Role of S-Nitrosothiols in Non-adrenergic Non-cholinergic Neurotransmission

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By

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Abbreviations

A	Absorption
Ach	Acetylcholine
BRP	Bovine retractor penis
DETCA	Diethyldithiocarbamate
EDRF	Endothelium-derived relaxing factor
GTN	Glyceryl trinitrate
Hb	Haemoglobin
HPLC	High performance liquid chromatography
IF	Inhibitory factor
L-NMMA	N^G-monomethyl-L-arginine
L-NOARG	N^G-nitro-L-arginine
NANC	Non-adrenergic, non-cholinergic
NO	Nitric oxide
NO_2	Nitrogen dioxide
P	Probability
P1	Peak 1 (in the HPLC)
P2	Peak 2 (in the HPLC)
P3	Peak 3 (in the HPLC)
RAR	Rabbit aortic ring
SOD	Superoxide dismutase

Summary

1. The aim of this study was to examine the possibility that S-nitrosocysteine or S-nitrosoglutathione rather than nitric oxide functions as the inhibitory non-adrenergic, non-cholinergic (NANC) neurotransmitter in the bovine retractor penis (BRP) muscle. This was investigated firstly, by examining whether free sulfhydryl groups are required for NANC relaxation to take place upon nerve stimulation in the BRP muscle and secondly, by examining the properties of the new relaxant produced when nitric oxide is reacted with a range of sulfhydryl compounds.

2. Treatment of BRP muscle with the sulfhydryl oxidising agent, diamide (1 mM), inhibited NANC relaxation induced by nerve stimulation. This effect was completely prevented and almost completely reversed by treating the tissue with the sulfhydryl compounds, L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM). The inhibition was not specific, however, since the oxidising agent also inhibited the relaxant actions of glyceryl trinitrate (0.01-0.1 μM) and isoprenaline (0.01-1 μM).

3. Treatment of BRP muscle with the sulfhydryl alkylating agent, N-ethylmaleimide (0.3 mM), inhibited NANC relaxation induced by nerve stimulation. This effect was completely prevented but not reversed by treating the tissue with the sulfhydryl compounds, L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM). As with diamide, inhibition was not specific, however, since the alkylating agent also inhibited the relaxant actions of glyceryl trinitrate (0.01-0.1 μM) and isoprenaline (0.01-1 μM).

4. The weak vasodilator activity of sodium nitrite (10 μM) was greatly enhanced by acidification. The optimal pH for enhancement of vasodilator activity was pH 1-2. Deoxygenation before acidification further enhanced and bubbling to saturation with

oxygen for 10 minutes before acidification inhibited relaxant activity. Increases in relaxant activity upon acidification were associated with decreases in nitrite content. Neutralisation of acidified samples led to a rapid loss of relaxant activity. These results are consistent with formation of nitric oxide upon acidification of nitrite and this is destroyed by oxygen and protected in an oxygen-free environment.

5. The first means adopted to form an S-nitrosothiol was to acidify nitrite (10 mM) in the presence of L-cysteine (1.5 M). The relaxant activity, assessed both in magnitude and duration, greatly exceeded that of equivalent solutions of acidified nitrite. Again maximum generation of relaxant activity occurred at pH 2, but unlike solutions of acidified nitrite, these solutions retained their relaxant activity upon neutralisation. These solutions were pink in colour and their relaxant activity was destroyed by the nitric oxide-binding substance, haemoglobin, but was unaffected by the inhibitor of nitric oxide synthase, N^G-nitro-L-arginine.

6. In the spectrophotometer at 190-900 nm, nitric oxide in the gas phase produced several narrow absorption bands at wavelengths less than 230 nm, whereas in aqueous solution it produced only a single peak at 190 nm. Upon admission of oxygen, nitrogen dioxide was rapidly formed in both the gas and liquid phases assessed by formation of its characteristic absorption peaks at 300-400 nm.

7. A second means adopted for the formation of S-nitrosothiols was by reacting nitric oxide gas with L-cysteine. In phenylephrine (0.3 μM)-contracted rabbit aortic rings denuded of endothelium and in BRP strips, nitric oxide (1-1,000 nM) alone induced transient relaxation in a concentration-dependent manner, whereas L-cysteine (0.15-4.5 mM) was without effect. When aqueous solutions of L-cysteine (15 mM) were reacted with nitric oxide (5 mM) in nominally oxygen-free conditions at pH 3 for 10 minutes followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation and added to achieve bath

concentrations equal to 10 nM nitric oxide and 30 μM L-cysteine, however, more powerful and prolonged relaxation was produced than could be attributed to nitric oxide alone.

8. In the HPLC, aqueous solutions of L-cysteine (10 mM) at pH 3.0 produced two peaks corresponding to L-cystine (~ 0.85 %, peak 1) and L-cysteine (~ 99.15 %, peak 2), respectively. After saturating this solution with nitric oxide gas for 10 minutes under nominal oxygen-free conditions, the solution produced three peaks in the HPLC corresponding to L-cystine (~ 0.85 %), L-cysteine (~ 98 %) and a new substance (~ 1.15%, peak 3) which was entirely responsible for relaxant activity. Re-run of the peaks in the HPLC revealed that L-cystine was stable, whereas L-cysteine and the new relaxant decayed slowly to form L-cystine.

9. Using a series of structural analogues of L-cysteine (all at 15 mM) it was found that removal of the carboxyl group (L-cysteamine), replacement of the carboxyl with an ester function (L-cysteine methyl ester) or substitution at the amino group (N-acetyl-L-cysteine) had no effect on the ability to generate new relaxant activity upon reaction with nitric oxide (0.1 mM). In contrast, substitution at the sulfhydryl group (S-methyl-L-cysteine, L-cysteinesulfinic acid and L-cysteic acid), or formation of disulphides (L-cystine and L-cystamine) led to a complete loss of ability to generate relaxant activity. L-glutathione (reduced) was also able to react with nitric oxide to produce new relaxant activity, and this too was blocked upon substitution of the free sulfhydryl group (S-methyl-L-glutathione) or formation of disulphides (oxidised L-glutathione). Furthermore, methionine which contains a substituted sulfhydryl group and the non-sulfhydryl-containing amino acids, L-arginine, L-glycine and L-glutamic acid, were all unable to react with nitric oxide to generate new relaxant activity. A free sulfhydryl group was therefore required to generate relaxant activity following reaction with nitric oxide.

10. UV-visible spectrophotometric analysis of this new compound revealed that it had three absorption bands with maxima at 218, 335 and 543 nm. The magnitude of absorption was: 218 > 335 > 543 nm.

11. Freeze drying of the relaxant under high vacuum overnight resulted in a complete loss of relaxant activity and the associated peak 3 in the HPLC, whereas storage at -20°C had little effect. Saturating the solution with pure oxygen for 10 minutes did not result in any change in its relaxant activity and height of peak 3. Treatment in a boiling bath for 10 minutes substantially reduced the relaxant activity and associated peak 3 in the HPLC, whereas treatment for 10 minutes at 70°C was without effect. After mixing with methanol (1:1, vol./vol.) for 10 minutes at 0°C, the relaxant activity and height of peak 3 were reduced by 50 % and three new peaks were generated. Thus, the new relaxant formed was relatively stable to heat and the presence of oxygen, but was destroyed by methanol.

12. Despite attempting to standardise reaction conditions, the amounts of relaxant activity generated differed enormously from experiment to experiment. Clearly, some variable factor was having an important effect upon the outcome of the reaction between nitric oxide and L-cysteine. We considered the possibility that our reaction conditions were not completely oxygen-free and that oxygen might play a part in the reaction. We therefore devised more rigorous procedures in which we first purified nitric oxide and oxygen-free nitrogen gases by purging through Fieser's solution and sodium hydroxide to remove traces of oxygen and nitrogen dioxide, respectively.

13. Under such rigorous oxygen-free conditions, bubbling nitric oxide gas to saturation for 10 minutes in solutions of L-cysteine (10 mM) at pH 3 followed by purging to remove free nitric oxide generated novel relaxant activity together with its associated peak 3 in the HPLC and absorption peaks at 218 and 335 nm but the

amounts formed were much smaller than when less rigorous oxygen-free conditions were employed. These solutions were colourless and no absorption peak at 543 nm was seen. Furthermore, these properties of the solution did not change upon exposure of the solution to the air.

14. In contrast, if under the same rigorous oxygen-free conditions, L-cysteine was reacted with nitric oxide and free nitric oxide was not removed from the solution before exposure to the air, the relaxant activity, magnitude of peak 3 in the HPLC and the absorption peaks at 218 and 335 nm were all some 40 times greater. Furthermore, upon exposure to the air, the solution instantly became pink in colour owing to the development of a new peak at 543 nm. Thus oxygen was critical for formation of the new relaxant activity and its associated peak 3 in the HPLC and its absorption peaks at 218, 335 and 543 nm.

15. In other experiments under rigorous oxygen-free conditions in a sealed cuvette when L-cysteine was reacted with nitric oxide and free nitric oxide was not removed by purging, absorbance peaks at 218 and 335 nm were small initially but grew with time. Furthermore, although no peak was visible initially at 543 nm, one began to appear by about 40 minutes and this too began to grow with time. These rises were likely to be due to entry of oxygen into the reaction chamber despite our rigorous efforts.

16. The new relaxant activity generated by reacting nitric oxide with L-cysteine or L-glutathione was abolished following treatment with the nitric oxide-binding agent, haemoglobin (3 mM), and the guanylate cyclase inhibitors, methylene blue (10 mM) and N-methylhydroxylamine (100 mM), but was unaffected by the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (30 μM). Thus, the new relaxant owed its activity to the release of nitric oxide which subsequently activated soluble guanylate

cyclase. It did not activate nitric oxide synthase in the NANC nerves and did not act as a substrate for the enzyme.

17. Two agents that generate superoxide anion, pyrogallol (0.1 mM) and hydroquinone (0.1 mM), also inhibited this new relaxant activity as well as that of authentic nitric oxide (0.3 mM), but as previously reported had no effect on relaxation induced by NANC nerve stimulation. Furthermore, superoxide dismutase (100 u ml^{-1}) potentiated the relaxation induced by the S-nitrosothiols, reversed the blockade induced by pyrogallol and hydroquinone but had no effect on NANC relaxation. Superoxide anion can therefore destroy the relaxant activity of nitric oxide and the S-nitrosothiols but has no effect on the NANC transmitter.

18. In conclusion, the reaction of nitric oxide with L-cysteine or L-glutathione generates relaxant activity which exceeds that of nitric oxide alone and probably results from formation of S-nitrosocysteine and S-nitrosoglutathione, respectively. The effects of pyrogallol and hydroquinone indicate that neither S-nitrosocysteine nor S-nitrosoglutathione are suitable candidates for the NANC inhibitory neurotransmitter in the BRP muscle and suggest that it is more likely to be a superoxide anion-resistant, nitric oxide-releasing molecule.

Chapter 1. INTRODUCTION

Body function is controlled and integrated mainly by the nervous system and the endocrine system. Control by the nervous system depends primarily on the rapid electrical conduction of information in nerve fibres, then between the nerve cells, and finally between the axone terminal of the last neurone in the chain and the effector cells it innervates. The signals crossing junctions between cells are usually carried by chemical substances rather than electrical impulses. This chemical transmission takes place through the release of small amounts of neurotransmitter from the nerve fibre terminals into the region of the synapse or junction. The chemical neurotransmitter diffuses to the postsynaptic cell and binds to a specialised receptor.

The peripheral nervous system can be divided into two major functional subdivisions, the autonomic division and the somatic division. The autonomic nervous system in turn consists of two major portions, the sympathetic division and the parasympathetic division, and was originally based on anatomical differences. On the other hand, a different classification of the autonomic nervous system can be made, based on the neurotransmitter released from the nerve fibre terminals. Classically the autonomic nervous system consisted of a cholinergic nerve division and an adrenergic division, based on the release of either acetylcholine or noradrenaline. When dealing with the post-ganglionic fibres, the cholinergic system corresponds, with few exceptions, to the parasympathetic division and likewise the adrenergic to the sympathetic division (Langley, 1921; Dale & Gaddum, 1930; Dale, 1935; Dale, *et al.*, 1936). The classical concept that the autonomic nervous system consisted of only cholinergic nerve and adrenergic nerve has, however, been challenged. Modern scientific developments in biochemistry, physiology and pharmacology have provided evidence for the existence or coexistence of many other neurotransmitters. For example, a variety of peptides such as substance P,

purines such as ATP and other amines such as dopamine are now established as neurotransmitters (Bell, 1991). Furthermore, the process of cotransmission whereby a single nerve releases two substances both of which affect post-synaptic tissues is now generally accepted (Cuello, 1982; Burnstock, 1985; 1986a; 1986b; Campbell, 1987; Furness, *et al.*, 1989). For example, neuropeptide Y is co-released with norepinephrine from sympathetic nerves in many arteries and potentiates the contractile effects of norepinephrine (Ekblad, *et al.*, 1984; Neild, 1987).

Part of the evidence for the existence of neurotransmitters other than noradrenaline and acetylcholine is a nerve response which is characteristically unaffected by either adrenergic or cholinergic blocking agents. The neurogenic origin of the response is proved by its abolition by tetrodotoxin. Such responses and the nerves producing them are referred to as non-adrenergic non-cholinergic (NANC) responses and nerves, respectively. A non-adrenergic non-cholinergic nerve innervation exists broadly in most peripheral smooth muscles including gastrointestinal tract, cardiovascular system, and the urinary and reproductive system and glands (Burnstock, 1969; 1972; 1975; 1978; Gillespie, 1972; 1987; Klinge & Sjostrand, 1974).

1.1. Historical Development of NANC Innervation

The phenomenon of atropine-resistant responses to nerve stimulation was reported as early as the late 19th century and implied a non-cholinergic innervation. Langley and Anderson (1895) reported that in the rabbit atropine could not inhibit penile erection produced by electrical stimulation of the sacral parasympathetic outflow via the pelvic nerves. Bayliss and Starling (1899) reported that vagal stimulation in the dog small intestine causes first relaxation and subsequently a powerful contraction. Both the relaxation and the contraction were unaffected by atropine. Henderson and Roepke (1933), using penile perfusion in dog, found that physostigmine potentiates

the effects of pelvic nerve stimulation on venous flow, while atropine has almost no effect. In 1934 they also reported that in the guinea pig the motor response of the urinary bladder to parasympathetic nerve stimulation is slightly reduced by low doses of atropine but the large residual motor effect is insensitive to even high doses. Henderson and Roepke (1934) suggested that atropine resistance in the bladder could be best explained by the release of a neurotransmitter other than acetylcholine or noradrenaline. Oppenheimer (1938) studied the cat retractor penis *in vivo*. He found that acetylcholine mimics the effect of this parasympathetic stimulation, however, atropine abolishes the effect of acetylcholine but not of parasympathetic nerve stimulation. He believed the relaxation of the retractor penis was caused by the release of the neurotransmitter acetylcholine. The resistance to atropine of the parasympathetic response was due to the proximity theory, the theory of Dale and Gaddum (1930) and reinvoked by Ambache (1955). The proximity theory was that the muscle cell membrane is in such close proximity to the nerve terminals as to deny access to exogenous blocking agents. However, electron-microscopical studies have disproved this theory because large gaps are found between the nerve terminal and the muscle cell membrane where the neurotransmitter is released and acts on the target membrane. Goldenberg (1965) found that in cat retractor penis injected nicotine produces relaxation which is blocked by hexamethonium but not by atropine. He believed the relaxation was not due to acetylcholine but to an unknown neurotransmitter released from the parasympathetic nerves stimulated by nicotine.

However, experimental proof of the existence of NANC nerves was delayed because no selective powerful adrenergic blocking drugs were available until the 1960s. When adrenergic neurone blocking drugs were introduced and applied to analyse the response of the stomach to nerve stimulation, it was found that they have no effect on the inhibitory response to vagal stimulation in the guinea pig (Martinson, *et al.*, 1963) and to transmural stimulation in the guinea pig and mouse

(Paton & Vane, 1963). The first definite proposal of the existence of non-adrenergic inhibitory nerves was based on the observation that nerve stimulation of the guinea pig taenia coli elicits a potent inhibitory response which is not blocked by bretylium (Burnstock, *et al.*, 1963; 1964). The response is blocked by low concentration of tetrodotoxin and therefore considered neurogenic in origin (Bulbring & Tomita, 1967).

Electropharmacological studies have also supported the existence of NANC inhibitory neurones. Following intramural nerve stimulation, large transient hyperpolarisations i.e. inhibitory junction potentials (IJPs) of up to 30 mV have been recorded in several isolated smooth muscle preparations including taenia coli (Burnstock, *et al.*, 1964; Bennett, *et al.*, 1966), jejumum (Kuriyama, *et al.*, 1967), caecum (Small, 1972) and colon (Furness, 1969). Summation of IJPs could be obtained by repetitive stimulation to produce maximum hyperpolarisations of up to 35 mV at an optimum frequency of 10 Hz. In contrast, no change in the membrane potential of smooth muscle cells or in mechanical activity is observed by single pulse stimulation of either extrinsic or perivascular sympathetic nerves. While the hyperpolarisations in gastrointestinal muscle induced by both sympathetic nerve stimulation and exogenously applied noradrenaline are due to an increase in both potassium and chloride conductance, the hyperpolarisations associated with stimulation of the non-adrenergic inhibitory nerves in the guinea-pig taenia coli are due to a specific increase in potassium conductance (Bennett, *et al.*, 1963; Tomita and Watanabe, 1973).

In the taenia coli of guinea pig the inhibitory relaxation is poorly sustained if continuous stimulation is applied (Burnstock, *et al.*, 1966). In contrast, in the sphincteric muscle of the gut, particularly the internal anal sphincter the response to continued stimulation is well maintained (Rayner, 1979). Furthermore, in the cat stomach relaxation mediated by non-adrenergic inhibitory nerves is not readily

fatigued (Abrahamson, 1973). Stimulation of the pelvic nerve brings about neither mechanical inhibition (Garry & Gillespie, 1955) nor inhibitory junction potentials in the smooth muscle cells of the large intestine (Furness, 1969), although transmural stimulation causes inhibitory junction potentials in both circular and longitudinal layers (Furness, 1969). These inhibitory junction potentials were reduced by hexamethonium suggesting an intrinsic cholinergic drive (Furness, 1970). Electrical and mechanical responses in the guinea pig taenia coli and urinary bladder are reduced by lowering the external calcium concentration (Burnstock, *et al.*, 1978) implying that transmitter release is calcium dependent and that the transmitter itself is probably held in membrane-bound storage vesicles, whose contents are released by exocytosis. In the anococcygeus muscle, responses to field stimulation of motor adrenergic nerves and NANC inhibitory nerves are potentiated by tetraethyl ammonium which is known to prolong the nerve action potentials so allowing more time for calcium entry (Gillespie & Tilmisany, 1976).

There have been many reports providing evidence for the existence of an NANC innervation in a wide variety of tissues. They include the lower oesophageal sphincter muscle (Rattan, *et al.*, 1977), oesophagus (Lund & Christensen, 1969), stomach (Martinson, 1965; Campbell, 1966), small intestine (Holman & Hughes, 1965; Day & Warren, 1967; 1968; Weston, 1971; Hirst & McKirdy, 1974), large intestine (Bianchi, *et al.*, 1968; Small 1972), taenia coli (Bennet, *et al.*, 1966), gall bladder (Davison, *et al.*, 1978), trachea (Coleman, 1973; Coburn & Tomita, 1973), BRP (Klinge, 1970a; Klinge & Sjostrand, 1974; Ambache & Killick, 1978), anococcygeus (Gillespie, 1972; 1987; Gillespie & McGrath, 1973; 1974; Gibson & Gillespie, 1973; Creed, *et al.*, 1977; Gibson & Mirzazadeh, 1989; Gibson, *et al.*, 1989; 1990; 1992; Gillespie, *et al.*, 1989; 1990; Li & Rand, 1989; Liu, *et al.*, 1991; Martin & Gillespie, 1991; Martin, *et al.*, 1993), rectococcygeus (King, *et al.*, 1977; King & Muir, 1977; 1981), and certain blood vessels including portal vein (Hughes & Vane, 1967; Hughes & Vane, 1969; 1970), penile artery (Dorr &

Brody, 1967; Klinge & Sjostrand, 1974; Liu, *et al.*, 1991) and cerebral artery (Toda & Okamura, 1990a; 1990b) of various species.

The NANC nerves can be divided into an excitatory division and an inhibitory division. The existence of NANC excitatory nerves has been found in several smooth muscles. For example, the motor responses induced by stimulation of the sacral parasympathetic outflow via pelvic nerves to the mammalian urinary bladder are only partially blocked by atropine (Langley & Anderson, 1895; Henderson & Roepke, 1934; 1935; Ambache, 1955). This atropine-resistance is generally accepted to be due to the release of an excitatory transmitter rather than acetylcholine from the excitatory nerves (Henderson & Roepke, 1934; Ambache & Zar, 1970; Burnstock, *et al.*, 1972) despite other explanations such as the proximity theory (Dale & Gaddum, 1930; Hukovic, *et al.*, 1965; Carpenter, 1977). One proposal was that the nerve was purinergic (Burnstock, *et al.*, 1978), but the response to nerve stimulation remains unchanged after the tissue is desensitised to ATP (Ambache, *et al.*, 1977). Atropine-resistant contractions have also been recorded in the oesophagus (Lund & Christensen, 1969), the stomach (Semba & Mizonishi, 1978) and the small intestine (Ambache & Freeman, 1968; Day & Warren, 1968).

Purinergic nerves constitute one group of NANC inhibitory nerve system. Burnstock (1972) proposed the existence of purinergic nerves which release adenosine triphosphate (ATP) or a related nucleotide as a transmitter. He found that ATP and the necessary enzymes for its synthesis are present in certain nerves. ATP is detected upon stimulation of these nerves and this is blocked by tetrodotoxin, implying a neurogenic origin. The enzymatic break down of ATP to ADP to AMP to adenosine which is subsequently taken up by tissues may represent its inactivation mechanism. In many tissues drugs have the same effects on responses to ATP and to nerve stimulation. Also, binding of ATP by quinacrine shows its existence in varicose nerve fibres in histochemical studies. The inhibitory purinergic nerves have

been shown to innervate many tissues including the gastrointestinal tract, trachea, and portal vein (Burnstock, 1972; 1975; 1978).

1.2. Anococcygeus and Retractor Penis Muscles

The BRP and rat and mouse anococcygeus muscles are typical examples of smooth muscles receiving an NANC inhibitory innervation (Gillespie, 1972; Gibson & Gillespie, 1973; Gibson & Yu; 1983; Klinge & Sjostrand, 1974). Sertoli (1883) first studied the retractor penis muscle of several species both *in vitro* and *in vivo*. He found the muscle exhibits spontaneous activity. In a warm environment it relaxes and in the cold contracts. These findings were confirmed by De Zilwa (1901) in the dog retractor penis *in vitro*. However, except for local anaesthetics and tetrodotoxin, none of the drugs blocking known neurotransmitters had any effect on the inhibitory response to the NANC nerve stimulation and no known neurotransmitter could mimic the relaxant response to NANC nerve stimulation in the retractor penis and anococcygeus muscles (Luduena & Grigas, 1966; 1972; Gillespie, 1972; 1980; 1987; Gibson & Gillespie, 1973; Klinge & Sjostrand, 1974; Creed, *et al.*, 1977; Gibson & Wedmore, 1981). One aim of this project was to identify the NANC inhibitory neurotransmitter in the bovine retractor penis muscle.

1.2.1. Innervation

The retractor penis muscle receives a double innervation. The motor sympathetic nerves originate from the spinal cord in the lumbar region. Most fibres emanate from the sacral ganglia of the sympathetic chain and reach the muscle via the pubic nerve. Some fibres seem to run in the hypogastric nerve and a few fibres may follow the pelvic parasympathetic nerves. The inhibitory nerves leave the sacral cord with their fibres in the pelvic parasympathetic nerves (Langley & Anderson, 1895). Studies in the pig, cat, dog, elk, hedgehog, ox and rat have all revealed a

common innervation of motor and inhibitory nerves (Langley & Anderson, 1895; Fletcher, 1898; Gruber, 1933). The motor response is blocked by α-adrenoceptor blocking agents and adrenergic neurone blocking agents and is therefore clearly adrenergic (Luduena & Grigas, 1966; Klinge & Sjostrand, 1974; Byrne & Muir, 1984), although in pig and sheep it might not entirely be adrenergic due to incomplete blockade by these drugs (Ambache & Killick, 1978).

The anococcygeus muscle, which extends to form the retractor penis (Gillespie, 1972), was initially described by Langley & Anderson (1895). However, it was Gillespie (1972) who reported that the rat anococcygeus muscle appears to possess many of the desirable features of an ideal innervated smooth muscle preparation for the study of adrenergic and NANC inhibitory neurotransmission. Like the retractor penis muscle, the anococcygeus muscle of the cat, mouse, rabbit and rat receives a dense adrenergic motor innervation via the lumbar sympathetic nerves and a NANC inhibitory innervation via the sacral parasympathetic nerves whose transmitter is unknown (Gillespie, 1972; Gillespie & McGrath, 1973; 1974; Creed, *et al.*, 1977; Gibson & Wedmore, 1981). Unlike the retractor penis muscle, the anococcygeus muscle of the rat lacks spontaneous tone (Gillespie, 1972). It was worth considering the possibility that NANC relaxation in the retractor penis and anococcygeus involved electrical rather than chemical neurotransmission. Electrical transmission of activity from one cell to another does occur in the giant motor synapse of the crayfish (Furshpan & Potter, 1959), and combined chemical and electrical transmission has been reported in the chick ciliary ganglion (Martin & Pilar, 1963a; 1963b). Such electrical transmission can only take place, however, where a well-defined ultrastructural organisation between pre- and post-synaptic elements with low resistance connections is present (Bennett, 1966). The lack of such a well defined ultrastructure in the anococcygeus and retractor penis muscles does not support the involvement of electrical transmission in these tissues (Gillespie & Lullman-Rauch, 1974; Eranko, *et al.*, 1976; Gibbins & Haller, 1979).

Histochemically, adrenergic, cholinergic and peptidergic nerves can all be identified in the retractor penis and anococcygeus muscles. A dense fluorescent adrenergic nerve net is observed in the both tissues in all species studied (Bell & McLean, 1970; Gillespie, 1972; Gillespie & McGrath, 1974; Creed, *et al.*, 1977; Gibson & Wedmore, 1981). There is also evidence from acetylcholinesterase staining for a terminal cholinergic network in the retractor penis of the bull (Klinge, *et al.*, 1970), dog (Bell & McLean, 1970), and in the anococcygeus of the cat (Garrett, *et al.*, 1972). However, no specific cholinesterase activity is observed in the rat anococcygeus (Gillespie, 1972). Vasoactive intestinal polypeptide (VIP)-containing terminal autonomic fibres representing peptidergic nerves are found in the human penis (Polak, *et al.*, 1981) and in the anococcygeus muscle of the cat, rabbit and rat where they are resistant to 6-hydroxydopamine (Hunter, *et al.*, 1984). Purinergic nerve terminals can also be traced by quinacrine which binds to ATP in rat anococcygeus (Burnstock, *et al.*, 1978a). Fine beaded fluorescent, presumably purinergic, nerve fibres are found sparsely in the rat anococcygeus (Iijima, 1983).

Ultrastructural studies have been made with the expectation of providing convincing evidence for the existence and nature of the NANC nerves in the anococcygeus and retractor penis muscles. In an early electron microscopic study of the rat anococcygeus muscle Gillespie and Lullman-Rauch (1974) described two types of varicosities, one with agranular vesicles and another with granular vesicles, corresponding to cholinergic and adrenergic nerves respectively. After exposure to 5-hydroxydopamine the number of the granular vesicles was greatly increased. There was no evidence of the nerve profiles containing predominantly large opaque vesicles (LOV) or p-type vesicles previously described as characteristic of purinergic nerves (Burnstock & Iwayama, 1971). In a subsequent study by applying a fixation technique not using permanganate, which was reported to destroy the axon profiles of the NANC nerves in the ureter (Hoyes & Barber, 1976), Gibbins

and Haller (1979) were able to observe three types of nerve profile in this tissue. They found that of the varicose fibres seen, about 60% contain mainly small dense cored vesicles which disappear after 6-hydroxydopamine treatment and correspond to adrenergic nerves, 5% contain mainly small clear vesicles resistant to 6-hydroxydopamine and correspond to cholinergic nerves, and the remaining 40% contain large uniformly stained dense cored electron-opaque, chromaffin-negative vesicles of 85-110 nm in diameter and are smaller than the LOV of the gut (Burnstock & Iwayama, 1971). The third nerve profile was not destroyed by pretreatment with 6-hydroxydopamine and was therefore taken to be the NANC nerves in the tissue. These three types of nerve profile are also found in the bovine retractor penis muscle (Eranko, *et al.*, 1976). Unfortunately, in a later report Gibbins (1982) revised his findings in the rat anococcygeus and described a range of varicosities with different proportions of small clear vesicles and small p-type vesicles which could not be divided clearly into two classes corresponding to adrenergic and NANC nerves. The small clear vesicles were at that time taken to be the likely source of the NANC transmitter.

1.2.2. Response to NANC inhibitory Nerve Stimulation

Electrical stimulation of the retractor penis muscle of several species *in vivo* and *in vitro* produces contraction and also sometimes relaxation (Sertoli, 1883). De Zilwa (1901) reported that *in vitro* the dog retractor penis muscle stimulated electrically produces contraction when it is in low tone but relaxation in high tone. When the tone is raised in the retractor penis and anococcygeus muscles, electrical field stimulation induces a biphasic response in which the initial motor component declines at higher levels of tone, leaving a powerful relaxation (Gillespie, 1972; Klinge & Sjostrand, 1974). If the adrenergic nerves are blocked with guanethidine, only a pure inhibitory response is observed. This relaxation is rapid and powerful. It is sometimes followed by a rebound contraction on stopping stimulation. In both

tissues of all species studied, maximum NANC relaxation is induced at low frequencies of 4-8 Hz, whereas the motor response is maximal at frequencies of 30 Hz or more (Gillespie, 1972; Klinge & Sjostrand, 1974; Gillespie & McGrath, 1974; Creed , *et al.*, 1977; Gibson & Wedmore, 1981).

The NANC inhibitory response in the retractor penis and anococcygeus muscles is neurogenically mediated since it is blocked by low concentrations of tetrodotoxin. Further, this relaxation is also produced by extrinsic nerve stimulation (Langley & Anderson, 1895; Gillespie & McGrath, 1973). It is not an example of co-transmission by the motor adrenergic nerves because the inhibitory response is unchanged after the adrenergic nerves are partially destroyed by immunosympathectomy or completely destroyed by 6-hydroxydopamine (Gibson & Gillespie, 1973). This is further supported by the separate spinal origin of the motor and inhibitory fibres (Langley & Anderson, 1895; Gillespie, 1972; Gillespie & McGrath, 1973).

The effects of inhibitory nerve stimulation on membrane potential, membrane conductance and accompanying mechanical relaxation have been studied in the anococcygeus of the rabbit and rat, and in the retractor penis of the bull (Creed, 1975; Creed, *et al.*, 1975; Creed & Gillespie, 1977; Byrne & Muir, 1984). In the rat anococcygeus there is little change in membrane potential upon NANC nerve stimulation, at most a few millivolts of hyperpolarisation are seen associated with a small rise in membrane conductance (Creed, *et al.*, 1975). Single pulse stimulation does not induce relaxation or change the membrane potential. There is no clear correlation between membrane hyperpolarisation and mechanical relaxation. In the rabbit anococcygeus muscle single pulse stimulation induces large hyperpolarisations of up to 30 mV and rises in membrane conductance, although they are not always associated with mechanical relaxation (Creed & Gillespie, 1977). The BRP resembles the rat anococcygeus in that field stimulation induces only small

hyperpolarisations in association with relaxation (Byrne & Muir, 1984). It appears that only in certain tissues does NANC nerve stimulation increase membrane permeability to ions, possibly potassium. Such finding suggest that hyperpolarisation is the main mechanism responsible for the mechanical relaxation (Creed & Gillespie, 1977; Byrne & Muir, 1984; Bolton & Large, 1986). In the rat anococcygeus muscle the absence of hyperpolarisation suggests that NANC relaxation must be the consequence of biochemical rather than electrical coupling.

1.2.3. Early Search for the NANC inhibitory Neurotransmitter

Generally the criteria for a substance to be established as a neurotransmitter are (Eccles, 1964): 1). It is synthesised and present in the nerve fibres; 2). It is released by nerve stimulation; 3). It should mimic the nerve response to electrical stimulation; 4). It combines with its receptor specifically; 5). Its mode of action and that of neurotransmitter of nerve should be the same; 6). Drugs which potentiate or block the nerve response should have the same effect on the putative neurotransmitter; 7). An inactivation mechanism should be present consisting either of enzymatic breakdown and / or uptake.

Drugs which selectively block a nerve response can often reveal information concerning the nature of the neurotransmitter involved. After tone of the BRP and the rat anococcygeus muscles is raised by guanethidine, 5-HT or furmethide, field stimulation of the muscles uncovers the inhibitory response. This response is not inhibited by any drugs which block known transmitters such as hexamethonium, guanethidine, phentolamine, phenoxybenzamine, propranolol, atropine, d-tubocurarine, diethylamide, mepyramine, and burimamide (Luduena & Grigas, 1966; 1972; Gillespie, 1972; Klinge & Sjostrand, 1974). Ethanol is one of the few chemicals found to inhibit selectively the relaxant response to NANC nerve stimulation in the rat and rabbit anococcygeus and BRP (Gillespie, *et al.*, 1982).

The NANC response is also not mimicked by acetylcholine, isoprenaline, GABA, glycine, glutamine, glutamate, glutamic acid, aspartic acid, asparagine, alanine, valine, tyrosine, N-acetyltyrosine, tryptophan, cysteine, methionine, histidine, proline, hydroxyproline, lysine, histamine, 5-HT , dopamine, prostaglandins and a variety of peptides including VIP, leucine- and methionine-enkephalin, neurotensin, and somatostatin (Gillespie, 1972; Gillespie & McGrath 1974; Klinge & Sjostrand, 1974; Creed, *et al.*, 1977; Sjostrand, *et al.*, 1981; Bowman & Gillespie, 1983). Although bradykinin causes relaxation at low concentrations in the anococcygeus of the cat, rabbit and rat (Gillespie & McKnight, 1978), it is also excluded since it induces contraction in the bovine retractor penis at low tone (Klinge & Sjostrand, 1974). Drugs such as haemoglobin and ethanol which are known to block the response to NANC inhibitory nerve stimulation in the anococcygeus and retractor penis do not block the response to bradykinin (Gillespie, *et al.*, 1981a; Bowman, *et al.*, 1982a). The rat anococcygeus is a particularly valuable tissue in evaluating any possible candidates as the NANC neurotransmitter since few substances can cause relaxation in this tissue.

Despite the finding that ATP causes contraction in the anococcygeus of the rat, and the retractor penis of the bull and dog (Luduena & Grigas, 1966; Klinge & Sjostrand, 1974), it was suggested to be the NANC inhibitory transmitter in the anococcygeus and BRP (Burnstock, *et al.*, 1978a). Burnstock *et al.* (1978) argued that the relaxant action of ATP is masked by its ability to produce contractile prostaglandins. However, indomethacin, a cyclo-oxygenase inhibitor, does not block the contractile response to ATP in the rat anococcygeus (Carpenter & Joshi, 1979) and contraction is only seen at high concentrations with no relaxation at low concentrations (Gillespie, 1972). Moreover, the relaxant response to NANC inhibitory nerve stimulation in the retractor penis of the bull and dog is unaltered after the tissue has developed tachyphylaxis to ATP following repeated exposure (Klinge & Sjostrand, 1974; Bowman & Gillespie, 1983). Furthermore, apamin, a

polypeptide from bee venom, blocks the relaxant responses to exogenous ATP and inhibitory purinergic nerve stimulation in gastrointestinal smooth muscle (Banks, *et al.*, 1979; Sjoqvist, *et al.*, 1980), but is without effect on NANC inhibitory relaxation in the rat anococcygeus and BRP (Bowman & Gillespie, 1982). In contrast, ethanol, at concentrations inhibiting the relaxant response to NANC inhibitory nerve stimulation in the rabbit anococcygeus, actually potentiates ATP-induced relaxation (Gillespie, *et al.*, 1982). Haemoglobin uniformly blocks the NANC inhibitory nerve response in the anococcygeus of the mouse, rat and rabbit and the retractor penis muscles of the bull, but is completely ineffective in blocking the responses induced by ATP in the anococcygeus (Bowman & Gillespie, 1981; 1982; 1983; Bowman, *et al.*, 1982b; Gibson & Tucker, 1982; Gibson & Yu, 1983). Also, it doesn't block the relaxation to inhibitory purinergic nerve stimulation in the taenia coli of the guinea-pig (Bowman & Gillespie, 1983). In addition, an ultrastructural study has shown that purinergic fibres are sparse in the rat anococcygeus and thus such an innervation is unlikely to account for the powerful and complete relaxation induced by the NANC nerves (Iijima, 1983). Clearly, these results convincingly reject ATP as the inhibitory neurotransmitter in the anococcygeus and retractor penis muscles.

Over the years, three potential candidates emerged for the NANC transmitter in the anococcygeus and the retractor penis muscles; the inhibitory factor (IF) extracted from the BRP, endothelium-derived relaxing factor (EDRF), and nitric oxide (NO) or NO-releasing compounds such as S-nitrosothiols, since they all cause relaxation of the muscles, act through stimulation of soluble guanylate cyclase, increase cGMP levels, and are blocked by haemoglobin.

1.3. Inhibitory Factor

One of the fundamental criteria to establish a substance as a neurotransmitter is to demonstrate its release upon nerve stimulation (Eccles, 1964). In this laboratory several attempts were made to try to detect the release of the NANC inhibitory transmitter in the rat anococcygeus and the BRP by bioassay. Although there was no difficulty in detecting EDRF released from the rabbit aorta using the same biodetection system (Gillespie & Sheng, 1988), all attempts to detect the NANC transmitter were without success. Therefore, efforts were made to try to extract the transmitter for subsequent identification. An early attempt failed to extract the transmitter from the rat anococcygeus with water or phosphate buffer (Gillespie, unpublished results). Later, Ambache and his colleagues (1975) were able to extract a substance from the BRP with dilute hydrochloric acid. This extract mimicked the relaxant response to NANC inhibitory nerve stimulation and its action was unaffected by drugs which block the effects of established transmitters. The physical, chemical and pharmacological properties of this activity termed inhibitory factor (IF) were further studied in an attempt to identify its chemical nature and to establish if it was indeed the transmitter (Gillespie & Martin, 1978; 1980; Bowman & Gillespie, 1981; 1982; Bowman, *et al.*, 1981; Gillespie, *et al.*, 1981; Crossley & Gillespie, 1983; Bowman, *et al.*, 1986; Martin, *et al.*, 1988).

1.3.1. Acid Activation

IF extracted with neutral buffer or methanol is biologically inactive (Gillespie & Martin, 1980), but its relaxant activity is generated by exposure to an acid pH. Some activity was generated at pH 4.0 and maximum activity was generated at pH 2.0. This occurred rapidly and within a few minutes but a standard time of 10 minutes was adopted before neutralisation and assay. Neutralisation of the acid-

activated IF does not lead to loss of its activity. This acid-activation explains Ambache's success in the original extraction of IF.

1.3.2. Solubility

IF does not partition into ether implying it is not a prostaglandin (Ambache, *et al.*, 1975). It is hydrophilic, soluble in water, methanol, less soluble in ethanol and insoluble in acetone, ether, chloroform (Gillespie, *et al.*, 1981b). Pure methanol can destroy the activity of IF but it is stable in solutions of 70% methanol or less.

1.3.3. Thermolability

The unactivated form of IF is stable but the acid-activated form loses its activity at room temperature in 24 hours (Gillespie & Martin, 1980; Gillespie, *et al.*, 1981b). This loss can be fully reversed by acidification for a second time, although only partial reversal is obtained if activity is lost by placing in a boiling water bath for 2 minutes. The acid-activated form of IF is stable on ice for several hours and this is the preferred means of storing the material during experiments. The thermolability of the acid-activated form of IF is observed in solution and is a result of a temperature-sensitive interaction with water. While lyophilised and sealed in ampoules, IF remains active at least for periods of up to three years.

1.3.4. Molecular Weight

The molecular weight of the unactivated and acid-activated forms of IF was assessed by their ability to pass through a series of ultrafiltration membranes (Gillespie, *et al.*, 1981b). Both forms of IF were found in the filtrate passing through membranes retaining molecules of 500 Daltons and no concentration was observed in the retentate. These findings were interpreted as suggesting the molecular weight of

both forms of IF was below this value. There was no evidence that the process of acid activation occurred with a change in molecular weight.

1.3.5. Electrical Charge

As IF was soluble in polar but not in non-polar solvents, it was likely to possess an electrical charge. This was examined by its retention and elution profile on ion-exchange resins (Gillespie, *et al.*, 1981b). IF, both in the inactive and acid-activated forms, was completely retained on an anion exchange column (Bio Rad AG1-X8, formate form) and was fully recovered upon elution with sodium chloride (500 mM). However, a cation exchange column (Bio Rad AG50-W, sodium form) was unable to retain IF in both the inactive and acid-activated forms. Therefore, IF in both the inactive and acid-activated forms is negatively charged and no evidence was obtained to suggest that the process of acid-activation occurred with a change in electrical charge.

1.3.6. Distribution

One of the conventional criteria necessary to establish a substance to be a neurotransmitter is to obtain evidence of its storage in nerve terminals. The possibility that IF, like established neurotransmitters, was held in storage vesicles was examined (Gillespie & Hunter 1982). If IF is the NANC transmitter, it should be present in storage vesicles and selectively concentrated in the high speed P_3 pellet after ultracentrifugation. BRP muscle was homogenised and separated into the conventional P_1, P_2, P_3 and S_3 fractions. The results showed activity in all fractions but with a seven-fold higher concentration in IF in P_3, suggesting storage in association with some form of sub-cellular particle.

If IF was the transmitter, it would be expected to be extracted only from tissues receiving NANC inhibitory innervation. IF has been extracted from the NANC innervated smooth muscles such as the BRP muscle and the rat anococcygeus muscle. It was also, however, extracted from the poorly innervated rat uterus and the uninnervated human umbilical artery. It was also extracted from the cat ventricle, skeletal muscle and liver, the tendon of the bovine hoof and the vitreous humour of the bovine eye (Gillespie & Martin, 1980; Gillespie, 1987). This widespread distribution of IF argues against it being the NANC inhibitory neurotransmitter. However, other established transmitters are present in non-neural tissue. For example, acetylcholine is present in the uninnervated placenta (Chang & Gaddum, 1933) and 5-hydroxytryptamine is found in blood platelets (Garattini & Valzelli, 1965).

1.3.7. Chemical Composition

An attempt was made to classify IF into one of the general classes of biological materials, i.e. protein, lipid or carbohydrate (Gillespie, *et al.*, 1981b). It was considered unlikely that IF was a protein or peptide since incubation with a range of proteases and peptidases such as the endopeptidases, pepsin, trypsin, and subtilisin and the exopeptidases, aminopeptidase or pyroglutamate aminopeptidase, did not lead to any loss of its inhibitory activity. IF was inactivated by carboxypeptidase but this was not an enzymatic action since it was time-independent and persisted after boiling the enzyme. Further evidence against it being a protein or peptide was obtained upon finding that the relaxant responses to IF and inhibitory nerve stimulation were not potentiated by the broad-spectrum protease inhibitor, Aprotinin, or the specific bradykinin/angiotensin converting enzyme inhibitor, SQ20881. The possibility that IF contained a carbohydrate group was suggested by the indirect evidence that the oxidant, periodic acid, and the reductant, sodium borohydride, destroyed IF (Gillespie, *et al.*, 1981b). The possibility existed,

however, that inactivation by these agents was simply due to more generalised reduction or oxidation reactions. The insolubility of IF in acetone or ether suggested that it was unlikely to be a lipid such as a prostaglandin.

Furchgott (1988) proposed that both EDRF and IF are nitric oxide and that the inactive form of IF is inorganic nitrite. He showed that the weak vasodilator activity of sodium nitrite is greatly enhanced by acidification, which is due to generation of nitric oxide from acidified nitrite according to the following chemical reactions (Sisler, 1956):

$$NO_2^- + H^+ \rightarrow HNO_2 \qquad (1)$$

$$2HNO_2 \rightarrow NO + NO_2 + H_2O \qquad (2)$$

$$2NO + O_2 \rightarrow 2NO_2 \qquad (3)$$

$$2NO_2 \leftrightarrow N_2O_4 \qquad (4)$$

$$2NO_2 + H_2O \leftrightarrow HNO_2 + HNO_3 \qquad (5)$$

This proposal was further supported by the evidence that the rank order of effectiveness of IF, EDRF and nitric oxide in relaxing the rabbit trachea and aorta, and the rat anococcygeus and BRP is similar (Gillespie & Sheng, 1988). Moreover, Martin and his colleagues (1988) reported that solutions of unactivated IF contain 4 μM nitrite. The activation of IF by exposure to acid at pH 2 for 10 minutes and subsequent neutralisation is in association with a 75% loss of nitrite, according to the above equation (2). Furthermore, the inactivation of the acid-activated IF by boiling was accompanied by an increase of nitrite content and the reactivation of this IF is linked with a further loss of nitrite content. However, an explanation by the above reactions is not completely consistent with certain properties of IF. For example, the relaxant activity of solutions of acid-activated and neutralised IF in open containers is stable for hours if kept on ice (Gillespie & Martin, 1980; Gillespie, *et al.*, 1981b), whereas that of acidified nitrite in open containers is

immediately lost upon neutralisation (Furchgott, 1988). Further differences are that nitric oxide is neutral but IF is negatively charged, and IF can be freeze dried and stored in the solid state (Gillespie & Martin, 1980; Gillespie, *et al.*, 1981b), whereas nitric oxide gas clearly can not. Martin and his colleagues (1988) believed that these apparent differences between IF and nitric oxide can be explained if IF extracted from the BRP contains an additional anionic substance which reversibly binds and stabilises nitric oxide. They suggested that unactivated IF extracted from the BRP is nitrite, whose acid-activated derivative is stabilised nitric oxide.

The proposed nitric oxide-stabilising substance in IF has been suggested to be a thiol such as L-cysteine and L-glutathione (Yui, *et al.*, 1989; Kerr, *et al.*, 1992). This was based on the finding that IF contains 8 μM nitrite and the loss of nitrite following acidification of IF is closely related to the content of sulfhydryl groups in the extract. Yui *et al.* (1989) proposed that the relaxant activity generated from IF by acidification was due to formation of S-nitrosothiols, although they neither monitored the formation of S-nitrosothiols nor measured relaxant activity. Kerr *et al.* (1992) confirmed that IF contains 1-10 μM nitrite and 40-200 μM total thiol. They found that S-nitrosothiols, but not nitric oxide from acidified nitrite, could reproduce the properties of IF including its smooth muscle relaxant activity, stability to argon purging and ability to stimulate cGMP formation by platelet-soluble guanylate cyclase. Other substances such as proteins, thiols and sugars have also been shown to reversibly bind and stabilise nitric oxide (Braughler, *et al.*, 1979).

1.3.8 Pharmacology

IF mimics the effects of NANC nerve stimulation in the BRP, the rat anococcygeus and the penile artery, not only in the degree of relaxation but also in the time course of the response (Bowman & Gillespie, 1983; Crossley & Gillespie, 1983). The

mode of action of IF and nerve stimulation is identical in these tissues. For example, both IF and nerve stimulation produce membrane hyperpolarisation in the BRP (Byrne & Muir, 1985). They cause relaxation, however, by raising cyclic GMP levels (Bowman & Drummond, 1984) and both effects are blocked by haemoglobin (Bowman & Gillespie, 1981; 1982; Bowman, *et al.*, 1982b), N-methylhydroxylamine (Bowman & Drummond, 1984) and methylene blue (Bowman, *et al.*, 1986) and potentiated by a selective inhibitor of cyclic GMP phosphodiesterase, M & B 22948 (Bowman & Drummond, 1984). Ethanol also selectively inhibits the relaxation of the rat anococcygeus and BRP to both NANC nerve stimulation and to IF without effect affecting the relaxant response to isoprenaline, sodium nitroprusside or the phosphodiesterase inhibitor, isobutylxanthine. The mechanism of action of ethanol might be to reduce calcium binding to cell membranes or to reduce the efficiency of receptor coupling (Gillespie, *et al.*, 1982).

Thus IF fulfils many of the requirements of the NANC inhibitory neurotransmitter in the BRP and rat anococcygeus, but the need for acid activation and its widespread presence in tissues lacking the appropriate NANC innervation are inconsistent with such a role (Gillespie & Martin, 1980).

1.4. Endothelium-Derived Relaxing Factor

1.4.1. Discovery

Endothelium-derived relaxing factor (EDRF) was first discovered by Furchgott and Zawadzki (1980) in experiments with isolated preparations of arteries. In such preparations the relaxant effect of acetylcholine was found to be strictly dependent on the presence of endothelial cells on the intimal surface of the arteries. This discovery resolved the paradox that acetylcholine though a powerful vasodilator *in*

vivo often produces no relaxation or even contraction on isolated preparations of arteries *in vitro*. The explanation was that established procedures for preparing arteries for tension recording usually led to the endothelium being rubbed off.

1.4.2. Detection of EDRF Release

EDRF released from donor tissues e.g. a rabbit aortic strip or ring with intact endothelium (Furchgott, 1984) or bovine cultured aortic endothelial cells (Cocks, *et al.*, 1985) has been detected by an assay tissue e.g. a rabbit endothelium-denuded aortic strip in a "sandwich" bioassay (Furchgott, 1984), and a rabbit endothelium-denuded coronary artery perfusion cascade (Griffith, *et al.*, 1984). These experiments demonstrated the humoral nature of EDRF.

1.4.3. Stability

It was only possible to analyse the stability of EDRF following its successful detection using a cascade bioassay system. By the use of this technique, it was established that EDRF is a very short-lived substance with a half-life of only about 3 to 50 seconds in oxygenated physiological salt solutions (Griffith, *et al.*, 1984; Forstermann, *et al.*, 1984; Cocks, *et al.*, 1985).

1.4.4. Endothelium-Dependent Relaxation

EDRF is a powerful vascular smooth muscle relaxant. It can be released from vascular endothelial cells of arteries, veins and microvessels following stimulation with acetylcholine, bradykinin, substance P and some related peptides, ATP and ADP, histamine, serotonin, thrombin, arachidonic acid and the calcium ionophore A23187 (Furchgott & Zawadzki, 1980; Furchgott, 1981; 1984; Altura & Chand, 1981; Chand & Altura, 1981), although differences exist in the responsiveness of

different species and blood vessels. Some other stimuli such as hypoxia, increased flow and electrical stimulation also cause EDRF-induced relaxation *in vitro* (Moncada, *et al.*, 1991). These initial observations led to the recognition that vasodilators could be classified into two groups, those that act via the endothelium and those such as nitrovasodilators and calcium channel blockers that do not require endothelial cells to produce their effect (Furchgott, 1984). Cross-tolerance occurs among endothelium-dependent relaxants but this tolerance does not affect endothelium-independent relaxants (Gold, *et al.*, 1989). After removal of the endothelial cells, the vasoconstrictive actions of noradrenaline and serotonin are significantly more powerful (Cocks & Angus, 1983), indicating that under normal circumstances contraction is depressed due to release of EDRF by these agonists. In addition to relaxing smooth muscle, EDRF also inhibits platelet aggregation and adhesion to vascular endothelium (Azuma, *et al.*, 1986; Radomski, *et al.*, 1987a; 1987b; 1987c).

The action of EDRF, like that of the NANC transmitter, is mediated through stimulation of soluble guanylate cyclase and consequent elevation of cyclic GMP levels within smooth muscle (Diamond & Chu 1983; Furchgott & Jothianandon 1983; Rapoport & Murad 1983a; 1983b).

The selective inhibitor of cyclic GMP phosphodiesterase M & B 22948 potentiates effects of EDRF (Martin, *et al.*, 1986), and superoxide dismutase can prolong relaxation of a bioassay tissue induced by EDRF released from a donor tissue (Gryglewski, *et al.*, 1986; Rubanyi & Vanhoutte, 1986). Cytochrome *C* and superoxide dismutase potentiate the activity of EDRF by scavenging superoxide anions (Moncada, *et al.*, 1986).

Drugs and experimental conditions which inhibit endothelium-dependent relaxation include anoxia (De Mey & Vanhoutte, 1983), methylene blue which inhibits soluble

guanylate cyclase, haemoglobin which efficiently binds EDRF (Martin, *et al.*, 1985), quinacrine which inhibits phospholipase A_2 (Furchgott, *et al.*, 1982; 1983; Singer & Peach, 1983), ETYA (5,8,11,14-eicosatetraynoic acid) which inhibits lipoxygenase and cyclooxygenase (Furchgott & Zawadzki, 1980), BPB (p-bromophenacylbromide) and NDGA (nordihydroguiaretic acid) which inhibit lipoxygenase and act as antioxidants (Furchgott, *et al.*, 1982; 1983), Fe^{2+} (Gryglewski, *et al.*, 1986), hyperoxia (Rubanyi & Vanhoutte, 1986), and a number of antioxidants including borohydride, hydroquinone and dithiothreitol (Griffiths, *et al.*, 1984;).

1.4.5. Identification as Nitric Oxide

Chemical identification of EDRF was difficult and some early research suggested that EDRF might be a product of arachidonic acid metabolism via lipoxygenase (Singer & Peach, 1983a; Forstermann & Neufang, 1984) or the cytochrome P-450 enzyme system (Pinto, *et al.*, 1985; MacDonald, *et al.*, 1986), or alternatively, a compound with a carbonyl group near its active centre (Griffith, *et al.*, 1984). Based on the similar pharmacological properties of EDRF and nitric oxide, Furchgott in 1986 suggested that EDRF might be nitric oxide (Furchgott, 1988). At the same meeting, Ignarro *et al.* (1988) also speculated that EDRF might be nitric oxide or a related substance.

Nitric oxide can be directly measured as a chemiluminescent product of its reaction with ozone (Downes, *et al.*, 1976). Using this technique, nitric oxide was detected in the fluid perfusing cultured porcine aortic endothelial cells following stimulation by bradykinin at concentrations that induce EDRF release (Palmer, *et al.*, 1987). Furthermore, the amounts of nitric oxide released by the cells were sufficient to account for relaxation of vascular strips.

The identification of EDRF as nitric oxide was further supported by their indistinguishable biological actions on vascular (Palmer, *et al.*, 1987; Gillespie & Sheng, 1988) and non-vascular smooth muscles (Gillespie & Sheng, 1988), and on platelets (Radomski, *et al.*, 1987b). They decay at the same rate during passage down a bioassay cascade (Palmer, *et al.*, 1987), their actions on vascular strips and on platelets are similarly potentiated by superoxide dismutase (SOD) and cytochrome *C* and inhibited by Fe^{2+} and some redox compounds including pyrogallol and hydroquinone which generate superoxide anion (Palmer, *et al.*, 1987; Hutchinson, *et al.*, 1987; Radomski, *et al.*, 1987b). The actions of these redox compounds as inhibitors of EDRF-induced and nitric oxide-induced vascular relaxation are attenuated by SOD to a similar extent.

1.4.6. Controversy of Nitric Oxide as EDRF

Although very strong evidence supports nitric oxide as EDRF, other studies have shown differences between nitric oxide and EDRF. The half-life of EDRF is variable from 3 to 50 seconds (Griffith, *et al.*, 1984; Forstermann, *et al.*, 1984; Rubanyi, *et al.*, 1985; Cocks, *et al.*, 1985; Gryglewski, *et al.*, 1986). However, this variation might be explained, as in the case of nitric oxide, in terms of differences in oxygen tension and superoxide anion concentrations in the experimental conditions in different laboratories. Early reports that EDRF generated from endothelium only relaxed vascular smooth muscle whereas exogenous nitric oxide relaxed vascular, tracheal and taenia coli smooth muscles might be due to the low concentrations of EDRF generated (Shikano & Berkowitz, 1987; Dusting, *et al.*, 1988a), since tracheal and taenia coli smooth muscle is less sensitive to nitric oxide and EDRF than vascular smooth muscle (Dusting, *et al.*, 1988b; Gillespie & Sheng, 1988). Moreover, EDRF and nitric oxide have been clearly shown to produce relaxation in vascular and non-vascular smooth muscle preparations in a bioassay system (Gillespie & Sheng, 1988; Angus & Cocks, 1989; Buga, *et al.*,

1989; Furchgott, *et al.*, 1990). Activity of EDRF was lost following application to an anion exchange column (Cocks, *et al.*, 1985; Long, *et al.*, 1987), whereas that of nitric oxide was not (Long, *et al.*, 1987) or was lost to a less extent (Khan & Furchgott, 1987). In these experiments the loss could have been non-specific since no group was ever able to subsequently elute EDRF from a column. The stabilisation of EDRF but not nitric oxide by acidification (Murray, *et al.*, 1986) might be explained by EDRF being a nitric oxide-releasing molecule rather than nitric oxide itself. Recently, Myers *et al.* (1990) suggested that EDRF is much more likely to be a nitrosylated compound, such as a nitrosothiol i.e. S-nitrosocysteine, than authentic nitric oxide. Indeed, a comparison of properties of nitric oxide, S-nitrosothiols and the NANC transmitter was a major objective of my studies.

