Non-Heme Iron Nitrosyls in Biology

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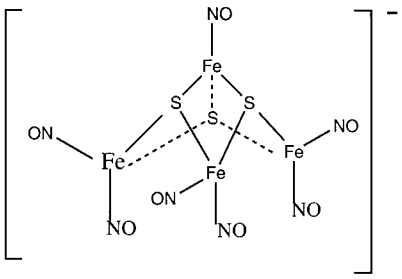
I. Introduction

Non-heme iron nitrosyls have been recognized by chemists for well over 100 years, but their place in biology was given serious attention only after the discovery of the biological role of nitric oxide in the 1980s. This review aims to bring together evidence from a wide range of sources to show that non-heme iron nitrosyls have biological properties that are defined by their chemical characteristics. They can act as NO-donor drugs, using the term drug in its widest sense, and they also form under physiological conditions. The sources are from the chemical, biological, and, occasionally, medical literature. There are, of course, many references to non-heme iron nitrosyls in the chemical literature, but we have restricted ourselves rigidly to those showing biological activity at physiologically relevant concentrations. Most references are taken from the last 20 years. We have, in some instances, proposed mechanisms of action based on recent information on the biological role of NO. We hope these proposals will be of value to physiologists as well as of interest to chemists.

II. Iron–Sulfur Cluster Nitrosyls

The two best-known species in this class are the anions first described[1] by the French chemist Roussin,[2] the eponymously named black and red salts (**1** and **2**) (Chart 1). The ester (**3**) is sometimes known as Roussin's red ester. Despite its structural complexity the synthesis of **1** is astonishingly simple. Reaction of iron(II) sulfate with a mixture of sodium nitrite and ammonium sulfide yields an intensely black solution from which the ammonium salt of **1** crystallizes.[3] Neither the stoichiometry nor the mechanism of this reaction is known with any certainty, and this lack of certainty is cloaked in the term "self-assembly".

Chart 1



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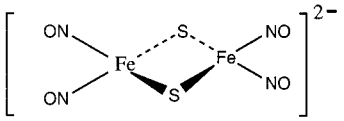

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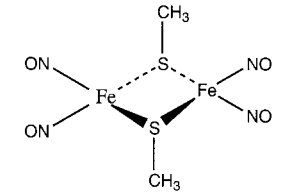

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Reaction of the ammonium salt of **1** with aqueous sodium hydroxide results in formation of $Na_2[Fe_2S_2(NO)_4]$[4] from which "esters" may be prepared by alkylation with an alkyl halide. Most other reactions of **2** result in conversion into **1**. Indeed, salts containing $[Fe_2S_2(NO)_4]^{2-}$ are converted into salts containing $[Fe_4S_3(NO)_7]^-$ simply by dissolving in methylene chloride[5,6] and the most stable of all the known iron−sulfur nitrosyls appears to be $[Fe_4S_3(NO)_7]^-$. The general chemical and physical properties of the known iron−sulfur nitrosyls have been described elsewhere,[7] and only those properties relevant to

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Ian L. Megson received his BSc in Physiology from the University of St. Andrews and continued his studies at the same university to be awarded a Ph.D. for his work on the biological activity of iron–sulfur cluster nitrosyls. In 1995, he was awarded a Research Fellowship by the British Heart Foundation to work with David Webb and Gillian Gray on the effects of nitric oxide in cardiovascular disease at the University of Edinburgh. In 1998, he was appointed as the Sir Stanley & Lady Davidson Lecturer in the Department of Medicine at Edinburgh and has since secured a lectureship in the Division of Biomedical & Clinical Laboratory Sciences at the same university. His professional interests lie in the role of nitric oxide in cardiovascular disease and in the development of novel nitric oxide donor drugs to help protect against heart disease and to improve outcome following surgical intervention.

their biological activity will be considered here. One unexpected physical property of the salts of $[Fe_3S_4(NO)_7]^-$ is their ready solubility in organic solvents, despite their ionic character. Indeed, during its synthesis the black salt may be extracted from the aqueous reaction mixture into diethyl ether, a convenient purification process.

For many years Roussin's black and red salts were chemical curiosities and the only studies were those directed toward the crystal structure of $[Fe_2(SEt)_2(NO)_4]$[8] and $Cs[Fe_4S_3(NO)_7]$.[9] The anti form of the former was observed as the sole species in the solid state. A more recent crystallographic study[10] of the tetraphenylarsonium salt of $[Fe_4S_3(NO)_7]^-$ has confirmed the essentially C_{3v} symmetry. The crystal structure of the tetraethylammonium salt has also been reported.[11] The structure of $[Fe_4S_3(NO)_7]^-$ is electron precise,[12−14] and electron removal by oxidation results in complete destruction of the framework and release of NO. Copper(II) salts are convenient oxidants, and the reaction works so well that it was recommended[1] as a way of generating NO when required. The release of NO on oxidation may be significant in the biological activity of Roussin's black salt. A detailed investigation[11] of the redox behavior of **1** by electrochemical techniques shows the existence of a four-step electron sequence, in which the charge changes from −1 to −4. The electrogenerated species $[Fe_4S_3(NO)_7]^{2-}$ and $[Fe_4S_3(NO)_7]^{3-}$ appear to be stable. The former has been isolated as the ammonium salt, for which a crystal structure has been obtained, confirming distortions of the $[Fe_4S_3(NO)_7]^-$ structure predicted by theory.[5]

