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N-Nitroso Products from the Reaction of Indoles with Angeli's Salt

Fabienne Peyrot,[†] *Bernadette O. Fernandez,*[‡] *Nathan S. Bryan,*[‡] *Martin Feelisch,*[‡]**

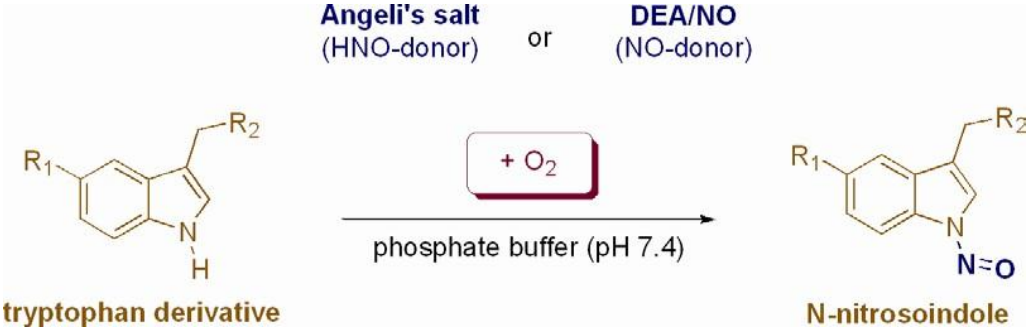
and Claire Ducrocq[†]**

Institut de Chimie des Substances Naturelles, C. N. R. S., Avenue de la Terrasse, F-91198, Gif-sur-Yvette, France, and Boston University School of Medicine, Whitaker Cardiovascular Institute, 650 Albany Street, Boston, MA 02118, USA

AUTHOR EMAIL ADDRESS *Claire.Ducrocq@icsn.cnrs-gif.fr* or *feelisch@bu.edu*

TITLE RUNNING HEAD Nitrosation of indolic compounds by Angeli's salt

TOC GRAPHIC


Angeli's salt
(HNO-donor)
or
DEA/NO
(NO-donor)
R1
R2
N
H
+ O2
phosphate buffer (pH 7.4)
R1
R2
N
N=O
tryptophan derivative
N-nitrosoindole

ABSTRACT

While nitroxyl (HNO) has been shown to engage in oxidation and hydroxylation reactions, little is known about its nitrosating potential. We therefore sought to investigate the kinetics of formation and identity of the reaction products of the classical nitroxyl donor Angeli's salt (AS) with three representative tryptophan derivates (melatonin, indol-3-acetic acid, and N-acetyl-L-tryptophan) *in vitro*. In the presence of oxygen and at physiological pH, we find that the major products generated are the corresponding N-nitrosoindoles with negligible formation of oxidation and nitration products. A direct comparison of the effects of AS, nitrite, peroxynitrite, aqueous $NO^{\bullet}$ solution and the NO-donor DEA/NO toward melatonin revealed that nitrite does not participate in the reaction and that peroxynitrite is not an intermediate. Rather, N-nitrosoindole formation appears to proceed via a mechanism that involves electrophilic attack of nitroxyl on the indole nitrogen, followed by a reaction of the intermediary hydroxylamine derivative with oxygen. Further *in vivo* experiments demonstrated that AS exhibits a unique nitrosation signature which differs from that of DEA/NO inasmuch as substantial amounts of a mercury-resistant nitroso species are generated in the heart whereas S-nitrosothiols are the major reaction products in plasma. These data are consistent with the notion that the generation of nitroxyl *in vivo* gives rise to formation of nitrosative post-translational protein modifications in the form of either S- or N-nitroso products, depending on the redox environment. It is intriguing to speculate that the particular efficiency of nitroxyl to form N-nitroso species in the heart may account for the positive inotropic effects observed with AS earlier.

KEYWORDS: nitrosation, nitroxyl (NO^-/HNO), indole, α-oxyhyponitrite, nitroside anion, tryptophan derivatives, nitration

FOOTNOTES

* Address correspondence to either *Claire.Ducrocq@icsn.cnrs-gif.fr*, Tel: (+33) 1 69 82 30 05, Fax: (+33) 1 69 07 72 47, or *feelisch@bu.edu*, Tel: (+1) 617-414 8150, Fax: (+1) 617-414 8151.

† Institut de Chimie des Substances Naturelles.

‡ Boston University School of Medicine.

[1] Abbreviations: RNOS, reactive nitrogen oxide species; NOS, nitric oxide synthase; NHE, normal hydrogen electrode; AS, Angeli's salt; DEA/NO, 2-(N,N-diethylamino)diazen-1-ium-1,2-diolate, diethylammonium salt; MelH, melatonin; DTPA, diethylenetriamine pentaacetic acid; MelNO, 1-nitrosomelatonin; ε, molar extinction coefficient; RSNO, S-nitrosothiols; RNNO, N-nitrosamines; DAF, 4,5-diaminofluorescein.

INTRODUCTION

Nitric oxide ($NO^{\bullet}$) has emerged as a major biological mediator in the cardiovascular, immune, and nervous system since the discovery of its biosynthesis two decades ago. Direct effects of $NO^{\bullet}$ are often attributed to its interaction with iron-heme-containing molecular targets, whereas indirect $NO^{\bullet}$ effects arise via intermediate formation of reactive nitrogen oxide species (RNOS[1]; e.g. $NO_2^{\bullet}$, $ONOO^-$, $ONOO^{\bullet}$, and N_2O_3) secondary to the reaction of $NO^{\bullet}$ with oxygen or oxygen-derived radicals *(1)*. Such RNOS have the ability to entertain nitrosative chemistry the outcome of which can affect protein structure and function. RNOS are generally more reactive than $NO^{\bullet}$ itself and can be divided into oxidizing, nitrating, and nitrosating agents. $NO^{\bullet}$ reacts with superoxide anion ($O_2^{\bullet -}$) at diffusion controlled rates yielding the potent oxidizing and nitrating species, peroxynitrite ($ONOO^-$, pK_a = 6.8) *(2)*. The nitrosating $NO^{\bullet}$-derived species (e.g., $ONOO^{\bullet}$, N_2O_3) are thought to be trapped by thiols to yield S-nitroso compounds, species believed to play a role in $NO^{\bullet}$ transport and storage *(3)*. Nitrite (NO_2^-), the end-product of $NO^{\bullet}$ autoxidation *(4)* and ubiquitous degradation product of $NO^{\bullet}$ under aqueous conditions *(5, 6)*, and nitrate (NO_3^-), the stable end-product of $NO^{\bullet}$'s reaction with oxygenated hemeproteins, both participate in $NO^{\bullet}$ elimination reactions.

In contrast to the aforementioned higher oxidation products such as N_2O_3 and peroxynitrite, the reactivity of the one-electron reduction product of $NO^{\bullet}$, nitroxyl (NO^-/HNO), towards organic biomolecules is largely unknown. This redox sibling of $NO^{\bullet}$ has recently been shown to have unique and promising pharmacological properties *(7)*. HNO and $NO^{\bullet}$ often induce discrete, orthogonal (i.e., of the same origin but not overlapping) reactions with heme centers as well as other biological targets that are highly dependent on reaction conditions *(8-10)*. Nitroxyl has been demonstrated to arise as a product of L-arginine oxidation by nitric oxide synthase (NOS)

under certain conditions *(11-15),* oxidation of N-hydroxyguanidines *(16),* thiolate-mediated decomposition of S-nitrosothiols *(17)* and as a possible intermediate of $NO^{\bullet}$ metabolism *(14),* although direct evidence for its formation *in vivo* is lacking. Recent evaluation of the physico-chemical characteristics of NO^{-}/HNO warrants exploration of the biological role of nitroxyl in order to complement the wealth of information on the physiological significance of $NO^{\bullet}$. The acid-base equilibrium of nitroxyl has been reevaluated, and the pK_a for HNO is now suggested to exceed 11 *(18, 19)* as opposed to 4.7 as determined by Grätzel and coworkers *(20).* Thus at physiological pH, HNO is the likely exclusive form of nitroxyl present in biological systems. This distinction is important since the reactivities of the protonated and unprotonated forms of nitroxyl vary substantially. The chemistry of HNO is primarily electrophilic in nature, whereas the anion NO^{-} is mainly involved in redox chemistry via outer-sphere electron transfer reactions *(1).* The redox potential of $NO^{\bullet}$, determined to be < -0.7 V (vs. NHE) *(18, 19),* makes electron transfer difficult to achieve and renders $NO^{\bullet}$ virtually inert to reduction within cells *(1).* Despite these thermodynamic hurdles, however, recent interest in nitroxyl has surged with possibilities of *in vivo* reduction of $NO^{\bullet}$ by superoxide dismutase *(21),* reactions with ferrous hemeproteins such as ferrocytochrome *c (22),* and the possibility that it may be formed via reaction of thiols with S-nitrosothiols *(17).*

Angeli's salt (AS; $Na_2N_2O_3$), a well known nitroxyl donor, has shown a large variety of biological effects. Similar to $NO^{\bullet}$, nitroxyl offers both protective *(7, 23)* as well as proinflammatory and cytotoxic effects *(24, 25),* depending on dose or concentration, and reaction conditions. In addition, it is a potent thiol oxidant *(25).* Whereas higher concentrations of AS have been shown to induce DNA double-strand breaks and base oxidation along with other oxidative damage *(15, 26),* more moderate pharmacological doses induce positive cardiac

inotropy and selective venodilation *in vivo (27).* AS is known to induce relaxation of vascular smooth muscle *in vitro* and to lower systemic blood pressure *in vivo*, effects which may be associated with the formation of iron-nitrosyl complexes *(28, 29).* Acute toxicity is often attributed to reductive nitrosylation of transition metals *(30, 31)*, thiol modification *(32)*, and to hydroxylation of aromatic compounds *(24).* While it has been observed that organ protection may occur after AS administration, there is no unifying mechanism to account for such an effect. Taken together, these studies indicate that while HNO is an integral component of the redox biology of $NO^{\bullet}$, its physiological chemistry is not well understood.

When dissolved in water, AS is in equilibrium with its conjugate acid ($HN_2O_3^-$; Eq. 1), the pK_a values of which have been reported to be 2.4-2.51 for the first deprotonation step and 9.35 or 9.7 for the second *(33, 34).* Thus, at physiological pH, it exists predominantly in its monobasic form, which is unstable and decomposes (at a rate of ~10^{-4} s^{-1} at 25°C) *(34)* to yield HNO as a singlet ground state and nitrite (Eq. 2).

$$Na_2N_2O_3 + H_2O \rightleftharpoons 2\,Na^+ + OH^- + HN_2O_3^- \quad (1)$$

$$HN_2O_3^- \rightleftharpoons {}^1HNO + NO_2^- \quad (2)$$

Deprotonation of HNO to the triplet ground state ($^3NO^-$) can only be achieved by crossing a large activation barrier due to the spin-forbidden reaction at physiological pH (at a rate of 5×10^4 M^{-1} s^{-1}) *(18).* Dimerization of ^{1}HNO can occur to generate a hydroxylating species (HON=NOH), which dehydrates to form nitrous oxide (N_2O) *(35).* The latter is frequently measured as a surrogate of HNO formation. In the presence of oxygen, peroxynitrite is believed to be formed to subsequently rearrange to nitrate with a rate constant of 1.2 s^{-1} (36).

