefore it is the aqueous chemistry of NO that should be referred to.

NO is a relatively unreactive free radical and due to its odd electron it is paramagnetic. Its structure can be represented by a number of canonical forms shown below.

The general distribution of the odd electron together with a bond order which remains the same if NO were to dimerise, probably explain why dimerisation of NO does not occur (Butler & Williams, 1993).

NO can be prepared by a number of methods: the action of sodium nitrite or nitrous acid on ascorbic acid (vitamin C) (**1**) and the reduction of nitric acid using copper (**2**). On the industrial scale it is made by the catalytic oxidation of ammonia as an intermediate in the synthesis of nitric acid (**3**). The direct formation of nitric oxide from nitrogen and oxygen is thermodynamically unfavourable, but does occur at high temperatures in lightning discharges and in the internal combustion engine (**4**).



R=$CH_2OH.CH(OH)$-

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

$$4NH_3 + 5O_2 \xrightarrow{Pt\text{-}Rh} 4NO + 6H_2O \quad (3)$$

$$N_2 + O_2 \longrightarrow 2NO \qquad \Delta G^\circ = 173 kJmol^{-1} \quad (4)$$

NO is rapidly oxidised to nitrogen dioxide (NO_2) in the gas phase. This is a classic example of a third order reaction, second order in NO (**5**).

$$2NO + O_2 \longrightarrow 2NO_2 \qquad Rate = k_3[NO]^2[O_2] \quad (5)$$

In aqueous solution, the final product of this reaction is nitrite ion (NO_2^-) or nitrous acid (HNO_2) depending on the pH, *but no nitrate* is seen. The rate law is the same as in the gas phase, but in aqueous media it would seem that NO is oxidised to NO_2 which reacts with more NO to yield N_2O_3, the anhydride of nitrous acid (**6-8**).

$$NO + 1/2O_2 \longrightarrow NO_2 \quad (6)$$

$$NO_2 + NO \longrightarrow N_2O_3 \quad (7)$$

$$N_2O_3 + H_2O \longrightarrow 2HNO_2 \quad (8)$$

This process has a third order rate constant of ~5 x 10^6 $dm^6mol^{-2}s^{-1}$ (Wink *et al.*, 1993; Awad *et al.*, 1993) and holds when either O_2 or NO are in excess. Calculations

based on these results show that even in aqueous media saturated with oxygen, physiological concentrations of NO have long half lives. For example a 10nM solution of NO has a half life of approximately *3 hours* (Butler & Williams, 1993).

It seems suprising that NO_2^- is the sole product from NO in an aerobic aqueous solution given that its oxidation product NO_2 produces an equimolar mixture of NO_2^- and nitrate ion (NO_3^-) (**9-10**).

$$2NO_2 \rightleftharpoons N_2O_4 \qquad (9)$$

$$N_2O_4 + H_2O \longrightarrow NO_2^- + NO_3^- + 2H^+ \qquad (10)$$

The conclusion must be that NO_2 reacts much faster with NO than it does with H_2O, whereas in the gas phase the reverse is the case. Pulse radiolysis studies have shown this to be true and Feelisch *et al.* (1991a) stated that NO_2 hydrolysis is about 10 fold slower than reactions of NO with NO_2 and eventual production of NO_2^-.

ii) NO^+ AND NO^-

Recent papers have suggested that the redox forms of NO, the nitrosonium ion (NO^+; Lipton *et al.*, 1993) and the nitroxide ion (NO^-; Fukuto *et al.*, 1992a,b & 1993) have physiological roles which up until recently have been confused with that of NO itself. Nitrosothiols have been implicated as molecules which possess the ability to release NO or NO^+ depending on chemical environment (**11** & **12**) (Lipton *et al.*, 1993). They can produce NO photolytically (see **Chapter 4**), or by a metal ion catalysed process (see **Chapter 3**) and the NO group can be transferred between the nitrosothiol and other molecules (eg thiols) in the form of NO^+ (see **Chapter 5**).

$$RSNO \longrightarrow RS^{\cdot} + NO^{\cdot} \qquad (11)$$

$$RSNO + X \longrightarrow RS^- + XNO \quad X = \text{eg.}R'S^- \qquad (12)$$

However, at no time can NO^+ exist freely in an aqueous environment such as in the cell as there will be reaction at a diffusion controlled rate to give nitrous acid or the nitrite ion depending on the pH.

$$H_2O + NO^+ \longrightarrow HNO_2 + H^+ \quad \downarrow \quad H^+ + NO_2^- \qquad (13)$$

Work is also being undertaken to investigate whether the complicated physiological action of SNP, a relatively long serving vasodilator for surgical operations is mediated by the dual action of NO and NO^+ (Lipton*et al.*, 1993).

The possibility that the anion NO^- has a physiological role has been little explained. Fukuto *et al.* (1992a) have shown that under certain conditions nitroxyl (HNO) can be released from N-hydroxyguanidine compounds such as N^{ω} hydroxy-L-arginine an intermediate in the L-arginine to NO biosynthetic pathway. He also proposed that some, if not all, the physiological action of EDRF could be accounted for by nitroxyl (Fukuto *et al.*, 1992b). A pK_a of 4.7 means that at physiological pH, HNO is dissociated to NO^- and H^+ and at physiological concentrations the rapid bimolecular reaction to form nitrous oxide (N_2O) and water will be very slow (**14**). These factors make it feasible that NO^- could well have a physiological role. However, if this is the case, a biosynthetic mechanism for its formation must be found.

$$2HNO \longrightarrow H_2O + N_2O \qquad (14)$$

Interestingly, if the interaction of the redox forms of NO with their most important target, the haem moiety of guanylate cyclase (or other haem containing proteins), is studied, there are a number of differences. The redox state of the haem iron is important. At physiological pH ferric haem has a net unitary positive charge (+3 from Fe^{3+} and -2 from the pyrrole nitrogens) whereas ferrous haem is neutral (+2 from

Fe^{2+} and -2 from the pyrrole nitrogens). Therefore, ferric haem will tend to react more strongly with anionic ligands such as NO^- because of electrostatic interaction, whereas (neutral) NO will be preferentially bound by ferrous haem due to the increased orbital interactions of the extra d-orbital electron. In fact looking at the binding of the redox forms of NO to ferrous haem, it is likely that the ligand field strength follows the order $NO>NO^+>NO^-$, as the isoelectronic compounds CO (=NO^+) and O_2 (=NO^-) follow this order (**Figure 1.5**).

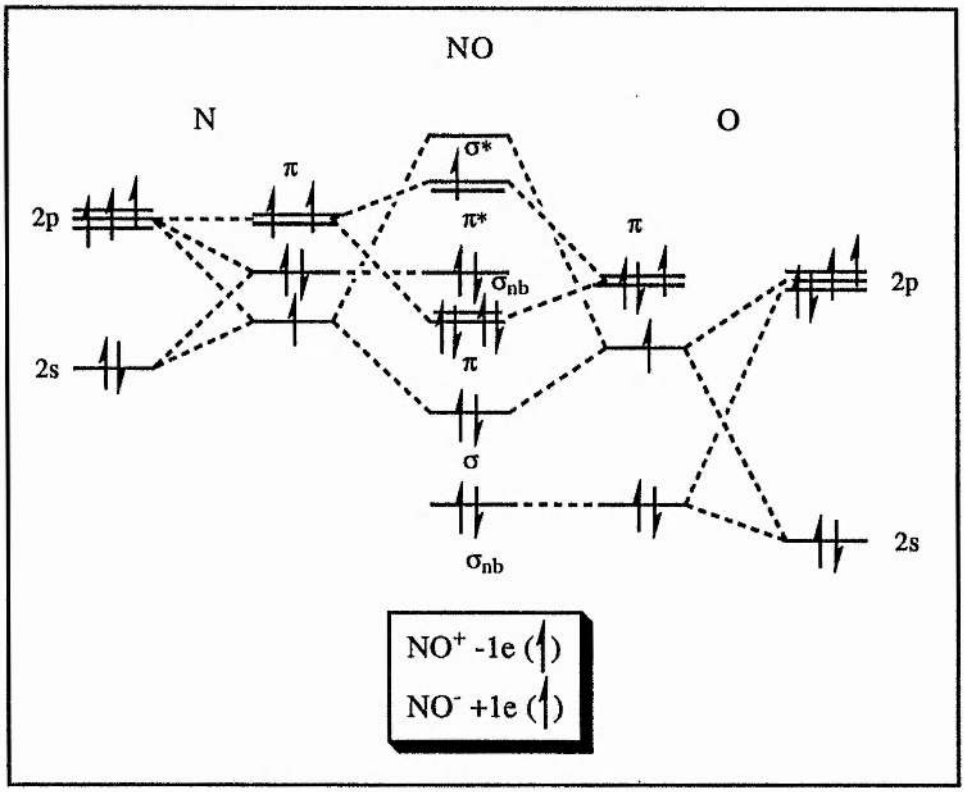


***Figure 1.5** An energy diagram of NO. Orbital hybridisation and energy promotion of the nitrogen atom relative to oxygen is shown. (Adapted from Huheey et al., 1983)*

O_2 binds differently to ferrous haem than CO, using a sp^2 hybridised orbital rather than an sp orbital for binding. This results in a bent Fe-O-O arrangement compared with the linear Fe-C-O. Therefore, the stronger bonding and the shorter bond length, coupled with the presence of 2 electrons in the antibonding orbitals of O_2 prohibiting backbonding from the iron, explain why CO binds to ferrous haem more strongly than O_2. Whether NO^+ and NO^- are analogous to CO and O_2 respectively in their binding

affinities for the ferrous haem remains to be determined. However, the protein environment of the rest of the enzyme must also be examined when looking at the relative ligand binding strengths of the redox forms of NO. Steric effects, solubility effects and other specific structural characteristics of the protein must be taken into account (Tsai, 1994).

1.10 NO: ITS TARGET MOLECULES AND INTRACELLULAR INTERACTIONS.

As previously mentioned in **section 1.7**, the principal target for NO action is its binding to the haem group of guanylate cyclase, the subsequent activation of the enzyme and production of the second messenger cGMP. There are, however, many other haemoproteins to which NO can be bound and thereby inactivate. Among the most important is its interaction with the O_2^- ligand of oxyhaemoglobin to produce the ferric form of haemoglobin, called methaemoglobin, and nitrate (Feelisch & Noack *et al.*, 1987). Since most cells are close to capillaries carrying erythrocytes loaded with Hb, NO cannot diffuse far without encountering oxyhaemoglobin. NO has a much lower affinity for methaemoglobin and other ferric (Fe3+) compounds than to ferrous (Fe2+) haemoglobin (see **section 1.9**). However, a nitrosyl haemoprotein adduct will form (metHbNO) which slowly converts to HbNO (Ignarro *et al.*, 1987, Chien, 1968).

Another aspect of NO metabolism *in vivo* is the formation of a variety of high and low molecular weight nitrosothiols. These species can be envisioned as arising via formal oxidation of NO to an NO^+ donor, followed by reaction with a thiol group. S-nitrosocysteine and S-nitrosoglutathione are among the biologically derived S-nitrosothiols that are postulated (Myers *et al.*, 1990), or known (Stamler et al., 1992b), to be carriers of NO in animals. More recently, extensive work by Stamler *et al.*, (1992a&b) has shown that protein thiols such as serum albumin, cathepsin B, and

tissue-type plasminogen activator (t-PA) react with NO to form very stable S-nitroso proteins. These S-nitrosoproteins have half-lives of over 24 hours in phosphate buffer and about 40 minutes in plasma. Stamler *et al.* (1992a) have shown that S-nitroso serum albumin is the major carrier of NO in blood plasma (~5.5μM) and has postulated that NO is stored by proteins such as this until required to aid vasodilator tone. His group has also shown that the S-nitroso protein can bring about vasodilation, as well as inhibition of platelet aggregation (Simon *et al,* 1993).

It has been proposed that NO may be rapidly bound and stabilised by low and high molecular weight iron-sulphur complexes (Vanin *et al.*, 1991). Dinitrosyl-iron (II)-L-cysteine has been shown to form a stable complex with protein thiols, which can act as an NO store in tissue and, by reaction with intracellular low molecular weight thiols, decompose and release low molecular weight dinitrosyl-iron (II)-dithiolate complexes into the extracellular space (Mulsch *et al.*, 1991). These compounds exhibit biological properties similar to EDRF. At the same time the interaction of NO produced by activated macrophages with iron-sulphur containing enzymes, such as aconitase and complex I &II, which are involved in the mitochondrial electron transport chain and the pathway for the synthesis of DNA, results in inhibition of the activity of these enzymes and resultant NO-mediated cytotoxicity (Hibbs *et al.*, 1990).

Superoxide (O_2^-) is an endogenous toxic species generated by several metabolic processes. It is implicated as a cause of acute cell injury in a variety of pathological conditions (Moncada *et al.*, 1991). Rubanyi and Vanhoutte (1986) have shown that exogenous addition of a superoxide generating system (eg pyrogallol or xanthine/xanthine oxidase) inactivated EDRF but in the presence of superoxide dismutase (SOD), a scavenger of O_2^- anions, the half-life of EDRF was substantially prolonged. Normal levels of oxygen radicals are controlled by enzymes such as SOD, minimising their cytotoxic effect. SOD converts superoxide into hydrogen peroxide (H_2O_2) and oxygen.

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (1)$$

Likewise, catalase and peroxidase control the levels of hydrogen peroxide and hence the formation of hydroxyl (HO·) an extremely reactive and potentially the most harmful radical, formed by the action of superoxide on H_2O_2 (Hogg *et al.*, 1992).

$$2H_2O_2 \xrightarrow[\text{catalase}]{\text{peroxidase/}} 2H_2O + O_2 \quad (2)$$

Despite the rapid activity of these enzymes, it is well documented that sufficient O_2^- exists in the intracellular environment to reduce NO stimulation of guanylate cyclase (Rubanyi & Vanhoutte., 1986; Gryglewski *et al.*, 1986b). Recent studies have suggested that NO reacts rapidly with superoxide anion to form peroxynitrite anion ($ONOO^-$) which, at physiological pH, is mainly in its protonated form, peroxynitrous acid (ONOOH; Beckman *et al.*, 1990).

$$NO + O_2^- \longrightarrow ONOO^- \quad (3)$$

$$ONOO^- + H^+ \longrightarrow ONOOH \quad (4)$$

Evidence has been presented to suggest that peroxynitrous acid can undergo homolytic cleavage to produce HO· and nitrogen dioxide (NO_2) (Beckman *et al.*, 1990; Hogg *et al.*, 1992), and it is thought that this reaction produces the toxic species (HO·) which may account for the pathological role evident when O_2^- is produced in reasonable concentrations. However, recombination of NO_2· and OH· to form nitrate (NO_3^-) and hydrogen ion (H^+) is likely. Theoretical and empirical calculations indicate that while hydroxyl radical formation is energetically allowed it is kinetically disfavoured. Thus the proposed production of HO· radicals from peroxynitrous acid may actually yield a species with an as yet undefined structure and reactivity approximately the same as hydroxyl radical (Koppenol & Beckman *et al.*, 1992).

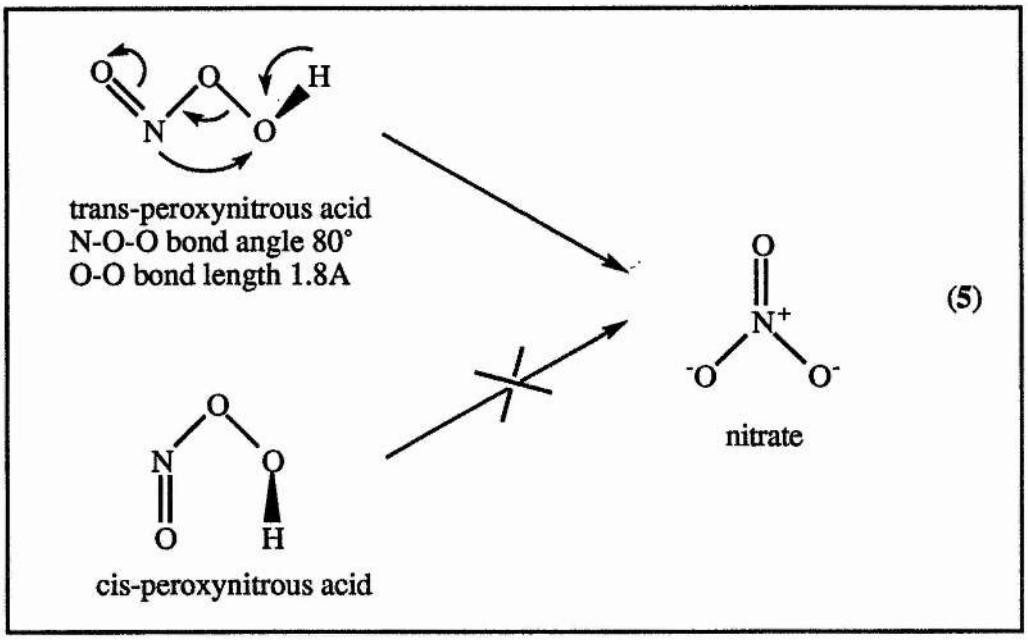
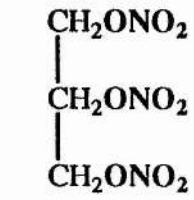


***Figure 1.6** Proposed formation of nitrate from peroxynitrous acid. (Adapted from Kerwin & Heller et al., 1994)*

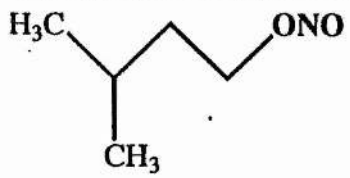
Another theory based on molecular mechanics calculations is that a potential transition state (*trans*-peroxynitrous acid) accounts for the oxidising power of peroxynitrous acid (Koppenol & Beckman *et al.*, 1992), suggesting that the decomposition of peroxynitrous acid is not a requirement for its oxidising behaviour. The observation that few hydroxyl radical-like products are trapped under optimal trapping conditions and that there is a high production of nitrate, can be rationalised from the *trans*-peroxynitrous acid theory which, due to its geometry, is theoretically better suited than the *cis*-isomer to undergo a concerted rearrangement to form nitrate (**Figure 1.6**).

1.11 NO: VASODILATION AND NO-DONOR DRUGS

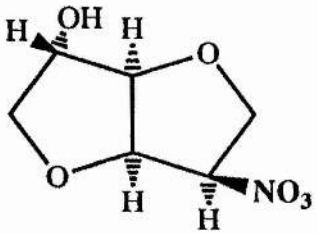
As described in **Section 1.8** NO release and dilator tone of arterioles can be enhanced by endothelium dependent vasodilators such as ACh and bradykinin. It has been recently discovered that nitrates and nitrites such as glycerol trinitrate (GTN) and amyl nitrite (see **Figure 1.7**), which have been therapeutic agents for the treatment of angina pectoris and congestive heart failure for over a hundred years (since 1867, after



Glyceryl Trinitrate
(GTN)

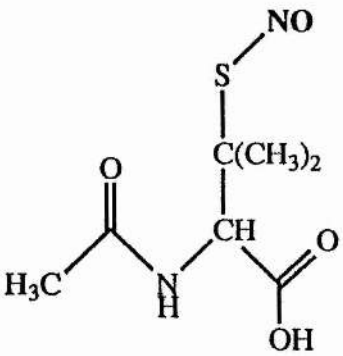


Amyl Nitrite
(AN)

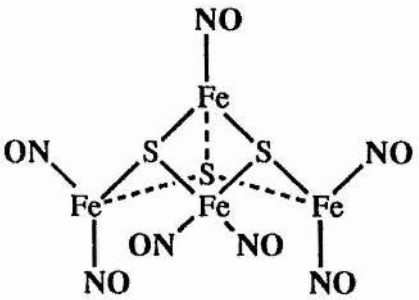


Isosorbide mononitrate
(ISMN)
(Isosorbide dinitrate metabolite)

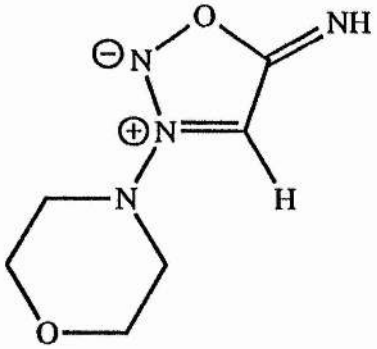
Sodium Nitroprusside
(SNP)



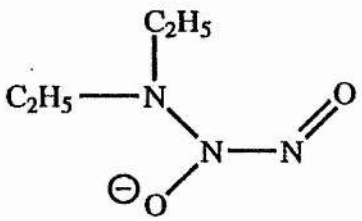
S-nitroso-N-acetyl penicillamine
(SNAP)



Roussins Black Salt
(RBS)



SIN-1
(Molsidomine metaboite)



Diethylamine/NO
(DEA/NO)
(NOnate)

Figure 1.7 *The different classes of NO donor drugs*

Sir Alfred Nobel made dynamite from GTN) liberate nitric oxide, which accounts for their biological action (Feelisch *et al.*, 1987). This exogenous production of NO, which is capable of stimulating guanylate cyclase in the same way as endogenously produced NO, implies that organic nitrates are endothelium independent vasodilators, and what are now termed as nitrovasodilators. Since this discovery the action of other long serving antihypertensive drugs such as SNP (see **Figure 1.7**) have been better understood. Moreover, this discovery has lead to research into other forms of NO-donor drugs such as S-nitrosothiols, the molsidomines, NOnates and iron-sulphur clusters which may have different or improved pharmacokinetic profiles and uses (see **Figure 1.7**).

There are many drugs with different mechanisms of action for treating hypertension and high blood pressure. Beta-blockers, calcium antagonists, diuretics and ACE (angiotensin converting enzyme) inhibitors being four of the most important. However, rapidly acting nitrates remain the 'gold standard' for treatment of all forms of angina pectoris. Relatively new nitrates such as isosorbide dinitrate and its active metabolite, isosorbide 5-mononitrate (see **Figure 1.7**)were developed in the 1940's and 50's and had slightly different and better pharmacokinetic profiles for the treatment of angina. The ease with which they can be delivered; chewable tablets, oral spray, sublingually or transmucosally, make them very attractive therapeutic agents. The major problem with nitrates has been the eventual, and often sustained, tolerance that these drugs induce (Needleman *et al.*, 1973), particularly when used frequently, in large doses or continuously. The discovery that they act by bioconversion to NO which activates guanylate cyclase, has given researchers an insight into how this family of drugs can be improved and the fundamental mechanisms which underlie nitrate tolerance.

It has been shown that nitrates undergo enzymatic (Chung & Fung, 1990; Feelisch & Kelm, 1991b; Kenkare & Benet, 1993) and nonenzymatic (Feelish & Noack, 1987)

decomposition to produce NO, which is responsible for their vasodilator and antiplatelet aggregating effects. Glutathione-S-transferase or cytochrome P_{450} reductases are thought to be responsible for enzymatic decomposition of nitrates in the liver and kidney and aorta (Kenkare & Benet, 1993), although they do not appear to be involved in the biotransformation of GTN to NO by smooth muscle cells or endothelial cells (Chung *et al.*, 1992; Gruetter *et al.*, 1985). The formation of two dinitrate metabolites (1,2- and 1,3- glyceryl dinitrates) has been found. This bioconversion takes place not only in the liver but also in VSM (Kawamoto *et al.*, 1987) and in human plasma (Fung *et al.*, 1986; Fung *et al.*,1989) and has been linked to decomposition of GTN by glutathione-S-transferases (Kenkare & Benet, 1993). The dinitrate metabolites elicit vasodilation and are acted upon to produce NO (Salvemisi & Anggard *et al.*, 1993).

Evidence has been presented to suggest that an enzyme-independent pathway for the production of NO from organic nitrates is elicited through a common intermediate which decomposes to release NO_2^- and NO (Feelisch & Noack, 1987). Thionitrates have been proposed as these intermediates and arise from transesterification between organic nitrate and thiol compounds by nucleophillic attack of a thiolate anion on the nitrogen atom of the ester group of the nitrate (Feelisch, 1991a).

Certain structural prerequisities of the thiol are thought to account for the finding that under physiological conditions only a limited number of sulphydryl containing compounds react with organic nitrates to form NO, whereas all thiols decompose organic nitrates to NO_2^-. Thiols such as cysteine are capable of carrying out this process (Feelisch & Noack, 1987; Feelisch, 1993). In contrast organic nitrites such as amyl nitrite react with all available thiol groups to form S-nitrosothiols which readily release NO.

Nitrate tolerance has been associated with a relative depletion or unavailability of thiol groups that are involved in the initial denitration of $RONO_2$. Sulphydryl groups (SH) are oxidised during this process. In addition, they are required for the production of S-nitrosothiols which are thought to be one of the end products of organic nitrate metabolism and necessary for guanylate cyclase activation. Many workers have shown that exogenous S-nitrosothiols can stimulate GTN tolerant vascular tissue both *ex vivo* and *in vivo* (Kowaluk *et al.*, 1987; Shaffer *et al.*, 1991; Bauer *et al.*, 1990; Horowitz *et al.*, 1983). It has also been shown that exposure of whole animals (rabbits and rats) to an infusion of S-nitroso-N-acetyl penicillamine (SNAP) does not bring about tolerance (Bauer *et al.*, 1990; Shaffer *et al.*, 1991).

Not all the literature is in agreement and many thiol donor studies have been unsuccessful in reducing tolerance (Gruetter *et al.*, 1986; Henry *et al.*, 1989; Keith *et al.*, 1981). Reasons for this are not clear but variable vascular tissue, sensitivity to thiols in different vascular beds and different animal species may explain these discrepancies.

The vasodilator action of sodium nitroprusside (SNP) is more difficult to understand as the only reaction leading to direct release of NO is photochemical decomposition (Wolfe & Swinehart, 1975). Experiments using a frogs heart showed that the action of SNP was greatly potentiated by exposure to laser light. (Flitney & Kennovin, 1987). It is known that after a multistep process NO is released from SNP on reaction with thiols (Butler *et al.*, 1988).

Recent evidence from our laboratory supports a proposed theory that S-nitrosothiols are intermediates in this process (Greig & Butler, unpublished results). Interestingly, evidence has been presented to suggest that SNP is metabolised to nitric oxide in vascular smooth muscle (VSM). This process was shown to be catalysed by a membrane-associated NO-generating activity which has a molecular weight of

approximately 4 to 11kDa and this activity could be enhanced by the addition of a reducing agent such as cysteine or NADPH (Kowaluk *et al.*, 1992).

A relatively new therapeutic agent for the treatment of coronary heart disease is N-ethoxycarbonyl-3-morpholino-sydnonimine, which prevents thrombus formation and possesses platelet anti-aggregating activity. It is metabolised in the liver to SIN-1 (3-morpholinosydnonimine) whereupon the 5-membered ring opens nonenzymatically at basic pH, forming SIN-1A (N-morpholino-N-nitrosoaminoacetonitrile), which is readily oxidised by oxygen to release NO (Kerwin & Heller, 1994; **Figure 1.8**).

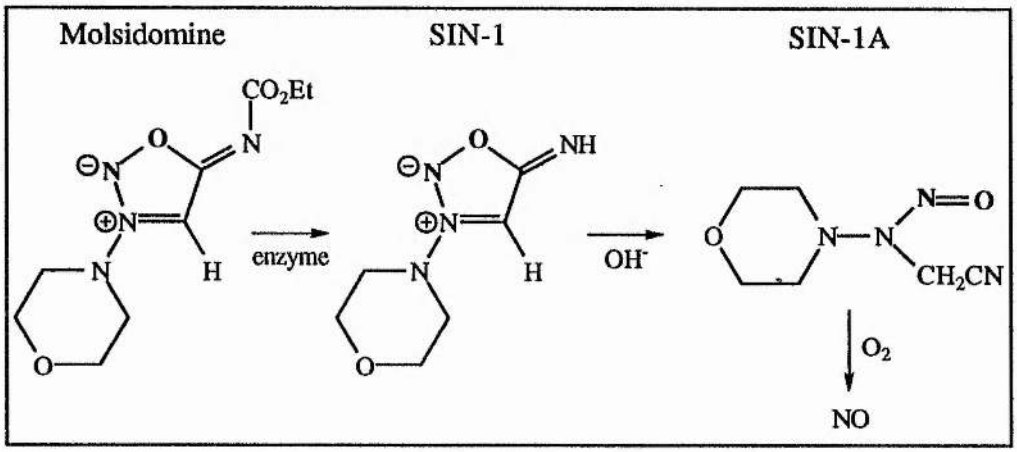


Figure 1.8 *The metabolic production of NO from molsidomine (adapted from Kerwin & Heller et al., 1994)*

A recently developed group of compounds, the NOnates are claimed to release predictable amounts of NO. These compounds are complexes of NO with nucleophiles of the general structure Nu-N(O^-)-N=O, where Nu is a nucleophile such as diethylamine or spermidine (Morley & Keefer *et al.*, 1991; see **Figure 1.9**).

According to Keefer, compounds based on this general structure can be readily tailored to specific medicinal applications as the rate which they release NO varies dramatically from compound to compound depending on the nucleophile present. NOnates spontaneously release NO under physiological conditions and therefore do not require

some sort of metabolic or electron transfer activation, which would limit their utility. However, this class of nitrovasodilator has been found to have a mutagenic effect on DNA (Wink & Keefer *et al.*, 1991).

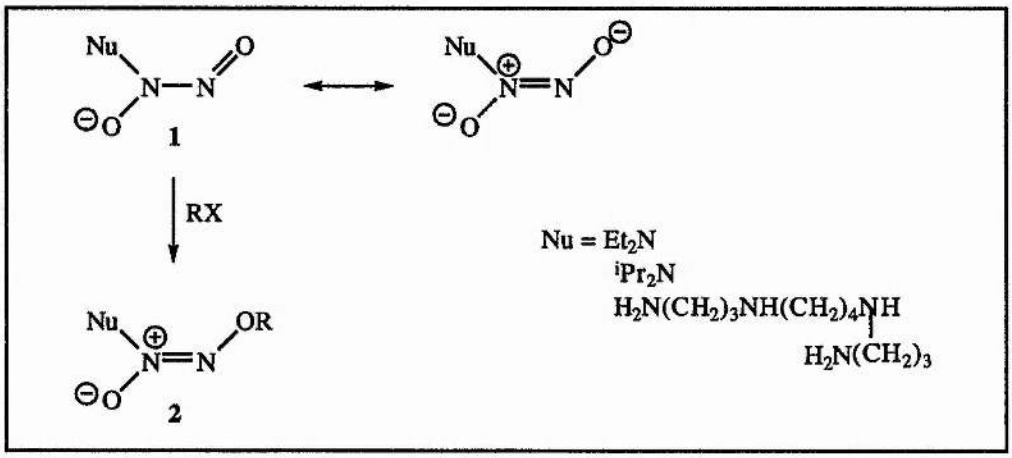


***Figure 1.9** The NOnates (1)which can be alkylated to form NOnate prodrugs (2) and which directly release NO under physiological conditions. (Adapted from Kerwin & Heller et al., 1994)*

As mentioned in **Section 1.10** iron-sulphur complexes are thought to be stores of NO in vascular tissue and have EDRF-like properties (Mulsch *et al.*, 1991). An iron sulphur cluster containing a number of NO ligands in each ion is the anion of Roussins Black Salt (RBS; $[Fe_4S_3(NO)_7]^-$ see **Figure 1.7**). This ion decomposes with release of NO by both chemical and photochemical routes and *ex vivo* experiments have shown it to be a highly reactive vasodilator. It has been shown to be taken up by endothelial cells due to its unusual solubility characteristics which although being ionic, make it more soluble in organic solvents than in water. The iron sulphur cluster nitrosyl remains in the endothelium for several hours giving a sustained release of NO which keeps the vascular tissue in a constant state of vasodilation (Flitney, Megson & Butler, 1992).

Another class of compounds with EDRF-like properties are S-nitrosothiols. These effective, fast acting vasodilators (see **Chapters 2 & 5**) release NO by a number of

chemical and physiological mechanisms, and certain S-nitrosothiols such as S-nitroso cysteine have a biological action very similar to that of NO itself. This is one reason why the controversy surrounding the chemical identity of the EDRF has been so difficult to resolve.(**Section 1.4**)

Biological thiols such as glutathione, cysteine and homocysteine comprise the majority of the mammalian fraction of sulphur that exists as free sulphydryl (Jocelyn, 1972) and react readily in the presence of NO_X under physiological conditions to form S-nitrosothiols (Ignarro *et al.*, 1981a&b; Ignarro *et al.*, 1989; Stamler *et al.*, 1992a,c; **Figure 1.10**). In addition, S-nitroso serum albumin has been found to be the most abundant S-nitrosoprotein in plasma (~5.5μM; Stamler *et al.*, 1992b). Non-endogenous S-nitrosothiols such as S-nitroso-N-acetyl penicillamine (SNAP; Field *et al.*, 1978) and S-nitrosocaptopril (SNoCap; Loscalzo *et al.*, 1989) have been synthesised as stable crystalline solids which also show marked biological activity. SNoCap acts by producing guanylate cyclase stimulated cGMP and by ACE inhibition (**Figure 1.10**).

The endogenous S-nitrosothiols have been implicated in a number of different biological roles. S-nitroso-N-acetyl cysteine and S-nitrosoglutathione have been proposed as the endogenous substance released by NANC (non-adrenergic, non-cholinergic) nerves in the mouse anococcygeus (Gibson *et al.*, 1992) and rat gastric fundus,respectively (Barbier & Lefebvre, 1994). Kerr *et al.* (1992) have evidence to suggest that the Inhibitory Factor (IF), an extract of the bovine retractor penis muscle which relaxes smooth muscle, is an S-nitrosothiol. Furthermore, since it has been realised that NO is a potent airway smooth muscle relaxant, it has been shown that S-nitrosothiols possess the ability to regulate airway tone by NO delivery which activates guanylate cyclase and that they are able to preserve the bioactivity of NO in the presence of high, ambient concentrations of oxygen and oxygen-derived free radicals (Jansen *et al.*, 1991). In 1985, Loscalso *et al.* showed that blood platelets incubated with GTN

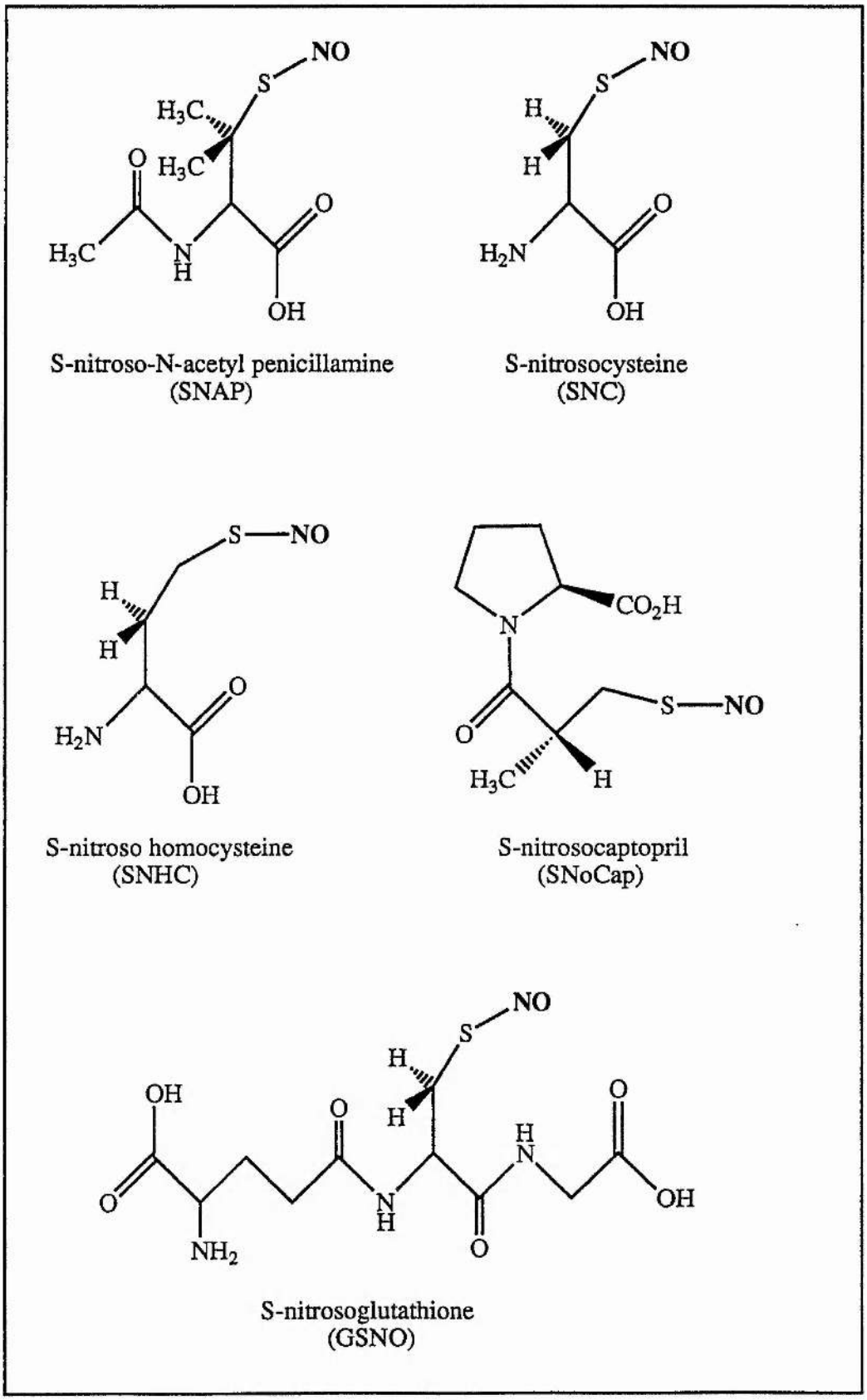


Figure 1.10 *Some synthetic and natually occuring S-nitrosothiols*

produced S-nitrosothiols, indicating that platelets are capable of producing S-nitrosothiols by some unknown mechanism, and it has been shown that S-nitrosothiols are potent inhibitors of platelet aggregation by many workers (see **Section 1.12**).

At present, analytical technology with sufficient sensitivity to detect the range of thiols, disulphides and S-nitrosothiols that are believed to circulate in plasma is still required. High Performance capillary Zone Electrophoresis has been developed (Stamler *et al.*, 1992c) and is capable of distinguishing between these three groups of compounds with rapidity and specificity (see **Chapters 3 & 5**). However, at the moment, the method lacks the sensitivity that is required for detection of S-nitrosothiols at levels present in humans. However, using a Gas Chromatography/High Performance Liquid Chromatography-photolysis-chemiluminesence system, Stamler and co-workers have been able to calculate the total concentrations of S-nitrosothiols, S-nitrosoproteins and free NO in plasma (Stamler *et al.*, 1992b).

The subject of this PhD thesis centres around understanding the mechanisms by which S-nitrosothiols release NO *in vitro* and *ex vivo*, giving an insight into how this class of NO-donor drug may be tailored to release NO at specific sites for specific purposes. Further background on S-nitrosothiols is presented in **Chapter 2.**

1.12 NO: PLATELET AGGREGATION AND NO-DONOR DRUGS

Blood platelets represent the first line of host defence when vessels are injured. Platelet adhesion to subendothelium (collagen fibrils, endothelial cell matrix and endothelium cell monolayers), aggregation and further platelet recruitment, culminate in 'haemostatic plug' formation which is accompanied by the consolidating effect of fibrin deposition on and between platelets.

Proximate endothelial cells possess three protective mechanisms (thromboregulators) for limiting the size of the haemostatic plug: Adenosine diphosphatease (ADPase), which removes secreted ADP and its prothrombotic effect; Eicosanoid metabolism, the major product of which is the prostaglandin PGI_2; and EDRF/NO (**Table 1.2**).

Type	Site of action	Mode of action	Inhibited by
PGI_2	Fluid phase autacoid	Elevation of platelet cAMP	Aspirin
EDRF/NO	Fluid phase autacoid	Elevation of platelet cGMP	Haemogobin (Hb)
ATPase --> AMP + Aden osine	Endothelial cell surface	Enzymatic removal of secreted ADP	ATPase inhibitors

***Table 1.2** Inhibitory thromboregulators associated with human endothelial cells (adapted from Marcus & Safier, 1993)*

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). Subsequently it was shown that EDRF itself was capable of inhibiting platelet aggregation *ex vivo* (Radomski *et al.*, 1987a) and *in vivo* (Hogan *et al.*, 1988). Radomski *et al.* showed that NO and prostacyclin, acting synergistically, had a superior antithrombotic effect (Radomski *et al.*, 1987b) and went on to show that both cAMP and cGMP regulated platelet aggregation (Radomski *et al.*, 1987c). However prostacyclin, which is a weak inhibitor of platelet adhesion, did not synergise with NO, suggesting that NO and consequently cGMP regulated platelet adhesion (Radomski *et al.*, 1987c,d).

In 1990, it became clear that platelets also generated NO and that the L-arginine/NO pathway responsible acts as a negative feedback mechanism to regulate platelet aggregation (Radomski *et al.*, 1990a). Aggregation induced by collagen caused an increase in intraplatelet levels of cGMP, which was inhibited by L-NMMA and enhanced by L-arginine (Radomski *et al.*, 1990b). The increase in cGMP led to

increased inhibition of platelet aggregation. L-arginine did not increase the basal levels of cGMP in unstimulated platelets, but the enhanced levels found in collagen stimulated platelets showed that this NO synthase could utilise exogenous substrate after it had been activated. These stimulated platelets possess 100-fold more intracellular Ca^{2+} than the resting platelet (Ware *et al.*, 1986) and it is likely that this increased level activates the NO synthase. The resulting increases in cGMP cause Ca^{2+} sequestration (Busse *et al.*, 1987) leaving less Ca^{2+} available for use in platelet aggregation. Therefore, as a thromboregulator, NO is produced by platelets from L-arginine and from vascular endothelium to inhibit platelet aggregation and adhesion to the cell wall of blood vessel. (**Figure 1.4**)

Endothelial dysfunction in a variety of clinical conditions such as hypercholesterolemia, atherosclerosis, hypertension and heart failure, increases the tendency for platelet aggregates to form and makes these patients susceptible to thrombotic events. Thus the potential clinical role of compounds capable of providing controlled therapeutic concentrations of NO could be great. However, the effect of nitrovasodilators on platelet aggregation has been poorly understood. It would seem that nitrovasodilators which release NO readily such as SNP, SIN-1, S-nitrosothiols and NOnates, are good therapeutic agents for the inhibition of platelet aggregation (Radomski *et al.*, 1992; Diodati *et al.*, 1993). Radomski *et al* (1992) have shown S-nitrosoglutathione to be a more potent inhibitor than NO itself. Furthermore, S-nitroso-N-acetylpenicillamine and other synthetic S-nitrosothiols have also been shown to be substantially more potent than all other classes of NO-donor drugs known to date (Salas, Askew & Radomski *et al.*, in press). Diodati *et al* (1993) has shown that NOnates (**Section 1.11**) can be synthesised with quite different pharmacokinetic profiles for preventing aggregation, which correlate with the rates at which they release NO. GTN, on the other hand, is almost inactive, suggesting that platelets lack the mechanisms required for the uptake and/or conversion of organic nitrates to NO (Gerzer *et al.*, 1988).

Recent work by Stamler *et al.* (1993) has shown that S-nitroso proteins such as S-nitroso serum albumin are potent inhibitors of platelet aggregation *in vitro*, *ex vivo* and *in vivo*. They are readily formed under physiological conditions (Stamler *et al.*, 1992a), and release NO at the platelet surface possibly to sulphydryl moieties (Stamler *et al* ., 1993). Furthermore, S-nitrosylated proteins can transfer NO to low molecular weight thiols to form S-nitrosothiols that can then enter the platelet or facilitate NO transfer at the platelet surface to inhibit aggregation (Stamler *et al.*, 1993). These data suggest that S-nitrosoproteins may serve as intermediates in the cellular metabolism of NO and support the view that S-nitrosothiols may serve a carrier role for NO as intermediates in the mechanism of organic nitrate and EDRF action.

1.13 OTHER PHYSIOLOGICAL FUNCTIONS OF NO

THE NERVOUS SYSTEM

a) ***Central Nervous System (CNS)***

Chemical messengers known as neurotransmitters allow messages to be passed between different nerve cells across synaptic contacts. When stimulation of presynaptic neurones *increases* the activity in a postsynaptic nerve cell it is known as an excitatory synapse. The amino acids glutamate and aspartate are two important excitatory neurotransmitters. In 1987, glutamate was shown to elevate cGMP levels in the postsynaptic nerve and give rise to an unstable intracellular factor (Garthwaite *et al.*, 1987). This factor was rapidly identified as having properties identical to EDRF or NO (Garthwaite *et al.*, 1988). In the following 2 years an isoform of NO synthase, dependent on Ca^{2+}/calmodulin and similar to that isolated from endothelium and platelets, was described, purified and cloned (Knowles *et al.*, 1989; Bredt *et al.*, 1990).

It appears that NO in the brain can act in at least two ways. Firstly it is formed in the postsynaptic nerve cell following activation by an amino acid neurotransmitter.

Stimulation of N-methyl-D-aspartate (NMDA) receptors causes the production of NO which diffuses out of the postsynaptic cell, on which it has no effect (possibly because high intracellular Ca^{2+} serves to inhibit guanylate cyclase activity), and acts upon one or more neighbouring structures, including the presynaptic nerve cell. For this reason NO has been proposed as a *retrograde* messenger, perhaps involved in the mechanism of long term memory (Gally *et al.*, 1990). However, there are a number of candidates for this role and support for NO as a retrograde messenger is speculative. Secondly, NO is found presynaptically in the climbing fibres of the cerebellum. The apparent effect of NO generated in these fibres is the long term depression of responses in Purkinjie fibres, since L-NMMA blocks the depression (Shibuki &Okada., 1991). It also seems possible that NO plays some part in the cerebral blood supply and it has been suggested that the over-production of NO maybe linked with certain degenerative conditions like senile dementia because of its radical nature and ability to generate hydroxyl radicals (Butler & Williams *et al.*, 1993).

2) *The peripheral nervous system (PNS)*

NO synthase has been discovered in a wide variety of tissues within the PNS. NO or a related compound such as an S-nitrosothiol (Gibson *et al.*, 1992; Barbier & Lefebvre, 1994), is now commonly termed a 'non-adrenergic, non-cholinergic' (NANC) neurotransmitter (Bult *et al.*, 1990). It is produced by the vagal nerves supplying gastrointestinal smooth muscle and in the corpus cavernosum of the human penis (Rajfer *et al.*, 1992). Here, NANC nerves are responsible for relaxing the muscle and allowing blood influx which results in the penile erection. There is evidence that the arginine to NO pathway is directly involved in this process (Rajfer *et al.*, 1992). NO-sensitive NANC nerves have been found in several other tissues including the cardiovasculature where they have a role in vasodilation and in the respiratory system. In all cases of NANC neurotransmission, competitive inhibitors of NO synthase block nerve induced relaxations.

THE PHYSIOLOGICAL & PATHOPHYSIOLOGICAL ROLE OF INDUCIBLE NO SYNTHASE (iNOS)

1) *Macrophages*

The immune response is the body's reaction to the destruction or neutralisation of 'foreign matter'. This can be done non-specifically throughout the body by cells known as macrophages which non-selectively protect the body against 'foreign substances' or cells. To do this substances known as cytokines stimulate the macrophage, resulting in the engulfment of the 'foreign matter' (phagocytosis), its subsequent neutralisation by cytotoxic substances (NO) injected by the macrophage and its removal.

The correlation between elevated urinary NO_2^- and NO_3^- levels and increased immuno-stimulation induced in lipopolysaccaride (LPS)-treated mice, suggested that macrophages were the most likely source of NO_2^- and NO_3^- (Stuehr & Marletta, 1985). At about the same time, the cytotoxic capability of activated macrophages against tumour target cells was shown to be dependent on the presence of L-arginine and accompanied by the production of L-citrulline and NO_2^- (Hibbs *et al.*, 1987). It was subsequently demonstrated that NO synthesised from L-arginine was indeed the precursor of the NO_2^- and NO_3^- in these cells (Marletta *et al.*, 1988).

The isoform of NO synthase in the macrophage was only found in the activated cells (activated by LPS or LPS and interferon-g (IFN-g); Stuehr & Marletta, 1985) and it required protein synthesis for its expression, which meant a time delay of up to 8 hours before NO_2^- and NO_3^- could be detected (Marletta *et al.*, 1988). This enzyme was subsequently found to be NADPH dependent, inhibited by L-arginine analogues, Ca^{2+}/calmodulin independent and possess long lasting release of nanomolar quantities of NO (**Section 1.5**).

As yet the mechanism by which NO produced by macrophage iNOS is cytotoxic is not fully understood. Three mechanisms have been proposed. Firstly, the NO radical is known to be destructive towards cell membranes and for this reason alone NO is cytotoxic. However, Beckman *et al.* (1990) has proposed that the reaction of NO with superoxide to form peroxynitrite, which has the potential to produce the extremely destructive hydroxyl radical, accounts for the cytotoxicity of NO (**Section 1.10**). Lastly, NO has been shown to react with enzyme iron sulphur centres necessary for metabolic activity to form iron-sulphur cluster nitrosyls (**Section 1.10**). This can be analysed by EPR (Butler *et al.*, 1985).

Whatever the mechanism by which NO produces its cytotoxic action, the large amounts of NO produced by the inducible enzyme (iNOS) can result in a dramatic reduction in blood pressure this can lead to hypotension when it is induced in the vascular endothelium due to infection. This condition is known as septic (or endotoxic) shock and can be fatal. The vascular relaxation caused by the over-production of NO and the resulting hypotension, is partly due to patient's unresponsiveness to conventional vasoconstrictors, such as dopamine and noradrenaline. Inhibition of both the inducible and constitutive enzymes by NO synthase inhibitors such as L-NMMA have been shown to restore the normal blood pressure and in some cases save lives (Petros *et al.*, 1991). However, the non-selective behaviour of this drug can be deleterious and requires the co-infusion of an NO donor drug such as SNAP to stop potentiation of the effects of septic shock in animal models. The NO donor preserves perfusion and replaces the vasodilator tone of the arteries (Nava, Palmer & Moncada, 1992; Wright, Rees & Moncada, 1992). For more selective behaviour glucocorticoids such as dexamethasone have the ability to inhibit the expression but not the activity of the inducible NO synthase. This gives them limited therapeutic usefulness in treating endotoxic shock (Wright, Rees, Moncada, 1992). In the future, selective inhibitors of the inducible enzyme will be more suitable for treating this and other conditions in which a similar profile of NO release occurs.

2) *Neutrophils*

Another part of the immune system is the collection of cells in blood known as leukocytes or white blood cells. Neutrophils are one type of leukocytes which have been shown to produce a substance that inhibits platelet aggregation (Salvemini *et al.*, 1989). Subsequently, it has been shown that neutrophils possess an inducible form of NO synthase (McCall *et al.*, 1991).

3) *NO and Immunological Responses*

Besides the role of NO in immune defence, it may also be involved with inflammatory and auto immune-mediated tissue destruction. Since NO, in addition to damaging DNA (causing mutagenisis; Wink *et al.*, 1991), is known to be a major cytotoxic effector in defence against a number of foreign bodies, bacteria and tumour cells, it is plausible that inappropriate or non-specific host cell attack may occur which may lead to inflammatory and auto immune response.

NO 'STORES' IN THE VASCULAR SMOOTH MUSCLE

Recently work by Megson et al., (1994) and Venturini, Palmer and Moncada (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 *et al.*, 1994). The repriming of the store is prevented by L-NMMA or haemoglobin and accelerated by NO donor drugs. Venturini *et al.* proposed that the 'store' is a nitrosothiol, a nitrosoprotein or an iron-sulphur nitrosyl complex. In addition, work by Megson *et al.* provides evidence that the photosensitive 'store' is a nitrosothiol or nitrosoprotein, since it is known that as ethacrynic acid a thiol alkylating agent, also prevents repriming of the 'store'.

NO IN EXHALED AIR OF HUMANS

Endogenous NO has been found to be produced by various cells within the lower respiratory tract including inflammatory and epithelial cells and is detectable in the exhaled air of normal human subjects. Using a chemiluminescence analyser to detect NO, Kharitonov *et al.* (1994) and Persson *et al.* (1994) have shown that asthma sufferers produce a greater concentration of NO in exhaled air than controls. The peak concentration of NO could be significantly reduced by L-NMMA and steroids. These observations may reflect induction of NO synthase which is known to be inhibited by steroids. Furthermore, Gaston *et al.* (1993) have shown that S-nitrosothiols can relax human bronchial smooth muscle. They suggest that these compounds preserve the bioactivity of NO in the airway and could have a potential role in the treatment of human bronchospasm.

1.14 THERAPEUTIC MANIPULATION OF THE NITRIC OXIDE PATHWAY

1) *NO donor drugs*

As mentioned in Section 1.11, nitrovasodilators based on nitrates are the mainstay of therapy in angina, and, to a lesser extent, congestive heart failure. S-Nitrosothiols have been discussed in relation to their potential as potent vasodilators and inhibitors of platelet aggregation and the newer group of nitrovasodilators, which includes the sydnonimines, is likely to expand. SIN-1 has been tested in patients suffering from erectile dysfunction and was shown to be dose dependent in its production of an erectile response offering an attractive alternative to current auto injection therapies (Kerwin & Heller, 1994). Very recently organic nitrates like GTN have been shown to ease uterine contractions of pregnant women and prevent premature birth.

The future development of nitrovasodilators and NO donor drugs lies in specific targeting of the site of metabolism in the vasculature or elsewhere, possibly producing a different effect or a greater potency of the drug. Reducing the development of nitrate tolerance by incorporating sulphydryl groups in its molecular structure could be a way to improve the pharmacokinetic profiles of organic nitrates.

2) *Agents which stimulate NO synthase*

Stimulii to NO release are diverse, from small molecules such as ACh to large peptides. Consequently, development of a drug that would increase endogenous production should be possible. This may have occurred already, by default, as the development of ACE inhibitors increase tissue concentrations of endogenous bradykinin, which is a potent stimulant of constitutive NO synthase.

3) *Inhaled NO gas*

As mentioned in the previous section, endogenous NO is a vasodilator of the pulmonary vasculature and inhibition of NO synthesis enhances pulmonary hypoxic vasoconstriction. Although NO has the advantage of a short half-life, and is therefore less susceptible than other pulmonary vasodilators to systemic hypotension, it can provoke respiratory distress syndrome if the tissue is over-exposed. For this reason, the therapeutic 'window' for inhaled NO is narrow and therapy requires caution. It could however be used to counteract the side effects of pulmonary hypertension caused by the use of non-selective NO synthase inhibitors in the treatment of septic shock.

4) *L-Arginine administration*

The use of exogenous L-arginine as a substrate for NO synthase has proved successful in treating patients with hypercholesterolaemia. It is thought that the added L-arginine may compete with endogenous arginine analogue inhibitors, such as L-ADMA (**Section 1.6**), rather than by replenishing low levels of L-arginine in plasma as concentrations of this amino acid are high anyway (10^{-4}M).

5) *NO synthase inhibitors*

The consequences of preventing NO synthesis are great, reflecting the diversity of NO as an intracellular signal. Consequently research into the synthesis of NO synthase inhibitors has been one of the more active fields since the L-arginine to NO pathway was discovered. For reasons mentioned in **Section 1.13 and 1.6** the search for selective inhibitors of the inducible isoforms of the enzyme is paramount and necessary for treatment of symptoms like septic shock (**Section 1.13**). Investigation into targeting the many cofactors of the NO synthases is another avenue of research being undertaken (**Section 1.6**).

6) *Steroids*

Glucocorticoids have been found to inhibit the induction of iNOS in both vascular and cardiac tissues without effecting the constitutive enzyme. The extent of this effect on the anti-inflammatory action of steroids, together with a number of other non specific functions this class of compounds posseses, is not yet known. However, glucocorticoids exert their beneficial effects only when given as pretreatment before enzyme induction is established, which obviously limits their therapeutic usefulness.

7) *Superoxide dismutase (SOD) therapy*

The use of SOD to remove superoxide anions and potentiate the lifetime and consequently the action of NO is another way in which the vasodilator tone arteries and other blood vessels could be improved. In other cases however, the use of SOD may exacerbate the effects of large amounts of NO produced by iNOS, enhancing the cytotoxic effects.

Conclusions

Manipulating the pathway in the right direction at the right time is the key to the future therapeutic management of the NO pathway, but the importance of NO biology to

therapeutics is such that many new approaches to treating patients are likely to come to light in the next decade.

CHAPTER 2

S-NITROSOTHIOLS; A NOVEL CLASS OF NO-DONOR DRUGS

2.1 INTRODUCTION

S-nitrosothiols or thionitrites were initially discovered as intermediates in the oxidation of thiols to disulphides. The fact that S-nitrosothiols are less stable than the corresponding oxygen analogues, such as alkyl nitrites, made them difficult to isolate and they are consequently less well investigated. Their chemical behaviour is very different from the oxygen analogues due to the inherent weakness of the sulphur-nitrogen bond compared with the oxygen-nitrogen bond of nitrites. This makes thionitrites more reactive and useful in organic synthesis as powerful nitrosating agents.

