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Chemical Evaluation of Compounds as Nitric Oxide or Peroxynitrite Donors using the Reactions with Serotonin

BEATRICE BLANCHARD, CLAUDINE SERVY and CLAIRE DUCROCQ*

Institut de Chimie des Substances Naturelles, C.N.R.S., Avenue de la Terrasse, F-91198 Gif sur Yvette (France)

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The aim of this work was to assess the capacities of some ·NO-donors to release ·NO, and consequently NOx in aerobic medium, or to give peroxynitrite. The method was based on the differential reactivity of serotonin (5-HT) with either NO_x or peroxynitrite, leading in phosphate-buffered solutions to 4-nitroso- and 4-nitro-5-HT formation, respectively. Yields and formation rates of 5-HT derivatives with ·NO-donor were compared to those obtained with authentic ·NO or peroxynitrite in similar conditions. Aside from the capacity of diazenium diolates (SPER/NO and DEA/NO) to release ·NO spontaneously, converting 5-HT exclusively to 4-nitroso-5-HT, all other ·NO donors must undergo redox reactions to produce ·NO. S-nitrosoglutathione (GSNO) and sodium nitroprusside (SNP) modified 5-HT only in the presence of Cu^{2+}, GSNO yielding 6 times more 4-nitroso-5-HT than SNP. Furthermore, in the presence of Cu^{+}, the yield of ·NO-release from GSNO was 45%. The molsidomine metabolite (SIN-1), which was presumed to release both ·NO and $O_2^{\cdot-}$ at pH 7.4, reacted with 5-HT differently, depending on the presence of reductant or oxidant. Under aerobic conditions, SIN-1 acted predominantly as a 5-HT oxidant and also as a poor ·NO and peroxynitrite donor (15% yield of ·NO-release and 14 % yield of peroxynitrite formation). The strong oxidant Cu^{2+}, even in the presence of air oxygen, accelerated oxidation and increased ·NO release from SIN-1 up to 86%. Only a small part of SIN-1 gave simultaneously ·NO and $O_2^{\cdot-}$ able to link together to give peroxynitrite, but other oxidants could enhance ·NO release from SIN-1.

Keywords: NO-donor, peroxynitrite, thionitrite, nitroprusside, NONOate, morpholinosydnonymine, nitrosation, nitration, hydroxytryptamine

Abbreviations: 5-HT, 5-hydroxytryptamine or serotonin; NONOate, diazenium diolate; SPER/NO, spermine bis (nitric oxide) adduct; DEA/NO, diethylamine bis (nitric oxide) adduct; GSNO, S-nitrosoglutathione; SNP, sodium nitroprusside; SIN-1, 3-morpholinosydnonymine; Hb Fe^{II}, hemoglobin; Hb Fe^{III}, methemoglobin; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione; ABTS, 2,2'-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid)

INTRODUCTION

Nitric oxide (·NO)† is a stable and free radical, currently obtained from a variety of chemical species, known as ·NO-donors [1]. Under oxygen, ·NO is converted to ·NO-derived nitrogen oxides that are more reactive towards proteins, lipids, nucleic acids and antioxidants [2]. Thus nitrite and nitrosothiol derivatives are the result of ·NO oxidation in water and medium containing free

* Author to whom correspondence should be addressed. Tel: 33 1 69 82 30 05. Fax: 33 1 69 07 72 47. E-mail: Claire.Ducrocq@icsn.cnrs-gif.fr

† The term peroxynitrite is used to refer to both $ONOO^-$ and ONOOH whose recommended names are oxoperoxonitrate (1-) and hydrogen oxoperoxonitrate, respectively. Nitrogen monoxide is currently named nitric oxide (·NO).

thiols, while 3-nitrotyrosine is formed in proteins by the peroxynitrite† ($ONOO^-$) provided by $^{\cdot}NO$ coupling with superoxide anion ($O_2^{\cdot-}$) [3].

Among the large variety of structures able to release $^{\cdot}NO$, some were used as drugs in the treatment of cardiovascular diseases long before the discovery of endogenous $^{\cdot}NO$ production [4, 5]. Such compounds are potentially beneficial in $^{\cdot}NO$-deficient conditions such as pulmonary hypertension, arteriosclerosis or reperfusion injury [6]. However, mistrust arose when some of them were shown to produce $ONOO^-$. Only sodium diazenium diolates (NONOates), which are adducts of two $^{\cdot}NO$ molecules on amine functions, spontaneously release $^{\cdot}NO$ in solution, at rates dependent on their structure and on the pH of the medium [7]. All other $^{\cdot}NO$-donors do not decompose spontaneously and have to be metabolized to release $^{\cdot}NO$, often through reductive pathways or under illumination [8–15]. Sodium nitroprusside (SNP) is the best known of the nitrosyl-iron complexes which can nitrosate amines and ketones [16]. SNP does not release $^{\cdot}NO$ spontaneously but through photochemical decomposition or by interaction with reducing agents like thiols, hemoglobin (Hb Fe^{II}) or ascorbic acid, to produce disulfide, methemoglobin (Hb Fe^{III}) or dehydroascorbic acid, respectively [8,9]. The molsidomine metabolite (SIN-1) is unique as, under aerobic conditions, it is able to release $^{\cdot}NO$, $O_2^{\cdot-}$ and products of oxidation and coupling simultaneously [17–19].