1.5. Nitric Oxide

1.5.1. Physical and Chemical Properties and Metabolism

Nitric oxide is a colourless neutral and lipophilic gas and thus readily permeates through biological membrane. Its taste and odour are not known. Its melting and boiling points are -163.6^{o}C and -151.8^{o}C, respectively (Hammond, 1966), so that it is difficult to liquefy. It is only very slightly soluble in water, 7.3 ml at 0^{o}C and 2.4 ml at 60^{o}C in 100 ml of water. It contains an odd number of electrons and is therefore paramagnetic and a free radical, but behaves very different from other molecules having an odd number of electrons. In particular it has a slight tendency to dimerize in the gaseous state or in solution and has relatively low chemical reactivity when compared to other free radicals (Cotton & Wilkinson, 1972). Its reactivity with other substances is very high since it is a free radical. It is thermodynamically unstable at 25^{o}C and 1 atm. At high pressures it readily decomposes:

$$3NO \rightarrow N_2O + NO_2$$

Because nitric oxide is both a Lewis acid and Lewis base, it can undergo numerous reactions even under biological conditions. Since nitric oxide, in an intermediate oxidation state, can donate and receive electrons, it behaves both as an oxidant and a reductant. Some of the important chemical reactions involving nitric oxide are discussed below (Committee on Medical and Biological Effects of Environmental Pollutants, 1977; Moody, 1991):

1.5.1.1. Reactivity with oxygen

The overall reaction of nitric oxide and oxygen is (Committee on Medical and Biological Effects of Environmental Pollutants, 1977; Olbergts, 1985):

$$2NO + O_2 \rightarrow 2NO_2$$

with the intermediate steps:

$$NO + O_2 \leftrightarrow O\text{-}O\text{-}N\text{-}O$$
$$O\text{-}O\text{-}N\text{-}O + NO \rightarrow 2NO_2$$

although no direct evidence supports the existence of the hypothetical transient species, O-O-N-O. The rate of nitrogen dioxide formation in the above reaction is given by the following third-order rate equation:

$$d[NO_2] = 2k[NO]^2[O_2]dt$$

i.e. the reaction is second order with respect to nitric oxide and first order with respect to oxygen. The reaction can take place in the gas phase or the liquid phase,

but the rate is much higher by a factor of about 4,000 in aqueous solution (Pogrebnaya, *et al.*, 1975). The dependence on the square of the nitric oxide concentration results in a marked change in the rate of the reaction with changes in the nitric oxide concentration. Thus, at low concentrations of nitric oxide the reaction is slow, but at higher concentrations it is much more rapid.

Nitrogen dioxide, which exists in equilibration with its dimeric form, dinitrogen tetroxide (N_2O_4), dissolves in water to form a mixture of nitric acid and nitrous acid, of which the latter undergoes further decomposition, eventually forming nitric acid (Cotton & Wilkinson, 1972; Moody, 1991):

$$2NO_2 + H_2O \rightarrow HNO_3 + HNO_2$$
$$3HNO_2 \rightarrow HNO_3 + 2NO + H_2O$$

However, nitrogen dioxide dissolves in alkaline solutions to yield a mixture of nitrite and nitrate (Keilin & Hartree, 1937; Kelm, *et al.*, 1988):

$$2NO_2 + 2OH^- \rightarrow NO_2^- + NO_3^- + H_2O$$

The inactivation of nitric oxide in oxygenated physiological salt solutions can be accounted for by spontaneous oxidation of nitric oxide to nitrite. In these salt solutions at least 90% of nitric oxide is converted to nitrite with little or no formation of nitrate (Feelisch, *et al.*, 1987; Kelm, *et al.*, 1988).

1.5.1.2. Reactivity with superoxide anion (O_2^-)

Nitric oxide reacts with superoxide anion to form the unstable intermediate peroxonitrite anion (^-OONO), which rearranges to yield nitrate (NO_3^-, Blough & Zafiriou, 1985):

$$NO + O_2^- \rightarrow {}^-OONO \rightarrow NO_3^-$$

This reaction represents one of only a few known examples of a radical-radical coupling of superoxide anion with another odd-electron species to generate a diamagnetic product.

Superoxide anion can rapidly and nearly completely destroy nitric oxide (Gryglewski, *et al.*, 1986; Moncada, *et al.*, 1986; Rubanyi & Vanhoutte, 1986; Ignarro, *et al.*, 1988) and superoxide dismutase protects nitric oxide by destroying superoxide anion (Gryglewski, *et al.*, 1986; Rubanyi & Vanhoutte, 1986; Ignarro, *et al.*, 1988).

1.5.1.3. Reactivity with ozone (O_3)

Nitric oxide reacts rapidly with ozone to yield an activated or high-energy state nitrogen dioxide. The excited nitrogen dioxide is of sufficient energy to dissociate to ground state nitrogen dioxide in association with the generation of light which can be detected by chemiluminescence (Fontijn, *et al.*, 1970; Zafiriou & McFarland, 1980):

$$NO + O_3 \rightarrow NO_2 \text{ (excited)} + O_2$$

$$NO_2 \text{ (excited)} \rightarrow NO_2 + h\nu$$

These principles have been applied to the measurement of nitric oxide in biological systems (Palmer, *et al.*, 1987)

1.5.1.4. Reactivity with oxyhaemoglobin

Nitric oxide reacts with oxyhaemoglobin to yield methaemoglobin and inorganic nitrate in association with the characteristic spectral changes from oxyhaemoglobin to methaemoglobin. This is the principle supporting a spectrophotometric measurement of nitric oxide using oxyhaemoglobin (Kelm, *et al.*, 1988). The overall reaction is:

$$HbO_2 + NO \rightarrow metHb + NO_3^-$$

The intermediate reactions are:

$$Hb^{3+}O_2^- + NO \rightarrow metHb + {}^-OONO$$

and

$${}^-OONO \rightarrow NO_3^-$$

Oxygen binds as superoxide anion to the haeme iron atom of haemoglobin (Wallace, *et al.*, 1974), and this species reacts rapidly with nitric oxide to yield the peroxonitrite anion, which rapidly isomerizes to nitrate (Blough & Zafiriou, 1985).

1.5.1.5. Reactivity with haeme iron

Nitric oxide reacts with haeme iron (Fe^{2+}) to yield nitrosyl-haeme adducts (Keilin & Hartree, 1937; Kon, 1968; Gibson & Roughton, 1957). Reduced iron (Fe^{2+}), complexed with protoporphyrin IX to form haeme, has a high binding affinity for and reactivity with nitric oxide and this is also true for haemoproteins. The biding affinity of haemoproteins is about 1,500 times higher for nitric oxide than for

carbon monoxide (Gibson & Roughton, 1957) and about 200 times higher for carbon monoxide than for oxygen (Giardina & Amiconi, 1981). Therefore, haemoproteins such as haemoglobin, myoglobin, and soluble guanylate cyclase react readily with nitric oxide to form the corresponding paramagnetic nitrosyl-haeme (NO-haeme) adduct. This explains why haemoglobin and myoglobin can block actions of nitric oxide. This reaction can take place *in vivo* and leads to the formation of relatively large amounts of nitrosyl haemoproteins in animals treated with nitric oxide or carcinogen (Woolin & Commoner, 1970; Oda, *et al.*, 1975; Case, *et al.*, 1979). Solutions of reduced haemoproteins, oxidised haemoproteins, and nitrosyl-haemoproteins are dark red, brown, and bright pink-red, respectively. The spectral characteristic changes from reduced or oxidised haemoproteins to nitrosyl haemoproteins have been applied in the spectrophotometric measurement of nitric oxide (Ignarro, *et al.*, 1987b). The pink-red colour of nitrosyl haemoproteins has been applied in the meat industry where the colour of freshly cut meat has been preserved by adding sodium nitrite and ascorbic acid to react with myoglobin to yield pink-red nitrosyl myoglobin. In addition, nitric oxide, like carbon monoxide, can form complexes with transition metals unassociated with haeme, such as in ferricyanide, and with non-haeme-iron-containing proteins such as lipoxygenase (Galpin, *et al.*, 1978).

1.5.1.6. Reactivity with thiols

Nitric oxide reacts with thiols (R-SH) to form S-nitrosothiols (R-SNO). Details of these reactions will be discussed in a separate section.

1.5.1.7. Some other reactions

Nitric oxide is also involved in numerous *N*-nitrosating reactions even in mammalian cells (Marletta, 1988). One of the well-known examples is the

formation of *N*-nitrosomorpholine from morpholine by cytotoxic murine macrophages activated by lipopolysaccharide and interferon γ, in which nitric oxide, nitrite and nitrate are the reaction products. Since some *N*-nitrosamines are well-known carcinogenic agents, *N*-nitrosating reactions have been extensively studied (for reviews, Ridd, 1961; Mirvish, 1975; Challis, 1981). Moreover, nitric oxide reacts with sulfanilic acid at acid pH to yield a diazo product which can be further coupled with *N*-(1-naphthyl)ethylenediamide to form an intense chromophore. This is the well-known diazo or Griess reaction for the measurement of nitric oxide and inorganic nitrite spectrometrically (Ignarro, *et al.*, 1987).

1.5.1.8. Half-life

The fate of nitric oxide depends mainly on its diffusion and reactions with other substances. The half-life of nitric oxide was reported to be 3 to 5 seconds at concentrations of 10-50 nM, but about 30 seconds or more at concentrations in excess of 300 nM (Ignarro, 1990). Under experimental conditions, the biological half-life of endothelium-derived nitric oxide is roughly about 3 to 5 seconds, which is identical to that of authentic nitric oxide at concentrations of 10 to 50 nM (Palmer, *et al.*, 1987; Ignarro, *et al.*, 1987). However, it has been observed that cultured endothelial cells, bovine pulmonary artery and human umbilical vein can produce large amounts of nitric oxide with a half-life in excess of 30 seconds (Ignarro, 1990).

Other factors could affect the biological half-life of nitric oxide. The haemoproteins could act as a sink for nitric oxide and rapidly bind it because of their extremely highly binding potency (Gibson & Roughton, 1957). Cellular oxidative systems could also facilitate the inactivation of nitric oxide. On the other hand, nitric oxide could be protected by nitric oxide-stabilising substances such as L-cysteine. It is

difficult to determine the biological half-life *in vivo* since it is very difficult to measure nitric oxide due to its labile nature and low concentrations.

1.5.2. Synthesis

1.5.2.1. Chemical synthesis

Nitric oxide can be synthesised in numerous chemical reactions which are most unlikely to take place in biological systems. Two of the most common reactions are (Cotton & Wilkinson, 1972):

$$Fe^{2+} + NO_2^- + 2H^+ \rightarrow Fe^{3+} + NO + H_2O$$

and

$$2NO_2^- + 2I^- + 4H^+ \rightarrow 2NO + I_2 + 2H_2O$$

1.5.2.2. Biosynthesis

Enzymatic formation of nitric oxide could occur in biological systems. Some enzymatic reactions known to generate nitric oxide involve azide anion (N_3^-), hydroxylamine (NH_2OH), hydrazine (NH_2NH_2), and L-arginine as substrates. Azide anion and hydroxylamine undergo nitrogen oxidation to nitric oxide in a complex enzymatic reaction catalysed by catalase (Keilin & Harttee, 1954; Nicholls, 1964). A similar reaction with hydrazine requires addition of a peroxide.

The enzymatic formation of nitric oxide plus L-citrulline from L-arginine in a reaction catalysed by nitric oxide synthase has been discovered in the last several years (Hibbs, *et al.*, 1988; Palmer, *et al.*, 1988a; Moncada & Palmer, 1990). Prior

to the discovery that vascular endothelial cells can synthesise nitric oxide (Palmer, *et al.*, 1988a), Stuehr and Marletta (1985) reported that murine macrophages synthesise nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. They further extended these observations to the activation of macrophages by *Mycobacterium bovis* BCG infection, lymphokines, or gamma interferon (Stuehr & Marletta, 1987). Although in these studies nitric oxide was not implicated even as a possible labile intermediate precursor of nitrite and nitrate, the same group did report an unrecognised clue that activated murine macrophages catalyse the nitrosation of amines (Miwa, *et al.*, 1987). At that time it was known that at neutral pH nitric oxide was a more powerful nitrosating species than nitrite and nitrate.

Soon afterwards, an L-arginine-dependent biochemical pathway was identified as the source of nitrite and L-citrulline in cultured macrophages (Hibbs, *et al.*, 1987a). This pathway was inhibited by N^G-monomethyl-L-arginine, a close structural analogue of L-arginine. The structural analogues of L-arginine, N^GN^G-dimethyl-L-arginine (asymmetrical) and N^GN^{1G}-dimethyl-L-arginine (symmetrical), were also found to block the formation of nitrite and L-citrulline, an effect competitively reversed by L-arginine (Hibbs, *et al.*, 1987b). Furthermore, another report showed that L-arginine was necessary for the formation of nitrite, nitrate and nitrosamines by cytotoxic activated murine macrophages (Iyengar, *et al.*, 1987). Employing gas chromatography-mass spectrometry and L-[*guanido*-^{15}N]arginine-loaded macrophages, it was established that the nitrogen of nitrite, nitrate and the nitroso moiety of *N*-nitrosamines was derived exclusively from one of the terminal guanidino nitrogens of L-arginine.

Nitric oxide was subsequently established to be the L-arginine-derived intermediate of nitrite and nitrate production by macrophages using a similar mass spectrometric technique (Iyengar, *et al.*, 1987). A study using $^{18}O_2$ and mass spectrometry showed that molecular oxygen is incorporated into both nitric oxide and L-citrulline

(Leone, *et al.*, 1991). By applying a radiochemical technique involving [^{3}H]arginine coupled with mass spectrometry, L-citrulline was confirmed to be a product derived from L-arginine in endothelial cells (Moncada & Palmer, 1990). This formation was specific since the metabolism of L-arginine was competitively blocked by N^G-monomethyl-L-arginine but not its D-isomer (Palmer, *et al.*, 1988). Further, endothelium-dependent relaxation is also inhibited by other L- but not D-analogues of L-arginine, an effect competitively reversed by L- but not D-arginine (Sakuma, *et al.*, 1988; Schmidt, *et al.*, 1988a; 1988b; Gold, *et al.* 1989). These inhibitors include N^G-nitro-L-arginine (L-NOARG), its methyl ester L-NAME, and N^G-iminoethyl-L-arginine, and their actions resemble those of L-NMMA in vascular tissue *in vitro* and *in vivo* (Moore, *et al.*, 1989; Ishii, *et al.*, 1990; Mulsch & Busse, 1990; Rees, *et al.*, 1990). Interestingly, N^G-N^G dimethylarginine (asymmetrical dimethylarginine, ADMA) and L-NMMA are both found in human urine and plasma (Kakimoto & Akazawa, 1970; Park, *et al.*, 1988), and both inhibit nitric oxide formation (Vallance, *et al.*, 1992).

Further evidence showing the critical role for L-arginine is that cultured bovine endothelial cells grown in its absence fail to produce nitric oxide in response to bradykinin or A23187, an effect reversed by restoring L- but not D-arginine (Palmer, *et al.*, 1988a). The associated L-arginine-dependent increase of cGMP was accompanied by the formation of L-citrulline from L-arginine in the vessel wall (Moncada & Palmer, 1990). This L-arginine-nitric oxide pathway is NADPH- and calcium/calmodulin-dependent (Mayer, *et al.*, 1989; Busse & Mulsch, 1990; Moncada & Palmer, 1990; Mulsch, *et al.*, 1989).

Biochemical evidence supports the existence of an enzyme system responsible for the nitric oxide formation from L-arginine. The simultaneous formation of nitric oxide and L-citrulline is catalysed by the soluble fraction from cytotoxic activated macrophages (Marletta, *et al.*, 1988). Subsequently, Palmer & Moncada (1989)

reported that a novel L-citrulline-forming enzyme activity was found in the cytosolic fraction of vascular endothelial cells, although the concomitant formation of nitric oxide was not demonstrated in the same reaction mixture. This nitric oxide-forming enzyme was termed nitric oxide synthase (Moncada & Palmer, 1990).

Nitric oxide synthase has been purified from brain (Bredt & Snyder, 1990) and vascular endothelial cells (Pollock, *et al.*, 1991). The purified enzyme is phosphorylated by cAMP-dependent protein kinase, protein kinase C (PKC) and calcium/calmodulin-dependent protein kinase II (Bredt, *et al.*, 1992). The regulation by PKC phosphorylation provides a potential "cross-talk" mechanism with another messenger system. Molecular cloning has revealed its structural similarity with cytochrome P-450 reductase, particularly in its C-terminal portion, and with bacterial sulphite reductase (Bredt, *et al.*, 1991b). The enzyme has an α helical, calmodulin-binding consensus sequence, a cAMP-dependent protein kinase phosphorylation sequence, and well-defined sites for binding of nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The purified enzyme from brain migrates as a single 150 kDa band on electrophoresis and appears to be a monomer (Bredt, *et al.*, 1991b). The nitric oxide synthase in macrophage has substantial homology with the enzyme in brain (Bredt & Snyder, 1992). It has been suggested that the previously reported NADPH-dependent monoxygenase in brain is in fact identical to nitric oxide synthase (Hibbs, *et al.*, 1988; Marletta, *et al.*, 1988), since nitric oxide synthase containing neurones co-localise with the staining for NADPH diaphorase (NDP, Bredt, *et al.*, 1991a; Dawson, *et al.*, 1991). Furthermore, nitric oxide synthase activity fully accounts for all NDP catalytic staining in experiments using cloned and expressed nitric oxide synthase (Dawson, *et al.*, 1991).

Generally there are two types of nitric oxide synthase, the constitutive nitric oxide synthase and the inducible nitric oxide synthase. Both are very similar. They use L-

arginine as their substrate, form nitric oxide with L-citrulline stoichiometrically, require NADPH as an electron donor and contain one molecule each of FAD and FMN probably forming an electron transport chain in each enzyme (Bredt, *et al.*, 1991b; Stuehr, *et al.*, 1991). However, it should be noted that L-citrulline formation should not be necessarily equated with nitric oxide synthase activity. For example, an enzymatic activity exists in the rat kidney 10,000 x g supernatant fraction which catalyses the direct conversion of N^G,N^G-dimethyl-L-arginine or N^G-monomethyl-L-arginine to L-citrulline plus dimethylamine and methylamine, respectively, without concomitant formation of nitric oxide (Ogawa, *et al.*, 1987). This citrulline-forming enzyme is not nitric oxide synthase.

The constitutive enzyme in endothelium is mainly membrane-bound (Boje & Fung, 1990; Forstermann, *et al.*, 1991; Mitchell, *et al.*, 1991), although it was initially reported to be cytosolic (Palmer & Moncada, 1989; Mulsch, *et al.*, 1989). In the brain, however, this enzyme is cytosolic (Schmidt, *et al.*, 1989). Formation of nitric oxide by the constitutive enzyme is found in the vascular endothelium (Mayer, *et al.*, 1989; Mulsch, *et al.*, 1989; Palmer & Moncada, 1989), the blood platelet (Radomski, *et al.*, 1990), the CNS (Bredt & Snyder, 1990; Knowles, *et al.*, 1990), the peripheral nervous system (Gillespie & Xiaorong, 1989; Gillespie, *et al.*, 1989; Gibson, *et al.*, 1989; 1990; Li & Rand, 1989), the adrenal gland (Palacios, *et al.*, 1989) , endocardial cells (Schulz, *et al.*, 1991), megakaryoblastic cells (Lelchuk, *et al.*, 1992), neuroblastoma cells (Ishii, *et al.*, 1989), kidney epithelial cells (Schroder & Schror, 1989; Ishii, *et al.*, 1989), mast cells (Salvemini, *et al.*, 1990), mesangial cells (Schultz, *et al.*, 1991) and the retina (Venturini, *et al.*, 1991).

The inducible enzyme exists in the cytosolic fraction of macrophages (Marletta, *et al.*, 1988). It is NADPH-dependent and was initially reported to be calcium-/calmodulin-independent. However, Nathan (1992) has shown that calmodulin is firmly bound to the inducible enzyme and is required for activity. L-Canavanine

inhibits this enzymatic activity but not that of the constitutive enzyme (Iyengar, *et al.*, 1987). Usually it is not detectable in cells (Stuehr & Marletta, 1985; 1987) but its expression is induced by lipopolysaccharide (LPS), γ-interferon (IFN-γ), tumour necrosis factor (TNF), granulocyte macrophage-colony stimulating factor (GNCSF) and interleukins (ILs)-1, 2 and 6 (Ding, *et al.*, 1988; Drapier, *et al.*, 1988; Werner-Felmayer, *et al.*, 1990; Beasley, *et al.*, 1991; Denis, *et al.*, 1991; Nussler, *et al.*, 1991; Schini, *et al.*, 1991). Furthermore, its expression is inhibited by transforming growth factor β, platelet-derived growth factor and interleukins(ILs)-4, 8 and 10 (Ding, *et al.*, 1990; Liew, *et al.*, 1991; Pfeilschifter & Vosbeck, 1991; McCall, *et al.*, 1992; Schini, *et al.*, 1992). Induction of this enzyme requires *de novo* protein synthesis (Marletta, *et al.*, 1988), and is inhibited by glucocorticoids (Di Rosa, *et al.*, 1990; Radomski, *et al.*, 1990b; Rees, *et al.*, 1990b). Induction of nitric oxide synthase has been found in macrophages (Hibbs, *et al.*, 1988; Stuehr, *et al.*, 1989), neutrophils (McCall, *et al.*, 1991a; 1991b), vascular endothelial cells (Knowles, *et al.*, 1990b; Radomski, *et al.*, 1990b; Rees, *et al.*, 1990b), vascular smooth muscle cells (Beasley, *et al.*, 1991; Busse & Mulsch, 1990b; Knowles, *et al.*, 1990; Rees, *et al.*, 1990; Schini, *et al.*, 1991), cardiac myocytes (Schulz, *et al.*, 1992), Kupffer cells (Billiar, *et al.*, 1989), hepatocytes (Curran, *et al.*, 1989), tumour cells (Amber, *et al.*, 1988; Radomski, *et al.*, 1991; Werner-Felmayer, *et al.*, 1992), megakaryoblastic cells (Lelchuk, *et al.*, 1992), fibroblasts (Werner-Felmayer, *et al.*, 1990), chondrocytes (Stadler, *et al.*, 1992), mesangial cells (Marsden & Ballerman, 1990; Pfeischifter & Schwarzenbach, 1990; Shultz, *et al.*, 1991), the liver (Knowles, *et al.*, 1990c), and lung (Knowles, *et al.*, 1990c).

1.5.3. Nitric Oxide in the Nerve System

1.5.3.1. The Central Nervous System

Early evidence implying the involvement of nitric oxide in the CNS is that nitric oxide stimulates soluble guanylate cyclase in the homogenates of mouse cerebral cortex (Miki, *et al.*, 1977). Moreover, the soluble fraction of rat forebrain contains a low molecular weight substance which activates soluble guanylate cyclase and whose action is blocked by haemoglobin (Deguchi, 1977). L-Arginine was subsequently identified as the endogenous activator of the soluble guanylate cyclase in brain (Deguchi & Yoshioka, 1982).

N-Methyl-D-aspartate (NMDA), a glutamate receptor agonist, stimulates rat cerebral cells and induces an increase in cGMP levels in association with the release of an EDRF-like substance (Garthwaite, *et al.*, 1988). The cGMP elevation in response to NMDA stimulation is enhanced by L-arginine and inhibited by L-NMMA (Bredt & Snyder, 1989; Garthwaite, *et al.*, 1989). Moreover, addition of L-arginine to rat synaptosomal cytosol in the presence of NADPH induces the formation of nitric oxide and L-citrulline by acting as a substrate for nitric oxide synthase (Knowles, *et al*, 1989).

Using antibodies raised against nitric oxide synthase purified from cerebellum, the enzyme was immunohistochemically localised exclusively in neurones (Bredt, *et al.*, 1990). Within the brain, no glia were stained. The highest densities of nitric oxide synthase were found in the cerebellum, olfactory bulb and the accessory olfactory bulb. This enzyme occurs mainly in processes of granule cells. It is also highly concentrated in basket cells but is absent from the Purkinje cells of the cerebellum.

The demonstration of long-term depression by exogenous nitric oxide or cGMP in the rat cerebral slices suggests the involvement of L-arginine: nitric oxide system in motor learning (Shibuki & Okada, 1991). Moreover, the neurotoxicity mediated by NMDA *in vitro* (Dawson, *et al.*, 1991) and *in vivo* (Mollace, *et al.*, 1991) is diminished by nitric oxide synthase inhibitors and potentiated by L-arginine. Nitric oxide is therefore involved in a number of fundamental process within the CNS.

1.5.3.2 The Peripheral Autonomic Nervous System

Experimental evidence has been accumulating to suggest that nitric oxide is also involved in the peripheral autonomic nervous system. Following the discovery that nitric oxide could account for the activity of EDRF (Palmer, *et al.*, 1987), we began to examine the possibility that it was also the NANC transmitter in the BRP. Initially we attempted to deplete BRP of L-arginine by applying continuous electrical field stimulation but no decline in relaxation was observed with time. We also stored BRP in the cold and although a decline in NANC relaxation was observed it was not reversed by addition of L-arginine and was probably due to denervation (Gillespie & Xiaorong, 1989). The involvement of nitric oxide in the NANC nerves was first revealed by ability of L-NMMA and N^G-nitro-L-arginine to block NANC relaxation in the anococcygeus of the rat and mouse, and the retractor penis of the bull (Gillespie & Xiaorong, 1989; Gillespie, *et al.*, 1989; 1990; Gibson, *et al.*, 1989; 1990; Li & Rand, 1989;). Gradually, similar abilities to block NANC relaxation were reported in various tissues including guinea-pig trachea (Tucker, *et al.*, 1990; Li & Rand, 1991), rat gastric fundus (Li & Rand , 1990), canine lower oesophageal sphincter (Man, *et al.*, 1991; Tottrup, *et al.*, 1991), canine ileocolonic junction (Bult, *et al.*, 1990), guinea-pig taenia coli (Gustafsson, *et al.*, 1990), canine proximal colon (Dalziel, *et al.*, 1991), dog (Toda & Okamura, 1990a), monkey (Toda & Okamura, 1990b) and porcine (Chen & Lee, 1990) cerebral artery and human (Holmquist, *et al.*, 1991; Goessel, *et al.*, 1992) and

rabbit corpus cavernosum (Ignarro, *et al.*, 1990; Goessel, *et al.*, 1992). The nitric oxide synthase inhibitors, L-NMMA and L-NOARG, act prejunctionally in blocking the NANC relaxation since they do not affect relaxation to exogenously applied nitric oxide or nitrovasodilators (Gillespie, *et al.*, 1989; Liu, *et al.*, 1991). Another effect of the inhibitors of nitric oxide synthase in the anococcygeus of the rat is a modest rise in tissue tone, suggesting that there is a basal release of nitric oxide from the nerves in the absence of stimulation.

Bult *et al.* (1990) have reported that electrical field stimulation of the NANC nerves in the canine ileocolonic junction induces the release of a factor whose biological actions, chemical instability, inactivation by superoxide anion and haemoglobin, inhibition by L-NOARG, and potentiation by L-arginine all suggest it to be nitric oxide. Moreover, an enzyme isolated from the soluble fraction of the homogenised BRP catalyses formation of nitric oxide and L-citrulline from L-arginine (Sheng, *et al.*, 1992) and is believed to be the nitric oxide synthase from the NANC inhibitory nerves.

The above evidence clearly demonstrated the involvement of nitric oxide in NANC neurotransmission. However, authentic nitric oxide is a highly lipophilic gas that could readily diffuse through lipid membranes. It could not fulfil one of the conventional requirements of any neurotransmitter i.e. that it is present in storage vesicles in nerve fibres. Furthermore, the superoxide anion generators, pyrogallol and hydroquinone, destroy the relaxant activity of nitric oxide in a superoxide dismutase-reversible manner, but have no effects on the NANC relaxation induced by nerve stimulation (Gillespie & Sheng, 1990). Nitric oxide, therefore, may be reversibly bound by a stabilising substance. The aim of this study was to identify the nature of this nitric oxide-containing transmitter.

1.6. S-Nitrosothiols

Descriptions of the physical and chemical properties of S-nitrosothiols differ enormously, which might reflect a variety of different conditions employed in the preparation and handling of these compounds in different laboratories.

Most S-nitrosothiols (also termed thionitrites) are unstable in the solid form or in aqueous solution and spontaneously decompose to nitric oxide gas and corresponding disulphides, in a process reported to be enhanced by heat, light, oxygen, and alkaline pH (Ignarro, 1990; Feelisch, 1991):

$$2R\text{-}SNO \rightarrow R\text{-}SS\text{-}R + 2NO$$

S-Nitroso-*N*-acetylpenicillamine (SNAP) is the only known exception, and is stable indefinitely as dry crystals at 0-5°C. The half-life of SNAP in aqueous solutions ranges from 4-5 hours in air at 37°C to 500 hours under nitrogen (Ignarro, *et al.*, 1981; Bauer & Fung, 1991). One group has, however, reported that S-nitrosoglutathione is also unusually stable; after storage for 30 days in the solid state under air nearly no decomposition was seen at 4°C and 25% decay was seen at room temperature (Park & Means, 1989). In contrast, S-nitrosocysteine in the crystal state rapidly decomposes and cannot been stored (Ignarro, 1990; Myers, *et al.*, 1990). When prepared in acidified methanol (1%[vol./vol.] 1N.HCl in absolute methanol) at -20°C it is stable indefinitely (Ignarro, 1990). When present at high concentration in aqueous solution it has a half-life of 1.1 hours under air at 37°C and 15 minutes under oxygen at 37°C, whereas at concentrations less than 1 μM in dilute oxygenated aqueous solutions at 37°C it has a half-life of 30 seconds or less (Ignarro, 1990; Myers, *et al.*, 1990). Others, however, have reported that the half-life of S-nitrosocysteine in aqueous solution to be between 60 minutes (Feelisch, 1991) and 7-10 days (Ignarro, *et al.*, 1980). The chemical stability of S-nitroso

derivatives of 2-mercaptoethylamine, 3-mercaptopropionic acid, penicillamine, dithiothreitol, glutathione, thioglucose, and *N*-acetylcysteine lies between that of SNAP and S-nitrosocysteine (Ignarro & Gruetter, 1980; Ignarro, *et al.*, 1980a; 1980b; 1981). Ignarro (1990) explained that the relative chemical stability of SNAP is due to its *N*-acetyl group which structurally hinders its spontaneous decomposition to the corresponding disulphide. This explanation seems not to apply, however, in the case of S-nitroso-*N*-acetylcysteine since it is unstable in the crystalline state (Ignarro, *et al.*, 1980b; Kowaluk & Fung, 1990).

S-Nitrosothiols have been reported to be readily formed by the reaction of thiols with nitric oxide (Ignarro, *et al.*, 1981), nitrogen dioxide (Myers, *et al.*, 1990), acidified nitrite (Saville, 1958), N-methyl-N'-nitro-N-nitrosoguanidine (Schultz, *et al.*, 1977; Ignarro, *et al.*, 1980a), nitroprusside (Ignarro, *et al.*, 1981), and organic nitrate or nitrite esters (Ignarro, *et al.*, 1981) under different reaction conditions. Two of the most commonly used reactions are:

$$1).\ R\text{-}SH + NO_2^- + H^+ \rightarrow R\text{-}SNO + H_2O$$

the intermediate reactions are:

$$NO_2^- + H^+ \rightarrow HNO_2$$
$$R\text{-}SH + HNO_2 \rightarrow R\text{-}SNO + H_2O$$

and

$$2).\ R\text{-}SH + NO \rightarrow R\text{-}SNO + H^+$$

However, the exact nature of the S-nitrosating species in these reactions remains a subject of much debate. For example, dinitrogen trioxide has been proposed in the

case of acidified nitrite (Leaf, *et al.*, 1990) and a higher oxide of nitrogen in the case of nitric oxide (Feelisch, 1991).

L-Cysteine has been proposed to directly react with nitric oxide under anaerobic conditions (Ignarro, *et al.*, 1981) but this has been challenged by Feelisch (1991) who says that oxygen is mandatory. Also, L-cysteine has been reported to react with acidified nitrite indirectly, via generation of nitrous acid in aqueous solution (Saville, 1958). In each case the S-nitrosocysteine formed is a pink substance, which has been monitored by spectrophotometrically at 545 nm (Ignarro, *et al.*, 1981) and has two other absorption peaks at 335 nm and 210 nm (Feelisch, 1991; Myers, *et al.*, 1990).

The pharmacology of S-nitrosothiols is indistinguishable from that of nitric oxide since they act by releasing nitric oxide. For example, they induce smooth muscle relaxation through activation of soluble guanylate cyclase and increases of cGMP levels (Ignarro & Gruetter, 1980; Ignarro, *et al.*, 1980a; 1980b; 1981; Ignarro, 1990; Myers, *et al.*, 1990; Feelisch, 1991).

1.7. Common Properties of the NANC Transmitter, Inhibitory Factor, Endothelium-Derived Relaxing Factor, Nitric Oxide, and S-Nitrosothiols

The NANC inhibitory transmitter, IF, EDRF, nitric oxide and S-nitrosothiols share many properties (Gillespie, *et al.*, 1990; Ignarro, 1990; Moncada, *et al.*, 1991), typically acting through stimulation of soluble guanylate cyclase and increases of cGMP levels (Murad, *et al.*, 1978; Rapoport & Murad, 1983b; Bowman & Drummond, 1984), in a manner potentiated by the phosphodiesterase inhibitor, M&B 22,948 (Bowman & Drummond, 1984), and superoxide dismutase, and inhibited by haemoglobin and methylene blue (Murad, *et al.*, 1978; Bowman &

Gillespie, 1982; 1983; Bowman, *et al.*, 1982b; Bowman & Drummond, 1984; Bowman, *et al.*, 1986; Martin, *et al.*, 1985; Gibson & Mirazadeh, 1989).

1.8. Aim

The aim of this study was to identify the inhibitory non-adrenergic, non-cholinergic (NANC) neurotransmitter in the bovine retractor penis (BRP) muscle. The objective was to determine whether free nitric oxide, or S-nitrosothiol such as S-nitrosocysteine or S-nitrosoglutathione could be candidates.

Chapter 2. MATERIALS AND METHODS

2.1. Preparation of Tissues and Tension Recording

2.1.1. Anatomy of BRP Muscle

The paired BRP muscles have their origin from the ventral surface of the first and second coccygeal vertebrae. From there the muscles run ventrally, one on either side of the colon, to pass the anal canal and proceed along the ventral surface of the bulbocavernosus muscle to the distal part of the penis where a broad insertion takes place. The paired muscles in the contracted state maintain the characteristic sigmoid flexure of the relaxed penis (Figure 1).

2.1.2. Preparation of BRP Strips

BRP muscles were obtained at Paisley Abattoir from bullocks of different breeds, weighing 200 to 1000 kg. The whole penis was removed at the level of the ischiocavernosus muscle and the bulbocavernosus muscle with the paired retractor penis muscles attached. The BRP muscles were dissected free from surrounding connective tissue and fat in the abattoir and only one of each pair was taken and transferred to the laboratory in a bottle containing freshly prepared Krebs' solution. The entire process from slaughter to preparation in the laboratory took place within 1 hour. In the laboratory, the muscles were stored in Krebs' solution at 4 ^{o}C in a refrigerator until required. Muscles were used either immediately or after storage for periods up to 2 days. There was no indication that storage for up to 2 days adversely affected the responses of the tissue.

BRP muscles were pinned out on a glass Petri dish lined with Sylgard containing Krebs' solution. Any remaining connective sheath covering the muscles was

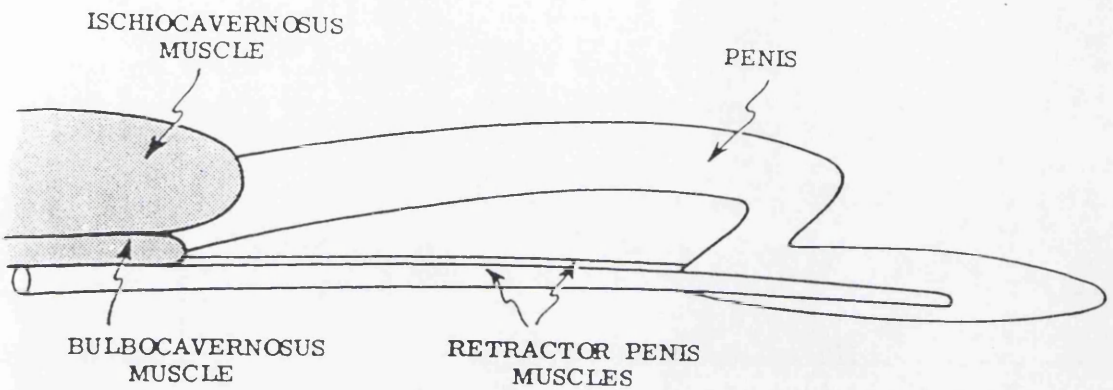


Figure 1. Schematic diagram of the contracted retractor penis muscles maintaining the relaxed penis of the bull with its characteristic sigmoid flexure.

removed to expose the muscle fibre bundles. Parallel bundles of muscle fibres were selected and longitudinal strips, 15 mm in length and 1.5 mm in width, were cut. Both ends of each strip were tied with 5:0 braided silk. One end was kept short for tying the preparation to a hook in an organ bath, and the other was left long for connection to a Grass FT 03 C isometric tension transducer. Each preparation was suspended in a 20 ml organ bath containing Krebs' physiological saline at 37°C and gassed with 5 % CO_2 and 95 % O_2. When responses to stimulation of intramural NANC nerve fibres were studied, one end of each strip was tied to a silver / silver chloride hook electrode and the muscle strip passed through a silver / silver chloride ring electrode embedded in Araldite (Burn & Rand, 1960) with the long thread tied to a Grass FT 03 C isometric tension transducer. Paired electrodes consisted of a ring electrode of a coil of 0.5 mm diameter silver wire, 4 mm in length and of 3 mm inside diameter embedded in Araldite epoxy resin, and a hook electrode of silver wire was placed 4 mm directly below the coil. In experiments in which the responses to exogenous drugs were studied in the absence of nerve stimulation, the BRP strips were mounted on glass hooks.

In all experiments BRP muscle strips were set up initially with no applied tension and allowed to equilibrate for 30 minutes. They were then placed under 2 g resting tension by repeated stretching with the degree being controlled by the tension displayed on the recorder.

2.1.3. Preparation of Rabbit Aortic Rings

In many experiments, rabbit aortic rings were used as an alternative to BRP strips as they were roughly equal in sensitivity to nitric oxide and nitric oxide-releasing agents as BRP strips (Gillespie & Sheng, 1988), but their tone was more stable. New Zealand white rabbits, 2 to 3 kg of either sex, were killed by a lethal injection of pentobarbitone and exsanguination. The chest and abdomen was opened. The

thoracic and abdominal aorta was carefully dissected and removed into Krebs' solution in a Petri dish. The aorta was cleared of adhering fat and connective tissue and cut into 2.5 mm wide transverse rings using a razor blade slicing device. Endothelial cells were removed from all rings by gently rubbing the luminal surface with moist filter paper. Rings were suspended under 2 g resting tension on stainless steel hooks in baths containing 20 ml of Krebs' solution at 37°C. Great care was taken during the whole procedure to avoid unnecessary stretch of the tissue. The rings were allowed to equilibrate for 60 to 90 minutes before experiments were begun. Removal of the endothelium was judged by the absence of relaxation to the endothelium-dependent relaxant, acetylcholine (0.1-1μM), against phenylephrine (0.3 μM)-induced tone.

2.1.4. Recording of Mechanical Responses

Mechanical responses of the BRP and rabbit aortic preparations were recorded isometrically by means of Grass isometric tension transducers (FT 03 C) and displayed on a six channel Grass Model 7D polygraph.

2.2. Experimental Conditions

2.2.1. Tone of the Preparations

When responses to stimulation of intramural NANC inhibitory nerve fibres in BRP muscle were to be studied, the adrenergic nerve blocking agent guanethidine (10μ M) was added to the preparations. Guanethidine is the most effective drug in uncovering the NANC inhibitory relaxation because it fulfils two major requirements: firstly, tone of the muscle must be raised and secondly the motor adrenergic responses must be blocked. In experiments in which responses to

exogenous drugs were to be studied in the absence of nerve stimulation, tone of the BRP strips was raised by phenylephrine (0.3μM).

When rabbit aortic rings were used, phenylephrine (0.3μM) was used to raise tone of the preparations as it maintained a stable tone.

2.2.2. Field Stimulation

Field stimulation of intramural nerves in BRP strips was applied through the silver / silver chloride electrodes (Burn & Rand, 1960) with rectangular shocks of 0.5 millisecond pulse width and supramaximal voltage. The pulses were simultaneously delivered from three Grass S88 stimulators with each channel controlling only one preparation. Where effects of drugs were examined on NANC responses to field stimulation, BRP muscle strips were stimulated with 20 pulses at 3 minute intervals. The frequency of the stimulation used was 2 Hz which is in the range of frequencies 1-8 Hz to induce optimal NANC relaxation.

2.2.3. Krebs' Solution

The composition (mM) of the Krebs' solution used was based on that described by Krebs and Henseleit (1932):

NaCl 118.0	KCl 4.8	$CaCl_2$ 2.5
$MgSO_4$ 1.2	KH_2PO_4 1.2	$NaHCO_3$ 24.0
Glucose 11.0		

With the exception of glucose, each component was prepared as a concentrated stock solution with distilled water. When the stock solution of $NaHCO_3$ was made up, it was bubbled with CO_2 gas (British Oxygen Company, UK) for 1 hour.

Glucose in solid form was added once all other components had been added. The final Krebs' solution was bubbled with 5 % CO_2 and 95 % O_2 for 30 minutes before use.

2.2.4. Temperature

The Krebs' solution in organ baths and in the tubes delivering Krebs to the baths was maintained at 37°C by a water jacket heated by a thermostatically controlled pump (Techne Tempette Junior TE-8J).

2.3. Preparation of Nitric Oxide Solutions and Synthesis of S-Nitrosothiols

2.3.1. Purification of Nitric Oxide and Nitrogen

Before attempting to prepare aqueous solutions of nitric oxide, it was first necessary to deoxygenated distilled water. This was achieved by bubbling with commercially available "oxygen-free" nitrogen gas (British Oxygen Company). This was found, however, to contain trace amounts of oxygen which were sufficient to convert substantial amounts of nitric oxide to nitrogen dioxide. It was essential to remove this remaining oxygen from the "oxygen-free" nitrogen gas.

Commercially available nitric oxide gas in steel cylinders is at most 99.9% pure. The major impurities are higher oxides of nitrogen, particularly nitrogen dioxide (Committee on Medical and Biological Effects of Environmental Pollutants, 1977). Since these higher oxides of nitrogen are much more reactive and soluble than nitric oxide (Hammond, 1966; Cotton & Wilkinson, 1972), their presence in proposed experiments involving reactions of nitric oxide with amino acids such as L-cysteine

and L-glutathione would greatly complicate matters (Hart, 1985; Hart, *et al.*, 1985; Wink, *et al.*, 1993).

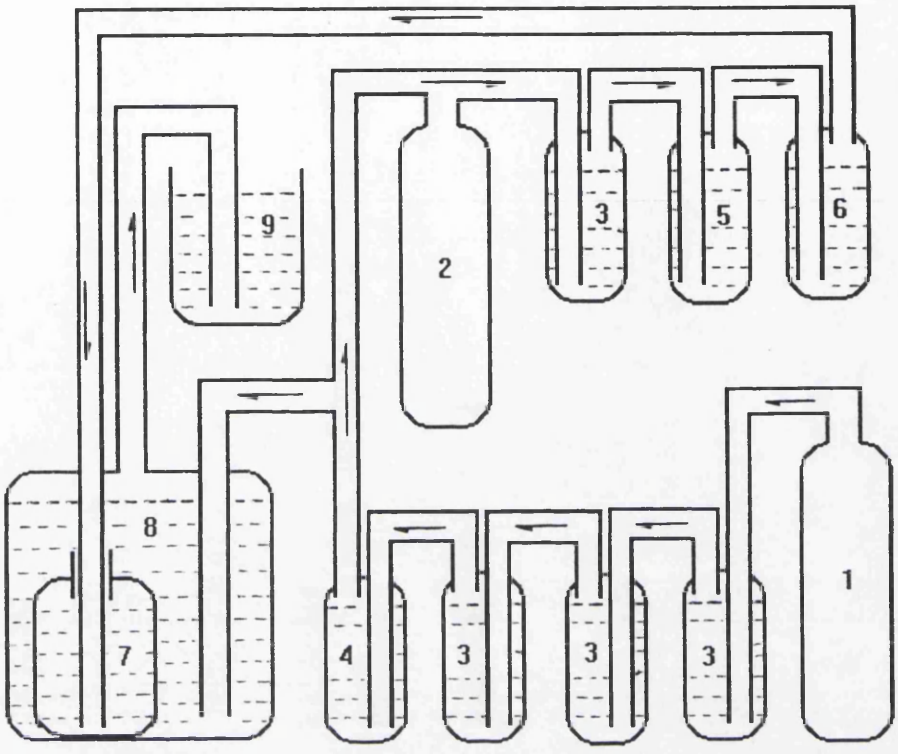
Furthermore, in these early experiments variable smooth muscle relaxant activity was generated in different experiments despite controlling the reaction conditions such as concentrations of L-cysteine and nitric oxide, reaction pH, time and temperature. The magnitude of a peak in the HPLC associated with this relaxant activity was also variable. Clearly, some important variable was affecting the outcome of experiments despite our attempts to keep conditions identical. In these experiments commercially available oxygen-free nitrogen was used to deoxygenate L-cysteine solutions. Furthermore, nitric oxide from a cylinder was transferred by a sample-lock gas syringe into the L-cysteine solutions in a brown bottle sealed with a silicon stopper while surrounded by air. It was possible, therefore, that despite our best efforts, variable amounts of oxygen could gain entry to the reaction chamber and dramatically affect the outcome of the reaction.

In preliminary tests massive increases in the relaxant activity and in the height of the associated peak in the HPLC were observed if small amounts of oxygen (0.01 mM) were introduced into the mixture of L-cysteine (10 mM, pH 3) and nitric oxide (0.1 mM). Therefore, it was essential in these experiments to purify nitric oxide gas in order to remove higher oxides of nitrogen and prevent contact with oxygen. More rigorous methods were developed to prevent introduction of oxygen and nitrogen dioxide during the reaction of L-cysteine with nitric oxide.

Removal of the remaining trace amounts of oxygen from commercial oxygen-free nitrogen (British Oxygen Company, UK) and nitrogen dioxide from nitric oxide (Air Products Ltd., UK) was performed by washing the gases with Fieser's solution and concentrated solutions (10%) of sodium hydroxide, respectively. Sodium hydroxide is an effective absorbent for nitrogen dioxide (Committee on Medical and

Biological Effects of Environmental Pollutants, 1977). Fieser's solution is an active absorbent to remove trace amounts of oxygen (Fieser & Fieser, 1967) and was prepared by dissolving 20 g of potassium hydroxide in 100 ml distilled water at 35°C and adding 2 g of sodium anthraquinone-β-sulfonate and 15 g of sodium dithionite ($Na_2S_2O_4$). The mixture was stirred until a clear, blood-red solution was obtained and cooled to room temperature. The sulfonate anthrahydroquinone dianion absorbs oxygen with great speed and is continually regenerated by the dithionite. Since the reagent operates on a catalytic principle, the original efficiency is retained to the point of exhaustion of the dithionite. When the solution has lost its ability to absorb oxygen, this is indicated by a change in colour from bright blood-red to dull red or brown. This is also associated with the appearance of a precipitate. When prepared as above, the solution has the capacity to absorb 788 ml of oxygen.

Obviously, deoxygenation of L-cysteine solutions, introduction of nitric oxide gas and the removal of freely dissolved nitric oxide should be performed in a closed system. This system should also made with oxygen-impermeable materials. Keeping a positive pressure in this closed system was also considered essential during these procedures. Any remaining trace amounts of oxygen present in commercial "oxygen-free" nitrogen gas and trace of nitrogen dioxide in commercial nitric oxide gas should be removed prior to their introduction into the solutions. Such a system is illustrated in Figure 2. HPLC stainless steel capillary tubing was used to connect the gas cylinder to washing bottles by piercing them through silicon rubber stoppers. Polythene tubing was not used since it absorbs and reacts with nitrogen dioxide and is permeable to oxygen. The end of the fine stainless steel tubing was pierced through a silicon stopper in the Amicon chamber and then passed through a common dynamic needle (21 gauge) into the reaction chamber which was also sealed with a similar stopper. To minimise the introduction of air into the reaction chamber particularly when spectrophotometric studies were to be carried out, the



1. Nitrogen cylinder
2. Nitric oxide cylinder
3. Fieser's solution
4. Lead acetate
5. Sodium hydroxide
6. Water
7. Reaction chamber
8. Amicon chamber
9. Water

Figure 2. Diagram of the closed system used to create rigorous oxygen- and nitrogen dioxide-free conditions in which aqueous solutions of L-cysteine or L-glutathione were gassed with purified nitrogen and nitric oxide. Arrows indicate the direction of gas flow.

reaction of L-cysteine with nitric oxide was conducted in a quartz cuvette (UV-visible, 1.5 ml, Hellma, UK). This cuvette is sealed with a silicon stopper. A small needle was inserted through this stopper to vent the chamber contents and this too could be sealed when required. The nitric oxide and nitrogen gases entering the Amicon chamber could escape through a silicon rubber tube whose far end was submerged in water in a beaker. High vacuum silicon grease was used to seal all joints.

Nitric oxide gas in fine bubbles was washed in a sealed environment by slowly passing through a series of washing solutions (all 100 ml); the first was a bottle of Fieser's solution to remove oxygen, the second was a bottle of sodium hydroxide (10%) to remove nitrogen dioxide and the last was a bottle of Milli-Q purified distilled deionised water to remove trace chemicals carried out from the other washing solutions. Oxygen-free nitrogen gas was purified in another sealed environment by passing through Fieser's solution to remove trace oxygen. Since a large amount of purified nitrogen gas was used to create an oxygen-free environment, it was passed through three consecutive bottles (all at 300 ml) of Fieser's solution to efficiently remove oxygen and finally a bottle (300 ml) of aqueous saturated lead acetate to remove trace amounts of H_2S generated from Fieser's solution. This purified nitrogen gas was used to create an external oxygen-free environment for a reaction chamber inside which nitric oxide would be reacted with L-cysteine. It was also introduced into the gas line supplying nitric oxide to prepare an oxygen-free pathway and into solutions of L-cysteine before the addition of nitric oxide. It was also, when stated, used to remove free nitric oxide from the reaction chamber after mixing L-cysteine with nitric oxide (see Results).

Usually the Amicon chamber, filled with distilled water, was deoxygenated by purified nitrogen gas for at least two hours. Solutions of L-cysteine in the sealed reaction chamber were submerged under water in the sealed Amicon chamber. The

solution was then deoxygenated by purified nitrogen delivered via a needle in the silicon stopper for 30 minutes before addition of purified nitric oxide gas either in measured volumes by a gas syringe or bubbled to saturation for 10 minutes. Finally, free nitric oxide was removed by purging with purified nitrogen for 10 minutes, except when it was required to remain present.

2.3.2. Synthesis of Nitrogen Dioxide

Nitrogen dioxide was prepared by two different methods, however, the experimental results obtained were similar in each case.

The first method chosen to prepare nitrogen dioxide was to mix nitric oxide gas (99.9%, Air Products Ltd., UK) with oxygen (British Oxygen Company, UK) in a ratio of 2:1 (vol./vol.) in a sample-lock gas syringe for 5 minutes before use. Rapid formation of a brown gas inside the syringe indicated formation of nitrogen dioxide:

$$2NO + O_2 \rightarrow 2NO_2$$

The second, and apparently more reliable means of preparing nitrogen dioxide was based on the following principles:

1). The thermal decomposition of the nitrates of metals other than those of Group I in the Periodic Table of the elements yields nitrogen dioxide, oxygen and the oxide of the metals (Moody, 1991). Lead(II) nitrate was used in the preparation of nitrogen dioxide because it is anhydrous, so avoiding a reaction between nitrogen dioxide and water. The following reaction takes place upon heating:

$$2Pb(NO_3)_2 \rightarrow 2PbO + 4NO_2 + O_2$$

To remove any traces of water, lead(II) nitrate crystal should be ground to a fine powder and dried at 150-200°C before use. The reaction proceeds with decrepitation, fragments of crystal being ejected from the mass, and gas being evolved from the fused solid.

2). The freezing point and the boiling point of nitrogen dioxide are -11.2°C and 21.2°C, respectively, whereas the freezing point and the boiling point of oxygen are -218.4°C and -183.0°C, respectively (Hammond, 1966). Thus, nitrogen dioxide can be easily separated from oxygen and collected by condensation at an appropriate temperature.

Specifically, nitrogen dioxide was generated by heating anhydrous fine powder of lead(II) nitrate (20 g) in a glass flask (200 ml) with a gas burner. The evolved gases passed through a glass cylinder (50 ml) cooled with crushed dry ice. Nitrogen dioxide condensed as a deep red-brown liquid first and was then frozen as a pale yellow solid, and oxygen remained in the gas phase and passed on. The solid nitrogen dioxide was stored under oxygen-free nitrogen in the sealed cylinder in a deep freeze at -20°C. When required, nitrogen dioxide gas was generated from the solid by gently heating the cylinder with warm water. After use, nitrogen dioxide was solidified again for future use.

2.3.3. Preparation of Solutions of Nitric Oxide

2.3.3.1. Preparation of solutions of nitric oxide under nominal oxygen-free conditions

At the beginning of this study, commercially available nitric oxide gas (99.9 %, Air Products Ltd., UK) and helium gas (British Oxygen Company) were used in preparation of nitric oxide solutions. Solutions of nitric oxide were prepared

immediately before use. One end of a piece of soft rubber tubing (inside diameter 3 mm) was connected to the cylinder containing nitric oxide gas and the other end submerged under water in a beaker. Enough gas was passed through the tube to clear the air from the tubing. A fine Hamilton air-tight syringe was then used to pierce the tubing wall near the cylinder and 4.5 µl of nitric oxide gas was drawn into the syringe and then immediately injected at atmospheric pressure into 20 ml of helium-deoxygenated distilled de-ionised water which filled completely a brown bottle sealed with a rubber seal. This produced a stock solution with a maximal nominal concentration of 10 µM. The solution of nitric oxide was protected from light.

2.3.3.2. Preparation of solutions of nitric oxide under rigorous oxygen-free conditions

Later in this study when it was realised that trace amounts of oxygen were still present in helium and commercially available "oxygen-free" nitrogen gases and nitrogen dioxide contaminated cylinders of nitric oxide gas, more rigorous procedures were used to prepare solutions of nitric oxide.

Solutions of nitric oxide gas (99.9%, Air Products Ltd., UK) were prepared immediately before use. They were prepared by submerging a brown bottle (20 ml) and a silicon rubber stopper in a beaker containing 300 ml of Milli-Q purified distilled deionised water and gassing with purified oxygen-free nitrogen (British Oxygen Company) for 1 hour. While still submerged, the rubber stopper was used to seal the bottle ensuring that no gas bubbles were left inside. One end of a long silicon rubber tube was connected using a "T" connector to the outputs of the gas washing bottles used to purify nitric oxide and nitrogen gases (see Figure 2), and the another end was submerged just under the water surface in another beaker. A sample-lock gas microsyringe (Dynatech series A, Precision Sampling Corporation,

USA) was used to pierce the tubing wall at a point near its middle. This part of tubing and the attached needle were then submerged under water in the beaker containing the sealed brown bottle. Sufficient volume of purified gases, first oxygen-free nitrogen and then nitric oxide, were passed through to purge the tubing and the syringe. 4.5 µl of nitric oxide gas was then drawn into the syringe and injected into the sealed bottle under water. This produced a stock solution of nitric oxide with a nominal concentration of 10 µM. These solutions of nitric oxide were made up daily and protected from light. They retained their relaxant activity for several hours while remaining sealed.

2.3.4. Synthesis of S-Nitrosothiols

Two methods were used to synthesise S-nitrosothiols, i.e. by a acidification of nitrite in the presence of thiols and by reacting nitric oxide with thiols. These methods were based on those previously reported (Saville, 1958; Ignarro, *et al.*, 1981). Since the yields of S-nitrosothiols were not known, their concentrations stated in this study were expressed as a function of the concentrations of each of the reactants.

2.3.4.1. Synthesis of S-Nitrosocysteine by Reacting L-Cysteine with Acidified Nitrite and Measurement of Nitrite Content

One method used to synthesise S-nitrosocysteine was by reacting L-cysteine with acidified sodium nitrite (Saville, 1958). Aqueous solutions of L-cysteine (1 M) were mixed with sodium nitrite (10 mM) at various pH values ranging from 7.4 to 1.0 for 10 minutes at 20^{o}C followed by neutralisation and bioassay of relaxant activity. The effect of pH on generation of S-nitrosocysteine was assessed by bioassay of relaxant activity. The reaction pH was maintained by the strong buffering capacity of L-cysteine in aqueous solutions pre-adjusted with 1 M hydrochloric acid to the

pH values required. The pH value was observed not to change following mixing with sodium nitrite in this study.

Also, this study investigated the relation between decreases in nitrite content and generation of relaxant activity in the acidified mixtures of L-cysteine and nitrite over a range of pH values. The nitrite content of the mixtures was measured by diazo formation according to the method previously reported (Martin, *et al.*, 1988). At 20°C, a sample (0.3 ml) was mixed with 0.3 ml of 1 % (w. /vol.) sulfanilic acid in 2 M hydrochloric acid. After 5 minutes, 0.3 ml of 1 % (w./vol.) aqueous solution of N-(1-napthyl)-ethylenediamine dihydrochloride was added, and the absorbance of the pink complex was measured at 548 nm by a Shimadzu UV-240 dual beam spectrophotometer connected to a Shimadzu Option Program / Interface OPI-2 which controlled the instrument. A standard concentration-absorbance curve was prepared with a series of standard solutions of sodium nitrite at concentrations of 0.00, 0.30, 1.00, 3.00, 10.00 and 30.00 μM. The content of nitrite in each sample was automatically determined by the spectrometer according to its absorbance against the standard curve.

2.3.4.2. Synthesis of S-nitrosothiols from reacting nitric oxide and nitrogen dioxide with L-cysteine and analogues

2.3.4.2.1. Synthesis of S-nitrosothiols under nominal oxygen-free conditions

The reactions between nitric oxide or nitrogen dioxide and L-cysteine or other compounds were studied. These other compounds included mainly analogues of L-cysteine and L-glutathione (reduced form), namely, N-acetyl-L-cysteine, L-cystamine, L-cysteamine, L-cysteic acid, L-cysteine methyl ester, L-cysteine ethyl ester, L-cysteinesulphinic acid, L-cystine, L-glutathione (oxidised form), L-

methionine, S-methyl-L-cysteine, and S-methyl-L-glutathione, and three other non-sulfhydryl-containing amino acids, L-arginine, L-glutamic acid and L-glycine.