Evidence for $ONOO^-$ formation can be obtained by measuring the transient absorption at 302 nm, and the rate of decay can be monitored in the presence and absence of CO_2, a known peroxynitrite scavenger, as well as by measurement of the stable end-product nitrate *(18, 37).* It has been suggested, however, that the oxidant species derived from the reaction of AS with oxygen is not necessarily peroxynitrite *(38-40).*

While the mechanism of nitrosation of thiols and amines by $NO^\bullet$ *in vitro* has been shown to be oxygen-dependent and to proceed via intermediate formation of N_2O_3 *(41)* little is known about the potential nitrosative and nitrative chemistry of nitroxyl and its biomolecular targets. Concerning the latter, both low molecular weight compounds and proteins have to be considered. In proteins, the sulfhydryl group of cysteine residues, the phenol ring of tyrosines and the indole nitrogen of tryptophans have been identified as major targets of ROS and RNOS. Recent observations about the physiological occurrence of N-nitroso species in human plasma *(42)* and the formation of N-nitroso proteins from $NO^\bullet$ in rodent tissues *(43)* has renewed the interest in tryptophan chemistry. The amino acid tryptophan is the precursor to melatonin (N-acetyl-5-methoxytryptamine) and indol-3-acetic acid, both of which are widely distributed throughout the animal and plant kingdom. The hormone melatonin, which is mainly produced by the pineal gland during hours of darkness, has been implicated in aging and senescence *(44)*, the regulation of seasonal reproductive cycles *(45)*, and other biological functions. In addition, it has been shown to be endowed with potent cardioprotective effects *(46)*, possibly by acting as an antioxidant to preserve mitochondrial integrity *(47).* Melatonin is also an effective scavenger of e.g. hydroxyl radicals ($OH^\bullet$) during a reaction with which it is oxidized to N-formylkynuramine, indol-2-one, pyrroloindoles, as well as hydroxylated and dimerized products *(48, 49).* Some of these species have been described as markers of oxidative stress. For example, pyrroloindole has

been detected in large amounts from the urine of rats and humans upon increased exposure to ionizing radiation *(50).* Transformations involving melatonin are typical of indoles in general and should help in clarifying what occurs with free tryptophan or at tryptophan residues when exposed to various NO_x species.

With questions arising as to the nature of the intermediate(s) in the AS decay route, analysis of the products of the AS reaction with various tryptophan derivatives should cast light on the effects of AS *in vitro* and *in vivo*. The objective of the present work is to investigate the chemistry of nitroxyl with particular emphasis on the generation of N-nitroso products. In addition, we aimed at comparing the outcome of *in vitro* reactions of nitroxyl and $NO^{\bullet}$ with tryptophan derivatives with the situation in an intact animal model *in vivo* using AS and DEA/NO as representative HNO and $NO^{\bullet}$ donors, respectively. The release characteristics of the respective NO_x species from each donor as well as their half-lives in solution are comparable ($t_{1/2}$ = 2.5 min at 37°C and 12-17 min at 25°C at pH 7.4 for DEA/NO) *(51),* allowing for a direct comparison of the redox siblings, HNO and $NO^{\bullet}$ *in vivo*.

EXPERIMENTAL PROCEDURES

Materials. Melatonin (MelH, $C_{13}H_{16}N_2O_3$, MW = 232), N-acetyl-L-tryptophan ($C_{13}H_{14}N_2O_3$, MW = 246), indol-3-acetic acid ($C_{10}H_9NO_2$, MW = 175), and diethylenetriamine pentaacetic acid (DTPA, $C_{14}H_{23}N_3O_{10}$, MW = 393) were obtained from Sigma. Sodium nitrite ($NaNO_2$, MW = 69) and sodium nitrate ($NaNO_3$, MW = 85) were obtained from Fluka. Isotopically labeled sodium nitrite, Na[^{15}N]-NO_2, anhydrous disodium hydrogenphosphate (Na_2HPO_4, MW = 142), sodium dihydrogenphosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$, MW = 156) and acetonitrile were obtained from Prolabo (France). Angeli's salt ($Na_2N_2O_3$, MW = 121) and 2-(N,N-diethylamino)diazen-1-ium-1,2-diolate, diethylammonium salt (DEA/NO, $C_4H_{10}N_3O_2 \cdot C_4H_{12}N$, MW = 206) were from Cayman Chemical. Stock solutions were prepared daily in 20 mM sodium hydroxide and kept on ice until use. Peroxynitrite synthesis was performed in a two-phase system using isoamyl nitrite and hydrogen peroxide following the method of Uppu et al. *(52)*. The product $ONOO^-$ was stored at –20 °C, and its concentration was determined by measuring the absorbance at 302 nm (ε = 1,670 $M^{-1}cm^{-1}$) in NaOH (0.02 N).

Reactions with Indolic Compounds. Unless stated otherwise, all reactions were performed at pH 7.5 (and 8.5) using phosphate buffered aqueous solutions (400 mM) at a temperature of 25°C. Ten equivalents of either AS or $ONOO^-$ and seven equivalents of DEA/NO were allowed to react with varying concentrations (1 mM and 100 μM) of the following tryptophan derivatives: melatonin, N-acetyl-L-tryptophan, or indol-3-acetic acid. Diluted $ONOO^-$ in NaOH (0.02 M) was added to each respective indole buffered solution either as a bolus or by infusion through a syringe at a flux rate of 1 μM/s while stirring vigorously. Reaction progress was monitored via HPLC and spectrophotometry.

For preparative purposes, an aliquot of AS (50 mg, 1 mM) was added to each indolic solution (100 μM) into a 400 mL 0.4 M phosphate-buffered solution. The final pH was determined to be 7.5. Absorption changes at λ_{max} = 346 nm or 335 nm were monitored. After one hour, the reaction mixture was subject to filtration using a 0.2 μM Acrodisc filter and injected into the preparative HPLC column.

Spectrophotometric Analysis. Absorption data were recorded either with a double-beam Uvikon 942 or Agilent 8453 UV-Vis spectrophotometer. One cm quartz cuvettes were used for all analyses. Under aerobic conditions, solutions were prepared using standard laboratory glassware, exposed to atmospheric conditions, and immediately transferred into the cuvette for analysis. Special precautions were taken for the anaerobic experiments. All solutions were deaerated by bubbling with oxygen-free argon for 15 minutes and kept sealed prior to measurement. The following absorbance values were measured and used to determine the concentrations of individual species: AS (ε (237 nm) = 6,100 M^{-1} cm^{-1}), 1-nitrosomelatonin (MelNO) (ε (346 nm) = 10,900 M^{-1} cm^{-1}), 1-nitrosoindol-3-acetic acid (ε (335 nm) = 4,900 M^{-1} cm^{-1}), and N-acetyl-1-nitroso-L-tryptophan (ε (335 nm) = 6,900 M^{-1} cm^{-1}).

Reversed Phase HPLC and Mass Spectrometry. The equipment and methods used for preparative and analytical liquid chromatography and mass spectrometry have been described previously *(53)*. The column was eluted using a 10- 50% gradient of acetonitrile in water for 60 min at a flow rate of 1 mL/min. Yields were evaluated by integrating the values obtained at 215 or 350 nm using external standards of synthesized nitroso compounds. In the case of melatonin, standards of oxidation and nitration derivatives used were those obtained from the reaction with

peroxynitrite. HPLC measurements allowed the main products to be identified and further characterization was obtained by NMR and MS analyses once aliquots were collected and lyophilized (-50°C, < 0.1 mbar). Molecular masses of all nitroso compounds were obtained using direct infusion of the methanolic solution.

NMR Measurements. All experiments were carried out at 25°C in a 600 MHz Bruker spectrometer. Chemical shifts are expressed as ppm relative to $SiMe_3$. All products isolated by preparative HPLC were dissolved in either CD_3OD or d^6-DMSO for NMR analysis. Standard N-nitroso compounds (Figure 1) were synthesized using methods described by Bravo et al. *(54)* for 1-nitrosomelatonin and by Bonnett et al. for N-acetyl-1-nitroso-L-tryptophan *(55)*. All products determined are mixtures of two conformers showing that the N-N=O bond is coplanar with the aromatic ring as shown for the crystal structure of 1-nitrosomelatonin *(45)*.

Insert Figure 1 here

^1H and ^{13}C NMR characteristics of 1-nitrosomelatonin (R_1=CH_3O; R_2=$CH_2NHCOCH_3$) have previously been described. *(50, 53)*. ^1H and ^{13}C NMR characteristics of N-acetyl-1-nitroso-L-tryptophan (R_1=H; R_2=$C(COOH)NHCOCH_3$) and 1-nitrosoindol-3-acetic acid (R_1=H; R_2=COOH) are available as supporting information.

In vivo Studies with AS and DEA/NO. Male Wistar rats (250-350g) were obtained from Harlan (Indianapolis, IN) and housed at a normal 12/12 light cycle 3 animals/cage with food and water *ad libitum*. DEA/NO and AS were dissolved at a concentration of 5 mg/mL in PBS immediately before intraperitoneal (ip) administration at a dose of 5 mg/kg. Heparinized (0.07 U/g ip) rats

were anaesthetized using diethylether and euthanized by cervical dislocation 15 min after compound administration. Following thoracotomy, a catheter was inserted into the infrarenal part of the abdominal aorta, and organs were flushed free of blood by retrograde *in situ* perfusion with air-equilibrated PBS supplemented with N-ethylmaleimide/EDTA (10 mM/ 2.5mM) at a rate of 10 mL/min essentially as described along with details on animal protocols, blood sampling, and organ harvest/homogenization elsewhere *(43)*.

Quantification of Nitrite, Nitrate, N_2O and Nitroso Species. Nitrite and nitrate were quantified by high pressure liquid ion chromatography employing on-line reduction of nitrate to nitrite and post-column derivatization with the Griess reagent (ENO20 Analyzer, Eicom, Kyoto, Japan) *(43, 56)*. The extent of nitrosation of endogenous biomolecules in blood and tissue homogenates was quantified using group-specific reductive denitrosation followed by chemiluminescent detection of $NO^{\bullet}$ in the gas phase (CLD77am sp, Eco Physics) as described in detail elsewhere *(43, 56)*. S-nitrosothiols (RSNO) and N-nitrosamines (RNNO) were differentiated by their mercury sensitivity where RSNO signifies Hg^{2+}-sensitive species and RNNO signifies Hg^{2+}-resistent species. The latter may include N-nitrosamines and metal nitrosyls. The formation of nitrous oxide (N_2O) from AS was quantified in the headspace of septum-sealed vials using gas chromatography essentially as described *(17)*.

