REACTION OF ORGANIC NITRATE ESTERS AND S-NITROSOTHIOLS WITH REDUCED FLAVINS: A POSSIBLE MECHANISM OF BIOACTIVATION

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ABSTRACT:
Organic nitrate esters, such as glyceryl trinitrate and isosorbide dinitrate, are a class of compounds used to treat a variety of vascular ailments. Their effectiveness relies on their ability to be bioactivated to nitric oxide (NO) which, in turn, relaxes vascular smooth muscle. Although there have been many biological studies that indicate that NO can be formed from organic nitrate esters in a biological environment, the chemical mechanism by which this occurs has yet to be established. Previous studies have implicated both flavins and thiols in organic nitrate ester bioactivation. Thus, we examined the chemical interactions of flavins and thiols with organic nitrate esters as a means of determining the role these species may play in NO production. Based on these studies we concluded that a reasonable chemical mechanism for organic nitrate ester bioactivation involves reduction to the organic nitrite ester followed by conversion to a nitrosothiol. The release of NO from nitrosothiols can occur via a variety of processes including reaction with dihydroflavins and NADH.

Glyceryl trinitrate (GTN), also referred to as nitroglycerin, was introduced over 100 years ago as a therapeutic agent for the treatment of angina pectoris (Murrel, 1879). Several other organic compounds with the nitrate ester functional group (-O-NO₂) such as isosorbide dinitrate and pentaerythritol tetranitrate, have similar pharmacological properties and, like GTN, are often prescribed in the treatment of a variety of heart and vascular ailments (Katzung and Chatterjee, 1989). Organic nitrate esters are characterized by their ability to relax vascular smooth muscle and thus, lead to vasodilation and unloading of the heart. Although the clinical effects of organic nitrate esters have been known for over a century, the biochemical mechanism by which they elicit smooth muscle relaxation became known only in the late 1970s. Organic nitrate esters are reductively metabolized to release nitric oxide (NO) which, in turn, leads to the activation of guanylate cyclase, which catalyzes the conversion of GTP to cyclic GMP (Arnold et al., 1977; Ignarro et al., 1981a; Kukovetz et al., 1979). The increase in cyclic GMP levels then leads to smooth muscle relaxation (for reviews see Waldman and Murad, 1987; Ignarro, 1989; Schmidt et al., 1993; Murad, 1994). Thus, the activity of organic nitrate esters is a result of metabolism to NO (Chung and Fung, 1990; Feelisch and Kelm, 1991), which then can participate in the endogenous pathway by which endothelial cells use NO to maintain vascular tone (Michel and Smith, 1993). Although it is clear that reductive metabolism of organic nitrate esters to NO is responsible for their biological activity, the metabolic pathway and mechanism by which NO is generated from organic nitrate esters have yet to be established.

One of the most troublesome aspects of organic nitrate ester therapy is the fact that patients can become refractory to their effects. That is, repeated and prolonged administration of organic nitrate esters results in the development of tolerance. The development of tolerance is characterized by a decrease in NO production. Interestingly, it has also been found that a decrease in tissue thiol levels is associated with the development of tolerance (Forster et al., 1991; Needleman and Johnson, 1973). The fact that tolerance can be reversed with the administration of thiol compounds (Torresi et al., 1985) indicates a critical role for thiols in the metabolism of organic nitrate esters to NO (Elkayam, 1991). Although there is a lack of conclusive data defining the role of thiols in organic nitrate ester bioactivation, S-nitrosothiols (RSNO compounds) have long been implicated as possible intermediates in these processes (Ignarro et al., 1981b; Kurz et al., 1991).

Previous studies indicate that the bioactivation of organic nitrate esters to NO may be an enzymatic process, possibly involving the cytochrome P-450 system (Servent et al., 1989; Schroder, 1992) and/or the thiol requiring glutathione (GSH) S-transferase (Nigam et al., 1993; Nigam et al., 1996; Simon et al., 1996). Moreover, it has been reported that nitrate ester bioactivation can be mediated by a flavoprotein (McGuire et al., 1994). The implication that flavins and thiols are involved in nitrate ester bioactivation prompted us to examine the intimate chemistry between these species. Herein we report that, indeed, flavin mononucleotide (FMN) is capable of catalyzing the reduction of organic nitrate esters by NAD(P)H to reduced nitrogen species, which may be precursors to NO formation. We further found that thiols may participate in this chemistry and thus enhance the generation of NO. This type of chemistry may serve as a basis for understanding the role of both flavins and thiols in the bioactivation of organic nitrate esters.

