# Photochemical reactions of thiols with organic nitrates — Oxygen atom transfer via a thionitrate[1]

**Jennifer L. Clarke, Irida Kastrati, Linda J. Johnston, and Gregory R.J. Thatcher**

**Abstract:** Nitroglycerin is an organic nitrate that has been used in the clinical treatment of angina for 130 years, yet important details of its mechanism of action remain unanswered. The biological activity of nitrates suggests that they are bioactivated to NO via a three-electron reduction. The involvement of free or bound protein thiols in this reduction has often been proposed. To examine the involvement of thiyl radicals in such a process, the photochemical generation of benzenethiyl radical from thiol and disulfide precursors was studied in the presence of isopropyl nitrate. Analysis of reaction products and kinetics led to the conclusion that photolysis of the nitrate to $NO_2$ dominated the observed photochemistry. Formation of sulfonothioate and NO as products, and trapping of $NO_2$ by 4-chlorophenol, indicated a mechanism involving oxygen atom transfer from N to S via a thionitrate intermediate. The results of the study did not indicate a rapid reaction between thiyl radical and organic nitrate. Despite weak nitrate absorption of light >300 nm and a relatively high BDE for homolysis to give $NO_2$, the photochemistry under thiyl-generating conditions was driven by nitrate photolysis to $NO_2$. A novel nitrate, containing a phenyl disulfanyl group linked to nitrate groups, did not undergo photolysis to $NO_2$ or generate sulfonothioate, but did yield NO. These observations suggest that reaction between thiyl radicals and nitrates leading to NO release is a viable pathway, but it is subservient to other competing reactions, such as photolysis, in the case of IPN, and reaction with thiolate, in the case of the novel nitrate.

*Key words:* nitrate, photolysis, thiyl radical, nitrogen dioxide, nitric oxide.

**Résumé :** Même si la nitroglycérine est un nitrate organique qui a été utilisé depuis 130 ans dans le traitement de l'angine, beaucoup de détails importants de son mode d'action restent sans réponse. L'activité biologique des nitrates suggère qu'ils sont biologiquement activés en NO par le biais d'une réduction à trois électrons. L'implication de groupes thiols de protéines libres ou liées dans cette réduction a été souvent proposée. Afin d'examiner l'implication des radicaux thiyles dans ce processus, on a étudié la génération photochimique du radical benzènethiyle à partir de précurseurs thiols et disulfures, en présence de nitrate d'isopropyle. L'analyse de la cinétique et des produits de la réaction conduit à la conclusion que la photolyse du nitrate en $NO_2$ domine la photochimie observée. La formation de sulfonothioate et de NO comme produits et le piégeage du $NO_2$ par du 4-chlorophénol suggèrent un mécanisme impliquant le transfert d'un atome d'oxygène du N vers le S par le biais d'un intermédiaire thionitrate. Les résultats de cette étude indiquent qu'il n'y a pas de réaction rapide entre le radical thiyle et le nitrate organique. Malgré la faible absorption de la lumière par le nitrate à des valeurs supérieures à 300 nm et une valeur relativement élevée du « BDE » pour l'homolyse conduisant au $NO_2$, la photochimie dans les conditions conduisant à la formation de thiyle est dominée par la photolyse du nitrate en $NO_2$. Un nouveau nitrate contenant du groupe phényl disulfanyle lié à des groupes nitrates conduit à du NO, mais ne donne pas lieu à une photolyse conduisant au $NO_2$ ou à la génération d'un sulfonothionate. Ces observations suggèrent que la réaction entre les radicaux thiyles et les nitrates est une voie viable pour la production de NO, mais elle est soumise à d'autres réactions de compétition, dont la photolyse dans le cas du nitrate d'isopropyle et d'une réaction avec le thiolate dans le cas du nouveau nitrate.

*Mots clés :* nitrate, photolyse, radical thiyl, bioxyde d'azote, oxyde nitrique.

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*This work is dedicated to Walter A. Szarek in celebration of his 65th birthday and his continuing contributions to medicinal and carbohydrate chemistry.*

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## Introduction

The synthesis of nitroglycerin (glyceryl trinitrate, GTN) was first reported in 1846. In 1878, it was demonstrated that small doses of GTN taken sublingually provided rapid and remarkable relief from the pain of angina. This led to clinical use of GTN in the treatment of angina pectoris that has continued to this day (1, 2). GTN is now applied in controlled hypotension during cardiac surgery and in congestive heart failure, whereas other organic nitrate vasodilators, including isosorbide dinitrate (ISDN), are used in the treatment of angina pectoris. ISDN, in a combination therapy, is entering the clinic for prevention of heart failure (3). Hybrid nitrates that conjugate a nitrate group to an established drug via a labile linker have been the subject of numerous preclinical and clinical studies (4), and novel nitrates are being explored beyond traditional cardiovascular applications (5). However, despite the historical and growing importance of nitrates in disease therapy, their mechanism of action is not well understood. One prevailing theory is that nitrates function as NO prodrugs, requiring bioactivation (or mechanism-based biotransformation) of the nitrate moiety ($RONO_2$) to NO (6, 7).

Nitrate esters (or organic nitrates) contain the nitrooxy functional group (-$ONO_2$); conversion of the nitrooxy group to NO is a three-electron reduction that must involve oxygen atom transfer. To date, no purified protein system has been demonstrated to mediate the direct reduction of nitrates to yield relevant quantities of NO, although this is widely held to be the biologically active product of nitrate bioactivation (8, 9). The biotransformation of GTN yields the dinitrate metabolites, glyceryl-1,2-dinitrate (1,2-GDN) and glyceryl-1,3-dinitrate (1,3-GDN), as products. Several proteins have been identified that are capable of mediating the denitration of GTN, yielding GDN and $NO_2^-$ as products, including hemoglobin, myoglobin, xanthine oxidoreductase, old yellow enzyme, glutathione *S*-transferase (GST), cytochrome P450 oxidase, and cytochrome P450 reductase (10–17). More recently, it was confirmed that ALDH2 (aldehyde dehydrogenase) was capable of mediating denitration of classical nitrates (18–20).

The vascular effects of nitrates are believed to be mediated primarily via activation of soluble guanylyl cyclase (sGC), which converts GTP to the secondary messenger, cGMP. sGC is activated by binding of NO to its ferrous-heme centre, inducing a conformational change that triggers activation (21, 22). However, in the context of nitrates and GTN, there is a disparity between sGC activity in intact tissue vs. both broken cell preparations and purified protein: only in intact tissue is GTN a potent sGC activator. In contrast, GTN and simple organic nitrates are incapable of activating sGC above basal levels, in vitro, in the absence of thiol adjuvants (23–25). GTN does react with cysteine in simple aqueous buffer to release NO, but the reaction is slow and does not yield a large flux of NO (26). Nevertheless, the reaction of an organic nitrate with a thiol, whether a free thiol or protein thiol such as in GST or ALDH2, does provide the simplest reaction leading to nitrate bioactivation. Study of the reaction of nitrates with thiols, and their oxidation and reduction products, is therefore important for understanding the mechanism of nitrate biotransformation and bioactivation.