$NO^{\bullet}$ Formation Upon Angeli's Salt Decomposition. Gas phase chemiluminescence techniques *(43)* were implemented to monitor $NO^{\bullet}$ formation from the decay of AS in 10 mM phosphate buffer with 1 mM EDTA (or 50μM DTPA) at neutral pH (7.5 and 25°C). Deoxygenated conditions were obtained by bubbling the samples and reaction chamber with N_2 (or Ar) for 15-

30 minutes. Oxygenated conditions implemented air-equilibrated samples (with $[O_2] \approx 0.24$ mM) while the reaction chamber was bubbled with compressed air for 15 minutes prior to addition of samples. $NO^{\bullet}$ was measured upon addition of AS while increasing concentrations of melatonin were added into the reaction chamber (0, 167, 333, 667 μM). In order to assess the effect of melatonin on the formation of $NO^{\bullet}$ as AS decays, the order of addition was reversed. This time, varying concentrations of melatonin were placed into the chamber after addition of AS and the formation of $NO^{\bullet}$ was monitored. The quantities of $NO^{\bullet}$ formed from deoxygenated and oxygenated conditions were compared by integrating the areas under the curve over a set period of time (2, 3, or 5 minutes).

RESULTS

Characterization of AS as a Nitroxyl Source – Effect of Oxygen. The classical view about the aerobic decomposition of AS is that it is associated with generation of equimolar amounts of nitrite and nitroxyl (Reactions 1 and 2) *(57, 58).* While nitrite is rather stable, nitroxyl is not and typically undergoes further reaction depending on the nature of the medium. At neutral pH and under aerated conditions, HNO consumes molecular oxygen with a rate constant of 3-8 $\times 10^3$ M^{-1} s^{-1} *(8, 37)* to yield $ONOOH/ONOO^-$ (or a related oxidant), which eventually re-arranges to form nitrate, NO_3^-, as a stable end product. The results of the present study are principally consistent with this route of AS decomposition. The half-life of AS at pH 7.5 and 37°C was 2.57 ± 0.43 min as determined by UV/Vis spectrophotometry, and did not differ between absence and presence of oxygen (n=3; data not shown). The concentrations of nitrite generated were roughly the same, approaching theoretical yields, under aerobic and anaerobic conditions (inset of Fig. 2). Formation of nitrate was negligible in the absence of oxygen, but approached ~ 20 mol% in its presence. The predominant end-product of nitroxyl is thought to be N_2O which forms with a rate constant of ~ 8 $\times 10^6$ M^{-1} s^{-1} *(18)*, via intermediate formation and subsequent dehydration of the HNO dimerization product, hyponitrous acid (HON=NOH) *(35, 59, 60).* Under anaerobic conditions, AS generated N_2O in amounts comparable to those of nitrite (90.5 mol% HNO with 100 μM AS as determined by gas chromatography). However, N_2O formation was substantially lower under aerobic conditions, corresponding to 70-80% inhibition by the presence of oxygen (n = 2; data not shown). Taken together, theses results confirm that the compound used in our subsequent studies behaves biochemically in a manner that is qualitatively and quantitatively consistent with what has been described for AS before *(30)*. In addition, the data reveal that under aerobic conditions molecular oxygen effectively competes with the dimerization reaction

of nitroxyl to form nitrous oxide by generating a reactive intermediate that decomposes to nitrate. Formation of the latter is consistent with, although no proof for, the involvement of peroxynitrous acid, ONOOH.

Insert Figure 2 here

N-nitrosation of Indoles by AS. The reaction of various indolic compounds (e.g., melatonin, indol-3-acetic acid, and N-acetyl-L-tryptophan) with AS in buffered aqueous solution under aerobic conditions was associated with prominent spectral changes between 250-450 nm. The spectral changes observed were qualitatively identical whether reactions were carried out at physiological pH or under more alkaline conditions (pH 7.5 and 8.5). Figure 3 illustrates the spectral changes observed upon reaction of melatonin with AS at pH 8.5. The disappearance of native melatonin is evident at 300 nm, while the appearance of a new species is shown by the increase in absorbance at λ_{max} = 346 nm. Similar spectral changes were observed with the other two tryptophan derivatives, except that λ_{max} was at 335 nm. In all cases, parallel analysis of the reaction mixtures by HPLC showed one major reaction product along with residual indolic compound(s). Although the major products of each respective indole examined were generally unstable (compounds typically decompose within 1-3 hours), we were able to identify them as the respective 1-nitrosoindole derivatives by their molecular mass as well as their UV-absorbance and NMR spectra (Fig. 1). Due to the labile nature of their $NO^{\bullet}$ moiety, mass spectra of 1-nitrosoindoles are notoriously difficult to obtain by traditional HPLC-MS. However, using direct injection of the methanolic solution of nitrosomelatonin, the complete fragmentation spectrum with peaks at m/z 254 $[M\text{-}NO+Na]^+$, 284 $[M\text{-}Na]^+$, 295 $[M\text{-}NO+CH_3CN+Na]^+$, 485 $[2(M\text{-}NO)+Na]^+$, 515 $[M+(M\text{-}NO)+Na]^+$, and 545 $[2M+Na]^+$ is revealed. With all three indoles

studied the yield of N-nitrosation increased with increasing concentrations of AS (Figure 4), which was accompanied by the mirror image disappearance of the starting compounds. With melatonin and a molar excess of AS, a 22% product yield was obtained at pH 7.5, with an observed rate constant (k_{obs}) of (7.6 ± 0.5) x 10^{-4} s^{-1} (inset of Figure 3). At pH 8.5, product yield doubled while k_{obs} was similar (6.4 ± 0.1x 10^{-4} s^{-1}). Trace secondary products in much less than 1% yield were identified as N-formylkynuramine (*N*-{3-[2-(formylamino)-5-methoxyphenyl]-3-oxopropyl}acetamide) (λ_{max} = 236, 265, 343 nm and *m/z* 287 $[M+Na]^+$) and 2,3-dihydro-2,3-epoxymelatonin (epoxide, λ_{max} = 260, 300 nm and *m/z* 271 $[M+Na]^+$ and 519 $[2M+Na]^+$). These compounds had been shown previously to be among the major reaction products of melatonin with peroxynitrite *(53)*. Control incubations with melatonin and AS in the presence of the metal chelator DTPA (20 μM) revealed no difference in either spectral changes or product formation confirming that N-nitrosoindole formation was not the result of Fenton-type chemistry due to contaminant trace metals in the buffer. Importantly, no formation of nitrosomelatonin was observed with melatonin and AS in the absence of oxygen, indicating that N-nitrosoindoles are not formed directly from nitrite or nitroxyl, but only after reaction with oxygen. Yields of N-nitrosation with indol-3-acetic acid and N-acetyl-L-tryptophan at pH 7.5 were 10% and 15%, respectively, with kinetics almost identical to melatonin. Since the pattern of reaction was similar with all three indoles investigated, melatonin was used as a representative compound in all subsequent mechanistic experiments.

Insert Figures 3 and 4 here

N-Nitrosation of Melatonin by Peroxynitrite. Peroxynitrite has been shown to exhibit potent reactivity towards a large range of molecules including the amino acids cysteine, methionine,

tyrosine, and tryptophan *(61)*. It has also been reported to be formed during the decay of AS in the presence of oxygen (from the reaction of HNO and O_2) *(37)*, and it was thus conceivable to assume that peroxynitrite formation may account for the N-nitrosation of melatonin observed in the present study. Indeed, we recently demonstrated that in phosphate buffered aqueous solution (pH 7.5) the reaction of peroxynitrite with melatonin produces 1-nitrosomelatonin *(53)*. Similar results were observed with N-acetyl-L-tryptophan *(62)*. However, depending on the chemical species (e.g. $ONOO^-$, ONOOH, or the CO_2 adduct, $ONOOCO_2^-$) and the reaction conditions involved peroxynitrite can act either as an oxidant or as a nitrosating and nitrating agent of aromatic, phenolic, and heterocyclic rings. Consistent with previous results *(53)*, but in contrast to AS, nitrosomelatonin was only one of many products formed from $ONOO^-$ with melatonin (Fig. 5).

Insert Figure 5 here

The formation of nitrosomelatonin by peroxynitrite was accompanied by formation of significant quantities of N-formylkynuramine. At pH 7.5, when $ONOO^-$ is added rapidly (as a bolus) to a solution of melatonin, similar yields were observed for nitrosomelatonin and N-formylkynuramine (19% and 18%, respectively). Consistent with the potent scavenging activity of HCO_3^-/CO_2 towards $ONOO^-$ ($k \sim 5.8 \times 10^4\ M^{-1}\ s^{-1}$) *(63)* product yields decreased to 8% for nitrosomelatonin and 7% for N-formylkynuramine in the presence of 60 mM bicarbonate. Increasing the pH to 8.5 increased overall product yields of nitrosomelatonin and N-formylkynuramine to 40 and 35%, respectively. The yields of the oxidation and nitration products identified were 7% for 2,3-dihydro-2,3-epoxymelatonin, 3% for 1-nitromelatonin, 2% for 3-nitromelatonin, and 3% for 4-nitromelatonin at pH 7.4.

Various factors influenced the reaction between peroxynitrite and melatonin. Increasing the concentration of melatonin (100, 500, and 1000 μM) while keeping the $ONOO^-$ concentration constant altered the product ratios, as did a change in the manner by which peroxynitrite was delivered (i.e., bolus vs. infusion; see Fig. 6). The latter is not surprising as the chemistry of peroxynitrite is known to differ depending on whether it is added as a bolus from a stock solution or produced *in situ* by cogeneration of $NO^{\bullet}$ and superoxide. However, its behavior was not the same for all reaction products. Bolus addition of $ONOO^-$ (700 μM) to increasing concentrations of melatonin increased the yields of both types of oxidation products (epoxide and N-formylkynuramine) as well as the nitrosation product (1-nitrosomelatonin) in a concentration dependent manner. In contrast, no significant change in product yield was observed with increasing melatonin for any of the nitration products (3-nitromelatonin, 6-nitromelatonin, 4-nitromelatonin, and 1-nitromelatonin). In an effort to mimic the hypothetical delivery that would be generated from AS and for comparison to the results obtained with bolus addition of peroxynitrite, an additional set of experiments was performed in which peroxynitrite was slowly infused into a melatonin-containing buffer solution. While little difference was observed in the yield and spectrum of oxidation products between bolus and infusion of $ONOO^-$ much less nitrosation and nitration products were generated when $ONOO^-$ was added to the melatonin solution by infusion.