2.1.1 *Nitrosation of thiols*

S-nitrosation of thiols occurs more readily than O-nitrosation due to the greater nucleophilicity of the sulphur atom. Evidence for the existence of S-nitrosothiols was reported by Tasker and Jones as early as 1909, when a transient red coloured solution (due to the presence of phenyl thionitrite) was seen when benzene thiol was treated with nitrosyl chloride. The colour rapidly disappeared due to the formation of diphenyl disulfide and NO (**1**).

$$PhSH + NOCl \longrightarrow [PhSNO] \longrightarrow PhSSPh + NO \qquad (1)$$

A number of S-nitrosothiols have since been prepared by treatment of thiols with nitrosating agents, such as acidified nitrite or nitrous acid, alkyl nitrites, dinitrogen tetroxide (N_2O_4), dinitrogen trioxide (N_2O_3) and nitrogen dioxide (NO_2) (for review see Oae & Shinhama, 1983). Until recently it was believed that the best yields of S-nitrosothiols were obtained by reacting the thiol with dinitrogen tetroxide N_2O_4 in equimolar amounts at -10°C, using an inert organic solvent eg hexane, acetonitrile etc (Oae *et al.*, 1978). However, Doyle *et al.* (1983) have shown that S-nitrosothiols can be conveniently synthesised using an alkyl nitrite, such as tertiary butyl nitrite, as the

nitrosating agent. The reaction is carried out in an inert organic solvent and can produce quantitative yields of S-nitroso product of tertiary butyl thiol and benzyl thiol.

Nucleophilic catalysis of amine nitrosation and nitrosation of other species is well known, and thiols can undergo the same catalysis by halide ions and thiocyanate ion (Morris & Williams, 1988) (**2**).

$$HNO_2 + H^+ + X^- \rightleftharpoons NOX + H_2O \qquad (2)$$

The extent of catalysis is principally governed by the size of the equilibrium constant for NOX formation and the well-established trend of reactivity ONCl>ONBr>ONSCN is found for the nucleophilic catalysis of thiol S-nitrosation.

The most stable thionitrites appear to be those with bulky substituents eg. t-butyl nitrosothiol, triphenyl methyl nitrosothiol and the stable solid, S-nitroso-N-acetyl-DL-penicillamine (SNAP), isolated and characterised by Field *et al.* (1978). Recent work by Moynihan & Roberts (1994) has determined the optimum conditions for SNAP preparation using a variation of the acidified nitrite nitrosation of acetyl penicillamine used by Field. They have also developed syntheseses for bis-thio-nitrosopenicillamine dipeptides which have comparable biological activity to GTN.

S-nitrosation of thiols is a rapid process and requires a fast technique such as stopped-flow spectrometry to enable rate constants to be measured. The rate of formation of nitrosothiols follows the rate law shown below (**3**) and applies to the nitrosation of a wide variety of substrates, including primary and secondary amines, alcohols etc, under certain experimental conditions (Williams, 1985).

$$\text{Rate} = k\,[\text{Thiol}]\,[H^+]\,[HNO_2] \qquad (3)$$

The nitrosation process can be generally interpreted in terms of a mechanism involving rate-limited attack by a positively charged species $H_2NO_2^+$ or NO^+. The high reactivity of thiols towards nitrosating agents makes them good scavengers of agents such as nitrous acid and they have been used to suppress nitrosamine formation by competing with amines for such compounds (Williams *et al.*, 1982).

2.1.2 *Reactions of S-nitrosothiols*

i) Thermal decomposition

S-Nitrosothiols readily thermally decompose to form the relevant disulphide and NO (**4**).

$$2RSNO \longrightarrow RSSR + 2NO \qquad (4)$$

Most S-nitrosothiols are unstable at room temperature although others, such as tertiary S-nitrosothiols require heating to produce the corresponding disulphide (Field *et al.*, 1978).

ii) Photochemical decomposition

S-Nitrosothiols have also been shown to decompose upon photolysis to give the corresponding disulphide and NO. Barrett *et al.* (1966) reported that the absorption of 3650A°-radiation by S-nitrosotoluene resulted in excitation to the extent of 79Kcal/mol, which was sufficient to cause fission of the S-N bond. The reaction can be formulated as follows (**5**).

$$\begin{aligned} PhCH_2SNO &\longrightarrow PHCH_2SNO^* \longrightarrow PhCH_2S^{\cdot} + NO^{\cdot} \\ PhCH_2S^{\cdot} + PhCH_2SNO &\longrightarrow PhCH_2SSCH_2Ph + NO^{\cdot} \end{aligned} \qquad (5)$$

iii) Oxidation

Oxidation of S-nitroso-tertiary butyl thiol (t-butylSNO) with fuming nitric acid forms t-butyl thionitrate (t-butylSNO_2) and the same product can be formed quantitatively by

reacting tertiary-butyl thiol (t-butylSH) with excess N_2O_4 (Oae & Shinhama, 1983). Subsequent quenching of the reaction with tertiary butanol (t-butOH) leads to the formation of the corresponding thiol sulphonate in good yields (RSO_2SR). Some S-nitrosothiols are also susceptible to air oxidation. With S-nitrosoethane thiol, the corresponding disulphide, diethyl disulphide, and N_2O_4 are obtained after about 3 hours of exposure to air (Lecher *et al.*, 1926) (**6**).

$$2EtSNO + O_2 \longrightarrow EtSSEt + N_2O_4 \qquad (6)$$

iv) Reduction

S-Nitrosothiols can be hydrolysed by Hg(II) ions to give the thiol. This reaction was used for analysis of SH group concentrations by Saville (1958) and his method has since been employed by many researchers to monitor free thiol concentrations in many different types of tissue (**7**).

$$\text{Cysteine-SNO} + H_2O \xrightarrow{Hg(II)} \text{cysteine-SH} + HNO_2 \qquad (7)$$

v) Reactions with thiols or sulphinic acids

The reaction of S-nitrosothiols with thiols or sulphinic acids gives good yields of unsymmetrical disulphides and thiolsulphonates respectively (Oae *et al.*, 1977a&b). This is the most convenient synthesis of mixed disulphides or thiolsulfonates as their preparation can be carried out as a one-pot synthesis by addition of an equimolar amount of N_2O_4 (in CCl_4) to an ethereal solution of the thiol. A greenish or reddish colour appears due to formation of the corresponding S-nitrosothiol and subsequent addition of an equimolar amount of another thiol or sulphinic acid results in the formation of the asymmetric product which can be obtained by evaporation of the solvents (**8**).

$$RSNO \begin{cases} \xrightarrow{R'SH} RSSR' \\ \xrightarrow{R''SO_2H} RSSO_2R'' \end{cases} \qquad (8)$$

vi) Reaction with amines

Treatment of S-nitrosothiols with cyclic secondary amines produce N-nitrosoamines (R_2NNO) (Oae *et al.*, 1977b). N-Methylaniline was converted to N-nitroso-N-methyl aniline with S-nitrosothiols in a good yield (**9**).

$$\text{PhNHMe} + \text{t-ButylSNO} \xrightarrow[\text{3 days, 92\%}]{\text{Et}_2\text{O, 25°C}} \text{PhNMeNO} \qquad (9)$$

S-nitrosothiols can also be used to prepare aryl halides in excellent yields by reaction with arylamines in the presence of copper (II) halides (Oae & Shinhama, 1983) (**10**).

$$\text{ArNH}_2 + \text{t-ButylSNO} \xrightarrow[\substack{\text{0-25°C} \\ \text{CH}_3\text{CN}}]{\text{CuX}_2} \text{ArX} + \text{N}_2 + \text{t-ButylSSButyl-t} + (\text{t-Butyl})_2\text{S}_3 \qquad (10)$$

2.2 S-NITROSO-N-ACETYL-DL-PENICILLAMINE (SNAP)

2.2.1 *History*

SNAP was first 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 (see **part 2** of **section 2.2**). It has been shown to be a potent relaxant of vascular smooth muscle and consequently a fast acting vasodilator (Ignarro et al., 1981b). 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).

SNAP is a single peptide based on penicillamine. In the solid state SNAP hydrogen bonds between the carboxyl and amide groups. It is readily reduced by tetrahydroborate to give the disulfide (Field *et al.*, 1978; **11**) and undergoes reduction

by carbon electrodes to form N-acetyl-DL-penicillamine (**12**) (Takeuchi & Osteryoung, 1985).

$$ONSCMe_2CH(NHAc)CO_2H \xrightarrow[EtOH\ 56\%]{NaBH_4\ in} [-SCMe_2CH(NHAc)CO_2H]_2 \qquad (11)$$

$$RSNO + H^+ \rightleftharpoons (RSNO)H^+ ; (RSNO)H^+ + e^- \rightleftharpoons RSH + NO \qquad (12)$$

It can also react with olefins such as methyl methacrylate in the presence of peroxides, such as benzoyl peroxide to form hard polymers (**13**). The same reaction can be initiated with UV radiation (Field *et al.*, 1978).

$$RSNO \longrightarrow RS^{\cdot} \xrightarrow{\text{=}\!\!\diagup\!\!\text{-}R'} RSCH_2\overset{R'}{\underset{|}{C}}H^{\cdot} \longrightarrow RS(-CH_2\overset{R'}{\underset{|}{C}}H-)_nSR \qquad (13)$$

$R = -CMe_2CH(NHAc)CO_2H$

The parent amino acid, penicillamine, has a strong binding affinity for certain metal ions, particularly copper. This property is utilised in the treatment of Wilson's disease, where the deposition of excessive amounts of copper causes damage to a wide variety of organs and can be fatal without treatment. D-penicillamine, but not L-penicillamine (due to its toxicity) is used to chelate the excess copper to treat this disease. If patients become tolerant to penicillamine, triethylenetetramine (TETA) is used as a substitute. This property of penicillamine could possibly explain why the stability of SNAP, one of its nitrosated derivatives, is dramatically affected by copper ions (see **Chapter 3**).

2.2.2 *The chemistry of SNAP in aqueous solution*

i) Results and Discussion

To determine if SNAP, like other S-nitrosothiols previously reported (see **reactions of S-nitrosothiols**), decomposed in aqueous solution to form its disulphide

(oxidised N-acetyl-DL-penicillamine) and NO, two methods were employed to identify the two proposed products.

a) *High performance liquid chromatography (HPLC)* was used to examine the purity of SNAP synthesised by a modified version of the method employed by Field *et al.* (1978) using acidified nitrite (**Figure 2.1**). HPLC traces of both the starting material, N-acetyl-DL-penicillamine, and the proposed product of decomposition of SNAP, oxidised N-acetyl-DL-penicillamine (**Figure 2.1**), were also obtained. A $5x10^{-4}$M solution of SNAP in distilled water was then left to decompose for 3 hours in the dark at 30°C (to mimic other experiments carried out on SNAP decomposition described in the forthcoming chapters). The HPLC trace of this solution was then obtained and compared to that of oxidised N-acetyl-DL-penicillamine synthesised by a method described by Field *et al.* (1978).

The results shown in **Figure 2.1** show that decomposed SNAP solution had a similar HPLC trace to that of the oxidised N-acetyl-DL-penicillamine, with the appearance of two peaks at similar retention times of 21.39 and 22.04 mins to that of 21.59 and 22.26 mins for oxidised N-acetyl-DL-penicillamine. The slight difference is almost certainly due to experimental error caused by manual operation of the detector component of the machine. The appearance of two peaks in both cases, is due to the formation of 2 diastereomers (LD and DL) and 2 enantiomers (DD and LL) present from the use of racemic N-acetyl-DL-penicillamine. The symmetry of the shapes and sizes of these peaks suggests that the enantiomers have the same retention time which is slightly different from that of the diastereomers.

b) *The Greiss Test* - The second product of SNAP decomposition is NO. As mentioned in **Chapter 1.9**, in aqueous solution NO is rapidly hydrolysed to form nitrite (NO_2^-), *but no nitrate* (NO_3^-). Consequently the use of the Greiss reagent (which uses diazotisation in a colorimetric assay of nitrite concentration) indirectly measures the

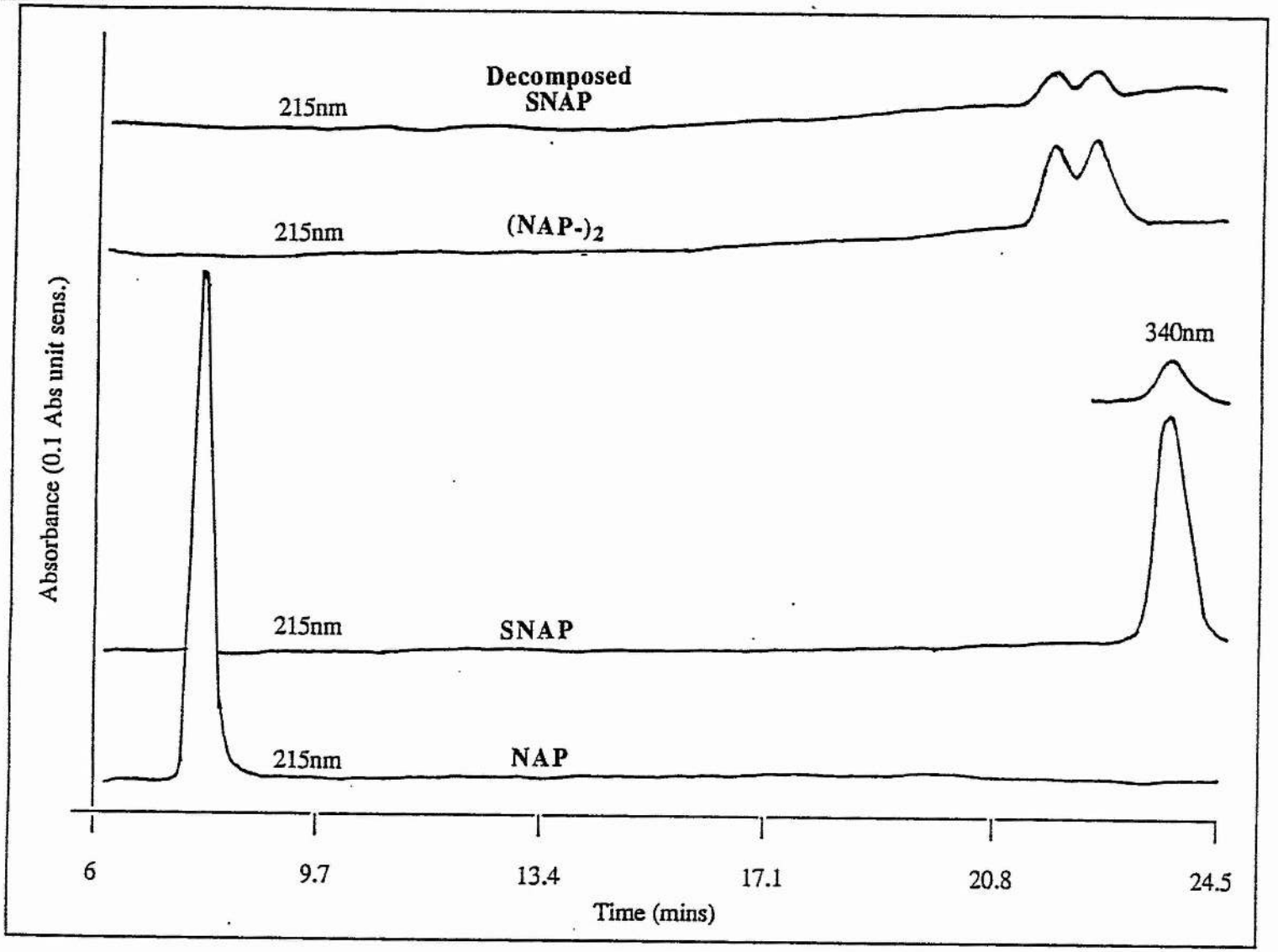


Figure 2.1 *HPLC traces of SNAP, N-acetyl-DL-penicillamine (both unknown concentrations), N-acetyl-DL-penicillamine disulphide ($5x10^{-4}M$), and the product of SNAP ($5x10^{-4}M$) left to decompose at 30 °C over 3 hours.*

concentration of NO produced. A standard line graph using different concentrations of sodium nitrite ($NaNO_2$) in excess quantities of Greiss reagent was constructed, after ensuring that the Greiss reagent itself did not change colour over the course of the reaction. Subsequently, concentrations of SNAP (from 60-500μM) were made up in distilled water. The solutions were kept in the dark to inhibit photolysis of the compound, but they were kept at 50°C to increase the rate of decomposition of SNAP (see **Chapter 4**). After 30 minutes the samples were diluted by a factor of 10 in Greiss reagent and the resulting solutions (≡6-50μM initial SNAP concentrations) were assayed at 549nm after 15 minutes for nitrite concentrations.

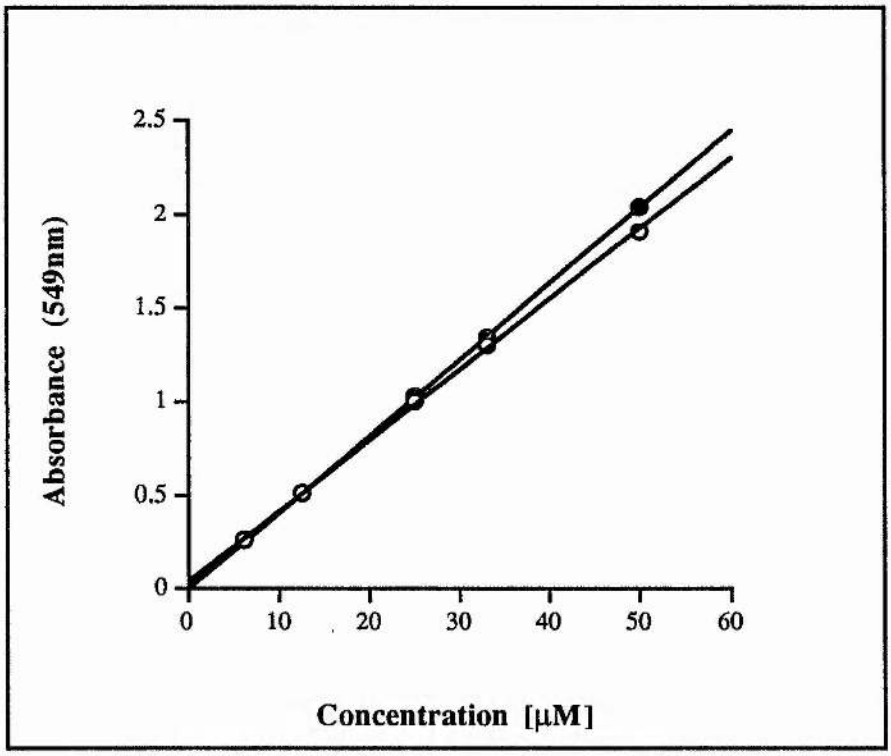


Figure 2.2** A graph using the Greiss test to show that there is 100% conversion of NO to NO_2^- from SNAP in aqueous solution, using $NaNO_2$ as the standard for the Greiss test.* (open circles***) *$NaNO_2$ y = 0.041x - 0.000, r=1.000.*
(***closed circles***) *SNAP y = 0.038x - 0.042, r=1.000.*

Figure 2.2 shows that there is a good correlation between the concentrations of nitrite expected and those obtained from the decomposition of SNAP. The small differences

are possibly due to experimental error or a fraction of the nitrite produced being oxidised to nitrate.

These two results prove that SNAP, like other nitrosothiols, decomposes to form its disulphide with loss of NO, even though initial molecular calculations carried out on penicillamine by C.H. Gorbitz (personal communication) suggest that its disulphide is unfavourable due to steric hindrance between the two sets of gem-dimethyl groups.

ii) Experimental

a)Syntheses

SNAP

N-acetyl DL penicillamine (5m mol; 0.96g) was dissolved in MeOH (10ml), 1M HCl (10ml) and conc. H_2SO_4 (1ml). $NaNO_2$ (10mmol; 0.69g) in H_2O (10ml) was added dropwise over 20 minutes with vigorous stirring at room temperature. After a further 15 minutes, SNAP was separated off, washed with water and air dried. All the solvents were cooled in water before the concentrated H_2SO_4 was added, to minimise the rise in temperature of the solution. The reaction vessel was covered in aluminium foil to exclude light from the reaction. On addition of the $NaNO_2$ a green colour was observed and SNAP was filtered off as green crystals with a red reflection. Mpt.149°C (lit. 152-154°C, Field *et al*, 1978); δH (300MHz,CD_3OD), 2.16 (3H,s,$COCH_3$), 2.20 (3H,s,CCH_3), 2.23 (3H,s,CCH_3), 5.51 (1H,s,CH); δC (300MHz,CD_3OD), 22.56 (CH_3CO), 26.09 (CH_3C), 27.54 (CH_3C), 59.00 (CH_3-C-CH_3), 61.46 (CH), 172.49 ($COCH_3$), 173.50 (CO_2H). Found: C, 37.8; H, 5.68; N, 12.6%. $C_7H_{12}O_4N_2S$ $0.14H_2O$ requires C, 37.8; H, 5.5; N, 12.3%. *m/z* (FAB) 221.0596 ($C_7H_{12}O_4N_2S$ requires 221.0596). One peak only on HPLC see **Figure 2.1.**

N-acetyl DL penicillamine disulphide (oxidised N-acetyl-DL-penicillamine)

This was formed by refluxing SNAP in MeOH (0.111M). After approx 2hrs the colour disappeared, and the disulphide was obtained by evaporation of the solvent, in 100%

yield. δH (300MHz,CD_3OD), 1.59-1.62 (12H,4xs,4xCH_3), 2.20 & 2.21 (6H,2xs,2x$COCH_3$), 4.72 & 4.75 (2H,2xs,2xCH); δC (300MHz,CD_3OD), 22.61 (2xCH_3CO), 24.77 (CH_3C), 25.00 (CH_3C), 27.21 (CH_3C), 27.40 (CH_3C), 51.96 (2xCH_3-C-CH_3), 61.27 (2xCH), 173.13 ($COCH_3$), 173.50 (CO_2H), 173.55 (CO_2H). *m/z (FAB)*, 403 (M^++ Na), 381 (M^++ H).

b) HPLC - The purity of SNAP was examined by reverse phase HPLC at 215 and 340nm and found to give only one peak for each wavelength at the same retention time. 0.5mM solutions of the synthesised N-acetyl-DL-penicillamine disulphide and the decomposed solution of SNAP were also injected into the machine under the same conditions and their HPLC traces were obtained. In addition, the trace of the reduced form of N-acetyl-DL-penicillamine was also run to compare the retention time differences between the different derivatives of acetyl penicillamine.

Apparatus -The HPLC system contained a Perkin-Elmer LC-235 Diode Array Detector and a Perkin-Elmer Series 410 LC pump system linked by a Lichroscorb RP-18 column (0.46 x 25cm, 10μm).

Conditions - The solvent system consisted of 0.05% trifluoroacetic acid/water (A) and 0.035% trifluoroacetic acid/acetonitrile (B) using a linear gradient of 90%(A) 10%(B) for 10 mins going to 80%(A) 20%(B) over the next 20 mins and then to 70%(A) 30%(B) over the following 10 mins. The solvent was monitored at 215 and 340nm with band widths of 15nm at each wavelength and with a sensitivity setting of 0.1 absorbance units.

c) Measurement of nitrite concentrations

1)The Greiss Test (developed by Saltzman, 1954)

All reagents were made from analytical grade chemicals and kept in stoppered brown bottles in the refrigerator to prolong their stability.

N-(1-Naphthyl)-ethylenediamine dihydrochloride (0.1%) - 0.1g of the reagent was dissolved in 100ml of water to produce the stock solution.

Absorbing (Greiss) Reagent - Sulphanilic acid (5g) was dissolved in almost one litre of water containing glacial acetic acid (140ml). 0.1% stock solution of N-(1-naphthyl)-ethylenediamine dihydrochloride (20ml) was added and the reagent was made up to one litre.

2) preparation of standard $NaNO_2$ samples

A 0.05M solution of $NaNO_2$ (0.345g in 100ml of distilled water) was made up and diluted 100 fold to obtain a 500μM stock solution. This was further diluted to make up 330, 250, 125, and 62.5μM solutions. Subsequently, 0.3ml of these solutions were diluted 10 fold in 2.7ml of Greiss reagent in the spectrometer cuvettes. The deep purple colour of diazosulphanilic acid, formed by the action of nitrite on sulphanilic acid, was allowed to develop to a maximum absorbance value over 15 minutes. The maximum absorbance values for all the $NaNO_2$ solutions were recorded and plotted against nitrite concentration to give the standard straight line graph shown in **Figure 2.2**.

3) preparation of SNAP samples

Five separate stock solutions of 500μM SNAP (0.011g in 100ml distilled water) were prepared and each one was further diluted to make up 330, 250, 125, or 62.5μM solutions of SNAP. These solutions were incubated in the dark at 50°C to increase the rate of decomposition of SNAP for 30 minutes until all the SNAP had disappeared. 0.3ml of these solutions were then added to 2.7ml of Greiss reagent to dilute the samples 10 fold in the same way as the $NaNO_2$ samples. The maximum absorbance values for all the concentrations were recorded and the straight line graph of nitrite produced versus absorbance was drawn (see **Figure 2.2**).

As a check, the Greiss reagent containing 0.3ml of only distilled water was left in the spectrophotometer for the 15 minute period and the absorbance at 549nm was

monitored. There was no increase in absorbance with time, indicating that there was no detectable nitrite present.

2.3 S-NITROSOGLUTATHIONE (GSNO); AN S-NITROSOTHIOL MORE STABLE IN AQUEOUS SOLUTION

2.3.1 *History*

S-nitrosoglutathione (GSNO) 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 glutathione (L-γ-glutamyl-L-cysteinyl glycine), which is found in numerous cellular systems and is considered an essential constituent of all living cells. Glutathione is generally the most abundant intracellular non-protein thiol (Meister & Anderson, 1983), with a concentration approaching 2-3mM in erythrocytes. Consequently, the discovery of EDRF and the suggestion that it is an S-nitrosothiol (see **Chapter 1.4**), together with evidence that the physiological half-life of NO can be stabilised by thiols (Ignarro *et al.*, 1980a; Stamler *et al.*, 1992a; Feelisch *et al.*, 1994), raises the possibility that S-nitrosoglutathione could be the most important S-nitrosothiol synthesised *in vivo* and involved in numerous physiological processes.

Since 1985, GSNO has been synthesised by other researchers using different methods (Park & Means, 1989; Clancy & Abramson, 1992). Clancy and Abramson used a column containing red agarose to synthesise GSNO. This was prepared by coupling 2,2-dithiobis(ethylamine) to Bio-Gel A through an amide linkage and after reduction to form reduced thiol using dithiothreitol, the immobilised thiol was treated with $NaNO_2$ (at pH 2) to form bound S-nitrosothiol. Addition of thiols, such as glutathione,to the column produced a transfer of the S-nitroso moiety from the immobilised thiol to the fluid phase thiol and stoichiometric amounts of S-nitrosoglutathione were formed.

GSNO has been shown to be a hypotensive drug as effective as sodium nitroprusside (Means & Park, US patent 1989) 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, although SNAP is more stable, GSNO kept at 4°C or below is stable over a number of months (Park & Means, 1989; Askew, unpublished results). A possible reason why pure S-nitrosothiols are so difficult to isolate in the solid state could be due to self-decomposition. It has been proposed that they decompose spontaneously by second order kinetics (Park, 1988) and so evaporation of the solvent leads to increased concentration and more chance of rapid decomposition to their disulphides.

A chemical and physiological comparison of the actions of GSNO and SNAP are the subjects of a large proportion of this thesis. Interesting and informative data have been compiled which gives an insight as to why these two compounds act differently and how S-nitrosothiols might be developed in the future to improve their pharmacokinetic profiles and target their actions better.

2.3.2 *The aqueous chemistry of GSNO compared with that of SNAP*

i) Results and Discussion

Many workers have shown that GSNO decomposes to form NO and oxidised glutathione under oxidative conditions (Park & Mean, 1989; Radomski *et al.*, 1992; Shaw *et al.*, 1990). However, the rate at which this process occurs in aqueous solution is far slower than for SNAP under the same conditions. The main reason for this will be discussed in the next chapter, but in repeated experiments following the decomposition profiles of SNAP and GSNO at 339nm (the wavelength at which the S-NO moiety of S-nitrosothiols absorbs and the λ_{max} of SNAP; see **Figure 2.3**) consistent absorbance profiles were observed for both SNAP and GSNO, with GSNO remaining stable over intervals during which SNAP completely decomposed. In fact

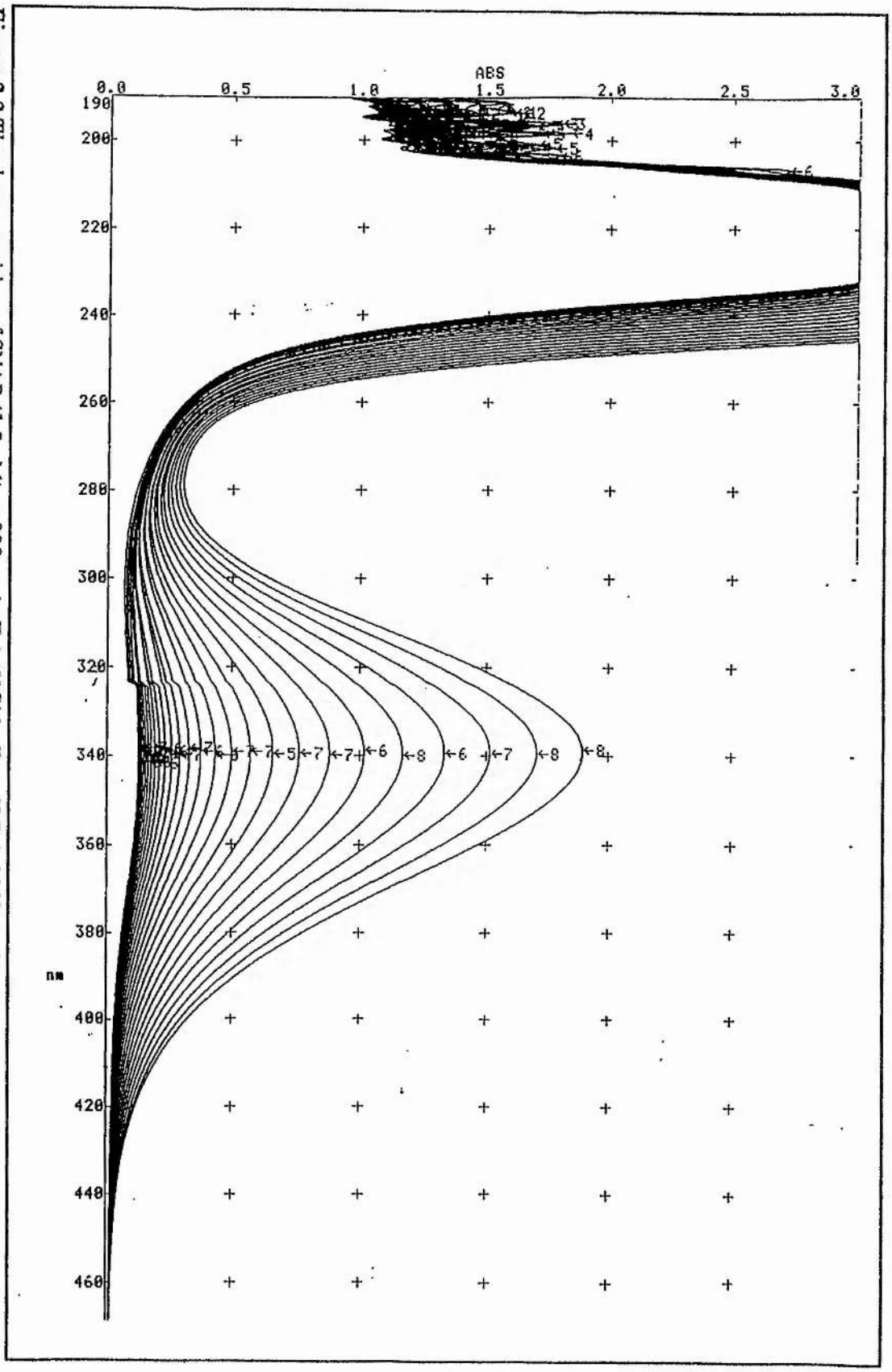


Figure 2.3 The decomposition of SNAP (1.5mM) at 339nm in Tris/HCl buffer pH 7.4, 30 °C, at 30 minute intervals

GSNO remains stable in the phosphate buffer (pH7.4) at 30°C for many hours and nearly 50% of the nitrosothiol remains after 35 hours under these conditions. **Figure 2.4** shows the marked difference in aqueous stabilities of these two S-nitrosothiols.

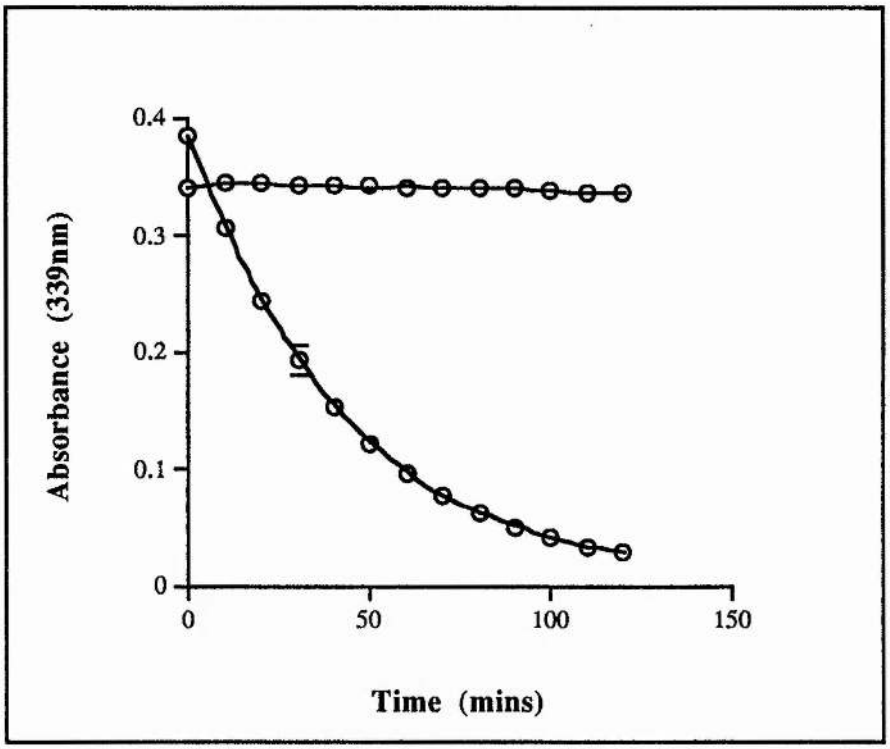


Figure 2.4 *A graph showing the relative decomposition profiles of GSNO and SNAP at 30°C in phosphate buffer pH7.4.*
(open circles) GSNO (0.4mM, n=6) *(closed circles) SNAP (0.4mM, n=11).*

ii) Experimental

GSNO synthesis

To a stirred ice-cold solution of glutathione (1.53g, 5mmol) in water (8ml) containing 2N HCl (2.5ml) and protected from light by aluminium foil, was added in one portion sodium nitrite (0.345g, 5mmol). After 60 minutes at 5°C, the red solution had developed into a red sludge and was filtered off and washed, successively, with ice cold water (3x1ml), acetone (3x10ml) and sodium-dried ether (3x10ml) to afford S-nitrosoglutathione (1.28g, 3.8mmol, 76%). λ_{max} (H_2O) 335, 545nm. Found: C, 35.2; H, 4.7; N, 16.3. $C_{10}H_{16}O_7N_4S$ $0.25H_2O$ requires C, 35.2; H, 4.9; N, 16.4%. *m/z*

(FAB) 337 (M^++ H). Only one peak was present on the HPLC trace at a retention time of 14.9 mins. (for HPLC details see experimental section of **Chapter 2.2**). The same solvent systems as those used for SNAP were used, but a different linear gradient of 100%(A) for 10 mins going down to 90%(A) 10%(B) over the next 10 mins and then to 80%(A) 20%(B) over the following 20 mins. The solvent was monitored at 215 and 340nm with band widths of 15nm at each wavelength.

Kinetic studies

The kinetics of decomposition of SNAP and GSNO were monitored by following the reduction in absorbance at 339nm using a Phillips PU8700 UV/Vis scanning spectrophotometer with a Pye Unicam cell temperature control unit. All experiments were carried out at 30°C in a phosphate buffer at pH 7.4. A stock solution of the buffer was made up from 250ml of 0.1M KH_2PO_4 (3.4g) and 195.5ml of 0.1M NaOH diluted to 500ml with distilled water.

GSNO (0.0135g) was diluted in 10ml of ice cold phosphate buffer (protected from photochemical decomposition by aluminium foil) to make up 4mM solution. 0.25ml aliquots of this solution were injected into spectrophotometer cuvettes containing 2.25ml of the phosphate buffer alone at 30°C in the cell programmer carriage, diluting the solutions to 0.4mM. The same process was carried out for SNAP using a 4mM stock solution (0.0088g in 10ml ice cold distilled water) protected from light. As soon as the cuvettes were full (2 for GSNO and 2 for SNAP), which was timed and took approximately 1 minute, the computerised timer was started and absorbance readings were taken at 10 minute intervals for all the cuvettes using the automated cell programmer. The buffer solution alone was used as the standard in the first of the 5 cuvettes.

2.4 A COMPARISON OF THE VASODILATOR PROFILES OF GSNO AND SNAP ELICITED IN THE RAT TAIL ARTERY

i) Experimental

Preparation

The vasodilator actions of SNAP and GSNO were determined by experiments performed on segments of tail artery taken from normotensive adult Wistar rat (250–460g) which were killed by cervical dislocation. A length of artery (0.8–1.5 cm) was dissected free from connective tissue and overlying skin, cannulated (Portex cannula) and transferred to a perspex bath (**Figure 2.5**).

Apparatus

The cannula (C) forms part of a constant flow perfusion driven by a peristaltic pump **P(1)** (Gilson Minipuls 2). The length of artery (**A**) was perfused internally (flow rate 2 $ml.min^{-1}$). The solution was pre-warmed to 31-33°C by passage through a heat exchanger. Drugs were introduced into the lumen of the artery by bolus injection (10µl) through a side tube (**I**)). The outer surface of the artery was superfused continuously with Krebs solution from a second peristaltic pump (**P(2)**). The temperature of the bath was held at 30–33°C by adjustment of the flow rate of the superfusing solution (ca. 8 $ml.min^{-1}$). This temperature range was chosen since phenylepherine-induced perfusion pressures remained more stable than at 37°C over the time periods (up to 8 hours) required to complete the experiments. A differential pressure transducer (**T**; sensym type SCX 150NC Farnell Electronic Components, Leeds, UK) detected changes in back pressure caused by changes in arterial tone. Responses were displayed on a computer and chart recorder.

Experimental Protocol

Arteries were perfused internally with 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

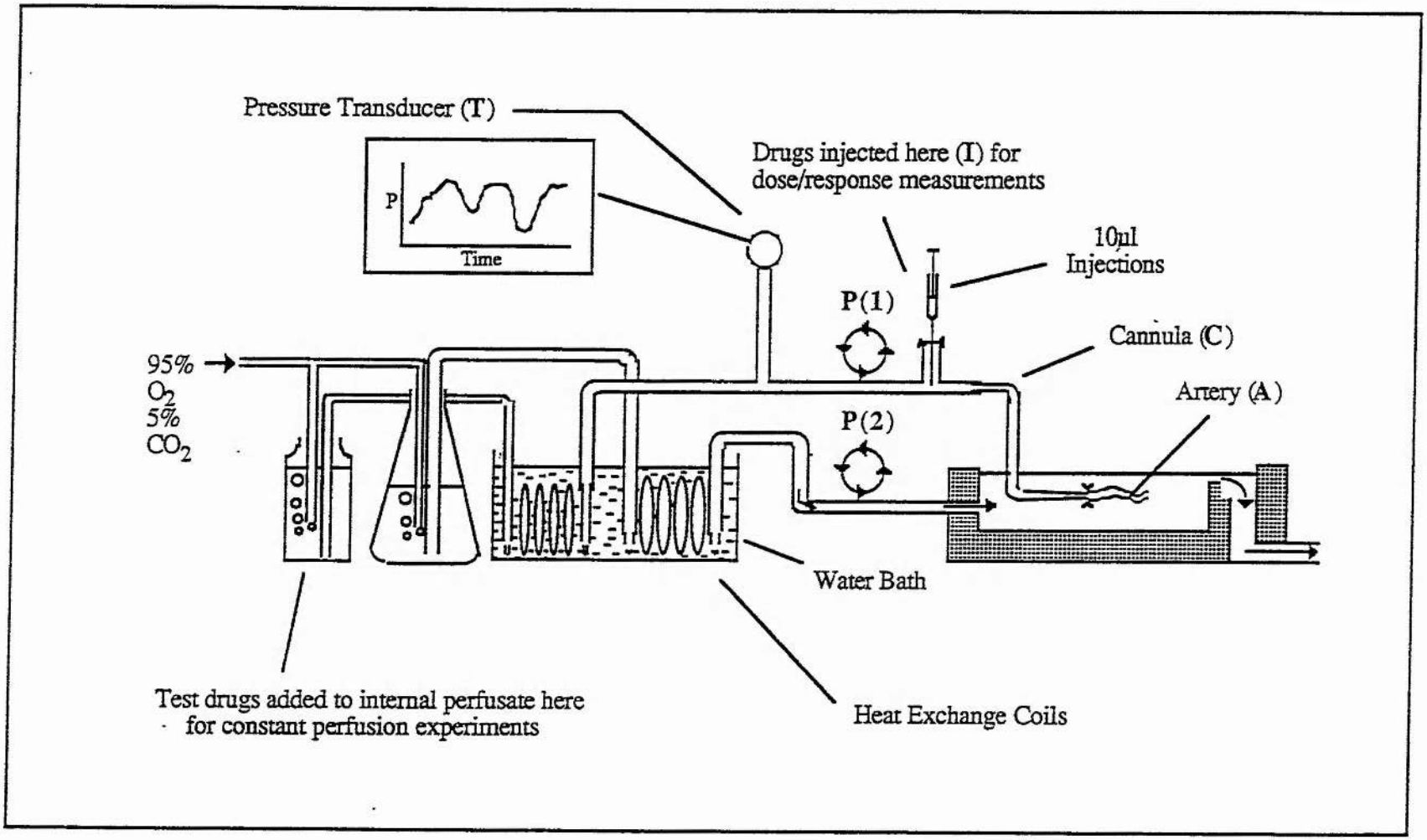


Figure 2.5 View of apparatus used for drug testing on the rat tail artery

with O_2/CO_2 (95%:5%) to maintain pH at 7.4). The initial flow rate was low but was gradually increased over 20 min to reach a final value 2 $ml.min^{-1}$. The preparation was allowed to stabilise for 20-30 min, after which the artery was precontracted by addition of phenylephrine hydrochloride to the Krebs buffer ([PE]: mean (± s.e.) = 2.7±0.3μM gave agonist induced perfusion pressure: mean (± s.e.) = 102±2.9mmHg)

Responses to **bolus** injections of SNAP and GSNO were recorded. All experiments were conducted in a darkened laboratory with a red safety light (60 W) as the sole means of illumination.

Drugs: use

SNAP & GSNO - 10^{-2}M solutions of SNAP and GSNO were made up in Krebs buffer and serially diluted to the concentration required for injection immediately prior to use. Both S-nitrosothiols solutions were kept in the dark and on ice at all times to minimise any type of decomposition.

N.B. The concentrations of the drugs injected (indicated in the figures) are virtually the same as the concentrations which reach the tissue due to the rapid injection time (<0.5 seconds) and the drug travelling through the narrow bore tubing as a bolus. However, a submaximal response produced by 7μM SNAP administered by **perfusion** was matched by a 3mM **injection** of SNAP. This suggests that less than 1/400th (1/428th) of the drug concentration delivered to the artery by injection (as a bolus) is reaching its site of action.

L-phenylephrine hydrochloride - (MW 203.7; Sigma Chemicals). Appropriate volumes of a 10mM stock solution were added to internal and external perfusates to produce the required concentration which gave the necessary active pressure.

Measurement of drug induced pressure changes for log dose/response curves

The pressure increase induced by the agonist, PE, was taken to be the active pressure (see **Figure 2.6**). This was calculated by subtracting the total pressure reading immediately before each injection of the test drug, from the pressure maintained by the artery before PE was added (termed the passive pressure). The pressure drop caused by the test drug, for each concentration, was measured and expressed as a % of the active pressure immediately prior to injection. The mean % relaxation and the standard error of the means (s.e.m.) for each concentration were calculated for the number of preparations n (indicated in the legends of the figures) and plotted against drug concentration (log dose /response curves).

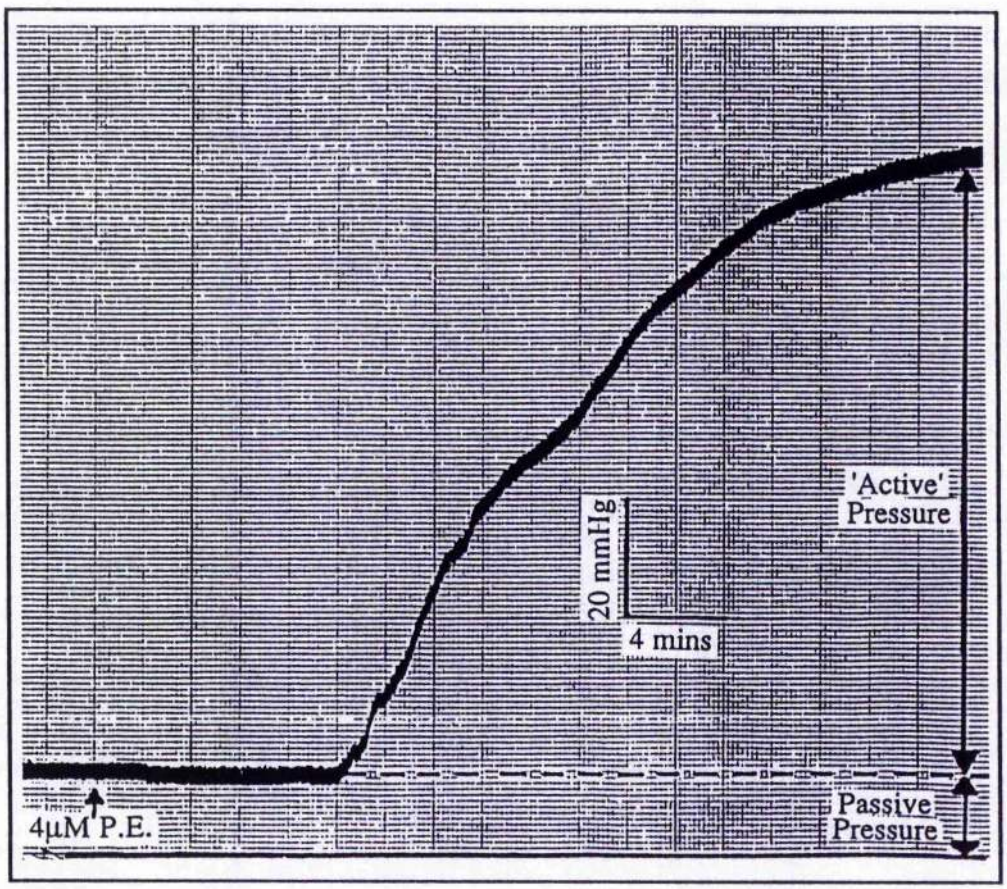


***Figure 2.6** Pressure recordings showing precontraction with PE. 4μM PE was added to the internal and external perfusate reservoirs at the time indicated. The pressure maintained by the vessel before PE addition is termed 'passive', whist agonist-induced pressure is 'active'. Expt. No 92/3/1.*

Statistical analysis of data in figures

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

ii) Differences between the perfused rat tail artery bioassay and the commonly used rabbit aortic ring bioassay.

The perfused rat tail artery as the bioassay system was used as an alternative to the commonly used organ bath containing rabbit aortic ring preparation . The apparatus is a modified version of that used by Flitney *et al.* (1992). Compared with the rabbit aorta rings system there are a number of important differences of this system. Firstly, the test drugs are injected through a side tube (**I**) located close to the cannula (**C**) which connects the rat tail artery (**A**). This means the drug passes through the artery, in contact with its internal surface, unlike the situation for the rings of rabbit aorta which are submerged in physiological buffer in which the test drug is injected. Consequently, both internal and external surfaces are exposed to the test drug, which would not be the case *in vivo*. Secondly, the injection of the test drug through into the internal perfusate, immediately before the cannula, means the drug reaches the rat tail artery in a matter of seconds as a bolus. This results in a larger dose of the drug being required to give the same response to that attained in the ring system, as only the small fraction of the bolus will be exposed to the tissue (see experimental section). However due to the 'flow through' nature of the internal circuit, the higher concentration of drug passes through the artery quickly (approx. 0.3 secs) and does not appear to damage the tissue as pressure rapidly returns to its pre-injection value (see **Figure 2.7**). When carrying out constant perfusion experiments, in which the artery is continuously exposed to the test drug from the internal perfusate, lower concentrations of the drug are required for maximal relaxation of the artery (**Chapter 7**).

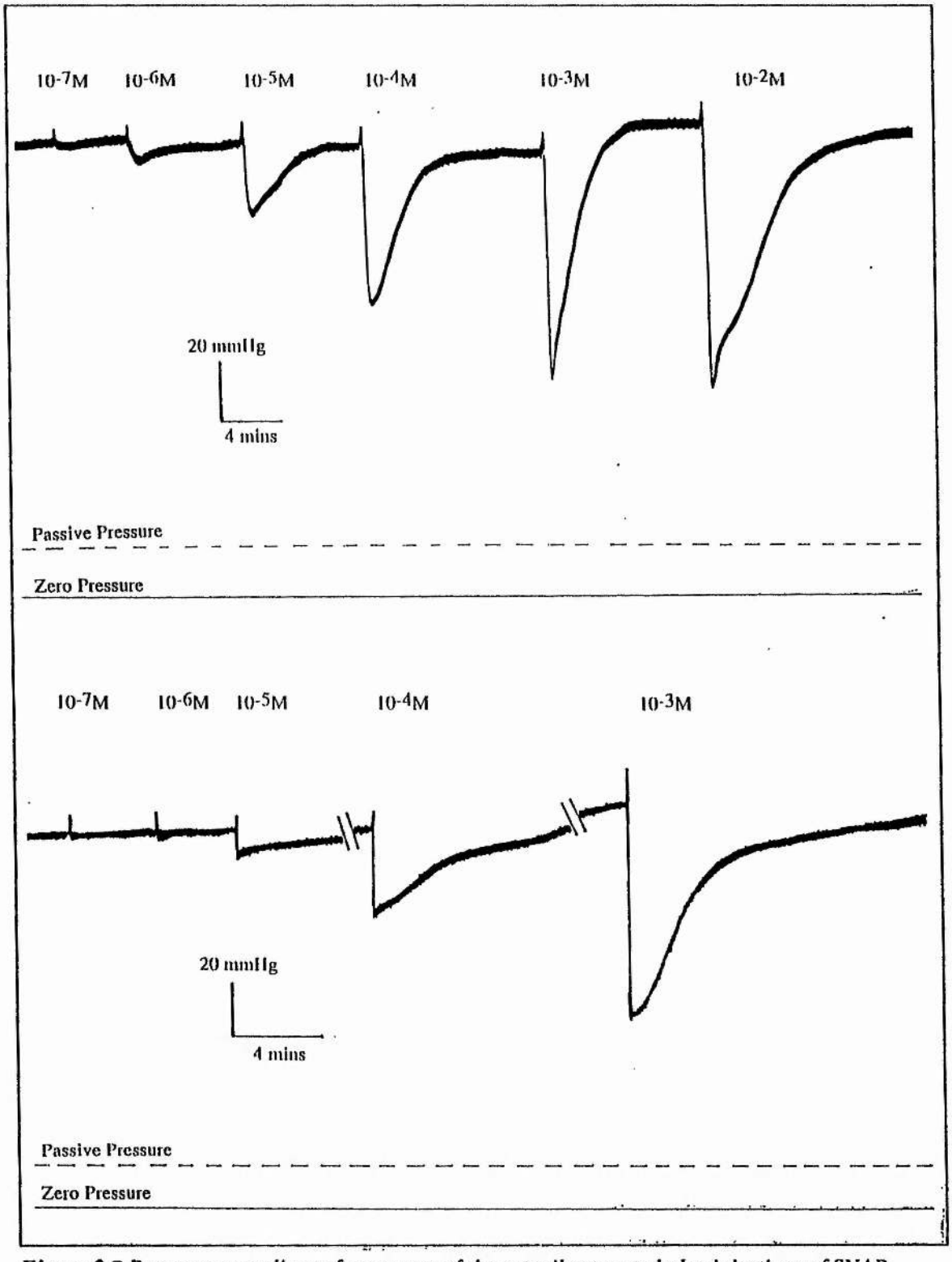


Figure 2.7 *Pressure recordings of responses of the rat tail artery to bolus injections of SNAP (Top trace) and GSNO (bottom trace) after precontraction with phenylephrine (PE) 4μM. (SNAP Expt. N° 91/11/9, GSNO Expt. No 93/4/23). See Experimental section for definitions of Passive and Active pressures.*

iii) Results and Discussion

Both SNAP and GSNO are fast acting and potent vasodilators. As can be seen in **Figure 2.7** the responses are sharp, appearing soon after injection, and they fully recover, in a matter of minutes, to the original precontracted tone of the artery.

When traces like those shown in **Figure 2.7** are translated into log/dose response curves like those shown in **Figure 2.8**, it is evident that SNAP is a more effective vasodilator. Arteries are approx. 18 fold more sensitive to SNAP than to GSNO with an ED_{50} value of 3.2μM compared to that of 59μM. The differences are statistically significant at the 99.9% confidence level, at 10μM and 100μM doses. The log/dose response curves are also significantly different at the 95% confidence level for doses of 1μM. The possible reasons why SNAP is a better vasodilator than GSNO will be discussed in later chapters.

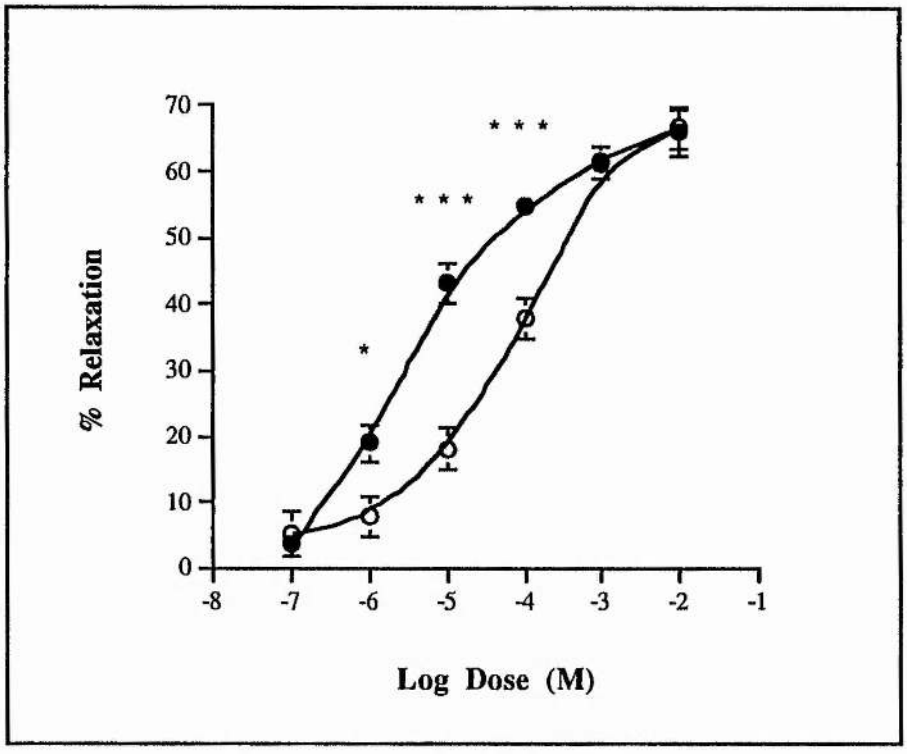


Figure 2.8 *Log [dose]/response curves comparing the vasodilator potencies of SNAP (closed circles, n=11-20) and GSNO (open circles, n=6-16).*

2.5 THE SYNTHESIS AND STABILITY OF SOME OTHER S-NITROSOTHIOLS

The syntheses of a number of other S-nitrosothiols derivatives of penicillamine, glutathione and cysteine were attempted using some of the nitrosation methods mentioned in **Chapter 2.1**. Comparisons of their aqueous stability and relative physiological properties as inhibitors of platelet aggregation and vasodilators were made in an effort to relate their structure differences with activity. Unfortunately the syntheses of these compounds proved difficult. Solid, pure, stable products could not be obtained in most cases as their disulphides were readily formed, as will be discussed later in this chapter. However, impure products containing small amounts of disulphide were still potent inhibitors of platelet aggregation (see **Chapter 6**). Their vasodilator action was also tested using rabbit aorta as a bioassay (see **Chapter 6**).

2.5.1 *Syntheses*

i) Results and Discussion

Methods used for S-nitrosation

Two methods were employed to nitrosate the different analogues of cysteine, penicillamine and glutathione; acidified nitrite (in an analogous method to that used by Hart (1985) and N_2O_3 produced by the action of concentrated sulphuric acid on sodium nitrite.

Nitrosation using acidified nitrite

The addition of sodium nitrite in one portion to an acidified solution of the thiol is advantageous for the quick nitrosation and formation of the less stable nitrosothiols as this can be conducted with minimal breakdown to the respective dimer. The methodology of acidified nitrite nitrosation developed by Field *et al.* (1978) uses sodium nitrite solution which is added dropwise over 20 minutes. This dilutes the solution so nitrosothiols that are on the borderline between being soluble and being

insoluble may not precipitate out of solution. At the same time, for unstable nitrosothiols in solution this method takes longer and would result in a less pure product. However, the main disadvantage with addition of solid sodium nitrite in one portion may lead to some of it remaining undissolved, resulting in contamination of the product.