The putative bioactivation of organic nitrate to NO is a three-electron reduction. If bioactivation is mediated by a thiol, of the three potential reducing agents, thiol, thiolate, and disulfide radical anion, the latter is clearly the best candidate simply based on reduction potentials (27).

$$[1a] \quad RS^{\cdot} + e^- \rightarrow RS^- \qquad E^\circ = 0.78 \text{ V}$$

$$[1b] \quad RS^{\cdot} + e^- + H^+ \rightarrow RSH \qquad E^\circ = 1.35 \text{ V}$$

$$[1c] \quad RSSR + e^- \rightarrow (RSSR)^{\cdot -} \qquad E^\circ = -1.42 \text{ V}$$

The reactions of nitrates with disulfide radical anions are worthy of study in this respect. Disulfide radical anions can be formed by the reversible combination of thiyl radical with thiolate (eq. [2]) and by reduction of disulfides (eq. [1*c*]).

$$[2] \quad RS^{\cdot} + RS^- \underset{k_{-f}}{\overset{k_f}{\rightleftharpoons}} (RSSR)^{\cdot -}$$

It is apparent that thiyl radicals are likely to be present in any system designed to generate a disulfide radical anion. It is also possible to draw a mechanism of reaction for nitrate with thiyl leading to NO.

$$[3] \quad R'S^{\cdot} + RONO_2 \rightarrow [RON(O)OSR']^{\cdot} \rightarrow R'SO^{\cdot} + RONO$$

$$[4] \quad RONO + 1e^- + H^+ \rightarrow ROH + NO$$

Therefore, it is essential to explore the reactivity of thiyl radicals themselves with nitrates. However, the clean generation of thiyl radicals (and disulfide radical anions) presents a challenge since there is no ready thermal source as has been described for other biological radicals, such as superoxide (28). Conversely, a number of photochemical systems for thiyl radical generation have been described. In this paper, thiyl radicals generated from disulfide and thiol photochemical precursors are studied in the presence of nitrates.

## Experimental section

All reagents were supplied by Sigma-Aldrich (Oakville, Ontario), with the exception of GTN and GT-130 that were synthesized by literature methods (29). All solvents used were HPLC grade. NO was supplied by Praxair Products Inc. (Mississauga, Ontario) and was used with a NO specific regulator. Phosphate buffer (PB) (0.1 mol/L, pH 7.4) was adjusted to pH and microfiltered before use.

All $^1$H NMR analyses were done on a Bruker Avance-400 (400 MHz) spectrometer. A Varian Cary 3 UV–vis diode array spectrometer was used, with 1 cm × 1 cm quartz cells. Kinetic measurements were performed on a HP 8452A diode array spectrophotometer at 40 °C. Chemiluminescence measurements were made using a SIEVERS 270B NO chemiluminescence analyzer and a SIEVERS 207B nitric oxide analyzer (Boulder, Colorado). The UV light sources used were 300, 350, and mixed 300/350 nm Rayonet reactors equipped with lamps from the Southern New England Ultraviolet Company (Brantford, Connecticut).

The lasers used in the laser flash photolysis experiments were an EX-530 Excimer-500 (Lumonics, Inc.), an UV-24 $N_2$ laser (Laser Photonics), and a HY 750 ytrium–aluminum–garnet (YAG) laser (Lumonics, Inc.). The detec-

tor consisted of a Digikrom 240 monochromator (CVI Instruments) with a six dynode photomultiplier tube. The oscilloscope used was a Tektronix 7912 AD programmable digitizer and the delay generator was a DG535 four-channel digital delay/pulse generator (Stanford Research Systems).

All gas chromatography (GC) work was done on a Hewlett Packard 5890 series II gas chromatograph with flame ionization detection. The column used was a HP-1 with dimensions 25 m × 0.2 mm × 0.33 μm, column pressure 20 psi He (1 psi = 6.894 757 kPa), and splitflow 30 cc/min. One microliter injection volumes were made by an autoinjector using a 10 μL syringe that was cleaned with acetonitrile (MeCN). The GC–MS used was a HP 5890 gas chromatograph with a HP 5970 series mass selective detector. The MS library used was the Wiley138 database. One microliter manual injections were made. The column used was a HP-5 with dimensions 25 m × 0.2 mm × 0.33 μm, column pressure 10 psi He, and splitflow 30 cc/min. The optimal GC method for analyzing the thiol-nitrate samples used an injection port temperature of 200 °C, a detector temperature of 300 °C, and an initial oven temperature of 50 °C with a gradient of 10 °C/min to a final temperature of 250 °C. This temperature was maintained for an additional 9 min.

All high-pressure liquid chromatography (HPLC) analyses were done at room temperature on Hewlett Packard series II 1090 liquid chromatographs using diode array spectrophotometers (HPIB 15). An autosampler was used with 25 μL injections. Both C8 and C18 reverse-phase columns were used. The C8 column was a Waters Nova-Pak HPLC column, with a 4 μm diameter and dimensions of 3.9 mm × 150 mm. The C18 column was a Waters Spherisorb ODS2 column with a diameter of 5 μm and dimensions of 4.6 mm × 250 mm. For all experiments, the eluant was a mixture of methanol (MeOH) and Millipore-filtered water, with a flow rate of 1 mL/min. Three HPLC methods were used, where A is $H_2O$ and B is MeOH. Method 1 used a gradient from 35% to 95% B over 16 min, followed by a plateau at 95% B for 6 min and gradient to 35% B over 2 min. Method 2 used a gradient from 35% to 95% B over 30 min, followed by a 5 min plateau. Products from the PhSH/IPN photoreaction were detected using HPLC Method 2 and the C18 column. HPLC Method 2 and the C8 column were used for the GT-130 photochemical and thermal product analysis. Detection of GDNs from GTN electron capture was done using Method 1 with the C18 column.

Benzenethiyl radical was generated by irradiation of benzenethiol (1) (PhSH, 10 mmol/L), diphenyl disulfide (PhSSPh, 4 or 1 mmol/L), or GT-130 (1 mmol/L) in the presence of 100 mmol/L 2,3-dimethyl-1,3-butadiene in oxygen- or nitrogen-saturated acetonitrile (MeCN/$N_2$) using either a 300 nm Rayonet reactor (arrangement of UV lamps) or a laser flash photolysis setup, respectively. Decane (3 mmol/L, anhydr.) was used as a standard for GC-FID analysis of the Rayonet reactions. GC–MS was first used to identify the 1,4- and 1,2-addition products, the identity of which was further confirmed by $^1$H NMR analysis and quantified by GC-FID.

Benzenethiyl radical was also generated in the presence of isopropyl nitrate (IPN, 100 mmol/L or 1.0 mol/L) by irradiating PhSH (10 mmol/L or 100 mmol/L) or PhSSPh (5 mmol/L) in nitrogen-saturated acetonitrile (MeCN–$N_2$) using 300 and 350 nm Rayonets, respectively. All experiments were done in duplicate. Reactions were analyzed by GC-FID using 3 mmol/L anhydrous decane as standard. Relative peak areas from the GC traces were calibrated and converted to concentration plots using GC calibration curves. These calibration curves were obtained using authentic PhSH, PhSSPh, and $PhSO_2SPh$. For $PhSO_2SPh$, a photodegradation calibration was necessary because it is photolabile and breaks down during the course of the experiment. The use of a photodegradation calibration run under identical conditions to the experiment provided an estimated correction. The calibrated concentrations were transformed to molar "% conversion" relative to the starting concentration of PhSH.

