The Physiological Role of Nitric Oxide

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1 Introduction

Even six years ago a review of nitric oxide in biology would have been very brief but, after the initial reports in 1987 of the necessary role of nitric oxide in vascular muscle relaxation, there has been an explosion of activity. At present more than 50 papers a month are published reporting the role of nitric oxide in a variety of physiological pathways, and these papers appear in a wide range of journals from medical and physiological to biochemical. The topic has been reviewed a number of times and among the most recent and significant is that by Moncada, Palmer, and Higgs.[1] In this review we will attempt to alert chemists to some of the remarkable developments in physiology linked to the chemistry of nitric oxide. A short explanation will be given of some terms to assist those unfamiliar with the vocabulary of physiology. In view of the large number of papers published and the limited number of references permitted in this review, much important work will not be mentioned or will be inadequately referenced. It is hoped, however, that a sufficient number of references is provided to allow the interested reader to gain access to the relevant literature. In 1994 there should be an extensive review, with greater emphasis on biological aspects, by Hodson of The Wellcome Research Laboratories in *Natural Product Reports*.

2 The Properties of Nitric Oxide

We will consider first the physical and chemical properties of nitric oxide which are relevant to its physiological action, but excluding its role in nitrosation and its properties as a ligand complexed with transition metals, which are discussed in Sections 4 and 6 respectively.

Nitric oxide is a colourless gas at room temperature with bp $-151.8\,^{\circ}C$ and mp $-163.6\,^{\circ}C$. The liquid and solid are also colourless when the material is pure (contrary to some literature reports which ascribe to it a blue colour, no doubt due to the presence of some N_2O_3). The solubility in water at 25 °C and 1 atmosphere pressure is 1.8×10^{-3} mol dm^{-3} which is unchanged within the pH range 2—13. For its physiological action nitric oxide is always present as an aqueous solution.

$$\cdot\overset{+}{N}-\overset{-}{O} \leftrightarrow \dot{N}=O \leftrightarrow \overset{-}{N}=\overset{+}{O}\cdot \leftrightarrow N\equiv O\cdot$$

Nitric oxide is one of the simplest odd-electron species and its structure has been the subject of much interest and debate. In valence bond terms it is best represented by the canonical forms shown. The presence of the unpaired electron effectively reduces the bond order to ~ 2.5 (it is much closer to 3 in NO^+), and the reluctance of nitric oxide to dimerize is related to the geometrical distribution of the odd-electron and also to the fact that the bond order would be virtually unchanged in the dimer. Nitric oxide is of course paramagnetic and its reactions with atoms and free radicals have been much studied. Its use as a free radical trap in gas-phase reactions is also well-known, and it is an efficient quencher of excited singlet states.

$$4NH_3 + 5O_2 \xrightarrow{Pt-Rh} 4NO + 6H_2O \qquad (1)$$

$$N_2 + O_2 = 2NO \qquad \Delta G^{\circ} = 173\ kJ\ mol^{-1} \qquad (2)$$

In the laboratory, nitric oxide can conveniently be prepared by the reduction of nitric acid (*e.g.* with Cu) or of nitrous acid (*e.g.* with iodide ion or ascorbic acid). On the industrial scale it is made by the catalytic oxidation of ammonia (equation 1). The bulk of nitric oxide made in this way is then oxidized to nitrogen dioxide and reacted with water to give nitric acid. The direct formation of nitric oxide from nitrogen and oxygen (equation 2) is thermodynamically unfavourable, but can be achieved to a small extent at high temperatures, such as in lightning discharges and, more importantly, in the internal combustion engine. In the absence of catalytic converters (which reduce NO back to N_2) the nitric oxide produced in this way is partially oxidized in air to nitrogen dioxide and produces air pollution problems (often ascribed to NO_x) in industrialized countries. The presence of nitrogen oxides in the air can cause major photochemical smogs, exemplified by the situation much of the time in the greater Los Angeles basin and in other places. There is also concern regarding the depletion of the ozone layer by reaction with nitric oxide emitted by the engines of supersonic aircraft.

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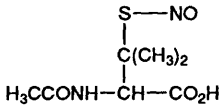
Lyn Williams was educated at University College London and obtained his Ph.D. in 1960 working with the late Professor P. B. D. de la Mare. After a short period as an ICI Fellow at University College Swansea he was appointed Lecturer in Durham University. He is currently the Head of Department and was promoted to a Chair in 1991. His research interests have always been in mechanistic organic chemistry, more recently in the chemistry of nitrosation reactions and of nitroso compounds.

The most well-known and studied reaction of nitric oxide is its oxidation to nitrogen dioxide which could, of course, occur *in vivo* In the gas phase it is the classical example of a third order reaction, second order in [NO] The observed rate constant decreases with increasing temperature The generally accepted explanation is that there is an initial equilibrium formation of a dimer (with a negative ΔH^0 value) which then reacts with oxygen in the rate limiting step (equation 3) but other explanations (such as that outlined in equation 4) have also been put forward

$$2NO \rightleftharpoons N_2O_2 \xrightarrow{O_2} 2NO_2 \qquad (3)$$

$$NO + O_2 \rightleftharpoons NO_3 \xrightarrow{NO} 2NO_2 \qquad (4)$$

The oxidation has also been studied kinetically in aqueous solution where the product is either nitrous acid or nitrite ion, depending on the pH, *but no nitrate* [2] The rate law for reaction in water[3] is the same as that found for gas-phase reaction and the most reasonable explanation (outlined in equations 5) is that NO is oxidized to NO_2 which reacts with more NO to yield N_2O_3, the anhydride of nitrous acid The value for the third order rate constant is $\sim 5 \times 10^6$ $dm^6mol^{-2}s^{-1}$,[3,4] which is unchanged in the pH range 1—13 The rate equation holds when either NO or O_2 is in excess Calculations based on these results show that, even in an aqueous medium saturated with oxygen, NO at a concentration of 10^{-8}M has a half-life of approximately 3 hours The yield of nitrate is undetectably low if precautions are taken to remove all traces of NO_2 Nitric oxide generated by the spontaneous decomposition of *S*-nitroso-*N*-acetylpenicillamine (SNAP) (1) in an aqueous buffer at pH ~ 7 also yields nitrite quantitatively [5]



