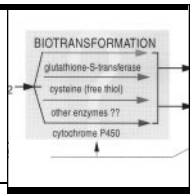
NO problem for nitroglycerin: organic nitrate chemistry and therapy



CHEMICAL SOCIETY REVIEWS

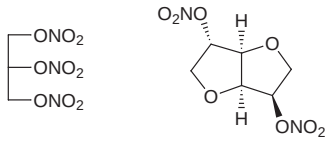
Gregory R. J. Thatcher* and Hazel Weldon

Department of Chemistry, Queen's University, Kingston, Canada ON K7L 3N6

Nitroglycerin (GTN) has been used clinically in the treatment of angina for over a century and is representative of the organic nitrates vasodilators. These are effective therapeutic agents that allow facile sublingual or transdermal administration. The vasodilatory mechanism involves activation of guanylate cyclase and is widely believed to involve biotransformation by chemical reaction of a nitrate with sulfhydryl or ferrous groups to yield nitric oxide. However, the chemistry of organic nitrates is poorly studied, provides scant support for these postulated reactions and provides a challenge for the chemist.

1 Introduction

'A trace of nitroglycerin was found in the wreckage of TWA Flight 800 but probably played no role in the explosion and may have simply come from a passenger's heart medicine.' 11/13/96 Associated Press. Nitroglycerin is a fascinating chemical that has both caused devastation and provided relief from suffering for very many people. The Italian chemist Sobrero, whose scarred face attested to his field of research, reported both the synthesis of nitroglycerin in 1846 and the headache that resulted from his attempt at oral characterization. Twenty years later, the taming of nitroglycerin in the form of dynamite was the basis of Alfred Nobel's fortune, but Nobel's brother was a fatal casualty of its explosive properties. Contemporary with Nobel's discovery, Thomas Brunton was forming relationships between angina pectoris and blood pressure and developing amyl nitrite for treatment of angina. Various reports of primitive clinical trials of nitroglycerin and the resulting headaches that ensued were clarified by the physician William Murrell, who demonstrated that smaller doses taken sublingually produced neither headaches nor dizziness, but provided rapid and remarkable relief from the intense pain of angina. Nitroglycerin was renamed glyceryl trinitrate (GTN), to avoid the anxiety associated with ingesting a high explosive, and has been used continuously in the treatment of angina since 1878. Used more recently for controlled hypotension during cardiac surgery, congestive heart failure and the treatment of anal fissures, GTN remains in the Top 100 prescribed drugs worldwide.



GTN ISDN

The remarkable therapeutic effectiveness of GTN is attested to by the relatively half-hearted attempts at finding an alternative organic nitrate vasodilator. Several simple organic nitrates including isosorbide dinitrate (ISDN) and mononitrate are used clinically. But as recently as 1993, 115 years after the advent of GTN therapy, simple nitrates such as hexane-α,ω-dinitrate and 2-phenyethyl nitrate were patentable as novel compounds and vasodilators. GTN and many nitrate esters have significant clinical attributes, one being facile delivery. Sublingual application of GTN tablets results in the onset of action and relief of anginal pain within 2 minutes. Furthermore, transdermal application *via* unguent or patch allows convenient slow release in the treatment of angina. Oral administration of GTN is ineffective because of rapid first pass metabolism, but with other convenient modes of delivery including sublingual and buccal, this does not present any obstacles.[1] The sole criticism of GTN in therapy is the onset of tolerance after repeated administration. The observation of tolerance scientifically has provided a fertile source for studies into the mode of action of GTN, but clinically in many patients tolerance is

Greg Thatcher is a native of Brighton, England. After completion of his BSc at the University of Manchester, he studied with Ron Kluger at the University of Toronto, completing his PhD in 1986. Following postdoctoral work with Mike Blackburn and a SERC Fellowship with Gordon Lowe he joined the faculty at Queen's University in 1988. Current research interests include: enzyme models for acyl and phosphoryl group transfer; inhibition strategies for phospholipases; biomolecular recognition and discrimination of anionic ligands; development of the chemistry of α-carbonyl phosphonates and of nitrates for use as therapeutic agents. He is a co-founder of GoBang Therapeutics Ltd.

Greg Thatcher

Hazel Weldon studied chemistry at Birmingham University and at Exeter University where she obtained her PhD working with Professor Stan Roberts on the synthesis of nitric oxide donors. After post-doctoral work with Dr Andrew Holmes at Cambridge University, Hazel joined Greg Thatcher at Queen's University where she has worked on the development of nitrate esters. Hazel's research interests lie in medicinal chemistry, in particular, nitric oxide research.

Hazel Weldon

simply overcome by the removal of treatment during rest periods (*i.e.* overnight).

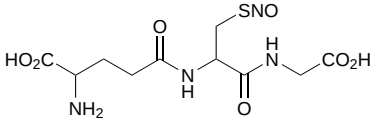
We will show that despite the beneficial therapeutic use of GTN for over a century, the mechanism of action remains to be solved. There has been a dogmatic belief over the past decade that GTN is a prodrug of nitric oxide, NO, and that GTN is biotransformed *via* a chemical reaction to NO. NO activates the enzyme guanylate cyclase (GCase), leading to smooth muscle relaxation. Direct chemical reactions of nitrates with ferrous and sulfhydryl groups have been proposed, but the complexities of GTN are intrinsically linked to NO and GCase. It is therefore necessary very briefly to review NO and GCase.