IPN (0.5 mol/L) with and without PhSH (10 mmol/L) in MeCN–$N_2$, GTN (5 mmol/L), and GT-130 (5 mmol/L) in MeCN–PB–$N_2$ (40:60), were each irradiated using Rayonets with 300 and (or) 350 nm lamps, and NO release was quantified using a NO chemiluminescence (CL) detector. CL was calibrated by the use of the diazeniumdiolate NO donor, SPE–NO: initial rates data were collected for NO release over 10 min and calibrated by comparison to the first-order rate constant obtained by spectrophotometric analysis of SPE–NO decay.

Solutions of 10 mmol/L PhSH, 1.0 mol/L IPN, and varying concentrations of 0–100 mmol/L 4-chlorophenol in MeCN–$N_2$ were irradiated for 0 and 10 min in a 300 nm Rayonet reactor. To determine the extent of photolysis at longer wavelength, solutions of 5 mmol/L PhSSPh, 1.0 mol/L IPN, and 0–100 mmol/L *p*-chlorophenol were deoxygenated with nitrogen and irradiated for 0 and 45 min in a 350 nm Rayonet reactor. Anhydrous decane was used as a standard in both experiments and product analysis was done by GC-FID.

Nitric oxide gas was purified according to the method of Bostrup et al. (30). Using this purification setup, three 25 mL portions of MeCN were each saturated with argon. One was flushed with purified NO for 25 min (until saturated) and another with unpurified NO. A stock solution was made with 20 mmol/L PhSH and 20 mmol/L decane standard. One milliliter of this solution was saturated with nitrogen and diluted with 1 mL of either (*i*) argon-saturated, (*ii*) purified NO-saturated, or (*iii*) unpurified NO-saturated MeCN, giving final concentrations of 10 mmol/L PhSH and 10 mmol/L decane. Another stock solution was made with 60 mmol/L *p*-chlorophenol and 20 mmol/L decane. This solution was deoxygenated with nitrogen and diluted with the satd. MeCN solutions similar to the above method. The resulting concentrations were 30 mmol/L and 10 mmol/L *p*-chlorophenol and decane, respectively. All samples were analyzed by GC-FID with decane as a standard.

## Theoretical methods

Geometry optimizations were performed with density functional theory using the (U)B3LYP function (31) with the 6-311++G(d,p) basis set implemented in the Gaussian 03 suite of programs (32). Calculation of structure and energy of the excited state of IPN was carried out in Spartan 04 (Wavefunction, Inc.) using TDDFT at the B3LYP/6-31G* level. For calculation of the thermodynamics of homolysis, minima were fully optimized and characterized by harmonic

vibrational frequency analysis, followed by further optimization and energy evaluation using the complete basis set (CBS-QB3) methodology, known to provide thermochemical estimates approaching experimental accuracy. Energy minimum structures were confirmed to have adequate convergence and zero imaginary vibrational frequencies. For the radical species, spin determinant calculations were evaluated to check for spin contamination.

## Results

The photochemical sources of thiyl radicals used were photolysis of the RS—H bond (eq. [5]) and photolysis of the S—S bond (eq. [6]) of the corresponding disulfide (33). Hydrogen abstraction from RSH by photochemically generated *tert*-butoxyl radicals (*t*-BuO$^{\cdot}$) is an alternative route (eq. [7]) that is complicated by the potential side reactions of the alkoxyl radical in solutions containing nitrates.

[5] $$RSH \xrightarrow{h\nu} RS^{\cdot} + {}^{\cdot}H$$

[6] $$RSSR \xrightarrow{h\nu} 2RS^{\cdot}$$

[7] $$t\text{-BuO–O-}t\text{-Bu} \xrightarrow{h\nu} t\text{-BuO}^{\cdot} \xrightarrow{RSH} RS^{\cdot} + t\text{-BuOH}$$

A restriction to these methods is the chromophore of the source, which is often weak. Alkyl thiyl radicals typically absorb at 330 nm, which is similar to the optical spectra of their sources, and have low extinction coefficients ($\varepsilon \sim 500$ (mol/L)$^{-1}$ cm$^{-1}$) (27). They are therefore difficult to detect and monitor using photochemical techniques (27, 34). Generation of aryl thiyl radicals is more facile owing to higher wavelength chromophores with higher extinction coefficients ($\varepsilon \sim 2500$ at 460 nm and 10 000 (mol/L)$^{-1}$ cm$^{-1}$ at 295 nm) (27). Consequently, aryl thiols and aryl disulfides are preferred as thiyl radical sources. A relatively clean method of generating thiyl radicals was found by irradiation in MeCN using a YAG laser ($\lambda = 355$ nm). Benzenethiyl radical (PhS$^{\cdot}$) was generated from the disulfide or thiol precursor and characterized by laser flash photolysis (LFP). The PhS$^{\cdot}$ spectrum obtained displayed the well-characterized absorbance maxima at 490 and 450 nm.

The thiyl radical was further characterized by measuring the kinetics of conjugate addition to 2,3-dimethyl-1,3-butadiene (DMB). Under anaerobic conditions, thiyl is known to add to olefinic bonds in anti-Markovnikov fashion (27), where the 1,4-adduct is expected to be the major product in the case of DMB (35). LFP was used to measure the addition rates of PhS$^{\cdot}$ to DMB in the presence and absence of oxygen. The rate constants thus derived for the addition of PhS$^{\cdot}$ to DMB under aerobic and anaerobic conditions were found to be $1.3 \times 10^8$ and $0.83 \times 10^8$ (mol/L)$^{-1}$ s$^{-1}$, respectively. These experiments validate and quantify the formation of thiyl radical, the rate of addition to the diene, and the utility of the diene trap in measuring thiyl formation.

### Generation of PhS$^{\cdot}$ in the presence of IPN — An intermolecular model

To facilitate product monitoring, photolysis of PhSH in the presence of DMB was carried out using a Rayonet photoreactor fitted with 300 nm lamps. In the absence of DMB, the only product observed by GC-FID analysis was disulfide. However, with excess quantities of DMB present, the yield of disulfide was reduced and both the 1,4- and 1,2-adducts were observed by GC-FID and $^1$H NMR analysis, with the major product being that of conjugate addition. Thermal generation of benzenethiyl at 95 °C and subsequent addition to DMB has previously been investigated, where the yield of the 1,4-adduct was found to be 98% (35). DMB is therefore an effective thiyl trap as predicted by the rate constants determined by LFP analysis. Addition of IPN to this reaction system led to a decrease in the yield of addition product in a concentration dependent manner (data not shown), suggesting that IPN inhibited reaction of thiyl radical with the conjugated diene.

To examine whether a reaction between benzenethiyl and IPN was responsible for the observed reduction in formation of the 1,4-DMB adduct, PhSH (10 mmol/L) was irradiated in the presence of IPN (1.0 mol/L) alone. A new product was observed in the GC-FID chromatogram, which was identified as a sulfonothioate (*S*-phenyl benzenesulfonothioate; $RS(O)_2SR$, R = Ph) by GC–MS and by comparison to an authentic sample. Formation of this product increased with irradiation time (Fig. 1); however, the sulfonothioate is a photolabile compound and its quantity is therefore underestimated by this assessment, especially at longer irradiation times. As a result, the sulfonothioate product stability was measured by GC-FID analysis as a function of irradiation time under the reaction conditions. This enabled construction of a corrected calibration curve that was used to more accurately estimate sulfonothioate formation (Fig. 1).