Insert Figure 6 here.

Mechanism of N-Nitrosation of Melatonin by AS - Roles of Peroxynitrite and Nitrite. Air-exposed phosphate-buffered aqueous solutions of $ONOO^-$ contain a mixture of $ONOO^-$, ONOOH, and $ONOOCO_2^-$ due the presence of adventitious CO_2 in air. The latter is an efficient

$ONOO^-$ scavenger which has been shown to reduce the yield of nitrosomelatonin while increasing the yield of 1- and 3-nitromelatonin from peroxynitrite *(53)*. In order to assess the role of $ONOOCO_2^-$ in N-nitrosation of melatonin by AS, separate incubations were performed in the presence of sodium bicarbonate (60 mM). More importantly, this addition altered neither the rate nor yield of nitrosomelatonin suggesting little to no involvement of peroxynitrite in its production by nitroxyl.

During AS decomposition, both HNO and the nitrite anion are formed (Eqs. 1 and 2). In order to assess the role nitrite may play in the nitrosation of melatonin, additional incubations with AS were carried out in the presence of an equimolar amount and a 10-fold excess of $NaNO_2$ at pH 7.5. In agreement with earlier findings *(64)*, no changes in either nitrosation rate or product yields were observed in the presence of additional nitrite. Moreover, mass spectrometric analysis of nitrosomelatonin produced in the presence of isotopically labeled nitrite ($^{15}NO_2^-$) revealed no incorporation of labeled ^{15}N. Taken together with the finding that N-nitrosation of melatonin by AS is dependent on the presence of oxygen (see above) these data unambiguously show that, under the conditions of this study, a nitroxyl oxidation product that is neither peroxynitrite nor nitrite is responsible for the nitrosation of the indole nitrogen of tryptophan derivatives by AS.

Mechanism of N-Nitrosation of Melatonin by AS – Possible Role of $NO^\bullet$. In order to contrast the reactions of nitroxyl and peroxynitrite directly with that of the $NO^\bullet/O_2$ system additional experiments were performed with i) a saturated aqueous solution of $NO^\bullet$ and ii) with the NO-donor DEA/NO. At pH 7.5 and 25°C, the half-lives of AS and DEA/NO are almost identical (~30 min), providing an ideal tool to directly compare the effects of nitroxyl with those of $NO^\bullet$.

Since DEA/NO releases 1.5 equivalents of $NO^{\bullet}$ per molecule, the amount of DEA/NO used was adjusted accordingly to generate ~10 equivalents of NO_x released for every melatonin. The rationale for the use of both aqueous $NO^{\bullet}$ solution and NO-donor were similar to that of comparing bolus addition and infusion of peroxynitrite. Where a 10:1 ratio of aqueous $NO^{\bullet}$:melatonin was applied, a 7:1 ratio of DEA/NO:melatonin was used in these studies. In agreement with earlier studies *(54, 64)* the data show that under both conditions melatonin required dioxygen to yield nitrosomelatonin as the exclusive organic reaction product. Interestingly, the HPLC traces (data not shown) and rates of formation of nitrosomelatonin from incubations of 100 μM of melatonin with DEA/NO were similar to those determined for AS ($\approx 1\times10^{-8}$ M s^{-1}) with an overall yield of 13% nitrosomelatonin (as compared to 22% with an excess of AS; see above).

In an attempt to explain the apparent similarity in profile of products and product yields, further experiments were performed to address under what conditions $NO^{\bullet}$ may be formed from AS. $NO^{\bullet}$ has been shown to be produced from AS in a pH-dependent manner, with highest yields under acidic conditions *(59)*. The results from the present study demonstrate that $NO^{\bullet}$ is generated from AS even at physiological pH (in the presence of either EDTA or DTPA as metal chelator), albeit in relatively low yield. Of note, $NO^{\bullet}$ production from AS was enhanced not only by molecular oxygen, but also by addition of melatonin (Fig. 7). Melatonin enhanced $NO^{\bullet}$ release from AS in a concentration-dependent manner both in the absence and presence of oxygen. Under aerobic (and anaerobic) conditions, the reaction of AS with melatonin does not appear to be straightforward as different rates of $NO^{\bullet}$ release were observed depending on the order of addition of reagents (data not shown). Taken together, these data suggest that small

amounts of AS-induced nitrosation of the indole nitrogen of melatonin may arise from the reaction of $NO^{\bullet}$ or HNO with O_2.

Insert Figure 7 here

Comparison of N-Nitrosating Potential of Nitroxyl and $NO^{\bullet}$ in vivo. *In vitro* experiments like the ones described above comparing the chemical reactivity of $NO^{\bullet}$ and HNO towards a common substrate (e.g. indoles) are crucial to understanding the differences between the two classes of N-oxides. However, they do not necessarily allow extrapolating results to the *in viv*o situation as the factors that govern the site-specificity of nitrosation processes are currently not well understood. Nevertheless, an extension of such a comparison to the *in vivo* situation may provide valuable insight into the potential physiological relevance of HNO. To this end, experiments were conducted in which 5 mg/kg of either DEA/NO or AS were administered via ip injection to male Wistar rats. After 15 minutes (corresponding to ~5 half-lives at 37°C), the concentrations of nitrite, nitrate, mercury-sensitive compounds (e.g. S-nitrosothiols; RSNO) and mercury-resistant compounds (e.g., N-nitrosoamines; RNNO) were measured via gas phase chemiluminescence and HPLC in red blood cells, plasma, and three exemplary tissues (i.e., brain, heart and liver). Figure 8 summarizes the net changes in the levels of these $NO^{\bullet}$/HNO related metabolites upon application of DEA/NO and AS *in vivo*. The administration of either AS or DEA/NO increased the levels of all $NO^{\bullet}$/HNO-related metabolites quantified (i.e., nitrite, nitrate, RSNO and RNNO) in blood and most tissues (with the brain representing a notable exception). Whereas the increases in nitrate concentrations were comparable in all compartments between AS and DEA/NO, nitrite levels after AS application were 2-5-fold higher in blood and two tissues (brain and heart, but not liver) compared to DEA/NO. More marked differences were seen in the nitrosation pattern elicited by either compound. Whereas AS and DEA/NO

produced roughly the same degree of S-nitrosation in all tissues, it preferentially formed RSNO in blood. In contrast, RNNO formation from either compound was comparable in blood, but substantially higher with AS than with DEA/NO in tissues. The most impressive elevation of mercury-stable nitroso species (presumed to represent primarily RNNO-type compounds) with AS was seen in the heart. Collectively, these data show that the biological chemistry of $NO^{\bullet}$ and nitroxyl differs substantially and that the nitrosative chemistry such endogenous target molecules are exposed to after administration of AS or DEA/NO *in vivo* depends on the biological compartment in which these species are generated and the biochemical makeup of the microenvironment. One of the outstanding features observed, which deserves further investigation, was the massive formation of mercury-stable nitroso species with AS in the heart.

Insert Figure 8 here

DISCUSSION

The present study focused on the kinetics of formation and the identification of the reaction products of indoles with nitroxyl generated from AS. Using three representative tryptophan derivatives (melatonin, indol-3-acetic acid, and N-acetyl-L-tryptophan) at physiological pH and in the presence of oxygen (O_2), we find negligible oxidation and nitration but N-nitrosoindoles as the primary reaction products. We further demonstrate that neither nitrite nor peroxynitrite are involved in N-nitrosoindole formation. In additional animal experimental work we demonstrate that AS exhibits a unique *in viv*o nitrosation signature, which differs from that of DEA/NO inasmuch as in the heart substantial amounts of a mercury-stable nitroso species are generated whereas in plasma the major reaction products are S-nitrosothiols (RSNO). Thus, the outcome of the nitrosation chemistry entertained by nitroxyl *in vivo* appears to depend on the redox environment in which it is generated.

During decomposition of AS, both HNO and nitrite are generated, and either species may potentially serve as a nitrosating agent. However, at physiological pH *in vitro* nitrite is a very poor nitrosating agent unless supported by enzymatic catalysis. In contrast, N_2O_3 has been shown to efficiently nitrosate not only thiols to form RSNO, but also secondary amines such as the indole nitrogen of tryptophan derivatives *(62).* Whether or not nitroxyl is capable of entertaining similar nitrosation chemistry at physiological pH is not known. In the first set of experiments, we therefore investigated the reactivity of AS towards melatonin and other tryptophan derivatives in order to provide insight into the NO^-/HNO mediated nitrosation reactions. In the presence of air, a single unique reaction product, i.e. the corresponding N-nitrosoindole, was found upon the incubation of AS with various indolic compounds while no nitrosation was observed in the absence of O_2. Formation of N-nitroso species was unexpected

since oxidation and hydroxylation products have been described to represent the predominant products of AS reaction with aromatic compounds in previous reports *(24, 38-40)*.

While the role of O_2 is significant, another possible intermediate, peroxynitrite ($ONOO^-$) does not appear to play a major role in AS-mediated nitrosation. Independent experiments with peroxynitrite reveal that the predominant products of these reactions are nitrosomelatonin and N-formylkynuramine whereas with AS, the only major product was nitrosomelatonin with yields differing by at least 10%. Furthermore, addition of a known peroxynitrite scavenger (HCO_3^-) into the reaction mixture containing AS and melatonin did neither alter rate nor yield of nitrosomelatonin, consistent with results reported for the oxidation of the fluorophore dihydrorhodamine (DHR) *(26)*.

Our studies suggest that there may be more than one route through which AS can elicit nitrosation, with O_2 playing a key role in all cases. Although AS is known to decompose into NO_2^- and HNO, the similarity in qualitative and quantitative results with either DEA/NO or AS and melatonin suggested involvement of a common intermediate in N-nitrosoindole formation with either donor. Given that the likelihood of peroxynitrite involvement is small, another conceivable intermediate was the one-electron redox sibling of nitroxyl, $NO^\bullet$. This suspicion was seemingly supported by the detection of free $NO^\bullet$ during AS decomposition using gas phase chemiluminescence.