2 NO: an endogenous vasodilator

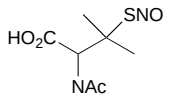
In 1979, work by Furchgott led to the discovery of a key role in relaxation of blood vessels for the endothelium and an endogenous substance, the endothelium derived relaxing factor (EDRF).[2] A great deal of effort and research was then directed towards the elucidation of the structure of this unknown vasodilator. However, it was not until 1987 that the groups of Moncada and Ignarro both independently identified EDRF as NO.[3,4] Part of the reason for this time lapse was due to the instability of NO and the subsequent isolation and characterization difficulties thus encountered. In addition, the known toxicity of NO undoubtedly led to a delay and trepidation in the identification of EDRF as NO.

The effects of endogenous EDRF and NO on relaxation of aortic and arterial strips were found to be indistinguishable.[3,4] Both were unstable with a half-life of 3–5 s, inactivated by superoxide anion, stabilized by superoxide dismutase and inhibited by oxyhaemoglobin. The reaction of EDRF and NO with haemoglobin gave, in both cases, nitrosylhaemoglobin and both caused the diazotization of sulfanilic acid. Thus EDRF was chemically identified as NO. Moncada and co-workers also used the chemiluminescence produced by reaction of NO with ozone to help identify EDRF.[3]

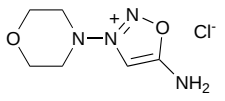
Despite this evidence some doubt has been cast on the chemical identity of EDRF. There have been reported discrepancies between the properties of EDRF and those of NO and it has been suggested, for example, that nitrosothiols or an iron complex having low molecular weight thiol ligands may account for the vasodilatory properties of EDRF. However, in 1994, Moncada and co-workers published a paper apparently clarifying the controversy as to the identity of EDRF and eliminating *S*-nitrosothiols, the dinitrosyl–iron–cysteine complex, sodium nitroxyl and hydroxylamine as EDRF candidates since in bioassay all are more stable than EDRF and less susceptible to inhibition by oxyhaemoglobin.[5] These workers maintain that EDRF is indeed NO, but recognition of the toxicity of NO and its short half-life *in vivo* has led to studies suggesting protein nitrosothiols as possible pools of EDRF.



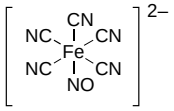
S-nitrosoglutathione (SNOG)



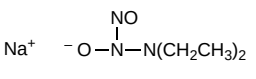
S-nitroso-*N*-acetylpenicillamine (SNAP)



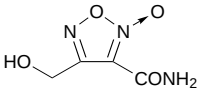
3-morpholinosydnonimine (SIN1)



Sodium nitroprusside (SNP)

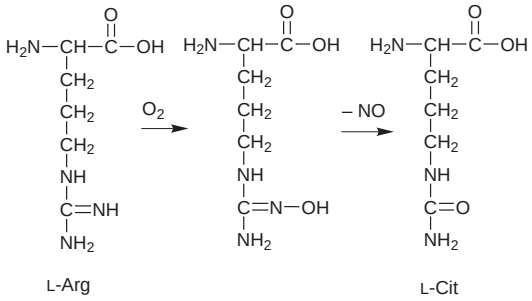


diethylamine NONOate (DENO)



4-hydroxymethylfuroxane-3-carboxamide

The biology and chemistry of NO has been the subject of numerous reviews.[6] Physiologically NO is produced enzymically from the terminal guanidino nitrogen of L-arginine, by nitric oxide synthase (NOS) (Scheme 1). Endothelial NOS (eNOS) releases NO which causes vasodilation or inhibition of platelet aggregation. Inducible NOS (iNOS) is found in macrophages and when induced, produces a large quantity of NO as part of the body's immune response. NO from neuronal NOS (nNOS) is involved in neurotransmission in the central and peripheral nervous systems.



Scheme 1

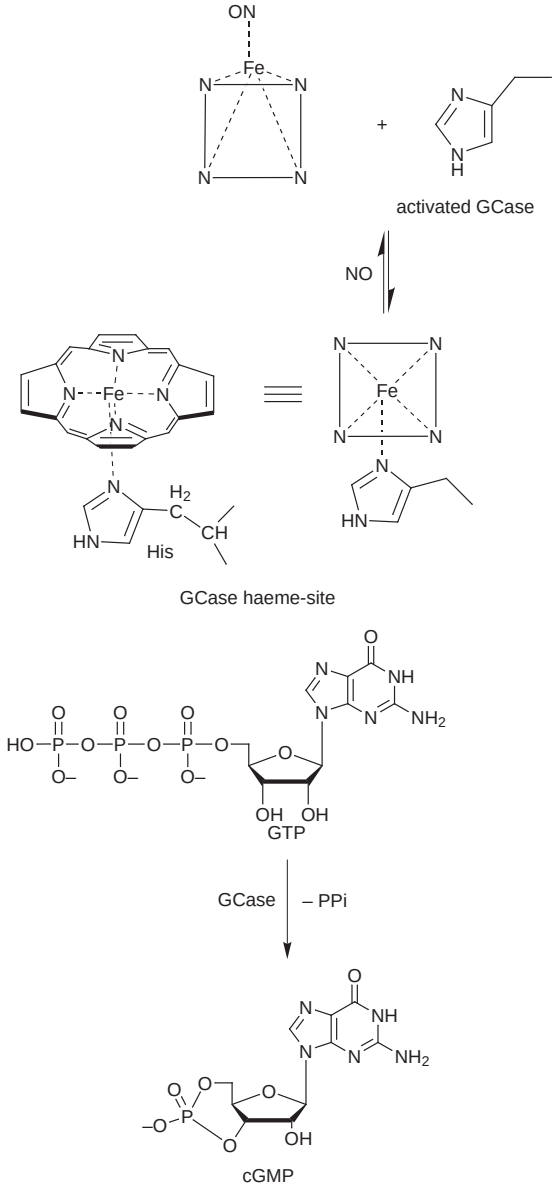
3 Guanylate cyclase and smooth muscle relaxation