Materials and Methods
Materials and Solutions. S-Nitroso-GSH (GSNO) was synthesized according to a previously reported method (Hart, 1985). GSH and n-butyl nitrate were

1 Abbreviations used are: FMN, flavin mononucleotide, fully oxidized; FMNH₂, dihydroflavinn mononucleotide, fully reduced; GSH, glutathione; GSNO, S-nitroso-GSH; GTN, glyceryl trinitrate/nitroglycerin; NO, nitric oxide; HNO, nitroxyl.
purchased from ICN Pharmaceuticals (Cleveland, OH). NADH and n-butyl nitrite were purchased from Aldrich Chemical Co. (Milwaukee, WI). Nitro-glycerin was a generous gift from Zeneca (Wilmington, DE). All solvents and reagents were obtained from commercial sources and were of the highest purity available. Authentic NO and N₂O gas were purchased from Matheson Gas Products (Cucamonga, CA). NO was passed through aqueous base before use to trap any contaminating nitrogen oxides. All solutions, unless specifically noted, were prepared in 100 mM, pH 7.4, potassium phosphate buffer containing 5 mM desferrioxamine and adjusted back to pH 7.4 with concentrated KOH or HCl. After the completion of reactions, the pH of the resultant solutions was determined and found not to differ by more than 0.2 pH units from the starting pH 7.4 in all cases.

**Analytical Techniques.** Analysis of n-butyl nitrate and n-butyl nitrite by HPLC. n-Butyl nitrate and n-butyl nitrite analysis was conducted on a Rainin HPLC system (Rainin Instrument Co., Woburn, MA) equipped with a 4.6 mm × 25 cm, 5-μm Beckman Ultrasphere reversed phase column (Beckman Instruments, Fullerton, CA), and a Spectra-Physics 100 UV-visible detector operating at 210 nm (Spectra-Physics Analytical, Mountain View, CA). The analytes were eluted using the following gradient: 0–7 min, 50% H₂O/50% acetonitrile to 100% acetonitrile at a flow rate of 1.5 ml/min. The retention times for n-butyl nitrate and n-butyl nitrite were 5.5 min and 6.2 min, respectively. Quantitation was accomplished by comparison with standard curves prepared with authentic n-butyl nitrate and n-butyl nitrite.

Analysis of inorganic nitrite. The method used in this study is a modification of a previously published method (Green et al., 1982). N-(1-Naphthyl)-ethylenediamine solution (0.1%, w/v) was prepared by the addition of 100 mg of N-(1-naphthyl)-ethylenediamine hydrochloride to 100 ml of deionized water. A 1% (w/v) sulfanilamide solution was prepared by the addition of 1 g of sulfanilamide to 5% H₃PO₄/distilled water solution. Shortly before analysis, the assay solution consisting of an equivalent volume mixture of both the N-(1-naphthyl)-ethylenediamine and 1% sulfanilamide solutions was prepared. A 200-μl aliquot (containing 20–200 nmol nitrite) of the sample to be analyzed was mixed with 1.8 ml of the assay solution. The samples were heated at 60°C for 1 min and then kept on ice. The absorbance at 546 nm of each sample was measured on a Beckman DU-30 UV/Vis spectrophotometer.

Comparisons of sample absorbances to those obtained from Greiss reaction mixtures with known nitrite concentrations were used for quantification.

**NO detection.** Possible evolution of NO from reaction solutions was determined using a method previously used in our laboratory (Wong et al., 1998). Briefly, through a 25-ml, three-neck flask equipped with a gas inlet adapter, a gas outlet adapter, and a septum sealed inlet was passed argon through a cold incoming glass stopcocks. Each flask contained 300 ml of 100 mM potassium phosphate buffer. The pH was adjusted to 7.4. Gas samples were collected by vacuum-nitrogen purge cycles at 0, 10, 30, 60, and 180 min, and the absorbance at 445 nm (λmax for FMN) and 710 nm (λmax for NADH) were monitored. Reaction of FMN/NADH (FMNH₂) with NO and GSNO.