Nitrosation using N_2O_3

It was hoped that this method would give purer products which contained no inorganic impurities. Experiments were conducted with the relevant thiol being dissolved in water, in ether, or in the solid form. A mixture of NO and NO_2 made by adding excess H_2SO_4, dropwise, to excess sodium nitrite, was carried in a continuous flow of nitrogen gas to a vessel containing the thiol on ice. Here, N_2O_3 is formed and nitrosates the thiol. For thiols having a free amine group, the technique seemed to have limited use since quantitative production of N_2O_3 proved difficult and therefore nitrosation of the free amine group was possible.

Penicillamine derivatives

The S-nitrosation of DL-penicillamine and N-formyl-DL-penicillamine were attempted using acidified nitrite and N_2O_3. Equimolar quantities of nitrite and thiol were used for nitrosation of the acidified thiol (as used by Hart in the synthesis of S-nitrosoglutathione), whereas excess N_2O_3 was produced by adding concentrated sulphuric acid to solid nitrite. Both methods were attempted on both thiols and resulted in nitrosation to form green solutions of varying shades. However when concentrated solutions were used, green suspensions were obtained which produced solids, in good yields, after filtration. Unfortunately the microanalyses of both the nitrosated derivatives did not correspond closely with expected values.

Glutathione derivatives

The N-acetyl and N-formyl analogues of glutathione were synthesised and the subsequent nitrosations attempted, with the intention of comparing them with the

penicillamine analogues. The parent thiols were synthesised from glutathione using acetic acid and formic acid respectively, in acetic anhydride. Both produced the expected products containing small amounts of dimethylformamide (DMF). S-Nitrosation of these compounds were carried out using acidified nitrite or N_2O_3 as the nitrosating agent (as above), this resulted in dark pink solutions of S-nitroso-N-acetylglutathione and S-nitroso-N-formylglutathione respectively. Both remained pink for several days with no signs of decomposition to the disulphide. However, isolation of the solid products was not attained as no pink solid precipitated in concentrated solutions. When the solutions were freeze-dried, a large portion of the solids formed were the respective disulphides.

Cysteine derivatives

The S-nitrosation of several cysteine analogues, N-acetyl-DL-cysteine, N-Boc-cysteine and DL-cysteine, using acidified nitrite and N_2O_3, was attempted. All produced deep blood red solutions characteristic of the S-nitrosated derivatives of cysteine. When concentrated solutions of the parent thiol were used S-nitrosocysteine produced a bright red solid obtained by filtration of the red suspension formed. However, the acetylated derivative and the N-Boc derivative only produced red solutions, even in concentrated solutions. Use of freeze-drying to isolate a solid produced the disulphide in both cases. S-nitrosation of N-Boc-cysteine using N_2O_3 produced a solid which rapidly decolourised to form the disulphide.

Microanalysis of the red solid obtained from S-nitrosation of DL-cysteine showed that some disulphide had formed in its isolation as indicated by the high carbon and low nitrogen values.

Summary

In most cases it seems apparent that when the solvent is removed to synthesise the solid S-nitrosothiol, rapid decomposition occurs, leading to the formation of the relevant

disulphide. As mentioned in the introduction to **Chapter 2.3**, a possible reason why pure S-nitrosothiols are so difficult to isolate in the solid state could be due to spontaneous decomposition. It has been proposed that they decompose by second order kinetics (Park, 1988) and therefore evaporation of the solvent would lead to increased concentration and more chance of rapid decomposition to their disulphides. However our finding that trace metal ions such as copper can catalyse the decomposition of S-nitrosothiols (discussed in **Chapter 3**), would suggest that evaporating the solvent merely concentrates these trace metals, promoting more rapid decompositon.

When some of these nitrosothiols were synthesised *in situ*, using equimolar quantities of thiol and nitrite (see **Section 2.5.2**), the resulting solutions were 100% nitrosated and in the case of S-nitrosoglutathione produced consistent λ_{max} absorbance values when compared to the same compound synthesised as the solid and diluted to the same concentration (see **Section 2.5.2**). It has previously been reported that the reaction of thiols with acidified nitrite is essentially irreversible and complete (Aldred *et al.*, 1982; Byler *et al.*, 1983; Mathews *et al.*, 1993) and it has been suggested that S-nitrosation and subsequent decomposition to yield the disulphide occurs only by a base catalysed reaction and is much inhibited by reduction of the pH below 4 (Pryor et al., 1982). It would seem that unstable S-nitrosothiols, or those which have insolubility problems when made in concentrated solutions, are best synthesised *in situ* in acidified solutions and the S-nitrosothiols formed then used in subsequent reactions by dilution in buffer solutions at the required pH.

ii) Experimental

Penicillamine derivatives

SNAP

See experimental of **Chapter 2.2**

S-nitroso-N-formyl-DL-penicillamine (SNFP)

a) Acidified Nitrite

N-formyl D-L penicillamine (0.53g, 3mmol; obtained from the Wellcome Laboratories) was added to a solution of water (9ml) and 2N HCl (1.5ml) on ice and the solution stirred until the solid dissolved (required heat from steam bath). $NaNO_2$ (0.207g, 3mmol) was added in one portion to the ice cold solution of N formyl D-L penicillamine and after 10-15 minutes a green precipitate of nitroso N-formyl D-L penicillamine was obtained. Found, C 27.19, H 3.75, N 10.16; $C_6H_{11}N_2O_4S$ requires; C 34.95, H 4.89, N 13.58.

b) Using N_2O_3

N-formyl D-L penicillamine (0.53g, 3mmol) was added to a volume of water (9ml) in a quickfit test tube with a stirrer. The solution was warmed until the N-formyl D-L penicillamine dissolved and then the test tube put in an ice/water bath. Excess conc. H_2SO_4 was added dropwise to excess sodium nitrite in a three necked flask and nitrogen was passed through the flask, blowing the NO and NO_2 produced through to the test tube containing the N-formyl D-L penicillamine solution via the third neck of the flask linked to the test tube by plastic tubing. The gases were cooled down by the ice/water bath to form the deep blue N_2O_3 solution. A green precipitate of S-nitroso-N-formyl D-L penicillamine was obtained.(75% yield). Microanalysis of the nitrosated compound did not correspond closely with expected values.

S-nitroso D-L penicillamine (SNPen)

a) Acidified nitrite

To a stirred ice-cold solution of D-L penicillamine (0.746g, 5mmol) in water (8ml) containing 2N HCl (2.5ml), was added one portion of sodium nitrite (0.345g, 5mmol). After 10 minutes the green compound was filtered off and washed with water (1x4ml). 0.29g (32.6% yield). Found; C 34.39, H 5.68, N 15.14, $C_5H_{10}N_2O_3S$ requires; C 33.70, H 5.66, 15.72.

b) Using N_2O_3

See Synthesis of SNFP. A green compound was formed but microanalysis of the nitrosated compound did not correspond closely with expected values.

Glutathione derivatives

SNOG

See experimental section **Chapter 2.3**

S-nitroso-N-acetylglutathione (SNAG)

a) Synthesis of N-acetyl glutathione

Acetic anhydride (1.5ml) was added to a solution of glutathione (1g) in acetic acid (3ml) at ~40°C. The reaction was followed by thin layer chromatography and was complete in 5 hours. A large excess of diethyl ether was added to precipitate the product which was separated, washed with ether and left in ethyl ethanoate overnight. The crude product was recrystallised from DMF (although absolute ethanol proved to be better) and ethyl ethanoate at ~-20°C (or 0°C for ethanol recrystallisation) 0.31g (28%yield); Found C 42.05, H 5.78, N 12.5, $C_{12}H_{19}N_3O_7S$ requires; C 41.26, H 5.48, N 12.03%. The difference was due to C_3H_7NO (DMF) detected by NMR.

b) Nitrosation of N-acetyl glutathione

Both methods of nitrosation were attempted but no solid product was obtained. The final product was a stable deep red solution.

S-nitroso-N-formyl glutathione (SNFG)

a) Synthesis of N-formyl glutathione

Acetic anhydride (1.5ml) was added to a solution of glutathione (1g) in formic acid (3ml) at ~26°C. The reaction, followed by thin layer chromatography, was complete in 5 hours. A large excess of diethyl ether was added to precipitate the product which was separated, washed with ether and left to solidify in ethyl ethanoate overnight. The crude product was recrystallised from DMF (although absolute ethanol proved to be

better) and ethyl ethanoate at ~-20°C (or 0°C for ethanol recrystallisation). 0.27g (24.75%). Found; C 41.39, H 6.41, N 14.01, $C_{11}H_{17}N_3O_7S$ requires, C 39.40, H 5.11, N 12.53%. The difference was due to 1.35moles of DMF (detected by NMR and calculated from a microanalysis fit program). δH (300MHz, D_2O), 1.9-2.3 (2H,m,$\underline{CH_2}$CHNH), 2.45 (2H,t,CO$\underline{CH_2}CH_2$CHNH), 2.95 (2H,d,$\underline{CH_2}$SH), 3.92 (2H,s,$\underline{CH_2}$COOH), 4.36 (H,q,NH$\underline{CH}CH_2$), 4.57 (H,t,$\underline{CH}CH_2$SH), 8.15 (H,s,NH$\underline{CH}$O). δC (300MHz, D_2O), 26 (CH_2SH), 27 ($\underline{CH_2}$CHNCOOH), 32 ($\underline{CH_2}CH_2$CHNCOOH), 41 ($\underline{CH_2}$COOH), 50 ($\underline{CH}CH_2$SH), 55 (NH$\underline{CH}$COOH), 161 (NCHO), 170,171,172,173 (2COOH & 2NHCO).

b) Nitrosation of N-formylglutathione

Both methods of nitrosation were attempted but no solid product was obtained, only a stable deep red solution.

Cysteine derivatives

S-nitroso-DL-cysteine (SNC)

a) Acidified nitrite

To a solution of cysteine (0.75g, 5mmol) in ice cold water (4ml) and 2N HCl (2.5ml) was added sodium nitrite (0.345g, 5mmol) in one portion and the product was isolated after 3-5mins giving a red crystalline powder. 0.47g (62.6% yield). Found; C 29.9, H 5.03, N 11.66, $C_3H_6N_2O_3S$ requires; C 24.0, H 4.03, N 18.66%

b) Using N_2O_3

See Synthesis of SNFP. A red compound was obtained but the microanalysis was a poor fit with expected percentages.

S-nitroso-N-acetyl cysteine (SNAC)

a) Acidified nitrite

N-acetylcysteine (1.676g) was dissolved in methanol (20ml), 1N HCl (20ml), and conc. H_2SO_4 (2ml). Sodium nitrite (1.38g) was added dropwise over 20 minutes and the solution was left to stir for another 15 minutes. A blood red solution was obtained

but no solid product. Precipitation with acetone gave a white solid which had a melting point of 300°C suggesting that the dimer had been formed.

b) Acidified nitrite (using acetone and not water as solvent)

To a solution of N-acetylcysteine (1.676g, 8.7mmol) in ice cold acetone (14ml) and 2N HCl (4.35ml) was added sodium nitrite (0.60g, 8.7mmol) in one portion and left to stir for 25 minutes at 5°C. No solid product was obtained. After removing the acetone under vacuum a white solid was formed (mpt. >300°C).

c) Using N_2O_3

See synthesis of SNFP. This method of synthesis gave a blood red solution which was freeze dried to remove the water and afforded a light pink solid. The microanalysis indicated that the product was mainly the N-acetylcysteine dimer.

S-nitroso-N-Boc cysteine (SNBC)

a) Acidified nitrite

See synthesis of S-nitroso D-L cysteine (SNC). A red solution was obtained and no solid product could be isolated.

b) Using N_2O_3

See synthesis of SNFP. A deep red solution was obtained but no solid product could be isolated. Synthesis of the nitrosothiol using the same method but no solvent resulted in rapid decolourisation of the red solid formed to the white disulphide.

2.5.2 The aqueous chemistry of S-nitrosothiols synthesised in situ.

i) Results and Discussion

Synthesis of GSNO *in situ* and measurement of its extinction coefficients at different wavelengths

GSNO was synthesised *in situ* by making up a 12.5mM solution in H_2SO_4 (0.4M) using equimolar concentrations of the thiol to solid sodium nitrite and 0.1ml of this

solution was added to 2.4ml of triple strength (i.e. 0.3M KH_2PO_4/0.3M NaOH instead of 0.1M of each solution) buffer at pH7.4. This gave a GSNO solution of 0.5mM with an absorbance reading of 0.5 at 339nm. When this was compared to the 0.5mM GSNO solution made up from dilution of the solid (synthesised as described in **Chapter 2.3**) in phosphate buffer at the same wavelength, it is evident that the absorbance value is the same (0.5; see **Figure 2.9**). When the same experiments are done using SNAP instead of GSNO, equivalent absorbance readings at 339nm of 0.56 are also attained for both 0.5mM solutions made up from SNAP synthesised *in situ* or from the pure solid (see **Figure 2.10** and **Figure 2.11** at T=0).

These results support the work of Aldred *et al.* (1982), Byler *et al.* (1983), Mathews & Kerr (1993) and Pryor *et al.* (1982), who propose that reaction of thiols with acidified nitrite is essentially irreversible and complete and that S-nitrosation and subsequent decomposition to yield the disulphide occurs only by a base catalysed reaction and reactions are much inhibited by reduction of the pH below 4.

The stability of S-nitroso-D,L-penicillamine (SNPen) and S-nitroso-D,L-cysteine (SNC) and their N-acetylated derivatives (SNAP and SNAC) in aqueous solution

The above mentioned nitrosothiols were synthesised *in situ* using equimolar quantities of thiol to sodium nitrite and H_2SO_4 (0.4M) as the acidified solvent. These solutions were diluted 25 fold in triple strength phosphate buffer (as above) to form 0.5mM solutions of the compounds and the decrease in their absorbance at 339nm were monitored (see **Figure 2.11**).

SNAC proved to be a very stable S-nitrosothiol in solution, far more stable than SNC, suggesting that there is a large stabilising effect from the acetyl group. Interestingly, SNAP is far less stable than SNAC and also less stable than SNAP synthesised as a pure solid and subsequently diluted to a similar concentration (see

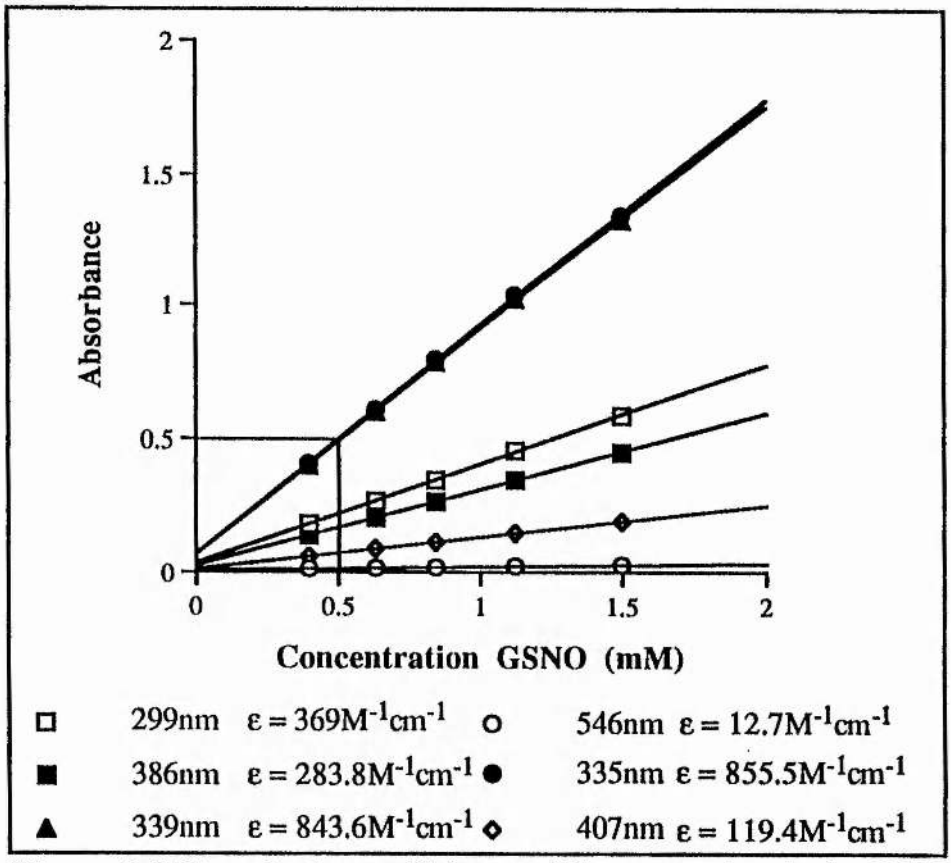


Figure 2.9 *The extinction coefficients at different wavelengths for GSNO (box indicates absorbance at 339nm of 0.5mM solution of GSNO)*

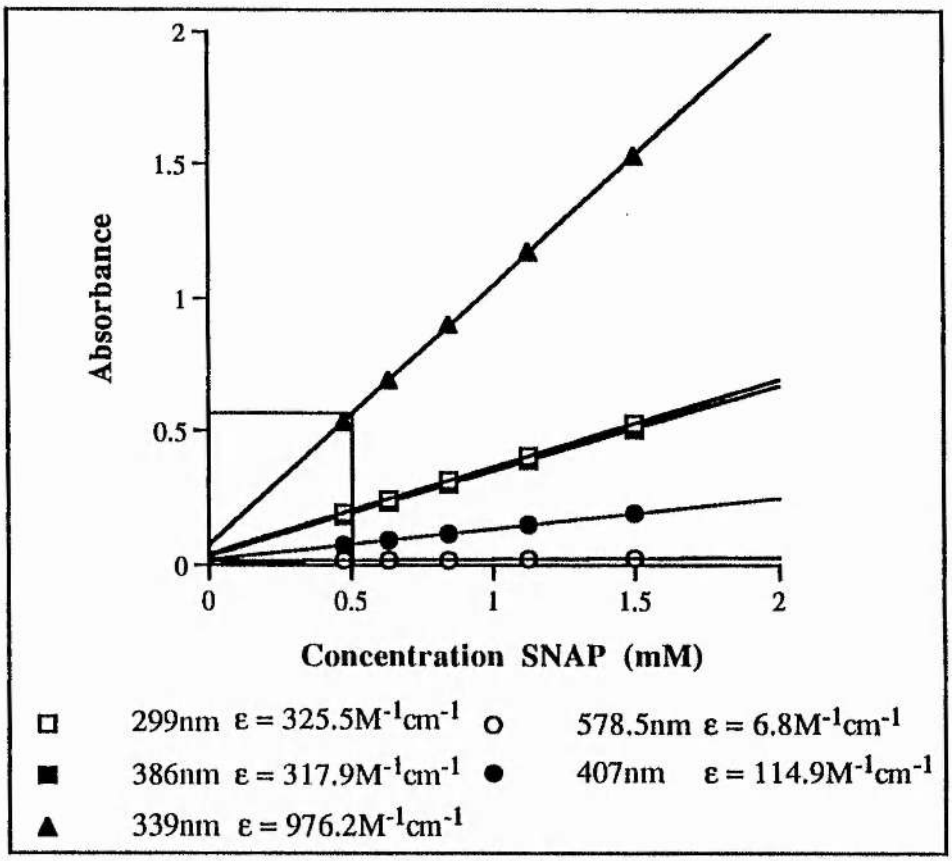


Figure 2.10 *The extinction coefficients at different wavelengths for SNAP (box indicates absorbance at 339nm of 0.5mM solution of SNAP)*

Figure 2.4). In this case, the SNAP had disappeared over 40 minutes whereas in **Figure 2.4** the average of 11 runs suggests that about 35% of SNAP still remains over the same time period. The only differences between these two experiments,other than the method of SNAP synthesis, is the increased buffer capacity used when making SNAP *in situ* to buffer against the addition of the acidic solution of S-nitrosothiol and the slightly higher concentration of nitrosothiol used in this experiment (0.5mM compared to 0.4mM). Both of these factors should not account for the large differences in SNAP decomposition rates. However, even with this increased rate of SNAP decomposition, a trend was evident as SNAP was still found to be more stable than the non-acetylated SNPen.

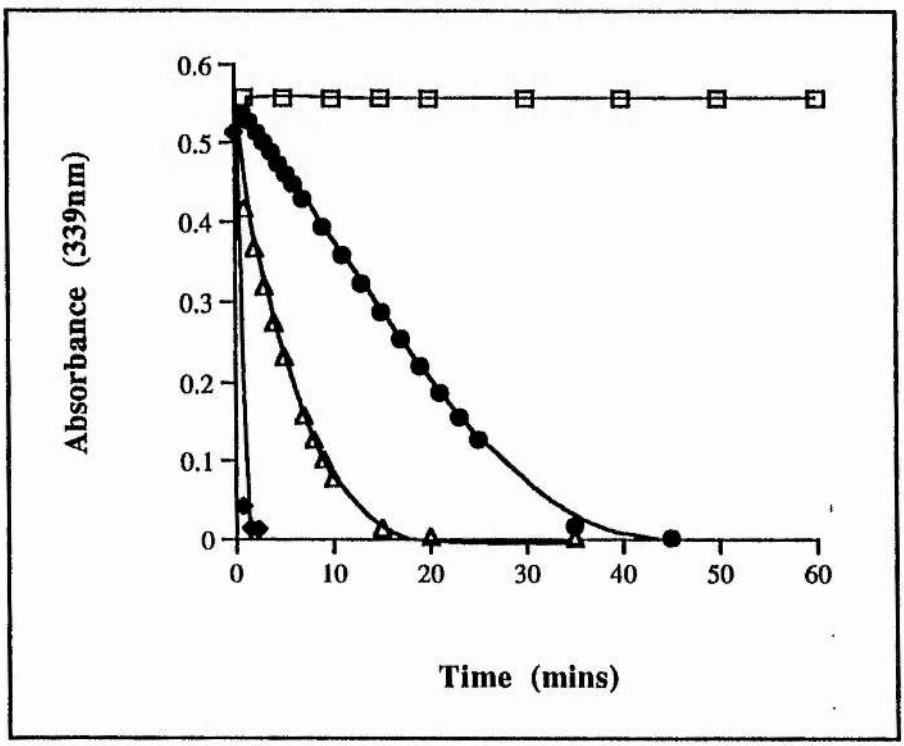


***Figure 2.11** The comparative stability of SNC, SNAC, SNPen, SNAP in phosphate buffer at pH7.4, comparing their absorbance profiles at 339nm. SNC, (closed diamond), SNAC (open square), SNPen (open triangle), SNAP (closed circles).*

It seems reasonable to conclude that the acetyl group has a stabilising effect on small S-nitrosothiols and the reasons why SNAC is more stable than SNAP (synthesised *in situ* or as a pure solid) will be discussed in more detail in **Chapter 3.**

ii) Experimental

Synthesis of GSNO and the other S-nitrosothiols *in situ*

0.4M H_2SO_4 (5ml) was added to glutathione (0.021g) and sodium nitrite (0.0044g) crystals in an aluminium foil covered vessel on ice. 0.1ml of the resulting pink/red solution was added to 2.4ml of phosphate buffer (0.3M KH_2PO_4/ 0.3M NaOH) at pH7.4 in a spectrophotometer cuvette at 30°C and the absorbance reading taken.

The same method was used for the other S-nitrosothiols mentioned in this section and the changes in absorbance at 339nm were monitored at timed intervals to gain their stability profiles in aqueous solution shown in **Figure 2.11.**

The calculation of extinction coefficients of SNAP and GSNO at different wavelengths

1.5mM solutions of SNAP (3.3mg in 10ml) and GSNO (5mg in 10ml) in phosphate buffer (0.1M KH_2PO_4/0.1M NaOH) pH7.4, 30°C, were made up in the presence of 1mM EDTA (3.7mg in 10ml) to stop metal ion catalysis see **Chapter 3.** These two solutions were diluted down further, using the buffer, to form solutions of concentrations 1.125mM, 0.844mM, 0.633mM and 0.399mM and the absorbances of the 5 concentrations for both GSNO and SNAP were monitored and recorded at 299,335 (GSNO only; λ_{max}), 339, 385, 407, 578.5 (SNAP only; λ_{max}) and 546nm (GSNO only; λ_{max}).

CHAPTER 3

THE METAL ION-CATALYSED DECOMPOSITION OF SNAP & GSNO

3.1 INTRODUCTION

Much of this work was carried out in collaboration with Prof. D.H.L. Williams's research group at Durham University. However any of the figures presented in this section have been constructed from data I have obtained.

In chapter 2 it was shown that S-nitrosothiols such as SNAP decompose in aqueous solution at physiological pH to produce their disulphide and nitrite (NO_2^-) quantitatively (**Figures 2.1 & 2.2**). If oxygen is vigorously excluded from the aqueous SNAP solution then no nitrite is formed and nitric oxide can be detected using an NO-probe (Askew & Barnett *et al.*, 1994 *in prep*). Furthermore, if SNAP is allowed to decompose in the presence of N-methylaniline by following the reaction at 275nm, a quantitative yield of N-methyl-N-nitrosoaniline was obtained (Askew & Barnett *et al.*, 1994 in prep). If oxygen was excluded no nitrosamine was formed.

These results can be rationalised in terms of release of nitric oxide, which in the presence of oxygen produces a reagent capable of electrophilic nitrosation. This is consistent with the mechanism postulated in **Chapter 1.9** proposing the formation of N_2O_3 as the nitrosating agent (**1**).

$$\begin{aligned} NO + 1/2O_2 &\longrightarrow NO_2 \\ NO_2 + NO &\longrightarrow N_2O_3 \end{aligned} \qquad (1)$$

As this species would not be formed in the absence of oxygen, it would be expected that no electrophilic nitrosation of N-methylaniline would occur. These results imply that the reaction of NO_2 with NO is much faster than the hydrolysis of NO_2, which would produce an equal mixture of nitrite and nitrate. As the sole product of SNAP decomposition is nitrite, N_2O_3 is the favoured species. However it has been postulated, that an, as yet, uncharacterised species $N_xO_y^{n-}$ could be formed and act as the nitrosating agent (Wink *et al.*, 1993).

Whatever the mechanism by which S-nitrosothiols like SNAP are decomposed to produce nitrite at physiological pH, the rate at which it occurs, or more correctly, the rate at which NO is released from the SNAP molecule was found to vary dramatically from experiment to experiment, under, what seemed the same conditions. Data were not reproducible between the two laboratories and the kinetic order was not consistent or clear. Occasionally, reasonable first order plots were obtained and at other times good half-order plots occurred. This pattern has been recorded in the literature; one reporter quoting a second order dependence (Park, 1988).

From the action of metal ions on thiol containing compounds used and studied in biochemistry, it was suggested that trace metal ions could be causing this erratic behaviour. Consequently, it was decided to investigate this possibility to determine whether trace metal ions contained in buffer solutions (or distilled water) could contribute to the decomposition of SNAP in aqueous media.

This chapter reports an investigation of the metal ion catalysed decomposition of SNAP and some other S-nitrosothiols, particularly concentrating on the effect of copper which was found to be the best metal for catalysis.

3.2 COPPER CATALYSIS OF SNAP DECOMPOSITION

3.2.1 Results and Discussion

Due to the strong binding affinity of penicillamine for copper ions, evident in the amino acid's use as a chelating agent for copper ions in the treatment of Wilson's disease (see **Chapter 5**), it was decided to investigate whether copper(II) ions had a similar binding affinity for SNAP, which may contribute to its decomposition and release of NO. By following the decrease in absorbance at 339nm, it was shown that addition of increasing concentrations of copper(II) ions to SNAP solutions brought about faster rates of decomposition (see **Figure 3.1**). Furthermore, it was found that the addition

of small quantities of EDTA (10μM), which has high formation constants with many metal ions, to aqueous solutions of SNAP, dramatically reduced the decomposition rate (see **Figure 3.1**). In another set of experiments, the first order plots of SNAP decomposition with increasing concentration of copper (II) ions were obtained and showed that there was clearly copper catalysis with good first order correlation (see **Figure 3.2**). The only exception to this was the experiment conducted without addition of extra copper (II) ions, over and above those present in the buffer/distilled water.

As initial evidence of copper catalysis had been demonstrated, it was decided to investigate the possible catalysis of S-nitrosothiol decomposition by other metal ions. The effects of iron (II), iron (III), silver and mercury were studied and preliminary results are shown in **Figures 3.3 and 3.4**. It is evident that the addition of extra iron(III) and silver to the aqueous SNAP solutions at pH 7.4 had no effect on the decomposition rate of SNAP. However, iron(II) and mercury(II) did alter the rate of decomposition. Iron(II) was found to be of similar effectiveness as copper(II) at decomposing SNAP, whereas the decomposition profile of SNAP in the presence of mercury (II) was drastically different to that of the other metal ions tested. Work carried out by the Durham group on SNAP has proved that mercury acts differently by coordinating to the sulphur group (Hg^{2+}--S) and expelling NO^{+}. No production of NO was picked up by an NO electrode and the binding of mercury(II) to SNAP was found to be irreversible and non-catalytic (see **Figure 3.5**). Further work by Barnett and Williams and by Neave and Butler, has shown that trace quantities of Zn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+} and Cr^{2+} do not effect the rate at which SNAP decomposes.

The catalysis of S-nitrosothiols by iron(II) salts was not investigated in this study and this will be the subject of further work in the group. However the concentrations of this metal in aqueous aerated buffer solutions would probably be quite small due to

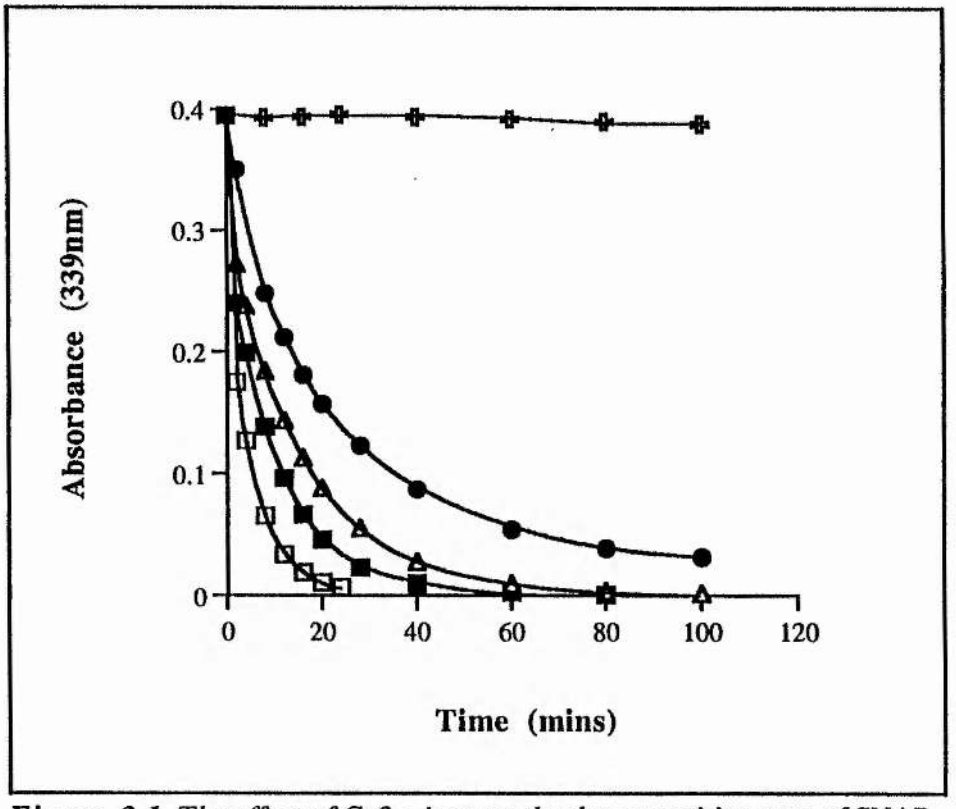


Figure 3.1 *The effect of Cu2+ ions on the decomposition rate of SNAP (0.4mM) [Cu2+] (open triangles) 5μM, (closed squares) 10μM, (open squares) 50μM. (closed circles) no added Cu2+, (open crosses) [EDTA] 50μM.30°C, KH_2PO_4 buffer pH 7.4.*

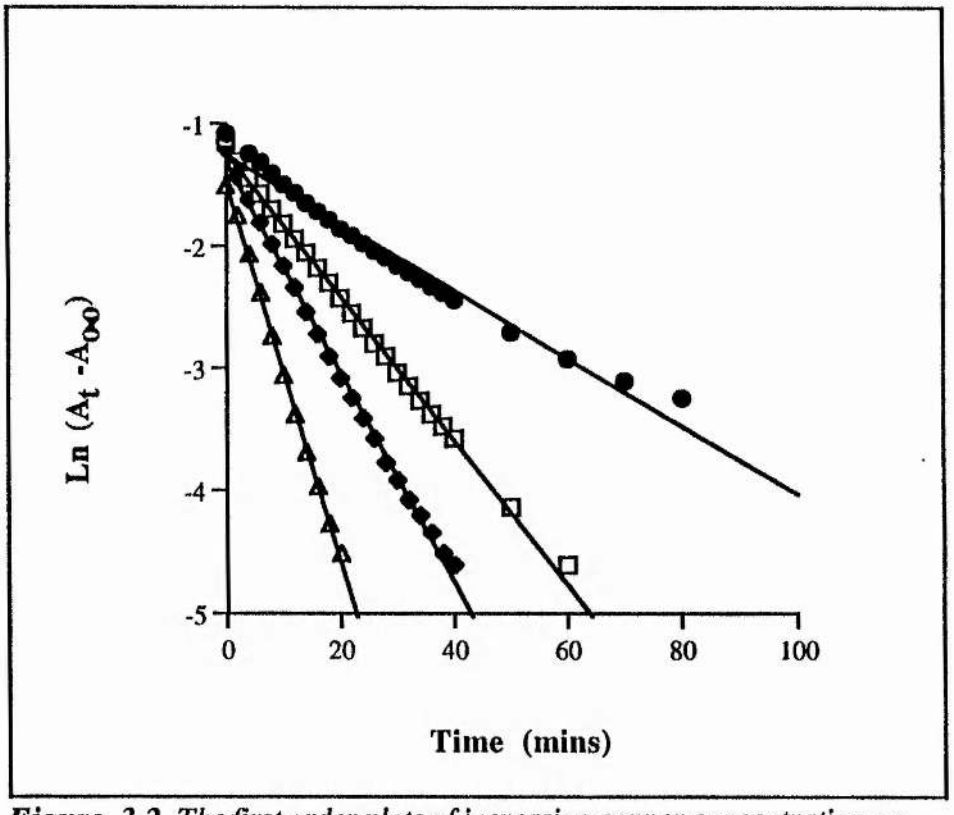


Figure 3.2 *The first order plots of increasing copper concentration on SNAP (0.4mM) decomposition rates.*

(closed circles) no added Cu^{2+}, y = -0.028x - 1.250, r=0.988, r^2=0.976

(open squares) 5μM Cu^{2+}, y = -0.059x - 1.231, r=0.999, r^2=0.997

(closed diamonds) 10μM Cu^{2+}, y = -0.086x - 1.303, r=0.999, r^2=0.997

(open triangles) 50μM Cu^{2+}, y = -0.155x - 1.477, r=0.999, r^2=0.999.

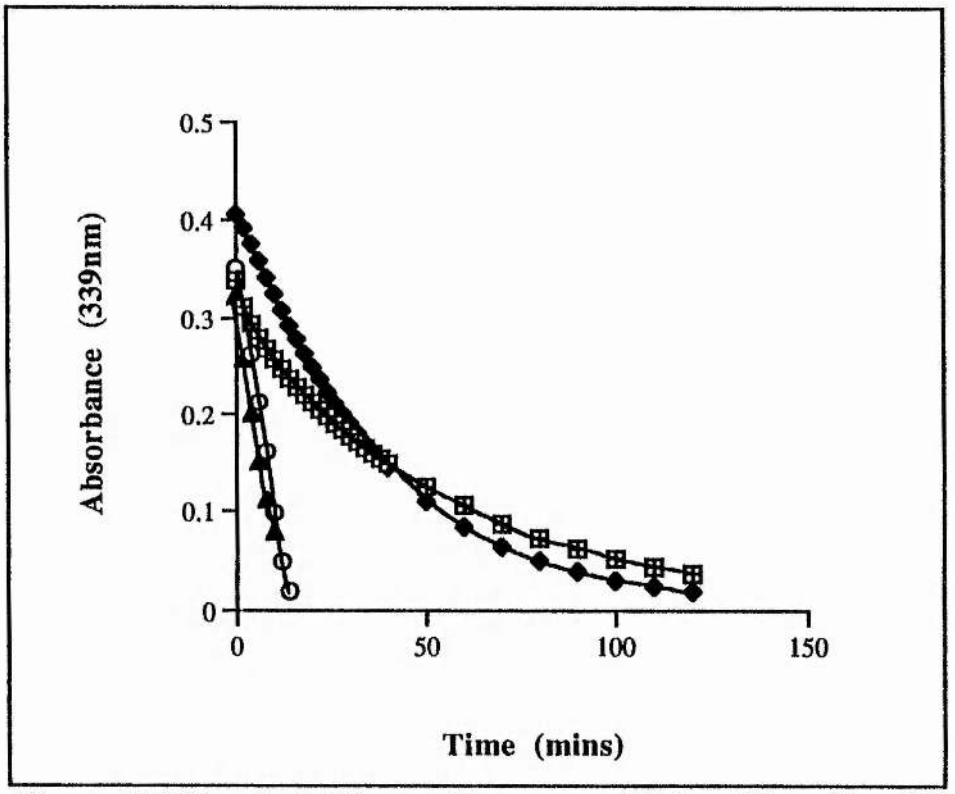


Figure 3.3 *The effect of the metal ions copper (II), iron (II) and iron (III) (0.1mM) on the decomposition rate of SNAP (0.4mM). (closed diamonds) no added metal ions, (squares with crosses) iron (III), (open circles) iron (II), (closed triangles) copper (II).*

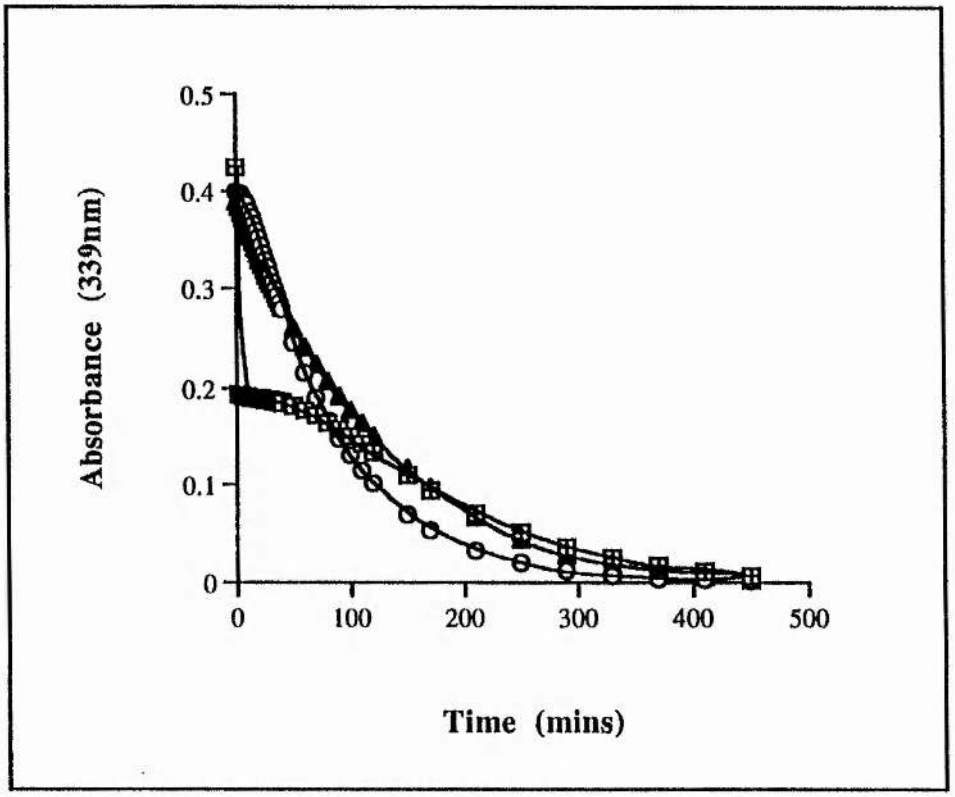


Figure 3.4 *The effect of the metal ions mercury (II) and silver(I) (0.1mM) on the decomposition rate of SNAP (0.4mM). (closed triangles) no added metal ions, (squares with crosses) mercury (II), (open circles) silver (I).*

oxidation to iron(III), which, as has been shown, does not effect the rate of SNAP decomposition.

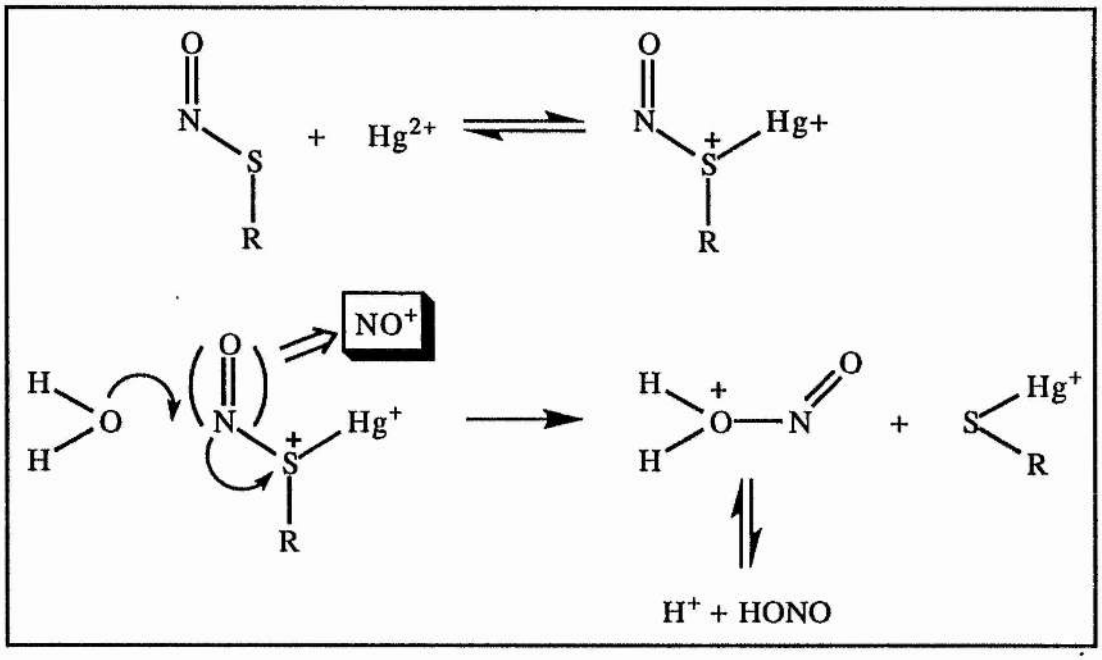


Figure 3.5 *The mechanism of action of mercuric salts on S-nitrosothiol decomposition*

Due to the effect of copper ions on SNAP decomposition, it was decided to calculate the metals concentration in the distilled water source and in the buffer constituents used. Using atomic absorption it was evident that the trace amounts of copper that were proposed to be responsible for the erratic decomposition of SNAP were not coming from the distilled water source. This had been the case for initial work conducted by the Durham group and reported in our first collaborative paper (McAninley *et al.*, 1993). The levels present in the distilled water used for all the experiments discussed in this thesis could not be measured on the parts per million (ppm) scale. However, it was likely that trace metal ions like copper were present in the other buffer constituents used. As most of the experiments were conducted at pH 7.4 in a phosphate buffer (0.1M KH_2PO_4/0.1M NaOH), it was decided to measure the total copper content of this buffer mixture. Unfortunately due to the high concentrations of sodium hydroxide used, atomic absorption could not be carried out on the sample unless ion exchange to

remove the sodium was done first. It was decided against carrying out this procedure due to possible further contamination of the buffer sample with metal ions.

To get an estimate of the maximum concentration of copper ions likely to be present in the buffer system, calculations were carried out using the maximum values of the trace amounts of copper written on the containers of the buffer constituents, supplied by the chemical companies. After taking concentrations and volumes into account, it was calculated that 0.03ppm of copper was present in the buffer solution at pH 7.4 (see experimental section), which converted into a maximum concentration of 0.47μM of copper, most of which came from the sodium hydroxide source, as Aristar grade phosphate was used. Therefore there will be enough copper ions present to catalyse the decomposition of SNAP.

From **Figure 3.1** it is evident that complexation of the metal by EDTA dramatically reduces the catalysed process. When copper (II) ions were added to solutions of SNAP in the presence of EDTA (10μM), only addition of concentrations of copper ions equal to or above the concentration of EDTA resumed the copper ion-catalysed decomposition (see **Figure 3.6**).

A more quantitative examination of the copper catalysed process was undertaken by following the decomposition of SNAP as a function of copper (II) ion concentration. By increasing the concentration of copper ions with respect to SNAP concentration, a 'window' of good first order kinetics was obtained. This 'window' varied slightly from one sample of SNAP to another. The reason for this is unclear but the kinetics did vary between the two laboratories. However, above and below the 'window' of good first order behaviour, poor first order fits were obtained. At higher concentrations the loss of first order linearity and erratic behaviour could be due to complex formation involving copper and the phosphate component of the buffer solution which forms an insoluble salt.

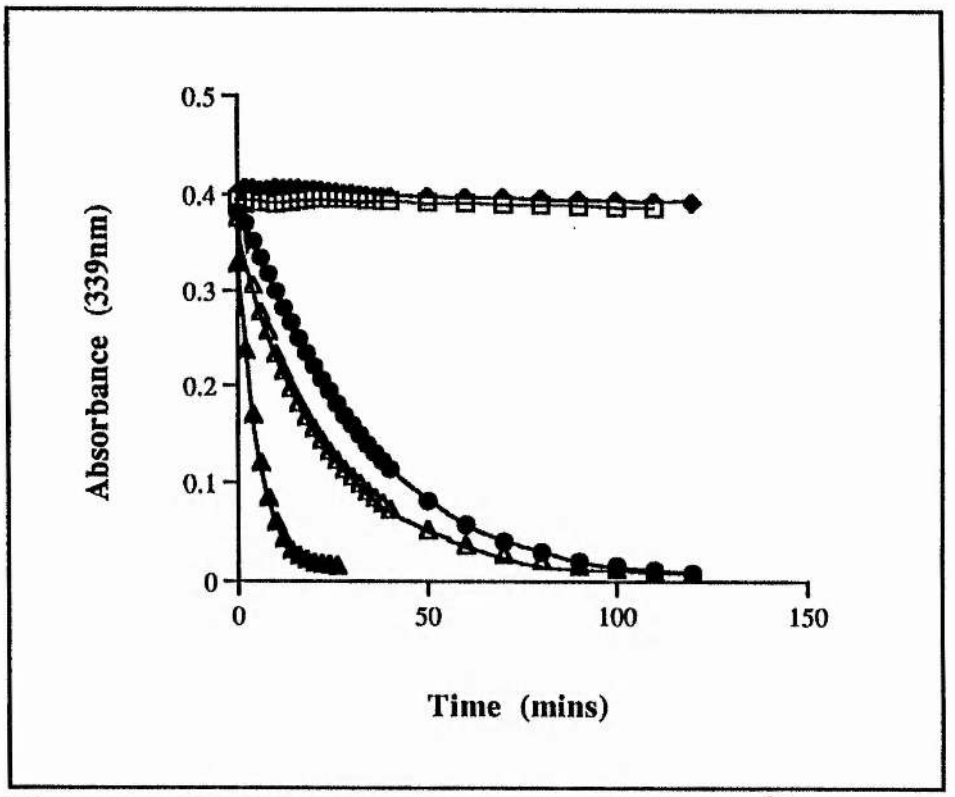


Figure 3.6 *The effect of increasing the concentration of Cu^{2+} ions with respect to EDTA (10μM) on the decomposition of SNAP (0.4mM). no added Cu^{2+} or EDTA (closed circles), EDTA + no added Cu^{2+} (open squares), EDTA + 5μM added Cu^{2+} (closed diamonds), EDTA + 10μM added Cu^{2+} (open triangles), EDTA + 100μM added Cu^{2+} (closed triangles) .*

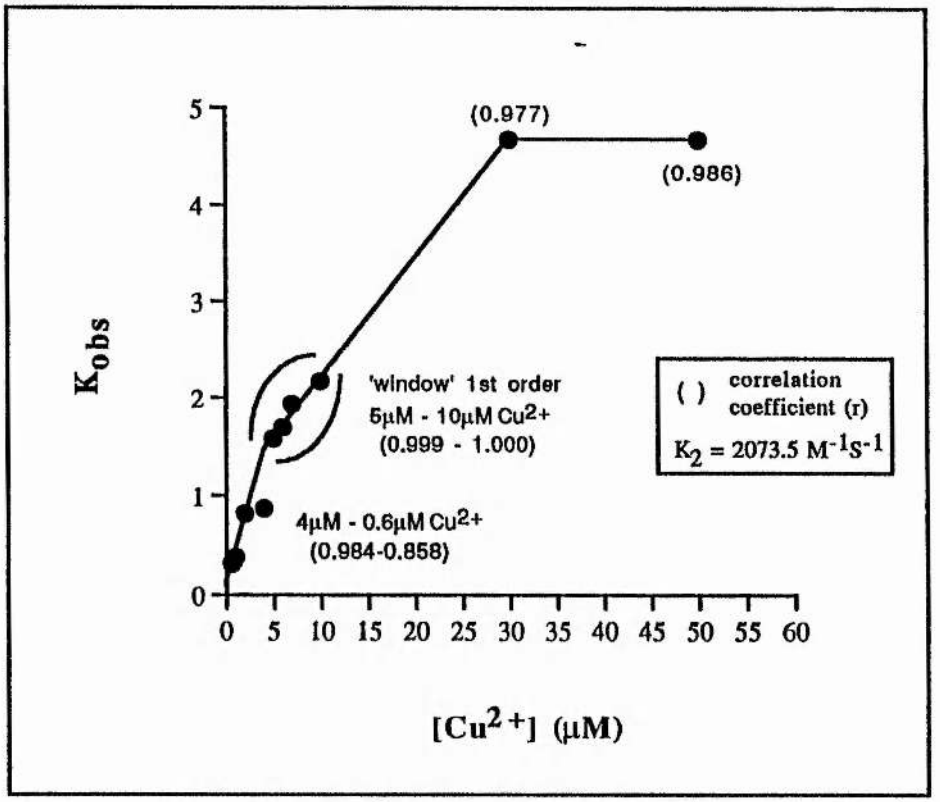


Figure 3.7 *The effect of Cu^{2+} ions on the decomposition of SNAP, a plot of k_{obs} vs $[Cu^{2+}]$. (closed circles) k_{obs}, k_2 = 2nd order rate constant calculated from the gradient of the straight line through the 1st order 'window'*

Figure 3.7 shows a graph of increasing copper concentration against k_{obs} for a range of experiments following the decomposition of SNAP at 339nm. A 'window' of 1st order kinetics was obtained between 5 and 10μM, which lay on the same straight line. The gradient of this line is equivalent to k_2, the second order rate constant and the results obtained were consistent with the rate equation (**2**). The small intercept, which was always present for all SNAP samples tested, probably represents the component of the reaction catalysed by the residual copper (II) ions present in the buffer system.

$$\text{Rate} = k_2 [\text{RSNO}] [Cu^{2+}] \qquad (2)$$

This rate equation has been shown to hold for a large range of S-nitrosothiol structures (Askew & Barnett *et al.*, 1994, *in prep.*).

It has also been shown that copper(I) ions can catalyse the decomposition of S-nitrosothiols. It is also evident from **Figure 3.8** that copper(1) is a better catalyst than copper (II). Sodium dithionite was used to reduce copper(II) nitrate to the copper(I) salt. When this was carried out in an areated environment a characteristic red colour appeared due to the formation of copper (I) oxide (Askew & Barnet *et al.*, 1994 *in press*). This rapidly disproportionates to copper(0) and copper(II). However, although disproportionation is favourable in aqueous solution, the low solubility of copper(I) in water renders this reaction less important than oxidation to copper(II). Therefore, in the graph presented in **Figure 3.8**, copper(I) was formed by reduction of copper(II) nitrate in a deareated environment to prolong the lifetime of the copper(I) species. In addition, complex formation notably increases the solubility and the stability of copper(I) salts, thus reducing disproportionation. As copper(I) is a soft acid it would be expected to readily form complexes with the phosphate buffer used. The probable equation for copper(I) formation is shown below.

$$2Cu^{2+} + S_2O_4^{2-} + 4OH^- \rightarrow 2Cu^+ + 2SO_3^{2-} + H_2O \qquad (3)$$

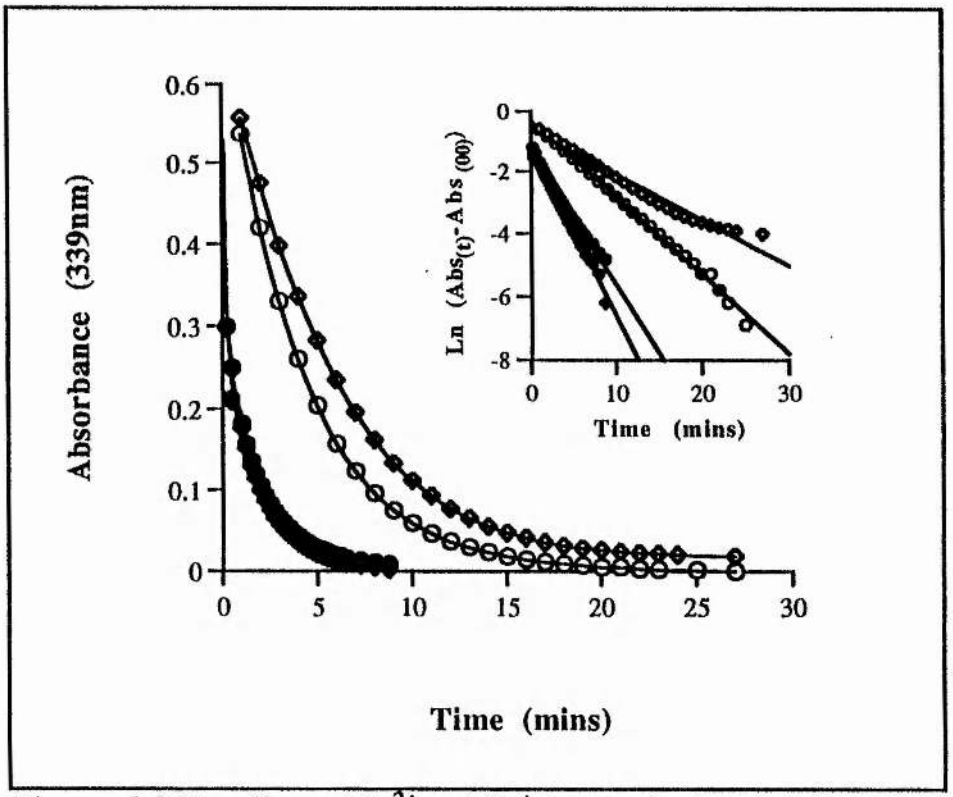


Figure 3.8 *The effects of Cu^{2+} and Cu^{+} ions on the decomposition rate of SNAP (0.5mM) in phosphate buffer (0.3M) pH7.4 in an oxygen free environment. (open symbols) Cu^{2+} (5μM), (closed circles & crossed diamonds) Cu^{+} (5μM).* ***Inset****; 1st order plots of these samples ,*
Cu^{2+} y = -0.333-0.249x, r = 0.998 & y = -0.639-0.147x, r = 0.985;
Cu^{+} y = -1.216-0.537x, r = 0.998 & y = -1.374-0.425x, r = 0.994.