Recently, we have described the reactivity of $^{\cdot}NO$ and its related oxides (NO_x, $ONOO^-$) with the indolic neurotransmitter 5-hydroxytryptamine (5-HT, serotonin) **1** [20]. $^{\cdot}NO$ is unreactive in an oxygen-free buffer, but at pH 7.4 in the presence of air a significant part of 5-HT is transformed into 4-nitroso-serotonin **3**, probably via N_2O_3 formation. On the other hand, $ONOO^-$ transforms 5-HT into the products of oxidation (5,5′-dihydroxy-4,4′-bitryptamine) **2**, a dimer and products of nitration (4-nitro-serotonin, **4**) [20]. Taking advantage of the differential reactivity of 5-HT towards NOx and $ONOO^-$, we describe here the chemical properties of $^{\cdot}NO$-donors with 5-HT by analyzing 5-HT derivatives formed in aerobic phosphate-buffered solutions at pH 7.4. This assay allowed simultaneous quantification of $^{\cdot}NO$ release and $ONOO^-$ formation from each $^{\cdot}NO$-donor, which is not possible with other available methods [21, 22, 23], some being suitable for $^{\cdot}NO$, others for $ONOO^-$.

MATERIALS AND METHODS

Chemicals

5-HT hydrochloride, ethylenediaminetetraacetic acid (EDTA), sodium nitroprusside (SNP), S-nitroso-glutathione (GSNO), glutathione (GSH), 3-morpholino-sydnonymine (SIN-1), superoxide dismutase (SOD) and cuprous chloride were from Sigma. L-cysteine hydrochloride, copper (II) sulfate, iron (II) sulfate, iron (III) chloride, disodium hydrogen phosphate and sodium dihydrogen phosphate were from Prolabo. Ascorbate was from Fluka Chemie, spermine-nitric oxide (SPER/NO) and diethylamine-nitric oxide (DEA/ NO) complexes were from Research Biochemicals International.

Reactions with $^{\cdot}NO$

A saturated $^{\cdot}NO$ solution was prepared by bubbling $^{\cdot}NO$ gas (Air Liquide, France), which had been previously passed through a degassed 10 M sodium hydroxide solution, through a degassed 0.4 M sodium phosphate buffer (pH 7.4). The final concentration of about 1 mM was measured relative to that of nitrite formed after injecting aliquots into oxygenated solutions of the Griess reagent. $^{\cdot}NO$ concentration was also measured by the oxidation of 2,2′-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS). Aliquots of $^{\cdot}NO$ solutions were injected into a 50 mM ABTS solution and increasing absorbances at 660 and 750 nm (ε=

12 000 and 15 000 $M^{-1}.cm^{-1}$, respectively) were measured [24].

The reaction of ·NO with 5-HT was realized by injecting the ·NO-saturated solution (from 0.1 to 0.9 mL) through a syringe into a stirred phosphate-buffered solution containing 5-HT, to obtain a final 1 mM 5-HT concentration in 1 mL. The mixture was left at 25°C, with or without vigorous stirring, and analyzed by HPLC.

Peroxynitrite synthesis

Was performed in a two-phase system using isoamyl nitrite and hydrogen peroxide following Uppu's method [25]. After decantation at −20°C, this yielded an oil containing up to 6 M peroxynitrite before MnO_2 treatment. The transformations of 5-HT are the same with this oil correctly diluted as with an oil less concentrated after MnO_2 treatment. We therefore preferred to use the very concentrated solution.

Reactions of ·NO-donors

Each NO-donor was first rapidly dissolved in 0.4 M phosphate buffer (pH 7.4) and subsequently diluted in a 5-HT open tube solution in the same buffer, to obtain a final concentration of 1 mM 5-HT in 1 mL. All experiments were carried out in the same size vessel with the same volume of solution. The mixture was homogenized and then stored at 25°C without stirring except when indicated for ·NO and SIN-1. SNP solutions were illuminated with a 150 W Xenophot lamp. When cations were added, they generally complexed 5-HT derivatives, showing characteristic spectral shifts and thus modifying the initial reactivity. Indeed, the order of addition of substrate, metal ions and ·NO-donor is of prime importance in our measurements. GSNO, SIN-1 or SNP was added to a mixture of 1 mM 5-HT and 1 mM metal salt. The results were obtained with or without addition of 1 mM EDTA, as indicated.

HPLC System

Aliquots of the incubation medium were analyzed in a Waters HPLC system using a Shandon Hypersil column (C 18 Silica gel, 5 µm, 250/4.6 mm). Columns were eluted using a gradient of 5%-35% acetonitrile in 0.05% TFA for 50 min at a flow rate of 1 $mL.min^{-1}$. Products were detected by measuring absorbance at 215 and 410 nm. Under these conditions, dimer **2**, 4-nitroso-5-HT **3** and 4-nitro-5-HT **4** were eluted at 11 min, 26 min, and 33 min, respectively. Integrations were calibrated using pure identified compounds as external standards.

5-HT Derivatives

The dimer **2**, 4-nitroso-5-HT **3** and 4-nitro-5-HT **4** were recovered by preparative HPLC of incubation mixtures of 5-HT treated with ·NO gas or peroxynitrite [20]. Lyophilization of the respective fractions produced trifluoroacetate salts of **2** containing 2.5 water molecules (*M*r 678), **3** containing 2 water molecules (*M*r 355) and **4** (*M*r 392). When obtained with NO-donors, products were compared with authentic 5-HT derivatives. Their identifications were confirmed by UV-visible spectroscopy (**2** presents a shoulder at 309 nm; **3** has characteristic absorbances at 385 nm and 515 nm independent of pH from 4 to 8; **4** absorbs specifically at 411 nm) and by molecular mass determination: m/z (M+1) 351 for **2**, 206 for **3** and 222 for **4**. Mass spectrometry was performed by J.-P. Le Caer (Ecole de Physique et Chimie Industrielles de la Ville de Paris) on a VG Platform mass spectrometer (VG Biotech, Manchester, UK) using an electrospray ion source and a quadrupole mass analyzer.