This method was based on that previously reported in synthesis of S-nitrosothiols by reacting thiols with nitric oxide (Ignarro, *et al.*, 1981). In early experiments we did not know that commercially available cylinders of nitric oxide and oxygen-free nitrogen gases contain traces of nitrogen dioxide and oxygen, respectively. Consequently the procedures used initially were similar to those used in the preparation of nitric oxide solutions under nominal oxygen-free conditions described above. Aqueous solutions of these compounds (10-15 mM, pH 3) were purged with commercially available "oxygen-free" nitrogen, then reacted either with given volumes of nitric oxide or nitrogen dioxide (at concentrations of 0.1 or 1 mM as indicated in Results) or saturated with nitric oxide or nitrogen dioxide for 10 minutes, and where necessary (as indicated in Results) purged with "oxygen-free" nitrogen again to remove free nitric oxide or nitrogen dioxide and neutralised.

In some experiments the effects of reactant concentrations, pH and oxygen tension were studied in more detail and these will be described in the Results .

2.3.4.2.2. Synthesis of S-nitrosothiols under rigorous oxygen-free conditions

In later experiments when it became clear that commercially available cylinders of nitric oxide and oxygen-free nitrogen contain traces of nitrogen dioxide and oxygen, respectively, far greater precautions were taken.

The reactions were carried out as far as possible under rigorous oxygen-free conditions in the closed system (see Figure 2) following the same procedures described above for purification of nitric oxide and nitrogen gases. Aqueous solutions of these compounds (10-15 mM, pH 3) were purged with oxygen-free

nitrogen, then reacted either with given volumes of nitric oxide or nitrogen dioxide (at concentrations of 0.1 or 1 mM as indicated in Results) or saturated with nitric oxide for 10 minutes, and where necessary (as indicated in Results) purged with purified oxygen-free nitrogen again to remove free nitric oxide and neutralised.

2.3.5. High Performance Liquid Chromatography Analysis

The HPLC analysis was performed using a system consisting of the following components:

1). Pump: A constant flow pump, Waters Model 6000A Solvent Delivery System, was used. Constant flow of the solvent was achieved with a pair of specially-driven positive-displacement pumping chambers.

2). Injector: A Rheodyne manual injector model 7125 was used.

3). Column and Guard Column: The Waters analytical and preparative reverse phase chromatography systems were used. The analytical system consisted of a μ Bondapac Guard-Pac C18 reverse-phase insert (particle size 10μm, pore size 125Å) in a Guard-Pak holder and a μBondapac Radial-Pac C18 reverse-phase cartridge (8 x 100 mm, particle size 10μm, pore size 125Å) in a RCM 8 x 10 cartridge holder. The preparative system consisted of a μBondapac Guard-Pac C18 insert (particle size 10μm, pore size 125Å) connected to a μBondapac Radial-Pac C18 reverse-phase cartridge (25 x 100 mm, particle size 10μm, pore size 125Å) in a RCM 25 x 10 cartridge holder.

4). Detector: A Waters UV absorbance detector model 440 was used. It has two separated detection systems which operate in two consecutive cells in series. The

wavelengths of the detector can be fixed at 254 or 280 nm, but in this study both were set at 254 nm.

5). Recorder: A Phillips Model PM8252 dual-pen standard compact recorder was used. It was connected to the output of the UV detector for recording of absorption.

6). Solvent: The solvent system used in experiments was varied during the course of this study. Methanol was used initially but as it destroyed the relaxant synthesised from the reaction of L-cysteine and nitric oxide (see Figure 27), it was abandoned. Phosphate buffer (pH 3.0, 1/15 M) failed to give adequate separation of peaks and was also abandoned. PIC A (tetrabutylammonium phosphate ion-pairing agent) was also rejected as a solvent since it reacted with samples generating numerous irregular peaks with high absorption, and the retention time of some of the peaks was often as long as an hour. Milli-Q purified water (pH 3.0), however, gave a good separation of peaks and produced low basal noise in the HPLC detection system. It was, therefore, used for most of the HPLC analyses.

Specifically, the purified HPLC grade water used in this study was prepared using glass-distilled water purified by passing through a Waters Milli-Q Reagent-Grade Water System which removed dissolved organic materials by absorption into activated charcoal, ionised compounds by mixed bed deionization and all micro-organisms larger than 0.22 μM by Millipore membrane filtration. The purified water was then adjusted to pH 3.0 with hydrochloric acid (1N, analytical grade). Finally, it was filtered through a Waters filtration membrane disc (47 mm, type HA, pore size 0.45 μM) and degassed under high vacuum in a Millipore all-glass filter system connected to a high vacuum pump (single stage model 1SC50, Edwards High Vacuum Limited, UK). During analysis the solvent was continuously degassed by bubbling with helium (British Oxygen Company).

HPLC detection was performed at 254 nm in both channels with a 10-fold difference in the two sensitivities. The absorption detected in each channel of the UV detector was continuously monitored on the dual pen recorder. When analysis was performed with the 8 x 100 mm analytical column, the flow rate was set at 1.5 ml per minute. Chart speed was set at 1.0 cm per minute and the sample volume was usually 10 µl. When analysis was performed with the 25 x 100 mm preparative column, the flow rate was set at 9.0 ml per minute. The chart speed was set at 2.0 cm per minute, and the sample volume was generally 1.0 ml

On rare occasions, small particles formed in samples of L-cysteine after reaction with nitric oxide or nitrogen dioxide. In order to avoid blockage of the column by these particles, such solutions were filtered through a Sartorius filter (Minisart NML, pore size 0.45 µM) and the filtrate was then loaded into the injector.

In order to maintain good resolution and column life, the column was washed after each experiment first with the normal solvent (Milli-Q purified water pH 3.0) and then with 50 % (vol./vol.) methanol in Milli-Q purified water for at least at five column volumes. At the beginning of each analysis the entire HPLC system was flushed and equilibrated with Milli-Q purified water (pH 3.0) for 30 minutes.

2.3.6. Ultra-violet and Visible Spectrophotometric Analysis

The absorption spectra of L-cysteine, nitric oxide, nitrogen dioxide, and products from reacting L-cysteine with nitric oxide or nitrogen dioxide were analysed by continuous scans at wavelengths from 900 to 190 nm in a Shimadzu UV-240 dual beam spectrophotometer connected to a Shimadzu Option Program / Interface OPI-2 which controlled the instrument. Two Hellma UV-visible quartz cuvettes (1.5 ml capacity) were used when making measurements. Where necessary to keep

deoxygenated samples from contact with air, the cuvettes were completely filled with the samples and sealed with silicon stoppers in an oxygen-free environment.

2.4. Drugs and Compounds

Acetylcholine chloride, N-acetyl-L-cysteine, L-arginine, L-cystamine, L-cysteamine, L-cysteic acid, L-cysteine, L-cysteine methyl ester, L-cysteinesulphinic acid, L-cystine, L-glutamic acid, L-glutathione, guanethidine sulphate, haemoglobin (bovine), hydroquinone, isoprenaline hydrochloride, L-methionine, S-methyl-L-cysteine, methylene blue, S-methyl-L-glutathione, N-methylhydroxylamine hydrochloride, N^G-nitro-L-arginine, phenylephrine hydrochloride and superoxide dismutase (bovine erythrocyte) were obtained from Sigma Chemical Company Limited (UK); diamide, dithiothreitol, N-ethylmaleimide, hydrochloric acid, methanol, potassium hydroxide, sodium anthraquinone-β-sulfonate, sodium dithionite, sodium hydroxide were obtained from Aldrich Chemical Company Limited (UK); pyrogallol was obtained from BDH (UK) and glyceryl trinitrate (10% w/w in lactose) was obtained from Napp Laboratories (UK).

Solutions of drugs and compounds were made up as concentrated stocks in deionised distilled water unless indicated otherwise. Serial dilutions to the required concentrations were made up from the stocks in distilled deionised water immediately before use. Usually they were all kept on ice during use. These drugs and compounds were added into 20 ml organ baths in volumes of 1 to 300 μl (usually 10μl) using micro syringes or Gilson automatic pipettes. The concentrations in the text and the Figures are the final concentrations in the baths, except where indicated.

Acetylcholine was made up as 10 mM stock solutions in phosphate buffer (10 mM, pH 4.0). The stock solution was divided into 1 ml aliquots put into small plastic

containers and stored in a deep freeze at -20°C for future use. Dilutions were made in distilled deionised water from the stock immediately before use.

Solutions of oxyhaemoglobin were prepared according to the procedures previously described (Bowman, *et al.*, 1982b). Human haemoglobin was obtained from the Sigma Chemical Company Ltd. (UK). It consists predominantly of methaemoglobin, as pure oxyhaemoglobin is unstable and readily oxidised when stored. Oxyhaemoglobin was prepared from the commercially available haemoglobin as follows: sodium dithionite 2 mg was added to 2 ml of an aqueous solution of "haemoglobin" (15 mg ml^{-1}). This reduced the methaemoglobin to deoxyhaemoglobin and the colour changed from brown to purple. Excess dithionite was removed immediately by passing the solution through a gel filtration column (Columns PD-10, 12 x 50 mm, Sephadex G-25M, Pharmacia Fine Chemicals, Sweden). During passage through the column, the deoxyhaemoglobin became oxygenated and its colour changed from purple to red as it separated from dithionite. The oxyhaemoglobin was eluted from the column with distilled deionized water. Its concentration was spectrophotometrically monitored against standard haemoglobin solution (Sigma Chemical Company Limited, UK). The resulting solution was stored in aliquots at -20°C for up to 14 days.

2.5. Statistics

The number of observations in each experiments was no less than 8. Sets of observations were expressed as the mean ± the standard error of the mean (s.e.m) in appropriate tables and charts. Differences between sets of means were analysed and estimated normally by using Student's t-test. A probability of 0.05 or less was considered significant.

Chapter 3. RESULTS

While non-adrenergic non-cholinergic (NANC) nerves are known to innervate the bovine retractor penis muscle (Klinge, *et al.*, 1970; Klinge & Sjostrand, 1974; Ambache & Killick, 1978) and the rat and mouse anococcygeus muscle (Gillespie, 1972; Gibson & Gillespie, 1973; Gillespie & McGrath, 1973; Gillespie & McGrath, 1974; Creed, *et al.*, 1977; Gibson & Wedmore, 1981; Gibson & Tucker, 1982; Gibson & Yu, 1983), the neurotransmitter was still unknown at the beginning of this study. Many substances especially some known neurotransmitters were rejected as the non-adrenergic non-cholinergic neurotransmitter (Klinge & Sjostrand, 1974; Gillespie, 1972; Gillespie, *et al.*, 1990). Many attempts were made to analyse and classify the non-adrenergic non-cholinergic neurotransmitter based on the effects of drugs on the NANC response (Klinge & Sjostrand, 1974; Gillespie, 1972; Martin & Gillespie; 1991). Other attempts were made to purify and chemically identify IF extracted from the BRP but with only partial success (Gillespie & Martin, 1978; 1980; Gillespie, *et al.*, 1981b; Martin, *et al.*, 1988). Attempts were also made to detect the NANC neurotransmitter released from a donor tissue i.e. a BRP or rat anococcygeus strip by an assay tissue such as a rabbit aortic ring or a BRP or rat anococcygeus strip but these were also unsuccessful. Now the most likely candidates are IF, EDRF, nitric oxide and S-nitrosothiols because they share many properties in common (Gillespie, 1987; Gibson, *et al.*, 1989; 1990; Gillespie, *et al.*, 1989; 1990; Li & Rand, 1989; Yui, *et al.*, 1989; Myers, *et al.*, 1990; Martin, *et al.*, 1991; Liu, *et al.*, 1991; Kerr, *et al.*, 1992).

The involvement of the L-arginine: nitric oxide system in the inhibitory NANC neurotransmission in the BRP and the rat and mouse anococcygeus has been well established (Gibson, *et al.*, 1989; 1990; Gillespie, *et al.*, 1989; 1990; Li & Rand, 1989; Liu, *et al.*, 1991). However, nitric oxide might not be the exact form of the transmitter since superoxide anion generators such as pyrogallol blocked exogenous

nitric oxide-induced relaxation but not the NANC inhibitory response to electrical field stimulation in the BRP and mouse anococcygeus (Gillespie & Sheng, 1990; Gibson, *et al.*, 1992). Furthermore, it has been suggested that EDRF is much more likely to be a nitrosylated compound, such as a nitrosothiol e.g. S-nitrosocysteine, than authentic NO (Myers, *et al.*, 1990). S-Nitrosothiols have also been proposed to act as active intermediates in vascular smooth muscle relaxation induced by organic nitrates, nitrites, nitroprusside and nitric oxide (Ignarro, *et al.*, 1981). Furthermore, activation of guanylate cyclase by glyceryl trinitrate specifically requires L-cysteine (Ignarro & Gruetter, 1980). Therefore, the roles of intracellular free sulfhydryl groups and S-nitrosothiols in the inhibitory NANC neurotransmission in the BRP are worthy of investigation.

3.1. Role of Free Sulfhydryl Groups in NANC Neurotransmission

If free sulfhydryl groups are involved in the inhibitory NANC neurotransmission in the BRP, the relaxant response to intramural nerve stimulation should be inhibited by depleting the tissue of free sulfhydryl groups. This is investigated by using two chemicals, diamide and N-ethylmaleimide, which oxidise and alkylate sulfhydryl compounds, respectively.

3.1.1. Inhibition of NANC Relaxation by Diamide

Diamide is a thiol-oxidising agent (Kosower, *et al.*, 1969) which converts free thiols to the corresponding disulphides:

$$2R\text{-}SH + (CH_3)_2NCON{=}NCON(CH_3)_2 \rightarrow R\text{-}S\text{-}S\text{-}R + (CH_3)_2NCONHNHCON(CH_3)_2$$

It has a particularly high affinity for L-glutathione, being some 10 time higher than for L-cysteine. As can be seen from the above equation, it stoichiometrically oxidises L-glutathione to the corresponding disulphide.

Diamide (1 mM) had two effects on the BRP muscle precontracted with guanethidine (10 μM, Figure 3). First, it partially inhibited (30-50 %) the NANC inhibitory relaxation induced by electrical field stimulation (2 Hz, 20 pulses) within 10-15 minutes. Secondly, it lowered guanethidine-induced tone in most preparations by around 20%, although no decrease or up to a 50% decrease of the tone was observed in a few preparations. The inhibition of both the NANC relaxation and guanethidine-induced tone was almost completely reversed by treatment with the sulfhydryl compounds, L-cysteine, L-glutathione or dithiothreitol all at 3 mM.

Pretreatment of the BRP preparations with L-cysteine, L-glutathione or dithiothreitol (all at 3 mM) prevented the diamide-induced inhibition both of the NANC response to intramural nerve stimulation and of guanethidine-induced tone (Figure 4).

Furthermore, these three sulfhydryl-containing compounds had a common additional effect (Figures 3 & 4): they all caused a transient decrease of guanethidine-induced tone of the BRP preparations lasting about 15 minutes prior to their reversal of the effects of diamide. When dithiothreitol (3 mM) was added to strips of BRP, it first caused a transient fall in tone, simultaneously reduced the NANC relaxant response by about 30 %, and then raised the tone to a level higher than before addition of dithiothreitol (Figure 4). The development of raised tone is consistent with inhibition of the NANC relaxation since both effects are also seen to occur following treatment with L-nitroarginine (Liu, *et al.*, 1991). Both of these effects of dithiothreitol were sustained, were not reversed by addition of diamide (1 mM, Figure 4) or superoxide dismutase (100-8,000 u ml^{-1}, Figure 5), and were not

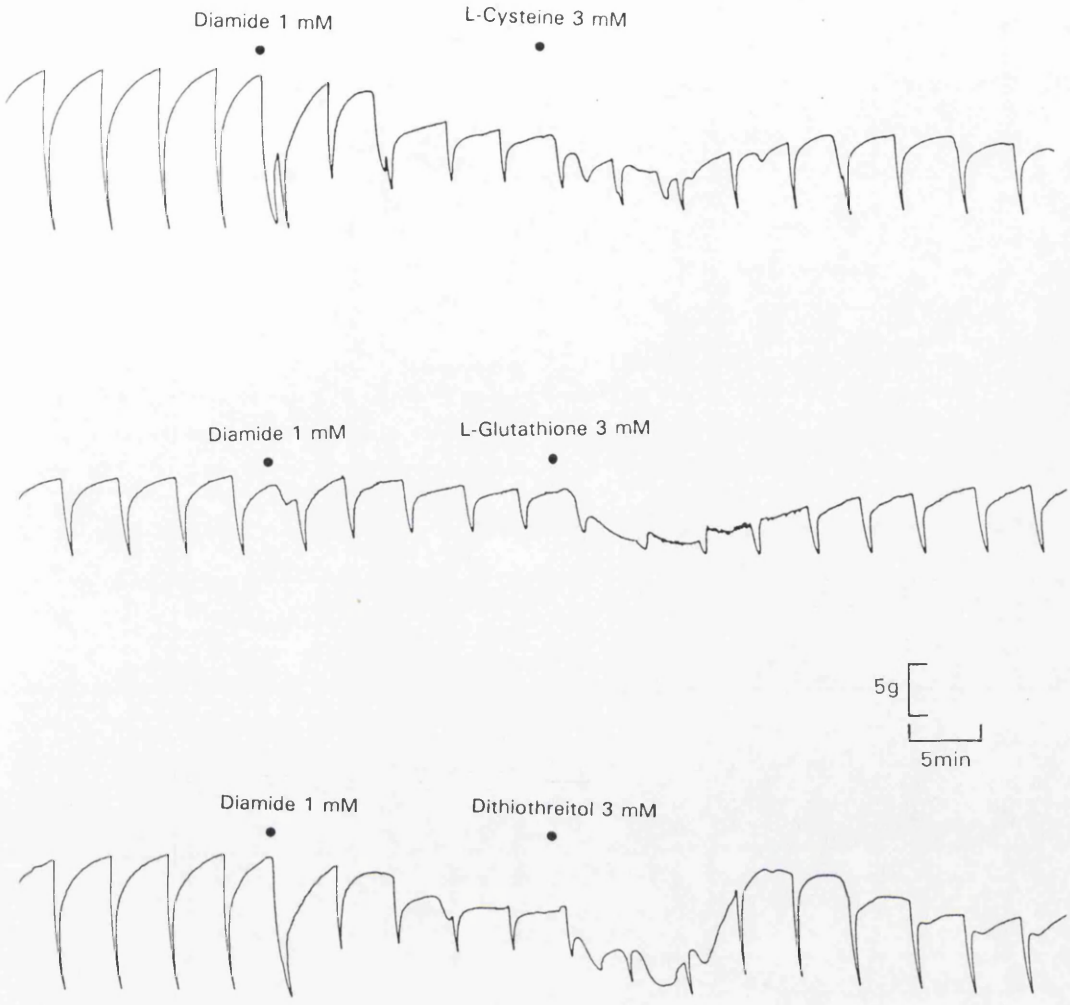


Figure 3. Treatment of BRP muscle with diamide (1 mM) partially inhibited NANC relaxation induced by field stimulation (2 Hz, 20 pulses, pulse width 0.5 ms and supramaximal voltage). This inhibition was reversed by treating the tissues with L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM). Tone of the BRP muscle was raised by guanethidine 30 μM.

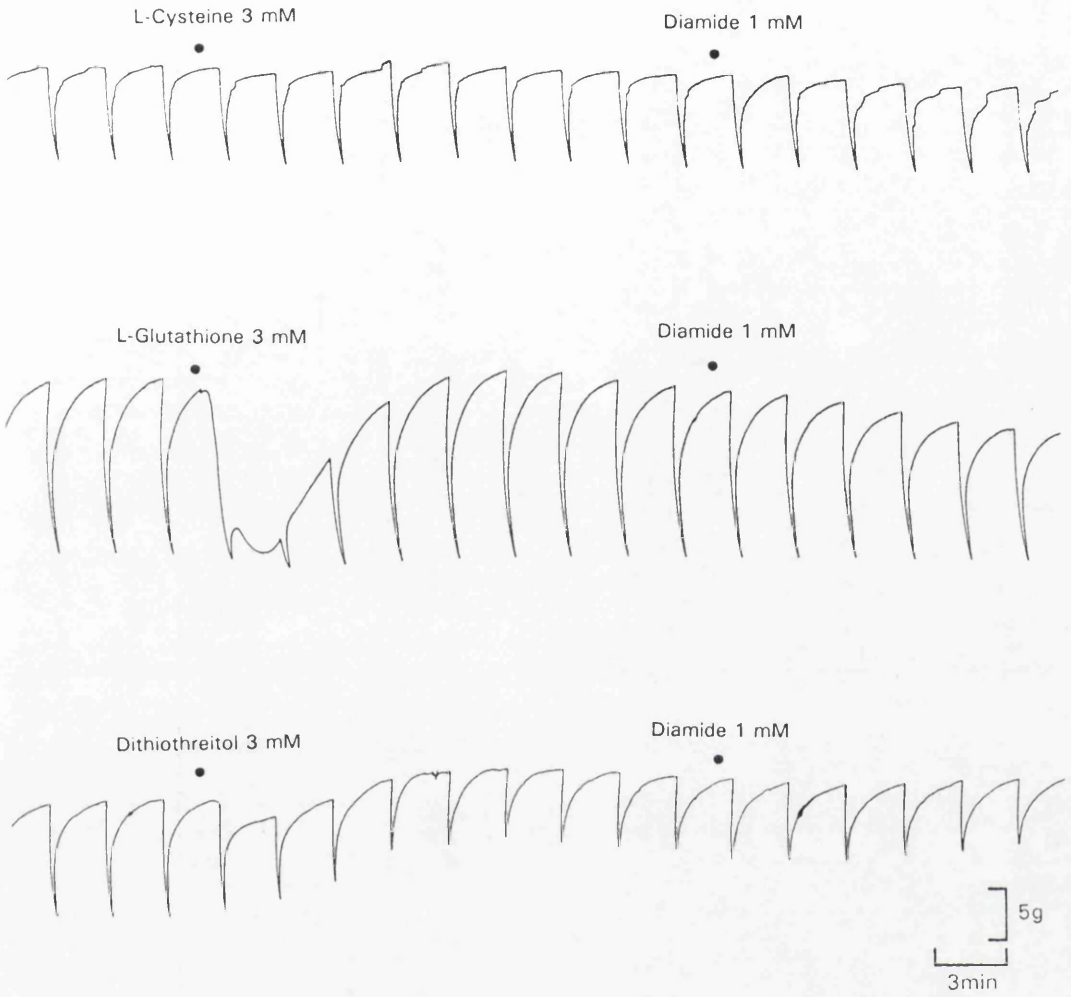


Figure 4. Pretreatment of BRP muscle with L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM) completely prevented inhibition by diamide (1 mM) of the inhibitory response to NANC nerve stimulation (2 Hz, 20 pulses, pulse width 0.5 ms and supramaximal voltage). Tone of the BRP muscle was raised by guanethidine 30 μM.

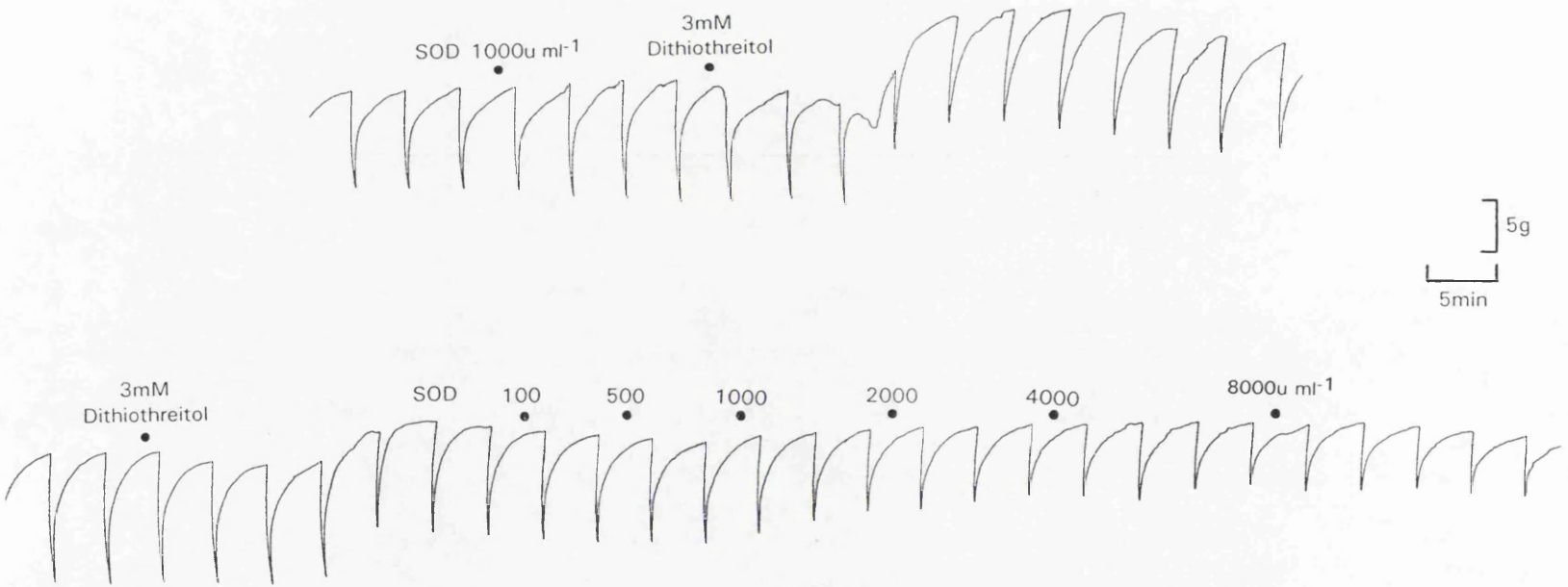


Figure 5. Treatment of BRP muscle with dithiothreitol (3 mM) partially inhibited NANC relaxation induced by nerve stimulation (2 Hz, 20 pulses, pulse width 0.5 ms and supramaximal voltage) and increased the tone raised by guanethidine (30 μM). These effects of dithiothreitol were neither prevented nor reversed by superoxide dismutase.

prevented by pretreatment with superoxide dismutase (1,000 u ml^{-1}, Figure 5). Furthermore, superoxide dismutase (1,000 u ml^{-1}) did not affect the NANC response to the nerve stimulation (Figure 5).

The selectivity of the ability of diamide to block NANC relaxation was examined by assessing its effects on relaxation induced by other stimuli. Pretreatment of BRP preparations with diamide at a concentration of 1 mM for 20 minutes completely blocked the relaxation induced by glyceryl trinitrate (10-100 nM) or isoprenaline (10-1000 nM) and this blockade was not reversed after addition of the free sulfhydryl-containing compounds L-cysteine, L-glutathione or dithiothreitol (all at 3 mM, Figure 6).

3.1.2. Inhibition of NANC Relaxation by N-Ethylmaleimide

N-Ethylmaleimide like diamide is a sulfhydryl-depleting agent but its mechanism of action is through alkylation of free sulfhydryl groups (Siegle, *et al.*, 1993).

N-Ethylmaleimide (0.3 mM) caused 50-70% inhibition of the NANC relaxation induced by field stimulation (2 Hz, 20 pulses) and gradually reduced guanethidine (10 μM)-induced tone in the BRP strips (Figure 7). Neither of these effects of N-ethylmaleimide was reversed by addition of the sulfhydryl compounds L-cysteine, L-glutathione or dithiothreitol (all at 3 mM). However, pretreatment of the BRP preparations with these three sulfhydryl-containing compounds completely prevented the ability of N-ethylmaleimide to inhibit NANC relaxation and to lower tone (Figure 8). Furthermore, at a concentration of 1 mM dithiothreitol itself did not either inhibit NANC relaxation or raise guanethidine-induced tone (Figure 8).

As with diamide, the ability of N-ethylmaleimide (0.3 mM) to inhibit NANC relaxation to nerve stimulation in the BRP muscle was not selective since this agent

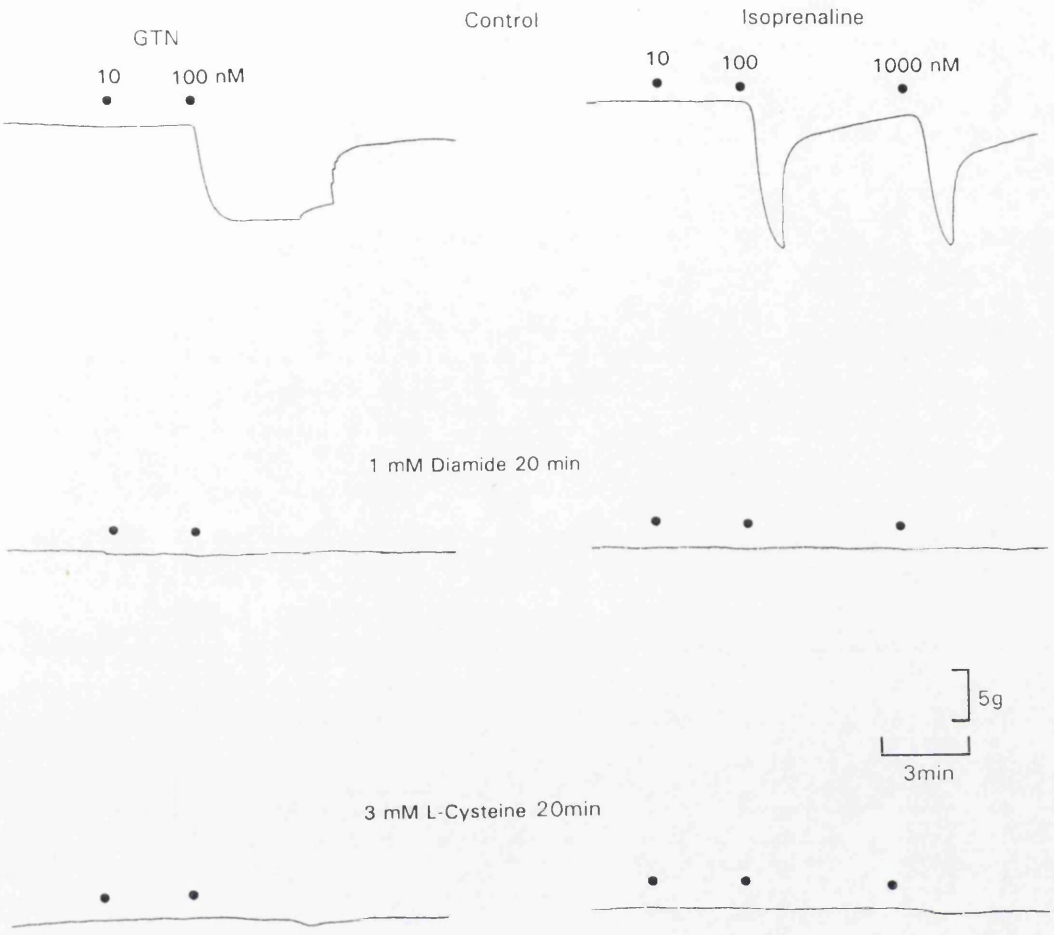


Figure 6. Treatment of BRP muscle with diamide (1 mM) for 20 minutes completely blocked relaxation induced by glyceryl trinitrate (10-100 nM) or isoprenaline (10-1000 nM). This blockade was not reversed after addition of L-cysteine (3 mM) for 20 minutes. Tone of the BRP muscle was raised by phenylephrine 0.3 μM.

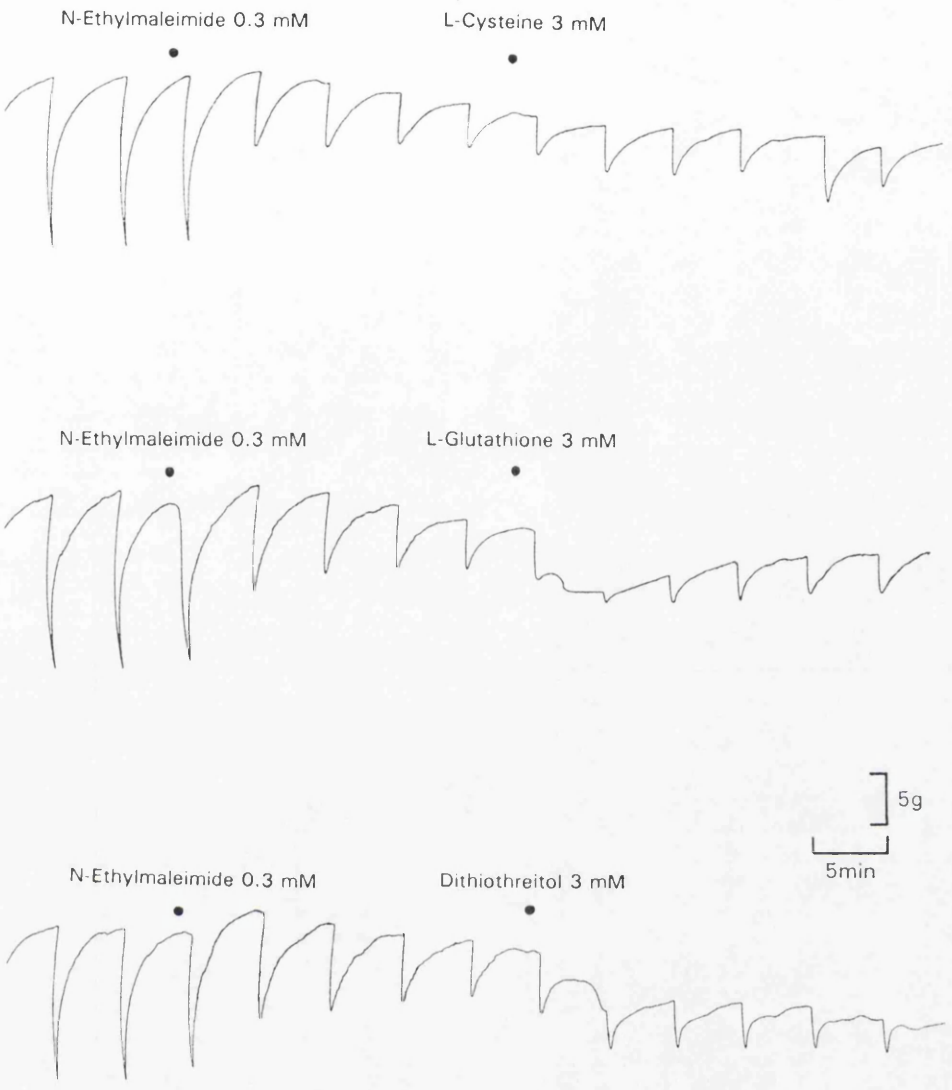


Figure 7. Treatment of BRP muscle with N-ethylmaleimide (0.3 mM) partially inhibited NANC relaxation induced by field stimulation (2 Hz, 20 pulses, pulse width 0.5 ms and supramaximal voltage). This inhibition was not reversed by treating the tissues with L-cysteine (3 mM), L-glutathione (3 mM) or dithiothreitol (3 mM). Tone of the BRP muscle was raised by guanethidine 30 μM.

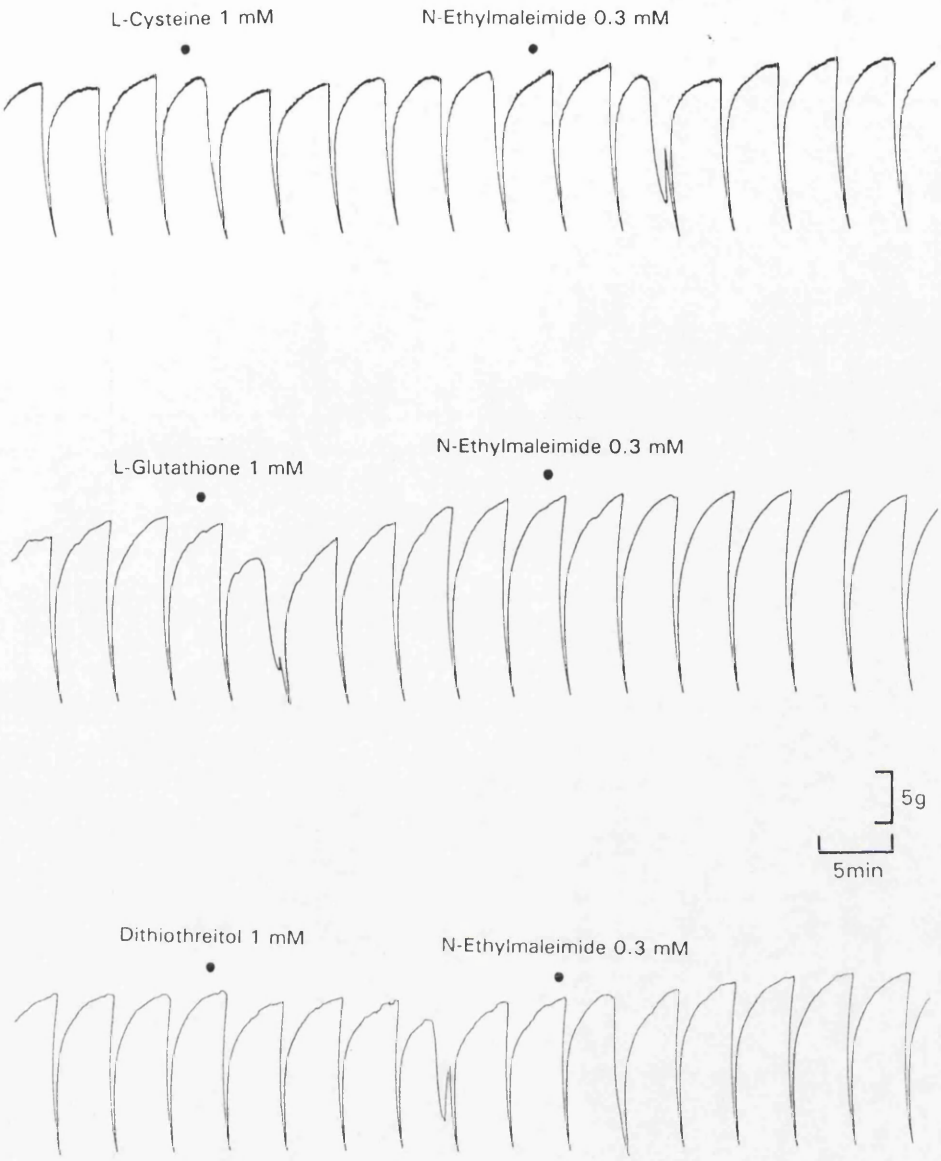


Figure 8. Pretreatment of BRP muscle with L-cysteine (1 mM), L-glutathione (1 mM) or dithiothreitol (1 mM) completely prevented inhibition of the inhibitory response to NANC nerve stimulation (2 Hz, 20 pulses, pulse width 0.5 ms and supramaximal voltage) by N-ethylmaleimide (0.3 mM). Tone of the BRP muscle was raised by guanethidine 30 μM.

also completely blocked the relaxation induced by glyceryl trinitrate (10-100 nM) or isoprenaline (10-100 nM) and this blockade was not reversed after addition of the free sulfhydryl-containing compounds L-cysteine, L-glutathione or dithiothreitol (all at 3 mM, Figure 9) .

3.2. Role of S-Nitrosothiols in NANC Neurotransmission

Nitric oxide is a highly lipophilic gas which readily diffuses across biological membranes. Owing to its odd electron, it is also chemically highly reactive with many intracellular and extracellular substances in the body such as oxygen. These properties make free nitric oxide an unlikely candidate for a neurotransmitter. However, these disadvantages of nitric oxide could be overcome if it binds to a carrier which protects it and delivers it to guanylate cyclase intact. Since S-nitrosothiols share many properties with the inhibitory NANC neurotransmitter an investigation was conducted to determine if such substances could act as an effective nitric oxide carrier.

3.2.1. Relaxation Induced by Solutions of Sodium Nitrite and Mixtures of Sodium Nitrite and L-Cysteine at Different pH Values

We first examined the relaxant activity of sodium nitrite with and without the presence of L-cysteine at different acid pH values in aqueous solutions by bioassay.

Figure 10 shows that the relaxant activity of sodium nitrite (10 μM) assessed on a rabbit endothelium-denuded aortic rings precontracted by phenylephrine (0.3 μM) was increased by lower pH. Given the same bath concentrations of 10 μM, sodium nitrite at pH 7.4 produced a small and slowly developing relaxation in the contracted ring, whereas when acidified to pH 4 or below, it added into baths, produced an additional quickly developing relaxation prior to the slow relaxation.

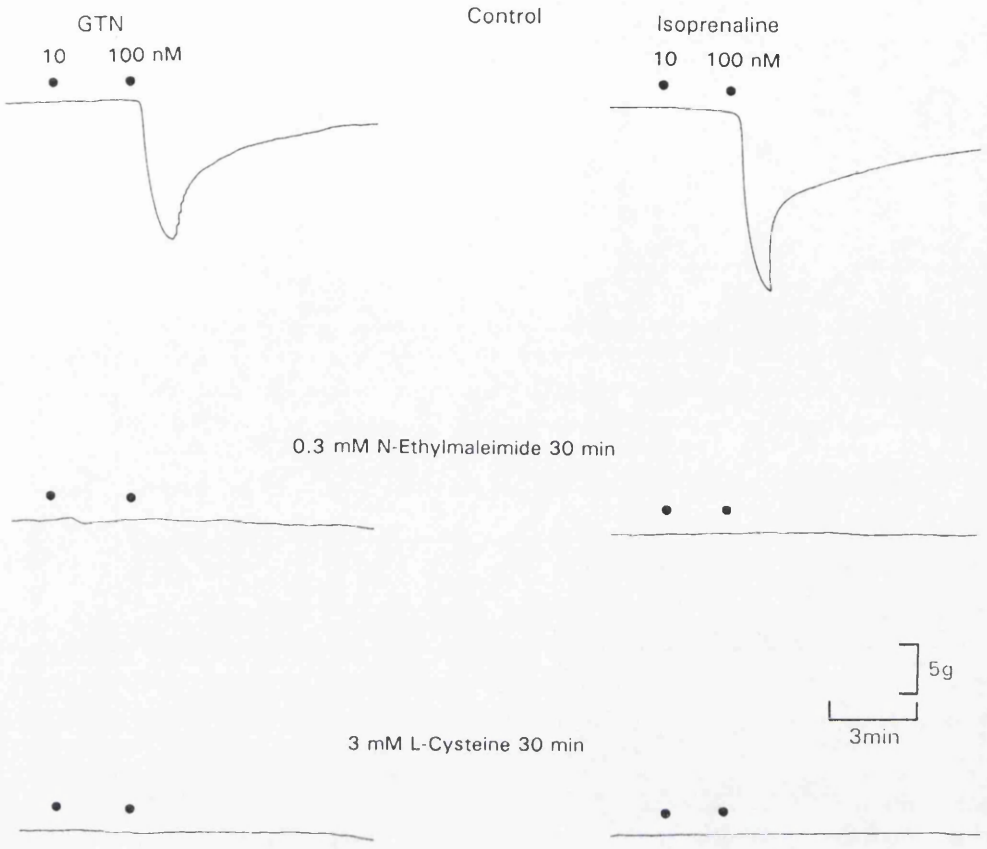
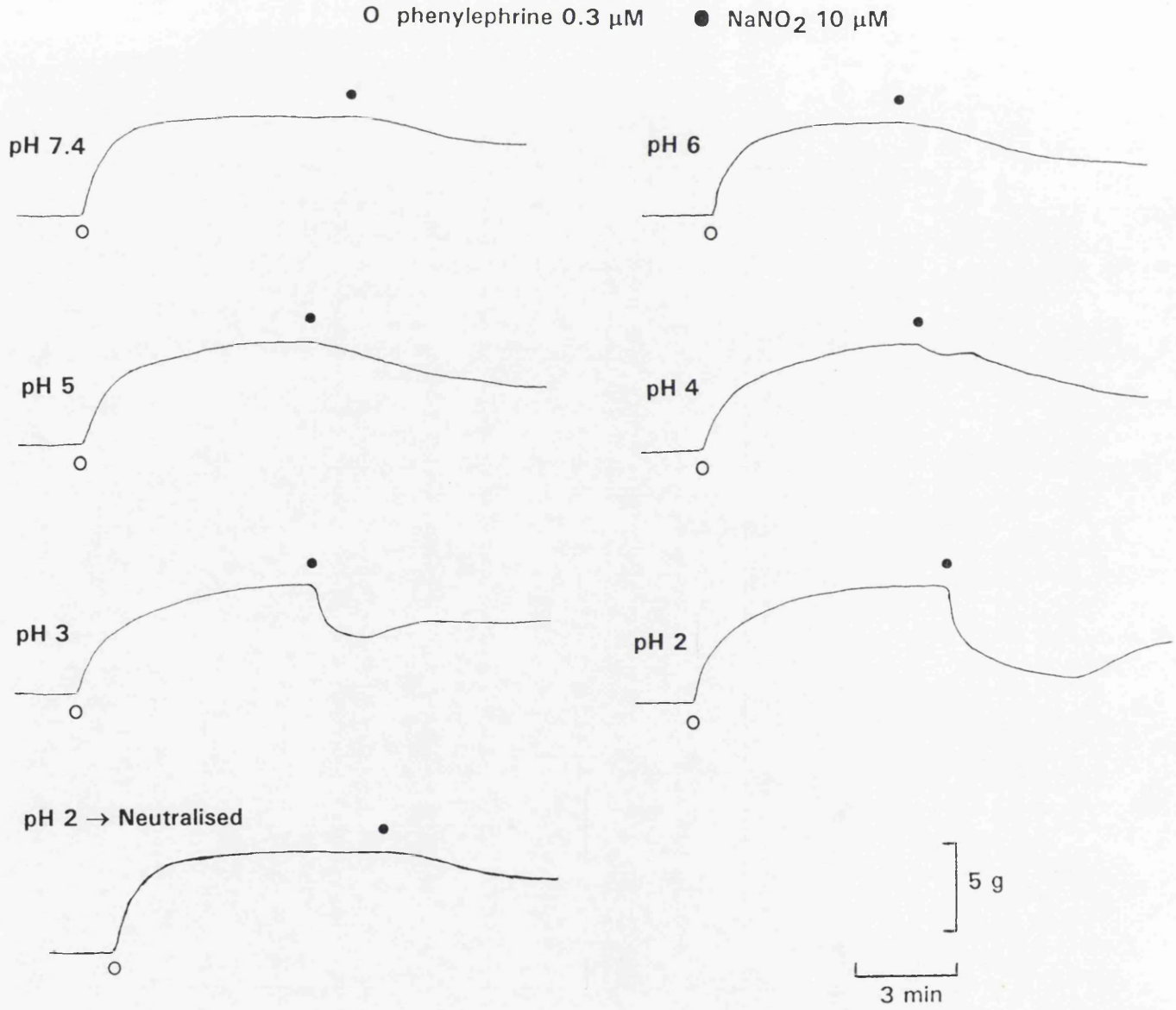


Figure 9. Treatment of BRP muscle with N-ethylmaleimide (0.3 mM) for 30 minutes completely blocked relaxation induced by glyceryl trinitrate (10-100 nM) or isoprenaline (10-100 nM). This blockade was not reversed after addition of L-cysteine (3 mM) for 30 minutes. Tone of the BRP muscle was raised by phenylephrine 0.3 μM.

Figure 10. Sodium nitrite (10 μM) had a weak relaxant activity when assayed on a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 μM). Acidification of these aqueous stock solutions (10 mM) resulted in the generation of a new relaxant activity in an acid pH-dependent manner; this was small and transient at pH 4, powerful and prolonged at pH 2, and was destroyed by neutralisation. However, the original weak relaxant activity of sodium nitrite remained unchanged upon acidification.


O phenylephrine 0.3 μM
● $NaNO_2$ 10 μM
pH 7.4
pH 6
pH 5
pH 4
pH 3
pH 2
pH 2 → Neutralised
5 g
3 min

The generation of this new relaxant activity was optimal at pH 2. The slowly developing relaxation was not affected by lowering the pH in the solutions of sodium nitrite. However, neutralisation of the acidified (pH 2) nitrite led to a rapid loss of the new relaxant activity. Simultaneous measurement of nitrite content in the stock solutions at various pH revealed that this new relaxant activity was associated with decreases in nitrite content (Figure 11). The powerful relaxant activity at pH 2 in the stock solution (Figure 10) was associated with a 27 % decrease in nitrite content (Figure 11), whereas at pH values above 4 where no new relaxant activity was generated (Figure 10) decreases in nitrite content were less than 3 % (Figure 11). Furthermore, the new relaxant activity was increased by purging the stock solution with oxygen-free nitrogen for 15 minutes before acidification in an open beaker, but was decreased by gassing the stock solution with oxygen for 15 minutes before acidification (Figure 12). Again, the slow relaxant activity was neither increased by purging the stock solution with oxygen-free nitrogen nor decreased by gassing the stock solution with oxygen.

When aqueous solutions of sodium nitrite (10 mM) were mixed with L-cysteine (1.5 M) at pH 7.4 and added to achieve bath concentrations equal to sodium nitrite 10 μ M and L-cysteine 1.5 mM, the relaxation produced was faster in onset and greater in magnitude (Figure 13) than that induced by sodium nitrite alone (Figure 10). Furthermore, when acidified to pH values of pH 6 to pH 2, more powerful and prolonged relaxant activities were produced in presence of L-cysteine (1.5 M, Figure 13) than in its absence (Figure 10). These greatly increased relaxant activities (Figure 13) were also associated with an acid pH-dependent decrease in nitrite content in the stock solutions of sodium nitrite (10 mM) and L-cysteine (1.5 M, Figure 14): the loss of nitrite was 68 % at pH 4 and almost 100 % at pH 3 and below (Figure 14). Unlike solutions of acidified sodium nitrite (10 mM), mixtures of L-cysteine (1.5 M) and sodium nitrite (10 mM) did not lose their relaxant activity when neutralised (Figure 13).

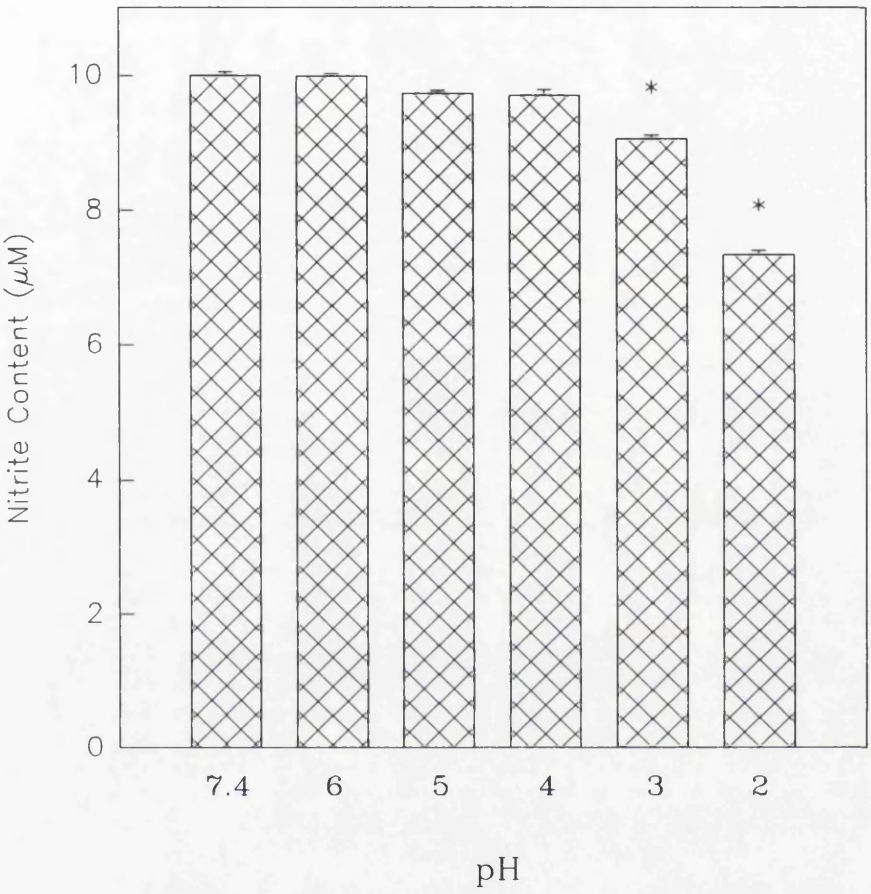


Figure 11. Acidification for 10 minutes led to pH-dependent decrease in nitrite content in aqueous solutions of sodium nitrite (10 μM). * $P<0.05$ indicates a significant reduction of nitrite content.

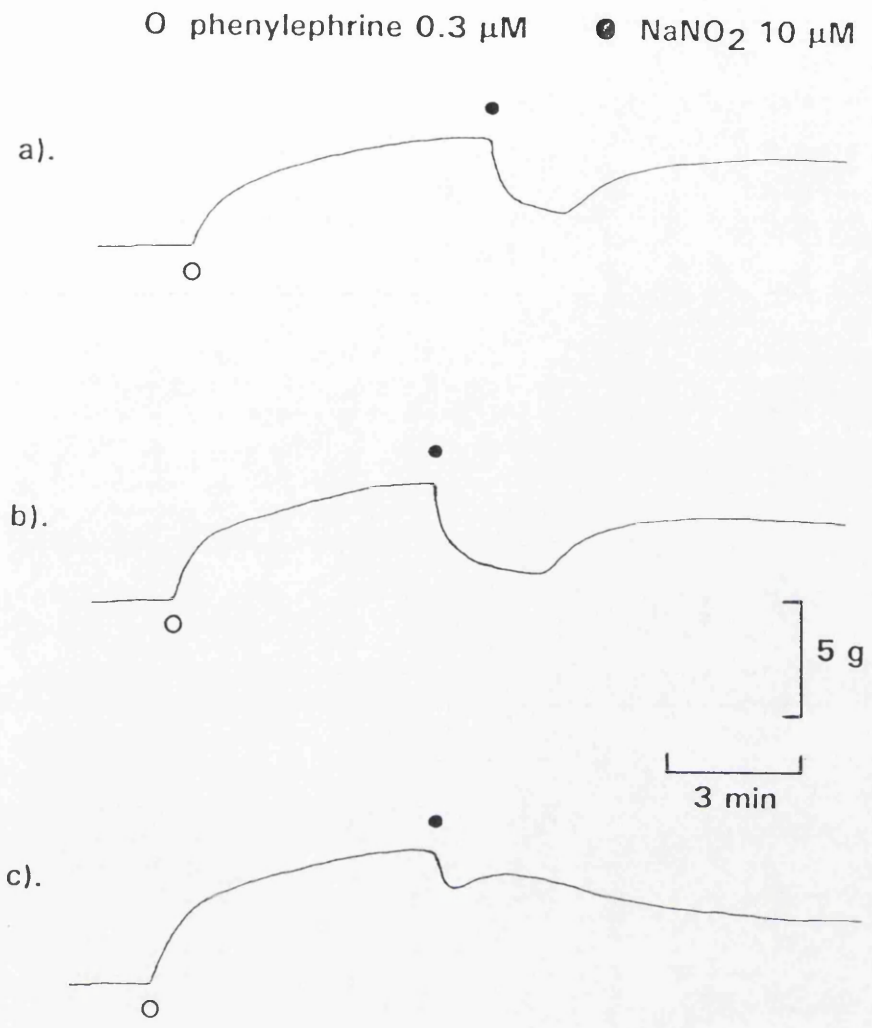
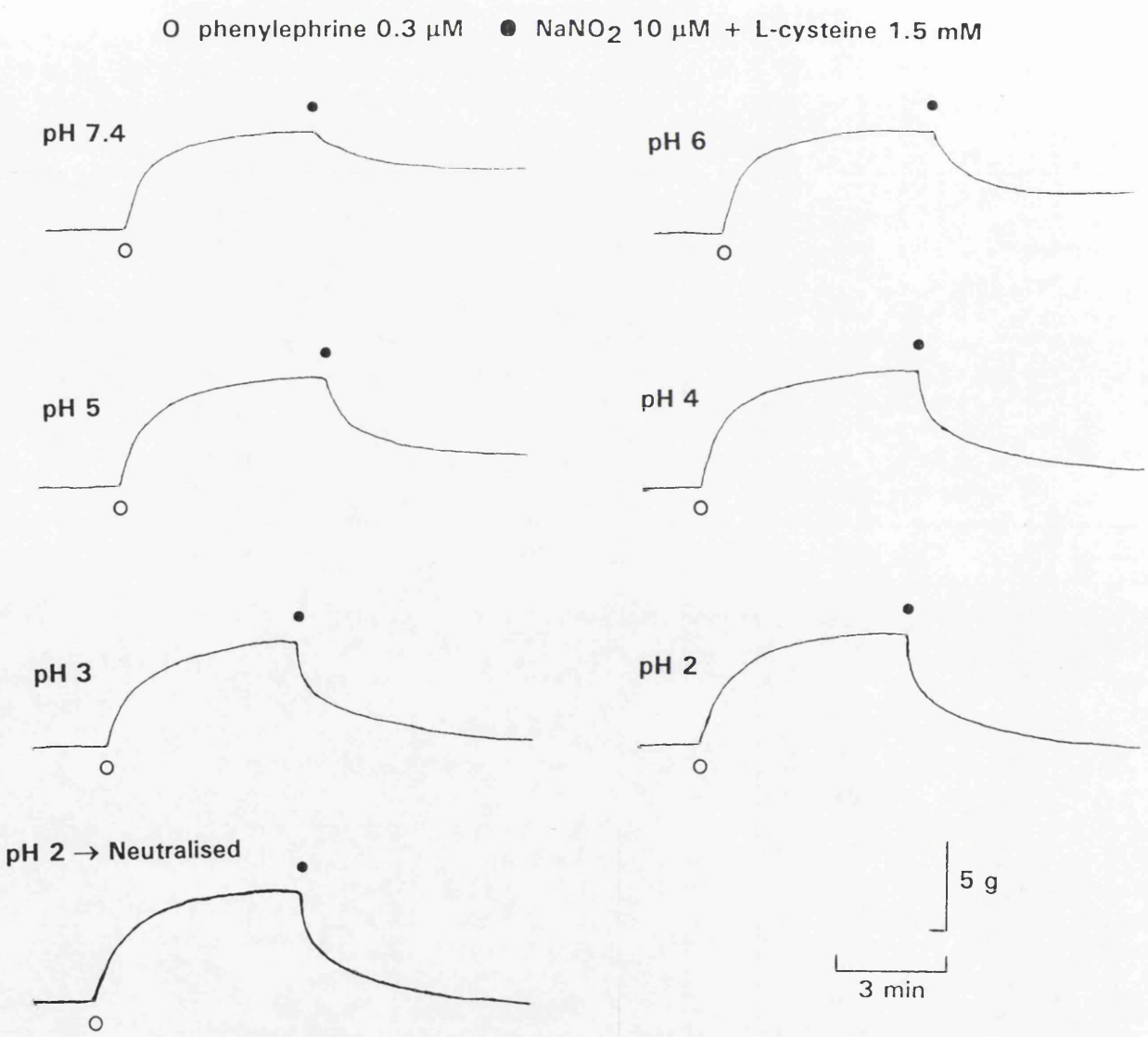


Figure 12. This figure shows the new relaxant activity generated by acidification (pH 2) of aqueous stock solutions of sodium nitrite (10 mM) at **a).** ambient oxygen tension; **b).** after purging with oxygen-free nitrogen for 15 minutes; **c).** after gassing with oxygen for 15 minutes. This activity was enhanced by deoxygenation but reduced by oxygenation of the solutions. The concentrations in the figure are final bath concentrations. Bioassay was performed on a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 μM).