SNAP

(1)

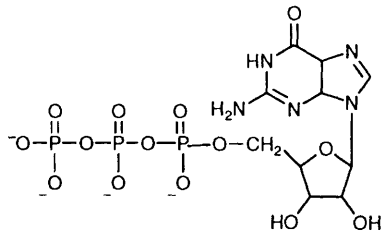
$$NO + \tfrac{1}{2}O_2 = NO_2$$
$$NO_2 + NO = N_2O_3 \qquad (5)$$
$$N_2O_3 + H_2O = 2HNO_2 = 2H^+ + 2NO_2$$

It is perhaps surprising at first sight that nitrite ion is the sole product from aerobic aqueous NO, given that the hydrolysis of its oxidation product NO_2 yields an equimolar mixture of nitrite and nitrate It must be that the reaction of NO with NO_2 is much faster than the hydrolysis of NO_2 Pulse radiolysis studies have shown that this is the case, since the rate constant for the reaction $NO + NO_2 = N_2O_3$ is very close to the diffusion controlled limit In aqueous solution the equilibrium favours N_2O_3, while in the gas phase the opposite applies

Recent work[4] has cast doubt on the interpretation based on the intermediacy of N_2O_3 however The results of scavenging experiments using azide ion are not consistent with the published rate constants for the reaction of N_2O_3 with N_3^- and for the hydrolysis of N_2O_3 However, to date, no alternative structure has been put forward which would account for all of the known results

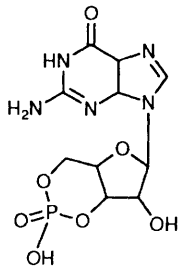
3 Physiology of Smooth Muscle Relaxation

According to structure, contractile properties, and control mechanism there are three types of muscle skeletal, smooth, and cardiac Sheets of smooth muscle surround hollow organs and tubes, most significantly blood vessels Contraction of vascular smooth muscle is one of the many factors controlling resistance to blood flow in the arterial system and sustained contraction, in the absence of compensation elsewhere in the body, can lead to high blood pressure (hypertension) Work by Murad in the early 1980s established that smooth muscle relaxation (which is a positive process rather than just the absence of contraction) requires activation of the enzyme guanylate cyclase and is accompanied by the conversion of guanosine triphosphate (GTP) (2) into cyclic guanosine monophosphate (cGMP) (3)



GTP

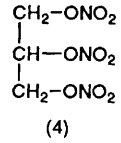
(2)



cGMP

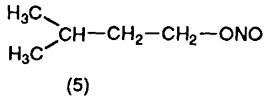
(3)

The process of relaxation can be triggered by a number of substances which occur in the body, such as acetylcholine and bradykinin, and it had been generally assumed that these chemicals act directly on the muscle cells of the vascular system, but a serendipitous discovery by Furchgott and Zawadzki[6] showed that this was not the case They were examining the effect of acetycholine on isolated rings of rabbit aorta (a major blood vessel coming direct from the heart) and found that the relaxing effect of acetylcholine (vasodilator action) was greatly attenuated if the endothelial cells (cells lining the inside of the aorta) had inadvertently been removed or damaged during preparation They concluded that acetylcholine [$CH_3CO-O-CH_2CH_2-\overset{+}{N}(CH_3)_3$] acted, not upon the muscle cells, but upon the endothelium which, in turn, produced a 'second messenger' This diffused from the endothelium into the underlying muscle cells and activated guanylate cyclase The second messenger became known as the endothelium-derived relaxing factor or EDRF During the succeeding years there was much speculation about the chemical identity of the EDRF and there were several claims which were later disproved or could not be substantiated In fact, there was enough information around to identify the EDRF correctly and the answer came to several people active in the field of muscle physiology at about the same time It had been known for some time that a group of compounds all containing the NO group in some form or other [glyceryl trinitrate (4), amyl nitrite (5), sodium nitroprusside (6), and nitric oxide itself] activate guanylate cyclase *in vitro* and it



GTN

(4)



amyl nitrite

(5)

$Na_2[Fe(CN)_5NO]$ SNP

(6)

was only a small but highly significant step to suggest that nitric oxide is the EDRF, the endogenous activator of guanylate cyclase That this was indeed the case was established[7] in 1987 independently by Palmer, Ferrige, and Moncada at The Wellcome Research Laboratories and by Ignarro *et al*

Both groups found that, in a bioassay, NO and the EDRF behaved identically Identifying the EDRF as NO also explained why the lifetime of the EDRF was prolonged by addition of the enzyme superoxide dismutase (SOD) (superoxide reacts with NO) and why the action of the EDRF was destroyed by haemoglobin (it binds NO very strongly) The most direct evidence that NO is formed *in vivo* also reveals the substrate from which NO is formed and comes from a study by Palmer, Ashton, and Moncada [8] Cultured endothelial cells were fed with L-arginine labelled with ^{15}N at the terminal position, perfused with a biological buffer and the perfusate purged with helium which was passed into a mass spectrometer After stimulation of the endothelial cells with bradykinin (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH) the helium was found to contain NO labelled with ^{15}N Reports of this and related work led to a flurry of activity on the L-arginine-to-NO pathway and some of this activity will be summarized in a later section of this review The enzyme or, more probably, the family of enzymes responsible for effecting the conversion is known as nitric oxide synthase (NO-synthase) A somewhat simplified view of what occurs when an endothelial cell is stimulated by the arrival of an endothelium-dependent vasodilator like acetylcholine is shown in Figure 1