Nitrosation Kinetics of 5-HT

Kinetics were studied by monitoring the formation of **3** at 385 nm ($\varepsilon = 2\ 900\ M^{-1}.cm^{-1}$) or 515 nm ($\varepsilon = 3\ 200\ M^{-1}.cm^{-1}$). The nitrosoderivative and Cu^{2+} formed a complex that absorbed at

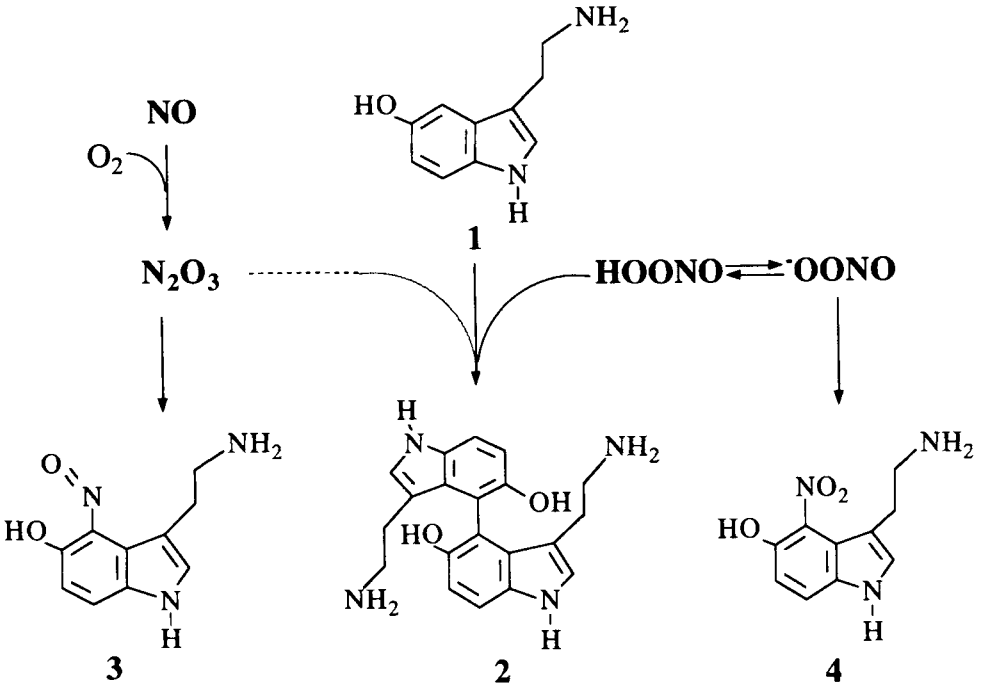


FIGURE 1 Scheme of 5-HT transformations by ·NO in the presence of oxygen or by peroxynitrite

450 nm ($\varepsilon = 5\ 500\ M^{-1}.cm^{-1}$) and 555 nm ($\varepsilon = 3\ 100\ M^{-1}.cm^{-1}$). All experiments were carried out at 25°C, using a 941 Kontron UV-visible spectrophotometer.

RESULTS AND DISCUSSION

We have previously reported that when pure NO was directly injected into a 5-HT solution under air, nitrosation was clearly the main reaction occurring at pH 7.4, while $ONOO^-$ was an oxidizing and nitrating agent of 5-HT [20]. Proposed mechanisms of these reactions are presented in Figure 1.

·NO and NONOates

To assess the amount and rate of reactions between NO_x and 5-HT, we based our estimations on the initial concentration of NO in solution. When 5-HT **1** was treated with NO in phosphate-buffered solution at pH 7.4, a significant transformation of 5-HT was observed by spectrophotometry and HPLC, showing 4-nitroso-5-HT **3** as the major product (Fig. 2a). Beside **3**, small amounts of dimer **2** and 4-nitro-5-HT **4** were also detected. Overall, 5-HT trapped 10% of NO_x, residual 5-HT being entirely recovered and residual NOx hydrolyzed to nitrite. Even if the transformations of 5-HT were limited, their yields evaluated by analytical HPLC were proportional to initial NO concentrations (Fig. 2a). Specifically, 1 mM NO yielded 60 μM **3**.

However, because NO is unreactive in oxygen-free buffered solutions, oxygen diffusion could be a rate-limiting step. Yield of **3** reached the same plateau after 2 hours and 15 minutes, respectively without and with constant stirring