**Reaction of FMN/NADH (FMNH₂) with GTN.** Two samples, each containing 1 ml each of 400 μM FMN and 4 mM NADH in 100 mM potassium phosphate buffer, pH 7.4, were placed into separate quartz cuvettes and stopped with rubber septa. The samples were deoxygenated on a vacuum line by several vacuum-nitrogen purge cycles. The samples were analyzed spectrophotometrically from 500 to 250 nm every 30 min. At 90 and 180 min, 100 μl of 1% GTN (44 mM) solution were added to the samples. The absorbances at 445 nm (λmax for FMN) and 710 nm (λmax for NADH) were monitored. Quantitation of FMN and NADH was then accomplished using a standard curve generated from authentic solutions of FMN and NADH.

**Reaction of FMN/NADH (FMNH₂) with GSH.** Two samples, each containing 1 ml each of 200 μM FMN and 2 mM NADH in 100 mM potassium phosphate buffer, pH 7.4, were placed into separate quartz cuvettes and stopped with rubber septa. The samples were deoxygenated on a vacuum line by several vacuum-nitrogen purge cycles. The samples were analyzed spectrophotometrically from 500 to 250 nm every 30 min. At 90 and 180 min, 100 μl of a 1% GTN (44 mM) solution were added to samples 1 and 2, respectively. The absorbances at 445 nm (λmax for FMN) and 710 nm (λmax for NADH) were used to determine FMN and NADH levels as described above.

**Reaction of FMN/NADH (FMNH₂) with NO and GSH.** Two samples, each containing 1 ml each of 400 μM FMN and 4 mM NADH in pH 7.4, 100 mM potassium phosphate buffer, pH 7.4, were placed into separate quartz cuvettes and stopped with rubber septa. The samples were deoxygenated on a vacuum line by several vacuum-nitrogen purge cycles. The samples were analyzed spectrophotometrically from 500 to 250 nm every 30 min. At 60, 120, and 180 min, 100 μl of pure NO gas (0.4 μmol) were added to the samples. The absorbance at 445 nm (λmax for FMN) was used to determine FMN levels using a standard curve of authentic FMN. The process was then repeated using successive 50-μl aliquots of 2 mM GSH.

**Reaction of n-butyl nitrate with FMN, NADH, and GSH.** Samples were prepared with either 200 μl of 75 mM FMN (15 μMol FMN), 100 μl of 200 mM (20 μmol) NADH, 1 ml of 200 mM GSH (100 μmol), or a combination of the reagents and diluted to 1.9 ml total volume with phosphate buffer solutions in 5 ml septum-stopped, pear-shaped flasks. The samples were deoxygenated on a vacuum line by several vacuum-nitrogen purge cycles. Then, 100 μl of 100 mM (10 μmol) of n-butyl nitrate in acetonitrile was injected into each sample and allowed to react for 2 h. The samples were then analyzed for n-butyl nitrate and n-butyl nitrite by HPLC using the procedures described above.

**Effect of NADH levels on n-butyl nitrate reduction.** Samples were prepared with 200 μl of 75 mM FMN (15 μMol) and 50, 100, or 250 μl of 200 mM (10, 20, or 50 μmol) NADH and buffer for a total of 1.9 ml. The samples were deoxygenated on a vacuum line by several vacuum-nitrogen purge cycles. A 100-μl aliquot of a 100 mM (10 μmol) n-butyl nitrate solution was then injected into each of the flasks. The samples were allowed to react for 2 h and then analyzed for n-butyl nitrate, n-butyl nitrite, inorganic nitrite, NO, and N₂O using the methods listed above. The experiment was repeated with the addition of GSH (100 mM) to all of the reaction solutions.

**Release of NO and N₂O from reaction of n-butyl nitrate, n-butyl nitrite, and inorganic nitrite with FMN/NADH.** Samples were prepared in 5-ml pear-shaped flasks equipped with glass stopcocks. Each flask contained 300 μl of 150 mM (45 μmol) FMN, 1.5 ml of 100 mM (150 μmol) NADH, and 1.1 ml of the potassium phosphate buffer, pH 7.4, solution (total volume 2.9 ml). The reaction vessels were then injected with 100 μl of 300-mM solutions of n-butyl nitrate in acetonitrile, n-butyl nitrite in acetonitrile, or sodium nitrite in buffer. The samples were allowed to react and headspace analyses for NO and N₂O were conducted at 30, 90, 180, and 360 min and at 24 h.