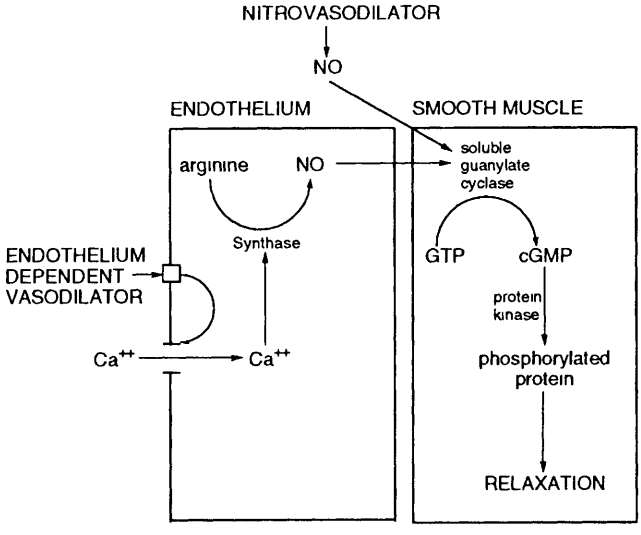


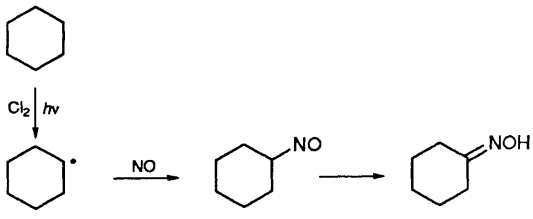
Figure 1

The identity of the EDRF as NO has been questioned That the final process in the activation of guanylate cyclase is transfer of NO to the enzyme cannot be doubted but NO could approach the enzyme, not as free NO, but bound to something else For example, Myers *et al* [9] suggested that the EDRF is *S*-nitrosocysteine but the validity of the experiments must be questioned as it is difficult to isolate *S*-nitrosocysteine in a pure form The search for an alternative to NO as the EDRF seems motivated partly by a distrust of a gas in solution as a messenger molecule, but NO has all the necessary qualities it is small and therefore very mobile, it is soluble in both water and lipid, as a radical species it is highly reactive but in isolation it is perfectly stable, and lastly another gas, ethene, is effective as a messenger molecule It is possible that NO is stored in the muscle cell as an *S*-nitroso-compound and Stamler *et al* [10] have reported that nitrosated thiol groups on proteins provide a vasodilator which is more stable than NO itself However it is not clear how the thiol groups are nitrosated because NO itself is not a nitrosating agent (see Section 4) Alternatives to NO as the EDRF are discussed in detail by Moncada, Palmer, and Higgs [1] In the judgment of the authors of this review there is no need to look beyond NO as the EDRF, but the ability of the oxides of nitrogen to act as nitrosating agents is worthy of further discussion It is relevant to the role of NO as a vasodilator and as a carcinogen (see later)

There appears to be another process which stimulates release of NO from the endothelium, it is shear stress [11] There is evidence that increased flow of blood automatically results in vascular smooth muscle relaxation and enlargement of the blood vessel and presumably, enhancement of protection against blood clots (see Setion 8)

4 Oxides of Nitrogen as Nitrosating Agents[12]

For electrophilic nitrosation the oxides of nitrogen must act as sources of NO^+ When pure and, in particular, when totally free from oxygen, nitric oxide is *not* an electrophilic nitrosating species Thus, with secondary amines no reaction occurs when oxygen is rigorously excluded, but nitrosamines are rapidly formed when air is admitted to the system [13] A lot of confusion has arisen in the past because reactions were carried out when all the oxygen had not been removed In the presence of copper(II) salts or molecular iodine, however, nitrosation of amines occurs readily, even in the absence of oxygen, and is brought about by the intermediacy of a copper–nitrosyl complex and nitrosyl iodide respectively Nitric oxide forms complexes with amines, the so-called Drago complexes, which can upon aerial oxidation lead to nitrosamine products [14] Nitroso products can also be obtained from nitric oxide and sources of free radicals, particularly carbon radicals One example is the formation of the oxime of cyclohexanone *via* the nitroso product arising from the reaction with the free radical derived from cyclohexane (equation 6) The reverse process could be a model for the *in vivo* production of NO from *N*-hydroxyarginine (see Section 13)



(6)

Because of nitrite formation (see Section 2) nitric oxide in aerated aqueous solution yields an effective nitrosating species and has been used to diazotize sulfanilamide and to nitrosate *N*-methylaniline and thiols Alkyl Grignard reagents react with nitric oxide (or with nitrosyl chloride) to yield *C*-nitroso products *via*, it is believed, the intermediacy of *N*-nitrosohydroxylamines [15]