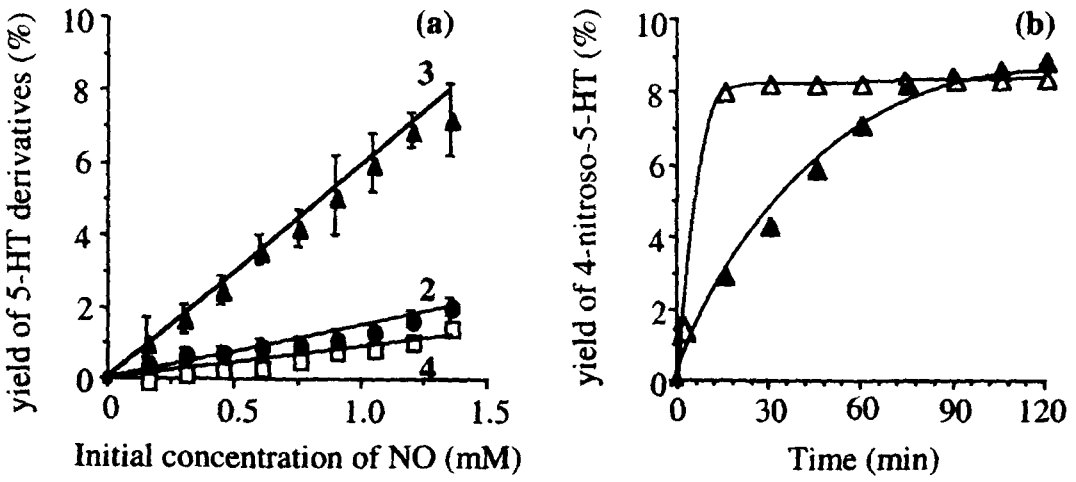


FIGURE 2 Transformations of 1mM 5-HT by ·NO in aerobic phosphate-buffered solutions at pH 7.4 and at 25°C. Yields of 4-nitroso-5-HT **3** (▲), dimer **2** (•) and 4-nitro-5-HT **4** (□) in 2-hour incubations. (**a**). Time-course of **3** formation with (△) and without (▲) stirring (**b**)

of the mixture at 25°C (Fig. 2b), suggesting the observed rate of 5-HT nitrosation is actually that of ·NO oxidation followed by 5-HT reaction. In order to compare 5-HT transformations with each ·NO-donor, controlled *in vitro* conditions were achieved using a 1 mM 5-HT mixture with the ·NO-donor added to the phosphate-buffered solution with stirring and then kept without stirring at 25°C. Under these conditions, evaluation of the yield of ·NO-release was based on the 60 μM **3** obtained from 1 mM ·NO.

Using NONOates (DEA/NO and SPER/NO), which are known to release 2 moles of ·NO per mole with first-order kinetics (corresponding to $t_{1/2}$: 16 min and 4 h for DEA/NO and SPER/NO, respectively, at pH 7.4 and 22°C [26]), the main reaction was clearly the 5-HT nitrosation. In both cases, formation of the dimer and of the nitro-compound was found to be negligible. The transformations lasted 4 and 8 hours, respectively (Fig. 3a, b), showing that the kinetic rate reflected the addition of ·NO-release,·NO oxidation and 5-HT nitrosation rates. Nevertheless, considering yields, the concentrations of the nitrosoderivative **3** were proportional to those of NONOate up to 6 equivalents (Fig. 3c, d) and reached 80% and 70% for DEA/NO and SPER/NO, respectively. The yield of **3** (60 μM) obtained with 0.5 mM of each NONOate (able to give 1 mM ·NO) was that obtained with 1 mM of authentic ·NO (Fig. 2). This demonstrated a quantitative yield of ·NO release from NONOate and no loss of ·NO and NOx from solutions. Furthermore, the ·NO delivered by a flux from NONOate or administered by one bolus gave the same 5-HT nitrosation yield.

S-Nitroso-glutathione

5-HT and S-nitrosoglutathione (GSNO) did not react with each other in buffered solutions deprived of transition metals. Literature findings suggest that Hg^{2+}, Cu^{+} and Cu^{2+} should be considered. S-nitrosothiol degradation by Hg^{2+} has been used as the basis for an analytical procedure for S-nitrosothiol determination [27]. In experiments using GSNO, 5-HT and Hg^{2+}, a 55% nitrosation yield of 5-HT was reached in 2 min