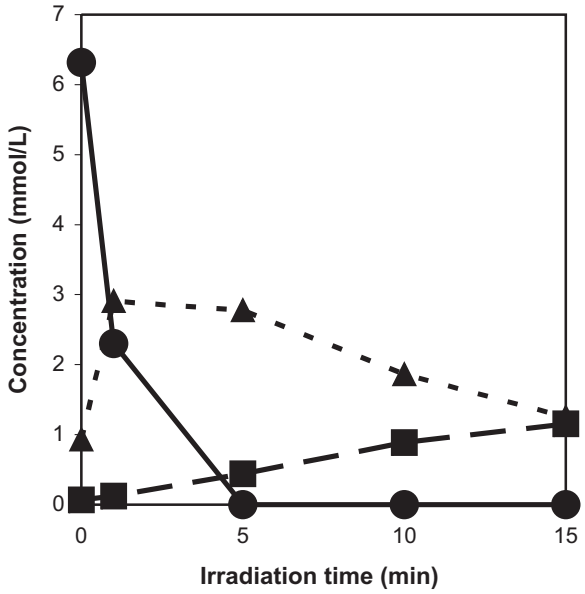
It is evident from Fig. 1 that in addition to a decrease in PhSH with irradiation time, a decrease in the disulfide product is also observed. The disulfide absorbs light at 300 nm and is cleaved into thiyl radicals, though requiring longer irradiation times than the corresponding thiol. This is compatible with the increase in sulfonothioate product observed at irradiation times where the PhSH source has been depleted. The sulfonate product therefore results from reaction of PhS$^{\cdot}$ formed from photolysis of both the thiol and the disulfide.

PhS$^{\cdot}$ was also generated from 5 mmol/L PhSSPh using LFP. A second-order fit to the decay trace (monitored at 460 nm) gave a rate constant for radical recombination of $2 \times 10^7$ (mol/L)$^{-1}$ s$^{-1}$. Addition of IPN had no effect on the decay rate of the radical, even when 80% IPN – 20% MeCN was used as the solvent, indicating that any reaction with IPN cannot compete with radical recombination. This effect was consistent under aerobic and anaerobic conditions, and questions the viability of a direct and rapid reaction between thiyl radical and nitrate.

### Sulfonothioate as a product of $NO_2$ reaction

The sulfonothioate product could potentially result from direct reaction between benzenethiyl radical and $NO_2$, since $NO_2$ is the expected product of IPN photolysis. Smaller amounts of sulfonothioate were observed in photochemical experiments where longer wavelength irradiation was used: reaction of PhS$^{\cdot}$ with IPN was performed using a 350 nm lamp and PhSSPh as the thiyl precursor. As shown in Fig. 2, much smaller amounts of sulfonothioate were detected, in comparison to the irradiation of PhSH and IPN at 300 nm. It should be noted that at $\lambda = 300$ nm, the relative molar

**Fig. 1.** Left: Decay of reactant (●) and growth of products (thiosulfonate (■) and disulfide (▲)) from irradiation of PhSH (10 mmol/L) in the presence of IPN (1.0 mol/L) in MeCN–$N_2$ using a 300 nm lamp, as assayed by GC-FID calibrated using authentic samples (average of two trials). Right: Identical reactant and product data incorporating a correction for the photochemical decomposition of the products under the reaction conditions.



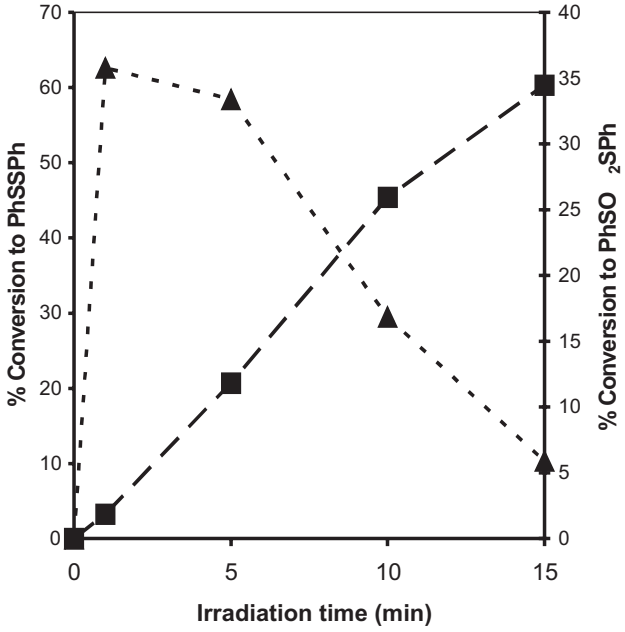
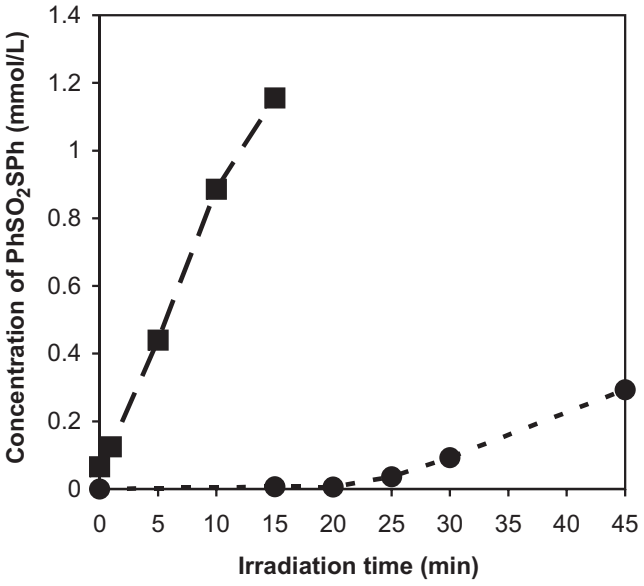




**Fig. 2.** Comparison of $PhS(O)_2SPh$ yield from IPN (1.0 mol/L) in MeCN–$N_2$. Irradiation of PhSH (10 mmol/L) at 300 nm (■); irradiation of PhSSPh (5 mmol/L) at 350 nm (●). Average of two trials.




absorbance of PhSH to IPN is approximately 10:1, whereas at 350 nm, the absorbance of IPN is lower by a factor of 40. Thus, it is expected that less $NO_2$, and consequently less sulfonate, would be generated at 350 nm as a result of decreased light absorption and photolysis of the nitrate. Evidence for thermal decomposition of IPN to $NO_2$ was seen in careful inspection of Fig. 1, which shows sulfonothioate product present at time zero. Sulfonothioate was formed owing to thermolysis in the high-temperature GC injection port (200 °C). To examine the photolysis of IPN, a $NO_2$ trapping experiment was designed using 4-chlorophenol. Photonitration of arenes has been known for many years and recent work has been published reporting the formation of 2- and 4-nitrophenol resulting from irradiation of nitrate or nitrite ion in the presence of phenol in aqueous solution (36, 37). The ortho-nitration of tyrosine is a well-known reaction (38), and the use of 4-chlorophenol has the advantage of limiting nitration to one isomeric product. Generation of 4-chloro-2-nitrophenol was shown to result from irradiation of 1.0 mol/L IPN in the presence of 10 mmol/L *p*-chlorophenol, where the product identity was confirmed by GC–MS analysis.