The photochemistry of Roussin's salts has been comprehensively studied by Bourassa et al.[15] Photolysis of the red salt in aerobic aqueous solution leads to quantitative production of the black salt, and $\Phi_I = 0.14$ for disappearance of the red salt:

$$[Fe_2S_2(NO)_4]^{2-} \xrightarrow{h\nu} [Fe_4S_3(NO)_7]^-$$

Real time detection of NO by electrochemical sensors in the photolysis solution demonstrated the release of NO with a quantum yield of 0.07. However, the black salt is much less photoactive ($\Phi_{II} = 1.1 \times 10^{-3}$) but does undergo photodecomposition in aerobic solution to give, eventually, iron(III) precipitates plus NO. The stoichiometry of the reaction is unexpected: 5.9(±0.2) mol of NO was obtained for each 1 mol of black salt.[16] These studies were undertaken in an attempt to find ways of delivering NO to biological targets.[17]

Iron−sulfur cluster nitrosyls display a range of biological activity because they can readily release NO. The biological activity of, particularly, the black salt is increased by its lipid solubility and by the enhancement of NO liberation through photolysis. There is also the possibility that the compounds may act as nitrosating agents as NMR spectroscopic studies have shown that the nitrosyl ligand is present as NO^+.[5] The ability of the black salt to act as a biologically acceptable NO-donor drug has been demonstrated in a number of areas.

The bactericidal effects of the black salt have been known for some time.[18] The toxicities of $[Fe_3S_4(NO)_7]^-$ and $[Fe_2(SCH_2CH_2OH)_2(NO)_4]$, a water-soluble "ester", toward *Clostridium sporogenes* have been investigated.[19,20] They are effective in the millimolar range. The black salt is also effective against both anaerobic and aerobic food spoilage bacteria. There are a number of possible targets for inhibition in bacterial cells: respiratory chain enzymes; iron−sulfur proteins; other metalloproteins; membranes; genetic apparatus.[21] The most significant ones have not be identified, but by comparison with other metal nitrosyl complexes, it appears that the presence of an NO^+ ligand leads to bactericidal activity. Roussin's black salt inhibits the growth of *Trichomonas vaginaliss*.[22] The ability of the black salt to bring about DNA damage in rat islets of Langerhans, where insulin is produced, as measured by the comet assay,

is greater that of other NO-donor compounds, possibly because of its lipid solubility.[23]

The photochemistry of the black salt has been exploited in a study of the role of NO in the relaxation of the smooth muscle in the guinea pig taenia caeci in vitro. Exposure to the black salt (50 μM) did not inhibit carbachol-mediated contraction, but subsequent illumination caused immediate relaxation, which depended upon the intensity, duration, and wavelength of the light.[24] Relaxation of vascular smooth muscle by Roussin's black salt, on the other hand, can occur in the dark and is sustained for several hours after washout, suggesting that the drug is retained within the intimal layers of the artery wall where it gradually releases NO.[25] Vascular relaxation in response to iron−sulfur cluster nitrosyls is enhanced by laser-induced photolysis due to oxygen-dependent decomposition of the compounds.[26] Similar experimental procedures have been used to show that NO, obtained by the photolysis of the black salt, causes calcium-independent release of [^{3}H]-dopamine[27−29] and acetylcholine[30] from rat striatum ex vivo. Addition of the black salt to a coculture of vascular endothelial and glioma cells, a model of the blood−brain barrier, resulted in a rapid and dose-dependent decline in the electrical resistance. There may be, therefore, a role for NO in blood−brain barrier function.[31] Preincubation of astrocyte-derived cells with the black salt resulted in the salt being taken up by cells, and subsequent illumination resulted in very effective inhibition of cellular respiration.[32] The black salt also inhibits platelet aggregation[33] and lymphocyte proliferation[34] in an NO-dependent manner. A study of the rat hippocampal slice showed that, following a flash of UV light, the black salt depressed transiently field excitatory postsynaptic potentials.[35] Hypoxic cells treated with the red salt in aqueous solution and then subjected to γ-radiation demonstrated lower survival rates when simultaneously exposed to white light irradiation than did control systems treated identically but in the dark.[15] Clearly photochemically released NO can be cytotoxic.

A rather different role for Roussin's ester comes from a major epidemiological study of the geographical distribution of different types of cancer in China showing a high, but very localized, incidence of esophageal cancer in the Linxian valley of Henan province in northern China.[11,36] The adult incidence of this type of cancer was as high as one in four. The probable role of local foodstuffs in the causation of this particular form of cancer was deduced from a study of domestic poultry. Chicken raised in Linxian commonly suffer from cancer of the gullet, analogous to human esophageal cancer. When a group from Linxian was rehoused some distance away and provided with poultry free of gullet cancer, feeding of these birds with food scraps prepared by methods normal in Linxian caused rapid development of gullet cancers. A distinctive item in the diet of Linxian is a form of pickled vegetable prepared by storage of cabbage in water high in nitrite and nitrate. Examination of this "pickled" vegetable by GC/MS showed that it contained unexpectedly high concentrations of the red ester $[Fe_2(SMe)_2(NO)_4]$.[37,38] This red ester has not been found in food from other parts of China, and it has been assumed that it is responsible for the local high incidence of esophageal cancer. By itself the red ester has only weak mutagenic properties,[39] but it acts as a promoter for the carcinogenic properties of other substances.[40,41] Reform of the eating habits of the inhabitants of Linxian has resulted in a lowering of the incidence of esophageal cancer; this worthy end has resulted in decreased interest in the carcinogenic properties of the red ester, and its precise role has not been elucidated. It could act as lipid-soluble nitrosating agent, reacting with secondary amines to give highly carcinogenic N-nitrosated secondary amines or it could release NO in a manner similar to that of the black salt. NO can itself, in an aerobic situation, act as a nitrosating agent. Interest in the cancer-causing properties of the red ester peaked before the physiological role of NO was discovered, and in the light of current knowledge, a reinvestigation of the biological activity of the red ester might be worthwhile.