Dinitrogen trioxide N_2O_3 is an effective nitrosating agent In the gas phase it breaks down to NO and NO_2, but exists as a molecular species in the liquid, solid, and solution forms In water, nitrous acid exists in equilibrium with N_2O_3 (equation 7) and the currently accepted value of the equilibrium constant is $3.0 \times 10^{-3} dm^3 mol^{-1}$ At reasonably high nitrous acid concentrations the blue colour of N_2O_3 may be detected by eye In aqueous solution N_2O_3 is a nitrosating agent, and at high concentrations of nitrous acid at low acidity it is the main reagent for the nitrosation of a range of substrates, as shown by the second-order kinetic dependence upon $[HNO_2]$ It is a very reactive nitrosating species in water, reacting for example, with amines with $pK_a > 5$ with a rate constant close to the diffusion limit For some of the most reactive substrates (*e g* aniline and azide ion), N_2O_3 formation from HNO_2 is the rate limiting step

$$2HNO_2 \rightleftharpoons N_2O_3 + H_2O \qquad (7)$$

Liquid N_2O_3 and solutions of N_2O_3 in non-aqueous solvents such as toluene and ether and also in aqueous alkali have all been used preparatively as reagents for effecting nitrosation for a wide

variety of substrates including amines, carbonyl compounds, alcohols, thiols, and alkenes These appear to be electrophilic reactions although no detailed mechanistic studies have been reported Some reactions of N_2O_3 in solution (and also in the gas phase) lead to nitro compounds, no doubt formed by way of free radical attack by NO_2 produced by dissociation of N_2O_3

The chemistry of N_2O_4 is closely linked with that of NO_2 since both species are related by the much studied equilibrium reaction given in equation 8 Below the freezing point (− 11 2°C) the solid consists entirely of N_2O_4 molecules and its structure is well-known as a planar molecule containing the N−N bond The NO_2 content increases with the temperature, being about 0 1% at the boiling point (21 5°C) in the liquid phase and 15 9% in the gas phase At 135°C there is virtually no molecular N_2O_4 The unpaired electron in NO_2 is more localized on the nitrogen atom than it is in NO, which may account for the greater ability of NO_2 to dimerize

$$2NO_2 \rightleftharpoons N_2O_4 \qquad (8)$$

Many reactions are initiated by NO_2 giving rise to nitro products by way of a free-radical pathway In addition, however, other reactions are best rationalized in terms of a heterolytic fission to give either NO_2^+ or NO^+ (equation 9) The latter reaction is of interest in terms of nitrosation This ionization mode is evident in media of high dielectric constant and studies in concentrated sulfuric and perchloric acids show that the conversion is virtually complete [12] For example the Raman spectrum of N_2O_4 in sulfuric acid clearly shows the NO^+ frequency of 2300 cm^{-1} and also NO^+ salts can be made from N_2O_4

$$N_2O_4 \rightleftharpoons NO_2^+ + NO_2$$
$$N_2O_4 \rightleftharpoons NO^+ + NO_3 \qquad (9)$$

N_2O_4 solutions in solvents such as CCl_4 or ether at low temperaturess have been much used synthetically to introduce the NO group, using amines, amides, alcohols, and thiols Reaction with an alkene in liquid ethane–propane solvent results in the formation of the nitroso nitrate adduct, probably *via* an electrophilic nitrosation reaction [12] Passing NO_2 gas into an aqueous solution of a thiol gives immediately the characteristic red colour of the thionitrites RSNO [16] These are unstable materials which are difficult to isolate, the product normally obtained is the disulfide Interestingly the same reaction with NO (with oxygen rigorously excluded) gave no colour evidence of the formation of the thionitrites The result with NO_2 suggests that enough N_2O_4 is present in solution to effect nitrosation

There have been suggestions that the reactive nitrosating species derived from N_2O_4 is in fact an isomer $ONONO_2$ This is an attractive idea but as yet there is no firm physical evidence for its involvment A much favoured preparative procedure for effecting nitrosation generally used by early workers was to pass 'nitrous fumes' into a solution containing the substrate These were generally prepared from nitric acid and a reducing agent or from sodium nitrite and nitric acid The composition has never been established but clearly will be a NO_2/NO mixture, which in solution could act as a nitrosating agent *via* the N_2O_3 or the N_2O_4 pathway

5 Activation of Guanylate Cyclase

Guanylate cyclase, the enzyme responsible for vascular muscle relaxation, is found in most cells and throughout the animal kingdom It exists in two forms a soluble enzyme inside the cell and in a membrane-associated (particulate) form The relative amounts of each within the cell vary with the cell type and its physiological state Normally purified soluble guanylate cyclase can be activated *in vitro* by NO-donating compounds such as sodium nitroprusside However, if the purification is sufficient to remove the haem component of the enzyme, activation is markedly reduced It can be restored by addition of haematin in the presence of a reducing agent It is generally assumed that NO activates guanylate cyclase by binding to the iron of the haem component and moving the iron out of the plane of the porphyrin ring Once the enzyme has been activated there is accumulation of cGMP which, in muscle cells, is accompanied by muscle relaxation In other cells the accumulation of cGMP is accompanied by different physiological effects, as will be described later

6 Nitric Oxide as a Ligand

NO-complexes of transition elements have been known for a long time, but there was a major expansion of interest in the 1960s and 1970s, principally because of their potential use as homogeneous catalysts for a range of reactions Well-known examples are the 'brown-ring' complex $[Fe(H_2O)_5NO]^{2+}$ in the classic test for nitrates, Roussin's red and black salts, and the nitroprusside anion $[Fe(CN)_5NO]^{2}$ Major reviews appeared in the 1970s, more recently a book has been published[17] devoted to these complexes

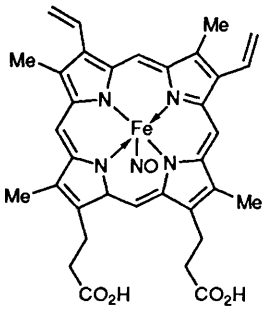
Nitrosyl complexes have now been synthesized and characterized for a large number of transition elements, including Fe, Mn, Cr, Co, Rh, Ir, Ni, Ru, Pt, V, Mo, Te, W, and Re Synthesis is usually by direct reaction with nitric oxide or a nitrosonium salt but sometimes other sources of the nitroso group such as acidified sodium nitrite, alkyl nitrites, *N*-nitroso compounds, or nitrosyl chloride have been successfully used Much effort has been directed at the establishment of the detailed structure and bonding of metal nitrosyls, using in particular *X*-ray crystallography, vibrational spectroscopy, photoelectron spectroscopy, electron spin resonance spectroscopy, and ^{15}N-NMR spectroscopy When NO is bound, for example, to the Fe^{II} atom in haem derivatives, it still has an unpaired electron and therefore can be detected by EPR [18] This use of a 'spin-labelled' ligand has been used to probe electronic structures of haem and other derivatives Ideas have been developed correlating the shape of the complex (*e g* linear or bent NO configuration) with the ability to transfer NO^+ The NO group can in some cases be oxidized by molecular oxygen in the presence of Lewis bases to give the corresponding nitro or nitrato complexes Other reactions are described in reference 17