Increasing concentrations of 4-chlorophenol were added to irradiated solutions of IPN with PhSH or PhSSPh to compete with $PhS^{\cdot}$ for any $NO_2$ generated by nitrate photolysis (Fig. 3). The decrease in relative peak area of 4-chlorophenol was the same as the increase observed in the relative peak area of 4-chloro-2-nitrophenol (data not shown), indicating that nitration was efficient and was the only reaction occurring with 4-chlorophenol. The observation of 4-chloro-2-nitrophenol formation confirmed the generation of $NO_2$ on irradiation, while nonirradiated solutions gave no nitration products. Clearly, $NO_2$ is generated by irradiation of 1.0 mol/L IPN at 300 nm and to a lesser extent at 350 nm. It also appears from Fig. 3 that the amount of 4-chloro-2-nitrophenol formed reaches a plateau. A simple rationale would be the lower yield of $PhS^{\cdot}$ and of $NO_2$ owing to competitive absorbance by 4-chlorophenol and 2-nitro-4-chlorophenol in the reaction solution. At 100 mmol/L 4-chlorophenol, $A$(300 nm) = 2.4 and $A$(350 nm) = 0.079; whereas for PhSH (10 mmol/L), $A$(300 nm) = 0.85; for PhSSPh (5 mmol/L), $A$(350 nm) = 0.75; and for IPN (1.0 mmol/L), $A$(300 nm) = 3.4 and $A$(350 nm) = 0.087.

Both the nitration of 4-chlorophenol and the formation of sulfonothioate appeared to result from the photolysis of IPN to $NO_2$. Commercial gaseous NO provides a further means

**Fig. 3.** Formation of 4-chloro-2-nitrophenol from irradiation of IPN (1.0 mol/L) in the presence of (top) 10 mmol/L PhSH and *p*-chlorophenol using a 300 nm lamp and (bottom) 5 mmol/L PhSSPh and *p*-chlorophenol using a 350 nm lamp. Averages of two trials.

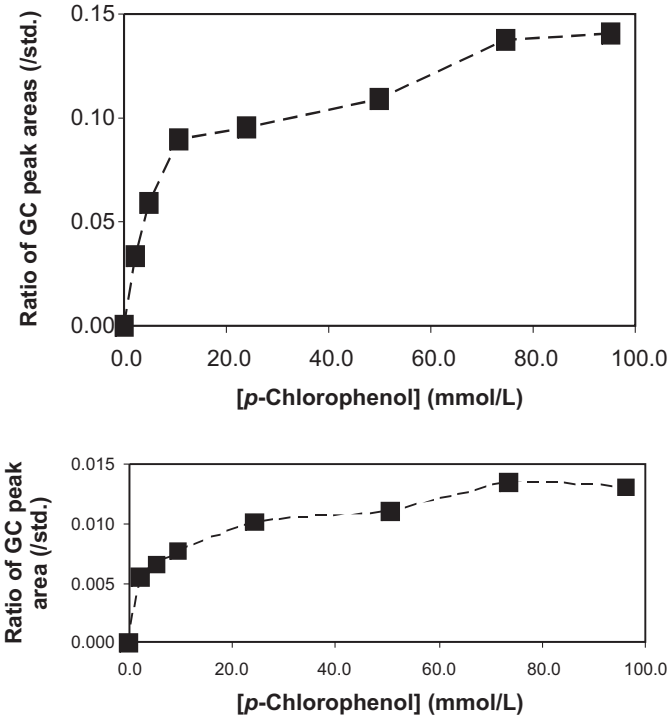
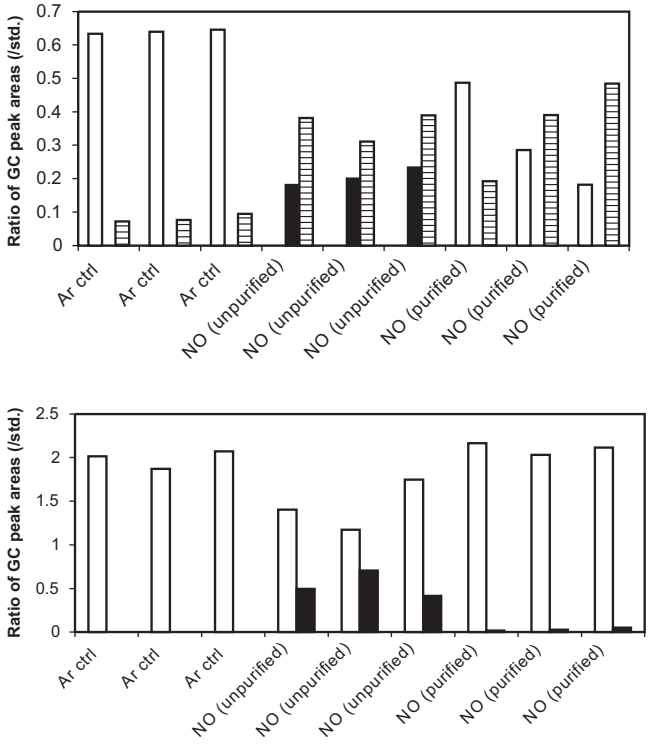


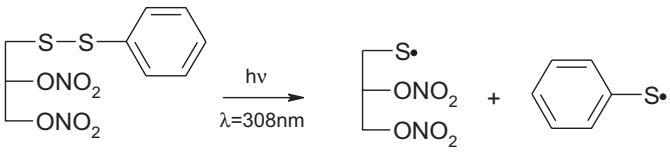

**Fig. 4.** Quantitation of products from reaction of NO gas with PhSH (10 mmol/L) in MeCN–$N_2$ by GC-FID. Argon, unpurified NO–$NO_2$ gas, or purified NO gas passed through alkaline scrubbers was bubbled through the reaction solution at comparable rates. Top: reactant PhSH (open bar), $PhS(O)_2SPh$ (solid bar), and PhSSPh (hashed bar) were measured. Bottom: the same reactions were carried out in the presence of 4-chlorophenol (open bar) and product 4-chloro-2-nitrophenol (solid bar) was measured. Triplicate reactions shown.




to test this hypothesis because the unscrubbed gas contains $NO_2$ as the major impurity. Nitration of 4-chlorophenol was measured in solutions of PhSH in MeCN–$N_2$ treated with gas streams of either argon, the unpurified NO–$NO_2$, or NO purified by passage through alkaline scrubbers (Fig. 4, bottom). Only in the samples treated with NO–$NO_2$ was significant nitration observed, indicating that this system is useful for studying reactions of $NO_2$ in the presence of other nitrogen oxide species and that 4-chlorophenol is an effective $NO_2$ trap. In parallel experiments, unreacted thiol, disulfide, and sulfonothioate were measured by GC-FID analysis in solutions of PhSH in MeCN–$N_2$ treated with gas streams of either argon, unpurified NO–$NO_2$, or purified NO. Treatment with purified NO resulted in significant oxidation of thiol to disulfide, but no sulfonothioate formation. In contrast, treatment with NO–$NO_2$ gas led to complete depletion of thiol, yielding both disulfide and sulfonothioate (Fig. 4, top). The nitration of 4-chlorophenol in PhSH solution flushed with NO–$NO_2$ was also monitored in a UV–vis spectrometer at 350 nm in which 4-chloro-2-nitrophenol was observed to form immediately upon flushing with the gas (data not shown).