Increased levels of cGMP within vascular smooth muscle result in vasodilation. The enzyme GCase catalyses the chemical conversion of GTP into cGMP (Scheme 2). cGMP production by GCase occurs at low basal levels unless the enzyme is activated by NO. The mechanism of GCase activation by NO involves binding of NO to a haeme-Fe centre, which is bound reversibly to the protein (Scheme 2).[7] NO has a high affinity for the Fe(II) haeme and has a labilizing effect on proximal ligands, displacing the proximal His-105 residue and thus moving the iron out of the plane of the porphyrin ring and activating the enzyme *via* conformational change.[7] Sodium nitroprusside (SNP), nitrosothiols (*e.g.* SNOG, SNAP) and other nitrovasodilators all activate GCase above basal levels. However, GTN and simple organic nitrates, in contrast, are incapable of activating soluble GCase above basal levels, *in vitro*, unless a thiol is added to the enzyme incubation.

Vascular smooth muscle is in a state of contraction in order to give resistance to blood flow. There is a continual release of NO which causes vasodilation and thus regulates vascular tone and assists in control of blood pressure. However, if there is damage to the endothelium and the production of NO is impaired a number of disease states may ensue. For example, sustained contraction of the smooth muscle of the blood vessel walls can be brought about by impaired NO production and may result in hypertension. Angina pectoris is a condition in which the arteries that supply blood to the heart are often narrowed and blood flow is restricted, resulting in lack of oxygen to the heart, especially during exercise or exertion. The ability of the heart to pump may be impaired, resulting in breathlessness and intense pain.

4 Chemistry of nitrate esters

Nitrate esters are subject to ionization in concentrated sulfuric acid giving rise to the production of nitronium ions.[8] However, nitrate esters are stable in dilute acid.[9] In strong alkaline solution, nitrate esters are known to undergo solvolytic decomposition for which three pathways are invoked, namely S_N2 nucleophilic substitution, β-hydrogen elimination and α-hydrogen elimination. (Scheme 3).[10] In the case of 1,2-dinitrates or β-hydroxy nitrates, oxirane formation is also possible under these strongly alkaline conditions (Scheme 3).[10] The largest body of mechanistic evaluation has simply used nitrate



Scheme 2

as a leaving group in solvolytic studies on the S_N2/S_N1 continuum.[11] No detailed chemical studies of the reaction of nitrate esters with other nucleophiles, such as mercaptans, have been pursued.

5 Biotransformation and tolerance

A substantial body of evidence supports the hypothesis that the vasodilatory activity of organic nitrates, and indeed other potent vasodilators such as SNP and SIN1, is primarily the result of activation of GCase, which mediates vascular smooth muscle relaxation.[1] We have already mentioned the problem of nitrate tolerance whereby long term or prolonged use of the drug leads to impairment of its vasodilatory effectiveness.[1] Tolerance has been thought to result from impaired biotransformation of GTN. Certainly, the observation of tolerance and the lack of *in vitro* GCase activation by GTN are the primary foundations for the dogma that GTN must be biotransformed to yield a chemical entity capable of GCase activation (Fig. 1). Clearly, any biotransformation pathway proposed, in addition to being chemically coherent, must also address the issues of tolerance. Futhermore, Bennett has usefully delineated clearance-based metabolism from mechanism-based biotransformation.[1] Clearance-based metabolism involves chemical reactions that degrade GTN to a product, usually nitrite ion, that does not influence vasodilation. Mechanism-based biotransformation requires a chemical mechanism to account for generation of a product that activates GCase, possibly NO.

Nucleophilic substitution:

(1) $HO^- + RONO_2 \longrightarrow ROH + NO_3^-$

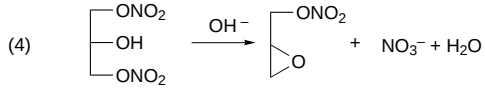
Elimination of β hydrogen:

(2) $HO^- + RCH_2CH_2ONO_2 \longrightarrow RCH{=}CH_2 + H_2O + NO_3^-$

Elimination of α hydrogen:

(3) $HO^- + RCH_2ONO_2 \longrightarrow RCH{=}O + H_2O + NO_2^-$

Hydrolysis of glycerol-1,3-dinitrate

(4) 

Scheme 3

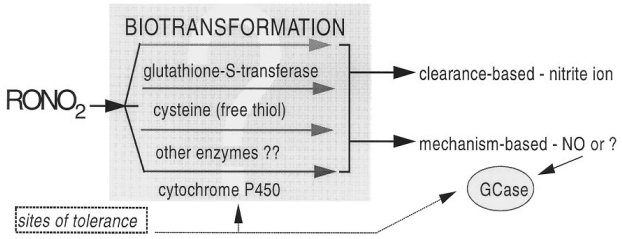


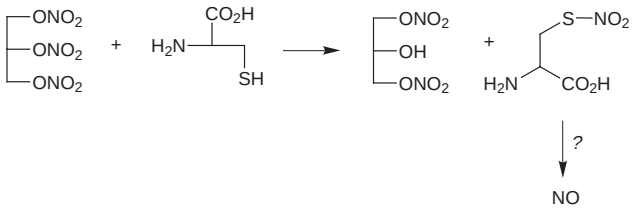
Fig. 1 Current state of knowledge concerning the pharmacology of organic nitrate vasodilators and possible biotransformation pathways. Activation of guanylate cyclase (GCase) leads to smooth muscle relaxation and vasodilation.