Thus, several scenarios were considered that would involve both $NO^\bullet$ and O_2. Two ways in which nitrosation at physiological pH can proceed (via the $NO^\bullet/O_2$ route) are through a concerted (involving N_2O_3) (Eq. 3) or a free radical pathway (involving $NO_2^\bullet/NO^\bullet$ or $ONOO^\bullet$) that may be competitive with the hydrolysis of N_2O_3 *(45)*.

$$NO^{\bullet} + O_2 \rightleftharpoons ONOO^{\bullet} \xrightarrow{NO^{\bullet}} 2\,NO_2^{\bullet}$$
$$NO_2^{\bullet} \xrightarrow{NO^{\bullet}} N_2O_3 \xrightarrow{H_2O} 2\,H^+ + 2\,NO_2^- \qquad (3)$$

While the concerted N_2O_3 pathway may account for the formation of N-nitrosoindoles from $NO^{\bullet}$ donors such as DEA/NO, it cannot account for that from AS since the nitrosation of all tryptophan derivatives was found to be first order with respect to AS (see Fig. 4). Since much of the chemistry involving the $NO^{\bullet}/O_2$ reaction is second order *(4)*, it is unlikely that N-nitrosation of indoles is caused by intermediates of the $NO^{\bullet}$ autoxidation reaction. However, the latter may well account for the elevated levels of NO_2^- found during AS decomposition in the presence of oxygen (Fig. 2 inset) whereas nitrate formation under the same conditions is likely to result from the reaction between O_2 and HNO (Eq. 4).

$$^1HNO + {}^3O_2 \longrightarrow \text{"}O_2NOH\text{"} \longleftrightarrow HNO_3 \qquad (4)$$

An alternative pathway for the nitrosation of melatonin that would involve both $NO^{\bullet}$ and O_2 might proceed via the intermediacy of the nitrosating peroxyl radical, $ONOO^{\bullet}$ which necessitates that the reaction is first order with respect to $NO^{\bullet}$ *(64)*. Although not thermodynamically favored by simple $NO^{\bullet}$ oxidation, $ONOO^{\bullet}$ can be stabilized by solvation in water *(65)*. Theoretically, this mild oxidant could react with melatonin to generate an adduct that might rearrange to form the 1-nitrosomelatonin product. However, little is known about the chemical properties of this peroxyl radical, other than that it is rather unstable *(66)*.

If true, how then is NO formed from AS? $NO^{\bullet}$ may be artifactually generated during AS decomposition due to the presence of trace metals in the solution *(30)*. With the metal chelator DTPA present, it is unlikely that the oxidation of AS will occur since the oxidation potential of $HN_2O_3^-$ about 0.9 ± 0.1 V (vs. NHE at pH 7) *(30)*. Another possibility why only small quantities of $NO^{\bullet}$ are detected is through the decomposition of the final N-nitrosoindole product rather than AS itself.

Since $NO^{\bullet}$ was observed to have formed, despite the presence of the metal chelator EDTA or DTPA, it most likely through the reaction with trace metals with HNO. In the presence of HNO, $NO^{\bullet}$ can form the hyponitrite radical $N_2O_2^{\bullet-}$ whose oxidation potential was determined to be 0.96 V and may act as the oxidant to react with melatonin to form the 1-nitrosomelatonin product in small quantities *(35)*.

Together with the fact that $NO^{\bullet}$ formation from AS was low, none of the above mechanisms involving the $NO^{\bullet}/O_2$ route is likely to account for the majority of the N-nitrosoindole product formed.

Since none of the routes above provide a viable mechanism to explain the bulk of 1-nitrosomelatonin product formed, an alternative model involving HNO is proposed (Scheme 1). In this Scheme, melatonin reversibly reacts with HNO to form an N-hydroxalamine intermediate (Intermediate 1). Addition of the powerful electrophile HNO to nitrogen-based nucleophiles is predicted to be highly favorable in solution *(67)*. This pathway is analogous to the proposed nitrosation of 4,5-diaminofluorescein (DAF) by AS *(40)*. Intermediate 1 can then react further

by either one of two routes; the first set of minor products can be generated via a radical pathway following homolytic cleavage of the N-N bond to form a melatoninyl radical species (Intermediate 2) and the hydroxylamine radical ($HNOH^{\bullet}$). The latter may further react with oxygen to generate $NO^{\bullet}$ and H_2O_2 while the melatoninyl radical (Intermediate 2) can trap $NO^{\bullet}$ to generate 1-nitrosomelatonin. The two-electron oxidation of Intermediate 1 by oxygen to yield the final products, 1-nitrosomelatonin and hydrogen peroxide (H_2O_2), is likely to be the more favored reaction pathway.

Insert Scheme 1 here.

Whatever the mechanism(s) involved in the N-nitrosation of indoles by nitroxyl and regardless of the similarities in product profile and yields between DEA/NO and AS *in vitro*, our animal experimental results indicate that the consequences of a generation of $NO^{\bullet}$ and HNO in terms of the post-translational protein modifications induced *in vivo* are vastly different. Specifically, S-nitrosothiols were elevated most profoundly with AS in plasma whereas mercury-resistant nitroso species (most likely, protein N-nitroso compounds) were elevated most dramatically in the heart, with a >30-fold difference between AS and DEA/NO induced N-nitrosation. Because the reported half-lives of the two donor compounds (~2.5 min at 37°C) are comparable, which translated into comparable levels of total NO_x in blood and tissues following their administration to rats, we conclude that the chemical fate of AS and DEA/NO at the level of the intact organism must differ substantially. Since facile redox interconversion between $NO^{\bullet}$ and HNO/NO^{-} is kinetically impossible, specific changes in redox states (dictated by the environment) of thiol, metal, or amine containing proteins provide an attractive setting for

regulation. Given the differences in the biological chemistry between HNO and $NO^{\bullet}$, we and others have observed, these redox siblings appear to represent ideal signaling agents for control of a variety of physiological processes.

While our experimental animal investigations do not provide any clues as to the nature of the reaction products in tissues, the data are consistent with the notion that the generation of nitroxyl *in vivo* can give rise to the formation of N-nitrosotryptophan derivatives. Such reaction products had previously been described only in the context of $NO^{\bullet}$ formation but not for nitroxyl. Further investigations are required to address the mechanism(s) of nitrosation by nitroxyl and its physiological/pharmacological implications. It is intriguing to speculate that the particular efficiency of this reaction and/or the accumulation of the reaction products in the heart may account for the positive inotropic effects observed with AS earlier. Clearly, considerable efforts will be required to identify and characterize which tissue targets are subject to N-nitrosation before the full the pharmacological potential of nitroxyl donors will be uncovered and potential therapeutic avenues for HNO donors (e.g. for the treatment of cardiovascular diseases such as heart failure *(1, 7, 27)* may be realized.

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Supporting Infonnation Available. ^{1}H and ^{13}C NMR characteristics ofN-acetyl-1-nitroso-L-tryptophan (R_1 H; R_2 C(COOH)NHCOCH$_3$) and 1-nitrosoindol-3-acetic acid (R_1 H; R_2 COOH). This material is available free of charge via the Internet at http://pubs.acs.org.

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FIGURE CAPTIONS

Figure 1. Structures of N-nitrosotryptophan derivatives (R_1=CH_3O, R_2=$CH_2NHCOCH_3$: 1-nitrosomelatonin; R_1=H, R_2=$C(COOH)NHCOCH_3$: N-acetyl-1-nitroso-L-tryptophan; R_1=H, R_2=COOH: 1-nitrosoindol-3-acetic acid).

Figure 2. Temporal spectral changes associated with Angeli's salt decomposition (100 μM) under aerobic conditions at 37°C using a phosphate-buffered aqueous medium (100 mM). The first 7 spectral traces were obtained at 60 s intervals. The two subsequent spectra are taken at 120 s intervals while the remaining spectra were recorded at 300 s intervals. The inset depicts the temporal changes in NO_2^- and NO_3^- concentrations generated from AS (10 μM) under aerobic and anaerobic conditions as quantified by ion chromatography. Depicted data are representative of 2-3 independent experiments and qualitatively identical for 3 to 300 μM AS.

Figure 3. Spectrophotometric analysis of the reaction between melatonin (100 μM) and AS (1 mM) in 0.4 M phosphate-buffered aqueous solution at pH 8.5 and 25°C. The absorption due to the decomposition of 1 mM AS under the same conditions was electronically subtracted from the spectra. The first spectrum was recorded just before addition of AS and every successive spectra were obtained in intervals of 5 minutes. The inset represents a comparison of the kinetics at pH 7.5 and 8.5 with λ_{max} = 346 nm illustrating the formation of nitrosomelatonin (MelNO) upon addition of AS (k_{obs} were determined to be (7.6 ± 0.5) × 10^{-4} s^{-1} at pH 7.5 and (6.4 ± 0.5) × 10^{-4} s^{-1} at pH 8.5).

Figure 4. Formation of 1-nitrosoderivatives from 3-substituted indoles at pH 7.5 and 25°C. All tryptophan derivatives (1 mM) were incubated with increasing concentrations of AS in 0.4 M phosphate-buffered aqueous solution under stirring. The concentrations of 1-nitrosoindoles formed were determined after 1 hour when the absorbance for the nitroso species had reached a maximum. 1-Nitrosomelatonin (diamonds) exhibited a λ_{max} at 346 nm while both N-acetyl-1-nitroso-L-tryptophan (squares) and 1-nitroso-indol-3-acetate (triangles) showed a λ_{max} at 335 nm.

Figure 5. Comparison of products generated after mixing melatonin with either peroxynitrite (A) or Angeli's salt (B). HPLC analysis of the reaction of melatonin (1 mM) in 0.4 M phosphate buffered solutions at pH 7.5 by (A) 15 min after bolus addition of $ONOO^-$ (10 mM), (B) 10 minutes after addition of AS (10 mM). Both conditions yielded nitrosomelatonin (t_R = 46 min), but the reaction with peroxynitrite generated a number of additional products. The identified products are as follows: 2,3-dihydro-2,3-epoxymelatonin (t_R = 19 min, ~1% yield), *N*-formylkynuramine (t_R = 23 min, ~0.5% yield), and 1-nitrosomelatonin (t_R = 46 min, 22% yield).

Figure 6. Effects of increasing concentrations of melatonin (100, 500, and 1000 μM) with $ONOO^-$ (700 μM) at pH 7.5. Three different sets of reaction products were generated: oxidation, nitration, and nitrosation products. Oxidation products included both 2,3-dihydro-2,3-epoxymelatonin (epoxide) and N-formylkynuramine, nitration products included 1-, 3-, 4-, and 6-nitromelatonin, and the only nitrosation product found was 1-nitrosomelatonin. Product yields

were found to depend on the method of addition (bolus vs. infusion at an approximate rate of 1 μM/s) and were determined by means of HPLC analysis. Product concentrations were determined using external standards. Depicted data represent means ± SD of at least n = 3 independent experiments.

Figure 7. Stimulation of $NO^{\bullet}$ formation from AS under aerobic conditions. Effects of increasing melatonin (MelH) under aerobic conditions as measured by gas phase chemiluminescence. The tracings depicted are representative of 2-5 individual experiments with qualitatively identical outcome. The solid line depicts the original tracing of $NO^{\bullet}$ formation and AS decay obtained from no melatonin present, while the dotted line represents the trace from 333 μM of melatonin. The inset depicts the melatonin dependence of $NO^{\bullet}$ formation on the system.