**Reaction of n-butylthiol with amylnitrite.** Amyl nitrite (134 μl, 1 mmol) was dissolved in 10 ml acetonitrile and sparged with nitrogen to remove oxygen in a 25-ml septum-capped, foil-covered Erlenmeyer flask. Then 113 μl of

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resulting in the NO detector reaction flask and NO evolution was monitored for 5 min. Then, 1 ml of 25 mM NADH solution was injected into the flask and NO evolution was followed for an additional 15 min.

The release of NO from GSNO and NADH and FMN was also examined using a slightly different procedure: An aliquot containing 1 ml of 50 mM GSNO and 50 mM FMN was injected into the NO detector reaction flask and NO evolution was monitored for 5 min. Then, 1 ml of 25 mM NADH solution of NADH was injected into the flask and NO evolution was followed for an additional 5 min.

Release NO and N<sub>2</sub>O from reaction of GSNO with NADH/FMN-24 h reaction. Samples containing 300 µl of 100 mM FMN solution, 1.5 ml of 100 mM NADH, or both were placed into 5-ml pear-shaped flasks equipped with glass stopcocks. The volume was adjusted to 2.7 ml with additional buffer. The samples were deoxygenated on a vacuum line by several vacuum-nitrogen purge cycles. Then an aliquot containing 300 µl of a 100-mM GSNO solution that had been deoxygenated by vacuum was injected via syringe into each of the samples. The samples were allowed to mix for 24 h. Headspace analysis for NO and N<sub>2</sub>O was conducted using the methods described above.

Arterial smooth muscle relaxation. The vasorelaxant activity of the model compounds used in this study was evaluated using an arterial smooth muscle preparation previously described (Fukuto et al., 1992). Briefly, a 2.5- to 3.5-kg New Zealand white male rabbit was sacrificed by lethal injection with pentobarbital (50 mg/kg). The thoracic aorta was carefully removed, cleared of fat and connective tissue, and cut into 3-mm rings. The rings were then mounted under 1 to 1.5 g of resting tension in 25-ml tissue baths in Krebs-bicarbonate solution (pH 7.4) at 37°C and challenged with phenylephrine. Changes in tension were measured isometrically on Grass Polygraphs after administration of n-butyl nitrate and n-butyl nitrite to the contracted tissues.

Results and Discussion

As mentioned previously, flavins have been implicated in the bioactivation of organic nitrate esters. This leads to the possibility that a reduced flavin species such as FMNH<sub>2</sub> may be interacting with the organic nitrate ester substrate directly. Thus, the direct chemical interaction between FMNH<sub>2</sub> and an organic nitrate ester was investigated.

Reaction of FMN with NADH, GSH and NADH + GSH. Figure 1 shows the time-dependent anaerobic reduction of FMN to FMNH<sub>2</sub> by NADH as measured by FMN loss. As expected, the FMN is reduced to the colorless FMNH<sub>2</sub> readily with the addition of excess (10 equivalents) NADH. This observation is in agreement with previous chemical model studies that have shown a direct transfer of a reduced flavin species such as FMNH<sub>2</sub> to FMNH<sub>2</sub> (data not shown). That is, GSNO was able to oxidize FMNH<sub>2</sub> as well (data not shown). The amount of FMNH<sub>2</sub> was rapidly oxidized, as indicated by the reformation of FMN (Fig. 1). GSNO and 50 mM FMN was injected into the NO detector reaction flask and NO evolution was monitored for 5 min. Then, 1 ml of 25-mM solution of NADH was injected into the flask and NO evolution was followed for an additional 5 min.