6 Sulfhydryl dependent pathways

In 1973, the observation that tolerance associated with prolonged exposure to GTN was accompanied by a decrease in the levels of tissue thiols led to a proposal that a sulfhydryl species was essential for the biotransformation of GTN and that the oxidation to a disulfide was the cause of tolerance.[12] In support of this theory it was reported that nitrate tolerance could be reversed by the addition of dithiothreitol, and indeed, the concurrent addition of thiols alongside GTN has been shown to circumvent the onset of tolerance.[13] However, it also has been stated that there is no correlation between the concentration of endogenous thiols and the state of tolerance.[14] Indeed, a large literature of contradictory observations exists on the role of thiols in the biotransformation of GTN.

Putting aside the pharmacological contradictions, sulfhydryl pathways require the chemical reaction of a thiol with an organic nitrate. The thiol may be free (*e.g.* cysteine), part of an enzyme, or the glutathione cofactor of glutathione-*S*-transferase.[1,12,15,16] It seems clear that GST has a role in clearance-based metabolism. Perhaps the only other undisputed observation is that activation of GCase *in vitro* by GTN requires addition of 'active' thiols [cysteine, *N*-acetyl cysteine (NAC), thiosalicylic acid (TSA)] but does not occur with other thiol adjuvants [*e.g.* dithiothreitol, (DTT)].[17] These thiols all possess a β-carboxylate group. *Is there a chemical mechanism for generation of NO from an organic nitrate such as GTN that represents the mechanism-based biotransformation pathway? If so, is there a role for neighbouring group participation by the intramolecular carboxy group of active thiols* (Scheme 4)?

In 1977 Murad suggested that NO might be the cause of the vasodilatory properties of GTN.[18] There followed the seminal



Scheme 4

1981 Ignarro hypothesis, well-accepted in the pharmacology community for a decade. GTN must first enter the smooth muscle cell, where it is then converted to nitrite ions by reaction with cysteine (depletion of which gives tolerance); nitrite then liberates NO *via* nitrous acid; NO combines with thiol to generate a nitrosothiol which activates GCase:[19]

$$RONO_2 \xrightarrow{2R'SH} ROH + R'SSR' + NO_2^- \overset{H^+}{\rightleftharpoons} HONO$$

$$HONO \longrightarrow NO \xrightarrow{R'SH} R'SNO$$

The concentration of nitrous acid at physiological pH always presented a problem in terms of the chemical mechanism. Similarly, the relatively high physiological concentrations of NO_2^- compared to GTN presented a pharmacological problem.[20] This Ignarro hypothesis had been superceded by the time that Williams had further shown that NO is not reactive towards simple thiols.[21]

Nevertheless, thiols do indeed react with organic nitrates to give disulfide and inorganic nitrite (NO_2^-) as products.[13,16,19] The specific products from GTN are the two glyceryl dinitrate isomers. Incubation of GTN in a phosphate buffer (pH 7.4) with cysteine and dithiothreitol yields ratios for 1,3 : 1,2-GDN of 1.6 : 1 and 1.7 : 1 respectively.[22] However, this reaction is very slow. For example, degradation of GTN (2 mM) by cysteine and NAC (5 mM) has proceeded only 10 and 1%, respectively, after 1 h at room temperature. In contrast, onset of vasodilation, *in vivo*, can occur within seconds after intravenous administration of GTN. Unfortunately, the most comprehensive studies of thiol reaction with GTN have used plasma as reaction solution, which does not allow assessment of simple chemical reactivity.[13]

The two simplest options for nucleophilic reaction of a thiol with an organic nitrate are substitution at C or at N. Attack at C must yield nitrate ion as product and therefore cannot provide the chemical pathway for mechanism-based biotransformation. Attack at N will yield a thionitrate ester ($RSNO_2$) as product (Scheme 4). Yeates and co-workers proposed formation of such a thionitrate from glutathione-*S*-transferase (GST)-mediated reaction of glutathione (GSH) with GTN, although only the disulfide final product was detected:[16]

$$RONO_2 \xrightarrow[GST]{GSH} G'S\text{–}NO_2 \overset{R'SH}{\rightleftharpoons} R'SSG + NO_2^-$$

In 1992 Yeates proposed that this thionitrate ester could undergo isomerization to a sulfinyl nitrite, homolytic decomposition of which would lead to the formation of NO:[16]

$$RONO_2 \xrightarrow{R'SH} R'SNO_2 \rightleftharpoons R'S(O)NO \longrightarrow NO$$

This pathway was in accord with the rising belief that mechanism-based biotransformation of GTN must yield NO. Although, no evidence for NO release from interaction of GTN with purified glutathione-*S*-transferase has been provided, the contemporary discoveries of the identity of EDRF and the biological role of NO led to wide acceptance that the vasorelaxant GTN was in fact an exogenous form of EDRF, that is a NO pro-drug. The reactions proposed appear chemically reasonable. However, the required confirmation was evidence for NO formation from GTN. *Is there direct chemical evidence for formation of NO from reaction of thiol with GTN in a simple aqueous medium?*