A number of nitrosyl complexes can act as direct electrophilic nitrosating agents Since this can be achieved in neutral or basic solution there is some synthetic potential here for situations where acidic conditions need to be avoided The most widely studied complex in this regard is the nitroprusside anion $[Fe(CN)_5NO]^{2-}$ (SNP) and the reactions, particularly with amines, have been studied mechanistically [12] The rate law is given in equation 10 and has been interpreted in terms of a mechanism in which a complex between SNP and the amine is rapidly and reversibly formed, which then breaks down in competing reactions by reaction with either another amine molecule or with water Similarly ketones yield the corresponding oximes but *S*-nitrosation does not occur with thiols, which is puzzling in view of the vasodilator action of nitroprusside (see Section 7)

$$\text{Rate} = k_1[\text{SNP}][\text{Amine}] + k_2[\text{SNP}][\text{Amine}]^2 \qquad (10)$$

Ruthenium and some other transition metal nitrosyls can also act as a source of NO^+, and reactions with azide ion, hydrazine, hydroxylamine, amines, alcohols, and β-diketones have all been recorded [12] Often the final product of nitrosation remains bound to the metal, but can sometimes react further For example, in the reaction with primary aromatic amines the bound diazonium group can bring about the normal azo coupling reaction with β-naphthol Nitrosyl haems can give nitrosamines from reaction with secondary amines [12]

It is certain that a haem function is a requirement for activation of guanylate cyclase (see Section 5) by NO and so it is very probable that a nitrosyl complex is first formed One such complex (7) is shown and has been characterized as the dimethyl



(7)

ester [19] The Fe^{II} form of protohaems generally react readily with NO (or HNO_2) to yield such complexes One familiar example is the pink coloured material which occurs in cured meats when treated with sodium nitrite An interesting reaction occurs between NO and oxyhaemoglobin (equation 11) which rapidly yields nitrate anion and methaemoglobin [20] This reaction is the basis of the analytical procedure using difference spectrophotometry, which allows the determination of NO down to 1 nM Incidentally there is a recent review[21] of analytical procedures for NO which includes chemiluminescence assay, diazotization assay, use of EPR, the nitrosyl-haemoglobin method, and microelectrode analyses

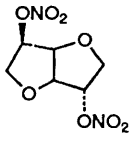
$$\overset{+2}{HbO_2} + NO \rightarrow \overset{+3}{MetHb} + NO_3 \qquad (11)$$

7 Nitrovasodilators

Long before NO was identified as the EDRF, a number of NO-donating compounds were recognized as vasodilators (substances which enlarge blood vessels) and used in the treatment of diseased conditions where increased blood flow will relieve the symptoms In the case of angina pectoris, arteries of the heart become constricted and the heart is unable to function effectively as a pump because of lack of oxygen When this happens physical activity is accompanied by intense pain in the chest and arm Sufferers are often given glyceryl trinitrate (4) and this was prescribed for Alfred Nobel who had made a fortune by incorporating it into porous silica as the explosive dynamite Amyl nitrite (5) and isosorbide dinitrate (8) have similar vasodilatory effects Nitrovasodilators act by by-passing the NO-generating system in the endothelium and delivering NO direct to muscle cells in the walls of the artery Organic nitrates and nitrites undergo a series of enzymatic transformation in the presence of thiol groups with eventual release of NO They are not effective as vasodilators unless given to whole animals [22] On the other hand, some NO-donating compounds, such as *S*-nitroso-*N*-acetylpenicillamine (SNAP) (1) and sodium nitroprusside (SNP) (7)[23] are effective in *ex vivo* experiments such as those involving pieces of isolated artery or a frog's heart The former compound releases NO in straightforward fission reaction, the other product of reaction is a disulfide

$$2RS{-}NO \rightarrow RS{-}SR + 2NO \qquad (12)$$

The action of sodium nitroprusside is more difficult to understand as the only reaction leading to direct release of NO is photochemical decomposition In experiments using a frog's heart it was found that the action of SNP was greatly enhanced by exposure to light from a laser [23] It is known that, in a fairly slow reaction, NO is also released from SNP on reaction with thiols [24] As SNP is often used for inducing low blood pressure (hypotension) during surgery on the vascular system, it is possible that the lighting in the operating theatre may play some part in providing NO, although there is also a non-photochemical reaction



isosorbide dinitrate

(8)

A compound containing a large number of NO ligands in each ion is the anion of Roussin's Black Salt (RBS), $[Fe_4S_3(NO)_7]$ This ion decomposes with release of NO by both chemical and photochemical routes and *ex vivo* experiments have shown it to be a highly effective vasodilator The effect of SNAP and SNP in such experiments is transient, but with RBS relaxation may persist for as long as six hours This is a consequence of the unusual solubility of RBS which, although ionic, is more soluble in organic solvents than in water RBS is rapidly taken into the endothelial cells because of its lipid solubility and remains there for several hours, slowly releasing NO [25]