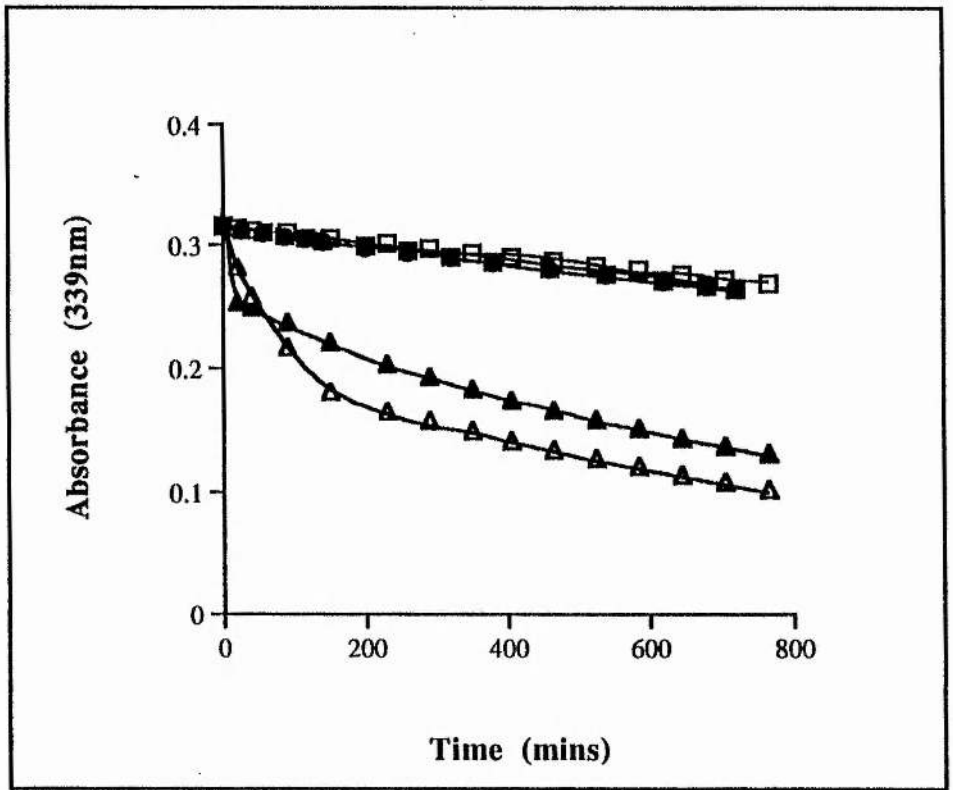


Figure 3.9 *The effects of Cu^{2+} and Cu^{+} ions on the decomposition rate of SNAP (0.4mM) in acetonitrile. (closed circles) no added Cu^{2+}/Cu^{+}, (open squares) Cu^{2+} (1μM), (open triangles) Cu^{2+} (100μM), (closed squares) Cu^{+} (1μM), (closed triangles) Cu^{2+} (100μM).*

When the decomposition rates of SNAP by copper(I) and copper(II) ions in acetonitrile were compared (used because it is a solvent which stabilises the copper (I) salt by forming ligands, such as $[Cu(NCMe)_4]^+$), it is noticeable that the two forms of copper ion effect the rate, even though the rates of decomposition are much slower in this solvent (possibly due to complexation of the metal ions to the solvent, which may stop them from acting on the S-nitrosothiol). These results are shown in **Figure 3.9.**

These findings suggest that copper ions may catalyse the decomposition of S-nitrosothiols such as SNAP by some sort of redox reaction, in which the copper changes its redox state between 1^+ and 2^+ to affect catalysis. Many bioinorganic reactions proceed this way and this could explain why copper is one of the only transition metal ions to effect catalysis of S-nitrosothiols. The mechanism by which copper brings about catalysis of S-nitrosothiols like SNAP is discussed in more detail in **Chapter 3.4.**

3.2.2 Experimental

Chemicals used - Analar grade copper(II) nitrate trihydrate and copper(I) chloride and aristar grade potassium dihydrogen orthophosphate were obtained from BDH Laboratory supplies, Poole, England. Analar grade iron(III) chloride, iron(II) sulphate acetonitrile and sodium hydroxide pellets were obtained from FSA laboratory supplies, Loughborough, England. EDTA, mercury(II) chloride and silver(I) chloride were obtained from Aldrich Chemicals, Dorset, England. The phosphate buffer was made up as described in the experimental section of **Chapter 4.3.** For experiments conducted using 0.3M phosphate buffer the quantities of KH_2PO_4 and NaOH were tripled. SNAP was synthesised as described in experimental section of **Chapter 2.2.2.**

Figure 3.1

A stock solution (6.25mM) of copper (II) nitrate trihydrate was made up (0.0151g in 10ml water), and diluted by a factor of 10 to form 0.625mM solution. An aliquot of

this solution (1ml) was equally diluted with water and 0.4ml of this solution were added to phosphate buffer (0.1M KH_2PO_4/0.1M NaOH; 2ml) at pH 7.4. When an aliquot (0.1ml) of SNAP solution (0.01M; 0.011g in 5ml buffer) was added to this solution in the spectrophotometer cuvette at 30°C, the final concentrations of SNAP and copper(II) ions in the cuvette were 0.4mM and 50μM 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 0.4mls to the cuvette and 0.1ml of the SNAP stock solution. Immediately after addition of the 0.1ml of SNAP (which had been kept on 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 (10.4μM) was made up in buffer (2.4ml) from a stock solution (0.0388g in 10ml buffer) which after addition of the stock SNAP solution (0.1ml) resulted in the final concentration of EDTA of 10μM.

Figure 3.2

The first order plots of the effects of increasing copper concentration on the decomposition of SNAP were obtained by subtracting the infinity absorbance reading from all the other readings of the samples described in **Figure 3.1**. A plot of ln ($A_{(t)}$ -$A_{(\infty)}$) with time was constructed.

Figures 3.3

Stock solutions (11mM) of iron(III) chloride (0.0178g in 1ml distilled water), iron(II) sulphate (0.0306g in 10ml distilled water), and copper(II) nitrate trihydrate (0.0266g in 10ml distilled water), were made up and diluted by a factor of 100 (0.1ml in 9.9ml) in phosphate buffer pH 7.4 to form 0.11mM solutions. Aliquots (2.25mls) of these solutions were added to the spectrophotometer cuvette with further addition of the SNAP solution (0.25ml of 4mM stock solution) to make up the required concentrations of 0.4mM SNAP and 0.1mM metal ion. The experiment was carried out as described for **Figure 3.1** using a reference cell containing the phosphate buffer at pH7.4.

Figure 3.4

The stock solutions (11mM), of silver (I) chloride (0.0158g in 10ml distilled water), and mercury (II) chloride (0.0299g in 10ml) were made up, diluted and used as described for **Figure 3.3**.

Figure 3.6

A stock solution (12.5mM) of EDTA (0.0465g in 10ml buffer) was made up and diluted 1000 fold to 12.5μM in phosphate buffer. 2ml of this solution was added to the spectrophotometer cuvette at 30°C in the spectrophotometer. A stock solution (6.25mM) of copper(II) nitrate trihydrate (0.0151g in 10ml distilled water) was diluted by a factor of 10 to form a 0.625mM solution, from which an aliquot (0.4ml) was added to the EDTA solution in the cuvette. After addition of an aliquot (0.1ml) of SNAP (4mM; 0.011g 1n 5ml buffer), the final concentrations of SNAP, Cu^{2+} ions and EDTA were 0.4mM, 0.1mM and 10μM respectively. The other concentrations of copper (II) ions were made up by further serial dilution of the stock solution of copper(II) nitrate, and for EDTA alone, an aliquot (0.4ml) of buffer not containing added copper, was mixed with the EDTA solution. The reference cell contained phosphate buffer and EDTA (10μM).

Figure 3.7

Serial dilution of a stock solution (25mM) of copper (II) nitrate trihydrate (0.0302g in 5ml distilled water) was used to make up the required concentrations of copper (II) ions presented in **Figure 3.7**. In each case aliquots (0.1ml) of the diluted copper solutions were added to the phosphate buffer pH7.4 (2.3ml). Aliquots (0.1ml) of the stock SNAP solution (12.5mM) were added to the resulting solutions in the spectrophotometer cuvette at 30°C, to make up the final solutions which contained 0.5mM SNAP. All samples were run against a reference cell containing phosphate buffer.

Figure 3.8

A stock solution (5.5mM) of sodium dithionite (0.0048g in 5ml of buffer) was made up in oxygen free buffer. An aliquot (0.1ml) was added to a deoxygenated solution

(9.9ml) of copper(II) nitrate (50μM) which was made up by serial dilution from a stock solution (5mM; 0.0060g in 5ml deoxygenated buffer). This was carried out to form copper(I) ions (50μM). An aliquot (0.25ml) of this solution was added to 2ml of deoxygenated phosphate buffer (0.3M KH_2PO_4/0.3M NaOH) in the spectrophotometer cuvette at 30°C. SNAP (0.25ml; 5mM) was added and the reaction was monitored immediately. The same process was carried out without the addition of dithionite to directly compare the action of copper(II) ions on SNAP decomposition. Both sets of experiments were run with a reference cell containing phosphate buffer (0.3M).

Figure 3.9

Stock solutions (11.1mM) of copper(I) chloride (0.011g in 10ml acetonitrile) and copper(II) nitrate trihydrate (0.276g in 10ml acetonitrile) were made up and serially diluted in acetonitrile to form 0.11mM and 1.11μM solutions. Aliquots (2.25ml) of these solutions were added to the spectrophotometer cuvette at 30°C and further addition of SNAP solution (0.25ml; 4mM) made up the final concentrations of copper(I) and copper(II) ions (0.1mM & 1μM) and SNAP (0.4mM). The reference cell contained acetonitrile.

Calculation of copper content in 0.3M KH_2PO_4/0.3M NaOH buffer pH 7.4.

Distilled water source = 0 ppm copper

NaOH source = 0.002% copper ≡ 20 ppm

mass of NaOH in 97.75ml of 0.3M NaOH within buffer solution = 1.173g

250ml buffer ∴ 20 x 1.173/250 = 0.09 ppm of copper from NaOH in buffer

KH_2PO_4 source = 0.005 ppm

mass of KH_2PO_4 in 125ml of 0.3M KH_2PO_4 within buffer solution = 5.10g

250ml buffer ∴ 0.005 x 5.10/250 = 0.1 ppb of copper from NaOH in buffer.

∴ Total copper present = 0.0901ppm ≡ 1.4μM

in 0.1M KH_2PO_4/0.1M NaOH; total copper present = 0.47μM.

3.3 THE BINDING OF COPPER IONS TO S-NITROSOTHIOLS

3.3.1 Results and Discussion

There was found to be a wide range of second order rate constants for the S-nitrosothiols tested, indicating substantial differences in reactivity towards copper(II) ions (Askew & Barnet *et al.*, 1994, *in press*). Three of the most reactive S-nitrosothiols were S-nitrosocysteine (SNC), S-nitrosopenicillamine (SNPen) and S-nitrosocysteamine (SNCA). These compounds all have free amine groups. An explanation for their fast decomposition in the presence of copper ions is that these ions become bidentately complexed with the nitrogen atoms of the nitroso group and the amino group, via a six membered ring shown in **Figure 3.10**.

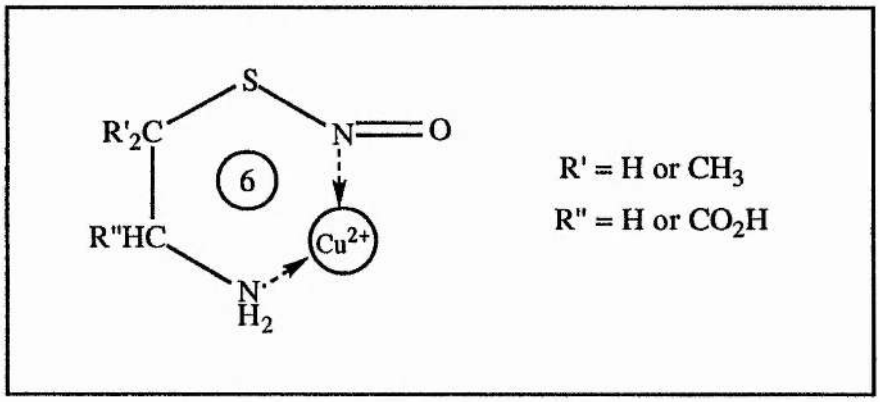


Figure 3.10 *The proposed six-membered ring intermediate formed when copper binds to SNPen, SNC and SNCA.*

Although the proposed binding structure of copper to the amino acids is presented as a 2 coordinate species, it is very likely that 4 or 6 coordination involving water molecules (or even the OH group at pH 7.4) occurs. These have not been included for simplicity of presentation. The first order dependence upon RSNO concentration argues against two molecules of S-nitrosothiol being involved in a four coordinate system.

The coordination chemistry of copper (II) is dominated by coordination to N and O sites (Hathaway, 1987; Stricks & Kolthoff, 1951), although coordination to sulphur and other elements is also well known. Much of the bioinorganic chemistry of copper (II) involves coordination compounds of this type, eg. with proteins and peptides (Pettit et al., 1985).

When amine and acid groups are absent, as in the case for S-nitroso-t-butyl thiol, the S-nitrosothiol is very stable and there was no measurable reactivity (Askew & Barnett *et al.*, 1994, *in prep*). Furthermore, increasing the chain length by one methylene group (as in S-nitrosohomocysteine) results in a large rate reduction, probably due to decomposition via the more unfavourable seven-membered ring intermediate (Askew & Barnett *et al.*, 1994, *in prep*). These variations will inhibit the formation of what seems like the more favoured six-membered ring intermediate.

Interestingly, when the decomposition rates of S-nitrosocysteine (SNC) and S-nitrosopenicillamine (SNPen) (which were made in situ see **Chapter 2**) were compared in the presence of increasing concentrations of EDTA (10μM-1mM), it is evident that EDTA has less of a stabilising effect on SNPen than SNC (see **Figures 3.11 & 3.12**). A possible explanation of this result is that the parent amino acid of SNPen, penicillamine, has a strong binding affinity for copper, more so than cysteine, and it has been shown that penicillamine can compete with EDTA for copper chelation (Hanaki & Sago, 1994; & **Chapter 5.1**). Whether this implies that the nitrosated derivative has similar binding affinities for copper has yet to be determined, but the fact that even 1mM concentrations of EDTA cannot abolish its decomposition, supports this theory. This finding may also explain why S-nitroso-N-acetyl cysteine (SNAC) was found to be substantially more stable than SNAP (see **Chapter 2 Figure 2.12**).

Further evidence supporting the theory that the S-nitroso derivatives of penicillamine compete better for copper ions in the presence of EDTA than the S-nitroso derivatives

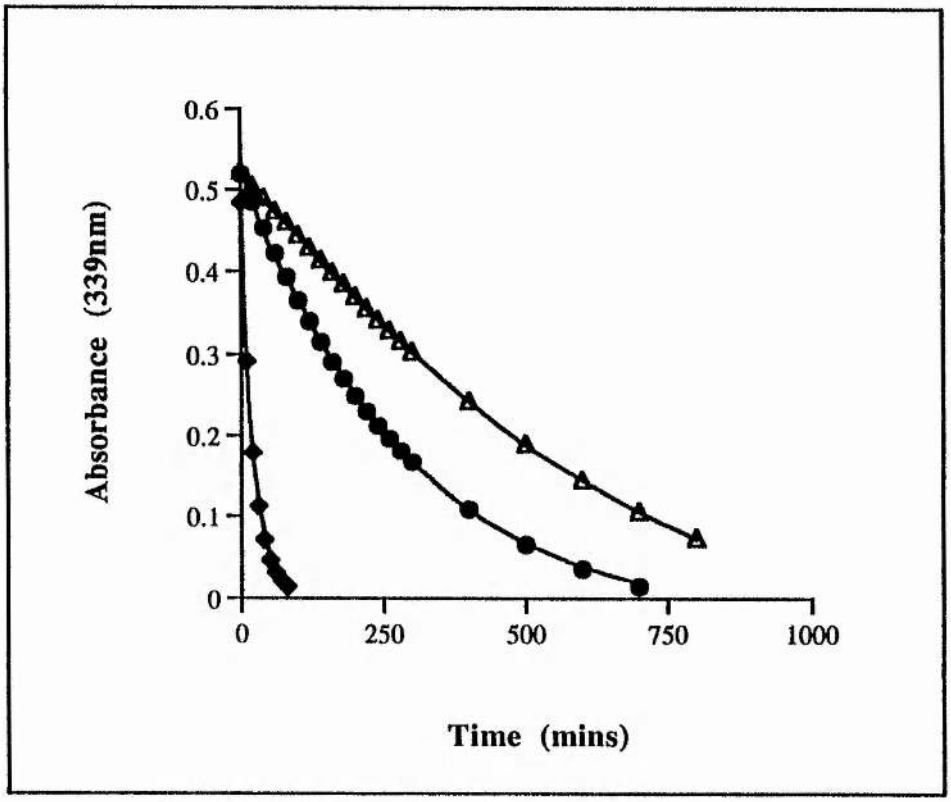


Figure 3.11 *The effect of increasing the concentration of EDTA (10-1000μM) on the decomposition rate of S-nitrosopenicillamine (SNPen, 0.5mM). (diamonds with crosses) 10μM EDTA, (closed circles) 100μM EDTA, (open triangles) 1000μM EDTA.*

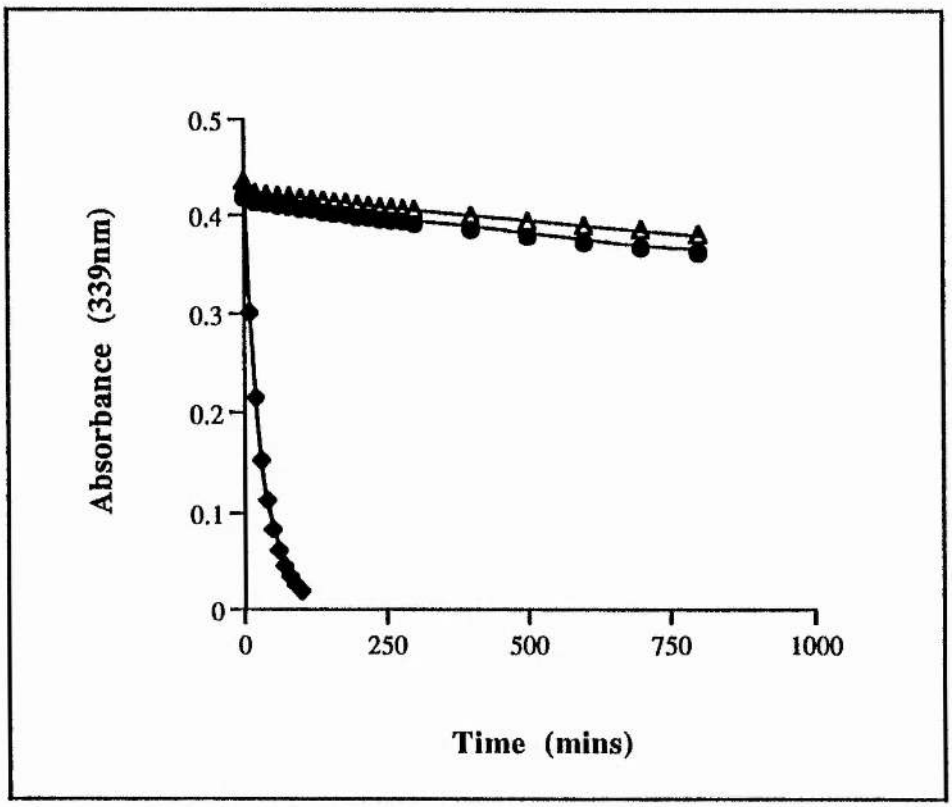


Figure 3.12 *The effect of increasing the concentration of EDTA (10-1000μM) on the decomposition rate of S-nitrosocysteine (SNC, 0.5mM). (diamonds with crosses) 10μM EDTA, (closed circles) 100μM EDTA, (open triangles) 1000μM EDTA.*

of cysteine is evident in **Figure 3.13.** SNAC was found to be much more resistant to decomposition than SNAP in the presence of EDTA (10μM) (and no added copper ions), having a similar stability to GSNO under the same conditions.

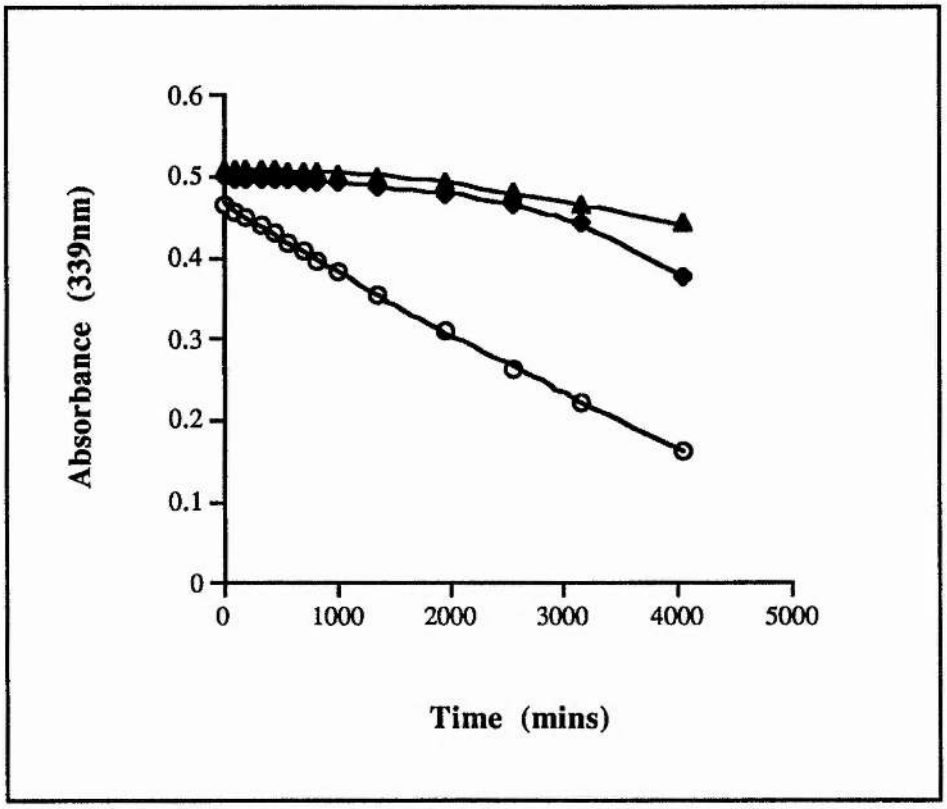


Figure 3.13 *The effect of EDTA (10M) on the decomposition rates of SNAP, GSNO and SNAC (all 0.5mM). SNAP (open circles), GSNO (diamonds with crosses), SNAC (closed triangles).*

N-acetylation of the amino group of SNC and SNPen to form SNAC and SNAP has been shown to have a dramatic effect on decomposition rates, reducing the reactivity in the case of SNAC to virtually unmeasurable levels (see **Chapter 2 Figure 2.12**) This is consistent with the expected large reduction in the electron density on the amino nitrogen atom due to its acetylation, which will make coordination with copper much weaker. The fact that SNAP does decompose in the presence of copper ions may have something to do with the argument presented in the previous paragraph, which could relate to the presence of the gem-dimethyl groups of the penicillamine amino acid. However, another possible theory is that it decomposes slowly through a seven-

membered ring intermediate involving coordination with the nitroso nitrogen atom and one of the oxygens of the carboxylate group (see **Figure 3.14**).

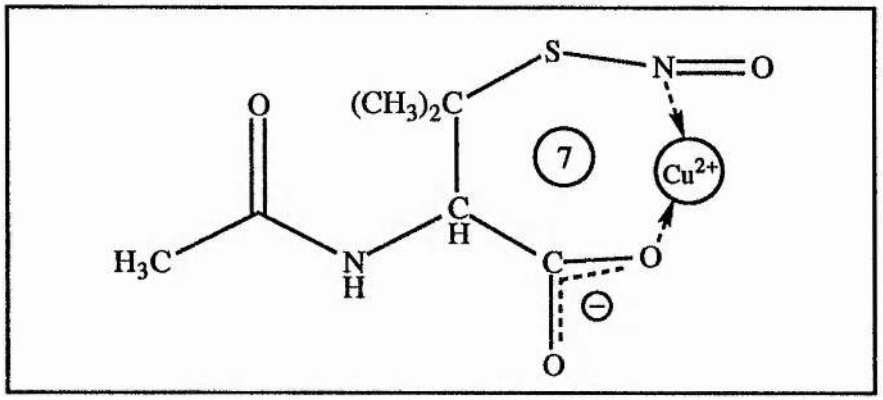


Figure 3.14 *The possible formation of the more unfavoured seven-membered ring when copper forms an intermediate with SNAP.*

To complicate the situation further, it is well known that coordination between copper (II) ions and carboxylic acids occurs (Hathaway, 1987). Indeed a number of copper (II) carboxylates have been isolated and examined structurally (Doedens, 1976). In the solid state they are binuclear with four carboxylate bridges (see **Figure 3.15**).

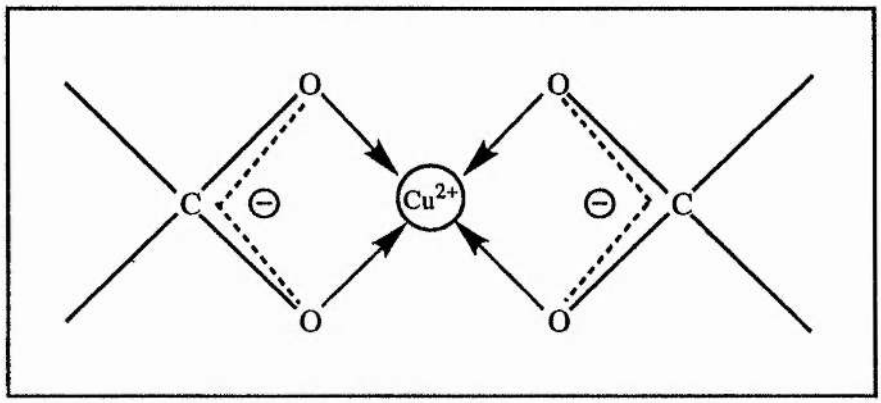


Figure 3.15 *The general binuclear structure of copper binding to carboxylate groups*

The nitrosated tripeptide, S-nitrosoglutathione, was found to be very resistant to copper catalysis even in the presence of a 50μM concentration of the metal (see **Figure 3.16**). From the theory already discussed, this finding is not suprising as the appropriate nitrogen atoms are acetylated. Furthermore, there are two carboxylate sites

in the molecule that will readily bind copper ions, which will reduce the number that can act on the 'active site' (the S-nitroso moiety). There is also a free amine group, with its substantial electron density, which will attract binding from metal ions such as copper, and two amide linkages which are known to have a high binding constants for copper (II) ions ($K_f \sim 10^5$; Pettit *et al.*, 1985).

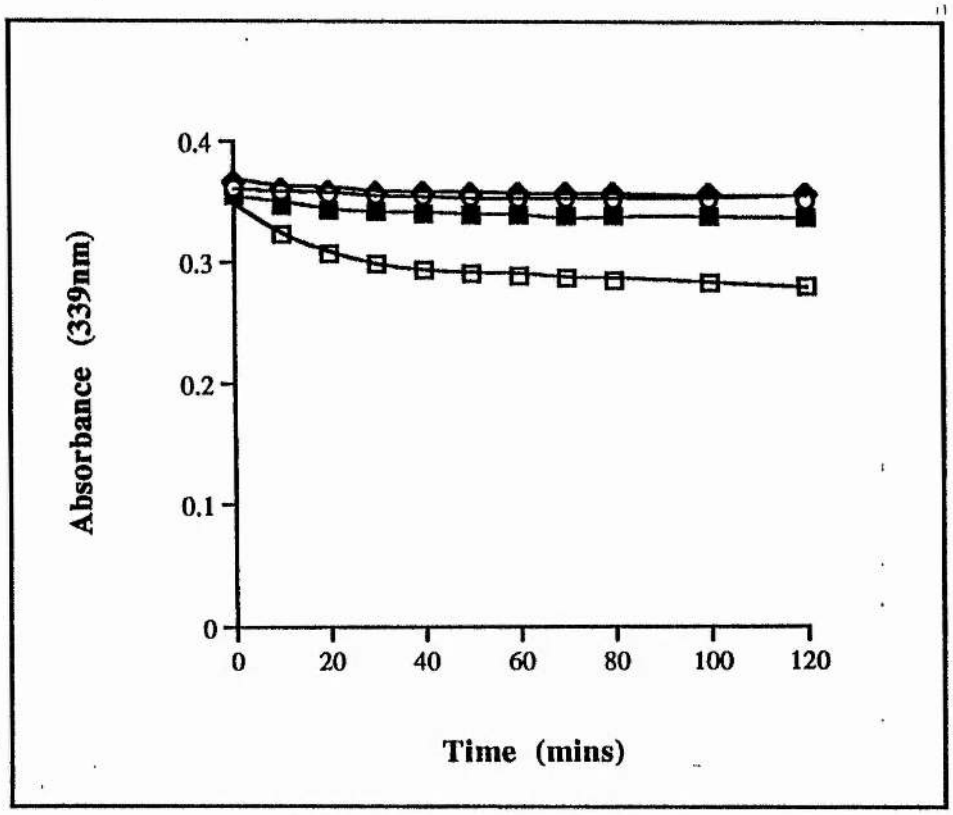


***Figure 3.16** The effect of Cu^{2+} ions on the decomposition rate of GSNO (0.4mM) [Cu^{2+}] 1μM (closed diamonds), 10μM (closed squares) ,50μM (open squares). no added Cu^{2+} (open circles), 30℃, KH_2PO_4 buffer pH 7.4.*

In conclusion, our results are consistent with the formation of a six-membered ring intermediate (shown in **Figure 3.10**), being the most favourable conformation to elicit copper ion catalysis of basic S-nitrosothiols, such as S-nitrosopenicillamine and S-nitrosocysteine. When structural changes are made to these S-nitrosothiols such as N-acetylation, removal of favoured ligands such as amine groups or carboxylates, or increasing the chain length between the two ligand binding sites, the stability of the S-nitrosothiol increases due to inhibition or reduction of the formation of this six-

membered ring intermediate. The larger the peptide chain, and the more carboxylate or free amine groups present, but removed from the S-nitroso-moiety, the greater the number of binding sites for copper, and the less chance of decomposition of the nitrosopeptide with loss of NO. For these reasons GSNO is more resistant than SNAP or most other S-nitrosothiols investigated.

3.3.2 Experimental

Figures 3.11 & 3.12

A stock solution of EDTA was made up and serially diluted to the required concentrations (10mM, 1mM and 0.1mM). By addition of an aliquot (0.25ml) of these solutions to spectrophotometer cuvettes containing 2.15ml of 0.3M KH_2PO_4/0.3M NaOH and aliquots (0.1ml) of the S-nitrosothiol (12.5mM) to be tested, the final concentrations of EDTA and S-nitrosothiol were 1mM-10μM and 0.5mM respectively. S-nitrosocysteine (12.5mM) and S-nitrosopenicillamine (12.5mM) were made up *in situ* by adding sulphuric acid (0.4M; 10ml) to L-cysteine (0.0151g) and L-penicillamine (0.0187g), both containing sodium nitrite (0.0086g). 0.1mls of these S-nitrosothiols were immediately added to the cuvettes containing EDTA and buffer (0.3M phosphate; 2.4ml). The reference cell contained buffer and the relevant concentrations of EDTA to be tested.

Figure 3.13

Stock solutions (12.5mM) of GSNO, SNAP and SNAC were made up by adding sulphuric acid (0.4M; 10ml) to glutathione (0.0384g), N-acetyl-DL-penicillamine (0.0239g) and N-acetyl-L-cysteine (0.0204g), all in the presence of sodium nitrite (0.0086g). Aliquots (0.1ml) of these samples were added to the spectrophotometer cuvette containing EDTA (10μM) in buffer (0.3M phosphate; 2.4ml). The reference cell contained buffer and EDTA (10μM).

Figure 3.16

The same experimental procedure was carried out as described for **Figure 3.1** in the previous experimental section. Aliquots (0.1ml) of the GSNO stock solution (10mM;

0.0168g in 5mls buffer) were added to 2.4ml of 0.1M phosphate buffer containing the relevant concentration of copper (II) ions. GSNO was synthesised as described in the experimental section of **Chapter 2.3.2.**

3.4 THE MECHANISM BY WHICH COPPER CATALYSES SNAP AND OTHER S-NITROSOTHIOL DECOMPOSITION

In this section the decomposition of SNAP by copper is discussed and used as a model for all S-nitrosothiols susceptible to copper catalysis.

3.4.1 Results and Discussion

In **Chapter 3.2** it was shown that copper(I) is a very effective catalyst of SNAP decompostion, and work carried out in the Durham laboratory has shown that this is also the case for other S-nitrosothiols (Askew & Barnett *et al.*, 1994 *in prep*). This brought into question whether SNAP decomposition could be catalysed by a redox process, in which there would be interconversion of copper between its Cu^{+} and Cu^{2+} states. Many of the bioinorganic reactions proceed this way and this could explain why copper is one of the only transition metal ions to effect catalysis of S-nitrosothiols. However, Barnett and Williams have examined the possibility of a redox $Cu^{2+} \leftrightarrow Cu^{+}$ interconversion by using ESR (Askew & Barnett *et al.*, 1994, *in prep*) and found no evidence for its presence. By following the ESR spectrum of copper(II) during the decomposition of SNAP in a static and a flow system, they were unable to detect a change in the spectrum during the reaction. In the absence of a buffer they attributed the spectrum to $Cu(H_20)_6^{2+}$ which was modified in the presence of a dimethylglutaric acid buffer system probably to a copper carboxylate species (see **Figure 3.15**). From these results it is possible to eliminate the quantitative conversion of copper (II) $\rightarrow$ copper (I) and its reappearance by oxidation.

An alternative possibility is that copper (II) ions coordinate to S-nitrosothiols such as S-nitrosocysteine or S-nitrosopenicillamine through the free amine group and the sulphur atom of the nitrosothiol moiety. This would result in the formation of a five-membered intermediate. As mentioned in **Chapter 5** and in **Chapter 1**, D-penicillamine is a well-known complexing agent for free copper (II) ions and is believed to be more efficient than EDTA. This is likely to involve a five membered ring species. **Figure 3.17 (A)** shows the visible traces of the absorbances between 438 and 900nm of the initial mixing and final absorbance after 16 hours of N-acetyl-DL-penicillamine (6.6mM) with copper (II) nitrate (6.6mM) in distilled water. The absorbance at 800nm is due to free copper (II) ions (aq), which have 9 d-electrons. **Figure 3.17 (B)** shows the visible absorbance trace of just copper (II) nitrate trihydrate (6.6mM) in distilled water and the absorbance of 0.085 at 804.8nm relates to an extinction coefficient of $12.88M^{-1}cm^{-1}$. It is evident that the absorbance at 800nm after initial mixing of N-acetyl-DL-penicillamine is substantially lower than this value at just over 0.06, which suggests that some binding of copper is occuring. It was noticeable that a rapid colour change transpired, producing a transient purple colour which is characteristic of copper-penicillamine complexes (Laurie, 1987). It is known that copper can cause the formation of the disulphides of thiols such as penicillamine (Laurie, 1987), and when the absorbance was monitored after 16 hours, it had returned to roughly the expected absorbance if all the copper was free. As binding of copper(II) ions to the disulphide is weak or nonexistent at the disulphide bridge and occurs only through the amine or carboxylate groups (Laurie, 1987), it is possible that more of the copper would be free and unbound.

Due to the fact that SNAP is N-acetylated and contains an NO group bound to the sulphur which is likely to have an adverse effect on the coordinating ability at sulphur, it is unlikely that copper(II) binds to form a five-membered ring intermediate. Furthermore, it is believed that the mercury(II)-catalysed and also the acid catalysed pathways for RSNO decomposition involve Hg^{2+}-S coordination (Saville, 1958) and

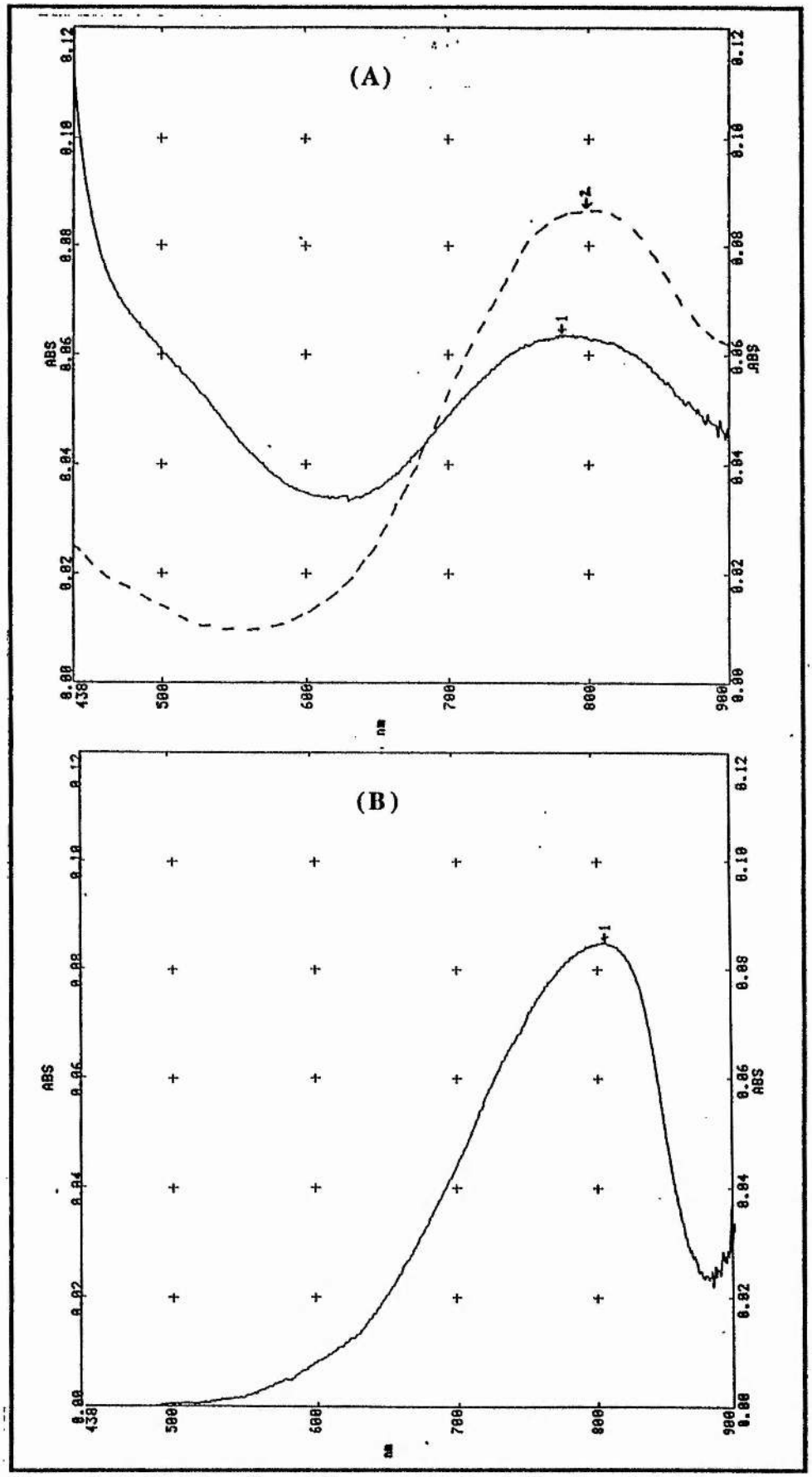


***Figure 3.17** (A) The effect of N-acetyl-DL-Penicillamine (6.6mM) on free copper (6.6mM copper (II) nitrate) absorbance at ~800nm in distilled water. (solid line) absorbance at t = 0, peak 1 at 780.8nm; (dotted line) absorbance at t = 16 hours, peak 2 at 798.4nm. (B) The absorbance profile of copper (II) nitrate (6.6mM) in distilled water, peak 1 at 804.8nm*

S-protonation (Al-Kaabi *et al.*, 1982) respectively. Both of which would lead to NO^+ expulsion and nitrous acid formation. Therefore no nitric oxide would be expected to form, contrasting with the copper(II) catalysed decomposition of SNAP in which NO was detected using an NO probe by Barnett & Williams (Askew, Barnett *et al.*, 1994, *in prep*).

When similar experiments as those conducted using equimolar quantities of copper (II) nitrate and N-acetyl-DL-penicillamine were carried out with SNAP, some interesting results were obtained. **Figure 3.18 (A)** shows the absorbance between 438 and 900nm of SNAP (6.6mM) in EDTA (0.5mM) with no added copper. A peak appears at 590nm due to the S-nitroso moiety of SNAP, but no peak appears at ~800nm for free copper (II) ions as they will be bound by EDTA. **Figure 3.18 (B)** shows the absorbance of SNAP (6.6mM) in the presence of copper (II) nitrate (6.6mM) after initial mixing of the two compounds. It is evident that little or no binding of the copper (II) ions is occuring as the absorbance at 800nm is around the value of that expected if all the copper was free. **Figure 3.19** shows the rapid decrease in SNAP absorbance at ~600nm taken at 2 minute intervals, due to decomposition. It is noticeable that there is little change in the free copper (II) absorbance at ~800nm. Therefore if complexation is occuring only a small amount of copper is bound at any time.

Having established the structural requirements for NO release from S-nitrosothiols (see sections 3.3 & 3.2) and from the work reported in this section , no more can be said, as yet, on the mechanistic detail as to the mode of NO release from the proposed cyclic intermediates for SNAP, and other S-nitrosothiols susceptible to NO decomposition. At this stage it is suggested that spontaneous break up of the ring occurs forming the thiol radical (RS·) which yields the disulphide (RSSR), free Cu^{2+} and NO, although the situation may be more complex than this.

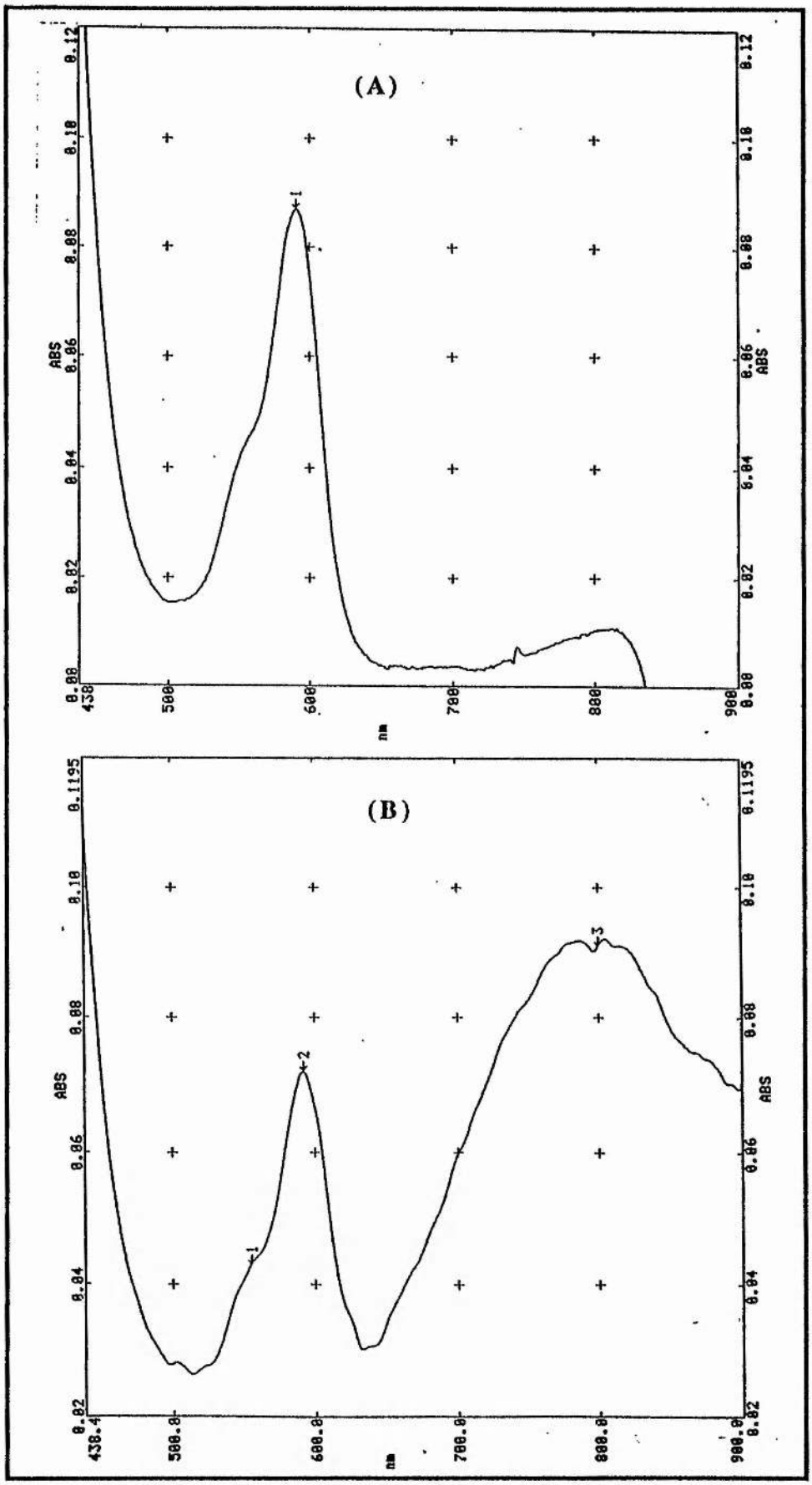


***Figure 3.18** (A) The effect of EDTA (0.5mM) on SNAP (6.6mM) in distilled water. Peak 1 at 590.5nm is due to SNAP. (B) The absorbance profile of SNAP (6.6mM) in the presence of copper (II) nitrate (6.6mM) in distilled water after initial mixing. Peak 1 & 2 at 554.6nm & 592.2nm are due to SNAP, Peak 3 at 799.9nm due to Cu (II) aq.*

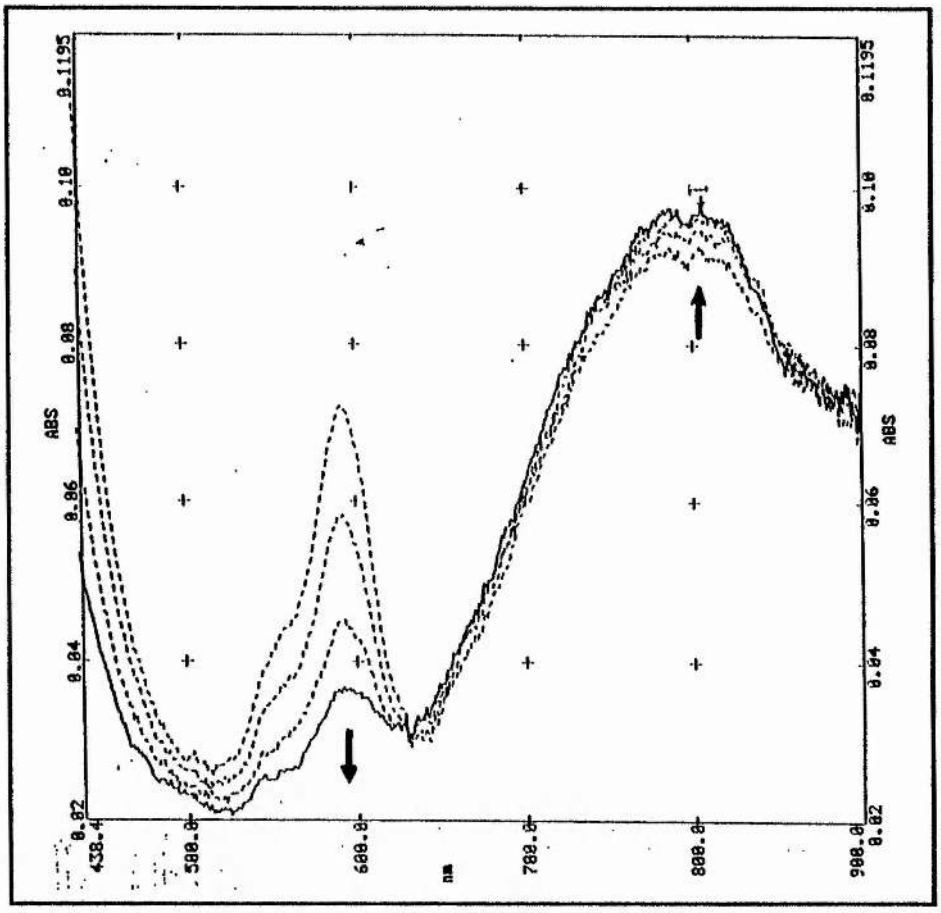


Figure 3.19 *The absorbance traces of SNAP (6.6mM) in the presence of copper (II) nitrate (6.6mM) taken at 2 minute intervals. Note the decrease in SNAP absorbance at 590nm and the slight change (increase) in absorbance at ~800nm due to free Cu (II) aq ions, possibly indicating small amounts of copper complexation.*

3.4.2 Experimental

Figures 3.17 - 3.21

SNAP (0.0145g) and N-acetyl-DL-penicillamine (0.0126g) were added to copper (II) nitrate trihydrate (0.0159g) and diluted in distilled water (10ml), to make up 0.0066M solutions of the amino acids with the metal. If EDTA (0.5mM; 0.0019g) was required this was also added to the 10ml solutions. The visible spectrum between 438nm and 900nm was measured immediately and in the experiments conducted using SNAP, the spectrum was taken continuously at 2 minute intervals.

CHAPTER 4

OTHER FACTORS AFFECTING THE DECOMPOSITION OF S-NITROSOTHIOLS IN AQUEOUS SOLUTION

4.1 INTRODUCTION

As well as the effect of trace metal ions present in buffer solutions there are a number of other chemical factors which can increase the rate of S-nitrosothiol decomposition to NO. These conditions maybe related to, or independent of, the metal ion catalysed process. In this chapter SNAP is used as the model S-nitrosothiol and its thermal and photochemical decomposition are discussed, together with its decomposition profile across a range of pH. In addition, two novel methods for following SNAP decomposition by monitoring the production of NO, directly and indirectly, are discussed. Finally, in a brief review, other methods of detection of NO and its metabolites are covered.

4.2 THE THERMAL DECOMPOSITION OF SNAP

i) Results and Discussion

As was mentioned in **Chapter 2**, SNAP, along with other S-nitrosothiols, is susceptible to thermal decomposition, releasing NO and forming its disulphide. For this reason, in all of the experiments carried out using S-nitrosothiols, the solutions made up for experimentation were stored on ice until immediately prior to use. Experiments carried out on SNAP at temperatures varying from 25°C to 50°C shown in **Figure 4.1** emphasise the dramatic effect temperature plays on the decomposition rate of SNAP. In these experiments, which were carried out before the metal ion catalysed decomposition of S-nitrosothiols was known, no precautions were taken to remove the trace quantities of metals present in the buffer solutions. This could suggest that it is the speed of this process which is enhanced when the temperature is increased. These results seem to support the case, as when precautions are taken to remove metal ions (using EDTA) there is a marked decrease in the rate of SNAP decomposition (see **Figure 4.2**). However, there is still a substantial difference in the thermal

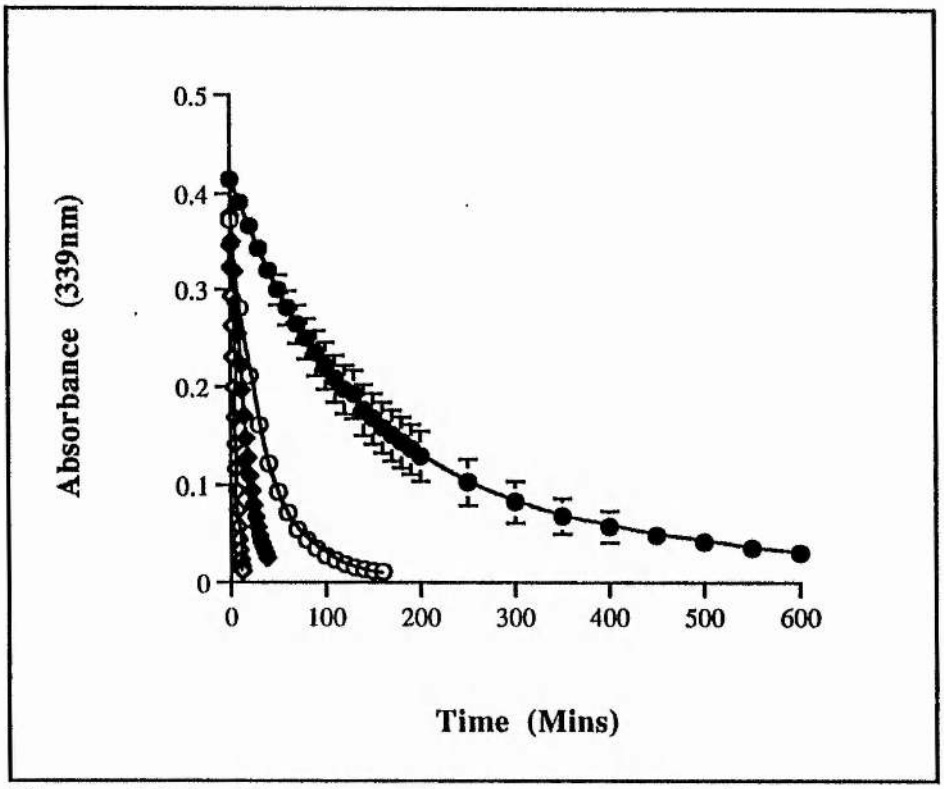


Figure 4.1 *The effect of increasing temperature on the decomposition rate of SNAP (0.4mM; n=5) in the presence of trace metal ions. 25 ℃ (closed circles), 30 ℃ (open circles), 37 ℃ (closed diamond), 50 ℃ (open diamond).*

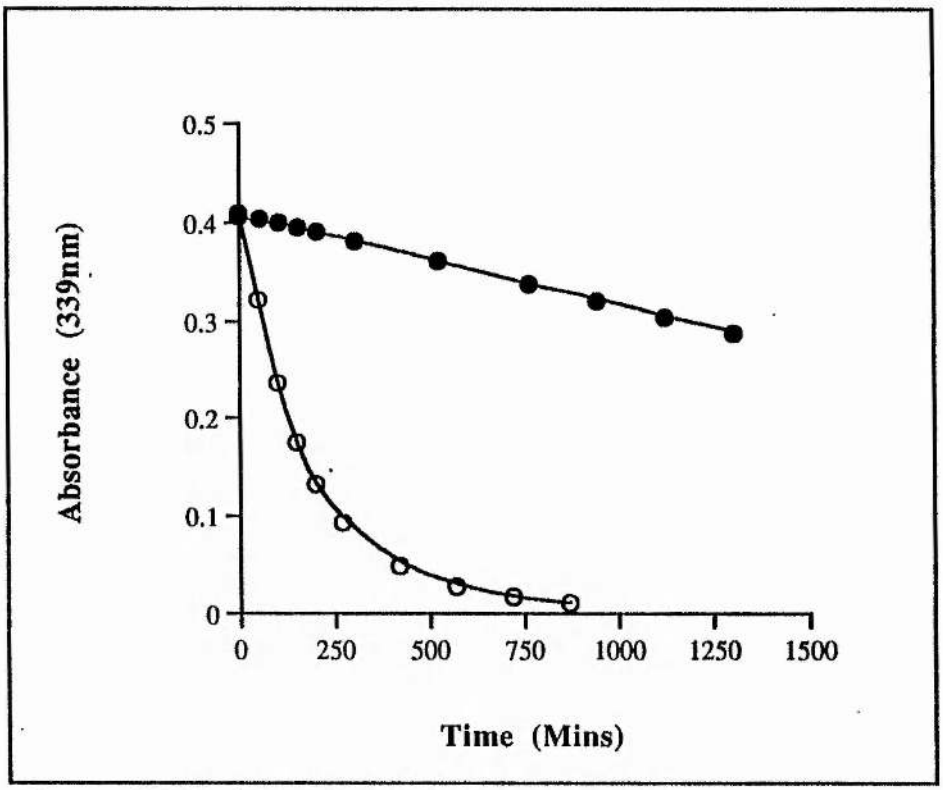


Figure 4.2 *The effect of increasing temperature on the decomposition rate of SNAP (0.4mM) in the absence of trace metal ions (removed by EDTA (10-5M). 30 ℃ (closed circles), 50 ℃ (open circles).*

decomposition rates over the 20°C range between 30 and 50°C, suggesting that thermal decomposition has a role in SNAP, and presumably other S-nitrosothiol's degradation.

ii) Experimental

(see **experimental section, Chapter 2.3.2** - *kinetics study*). For the experiment presented in **Figure 4.1** the same procedure was carried out as described, with adjustment of the temperature setting. In the case of the experiment shown in **Figure 4.2** a 25mM solution of EDTA was made up (0.0931g in 10ml buffer), diluted 100 fold to produce 0.25mM solution. An aliquot (0.1ml) of this solution was diluted 25 fold (10μM) in the spectrophotometer cuvette, prior to the addition of SNAP (0.25ml, 0.4mM).