Detection and quantification of NO remains a challenge. Methods available include several potentiometric devices involving NO-selective electrodes, spin-trapping/ESR detection, chemiluminescence detection of NO_2 formed by reaction of NO gas from the reaction headspace with O_3, and trapping of NO by Fe(II)-oxyhaemoglobin (oxyHb). In the last method, oxyHb is oxidized to Fe(III)-methaemoglobin which can be monitored spectrophotometrically. Feelisch and Noack reported a good correlation between the rate of NO production and GCase activation, from solutions of organic nitrates with added thiols, using the oxyHb method.[17] Furthermore, similar rates of NO release were reported from GTN + thiol in phosphate buffer, using chemiluminescence detection.[23] This work of Feelisch and Noack is widely cited as evidential proof for generation of NO from reaction of thiol with GTN. However, these papers must be viewed with more circumspection than has been the case. The oxyHb assay, in which an initial rate of Fe(II)-oxyHb oxidation is spectrophotometrically monitored, is not entirely specific for NO.[24,25] Moreover, in a detailed study using chemiluminescence detection, Fung and co-workers reported that no measurable quantity of NO could be detected from GTN + thiol in phosphate buffer, except under anaerobic conditions with the addition of superoxide dismutase (to scavenge for superoxide radical that would otherwise rapidly degrade NO).[13] Even under these conditions, NO generation from GTN + cysteine in buffer was 5% of that observed from GTN in plasma. In addition, NO cannot be detected from the reaction of GTN ($\leqslant$2 mM) with cysteine ($\leqslant$50 mM) by an NO-specific electrode with detection limits for NO production of 1.5 nM s^{-1}.[24]

The rate of breakdown of GTN in the presence of thiol depends upon the identity of the thiol and furthermore does not correlate with the rate of activation of GCase in the presence of GTN and thiol. For example, DTT reacts much more rapidly with GTN than does *N*-acetyl cysteine, but GCase is activated by GTN in the presence of *N*-acetyl cysteine, but not DTT. Moreover, the ratio of nitrite ion production to the rate of oxyHb oxidation by GTN varies depending on the thiol adjuvant, for example the ratio is 5 times larger for cysteine than for *N*-acetyl cysteine.[17,23] We have seen the initial formation of a thionitrate from the transesterification reaction of thiol with GTN proposed previously. Thus, it is reasonable to suggest partitioning of this common intermediate between two pathways, clearance-based to nitrite ion and mechanism-based to yield a species capable of activating GCase. A number of groups have proposed rearrangement of the thionitrate intermediate to either a sulfinyl or sulfenyl nitrite:[16,26]

$$RONO_2 \xrightarrow{R'SH} R'SNO_2 \rightarrow\rightarrow R'S\text{-}ONO \rightarrow\rightarrow R'S\text{-}(O)NO \overset{R'SH}{\rightarrow} \begin{matrix} R'SNO \\ NO \end{matrix}$$

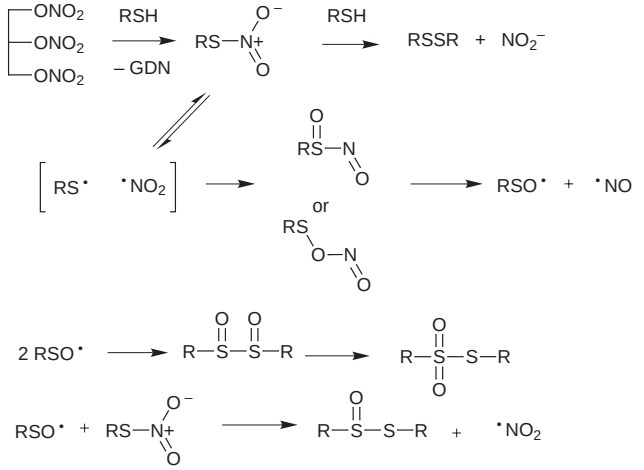
In simile with the facile homolytic fission of *tert*-butyl sulfinyl nitrate to NO_2 and the ready formation of sulfinyl radicals, release of NO from this rearrangement pathway appears feasible.

7 Thionitrates

In 1932 *tert*-butyl thionitrate was synthesized by oxidation of the corresponding nitrosothiol employing fuming nitric acid as the oxidant. It was reported that the resulting thionitrate was more stable than the initial nitrosothiol.[27] It was not until 1978 that an alternative synthesis *via* N_2O_4 was published and the same group later reported the synthesis of the unstable aryl esters and data on thermolytic decomposition of thionitrates.[28] Neither sulfenyl nor sulfinyl nitrites have been isolated. High level theoretical calculations on the stability of methyl sulfenyl nitrite showed a marginally higher energy than the corresponding thionitrate, but also showed that the rearrangement from

thionitrate to sulfenyl nitrite was thermodynamically accessible.[26] Further calculations on *tert*-butyl thionitrate show that this sulfinyl nitrite is of comparable stability to the sulfenyl nitrite.[24]

Despite the thermodynamic accessibility of the rearrangement process and low barriers to subsequent homolytic fission of the sulfenyl nitrite to give NO, the calculations revealed a substantial barrier to concerted rearrangement. A homolytic rearrangement mechanism was proposed *via* a geminate radical pair, {RS··NO$_2$}, presenting the possibility of release of NO$_2$.[24] An experimental study of the hydrolysis of *tert*-butyl thionitrate supported this mechanism, but requires that the radical pair recombine to give the sulfenyl nitrite much more rapidly than dissociation.[26] The reaction of this thionitrate is remarkably clean. Only di-*tert*-butyl thiosulfinate [Bu^{t}S(O)S–Bu^t] and di-*tert*-butyl thiosulfonate [Bu^{t}S(O)$_2$S-Bu^t] are detected as organic reaction products, with no sign of any disulfide formation (Scheme 5). NO is also detected by an NO-specific electrode. *So, is this thionitrate rearrangement the mechanism-based biotransformation pathway*?



Scheme 5 Detailed mechanism for reaction of organic nitrate with thiol and subsequent thionitrate rearrangement.

Perplexingly, *tert*-butyl thionitrate which releases NO and oxidizes oxyHb, was not found to activate GCase! Nor did the thionitrate inactivate GCase to activation by other nitrovasodilators. This highlights a significant problem with data on nitrovasodilator activation of GCase. All such GCase experiments have been carried out with a partially purified tissue homogenate that contains DTT and other components. Thionitrate esters are very reactive towards thiols, being converted quantitatively to disulfide and nitrite ion and may be decomposed rapidly in the assay medium (Scheme 5). GTN does not activate GCase in the presence of Bu^{t}SH and it is possible that the β-carboxy group of active thiols, *vide supra*, either stabilizes the resulting thionitrate to decomposition, or accelerates partitioning to NO. The latter would seem chemically reasonable and better precedented.