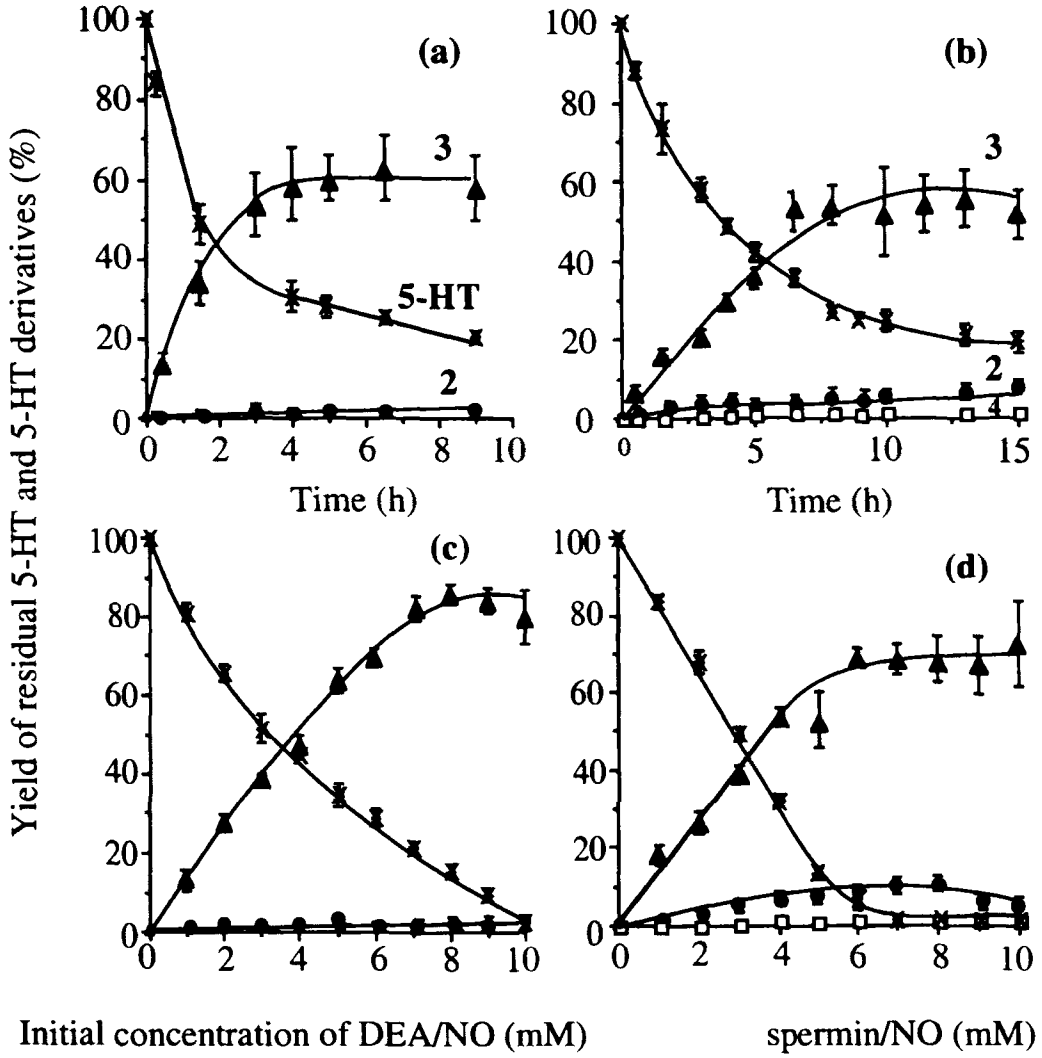


FIGURE 3 Time-course and yield of 1mM 5-HT transformations by diazeniumdiolates in aerobic phosphate-buffered solutions at pH 7.4 and 25°C. Time-course of residual 5-HT (*), 4-nitroso-5-HT **3**(▲), dimer **2** (•) and 4-nitro-5-HT **4** (□) formation obtained with 5mM DEA/ NO (**a**) or with 5mM spermine/NO (**b**). Yields obtained with each concentration of DEA/NO after 4-hour incubation (**c**) and spermine/NO after 8-hour incubation (**d**)

(Fig. 4a). Such a yield and rate were not achievable by quantitative release of ·NO from 5 mM GSNO. All these observations are in agreement with the formation of a $[GS(Hg)NO]^{2+}$ complex unable to release free ·NO, but able to nitrosate water to nitrite [27] or 5-HT to **3**:

$$[GS(Hg)NO]^{2+} + 5 - HT \longrightarrow [GSHg]^{+} + 4 - nitroso - 5 - HT + H^{+}$$

S-nitrosothiol decomposition to ·NO has been found to be catalyzed by Cu^{2+} and Cu^{+} [13–15]. Beside acting on the degradation pathway of GSNO into GSSG and ·NO release, Cu^{2+} also catalyzed the slow oxidation of 5-HT (4% of **2** was obtained in 6 hours instead of 0.1% in the absence of Cu^{2+} ions at pH 7.4).

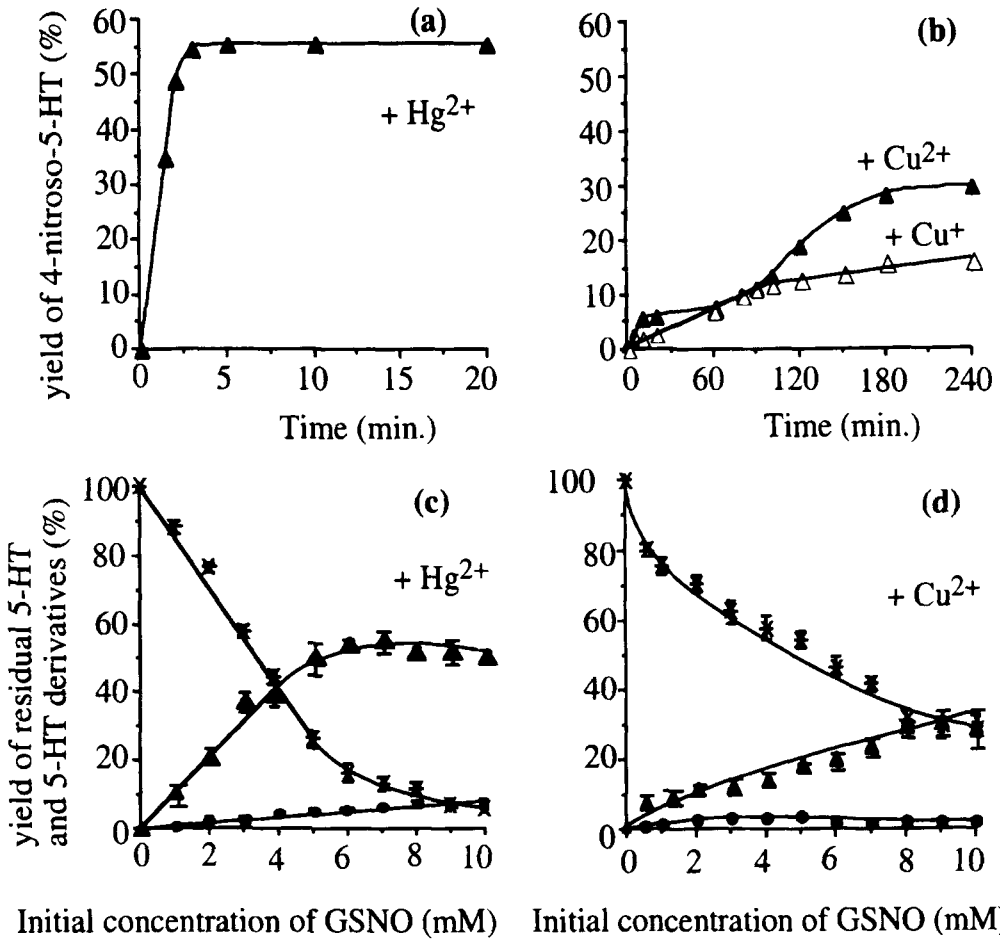
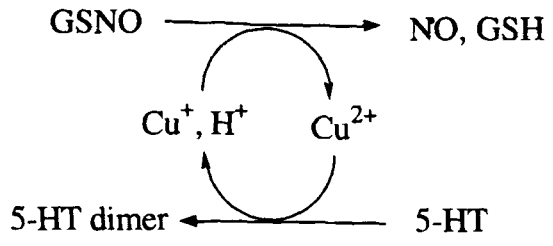