Figure 13. This figure shows that the relaxant activity of aqueous mixtures of sodium nitrite (10 mM) and L-cysteine (1.5 M) was greatly enhanced by acidification for 10 minutes. This relaxant activity remained unchanged upon neutralisation. The concentrations in the figure are final bath concentrations. Bioassay was performed on a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 μM).


O phenylephrine 0.3 μM ● $NaNO_2$ 10 μM + L-cysteine 1.5 mM
pH 7.4
pH 6
pH 5
pH 4
pH 3
pH 2
pH 2 → Neutralised
5 g
3 min

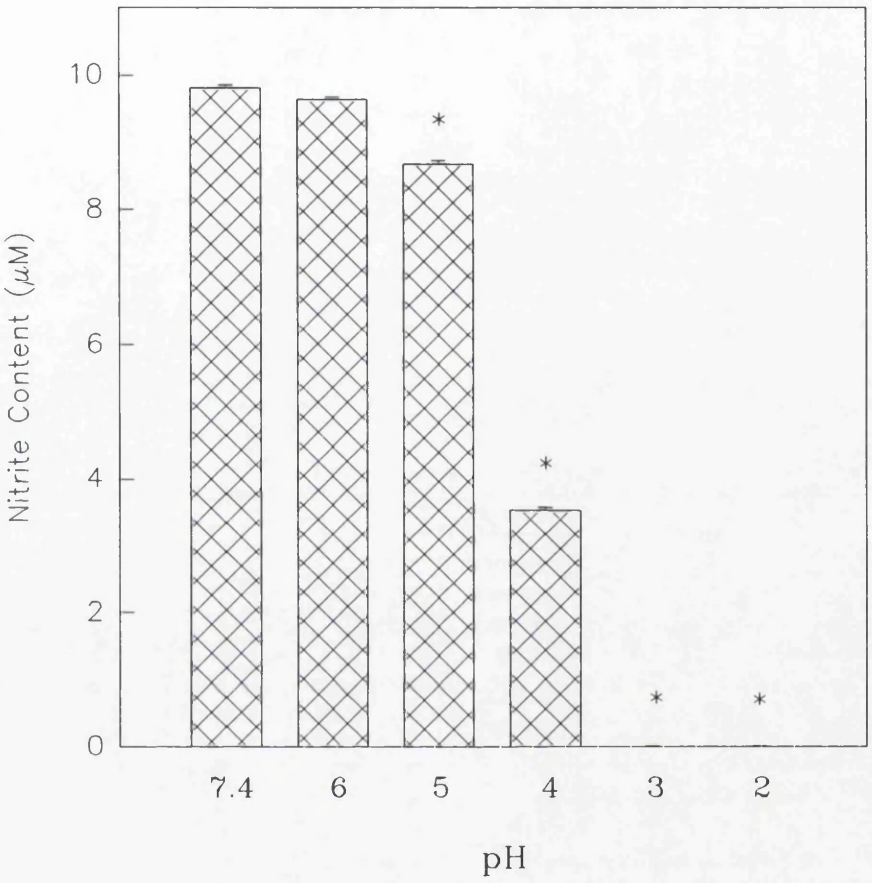


Figure 14. Acidification for 10 minutes led to a pH-dependent decrease in nitrite content in aqueous mixtures of L-cysteine (1.5 mM) and sodium nitrite (10 μM). At pH 3 and pH 2 nitrite was almost completely lost. * $P<0.05$ indicates a significant reduction of nitrite content.

At bath concentrations equal to sodium nitrite 10 μM and L-cysteine 1.5 mM, this new powerful relaxant activity generated by mixing sodium nitrite (10 mM) with L-cysteine (1.5 M) at pH 2 was completely blocked by haemoglobin (3 μM) but not by pretreatment of the rabbit aortic ring with L-nitroarginine for 15 minutes (Figure 15).

Thus, acidified nitrite in the presence of L-cysteine greatly enhanced relaxant activity. If S-nitrosothiols were produced *in vivo*, they would be formed from the reaction of nitric oxide and not nitrite with thiols. Consequently, a study was conducted to investigate the reactions of nitric oxide with thiols.

3.2.2. Relaxation Induced by Nitric Oxide and Mixtures of Nitric Oxide and L-Cysteine or L-Glutathione

Based on the report that the properties of S-nitrosocysteine more closely resembled those of EDRF than authentic nitric oxide (Myers, *et al.*, 1990) and that L-cysteine and L-glutathione are two of the most abundant free sulfhydryl-containing substances in the body, L-cysteine and L-glutathione were chosen to be studied.

When added into the bath, nitric oxide prepared in oxygen-free Milli-Q purified water induced a rapid concentration (1-1000 nM)-dependent transient relaxation of rabbit aortic rings denuded of endothelium and precontracted by phenylephrine (0.3 μM, Figure 16). The tone of the tissues was not affected by L-cysteine at concentrations of 0.15 to 4.5 mM but was slowly reduced at a concentration of 15 mM. L-Cysteine (1 mM) slightly reduced the magnitude but significantly prolonged the duration of subsequent exogenous nitric oxide (10 nM)-induced relaxation. It also changed the time course of the relaxation both in onset and offset from fast to slow. After washing L-cysteine out of the bath five times in 10 minutes, the

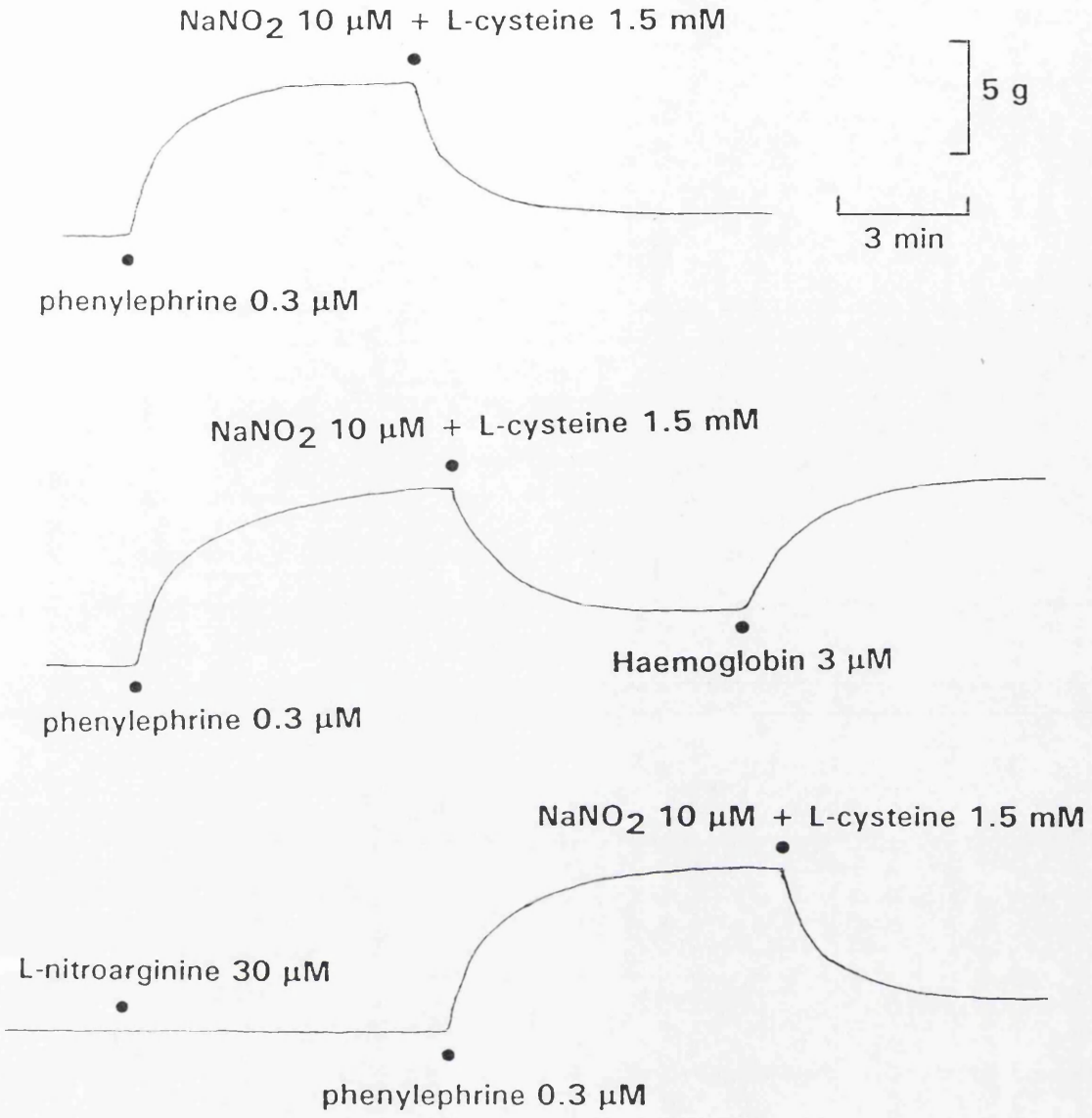


Figure 15. The powerful relaxant activity generated by mixing sodium nitrite (10 mM) with L-cysteine (1.5 M) in aqueous stock solutions at pH 2 for 10 minutes followed by neutralisation was completely blocked by haemoglobin (3 μM) when assayed on a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 μM). However, pretreating the tissue with L-nitroarginine (30 μ M) for 15 minutes did not affect the relaxant activity. The concentrations in the figure are final bath concentrations.

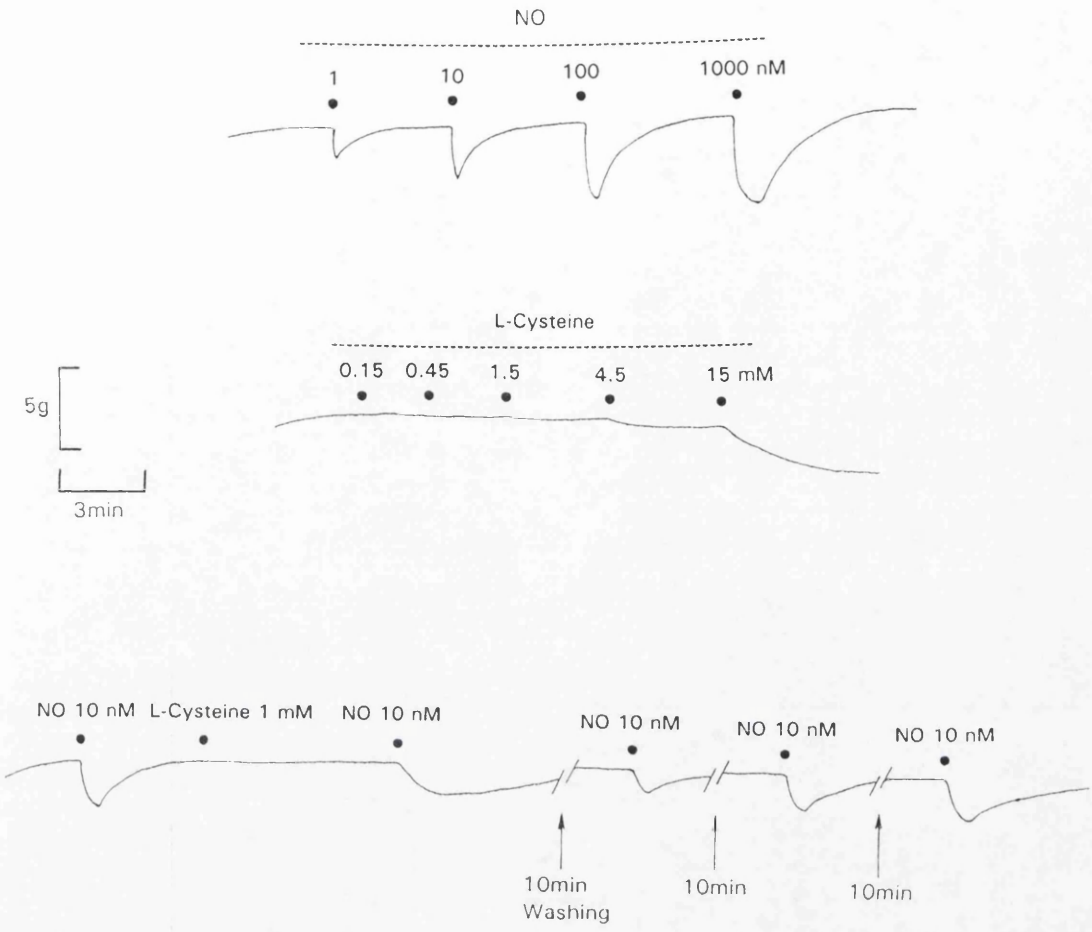


Figure 16. Nitric oxide (1-1000 nM), but not L-cysteine (0.15-15 mM), induced in a concentration-dependent manner transient rapid relaxation in rabbit phenylephrine (0.3 μM)-contracted aortic rings denuded of endothelium. In the presence of exogenous L-cysteine (1 mM) relaxation induced by exogenous nitric oxide (10 nM) was slightly reduced in magnitude and the time course of both onset and offset was slowed. After L-cysteine was washed out by changing the Krebs' solution 5 times in 10 minutes, the magnitude and duration of nitric oxide-induced relaxation were reduced. These effects of L-cysteine were slowly reversed within 30 minutes of washing.

magnitude and the duration of nitric oxide-induced relaxation were greatly reduced. This reduction in magnitude and duration slowly recovered within 30 minutes of washing.

Mixing nitric oxide (5 μM) with L-cysteine (0.75 M) in nominally oxygen-free aqueous solution at pH 2 followed by removal of free nitric oxide by purging with oxygen-free nitrogen and neutralisation with sodium hydroxide (1 N) and giving final bath concentrations equivalent to 10 nM nitric oxide and 1.5 mM L-cysteine produced more powerful and sustained relaxation than nitric oxide (10 nM) alone (Figure 17).

Since neither nitric oxide (10 nM) nor L-cysteine (1.5 mM) alone could account for such powerful and sustained relaxation, it was possible that a new substance had been generated from this mixture which was responsible for the relaxation. If this were true, then this new substance might be separable from the mixture by HPLC and only this new substance collected from HPLC should induce relaxation on a bioassay tissue. L-Cysteine (10 mM) produced two peaks in the HPLC (Figure 18), the first one (peak 1) corresponded to its oxidised form, L-cystine. L-Cysteine is slowly oxidised to L-cystine during storage and the retention time of peak 1 in L-cysteine solutions was identical with that of standard L-cystine solutions (Figure 19). The second peak (peak 2) corresponded to authentic L-cysteine (Figure 18). Mixing L-cysteine (10 mM) with nitric oxide (50 μM) in nominally oxygen-free aqueous solution at pH 3 followed by removal of free nitric oxide with oxygen-free nitrogen produced three peaks; the first two peaks corresponded to oxidised and reduced forms of L-cysteine respectively, with the third peak (peak 3) being the new substance. This new substance collected from the HPLC exhibited powerful and sustained relaxant activity when assayed on phenylephrine (0.3 μM)-precontracted rabbit thoracic aortic rings, whereas the reduced or oxidised forms of L-cysteine had no relaxant activity. There was no difference in the height of peak 1

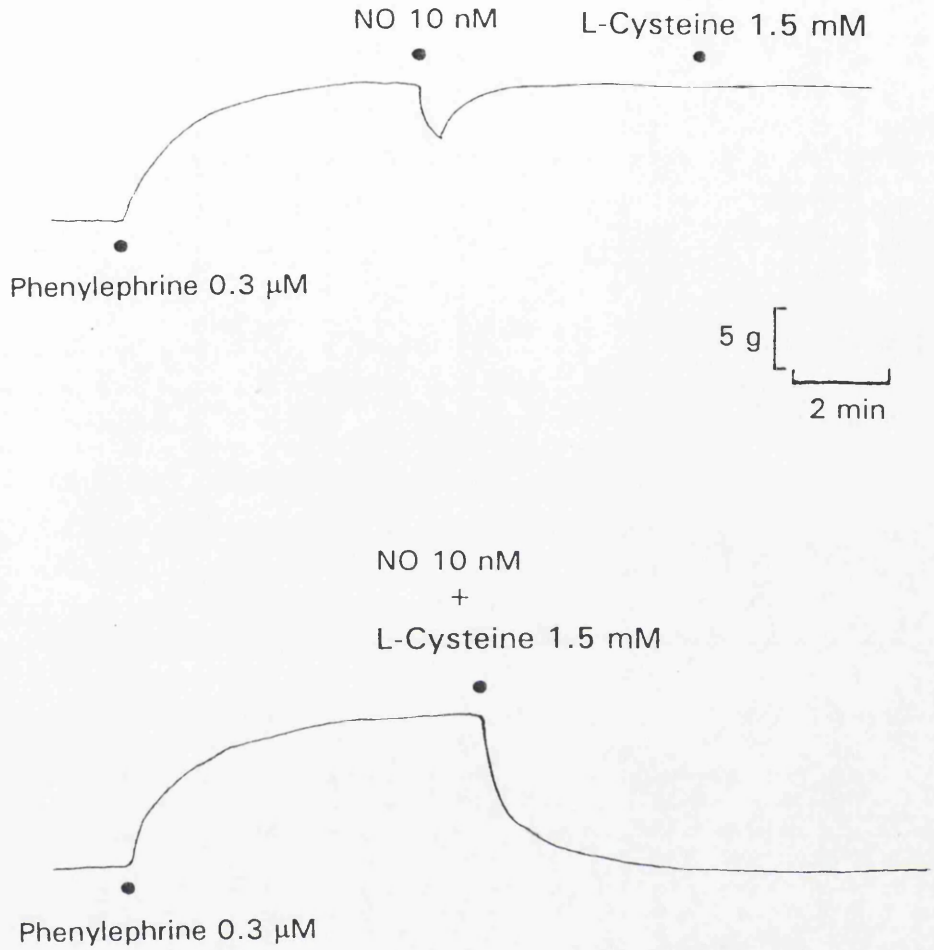


Figure 17. Nitric oxide (10 nM) induced a transient relaxation on rabbit endothelium-denuded aortic rings precontracted by phenylephrine (0.3 μM), whereas L-cysteine (1.5 mM) had no effect. Given the same bath concentrations, L-cysteine (0.75 M) mixed with nitric oxide (5 μM) in aqueous deoxygenated solution at pH 2.0 for 10 minutes followed by purging with oxygen-free nitrogen and neutralisation produced complete and sustained relaxation of the tissue.

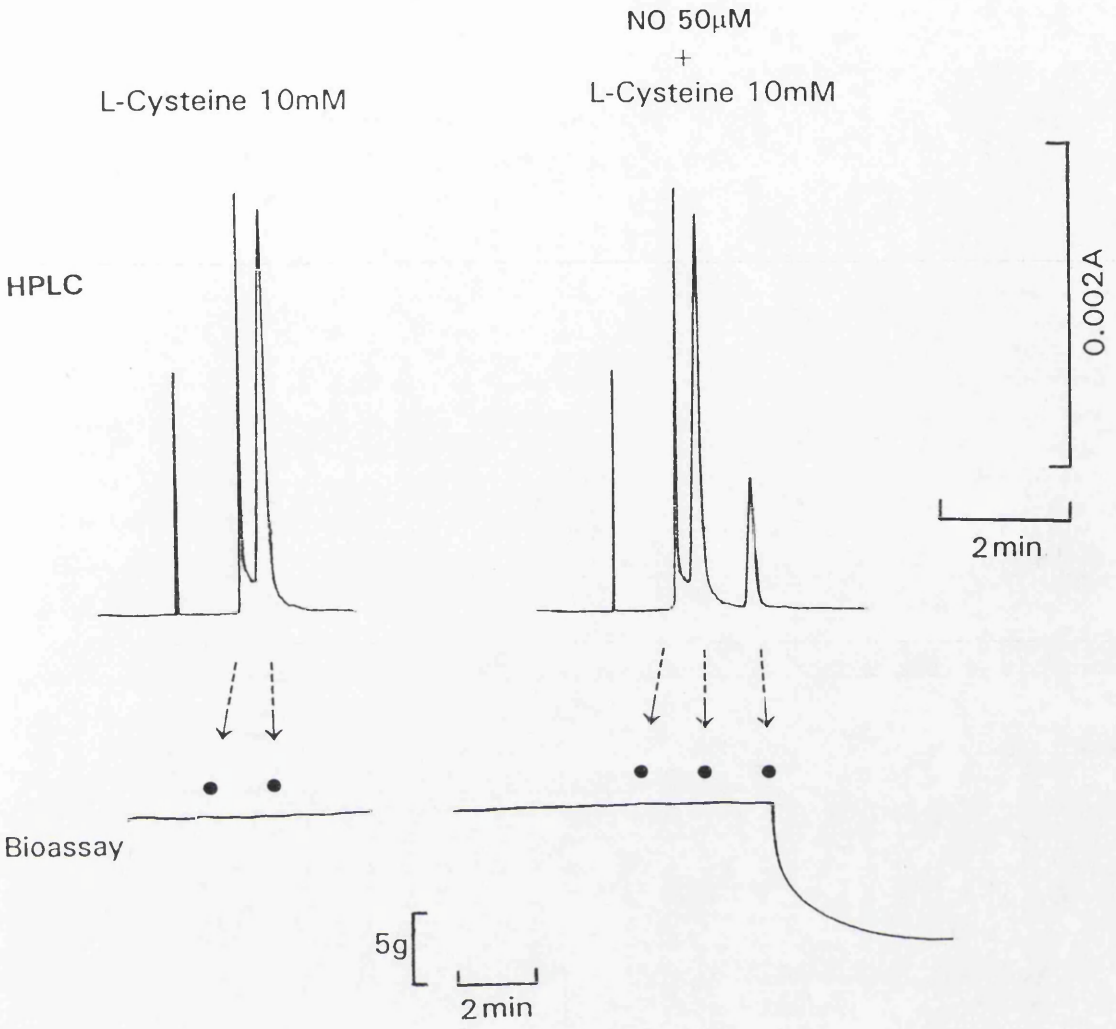


Figure 18. In the HPLC L-cysteine (10 mM) in aqueous solution at pH 3.0 produced two peaks, the first representing its oxidised form, L-cystine, and the second L-cysteine. After mixing this solution with nitric oxide (50 μM) under nominally oxygen-free conditions at pH 3.0 for 10 minutes followed by purging with oxygen-free nitrogen, this solution produced a new peak associated with powerful and sustained relaxant activity in the bioassay. HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent. Bioassay was conducted on a rabbit endothelium-denuded aortic ring precontracted by phenylephrine 0.3 μM.

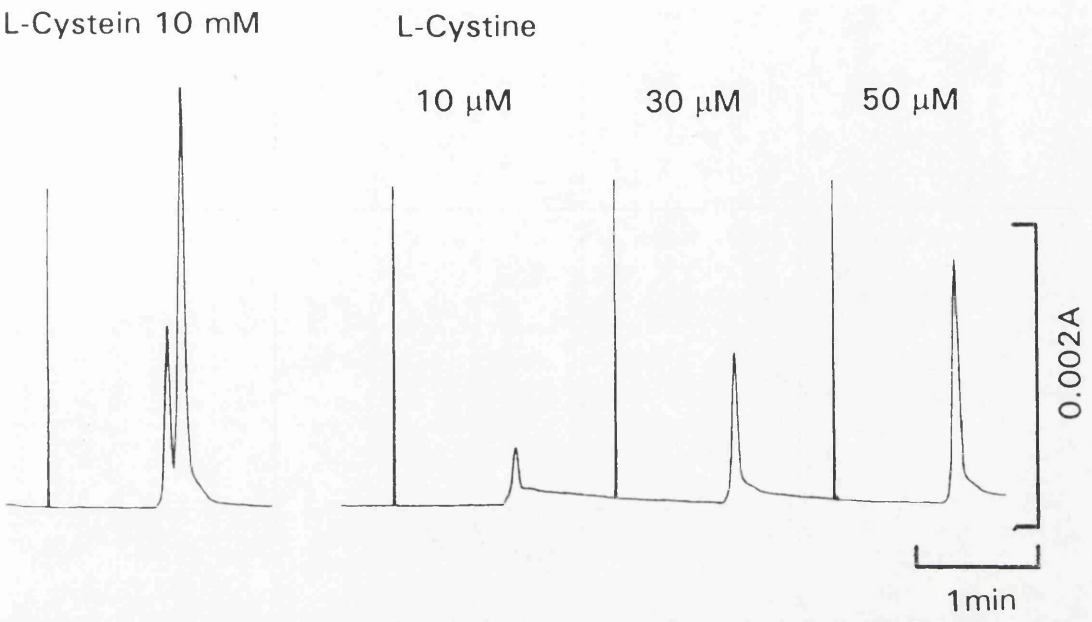


Figure 19. In the HPLC L-cysteine (10 mM) in aqueous solution at pH 3.0 produced two peaks; peak 1 was identical in retention time to that of standard solutions of L-cystine (pH 3.0). The magnitude of peak 1 was equivalent to L-cystine at around 40 μM. HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

before and after mixing with nitric oxide, whereas the height of peak 2 was reduced by 2% after mixing with nitric oxide.

L-Glutathione (10 mM) in aqueous solution (pH 3.0) produced one peak in HPLC (Figure 20). Mixing L-glutathione (10 mM) with nitric oxide (50 μM) in nominally oxygen-free aqueous solution at pH 3 followed by removal of free nitric oxide by purging with oxygen-free nitrogen produced two peaks; the first peak corresponded to L-glutathione in retention time and the second peak corresponded to a new substance. When bioassayed on rabbit aortic rings precontracted by phenylephrine (0.3 μM), this new substance collected from the HPLC exhibited powerful and sustained relaxant activity, whereas L-glutathione produced no relaxation. The peak height of L-glutathione after mixing with nitric oxide was reduced by an amount equivalent to the height of the new peak. Thus, the new relaxant appeared to have a similar extinction coefficient to L-glutathione.

3.2.3. Characteristics of the Relaxant Generated in Mixtures of Nitric Oxide and L-Cysteine

L-Cysteine (10 mM) was made up with Milli-Q purified water and adjusted to pH 3 with hydrochloric acid (1 N). An aliquot of this solution was gassed to saturation with nitric oxide under nominally oxygen-free conditions at pH 3 for 10 minutes followed by removal of free nitric oxide by purging with oxygen-free nitrogen. Separation of the two peaks in the original solution of L-cysteine and the three peaks following mixing nitric oxide with L-cysteine was performed in the HPLC. The five separated samples were collected from only the top 5% of each peak to ensure a high degree of purity.

3.2.3.1. Peak Maxima in UV-visible Spectrophotometry

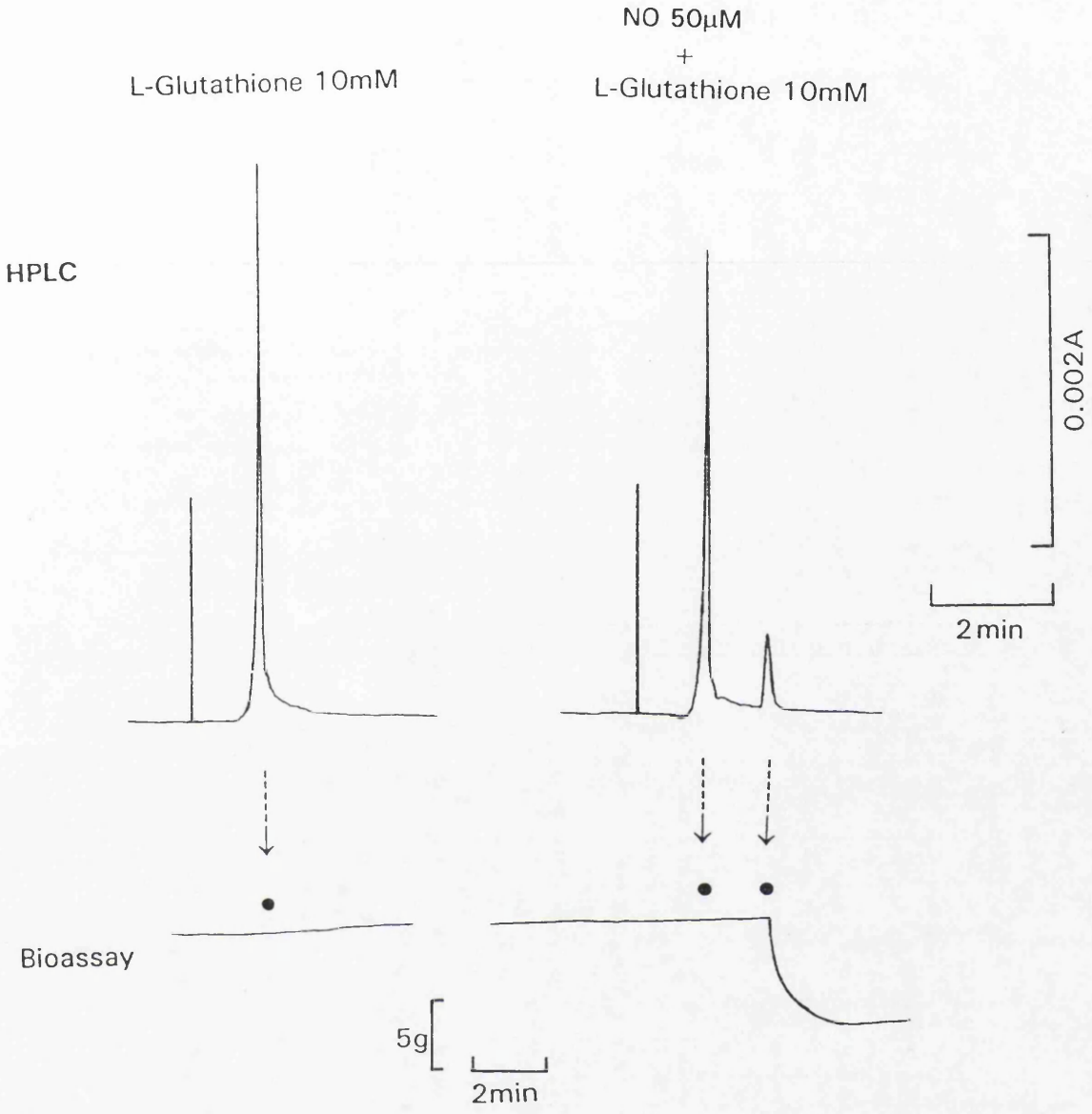


Figure 20. L-Glutathione (10 mM) in aqueous solution at pH 3.0 produced one peak in the HPLC. After mixing with nitric oxide (50 μM) under nominally oxygen-free conditions at pH 3.0 for 10 minutes followed by purging with oxygen-free nitrogen, this solution produced a new peak associated with powerful and sustained relaxant activity in the bioassay. HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent. Bioassay was conducted on a rabbit endothelium-denuded aortic ring which was precontracted by phenylephrine 0.3 μM.

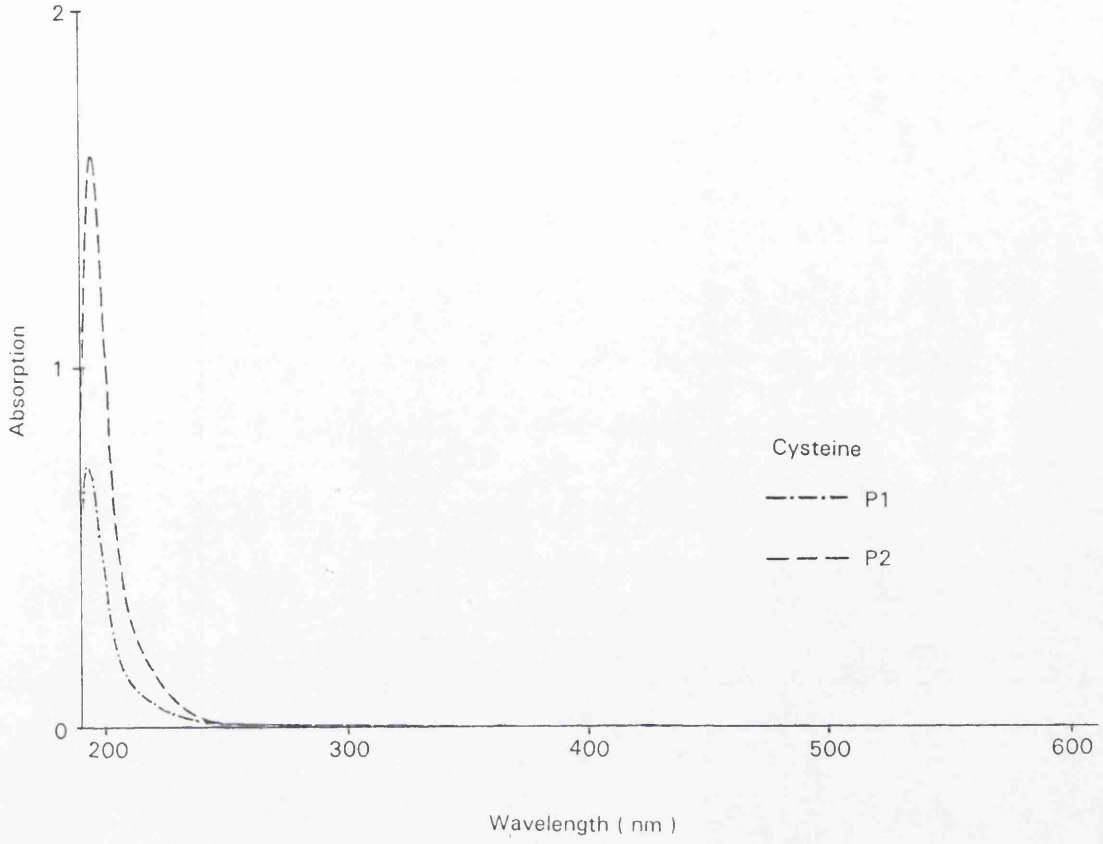
The UV-visible spectrophotometric characteristics of each of the five peaks were investigated by differential absorbance against the solvent Milli-Q water (pH 3) in a continuous scan at wavelengths from 900 nm down to 190 nm. HPLC peak 1 and peak 2 each contained only one absorption peak with maxima at 193 nm and 195 nm, respectively. The results were the same whether they were obtained from solutions of L-cysteine or from nitric oxide-bubbled solutions of L-cysteine (Figure 21). HPLC peak 3 produced three absorption peaks with maxima at 193, 218 and 335 nm; the highest was at 218 nm and the lowest was at 335 nm.

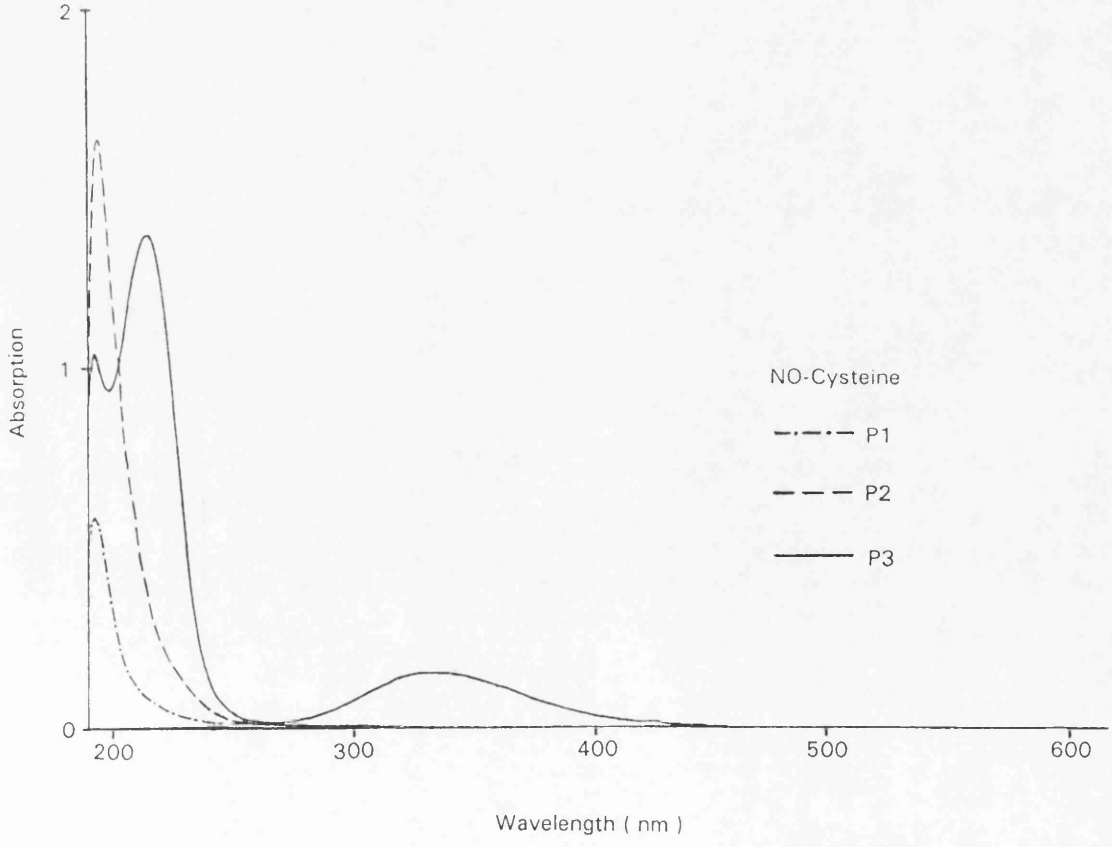
3.2.3.2. Stability

When each of the three separated peaks from nitric oxide-bubbled L-cysteine solutions was collected and immediately re-run in the HPLC, it was revealed that peak 1 was very stable but peaks 2 and 3 were not (Figure 22); when re-run, peak 1, previously identified as L-cystine, gave only one peak at the same retention time. Peak 2, previously identified as L-cysteine, was split into two peaks whose retention times were equal to those of peak 1 and peak 2, respectively. Peak 3, previously identified as the novel relaxant, also generated another peak whose retention time was equal to that of peak 1. Thus, both peak 2 (L-cysteine) and peak 3 (novel relaxant) decayed to give peak 1 (L-cystine).

Peaks 1, 2 and 3 were collected and stored in glass test tubes at -20^{o}C in the deep freeze for 12 hours and then retested in the HPLC and bioassay. Peak 1 still ran as a single peak, peak 2 partially decayed to peak 1, and peak 3 also partially decayed to peak 1 but retained much of its relaxant activity when assayed on phenylephrine (0.3 μM)-contracted BRP strips (Figure 23). Following freeze drying for 12 hours and reconstitution in an identical volume, peak 1 still ran as a single peak in the HPLC, peak 2 partially decayed to peak 1, but peak 3 completely decayed to peak 1 and this was associated with loss of all of its relaxant activity. Thus the novel

Figure 21. Differential spectrophotometry of the peaks collected following HPLC separation of L-cysteine (10 mM) before (Cysteine) and after (NO-Cysteine) gassing with nitric oxide in aqueous deoxygenated solutions at pH 3.0 for 10 minutes and followed by purging with oxygen-free nitrogen. HPLC peaks 1 (P1) and 2 (P2) each had one absorption peak with a maximum at 193 nm and 195 nm, respectively. HPLC peak 3 (P3) had three absorption peaks with maxima at 193, 218 and 335 nm.


2
1
0
Absorption
200
300
400
500
600
Wavelength (nm)
Cysteine
P1
P2


2
1
0
Absorption
200
300
400
500
600
Wavelength (nm)
NO-Cysteine
P1
P2
P3

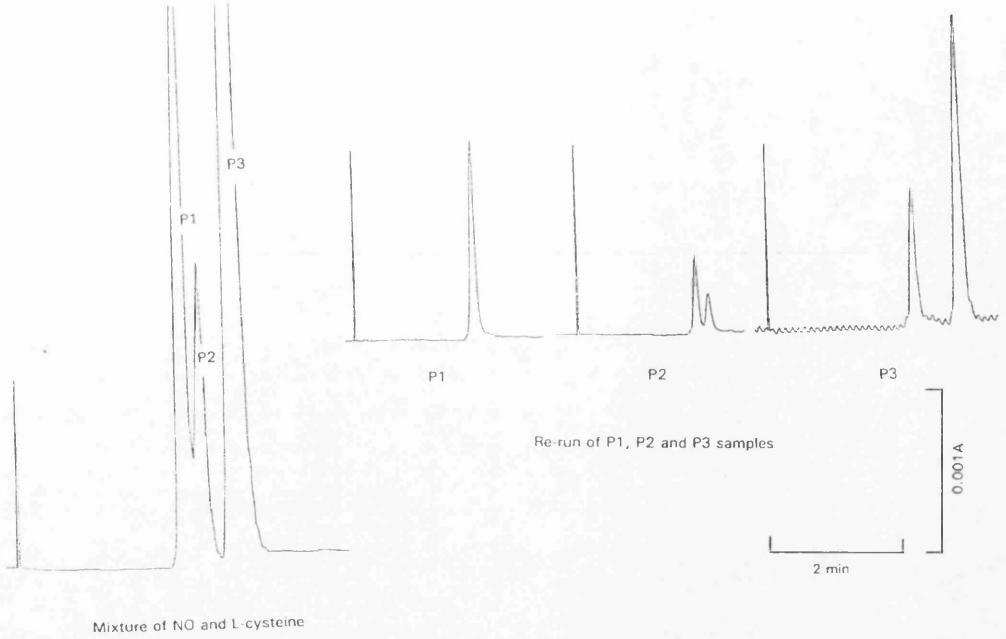


Figure 22. In the HPLC the three peaks (P1, P2 and P3) were separated from a solution in which L-cysteine (10 mM) at pH 3.0 was bubbled to saturation with nitric oxide for 10 minutes under nominal oxygen-free conditions followed by purging with oxygen-free nitrogen. Re-run of P1, P2 and P3 collected from the top 5% of each peak showed that P1 was stable, and that P2 and P3 partially decayed to P1. HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

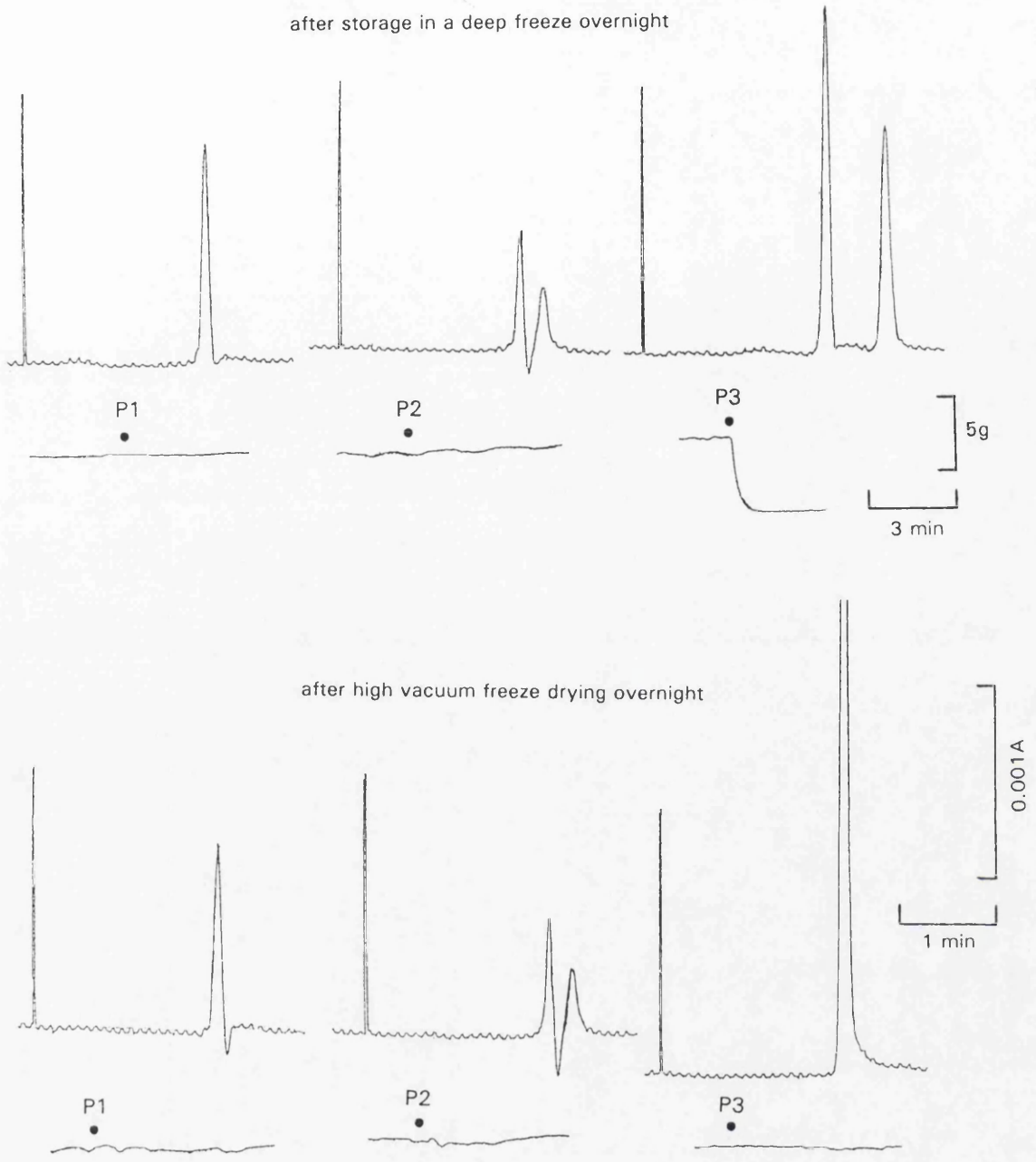


Figure 23. Following separation by HPLC, peaks 1 (P1), 2 (P2) and 3 (P3) were collected and stored in the deep freeze (-20^{o}C) or freeze dried overnight. Peak 1 was stable after freezing or freeze drying. Peak 2 partially decayed to peak 1 after freezing or freeze drying. Peak 3 gradually decayed to peak 1 after freezing but retained most of its relaxant activity. After freeze drying all of peak 3 decayed to peak 1 and this was associated with a complete loss of its relaxant activity as assessed on phenylephrine (0.3 μM)-precontracted strips of BRP. HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

relaxant contained in HPLC peak 3 is relatively stable in the freezer, but is completely destroyed by freeze drying.

The relaxant activity contained in the HPLC peak 3 was not sensitive to oxygen (Figure 24). After gassing with pure oxygen for 10 minutes at room temperature, the behaviour of peak 3 did not change in the HPLC or bioassay; like the control, it partially decayed into peak 1.

Treatment of the peak 3 sample at 70°C for 10 minutes had little effect on its relaxant activity as assessed on phenylephrine (0.3 μM)-contracted BRP strips but a small reduction in peak 3 and a slight increase in peak 1 were seen when the sample was re-run in the HPLC (Figure 25). However, when the peak 3 sample in an open glass test tube was placed in boiling water for 10 minutes, much of its relaxant activity was lost when bioassayed (Figure 26). Furthermore, its peak 3 was almost completely lost and this was accompanied by a large increase in peak 1 in the HPLC. Thus, the new relaxant is relatively stable at 70°C but not when boiled.

The new relaxant activity was destroyed by methanol (Figure 27); mixing the peak 3 sample with pure methanol (HPLC grade, 1:1) at 0°C for 10 minutes resulted in a significant decrease in relaxant activity, a reduction of its peak 3 component by 50 %, an increase in the height of its peak 1 component and generation of three more new peaks in the HPLC.

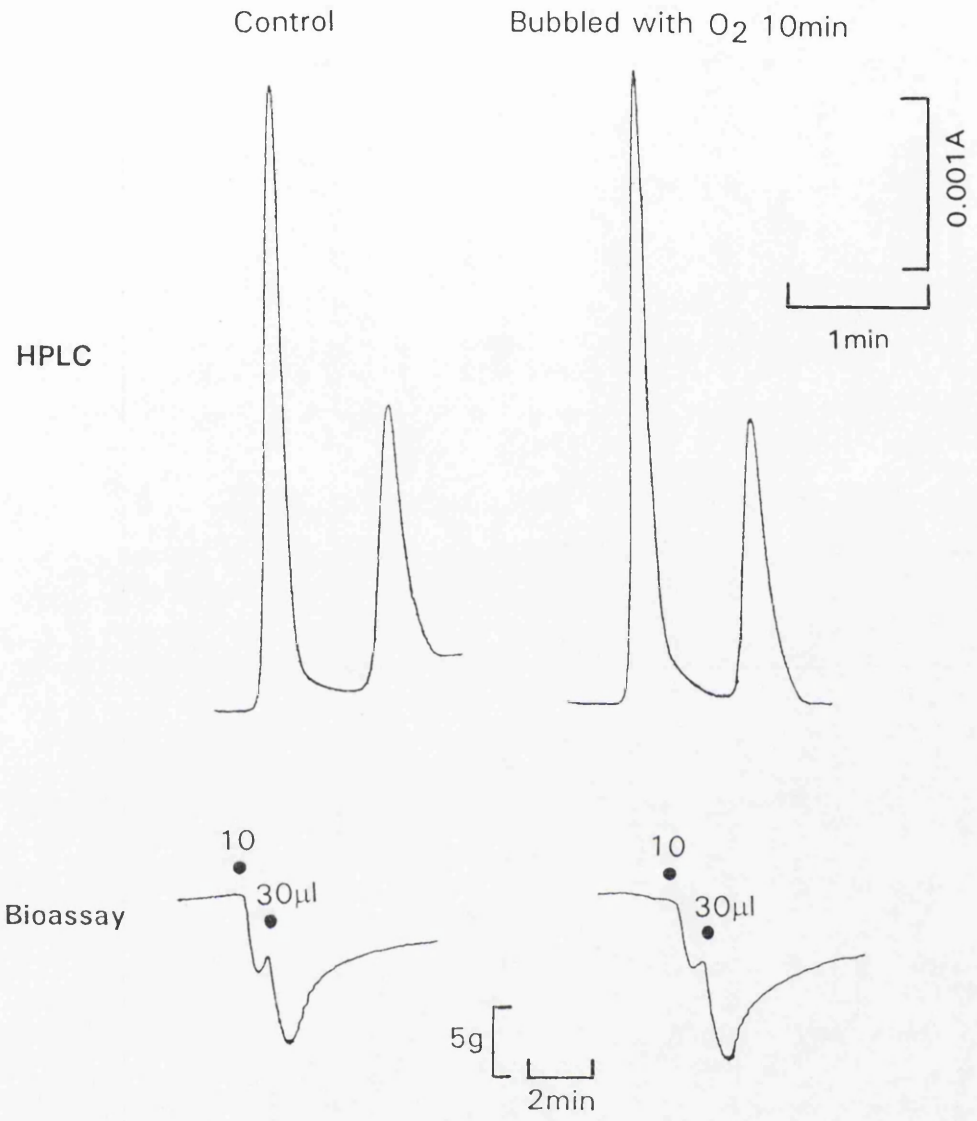


Figure 24. Bubbling the peak 3 relaxant fraction collected from the HPLC with oxygen for 10 minutes changed neither its behaviour in the HPLC nor its relaxant activity. Tone of the BRP muscle was raised by phenylephrine (0.3 µM). HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

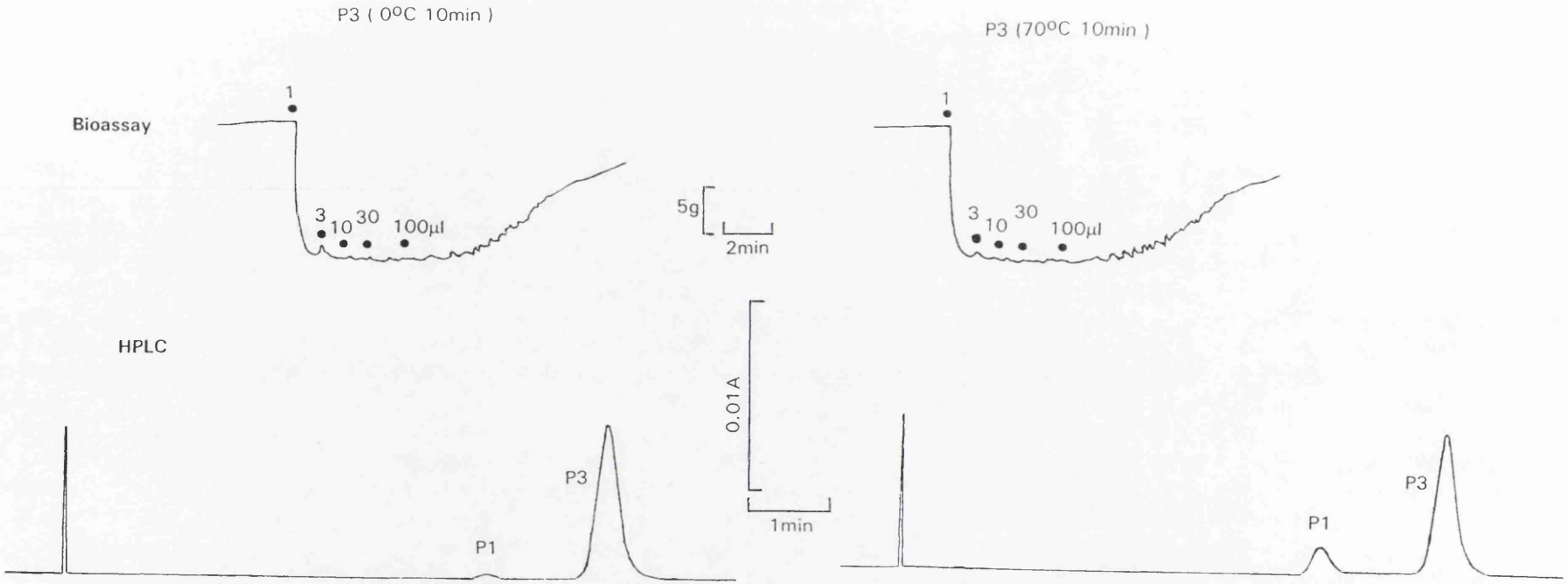


Figure 25. Treatment of the peak 3 relaxant fraction collected from the HPLC in an open test tube at 70°C for 10 minutes resulted in no change in its relaxant activity but slightly reduced the height of peak 3 (P3) and increased the height of peak 1 (P1) in the HPLC. Tone of the BRP muscle was raised by phenylephrine (0.3 μM). HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

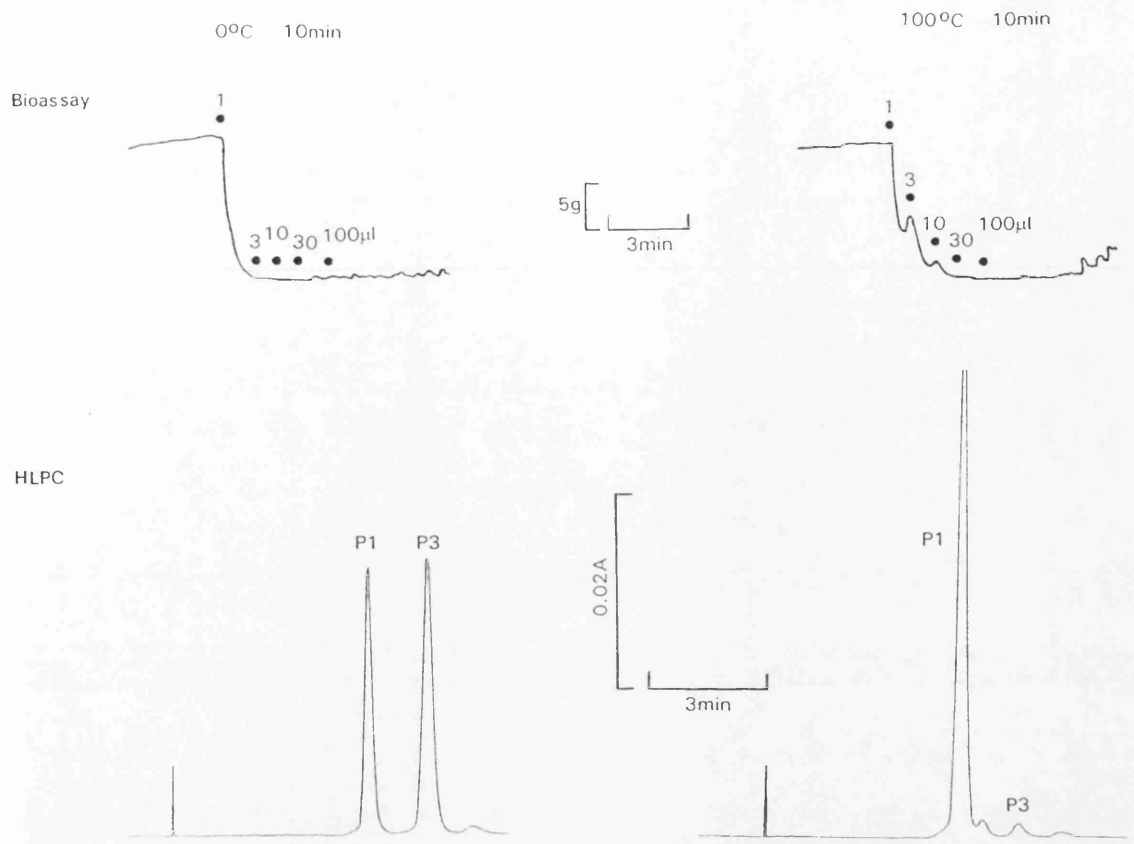


Figure 26. Treatment of the peak 3 relaxant fraction collected from the HPLC in an open test tube at 100°C for 10 minutes resulted in a decrease in its relaxant activity in association with a nearly complete conversion of peak 3 (P3) to peak 1 (P1) in the HPLC. Tone of the BRP muscle was raised by phenylephrine (0.3 µM). HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

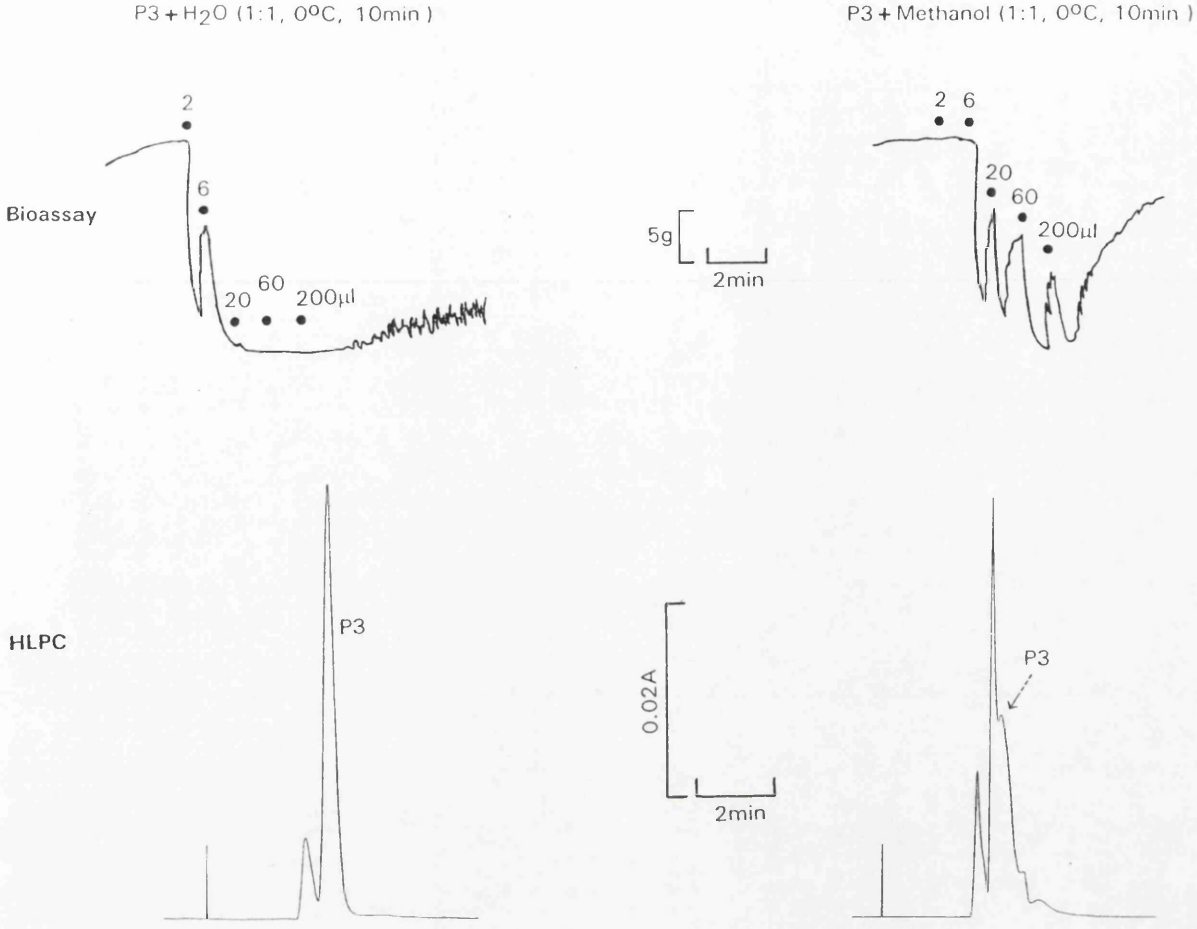


Figure 27. Treatment of the peak 3 relaxant fraction collected from the HPLC with methanol (1:1 in volume) for 10 minutes resulted in a decrease in its relaxant activity in association with a 50% decrease in the height of peak 3 (P3), a two-fold increase in the height of peak 1 and the generation of three other peaks in the HPLC. Tone of the BRP muscle was raised by phenylephrine (0.3 µM). HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

3.2.4. Conditions Favouring the Formation of the New Relaxant in Mixtures of Nitric Oxide and L-Cysteine

3.2.4.1. Acid pH-Dependence

Under nominally oxygen-free conditions formation of the powerful new smooth muscle relaxant in mixtures of nitric oxide and L-cysteine was strictly acid pH-dependent. Figure 28 shows a bioassay on a rabbit phenylephrine (0.3 μM)-contracted aortic ring of mixtures of nitric oxide (100 μM) and L-cysteine (15 mM) in aqueous solutions at pH values ranging from 7.4 to 1.0. After 10 minutes these solutions were purged with oxygen-free nitrogen to remove free nitric oxide and then adjusted to pH 7.4 respectively. The lower the reaction pH, the higher was the relaxant activity; at pH 7.4 little relaxant activity was generated and at pH 1.0 maximal relaxant activity was generated.