8 Alternative sulfhydryl pathways

In some quarters there is a belief that the significant differences between organic nitrates and other nitrovasodilators indicate that GTN is biotransformed to a nitrosothiol rather than NO.[29] Nitrosothiols are effective nitrovasodilators and activators of GCase. Fung has argued against a requirement for conversion of NO to nitrosothiol in the biotransformation of organic nitrates, but he and others have also suggested that GTN biotransformation yields a nitrosothiol.[30] It has been suggested that initial reduction of an organic nitrate would afford an organic nitrite ester which would subsequently react with a thiol to yield a nitrosothiol:

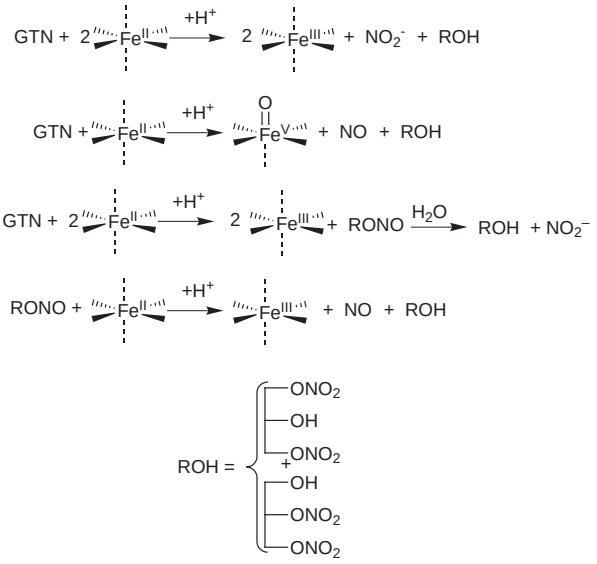
$$RONO_2 \longrightarrow RONO \xrightarrow{R'SH} R'SNO \longrightarrow NO$$

There is neither evidence nor mechanism for the initial reduction and the putative nitrite intermediate from GTN has been studied and shown to be very hydrolytically labile.[31] Yeates *et al.* have speculated that organic nitrates are first reduced to organic nitrites prior to GST enzyme-mediated reaction with glutathione to afford *S*-nitrosoglutathione which could subsequently release NO.[35] Another speculative proposal has an unknown reduction process converting the thionitrate intermediate directly to a nitrosothiol:

$$RONO_2 \xrightarrow{R'SH} R'SNO_2 \longrightarrow R'SNO \longrightarrow NO$$

9 Metal-ion/haeme dependent pathways

Conversion of an organic nitrate to NO is a 3e$^-$ reduction and GTN has been shown to react with the ferrous-haeme moieties of haemoglobin and myoglobin to give both GDN regioisomers.[1] However, the reaction of GTN with deoxyHb itself yields only nitrite ion. Nitrate reductase will reduce inorganic nitrate (NO_3^-), and a Mo-complex has been reported to yield nitrogen dioxide from NO_3^-. Doyle has studied the reaction of organic nitrites with haemoglobin and notes binding at the haeme site, leading to formation of NO and alcohol.[32] Binding and reduction of lipophilic GTN (in place of O_2 or H_2O_2) at the active site of cytochrome P450 is easily visualized, especially under anaerobic conditions.[1] Thus direct reaction of ferrous-haeme proteins with organic nitrates to yield NO is not chemically unreasonable (Scheme 6). Indeed, the first chemical evidence for this has been provided by observation of the rapid release of NO and GDN from reaction of GTN with an Fe(II)-tetraphenylporphyrin bearing *N*-methylimidazole ligands.[22]



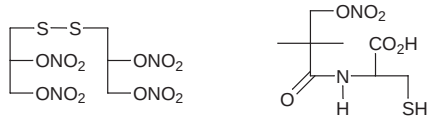
Scheme 6 Possible mechanisms for reaction of organic nitrate (GTN) with Fe(II)-porphyrin producing NO and nitrite ion.

The ferrous-haeme site of GCase binds NO with high affinity.[7] Direct reaction of GTN at this site has not been seriously considered, probably because of the requirement for added active thiol for GCase activation. However, it is simple to theorize on a mechanism whereby the role of the thiol is, (a) as a reducing agent to cycle either the haeme-Fe or an essential protein thiol, or (b) as an allosteric activator specific to nitrates. The conserved structure of the active thiols may be required for appropriate binding to GCase. If this theory is correct, then depletion of active thiol (or an *in vivo* reducing equivalent) would lead to tolerance, but GCase desensitization would not necessarily be a tolerance factor.

A mixed haeme-sulfhydryl pathway is relevant to the postulate of direct reaction of GTN with GCase and other theories. Reaction of GTN with a cysteine residue of GCase could yield a GCase-thionitrate which might interact directly with the Fe-haeme site or indirectly *via* NO. Doyle proposed a mixed haeme-sulfhydryl pathway for deoxyHb + GTN, since the Hb-β-93 cysteine thiol residue may participate by means of nitrosyl exchange with organic nitrite to afford to nitrosothiol capable of NO release.[32] Furthermore, nitrosyl exchange of organic nitrites with thiols to produce nitrosothiols is well documented.[21] However, in order that this specific theory can be applied to organic nitrates, it is required that an organic nitrate be converted to an organic nitrite. Reaction of GTN with haemoglobin is observed to lead only to the production of nitrite ions, discounting a mixed haeme-sulfhydryl dependent theory for this, but not other haeme-proteins.