FIGURE 4 Time-course and yield of 1mM 5-HT transformation by S-nitrosoglutathione in aerobic phosphate-buffered solutions at pH 7.4 and 25°C. Time-course of residual 5-HT (*), 4-nitroso-5-HT **3**(▲) and dimer **2** (•) formation obtained by 5mM S-nitrosoglutathione in the presence of mercuric (**a**) or copper ions (**b**). Yields were obtained with each concentration of S-nitrosoglutathione after 2-hour incubation in the presence of copper sulfate (**d**) and after 5 minutes in the presence of mercuric chloride (**c**)



In incubation mixtures involving 5-HT, GSNO and Cu^{2+}, 5-HT was mostly nitrosated but also oxidized by Cu^{2+} at the same rate as without nitrosothiol (dashed line in Fig. 6b). Nitrosation increased with concentrations of GSNO up to 8 mM (Fig 4d). At 5 mM GSNO, the yield of **3** was 25% in the presence of Cu^{2+} and 15% in the presence of Cu^{+} (Fig. 4b). Taking into account that Cu^{+} has been recognized as the efficient reagent accounting for ·NO release from GSNO, and that Cu^{2+} was reduced by 5-HT to Cu^{+}, the increase in nitrosation yield observed in the presence of Cu^{2+} could be attributed to the formation of a nitrosating $[GS(Cu)NO]^{2+}$ complex (Fig. 5) in the

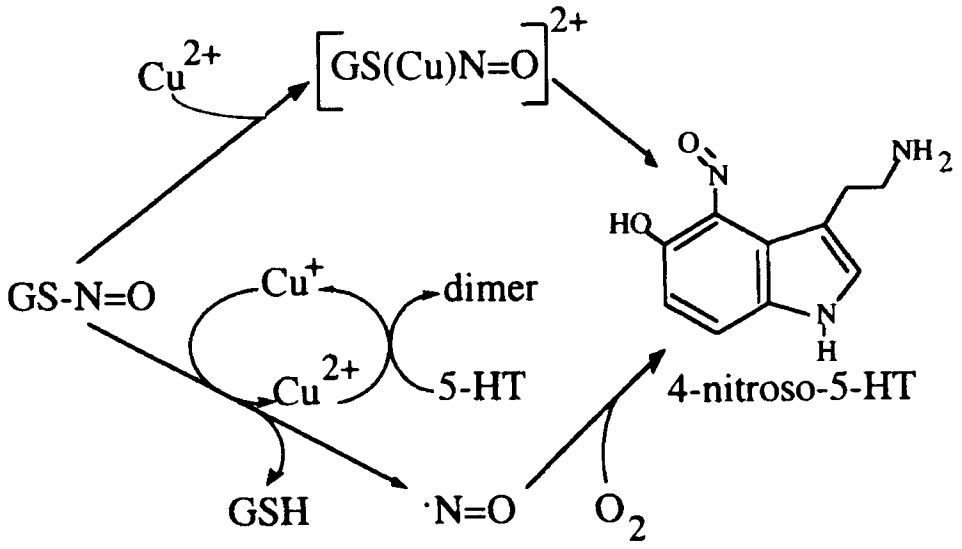


FIGURE 5 Proposed scheme of 5-HT transformations by GSNO in the presence of Cu^{2+}

same manner as with Hg^{2+}, whereas Hg^{2+} was regarded as more effective than Cu^{2+}. The multiple reactions of Cu^{2+} with 5-HT and GSNO could also account for the complex nitrosation kinetics observed (Fig. 4b). Only in the presence of Cu^{+} can we calculate a real ·NO-release yield (45%) in comparison with authentic ·NO.

When GSH or ascorbate was added to the mixture of 5-HT, GSNO and metal cations, none of the 5-HT derivatives could be detected, suggesting an efficient quenching of the oxidized derivatives of ·NO by GSH as well as by ascorbate (results not shown).

Sodium Nitroprusside

Degradation pathways of sodium nitroprusside (SNP) to ·NO under illumination, have been described [28].

$$[Fe_{III}(CN)_5NO]^{3-} \xrightarrow{h\nu} [Fe_{II}(CN)_5NO]^{4-} \longleftrightarrow [Fe_{II}(CN)_4NO]^{3-} \longrightarrow NO$$

Under illumination, the main and first reaction of 5-HT was an oxidation to **2** in 4 hours (17% corresponding to 34% of consumed 5-HT obtained with 5 equivalents of SNP). A minor part of this oxidation was related to illumination (2% in 4 hours). Besides oxidation, significant amounts of nitrosation and nitration were detected (8% and 4% of 5-HT, respectively, with 5 equivalents of SNP) (Fig. 6a). Nitrosation could account for a slow and small release of ·NO.

Reductive metabolism of SNP under aerobic conditions has been shown previously to be accompanied by the activation of molecular oxygen [9]. In accordance with this observation, the detection of **4** should be due to $ONOO^-$ formation under our conditions. Generation of $O_2^{\cdot-}$ and further oxidation of ·NO is the most likely mechanism of $ONOO^-$ formation through photolytic decomposition of SNP, in the same manner as with GSNO used under similar conditions [29].