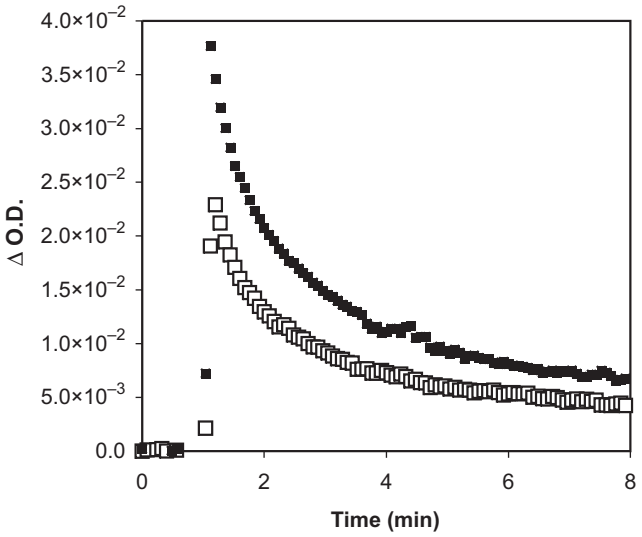
### An intramolecular model for thiyl-nitrate interactions — GT-130

The phenyldisulfanyl dinitrate (GT-130) contains both disulfide and nitrate moieties, and is therefore a suitable candidate for studying the likelihood of thiyl radical reactivity with nitrates owing to the proximity of the two reactive groups. In the presence of DMB and under identical conditions to those used for the study of PhSH–IPN, irradiation of GT-130 resulted in formation of the same 1,4-thiyl-DMB adduct, verifying that $PhS^{\bullet}$ was produced and therefore confirming photolysis of the S—S bond.




Using an Excimer laser (308 nm) to excite GT-130, $PhS^{\bullet}$ was again identified by LFP analysis of its characteristic spectrum and shown to be a product of GT-130 photolysis. The decay curves of $PhS^{\bullet}$ generated by photolysis of GT-130 and $Ph_2S_2$ at equal concentrations are shown in Fig. 5. As expected, the change in optical density from $Ph_2S_2$ photolysis was approximately twice that from GT-130 photolysis, since twice as many $PhS^{\bullet}$ radicals would result from photolysis of the S—S bond. The decay rate constants measured for each curve were second order and were found to be similar, indicating that the recombination pathway for the GT-130 radicals has similar kinetics to the radical–radi-

**Fig. 5.** Decay curves of $PhS^{\cdot}$ monitored at 450 nm by LFP. Formed by irradiation of 1 mmol/L GT-130 (□) or 1 mmol/L PhSSPh (■) in MeCN–$N_2$ using an Excimer laser (308 nm).




cal recombination of $PhS^{\cdot}$ forming PhSSPh. This suggests that the $PhS^{\cdot}$ radical formed via photolysis undergoes recombination to GT-130 with a rate similar to that of self-combination to PhSSPh.

To explore possible nitrate photolysis of GT-130, irradiation was performed at 300 nm in the presence of the 4-chlorophenol trap. Nitration was not observed (data not shown), indicating that $NO_2$ either was not formed or was trapped more rapidly by another species. A reasonable explanation is the low absorbance associated with the nitrate groups in GT-130 (5 mmol/L), which is substantially less than in experiments with IPN (1.0 mol/L). Disulfide cleavage is therefore the dominant route of photolysis upon irradiation of GT-130 at 5 mmol/L.

## NO release from reaction of GT-130

The thermal reaction of GT-130 with thiols has been reported to result in production of NO (29). The photolysis of GT-130 was compared with that of GTN by chemiluminescence detection of NO in headspace gas of the reaction mixtures (Fig. 6). Small, submicromolar quantities of NO were observed from GTN photolysis, but GT-130 photolysis generated more than 20 μmol/L NO after 20 min irradiation. However, comparison of NO release from thermal reaction of GT-130 with cysteine vs. photochemical reaction of GT-130 showed that the thermal reaction is more efficient in generating NO (29). Irradiation of GT-130 under aerobic or anaerobic conditions exhibited no change in the NO response (data not shown).

Interestingly, irradiated solutions of PhSH–IPN in MeCN–$N_2$ also generated NO, with a linear rate of NO release for at least the first 20 min of reaction (Fig. 7). As shown, photolysis of IPN alone did not produce NO within the detection limits.

Once it was established that irradiation of GT-130 resulted in efficient S—S bond cleavage, the nature of the resulting organic products was investigated. The products resulting from thermal reaction of GT-130 with PhSH in neutral aqueous solution were analyzed by HPLC with UV detection and

**Fig. 6.** Comparison of cumulative NO response. Irradiation of 5 mmol/L GTN (○) and 5 mmol/L GT-130 (■) in MeCN–PB–$N_2$ (40:60) using mixed 300 and 350 nm lamps (average of three trials).

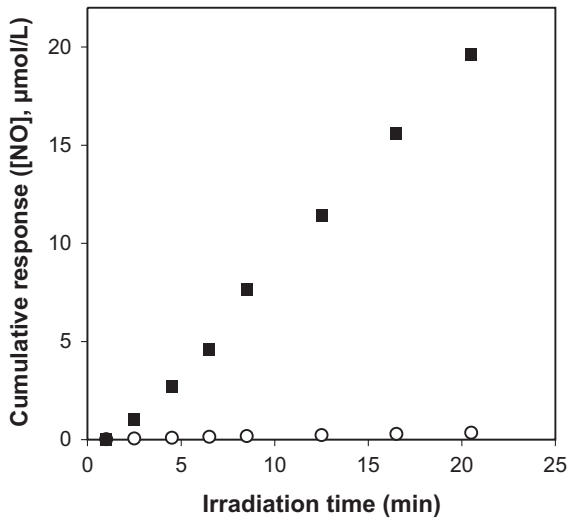
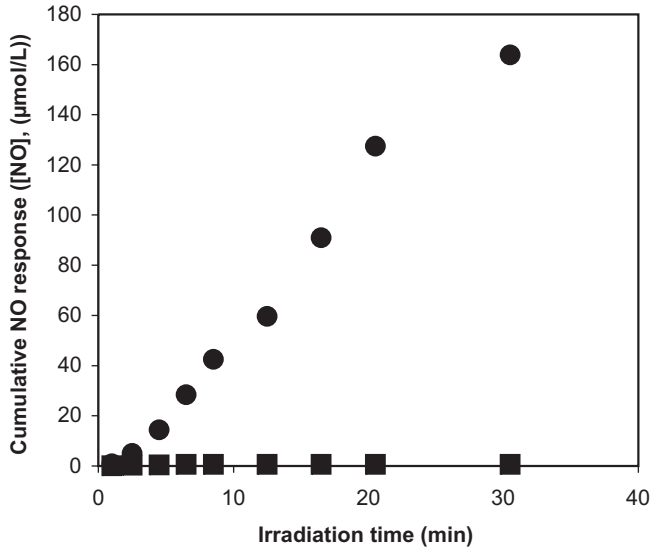
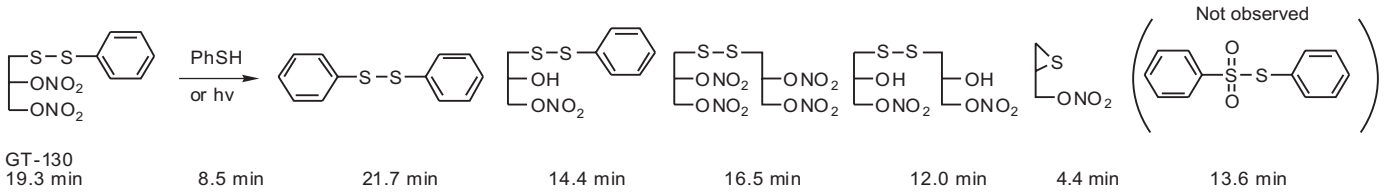