Figure 8. Net changes in tissue concentrations of nitrite, nitrate, S-nitroso (RSNO) and N-nitroso (RNNO) products after ip administration of either DEA/NO or AS (5 mg/kg). Animals were sacrificed after 15 minutes, and blood (plasma and RBC) and three representative tissues (brain, heart, and liver) were analyzed by HPLC and chemiluminescence (means ± SEM; n=3). Untreated animals served as controls and had typically less than 1% of $NO^{\bullet}$/HNO-induced nitroso products of the AS and DEA/NO-treated animals in their blood and tissues.

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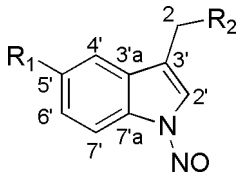

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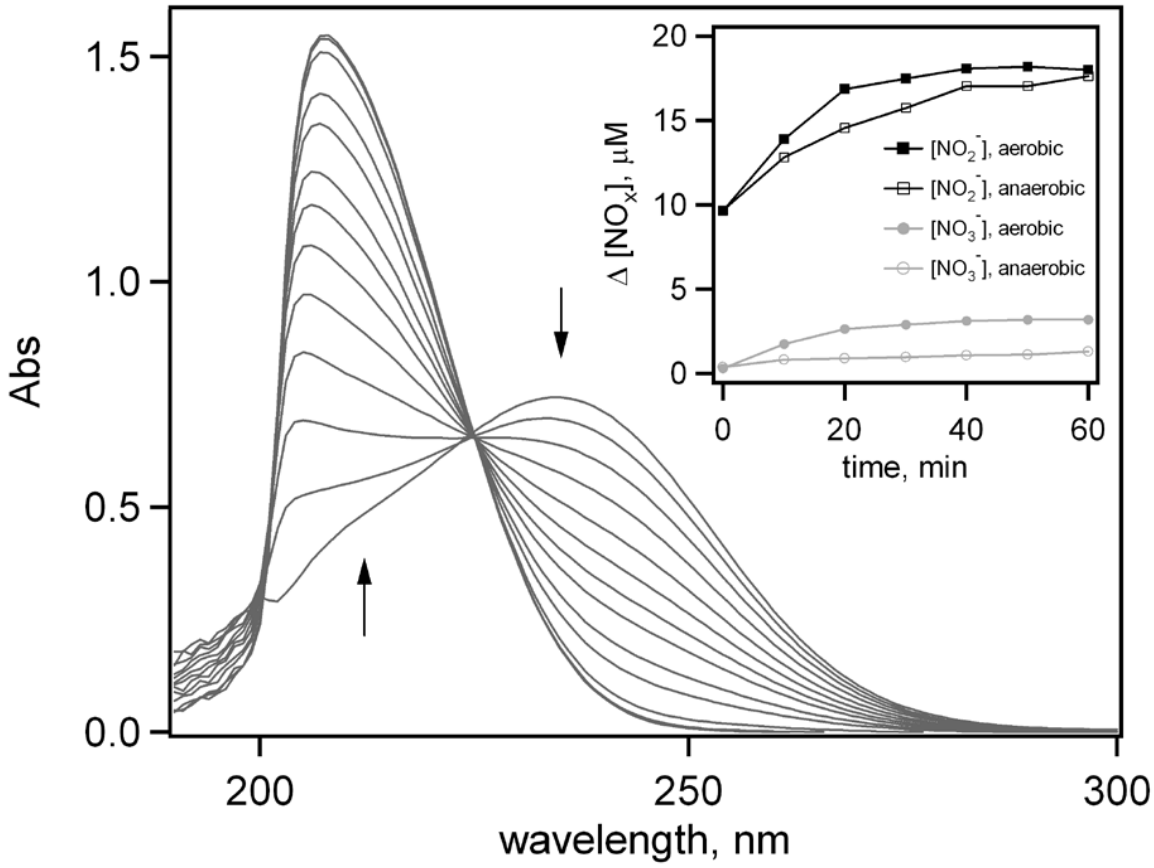

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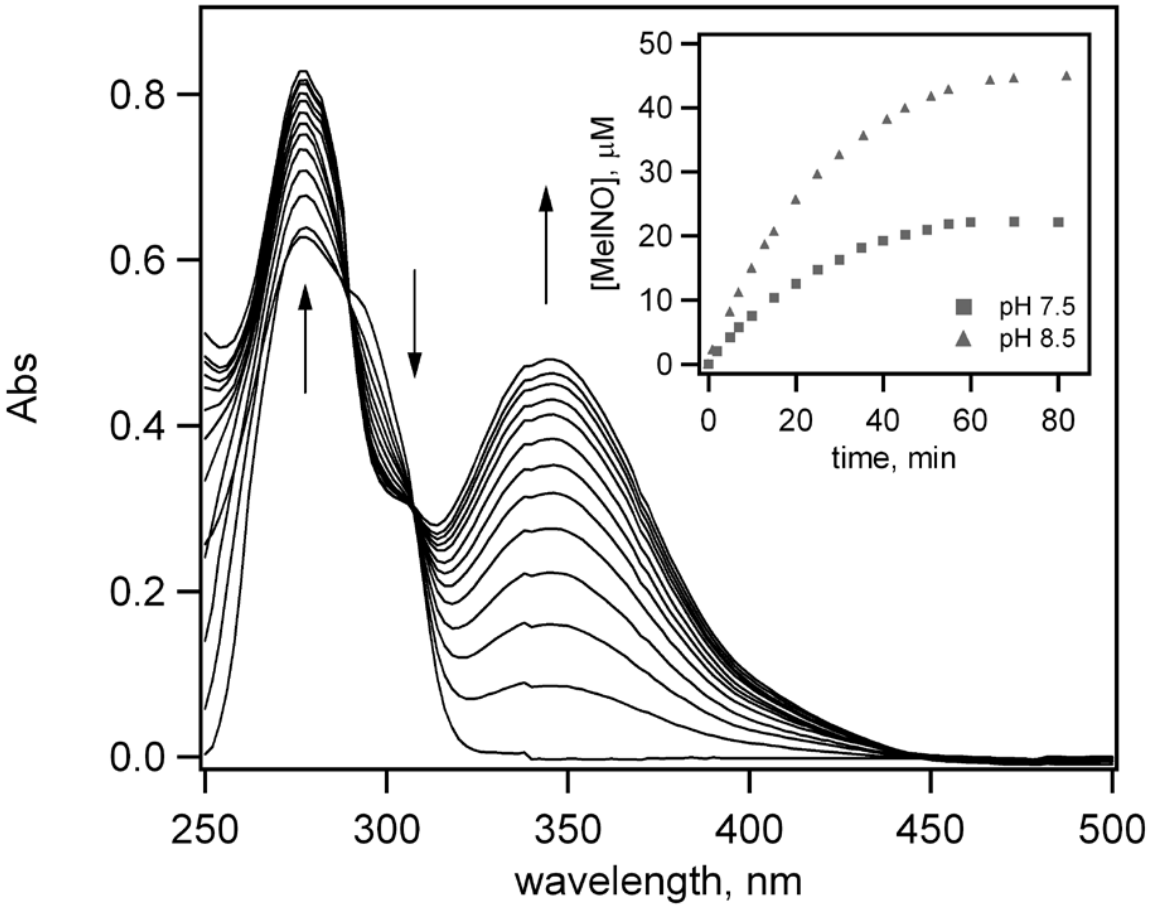

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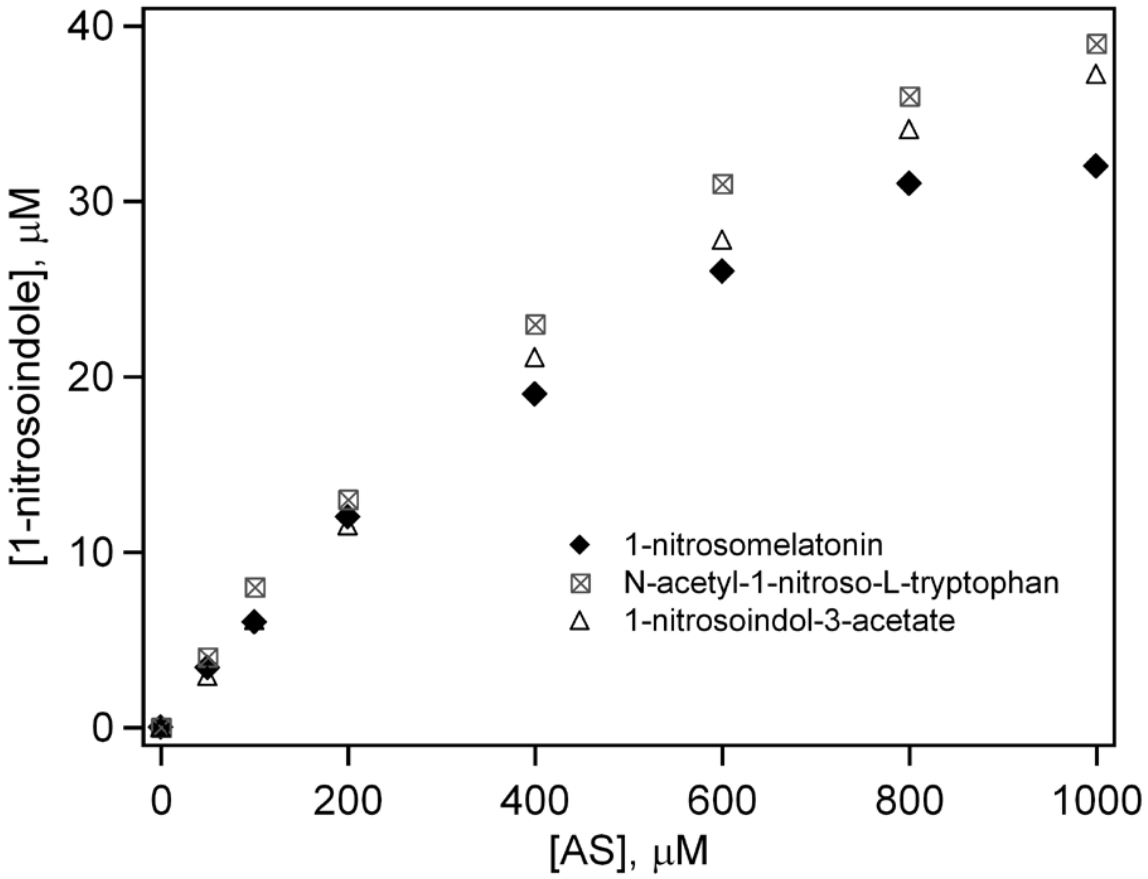