Reactivity of FMN with NADH and GTN. Figure 2 shows the conversion of FMN to FMNH<sub>2</sub> by NADH as measured by loss of FMN. Additionally, the loss of NADH, due to its oxidation to NAD<sup>+</sup>, is shown in Fig. 2. With the addition of GTN at 90 and 180 min, FMNH<sub>2</sub> was rapidly oxidized, as indicated by the reformation of FMN (Fig. 2). Also, NADH consumption in these experiments increased slightly upon the addition of GTN (Fig. 3). GTN alone was shown not to affect NADH levels (data not shown). Thus, these data indicate that FMNH<sub>2</sub> is capable of reacting with GTN resulting in oxidation of FMNH<sub>2</sub> to FMN and, presumably, GTN is subsequently reduced in the process. Similar results were seen upon addition of GSNO to the FMNH<sub>2</sub>, (data not shown). That is, GSNO was able to oxidize FMNH<sub>2</sub> with subsequent reduction of GSNO (to be discussed later). Also, NO was capable of oxidizing FMNH<sub>2</sub> as well (data not shown).

Reaction of n-butyl nitrate with FMN, NADH, and GSH. Figure 4 shows the amount of n-butyl nitrate remaining after its reaction with FMN, NADH, GSH, or a combination of the reagents. n-Butyl nitrate does not appear to react with any of the reagents alone. However, its level decreases greatly with the combination of FMN and NADH, indicating its reaction with a reduced flavin species. GSNO has no effect on n-butyl nitrate levels except in the presence of both FMN and NADH. The observation that n-butyl nitrate appears to react only with a reduced flavin species formed from the reaction of FMN and NADH, is similar to those seen in the reduction of aryl-nitroso compounds by reduced flavins and pyridine nucleotides (Leskovac et al., 1989). In that particular study, the 2-electron reduction of aryl-nitroso compounds to their corresponding N-hydroxy analog was investigated. The ability of pyridine nucleotides, such as NADH and reduced flavins, to perform this reaction was compared and it was found that although both NADH and reduced flavins were able to reduce the aryl-nitroso compounds, reduced flavins were much more efficient reductants. Thus, our results are consistent with these previous observations.

It is clear that the reduced flavin FMNH<sub>2</sub> is capable of reacting with organic nitrate esters as evidenced by both a loss of nitrate ester (Fig. 4) and a corresponding generation of FMN (Fig. 2). Furthermore, the presence of a thiol enhances this process of flavin catalyzed reduction of nitrate esters by NADH (Fig. 4).
reaction volume was 2 ml. At 90 and 180 min, 10 mM alcohol and nitrite (reaction 5), a reaction known to occur at a

Conceivable that nitrate esters can be reduced by 2 electrons to form a 1% GTN solution was added to anaerobic reaction mixtures at 90 and 180 min, the corresponding nitrite ester (reaction 4).

![Figure 2](image)

**Fig. 2.** Effect of GTN on conversion of FMN (100 μM initial concentration) to FMNH$_2$ by NADH (1 mM) under anaerobic conditions.

FMN levels were monitored spectrophotometrically at 445 nm. Ten microliters of a 1% GTN solution was added to the anaerobic reaction mixtures at 90 and 180 min, respectively (as indicated by arrows).

![Figure 3](image)

**Fig. 3.** Effect of GTN on conversion of NADH to NAD$^+$ in the presence of FMN.

Concentration of NADH (1 mM initial concentration) remaining after reaction with FMN (100 μM) was monitored spectrophotometrically at 710 nm. Total reaction volume was 2 ml. At 90 and 180 min, 10 μl of a 1% GTN solution was added to anaerobic reaction mixtures.

**Product Analysis of FMN-Catalyzed Reduction of Organic Nitrate Esters by NADH.** The reduction of organic nitrate esters can result in the generation of a variety of products. For example, it is conceivable that nitrate esters can be reduced by 2 electrons to form the corresponding nitrite ester (reaction 4).

\[
R-O-NO_2 + 2e^- + 2H^+ \Rightarrow R-O-NO + H_2O \quad (4)
\]

Hydrolysis of this species would then lead to the generation of the alcohol and nitrite (reaction 5), a reaction known to occur at a significant rate (k ~ 10$^5$ M$^{-1}$ s$^{-1}$) under conditions similar to those employed in these studies (Allen, 1952).

\[
R-O-NO + H_2O \Rightarrow ROH + NO_2^- + H^+ \quad (5)
\]