III. Sodium Nitroprusside

Nitroprusside $[Fe(CN)_5NO]^{2-}$ has a special place among iron−nitrosyl complexes with biological activity. It was first prepared in the middle of 19th century by the St. Andrews chemist Playfair.[42] Unlike most nitrosyl ligands, the NO of nitroprusside is very reactive. There is a comprehensive review of its in vitro chemistry by Swinehart,[43] which, although over 30 years old, is still valuable. What gives nitroprusside its current prominence is the discovery, in 1929, that it is a highly effective hypotensive agent with few undesirable side effects. The chemistry of its hypotensive action was fully discussed, in a manner appropriate at the time (1987), by Butler and Glidewell.[44] The possibility of cyanide release will be discussed later. In view of what we now know about the biological effect of NO, it is generally assumed that nitroprusside is hypotensive because it is an NO-donor drug. It should, therefore, have biological activity in all those areas where NO is active and described elsewhere in this volume. Although this hypothesis has not been comprehensively tested, nitroprusside does have a wide range of biological activity. There are over 1000 references to the use of nitroprusside in physiological and medical experimentation since 1981, and it would be unprofitable to list and describe even a fraction of them. The following selection gives a flavor of the living processes upon which nitroprusside has some impact.

In a study[45] of the effect of a number of NO-donor drugs, nitroprusside was found to be more effective than NONOates (diazeniumdiolates) in dilation of the rabbit basilar artery, and its effect is not modified by removal of endothelial cells. It also increases cardiac output in failing hearts.[46] Not surprisingly nitroprusside effects relaxation of nonvascular smooth muscle such as guinea pig lung strips and bronchial rings,[47] mouse trachea,[48] longitudinal esophageal muscle,[49] and muscle cells of the mouse anococcygeus.[50] ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), a selective inhibitor of soluble guanylate

cyclase, prevents the nitroprusside-induced relaxation of guinea pig trachea.[51] Nitroprusside also parallels some of the effects of NO in the immune system. It induces apoptosis in neuroblastoma cells[52] and has a profound effect upon human brain tumor cells.[52] It has a radiosensitizing potency toward human pancreatic tumor cells[53] and inhibits falcipain, the papain-like cysteine protease involved in haemoglobin degradation by *Plasmodium falciparum*, a process which provides nutrients for the malarial parasite.[54] Nitroprusside very effectively inhibits proliferation of human endothelial cells,[55] as does NO, and also impacts on the renal functions of dogs.[56] The effect of nitroprusside on sperm mobility and viability has been studied,[57] and the observed effect may be due to the reduction of lipid peroxidative via the reaction of NO with reactive oxygen species essential to the process. Topical application of sodium nitroprusside solution to the round window membrane of the guinea pig ear has an acute ototoxic effect.[58] Although it is experimentally difficult to examine the effect of nitroprusside on nerve and brain, limited research shows it resembles the effect of NO. It increases pacemaker rhythm in the guinea pig sinoatrial node[59] and enhances synaptic plasticity.[60] Sodium nitroprusside, when injected into the central nervous system of male rats, was found to induce both yawning and penile erection (an unflattering combination), two processes known to be mediated by NO.[61]

Sodium nitroprusside has a number of clinical uses, the most common of which is hypotensive anaesthesia during surgery. Although there is some concern that prolonged or high-dose treatment with sodium nitroprusside might lead to cyanide poisoning, the major limitations for its use in a clinical setting are that it generally requires intravenous administration and that it is so powerful a vasodilator it is difficult to titrate. Nevertheless, it has been used recently in the treatment of erythromelalgia, a rare condition of children[62,63] involving swollen and painful extremities and it is routinely used in clinical studies as the "gold-standard" NO-donor drug.

Most of what has been described above suggests that the biological effect of nitroprusside is due to the release of NO, and it is generally described in the biological and medical literature as an NO-donor drug and set alongside spontaneous NO generators, including the NONOates and *S*-nitrosothiols, and NO donors that require metabolism, like the organic nitrates. Much of the literature does not address the question of how NO is derived from sodium nitroprusside but in general, it is intimated that spontaneous release of NO is responsible for its biological actions. However, spontaneous release of NO is not a conspicuous reaction of nitroprusside under most in vitro circumstances. Both as a solid and in solution sodium nitroprusside is completely stable in the absence of light. Photochemistry is often overlooked in assessing the biological activity of nitroprusside, and so we will now consider this in some detail before attempting to delineate the mechanism of nitroprusside's biological action.

The primary photochemical products from nitroprusside in aqueous solution, independent of both pH and photolysis wavelength over a wide range, are the aqua-complex $[Fe(CN)_5H_2O]^{2-}$ and NO:[64]

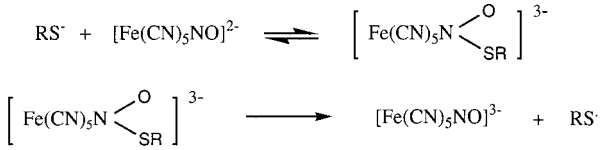
$$[Fe(CN)_5NO]^{2-} + H_2O \rightarrow [Fe(CN)_5H_2O]^{2-} + NO$$