4.3 THE EFFECT OF PH ON SNAP DECOMPOSITION RATES

i) Results and Discussion

There is a dramatic effect of pH on the stability of SNAP. At high and low pH SNAP is very stable (see **Figure 4.3**), whereas at physiological pH the rate of SNAP decomposition is dramatically increased (see **Figure 4.4**). The reasons for these observations probably lies with the metal ion catalysed decomposition. The metal, and metal ligands, will have different chelating abilities in the protonated and deprotonated environments of acid and basic solutions respectively. For instance, at high pH amine groups and amide linkages between peptides will be very good ligands for the positively charged metal ion like Cu^{2+} as it will readily deprotonate these species when it chelates (Pettit *et al.*, 1985) For these reasons it would be expected that the rate of SNAP decomposition would increase, however, in the case of copper catalysis the copper ion will be in a different aqueous form than at neutral pH, with a general formula $Cu(OH)_x(H2O)_y$. It is known that the reaction $[Cu(OH_2)_6]^{2+} \leftrightarrow [Cu(OH_2)_5OH]^{+} + H^{+}$ has a pK_a of 6.8. The stronger binding of OH ligands to Cu^{2+} makes x binding sites on the copper ion unavailable for binding to the peptide, and

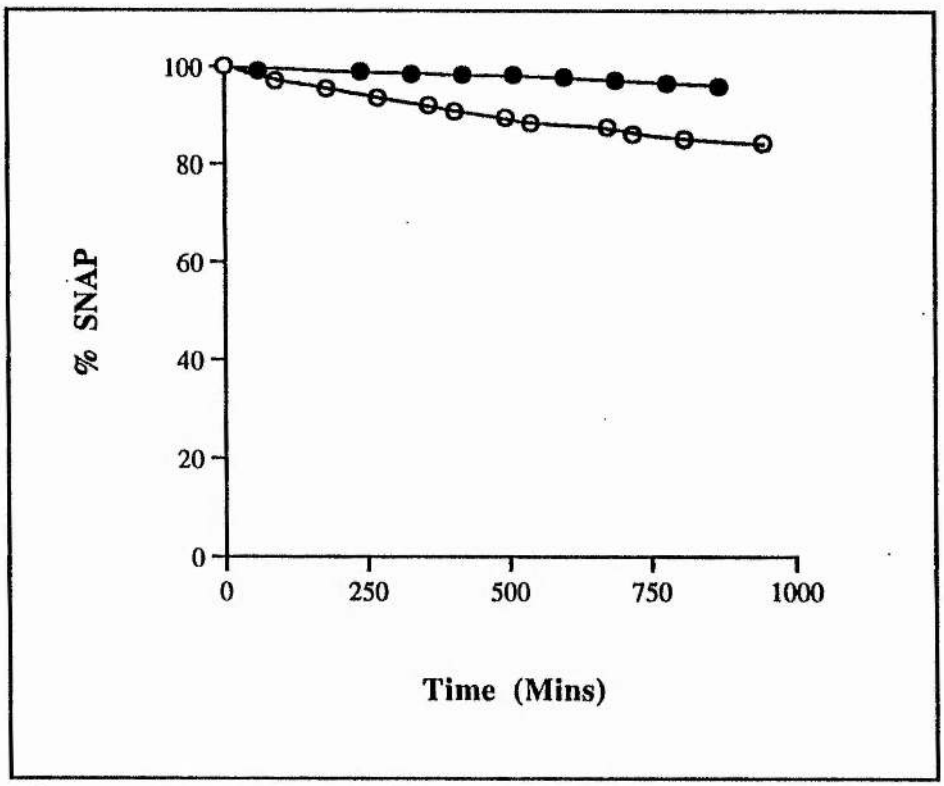


Figure 4.3 *A comparison of the rates of SNAP decomposition under highly acidic (pH2.2) and highly basic (pH10.2) conditions at 30℃. pH2.2 - glycine /HCl (closed circles), pH10.2 - glycine/NaOH (open circles). % SNAP represents a direct comparison to absorbance, with absorbance at t=0 representing 100% SNAP .*

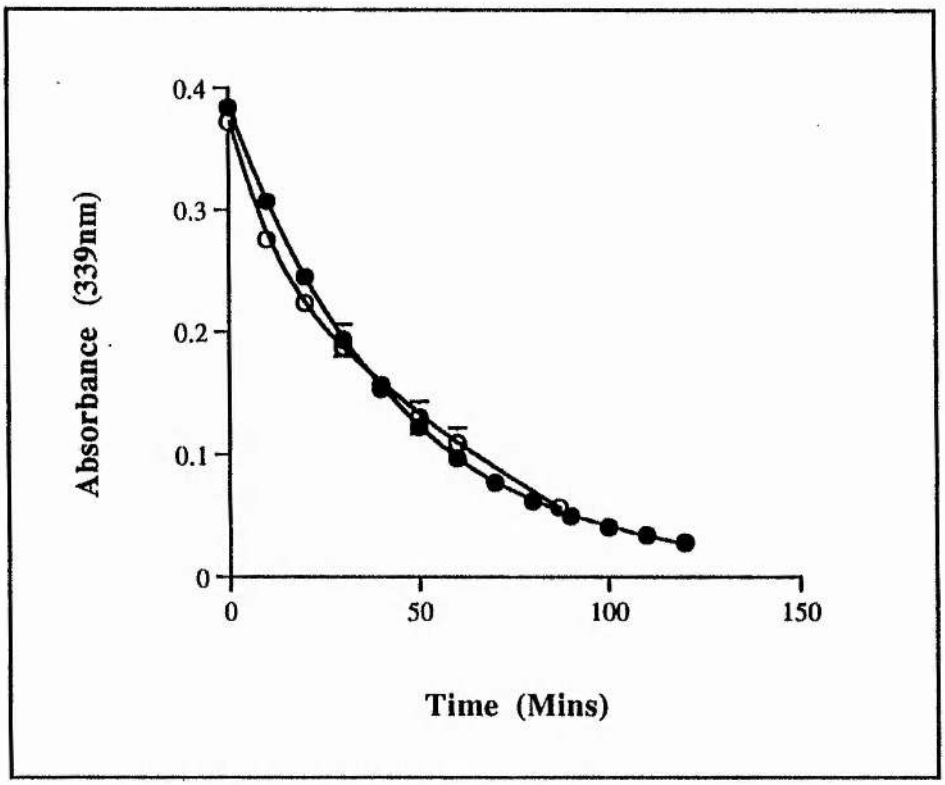


Figure 4.4 *A comparison of the rates of SNAP (0.4mM) decomposition at pH7.4 in KH_2PO_4/NaOH (closed circles; n=12) and dimethylglutaric acid (DGA)/NaOH (open circles; n=5) at 30℃*

experimental results suggest this probably overrides the fact that the free amine groups, or amide linkages are readily available for chelation.

At low pH most of the possible ligands for chelation to copper ions are protonated producing electrostatic repulsion between the positively charged copper ion and its binding sites. This would be expected to result in little metal ion catalysis and greater stability of SNAP. At physiological pH, the aqueous environment of the copper species, together with the chelating affinities of its ligands from the S-nitrosothiol, seem to be in more amenable forms.

To complicate this study, all buffers will chelate metal ions to different extents at different pH's. Therefore, the different binding affinities of the buffer constituents must be taken into account, as they may remove or reduce the chelation of the metal ions to the S-nitrosothiol. Interestingly, when comparing the buffers KH_2PO_4/NaOH and dimethylglutaric acid (DMGA)/NaOH at the same buffer capacities, little difference in the SNAP decomposition rates were seen (see **Figure 4.4**). However, initial evidence from experiments following the decomposition rate of SNAP in tris/HCl buffer over increasing pH (see **Figure 4.5**) suggests that the tris/HCl buffer system has less chelating ability at physiological pH than the other two buffer systems. The rate of decomposition is faster at pH7.5 for tris/HCl, than at pH7.4 for DMGA/NaOH and KH_2PO_4/NaOH. **Figure 4.5** shows the dramatic effect a change of 2 pH units (7.1-8.9) has on the decomposition rate of SNAP, without the complication of different buffer systems and their different chelating affinities. **Figure 4.6** shows that when metal ions are removed by EDTA the rates of decomposition of SNAP in the same buffer system are dramatically reduced, and are very similar over the first 1000 minutes and the same pH range.

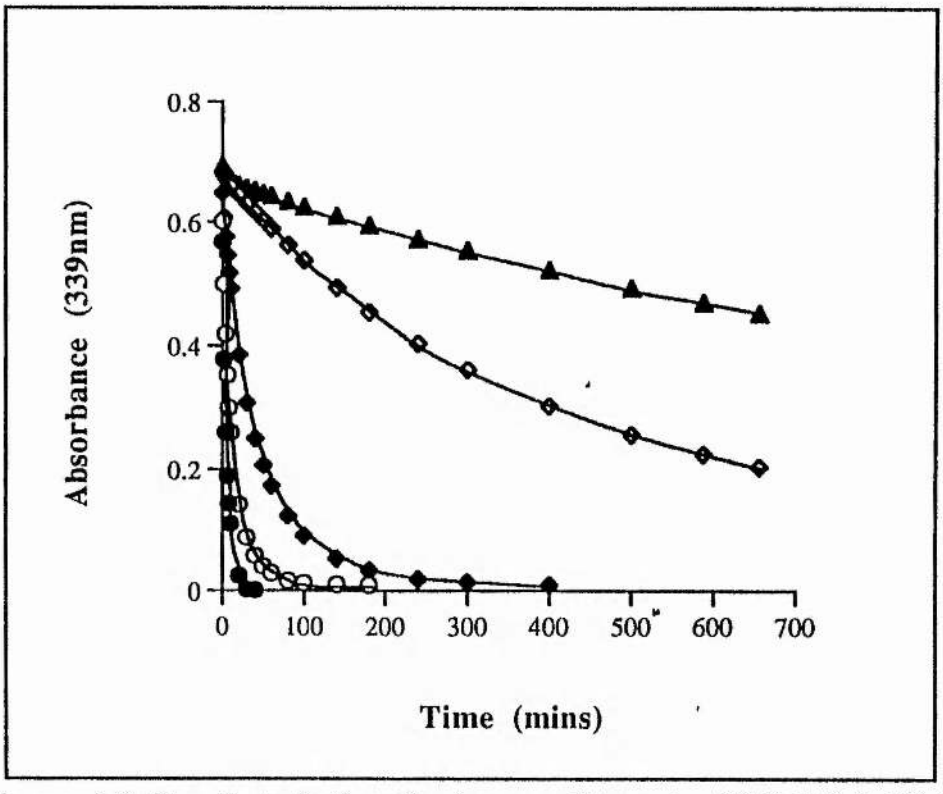


Figure 4.5 *The effect of pH on the decomposition rate of SNAP (0.8mM) in the presence of trace metal ions. Tris/HCl buffer, 30 ℃, pH7.1(closed circles), pH7.5 (open circles), pH8 (closed diamond), pH8.5 (open diamond), pH8.9 (closed triangle).*

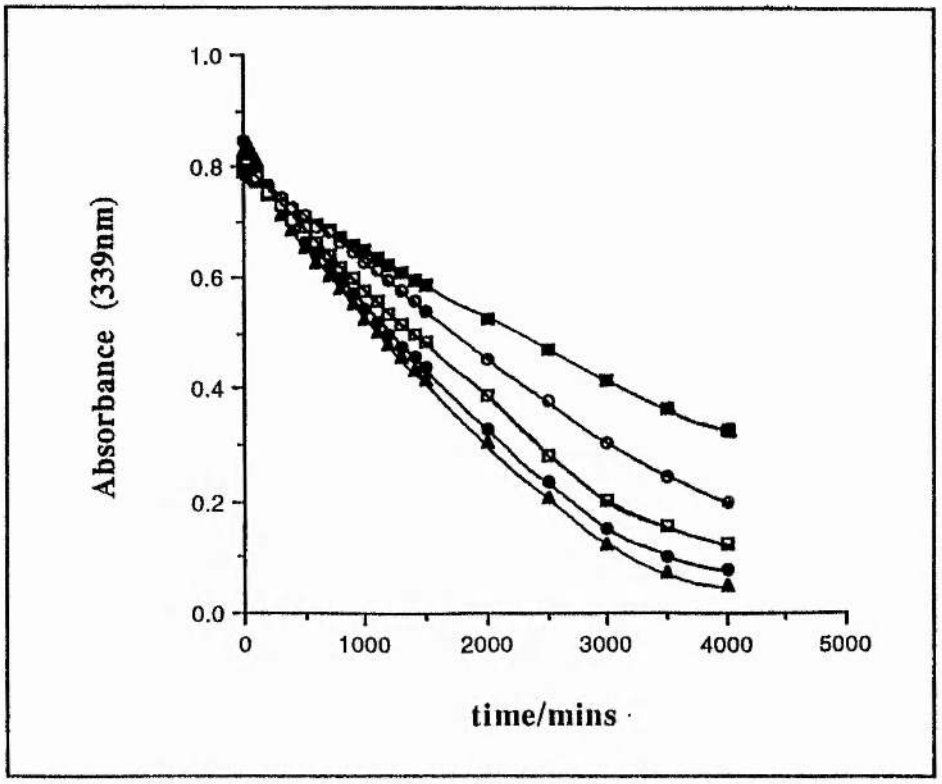


Figure 4.6 *The effect of pH on the decomposition rate of SNAP (0.8mM) in the absence of trace metal ions (complexed out by EDTA, 10-5M). Tris /HCl buffer, 30 ℃, pH7.1 (closed squares), pH7.5 (open circles), pH8 (closed circles), pH8.5 (closed triangles), pH8.9 (open squares). (Graph taken from Neave W.E., senior honours project, 1994).*

ii) Experimental

(see **experimental section, Chapter 2.3.2** - *kinetics study*). For the experiments presented in **Figure 4.3-4.6** the same procedure was carried out as described with adjustment of pH and the use of different buffer systems.

Buffer systems used

KH_2PO_4/NaOH pH7.4

To 50ml of 0.1M KH_2PO_4 (13.60g/l), was added 39.1ml of 0.1M NaOH and this was diluted to 100ml with distilled water.

β,β-Dimethylglutaric acid (DMGA)/NaOH pH7.4

To 50ml of 0.1M β,β-Dimethylglutaric acid (16.02g/l), was added 46.6ml of 0.2M NaOH and this was diluted to 100ml with distilled water.

Glycine/HCl pH 2.2

To 25ml of 0.2M glycine (15.01g/l), was added 22ml of 0.2M HCl and this was diluted to 100ml with distilled water.

Glycine/NaOH pH 10.2

To 25ml of 0.2M glycine (15.01g/l), was added 16ml of 0.2M NaOH and this was diluted to 100ml with distilled water.

Tris(hydroxymethyl)aminomethane buffer solutions pH7.1 - 8.9

To 50ml of Tris (12.114g/l), was added *x*ml of 0.1M HCl and this was diluted to 100ml with distilled water.

x = 45.7ml (pH7.1), 40.3ml (pH7.5), 29.2ml (pH8), 14.7ml (pH8.5) & 7ml (pH8.9). As the Tris buffer system changes its pH with temperature (Dawson, 1990; dpH/dt ~ -0.028pH units/deg) and the values quoted above are set for a temperature of 25°C, slight adjustments to the stock solutions of buffer were made to compensate for the slight drop in pH by raising the temperature to 30°C.

4.4 THE PHOTOCHEMICAL DECOMPOSITION OF SNAP

i) Results and Discussion

As was mentioned in the introduction to **Chapter 2,** SNAP like other S-nitrosothiols is susceptible to photochemical decomposition to form NO and its disulphide. Consequently, in all experiments carried out using S-nitrosothiols, solutions were protected from light by aluminium foil. Due to the unavailability of a tuneable and constant intensity light source, across wavelength, a detailed study of the photochemistry of SNAP (and GSNO) was not undertaken. However, in a qualitative experiment using daylight as the light source, the decomposition rate of SNAP was shown to be photosensitive (see **Figure 4.7**). The same result was obtained using NO chemiluminescence to monitor the production of NO from SNAP, when the S-nitrosothiol was exposed to and protected from light (see **Figure 4.9**). This will be discussed in more detail in the next section.

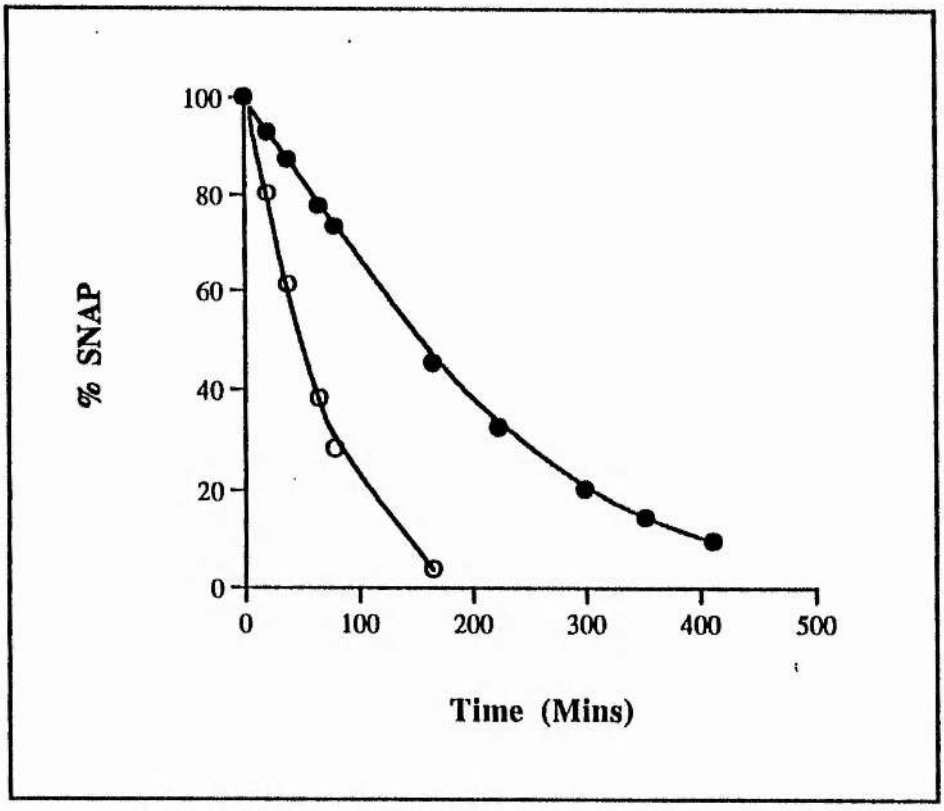


***Figure** 4.7 A comparison of decomposition rates of SNAP (1.5mM) in distilled water in the presence and absence of light (daylight) at 30 ℃. light (open circles), no light (closed circles). % SNAP represents a direct comparison to absorbance, with absorbance at t=0 representing 100% SNAP.*

ii) Experimental

(see **experimental section, Chapter 2.3.2** - *kinetics study*). For the experiments presented in **Figure 4.7** the same procedure was carried out as described. However, manual rather than automated use of the spectrophotometer was implemented as both the test solutions of SNAP in distilled water (one of which was covered in tin foil) were kept at 30°C in a water bath exposed to daylight.

4.5 NOVEL METHODS FOR MONITORING NO PRODUCTION FROM S-NITROSOTHIOLS SUCH AS SNAP

The experiments described in this section were carried out at the Wellcome Laboratories, before the metal ion catalysed process was known. They have been included in this section to present other possible methods which could be used to follow S-nitrosothiol decomposition *in vitro* or possibly *ex vivo/in vivo*, by monitoring the production of NO (by NO chemiluminescence), or its reaction with oxyhaemoglobin, rather than the removal of S-nitrosothiol. In parts **4.5.1** and **4.5.2** the experimental sections precede the results and discussion for clarity.

4.5.1 Nitric Oxide Chemiluminescence (Downes et al., 1976)

This technique follows the reaction between NO and ozone (O_3) which produces a chemiluminescent product of excited NO_2, this gives out light when it relaxes to its ground state (**1**).

$$\begin{aligned} NO + O_3 &\longrightarrow NO_2^* + O_2 \\ NO_2^* &\longrightarrow NO_2 + \text{light} \end{aligned} \qquad \textbf{(1)}$$

Many chemiluminescent analysers are now available for quantification of this reaction and the technique is highly sensitive (detection limit 1nM) and linear over a wide range.

1 to 1000nM can easily be covered without recalibration of equipment. Analysers are rapid and highly reproducible.

i) Experimental

Apparatus - NO chemiluminescence apparatus located at the Wellcome Laboratories and the same apparatus used by Palmer *et al.* (1987).

Procedure

To 149ml of deionised, distilled water in a conical flask, degassed with argon and set at pH 7.5, was added 1ml of 1.5mM SNAP solution in the same aqueous medium forming a 10μM concentration in the flask. This solution was immediately bubbled vigorously and continuously with argon to remove NO as it was formed from SNAP decomposition, and to keep the pH at 7.5 by not allowing resolvation of acidic gases such as CO_2. Argon was chosen as the bubbling gas due to its heavier molecular weight than nitrogen, allowing it to remain just above the aqueous layer. The conical flask was contained in a water bath at 37°C. The same procedure was carried out on another conical flask containing the same solution only covered in aluminium foil to protect the SNAP solution from the daylight.

50μl aliquots of the SNAP solution were removed, at timed intervals, from the stock solution for injection into the three necked flask of the chemiluminesence analyser (**Figure 4.8**). The flask contained a strongly heated solution of 75% acetic acid/25% sodium iodide (~80ml). This was done to measure the amount of SNAP remaining. Each SNAP molecule was immediately decomposed to produce one NO molecule which, when formed, was immediately blown from the acetic acid / sodium iodide solution by nitrogen, which is continuously bubbling through the solution at a rate of 100ml/min (controlled by a Platon flow meter), through one of the three necks on the flask. The NO in the nitrogen gas passes up through a condenser, through a solvent trap and into the chemiluminescence analysing chamber. Here it initially reacts with

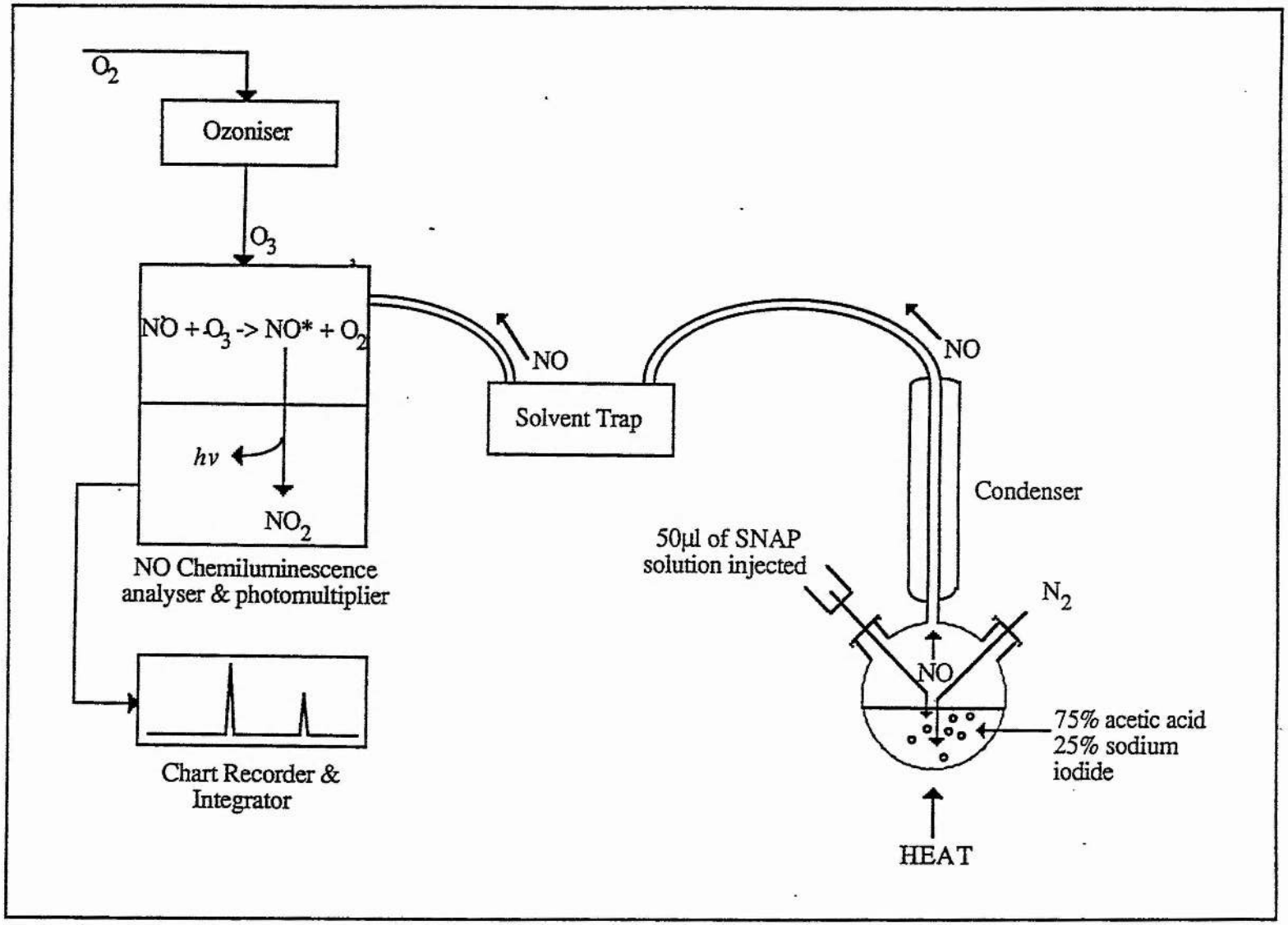


Figure 4.8 The NO chemiluminescence apparatus

excess ozone, formed from an ozoniser supplied with oxygen at a rate of 100ml/min (controlled by a Platon flow meter), to form excited NO_2 which chemiluminesces to the ground state NO_2. The chemiluminescence was picked up by a NO chemiluminescence photomultiplier system Model MK1A, which was linked to a Gould BS-272 chart recorder and a Schimadzu C-R3A chromatopac (integrator). This calculates the area under the peak formed from the chemiluminescence due to NO. Both the oxygen and nitrogen used were purified by a Supeloo carrier gas purifier.

By injecting into the three necked flask 10,30 and 50ml aliquots of a 10mM solution of sodium nitrite, a calibrated linear response was obtained allowing a straight line graph of area under the chemiluminescence peak versus moles of NO, to be calculated. The peak areas produced by these standard nitrite samples are very reproducible. This was used to quantify the number of moles of NO produced from SNAP. The injections of SNAP samples were repeated until the peaks being registered on the integrator were too small to be measured accurately, as the amount of SNAP remaining was so small.Then a graph of 'moles of SNAP versus time' was plotted (see **Figure 4.9**).

ii) Results and Discussion

Figure 4.9 shows the decomposition profiles obtained by this method in the presence and absence of daylight. As in the similar experiment carried out using UV/Vis spectrophotometry to monitor the decrease in absorbance of SNAP in the presence and absence of daylight (see **Figure 4.7**), this experiment shows that SNAP is very photosensitive. As expected from the unknown light intensities and temperature differences, the two decomposition profiles of SNAP exposed to light are different, the experiment shown in **Figure 4.7**, using UV/Vis spectrophotometry to monitor decomposition, being the slower. Furthermore, the decomposition profile of SNAP at 37°C in the absence of light, monitored by NO chemiluminescence, is significantly slower than those obtained at a similar pH and the same temperature, in phosphate buffer, monitored by UV/Vis spectrophotometry (see **Figure 4.1**). A possible

explanation for the slower rate of decomposition, is that the water used for the experiment using the NO chemiluminescence technique was milliQ water which contains no detectable metal ions. The only possible metal ions present would come from the alkali or acid used to adjust the pH of the water to 7.5. This would suggest a slower rate of decomposition of SNAP, compared to the presence of a possible concentration of 1.4μM Cu^{2+} ions in the phosphate buffer used in experiments represented in **Figure 4.1**.

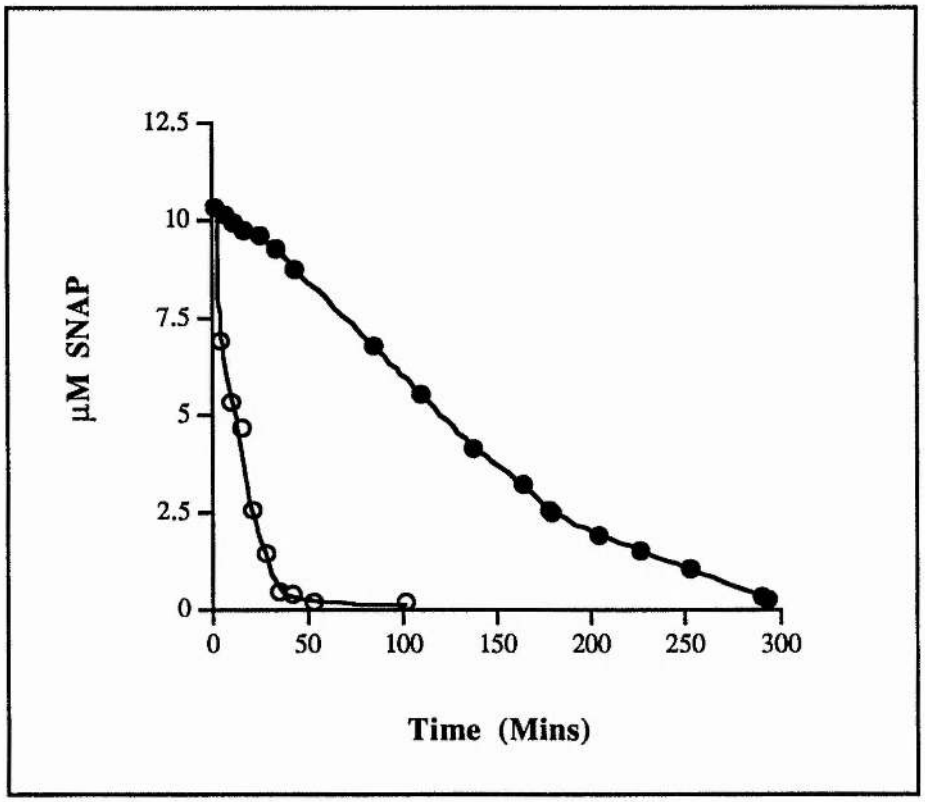


Figure 4.9 *The photochemical decomposition of SNAP (10M) measured qualitatively using daylight as the light source and monitored by NO chemiluminescence. Presence of light (open circles); absence of light (closed circles).*

Alternatively, if the decomposition of S-nitrosothiols is second order, as proposed by Park (1989), the lower concentration of SNAP used in this experiment (40 fold lower) compared with that used for the experiments monitored by the UV/Vis spectrophotometric method, would suggest a slower rate of decomposition.

4.5.2 *The use of oxyhaemoglobin to determine the rate of decomposition of SNAP*

Another spectrophotometric method used to monitor NO production involves the use of oxyhaemoglobin (HbO_2) and its conversion to methaemoglobin (MetHb) and nitrate in the presence of NO (**2**).

$$Hb^{2+}O_2 \quad + \quad NO \quad \longrightarrow \quad MetHb^{3+} \quad + \quad NO_3^- \qquad (2)$$

This process is monitored by a dual beam double wavelength spectrophotometer monitoring the difference in absorbances at 401 and 421nm. The conversion is pH-dependent and displays the following characteristics at pH7.7. At 421nm, oxy-haemoglobin absorbs, and its reaction with NO produces a peak at 401nm due to the formation of metHb. A representation of the process is presented in **Figure 4.10**, where the isosbetic point at 411nm is represented at zero absorbance. This technique is very sensitive and can detect picomolar quantities of NO.

i) Experimental

The difference spectrum of methaemoglobin versus oxyhaemoglobin, was monitored using a double-beam, dual wavelength spectrophotometer (Schimadzu UV 300 with temperature control unit TB 95). By continuous recording of the absorbance difference between the disappearance of the oxyHb peak at 421nm and the formation of the metHb peak at 401nm a measure of the nitric oxide development per unit of time can be calculated and hence the decomposition rate of SNAP.

Procedure

In these experiments deionised, distilled water was bubbled with argon for about 20-25 mins to remove any dissolved oxygen/carbon dioxide. The pH was then set at 7.5 at 37°C, using HCl/NaOH solutions of weak concentrations. 1.1µl (5mM) of oxygenated

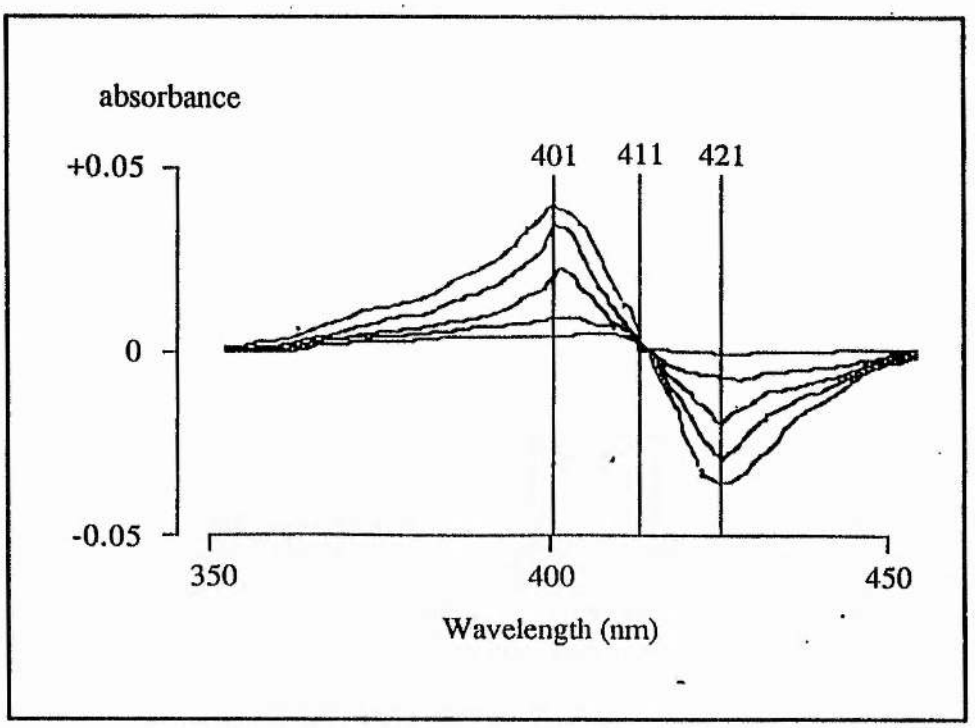


Figure 4.10 *A representation of the disappearance of oxyHb (peak at 421nm) and the appearance of the metHb (peak at 401nm) due to reaction with NO (produced from GTN in the presence of cysteine) measured by difference spectrophotometry. An isosbestic point was found at 411nm and represented here at zero absorbance. (taken from Feelisch & Noack, 1987).*

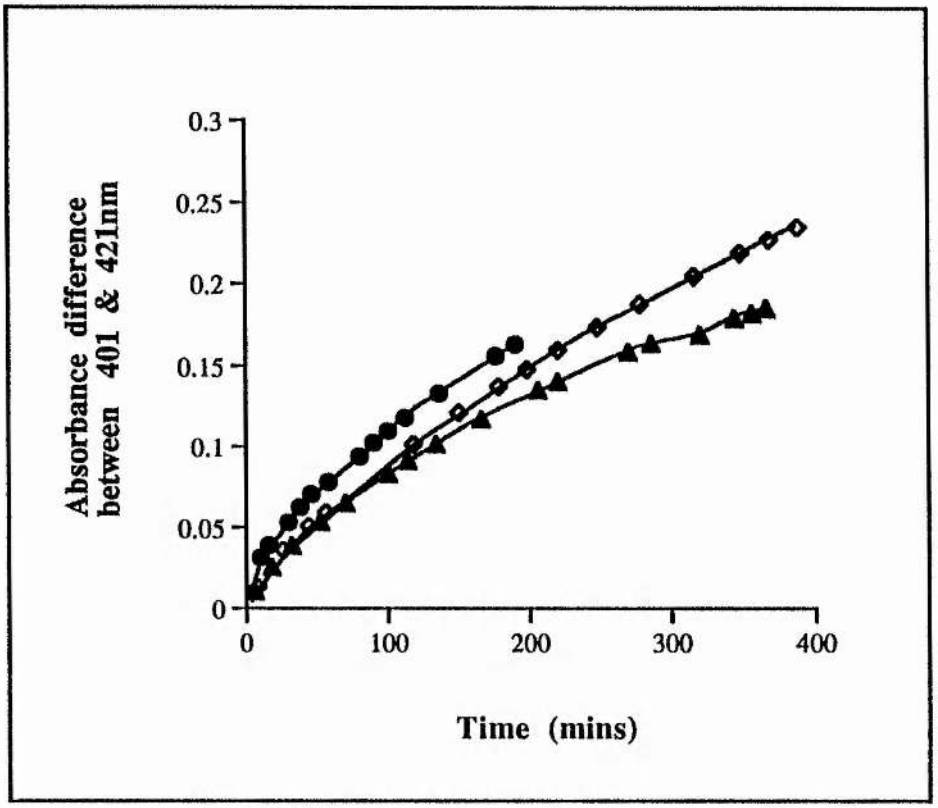


Figure 4.11 *A graph showing the formation of metHb from O_2Hb due to NO produced from SNAP (0.1μM) using different concentrations of O_2Hb. (closed triangle) 4.9nM, (open diamond) 10nM, (closed circle) 0.1μM*

haemoglobin (see *materials* for preparation), was transferred to the spectrophotometer cuvette by syringe and 493.9µl of the argon bubbled water at pH 7.5 and 37°C was added to it. This was then inserted into the spectrophotometer mentioned above and allowed to equilibrate for 2-3 mins and then zeroed. 5µl of SNAP solution (10µM), made up in the same water, was then immediately added to the vial to make up a final concentration of 0.1µM SNAP, and the experiment was started. Various concentrations of oxyHb were made up at by adjusting the volume of oxyHb added and compensating by reducing the amount of water.

Materials

The oxyhaemoglobin solution was prepared as follows: dissolution of haemoglobin crystals in neutral distilled water, subsequent gassing with oxygen for 3 minutes, reduction with excess sodium dithionite, thereafter gassing with oxygen again for 15 mins; desalting and purification by passing the solution through a Sephadex G 25 column. Identification and adjustment were performed spectrophotometrically. Each batch prepared was stored at -80°C to avoid contamination.

ii) Results and Discussion

It has been shown that it takes less than 100 milliseconds for the NO radical to react with oxyHb (Kelm & Feelisch *et al.*, 1988) and so the rate of NO production should be a good approximation to the rate of SNAP decomposition. Different concentrations of oxyHb were used to see if it would effect the rate of decomposition of SNAP by scavenging NO from the thiol itself. This did not seem to be the case as **Figure 4.11** shows the rates of NO production were similar across the concentration range studied. However at higher concentrations of oxyHb (0.3µM), in which oxyHb was in excess versus SNAP the rate of NO production was very slow and therefore not monitored.

The possible reasons for the overall slowness of SNAP decomposition monitored by this technique (possibly even accounting for the very slow decomposition rate at 0.3µM

oxyHb) are based on the concentration of SNAP used and the metal ion catalysed process. Both the experiments carried out using NO chemiluminescence (**Figure 4.9**) and UV/Vis spectrophotometry (**Figure 4.1**), at similar pH, show faster rates of decomposition of SNAP than using this technique. The SNAP concentration of 0.1μM used in experiments monitored by difference spectrophotometry is 100 fold lower than that used in the experiment monitored by NO chemiluminescence and 4000 times lower than that used in experiments followed by UV/Vis spectrophotometry shown in **Figure 4.1**. A possible explanaion is that if the decomposition of SNAP is second order, as proposed by Park (1989), then a much slower rate of NO production from SNAP would be expected, as the concentration of SNAP used is lowered. However, in light of the discovery of the metal ion-catalysed decomposition of SNAP another possible explanation can be put forward. Haemoglobin, being a large protein contains many amide bonds which have been shown to have a high formation constant with metal ions such as copper ($K_f \sim 1x10^5$; Pettit *et al.*, 1985). Therefore any metal ions present (added in the acid /alkali used to adjust water pH) will have a high chance of being complexed and consequently being less available for chelation with SNAP to instigate production of NO.

4.6 OTHER TECHNIQUES USED TO DETECT AND MEASURE NO DIRECTLY AND INDIRECTLY

NO chemiluminescence and oxyHb detection of NO produced from S-nitrosothiols are only two of the sensitive techniques for measuring NO. Both techniques have been used to detect NO from liquid samples taken from biological media (Palmer *et al.*, 1987; Kelm & Feelisch *et al.*, 1988; Meyer *et al.*, 1994 *in press*).

Chemiluminescence specificity is high in most systems because the majority of other molecules potentially able to chemiluminescence with ozone (amines, DMSO etc) are not volatile or do not occur in biological systems. This can be demonstrated by passing

samples through solutions of Fe^{2+} salts which will remove the NO contribution to the signal, or by using low temperature traps (see **Figure 4.8**) to remove contaminants from the sample.

4.6.1 Direct measurements of NO

NO Probes

Other than chemiluminescence the other main direct measure of NO concentration is the NO probe. Two main probe systems have been developed (Shibuki, 1990; Malinski & Taha, 1992) which rely on the electrochemical oxidation of NO to generate electric current. They can be placed in *in vitro* systems to determine the exact locality at which NO exists. The specificity of these probes depends on their ability to exclude other molecules that might give rise to current. Shibuki's probe is a modified oxygen electrode, which is robust and sensitive down to 0.5μM. However most versions of the probe are several millimetres in diameter and do not allow particularly accurate positioning in a cellular system. Malinski has developed a probe which uses a polymeric metalloporphyrin to catalyse the electrochemical oxidation of NO. This is covered by a thin layer of the cation exchanger Nafion which eliminates anionic interference from nitrite. The high sensitivity, small diameter (0.2-1.0μM) and fast response time (10ms) are necessary features for NO detection in microsystems such as a single cell. It also has a detection limit of 10nM and a wide and linear current-concentration relationship (10nM-3mM). However, it is very delicate and requires considerable technical skill to build and operate.

4.6.2 Indirect measurement of NO

These techniques are widely used in the more complex *in vivo* detection of NO. Often a combination of indices are used for more accurate measurement.

i) *Bioassay*

The earliest measurement of NO production involved bioassays able to provide units of response in particular biological systems. This technique is primarily qualitative. Many of the biological parameters regulated by NO are also controlled by other molecules and so specificity is a potential problem. However use of drugs which inhibit NO production as well as inhibitors of other factors with similar biological actions can usually target the cause of a response.

ii) *Cyclic GMP*

The production of the second messenger cGMP from stimulated guanylate cyclase is a possible index of NO production. However many molecules activate guanylate cyclase, which will effect the specificity of this technique.

iii) *Nitrite & Nitrate*

The short lived nature of NO means rapid conversion to a range of breakdown products which include nitrite and nitrate. These indices of NO are relatively stable and a wide range of techniques have been developed to measure them. Most of these techniques work well in oxygenated aqueous solution where the ratio of nitrite and nitrate is predictable and other sources of these anions are easily controlled. However, *in vivo* work is more complicated as the ratio of nitrite to nitrate may vary considerably due to the reaction of NO with molecules such as haemoglobin, superoxide anions and hydroxyl radicals. In addition, other sources of nitrite and nitrate are difficult to control.

GCMS

Gas chromatography mass spectrometry (GCMS) is a very sensitive and specific method for determining nitrite and nitrate levels in plasma. Furthermore, gas chromatography combustion isotope mass spectrometry (GCIRMS) is under development and has the great advantage of providing isotopic analyses, so that studies can be performed on stable isotopes. However, nitroaromatic measurement by mass spectrometry is not suitable for routine analyses as nitrite and nitrate levels must be

measured separately, with nitrite being converted to nitrate and both anions further converted to a nitroaromatic. This increases the chances of contamination.

The Greiss Test

This assay can be used to detect nitrite (see **Chapter 2**)

High Performance Liquid Chromatography (HPLC)

This technique can be used to measure nitrite and nitrate in a single run. However extensive pretreatment of samples is required before analysis which risks contamination with nitrite and nitrate.

Capillary Zone Electrophoresis (CZE)

Is a rapid and user friendly technique for measuring nitrite and nitrate simultaneously with minimal sample preparation, high resolving power, rapid analyses and low sample consumption (Leone *et al.*, 1994 *in press*). Although sensitivity has not been a particular strength of this technique there have been recent advances towards improving this aspect of the technology. HPCE is performed by application of high voltage (10-30kV) across narrow bore fused silica capillaries and samples migrate down the capillary at different speeds depending on their charge to mass ratio. This technique is used and discussed in more detail in **Chapter 5**.

iv) *Electron spin resonance (ESR)*

NO has an unpaired π orbital electron that can be excited by microwave and magnetic energy giving rise to a characteristic spectrum on its return to the ground state. This is detectable by ESR. NO will, however, react rapidly with oxygen and radicals, so spin traps such as nitroxides or haemoglobin are used. The complex formed between NO and reduced haem iron (FeII) of haemoglobin has well defined spectroscopic properties producing a characteristic 3-line hyperfine pattern which is not seen when Hb binds nitrite or other nitrogen containing compounds.

Although Hb has been used as a spin trap and reported to have been able to detect NO at 0.1nM levels (Greenberg *et al.*, 1990), others have been unable to detect basal

HbNO in various animal models (Wang *et al.*, 1991). For this reason, together with the technical complexity of analyses, the use of ESR as an index of NO detection is problematic.

CHAPTER 5

FACTORS EFFECTING NO RELEASE FROM S-NITROSOTHIOLS *EX VIVO*

5.1 INTRODUCTION

In **Chapters 1 to 3** the mechanisms of S-nitrosothiols chemical decomposition were discussed by examining the stabilities of SNAP and to a lesser extent GSNO under different experimental conditions. It was found that GSNO is more stable than SNAP at physiological pH and is far less susceptible to metal ion catalysis. In **Chapter 1**, a comparison of the vasodilator effectiveness of SNAP and GSNO on the rat tail artery was briefly investigated and experimental results suggested that SNAP is about 18-fold more effective than GSNO.

These findings are in line with the theory that guanylate cyclase stimulation by S-nitrosothiols is inversely proportional to their stability (Feelisch, 1993; Ignarro & Gruetter, 1980a). However, in *ex vivo* and *in vivo* situations, many other factors may influence NO release from S-nitrosothiols. In this chapter some of these factors are investigated. The findings suggest that different modes of NO production may go some way towards explaining the relative effectiveness of these two S-nitrosothiols as vasodilators and inhibitors of platelet aggregation.

For ease of comprehension, the experimental sections come before the results and discussion sections in this chapter.

5.2 SNAP & GSNO; ENDOTHELIUM INDEPENDENT VASODILATORS ?

5.2.1 Experimental

The effect of L-NAME and ferro-haemoglobin on SNAP responses

The trace shown in **Figure 5.1** was obtained using the rat tail artery preparation described in **Chapter 2.4.** The same protocol was followed. After addition of PE

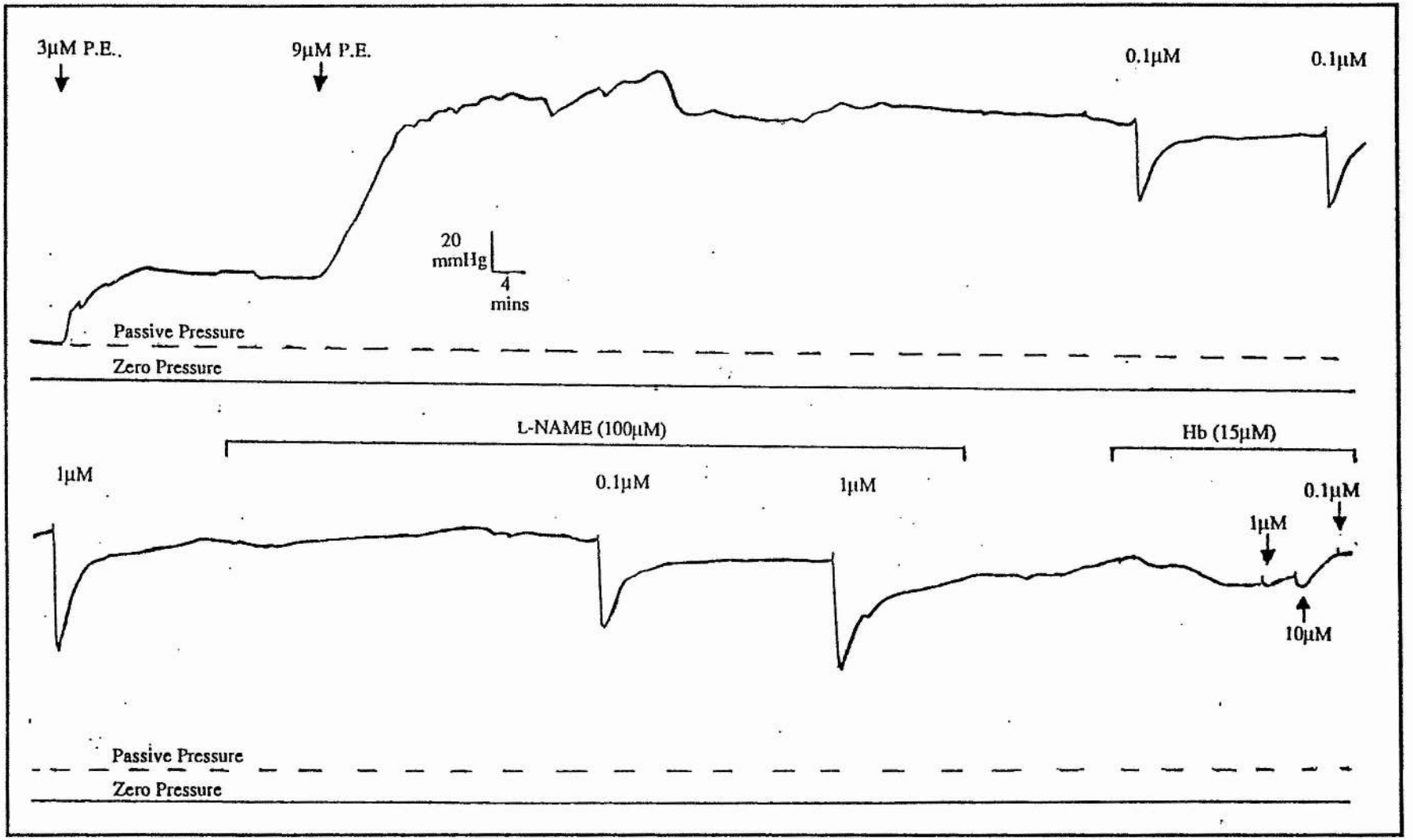


Figure 5.1 The effect of L-NAME and ferro-haemoglobin on the vasodilator response of the rat tail artery to bolus injections of SNAP, showing SNAP is an endothelium-independent vasodilator

(12μM) to give an active pressure of 115mmHg, SNAP was injected at position **I** (see **Figure 2.5**) at concentrations of 0.1 and 1μM. L-NAME (100μM) was added to the internal perfusate (see **Figure 2.5**) at the time indicated on the trace in **Figure 5.1.** This solution was continuously perfused through the artery as indicated by the horizontal bar, and the SNAP injections were repeated. The system was then washed out for 15 minutes with the original Krebs solution (containing 12μM P.E.) at which point ferro-haemoglobin (3ml, 1mM) was added to 200ml of Krebs solution (containing 12μM P.E.) in the internal perfusate to make up a 15μM solution, which was perfused for the time indicated. Doses of SNAP (0.1-10μM) were injected and the response recorded.

Ferro-haemoglobin (MW 64500) was prepared by reduction of 100mls of 1mM bovine haemoglobin using 10mM sodium dithionite. The dithionite was dialysed out of solution against 3x2 litre volumes of deareated distilled water at 0-5°C. The stock ferro-haemoglobin was then split into 3ml samples and frozen (-10°C). Samples were used within 2 weeks.

Responses of rat aortic rings, with and without endothelium, to SNAP and GSNO

Male Wistar rats (250-300g) were killed by cervical dislocation. The thoratic aorta was removed, trimmed free of adhering fat and connective tissue and cut into 4mm rings. The endothelium was removed from some rings by gently rubbing the intimal surface with a pipe cleaner. The rings were mounted on stainless steel hooks in 20ml organ baths containing Krebs buffer. Indomethacin (5μM) was added to the Krebs buffer to inhibit the synthesis of cyclo-oxygenase products. The tissues were then subjected to an initial tension (1.0g) and allowed to equilibrate (75mins), during which time, the Krebs buffer was changed at 15 minute intervals. Responses were measured isometrically using Grass FT03 force-displacement transducers and recorded on a 6-channel multipen recorder (Rikadenkil).

The failure of ACh (1.28μM) to induce relaxation was taken as an indication that removal of the endothelium had been effective and this was confirmed in some experiments by scanning electron microscopy. In intact tissues, cumulative relaxation curves to ACh were obtained in each ring to assess the integrity of the endothelium. Rings showing <65% relaxation were discarded. After washout the tissues were allowed to equilibrate for a further 45 minutes during which time the Krebs buffer was changed at 15 minute intervals. The tissues were again contracted submaximally by addition of PE (approx EC_{90}). When a stable contraction was obtained, SNAP and GSNO doses were added to the organ baths.

5.2.2 Results & Discussion

S-Nitrosothiols such as SNAP and GSNO are NO donor drugs which release NO readily in tissue environments in a manner which does not require biotransformation, as is the case with organic nitrates. An experiment was carried out to show that SNAP causes vasodilation by an endothelium-independent mechanism. The responses elicited in rat tail artery preparations by bolus injections of SNAP (10μl) in the absence and presence of L-NAME, an NO synthase inhibitor, and the absence and presence of ferro-haemoglobin, an efficient NO scavenger, were compared. These were added to the internal perfusate (see apparatus **Figure 2.5**), .

The results are shown in **Figure 5.1**. In the presence of L-NAME there was no significant difference in the amplitude of response caused by 1 and 0.1μM injections of SNAP (0.1μM - 34% relaxation, 1μM - 52% relaxation) compared with those obtained in the absence of L-NAME (0.1μM - 36% relaxation, 1μM - 52% relaxation). In the presence of ferro-haemoglobin the responses to SNAP were dramatically reduced for 10μM injections (0.1μM & 1μM - 0% relaxation, 10μM - 6%), indicating the liberation of NO from the S-nitrosothiol.

These observations suggests that SNAP induced vasodilation is due to spontaneous release of NO and not due to stimulation of NO synthase. Further work carried out at Wellcome in collaboration with Rees, using rat aorta rings showed that SNAP and GSNO gave enhanced responses in the absence of the endothelium (see **Table 5.1**). Responses may be enhanced because of the supersensitivity of guanylate cyclase to NO which develops following a period of NO deprivation (Moncada *et al.*, 1991b).

Compound	n	ED_{50} (+E)	ED_{50} (-E)	Comments
SNAP	5	0.31+/- 0.15	0.056+/-0.0003	Fast, short acting response, full relaxation.
GSNO	1	0.15	0.074	intermediate rate of onset and duration, full relax.

***Table 5.1** A comparison of the vasodilator effectiveness of SNAP and GSNO on rat aorta rings showing that both S-nitrosothiols are endothelium-independent vasodilators. (+E) = + endothelium, (-E) = - endothelium.*

5.3 SPONTANEOUS/METAL ION CATALYSED RELEASE OF NO FROM SNAP

5.3.1 Experimental

The preparation, experimental procedure and SNAP sample preparation were carried out as described in the experimental section of **Chapter 2.4**. Two samples of SNAP (0.5mM) were prepared in phosphate buffer (0.1M KH_2PO_4/0.1M NaOH; see experimental section **Chapter 4.3**) and stored on ice at 0°C, protected from light. When the artery had stabilised after contraction with PE (about 30 minutes), 0.1ml of a solution of copper sulphate (1mM) was added to 9.9mls of one of the SNAP solutions to produce a solution containing 10μM of copper (II) ions. The clock was started and 10μl of the two SNAP solutions (one with added copper and one without) were injected into the artery at timed intervals, allowing full recovery between injections. This was carried out until the responses to injection of the SNAP/Cu(II) ion solution were minimal and unchanging.

Control injections of phosphate buffer alone and the phosphate buffer containing 10μM copper (II) sulphate were administered. Neither produced a response. Ferro-haemoglobin was made up as described in the experimental section of **Chapter 5.2.**

5.3.2 Results and Discussion

In **Chapter 3** it was shown that SNAP, unlike GSNO, is far more susceptible to decomposition by metal ion catalysis. Numerous other workers have suggested that S-nitrosothiols spontaneously decompose to release NO, and this is responsible for the physiological action of these compounds as vasodilators and smooth muscle relaxants. The often conflicting results obtained by different research groups have been attributed to the different preparations used to test the drugs. However, it is possible that the explanation may lie in differing amounts of trace metal ions present in the physiological buffer solutions (such as Krebs), required to keep the tissue viable. The constituents of these buffers will contain trace amounts of metal ions which can contaminate the perfusate and destabilise certain S-nitrosothiols (see **Chapter 3**).

Unfortunately, the use of EDTA to chelate metal ions from physiological buffers would also complex metal ions required for the essential functioning of the tissue. Therefore, quantification of the effect of trace metals on the physiological (vasodilator) responses of smooth muscle, to S-nitrosothiols is difficult to obtain and will depend on the time the drug is exposed to the buffer before it reaches the tissue.

A series of experiments were undertaken in which the decomposition rate of SNAP was compared to its vasodilator response on the rat tail artery, in the presence of trace amounts of copper ions (0.1μM-100μM), to see if the presence of copper ions affected vasodilator responses. Unfortunately, addition of copper to the internal perfusate greatly effected the precontracted (PE-induced) tone of the artery. Injections of increasing concentrations of SNAP into the perfusate containing copper did not fully recover. The progressive loss of tone after several injections resulted in an active

pressure which was too low to be physiologically relevant and indicated that continuous exposure of the artery to copper ions was possibly toxic for the preparation.

Due to this complication in the experimental protocol, experiments were carried out to investigate the effect of addition of copper(II) (10μM) to SNAP solutions (0.5mM) on the vasodilator responses obtained from bolus injection of this solution (10μl), at timed intervals. **Figure 5.2** shows an original trace obtained of vasodilator response versus time. The decomposition rate of SNAP was also monitored under the same conditions to compare the vasodilator response of SNAP to the rate at which it decomposes. It was hoped that this would give an insight into whether SNAP, NO, or both are responsible for the vasodilator response.