**Fig. 7.** Comparison of NO response. Irradiation of 10 mmol/L PhSH + 0.5 mol/L IPN (●) vs. irradiation of 0.5 mol/L IPN (■) in MeCN–$N_2$ (average of three trials).




have been reported previously (29). The major products were identified by comparison of HPLC chromatograms with authentic samples, and were found to be PhSSPh and 2-denitro-GT-130, with an equal amount of unreacted GT-130 present (Scheme 1). Of numerous minor products, the cyclic thiirane was the most abundant and the symmetrical disulfide 1,2-di(3-nitrooxy-2-hydroxypropyl)disulfane was also identified (Scheme 1). The products identified in the photolysis of GT-130 (excluding the starting material) were, in order of abundance, diphenyldisulfide > 2-denitro-GT-130 > 1,2-di(2,3-dinitrooxypropyl)disulfane > 3-nitrooxypropylthiirane > 1,2-di(3-nitrooxy-2-hydroxypropyl)disulfane (Scheme 1). In the context of this work, it is important to note that no $PhS(O)_2SPh$ was detected in either the thermochemical or photochemical reactions of GT-130. In

**Scheme 1.** HPLC retention times are annotated under the conditions described in the text.




contrast, HPLC analysis of reaction mixtures of PhSH and IPN did reveal sulfonothioate after irradiation.

Irradiation of GT-130 for 20 min at 300 nm was sufficient to identify the photoproducts. Further experiments revealed no change in photochemical products from nitrogen-deoxygenated to air-equilibrated solutions. It can therefore be confirmed that oxygen has no effect on the photochemical reaction of GT-130 leading to the products shown.

## Discussion

The release of NO from organic nitrates in complex biological systems can often be observed using a variety of different methods. In human plasma, NO release was observed from solutions of nitrates and thiosalicylic acid using amperometric detection (39). In plasma, the series of 1,2-dinitrooxy propane derivatives including GTN were all observed to release NO in the presence of TSA with an initial rate of ~4–80 nmol/L $s^{-1}$ at 0.25–0.5 mmol/L nitrate concentration. At physiological pH, the reactivity of free thiols towards nitrates is dependent on thiol p$K_a$ and is too slow to account for the rapid bioactivation of GTN; for example, only 10% reaction of GTN (1 mmol/L) with cysteine (2 mmol/L) is observed in aqueous solution at pH 7.4, 37 °C, after 1 h (26). Using earlier electrochemical devices, this reaction was not found to produce NO within the detection limits, while chemiluminescence was able to detect the low levels of NO produced in these mixtures (26, 40). Nevertheless, catalysis of the reaction between cysteine thiol and nitrate at an enzyme active site or at other protein sites containing reactive thiols, such as thioredoxin folds, may lead to acceleration of reaction and an increased flux of NO (6). The most popular, contemporary theory for GTN bioactivation invokes ALDH2, which contains three cysteines at the active site (41, 42). ALDH2 and several other proteins are capable of accelerating reductive denitration of nitrates to inorganic nitrite (eq. [8]), however, conversion to NO requires further reduction.

[8] $RONO_2 + 2e^- + 2H^+ \rightarrow ROH + NO_2^-$

[9] $NO_2^- + e^- + H^+ \rightarrow HO^- + NO$

A chemical mechanism that identified an organic thionitrate ($RSNO_2$) as the common intermediate in a mechanism leading to either $NO_2^-$ or NO from the reaction of thiol with nitrate was first described by Yeates (43). Later computational and experimental approaches to this reaction yielded a cohesive thionitrate rearrangement pathway for a thiol-nitrate reaction leading to NO release (eqs. [10]–[13]) (44, 45). In an investigation of the aqueous decomposition of *tert*-butyl thionitrate, the only organic products observed were sulfonothioate and sulfinothioate, which were proposed to result from radical combination based on computational studies (eqs. [14] and [15]). The lack of disulfide product formed from reaction of pure samples of thionitrate indicated that rxns. [16]–[18] (and thiyl radical recombination) were not competitive under the reaction conditions.

[10] $RSH + R'ONO_2 \rightarrow RSNO_2 + R'OH$

[11] $RSNO_2 \rightarrow [RS^{\cdot}NO_2^{\cdot}]$

[12] $[RS^{\cdot}NO_2^{\cdot}] \rightarrow RSONO$ or $RS(O)NO$

[13] $RSONO \rightarrow RSO^{\cdot} + NO$

[14] $2RSO^{\cdot} \rightarrow RS(O)_2SR$

[15] $RSO^{\cdot} + RS^{\cdot} \rightarrow RS(O)SR$

[16] $[RS^{\cdot}NO_2^{\cdot}] \rightarrow RS^{\cdot} + NO_2^{\cdot}$

[17] $RS^- + NO_2^{\cdot} \rightarrow RS^{\cdot} + NO_2^-$

[18] $RS^- + RSNO_2 \rightarrow RSSR + NO_2^-$

GT-150 (2,3-dinitrooxypropane-1-thiol) contains a thiol group *vicinal* to a nitrate group. This compound was shown to spontaneously generate NO in neutral aqueous solution, as was the related disulfanyl nitrate GT-130 on addition of thiol (Scheme 1) (29), with no evidence being obtained for sulfonyl or sulfinyl products (S=O containing). Reaction of the disulfanylnitrates was unaffected by $O_2$, and other reductants studied did not facilitate NO release. The simplest mechanism, compatible with these observations was proposed to be a thiol-disulfide exchange.

[19] $RSSCH_2CHRONO_2 + R'S^- \rightarrow RSSR' + {}^-SCH_2CHRONO_2$

[20] ${}^-SCH_2CHRONO_2 \rightarrow \rightarrow NO$

A sequence of reactions postulated to account for NO production in the absence of S=O containing products proceeds via a sulfenate ($RSO^-$) and a nitrosothiol (RSNO) intermediate (eqs. [21]–[24]). The spontaneous release of NO from nitrosothiols in the presence of thiol is a well-studied phenomenon (46, 47).

[21] $RS^- + R'ONO_2 \rightarrow RSO^- + R'ONO$

[22] $RSO^- + RSH + H^+ \rightarrow RSSR + H_2O$

[23] $R'ONO + RSH \rightarrow RSNO + R'OH$

[24] $RSNO \rightarrow RS^{\cdot} + NO$

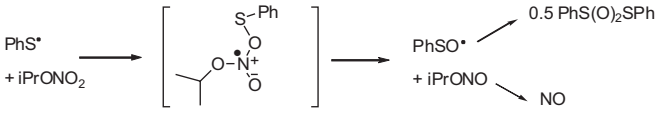
Alternative mechanisms were considered, including an inner-sphere electron-transfer process via an intermediate in thiol-disulfide exchange or a disulfide radical anion. Since

the overall reaction is a $3e^-$ reduction, radicals in addition to NO must be involved or released at some stage of the reaction. In many such reaction mechanisms a thiyl radical is a by-product.

To initiate studies into the reactivity of S-centred radicals with nitrates, thiyl radical, the simplest of such radicals, was studied using photochemical systems to generate the radical in the presence of a simple aliphatic nitrate (IPN). The $PhS^{\bullet}$ radical was generated by photolysis of benzene thiol or the disulfide and characterized by LFP analysis of its characteristic spectrum and decay rates in the presence of DMB. The conjugate addition of the thiyl radical to the DMB diene was also used as a measure of the ability of the nitrate to react with thiyl radical and inhibit the addition reaction. Significant inhibition of the formation of addition product from reaction of $PhS^{\bullet}$ with DMB was only observed at high concentrations of the nitrate ([IPN] = 1.0 mol/L). The reaction of the benzene thiyl radical with IPN at this high concentration was studied by identifying the reaction products and quantifying formation as a function of irradiation time. In addition to the expected disulfide product of thiyl radical combination, the sulfonothioate ($PhS(O)_2SPh$), was observed. The observation of this product under the anaerobic reaction conditions demonstrated that the photochemical reaction of thiyl with nitrate involved O atom transfer from nitrogen to sulfur.