Certainly, reduction products other than those indicated above are possible (i.e., NO, N$_2$O, etc.). That is, further reduction of NO$_2^-$ or R-O-NO can lead to the formation of other reduced nitrogen species (mechanisms for these possibilities will be discussed later). Thus, to examine the possibility that reactions 4 and 5 (and others) were occurring, the products of the FMN-catalyzed reduction of the organic nitrate ester by NADH were examined. Thus, reactions were analyzed for formation of NO, N$_2$O, R-O-NO, and NO$_2^-$. Also, the effect of reducing agent (NADH) equivalents on the formation of these products was determined. Figure 5 graphically depicts the results. It appears that increasing amounts of NADH leads to both increasing loss of n-butyl nitrate and the formation of inorganic nitrite. Interestingly, inorganic nitrate (NO$_3^-$) and n-butyl nitrite were not detected. Moreover, NO levels were less than 1% of the starting n-butyl nitrate. At a high NADH/organic nitrate ester ratio, a small but significant amount of N$_2$O was formed. The effect of GSH on product formation from the FMN-catalyzed reduction of the organic nitrate ester by NADH was determined (Fig. 6). GSH clearly enhanced the formation of the reduction products NO$_2^-$ and N$_2$O compared with reactions performed in the absence of the thiol (Fig. 5).

The fact that the organic nitrite intermediate was not detected may indicate that the hydrolysis of the nitrite ester (reaction 5) is faster than the reduction of the organic nitrate ester to the nitrite ester (reaction 4). The observed enhancement of reduced nitrogen products by GSH can be explained by the previous observation indicating that GSH enhanced the reduction of FMN by NADH (Fig. 1). Thus GSH may enhance the rate of regeneration of the catalytically reactive species (reactions 2 and 3).

The generation of N$_2$O from these reactions requires the formation of a nitrogen-nitrogen bond. That is, two intermediate nitrogen species need to combine to form N$_2$O, because the starting nitrate esters contain only a single nitrogen atom. One established way to form a nitrogen-nitrogen bond is via dimerization of nitroxyl (HNO) to give initially hyponitrous acid that will then spontaneously dehydrate to give N$_2$O and water (reaction 6).

\[
2\text{HNO} \Rightarrow \text{HON} = \text{NOH} \Rightarrow \text{N}_2\text{O} + \text{H}_2\text{O} \quad (6)
\]

Thus, the detection of N$_2$O is often used as an indication of in situ HNO formation. The generation of HNO represents a formal 4-electron reduction of an organic nitrate ester (i.e., R-O-N$^{3-}$O$_2$ to HN$^{(+1)}$O). Because an organic nitrite ester or nitrite ion represents a 2-electron reduction of an organic nitrate ester (i.e., R-O-N$^{5-}$O$_2$ to R-O-N$^{3-}$O or $^{4-}$ON$^{(+3)}$O$_2$), it is possible that HNO could have formed as a result of reduction of any three of these species, R-O-NO$_2$, R-O-NO, or NO$_2^-$. Therefore, the generation of HNO (as detected by N$_2$O formation) was examined from reactions of all of these species. Also, possible NO generation was monitored as well.

**Formation of NO and N$_2$O by Reaction of n-Butyl Nitrate, n-Butyl Nitrite, and Inorganic Nitrite with FMN/NADH.** n-Butyl nitrate, n-butyl nitrite, and inorganic nitrite were reacted separately with both FMN and NADH. Headspace analysis was conducted at various time points to determine NO and N$_2$O production. NO levels in all three samples were fairly low (<1%) and decreased over time (data not shown). N$_2$O levels (Fig. 7), however, increased slowly with inorganic nitrite releasing the greatest amount of N$_2$O. This observation is consistent with the idea that N$_2$O is formed from the reduction of inorganic nitrite by FMNH$_2$. Thus, a plausible pathway for the

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generation of HNO in these systems is indicated in Scheme 1. One-electron reduction of the nitrate ester generates the nitroxide intermediate, which undergoes further 1-electron reduction to give a hydrated nitrite ester, which loses water to give the nitrite ester. Hydrolysis of the nitrite ester generates NO$_3^-$, which is then subject to further 2-electron reduction to HNO.