In the dark, 5-HT was unable to reduce SNP and thus release ·NO. Surprisingly, among several metal ions assayed, only Cu^{2+} allowed sig-

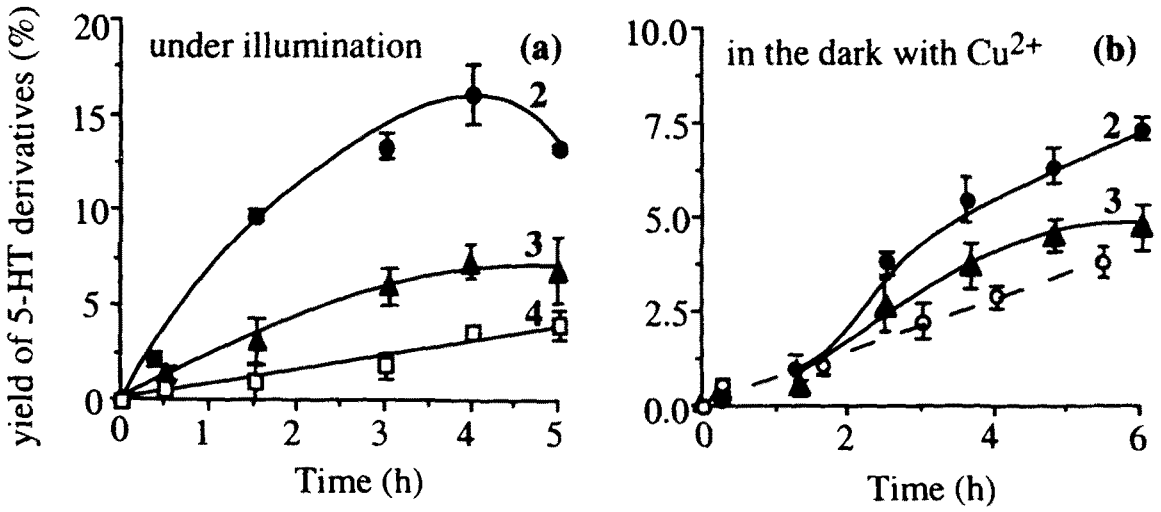


FIGURE 6 Time-course of 1 mM 5-HT transformation by SNP in aerobic phosphate-buffered solutions at pH 7.4 and 25°C. Formation of dimer **2** (•), 4-nitroso-5-HT **3** (▲) and 4-nitro-5-HT **4** (□) by 5mM SNP, under illumination (**a**) and in the dark and the presence of 1mM copper sulfate and 1mM EDTA (**b**). The time-course of oxidation of 1mM 5-HT with 1mM copper sulfate (◦), in the dark, is also shown (dashed line, (**b**))

nificant nitrosation and oxidation of 5-HT (Fig. 6b). Less than 2% of 5-HT transformations were observed in the presence of Fe^{2+}, Fe^{3+} or Cu^{+} (data not shown). In the presence of Cu^{2+} ions, 5-HT dimerization was catalyzed (increasing linearly with time to 4% in 6 hours) and increased with SNP. It was accompanied in that case by nitrosation (5% in 5 hours), showing the cupric cations could interfere with SNP.

The Molsidomine Metabolite, SIN-1

SIN-1 has to undergo an oxidative step by molecular O_2 to give $O_2^{\cdot-}$ and the SIN-1A radical which is in turn able to release $^{\cdot}NO$ spontaneously [17–19]. We verified that O_2 was obligatory for observation of any transformation of 5-HT by SIN-1 (Fig. 8). Under air, SIN-1 decomposed and led to oxidation, nitrosation and nitration of 5-HT in yields dependent on the concentration of SIN-1 (Fig. 7b). The main reaction, dimerization, was maximal with a SIN-1/5-HT ratio of 3 (Fig. 7b). Nitrosation and nitration yields indicated that NO and $ONOO^{-}$ were formed both at low level from SIN-1 (1% of 5-HT) (Fig. 7a), corresponding to a 15% yield of $^{\cdot}NO$-release and a 14% yield of $ONOO^{-}$-formation, in comparison with data obtained from authentic $^{\cdot}NO$ or peroxynitrite. This low nitration yield could not be attributed to an eventual exhausting of dissolved oxygen, whereas with constant stirring transformations of 5-HT gave the same yields (results not shown). Nitration was due to $ONOO^{-}$ formation because it was inhibited by the addition of 1000 units/ml of superoxide dismutase, without any change in oxidation and nitrosation. 5-HT oxidation by SIN-1 could be hypothesized in which the SIN-1A radical gives an electron to 5-HT regenerating SIN-1A (Fig. 8). This would also explain the slower rate of SIN-1 decay, which is halved in the presence of 5-HT (results not shown).

An equimolecular concentration of the strong oxidant Cu^{2+} led to a more rapid conversion of 5-HT into **2** and an enhanced yield of **3**, without change in **4** formation (Fig. 7a). Under these con-