Direct reaction of the electrophilic thiyl radical with the HOMO of the nitrate (localized on the nonbonding orbitals of the terminal oxygens) would yield a radical intermediate leading to O atom transfer (eq. [25]). Homolysis of this intermediate yields sulfinyl radical and organic nitrite, with subsequent sulfinyl radical combination leading to the observed sulfonothioate product. An important feature of this mechanism is that the organic nitrite product is a known source of NO (48).

[25]




However, a sulfonothioate is also the major product of the thermal decomposition of *tert*-butyl thionitrate (*t*-$BuSNO_2$) in neutral aqueous solution at room temperature (44), hinting at the formation of $PhSNO_2$ as an intermediate in the photochemical reaction of $PhS^{\bullet}$ with IPN. The simplest route to thionitrate is by the rapid radical combination of $NO_2$ with thiyl radical.

[26] $PhS^{\bullet} + NO_2^{\bullet} \rightarrow PhSNO_2$

[27] $PhSNO_2 \rightarrow PhSONO \rightarrow PhSO + NO$

[28] $2PhSO \rightarrow 0.5PhS(O)_2SPh$

Evidence against the direct reaction of thiyl with nitrate was seen in the photochemical generation of $PhS^{\bullet}$ from irradiation of PhSSPh at 350 nm in which sulfonothioate formation was greatly reduced from the 35% yield observed on irradiation of PhSH at 300 nm. This observation is compatible with thionitrate formation from $NO_2$ formed by the initial photolysis of IPN, which would be greatly reduced owing to the negligible absorbance of organic nitrates at 350 nm compared with 300 nm.

Nitration of 4-chlorophenol was used to confirm the nitrate photolysis pathway. The nitration of phenols by $NO_2$, involving oxidation and radical addition, is a well-known reaction that leads to tyrosine nitration in vivo. 4-Chlorophenol was used as a $NO_2$ trap, where formation of 4-chloro-2-nitrophenol revealed release of $NO_2$ upon irradiation of PhSH–IPN at 300 nm. In contrast, significantly less $NO_2$ was observed from irradiation of PhSSPh–IPN at 350 nm, compatible with the very weak absorbance of nitrates at higher wavelength.

The use of the 4-chlorophenol trap was validated by observation of nitration by unpurified NO gas containing $NO_2$ impurity, whereas purified NO gave almost no nitration product. Treatment of a PhSH solution with NO–$NO_2$ gave substantial formation of $PhS(O)_2SPh$, which was not observed with purified NO gas, supporting photolysis of IPN to $NO_2$ as the source of sulfonothioate. The thermal experiments with the gaseous NO–$NO_2$ source confirm that reaction of $NO_2$ with thiyl radical (eq. [26]) competes both with thiol oxidation and with radical combination with NO (eqs. [29]–[31]), which is in accord with literature rate data.

[29] $RSH + NO_2 \rightarrow RS^{\bullet} + HNO_2$

[30] $RS^{\bullet} + NO_2 \rightarrow RSNO_2$

[31] $NO + NO_2 \rightarrow N_2O_3$

In addition to photolysis to $NO_2$, the irradiation of PhSH–IPN solutions generated a reasonable flux of NO, with the presence of thiol being a requirement. This novel observation is entirely compatible with the rearrangement of the thionitrate intermediate to yield NO (eq. [27]), which we have previously reported in the context of the thermal decomposition of a thionitrate (44).

A large part of the early work on organic nitrate reactivity, including photochemistry, is part of the impressive ouevre of Canadian carbohydrate chemists, and published in this journal (49, 50). From these and other studies, it is seen that various benzyl nitrates are photolabile, whereas simple aliphatic nitrates require extended reaction times to observe photolysis products (51, 52). Work by Csizmadia and Hayward investigating the photolysis of hydrobenzoin and acenaphthenediol dinitrates in benzene found the predominant process to be photolysis to $NO_2$ (49, 50). This was validated by the detection of $NO_2$ as a reaction product using ESR spectroscopy (49). There is no evidence for direct photolysis leading to NO.

Calculation of the thermodynamics for homolysis of IPN reveals the BDE and $\Delta G$ of reaction to be 43.7 and 31.6 kcal/mol, respectively, (1 cal = 4.184 J) employing CBS-QB3//B3LYP methodology. The structure of the first excited state was calculated and the energy shown to be 54 kcal/mol above the ground state (Table 1). The calculated BDE for a nitrate is substantially larger than that for homolysis of the S—N bond of a nitrosothiol as shown by comparison of the thermochemical data calculated for $MeONO_2$ and MeSNO, which reveals a BDE for the nitrate greater by 11 kcal/mol. Nitrosothiols are well-known to be labile to photolysis to NO. Nitrosothiol photolysis is facili-

**Table 1.** Calculated homolysis thermodynamics for IPN compared with methyl nitrate and thionitrite.

| | Energy (kcal/mol) | | |
|---|---|---|---|
| Isodesmic reaction | $E^{o a}$ | BDE | $\Delta G$ |
| *i*-$PrONO_2$ → *i*-PrO + $NO_2$ | 44.0 | 43.7 | 31.6 |
| $MeONO_2$ → MeO + $NO_2$ | 42.0 | 43.2 | 30.6 |
| MeSNO → MeS + NO | 31.0 | 31.9 | 20.9 |

**Note:** Calculated using CBS-QB3//B3LYP/6-311++G**.
[a]Total energy including scaled zero-point energy.

tated by significant absorbance and a $\lambda_{max}$ above 300 nm, in contrast to nitrates.

The disulfanyl dinitrate (GT-130) might be anticipated to undergo competitive photolysis to yield either PhS$^{\cdot}$ or $NO_2$. Good evidence for efficient thiyl radical formation was obtained, but no evidence for formation of $NO_2$ was found. This novel nitrate, containing a phenyl disulfanyl group linked to an organic nitrate, introduces the possibility of an intramolecular reaction between thiyl radical and the nitrate functionality. Photolysis of GT-130 (5 mmol/L) was observed to give a reasonable rate of NO flux, but the rate was lower than from the thermal reaction of GT-130 with thiol.

Taken together, the results of this study provide no support for a rapid reaction between thiyl radical and organic nitrate. Despite weak absorption of light >300 nm and a relatively high BDE for homolysis, the dominant photochemistry under conditions required to generate thiyl radical was driven by nitrate photolysis to $NO_2$. The observation of both sulfonothioate and NO as products of reaction is readily explained by the formation of a thionitrate intermediate and provides further evidence that thionitrates are a source of NO. A novel nitrate containing a phenyl disulfanyl group did not undergo photolysis to $NO_2$, nor generate sulfonothioate, but did yield NO. The observations with the novel nitrate suggest that reaction between thiyl radicals and nitrates leading to NO release is a viable pathway, but is subservient to other competing reactions, such as photolysis in the case of IPN and reaction with thiolate in the case of the novel nitrate.

## Acknowledgements

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