10 Novel nitrate esters

Activation of GCase *in vitro* by organic nitrates requires an active thiol such as cysteine, anticipating the development of nitrate esters containing a cysteine moiety. Studies on such a family of compounds have been reported.[33] Although such cys-nitrates have been shown to circumvent tolerance, similar behaviour of control compounds that do not contain cysteine casts ambiguity on these data. In the cys-nitrates, the nitrate N



S-nitrate

cys-nitrate

atom is nine atoms removed from the cysteinyl-S making the simple intramolecular chemical reaction between these centres unfavourable. A different family of S-containing organic nitrates has also been studied.[34] Many S-nitrates have the potential for rapid intramolecular reaction *via* 5- and 6-membered rings to form thionitrates. In studies on aortic tissue relaxation, tolerant-tissue and GCase activation, these S-nitrates show properties very different from GTN itself.

11 Potential new therapies

NO has a multitude of biological roles and with NO synthase dysfunction is associated with many disease states. Organic nitrates appear in many respects to act as exogenous NO sources, which suggests many potential medicinal applications. Circumventing nitrate tolerance in current cardiovascular therapies would be beneficial. Perhaps more exciting is the mounting evidence that organic nitrates have significant neuroprotective effects, which may provide the basis for new treatments, for example, cerebrovascular therapeutics for management of stroke. It has been proposed in the literature that GTN itself has neuroprotective effects due to interaction with the redox regulatory site of the NMDA receptor, which is a thiol–disulfide couple.[35] Again there exists the possibility of a direct chemical reaction between a thiol and GTN mediating a potent biological response, although simple non-covalent binding interactions of the nitrate group with an allosteric site on glutamate receptors cannot be ruled out. Evidence presented to date is promising for novel therapeutic applications of organic nitrates.

12 Summary of pharmacological data

In contrast to the scant literature on organic nitrate chemical reactivity, there is a vast literature on pharmacological activity. This review, because of its nature, has presented very little pharmacological data, but the limited references provided should provide a starting point for the interested reader. The pharmacological literature holds much of relevance to the chemist but is daunting in both its volume and its highly contradictory nature. The following summary provides a partial listing of the contradictions encountered, but the interested reader is encouraged to delve more deeply into the primary literature. (a) Quantification of NO release remains a problem, because of the relatively high detection thresholds in NO-selective electrodes and chemiluminescence; (b) levels of cGMP from GCase activation required for vasodilation may be so low that the corresponding increase in levels of cGMP is at detection limits; (c) *in vivo* GTN concentrations required for vasodilation (nanomolar) are substantially lower than EC_{50} values measured for GCase activation in broken cell preparations; (d) 'active' thiols are required for *in vitro* activation of GCase, but evidence is poor for such an absolute requirement *in vivo*; (e) tissue relaxation studies are highly dependent on time of incubation, dose and precontraction conditions; (f) many pharmacological studies on GTN are carried out in anaerobic conditions, in complex media (*e.g.* plasma), and/or in the presence of SOD and catalase; (g) differentiation of 'mechanism-based' and 'clearance-based' biotransformation pathways is difficult: *e.g.* loss of stereoselectivity of biotransformation often accompanies tolerance and is used as an indicator for tolerance, but is this loss of stereoselectivity associated with a clearance-based or mechanism-based pathway?; (h) inhibitors of redox processes and haeme-proteins, for example cytochrome P450, are rarely specific and the number of redox and haeme-proteins that may be involved complicates the problem (*e.g.* P450, P450 reductase, deoxyHb, GCase).

13 Conclusions

Sobrero is quoted, 'When I think of all the victims killed during nitroglycerine explosions, and the terrible havoc that has been wreaked, which in all probability will continue to occur in the future, I am almost ashamed to admit to be its discoverer'. However, nitroglycerin and other organic nitrates are established and very important cardiovascular drugs. Moreover, there appears exciting promise for therapeutic application in other disease states, including cerebrovascular and neurological disorders. Features such as high lipophilicity, facile administration and low toxicity are clearly beneficial. Nevertheless, more than a century after Murrell's clinical introduction of GTN, the chemical mechanism underlying vasodilation remains unproven. It is certain that without more research by chemists to increase our knowledge of the chemistry, structure and reactivity of the organic nitrate functional group, that this unsatisfactory situation will continue. To summarise our knowledge of the biological chemistry:

1. Organic nitrates are widely believed to undergo mechanism-based biotransformation *in vivo* to yield NO or a nitrosothiol. Chemical mechanisms proposed involve reaction with a ferrous or a sulfhydryl functionality.
2. The reaction of organic nitrates with many thiols at physiological pH is slow and yields disulfide and nitrite ion as major products.
3. Thionitrate esters are putative intermediates in the reaction of thiols with organic nitrates; reaction of thionitrates with thiols yields disulfides; thionitrates undergo hydrolysis at physiological pH to yield sulfinyl radical products and NO.
4. Reaction of nitrate esters with the ferrous group of Fe(II)-porphyrins can be rapid: reaction with deoxyHb yields nitrite ion as product, whereas reaction with a simple Fe(II)-porphyrin yields NO.
5. The reactivity and biological activity of organic nitrates is very different to nitrosothiols, NO-releasing NONOates and other nitrovasodilators.
6. There is little unambiguous evidence that organic nitrates act as NO pro-drugs and it is possible that NO release occurs subsequent or consequent to GCase activation.

7. It is very likely that more than one mechanism-based biotransformation pathway is in operation *in vivo* to produce the potent vasodilation invoked by nitrate esters.