8 Platelet Aggregation

NO is involved in another important aspect of the blood supply In blood there are numerous colourless cell fragments, containing granules, known as platelets They are much smaller than red blood cells When a blood vessel is damaged, excessive bleeding is prevented by platelet aggregation to form a plug which adheres to the wall of the blood vessel Further aggregation occurs in blood coagulation to form a clot, which is the essential defence against bleeding after injury Myocardial infarction ('heart attack') may be caused by abnormal clotting of the blood in a coronary vessel coated with atherosclerotic plaque (a coating on the inside of a blood vessel containing quantities of cholesterol) Concurrently with the discovery of the role of NO in effecting vascular muscle relaxation came the discovery that NO inhibits both platelet aggregation and adhesion [26] Prostacyclin and NO act synergistically to inhibit platelet aggregation and to disaggregate platelets, but there is no parallel synergism in platelet adhesion [26] The role of NO in this area seems to be as a feedback mechanism to counteract the effect of substances in the body, produced after injury, which promote aggregation and adhesion The NO utilised by the platelets is derived from endothelial cells with which the platelets come in contact, but there is also an enzyme system in the platelets themselves which acts on arginine to produce NO

9 Macrophage Activity

The immune response is the body's reaction by which foreign matter, both living and non-living, is neutralized or destroyed The non-specific immune response non-selectively protects against foreign substances or cells without having to recognize their specific identities, and a key part of that response comes from a set of cells known as macrophages which are found in virtually all organs and tissues, their structure varying somewhat from location to location For macrophages to respond they have to be activated by substances known as cytokines The role of macrophages is quite extensive and includes engulfing foreign matter (phagocytosis) and, if necessary, killing it by injection of cytotoxic substances Macrophages can also kill invading microbes by contact without phagocytosis It is the killing process which appears to involve nitric oxide

Before this was discovered it had been known for some time that there is a correlation between activity of the immune system and elevated nitrate levels in the urine More recent work[27] had shown that cultured macrophages from a mouse generate substantial amounts of both nitrite and nitrate after activation It had also been shown that the cytotoxicity of macrophages against tumour target cells depended upon the presence of L-arginine (9) and that activity was again accompanied by the formation of nitrite and citrulline (10) [28] Once the production of NO from L-arginine in endothelial cells had been established it became clear that a similar process occurs in activated macrophages, and that both nitrite and nitrate come from the common

precursor, NO However, in view of the recently reported exclusive formation of nitrite from NO in oxygenated, aqueous solution, this view may have to be modified (see Section 8), but nitrate is readily formed from nitrite by oxidation in blood NO production was confirmed independently by three groups[29] and it now seems certain that in activated macrophages there is a process occurring which parallels that taking place in endothelial cells Nature appears to have been economical in using the same enzyme system for two entirely different tasks, but clearly the properties of NO are special enough to make this profitable However, it does pose a problem If any part of the body suffers from a massive infection there will be much macrophage activity The NO produced as a consequence will have not only substantial cytotoxicity, *i e* it kills the invading cells, but will also bring about massive hypotension This condition is known as septic shock and can be fatal Now that the nature of the species responsible for septic shock may have been identified there is a chance that improved treatment can be developed

Why is NO toxic towards invading bacteria and unhealthy host cells? It could be that, as a radical species, NO is destructive towards the lipid cell membrane and that this alone is sufficient to explain its action but there is, at the moment, little direct experimental evidence to substantiate this view Beckman *et al*[30] suggested that NO reacts with superoxide, also produced during macrophage activity, in the following way

$$\begin{aligned} NO + O_2^- &\rightarrow ONOO \\ ONOO^- + H^+ &\rightarrow ONOOH \\ ONOOH &\rightarrow NO_2 + HO \end{aligned} \qquad (13)$$

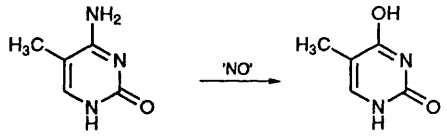
Peroxynitrite (ONOOH) is a weak acid and the anion will be protonated at physiological pH and could then fragment to give nitrogen dioxide and hydroxyl radicals, which are known to be very destructive towards lipid membranes and DNA However, peroxynitrite also rearranges to the more stable and non-toxic nitrate and so the generation of hydroxyl radicals by this route remains speculative A third possibility is that NO reacts with an enzyme iron–sulfur centre that is essential for metabolic activity to give an iron-sulfur cluster nitrosyl In solution, iron–sulfur cluster nitrosyls may dissociate to give EPR-active species and analysis of these spectra[31] has permitted detection of similar species in tumour target cells co-cultured with activated macrophages [32] Similar EPR signals are obtained when NO from activated macrophages acts upon aconitase, an enzyme in the citric acid cycle which is known to contain an iron–sulfur centre

10 Neutrophils

Another part of the body's immune system is the collection of cells in blood known as leukocytes or white blood corpuscles Human neutrophils are one type of leukocyte and are known to produce a substance which inhibits platelet aggregation [33] The biological significance of NO production in neutrophils has yet to be elucidated

11 NO as a Carcinogen

Although NO can be cytotoxic towards early tumour cells it seems that it can also act as a carcinogen or cancer-causing agent It is known that, under physiological conditions, NO can nitrosate natural secondary amines to form carcinogenic *N*-nitroso compounds From a chemical point of view this is somewhat puzzling as NO is not a nitrosating agent, but how it might be converted into a positive nitrosation species is discussed in Section 4 Also there appears to be another, more direct, cancer-inducing process for which NO is responsible Keefer *et al*[34] have shown that NO can effect mutagenesis (a change in base-pair sequence in DNA) in *Salmonella typhimurium* by the conversion of 5-methylcytosine into thymine (equation 14) This is nitrosative deamination and must again involve conversion of NO into an electrophilic nitrosating agent



(14)