The results of three experiments are shown in **Figures 5.3** and **5.4. Figure 5.3** shows that when a single concentration of SNAP is stored at pH 7.4 on ice, in the presence of only the trace metal ions found in the phosphate buffer, its physiological effect hardly changed over the duration of the experiment (~200 mins). If this is compared with the decomposition rate of SNAP under the same conditions (on ice), it is evident that virtually no SNAP has decomposed over the same time period (**Figure 5.4**). This suggests that SNAP, or NO produced from SNAP in the presence of the artery, is producing the vasodilator response. However, when copper (II) ions (10μM) are added to the buffer solution containing SNAP (over and above those already present from the buffer itself) the vasodilator response of the resulting solution is dramatically reduced over the same time period (**Figure 5.3**). Comparison of this vasodilator response profile with the decomposition rate of SNAP under the same conditions (**Figure 5.4**), shows a large discrepancy as SNAP has fully decomposed over 40 minutes but is still capable of causing approx. 52% vasodilation. The amplitude of this response decreases with time but is still present after 150 minutes. These results suggest that the decomposition products of SNAP are also giving a response. Ferro-haemoglobin abolished most of the response indicating that it is an

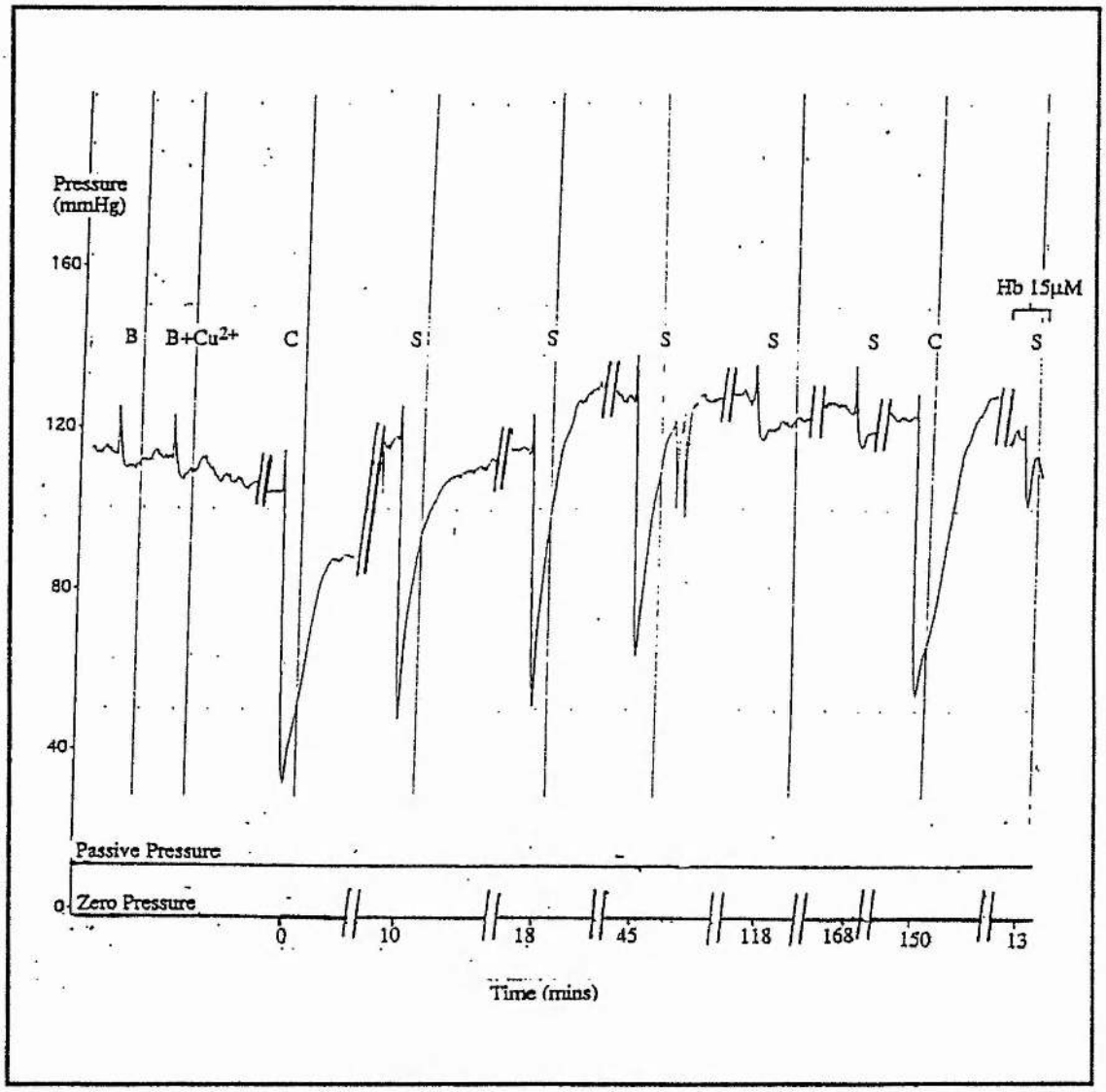


Figure 5.2 *A trace showing injections of a solution of SNAP (0.5mM) and Cu^{2+} ions ($CuSO4$; 10µM) kept on ice, with time. B = phosphate buffer only; B + Cu^{2+} = buffer + 10µM Cu^{2+} ions only; C = control, SNAP (0.5mM) & no added Cu^{2+} ions; S = sample, SNAP (0.5mM) & 10µM Cu^{2+} ions. Another sample (S) was injected into the artery perfused with 15µM Hb after 13 minutes of incubation with the Cu2+ ions (10µM). (Expt. N°94/4/27, PE 2.5µM)*

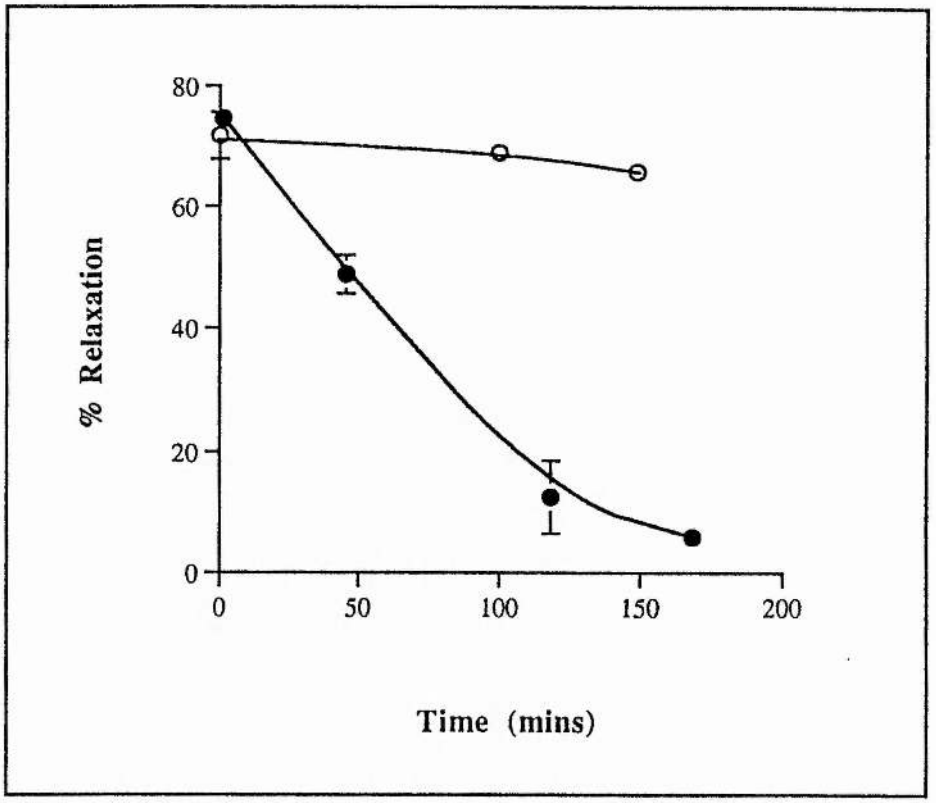


Figure 5.3 *The effect of copper ions (CuSO4; 10μM) on the vasodilator action of SNAP (0.5m; kept at 0 ℃) over time.(open circles) no added Cu(II) ions; (closed circles) 10μM Cu(II) ions added*

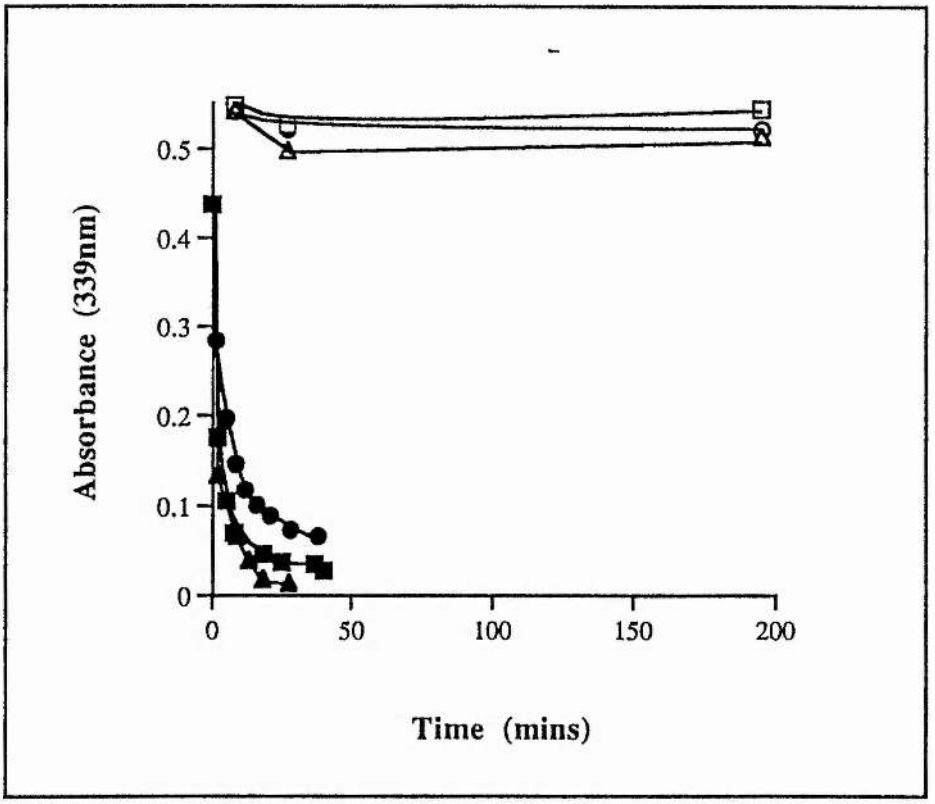


Figure 5.4 *The effect of copper(II) ions (CuSO4; 10μM) on the decomposition of SNAP at 0 ℃. (filled symbols) Cu(II) ions added; (open symbols) no added Cu(II) ions.*

NO-mediated response, suggesting that a significant amount of NO survives for long enough to cause vasodilation of the artery.

These results suggest that preformed NO from copper ion catalysis, NO produced from SNAP in the presence of the tissue, or even SNAP itself can produce vasodilation of the rat tail artery. It also demonstrates that metal ions, like copper, can affect the vasodilator response of SNAP.

Can metal ion catalysed decomposition of S-nitrosothiols occur in vivo?

Copper is the third most abundant heavy metal ion in the human body, at levels of between 1.4-2.1mg/kg body weight (Hughes, 1987; Howard-Lock & Lock, 1987). It is an essential metal and is present in trace amounts in all tissues for normal metabolism. The highest levels in man are found in the liver, brain, lung and kidney. Deficiencies in copper result in increased fragility of bones, aneurysm formation in arteries and loss of lysyl oxidase activity in cartilage. These deficiencies are brought about by a number of factors;

1) Low intake - due to malnutrition
2) High loss - due to cystic fibrosis and nephrotic syndromes
3) Genetic factors such as Menke's disease

Menke's disease is an inborn error of copper metabolism leading to a life expectancy of less than three years due to low activity of copper-requiring enzymes. The lethal deficiency occurs in the brain and a characteristic feature of the disease is 'steely' hair structure, due to the presence of free SH groups as lysyl oxidase fails to form disulphide links.

At the other end of the scale, an excess of copper, particularly in the liver is a symptom of Wilson's disease. Low levels of the copper transport protein ceruloplasmin result in excess copper in the liver and progressive liver disease. D-penicillamine or

triethyltetramine (TETA) are used as therapeutic agents to chelate excess copper (more about chelation is discussed in **Chapter 3**).

Of the 5mg of circulating copper in human serum approximately 90-95% of it is tightly and irreversibly bound to the protein ceruloplasmin (Hughes, 1987; Howard-Lock & Lock, 1987), a blue-coloured oxidase enzyme that plays a major role in copper transportation. Ceruloplasmin is a single polypeptide chain of molecular weight 130,000D containing 7 copper binding sites (Hughes 1987). These 7 copper ions are in different enzymatic sites. Two are in type 1 sites which are responsible for electron transfer reactions, the nature of the coordination site ensures a minimal activation energy for copper to change between its two oxidation states. One copper ion is in a type 2 site, which works in conjunction with the type 1 sites in the overall electron transfer process and may represent a substrate binding site. There are also four type 3 sites which consist of Cu(I) and binuclear coupled Cu(II) ions which are used to bind and reduce dioxygen.

The remaining copper in human serum is reversibly bound to the protein albumin or histidine, particularly in the gut. **Figure 5.5** shows a tripeptide mimic to the Cu(II) binding site of albumin (Iyer *et al.*, 1978). It has been postulated that the copper ion can be rapidly transferred from albumin to an amino acid eg. histidine, and then to its free unbound state (Sarker, 1981). Albumin is thought to be a transporter of copper whereas the small size of amino acids like histidine make them membrane diffusible, allowing copper to enter cells (**Figure 5.5**). This mechanism has not been satisfactorily proved due to the small amount of copper involved (1μM) which is difficult to measure experimentally. It is also reasonable to assume that receptor sites exist in the various target tissues for copper ions and the possibility of copper release by a reductive method from ceruloplasmin or albumin. If this occurs at the cell membrane then there may be an intracellular receptor for Cu(I) which, as discussed in **Chapter 3,** also catalytically decomposes SNAP.

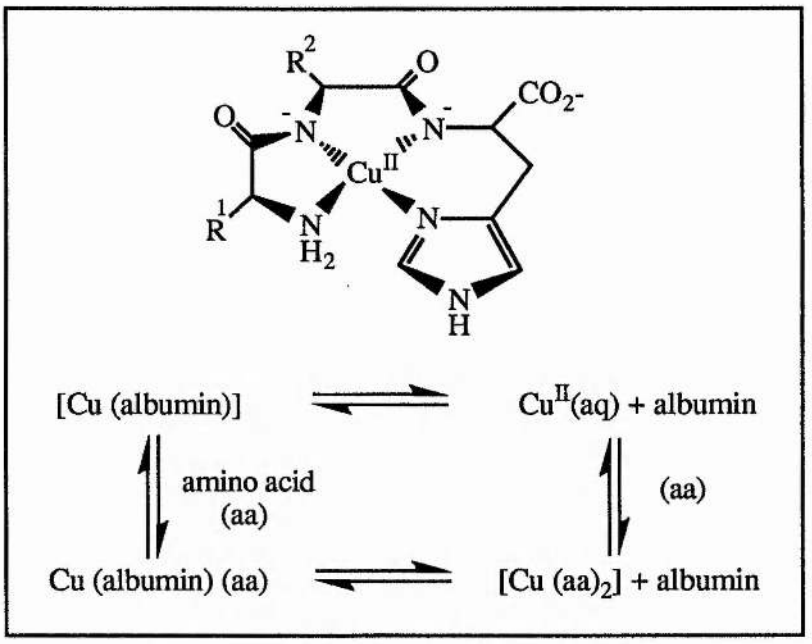


Figure 5.5 *A model for the copper (II) binding site of albumin by the tripeptide l-Gly-L-Asp-L-His and the different environments postulated for copper in the presence of albumin and histidine. (adapted from Comprehensive Coordination Chemistry chapter 20.2).*

Whatever environment the copper ions are in, the quantity required to catalyse the decomposition of S-nitrosothiols is very small, in the region of 1μM which as mentioned is difficult to detect experimentally. Furthermore, it has not been determined if copper bound to albumin or ceruloplasmin or the peptide histidine, can effect catalysis of S-nitrosothiols by ligand replacement. It is also possible that one or more of the different copper sites on ceruloplasmin could produce NO from S-nitrosothiols. These factors must be examined to determine whether trace metal ion catalysis by copper ions has a role in the *in vivo* metabolism of S-nitrosothiols.

5.4 IS SNAP DECOMPOSED ENZYMATICALLY TO NO *EX VIVO*

5.4.1 Experimental

a) *Synthesis of D-SNAP (S-nitroso-N-acetyl-D-penicillamine)*

The same procedure was followed as for the synthesis of S-nitroso-N-acetyl-DL-penicillamine, using N-acetyl-D-penicillamine (see experimental section **Chapter 2.2.2**). Mpt. (149°C), $[\alpha]^{20}_{D}$ (c=0.088, methanol) = -80.1°

b)*Synthesis of L-SNAP (S-nitroso-N-acetyl-L-penicillamine)*

N-acetyl L penicillamine is not available commercially and therefore had to be synthesised in the laboratory as follows.

i). *Preparation of N methoxydiacetimide*

Triethylamine (239mmol, 24.14g) was mixed with methoxyamine hydrochloride $MeONH_2HCl$ (59.7mmol, 4.98g) in CH_2Cl_2 (50ml) with cooling. Acetic anhydride (149mmol, 15.2g) was added dropwise over a period of 30 mins. The reaction mixture was stirred overnight at room temperature. The salt formed was filtered off and the solution washed with 5% bicarbonate (3x35ml), 0.1M HCl (2x30ml), H_2O (1x30ml) and then dried over magnesium sulphate. The solvent was removed under reduced pressure to give an oil that was distilled (72-74°C 12mmHg) to give the product as a colourless oil. 5.77g (73.7% yield) V_{max} (Nujol) 1720cm^{-1} (amide CO), δH (200MHz, $CDCl_3$) 2.31 (s,6H,CH_3CO) & 3.74 (s,3H,OCH_3), *m/z* (EI) 131 (M^+, 1.92%), 89 (M^+H-CH_3CO, 38.17%), 43 (CH_3CO, 100%),

ii) *Preparation of N-acetyl-L-penicillamine*

To L-penicillamine (1mmol, 0.149g) in dioxane (4ml) was added 1 equivalent of sodium hydroxide solution (2M, 0.5ml) followed by N-methoxydiacetimide (262mg, 2mmol). The solution was stirred at room temperature for 27 hours approx. after which time the mixture was diluted with H_2O (4ml), pH adjusted to 4 with glacial acetic acid and extracted with ethyl acetate (3x20ml). The organic phase was dried over $MgSO_4$ and the solvent removed under reduced pressure to give a white solid. This solid was

then suspended in ether, filtered, washed with ether (15ml) and dried to give N-acetyl-L-penicillamine as a white solid. 0.067g (35% yield). $[\alpha]^{20}_D$ (c=1, ethanol/water 1:1) = -11.28° (lit -10.5±1°, Field *et al.*, 1978) δH (200MHz,d_6-DMSO) 1.4 (6H,s,2xCH_3), 1.95 (3H,s,OCH_3), 4.44 (1H,d,CH), 8.15 (1H,d,NH), V_{max} (nujol) 3600-3200cm^{-1} (OH), 3350 (NH), 1720 & 1640 (C=O).

iii).Preparation of L-SNAP (S-nitroso-N-acetyl-L-penicillamine)

The same procedure as the synthesis of S-nitroso-N-acetyl-DL-penicillamine was followed, only using N-acetyl-L-penicillamine. Mpt. (149°C), $[\alpha]^{20}_D$ (c=0.088, methanol) = +75.0°

Physiological Testing

The same procedure was carried out as described in the experimental section of **Chapter 2.4.**

5.4.2 Results and Discussion

Another possible mechanism in which S-nitrosothiols like SNAP can be decomposed *ex vivo* and *in vivo* is by an enzymatic process. To investigate this possibility, the two enantiomers of SNAP (L & D) were synthesised and their pharmacological properties compared using the rat tail artery preparation. S-nitroso-N-acetyl-D-penicillamine was synthesised directly from N-acetyl-D-penicillamine by nitrosation using acidified nitrite. S-nitroso-N-acetyl-L-penicillamine was synthesised from L-penicillamine by acetylation using N-methoxydiacetimide for acetylation, and subsequent nitrosation by the same method. Optical rotation readings showed that the opposite chirality of the two compounds had been retained. The samples made up for testing on the rat tail artery were stored on ice for the period of the experiment to minimise metal ion catalysed decomposition (see **Figure 5.3**).Solutions of SNAP (0.1μM - 10mM) were injected into the artery as a bolus. The narrow bore of the cannula, the short time period between injection and reaching the tissue, and the bolus nature of drug delivery serve to minimise the metal ion catalysed decomposition of SNAP. Therefore, if there

was an enzymatic process which contributed to SNAP decomposition, a difference should be seen in the log dose/response curves. These log dose/response curves are shown in **Figure 5.6** and compared with those obtained for the racemic mixture of DL SNAP, tested on the same arteries. The results show no statistically significant difference between any of the SNAP samples tested, suggesting that enzymatic decomposition of SNAP is not a factor determining the rate of SNAP decomposition in the rat tail artery. Responses were not examined in other physiological models to see if this finding was consistent across tissue types.

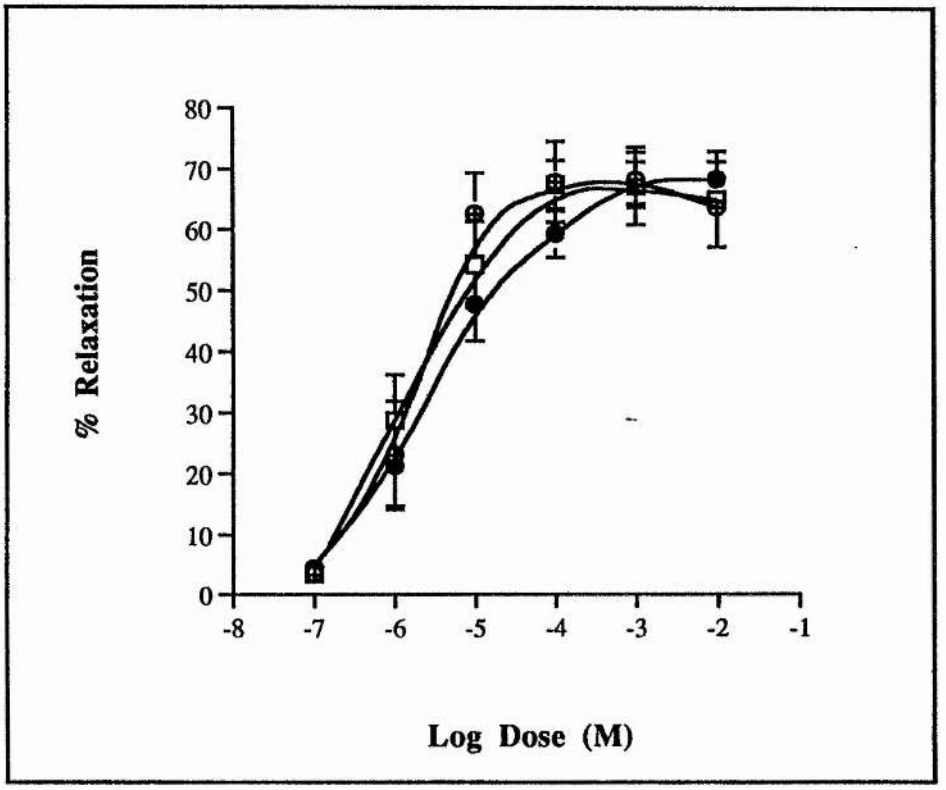


Figure 5.6 *Log dose/response curves comparing vasodilator responses elicited in the rat tail artery by injections (10μl) of DL SNAP (closed circles, n=3-5), D SNAP (open circles with crosses, n=3-5), and L SNAP (open squares, n=3-5).*

Experiments were also conducted by continuous perfusion of the artery with the enantiomers of SNAP (see **Chapter 7** for the experimental procedure) and no difference in the responses obtained was evident. These results are not shown as decomposition of SNAP using this method of delivery is more susceptible to metal ion

catalysis than that of delivery by bolus injection. This puts the results obtained under question.

5.5 THE EFFECT OF γ-GLUTAMYL TRANSPEPTIDASE ON GSNO DECOMPOSITION

5.5.1 Introduction

In **Chapters 2 and 3** it was shown that GSNO is a stable S-nitrosothiol in aqueous solution at physiological pH and is essentially resistant to trace metal ion catalysis. However when exposed to the rat tail artery, *ex vivo*, it turns out to be a good vasodilator. If it is assumed that NO released from GSNO activates guanylate cyclase to produce relaxation, then this suggests that the tissue possess the ability to cleave NO from the tripeptide. Possible tissue candidates for carrying out this action will be discussed in this section and **section 5.6**.

In **section 5.4**, experimental evidence suggested that SNAP is not decomposed by an enzymatic process. However, SNAP is a synthetic S-nitrosothiol which is not endogenous to living cells. GSNO on the other hand, a derivative of the endogenous peptide glutathione, has been detected *in vivo* at concentrations of approximately 0.25μM in venous plasma and up to 1.2μM in arterial plasma (Stamler *et al.*, 1992; Meyer *et al.*, 1994). Therefore it is more likely that this S-nitrosothiol could be acted upon enzymatically to produce NO and vasodilation.

One possible candidate is γ-glutamyl transpeptidase (γ-GT), a cell surface enzyme thought to be of particular importance in the hydrolysis of GSH and GSH conjugates. The enzyme is able to catalyse both the hydrolysis of the γ-glutamyl group of GSH (or other γ-glutamyl peptides), autotranspeptidation with glutathione as the acceptor and γ-glutamyl transfer to other suitable acceptors (see **Figure 5.7**). Work carried out by incubating the purified enzyme with glutathione and a mixture of amino acids, that

closely approximates the amino acid composition of blood plasma, showed that transpeptidation is a significant function of the enzyme. This was determined by quantitative measurement of the products formed (Allison & Meister, 1981). At physiological pH (7.4), about 50% of the glutathione that was utilised, participated in transpeptidation. Studies in which the formation of individual γ-glutamyl amino acids was monitored showed that L-cystine and L-glutamine are the most active amino acid acceptors.

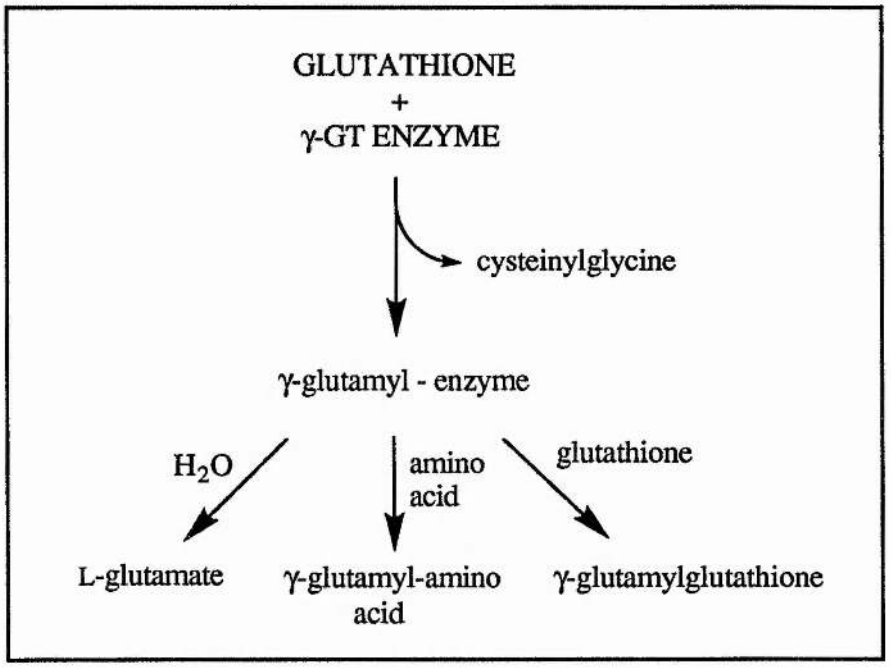


***Figure 5.7** Reaction pathways catalysed by γ-glutamyl transpeptidase*

The enzyme mechanism

The mammalian γ-glutamyl transpeptidases exhibit similar catalytic properties which are specific for γ-glutamyl compounds. Three separate subsites exhibiting characteristic preferences can be distinguished at the active site. Binding of L-γ-glutamyl, D-γ-glutamyl and L-γ-(α-methyl) glutamyl compounds occurs at the γ-glutamyl binding subsite (Griffith & Meister, 1977). On the other hand, the acceptor site, consisting of subsites for cysteinyl and glycine moieties, has restricted stereo-specificity in that only L-amino acids and dipeptides, in which both amino acids are of L-configuration, serve

as acceptors (Tate & Meister, 1974). Kinetic and specificity studies indicate that a large variety of dipeptide acceptors bind to the Cys-Gly site, interacting with the cysteinyl subsite which prefers neutral amino acids such as cystine and glutamine. Kinetic studies are also consistent with a ping-pong mechanism involving a γ-glutamyl-enzyme intermediate (see **Figure 5.8**) allowing transpeptidation or hydrolysis (Tate & Meister, 1974).

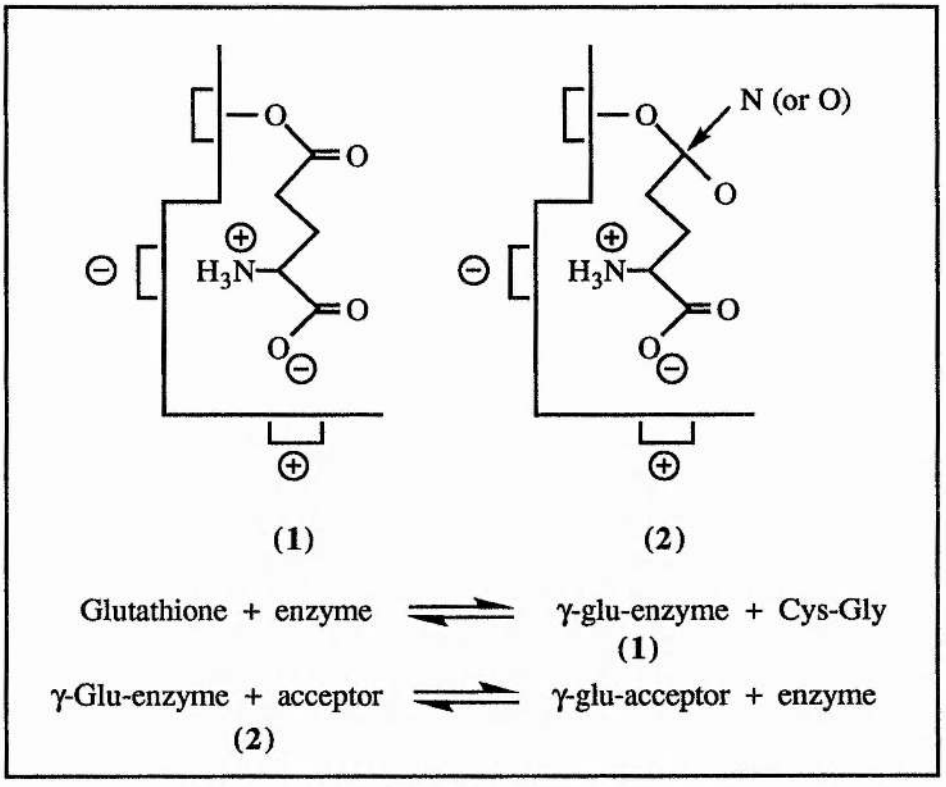


***Figure 5.8** A schematic representation of the active site of γ-glutamyl transpeptidase depicting the γ-glutamyl-enzyme intermediate **(1)** and the expected tetrahedral transition state intermediate that would be formed during the transfer of the enzyme bound γ-glutamyl moiety to the α-amino acid group of the acceptor or to water **(2)**.*

γ-GT is present on the *external* surface of certain cell membranes. In most mammals the kidney exhibits the highest activity, but the enzyme is found in many different types of cells and has been detected in bile, seminal fluid and, more importantly for this study, in human blood serum. (Tate & Meister, 1981). It is in contact with many amino acids, including glutathione which is translocated across cell membranes.

Therefore, it would seem that γ-glutamyl amino acids must be continually produced and transported into cells. The enzyme may also play a role in the extracellular metabolism of glutathione.

The subject of this study was to see if γ-GT could bring about decomposition of GSNO, by the same mechanism of γ-glutamyl cleavage. This would form an S-nitrosothiol which is likely to have greatly reduced stability, compared with GSNO and which could readily release NO. Evidence that this mechanism is possible, may imply that this enzyme, or a closely related enzyme such as the γ-glutamyl transpeptidase related enzyme which has 40% homology to γ-GT (Heisterkamp *et al.*, 1991), 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.

5.5.2 Experimental

Solution preparation

A γ–glutamyl-transpeptidase solution (Sigma, 2.1mg in 1.5ml distilled water; 35units/ml) was diluted to the concentrations indicated in **Figures 5.10 & 5.11** by serial dilution into spectrophotometer cuvettes containing phosphate buffer pH7.4 at 30°C. An aliquot (0.1ml) of GSNO (0.0168g in 5ml buffer) was added to the cuvette to make up a 0.4mM solution. If EDTA (50μM) was required, 2ml of 12.5μM solution in phosphate buffer pH7.4, was used in the cuvette instead of buffer alone. When this was diluted to 2.5ml by the enzyme solution and GSNO the required concentration of EDTA was attained.

Kinetic studies

The kinetics of GSNO decomposition was monitored by following the reduction in absorbance at 339nm using a Phillips PU8700 UV/Vis scanning spectrophotometer with a Pye Unicam cell temperature control unit. All experiments were carried out at 30°C in a phosphate buffer at pH 7.4 (0.1M KH_2PO_4/ 0.1M NaOH) made up using

distilled water which had undetectable copper and iron content on the ppm (parts per million) scale. Therefore, trace metal ions present in the buffer solution must come from the buffer system itself.

Enzyme kinetics

The activity of the enzyme γ–glutamyltranspeptidase was tested by the use of γ–glutamyl-*p*-nitroanilide as the standard substrate and showed expected activity. Measured amounts of the enzyme (see **Figures 5.10 & 5.11**) were then added to buffered solutions of GSNO, in the absence and presence of glutamine (0.4mM), and the decomposition monitored spectrophotometrically as above.

Capillary Zone Electrophoresis

The apparatus used was a Biofocus 3000 Capillary Electrophoresis System (Bio–Rad, Richmond, CA) fitted with a polyacrylamide coated capillary (17 cm x 25μm). Electrophoretic separations were detected on–line by computer. The analyses were performed at 20°C. To initiate analyses the capillary and electrode reservoirs were filled with electrophoresis buffer (0.01M Na_3PO_4/0.03 HCl, pH 2.3, as used by Stamler *et al.*, 1992). The capillary was first flushed with HPLC-grade water (Rathburn Chemicals, Walkerburn, Scotland), secondly with capillary wash (Bio–Rad), and thirdly by the electrophoresis running buffer. This procedure was carried out between each sample run. The voltage field strength was 11kV and the polarity of the internal power supply was set for migration of cations towards the detector (positive polarity). The samples were diluted 1:10 with HPLC water. The detector wavelength was set at 200nm. The decomposition of GSNO was also monitored at 335nm (the absorption maximum of GSNO).

5.5.3 Results and Discussion

GSNO was incubated with γ–glutamyltranspeptidase for 30–60 mins and the resulting solution examined by capillary zone electrophoresis (CZE) (**Figure 5.9**). Peaks were

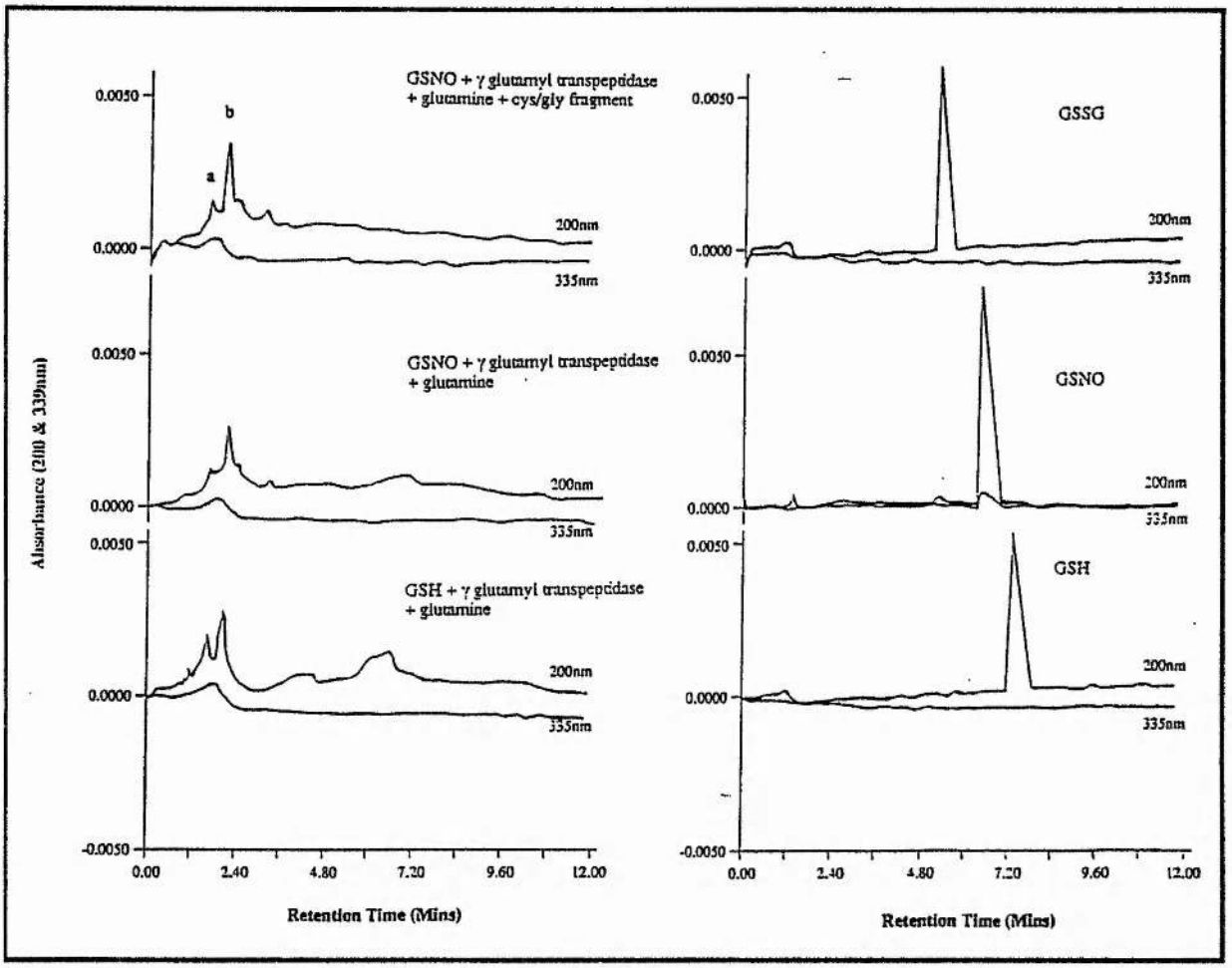


Figure 5.9 *Capillary Zone Electrophoresis traces for glutathione (GSH), S-nitrosoglutathione (GSNO) and oxidised glutathione (GSSG) (all 1mg/ml), comparing them to the γ-glutamyl transpeptidase enzyme (3.3 units/ml) digest products of glutathione and S-nitrosoglutathione in the presence of the acceptor amino acid glutamine (1mg/ml). The L-cysteinylglycine (cys/gly) fragment of glutathione was also used to spike the enzyme digest of S-nitrosoglutathione and glutamine, giving rise to an increase in peaks a & b. It was shown that a & b were the reduced and oxidised forms of the cys/gly fragment. Note the complete disappearance of the GSNO peak and no formation of the peak corresponding to GSSG. This also indicates that cleavage of the glutamyl/cystyl peptide bond has taken place.*

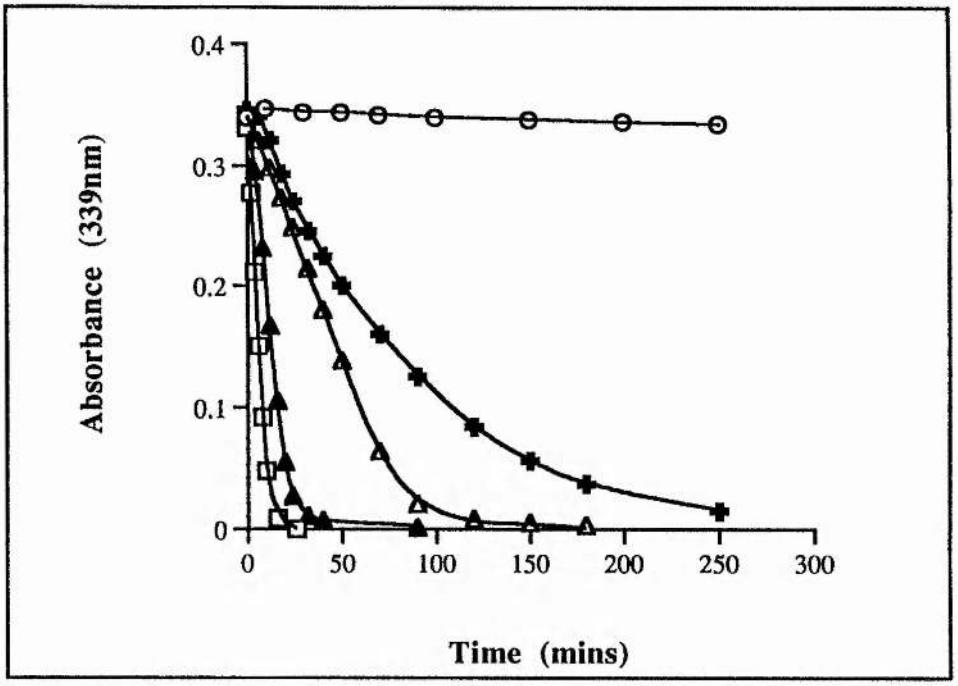


Figure 5.10 *The effect of the enzyme γ-glutamyl transpeptidase (GGT) on the decomposition rate of GSNO (0.4mM), in the presence and absence of EDTA. (open circles) GSNO, (open triangles) GSNO/GGT (0.7 units/ml), (closed triangles) GSNO/GGT (2.8 units/ml), (open squares) GSNO/GGT (5.6 units/ml), (black crosses) GSNO/GGT (5.6 units/ml) + EDTA (50μM). Conditions; 30°C, KH_2PO_4 buffer pH 7.4*

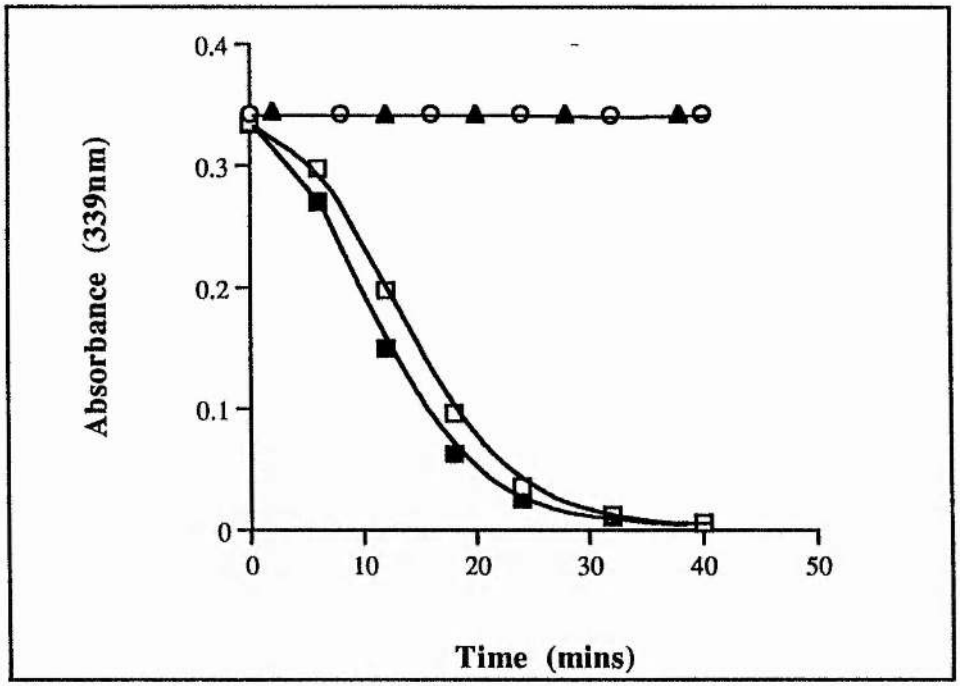


Figure 5.11 *The effect of the enzyme γ-glutamyl transpeptidase (GGT) on the decomposition rate of GSNO (0.4mM) in the presence and absence of an acceptor peptide (glutamine), for the transfer of the glutamyl fragment of GSNO. (open circles) GSNO, (closed triangles) GSNO+glutamine (0.4mM), (open squares) GSNO/GGT (2.1units/ml), (closed squares) GSNO/GGT (2.1units/ml) + glutamine (0.4mM). Conditions; 30°C, KH_2PO_4.*

observed at the elution times corresponding to L-cysteinylglycine and the disulfide of L-cysteinylglycine. However, no peaks were observed corresponding to GSNO, glutathione, and the disulphide of glutathione. The absence of a GSNO peak shows that decomposition had occurred. However this appears not to have resulted in release of NO from GSNO since such a reaction would have led to formation of the disulphide of glutathione. However, if the enzyme had effected cleavage of the cysteine–glutamic acid peptide bond to give S–nitroso-L-cysteinylglycine, which then decomposed, releasing NO, formation of the disulphide of L-cysteinylglycine would be expected. Subsequently, the kinetics of breakdown of GSNO were examined by monitoring the disappearance of the absorption peak at 335nm, as a function of enzyme concentration. The results are displayed in **Figures 5.10** and **5.11**. Clearly the rate of GSNO decomposition was enzyme dependent and occurred by hydrolysis (**Figure 5.10**), or in the presence of the amino acid acceptor of the glutamyl fragment, glutamine (**Figure 5.11**).

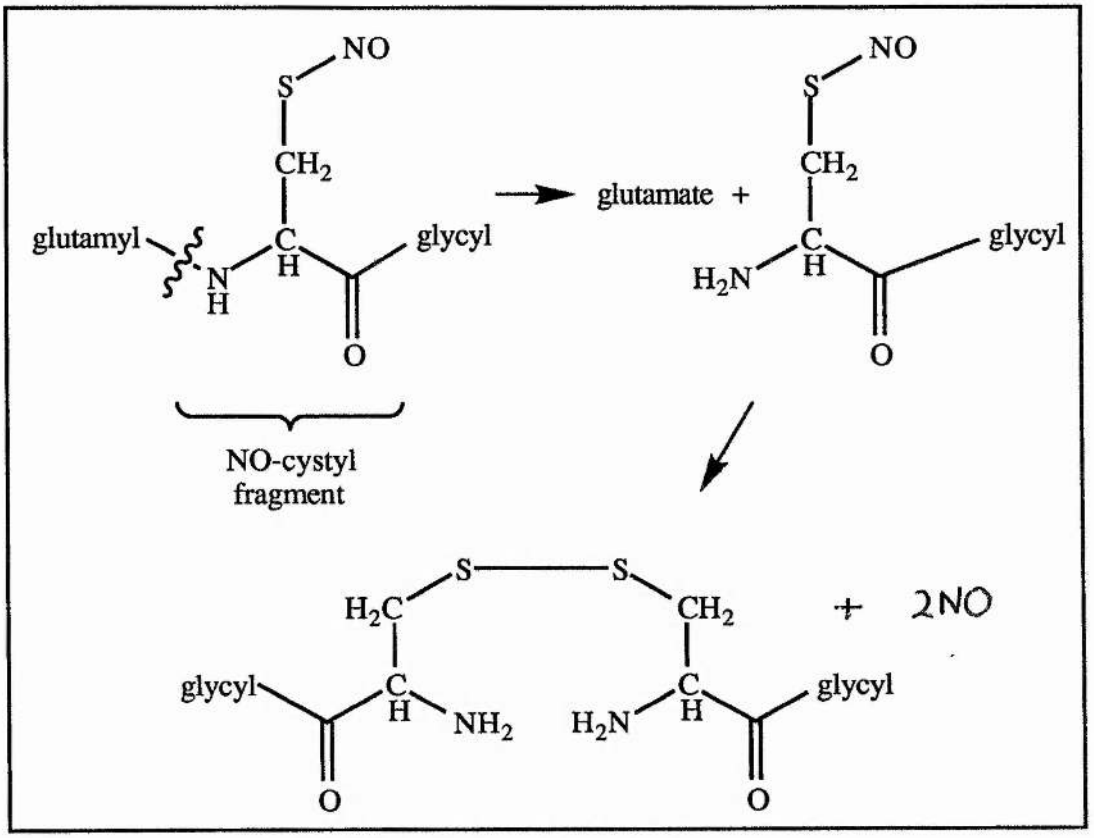


***Figure 5.12** The proposed mechanism of action of γ-glutamyl transpeptidase on the decomposition rate of GSNO, postulated from experimental results.*

The results obtained are consistent with the reaction shown in **Figure 5.12**. One would expect the rate of release of NO from S-nitroso-L-cysteinylglycine to be greater than from GSNO as the catalysing metal can complex with two favourable binding sites in the former ($-NH_2$ and $-SNO$) while the latter is resistant to metal ion catalysis, as discussed in **Chapter 3**.

Figure 5.10 shows metal ion catalysed NO release from GSNO occurred. Addition of EDTA to a mixture of GSNO and γ–glutamyltranspeptidase diminished the rate of decomposition of the S-nitrosothiol to NO, while having no effect on the action of the enzyme (results not shown).

5.6 TRANSNITROSATION (NO TRANSFER) FROM SNAP AND GSNO TO OTHER THIOLS

5.6.1 Introduction

The effect of trace metal ion catalysis on the decomposition rate of SNAP, and the effect of enzymes such as γ-glutamyl transpeptidase on GSNO metabolism, are supplemented by possible NO transfer to one or more of numerous sulphydryl containing molecules present *in vivo*. Clearly, the process of transnitrosation can also effect the nature and stability of GSNO and SNAP. The sulphydryl groups present *in vivo* are contained in molecules which are either membrane bound, or free, either in the intracellular matrix (eg. cysteine, glutathione) or in the plasma (albumin). These sulphydryl containing molecules, together with tissue-type plasminogen activator and cathepsin B, are susceptible to nitrosation and NO transfer from S-nitrosothiols under physiological conditions (Stamler *et al.*, 1992). 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 in 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 demonstrated that bioactive NO

equivalents in plasma are bound predominantly to thiol groups of proteins and that this reservoir serves a role in modulating vasodilator tone (Stamler *et al.*, 1992a&b).

If transnitrosation occurs from a relatively stable S-nitrosothiol 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-nitrosothiol, S-nitrosocysteine, could facilitate the release of NO and account in part for the biological action of these S-nitrosothiols. This possibility is discussed in this section.

5.6.2 Experimental

Kinetics study

0.25ml of 10mM L-Cysteine (0.0121g in 10ml buffer) was added to phosphate buffer pH7.4 (2ml) at 30°C in a spectrophotometer cuvette. An aliquot (0.25ml) of SNAP (0.0088g in 10ml buffer; 4mM) was added to the cuvette and the absorbance decrease at 339nm was measured as a function of time for the 1mM cysteine/0.4mM SNAP solution, versus a standard cuvette containing all but the S-nitrosothiol at the same concentrations. The same experiment was conducted using S-methylcysteine (0.0135g in 10ml buffer; 10mM) substituted for cysteine with SNAP.

Both experiments were repeated using GSNO (0.0134g in 10ml buffer; 4mM) instead of SNAP.

Physiological testing

The same procedure was carried out as described in the experimental section of **Chapter 2.4**. Bolus injections of GSNO (0.1μM - 10mM) were administered to the artery via the injection port **I** (see **Figure 2.5**) to obtain a control dose response curve. L-cysteine solution (1mM) was then perfused through the artery in the internal Krebs buffer supply and the GSNO injections were repeated. The mean response

amplitudes of nine experiments were obtained for each concentration and statistical analysis of the results was carried out.

Statistical analysis of data in figures

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

5.6.3 Results and Discussion

Addition of a 20mM cysteine solution to a 20mM solution of GSNO produced a change in colour from pink to a red solution, with an absorption spectrum identical to that of S–nitrosocysteine (made by the action of acidified sodium nitrite upon cysteine). This effect could not be observed visually at 0.4mM concentrations of GSNO but the greatly enhanced rate of decomposition (monitored spectrophotometrically) of GSNO in the presence of cysteine was evidence that it occurred (**Figure 5.13**). There was no difference in decomposition rate for GSNO, compared with GSNO in the presence of 1mM S-methylcysteine (**Figure 5.13**), implying that NO transfer can occur only if there is a free thiolate group. A similar effect was seen on the decomposition rate of SNAP on addition of cysteine (**Figure 5.14**). In contrast, addition of S-methylcysteine to SNAP caused a small decrease in the rate of NO release. This is consistent with the removal of adventitious metal ions in the buffer solution due to binding to S-methylcysteine, leaving less available to catalyse the decomposition of SNAP (see **Chapter 3**).

Transnitrosation reactions are known to be rapid (Park, 1988). Therefore, the rate-determining step in the cysteine catalysed decomposition of GSNO must be loss of NO from S–nitrosocysteine. Formally, transnitrosation may be seen as transfer of NO^+ from GSNO to cysteine (**Figure 5.15**) and here the suggestion by Lipton (1993) that

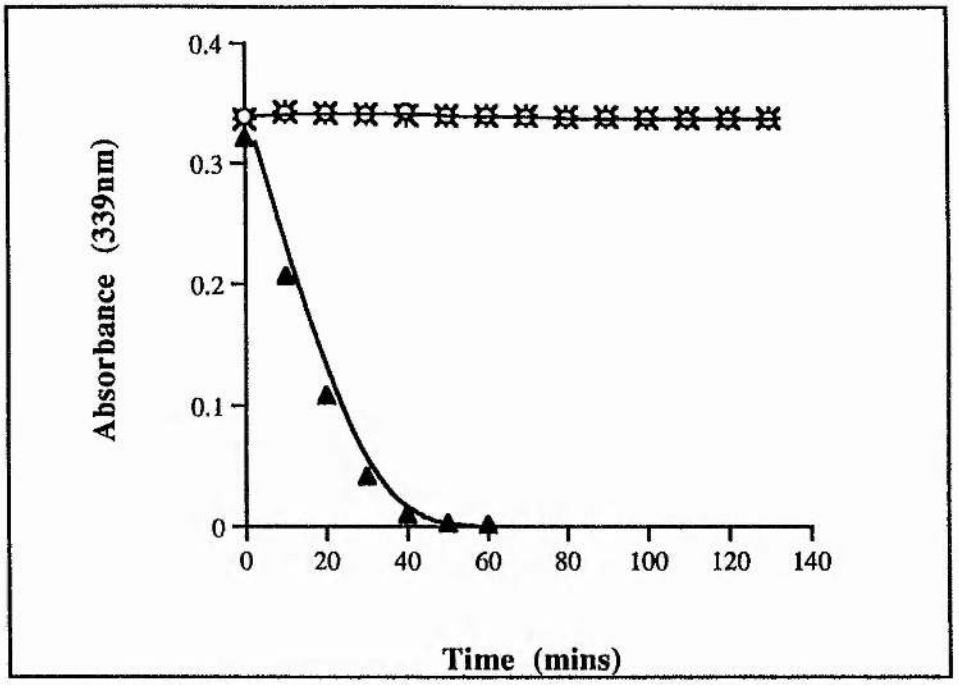


Figure 5.13 *The effect of cysteine (1mM) and S-methylcysteine (1mM) on the decomposition of GSNO. GSNO (open circles, n=6), GSNO + cysteine (closed triangles, n=4), GSNO+S-methylcysteine (black crosses, n=2). GSNO+cysteine data is significantly different from GSNO and GSNO+S-methylcysteine data at P=0.001 (***) confidence level. Conditions; 30 ℃, KH_2PO_4 buffer pH 7.4*

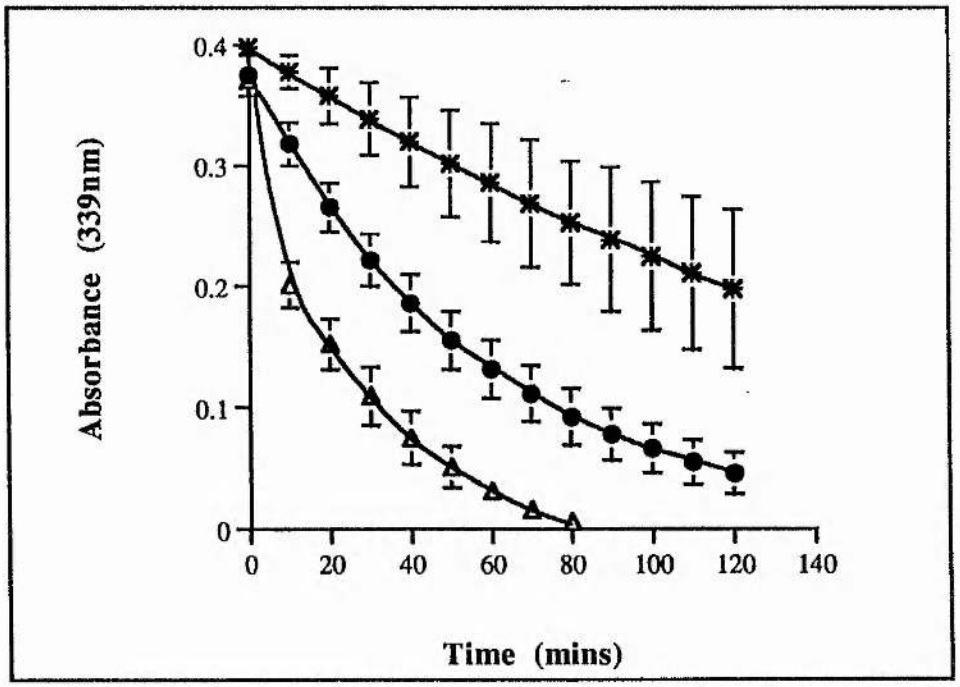


Figure 5.14 *The effect of cysteine (1mM) and S-methylcysteine (1mM) on the decomposition of SNAP. SNAP (closed circles, n=9), SNAP+cysteine (open triangles, n=7), SNAP+S-methylcysteine (black crosses, n=4). SNAP+cysteine data is significantly different from SNAP and SNAP+ S-methylcysteine data at P=0.01 (**) & at P=0.001 (***) confidence level respectively, & SNAP data is significantly different from SNAP+ S-methylcysteine data at P=0.01 (**) confidence level . Conditions; 30 ℃, KH_2PO_4 buffer pH 7.4*

NO^+, as well as NO, has a role in animal physiology, is acknowledged. However, the free nitrosonium ion (NO^+) can never exist in an aqueous physiological environment due to its rapid conversion to nitrous acid.