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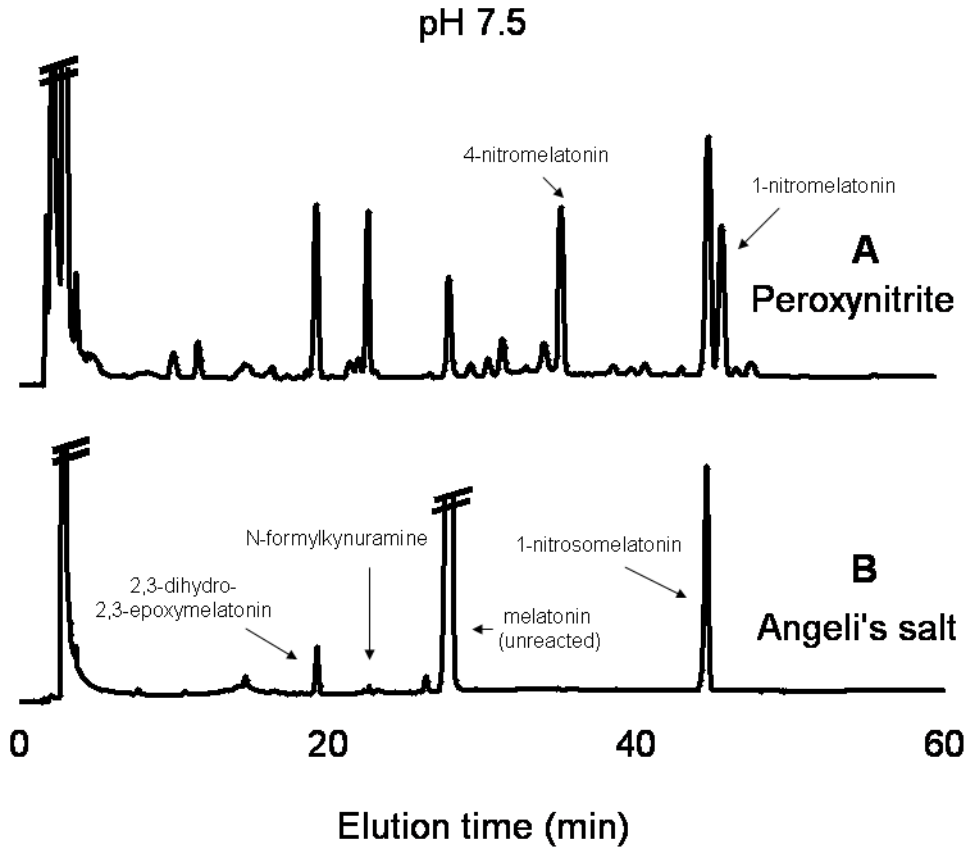

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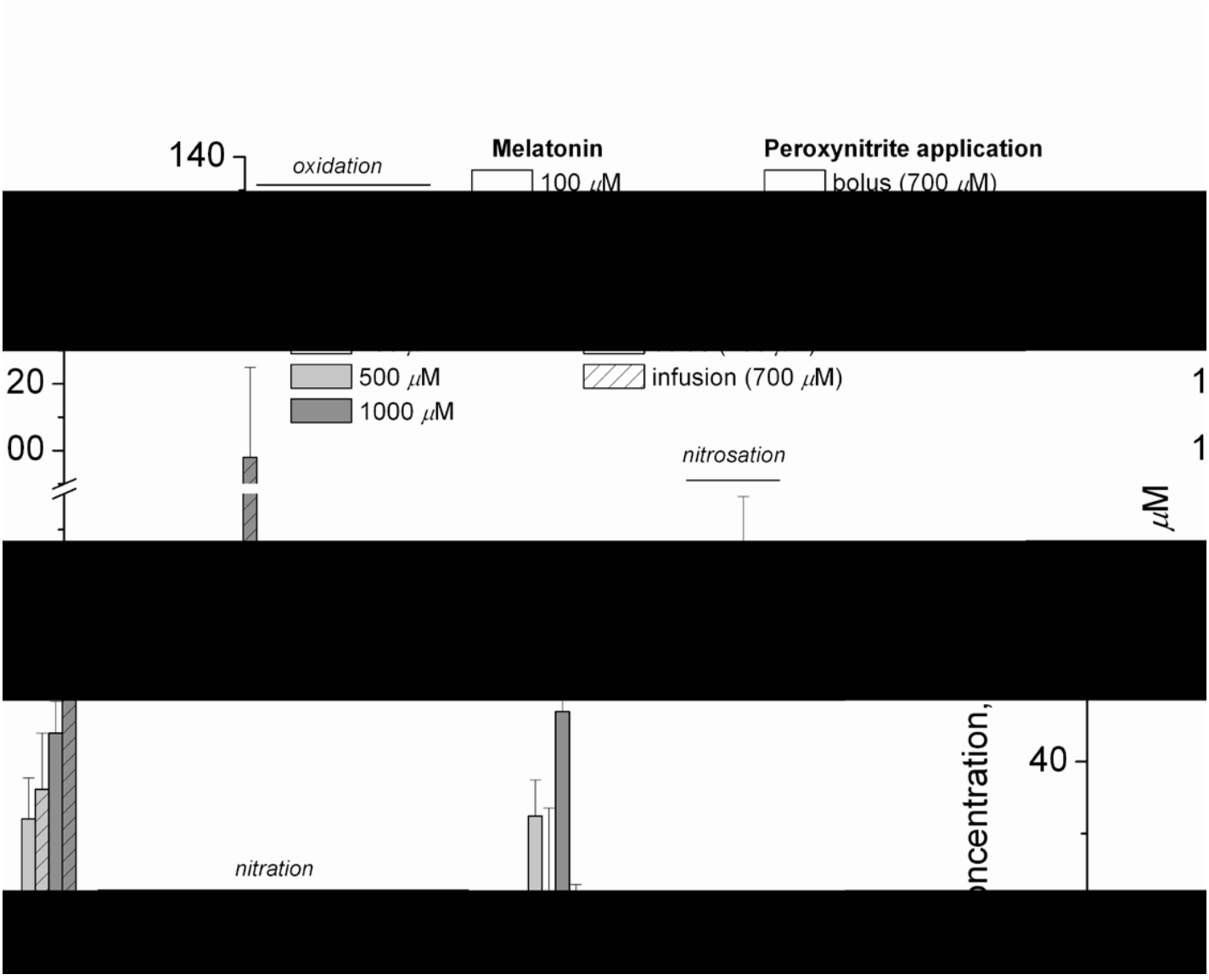

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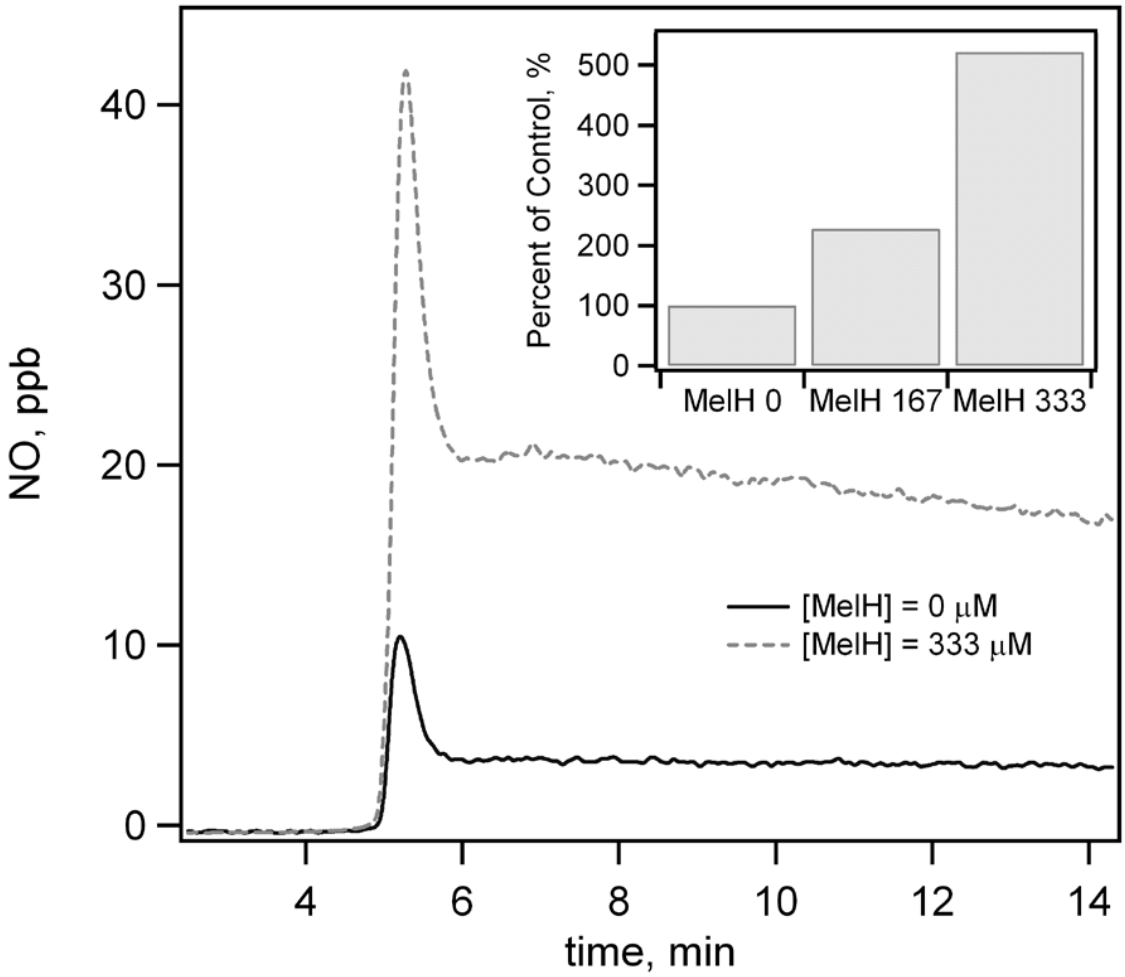