Figure 29 shows that in the HPLC the formation of the new relaxant in nominally oxygen-free L-cysteine aqueous solutions following mixing with nitric oxide was not only acid pH-dependent but also dependent upon the concentration of nitric oxide added. L-Cysteine (10 mM) alone gave a single peak in the HPLC rather than two peaks because the efficiency of the column was poor on this day. The new relaxant was therefore represented here as peak 2 rather than peak 3. At pH 5 and pH 7 more L-cysteine was oxidised to L-cystine which has 125 times the absorption of L-cysteine and this accounts for the high absorption of peak 1 at these pH values. At pH 7 the new relaxant (peak 2) was only detected when solutions of L-cysteine (10 mM) were gassed with nitric oxide for 10 minutes but not if nitric oxide was added at a concentration of 50 or 500 μM. At pH 5 all of these concentrations of nitric oxide generated peak 2 following reaction with L-cysteine (10 mM) and the size of the peak was proportional to the amounts added. At pH 3 again the

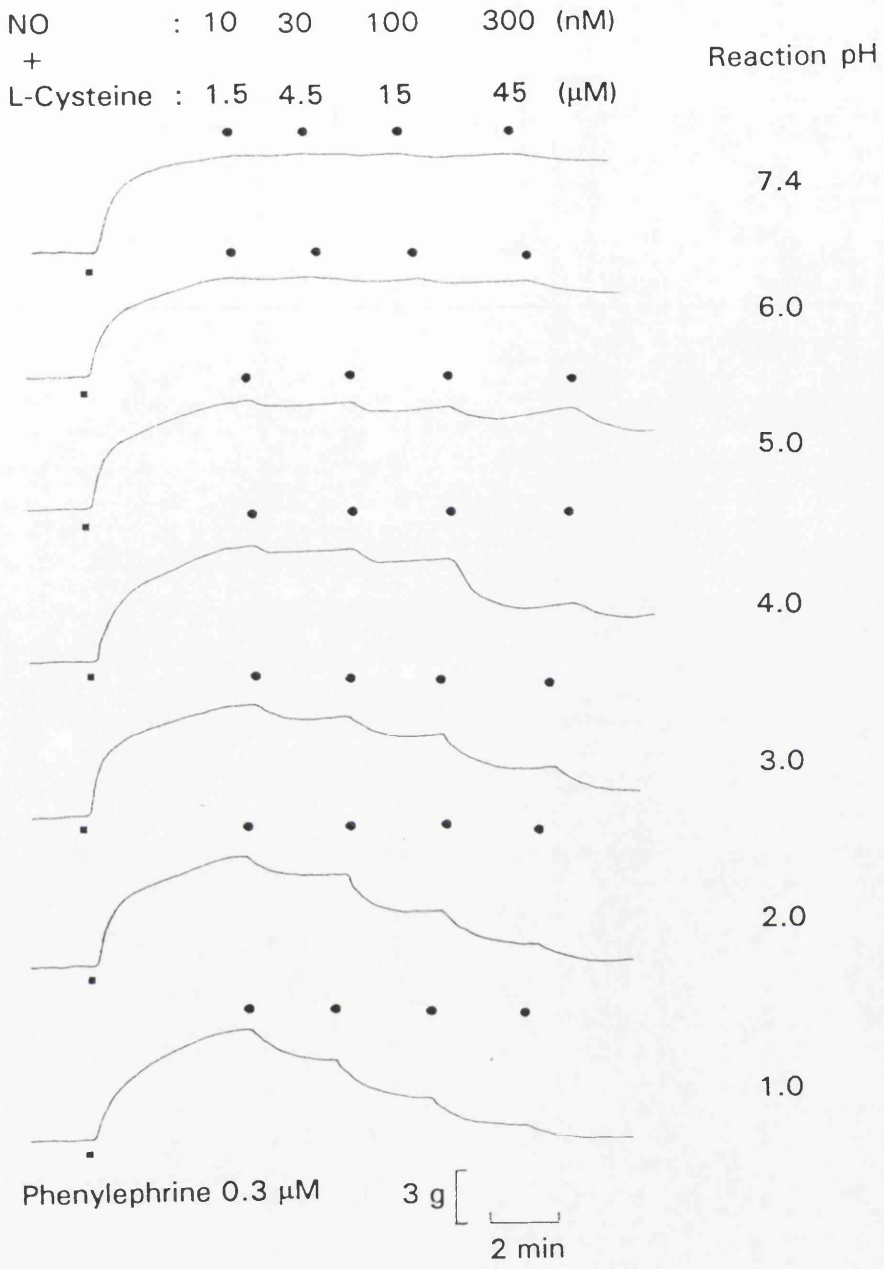
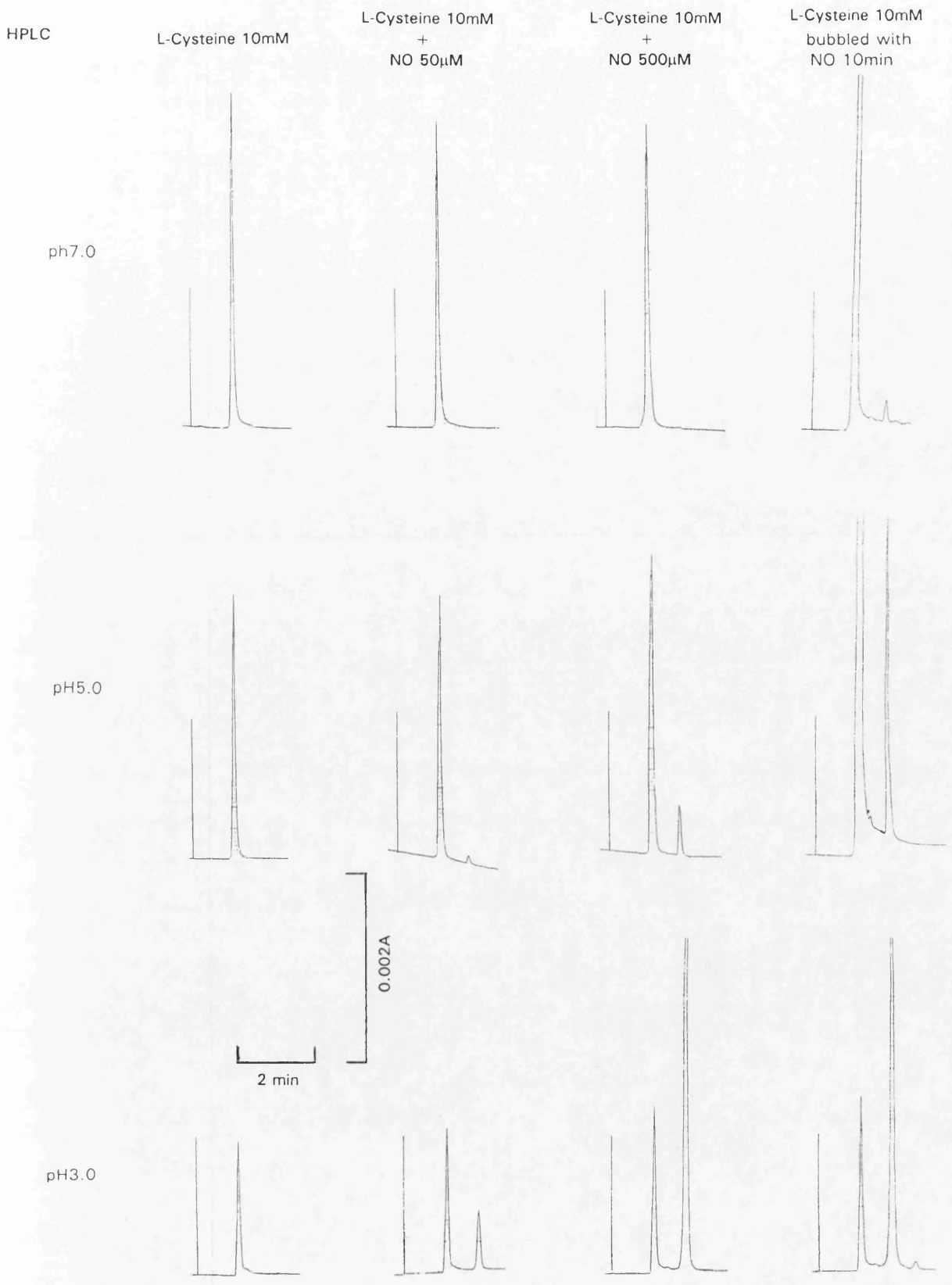


Figure 28. Generation of powerful relaxant activity from the reaction of nitric oxide (100 μM) and L-cysteine (15 mM) was strictly pH-dependent; following removal of free nitric oxide by purging with oxygen-free nitrogen and neutralisation, little activity was generated at pH 7.4 and maximal activity was achieved at pH 1.0. Bioassay was performed in a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 μM). The concentrations refer to the final bath concentrations of the reactants.

Figure 29. HPLC analysis showed that the formation of the new relaxant following reaction of L-cysteine (10 mM) and nitric oxide (50 μM to saturation) was acid pH- and concentration-dependent. In this experiment the efficiency of the column was lower than on previous days and L-cysteine and its oxidised form, L-cystine, eluted as a single rather than a double peak. The new relaxant formed by reacting L-cysteine and nitric oxide was therefore eluted as a second rather than a third peak. At pH 7 the novel relaxant represented by peak 2 was only formed if nitric oxide was bubbled to saturation but not if it was added at 50 or 500 μM. At pH 5 peak 2 was formed with all three concentrations of nitric oxide with the amounts formed being proportional to the concentrations of nitric oxide added. At pH 3 the height of peak 2 was again proportional to the concentrations of nitric oxide and the amounts formed were greater than at pH 7 or pH 5. HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.


HPLC
L-Cysteine 10mM
L-Cysteine 10mM + NO 50μM
L-Cysteine 10mM + NO 500μM
L-Cysteine 10mM bubbled with NO 10min
ph7.0
pH5.0
pH3.0
0.002A
2 min

magnitude of peak 2 was proportional to the amount of nitric oxide added, but the peak sizes were greater than at pH 7 or pH 5.

3.2.4.2. Oxygen Dependence

Although all previous experiments involving the reaction of nitric oxide and L-cysteine were conducted under nominally oxygen-free conditions, we suspected that the techniques used were not sufficient to completely exclude oxygen. We therefore adopted the more rigorous procedures described in Materials and Methods (see section 2.3.1. and Figure 2) in which trace amounts of oxygen and nitrogen dioxide were removed from nitric oxide and commercial "oxygen-free" nitrogen gases using Fieser's solution and sodium hydroxide respectively.

3.2.4.2.1. Assessment of Ability to Exclude Oxygen

Before investigating the reaction of nitric oxide with L-cysteine in the absence and presence of oxygen, it was essential to ensure that no oxygen or nitrogen dioxide was present in the reactants and the apparatus. Nitric oxide readily reacts with oxygen to form nitrogen dioxide and both oxides of nitrogen can be detected upon spectrophotometric examination. Under rigorous oxygen-free conditions, purified oxygen-free nitrogen gas was bubbled into 1 ml of purified Milli-Q water in a cuvette for an hour and then the solution was bubbled to saturation for 30 minutes with purified nitric oxide gas. Figure 30 shows the results of scanning the solution in a spectrophotometer between 190 to 410 nm. Nitric oxide (saturated) in water had only one absorption peak with a maximum at 190 nm. The stopper was then removed exposing the solution to the air and immediately replaced back on the cuvette. The nitric oxide solution was then mixed with the introduced air by inversion 5 times every 10 seconds with introduction of air again after every minute. Formation of nitrogen dioxide was indicated by its characteristic absorption

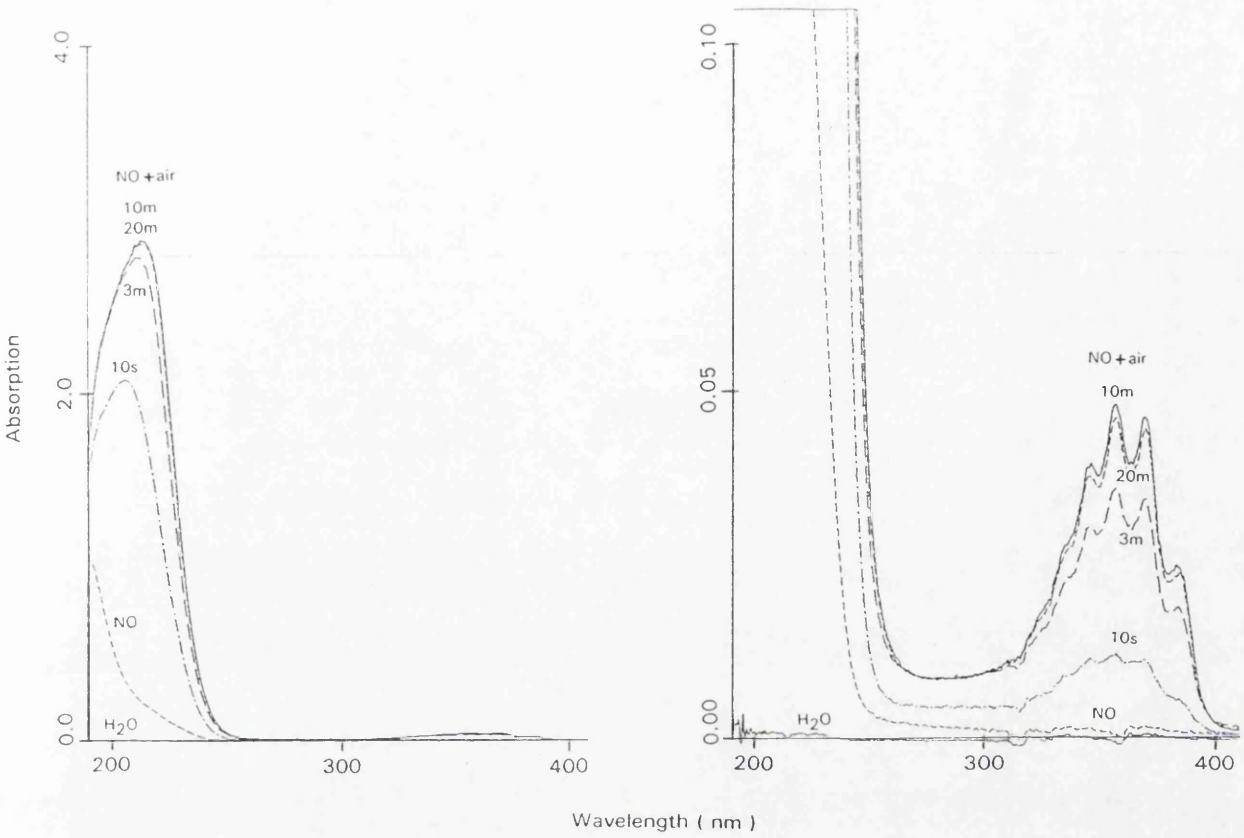


Figure 30. Differential spectrophotometry of nitric oxide-saturated water under rigorous oxygen-free conditions and its spectrophotometric development of absorption after mixing with air for 10 seconds (10s), 3 (3m), 10 (10m) and 20 (20m) minutes. Nitric oxide-saturated water had an absorption peak with a maximum at 190 nm. The appearance of the characteristic absorption in the 300-400 nm wavelength region indicated formation of nitrogen dioxide in water. Also the maximum at 190 nm was gradually shifted toward 232 nm after introduction of air. Note the two different scales used to measure absorbance.

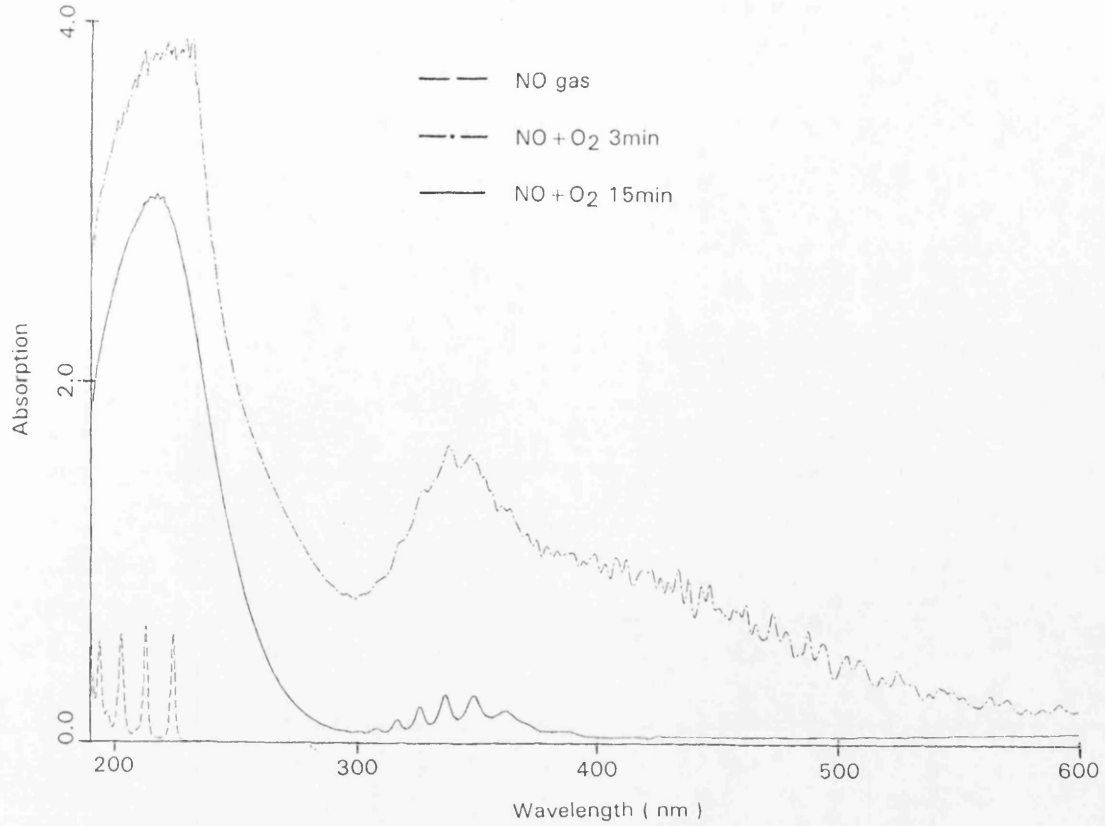
(multiple peaks) at 300-400 nm. Also, the peak maximum at 190 nm gradually shifted toward 232 nm. Significant absorption changes were measured even with 10 seconds of mixing with air and maximal changes were achieved in 10 minutes. Thus, if any trace of oxygen is present during preparation of nitric oxide solutions, there will be a shift of maximum at 190 nm.

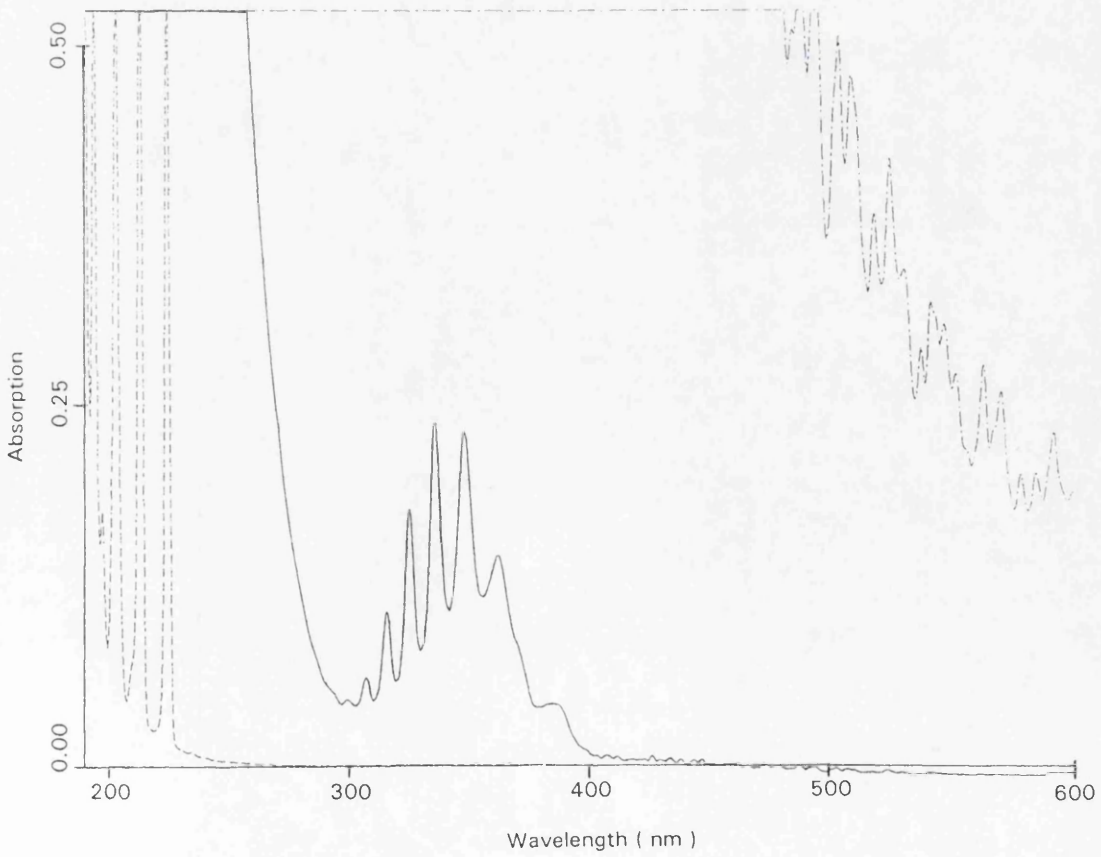
Figure 31 shows the differential spectrophotometric absorption of nitric oxide under rigorous oxygen-free conditions in the gas phase against air. The absorption characteristics are identical to those described for nitric oxide in the gas phase (Committee on Medical and Biological Effects of Environmental Pollutants, 1977). The first band of absorption (multiple peaks) appears at wavelengths less than 230 nm. Following introduction of oxygen (1:1), a deep red-brown gas was immediately formed in the cuvette and the spectrophotometric absorption characteristics of nitrogen dioxide (multiple peaks at 300 to 400 nm) appeared.

3.2.4.2.2. Reaction of L-Cysteine and Nitric Oxide at Acid pH in Oxygen-free Conditions

All experiments were conducted using the rigorous technique for removing oxygen and nitrogen dioxide described in Materials and Methods (see section 2.3.1. and Figure 2). In these experiments aqueous solutions of L-cysteine (10 mM, pH 3.0) were bubbled to saturation with nitric oxide for 10 minutes. They were then purged with purified oxygen-free nitrogen to remove free nitric oxide and kept in the sealed cuvette (Figure 32). These solutions did relax phenylephrine (0.3 μM)-precontracted rings of rabbit aorta but their relaxant activity was much lower than that observed under less rigorous oxygen-free conditions (Figure 17). This relaxant activity was associated with a small peak 3 in the HPLC (Figure 33) and two low absorption peaks with maxima at 218 and 335 nm in the spectrophotometer (Figure 34). The relaxant activity in the bioassay, the height of peak 3 in the HPLC, and the

Figure 31. Differential spectrophotometry of nitric oxide in the gas phase under rigorous oxygen-free conditions and the change of absorption after mixing with oxygen (1:1 vol./vol.) for 3 and 15 minutes. In the UV-visible region the first electronic absorption band of nitric oxide in the gas phase occurred at wavelengths less than 230 nm. Formation of nitrogen dioxide was indicated by the appearance of its characteristic absorption (multiple peaks) in the 300-400 nm wavelength region. Note the use of two different absorption scales.


NO gas
NO + O2 3min
NO + O2 15min
Absorption
4.0
2.0
0.0
200
300
400
500
600
Wavelength (nm)


Absorption
0.50
0.25
0.00
200
300
400
500
600
Wavelength (nm)

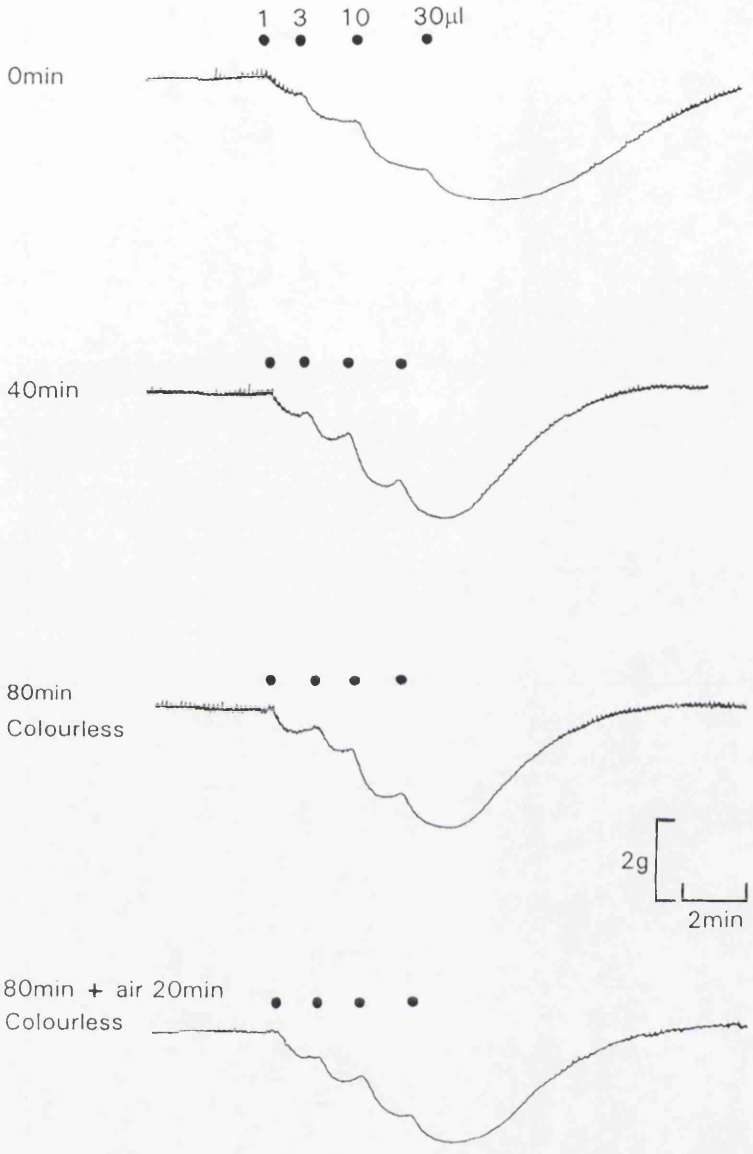


Figure 32. No changes were observed within 80 minutes in the relaxant activity of aqueous solutions of L-cysteine (10 mM, pH 3.0) bubbled to saturation with nitric oxide for 10 minutes under rigorous oxygen-free conditions and purged with oxygen-free nitrogen in a sealed cuvette. At the end of this time the solution was mixed with air for 20 minutes but again relaxant activity remained unchanged. Bioassay was carried out on a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 µM).

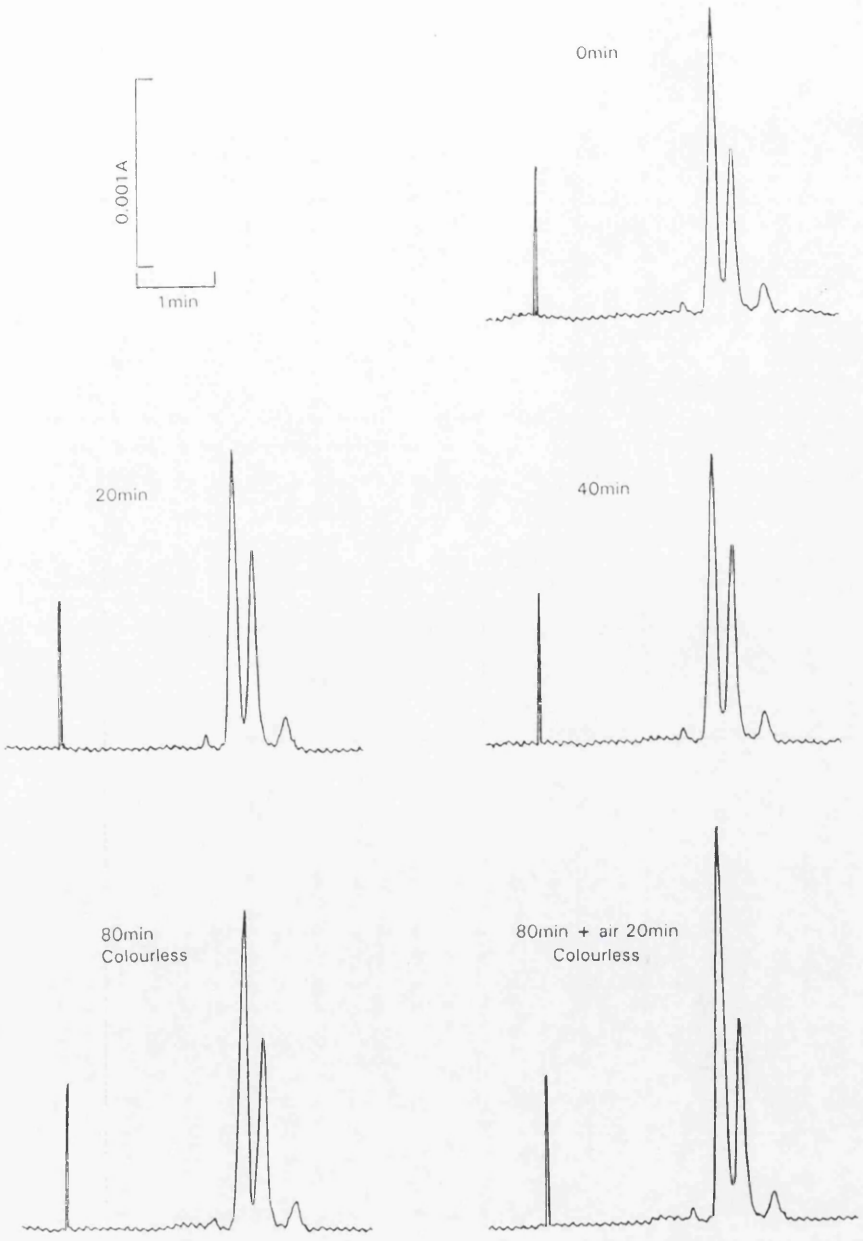


Figure 33. In the HPLC no changes in peak 3 within 80 minutes were observed in aqueous solutions of L-cysteine (10 mM, pH 3.0) bubbled to saturation with nitric oxide for 10 minutes under rigorous oxygen-free conditions and purged with oxygen-free nitrogen in a sealed cuvette. Peak 3 also did not change when the solution was mixed with air for 20 minutes. HPLC analysis was performed with an analytical column using water at pH 3 as the solvent.

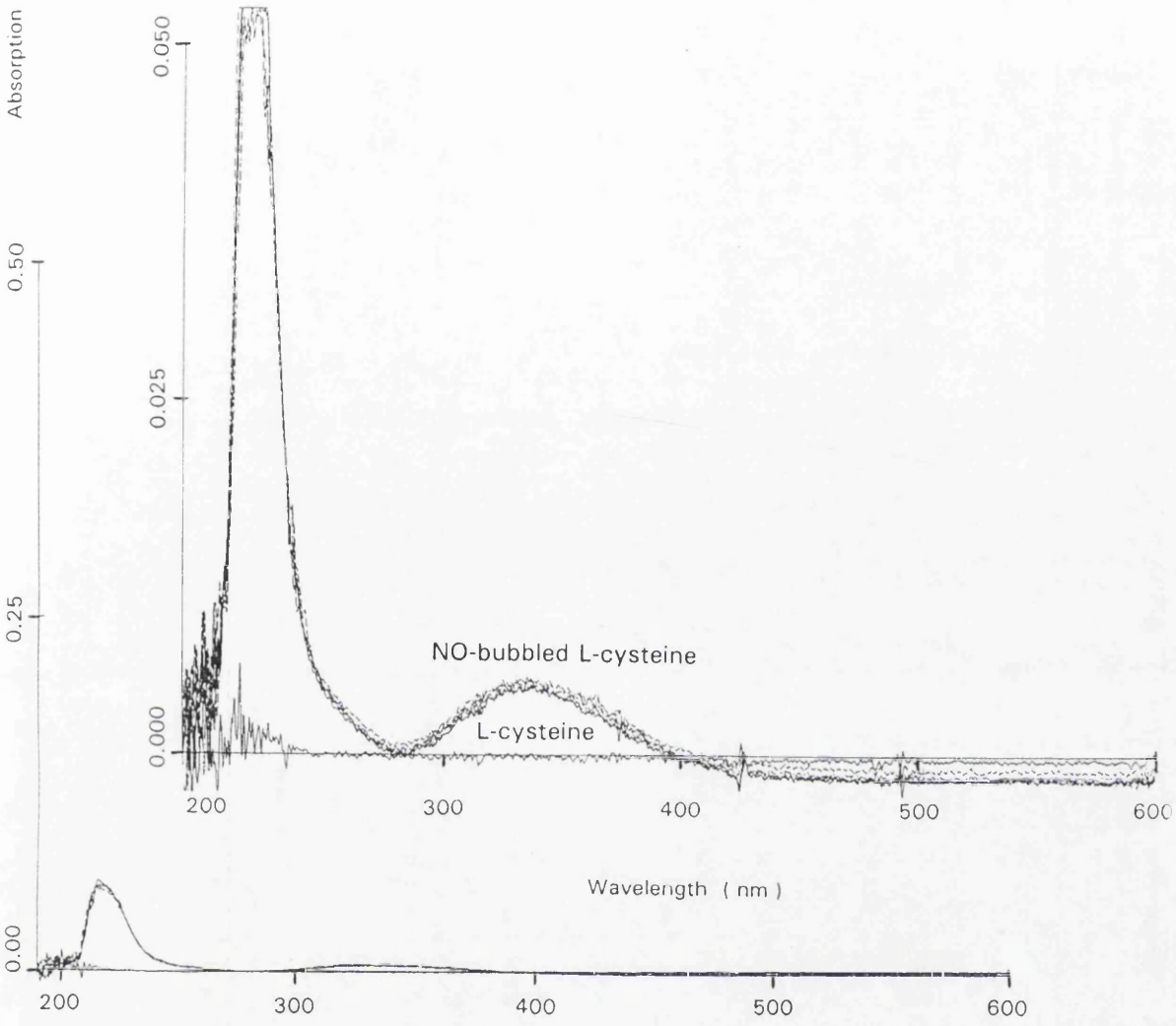


Figure 34. Using differential spectrophotometry aqueous solutions of L-cysteine (10 mM, pH 3.0) bubbled to saturation with nitric oxide for 10 minutes under rigorous oxygen-free conditions and purged with oxygen-free nitrogen in a sealed cuvette had two absorption peaks with maxima at 218 and 335 nm. These peaks were not changed for up to 80 minutes while the cuvette was sealed and were not changed after mixing with air for 20 minutes. Note the use of two different absorption scales.

absorption peaks in the spectrophotometer remained unchanged if the solutions were kept sealed for up to 80 minutes and these properties did not change when the solutions were mixed with air for 20 minutes. The solutions remained colourless throughout the experiment and no absorption bands were present in the visible region (>500 nm). In the HPLC peaks 2 and 3 were very stable, whereas peak 1 increased in height after mixing with air for 20 minutes.

If nitric oxide dissolved in the L-cysteine solution and that free in the head space in the cuvette was not removed after mixing, the relaxant activity generated was much greater (Figures 35 & 36) than when nitric oxide was removed by purging (Figure 32). This was not surprising since this solution contained about 1-3 mM dissolved nitric oxide when saturated and free nitric oxide was also a very powerful relaxant (Figures 16 & 17). When assayed on phenylephrine (0.3 μM)-contracted aortic rings (Figure 35) or BRP strips (Figure 36) the magnitude of the relaxation induced by the nitric oxide-saturated L-cysteine (10 mM) solution remained unchanged for up to 80 minutes in the sealed cuvette and did not change even after mixing with air for 20 minutes. However, the duration of the relaxation was greater when assayed on rabbit aortic rings after exposed to the air (Figure 35) but this was not seen on BRP strips (Figure 36). This solution at zero time produced four peaks in the HPLC; peaks 1, 2, 3 and 4 corresponding to L-cystine, L-cysteine, the new relaxant and nitric oxide in retention time, respectively (Figure 37). When the solution was kept sealed in the cuvette for up to 80 minutes, peaks 1 and 3 gradually increased in size, and peaks 2 and 4 remained unchanged. After the solution was mixed with air for 10 minutes, peaks 1 and 3 increased massively, peak 2 was slightly reduced and peak 4 disappeared. In the spectrophotometer there were two low absorption peaks at 218 and 335 nm in this sealed solution at zero time (Figure 38). The height of these peaks increased slowly during the 80 minutes before the solution was mixed with air but increased massively after mixing. The peak at 218 nm was also shifted towards 232 nm. While remaining sealed, a third peak at 545 nm became

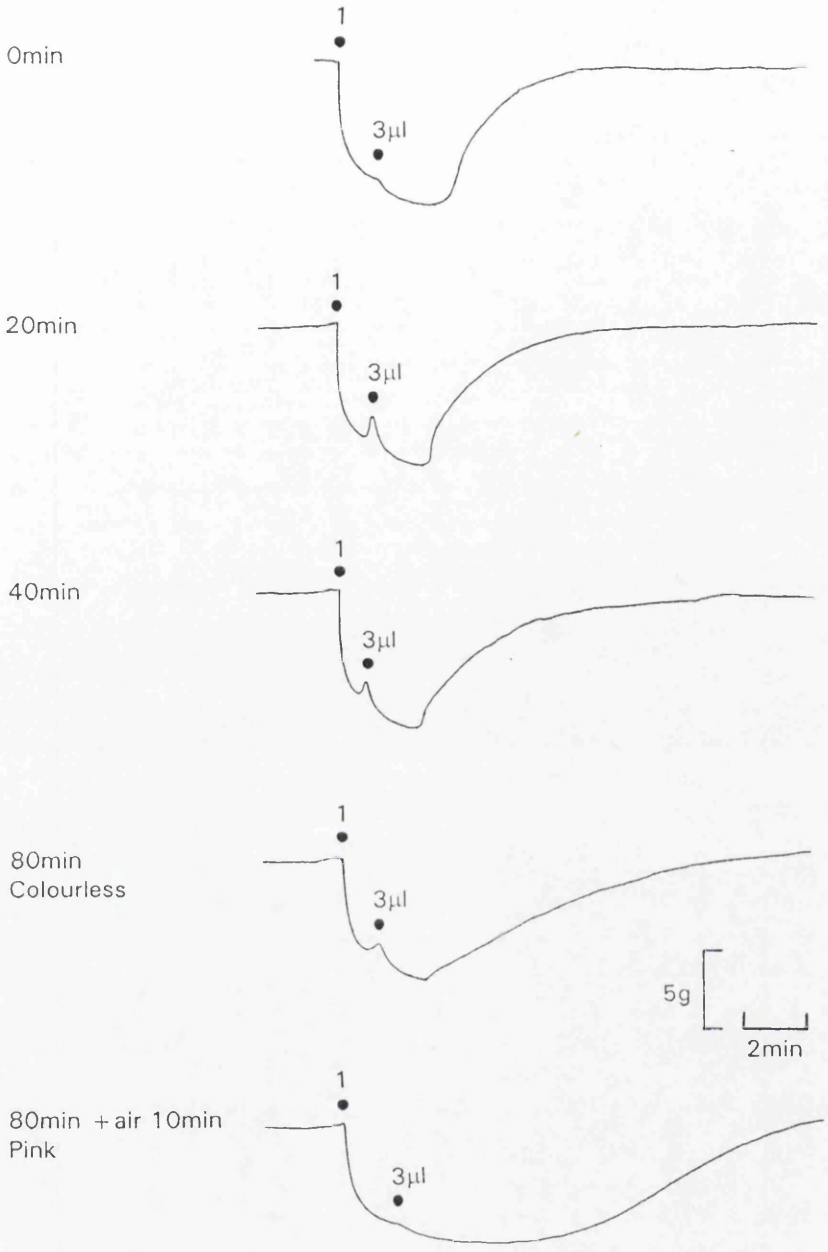


Figure 35. This figure shows the relaxant activity generated under rigorous oxygen-free conditions when aqueous solutions of L-cysteine (10 mM, pH 3.0) were bubbled to saturation with nitric oxide for 10 minutes in a sealed cuvette. These solutions were not purged to remove free nitric oxide. The relaxant activity of these solutions did not change during 80 minutes while sealed. After mixing with air the solution instantly became pink in colour and 10 minutes later its relaxant activity was equal in magnitude to that before exposure to the air but was longer in duration. Bioassay was carried out on a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 μM).

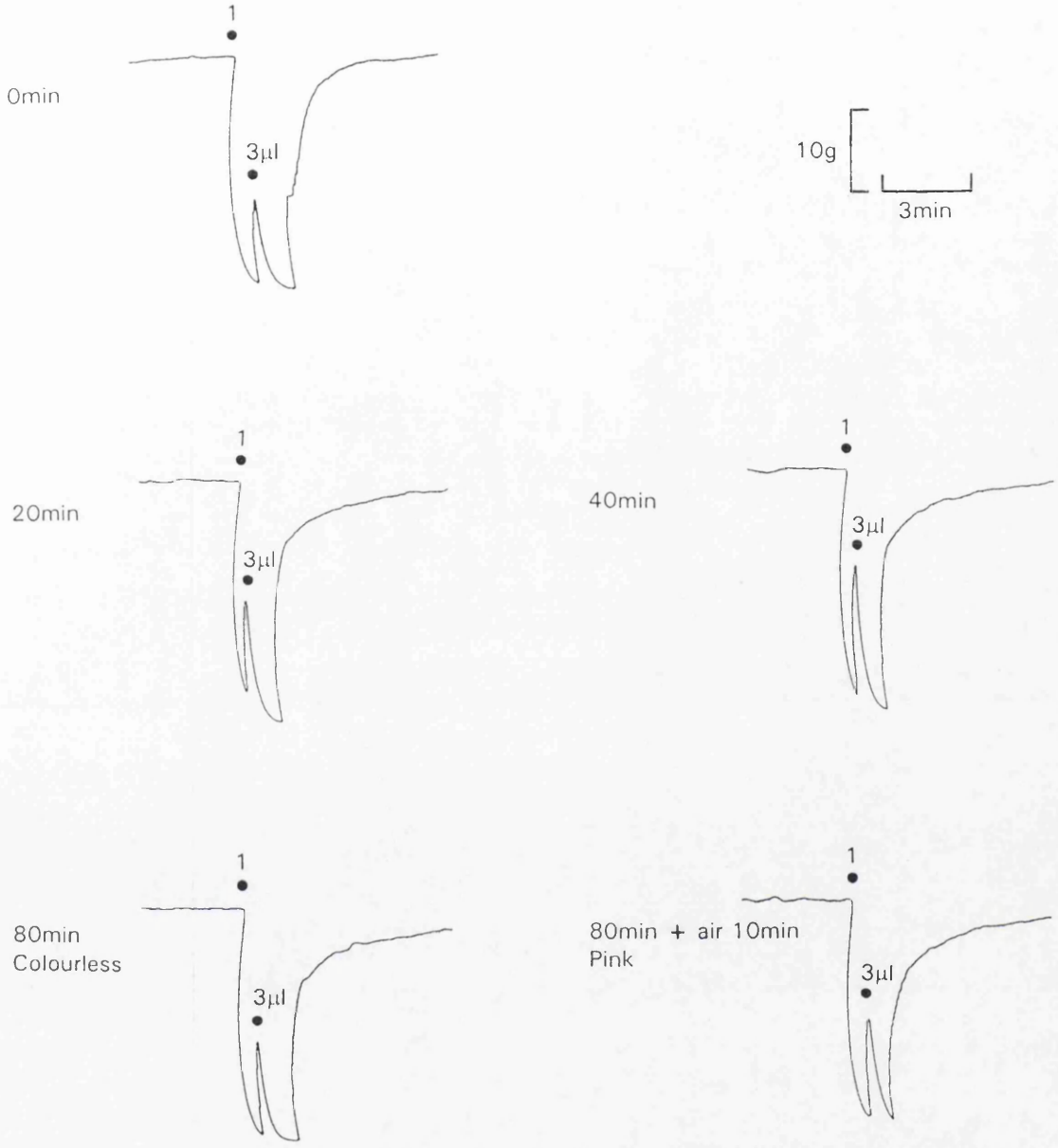


Figure 36. This figure shows the relaxant activity generated under rigorous oxygen-free conditions when aqueous solutions of L-cysteine (10 mM, pH 3.0) were bubbled to saturation with nitric oxide for 10 minutes in a sealed cuvette. These solutions were not purged to remove free nitric oxide. The relaxant activity of these solutions did not change during 80 minutes while sealed. After mixing with air the solution instantly became pink in colour but the relaxant activity remained unchanged. Bioassay was carried out on a BRP strip submaximally contracted by phenylephrine (0.3 µM).

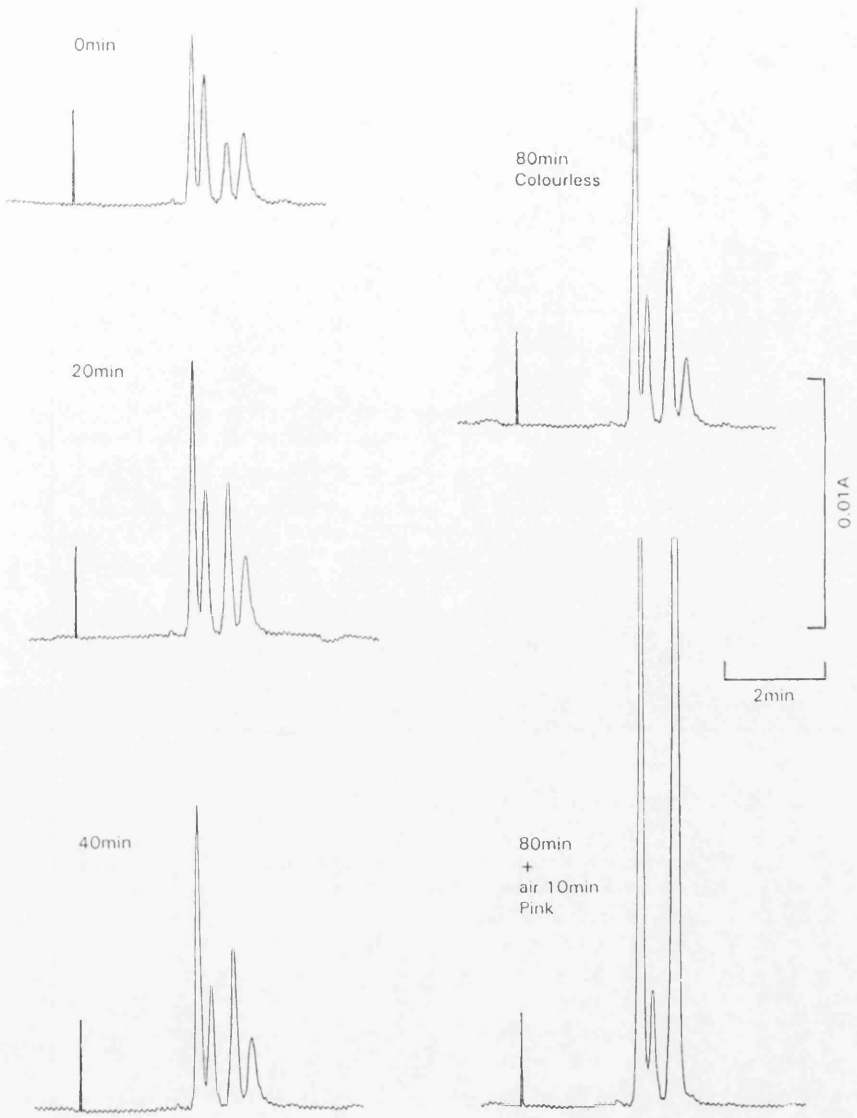


Figure 37. This figure shows the HPLC analysis carried out on the novel relaxant formed under rigorous oxygen-free conditions when aqueous solutions of L-cysteine (10 mM, pH 3) were bubbled to saturation with nitric oxide for 10 minutes and free nitric oxide was not removed by purging with nitrogen. In the HPLC peaks 1 and 3 gradually increased but peaks 2 and 4 remained unchanged over 80 minutes while the cuvette remained sealed. After this time the solution was opened to the air and it turned pink and following mixing with air for 10 minutes, peaks 1 and 3 were increased massively, peak 2 was reduced slightly and peak 4 disappeared. HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

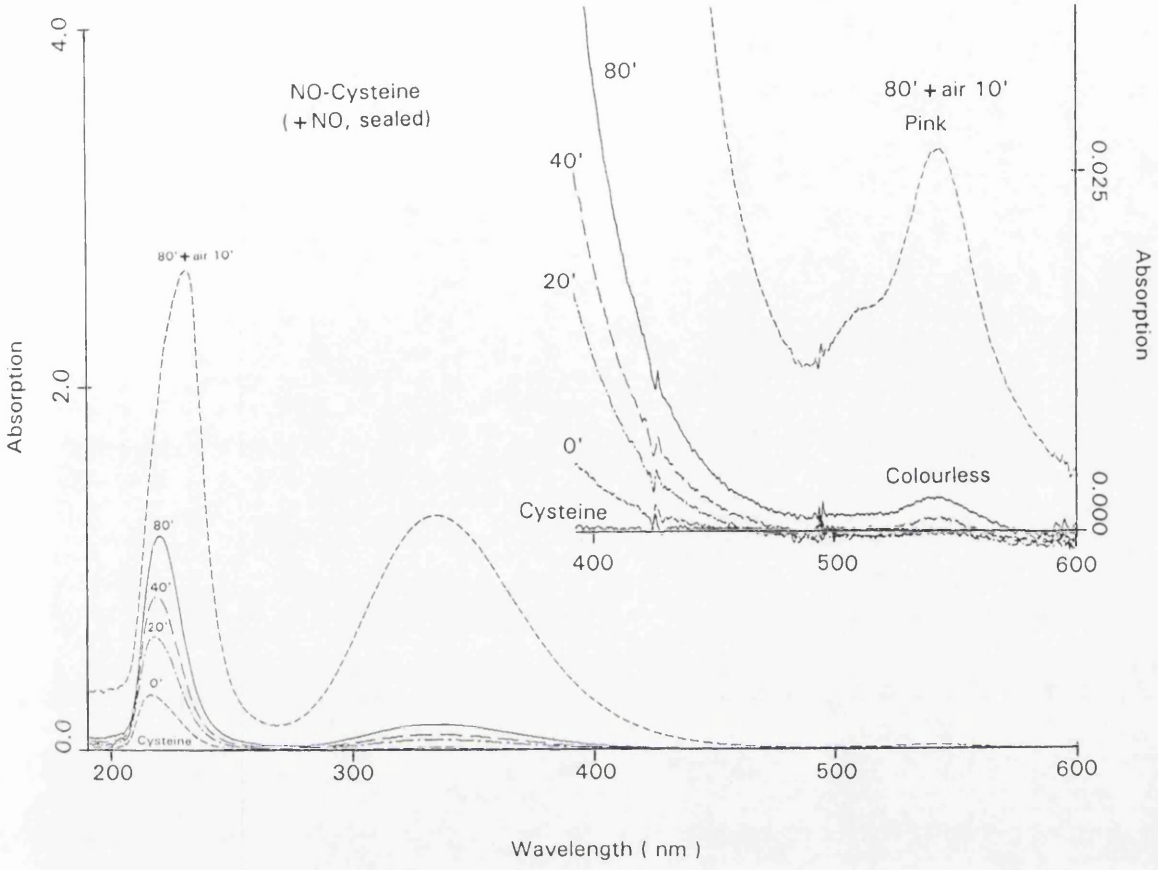


Figure 38. This figure shows the differential spectrophotometric analysis carried out on the novel relaxant formed under rigorous oxygen-free conditions when aqueous solutions of L-cysteine (10 mM, pH 3) were bubbled to saturation with nitric oxide for 10 minutes and free nitric oxide was not removed by purging with nitrogen. At time zero two absorption peaks with maxima at 218 and 335 nm were seen in the sealed cuvette and these increased in size over 80 minutes. After 40 minutes a new peak at 543 nm became measurable if the sensitivity was increased to 0.05 absorption units full scale (insert) and this increased slightly by 80 minutes. The height of three peaks increased massively after the solution was opened to the air and mixed for 10 minutes. The solution turned pink immediately following mixing with air. The peak at 218 nm was slowly shifted toward 232 nm, but no shift was seen in the peaks at 335 and 543 nm.

measurable after 40 minutes but only if the sensitivity was increased 40 times from 2.0 to 0.05 absorption units full scale. This third absorption peak rose further by 80 minutes, and increased dramatically when the solution was mixed with air for 10 minutes. This rise explains why the solution instantly became pink in colour when exposed to the air.