A recent study[65] has confirmed, by spectrometric techniques, formation of NO but also detected HCN and cyanogen as gaseous products. Duchstein and Riederen[66] report that, in the absence of light, nitroprusside is stable but illumination causes immediate formation of NO, detected by chemiluminescence after reaction with ozone. Reduction and illumination together enhance NO formation. The NO released from nitroprusside upon illumination has also been detected as the adduct with nitronyl nitroxide to give an imino nitroxide, which is EPR active.[67] The quantum yield for the production of NO was measured as 0.20 and 0.32 at 420 and 320 nm, respectively. Photolytically released NO has also been detected in the cortical tissue of cats by use of a NO electrode.[68] Benzophenone sensitizes the photoreaction of nitroprusside in methanol and methanol/propan-2-ol mixtures giving the ligand substitution product $[Fe(CN)_5CH_3OH]^{2-}$ with $[Fe(CN)_4NO]^{2-}$ as the reduction product through diphenylketyl radicals. Ligand substitution takes place from the triplet $d_{xz,yz} \rightarrow \pi(NO)$ state, and the lower quantum yield for this process, compared with reaction from the singlet state in direct photolysis, is due to the favorable bimolecular deactivation of the triplet state.[69]

Clearly photolysis could be a source of NO in biological experimentation using nitroprusside, but it is rarely considered. Flitney et al.[70] found that ambient light was sufficient to activate sodium nitroprusside in isolated frog heart preparations and that preparations shielded from light altogether failed to respond to nitroprusside. This work was carried out in Scotland, which is hardly famous for its dazzling sunshine. Furthermore, Hurst et al.,[32] in a study of the regulation of cell respiration by NO, showed that intense light was necessary to produce an inhibitory effect with sodium nitroprusside, whereas Roussin's black salt had an effect with ordinary laboratory lighting. So ordinary laboratory illumination may play a part in nitroprusside's biological activity and the consequence of shielding the experimental setup from light should always be considered as illumination may vary from time to time and thus cause lack of reproducibility. However, in mammalian in vitro preparations and in vivo, the use of sodium nitroprusside gives highly reproducible result, irrespective of lighting conditions. Clearly light is something to be considered, particularly when using amphibian tissue, but other mechanisms of NO release must normally dominate to release NO from nitroprusside in mammalian tissue.

Although the photochemical reaction unambiguously releases NO and will therefore have biological consequences, it is not the only reaction of nitroprusside which is biologically significant; nitroprusside is a dilator of mammalian artery even in the complete absence of light, albeit somewhat reduced from the effect observed in illuminated conditions.[71] The quan-

Scheme 1



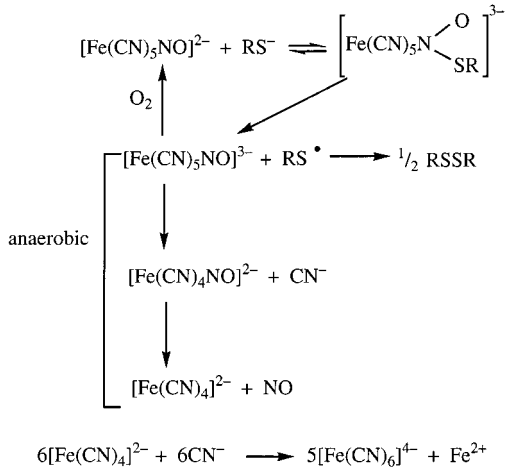
tum yield for the release of NO from nitroprusside is low;[67] in many laboratory conditions where nitroprusside is active, no NO will be produced photochemically, and so postulation of a "dark reaction" is required. In vitro nitroprusside reacts with a range of nucleophilic species (hydroxide, amines, carbanions, and thiols), and for the first three the reaction rates are slow. With hydroxide the product is the nitrito-complex $[Fe(CN)_5NO_2]^{3-}$;[72] toward amines nitroprusside acts as a nitrosating agent,[73] and with carbanions an oxime is formed.[74]

None of these seems a likely candidate for the dark reaction. It is the reaction with thiols, which is both fast and complex, which may contribute to the dark reaction mentioned above, as many thiols are present in tissue. Mulvey and Waters[75] suggested that nitroprusside reacts with thiols to give, first, a highly colored adduct and then the reduced ion $[Fe(CN)_5NO]^{3-}$ (Scheme 1).

The reduced species, first suggested by Griffiths et al.,[76] was thought by Mulvey and Waters to be consistent with the observed EPR spectrum. Much more is now known about this reaction. Adduct formation is very fast,[77] and the kinetics of this process were studied in detail by Johnson and Wilkins.[78] The results indicate that there is an equilibrium. The thiyl radical formed immediately dimerizes to give the disulfide. The reduced species $[Fe(CN)_5NO]^{3-}$ is readily oxidized by air back to $[Fe(CN)_5NO]^{2-}$, which reacts with further thiol to give more colored adduct. Thus, nitroprusside is cycled and the overall reaction is conversion of thiol to disulfide. The identification of the of the EPR signal observed by Mulvey and Waters ($g = 2.024$, $A(^{14}N) = 15.2$G) with $[Fe(CN)_5NO]^{3-}$ was questioned by Glidewell and Johnson.[79] By use of 90% ^{13}C-labeled nitroprusside the signal was reassigned to $[Fe(CN)_4NO]^{2-}$ and the discovery of this intermediate leads to a more complete account of the reaction of nitroprusside with thiols. If the reaction mixture is allowed to stand for some time under anaerobic conditions, and then purged with nitrogen, NO is detected in the effluent gas. Examination of the final reaction mixture, having started with 90% ^{13}C-labeled nitroprusside, indicated the presence of $[Fe(CN)_6]^{4-}$ but no free cyanide.[80] The overall reaction scheme emerging from these studies is shown in[81] Scheme 2.