14 Acknowledgements

We are grateful to the Heart and Stroke Foundation of Ontario (Grant #A2259) and GoBang Therapeutics Ltd for financial support and Dr Brian Bennett for advice and collaboration.

15 References

1 B. M. Bennett, B. J. McDonald, R. Nigam and W. C. Sinon, *Trends Pharmacol Sci.*, 1994, **15**, 245, and references therein.
2 R. F. Furchgott and J. V. Zawadzki, *Nature*, 1980, **288**, 373, and references therein.
3 R. M. J. Palmer, A. G. Ferrige, and S. Moncada, *Nature*, 1987, **327**, 524.
4 L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns and G. Chaudhuri, *Proc. Natl. Acad. Sci. USA*, 1987, **84**, 9265.
5 M. Feelisch, M. te Poel, R. Zamora, A. Deussen and S. Moncada, *Nature*, 1994, **368**, 62.
6 A. R. Butler and D. L. H. Williams, *Chem. Soc. Rev.*, 1993, 233, and references therein.
7 J. R. Stone and M. A. Marletta, *Biochem.*, 1996, **35**, 1093.
8 L. P. Kuhn, *J. Am. Chem. Soc.*, 1947, **69**, 1974.
9 J. Honeyman and J. W. W. Morgan, *Adv. Carbohydr. Chem.*, ed. M. L. Wolfram and R. S. Tipson, New York, 1957.
10 J. W. Baker and D. M. Easty, *J. Chem. Soc.*, 1952, 1193; C. Capellos, W. J. Fisco, C. Ribaudo, V. D. Hogan, J. Campisis, F. X. Murphy, T. C. Castorina and D. H. Rosenblatt, *Int. J. Chem. Kin.*, 1984, **16**, 1027.
11 R. S. Robertson, K. M. Koshy, A. Annessa, J. N. Ong, J. M. W. Scott and M. J. Blandamer, *Can. J. Chem.*, 1982, **60**, 1780, and references therein.
12 P. Needleman and E. M. Johnson, Jr., *J. Pharmacol. Exp. Ther.*, 1973, **184**, 709.
13 S. Chong and H. L. Fung, *Biochem. Pharmacol.*, 1991, **42**, 1433.
14 C. A. Gruetter and S. M. Lemke, *Can. J. Physiol. Pharmacol.*, 1986, **64**, 1395.
15 S.-J. Chung and H.-L. Fung, *Biochem. Pharmacol.*, 1993, **45**, 157.
16 R. A. Yeates, *Arzneim.-Forsch./Drug Research*, 1992, **42**, 1314 and references therein.
17 M. Feelisch and E. A. Noack, *Eur. J. Pharmacol.*, 1987, **139**, 19.
18 W. P. Arnold, C. K. Mittal, S. Katsuki and F. Murad, *Proc. Natl. Acad. Sci. USA*, 1977, **74**, 3203.
19 L. J. Ignarro, H. Lippton, J. C. Edwards, W. H. Baricos, A. L. Hyman, P. J. Kadowitz and C. A. Gruetter, *J. Pharmacol. Exp. Ther.*, 1981, **218**, 739.
20 B. M. Bennett and G. S. Marks, *Trends Pharmacol. Sci.*, 1984, 329, and references therein.
21 A. R. Butler, F. W. Flitney and D. L. Williams, *Trends Pharmacol. Sci.*, 1995, **16**, 18, and references therein.
22 J. D. Artz and G. R. J. Thatcher, *Chem. Res. Toxicol.*, submitted.
23 M. Feelisch and E. Noack, *Eur. J. Pharmacol.*, 1987, **142**, 465.
24 J. D. Artz, K. Yang, J. Lock, C. Sanchez, B. M. Bennett and G. R. J. Thatcher, *Chem. Commum.*, 1996, 927.
25 K. Schmidt, P. Klatt and B. Mayer, *Biochem. J.*, 1994, **301**, 645.
26 D. R. Cameron, A. M. P. Borrajo, B. M. Bennett and G. R. J. Thatcher, *Can. J. Chem.*, 1995, **73**, 1627.
27 H. Rheinboldt and F. Mott, *Chem. Berichte*, 1932, 1223.
28 S. Oae, K. Shinhama, K. Fujimori and Y. H. Kim, *Bull. Chem. Soc. Jpn.*, 1980, **53**, 775.
29 G. S. Marks, B. E. McLaughlin, S. L. Jimmo, M. Poklewska-Koziell, J. F. Brien and K. Nakatsu, *Drug Metab. Dispos.*, 1995, **23**, 1248.
30 H. L. Fung, S. J. Chung, J. A. Bauer, S. Chong and E. A. Kowaluk, *Am. J. Cardiol.*, 1992, **70**, 4B.
31 F. Buckell, J. D. Hartry, U. Rajalingam, B. M. Bennett, R. A. Whitney and G. R. J. Thatcher, *J. Chem. Soc. Perkin Trans. 2*, 1994, 401.
32 M. P. Doyle, R. A. Pickering and J. D. Conceicao, *J. Biol. Chem.*, 1984, **259**, 80, and references therein.
33 J. Zanzinger, M. Feelisch and E. Bassenge, *J. Cardiovasc. Pharmacol.*, 1994, **23**, 772.
34 K. Yang, J. D. Artz, J. Lock, C. Sanchez, B. M. Bennett, A. B. Fraser and G. R. J. Thatcher, *J. Chem. Soc., Perkin Trans. 1*, 1996, 1073.
35 S. A. Lipton, Y. B. Choi, N. J. Sucher, Z. H. Pan and J. S. Stamler, *Trends Pharmacol. Sci.*, 1996, **17**, 186, and references therein.

Received, 9th March 1998
Accepted, 28th April 1998