In a similar way, incubation of a human cell line with NO leads to mutation [35] It is difficult at the moment to define precisely the physiological conditions under which NO will act either as a cause of or a cure for cancer The role of NO as a carcinogen has environmental consequences (see Section 2) It is present at quite high concentrations in cigarette smoke but, as there are so many other carcinogens present already, an additional one hardly elevates the health risk significantly, but it could contribute to the dangers of passive smoking

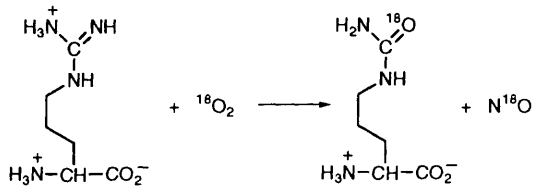
12 NO as an Antiparasitic Agent

In the same way as NO is toxic towards bacteria as part of the body's immune system, so NO will kill non-bacterial parasites, and there is some evidence that it is part of the body's natural defence mechanism Macrophages from a mouse incubated with the cytokine interferon-γ are effective in killing the protozoal parasite *Leishmania major* and the effect is diminished if an inhibitor of NO synthase is added [36] NO itself did not inhibit the growth of the malarial parasite *Plasmodium falciparum* but *S*-nitrosocysteine and *S*-nitrosoglutathione are effective at very low concentrations [37] Nitroprusside and Roussin's Black Salt are also toxic towards *Plasmodium berghei*, with the former being the more effective, although NO is more readily obtained from the latter If NO is part of the body's natural defence against the malarial parasite it is a matter of great importance as it could initiate a new class of antimalarial drugs, desperately needed in view of the catastrophic increase in the incidence of falciparum malaria, particularly in Africa and Southeast Asia

13 NO-Synthase

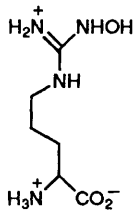
The enzyme responsible for the production of NO *in vivo* has been the object of considerable scrutiny The enzymes from the brain,[38a] macrophages,[38b] and neutrophils,[38c] *inter alia*, have been isolated and purified and, more recently, the enzyme has been the target for the techniques of modern molecular biology Fair quantities are now available for study There are two, or more probably three, distinct enzymes which can effect NO formation The exact nature of the enzyme depends on the tissue from which it was obtained Although there is a large measure of homology the different members of the family exhibit important physiological differences All show a degree of homology with P-450 reductase The substrate for the enzymes is L-arginine (9) and the products of reaction are citrulline (10) and NO

Definitive insight on the mechanism of enzyme action has come from Moncada and co-workers [39] They have shown that the source of oxygen in both NO and citrulline is molecular oxygen (Figure 2) and so NO synthase is correctly described as a dioxygenase This discovery eliminated a number of pathways which had been proposed previously It is known that ω-hydroxy-L-arginine is a vasodilator and so it is assumed that this molecule is an intermediate on the pathway A number of oxidizing agents are known to convert *N*-hydroxy-L-arginine



(9) (10)

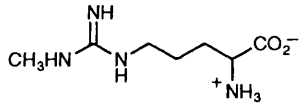
Figure 2 Isotopic study of the enzyme NO-synthase



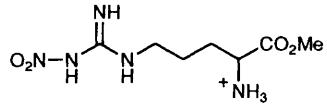
N-hydroxy-L-arginine

(11)

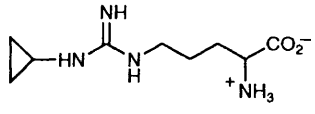
(11) into citrulline and NO Compounds related to arginine are effective inhibitors of NO-synthase (Figure 3) and their use has proved invaluable in many investigations on the biology of NO



N-monomethy-L-arginine (L-NMMA)



N-nitro-L-arginine methyl ester (L-NAME)



N-cyclopropyl-L-arginine

Figure 3 Some inhibitors of NO-synthase

NO-synthase in endothelial cells is a constitutive enzyme This means it is present all the time and responds rapidly to activation There appears to be a basal production of NO from endothelial cells which continuously acts against constriction of the vascular smooth muscle On the other hand, the enzyme in macrophages is inducible, that is, it is not present until the macrophages have been activated by a cytokine There is a delay of some hours between activation and the appearance of nitrate and nitrite, indicative of macrophage activity Other differences between the inducible and the constitutive enzyme are shown in Table 1 (modified from reference 1)

Table 1 Similarities and differences between the two NO-synthases

Constitutive	Inducible
Dioxygenase	Dioxygenase
NADPH dependent	NADPH dependent
Ca^{2+}/calmodulin dependent	Ca^{2+}/calmodulin independent
Picomoles NO released	Nanomoles NO released
Short-lasting release	Long-lasting release
Inhibited by L-arginine analogues	Inhibited by L-arginine analogues

Some of the differences between the two enzymes can be understood in terms of their different physiological roles NO is cytotoxic only when large quantities are produced and this explains why the NO used to activate guanylate cyclase in muscle cells has no adverse effects Macrophage activity must, necessarily, be long-lasting to rid the body of infection but, in order to respond to the rapidly changing needs of the body, the effect of NO in muscle cells must be short-term

Calmodulin is a protein which binds calcium ions, and most enzymes which are activated by calcium ions are also calmodulin dependent Very recent work suggests that the apparent Ca^{2+}/calmodulin independence of the inducible enzyme may be due to the very strong binding between the enzyme and calmodulin, so strong that it is impossible to obtain the enzyme without calmodulin

There is much activity in the design of inhibitors of both the inducible and constitutive enzymes If the former could be selectively inhibited then the condition of septic shock could be managed Equally, selective stimulation of the constitutive enzyme in blood platelets could be an approach to the treatment of clotting without overstimulating the immune system There should be many exciting developments during the next few years