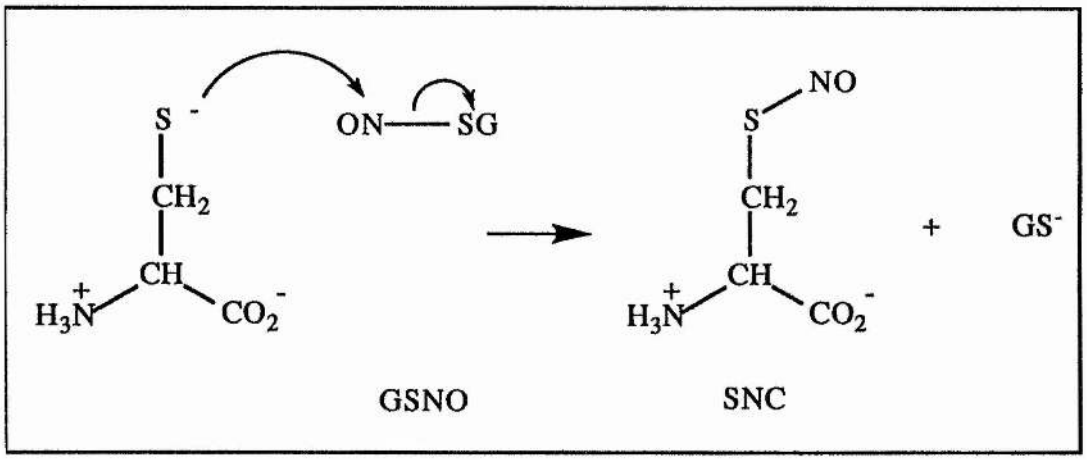


Figure 5.15 *The transfer of NO (as NO^+) from GSNO to L-cysteine*

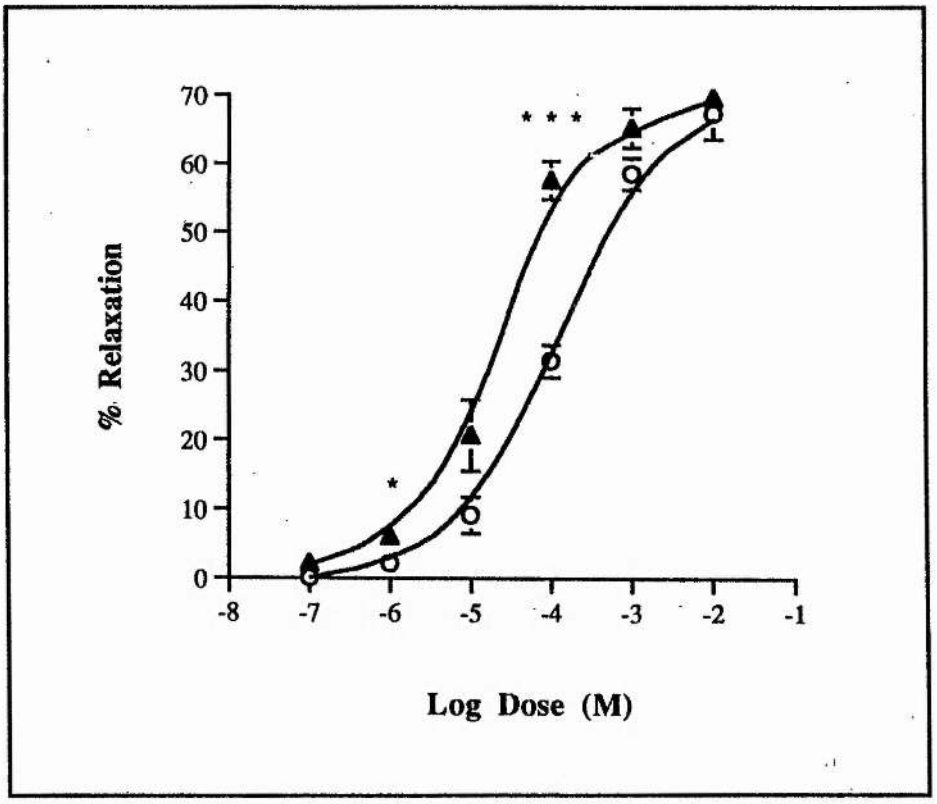


Figure 5.16 *The effect of adding cysteine (1mM) to the internal perfusate on the vasodilator responses to bolus injections of GSNO. GSNO (open circles, n=5-9), GSNO/L-cysteine (closed triangles, n=5-9). ED_{50} GSNO = 120μM, ED_{50} GSNO/L-cysteine = 19μM.*

The *ex vivo* vasodilator action of bolus injections of GSNO was enhanced when cysteine was allowed to perfuse through the artery via the internal Krebs buffer supply (**Figure 5.16**). The ED_{50} value for microinjections of GSNO was found to be 120μM compared with 19μM in the presence of 1 mM cysteine. As GSNO has only 3-5 seconds to react with cysteine before reaching the artery, it is reasonable to suppose that NO^+ transfer to form a less stable thiol occurs rapidly.

5.7 SITES OF NO PRODUCTION *EX VIVO* BY GSNO AND SNAP

5.7.1 Introduction

S-nitrosothiols are endothelium-independent vasodilators which are susceptible to decomposition by a number of mechanisms. The rate of decomposition of SNAP *ex vivo* has been shown to be influenced by trace metal ion catalysis, and also by the presence of other thiols such as cysteine, by production of the very unstable S-nitrosothiol, S-nitrosocysteine. On the other hand, GSNO is more stable in solution, being more resistant to trace metal ion catalysis, but its decomposition rate is affected by the enzymatic action of γ-glutamyl transpeptidase (or other possible γ-glutamyl cleaving enzymes) and by thiols such as cysteine.

The extent to which each of these different mechanisms cause decomposition of S-nitrosothiols *ex vivo* is difficult to ascertain quantitatively. The relative degree of NO production from GSNO and SNAP at different sites within or around the tissue, would give an indication as to which mechanisms are most important in this process. The use of the NO scavenger ferro-haemoglobin (Chies, 1969; Dickson & Chies, 1971; Martin *et al.*, 1985) or superoxide dismutase, an enzyme which scavenges superoxide ions and consequently prolongs the half-life of NO, added to the internal perfusate of the rat tail artery preparation, should give an indication as to whether NO produced from SNAP and GSNO is released in solution, in the presence of the tissue, or both.

5.7.2 Experimental

The same procedure was carried out as described in the experimental section of **Chapter 2.4**. Bolus injections of GSNO or SNAP (0.1μM - 10mM) were administered to the artery via the injection port **I** (see **Figure 2.5**) to obtain a control dose response curve. Ferro-haemoglobin (15μM) and superoxide dismutase (150 units ml^{-1}; Sigma Chemicals) were then perfused through the artery in the internal Krebs buffer supply and the GSNO or SNAP injections were repeated. The meaned response amplitudes of experiments were obtained for each concentration and statistical analysis of the results was carried out.

Statistical analysis of data in figures

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

Drugs: Synthetic procedures and use

SNAP & GSNO - 10^{-2}M solutions of SNAP and GSNO were made up in Krebs buffer and serially diluted to the concentration required for injection immediately prior to use. Both S-nitrosothiols solutions were kept in the dark and on ice at all times to minimise their decomposition.

Ferro-haemoglobin (Mr 64500) was prepared by reduction of 100mls of 1mM bovine haemoglobin using 10mM sodium dithionite. The dithionite was dialysed out of solution against 3x2 litre volumes of deareated distilled water at 0-5°C. The stock ferro-haemoglobin was then split into 3ml aliquots and frozen (-10°C). Aliquots were added to 200ml of Krebs buffer used for the internal perfusate of the artery, immediately prior to perfusion, to make up the 15μM concentration required. Samples were used within 2 weeks.

5.7.3 Results and Discussion

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, 1989b). Typical traces showing the effect of perfusing ferro-haemoglobin (Hb, 15μM) through the internal circuit, on SNAP and GSNO vasodilator responses are shown in **Figures 5.17** and **5.18** respectively. The vasodilator action of GSNO was substantially less affected by ferro-haemoglobin than that of SNAP (see **Figures 5.19 & 5.20**). Ferro-haemoglobin reduced the ED_{50} value for GSNO by a factor of only **11** whereas that for SNAP is reduced by a factor of **73**. 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 both SNAP and GSNO are only partially inhibited by 15μM 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 & Fung, 1990).

When rat tail artery perfused with Krebs buffer containing superoxide dismutase (SOD, 150 units ml^{-1}) is given a bolus injection of GSNO there is no potentiation of the vasodilator response (**Figure 5.21**). The ED_{50} value for GSNO of 17μM remained unchanged in the presence of SOD. This observation is consistent with the view that GSNO does not release NO spontaneously in the lumen of the artery but requires tissue interaction to effect its release.

On the other hand, the vasodilator action of SNAP was slightly but significantly enhanced at lower concentrations by SOD (**Figure 5.22**). A typical trace is shown in **Figure 5.23**. This further suggests that SNAP releases NO in the lumen, where it can then be destroyed by superoxide. Release of NO from SNAP in the lumen could be due to the presence of metal ions.

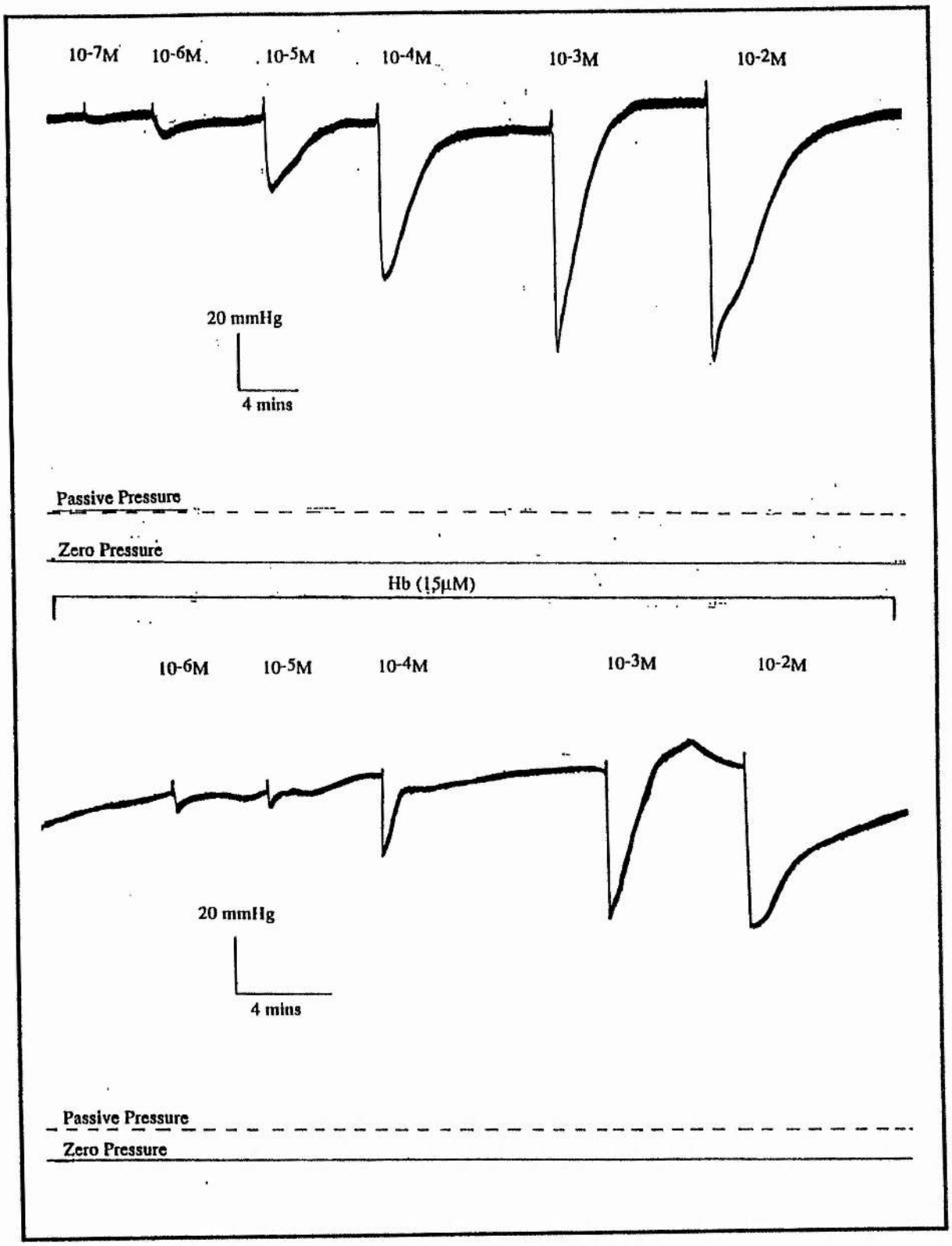


***Figure 5.17** A comparison of pressure recordings of SNAP injections from typical traces in the presence and absence of ferro-haemoglobin (Hb; 15μM) in the internal perfusate after precontraction with phenylephrine (PE). (SNAP Expt. N° 91/11/9, 4μM PE; SNAP + Hb Expt. N° 93/11/5, 3.75μM PE).*

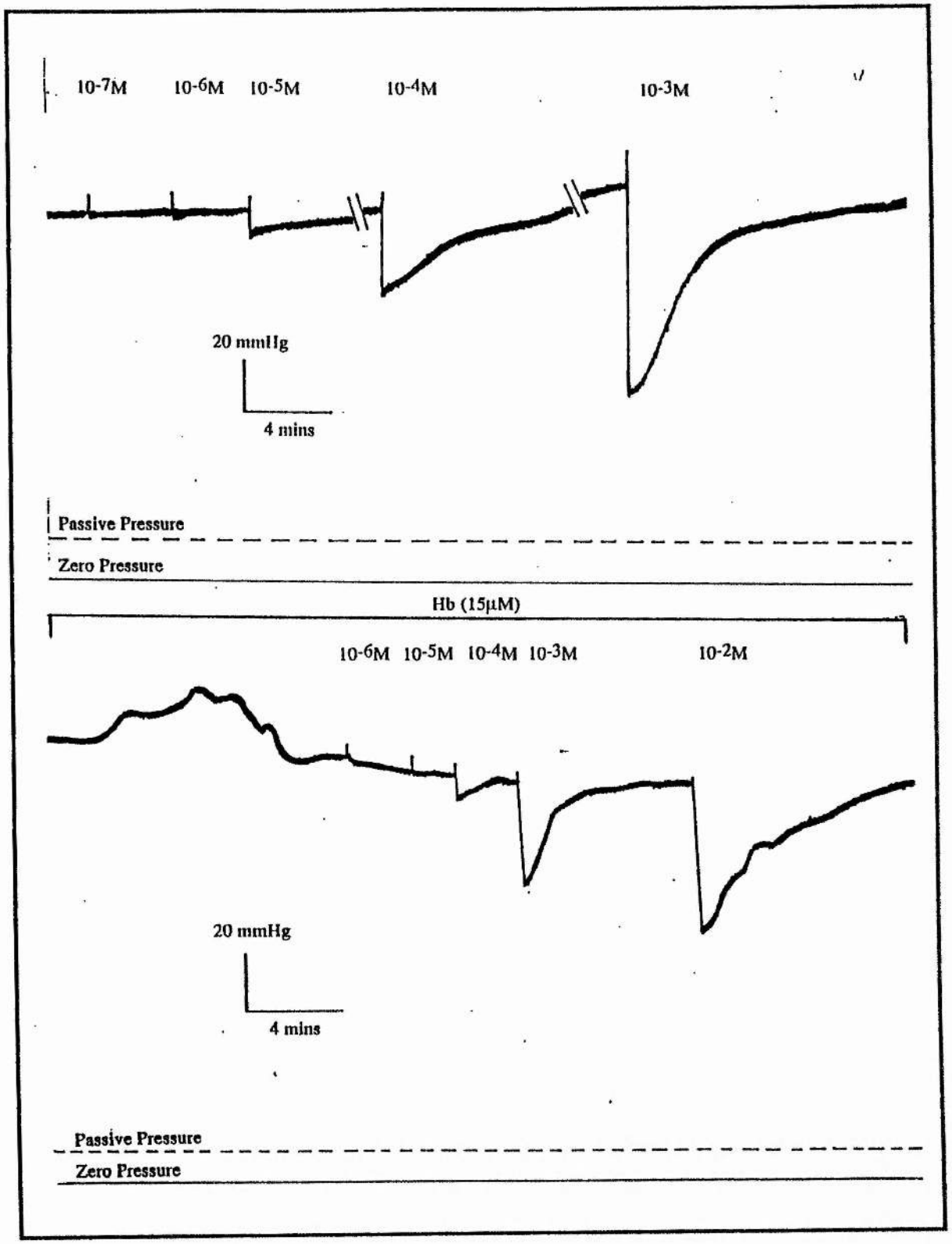


***Figure 5.18** A comparison of pressure recordings of GSNO injections from typical traces in the presence and absence of ferro-haemoglobin (Hb; 15μM) in the internal perfusate after precontraction with phenylephrine (PE). (GSNO Expt. N° 93/4/23, 4μM PE; GSNO + Hb Expt. N° 93/11/5, 3.75μM PE).*

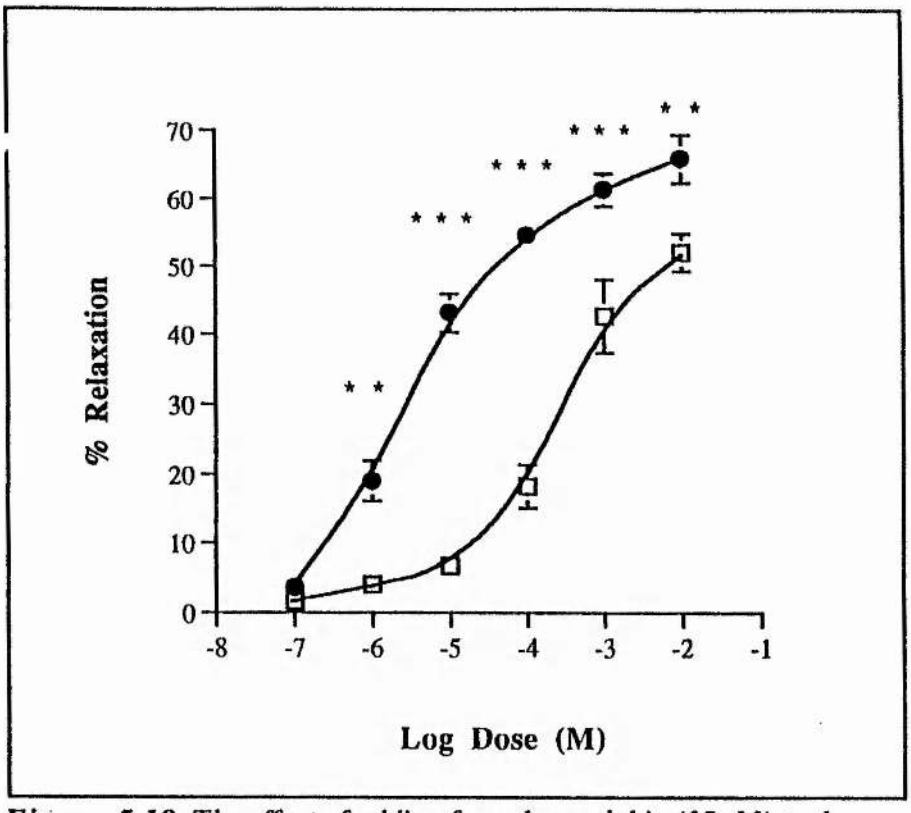


Figure 5.19 *The effect of adding ferro-hemoglobin (15μM) to the internal perfusate on the vasodilator reponses to bolus injections of SNAP. SNAP (closed circles, n=20) SNAP/Hb (open squares,n=10).* ED_{50} *SNAP = 3.2μM* ED_{50} *SNAP/Hb = 230μM*

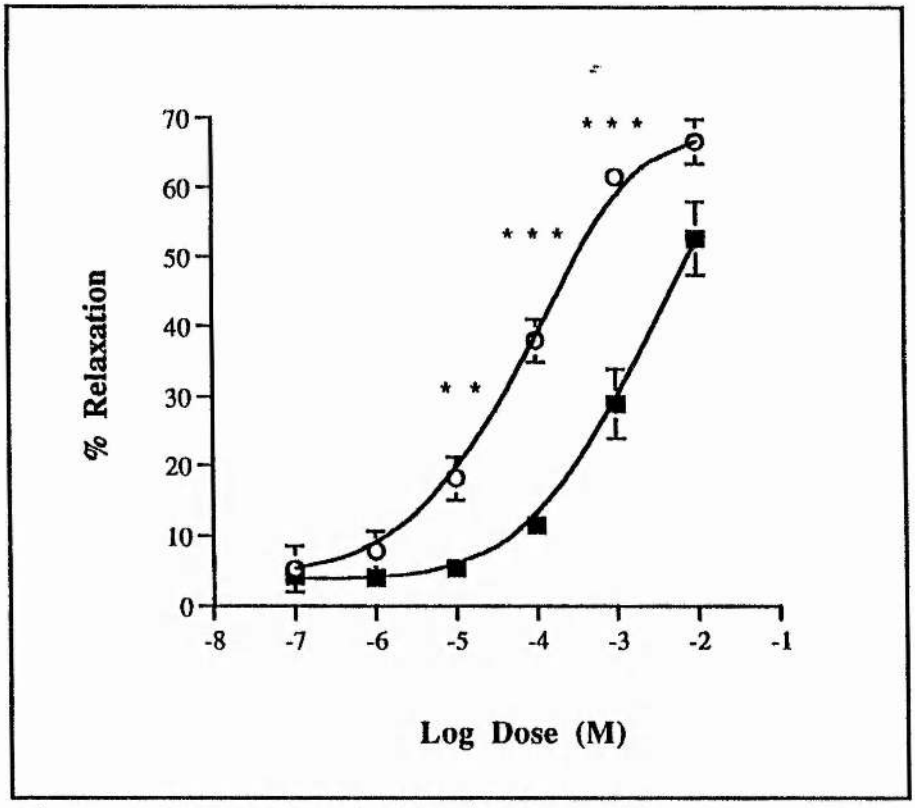


Figure 5.20 *The effect of adding ferro-hemoglobin (15μM) to the internal perfusate on the vasodilator reponses to bolus injections of GSNO. GSNO (open circles, n=16) GSNO/Hb (closed squares,n=8).* ED_{50} *GSNO = 59μM* ED_{50} *GSNO/Hb = 660μM*

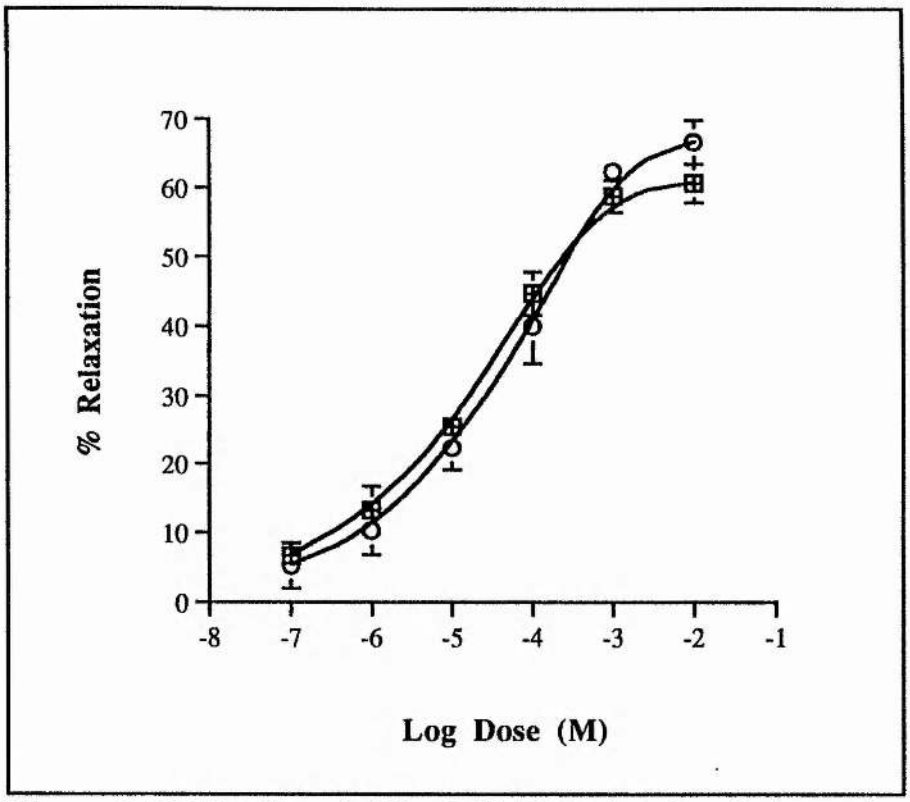


Figure 5.21 *The effect of adding SOD (150 units/ml) to the internal perfusate on the vasodilator response to bolus injections of GSNO. GSNO(open circles, n=5) GSNO/SOD (crossed squares,n=5) ED_{50} GSNO & GSNO/SOD = 17μM*

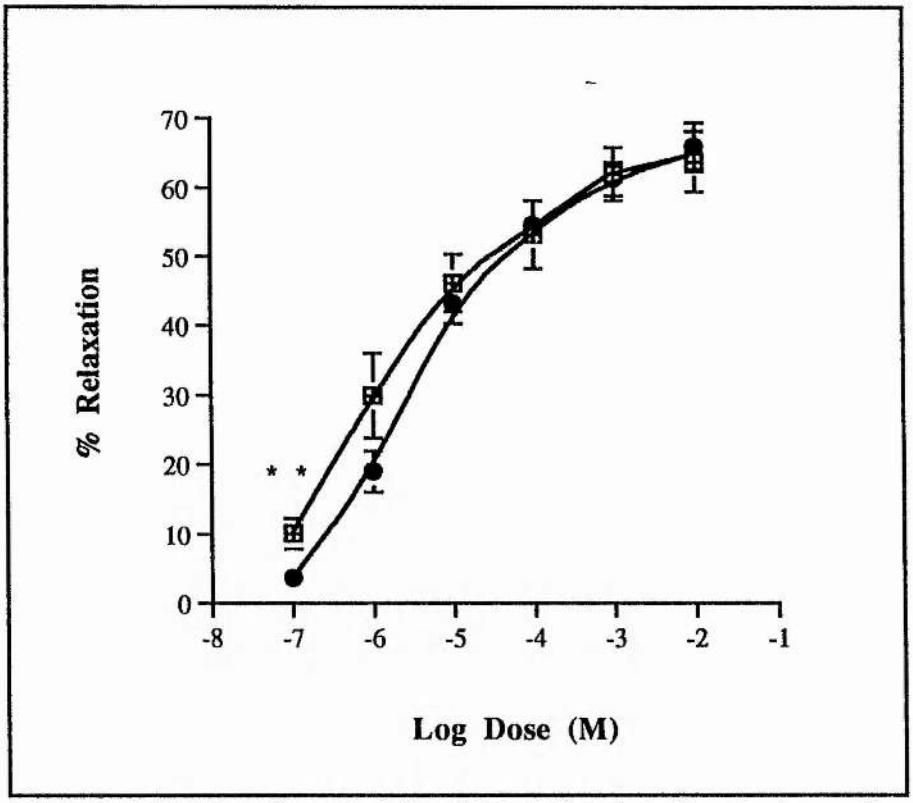


Figure 5.22 *The effect of adding SOD (150 units/ml) to the internal perfusate on the vasodilator response to bolus injections of SNAP. SNAP (closed circles, n=5) SNAP/SOD (crossed squares, n=5) ED_{50} SNAP = 3.2μM ED_{50} SNAP/SOD = 1.5μM*

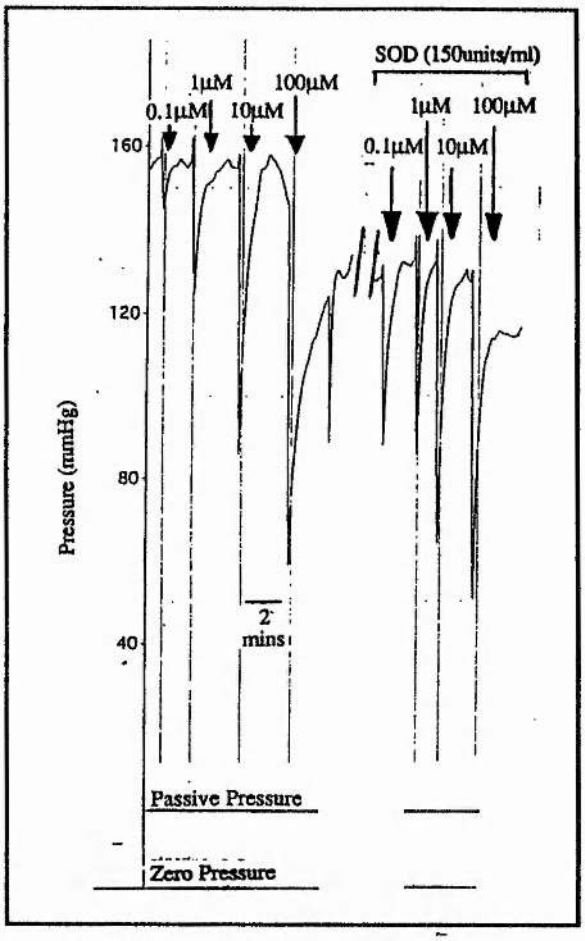


Figure 5.23 *Pressure recordings of responses of the artery to bolus injections of SNAP comparing typical vasodilator responses in the absence and presence of SOD (150 units/ml) in the internal perfusate (Expt. Nº 94/1/11, 2μM).*

A further possibility that cannot be excluded is that GSNO and SNAP enter vascular smooth muscle cells and activate guanylate cyclase directly. However, after treatment of isolated rat tail artery with ethacrynic acid, a known alkylator of thiol groups, there was complete inhibition of the vasodilator action of GSNO and so direct activation appears unlikely. Attenuation of responses to 'authentic' NO also occurred, but this was not unexpected as the activation of guanylate cyclase is critically dependent upon SH groups (Waldeman & Murad, 1987). This finding suggests that SH groups could play a major, if not essential, role in the vasodilator action of GSNO.

A detailed study carried out by Kowaluk & Fung (1990) on the liberation of NO from S-nitrosothiols, found that spontaneous liberation of nitric oxide cannot account for *ex vivo* vascular relaxation by S-nitrosothiols. They found that a range of S-nitrosothiols, from the small unstable amino acid, S-nitrosocysteine, to the bulky and polar S-nitroso-coenzyme A (MW 797), had similar relaxation potencies on rat aortic rings, even though they released NO at different rates. This indicated that entry of intact S-nitrosothiols into cells is unlikely to be a requirement for activity. Furthermore, the polar, and probably ionised, nature of these compounds at physiological pH, makes it unlikely that they would traverse cell membranes readily, which supports this hypothesis.

Working on bovine coronary arterial smooth muscle subcellular fractions, they found that the tissue exhibited substantial catalytic activity for NO generation from SNAP and deduced that this activity might be associated with cellular membranes. This raises the possibility that denitrosation of S-nitrosothiols might take place at external vascular membranes.

Similar results were obtained by Mathews & Kerr (1993) when they found that glutathione and the monoethyl ester of glutathione, which has been shown to enter cells much more readily than glutathione, were equipotent in the rabbit aortic ring assay. They also showed that the stabilities of the S-nitrosothiols tested had no correlation with their potencies on three different test tissues, or with their ability to inhibit platelet aggregation in whole blood. The relative potencies were also found to be different on the different tissues and blood platelets. They concluded that the R group in RSNO determined the biological effectiveness of the S-nitrosothiol and that generation of NO at the cell surface, either enzymatically or nonenzymatically, was the most likely mechanism of RSNO activity. 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. Alternatively, the target cell may have a

transport system that could allow entry of a specific RSNO into the cell making it more effective on that tissue than on another tissue in which it is not transported.

Comparing these findings with those of the rat tail artery experiments, in which the endothelium was present and intact, there is a question as to whether the endothelium can denitrosate S-nitrosothiols. Experiments carried out using the same model tissue and the same system with Roussins Black Salt, a potent NO donor drug, which was found to be taken up by the endothelium, demonstrated that ferro-haemoglobin *completely* abolished the response of the artery to the drug (0.5mM) by scavenging NO (Flitney*et al.*, 1992). This suggests that any NO produced from within the endothelium will be scavenged by ferro-haemoglobin. Therefore, the responses seen for SNAP and GSNO during perfusion of the artery with ferro-haemoglobin are possibly due to interaction with the endothelial cells, or smooth muscle cells of the rat tail artery, in which NO does not become 'free'. From the work carried out by Kowaluk and Fung (1990) it could be hypothesised that it is the external smooth muscle membranes which catalyse denitrosation of SNAP and GSNO. However, as the drugs initially come into contact with the endothelium this may also play a role in S-nitrosothiol metabolism, possibly by removing NO from the drug and transferring it to smooth muscle cells without NO becoming free. Catalytic activity at the vascular membranes could be due to NO transfer to thiol containing molecules, bound, or located free on the other side of the membrane, which transport NO to guanylate cyclase for subsequent activation of the enzyme.

In summary, it would seem that SNAP can spontaneously release NO without the presence of tissue. Its response is not completely abolished by ferro-haemoglobin and a significant percentage of the drug, or 'bound NO' from interaction of SNAP with tissue such as the endothelium, reaches the vascular smooth muscle cells. GSNO seems to require tissue to be biologically active. As a vasodilator in the rat tail artery preparation its action is diminished, but not abolished, by ferro-haemoglobin. Its

vasodilator effects are less diminished than those of SNAP, suggesting that a greater percentage of the drug or 'bound NO' produced from interaction of GSNO with tissue, reaches the vascular smooth muscle cells to exert its effect. The diminished response in the presence of ferro-haemoglobin could be due to 'free' NO release due to reaction at the surface of endothelial cells.

CHAPTER 6

S-NITROSOTHIOLS; DISAGGREGATORS OF PLATELETS AND INHIBITORS OF PLATELET AGGREGATION

6.1 INTRODUCTION

As well as being very effective vasodilators, S-nitrosothiols have also been shown to be good inhibitors of platelet aggregation (see **Chapter 1.12** for background). *In vitro*, S-nitrosation of molecules containing functional thiol groups leads to the formation of S-nitrosothiols. Although the biological significance of S-nitrosation *in vivo* is still uncertain, this reaction may result in stabilisation of the chemical and pharmacological properties of NO and generation of compounds with NO-donating properties (Stamler *et al.*, 1992; Venturini *et al.*, 1993). A recent study has suggested that NO may be released from the endothelium in the form of an S-nitrosothiol adduct (Myers *et al.*, 1990). Furthermore, sulphydryl groups in proteins represent an abundant source of reduced thiol for interaction with NO (or, by way of its derivative cation, the nitrosonium ion, NO^+ described in **Chapter 1.9**). and S-nitrosoproteins form readily under physiological conditions (Stamler *et al.*, 1992).

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 antiplatelet effects with IC_{50}'s in the range of 1.5μM (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).

All this evidence suggests that S-nitrosothiols have a possible role as thromboregulators controlling platelet aggregation. The subject of this study was to investigate the possibility that synthesised exogenous S-nitrosothiols based on penicillamine are also potent inhibitors of platelet aggregation. The study includes a comparison of these S-nitrosothiols with the platelet action of GSNO, an endogenous S-nitrosothiol, and SIN-1, another class of NO-donor drug which has been used as a thromboregulator. The pharmacological actions of SNAP and analogues of SNAP, S-nitroso-DL-penicillamine

(SNPen) and S-nitroso-N-formyl-DL-penicillamine (SNFP; see **Figure 6.1**), were examined on human platelets *in vitro*. Most of the testing of these drugs was carried out by Radomski at the Wellcome Laboratories, although the method described below was used by the author in collaboration with Radomski.

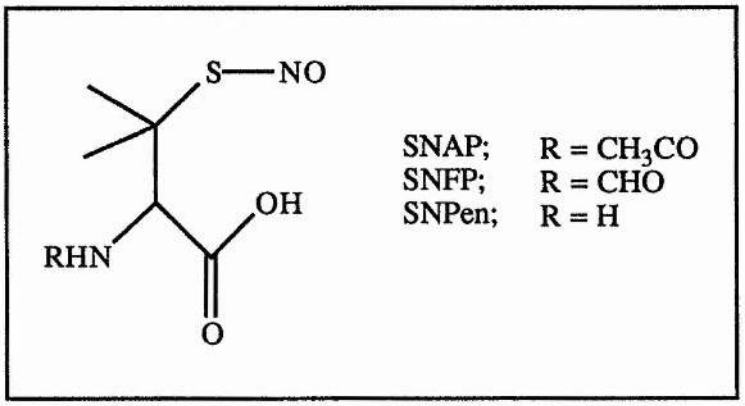


Figure 6.1 *The structures of the three S-nitrosothiols based on penicillamine*

6.2 THE EXPERIMENTAL METHOD USED TO EXAMINE THE PHARMACALOGICAL EFFECTS OF S-NITROSOTHIOLS ON PLATELET-RICH PLASMA (PRP) AND WASHED PLATELET (WP) SUSPENSIONS

Two different studies were carried out to examine the effect of S-nitrosothiols on human platelets. Firstly, the inhibition of platelet aggregation by these compounds was studied and secondly the disaggregation of aggregated platelets by S-nitrosothiols was investigated. In both studies the experimental procedure, based on a method developed by Born & Gross (1963), comprised the examination of both optical aggregation, following absorbance changes, and looking at ATP release from the aggregating platelets, using Luciferin-Luciferase (L.L) (from the fire fly) and monitoring the chemiluminescence produced.

The light beam from the spectrophotometer passed through the sample cuvette containing a stirring solution of WP and LL and the absorbance noted. Collagen, added to washed platelets, or ADP added to platelet rich plasma, were used to aggregate the platelets. This caused a dramatic decrease in absorbance due to reduced light scattering by the aggregating platelets. Example traces are shown in **Figure 6.2.** At the same time, aggregation of platelets produces ATP which is monitored using its reaction with LL which results in chemiluminescence. Various concentrations of the S-nitrosothiol to be tested were injected into the cuvette prior to or after the addition of collagen/ADP. If the S-nitrosothiol is added after collagen, an increase in absorbance is seen due to dispersion of the aggregates (see **Figure 6.2a**). Addition of collagen to washed platelets, after the S-nitrosothiol, however, produced a slow decrease in absorbance depending on the S-nitrosothiol concentration (see **Figure 6.2b**). The chemiluminescence from LL increased with increasing aggregation (**Figure 6.2d** shows a representative trace).

6.3 EXPERIMENTAL

Platelet-rich plasma (PRP) and washed platelet suspensions (WP):

Human blood was collected and PRP and prostacyclin (PGI_2)-washed platelet suspensions were prepared as described before (Radomski & Moncada, 1983).

Platelet aggregation and release of ATP

Platelet aggregation and release of ATP were measured in a platelet-ionised calcium lumi-aggregometer. S-nitrosothiols were incubated with WP for 1 min prior to the addition of collagen (1μg/ml) and their effects on platelet aggregation studied for 3 minutes. In some experiments, haemoglobin (5μM) was added 1 min before the S-nitrosothiol. In other experiments the compounds (all at 1μM) were incubated for 60 mins at 37°C.

Platelet disaggregation

Platelet disaggregation was measured in PRP following initiation of aggregation by ADP (3-5μM). S-nitrosothiols were added 1 minute after ADP and their effects studied for 5 mins.

Intraplatelet cyclic GMP levels

Intraplatelet cyclic GMP levels were measured in WP by enzyme immunoassay as described previously (Radomski *et al.*, 1992).

^{125}I-fibrinogen binding to platelets

^{125}I-fibrinogen binding to platelets was measured as described by Marguerie *et al.* (1979) with some modifications. Washed platelets (10^8ml^{-1}) were incubated with the compounds in Tyrode's solution for 15 minutes. Binding was initiated by adding ^{125}I-fibrinogen (50μg/ml) and ADP (50μM) in a final volume of 500μl. After 15 minutes samples (50μl) of the reaction mixture were layered on 200μl of 15% sucrose solution and spun for 1 minute at 10,000 x g. The samples were immediately frozen in dry ice and the radioactivity associated with the platelet pellet counted in a γ-counter.

Release of NO from S-nitrosothiols

The release of NO from S-nitrosothiols was measured by the method described by Feelisch and Noack (1987). The compounds were incubated for 5 mins at 37°C with or without intact platelets, platelet lysate, platelet cytosol (100,000 x g for 30 mins prepared from 2.5 x 10^8 platelets), reduced glutathione (30μM), ascorbic acid (30μM) and superoxide dismutase (20Uml^{-1}) and the rate of NO release was measured using a dual wave spectrophotometer (Shimadzu; see **Chapter 4**).

S-nitrosothiols:

SNAP was synthesised by the method of Field *et al* (1978), and SNPen and SNFP were synthesised according to the method of Hart (1985) as described in **Chapter 2.**

Reagents

Human haemoglobin was prepared by the method of Paterson *et al.* (1976). Adenosine diphosphate (ADP), adenosine triphosphate (ATP), luciferin-luciferase reagent, sucrose, superoxide dismutase, Arg-Gly-Asp-Ser (Sigma), prostacyclin sodium salt (Wellcome), L-ascorbic acid (BDH), collagen (Hormon-Chemie), ^{125}I-fibrinogen (Amersham), cyclic GMP enzyme immunoassay (Amersham) and GMP-140 enzyme immunoassay (British Biotechnology) were obtained from the sources indicated. Solutions of NO gas were prepared as described by Palmer *et al.* (1987). Briefly, a glass bubble was filled with NO (British Oxygen) from a cylinder and sealed with silicone rubber injection septa. An appropriate volume was removed with a syringe and injected into another gas bulb filled with H_2O, which had been deoxygenated by gassing with Helium for 1 hour, to give stock solutions of NO of 0.01-1.0% (v/v).

Statistics

Results are mean ± s.e. mean of at least 3 separate experiments. They were compared by analysis of variance and $p<0.05$ was considered as statistically significant.

6.4 RESULTS & DISCUSSION

a) *Effects of RSNO on platelet aggregation, ATP release and disaggregation*

Incubation of the S-nitrosothiol derivatives of penicillamine (0.01-10μM) with washed platelets resulted in a concentration-dependent inhibition of collagen-induced aggregation and ATP release from the platelets (see **Figure 6.2** and **Table 6.1**). SNAP and SNFP were significantly more potent than SNPen ($p<0.05$, n=3-7) as inhibitors of platelet aggregation. However there were no significant differences found in the action of the three compounds in inhibiting ATP release from the platelets. The inhibitory activity of these compounds was reversed by haemoglobin (5μM) (**Figure 6.2c**), indicating the involvement of NO in the process. The addition of the S-

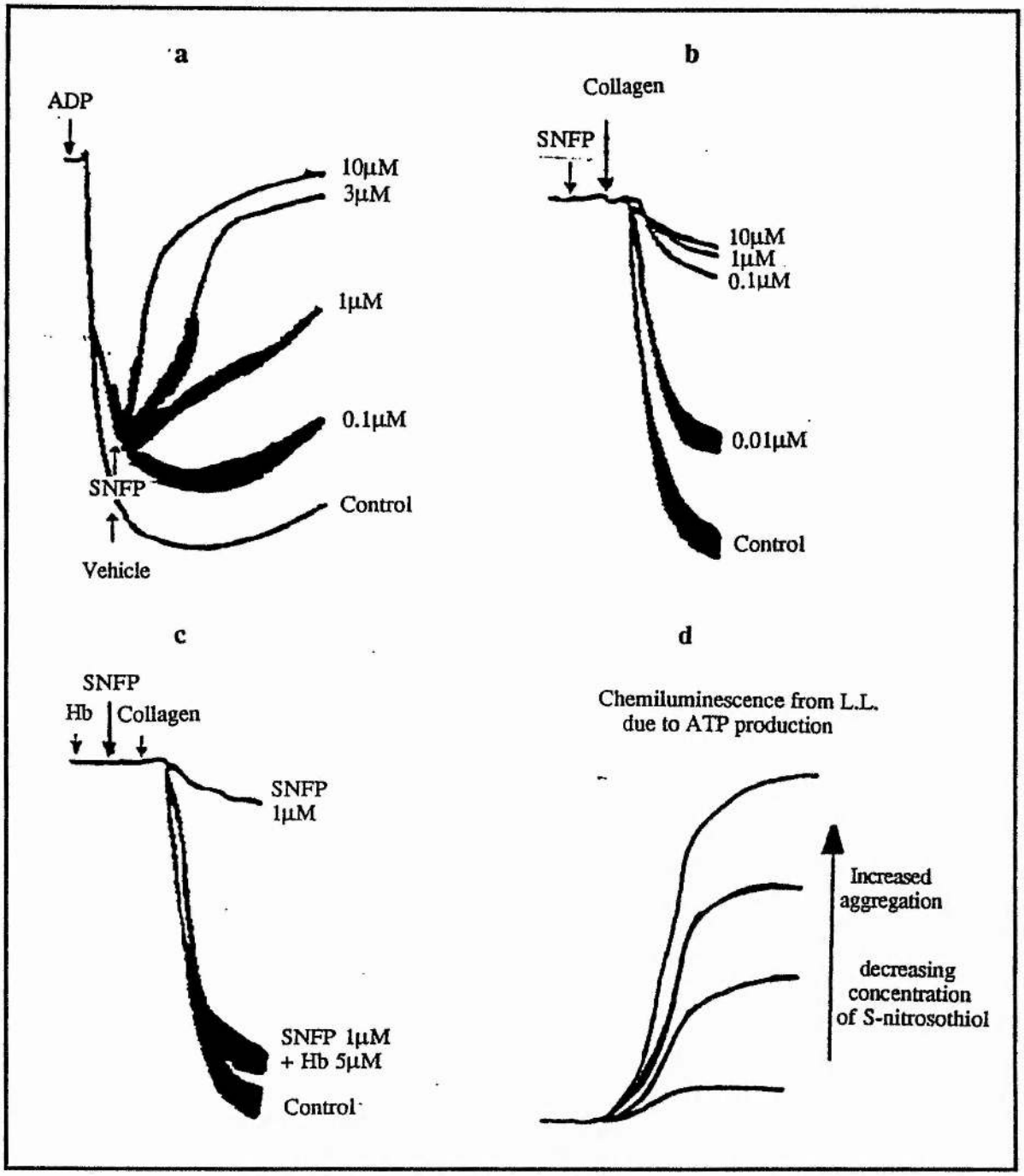


Figure 6.2 *The effects of SNFP on platelet aggregation.* ***a.*** *stimulation of disaggregation of platelets aggregated by ADP (5μM) in PRP,* ***b.*** *Inhibition of collagen (1μg/ml)-induced aggregation in WP,* ***c.****and its reversal by haemoglobin (5μM),* ***d.*** *A schematic diagram showing the increase in chemiluminescence from L.L. due to ATP production by aggregating platelets as S-nitrosothiol concentration is reduced.*

nitrosothiol (0.01-10μM) one minute after induction of platelet aggregation with ADP (3-5μM) in platelet rich plasma resulted in a concentration-dependent induction of platelet disaggregation (**Figure 6.2a** and **Table 6.1**). The effectiveness of SNAP, SNFL and SNPen as inducers of disaggregation was similar. However, in most cases higher concentrations of S-nitrosothiols were required than for inhibition of aggregation.

When these three compounds are compared with the two S-nitrosothiols, GSNO and S-nitrosocysteine, which have been found *in vivo*, they collate favourably having IC_{50}'s of the same or similar orders of magnitude, although GSNO appears to be more effective over the two test methods (see **Table 6.1**). When compared with authentic NO, only SNPen seems less effective at inhibiting platelet aggregation, and all the S-nitrosothiols tested are far more potent (at least six fold) than the other NO-donor drug SIN-1 (see **Table 6.1**).

Compound (n=3-7)	Inhibition of Collagen-induced aggregation (IC_{50},μM)	Inhibition of Collagen-induced release of ATP (IC_{50},μM)	Stimulation of disaggregation of platelets aggregated by ADP (IC_{50},μM)
SNAP	0.12 ± 0.02	0.18 ± 0.11	1.09 ± 0.32
SNFP	0.05 ± 0.01	0.15 ± 0.07	0.63 ± 0.21
SNPen	0.38 ± 0.05	0.59 ± 0.13	0.25 ± 0.01
GSNO	0.12 ± 0.04	0.09 ± 0.04	
SNC	0.11 ± 0.05	0.75 ± 0.59	
NO	0.36 ± 0.10	0.30 ± 0.09	
SIN-1	2.16 ± 0.41	3.41 ± 0.49	

***Table 6.1** A comparison of the relative effectiveness at inhibiting platelet aggregation and disaggregating platelets of a range of S-nitrosothiols and other NO donors.*

Work carried out by M.W.Radomski

b) *Cyclic GMP and ^{125}I-fibrinogen binding assay*

The basal levels of cyclic GMP in platelets were 131 ± 33 fmol/10^8 platelets, (n=4). Incubation of platelets with SNAP, SNFP and SNPen (0.01-10μM) caused a concentration-dependent increase in intraplatelet cyclic GMP to a maximum of 1643 ± 133, 1599 ± 123 and 1611 ± 131 fmol/10^8 platelets respectively. These values were not significantly different from each other.

All three S-nitrosothiols caused a concentration-dependent inhibition of ^{125}I-fibrinogen binding to the platelets with an IC_{50}'s shown in **Table 6.2.** There was a significant correlation between the concentrations of S-nitrosothiol needed for half-maximal stimulation of intraplatelet cyclic GMP and those required for inhibition of fibrinogen binding (see **Table 6.2**).

RSNO (n=3-7)	Half-maximal stimulation of intraplatelet cGMP (EC_{50}, μM)	Inhibition of ^{125}I-fibrinogen binding (IC_{50}, μM)	Correlation between cGMP and fibrinogen binding (r)
SNAP	0.40	0.34 ± 0.07	0.97
SNFP	0.35	0.64 ± 0.13	0.88
SNPen	0.29	0.34 ± 0.07	0.95

***Table 6.2** The correlation between cyclic GMP and fibrinogen binding in platelets for the S-nitrosothiols based on penicillamine.*

There was good correlation between inhibition of fibrinogen binding to platelets and the formation of cGMP by S-nitrosothiols, at concentrations that also inhibited platelet aggregation and caused disaggregation. This suggests that the platelet-inhibitory activity of RSNO depends on cyclic GMP-mediated inhibition of fibrinogen binding to platelets. Similar observations have been published recently by Mendelsohn *et al.* (1990) working on S-nitroso-N-acetylcysteine.

c) *Release of NO*

When SNPen was dissolved in Tyrode's solution, NO was released in a concentration-dependent manner (**Table 6.3**), which was detected at concentrations as low as 0.1μM. However, the release of NO from SNAP and SNFP was not detected at concentrations lower than 10μM. The addition of platelet lysate, intact platelets, platelet cytosol, reduced glutathione, ascorbic acid or superoxide dismutase did not increase the rate of NO release from these compounds at concentrations up to 10μM (n=3).

In the experiments testing the effects of these three S-nitrosothiols on platelets, there was good correlation between NO release from SNPen and the pharmacological effects of this S-nitrosothiol. However using the oxyhaemoglobin assay (see **Chapter 4** for experimental details), NO release was not detected at concentrations below 10μM, a concentration capable of producing both anti-platelet effects and stimulation of guanylate cyclase for SNAP and SNFP. Even by the addition of platelet fractions and reducing agents known to enhance the rate of NO release from other NO donors (Feelisch & Noack, 1987; Radomski *et al.*, 1992), NO could not be detected.

RSNO	NO release (pmol/min)		
(μM, n=4)	SNPen	SNFP	SNAP
0.01	nd	nd	nd
0.1	1.29 ± 0.02	nd	nd
1.0	52.34 ± 1.23	nd	nd
3.0	253.70 ± 2.73	nd	nd
10.0	743.00 ± 5.60	2.15 ± 0.02	5.15 ± 0.64

***Table 6.3** Release of NO from S-nitrosothiols. nd: not detectable (< 0.1 pmol/min)*

It has also been shown that release of NO from GSNO (10μM) under the same conditions was very low (10 ± 10 pmol/min; Radomski *et al.*, 1992). This rate of NO release was enhanced by addition of platelet lysate but not by intact platelets or cytosol.

6.5 CONCLUSIONS

It is evident that S-nitrosothiols, as a class of NO donor drugs, are very effective at inhibiting platelet aggregation and at disaggregating platelets. Whether endogenously or exogenously produced, they are far more potent than other commercially available NO-donor drugs like SIN-1 and, but for a couple of exceptions, they were found to be more potent than NO itself.

The fact that NO could not be detected from SNAP and SNFP in the presence of platelets, indicates that either the release of NO from SNAP and SNFP occurs inside the platelet, or that this release is not a necessary prerequisite for the stimulation of the soluble guanylate cyclase, as the enzyme can also be stimulated directly by these compounds. However, the abolished inhibitory effect on platelet aggregation of the S-nitrosothiols by ferro-Hb, suggests that NO is the ultimate activator of soluble guanylate cyclase in the platelet. On the other hand, NO was found to be produced spontaneously from SNPen, even in the absence of platelets, and it was not found to be as effective as its formyl or acetyl derivatives. This might indicate that release of NO in the platelet is more effective than outside, and that the introduction of formyl or acetyl groups to the structure of S-nitroso-DL-penicillamine leads to a chemical stabilisation of the resultant S-nitrosothiols without affecting their pharmacological properties as inhibitors of platelet function.

The results obtained by Radomski *et al.* (1992) which showed that NO release from GSNO was low but was significantly enhanced in the presence of platelet lysate, suggest that release of NO from GSNO may be catalysed by an enzyme present in platelet membranes which become activated following platelet stimulation. This theory is consistent with the finding by Kowaluk & Fung (1990) that an enzymatic/protein effect rather than spontaneous liberation of NO accounts for the relaxation of rat aortic rings by GSNO. Work presented in **Chapter 5** showed that GSNO is susceptible to

enzymatic decomposition by γ-glutamyl transpeptidase which has been found in human serum (Goldberg, 1980; Rosalki, 1975), although it has not been determined as to whether this or a related enzyme is present in platelet lysate.

An alternative explanation is that S-nitrosothiols such as GSNO, SNFP and SNAP, release NO at the platelet surface to sulphydryl groups as this is a source of reduced thiol equivalents (Simon *et al.*, 1993). These authors showed that S-nitroso-bovine serum albumin, another stable S-nitrosothiol, was susceptible to this mechanism and the results discussed here are also consistent with this theory.

CHAPTER 7

CYCLODEXTRINS; A WAY OF STABILISING S-NITROSOTHIOLS ? AND A COMPARISON OF THE EFFECTIVENESS OF S-NITROSOTHIOLS WITH OTHER CLASSES OF NO-DONOR DRUGS

7.1 CAN THE STABILITY OF SNAP BE ENHANCED BY β-CYCLODEXTRIN ?

7.1.1 Introduction

The main restriction on using S-nitrosothiols as a class of therapeutic agents for the delivery of NO is their inherent instability at physiological pH. For therapeutic purposes, completely stable drugs are required which can be made to act on a specific target site. The instability of most S-nitrosothiols means that NO will be delivered to many sites with little specificity for the intended target. SNAP has been shown to be a potent vasodilator which produces less pharmacological tolerance than GTN (Bauer & Fung, 1991a), but has a short half-life in solution. A paper published by Bauer & Fung (1991b) has claimed that SNAP can be stabilised in solution through the use of cyclodextrin to form a complex which was found not to affect the pharmacological activity of SNAP. The authors investigated the stabilising effects of a variety of cyclodextrins, and studied their effects on the physiological activity of SNAP on rat aortic rings. They found that the parent cyclodextrin, β-cyclodextrin, had the greatest stabilising effect. It was decided to re-investigate the proposed stabilising effects of cyclodextrins due to the discovery of trace metal ion catalysis following the same conditions used in Bauer & Fung 's experiments. The rate of SNAP decomposition was monitored, in the presence and absence of copper (II) ions, and at different pH, to see if the cyclodextrin stabilisation was affected.

7.1.2 Results and Discussion

Cyclodextrins are cyclicoligosaccharides composed of 7 D-glucose units which are known to form inclusion compounds. They are of pharmaceutical interest primarily because of their ability to enhance the aqueous solubility of lipophilic compounds (Pitha *et al.*, 1988), an improvement which does not require use of cosolvents or structural modification of the molecule. Improvements in the adsorption, bioavailability and drug

dissolution rates have all been observed for selected compounds when incorporated with cyclodextrins (Pitha *et al.*, 1986; Frijlink *et al.*, 1990).

Evidence has been presented to suggest that SNAP forms an inclusion compound with cyclodextrins and seems to do so by a single complexation species, as a linear relationship was found between SNAP solubility and cyclodextrin concentration (Bauer & Fung, 1991b). Since inclusion complex formation protects the guest molecule from the surrounding media, its stability is often improved as well (Golmot *et al.*, 1988).

Due to the discovery of trace metal ion catalysis by metals such as copper, it was decided to investigate whether the stabilising effect of cyclodextrin was due to the formation of an inclusion compound or whether the cyclodextrin, along with the vehicle (5% dextrose) which was used by Bauer and Fung (1991b), stabilised SNAP by complexing these metal ions. The use of cyclodextrin in dextrose substantially lowers the pH (to ~ 3.5) compared with the physiological pH of 7.4, in which most of the cyclodextrin-free experiments were conducted. As has been discussed in **Chapter 4**, pH plays a major role in the rate at which SNAP decomposes and may also play a role in the stabilising effect of β-cyclodextrin.