**Reaction of t-Butylthiol with Amyl Nitrite.** Herein, we have found that thiols (i.e., GSH) can enhance the reduction of organic nitrate esters, possibly by assisting in the generation of the FMNH$_2$. However, this clearly is not the only effect thiols can have in these reaction systems. Previous labs have proposed that thiols such as GSH may react with organic nitrite esters to the corresponding S-nitrosothiol which, in turn, serves as a precursor to NO formation (reaction 7) (Meyer et al., 1994; Ji et al., 1996).

$$\text{RONO} + \text{R'}\text{SH} \rightarrow \text{R'}\text{SNO} + \text{ROH}$$  \hspace{1cm} (7)

However, the rapid rate of hydrolysis of $n$-butyl nitrite and the probability that any RSNO formed would be reduced by FMNH$_2$ rapidly (described below) make reaction 7 difficult to monitor. Therefore, to test the feasibility of reaction 7, the reaction of a $t$-butyl thiol and amyl nitrite was examined in nonaqueous conditions. The reaction
of equimolar amounts of t-butyl thiol with amyl nitrite in acetonitrile produced the S-nitroso-t-butyl thiol with 100% conversion. This result shows that reaction 7 can occur and provides a possible link between an organic nitrate ester reduction product and S-nitroso thiol generation, which, as discussed later, readily releases NO under a variety of conditions. Significantly, previous studies have shown that GSH S-transferase can catalyze reaction 7 (Meyer et al., 1994; Ji et al., 1996).

These chemical studies provide strong evidence that organic nitrate esters can be reduced in the presence of pyridine nucleotides and flavins to the organic nitrite ester. This probable "first step" in the bioactivation of organic nitrate esters integrates the observation that flavoproteins are involved and answers the question why organic nitrates are more potent than nitrates. However, given the fact that organic nitrate esters are more stable to hydrolysis than nitrites (Allen, 1952; Baker and Easty, 1952), the role of the nitrate ester may be to act as a "shield" for the organic nitrite ester until it gets into a cell. After being formed, the nitrite ester may hydrolyze to give inorganic nitrite or react with GSH to form GSNO. This possibility integrates the idea that GSH S-transferases can be involved through their ability to catalyze S-nitrosothiol formation from endogenous GSH and organic nitrite esters. This utilization of GSH provides a mechanism by which thiol levels can be depleted. This occurrence may explain the role of thiols in the development of tolerance. Based upon these observations, a possible mechanism for the bioactivation of organic nitrate esters is summarized in Scheme 2 and described as follows: 1) Organic nitrate esters are taken up by smooth muscle cells. 2) After the nitrate ester diffuses into the cell, a flavoprotein, possibly membrane bound (Seth and Fung, 1993), reduces it into an organic nitrite ester. 3) The reaction between endogenous GSH and the organic nitrite ester to form GSNO is catalyzed by GSH S-transferase. 4) GSNO then releases NO by variety of mechanisms (described below).

Although the evidence presented in this and in previous studies (Ignarro et al., 1981b) indicate the involvement of S-nitrosothiols as active intermediates in the bioactivation of organic nitrate esters, the exact mechanism by which NO is released from them has yet to be fully elucidated. Previous studies have indicated that biological reducing agents such as ascorbate (Kashiba-Iwatsuki et al., 1996), thiols (Scorza et al., 1997), and metals (Singh et al., 1996) are capable of liberating NO from S-nitrosothiols. However, the role of pyridine nucleotides and flavins in this process has yet to be determined and was investigated in the following experiments.

**Formation of NO from Reaction of GSNO with FMN/NADH.**

Figure 8 shows the release of NO from GSNO upon the addition of NADH. NO release is proportional to the amount of NADH added. The presence of FMN in this system greatly enhances NO release. NO may be generated by two different mechanisms. When only NADH is present, the hydride from the pyridine nucleotide reacts with the S-nitrosothiol to afford a free thiol and HNO (reaction 8).

\[
\text{GSNO} + \text{NADH} \rightarrow \text{NAD}^+ + \text{GS}^- + \text{HNO}
\]  

It has been previously shown that HNO can liberate NO from GSNO presumably via initial attack of HNO on the electrophilic nitrogen of the nitrosothiol followed by expulsion of thiolate (Wong et al., 1998) (reaction 9).

\[
\text{GSNO} + \text{HNO} \rightarrow \text{GSN(OH)NO} \rightarrow \text{GSH} + 2\text{NO}
\]
by flavins can be a result of two possible reactions. FMNH$_2$ or its semiquinone intermediate may directly reduce GSNO to generate NO (reactions 10 and 11).