The last reaction amounts to ligand reorganization, a process that also occurs in the reaction of nitroprusside with acetylacetone.[82] This scheme accounts for all the identified products of reaction, viz., disulfide, $[Fe(CN)_6]^{4-}$, Fe^{2+}, and NO, and the absence of any free cyanide. Although this process releases NO, it is unlikely to be the complete dark reaction responsible for the biological activity of nitroprusside as the NO-releasing process occurs only under anaerobic conditions. It has been suggested[83] that cyanide release always accompanies NO formation, but we could find no evidence for this. If this were correct, then the biological effect of nitroprusside would always be complicated by the biological effect of cyanide. The final cyanoferrate formed $[Fe(CN)_6]^{4-}$ is biologically inert and is unlikely to cause cyanide poisoning. However, it has been claimed that when nitroprusside is used clinically as a hypotensive agent the patient is in danger of cyanide poisoning, and this has restricted its use in medicine.[84] There are reported deaths of patients while receiving nitroprusside.[85] In an effort to understand this process an in vitro study suggested that when nitroprusside is mixed with erythrocytes there is rapid and complete conversion into iron ions, NO, and free cyanide.[86] This is a surprising reaction as the formation constants of cyanoferrates are very high; e.g., β_6 for hexacyanoferrate(II) is 10^{36}. Butler et al.[87] examined this claim with a nonintrusive analytical procedure using 90% ^{13}C-labeled nitroprusside. This species has a very distinct ^{13}C NMR spectrum which remained unchanged over several hours after mixing with whole blood. The cyano ligands in nitroprusside are inert, i.e., they do not exchange, but in some of the complexes formed from nitroprusside, e.g. $[Fe(CN)_5H_2O]^{2-}$ and $[Fe(CN)_5NOSR]^{3-}$, the ligands are labile. Also oxidation of nitroprusside can lead to an iron(III) complex with labile cyanide ligands.[88] Such reactions may explain why cyanide can be extracted from biological media containing nitroprusside and why patients treated with nitroprusside occasionally die of cyanide poisoning. The body can tolerate a certain amount of cyanide as there is an enzyme, rhodanase,[89] which will convert it into the nontoxic thiocyanate:

Scheme 2



$$CN^- + S_2O_3^{2-} \rightarrow CNS^- + SO_3^{2-}$$

Thiosulfite is an example of a sulfur donor species. It is very likely that today, in view of the stringent requirements of drug safety, nitroprusside would not be given permission for use in clinical medicine. However, despite the dangers it is an excellent hypotensive agent. If cyanide is released, as has been suggested, it is surprising that more patients do not

die, but this is an area where further direct experimentation is inappropriate. The use of nitroprusside in physiological experimentation as a source of NO, where cyanide release is less significant, is still hazardous in that the correct conditions for NO may not exist. This could result in erroneous conclusions being drawn. The fact that nitroprusside does not release NO in the direct way that NONOates and nitrosothiols do means that its physiological impact may be markedly different from that of the other NO-donor drugs. For example, Lovren and Triggle[90] compared the effect of authentic NO and nitroprusside upon precontracted human umbilical artery and found significant differences. Sato et al.[91] studied the inhibitory effect of a number of NO-donor drugs upon purified Na^+, K^+ ATPase activity. The behavior of nitroprusside was different from the others. It is known that NO regulates nitric oxide synthase (NOS) through negative feedback.[92] Nitrosothiols and nitroprusside affect NOS in neuroblastoma cells very differently, consistent with different routes for NO generation.[93] Sodium nitroprusside appears to be transformed into dinitrosyliron complexes in murine tumor tissue.[94]

A recent paper by Szacilowski et al.[95] provides evidence of a complication to the picture of the in vitro reaction given above. There is photochemical release of nitrosothiol from the nitroprusside−thiol adduct by illumination at 526 nm. This should result in release of NO by homolysis of the sulfur−nitrogen bond (Scheme 3).

Scheme 3

$$[Fe(CN)_5NOSR]^{3-} \xrightarrow{h\nu} [Fe(CN)_5]^{3-} + RSNO$$

$$[Fe(CN)_5]^{3-} + H_2O \longrightarrow [Fe(CN)_5H_2O]^{3-}$$

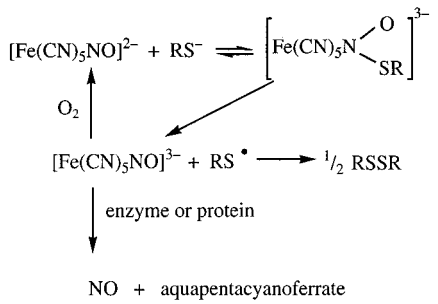
$$RSNO \longrightarrow {}^1/_2\,RSSR + NO$$

Such a possibility does not change matters to any great extent as the products are still disulfide and NO. This appears to be the only report of formation of a nitrosothiol from reaction of nitrosprusside with thiols, although this route to the formation of NO from nitroprusside has been postulated, but without compelling experimental evidence, elsewhere.[44]

All the mechanistic work described so far is based on experiment in vitro. In biological fluids or tissue, other reactions may come into play, and various possibilities have been discussed by a number of authors. Ramakrishna et al.[96] described a reductive process which may occur in rat hepatocytes and human erythrocytes and suggest that this reduction occurs in the presence of NADH or NADPH. Nitroprusside can pass through cell membranes, and so there is no intrinsic difficulty in this suggestion. There is direct evidence[80] to support the in vivo occurrence of the reactions shown in Scheme 3. Spin echo techniques in NMR spectroscopy were used to demonstrate the conversion by nitroprusside of glutathione into glutathione disulfide within erythrocytes.