There were two possible explanations for the slow increase with time in the magnitude of these three peaks at 218, 335 and 543 nm. One was that the reaction between nitric oxide and L-cysteine was slow, but that seemed unlikely. A more likely explanation was that despite our rigorous precautions oxygen slowly penetrated through the sealed container and increased the rate of the reaction. This latter possibility could also explain why the relaxant activity was stable whereas peak 3 in the HPLC and the absorption peaks at 218, 335 and 543 nm in the spectrophotometer increased slowly with time in this particular experiment (Figures 35-38). Our hypothesis was that because the bioassay was performed on an oxygenated conditions, it was possible that when the contents of the cuvette were added to the bath, a further rapid reaction with oxygen could take place causing a large increase in relaxant activity, whereas HPLC and spectrophotometric analysis were performed under oxygen-free conditions. Experiments were therefore designed to test whether oxygen played a critical role in the reaction of nitric oxide with L-cysteine. Solutions of L-cysteine (10 mM) were bubbled with nitric oxide gas to saturation under stringent oxygen-free conditions followed by purging with oxygen-free nitrogen to remove free nitric oxide, neutralisation and exposed to the air. These solutions had very low relaxant activity (Figure 39), were colourless but contained two small absorption peaks at 218 and 335 nm. If, however, nitric oxide was bubbled to saturation in solutions of L-cysteine (10 mM) and the contents were exposed to the air before purging and neutralisation, the relaxant activity generated was greatly (about 40-fold) increased and proportionate rises seen in the absorption peaks at 218 and 335 nm. Furthermore, a new peak became visible at 543 nm which

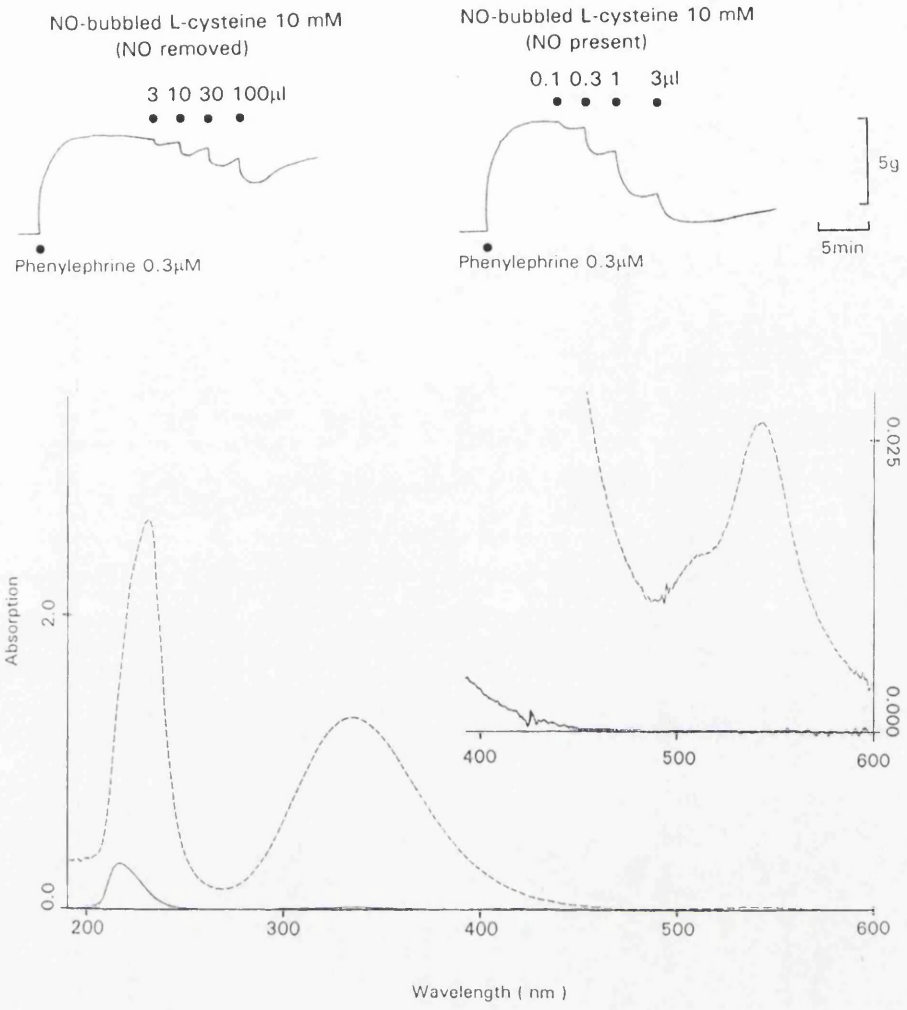


Figure 39. Bubbling nitric oxide gas to saturation for 10 minutes in solutions of L-cysteine (10 mM) in stringent oxygen-free conditions followed by purging with oxygen-free nitrogen to remove free nitric oxide (NO removed), neutralisation and exposed to the air produced low relaxant activity as assessed on phenylephrine (0.3 µM)-contracted, endothelium-denuded rings of rabbit aorta. These solutions were colourless but contained two small absorption peaks at 218 and 335 nm (continuous line). If, however, nitric oxide was bubbled to saturation for 10 minutes in solutions of L-cysteine (10 mM) and the reaction chamber was opened to the air before neutralisation and purging (NO present), the relaxant activity generated was greatly (≈40-fold) increased. The absorption peaks at 218 and 335 nm (discontinuous line) increased by a proportionate degree and a new peak (insert) became visible at 543 nm which was responsible for the development of a pink colour. Note the use of two absorption scales.

was responsible for the development of a pink colour. These findings suggested that relaxant activity and magnitude of the absorption peaks at 218, 335 and 543 nm were strictly related. They also implied a critical involvement of oxygen in the reaction of nitric oxide with L-cysteine and in the slow increase of absorption in the sealed cuvette containing L-cysteine and nitric oxide (Figure 38).

A different type of experiment was conducted to establish if the slow rise in absorbance when saturated nitric oxide was left in sealed solutions of L-cysteine was due to a slow rate of reaction of nitric oxide with L-cysteine or to a slow entry of oxygen. Two samples were prepared by the same rigorous procedures to obtain oxygen-free conditions. One sealed sample was placed in the spectrophotometer and readings taken for up to 24 hours and the other was kept under water which was continuously bubbled with purified oxygen-free nitrogen in the sealed Amicon chamber for 24 hours before it was removed for spectrophotometric measurement. If the absorption spectra of two samples were identical at 24 hours, then the increase of absorption would be due to the reaction between nitric oxide and L-cysteine occurring at a slow rate. Alternatively, if the absorption spectra of the sample kept in a sealed cuvette under water which was deoxygenated by oxygen-free nitrogen in an Amicon chamber was lower at 24 hours than that of the sample placed in the spectrophotometer, then the increase of absorption would be due to oxygen penetrating through the container.

The details of the experiment were as follows. Two cuvettes, stoppers and needles were degassed under 0.06 mmHg pressure in a high vacuum freeze dryer for 24 hours to maximally remove air in these materials. Before being taken out of the vacuum, the flask in which they were placed was filled with oxygen-free nitrogen. Then they were immediately removed from the freeze dryer into aqueous solutions of L-cysteine (10 mM, pH 3) that had been deoxygenated by purified oxygen-free nitrogen for 2 hours. These two cuvettes were completely filled with the solution,

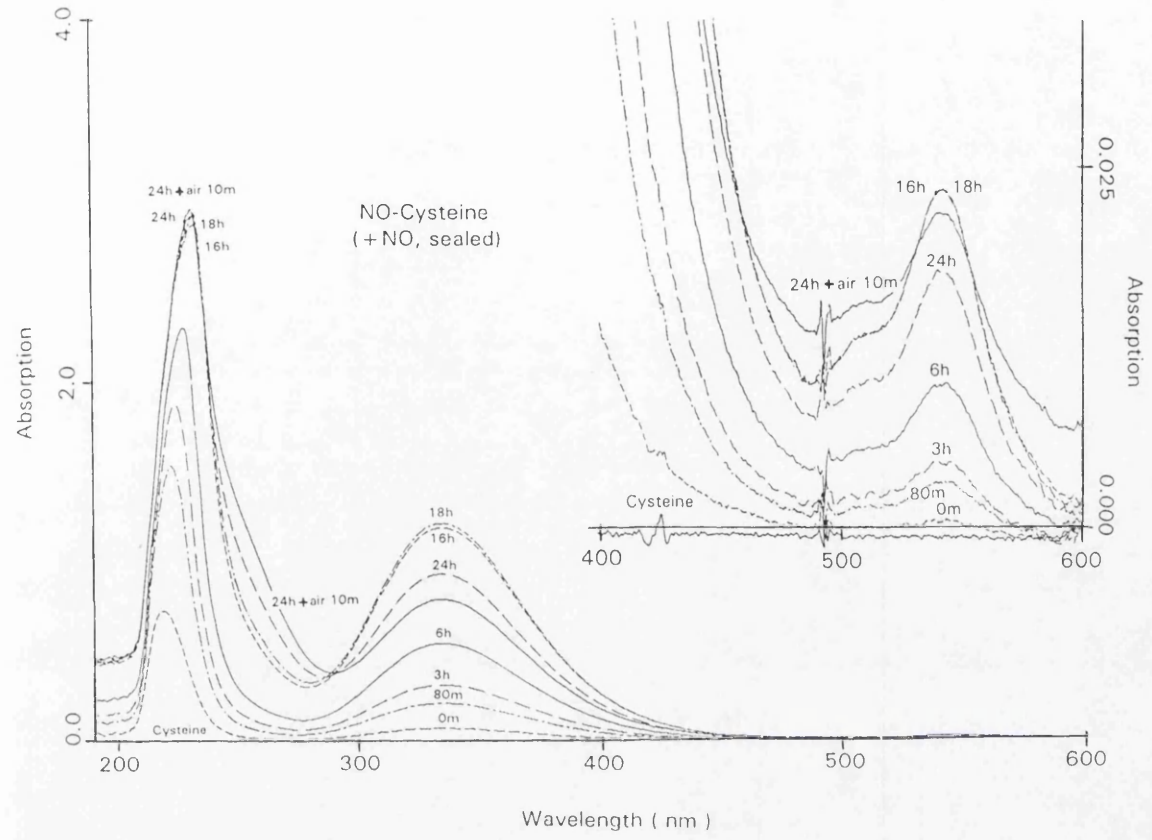
sealed and quickly transferred into the Amicon chamber and submerged under Milli-Q purified water that had been deoxygenated by oxygen-free nitrogen for two hours. The Amicon chamber was then closed and the solutions in the cuvettes were gassed with oxygen-free nitrogen for 1 hour. The solutions in the cuvettes were then gassed to saturation with purified nitric oxide for 10 minutes. One sealed cuvette remained in the Amicon chamber under water continuously gassed with oxygen-free nitrogen for 24 hours and the other was removed and the seal enhanced by pouring melted wax over the silicon stopper before placing the cuvette in a spectrophotometer. The absorption spectra of this sample were measured from 900 nm down to 190 nm at time zero (nominally), 80 minutes, 3, 6, 16, 18 and 24 hours and after the solution was exposed to the air and mixed for 10 minutes. Figure 40 shows that while sealed, the absorption peaks at 218, 335 and 543 nm increased with time but changed little when the cuvette was opened to the air at 24 hours. In contrast, the absorption peaks at 218, 335 and 543 of the sealed sample which remained in the Amicon chamber for 24 hours while under water bubbled with oxygen-free nitrogen were much lower. Furthermore, when this second sample was exposed to the air for 10 minutes, all three peaks increased in magnitude. These results suggested that the slow rise in the three absorption peaks with time even when the cuvette was sealed was due to the slow entry of oxygen into the reaction chamber and that oxygen was a vital component in the reaction.

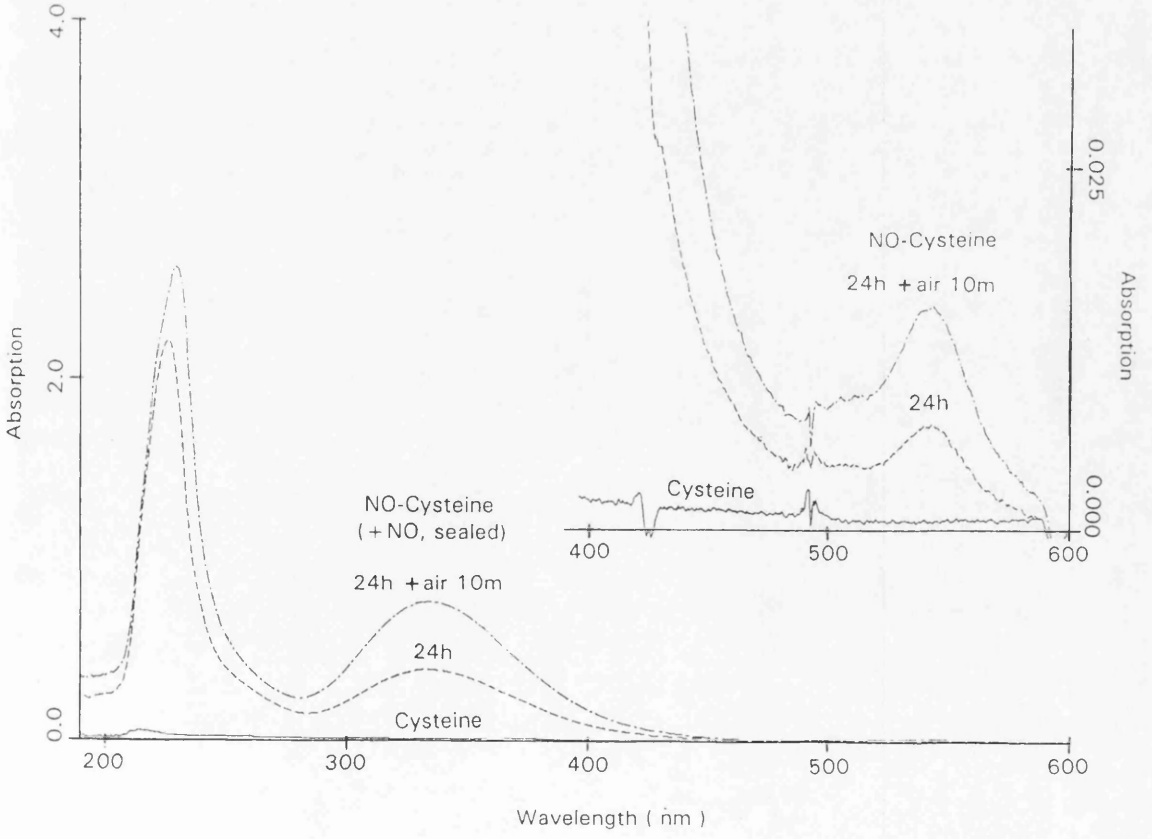
3.2.4.3. Investigation of Involvement of Nitrogen Dioxide

Since formation of the new relaxant in the reaction of nitric oxide with L-cysteine was enhanced in the presence of oxygen and since nitrogen dioxide is formed from the reaction of nitric oxide with oxygen, the possibility was examined that nitrogen dioxide was the species which reacted with L-cysteine to form the new relaxant.

Figure 40. The top panel shows the differential spectrophotometric trace of an aqueous solution of L-cysteine (10 mM) at pH 3.0 bubbled to saturation with nitric oxide for 10 minutes in a sealed cuvette. At time zero there were two absorption peaks with maxima at 218 and 335 nm. After 80 minutes a new peak at 543 nm became measurable at an enhanced sensitivity of 0.05 absorption units full scale (insert). All peaks increased slowly until 18 hours. The peak with a maximum at 217 nm shifted slowly towards 232 nm, whereas those at 335 nm and 542 nm did not shift. At 24 hours the peak absorption remained unchanged at 232 nm but those at 335 and 543 nm decreased slightly. After the solution was opened and mixed with air, the absorption at 232 and 543 nm increased slightly but that at 335 nm decreased slightly. m=minutes; h=hours.

The bottom panel shows the differential spectrophotometric trace of an aqueous solution of L-cysteine (10 mM) at pH 3 bubbled to saturation with nitric oxide for 10 minutes in a sealed cuvette and kept for 24 hours under water which was continuously gassed with oxygen-free nitrogen in a sealed Amicon chamber. At 24 hours and while sealed the three absorption peaks at 232, 335 and 543 nm were much lower than in the sample placed in the spectrophotometer for 24 hours. After the solution was opened and mixed with air, the magnitude of all three peaks was increased.


Absorption
4.0
2.0
0.0
NO-Cysteine
(+NO, sealed)
24h+air 10m
24h
18h
16h
6h
3h
80m
0m
Cysteine
200
300
400
500
600
Wavelength (nm)
0.025
0.000


Absorption
4.0
2.0
0.0
NO-Cysteine
(+NO, sealed)
24h +air 10m
24h
Cysteine
200
300
400
500
600
Wavelength (nm)
0.025
0.000

This investigation was performed by comparing the relaxant activity in the bioassay, the height of peak 3 in the HPLC and the spectrophotometric characteristics of mixtures of nitric oxide and L-cysteine and mixtures of nitrogen dioxide and L-cysteine at the same concentrations and at pH 3.0 under rigorous oxygen-free conditions. All solutions were purged with oxygen-free nitrogen to remove the free gases before bioassay or analysis in the HPLC and spectrophotometer. At a concentration of 0.1 mM nitric oxide, but not nitrogen dioxide, reacted with L-cysteine (10 mM) producing relaxant activity as assayed on a phenylephrine (0.3 μ M)-contracted BRP strip (Figure 41). The associated relaxant peak 3 in the HPLC and the characteristic absorption peak at 335 nm corresponding to the relaxant in the spectrophotometer (Figures 41-43) were also formed only with nitric oxide. If the concentration of the two gasses was increased to 1 mM, relaxant activity and the associated third peak in the HPLC were also produced with nitrogen dioxide, but the magnitude of these was lower than that for nitric oxide (Figure 44). If L-cysteine solutions (10 mM) were gassed to saturation with nitric oxide or nitrogen dioxide for 10 minutes followed by removal of the gasses by purging with nitrogen, complete relaxation of a phenylephrine (0.3 μM)-contracted BRP strip was obtained by adding 10 μl of either solution, but the duration of the relaxation induced by the solution bubbled with nitrogen dioxide was longer than that induced by the solution bubbled with nitric oxide (Figure 45). In each case the relaxant activity was associated with the characteristic peak 3 in the HPLC. In the case of bubbling to saturation with nitrogen dioxide numerous other peaks were eluted from the column after peak 3 and were thus more lipophilic. Due to the high concentrations of L-cystine generated in both solutions, peak 1 and peak 2 merged (Figure 45). Clearly, nitrogen dioxide had the ability to generate many more new substances than nitric oxide. Furthermore, the solution bubbled with nitrogen dioxide was pinkish red, whereas that bubbled with nitric oxide was colourless. Even when the solution was kept in a sealed brown bottle at $4^{o}C$ for up to 7 days, further reactions continued to

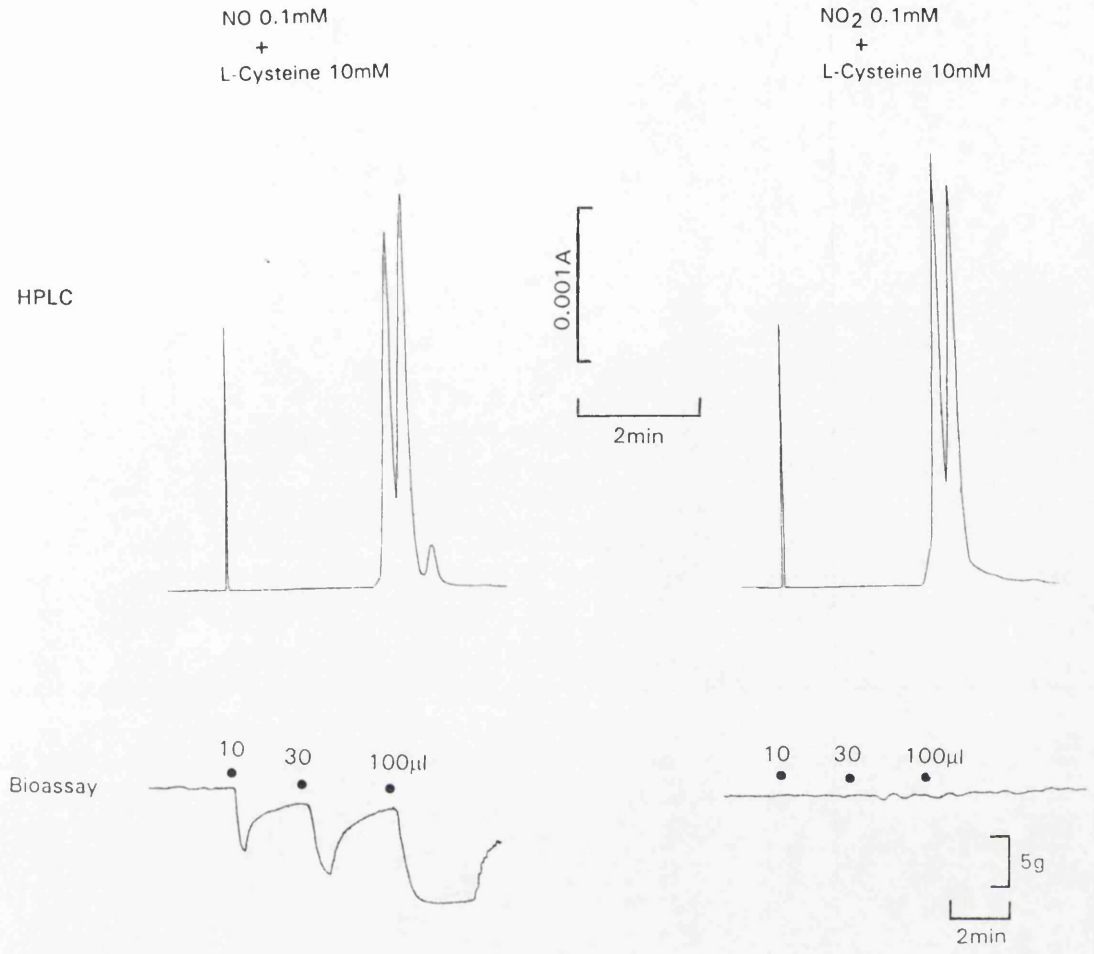


Figure 41. The relaxant activity in the bioassay and the associated peak 3 characteristic of the relaxant in the HPLC were generated under rigorous oxygen-free conditions from L-cysteine (10 mM) reacting with nitric oxide (0.1 mM), but not with nitrogen dioxide (0.1 mM), in aqueous solutions at pH 3 for 10 minutes followed by purging with oxygen-free nitrogen. Bioassay was carried out on a BRP strip submaximally contracted by phenylephrine (0.3 µM). HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

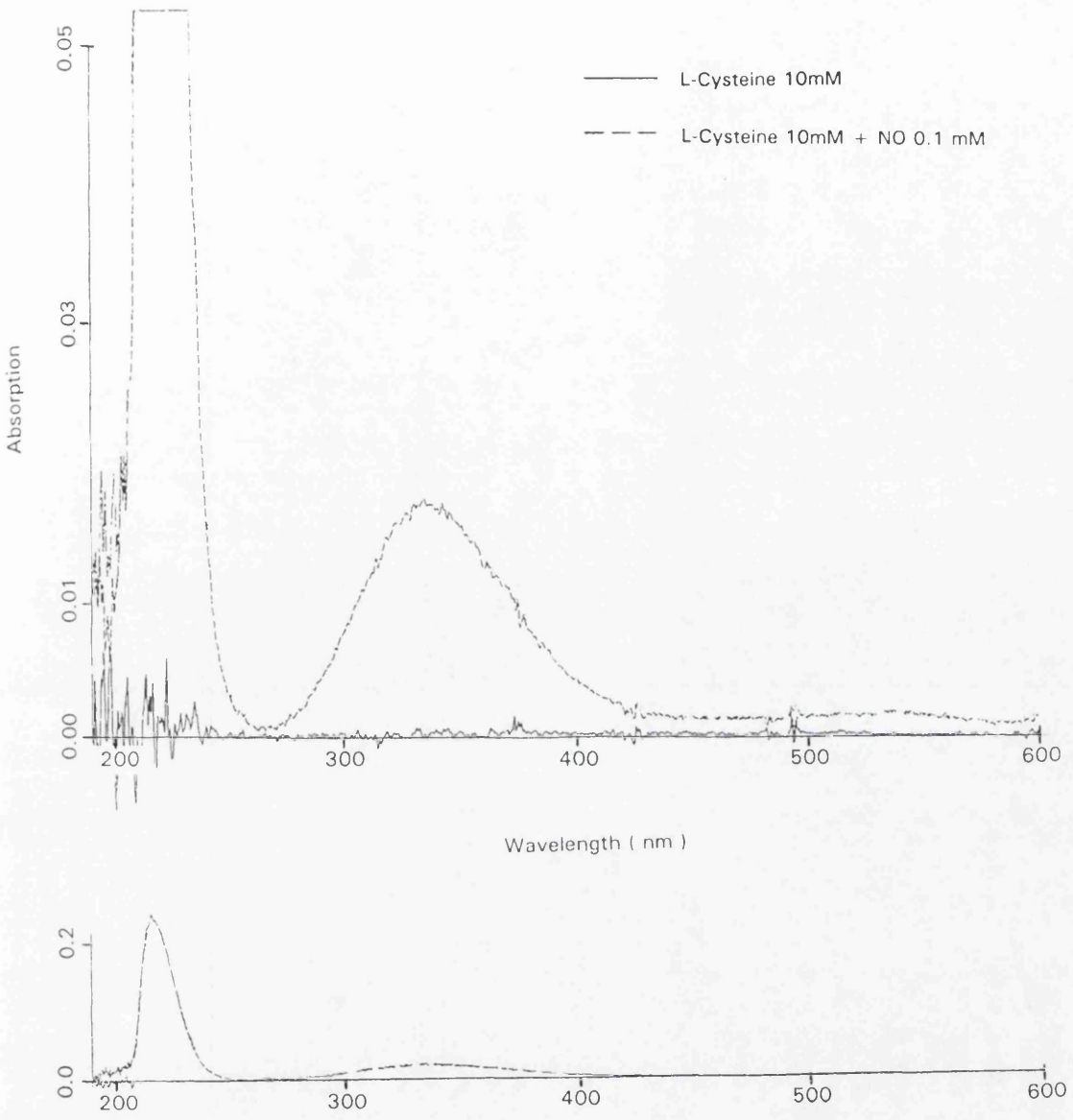


Figure 42. Differential spectrophotometry shows that the characteristic absorption of the new relaxant with a peak at 335 nm was observed under rigorous oxygen-free conditions when aqueous solutions of L-cysteine (10 mM) at pH 3 were reacted with nitric oxide (0.1 mM) for 10 minutes followed by purging with oxygen-free nitrogen in a sealed cuvette. Note the use of two absorption scales.

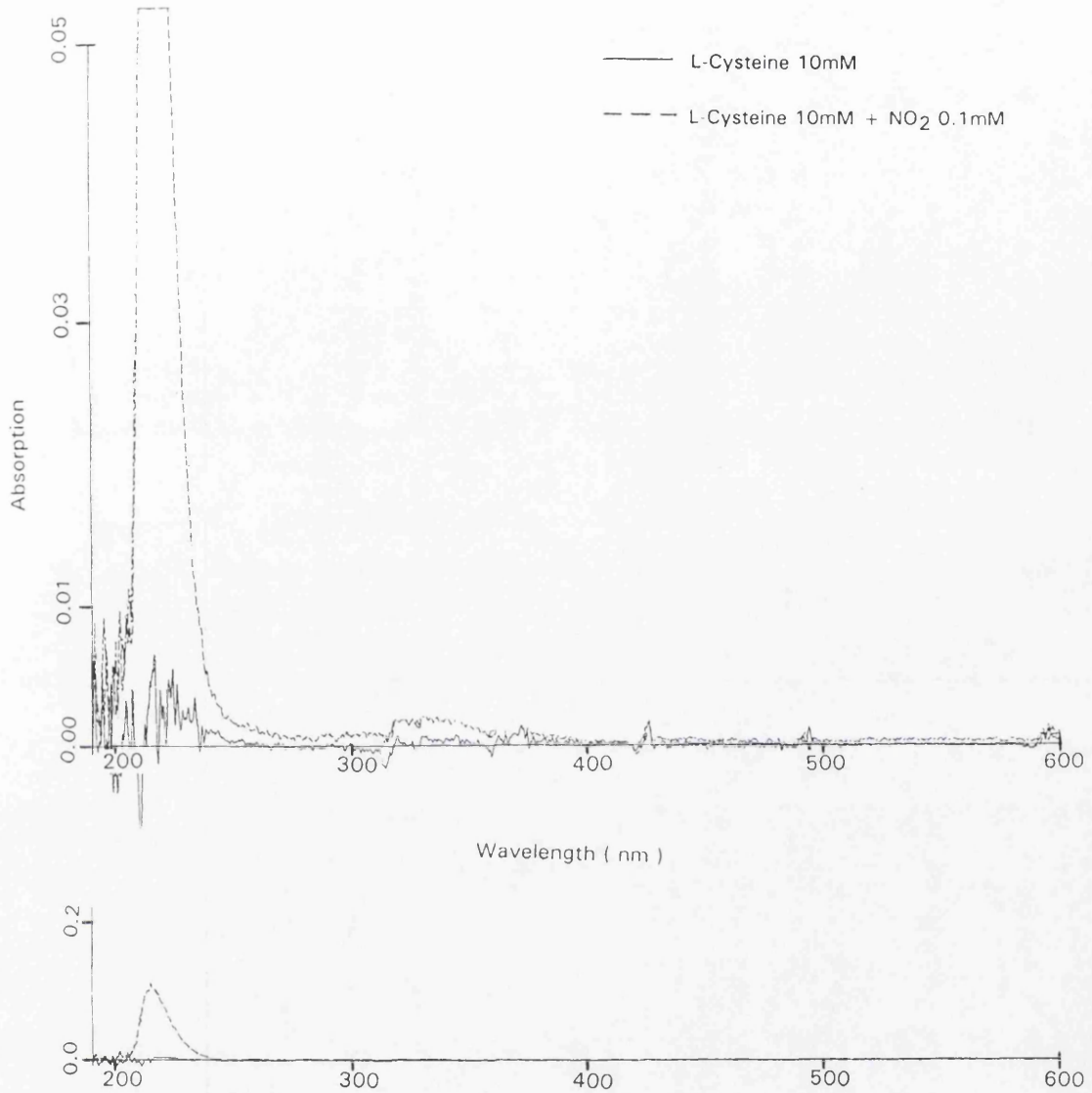


Figure 43. Differential spectrophotometry shows that the characteristic absorption of the new relaxant with a peak at 335 nm was not observed under rigorous oxygen-free conditions when aqueous solutions of L-cysteine (10 mM) at pH 3 were reacted with nitrogen dioxide (0.1 mM) for 10 minutes followed by purging with oxygen-free nitrogen in a sealed cuvette. Note the use of two absorption scales.

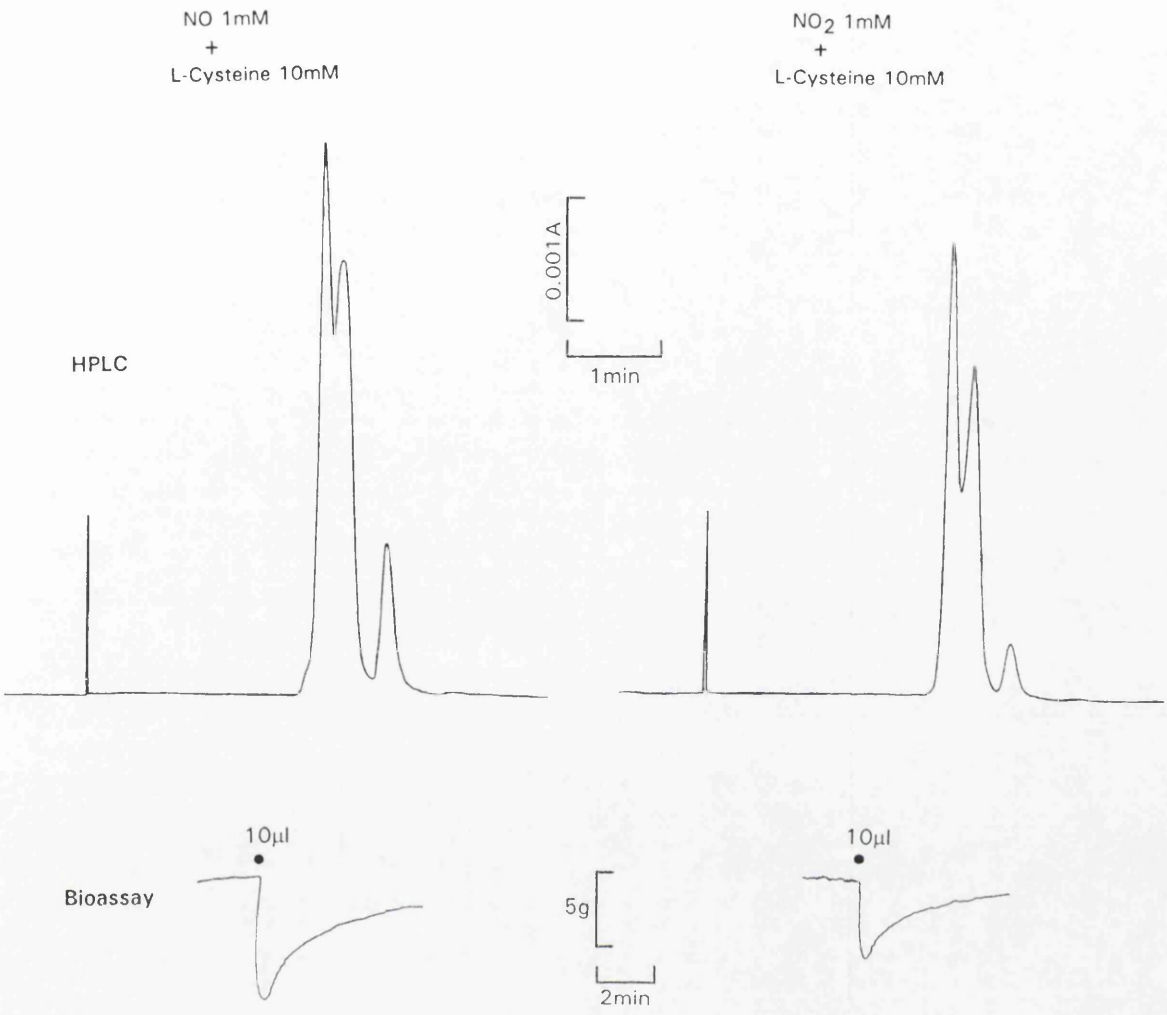


Figure 44. The relaxant activity in the bioassay and the associated peak 3 characteristic of the new relaxant in the HPLC were generated under rigorous oxygen-free conditions when nitric oxide (0.1 mM) and nitrogen dioxide (0.1 mM) respectively reacted with L-cysteine (10 mM) in aqueous solutions at pH 3 for 10 minutes followed by purging with oxygen-free nitrogen. Nitric oxide generated greater relaxant activity and a larger peak 3 than nitrogen dioxide. Bioassay was carried out on a BRP strip submaximally contracted by phenylephrine (0.3 µM). HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

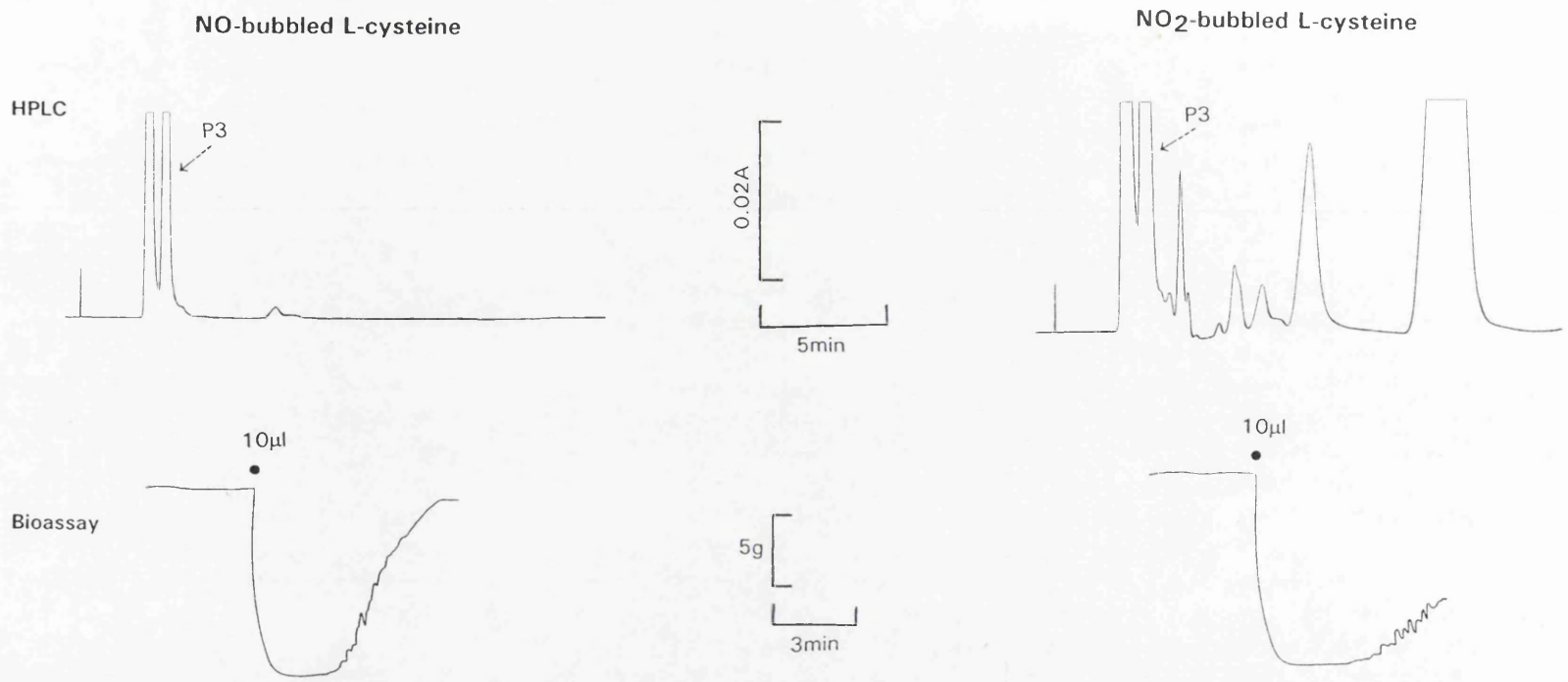


Figure 45. Powerful relaxant activity in the bioassay and the associated peak 3 characteristic of the new relaxant in the HPLC were generated under rigorous oxygen-free conditions when an aqueous solution of L-cysteine (10 mM at pH 3.0) was bubbled to saturation with nitrogen dioxide for 10 minutes followed by purging with oxygen-free nitrogen for 10 minutes. HPLC also shows many other peaks generated in this solution which were eluted later than peak 3 (P3) indicating their lipophilic nature. Bioassay was carried out on a BRP strip submaximally contracted by phenylephrine (0.3 µM). HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

take place as judged by the continued formation of new peaks in the HPLC (Figure 46). However, during these 7 days peak 3 was gradually reduced in magnitude.

Since nitrogen dioxide at high concentrations was able to react with L-cysteine to form the new relaxant, the possibility was considered that it was only able to do so after dissolving in solution to form nitrous acid which would then under acid conditions decay to form nitric oxide. Experiments were then conducted under rigorous oxygen-free conditions in which nitric oxide and nitrogen dioxide were gassed to saturation in distilled water and the contents assessed by bioassay and by HPLC. Figure 47 shows that water bubbled to saturation with nitric oxide or nitrogen dioxide for 10 minutes exhibited similar powerful relaxant activity when assayed on phenylephrine (0.3 μM)-contracted BRP strips. In the HPLC nitrogen dioxide-gassed water produced two peaks, the second of which was identical in retention time to the single peak observed for nitric oxide-gassed water. The relaxant activities in the bioassay were consistent with the height of the nitric oxide-related peak in the HPLC. Thus, solutions bubbled to saturation with nitrogen dioxide do generate nitric oxide and this may explain why at high concentrations nitrogen dioxide can react with L-cysteine to form the new relaxant and the associated peak 3 in the HPLC.

3.2.4.4. Further Studies of the Reactions of L-Cysteine with Nitric Oxide or Nitrogen Dioxide

Since the above studies on the reactions between L-cysteine and nitric oxide or nitrogen dioxide were shown to be optimal at acid pH and in the presence of oxygen, it was important to determine if the new relaxant could be formed under more physiological conditions. A series of experiments was therefore conducted to examined if the new relaxant could be formed under ambient oxygen conditions at neutral pH and at physiological concentrations of L-cysteine.

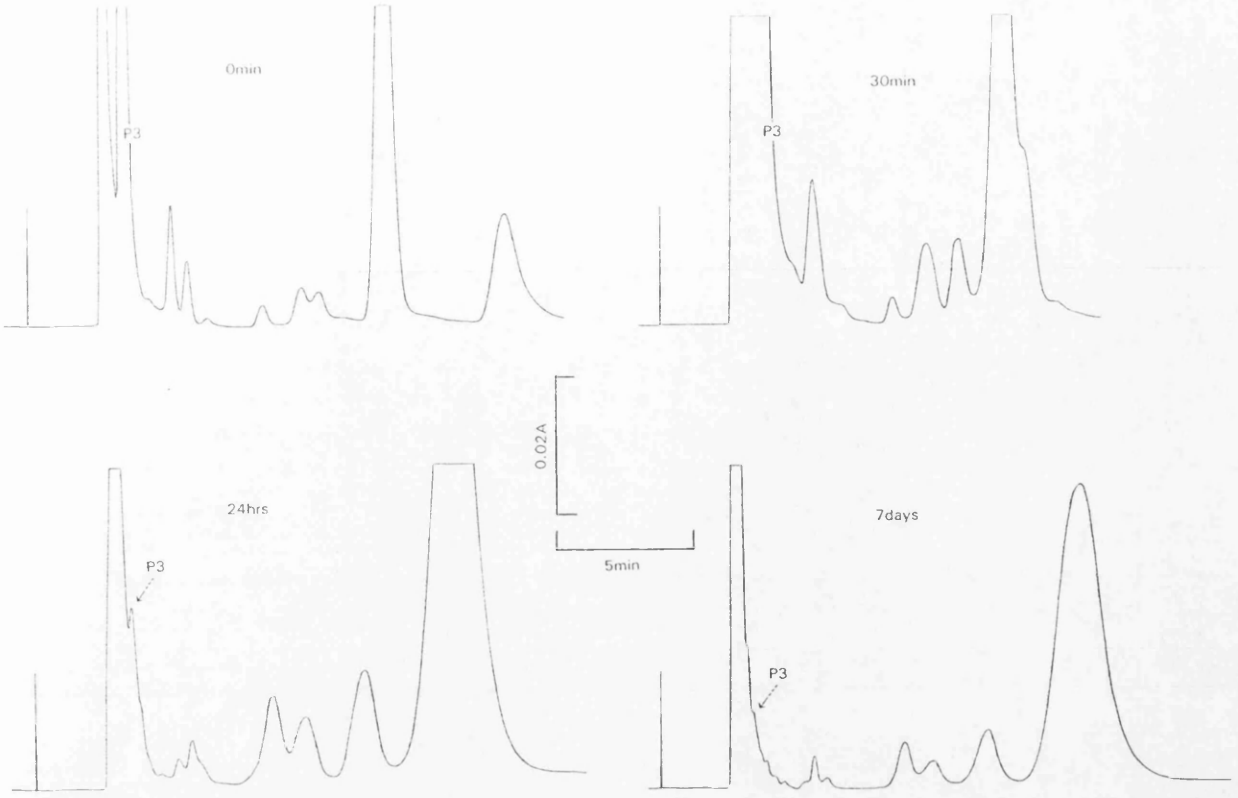


Figure 46. These HPLC traces show that under rigorous oxygen-free conditions when aqueous solutions of L-cysteine (10 mM) at pH 3 were bubbled to saturation with nitrogen dioxide for 10 minutes followed by purging with oxygen-free nitrogen for 10 minutes the peak 3 (P3) characteristic of the new relaxant was formed. Many other peaks were also formed and reactions continued to occur for up to 7 days as indicated by the appearance of further new peaks when the solution was stored at 4^{o}C in a brown bottle. Bioassay was carried out on a BRP strip submaximally contracted by phenylephrine (0.3 μM). HPLC analysis was performed with an analytical column using water at pH 3.0 as the solvent.

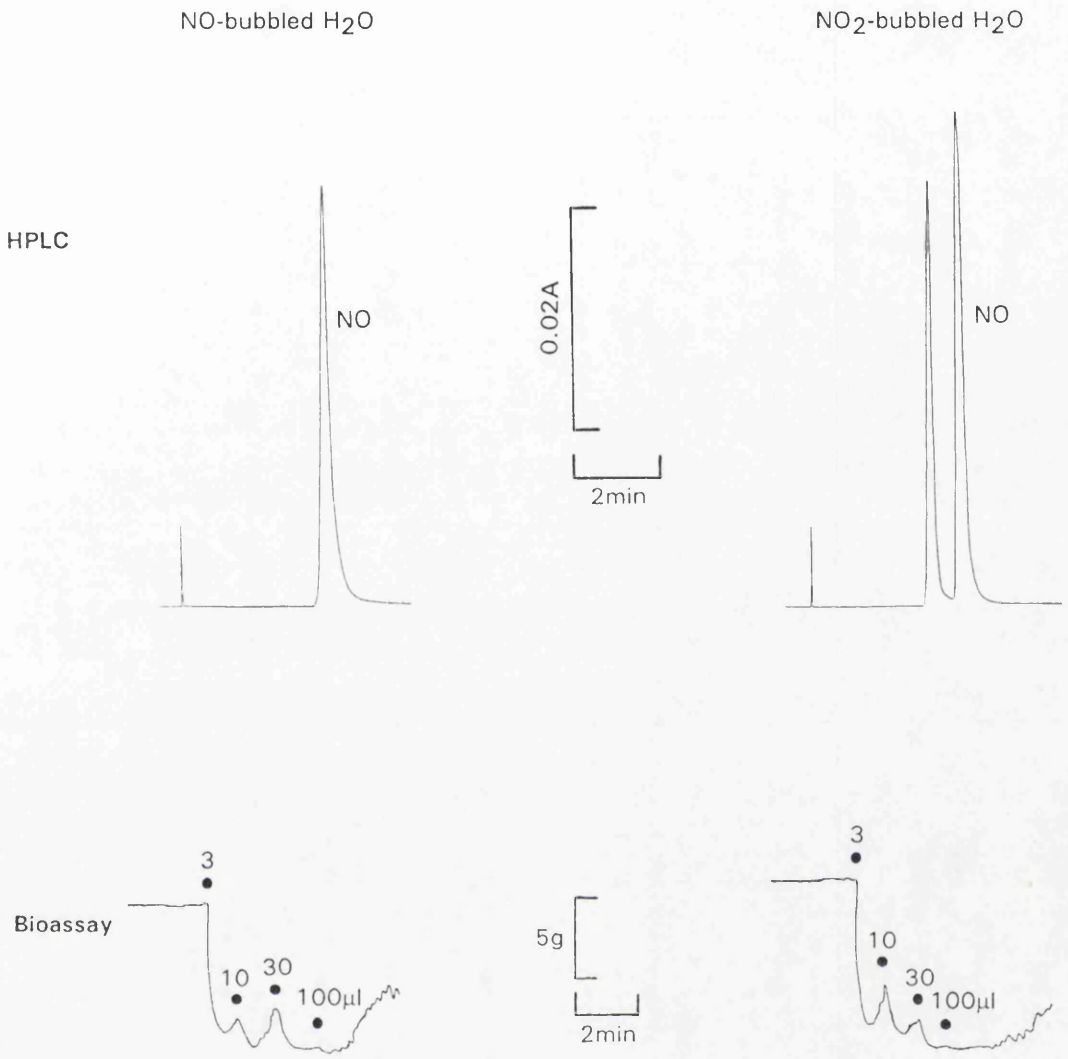


Figure 47. Deoxygenated water bubbled to saturation with nitric oxide or nitrogen dioxide for 10 minutes exhibited similar powerful relaxant activity on a BRP strip contracted by phenylephrine (0.3 µM). In the HPLC nitrogen dioxide-gassed water produced two peaks, the second of which was identical in retention time to the single peak of nitric oxide-gassed water. The relaxant activities in the bioassay were consistent with the height of the peak associated with nitric oxide in the HPLC. HPLC analysis was performed with an analytical column using water at pH 3 as the solvent.

At ambient oxygen tension and at pH 3.0 relaxant activity and the associated characteristic absorption of the relaxant at 335 nm in the spectrophotometer were generated in aqueous solutions of L-cysteine (10 mM) following reaction with nitric oxide (0.1 mM) or nitrogen dioxide (0.1 mM), but the magnitude of these was higher for nitric oxide than for nitrogen dioxide (Figure 48). Even at neutral pH relaxant activity was generated when solutions of L-cysteine (10 mM) were reacted with nitric oxide (0.1 mM) or nitrogen dioxide (0.1 mM, Figure 49). Again, the activity and the associated absorption peak at 335 nm was greater when the solution was reacted with nitric oxide than with nitrogen dioxide. These results further support the oxygen- and acid pH-dependence of the formation of the new relaxant in the reaction of L-cysteine with nitric oxide or nitrogen dioxide. They also indicate that the new relaxant can be formed at physiological pH and ambient oxygen tension.

The reaction discussed above involved L-cysteine at a concentration of 10 mM but this is greatly in excess of its physiological concentration (0.15 mM in plasma, Ramsay, 1985). Also, the concentration of nitric oxide (0.1 mM) was greater than would expected physiologically. Consequently, an experiment was conducted in which more physiological concentrations of L-cysteine (0.15 mM) and nitric oxide (1 μM) were reacted and formation of the new relaxant assessed by bioassay and by the characteristic absorption peak at 335 nm in the spectrophotometer. Figure 50 shows that even when under these sub-optimal conditions some relaxant activity and the associated absorption peak at 335 nm were observed. Thus, these studies suggest that the new relaxant can be formed when nitric oxide reacts with L-cysteine under ambient oxygen tension and at physiological pH and concentrations. Its formation is far from optimal under these conditions, however.

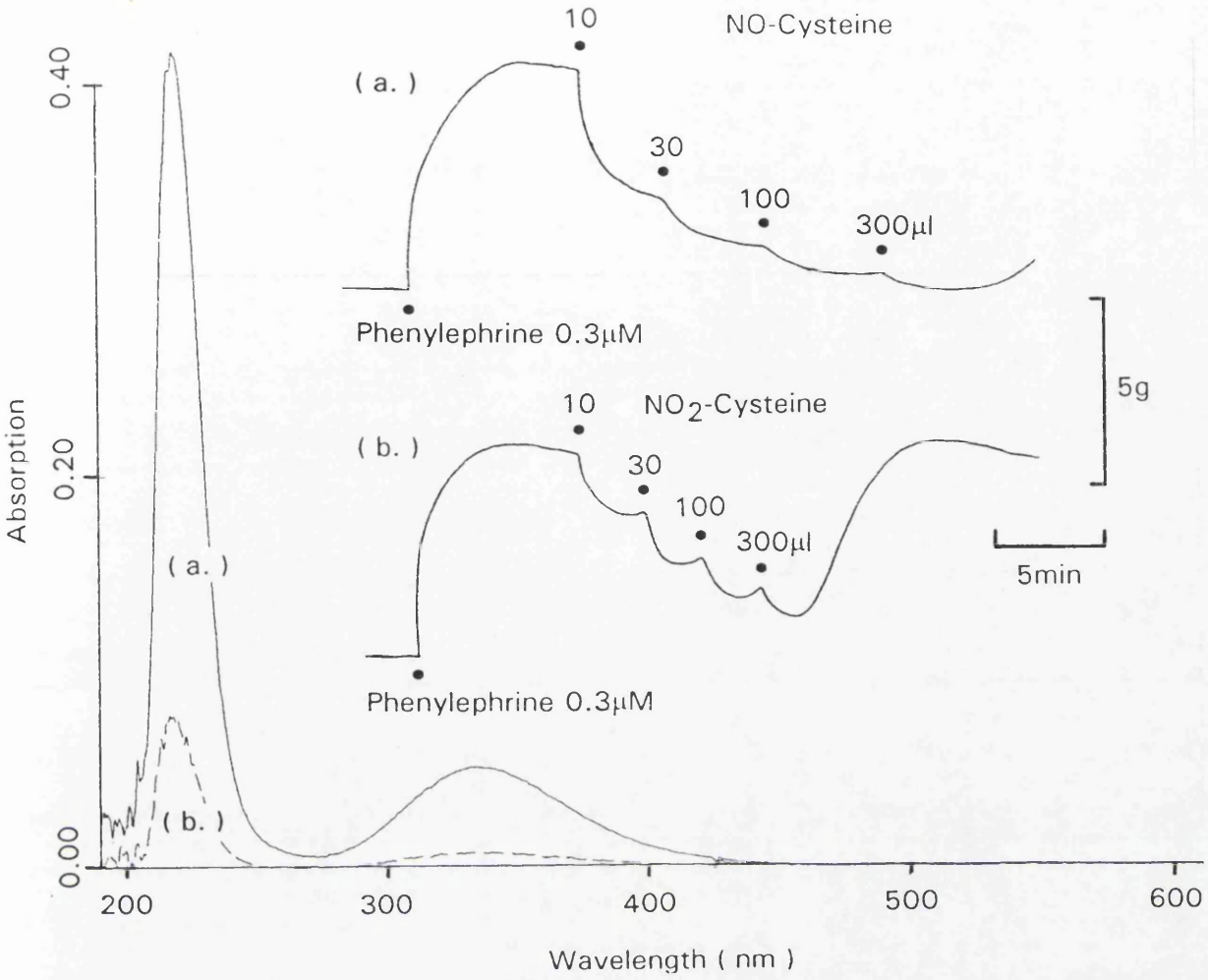


Figure 48. At ambient oxygen tension and at pH 3.0 relaxant activity in the bioassay and the associated characteristic absorption peak of the relaxant at 335 nm in the spectrophotometer were generated in aqueous solutions of L-cysteine (10 mM) reacting with (a.) nitric oxide (0.1 mM) or (b.) nitrogen dioxide (0.1 mM), but the magnitude of these was higher for nitric oxide than for nitrogen dioxide. These solutions were purged with oxygen-free nitrogen after the reaction to remove free gas. Bioassay was carried out on a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 µM). Differential absorption of the solutions was performed.

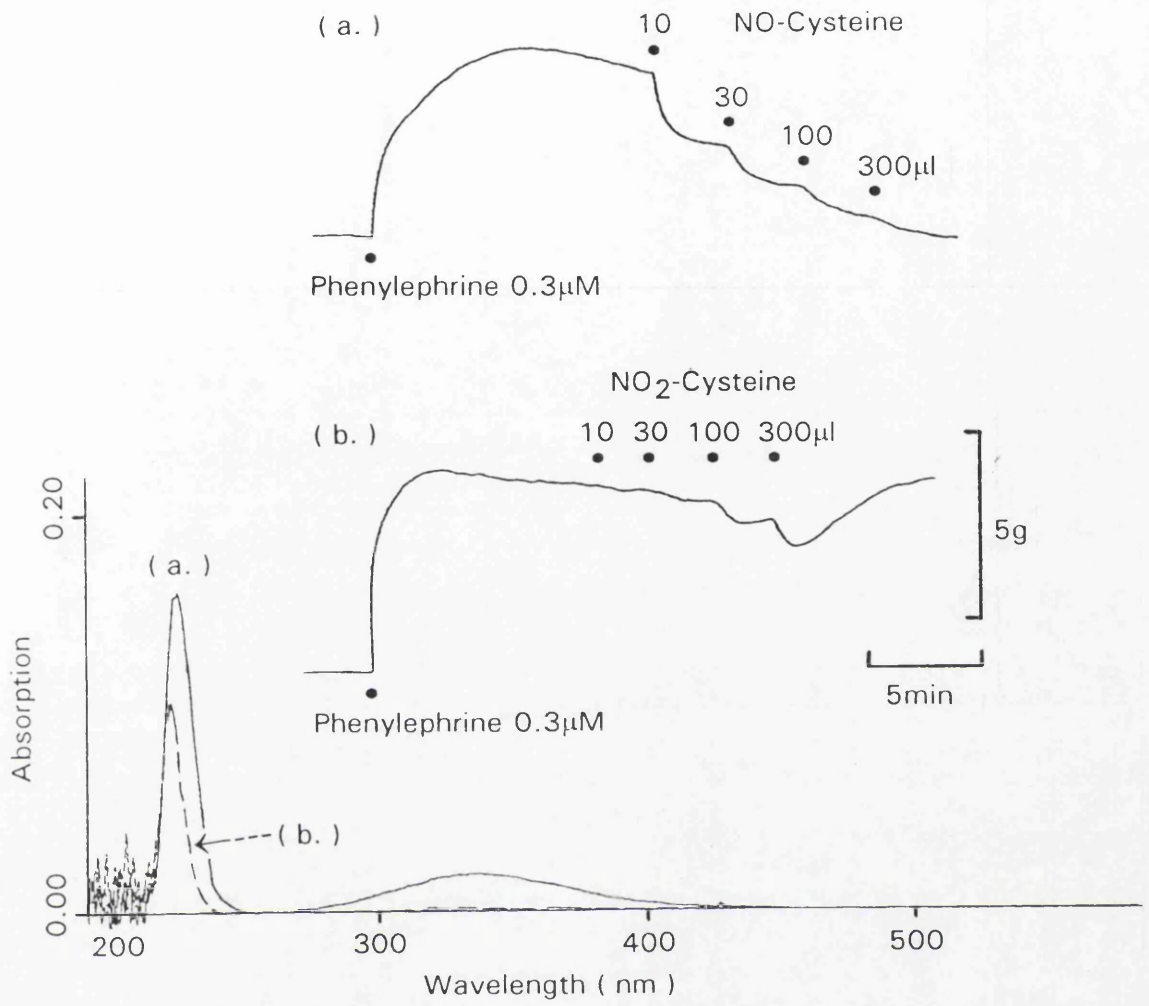


Figure 49. At ambient oxygen tension and at pH 7.0 relaxant activity in the bioassay and the associated characteristic absorption peak of the relaxant at 335 nm in spectrophotometer were generated in aqueous solutions of L-cysteine (10 mM) reacting with (a.) nitric oxide (0.1 mM) or (b.) nitrogen dioxide (0.1 mM), but the magnitude of these was higher for nitric oxide than for nitrogen dioxide. These solutions were purged with oxygen-free nitrogen after the reaction to remove free gas. Bioassay was carried out on a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 μM). Differential absorption of the solutions was measured.