$$\text{GSNO} + \text{FMNH}_2 \rightarrow \text{GS}^- + \text{FMN}^- + \text{NO} + 2\text{H}^+ \tag{10}$$

$$\text{GSNO} + \text{FMN}^- \rightarrow \text{GS}^- + \text{FMN} + \text{NO} \tag{11}$$

Alternatively, the increase in NO production may be due to a reaction between HNO, generated via reaction 8 and FMN whereby HNO donates an electron to FMN (reaction 12).

$$\text{HNO} + \text{FMN} \rightarrow \text{NO} + \text{FMN}^- + \text{H}^+ \tag{12}$$

The oxidation of HNO to NO by FMN has been previously reported by us (Fukuto et al., 1993).

**Formation of NO and N$_2$O from Reaction GSNO with FMN and NADH (24 h).** Figure 9 shows the formation of NO and N$_2$O from the reaction of GSNO with NADH, FMN, or both NADH and FMN. Appreciable amounts of NO release occur only during the 5-min time point giving a final reaction volume of 2 ml.

The HNO produced can then dimerize to form nitrous oxide as previously mentioned.

Although these in vitro experiments clearly show that pyridine nucleotides and reduced flavin species are capable of liberating NO from GSNO, their actual contribution to the metabolism of GSNO to NO in vivo will require further investigation.

**Biological Activity of n-Butyl Nitrate and n-Butyl Nitrite.** Most clinically used organic nitrate esters (i.e., GTN, isosorbide dinitrate, etc.) contain multiple nitrate ester functions. Because the model compounds used in this study contain only one nitrate ester function, there is the possibility that significant physiological NO release requires multiple nitrate esters on the same molecule. Therefore, to address this issue and test the feasibility of n-butyl nitrate as an appropriate model compound, the ability of n-butyl nitrate and its organic nitrite ester analog, n-butyl nitrite, to elicit vasodilation in smooth muscle tissue was examined. In rabbit aorta challenged with phenylephrine, both n-butyl nitrate and n-butyl nitrite elicited vasorelaxation. The EC$_{50}$s of the compounds were $2.2 \times 10^{-3}$ and $7.8 \times 10^{-3}$ M, respectively. The biological assays with n-butyl nitrate and n-butyl nitrite indicate that both compounds do have vasodilatory properties, albeit not as potent as GTN itself (Kowaluk and Fung, 1991). The n-butyl nitrite ester used in our study does show increased potency over that of the nitrate, however, the difference is not as dramatic as seen in previous studies (Zimmermann et al., 1991). Nevertheless, the increased potency of the nitrite ester versus that of the nitrate ester is consistent with a possible role of the nitrite ester as an intermediate in bioactivation of organic nitrate esters.

**Summary.** Through the use of biological reducing agents, this study was able to determine a possible mechanism by which organic nitrate esters can be bioactivated to NO. Organic nitrate esters are readily reduced to organic nitrite esters in the presence of a reduced flavin species. GSH can then react with the organic nitrite ester to form an S-nitrosothiol, which has been shown to release NO through a variety of mechanisms. This chemical hypothesis thus reconciles previous studies that have implicated both flavoproteins and thiols in the bioactivation of organic nitrate esters to NO. It should be emphasized that the metabolic pathway described herein for the generation of NO from organic nitrate esters is likely to be only a minor physiological event. That is, the major metabolic fate of organic nitrate esters in vivo is not via pathways that will easily evolve NO but rather pathways that generate, for example, NO$_3^-$. However, because NO is an extremely potent vasorelaxant, even low metabolic yields of NO from organic nitrate esters allows these compounds to be therapeutically viable.

![Figure 8](image-url) Release of NO from reaction of either GSNO (50 μmol) or GSNO/FMN (50 μmol each) with 12.5, 25, and 50 μmol of NADH.

Reactions were carried out under anaerobic conditions. NADH was added at the 5-min time point giving a final reaction volume of 2 ml.

![Figure 9](image-url) Release of NO and N$_2$O from the reaction of 30 μmol of GSNO (10 mM) with either 150 μmol of NADH (50 mM), 30 μmol of FMN (10 mM), or both under anaerobic conditions after 24 h.

N$_2$O values were doubled to account for two nitrogen atoms in molecule.
References