Although thiols are ubiquitous, they are unlikely to be exclusively responsible for the biological activity of sodium nitroprusside as it is inactive in the frog

Scheme 4

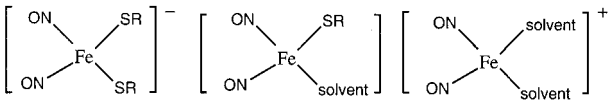


heart unless exposed to light. In contrast nitroprusside is active in mammalian arteries even in the dark. Furthermore, Sogo et al.[97] could not detect NO generation from sodium nitroprusside in human plasma containing cysteine, glutathione, homocysteine, and reduced cysteine residues in albumin. This suggests that although reaction with thiol is a necessary step in the vasodilator action of nitroprusside, it is not sufficient. There must be a unique component of mammalian tissues which is also involved in the release of NO from nitroprusside, but its involvement must come after reaction with thiol. Crucially, the work of Kowaluk et al.[98] reports that nitroprusside is readily metabolized to NO in subcellular fractions of bovine coronary arterial smooth muscle and the dominant site of metabolism is in the membrane fraction. This led to the isolation of a small membrane-bound protein or enzyme that can convert nitroprusside into NO. We therefore propose Scheme 4 for in vivo action of nitroprusside. In medicine the danger of producing labile cyano ligands is a real one.

IV. Dinitrosyliron Complexes

It was first proposed in 1965[99] that reaction between iron salts and NO in the presence of anioinic ligands gave rise to four-coordinated complexes of general stoichiometry $[Fe(NO)_2L_2]^{x+}$. These species are characterized by EPR spectra with isotropic g values of around 2.03. In the specific cases of L = cysteine and penicillamine, the identification of the stoichiometry was particularly clear, and it was further inferred that the iron was formally present as $d^9(Fe^{-I})$. In subsequent studies, although L was varied considerably, the g value was found to remain around 2.03.[100] The observed value of g is a reliable probe for establishing the formation of dinitrosyliron complexes (DNICs) of this type. The electronic and geometric structures of a number of paramagnetic iron dinitrosyl complexes were investigated by Bryar amd Eaton,[101] and the authors conclude that the structures are best described as 17 electron complexes with a d^9 configuration. The results are consistent with the view that the structures have distorted tetrahedral geometry, the extent of the distortion depending on the ligand. The anisotropy of the g values, determined from ESR studies using frozen solutions, varies considerably according to the nature of the ligand. The synthesis, structure, and ESR spectrum of $Fe(NO)_2(1\text{-MeIm})_2$ have been reported.[102] The spectrum obtained suggests that DNICs observed in biological fluids with $g = 2.03$ may have ligands other than thiols.

Chart 2



DNICs form readily from Roussin's red esters (**3**) by reaction with thiols in polar, coordinating solvents.[103] The EPR spectra of these complexes were fully characterized, and the structure of this family of DNICs was established as shown in Chart 2. Similar paramagnetic iron−nitrosyl complexes, characterized by anisotropic *g* values around 2.03, have been observed[104−106] in extracts of rat liver following administration of certain chemical carcinogens. These spectra have also been observed in extracts from organs of experimental animals maintained on a normal diet supplemented by iron(II) sulfate and sodium nitrite.[107,108] The DNICs from ferritin constitute a very interesting class. Lee et al.[109] studied the ESR signals ascribed to complexes made with horse spleen apo- and holoferritin, with chemically modified protein, and with recombinant human H-chain apoferritin and its site-directed mutants. Three types of complex were obtained, and the ESR spectra indicate the involvement of the imidazole group of histidine, thiol groups of cysteine, and carboxylate groups of aspartate and glutamate. This identifies the groups on ferritin involved in iron−nitrosyl formation.

As it is now known that NO plays a part in many physiological processes, there is renewed interest in DNICs and some evidence that they are biologically important. For example, after stimulation of murine macrophages to produce NO and coculturing with tumor-target cells, which do not produce NO, an EPR signal characteristic of DNICs was observed at 77 K.[110] Clearly nitrosation of iron is one consequence of cell-to-cell contact in this system. The use of EPR spectroscopy for the detection of DNICs in whole cells that are targets for NO has been reviewed.[111,112] Muller, Kleschyov, and Stoclet[113] established that storage of NO occurs in lipopolysaccharide-treated rat aorta. The EPR signal observed is characteristic of a DNIC and enhanced by addition of *N*-acetylcysteine, a ligand for DNICs. The iron of naturally occurring DNICs is thought to come from non-heme sources.[114]

DNICs have biological activity suggesting that they are ready sources of NO. They have a vasodilatory effect,[115,116] will *S*-nitrosate protein[117] and hemoglobin,[118] and induce heat-shock protein synthesis[119] and the DNA repair response in *Escherichia coli*.[120,121] The tissue distribution of DNIC after intravenous administration has been studied.[122] DNIC stability in living systems is strongly influenced by the concentration of free thiol, the redox potential of the environment, and the oxygen concentration.[123] Cellular non-heme iron content of tissue, which determines the level of DNIC formation on exposure to NO, is a factor in determining the cytotoxic action of NO (either necrosis or apoptosis). At higher concentrations of iron there is delayed NO-induced cell death, which then occurs in the absence of caspace-3 activation. If S-nitrosation of protein or enzyme is a step in NO-mediated cell death, then the ready formation of DNICs, which are nontoxic, could weaken NO cytotoxicity.[124] DNICs also form in liver cells during oxidative stress.[125]

Although EPR spectroscopy is the normal method of detection of DNICs, there is a recent report describing the use of electrospray ionization−mass spectrometry for characterizing protein-bound DNICs formed from iron−sulfur clusters. Solvent accessibility appears to be an important, but not necessary, factor for [Fe−S] cluster degradation by NO.[126] DNICs may also be detected by proton−electron double-resonance imaging.[127] The role of DNICs in biology has been reviewed.[128]