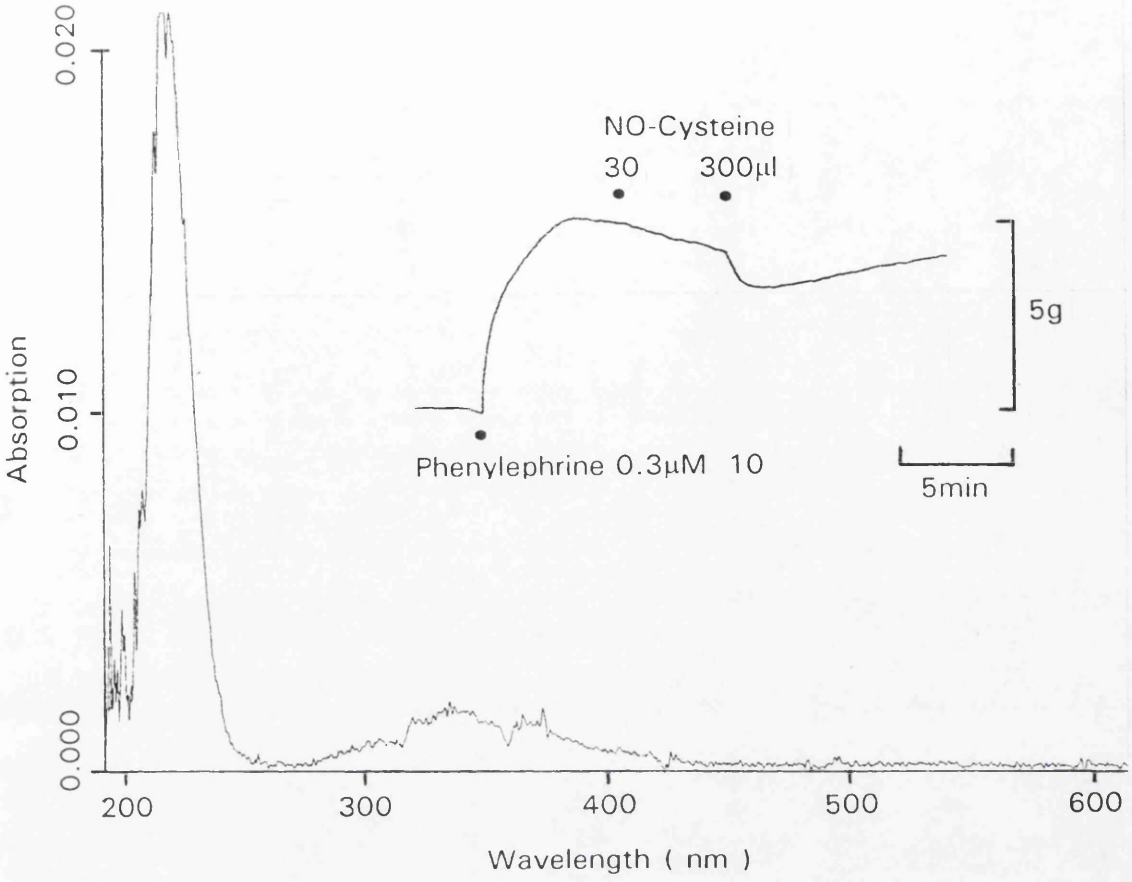


Figure 50. At ambient oxygen tension and at pH 7.0 relaxant activity in the bioassay and the associated characteristic absorption peak of the relaxant at 335 nm in the spectrophotometer were generated in aqueous solutions of L-cysteine (150 µ M) reacting with nitric oxide (1.0 µM). This solution was purged with oxygen-free nitrogen after the reaction to remove any free nitric oxide. Bioassay was carried out on a rabbit endothelium-denuded aortic ring submaximally contracted by phenylephrine (0.3 µM). Differential absorption of the solution was measured.

3.2.5. Identity of the Relaxant Generated by Reacting L-Cysteine With Nitric Oxide

The reaction of nitric oxide with L-cysteine generates a powerful new relaxant which may be important physiologically. It is therefore important that this novel substance be identified chemically.

3.2.5.1. Functional Groups Reacting with Nitric Oxide

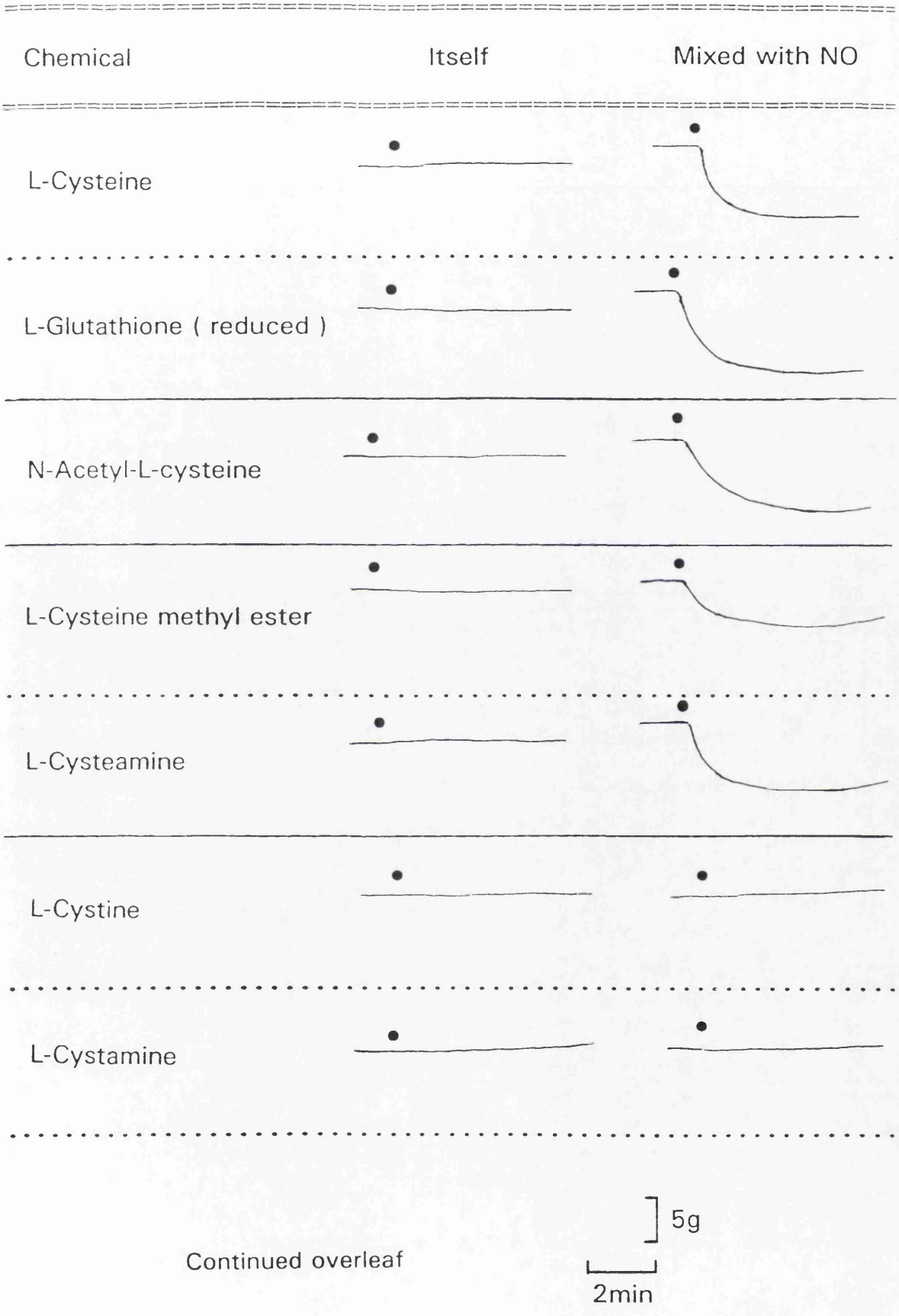
The L-cysteine molecule has three functional groups, namely, the amine, carboxyl and sulfhydryl groups. The formation of the new relaxant must take place when nitric oxide reacts with one or more of these groups of L-cysteine. Therefore, removal, replacement or substitution of these functional groups on the L-cysteine molecule may affect the formation of the new relaxant and may provide information on its chemical nature. The study was extended also to L-glutathione, another abundant sulfhydryl-containing substance which has these three functional groups, and to its structural analogues as well as to a group of common non-sulfhydryl-containing amino acids.

In these experiments the compounds used were classified into five groups according to their structure. The first group comprised those compounds with all three functional groups intact. This group included L-cysteine and L-glutathione. The second group comprised only N-acetyl-L-cysteine whose amine group was substituted but whose carboxyl and sulfhydryl groups were free. The third group included those compounds whose carboxyl groups were removed or replaced with an ester function but whose amine and sulfhydryl groups were free. This group included L-cysteamine, L-cysteine ethyl ester and L-cysteine methyl ester. The fourth group included those compounds whose sulfhydryl groups were substituted or oxidised to the corresponding disulphide but whose amine and carboxyl groups were

free. This group included L-cystamine, L-cysteic acid, L-cysteinesulphinic acid, L-cystine, L-glutathione (oxidised form), S-methyl-L-cysteine, S-methyl-L-glutathione and L-methionine. The final group included L-arginine, L-glutamic acid and L-glycine, all of which contain amine and carboxyl groups but not a sulfhydryl group. L-Arginine was also chosen since it is the precursor of nitric oxide in the body. L-Cysteine, L-glutamic acid and L-glycine are the amino acids which together comprise L-glutathione.

None of these compounds at a bath concentration of 0.15 mM had any smooth muscle relaxant activity when bioassayed on rabbit phenylephrine (0.3 μM)-contracted aortic rings (Figure 51). When aqueous deoxygenated solutions of these compounds (all at 15 mM) were mixed with nitric oxide (50 μM) at pH 2.0 for 10 minutes followed by removal of free nitric oxide by purging with oxygen-free nitrogen and neutralisation with sodium hydroxide (1 N), relaxant activity was generated in the solutions of those compounds with free sulfhydryl groups (Figure 51). Thus, L-glutathione, N-acetyl-L-cysteine, L-cysteamine, L-cysteine ethyl ester and L-cysteine methyl ester generated novel relaxant activity similar to that generated with L-cysteine. No relaxant activity was generated when the sulfhydryl group was removed, oxidised or substituted. Thus, L-cystamine, L-cysteic acid, L-cysteinesulphinic acid, L-cystine, L-glutathione (oxidised form), S-methyl-L-cysteine, S-methyl-L-glutathione, L-methionine, L-arginine, L-glutamic acid and L-glycine did not generate relaxant activity when reacted with nitric oxide. Thus, removal, replacement or substitution of amine or carboxyl groups in these compounds does not block the formation of new relaxant activity, whereas oxidation, removal or substitution of the free sulfhydryl group completely blocks the generation of new relaxants from these compounds. A summary of these results is shown in Table 1.

Figure 51. Analysis of the functional group, i.e. amine, carboxyl or sulfhydryl group, in the L-cysteine molecule that reacted with nitric oxide in the formation of the new smooth muscle relaxant. None of these compounds at bath concentration of 0.15 mM had any relaxant activity by themselves when assayed on rabbit phenylephrine (0.3 μM)-contracted aortic rings. When aqueous deoxygenated solutions of these compounds (all at 15 mM) were mixed with nitric oxide (50 μM) at pH 2.0 for 10 minutes followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation with sodium hydroxide (1 N), relaxant activity was generated in the solutions of those compounds with free sulfhydryl groups but not in those of compounds whose sulfhydryl groups were removed, substituted or oxidised to the disulphide. Removal, replacement or substitution of the amine or carboxyl groups did not block the formation of new relaxant activity, whereas oxidation, removal or substitution of the free sulfhydryl group completely blocked the generation of new relaxant activity.



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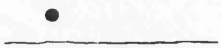
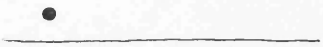
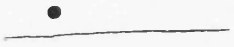
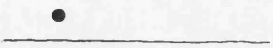
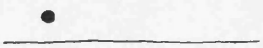
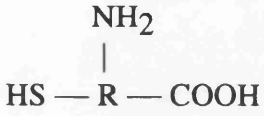
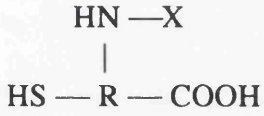
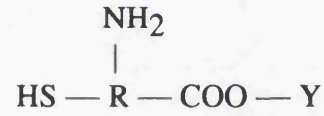
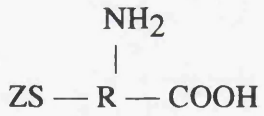
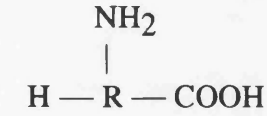
Chemical	Itself	Mixed with I
L-Cysteic Acid		
L-Cysteine sulphinic acid		
S-Methyl-L-cysteine		
L-Methionine		
L-Glutathione (oxidized)		
S-Methyl-L-glutathione		
L-Arginine		
L-Glycine		
L-Glutamic Acid		

Table 1. Classification of compounds used to identify the functional group of L-cysteine that reacts with nitric oxide

common structure	relaxant activity	relaxant activity after reacting with nitric oxide
	no	yes
	no	yes
	no	yes
	no	no
	no	no

This structure-activity analysis by using various structural analogues of L-cysteine and L-glutathione indicates that the reactions with nitric oxide take place at the sulfhydryl group of the molecules. Therefore, the new relaxants generated are most likely to be the S-nitroso derivatives of these thiols.

3.2.6. Effects of Drugs on Relaxation Induced by Nitric Oxide, NANC Nerve Stimulation, and the New Relaxant Generated by Reacting Nitric Oxide with L-Cysteine or L-Glutathione

Nitric oxide, the NANC transmitter and the novel compounds (S-nitrosocysteine and S-nitrosoglutathione) generated from nitric oxide reacting with L-cysteine or L-glutathione are all powerful relaxants. There is controversy, however, as to whether EDRF is nitric oxide (Palmer, *et al.*, 1987) or an S-nitrosothiol (Myers, *et al.*, 1990). By analogy, we wanted to establish whether nitric oxide or an S-nitrosothiol more closely resembles the NANC transmitter in the BRP. A comparison of the effects of drugs on relaxation induced by nitric oxide, NANC nerve stimulation and the new relaxants generated by reacting nitric oxide with L-cysteine or L-glutathione was therefore made. It was hoped that this study would provide a vital clue to the precise nature of the inhibitory NANC neurotransmitter in the BRP.

3.2.6.1. Effects of N^G-Nitro-L-arginine

In order to investigate the unlikely possibility that the novel relaxant generated from the reaction of L-cysteine with nitric oxide induced relaxation by stimulating the NANC nerves, the effects of a nitric oxide synthase inhibitor was examined.

N^G-Nitro-L-arginine, an analogues of L-arginine, is a powerful inhibitor of nitric oxide synthase (Gibson, *et al.*, 1989). It inhibits endothelium-dependent relaxation in vascular smooth muscle (Moore, *et al.*, 1989; Mulsch. & Busse, 1990) and

NANC relaxation induced by nerve stimulation in the BRP and the rat and mouse anococcygeus muscles (Gibson, *et al.*, 1989; 1990; Liu, *et al.*, 1991).

Pretreatment of a rabbit aortic ring denuded of endothelium with N^G-nitro-L-arginine (30 μM) for 15 minutes did not inhibit the relaxation on a phenylephrine (0.3 μM)-contracted ring induced by the relaxant generated by reacting nitric oxide (5 μM) with L-cysteine (15 mM, Figure 52). Thus, the new relaxant does not produce relaxation by activating the NANC nerves.

3.2.6.2. Effects of Haemoglobin

If the relaxant action of the new relaxant formed by reacting nitric oxide with L-cysteine is derived from the nitric oxide it releases, then its activity should be blocked by haemoglobin.

Haemoglobin binds nitric oxide avidly (Gibson & Roughton, 1957). It is a powerful inhibitor of EDRF-dependent relaxation (Martin, *et al.*, 1985) and inhibitory NANC relaxation in the BRP (Bowman, *et al.*, 1982b). In a BRP strip contracted by guanethidine (10 μM) oxyhaemoglobin at a concentration of 3 μM completely blocked the relaxation induced by authentic nitric oxide (0.3 μM), the new relaxant (30 μl into a 20 ml bath) generated from L-cysteine (10 mM) solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 and purged with oxygen-free nitrogen to remove free nitric oxide, and NANC nerve stimulation (2 Hz, 20 pulses) (Figure 53). It is therefore likely that the new relaxant formed from reacting nitric oxide with L-cysteine induces relaxation by the liberation of nitric oxide.

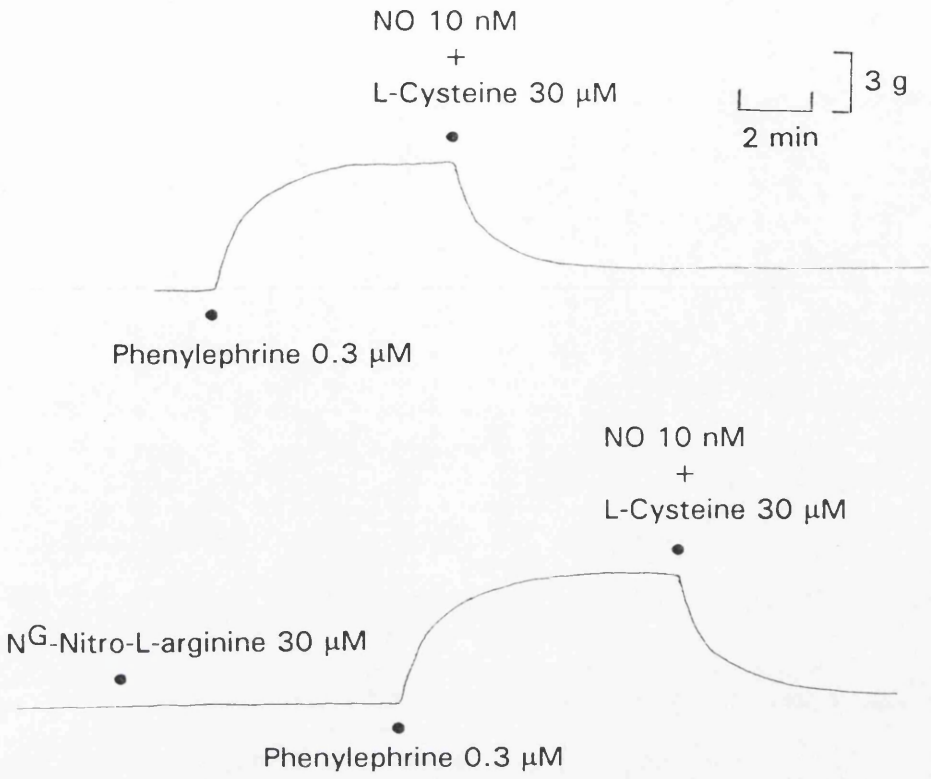


Figure 52. Pretreatment of a rabbit endothelium-denuded aortic ring with N^G-nitro-L-arginine (30 μM) for 15 minutes did not inhibit the relaxation induced by the relaxant generated from L-cysteine (15 mM) reacting with nitric oxide (5 μM) in aqueous deoxygenated solutions at pH 3 for 10 minutes followed by purging with oxygen-free nitrogen and neutralisation. The rabbit aortic ring was submaximally contracted by phenylephrine (0.3 μM). The concentrations given on the figure are final bath concentrations.

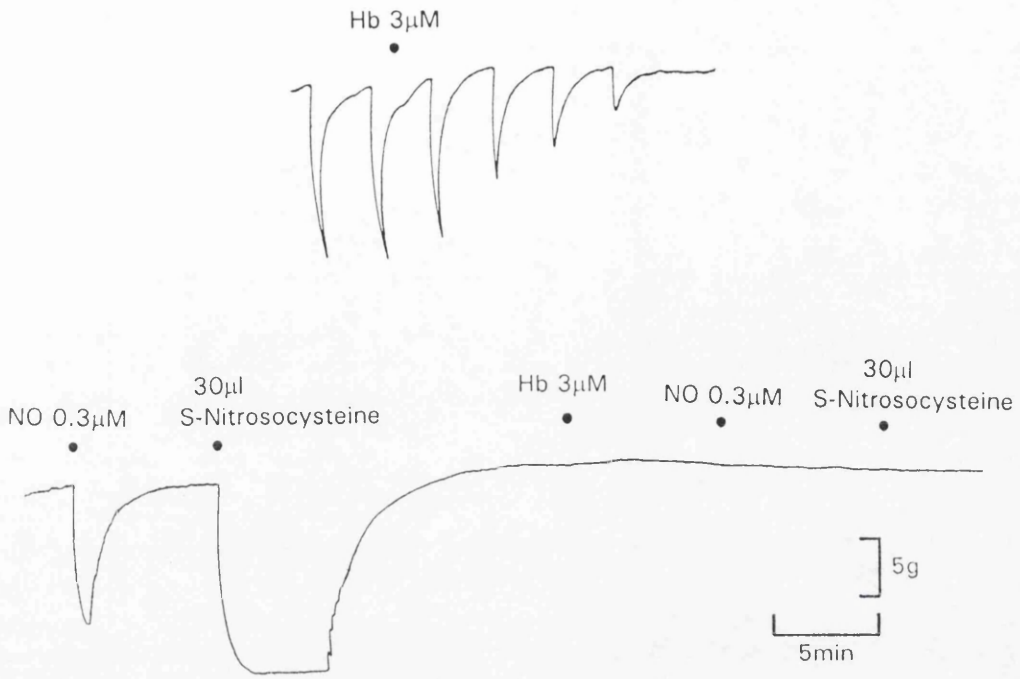


Figure 53. In BRP muscle precontracted by guanethidine (10 μM) oxyhaemoglobin (Hb 3 μM) completely blocked the relaxation induced by NANC nerve stimulation (2 Hz, 20 pulses), nitric oxide (0.3 μM) and S-nitrosocysteine generated from L-cysteine (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation.

3.2.6.3. Effects of Methylene Blue and N-Methyl-hydroxylamine

Methylene blue and N-methyl-hydroxylamine are both inhibitors of soluble guanylate cyclase (Martin, *et al.*, 1985; Bowman & Drummond, 1984). If the novel relaxant formed from reacting nitric oxide with L-cysteine induces relaxation by activation of soluble guanylate cyclase, then its actions should be inhibited by these compounds. Methylene blue (10 μM) and N-methyl-hydroxylamine (1 mM) were found to powerfully block the relaxation induced by nitric oxide (0.3 μM), the new relaxant (1-10 μl into a 20 ml bath) generated from L-cysteine (10 mM) solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 and purged with oxygen-free nitrogen to remove free nitric oxide, and NANC nerve stimulation (2 Hz, 20 pulses) in BRP strips contracted by guanethidine (10 μM) (Figures 54 & 55). In addition, methylene blue (10 μM) also produced a further increase in the guanethidine-induced tone.

3.2.6.4. Effects of Pyrogallol, Hydroquinone and Superoxide Dismutase

Pyrogallol and hydroquinone are closely related structurally and can both produce superoxide anions. Furthermore, superoxide dismutase is a scavenger of superoxide anion and can be used to reverse or block the actions of pyrogallol and hydroquinone. Work from our laboratory and elsewhere has shown previously that although pyrogallol and hydroquinone can inhibit the relaxant actions of nitric oxide, they do not block those to NANC stimulation in the BRP (Gillespie & Sheng, 1990) or mouse anococcygeus (Gibson, *et al.*, 1992). These findings suggested that the NANC transmitter was a superoxide anion-resistant, nitric oxide releasing molecule rather than free nitric oxide. We therefore examined whether or not the new relaxant formed in the reaction of nitric oxide with L-cysteine or L-glutathione shared the property of being resistant to destruction by superoxide anion.

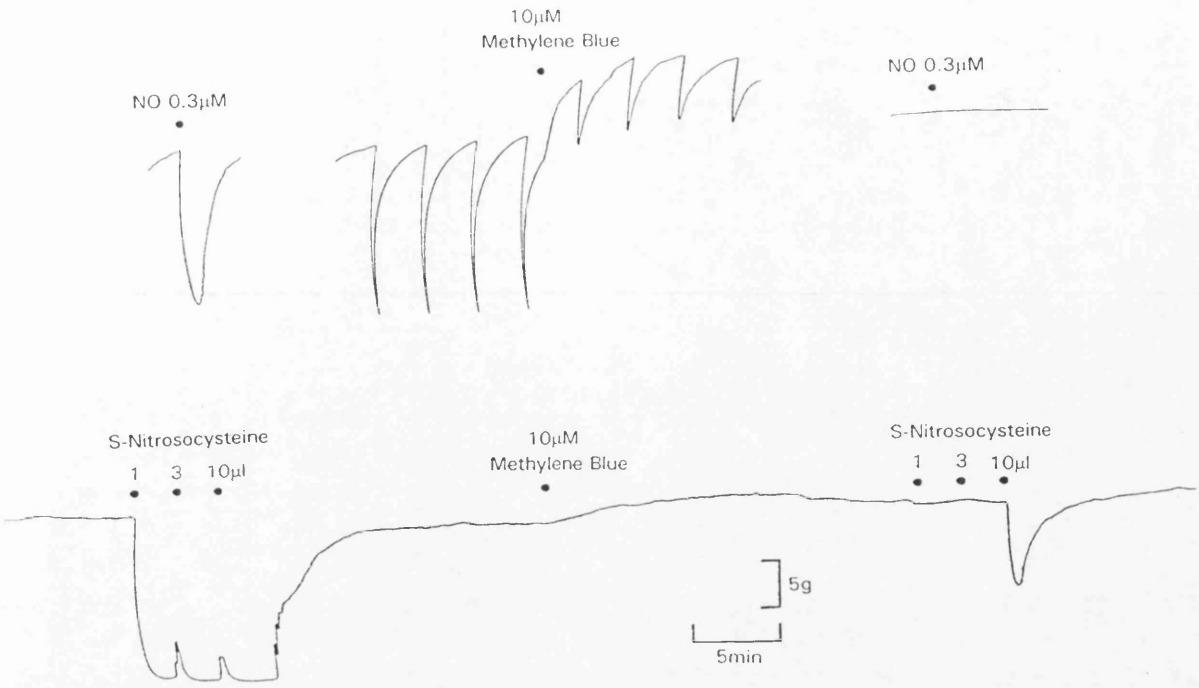


Figure 54. In BRP muscle precontracted by guanethidine (10 μM) methylene blue (10 μM) powerfully blocked the relaxation induced by NANC nerve stimulation (2 Hz, 20 pulses), nitric oxide (0.3 μM) and S-nitrosocysteine generated from L-cysteine (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation.

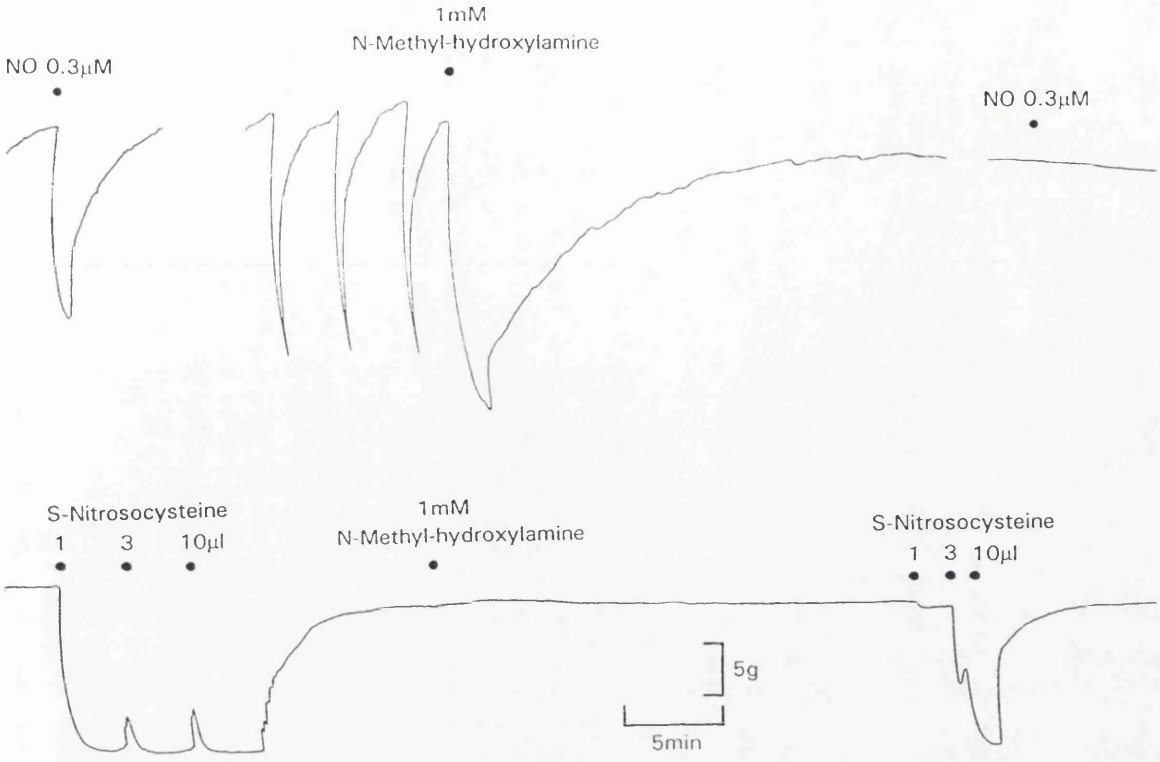


Figure 55. In BRP muscle precontracted by guanethidine (10 μM) N-methylhydroxylamine (1 mM) powerfully blocked the relaxation induced by NANC nerve stimulation (2 Hz, 20 pulses), nitric oxide (0.3 μM) and S-nitrosocysteine generated from L-cysteine (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation.

In BRP muscle precontracted by guanethidine (10 μM) or phenylephrine (0.3 μM) pyrogallol at a concentration of 0.1 mM completely blocked nitric oxide (0.3 μM)-induced relaxation but had no effect on the NANC relaxation induced by field stimulation (Figure 56). Furthermore, pyrogallol (0.1 mM) powerfully blocked the relaxation induced by the new relaxant (3-100 μl in a 20 ml bath) generated when L-cysteine (10 mM) solutions were bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging to remove free nitric oxide (Figures 56 & 57). The blockade of the relaxation induced by the new relaxant was reversed by subsequent addition of and prevented by pretreatment with superoxide dismutase (100 u ml^{-1}) in phenylephrine (0.3 μM)-contracted BRP muscle (Figures 57 & 58). In the absence of pyrogallol superoxide dismutase (100 u ml^{-1}) powerfully potentiated the activity of the new relaxant (Figure 58) but had no effect on the relaxation to NANC nerve stimulation (Figure 5).

In phenylephrine (0.3 μM)-contracted BRP muscle hydroquinone (0.1 mM) produced inhibition of the relaxation induced by the new relaxant generated from L-cysteine (10 mM) solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging to remove free nitric oxide with oxygen-free nitrogen (Figure 59). The inhibition was, however, less powerful than that induced by pyrogallol (Figure 57). Treatment of the tissue with superoxide dismutase (100 u ml^{-1}) not only reversed this inhibition by hydroquinone but also potentiated the relaxation induced by the new relaxant (Figures 59 & 60). Furthermore, pretreatment of the tissue with superoxide dismutase (100 u ml^{-1}) also prevented this inhibition by hydroquinone (Figure 60).

In a separate series of experiments the new relaxant (100 μl into a 20 ml bath) generated from L-cysteine (10 mM) solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove

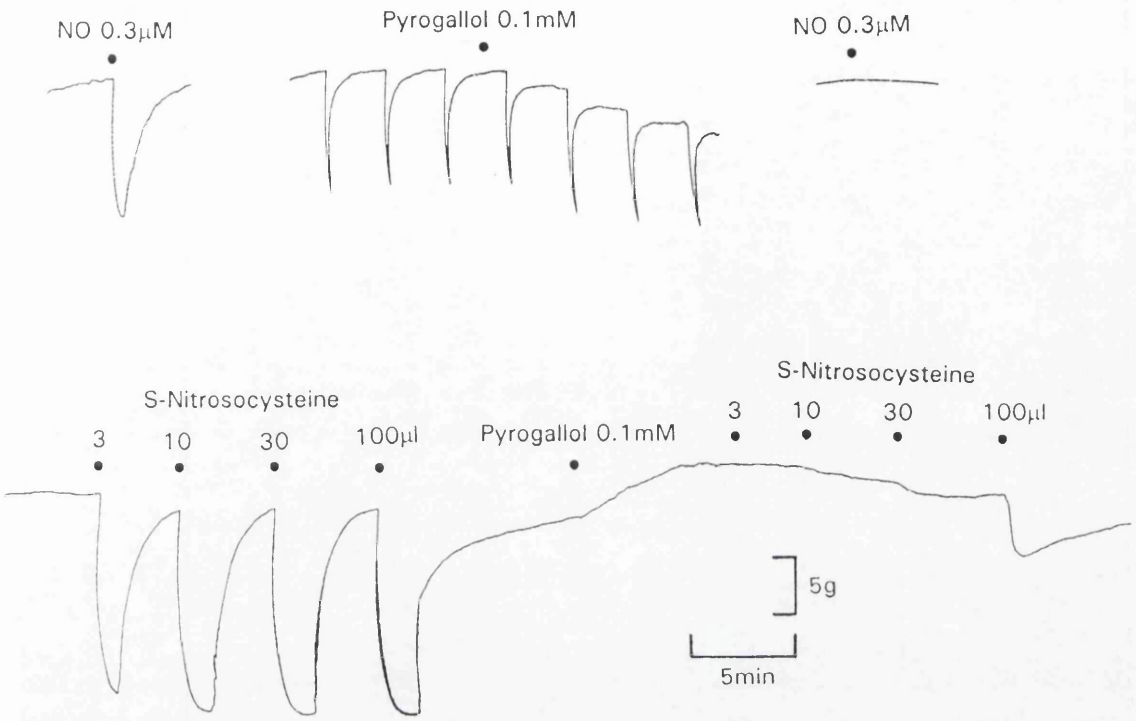


Figure 56. On BRP muscle precontracted by guanethidine (10 μM) pyrogallol (0.1 mM) powerfully blocked the relaxation induced by nitric oxide (0.3 μM) and S-nitrosocysteine generated from L-cysteine (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation, but it did not inhibit the NANC relaxation induced by field stimulation (2 Hz, 20 pulses).

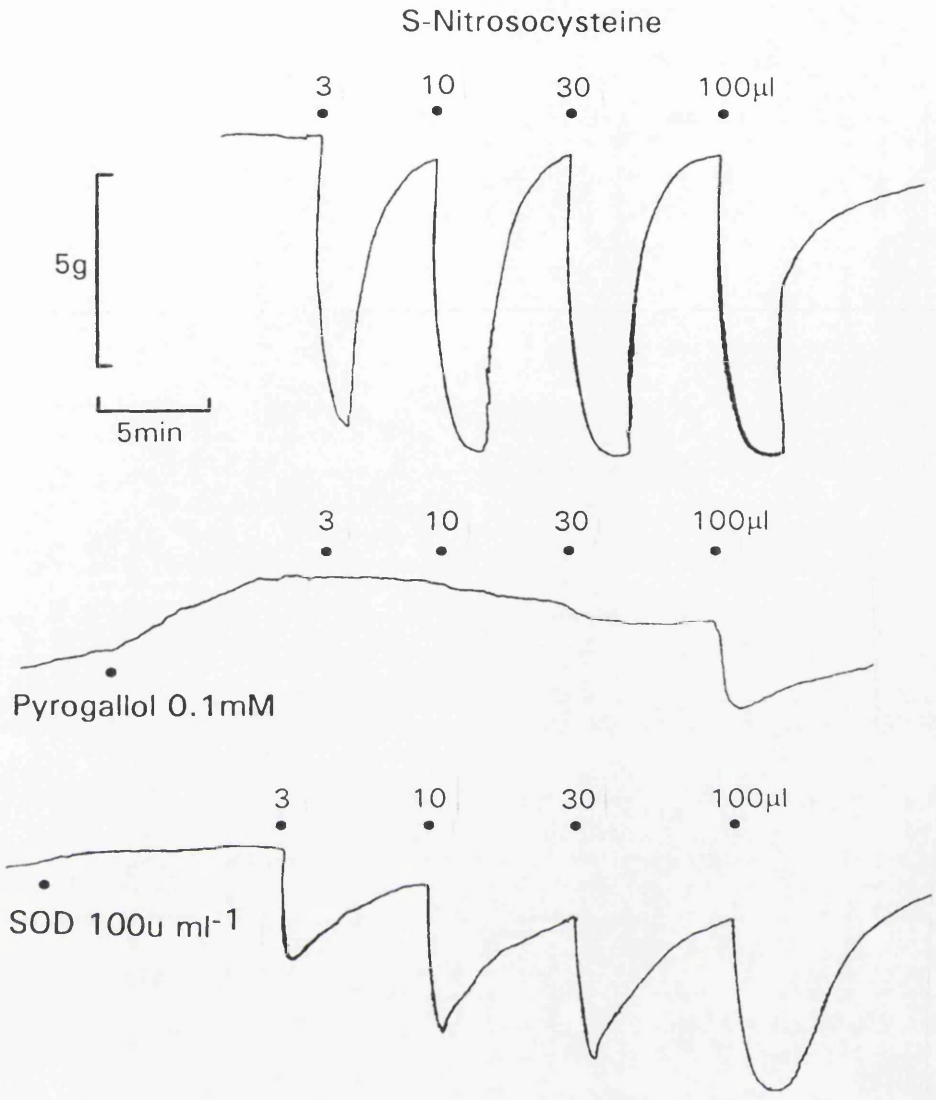


Figure 57. In BRP muscle precontracted by phenylephrine (0.3 µM) pyrogallol (0.1 mM) powerfully blocked the relaxation induced by S-nitrosocysteine generated from L-cysteine (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation. This blockade was reversed by addition of superoxide dismutase (SOD, 100 u ml^{-1}) to the assay tissue.

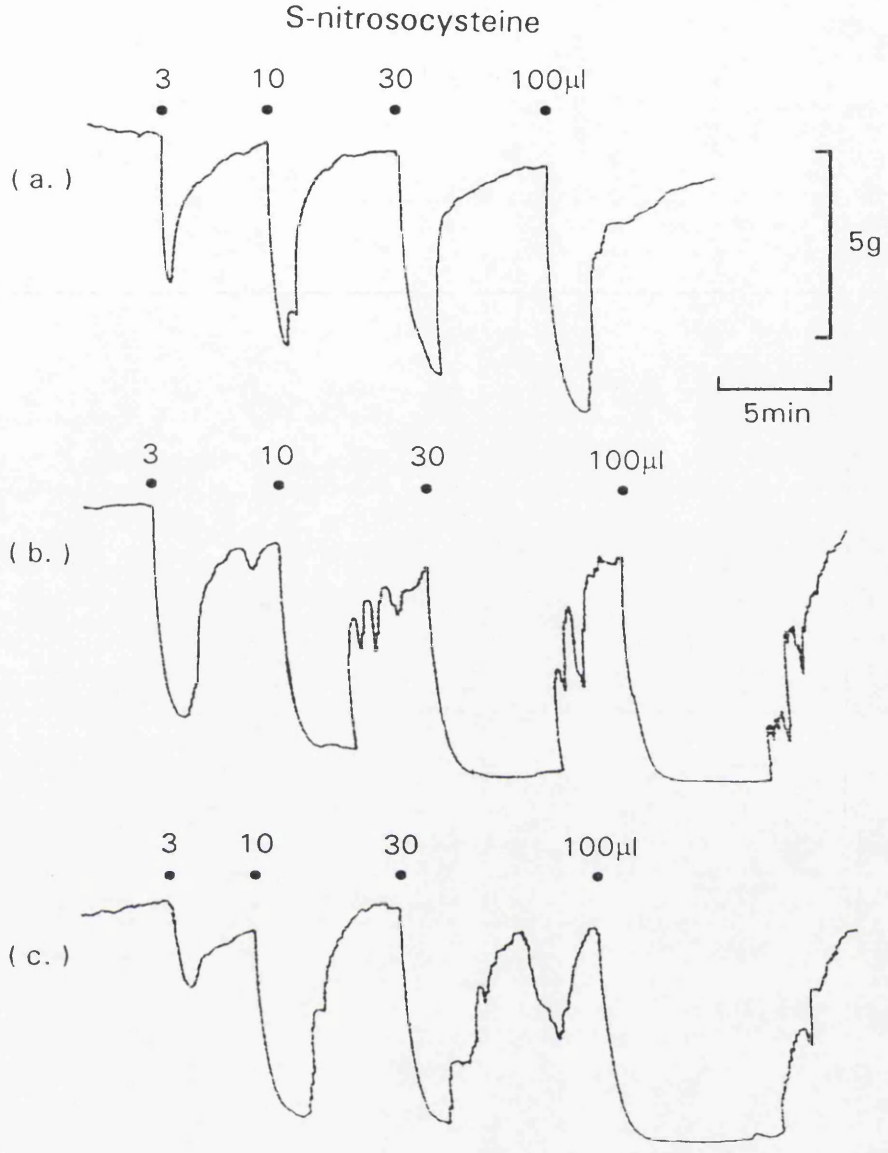


Figure 58. (a.) Concentration-response of S-nitrosocysteine generated from L-cysteine (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation. (b.) Pretreatment with superoxide dismutase (100 u ml^{-1}) potentiated the activity of S-nitrosocysteine. (c.) In the presence of superoxide dismutase (100 u ml^{-1}) the activity of S-nitrosocysteine was not inhibited by pyrogallol (0.1 mM). Bioassay was carried out on a BRP strip submaximally contracted by phenylephrine (0.3 µM).

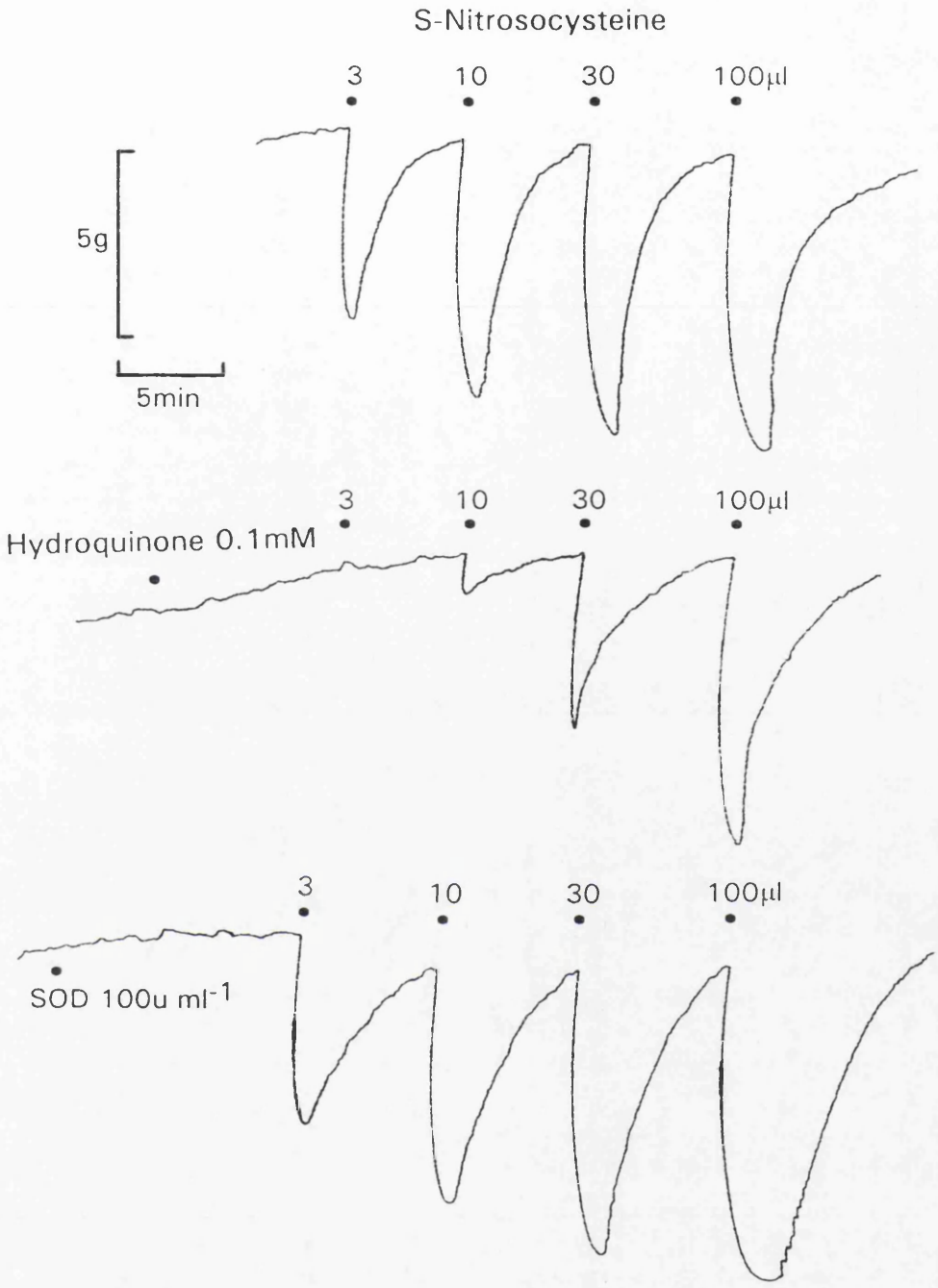


Figure 59. In BRP muscle precontracted by phenylephrine (0.3 µM) hydroquinone (0.1 mM) partially blocked the relaxation induced by S-nitrosocysteine generated from L-cysteine (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation. Superoxide dismutase (SOD, 100 u ml^{-1}) not only reversed this blockade by hydroquinone but also potentiated the relaxation induced by S-nitrosocysteine.

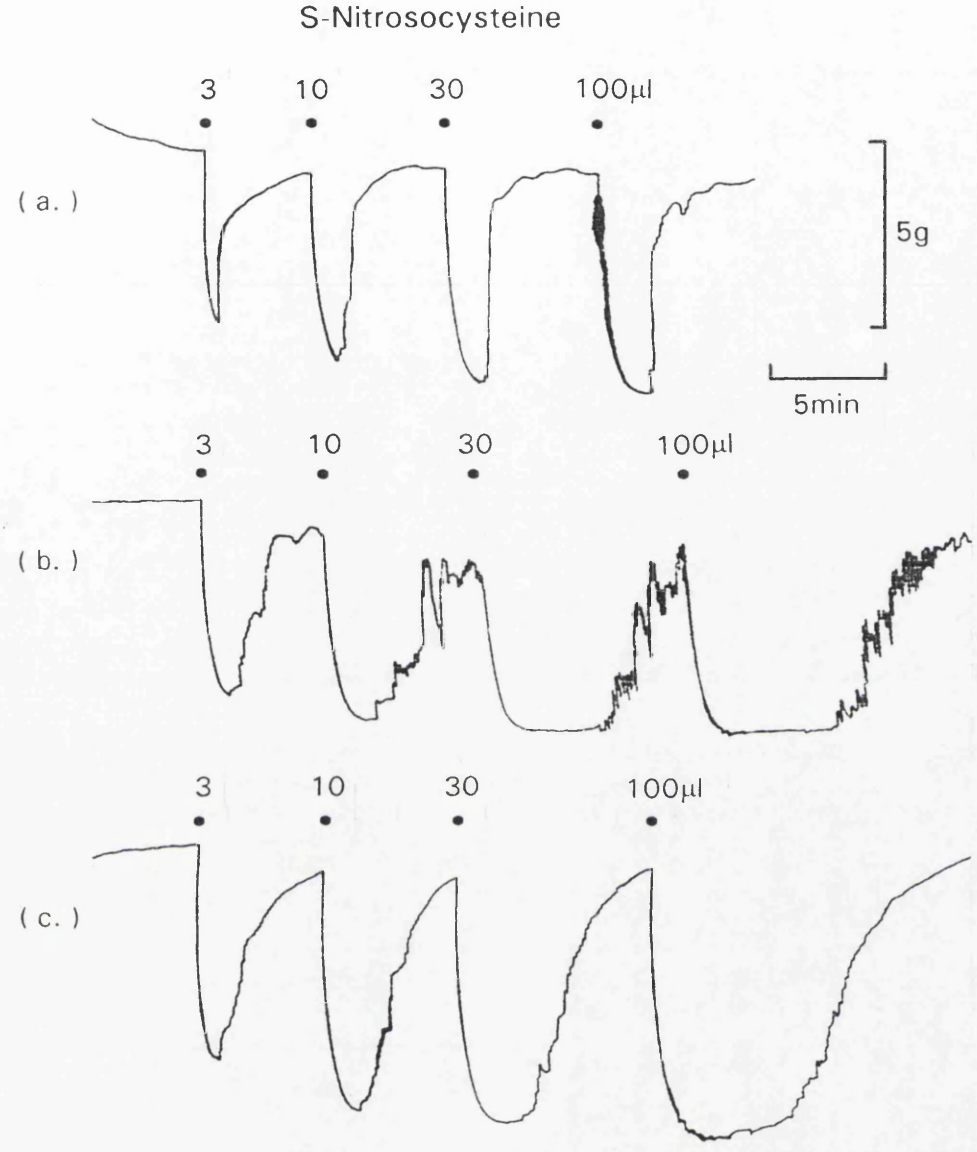


Figure 60. (a.) Concentration-response of S-nitrosocysteine generated from L-cysteine (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation. (b.) pretreatment with superoxide dismutase (100 u ml^{-1}) potentiated the activity of S-nitrosocysteine. (c.) In the presence of superoxide dismutase (100 u ml^{-1}) the relaxation induced by S-nitrosocysteine was not inhibited by hydroquinone (0.1 mM). Bioassay was carried out on a BRP strip submaximally contracted by phenylephrine (0.3 µM).

free nitric oxide induced a powerful relaxation in phenylephrine (0.3 μM)-contracted BRP muscle strips (Figure 61). Once established, this relaxation was reversed quickly by pyrogallol or slowly by hydroquinone both at a final bath concentration of 0.1 mM (20 μl of 0.1 M into a 20 ml bath). The reversal of the relaxation induced by hydroquinone, but not by pyrogallol, was reversed after adding superoxide dismutase (100 u ml^{-1}) to the tissue. Furthermore, when the relaxant (100 μl) was mixed with either pyrogallol (20 μl of 0.1 M) or hydroquinone (20 μl of 0.1 M) for 10 minutes prior to their addition into the bath, almost all of their relaxant activity was destroyed even when assayed in tissues pretreated with superoxide dismutase (100 u ml^{-1}, Figure 61).

When assayed on phenylephrine (0.3 μM)-contracted BRP muscle pyrogallol (0.1 mM) and hydroquinone (0.1 mM) also powerfully blocked the relaxation induced by the new relaxant generated from L-glutathione (10 mM) solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide (Figures 62 & 63). Superoxide dismutase (100 u ml^{-1}) not only reversed this blockade by pyrogallol and hydroquinone but also potentiated the relaxation induced by the new relaxant.

Thus, superoxide anion was able to destroy the relaxant activity of nitric oxide and of the new relaxants formed following the reaction of nitric oxide with L-cysteine or L-glutathione but was unable to destroy the NANC transmitter in the BRP.

3.2.6.5. Effects of DETCA

Clearly, pyrogallol and hydroquinone are unable to block the effects of the inhibitory NANC transmitter but they can destroy the actions of nitric oxide or the new relaxants generated from L-cysteine or L-glutathione following reaction with nitric oxide. These findings make it unlikely that nitric oxide or the new relaxants

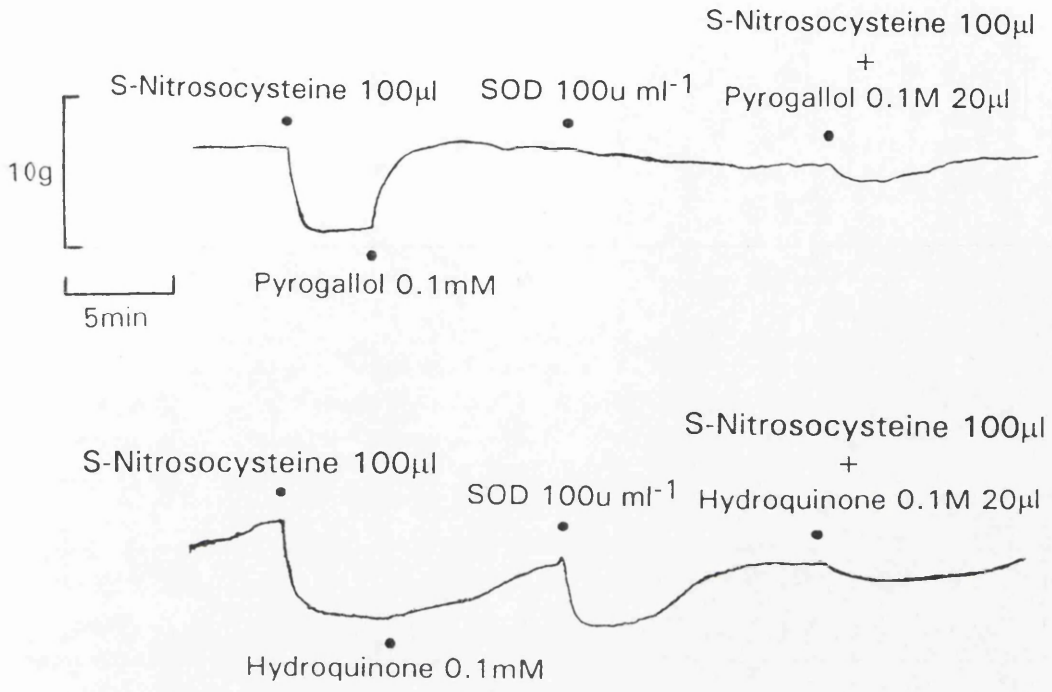


Figure 61. S-Nitrosocysteine (100 µl into a 20 ml bath) generated from L-cysteine (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation induced a powerful relaxation in phenylephrine (0.3 µ M)-contracted BRP muscle. This relaxation was blocked quickly by pyrogallol and slowly by hydroquinone both at final bath concentrations of 0.1 mM (20 µl of 0.1 M into the 20 ml bath). The blockade of the relaxation by hydroquinone, but not that by pyrogallol, was reversed after addition of superoxide dismutase (SOD, 100 u ml^{-1}) into the bath. When S-nitrosocysteine (100 µl) was mixed with pyrogallol (20 µl of 0.1 M) or hydroquinone (20 µl of 0.1 M) for 10 minutes before addition to the bath, the relaxant activity of S-nitrosocysteine was almost completely destroyed even in the presence of superoxide dismutase (100 u ml^{-1}) in the tissue.

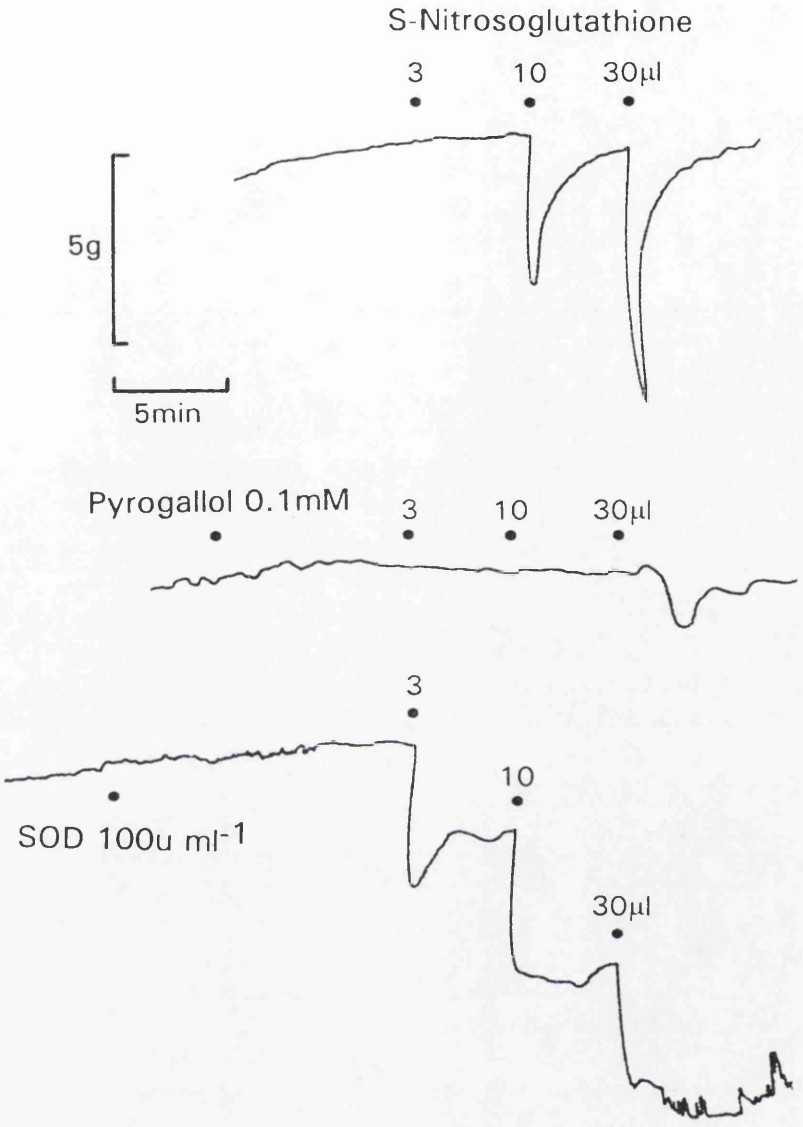


Figure 62. On BRP muscle precontracted by phenylephrine (0.3 µM) pyrogallol (0.1 mM) powerfully blocked the relaxation induced by S-nitrosoglutathione generated from L-glutathione (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation. Superoxide dismutase (100 u ml^{-1}) not only reversed this blockade by pyrogallol (0.1 mM) but also powerfully potentiated the relaxation induced by S-nitrosoglutathione.