The effect of pH was investigated first, by comparing the decomposition rates of SNAP in the presence of β-cyclodextrin and 5% glucose (pH3.5) and in the presence of β-cyclodextrin and 5% glucose in phosphate buffer at pH 7.4. This was done by monitoring the change in absorbance at 339nm. The results are shown in **Figures 7.1 & 7.2** respectively. As can be seen from **Figure 7.1**, the half-life of SNAP is substantially longer in the presence of 5% glucose and enhanced even more by β-cyclodextrin when compared with the decomposition rate of SNAP in phosphate buffer at pH7.4. The concentration of 1.2mM β-cyclodextrin to 0.4mM SNAP is virtually the same ratio of β-cyclodextrin to SNAP which was shown by Bauer & Fung (1991b) to stabilise SNAP. It is evident that the increasing ratio of cyclodextrin (0.4-5.0mM) to

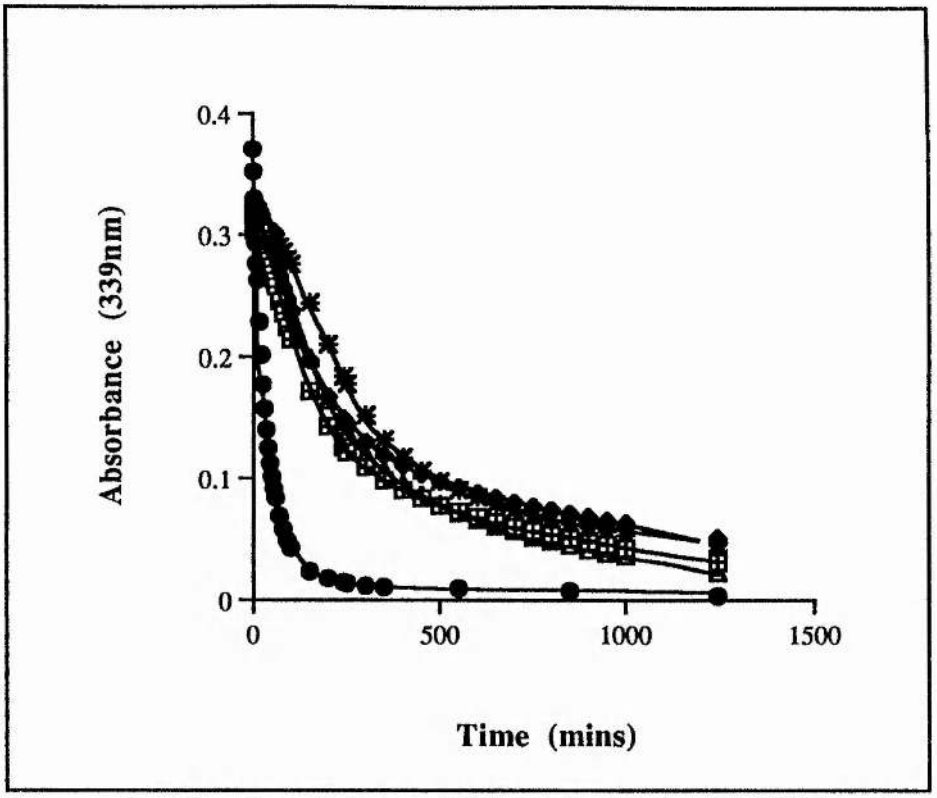


***Figure** 7.1 The effect of glucose (5%) and β-cyclodextrine (CD) on the decomposition rate of SNAP (0.4mM) at pH 3.5, compared to that of SNAP at physiological pH (7.4). SNAP pH7.4 (closed circles), glucose pH3.5 (squares with crosses), glucose + 0.4mM CD pH 3.5 (closed diamonds), glucose + 1.2mM CD pH 3.5 (open triangles), glucose + 5.0mM CD pH 3.5 (black stars).*

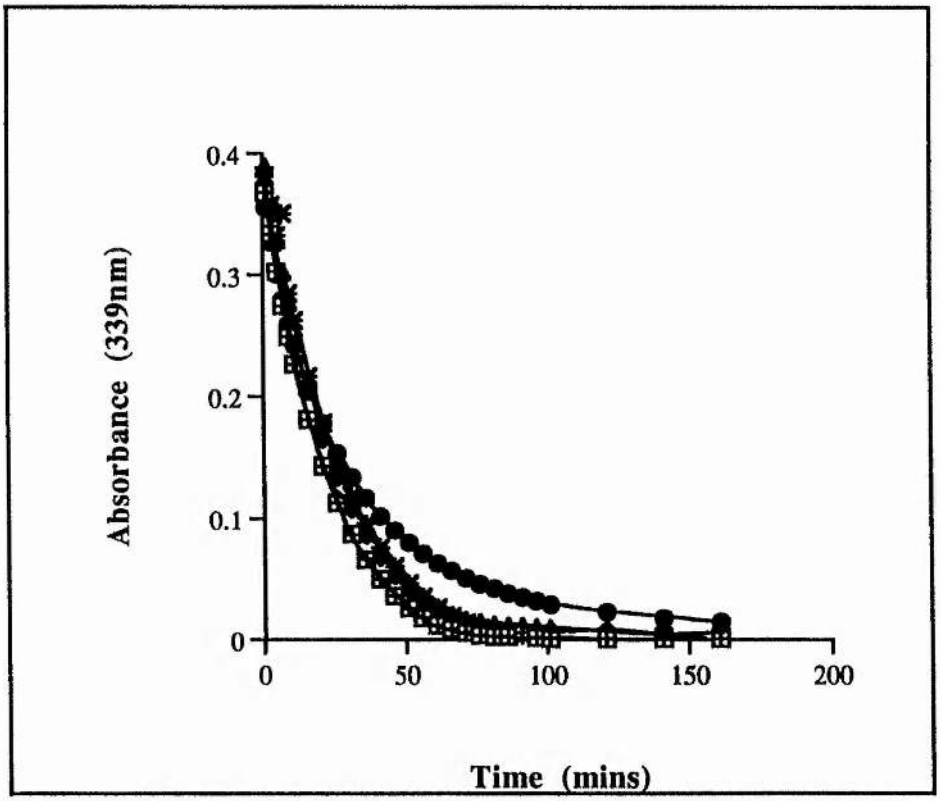


***Figure** 7.2 The effect of glucose (5%) and β-cyclodextrine (CD) on the decomposition rate of SNAP (0.4mM) at pH 7.4. SNAP pH7.4 (closed circles), glucose pH7.4 (squares with crosses), glucose + 0.4mM CD pH 7.4 (closed diamonds), glucose + 1.2mM CD pH 7.4 (open triangles), glucose + 5.0mM CD pH7.4 (black stars).*

SNAP concentration does not dramatically increase the stability of SNAP over and above that produced by 5% glucose. Furthermore, when the stabilising effect of β-cyclodextrin and glucose are examined at physiological pH (see **Figure 7.2**), it is clear that it is the difference in pH (3.5 compared to 7.4) which accounts for the apparent stabilising effect of these two compounds on SNAP.

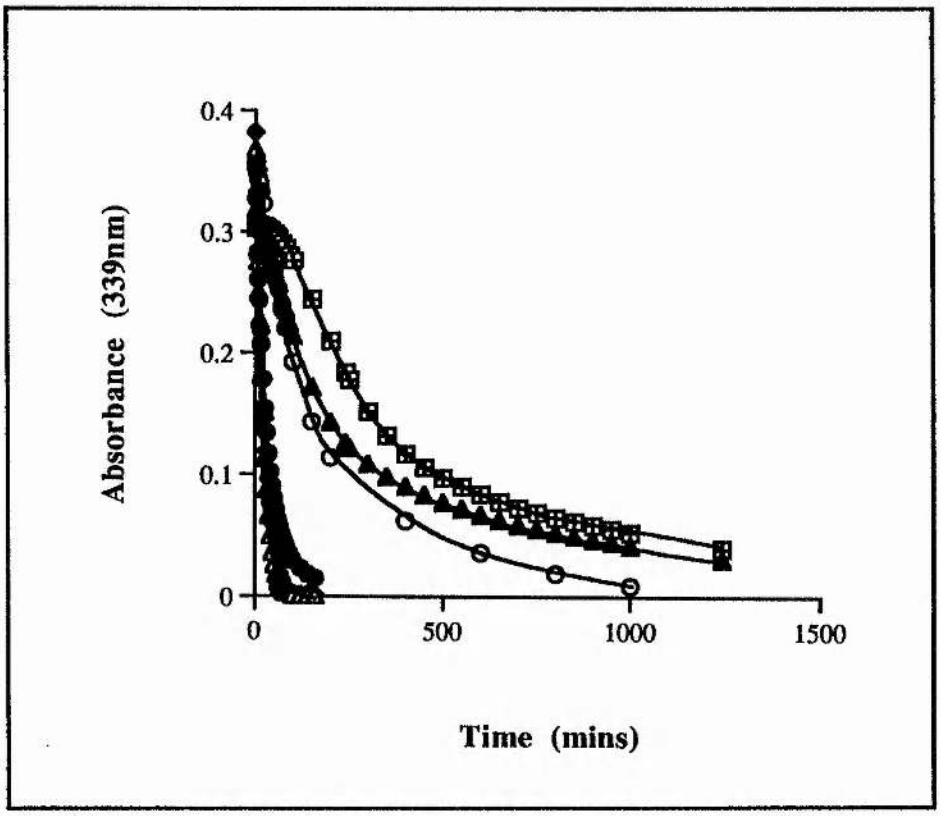


Figure 7.3 *A comparison of the stabilising effect of glucose (5%) and β-cyclodextrin (CD) on the decomposition rates of SNAP (0.4mM) at pH 3.5 and 7.4. SNAP pH 3.5 (open circles) , glucose pH 3.5 (closed triangles), glucose + CD pH 3.5 (squares with crosses), SNAP pH 7.4 (closed circles), glucose pH 7.4 (open triangles), glucose + CD pH 7.4 (diamonds with crosses).*

To check whether there was a stabilising effect of glucose and cyclodextrin at pH 3.5, the decomposition rates of SNAP in glucose and SNAP in glucose in the presence of β-cyclodextrin (5mM), were compared with that of SNAP in distilled water at pH 3.5. These decomposition rates were also compared with those of the same constituents at physiological pH. The results presented in **Figure 7.3** show that there was a

stabilising effect of glucose, which was further enhanced in the presence of β-cyclodextrin compared with SNAP in distilled water at pH 3.5. No stabilising effect was seen at pH 7.4.

It was decided to determine whether the stabilising effect of β-cyclodextrin at pH 3.5 was due to binding of trace metal ions, or to formation of the inclusion complex postulated by Bauer and Fung (1991b). To test this theory, copper (II) ions (1-100μM) were added to solutions of SNAP/β-cyclodextrin (1.2mM) at pH 3.5. **Figure 7.4** shows that the decomposition rate of SNAP in β-cyclodextrin is dramatically increased by copper (II) ions even at pH 3.5. This was also found to be the case at pH 7.4 (see **Figure 7.5**).

In conclusion, it is apparent that if an inclusion complex of SNAP is formed with cyclodextrin, any stabilising effect, such as that described by Bauer & Fung, is solely due to the pH of the vehicle solution in which it was examined. Furthermore, whether an inclusion compound is formed or not, trace metal ions such as copper can still decompose SNAP. It would seem that the slight stabilising effect of β-cyclodextrin on SNAP at pH 3.5 is probably due to metal ion complexation. Consequently, it is unlikely that cyclodextrins will be useful in delivery systems to prolong and improve the pharmacokinetics of S-nitrosothiols like SNAP.

7.1.3 Experimental

Kinetic Studies; **Figures 7.1, 7.2 & 7.3.**

β-cyclodextrin hydrate (0.1135g) was dissolved in 10ml of 0.1M KH_2PO_4/ NaOH buffer or distilled water to make up 0.01M stock solutions at pH 7.4 and 3.5 respectively. The required concentrations of β-cyclodextrin (indicated in the figures) were obtained by adding different volumes (0.1-1.25ml) of the stock solutions to the spectrophotometer cuvette at 30°C, which contained 1.0ml of 12.5% glucose (12.5g in

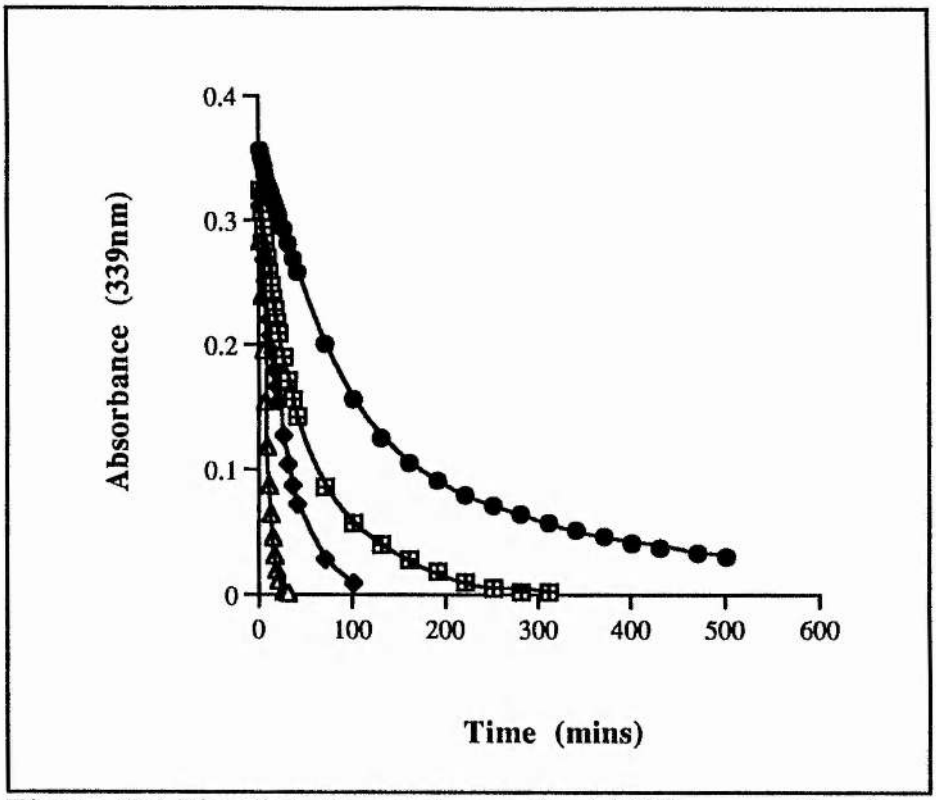


***Figure** 7.4 The effect of copper ions on the stabilising properties of β-cyclodextrine (1.2mM) of SNAP (0.4mM) at pH 3.4. (closed circles) SNAP + CD, (squares with crosses) 1μM Cu^{2+}, (closed diamonds) 10μM Cu^{2+}, (open triangles) 100μM Cu^{2+}.*

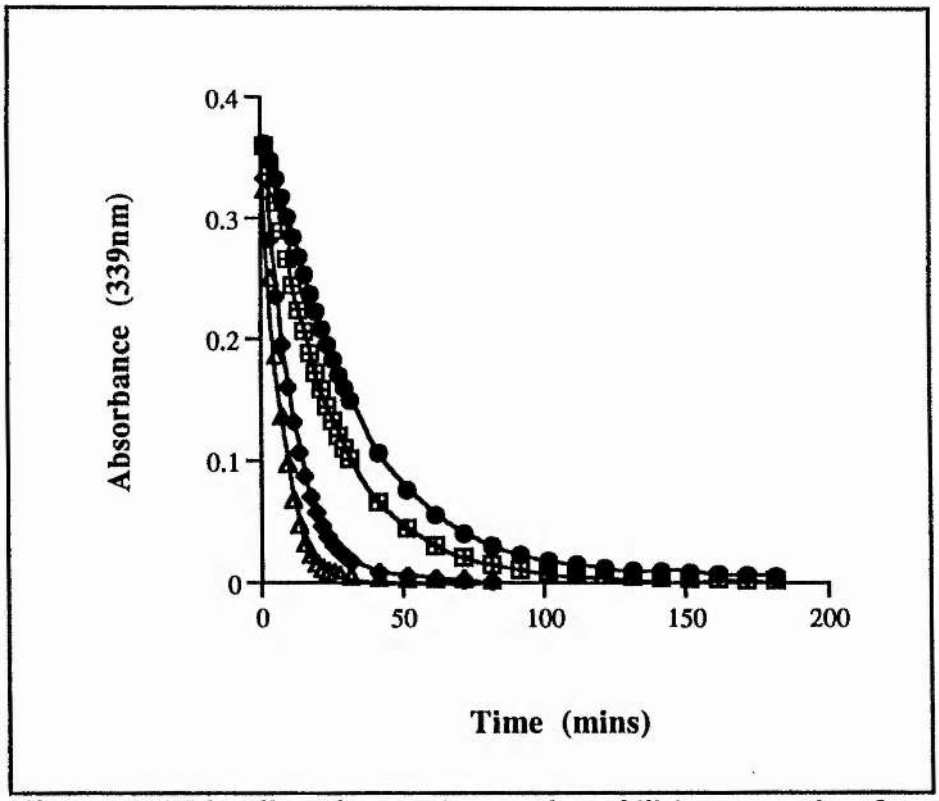


***Figure** 7.5 The effect of copper ions on the stabilising properties of β-cyclodextrine (1.2mM) of SNAP (0.4mM) at pH 7.4. (closed circles) SNAP + CD, (squares with crosses) 1μM Cu^{2+}, (closed diamonds) 10μM Cu^{2+}, (open triangles) 100μM Cu^{2+}.*

100ml distilled water). These solutions were made up to 2.25ml in cuvettes with either the phosphate buffer or distilled water (depending on the pH required). After 0.25mls of SNAP solution (0.0088g in 10ml buffer/distilled water; 4mM) were added to the cuvettes, the final concentrations of SNAP, glucose and β-cyclodextrin indicated in the figure legends were obtained.

Kinetic Studies; **Figures 7.4 & 7.5**

β-cyclodextrin hydrate (0.1135g) was dissolved in 10ml of 0.1M KH_2PO_4/ NaOH buffer or distilled water to make up 0.01M stock solutions at pH 7.4 and 3.5 respectively.

The required concentrations of β-cyclodextrin (indicated in the figures) were obtained by adding different volumes of the stock solutions to the spectrophotometer cuvette at 30°C. These contained 0.25ml of copper (II) nitrate solutions (1-100μM), obtained by serial dilution from a 1mM stock solution (0.0242g $Cu(NO_3)_2$ (Analar grade) in 100ml of buffer/distilled water). These solutions were made up to 2.25ml in the cuvettes with either the phosphate buffer or distilled water (depending on the pH required). After 0.25mls of SNAP solution (0.0088g in 10ml buffer/distilled water; 4mM) were added to the cuvettes, the final concentrations of SNAP, copper and β-cyclodextrin indicated in the figure legends were obtained.

7.2 A COMPARISON OF THE VASODILATOR RESPONSES OF RAT TAIL ARTERY PREPARATIONS TO CONTINUOUS PERFUSION OF SNAP, GSNO AND OTHER CLASSES OF NO-DONOR DRUG,

7.2.1 Introduction

SNAP and GSNO are both fast acting and effective vasodilators (**Chapters 2 & 5**). Here, the *ex vivo* vasodilator action of these S-nitrosothiols on the rat tail artery bioassay are compared with the vasodilator profiles of other more established NO-

donor drugs which have been used clinically. Isosorbide-5-mononitrate (ISMN) was chosen as an example of one of the novel nitrate drugs used in the treatment of angina as it is a metabolite of isosorbide dinitrate. Comparisons were also made with sodium nitroprusside (SNP), a fast-acting vasodilator used clinically, to rapidly lower blood pressure. The structures are shown in **Figure 7.6.**

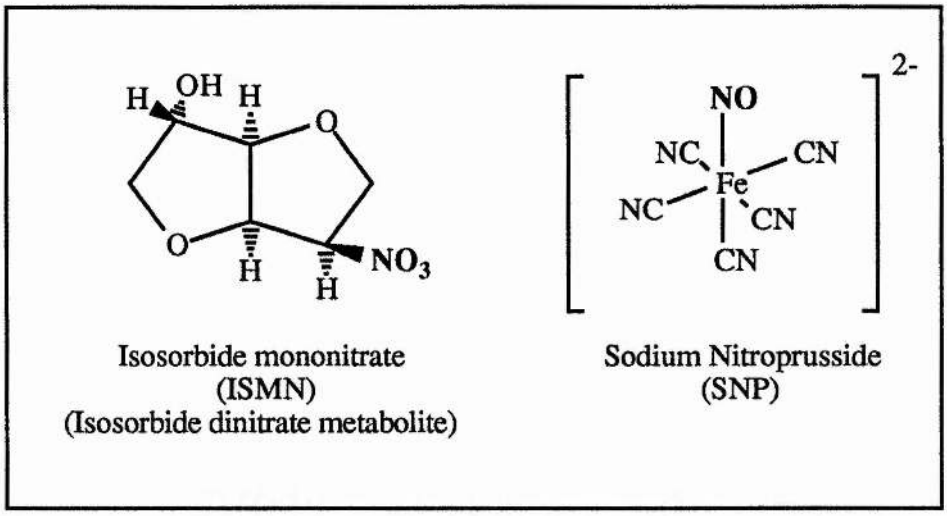


***Figure 7.6** The structures of two clinically used NO-donor drugs*

In **Chapter 2** the log-dose response curves of GSNO and SNAP are presented (**Figure 2.8**). Similar response profiles have been shown for sodium nitroprusside, administered in the same way (Megson, PhD thesis 1993). These responses are longer lasting but fully reversible like GSNO and SNAP. Comparatively, isosorbide mononitrate produced no responses from the rat tail artery, over concentration (0.1μM-1mM) when administered by injection (results not shown). The possible reasons for these observations will be discussed in this chapter. However, attention will focus on the administration of these drugs by continuous perfusion through the artery. As has been discussed previously (**Chapter 2.4**), administering the drug by this method requires much lower concentrations than by bolus injection to elicit responses of the same amplitude. A submaximal response produced by 7μM SNAP administered by **perfusion** was matched by a 3mM **injection** of SNAP. This suggests that less than 1/400th (1/428th) of the drug concentration delivered to the artery by injection (as a

bolus) is reaching its site of action. The main reason for administering the drugs in this way was to see how it would effect the response elicited by the artery, and to establish the vasodilator profile of S-nitrosothiols with the two previously mentioned NO-donor drugs.

7.2.2 Experimental

Continuous perfusion

The rat tail artery was dissected, cannulated and prepared for drug testing in the same way as previously described in **Chapter 2.** The test drugs (1ml) of concentrations 1000 fold greater than that intended for experimentation were added to 1 litre of internal perfusate (Krebs buffer). The drug solution would then pass through the tubes of the perfusion circuit at a flow rate of $2mlmin^{-1}$ to the artery and then on to waste (it was not recirculated). When the experiment is complete the artery is washed out with the original concentration of PE/Krebs solution used to precontract the artery before the experiment began. After a ~30 minute wash-out period, the next drug was tested following the same procedure. **Chapter 2.4, Figure 2.5** shows the experimental set up used and the point of drug addition for continuous perfusion.

Drugs used

Isosorbide-5-mononitrate was obtained from Janssen Chimica, Belgium and sodium nitroprusside from BDH Chemicals, Poole, England.

7.2.3 Results and Discussion

Identical concentrations of SNAP, SNP or ISMN were added to the internal perfusate (see **Chapter 2, Figure 2.5** for the position of administration) to make up a final concentration of 0.73μM. The responses of the precontracted rat tail artery were compared for these three drugs and an example trace is plotted in **Figure 7.7.**

The plotted trace shows that SNAP produces the largest initial vasodilation, but that it recovers rapidly (~45 minutes) to its precontracted pressure during the period of

perfusion. However, SNP produces a vasodilation (of smaller amplitude than SNAP) which is maintained throughout the period of SNP perfusion (~ 3 hours 20 minutes). ISMN produces no vasodilator response at this concentration, as was expected from the experiments conducted previously using bolus injections.

The responses of the same artery to these NO-donor drugs are markedly different, indicating different modes of action. The rapid recovery to SNAP appeared to be due to desensitisation of the artery to the drug. Similar vasodilator profiles were seen for GSNO, with a rapid recovery towards the precontracted pressure of the artery (see **Figure 7.8**). As GSNO has been shown to be stable in aqueous solution the rapid return towards the precontracted pressure cannot be accounted for by thermal or metal-ion catalysed decomposition of the drug *en route* to the tissue. In the case of the action of SNAP, drug degradation could explain recovery of vasodilator response during the perfusion period. In experiments in which the vasodilator profiles of SNAP and GSNO were compared (**Figure 7.8**), there is evidence to suggest that the different chemical stabilities of GSNO and SNAP have an effect on their vasodilaton when continuously perfused. The plotted traces show that over the sixty minute period examined, the artery had fully recovered to its precontracted pressure in response to SNAP perfusion, whereas GSNO perfusion still produced a vasodilation of ~35% relaxation. These results are consistent with the hypothesis that the response profile obtained with SNAP perfusions is due, at least in part, to its degradation *en route* to the vessel. However, the profile obtained during GSNO perfusion is likely to be due to desensitisation (or 'tolerance') of the vessel to the drug (Needleman *et al.*, 1973).

The vasodilator response obtained on perfusion of SNP through vessels showed no signs of recovery during perfusion but fully reversed on washout. However, all experiments were carried out in the dark in Krebs solution buffered to pH 7.4, conditions under which SNP is known not to release NO *in vitro* (Wolf & Swinehart, 1975; Rucki, 1977) or even ex vivo, when its effects on frog heart muscle contractility

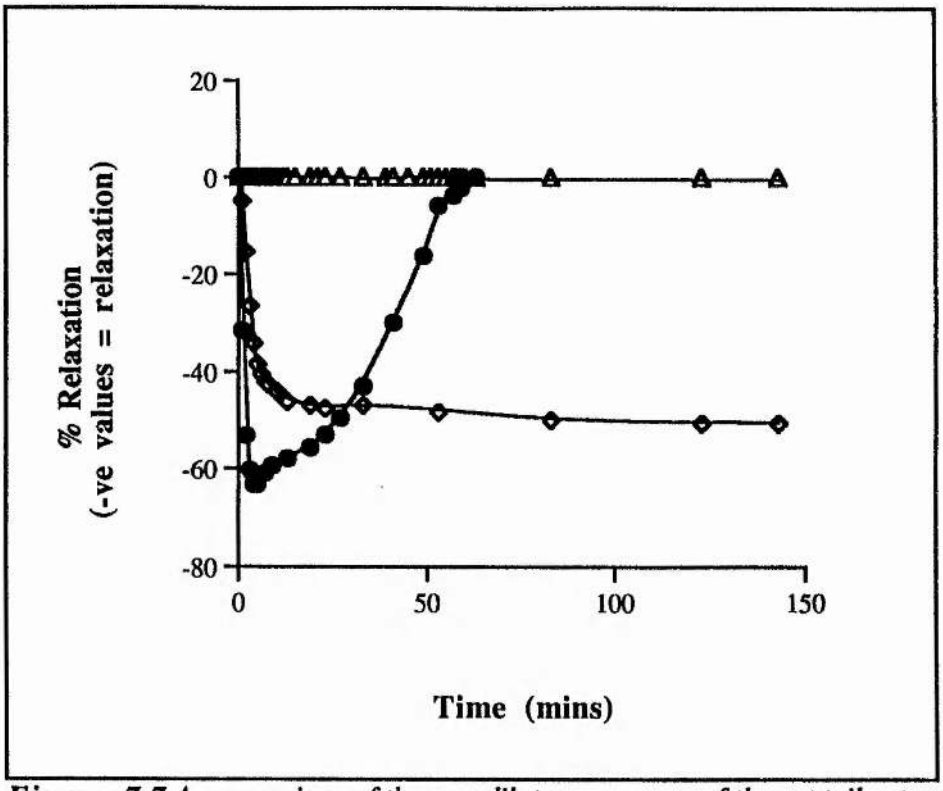


***Figure** 7.7 A comparison of the vasodilator responses of the rat tail artery to three NO-donor drugs (0.73μM) delivered by continuous perfusion. SNAP (closed circles) , sodium nitroprusside (open diamonds) , isosorbide -5-mono-nitrate (open triangles). This is a representative trace of three experiments. (Expt. N° 20/4/92, PE 6μM).*

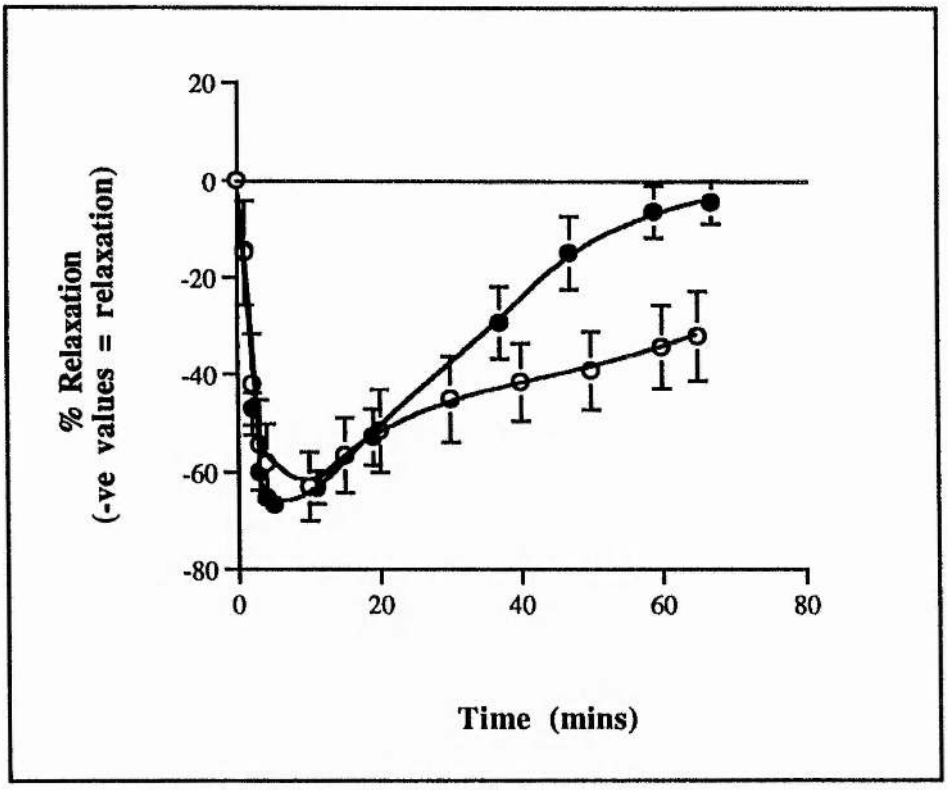


***Figure** 7.8 A comparison of the vasodilator responses of the rat tail artery to continuous perfusion of SNAP (0.5μM) and GSNO (0.5μM). SNAP (closed circles, n=3), GSNO (open circles, n=4).*

were studied (Flitney & Kennovin, 1987). Previous experiments in this laboratory using bolus injection of SNP in the absence of light, have shown that SNP-induced relaxations are not significantly inhibited by Hb in the rat tail artery preparation (Megson, unpublished data). This evidence, along with irregularities with regard to the amplitude of SNP-induced relaxations and the rise in intracellular cGMP (Feelisch, 1991a), suggests that SNP may act through mechanisms other than that mediated by NO.

In light of this knowledge, the response caused by SNP reported here (**Figure 7.7**), which showed no tendency to recover during the perfusion period, may also indicate a different mode of action when compared with both SNAP and GSNO.

ISMN, not surprisingly, in light of results using bolus injections, produces no relaxation of the rat tail artery from micromolar quantities of the drug administered by continuous perfusion. This is possibly due to the multistep nature of conversion of nitrates to NO which has been outlined in **Chapter 1.11**, requiring several enzymes which may not be found in the rat tail artery.

In conclusion, it is evident that prolonged exposure to S-nitrosothiols like SNAP and GSNO brings about tolerance or desensitisation of the artery to the drugs. What causes this is unknown, but it could be either the depletion of thiol groups which has been put forward to explain nitrate tolerance, or down-regulation of guanylate cyclase.

CHAPTER 8

CONCLUSIONS
&
SCOPE FOR
FURTHER WORK

8.1 CONCLUSIONS

It has been demonstrated in this thesis that S-nitrosothiols are effective NO-donating drugs which can elicit vasodilation and disaggregate or inhibit the aggregation of blood platelets. Most of this study deals with the chemical mechanisms which decompose SNAP and GSNO in aqueous solution and *ex vivo*, and may contribute to the biological effects of these two S-nitrosothiols.

It is evident from the work presented that they are both endothelium-independent, fast-acting, and effective vasodilators of the perfused rat tail artery, and decompose to form their disulphide and NO (leading to nitrite) in aqueous solution (**Chapters 2 & 5**). GSNO was found to be significantly more stable than SNAP at physiological pH.

In **Chapter 3** evidence is put forward to suggest that trace metal ions, such as copper(II), present in buffer solutions, are largely responsible for the difference in stabilities of these two S-nitrosothiols. This may also be the case in plasma. It is postulated that a six-membered ring intermediate (see **Figure 3.10**) containing a copper(II) ion is the most favourable conformation to elicit copper ion catalysis of the decomposition of small S-nitrosothiols like SNPen and SNC. Changing the structure of these S-nitrosothiols, by, for example, N-acetylation, removal of favourable ligand groups such as amines or carboxylates, or increasing the chain length between the two ligand binding sites (Askew & Barnet *et al.*, 1994 *in prep*) stabilises the S-nitrosothiol by reduction or inhibition of the formation of this six membered ring intermediate. This theory is supported by the finding that SNAP and SNAC show increased stability compared with their analogues SNPen and SNC (**Chapter 2**). GSNO was found to be very resistant to metal ion catalysis possibly due to its increased number of possible copper binding sites (i.e. two carboxylates, an amine group and amide linkages) removed from the S-nitroso moiety.

The exact mechanism by which copper catalyses the decomposition of SNAP and other S-nitrosothiols (RSNO) is not fully understood, but evidence is presented to suggest spontaneous break up of the ring 'intermediate' with the production of NO, the thiol radical (RS·), which forms the disulphide (RSSR), and free copper(II).

In **Chapter 4** other chemical mechanisms which bring about SNAP decomposition are discussed. Evidence is presented to suggest that thermal and photochemical decomposition of SNAP are possible. The thermal decomposition of SNAP was found to be enhanced by the presence of trace metal ions. pH was also shown to have a large effect on the stability of S-nitrosothiols. It is deduced that the effect of pH, which at physiological pH (7.4) produces the fastest rate of SNAP decomposition, but stabilises the S-nitrosothiol under acid or alkaline conditions, is probably related to the metal ion catalysed process. The relative solvated form of the metal (copper) ions or the protonation state of the ligands, possibly contribute to reduced coordination under alkali or acid conditions.

Results presented in **Chapter 5** indicate that copper can reduce the vasodilator action of SNAP on the perfused rat tail artery over time, suggesting that the removal of NO from the S-nitrosothiol and subsequent oxidation and reaction to form nitrite is the cause. Further work on the vasodilator action of SNAP elicited in the rat tail artery found no evidence for enzymatic decomposition of the compound, as no significant differences were found for the responses of D, L, and DL SNAP. However, chemical evidence suggests that SNAP can transnitrosate with other thiol containing compounds like cysteine. This supports the theory that transnitrosation may play a role in the vasodilator action of SNAP, as it can still elicit a response in the rat tail artery even in the presence of ferro-haemoglobin. The same process is possible for GSNO which is shown to elicit a significantly increased vasodilator response in the same bioassay when injected in the presence of cysteine. Chemical evidence demonstrates that this is due to the formation of the significantly more unstable S-nitrosothiol, SNC, by NO-transfer

from GSNO. As GSNO is far more resistant to copper ion catalysis than SNAP it does not readily decompose in buffer solutions which contain the metal. However, the enzyme γ-glutamyl transpeptidase (γ-GT) was shown to decompose the nitrosated tripeptide. Evidence is presented to suggest that this decomposition results in the formation of an S-nitrosothiol, S-nitrosocysteinylglycine, which would be expected to be far more susceptible to metal (copper) ion catalysis and therefore readily produce NO. As this enzyme has been found to be present in numerous tissue types (Rosalki, 1975; Goldberg, 1980) it could present a viable mechanism which would elicit release of NO from GSNO.

In **Chapter 6** it is shown that these S-nitrosothiols are very effective at modulating the behaviour of platelets, by inhibiting their aggregation or disaggregating aggregated platelets. As a class of NO-donor drugs, S-nitrosothiols are far more effective than other classes of NO-donor drugs, such as the molsidomines (SIN-1), which have been reported as effective inhibitors of platelet aggregation (Wautier *et al.*, 1989). In fact, they were found to be equally effective, or even more effective, than 'authentic' NO. SNAP and SNFP were found to be more effective than SNPen and yet the production of NO was not detected from these two S-nitrosothiols, unlike SNPen. However, the effect of ferro-haemoglobin which induced aggregation, even in the presence of SNAP and SNFP, suggested that NO was ultimately released, probably within the platelet or to sulphydryl groups on the platelet surface (Simon & Stamler *et al.*, 1993). It seems that NO released from the S-nitrosothiol in ways similar to these, is more effective than spontaneous release of NO outside the platelet, at modulating their behaviour. Evidence recently presented has shown that the effective inhibition of platelet aggregation by GSNO is enhanced by platelet lysate, suggesting that NO production from GSNO is catalysed by the platelet membranes (Radomski et al., 1992). From work presented in this thesis and the fact that γ-GT is present in human serum (Rosalki, 1975; Goldberg, 1980), this enzyme, or a related enzyme could be a possible candidate for catalysing GSNO decomposition.

The final chapter investigates the possibility of stabilising these relatively unstable NO-donor drugs with cyclodextrins, as a follow up study to one recently done by Bauer and Fung, (1991b). This class of compounds has been used to improve the pharmacokinetic profiles of other classes of drugs by improving their stability and delivery (Pitha *et al.*, 1986; Frijlink *et al.*, 1990). In the experiments carried out it was found that there was no enhanced stabilisation of SNAP, and that copper could still catalyse its decomposition.

As a class of NO-donor drugs, S-nitrosothiols like SNAP and GSNO were shown to be more instantly effective and as fast acting as SNP in the perfused rat tail artery bioassay, although their effects were short lived in comparison (**Chapter 7**).

The findings presented in this thesis are an attempt to understand the mechanisms underlying the chemical and physiological properties possessed by the S-nitrosothiols, SNAP and GSNO. It can be concluded that although SNAP and GSNO, like other S-nitrosothiols, undergo decomposition by similar mechanisms, such as by thermal and photochemical means, they are also susceptible to decomposition by NO-transfer (transnitrosation) to thiol groups such as cysteine. Furthermore, they can be decomposed by different, specific mechanisms. SNAP is very susceptible to copper ion catalysis whereas GSNO is not, but GSNO is decomposed by the enzyme γ-GT. These differences may play a part in explaining their different biological effectiveness as vasodilators and modulators of platelet behaviour.

8.2 SCOPE FOR FURTHER WORK

Due to the broad field of study undertaken and reported in this thesis there are many areas of work which can be further researched. Some of these are categorised into different subjects and discussed below.

Chemistry

1) Having investigated the effects of Cu(II) ions on the decomposition rates of SNAP and GSNO, further investigation of the general mechanism by which this metal brings about release of NO from S-nitrosothiols can be undertaken. By synthesising analogues of these S-nitrosothiols and varying ligands in close proximity to, or distant from, the S-nitroso moiety the effects on metal binding can be compared.

2) Initial results presented in **Chapter 2** show that Fe(II) ions also have a dramatic effect on the stability of SNAP and presumably other S-nitrosothiols. As iron is present in larger concentrations than copper *in vivo*, a full study on the effect of Fe(II) ion on S-nitrosothiols is needed, and a comparison with copper using the same model S-nitrosothiols may give an insight into whether the mechanisms by which they act are the same.

3) The recent purchase of an NO electrode will allow the study of the decomposition of S-nitrosothiols by spectrophotometric techniques, to be supplemented with the monitoring of NO production from these compounds. This instrument could be used to re-examine the effects of thermal decomposition and pH on S-nitrosothiol decomposition and to conduct an in depth study of the photochemical decomposition of this class of compounds.

4) The discovery of trace metal ion catalysis, and the subsequent study of the effect of copper(II) on different S-nitrosothiols conducted by this laboratory and the Durham group, has resulted in the identification of factors which make S-nitrosothiols susceptible or resistant to decomposition. The synthesis of di- and tripeptides, which have alternative metal ion binding sites away from the S-nitroso moiety, would produce more robust S-nitrosothiols which may have improved pharmacokinetic profiles when biologically tested. If these peptides incorporate a γ-glutamyl group in the structure, particularly if it is situated next to the thiol containing amino acid which is nitrosated,

then there is the possibility of improved targeting of the S-nitrosothiol to γ-glutamyl transpeptidases. The resultant cleavage of the γ-glutamyl group would leave an S-nitrosothiol with a terminal amino acid containing the S-nitroso moiety, susceptible to metal ion catalysis. If it is proved that γ-GT or a related enzyme is responsible for the catalytic decomposition of GSNO in platelet lysate (Radomski *et al.*, 1992; see later for experimental determination), then this may present a way to improve and increase specificity for the action of this class of NO-donor drugs as modulators of platelet behaviour.

5) As all the work conducted and presented in this thesis deals with the decomposition rates of S-nitrosothiols like SNAP and GSNO in physiological buffer, the natural progression would be to monitor the decomposition of S-nitrosothiols in 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) The effect on S-nitrosothiol stability of iron-sulphur clusters in enzymes and proteins which are contained in many tissue types, has not been investigated. An initial study using model iron-sulphur clusters with a range of S-nitrosothiols, would enable us to determine whether this class of compounds has a role to play in S-nitrosothiol metabolism and whether a more detailed study is required.

Physiology and Biochemistry

7) It has been shown that GSNO is rapidly decomposed by γ-GT (this thesis), and that the release of NO from the S-nitrosothiol is significantly enhanced in the presence of platelet lysate (Radomski *et al.*, 1992). Therefore, it is necessary to verify whether, or not, platelet lysate contains γ-GT or a related enzyme and whether this is responsible, at least in part, for the release of NO from GSNO. It has been shown that the most

effective and sensitive method for determining the activity of γ-GT in serum or plasma, is by the use of γ-glutamyl-*p*-nitroanilide as a substrate, together with glycylglycine as the acceptor (Dimov & Kulhanek, 1967). Using this method on platelet lysate would prove if a γ-GT-type mechanism and therefore γ-GT or a related enzyme is present. In addition, biochemical techniques such as electrophoresis could be used to separate the protein components of the platelet lysate. These could be assayed for activity of γ-GT enzymes already known, selected marker enzymes such as cytochrome C oxidase, NADPH-cytochrome C reductase and others.

7) The use of a more specific alkylating agent than ethacrynic acid for modification of thiol groups (protein-bound or free) would give a better insight as to whether NO-transfer between S-nitrosothiols like SNAP and GSNO, to thiol containing molecules in tissue, plays an essential role in the biological activity of these drugs as vasodilators or modulators of platelet behaviour. The blocking of specific thiol groups would eliminate or support the relevant thiol containing compound as being involved, depending on the biological response. In addition, the use of Ellmans reagent (5,5'-dithiobis(2-nitrobenzoic acid), DTNB; Ellman, 1959), can be used to detect the concentration of reduced thiol present before and after treatment with an alkylating agent by a colourimetric assay. Its only draw back is that it is not specific for individual thiols. However, if the thiol is protein bound, methods published by Kowaluk et al. (1992) and Kowaluk & Fung, (1990) should allow the determination of the site and weight of the protein involved to be determined. Likewise, these methods could also be used to determine any catalytic activity existing in vascular smooth muscle or endothelial cells, which would bring about decomposition of S-nitrosothiols like SNAP and GSNO.

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APPENDICES
TO
DOSE-RESPONSE CURVES

APPENDICES TO DOSE RESPONSE CURVES

APPENDIX TO FIGURE 2.7

Below are the peak amplitude (% Active pressure) of responses to 10µl injections of SNAP& GSNO

SNAP

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
91/11/4	7.9	16.0	36.7	43.7	48.2	54.1
91/11/5	8.8	38.8	59.8	61.6	70.0	68.3
91/11/9	0.0		41.3	53.4	60.4	62.5
91/11/14	5.2	33.7	58.8	65.6	72.0	72.0
93/1/12	2.9	9.5	49.1	57.5	57.2	
93/1/13i	2.9	6.8	39.6	65.4	74.5	
93/1/13ii	4.1	13.7	34.0	47.0	58.1	
93/1/19	9.9	12.3	64.6	64.7	73.5	70.5
93/4/23i	0.0	7.1	32.9	55.2	63.3	
93/4/23ii	0.0	7.1	23.6	45.3	54.3	
93/4/23iii	0.0	8.6	32.4	46.8	50.4	
93/4/23iv	0.0	3.8	22.6	41.5	50.9	
93/12/10i	6.5	10.1	37.4	52.5	59.8	69.3
93/12/10ii	4.6	41.4	53.5	65.0	68.9	79.5
94/1/12i	3.8	25.8	43.3	49.7	57.9	65.1
94/1/12ii	4.1	32.2	52.8	57.6	64.1	
94/1/12iii	4.2	16.4	40.8	55.8		
94/1/13i	4.0	32.7	58.1	68.5	76.9	74.4
94/1/13ii	0	34.9	55.1	60.0	68.0	71.8
94/1/13iii	2.8	9.6	26.0	34.9	36.7	37.2
MEAN	**3.6**	**19.0**	**43.1**	**54.6**	**61.3**	**65.9**
S.E.	**0.7**	**2.9**	**2.8**	**2.1**	**2.4**	**3.5**

GSNO

* = statistically significant from SNAP responses above at P=0.05 confidence level

*** = statistically significant from SNAP responses above at P=0.001 confidence level

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
93/4/23i	0.0	0.0	6.9	26.5	58.7	
93/4/23ii	0.0	0.0	5.0	24.4	56.3	
93/4/23iii	0.0	2.6	4.7	30.5	65.2	
93/4/23iv	0.0	0.0	4.2	26.3	48.7	
93/5/27i	2.9	5.4	27.0	47.2	64.0	
93/5/27ii	1.5	3.4	17.9	63.0	64.0	
93/5/27iii	3.6	6.4	26.6	50.0	64.0	
93/11/5ii	0.0	3.6	14.9	47.0	61.9	
93/11/5iii	33.3	44.9	45.9	45.8	59.2	
93/11/5iv	10.8	14.4	20.6	28.4	68.4	
93/11/4ii		17.6	30.3	49.1	62.2	
93/11/4iii		5.0	23.4	46.7	59.6	
93/12/9ii		3.4	23.3	33.3	62.8	64.7
93/12/9iii		2.8	7.5	24.0		80.9
93/12/9iv		6.7	12.8	39.1	60.4	68.9
93/12/7ii				38.6		59.1
93/12/7iii				37.2		64.0
94/1/12				24.4		61.8
MEAN	**5.2**	**7.7***	**18.1*****	**37.9*****	**61.5**	**66.6**
SE	**3.3**	**2.9**	**3.1**	**2.7**	**1.4**	**3.2**

APPENDIX TO FIGURE 5.6

Below are the peak amplitude (% Active pressure) of responses to 10µl injections of DL-SNAP, D-SNAP & L-SNAP on the same arteries

DL-SNAP

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
91/11/9	0	38.8	41.3	53.4	60.4	62.5
91/1/13	4.1	13.7	34.0	47.0	58.1	
93/1/14i	5.2	33.7	58.8	65.6	72.0	72.0
93/1/14ii	2.9	6.8	39.6	65.4	74.5	
93/1/19	9.9	12.3	64.6	64.7	73.5	70.5
MEAN	**4.4**	**21.1**	**47.7**	**59.2**	**67.7**	**68.3**
SE	**1.6**	**6.4**	**5.9**	**3.8**	**3.5**	**2.9**

D-SNAP

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
91/11/9	4.2	11.9	37.1	41.3	61.5	62.0
91/1/13	2.7	50.1	67.8	72.2		
93/1/14i	3.7	38.3	71.7	76.8	77.0	58.9
93/1/14ii	1.0	6.7	76.3	75.7	91.7	
93/1/19	3.7	8.2	59.8	72.9	66.1	65.1
MEAN	**3.3**	**23.0**	**62.5**	**67.8**	**68.2**	**63.6**
SE	**1.2**	**8.9**	**6.9**	**6.7**	**4.6**	**1.6**

L-SNAP

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
91/11/9	5.7	13.4	35.4	54.4	56.2	57.2
91/1/13	3.1	19.6	66.6	73.0		
93/1/14i	1.3	57.3	73.8	74.5	78.3	
93/1/14ii	0.0	27.2	51.4	61.5		
93/1/19	6.5	26.0	44.0	73.4	67.0	73.0
MEAN	**3.3**	**28.7**	**54.2**	**67.4**	**67.2**	**65**
SE	**1.2**	**7.6**	**7.1**	**4.0**	**6.8**	**7.9**

APPENDIX TO FIGURE 5.16

Below are the peak amplitude (% Active pressure) of responses to 10µl injections of GSNO and GSNO with Cysteine (1mM) continuously perfusing through the internal perfusate. Experiments were conducted on the same arteries.

GSNO

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
93/4/23i	0.0	0.0	6.9	26.5	58.7	
93/4/23ii	0.0	0.0	5.0	24.4	56.3	
93/4/23iii	0.0	2.6	4.7	30.5	65.2	
93/4/23iv	0.0	0.0	4.2	26.3	48.7	
93/12/9i	0.0	3.4	23.3	33.3	62.8	64.7
93/12/9ii	0.0	2.8	7.5	24.0		80.9
93/12/9iii	0.0	6.7	12.8	39.1	60.4	68.9
93/12/7i				38.6		59.1
93/12/7ii				37.2		64.0
MEAN	**0.0**	**2.2**	**9.2**	**31.5**	**58.7**	**67.5**
S.E.	**0.0**	**0.9**	**2.6**	**2.3**	**2.4**	**3.7**

GSNO in the presence of Cysteine

* = statistically significant from GSNO responses above at P=0.05 confidence level

*** = statistically significant from GSNO responses above at P=0.001 confidence level

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
93/4/23i	6.0	8.3	22.2	42.3	56.3	
93/4/23ii	0.0	6.6	9.6	64.6	74.1	
93/4/23iii	2.9	6.0	18.9	57.8	60.5	
93/4/23iv	0.0	0.0	11.6	54.5	66.9	
93/12/9i	3.9	10.0	50.0	53.3	63.0	64.5
93/12/9ii	0.5	6.2	11.9	56.2	60.4	73.0
93/12/9iii	2.2	6.4	21.0	55.6	76.6	69.1
93/12/7i				72.0		71.7
93/12/7ii				62.6		71.0
MEAN	**2.2**	**6.2***	**20.7**	**57.7*****	**65.4**	**69.9**
S.E.	**0.9**	**1.4**	**5.2**	**2.8**	**2.8**	**1.5**

APPENDIX TO FIGURE 5.19

Below are the peak amplitude (% Active pressure) of responses to 10µl injections of SNAP and SNAP with ferro-haemoglobin (15µM) continuously perfusing through the internal perfusate.Control experiments were not significantly different to the results presented in the appendix for **Figure 2.7.**

SNAP in the presence of Hb

** = statistically significant from SNAP responses (Fig 2.7) at P=0.005 confidence level

*** = statistically significant from SNAP responses (Fig 2.7) at P=0.001 confidence level

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
93/1/12	0.0	0.0	3.3	23.7	64.9	64.8
93/1/13ii	0.0		1.7	22.7	45.4	50.0
93/1/14i			13.6	33.8	66.7	62.5
93/11/5iii	0.0	6.8	0.0	12.2	40.8	55.4
93/11/5iv	6.7	4.8	6.1	26.3	45.8	51.3
93/11/4ii			14.0	9.9	44.0	54.5
93/11/4iv		7.7	6.5	27.6	52.0	57.3
93/12/9ii		3.2	10.6	11.7	20.0	35.9
93/12/9iii		1.8	8.0	2.5	13.8	43.1
93/12/9iv			2.7	11.7	33.6	46.0
MEAN	**1.7**	**4.1****	**6.6*****	**18.2*****	**42.7*****	**52.1****
S.E.	**1.7**	**1.2**	**1.5**	**1.5**	**5.4**	**2.8**

APPENDIX TO FIGURE 5.20

Below are the peak amplitude (% Active pressure) of responses to 10µl injections of GSNO and GSNO with ferro-haemoglobin (15µM) continuously perfusing through the internal perfusate.Control experiments were not significantly different to the results presented in the appendix for **Figure 2.7.**

GSNO in the presence of Hb

** = statistically significant from GSNO responses (Fig 2.7) at P=0.005 confidence level

*** = statistically significant from GSNO responses (Fig 2.7) at P=0.001 confidence level

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
93/11/5ii	0.0	3.8	5.1	6.3		
93/11/5iii	5.0	4.4	9.9	17.1	29.1	64.7
93/11/5iv	7.5	3.6	14.9	11.9	34.2	64.9
93/11/4ii		9.7	3.3	10.9	37.2	60.7
93/11/4iv		2.8	0.0	13.1	48.9	58.6
93/12/9ii		4.2	2.0	10.1	14.4	35.2
93/12/9iii		3.2	3.2	8.2	11.1	26.1
93/12/9iv		0.0	3.4	14.2	27.4	46.2
MEAN	**4.2**	**4.0**	**5.2****	**11.5*****	**28.9*****	**52.6**
S.E.	**2.2**	**1.0**	**1.7**	**1.2**	**4.9**	**5.3**

APPENDIX TO FIGURE 5.21

Below are the peak amplitude (% Active pressure) of responses to 10µl injections of GSNO and GSNO with SOD (150 units/ml) continuously perfusing through the internal perfusate.

GSNO

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
93/11/5ii	0.0	3.6	14.9	47.0	61.0	68.9
93/11/5iv	10.8	14.4	20.6	28.4	68.4	81.4
93/11/4i	5.4	17.6	30.3	49.1	62.2	58.1
93/11/4ii	1.0	5.0	23.4	46.7	59.6	60.0
94/1/12	9.4				62.3	61.8
MEAN	**5.3**	**10.2**	**22.3**	**39.1**	**62.3**	**66.8**
S.E.	**2.2**	**3.4**	**3.2**	**5.2**	**1.3**	**3.8**

GSNO in the presence of SOD

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
93/11/5ii	7.9	11.3	29.8	52.2	61.9	
93/11/5iii	6.4	14.7	25.0	44.8	53.7	
93/11/5iv	15.7	21.6	21.1	36.1	55.3	
93/11/4i	7.0	13.2	30.8	54.5	66.2	
93/11/4ii	2.0	5.2	18.9	44.0	57.1	
93/10/12iii	1.6					57.5
93/10/12iv	9.2					66.2
94/1/12ii	3.7		27.4	36.6		58.5
MEAN	**6.7**	**13.2**	**25.5**	**44.7**	**58.8**	**60.7**
S.E.	**1.6**	**2.7**	**1.9**	**3.1**	**2.3**	**2.7**

APPENDIX TO FIGURE 5.22

Below are the peak amplitude (% Active pressure) of responses to 10µl injections of SNAP and SNAP with SOD (150 units/ml) continuously perfusing through the internal perfusate.Control experiments were not significantly different to the results presented in the appendix for **Figure 2.7.**

SNAP in the presence of SOD

** = statistically significant from SNAP responses (Fig 2.7) at P=0.005 confidence level

Expt No	10^{-7}M	10^{-6}M	10^{-5}M	10^{-4}M	10^{-3}M	10^{-2}M
93/12/10iii	3.9	21.4	41.3	54.0	62.2	62.7
93/12/10iv	11.7	18.0	48.8		70.0	70.5
94/1/12ii	8.4	27.8	47.4	54.9	60.2	64.3
94/1/12iii	11.6	29.5	40.6	49.4	60.4	65.9
94/1/12iv	14.5	35.6	51.8	60.4	68.3	69.5
MEAN	**10**	**26.4**	**46.0**	**54.7**	**64.2**	**66.6**
S.E.	**2.3**	**3.1**	**2.2**	**2.3**	**2.1**	**1.5**

APPENDICES TO CONTINUOUS PERFUSION GRAPH-FIGURE 7.8

Below are the peak amplitude (% Active pressure) of the responses of the artery to SNAP and GSNO at timed intervals (mins) from continuous perfusion of the artery with 0.5μM of the drug through the internal perfusate.

SNAP

TIME (mins)	Expt No 92/5/7i	Expt No 92/5/7ii	Expt No 92/5/7iii	MEAN	S.E.
0	0.0	0.0	0.0	**0.0**	**0.0**
1	13.2	17.6	12.8	**14.5**	**1.5**
2	52.9	47.2	41.6	**47.2**	**3.3**
3	64.5	59.1	56.8	**60.1**	**2.3**
4	70.2	63.0	62.4	**65.2**	**2.5**
5	72.9	63.8	64.0	**66.9**	**3.0**
11	69.8	59.9	59.6	**63.1**	**3.4**
19	63.6	51.0	44.0	**52.9**	**5.7**
37	41.3	31.0	15.6	**29.3**	**7.5**
47	20.7	23.9	0.0	**14.9**	**7.5**
59	2.1	17.1	0.0	**6.4**	**5.4**
67	0.0	13.2	0.0	**4.4**	**4.4**

GSNO

TIME (mins)	Expt No 93/5/5i	Expt No 93/5/5ii	Expt No 93/5/5iii	Expt No 93/5/5iv	MEAN	S.E.
0	0.0	0.0	0.0	0.0	**0.0**	**0.0**
1	46.9	4.4	4.1	4.2	**14.9**	**10.7**
2	70.3	45.7	26.0	26.3	**42.1**	**10.5**
3	76.5	61.9	35.7	43.7	**54.5**	**9.2**
4	79.0	61.9	44.9	47.1	**58.2**	**7.9**
10	80.2	68.6	52.0	51.1	**63.0**	**7.0**
15	75.9	61.4	42.9	46.1	**56.6**	**7.6**
20	72.8	56.6	35.2	41.7	**51.6**	**8.4**
30	67.0	51.0	27.6	34.7	**45.1**	**8.8**
40	62.0	45.7	25.0	33.7	**41.6**	**8.0**
50	61.1	41.0	23.5	31.0	**39.2**	**8.2**
60	59.9	27.6	20.4	29.3	**34.3**	**8.7**
65	59.3	21.9	18.6	28.3	**32.0**	**9.3**