Sodium nitrite has been used for many years for the inhibition of food spoilage agents, such as *Clostridium* species, in preserved food like tinned meat. Indeed, the color of tinned meat is due to the interaction of nitrite with a heme species. The mode of action of sodium nitrite as an inhibitory agent is still not fully understood as nitrite alone is only mildly cytotoxic. One key feature is the loss of nitrite and formation of a bacterial inhibitor more potent than nitrite during pasteurization. This phenomenon is known as the Perigo effect.[129−131] One observation may provide a clue to an understanding of the Perigo effect. Reddy, Lancaster, and Cornforth[132] noted that treatment of vegetative cells of *C. botulinum* with nitrite in the presence of ascorbic acid caused replacement of the EPR signal at $g = 1.94$, characteristic of a reduced form of a {4Fe−4S} iron−sulfur center, by a signal at $g = 2.035$, characteristic of a DNIC. A similar transformation occurs using synthetic models for both the {4Fe−4S} and {2Fe−2S} centers of redox proteins.[133] With the synthetic Fe−S cluster, reaction with nitrite leads eventually to formation of Roussin's black salt (**1**). Thus, the Perigo effect could be due to formation of **1** during heat treatment as **1** does have antibacterial action (see above). However, the same specificity against *Clostridium* species is not obtained when synthetic Roussin's salt is added to preserved meat products. It seems most likely that the Perigo effect is due to formation of a number of iron−sulfur nitrosyls, including cluster compounds and DNICs. The latter are known to react with the phosphoclastic system of *C. sporogenes*.[134]

One factor in understanding the formation of such compounds during the heat-treatment of food is deciding on the source of the sulfur. Glidewell and Glidewell[135] have shown, by a range of analytical techniques, that there is sulfur capture from several cysteine sources. They also demonstrated that, in the presence of iron(II) salts and under conditions relevant to food processing, nitrite reacts with methionine and some of its derivatives by $-SCH_3$ transfer to yield the neutral species Roussin's red ester (**3**), a further candidate for the Perigo effect.

V. Interaction of NO with Nonheme Iron in Biological Systems

The biological properties of NO are generally attributed to its interaction with iron in the heme groups of key cellular enzymes. Nitrosation of the heme moiety of soluble guanylate cyclase is the trigger for enzyme activation and increased cGMP

generation that underpins the majority of the physiological processes governed by NO.[136,137] Similarly, nitrosation of the heme moiety of complex IV in the respiratory chain has long been recognized to result in powerful inhibition of respiration and to partially account for the cytotoxic effects of NO. Unsurprisingly, however, it is becoming increasingly clear that NO interacts with a wide range of other cellular components, many of which do not contain heme and some of which do not contain iron (Figure 1). This section will focus on those iron-containing cellular structures that have so far been identified as legitimate targets for NO or higher oxides of nitrogen and particularly peroxynitrite ($ONOO^-$).

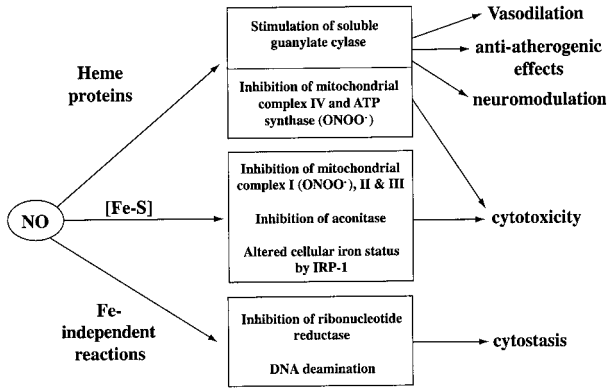


Figure 1. Cellular actions of NO. NO is best recognized as a powerful stimulator of soluble guanylate cyclase activity, resulting in well-documented effects in the cardiovascular and nervous systems. NO, together with related species including RS−NO and $ONOO^-$, is now also recognized to interact with other cellular components including non-heme metalloproteins that contain [Fe−S] centers. In general, relatively high concentrations of NO are required to influence the activity of these proteins, suggesting that the primary role of this interaction in vivo is during inflammatory responses where large quantities of NO are generated by inducible nitric oxide synthase to destroy invading pathogens.

A. [Fe–S] Centers of the Respiratory Chain

Recent evidence has revealed that inhibition of complex IV of the respiratory chain does not fully account for the cytotoxic effects of NO. Indeed, it is now apparent that the interaction of NO with complex IV gives rise to an inhibitory effect that is rapidly reversible,[138] while inhibition of respiration by NO often only reverses slowly or is irreversible.[139,140] An alternative mechanism by which respiration might be inhibited irreversibly has now been attributed to NO-mediated modification of [Fe−S] centers essential for the transfer of electrons through complex II and is characterized by the disappearance of the EPR spectra for these centers and the appearance of EPR-detectable iron−NO.[139] The effect is only seen with very high concentrations of NO (>1 mM) and is apparently irreversible. Similar concentrations of NO have also been found to reversibly alter the structure of the Rieske [Fe−S] center in complex III without the loss of iron.[139] It is interesting to note that $ONOO^-$ (100−500 mM) is also capable of causing irreversible inhibition of respiration via complex IV-independent mechanisms,[140−142] some of which might involve destruction of [Fe−S] centers in complex I and II.[143]

B. Mitochondrial Aconitase

Mitochondrial aconitase is an enzyme that catalyses isomerization of citrate to isocitrate in the citric acid (Krebs) cycle, a major source of reduced electron carriers (NADH, $FADH_2$) that are critical for respiration and the activity of cellular reductases. Aconitase contains a [Fe−S] center that is essential for complexing with citrate. $ONOO^-$ has been shown to inhibit the activity of isolated aconitase[144−146] by a mechanism that is reversed by addition of iron and thiols, prompting speculation that removal of the α-Fe of the [Fe−S] center is responsible for the inhibitory effect. High concentrations of NO (>100 μM) can only cause a small, reversible inhibition of aconitase, and *S*-nitrosothiols inhibit the enzyme via a NO-independent mechanism.[144−146] This, together with the fact that the citric acid cycle is only one of several potential sources of reduced electron carriers, has called into question the importance of the interaction of NO with the [Fe−S] center of aconitase in the inhibition of respiration under physiological or even inflammatory conditions. Furthermore, it is unclear whether nitrosation of the [Fe−S] center, or its destruction by NO, is a feature of the inhibitory effect of aconitase on $ONOO^-$.