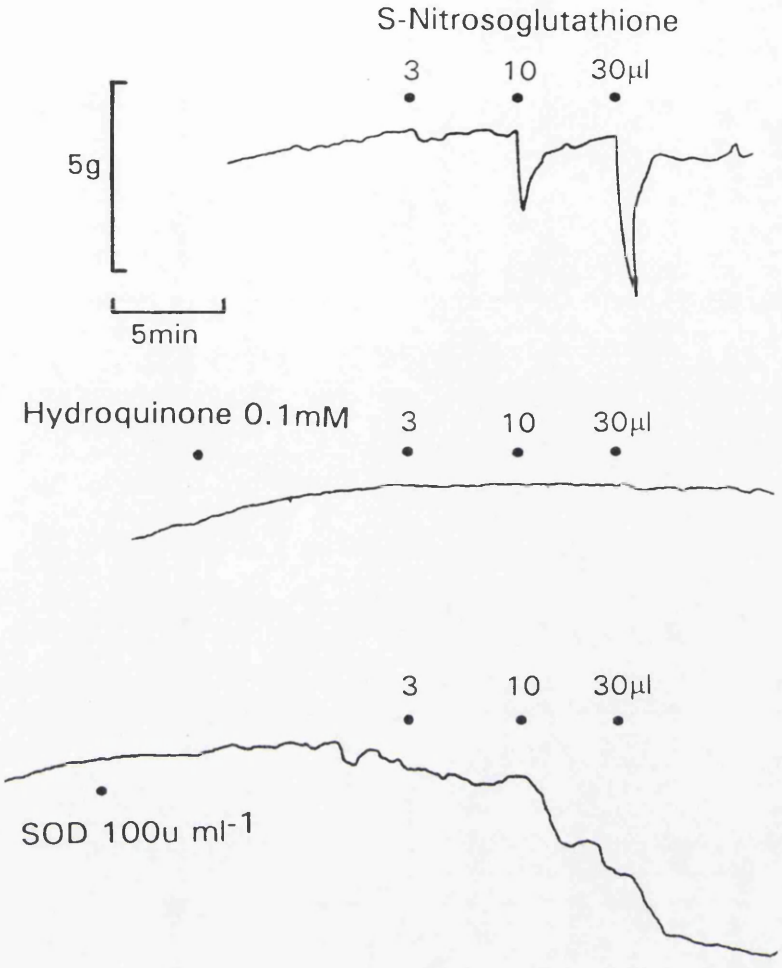


Figure 63. On BRP muscle precontracted by phenylephrine (0.3 µM) hydroquinone (0.1 mM) completely blocked the relaxation induced by S-nitrosoglutathione generated from L-glutathione (10 mM) aqueous deoxygenated solutions bubbled to saturation with nitric oxide for 10 minutes at pH 3.0 followed by purging with oxygen-free nitrogen to remove free nitric oxide and neutralisation. Superoxide dismutase (100 u ml^{-1}) not only reversed this blockade by hydroquinone but also potentiated the relaxation induced by S-nitrosoglutathione.

act as the NANC neurotransmitter in the BRP. However, if a mechanism exists in the tissue to prevent the transmitter from being destroyed by superoxide anions, then nitric oxide, S-nitrosocysteine and S-nitrosoglutathione could still be regarded as candidates. Such a mechanism could be possible if the BRP contained high superoxide dismutase activity. Mammalian tissues contain Cu^{++}, Zn^{++} superoxide dismutase (CuZnSOD) and this is inactivated by the copper chelator diethyldithiocarbamate (DETCA, Halliwell & Gutteridge, 1989). An experiment was therefore conducted in which strips of BRP muscle were treated with DETCA to determine if this would make the NANC relaxation inhibitable by pyrogallol.

In guanethidine (10 μM)-contracted BRP strip preparations pyrogallol (10-100 μM) did not inhibit the NANC relaxation induced by field stimulation (2 Hz, 20 pulses) although it reduced the tone at concentrations of 100 μM (Figure 64). DETCA at a concentration of 10 mM neither inhibited the NANC relaxation nor affected the tone of the tissue. After treatment of the BRP muscle with DETCA (10 mM) for 30 minutes pyrogallol (100 μM) remained unable to inhibit the NANC relaxation.

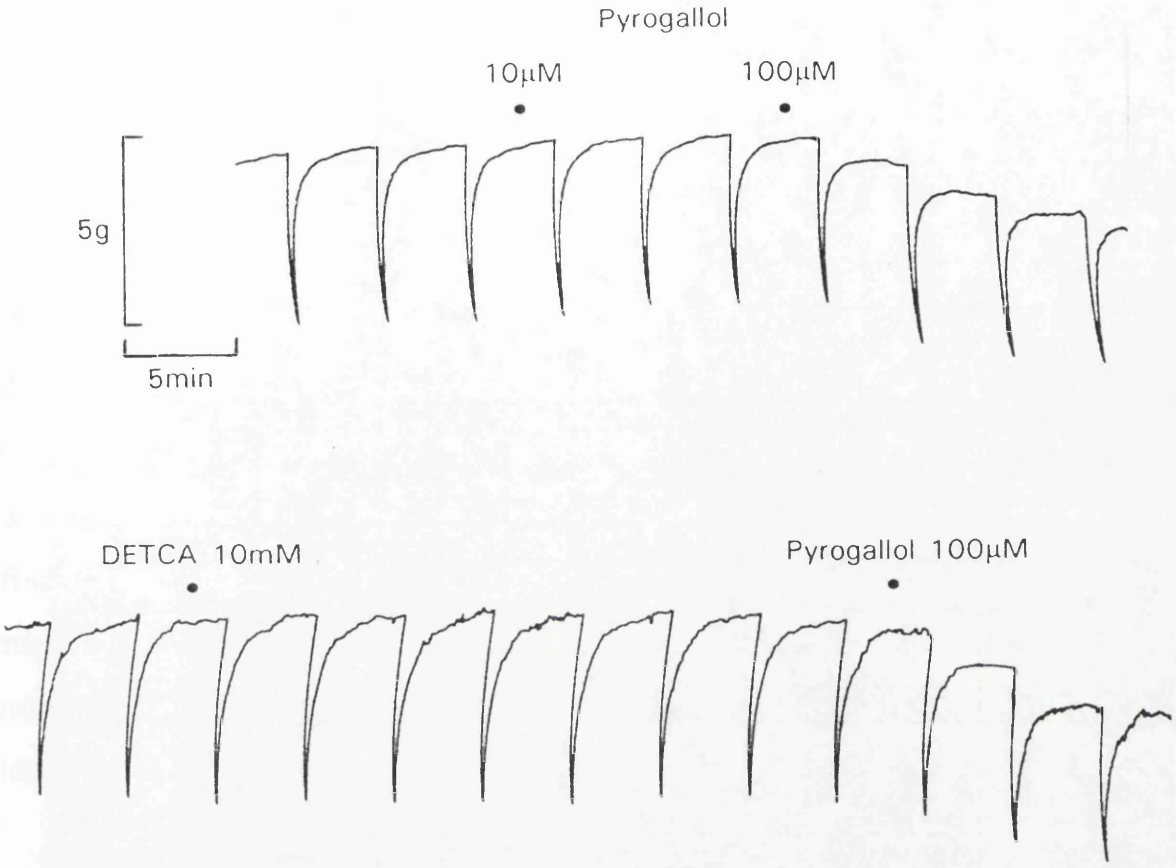


Figure 64. On a guanethidine (10 μM)-contracted BRP strip pyrogallol (10-100 μ M) did not inhibit the NANC relaxation induced by field stimulation (2 Hz, 20 pulses) but reduced the tone at a concentration of 100 μM. DETCA at a concentration of 10 mM neither inhibited the NANC relaxation nor affected the tone of the tissue. After treating the BRP muscle with DETCA (10 mM) for 30 minutes pyrogallol (100 μM) remained unable to inhibit the NANC relaxation.

Chapter 4. DISCUSSION

The phenomenon of atropine-resistant penile erection produced by electrical stimulation of the sacral parasympathetic outflow via the pelvic nerves in the rabbit (Langley & Anderson, 1895) can now been regarded as the first demonstration of the existence of a non-adrenergic, non-cholinergic innervation although at that time concept of chemical neurotransmission had not been established. The first major observation that provided firm evidence for the existence of NANC nerves was the finding that bretylium did not block the powerful inhibitory response to nerve stimulation of the guinea pig taenia coli (Burnstock, *et al.*, 1963; 1964). The neurogenic origin of the response was verified by its blockade by tetrodotoxin at low concentrations. (Bulbring & Tomita, 1967).

The retractor penis muscle possesses a typical NANC inhibitory innervation (Klinge & Sjostrand, 1974). Anatomically, it is an extension of the anococcygeus muscle (Gillespie, 1972). Both muscles receive a double innervation, the adrenergic motor sympathetic nerves from the spinal cord in the lumbar region with most fibres travelling via the pubic nerve, and the NANC inhibitory nerves from the sacral cord with their fibres in the pelvic parasympathetic nerves whose transmitter was unknown at the beginning of this study (Langley & Anderson, 1895; Langley & Anderson, 1895; Fletcher, 1898; Gruber, 1933; Luduena & Grigas, 1966; Gillespie, 1972; Gillespie & McGrath, 1973; 1974; Klinge & Sjostrand, 1974; Creed, *et al.*, 1977; Gibson & Wedmore, 1981; Byrne & Muir, 1984). Until recently, except for local anaesthetics and tetrodotoxin, none of the drugs which block the actions of known neurotransmitters had any effect on the inhibitory response to the NANC nerve stimulation and no known neurotransmitter could mimic the relaxant response to NANC nerve stimulation in both tissues (Luduena & Grigas, 1966; 1972; Gillespie, 1972; Klinge & Sjostrand, 1974; Creed, *et al.*, 1977).

Over the years, three potential candidates emerged for the NANC transmitter in the anococcygeus and the retractor penis muscles: the inhibitory factor (IF) extracted from the BRP, endothelium-derived relaxing factor (EDRF), and nitric oxide (NO). According to the conventional concept of neurotransmission, a substance must fulfil the following criteria before being established as a neurotransmitter (Eccles, 1964): 1). It is synthesised and present in the nerve fibres; 2). It is released by nerve stimulation; 3). It should mimic the nerve-induced response to electrical stimulation; 4). It combines with its receptor specifically; 5). Its mode of action and that of the neurotransmitter released from nerve should be the same; 6). Drugs which potentiate or block the nerve-induced response should have the same effect on the putative neurotransmitter; 7). An inactivation mechanism should be present consisting either of enzymatic breakdown and / or uptake. IF, EDRF and nitric oxide share many properties with the NANC transmitter and fulfil many of these criteria. For example, all act through stimulation of soluble guanylate cyclase and increases of cGMP levels (Murad, *et al.*, 1978; Rapoport & Murad, 1983b; Bowman & Drummond, 1984) in a manner potentiated by the cGMP-selective phosphodiesterase inhibitor, M&B 22,948 (Bowman & Drummond, 1984), and inhibited by haemoglobin and methylene blue (Murad, *et al.*, 1978; Bowman & Gillespie, 1982; 1983; Bowman, *et al.*, 1982b; Bowman & Drummond, 1984; Bowman, *et al.*, 1986; Martin, *et al.*, 1985; Gibson & Mirazadeh, 1989).

EDRF was first discovered by Furchgott and Zawadzki (1980) and was suggested to be nitric oxide by Furchgott (1988) and Ignarro *et al.* (1988). Following this suggestion, nitric oxide was detected in the fluid perfusing cultured porcine aortic endothelial cells following stimulation by bradykinin at concentrations that induce EDRF release (Palmer, *et al.*, 1987). The amount of nitric oxide released by the cells was sufficient to account for the relaxation of vascular strips by EDRF. A study using $^{18}O_2$ and mass spectrometry showed that molecular oxygen is

incorporated into both nitric oxide and its co-product L-citrulline (Leone, *et al.*, 1991). By applying a radiochemical technique involving [^{3}H]arginine coupled with mass spectrometry, L-citrulline was confirmed to be a product derived from L-arginine metabolism (Moncada & Palmer, 1990). This formation was specific since the metabolism of L-arginine was competitively blocked by N^G-monomethyl-L-arginine but not its D-isomer, an effect competitively reversed by L- but not D-arginine (Palmer, *et al.*, 1988).

The clear involvement of nitric oxide in NANC neurotransmission was first revealed by the ability of L-NMMA , but not D-NMMA, to block NANC relaxation in the anococcygeus of the rat and mouse, and the retractor penis of the bull, an effect reversed by L-arginine, but not its D-isomer (Gibson, *et al.*, 1989; 1990; Gillespie & Xiaorong, 1989; Gillespie, *et al.*, 1989; 1990; Li & Rand, 1989). It is also widely accepted that the L-arginine: nitric oxide system mediates NANC inhibitory transmission in many other tissues including the canine ileocolonic junction (Boeckxstaens, *et al.*, 1991), the guinea-pig trachea (Li & Rand, 1991), the human corpus cavernosus (Ignarro, *et al.*, 1990; Pickard, *et al.*, 1991), the dog and monkey cerebral artery (Toda & Okamura, 1990a; 1990b), and the bovine penile artery (Liu, *et al.*, 1991). However, nitric oxide binds to and / or reacts at great speed with many substances normally present in the body and thus its action on soluble guanylate cyclase could be blocked by these substances preventing it from reaching its target cells. For example, haeme has a high binding affinity for and reactivity with nitric oxide and this is also true for haemoproteins. The binding affinity of haemoproteins is about 1,500 times higher for nitric oxide than for carbon monoxide (Gibson & Roughton, 1957) and about 200 times higher for carbon monoxide than for oxygen (Giardina & Amiconi, 1981). It is difficult to imagine how nitric oxide produced in one cell could travel a distance and activate soluble guanylate cyclase in a distant cell without binding to other haemoproteins such as haemoglobin and myoglobin. Moreover, nitric oxide reacts readily with

oxygen according to the following equation (Committee on Medical and Biological Effects of Environmental Pollutants, 1977; Olbergts, 1985):

$$2NO + O_2 \rightarrow 2NO_2$$

The rate of the above reaction is given by the following third-order rate equation:

$$d[NO_2] = 2k[NO]^2[O_2]dt$$

The reaction takes place at a rate which is about 4,000 times higher in aqueous solution than in the gas phase (Pogrebnaya, *et al.*, 1975). That nitric oxide may not be the exact form of the NANC transmitter was supported by the evidence that the superoxide anion-generating drugs, pyrogallol and hydroquinone, inhibit the relaxant actions of authentic nitric oxide but not those of NANC nerve stimulation in the BRP and mouse anococcygeus (Gillespie & Sheng, 1990; Gibson, *et al.*, 1992).

The effects of pyrogallol and hydroquinone suggest that the NANC neurotransmitter in these tissues may be a superoxide anion-resistant, nitric oxide-releasing molecule rather than free nitric oxide. Indeed, an analogous debate continues concerning the precise nature of EDRF: Palmer *et al.* (1987) reported that the levels of nitric oxide produced by endothelium as measured by chemiluminescence could account for the biological activity of EDRF, but Myers *et al.*, (1989), using the same technique, found that the levels of nitric oxide detected were roughly 10-fold lower than required to account for its activity. In fact, Myers *et al.* (1990) suggest that EDRF more closely resembles S-nitrosocysteine than nitric oxide. If a compound does exist which binds nitric oxide and acts as a delivery system between synthesis in the nerve endings or endothelium and action on soluble guanylate cyclase in the effector cell it would ideally meet certain conditions. Specifically, it would bind nitric oxide

securely enough to prevent non-specific interactions with proteins and other substances and prevent destruction by oxygen and superoxide anion. Furthermore, the binding must be reversible so as to deliver nitric oxide to soluble guanylate cyclase in the target cell.

This study investigated the possibility that the NANC inhibitory neurotransmitter in the BRP was S-nitrosocysteine or S-nitrosoglutathione rather than authentic nitric oxide. This was done firstly, by examining whether free sulfhydryl groups are required for NANC relaxation to nerve stimulation in the BRP muscle, and secondly, by examining the properties of S-nitrosocysteine and S-nitrosoglutathione and finally, by comparing the effects of drugs on the properties of the S-nitrosothiols and the NANC transmitter. In this study the initial investigation into the possibility of an S-nitrosothiol being the NANC transmitter in the BRP muscle was based on: 1). The NANC inhibitory transmitter, IF, and S-nitrosothiols share many properties, typically stimulating soluble guanylate cyclase and relaxing smooth muscle (Gillespie, *et al.*, 1990; Ignarro, 1990; Moncada, *et al.*, 1991). 2). Thiols are required for relaxation of vascular smooth muscle (Needleman, *et al.*, 1973) and for the activation of soluble guanylate cyclase by certain nitrovasodilators including glyceryl trinitrate (Ignarro & Gruetter, 1980; Ignarro, *et al.*, 1981b). 3). The unactivated form of IF contains 4μM nitrite and the activation of IF by exposure to acid at pH 2 is associated with a 75% loss of nitrite (Martin, *et al.*, 1988). 4). The relaxant activity and loss of nitrite in solutions of activated IF is closely related to the content of sulfhydryl groups in general and L-cysteine in particular (Yui, *et al.*, 1989; Kerr, *et al.*, 1993). 5). S-Nitrosothiols are synthesised by acidification of sodium nitrite in the presence of thiols (Gibson, *et al.*, 1992; Kerr, *et al.*, 1993). In the study reported here, L-cysteine and L-glutathione were chosen since they are the most abundant sulfhydryl-containing substances in the body, for example, blood plasma contains L-cysteine at 150 μM and the intracellular concentration is several times higher than this (Apps & Harnden, 1985).

Free sulfhydryl inactivating agents have previously been employed to investigate the involvement of S-nitrosothiols in the activity of EDRF, but with equivocal results (Siegle, *et al.*, 1993; Minor, *et al.*, 1989). Minor *et al.* (1989) reported that basal EDRF release was inhibited by depletion of L-cysteine from endothelial cells, whereas Siegle *et al.* (1993) found that sulfhydryl depleting agents inhibited stimulated but not basal EDRF release from cultured endothelial cells. We examined the actions of two sulfhydryl-depleting agents, diamide which oxidises free sulfhydryl groups to the corresponding disulphide (Kosower, *et al.*, 1969) and N-ethylmaleimide which covalently alkylates free sulfhydryl groups (Siegle, *et al.*, 1993), on NANC relaxation to nerve stimulation in the BRP muscle. Consistent with involvement of an S-nitrosothiol in NANC neurotransmission, we found that both agents blocked NANC relaxation and this was prevented by pretreating the tissues with excess sulfhydryl groups in the form of L-cysteine, L-glutathione or dithiothreitol. These three sulfhydryl compounds were also effective in reversing the actions of diamide, presumably through reduction of the -S-S- bonds formed following its action (Kosower, *et al.*, 1969). The actions of N-ethylmaleimide were not reversed, suggesting that blockade had not resulted from covalent inactivation of the sulfhydryl groups of endogenous L-cysteine or L-glutathione. The most compelling evidence against the blockade having resulted from loss of the ability to form S-nitrosothiols was obtained when we found that relaxation to glyceryl trinitrate (GTN) and isoprenaline was also blocked by these two sulfhydryl inactivating agents and was not reversed by the three sulfhydryl-containing compounds. Since GTN and isoprenaline activate soluble guanylate cyclase and adenylate cyclase, respectively, the sulfhydryl inactivating agents were clearly blocking the effector pathways for these diverse relaxants and that any additional action in preventing formation of S-nitrosothiols by the NANC nerves would be impossible to discern. However, the involvement of free sulfhydryl groups in the NANC relaxation is still suggested by the evidence that the sulfhydryl compounds

partially reversed the blockade by diamide on relaxation to NANC nerve stimulation but not to GTN and isoprenaline. Because the relaxant actions of both NANC transmitter and GTN are via activation of soluble guanylate cyclase and increases in cGMP levels, at least two different subtypes of soluble guanylate cyclase in the BRP muscle, one responding to NANC transmitter and the other responding to GTN, were suggested since we found that the sulfhydryl compounds, L-cysteine, L-glutathione and dithiothreitol, reversed the blockade by diamide on the relaxation induced by NANC nerve stimulation but not by GTN. These results extend the previous finding (Needleman, *et al.*, 1973) that the sulfhydryl-depleting agent, ethycrinic acid which covalently alkylates free sulfhydryl groups, blocked the vasorelaxant action of GTN in rabbit aortic strips, and also provides further evidence in support of a common mechanism of free sulfhydryl-requirement in smooth muscle relaxation (Needleman, *et al.*, 1973).

It has been reported that various disulphides such as L-cystamine, -SH oxidants such as diamide and thiol alkylating agents inhibit both basal activity of partially purified hepatic soluble guanylate cyclase and activity stimulated by nitric oxide and S-nitrosocysteine (Ignarro, *et al.*, 1981b). The -SH groups at the catalytic site in the enzyme are believed to interact with enzyme activators since preincubation of the enzyme with enzyme activators markedly protects the enzyme against inhibition by various thiol reactive agents. Two closely juxtaposed -SH groups at the catalytic site are proposed since 2,3-dimercaprol, which possesses vicinal dithiols, but not dithiothreitol, markedly inhibited enzyme activity (Ignarro, *et al.*, 1981b). The oxidation-reduction reactions involving -SH groups were suggested to govern guanylate cyclase activity (DeRubertis & Craven, 1977; Goldberg & Haddox, 1977; Craven & DeRubertis, 1978b), with -SH to -S-S- transformation as a potential mechanism of enzyme activation (DeRubertis & Craven, 1977; Craven & DeRubertis, 1978b). Although Ignarro *et al.* (1981b) also supported the involvement of this redox system governing activation of the enzyme, they

proposed, in contrast, that -S-S- to -SH transformation activates the guanylate cyclase (Ignarro, *et al.*, 1981b) since various oxidants inhibit activation of the enzyme by nitric oxide (Ignarro & Gruetter, 1980; Ignarro, *et al.*, 1980b; 1981b). In this study, our findings were not completely consistent with either of these proposals regarding the activation of guanylate cyclase. The proposed mechanism of -S-S- to -SH in the activation of guanylate cyclase was supported by our findings in this study that the sulfhydryl-containing compounds, L-cysteine, L-glutathione and dithiothreitol, all produced relaxation lasting for 10 minutes in contracted BRP strips. These free sulfhydryl compounds may have reduced -S-S- to -SH in soluble guanylate cyclase and thereby activated the enzyme in the BRP muscle. It may also explain why GTN-induced relaxation was blocked by sulfhydryl oxidising and alkylating agents in this study if the action of GTN via stimulation of soluble guanylate cyclase involves an -S-S- to -SH transformation (Ignarro, *et al.*, 1981b). It is not clear why the blockade of GTN-induced relaxation by diamide was not reversed after treating the tissue with the sulfhydryl compounds, L-cysteine, L-glutathione and dithiothreitol in this study. Thus, this blockade may not simply have been due to oxidation of sulfhydryl groups but may have involved other mechanism. In contrast, the proposed mechanism of -SH to -S-S- transformation in the activation of guanylate cyclase was also supported by our findings in this study that in addition to its small and transient relaxant action, dithiothreitol at high concentrations subsequently produced small but sustained inhibition of NANC relaxation to nerve stimulation and contraction of the BRP muscle. Although inhibition by dithiothreitol of endothelium-dependent relaxation (Griffiths, *et al.*, 1984) was reported to be due to generation of superoxide anion (Moncada, *et al.*, 1991), the inhibition of NANC relaxation to nerve stimulation and the associated increase in tone by dithiothreitol in the BRP muscle was not through generation of superoxide anion since its actions were not prevented or reversed by superoxide dismutase.

Our next approach was to examine the properties of S-nitrosothiols. A number of methods are available for the synthesis of these compounds, including the reaction of L-cysteine and nitrogen dioxide in cold methanol (Myers, *et al.*, 1990), acidification of sodium nitrite in the presence of L-cysteine and other sulfhydryl compounds (Kowaluk & Fung, 1990; Gibson, *et al.*, 1992; Kerr, *et al.*, 1993), and the reaction of nitric oxide with L-cysteine and other sulfhydryl compounds under anaerobic conditions (Ignarro & Gruetter, 1980; Ignarro, *et al.*, 1981). Although differences exist in the properties of S-nitrosothiols generated by these methods, one of the properties in common is that the S-nitrosothiols produced by each of these routes are powerful smooth muscle relaxants whose actions both in magnitude and time course can not be attributed to nitric oxide alone. The S-nitrosothiols produced by each of these routes are normally pink in colour and have been monitored spectrophotometrically at wavelengths of 210, 335 and 545 nm (Ignarro & Gruetter, 1980; Ignarro, *et al.*, 1981; Myers, *et al.*, 1990; Feelisch, 1991). There is, however, enormous variability in the reported stability of the S-nitrosothiols produced by these methods. For example, Myers *et al.* (1990) reported that S-nitrosocysteine is extremely unstable with a half-life of about 30 seconds and must be kept under nitrogen in the dark; Feelisch (1991) said that S-nitrosocysteine must be kept from light and has a half-life of about 60 minutes; whereas Ignarro and Gruetter (1980) reported that S-nitrosocysteine has a half-life of 7-10 days. S-Nitrosoglutathione has also been described as very unstable (Hart, 1985) or unusually stable for 32 days with only 25% loss at room temperature (Park & Means, 1989). It was possible therefore that all of these groups did indeed synthesise S-nitrosothiols but that their reaction conditions differed sufficiently to account for the different half-lives reported. Our initial approach was to use the simple technique of generating S-nitrosocysteine with acidified nitrite in the presence of L-cysteine.

We confirmed the finding that the weak vasodilator activity of sodium nitrite is greatly enhanced by acidification (Furchgott, 1988); little activity was generated at pH values above 5.0 and maximum generation occurred at pH 2. This was probably due to the acid-pH dependent generation of the relaxant, nitric oxide, according to the following chemical reactions (Sisler, 1956):

$$NO_2^- + H^+ \rightarrow HNO_2 \quad (1)$$

$$2HNO_2 \rightarrow NO + NO_2 + H_2O \quad (2)$$

$$2NO + O_2 \rightarrow 2NO_2 \quad (3)$$

$$NO + NO_2 \leftrightarrow N_2O_3 \quad (4)$$

$$2NO_2 + H_2O \leftrightarrow HNO_2 + HNO_3 \quad (5)$$

The activity generated in this study in aqueous solutions of sodium nitrite at pH 2.0 was greater under deoxygenated conditions than under oxygenated conditions, probably because oxygen destroys nitric oxide, according to equation (3). The increase in relaxant activity upon acidification was associated with decrease in nitrite content, according to equation (2), as previously reported (Martin, *et al.*, 1988). Moreover, the relaxant activity was greatly increased and prolonged by acidifying nitrite in presence of L-cysteine. Again, optimum generation of the relaxant was obtained at pH 2. The relaxant activity was not abolished upon neutralisation whereas that of acidified nitrite decayed rapidly. Furthermore, the generation of powerful relaxant activity was immediately associated with an even greater decrease in nitrite content than in the absence of L-cysteine and the development of a pink colour. These observations can be explained by the generation of the powerful relaxant, S-nitrosocysteine, upon acidification of sodium nitrite in the presence of L-cysteine (Kowaluk & Fung, 1990; Gibson, *et al.*, 1992; Kerr, *et al.*, 1993). We found that this relaxant activity was blocked by haemoglobin, methylene blue and N-methylhydroxylamine but not by N^G-nitro-L-arginine confirming that its action is through liberation of nitric oxide and activation

of soluble guanylate cyclase (Ignarro, *et al.*, 1981b) but that it is not a substrate or stimulant of nitric oxide synthase (Gibson, *et al.*, 1989). This process is reminiscent of the "acid-activation" of inhibitory factor extracted from BRP (Gillespie & Martin, 1980). The unactivated form of inhibitory factor contains inorganic nitrite and upon acidification generates nitric oxide which binds to a substance that stabilises its activity (Martin, *et al.*, 1988). It has recently been proposed that the acid-activated inhibitory factor is S-nitrosocysteine or S-nitrosoglutathione (Yui, *et al.*, 1989; Kerr, *et al.*, 1993). Yui *et al.* (1989) found that IF contained 8 μM nitrite and the loss of nitrite in the activated IF was closely related to the content of both sulfhydryl groups and L-cysteine in an HPLC analysis. However, they neither monitored the formation of S-nitrosothiols nor measured biological relaxant activity. A correlation of nitrite loss with the formation of S-nitrosothiols and biological activity was not made, and therefore IF could not be verified conclusively as an S-nitrosothiol in their study although this seems likely. More recently, Kerr *et al.* (1992) confirmed that IF contains 1-10 μM nitrite and 40-200 μM thiol. They found that S-nitrosothiols, but not nitric oxide from acidified nitrite, could reproduce similar effects to those of IF including smooth muscle relaxant activity, stability to argon purging and ability to stimulate cGMP formation by platelet soluble guanylate cyclase. Our results supplement their findings and support their proposal that IF is an S-nitrosothiol.

Acidification of sodium nitrite in the presence of thiols in aqueous solution has been adopted by many groups as a means of generating S-nitrosothiols (Kowaluk & Fung, 1990; Gibson, *et al.*, 1992; Kerr, *et al.*, 1993). However, the precise nitrosating species in the chemical reaction is far from clear. Obviously, nitrous acid, nitric oxide, nitrogen dioxide, dinitrogen trioxide are present in the aqueous solutions, according to the above reaction equations. We adopted an alternative method of synthesising S-nitrosothiols by reacting thiols and nitric oxide under oxygen-free conditions (Ignarro & Gruetter, 1980; Ignarro *et al.*, 1980a) since we

wished to determine if nitric oxide itself rather than higher oxides of nitrogen could react with the -SH moiety of L-cysteine. Also, this method would simplify the reaction because nitric oxide cannot generate nitrous acid, nitrogen dioxide and dinitrogen trioxide under oxygen-free conditions in aqueous solutions, according to the above reaction equations.

We confirmed nitric oxide is a powerful smooth muscle relaxant. Nitric oxide induced a rapid concentration-dependent transient relaxation of strips of BRP and rabbit aortic rings denuded of endothelium, whereas L-cysteine at concentrations less than 4.5 mM produced no relaxant activity. We found that in the presence of L-cysteine in the baths, exogenous nitric oxide-induced relaxation was slightly reduced in magnitude and significantly prolonged in duration. Also, the time course of the relaxation both in onset and offset was changed from fast to slow. After washing L-cysteine out of the bath, the magnitude and the duration of exogenous nitric oxide-induced relaxation were greatly reduced but they slowly recovered. The finding implied that nitric oxide is not the NANC transmitter since L-cysteine was without effect on the NANC relaxation to nerve stimulation.

Furthermore, we found that mixing nitric oxide with L-cysteine in nominally oxygen-free aqueous solution at pH 2 followed by removal of free nitric oxide and neutralisation produced a more powerful and sustained relaxation than nitric oxide alone could account for. The possibility that a new powerful relaxant had been generated from this mixture was investigated. HPLC analysis confirmed the generation of a novel compound that was entirely responsible for relaxant activity in the mixture of nitric oxide and L-cysteine. L-Cysteine produced two peaks in the HPLC, the first one corresponded to its oxidised form, L-cystine. The retention time of peak 1 in L-cysteine solutions was identical to that of standard L-cystine solutions. The second peak corresponded to authentic L-cysteine. Mixing L-cysteine with nitric oxide in oxygen-free aqueous solution at pH 3 followed by removal of

free nitric oxide by purging with oxygen-free nitrogen produced three peaks; the first two peaks corresponded to oxidised and reduced forms of L-cysteine, respectively, with the third peak being the new substance. This new substance collected from the HPLC exhibited powerful and sustained relaxant activity when assayed on rabbit thoracic aortic rings, whereas the reduced and oxidised forms of L-cysteine had no relaxant activity. There was no difference in the height of peak 1 before and after mixing with nitric oxide, whereas the height of peak 2 was reduced by 2% after mixing with nitric oxide. This would indicate that the amount of the new substance represented by peak 3 was no more than 2% of the original content of L-cysteine. The height of peak 3, however, was 22% that of peak 2, suggesting that the new substance had a higher extinction coefficient than L-cysteine. By comparing the height of peak 1 in solutions of L-cysteine with that of standard L-cystine solutions, the extinction coefficient of L-cystine was found to be 125 times that of L-cysteine. Thus, although peak 1 was often equal to or greater than peak 2 in height, it normally represented a content of 1-2 % of the oxidised form.

We extended these studies to L-glutathione, another abundant sulfhydryl compound. L-Glutathione itself produced a single peak in the HPLC and had no relaxant activity. After mixing with nitric oxide, it produced a new peak associated with powerful relaxant activity. Thus, both L-cysteine and L-glutathione could react with nitric oxide to generate a powerful new relaxant.

Spectrophotometric studies revealed that the new relaxant formed from reacting nitric oxide with L-cysteine had three absorption peaks with maxima at 193, 218 and 335 nm, whereas L-cystine and L-cysteine each produced only one absorption peak with maxima at 193 nm and 195 nm, respectively. These findings could explain why the new relaxant had been measured at 210 nm (Myers, *et al.*, 1990) and 335 nm (Feelisch, 1991). However, in our study under nominal oxygen-free

conditions no absorption was observed around 540-550 nm and no pink colour was seen in contrast to the report of Ignarro, *et al.* (1981).

We found that the new relaxant was unstable. HPLC analysis revealed that the new relaxant continuously decomposed to form L-cystine. This process was slow in aqueous solutions at -20°C but enhanced by freeze-drying under high vacuum. These findings are therefore consistent with the reported stability of S-nitrosocysteine in aqueous solution and with its instability in the solid form (Ignarro & Gruetter, 1980; Ignarro, *et al.*, 1981). These properties can be explained by spontaneous decomposition of the relaxant to nitric oxide gas and corresponding disulphide L-cystine (Ignarro, 1990; Feelisch, 1991):

$$2R\text{-}SNO \rightarrow R\text{-}SS\text{-}R + 2NO$$

In the HPLC the relaxant continuously generated L-cystine, implying its ability to release nitric oxide. The release of nitric oxide from the relaxant was consistent with the role of L-cysteine acting as a carrier which reversibly binds and releases nitric oxide and thus with the possibility of S-nitrosocysteine being the NANC transmitter. We also found that the activity of the new relaxant was unchanged after bubbling with oxygen for 10 minutes. This would be a valuable asset for a substance proposed as the NANC transmitter since this would mean that it would not be destroyed in oxygenated environments within the body.

We found that placing the new relaxant in a boiling water bath for 10 minutes resulted in substantial reduce of its activity in the bioassay and its associated peak 3 in the HPLC, whereas at 70°C little decay was observed. Neutralisation of solutions of the new relaxant did not affect their relaxant activity. Thus, the new relaxant was similar in this respect to IF (Gillespie & Martin, 1980; Gillespie, *et al.*, 1981b) but was different from authentic nitric oxide (Furchgott, 1988).

In this study we found that S-nitrosocysteine generated from the reaction of nitric oxide and L-cysteine was destroyed by methanol (50 %, vol./vol.), and this is consistent with the earlier finding that IF is destroyed by methanol (Gillespie, *et al.*, 1981b).

Another important but unexpected finding in this study was that the reaction of nitric oxide with L-cysteine was pH dependent: little relaxant activity was generated at pH 7.4, but as the pH was lowered, relaxant activity was increased until an optimum was reached at pH 1. The pH dependence in the formation of the new relaxant was further confirmed by the finding that the height of the associated peak 3 in the HPLC was increased in parallel with relaxant activity. Although it is well established that acidification of nitrite leads to liberation of nitric oxide (Furchgott, 1988), it is unclear why acidification should have the additional effect of catalysing the reaction of nitric oxide with L-cysteine.

At the beginning of this study, commercially available "oxygen-free" nitrogen gas (British Oxygen Company) was used to deoxygenate aqueous solutions of L-cysteine in which nitric oxide gas (99.9 %, Air Products Ltd., UK) was to be mixed with L-cysteine. However, there was a numerous variation in the amount of relaxant activity and the associated peak 3 in the HPLC generated despite our best efforts to keep reaction conditions constant and prevent introduction of oxygen. Thus, some critical variable was having a profound effect upon the outcome of the reaction of nitric oxide and L-cysteine under nominally oxygen-free conditions.

Later, we found that commercially available oxygen-free nitrogen contains trace amounts of oxygen so our reaction conditions were not strictly oxygen-free. Furthermore, we learned that commercially available nitric oxide gas (99.9%) in steel cylinders also contains impurities including higher oxides of nitrogen,

particularly nitrogen dioxide (Committee on Medical and Biological Effects of Environmental Pollutants, 1977). These higher oxides of nitrogen are much more reactive and soluble than nitric oxide (Hammond, 1966; Cotton & Wilkinson, 1972), so their presence in proposed experiments involving the mixing of nitric oxide with amino acids such as L-cysteine and L-glutathione in aqueous solution would result in extremely complicated reactions, even chain reactions with numerous uncharacterised products (Hart, 1985; Hart, *et al.*, 1985; Wink, *et al.*, 1993).

Consequently, methods were sought to remove the remaining trace amounts of oxygen from commercial oxygen-free nitrogen (British Oxygen Company, UK) and nitrogen dioxide from commercial nitric oxide (Air Products Ltd., UK). This was achieved by washing the gases through Fieser's solution and concentrated solutions (10%) of sodium hydroxide, respectively, since Fieser's solution is an active absorbent for even trace amounts of oxygen (Fieser & Fieser, 1967) and sodium hydroxide is an effective absorbent for nitrogen dioxide (Committee on Medical and Biological Effects of Environmental Pollutants, 1977). Apart from purification of nitrogen and nitric oxide gases, it was absolutely vital that atmospheric oxygen was prevented from entering the reaction chamber. To this end, we devised a system (illustrated in Figure 2) in which a sealed reaction chamber was immersed under water deoxygenated by purified oxygen-free nitrogen in an oxygen-impermeable Amicon chamber under positive pressure, purified oxygen-free nitrogen and nitric oxide gasses were delivered to the reaction chamber and the Amicon chamber via stainless steel tubing rather than polythene tubing which is permeable to oxygen and reacts with nitrogen dioxide. By using purified nitric oxide and nitrogen in this system, nitric oxide in water produced only one low absorption peak at 190 nm when tested within 10 minutes of preparation. In contrast, if either of the gases had not been purified, the presence nitrogen dioxide was indicated by a shoulder in the 210 nm region, a shift of the peak from 190 nm towards 232 nm and the

characteristic multiple peaks at 300-400 nm (Committee on Medical and Biological Effects of Environmental Pollutants, 1977). Thus, the new rigorous system for generating an oxygen-free environment appeared to be satisfactory, at least within commitments of preparing samples.

Using this system, we found that if nitric oxide was bubbled to saturation in solutions of L-cysteine under stringent oxygen-free conditions in sealed cuvettes and free nitric oxide was not removed by purging, then the absorption peaks at 218 and 335 nm grew slowly with time over a period of 80 minutes, but if the solutions were opened to the air, they rose roughly 40-fold within a few seconds to stable new values and the solution became pink in colour owing to a new absorbance peak developing at 543 nm. The peaks at 335 and 543 nm did not shift, but that at 218 nm shifted to 232 nm with increased absorption. The increase in absorption was associated with an increase of peak 3 in HPLC. In contrast, however, the relaxant activity which was powerful at zero time did not change over 80 minutes while sealed and also did not change when the solution was exposed to the air. These findings initially suggested to us that relaxant activity could be dissociated from peak 3 in the HPLC and the absorption peaks at 218, 335 and 543 nm. There were two possibilities for the slow increases in absorption peaks while the cuvette remained sealed in presence of nitric oxide: one explanation was that it was possible that the reaction between nitric oxide and L-cysteine took place at a very slow rate, but this was unlikely since chemical reactions proceed extremely fast. Alternatively, it was possible that despite our rigorous efforts oxygen was able to slowly enter the reaction chamber and play a critical role in the reaction of nitric oxide with L-cysteine. This was more likely since nitric oxide is less active than higher oxides of nitrogen such as nitrogen dioxide which could be formed in the presence of oxygen (Cotton & Wilkinson, 1972; Committee on Medical and Biological Effects of Environmental Pollutants, 1977; Moody, 1991).

By using the closed system described above and purified gases, we found that the growth of absorption peaks at 218, 335 and 543 nm with time following the reaction of nitric oxide and L-cysteine when free nitric oxide was not removed by purging was not due to slow rate reaction. The growth of peaks was much faster if the mixture contained in a sealed cuvette was surrounded by air for 24 hours than if the sealed cuvette was submerged under stringent oxygen-free water. It was almost certain therefore that the more rapid rises in the absorption peaks in the sealed cuvette in air were due to penetration of oxygen through the cuvette.

Therefore, the presence of oxygen was vital in the reaction of nitric oxide with L-cysteine in generation of absorption peaks at 218, 335 and 543 nm and in the generation of peak 3 in the HPLC. What was less clear, however, was whether these absorption peaks and peak 3 in the HPLC were related to the relaxant activity. Previous experiments suggested that there was no relationship because when nitric oxide and L-cysteine were reacted together and free nitric oxide was not removed by purging, exposure to the air led to massive rises (≈40-fold) in the absorption peaks and the magnitude of peak 3 in the HPLC but relaxant activity did not change. We considered the possibility that the apparent lack of relationship was not correct. Our reason was that although spectrophotometry and HPLC analysis could be conducted unaerobically, the bioassay required us to add the new relaxant into an oxygenated tissue bath. It was therefore possible that the sample taken from the sealed cuvette reacted rapidly with oxygen in the bath to generate a more powerful relaxant than was originally present. This possibility was tested in a number of experiments in which purified nitric oxide was bubbled to saturation in solutions of L-cysteine followed by purging to remove free nitric oxide before exposure to the air and the relaxant activity and the magnitude of absorption peaks at 218, 335 and 543 nm were compared with solutions which were not purged to remove free nitric oxide before exposure to the air. It was absolutely clear that those solutions in which nitric oxide was removed by purging before exposure to the air had low

relaxant activity and the magnitude of the absorption peaks at 218, 335 and 543 nm were corresponding low. In sharp contrast, however, these solutions which were not purged had much greater (≈40-fold) relaxant activity and proportionately higher peaks at 218, 335 and 543 nm. Under controlled conditions therefore, there was a strict relationship between relaxant activity and the magnitude of the absorption peaks generated. Clearly, oxygen was a vital component in the generation of the new relaxant following reaction of nitric oxide with L-cysteine.

The requirement for oxygen in the development of relaxant activity and associated absorption peaks suggested that nitric oxide itself was unable to react with L-cysteine to form the new relaxant and that a higher oxide of nitrogen was the reactive species. Indeed the method of Ignarro *et al.* (1981b) of reacting nitric oxide with L-cysteine under anaerobic conditions adopted by us has been criticised by Feelisch (1991) who reported that the presence of oxygen was a prerequisite for nitrosation to proceed, and our later observations supported this. There is debate over the precise nature of the nitrosating species; some have suggested that it is N_2O_3 or N_2O_4 (Leaf, *et al.*, 1990). Wink *et al.* (1993), however, disagree and claim that none of these species is the nitrosating agent in the reaction. They investigated the reaction kinetics of nitric oxide oxidation in aerobic aqueous solutions by employing various techniques including production of nitrite and trapping of the strongly oxidising and nitrosating intermediates formed in this reaction. They found that the key oxidising and nitrosating intermediates are none of those commonly proposed, including NO^+, NO_2, N_2O_3 and O_2NO^- and suggest that as yet uncharacterised NO_x species are involved. We also dismissed the possibility that it was nitrogen dioxide, since this gas generated less relaxant activity than nitric oxide following reaction with L-cysteine either at neutral pH or at acid pH (pH 3.0) with or without presence of oxygen. It was not surprising to find that the relaxant activity and the associated peak 3 in the HPLC were generated in aqueous solutions of L-cysteine gassed to saturation with nitrogen dioxide for 10

minutes since nitrogen dioxide dissolves in aqueous solutions to generate HNO_2, NO, NO_2, N_2O_4 and N_2O_3, according to the following reaction equations:

$$2NO_2 + H_2O \leftrightarrow HNO_2 + HNO_3 \quad (1)$$

$$2HNO_2 \rightarrow NO + NO_2 + H_2O \quad (2)$$

$$NO + NO_2 \leftrightarrow N_2O_3 \quad (3)$$

$$2NO_2 \leftrightarrow N_2O_4 \quad (4)$$

Consistent with the generation of nitric oxide in the above reactions, we found that nitrogen dioxide-saturated water produced two peaks in the HPLC, one of which was identical to that of nitric oxide-saturated water in retention time. Furthermore, in the bioassay, the relaxant activity of saturated solutions of nitrogen dioxide could be accounted for by the height of the peak with the retention time of nitric oxide.

In our experiments optimal generation of the new relaxant occurred at pH 1-2 following the reaction of high concentrations of nitric oxide (saturated ≈3 mM) and L-cysteine (10 mM). If the new relaxant was found in the body and acted as an important mediator, it would have to be formed under more physiological conditions. We therefore examined whether the new relaxant could be formed at neutral pH and at more reasonable concentrations of nitric oxide and L-cysteine. We found that in aqueous solution L-cysteine (10 mM) could react sufficiently with nitric oxide to produce relaxant activity and the associated absorption peaks at 218 and 335 nm at pH 7 but the magnitude of these was much less than at pH 3. Again, the presence of oxygen increased the amount of the new relaxant generated both in the bioassay and in the spectrophotometer. L-Glutathione (10 mM) was also shown to react with nitric oxide at pH 7 to generate new relaxant activity and the associated absorption peaks in the spectrophotometer. Thus, new relaxant activity can be formed at neutral pH from the reaction of nitric oxide with L-cysteine and L-glutathione.

Our next step was to determine if new relaxant activity could be generated at neutral pH when physiological concentrations of nitric oxide and L-cysteine were allowed to react. It is not inconceivable that concentrations of 1 μM nitric oxide could be produced locally under physiological conditions and L-cysteine is normally presented in blood plasma at 150 μM with that in cells being several times higher (Apps & Harnden, 1985). We found that when nitric oxide (1 μM) was mixed with L-cysteine (150 μM) at neutral pH under ambient oxygen tension, some new relaxant activity and its associated absorption peaks at 218, 335 and 543 nm were generated. Although these conditions were far from optimal, it is possible that this new relaxant activity could be formed *in vivo*. It is unlikely that such an inefficient process would be used physiologically as the basis of NANC neurotransmitter. Formation may be particularly important, however, in pathophysiological conditions such as septic shock where massive quantities of nitric oxide is produced by activated macrophages and could contribute to the associated hypotension (Petros, *et al.*, 1991). Furthermore, Gaston *et al.* (1993) reported that S-nitrosothiols, predominantly the adduct with L-glutathione, have been measured at micromolar concentrations in the airways of normal subjects. They have used these finding to propose that S-nitrosothiols are endogenous bronchodilators in human airways. Therefore, further investigation is needed into the potential roles of S-nitrosothiols in the airways of normal subjects and in other physiological and pathophysiological processes.

We have assumed that the new smooth muscle relaxants generated from the reaction of nitric oxide and L-cysteine or L-glutathione are the corresponding S-nitrosothiols, S-nitrosocysteine and S-nitrosoglutathione, respectively. We have made this assumption in the basis of previous reports in the synthesis and properties of these compounds (Ignarro & Gruetter, 1980; Ignarro, *et al.*, 1981). For example, in infra-red spectrophotometry the characteristic absorption of the nitroso

moiety as a sharp peak at 1440-1490 cm^{-1} is present following reaction of nitric oxide with thiols but is absent in the corresponding thiols. Furthermore, the characteristic peak of the sulfhydryl moiety at 2540-2580 cm^{-1} present for the thiols but is absent from the corresponding S-nitrosothiols (Ignarro & Gruetter, 1980). However, N-nitrosation of amines by nitrosating species such as nitrogen dioxide and acidified nitrite has been described (World Health Organization, 1977). Thus, it was necessary for us to further identify precisely the chemical structure of the new relaxant generated from the reaction of nitric oxide and L-cysteine or L-glutathione.

We established a collaboration with chemists to identify the new relaxant using IR spectroscopy and mass spectroscopy but these were unsuccessful because we were unable to prepare sufficient material in concentrated form. We therefore adopted another strategy by using a series of analogues of L-cysteine and L-glutathione in order to determine the functional group in the molecule with which nitric oxide reacts. L-Cysteine and L-glutathione have three functional groups in the molecule which could potentially react, namely, the sulfhydryl, the amine and the carboxyl groups. Five groups of analogues were chosen for study. The first group comprised those compounds with all three functional groups intact. This group included L-cysteine and L-glutathione. The second group comprised only N-acetyl-L-cysteine whose amine group was substituted but whose carboxyl and sulfhydryl groups were free. The third group included those compounds whose carboxyl groups were removed or replaced with an ester function but whose amine and sulfhydryl groups were free. This group included L-cysteamine, L-cysteine ethyl ester and L-cysteine methyl ester. The fourth group included those compounds whose sulfhydryl groups were substituted or oxidised to the corresponding disulphide but whose amine and carboxyl groups were free. This group included L-cystamine, L-cysteic acid, L-cysteinesulphinic acid, L-cystine, L-glutathione (oxidised form), S-methyl-L-cysteine, S-methyl-L-glutathione and L-methionine. The final group included L-arginine, L-glutamic acid and L-glycine, all of which contain amine and carboxyl

groups but not a sulfhydryl group. L-Arginine was chosen since it is the precursor of nitric oxide in the body. L-Cysteine, L-glutamic acid and L-glycine are the amino acids which together comprise L-glutathione. The findings were clear cut. Those compounds in which the amine group or the carboxyl group was removed or substituted but in which the sulfhydryl was intact were all able to react with nitric oxide to form new relaxant activity. In contrast, if the sulfhydryl group was removed, substituted or oxidised to disulphide, the ability to form new relaxant activity was completely lost. Thus, it was clear that the new relaxants formed were the corresponding S-nitrosothiols and that N-nitrosation or nitrosation of the carboxyl group was not involved in the generation of relaxant activity. Furthermore, the spectral absorption characteristics of the relaxant generated from the reaction of nitric oxide with L-cysteine were identical to those previously reported (Ignarro, *et al.*, 1980; Feelisch, 1991). We felt confident in our conclusion that the relaxants produced were the respective S-nitrosothiols.

We confirmed that the smooth muscle relaxant action of the S-nitrosothiols (Ignarro, *et al.*, 1981; Gibson, *et al.*, 1992) is blocked by haemoglobin, which binds and inactivates nitric oxide (Bowman & Drummond, 1984; Martin, *et al.*, 1985), and by methylene blue and N-methylhydroxylamine, which inhibit soluble guanylate cyclase (Bowman & Drummond, 1984; Martin, *et al.*, 1985). The blockade by haemoglobin suggests that the action of S-nitrosothiols is via dissociation of nitric oxide from the S-nitrosothiols which subsequently activates soluble guanylate cyclase. Whether S-nitrosothiols can activate soluble guanylate cyclase directly without release of nitric oxide has not yet been established. S-Nitrosothiols thus fulfil one more of the requirements for consideration as the NANC neurotransmitter in the BRP since its mode of action through activation of soluble guanylate cyclase is the same as that of the NANC transmitter (Bowman & Gillespie, 1982; 1983; Bowman, *et al.*, 1982b; Bowman & Drummond, 1984; Bowman, *et al.*, 1986).

Haemoglobin competes with soluble guanylate cyclase for the binding nitric oxide, EDRF, IF and the NANC transmitter (Miki, *et al.*, 1977; Murad, *et al.*, 1978; Bowman & Gillespie, 1981; 1982; Bowman, *et al.*, 1982b; Martin, *et al.*, 1985) since both guanylate cyclase and haemoglobin contain the same active site i.e. a ferrous haeme receptor moiety (Craven & De Rubertis, 1978). The action of haemoglobin is selective since it does not affect relaxation induced by stimulation of adenylate cyclase and increases of cAMP levels such as by forskolin, PGE_1 or VIP (Bowman & Drummond, 1984).

It has been reported that activation of soluble guanylate cyclase isolated from lung, liver or platelet by nitric oxide, S-nitrosothiols, and other nitric oxide-generating chemicals such as sodium nitroprusside is haeme-dependent since haeme deficient purified soluble guanylate cyclase is not activated by these agents but is activated either by addition of nitric oxide and haeme or addition of preformed NO-haeme complex (Ignarro, 1991; Ignarro, *et al.*, 1983). Reduced iron (Fe^{2+}) binds with protoporphyrin IX to form haeme. Nitric oxide reacts with haeme iron (Fe^{2+}) to yield nitrosyl-haeme adducts (Keilin & Hartree, 1937; Kon, 1968; Gibson & Roughton, 1957). The requirement of haeme in soluble guanylate cyclase is attributed to the formation of the paramagnetic nitrosyl-haeme (NO-haeme) adduct, which is responsible for enzyme activation (Craven & DeRubertis, 1978; Craven, *et al.*, 1979). The molecular mechanism by which NO-haeme activates guanylate cyclase is unknown but is indistinguishable from that of protoporphyrin IX (Ignarro, *et al.*, 1982b; Wolin, *et al.*, 1982): the binding of haeme to guanylate cyclase involves the complexing of iron to the enzyme protein, and this binding prevents enzyme activation. When nitric oxide reacts with haeme iron to form NO-haeme, the bond between haeme iron and enzyme protein is broken but the porphyrin ring remains tightly bound to the enzyme. Thus, a protoporphyrin IX-like binding interaction occurs, and this is responsible for enzyme activation. Consequently, the increased cGMP in the target cells stimulates extrusion and binding of intracellular

free calcium, and prevents calcium entry, resulting in smooth muscle relaxation (Ignarro, 1990).

The superoxide anion-generating agents, pyrogallol and hydroquinone, have previously been reported to destroy the activity of authentic nitric oxide, but do not affect NANC relaxation in the BRP or mouse anococcygeus (Gillespie & Sheng, 1990; Gibson, *et al.*, 1992), suggesting that the NANC transmitter involved is a superoxide anion-insensitive, nitric oxide-releasing molecule rather than free nitric oxide. We found that pyrogallol and hydroquinone destroyed the relaxant activity of S-nitrosocysteine, S-nitrosoglutathione and nitric oxide when added to their respective solutions or to the BRP assay tissue, but as before, neither affected relaxation to NANC nerve stimulation. The blockade of the relaxant activity of the S-nitrosothiols by pyrogallol and hydroquinone was completely prevented and reversed by superoxide dismutase, implying that the blockade was indeed via the generation of superoxide anion. Furthermore, superoxide dismutase potentiated the relaxant actions of nitric oxide and the S-nitrosothiols but had no effect on those of NANC transmitter. Our findings confirm those of Gibson *et al.* (1992) who also found that hydroquinone inhibits the relaxant activity of S-nitrosocysteine, but not of NANC nerve stimulation in the mouse anococcygeus. In contrast to our findings, however, they reported that hydroquinone did not block the actions of S-nitrosoglutathione. Whether this disparity is due to differences in concentrations of drugs used or methods in preparing the nitrosothiol is unclear at present. We are in agreement, however, that the ability of free radical-generating drugs to destroy the activity of S-nitrosocysteine precludes the involvement of this substance in NANC transmission in the BRP and anococcygeus muscles.

Superoxide anion can rapidly and nearly completely destroy nitric oxide (Gryglewski, *et al.*, 1986; Moncada, *et al.*, 1986; Rubanyi & Vanhoutte, 1986; Ignarro, *et al.*, 1988). Moreover, superoxide dismutase protects nitric oxide by

destroying superoxide anion (Gryglewski, *et al.*, 1986; Rubanyi & Vanhoutte, 1986; Ignarro, *et al.*, 1988). There is no clear data *In vivo* to show the extent to which superoxide anion contributes to inactivation of nitric oxide. However, in physiological environments it is unlikely that superoxide anion contributes significantly to the inactivation of nitric oxide for a number of reasons. Firstly, the widespread distribution and high activity of superoxide dismutase in tissues make it unlikely that sufficient levels of superoxide anion are present to destroy nitric oxide. Secondly, in physiological conditions about 90% nitric oxide is converted to nitrite (Feelisch, *et al.*, 1987; Kelm, *et al.*, 1988) whereas the oxidation of nitric oxide by superoxide anion leads to the production of nitrate (Blough & Zafiriou, 1985).

The inability of pyrogallol and hydroquinone to block the inhibitory NANC relaxation but not the relaxation induced by nitric oxide or the new relaxants generated from L-cysteine or L-glutathione reacting with nitric oxide suggests that the NANC neurotransmitter in the BRP is unlikely to be nitric oxide, S-nitrosocysteine or S-nitrosoglutathione. However, if a mechanism exists in the tissue to prevent the transmitter from being destroyed by superoxide anions generated from pyrogallol or hydroquinone, then nitric oxide, S-nitrosocysteine and S-nitrosoglutathione could not be ruled out as candidates. Such a mechanism could exist if high levels of superoxide dismutase were present in the BRP which could inactivate any superoxide anion generated within the tissue and so protect any endogenously produced nitric oxide or S-nitrosothiols. The endogenous superoxide dismutase would clearly be unable to remove superoxide anion generated in the tissue bath and so be unable to prevent destruction of exogenously added nitric oxide or S-nitrosothiols. Consequently, attempts were made to re-examine the effects of pyrogallol on NANC relaxation in the BRP muscle after the activity of superoxide dismutase had been inhibited. Mammalian tissues contain Cu^{++}, Zn^{++} superoxide dismutase (CuZnSOD, Halliwell & Gutteridge, 1989). This enzymatic activity is inhibited by very few substances. Cyanide is one such inhibitor

and is effective at millimolar concentrations. However, it could not be used for this purpose in the BRP since at these concentrations it causes complete loss of tone. Diethyldithiocarbamate (DETCA) is another inhibitor of superoxide dismutase (Halliwell & Gutteridge, 1989; Omar, *et al.*, 1991). It has been reported to inhibit CuZnSOD activity in isolated erythrocytes, intestinal cells, and in whole animals, by binding and removing the copper from the active site on the enzyme (Halliwell & Gutteridge, 1989). Furthermore, at a concentration of 10 mM it has been shown to inhibit acetylcholine-induced endothelium-dependent relaxation in the isolated bovine coronary arteries (Omar, *et al.*, 1991). This inhibition was likely to have occurred due to build up of superoxide anion which would destroy EDRF. Therefore, we wished to test the hypothesis that the inability of pyrogallol to inhibit NANC relaxation of BRP was due to high endogenous levels of superoxide dismutase by inhibiting the enzyme with DETCA. We found, however, that after treatment of the BRP muscle with DETCA (10 mM) for 30 minutes, pyrogallol (100 μM) remained unable to block NANC relaxation. We therefore have no evidence that the inability of pyrogallol to inhibit NANC relaxation can be explained by high endogenous levels of superoxide dismutase. Our experiments could be criticised, however, since we had no independent evidence that we had indeed blocked activity of superoxide dismutase in the BRP. The concentration used in our study has, however, been shown to be effective in other tissues (Omar, *et al.*, 1991).

In conclusion, the major aim of this study was to determine whether an S-nitrosothiol rather than nitric oxide functions as the NANC transmitter in the BRP. Although the sulfhydryl inactivating agents, diamide and N-ethylmaleimide, inhibit NANC relaxation in the BRP, this occurs through a non-selective action rather than from an inability to form an S-nitrosothiol since the relaxant actions of glyceryl trinitrate and isoprenaline are also blocked. Nitric oxide can react in the presence of oxygen and at acid pH with sulfhydryl compounds to produce S-nitrosothiols which

are potent, long-lasting smooth muscle relaxants. The ability of superoxide anion-generating drugs to inhibit the relaxant actions of these S-nitrosothiols as well as of nitric oxide but not of the NANC transmitter in the BRP, suggests that these substances are not candidates for the NANC inhibitory transmitter. The data suggest that the NANC neurotransmitter is more likely to be a superoxide anion-resistant nitric oxide-releasing molecule.

Appendix. REFERENCES

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