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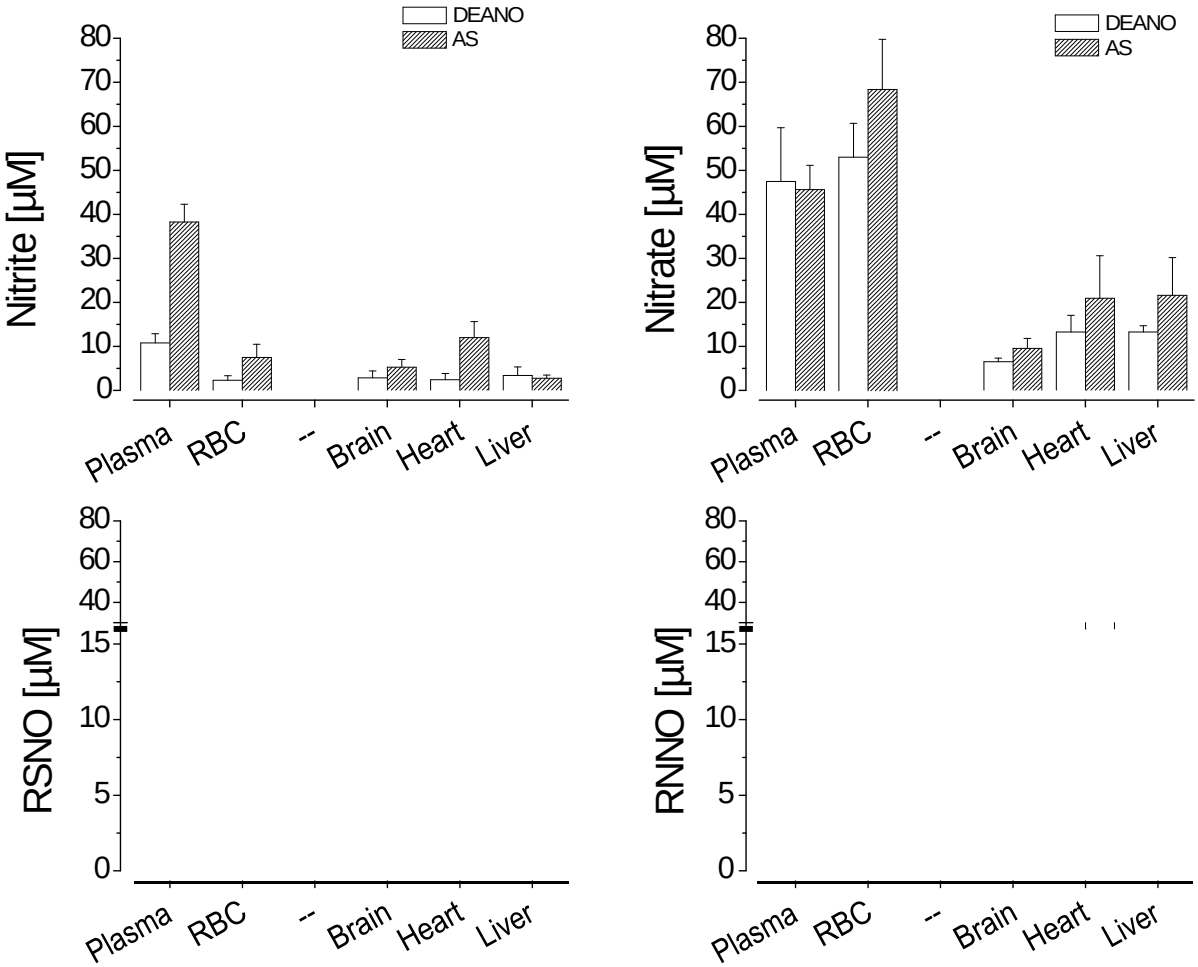
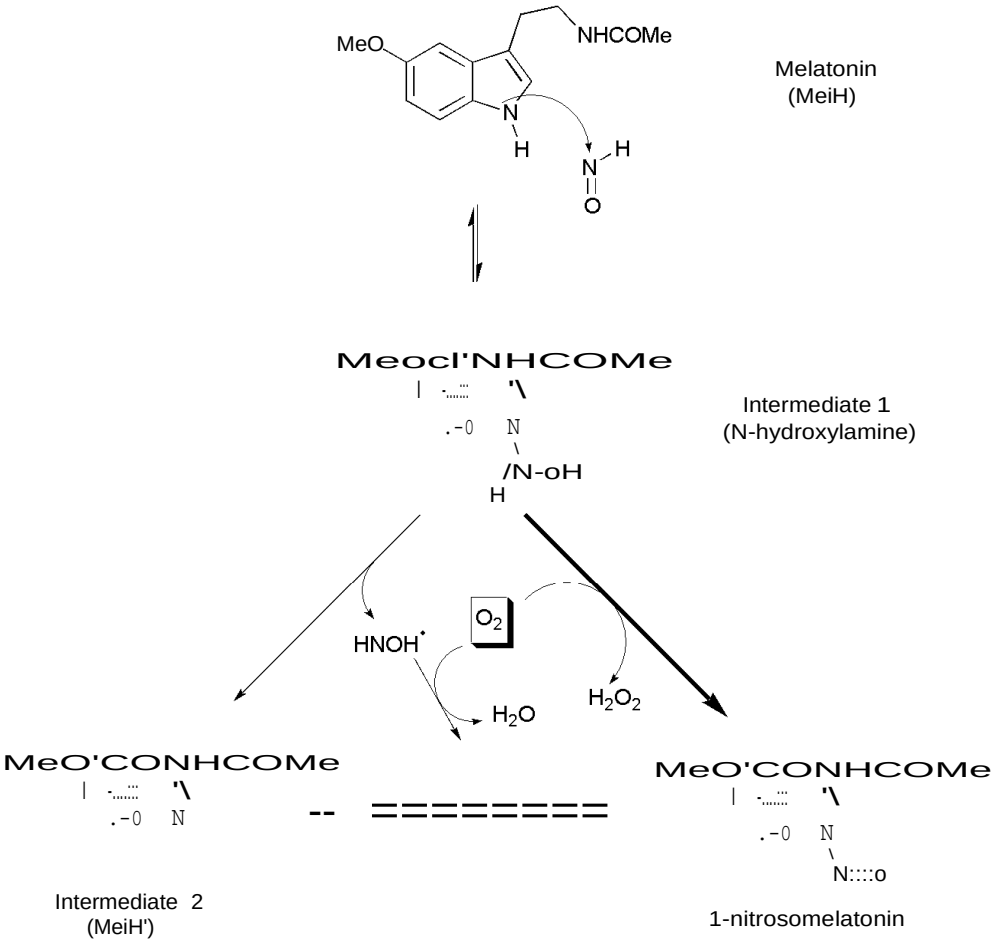


SCHEME TITLES

Scheme 1. Proposed mechanism(s) for the nitrosation of melatonin using Angeli's salt.

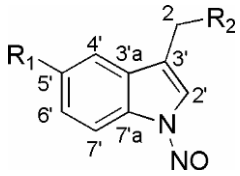


Supporting Information for

N-Nitroso Products from the Reaction of Indoles with Angeli's Salt

Fabienne Peyrot,[†] Bernadette O. Fernandez,[‡] Nathan S. Bryan,[‡] Martin Feelisch,[‡]* Claire Ducrocq[†]*

^{1}H and ^{13}C NMR characteristics of N-acetyl-1-nitroso-L-tryptophan (R_1=H; R_2=C(COOH)NHCOCH$_3$) and 1-nitrosoindol-3-acetic acid (R_1=H; R_2=COOH).



δ (ppm) of N-acetyl-1-nitroso-L-tryptophan (R_1=H; R_2=C(COOH)NHCOCH$_3$) in CD$_3$OD: A : ^{1}H NMR: 1.90 (3H, s, -NHC(O)CH$_3$), 3.09 (dd, J = 15.0, 8.3 Hz, H_{2a}), 3.31 (dd, J = 15.0, 4.9 Hz, H_{2b}), 4.77 (1H, dd, J = 8.3, 4.9 Hz, H_1), 7.42 (1H, dd, J = 7.5, 7.5 Hz, $H_{5'}$), 7.49 (1H, dd, J = 7.8, 7.5 Hz, $H_{6'}$), 7.66 (1H, s, $H_{2'}$), 7.68 (1H, brd, J = 7.5 Hz, $H_{4'}$), 8.13 (1H, brd, J = 7.8 Hz, $H_{7'}$). ^{13}C NMR: δ = 22.5 (-NHC(O)CH$_3$), 28.0 (C_2), 53.8 (C_1), 112.0 ($C_{7'}$), 113.9 ($C_{2'}$), 120.9 ($C_{4'}$), 123.7 ($C_{3'}$), 126.7 ($C_{5'}$), 127.5 ($C_{6'}$), 130.8 ($C_{3'a}$), 136.9 ($C_{7'a}$), 173.2 (-NHC(O)CH$_3$), 174.3 (-COOH). B: ^{1}H NMR: 1.94 (3H, s, -NHC(O)CH$_3$), 3.17 (dd, J = 15.0, 8.3 Hz, H_{2a}), 3.37 (dd, J = 15.0, 5.1 Hz, H_{2b}), 4.84 (1H, dd, J = 8.3, 5.1 Hz, H_1), 7.39 (1H, dd, J = 7.5, 7.5 Hz, $H_{5'}$), 7.43 (1H, dd, J = 7.8, 7.5 Hz,

$H_{6'}$), 7.62 (1H, brd, J = 7.5 Hz, $H_{4'}$), 8.18 (1H, s, $H_{2'}$), 8.29 (1H, brd, J = 7.8 Hz, $H_{7'}$). ^{13}C NMR: δ = 22.5 (-NHC(O)CH_3), 28.0 (C_2), 53.4 (C_1), 116.3 ($C_{7'}$) 119.9 ($C_{4'}$), 122.0 ($C_{3'}$), 122.0 ($C_{6'}$), 128.0 ($C_{2'}$), 128.0 ($C_{5'}$), 129.6 ($C_{7'a}$), 130.5 ($C_{3'a}$), 173.2 (-NHC(O)CH_3), 174.5 (-COOH).

δ (ppm) of 1-nitrosoindol-3-acetic acid (R_1=H; R_2=COOH) in DMSO-d^6: A: ^{1}H NMR: 3.75 (2H, s, H_2), 7.44 (1H, dd, J = 8.0, 8.0 Hz, $H_{5'}$), 7.52 (1H, dd, J = 8.0, 8.0 Hz, $H_{6'}$), 7.66 (1H, d, J = 8.0 Hz, $H_{4'}$), 7.87 (1H, s, $H_{2'}$), 8.16 (1H, d, J = 8.0 Hz, $H_{7'}$). ^{13}C NMR: δ = 30.3 (C_2), 111.1 ($C_{7'}$), 114.1 ($C_{2'}$), 120.8 ($C_{4'}$), 121.1 ($C_{3'}$), 125.9 ($C_{5'}$), 126.7 ($C_{6'}$), 129.3 ($C_{3'a}$), 135.0 ($C_{7'a}$), 171.2 (C_1). B: ^{1}H NMR: 3.79 (2H, s, H_2), 7.44 (1H, dd, J = 8.0, 8.0 Hz, $H_{6'}$), 7.46 (1H, dd, J = 8.0, 8.0 Hz, $H_{5'}$), 7.61 (1H, d, J = 8.0 Hz, $H_{4'}$), 8.26 (1H, dd, J = 8.0, 1.3 Hz, $H_{7'}$), 8.47 (1H, s, $H_{2'}$). ^{13}C NMR: δ = 30.1 (C_2), 115.1 ($C_{7'}$), 119.8 ($C_{3'}$), 119.8 ($C_{4'}$), 126.4 ($C_{2'}$), 127.2 ($C_{6'}$), 127.4 ($C_{5'}$), 127.9 ($C_{7'a}$), 129.0 ($C_{3'a}$), 171.5 (C_1).