C. Regulatory Proteins

Iron regulatory proteins (IRP) 1 and 2 are cytoplasmic mRNA-binding proteins that control intracellular iron homeostasis by regulating the translation of ferritin mRNA and the stability of transferrin receptor mRNA. Ferritin and transferrin play vital roles in binding Fe^{2+} and Fe^{3+} ions to prevent generation of highly cytotoxic hydroxyl radicals via Fenton chemistry and have themselves been shown to interact with NO to produce paramagnetic species.[110] The structure and function of the products of the interaction of NO with ferritin and transferrin have yet to be elucidated.

Although structurally and functionally similar, the two IRPs are different in their mode of regulation, pattern of tissue expression, and modulation by multiple factors, including NO. IRP1 is a cytoplasmic protein with close homology to mitochondrial aconitase and indeed is active as aconitase when iron levels are in balance.[147] However, when cellular iron levels fall, IRP1 activity switches to regulate ferritin and transferrin expression by binding to mRNA and modulating translation. It is recognized that the [Fe−S] center is pivotal in determining which of these quite different functions is performed by this bifunctional protein.[147,148] Experiments have demonstrated that expression of inducible NO synthase (iNOS) in activated macrophages is inversely correlated to aconitase activity but shows a positive correlation to RNA binding activity of IRP1.[149,150] NO, therefore, apparently drives IRP1 activity toward regulation of proteins related to iron sequestration, a finding that has been supported by evidence that stimulation of neuronal NO synthase has a similar effect on neurons

in the brain.[151] Although the [Fe−S] cluster in IRP1 has been strongly implicated as the key for conversion from aconitase to RNA-binding activity, the nature of the NO-related species that can mediate the conversion and its relative importance in vivo is still to be determined.[152] However, work with a "prototypical" [Fe−S]-containing protein, high potential iron protein (HiPIP) from *Chromatium vinosum* suggests that NO readily reacts with the [Fe−S] center to form a dinitrosyliron complex (DNIC) with a characteristic $g = 2.03$ EPR signal, providing the center is solvent-accessible. The [Fe−S] center of IRP1 is solvent-accessible and is therefore likely to react readily with NO.[153] The complex nature of IRP1 and its interaction with biologically relevant reactive species has prompted speculation that the protein might exist in at least three additional forms, interconversion of which is strongly influenced by NO, $ONOO^-$, and oxygen-derived radicals.[154] However, the precise mechanism underlying this interaction remains to be fully elucidated.

IRP2 does not have aconitase activity but might nevertheless contain an [Fe−S] cluster. Given that IRP2 activity is also modulated by NO, it is possible that interaction with the [Fe−S] center of IRP2 is central to the activity of this protein. NO-mediated activation of IRP2 has been shown to regulate hepatic iron regulation during acute liver inflammation.[155]

Gene transcription is a complex process which is regulated by specific regulatory proteins that bind to the promoter regions upstream of the gene. In the bacteria *E. coli*, a regulon known as SoxRS controls the expression of several genes involved in defense against activated macrophage-induced oxidative and nitrosative damage. The [Fe−S] center in the regulatory protein, SoxR, is essential for activation of transcription[156] and is sensitive to reactive oxygen species and NO.[157] This finding is somewhat surprising because nitrosation of [Fe−S] centers is usually thought to inactivate proteins, but in this case, there is clear evidence from EPR studies that, in both the isolated protein and in intact *E. coli*, the [Fe−S] center is *activated* by nitrosylation.[158] The nitrosylated [Fe−S] is stable in purified SoxR but is short-lived in intact bacteria, suggesting that a specific mechanism exists in bacteria to reverse nitrosylation.[158] Several other nonheme iron-containing proteins that are involved in bacterial gene regulation are potential targets for NO modulation. Of these DtxR and FNR are best known, but neither has yet been shown to interact with NO.[154]

The interaction of NO with non-heme metalloproteins is an area of NO biochemistry in its infancy with many questions still to be answered, but it is already clear that while interactions of NO with heme-containing proteins have rightly dominated the NO-related literature, interactions with other Fe-containing metalloproteins are also important in modulating cellular function. Of particular interest are those proteins that contain [Fe−S] centers because it is now evident that NO, along with oxygen-derived free radicals, can modulate the function of these proteins by nitrosylating or disassembling [Fe−S] centers. To date, it is apparent that, in most instances, very high concentrations of NO are required to affect [Fe−S] centers, suggesting that modulation will only occur in inflammatory conditions when inducible iNOS expression leads to generation of large quantities of NO. However, it appears increasingly likely that a further level of regulation might also arise from higher oxides of nitrogen, which can have different effects on [Fe−S] centers from NO itself. [Fe−S] centers of metalloproteins are likely, therefore, to operate as complex sensors for oxidative and nitrosative stress in both prokaryotic and eukaryotic cells.

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