14 Central Nervous System

Messages are conveyed along nerve cells as electrical impulses but, for movement across the gaps between nerve cells (synapses), there are chemical messengers, known as neurotransmitters When a synapse increases the activity in a postsynaptic nerve cell it is known as an excitatory synapse Among the major excitatory neurotransmitters are the amino acids aspartate and glutamate It has been known for some time that, within the central nervous system, activity in excitatory pathways leads to enhanced levels of cGMP, paralleling what occurs during relaxation for vascular smooth muscle It was also known that arginine is the endogenous precursor for activation of rat forebrain guanylate cyclase In view of the similarities with smooth muscle relaxation it seemed reasonable to look for NO In 1988 Garthwaite *et al*[40] reported that cultured brain cells produced an EDRF-like substance when stimulated by *N*-methyl-D-aspartate, and in 1989 Knowles, Palacios, Palmer, and Moncada[41] isolated, from the rat forebrain, an enzyme which catalyses the formation of NO from arginine Confirmation of this pathway in the brain came from a study by Bredt and Snyder[42] of the activation and inhibition of NO-synthase in brain slices That the role of NO is intercellular was shown by Southam and Garthwaite[43] by the inhibiting action of haemoglobin, which is an extracellular agent

It appears that NO in the brain acts in two ways In the first, NO is formed in the postsynaptic nerve cell following activation by an amino acid neurotransmitter NO does not act upon guanylate cyclase within the cell where it was produced but diffuses out and acts upon one or more neighbouring structures, including the presynaptic nerve cell and so strengthening the connection between the cells on the two sides of the synapse Thus NO is part of a feedback loop or a retrograde messenger In one model of brain activity such a retrograde messenger is the way in which long term memory is built up NO is not the only candidate as the retrograde messenger and so we must wait for further investigation before we can be certain that NO is involved in the process of memory The second role of NO in the brain is more like that of a normal neurotransmitter in that it acts upon nerve cells other than the presynaptic nerve cell It also seems probable that NO in the brain plays some part in the cerebral blood supply It has also been suggested that overproduction of NO could be responsible for brain damage and certain degenerative conditions such as senile dementia because of its radical nature, or because it can generate hydroxyl radicals

As well as in the brain, NO appears to have role in the peripheral nervous system There is a set of nerves in the body known as NANC nerves (non-adrenergenic non-cholinergic, an inelegant description as it merely specifies what the neurotransmitter is not) in which the neurotransmitter has not been identified with any certainty but evidence is growing that, in some cases, it is NO Recent studies of the canine colonic sphincter muscle, a valve in the gut that is activated by a NANC nerve, have demonstrated that stimulation is the result of the release of NO [44] In this instance release of NO is prompted by an

electrical impulse in the nerve rather than a chemical messenger (acetylcholine) as in the case of relaxation of vascular smooth muscle The role of NO in NANC nerve cells may turn out to be a very general phenomenon The male penile erection is brought about by relaxation of muscles in the corpus cavernosum and the inflow of blood A NANC nerve is responsible for this process and there is now direct evidence that the arginine-to-NO pathway is directly involved Muscle relaxation in the corpus cavernosum can be effected by NO-donor drugs [45]

15 Other Sources of NO

Although it may already seem that NO is unexpectedly widespread in the body there are further experimental observations to suggest that it occurs even more generally Kupffer cells (another part of the non-specific immune system) produce NO when stimulated with lipopolysaccharide Some muscles, although normally activated by NO produced in proximate endothelial cells, appear also to contain an inducible NO-synthase, but its effect may not be observed for some hours Adrenal glands produce NO but the reason for this is not clear NO appears to play some part in vision and the fluid in arthritic joints has an abnormally high level of nitrite Details of these little-explored areas of NO activity can be found in the review of Moncada, Palmer, and Higgs [1] NO formed in the liver has been trapped as a complex with diethyldithiocarbamate and identified by ESR spectroscopy [46]

16 Clinical Aspects

It is unlikely that a discovery of such fundamental importance as that of the role of NO in human biology will not have a profound effect on the drugs used to treat a number of diseased conditions Because of the large number of drugs already available for the treatment of high blood pressure it is unlikely that there will be a major impact in this area Platelet aggregation and adhesion are more fruitful topics As NO has so many roles it is difficult to see how an NO-donor drug affecting only one metabolic function could be devised One of the major challenges to medicinal chemists is the simplicity of the NO molecule How do you modify such a simple molecule to make its action more specific? On the other hand, NO binds to a number of metals (see Section 6) and there may be possibilities for inorganic drugs containing NO as a ligand

There have already been some clinical uses made of NO The NO-synthase inhibitor L-NMMA will bring about a decrease in blood pressure in the human forearm and this effect has been used in a study of essential hypertension (elevated blood pressure due to a fault in the basal production of NO) [47] Inhaled NO has been used successfully in the treatment of severe adult respiratory distress syndrome (ARDS) although the toxicity of NO make this an unattractive clinical procedure [48] One of the biggest clinical challenge is the management of the hypotension associated with septic shock What may be required is control of the amount of NO present rather than its complete removal and this will not be easy to achieve

17 Conclusion

Few subjects have grown as rapidly in importance as has the biological role of NO If only half the results now appearing stand the test of time NO is still a major mediator of physiological control NO has appeared in the quality press in January 1993 it was featured as 'Molecule of the Month' in the Science and Medicine page of *The Independent* and it has been mentioned in *Cosmopolitan* In October 1993 the third international conference on nothing but NO in biology takes place in Cologne What the future holds is difficult to predict greater insight into how our bodies work and some progress, and also some false hopes, in new drugs or new clinical procedures Although research on NO is now worldwide, much of the early pioneering work, when a biological role for NO seemed unlikely, took place in Britain and this is something of which British science should be proud

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