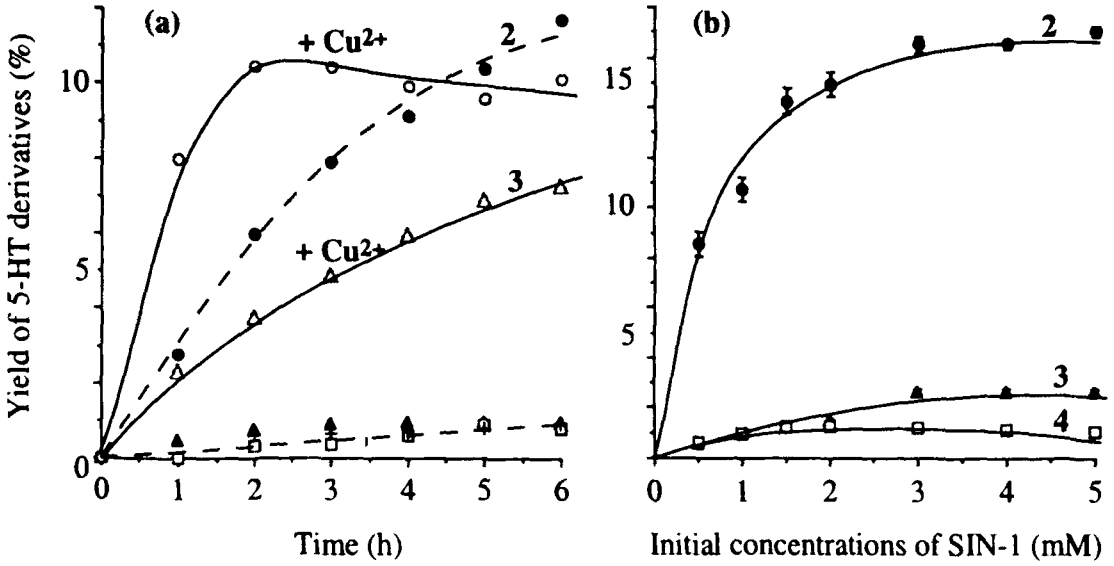


FIGURE 7 Time-course and yield of 1mM 5-HT transformations by SIN-1 in aerobic phosphate-buffered solution at pH 7.4 and 25°C. Formation of dimer **2** (•, ◦), 4-nitroso-5-HT **3** (▲, △) and 4-nitro-5-HT **4** (□, +) obtained by the reaction of 1mM SIN-1, in the absence (dashed line) and presence (full line) of 1mM copper sulfate and 1mM EDTA (**a**). Yields were evaluated for each SIN-1 concentration, with continuous stirring or without, in the absence of cupric ions, in a 6-hour incubation (**b**)

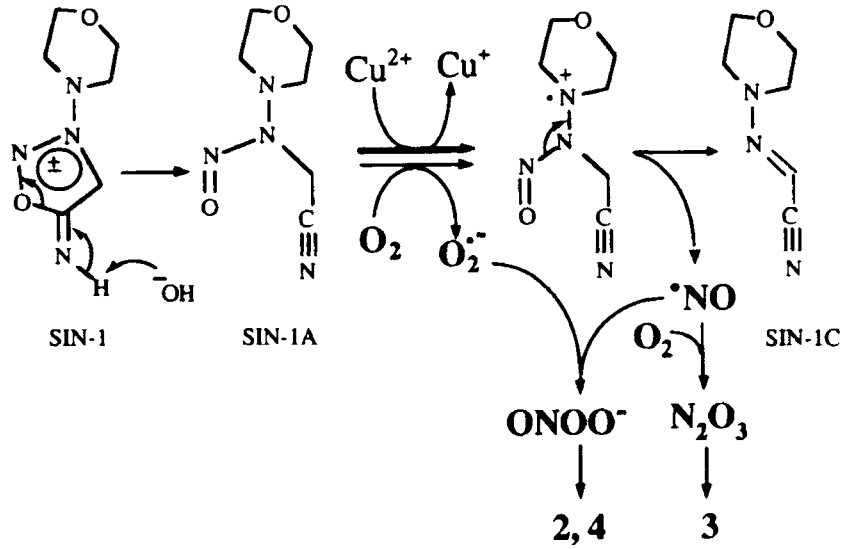


FIGURE 8 Proposed scheme of SIN-1 degradation and 5-HT transformations

ditions, considering the extent of nitrosation, the yield of $^{\cdot}NO$ release from SIN-1 increased up to 6-fold, corresponding to an 86% $^{\cdot}NO$-release yield from SIN-1. Beside the route of spontaneous oxidation of SIN-1A by O_2, another type of oxidation by Cu^{2+} was superimposed leading to $^{\cdot}NO$-release without $ONOO^-$ formation (Fig. 8). Indeed, SIN-1 behaved as a real $^{\cdot}NO$-donor in the presence of Cu^{2+}. This effect of Cu^{2+} has already been suggested [17–19] without the idea of the ratio of $^{\cdot}NO$ and $ONOO^-$ produced.

In comparison with 5-HT, the ability of SIN-1 to dimerize and nitrate the free tyrosine has been considered [30]. The oxidative action of SIN-1 was observed by dityrosine formation. The dityrosine / 3-nitrotyrosine ratio was reported to be higher with SIN-1 than with $ONOO^-$ [31], in the same manner that the **2 / 4** ratio was found to be 10 and above with SIN-1 (Fig. 7) and 1 with $ONOO^-$ [20].

Recently, oxidation of tyrosine by low concentration of $ONOO^-$ was shown to be the predominant pathway of the $ONOO^-$ action [32]. Such an oxidation leading to dityrosine formation and protein cross-linking, was an important feature of the action of *in vivo* $ONOO^-$ which was detected in numerous diseases associated with oxidative stress.

CONCLUSION

We have compared $^{\cdot}NO$-donors and predicted peroxynitrite effects for some of them, through the reactions they undergo with 5-HT. As $^{\cdot}NO$ oxidation and subsequent 5-HT reactions with the resulting nitrogen oxides (NO_x) formed in aerobic neutral aqueous solutions were fully reproducible, our results reflect the differences in the rate and level of $^{\cdot}NO$ delivery from each $^{\cdot}NO$-donor. Specifically, the delivery of $^{\cdot}NO$ from diazeniumdiolates, referred to as NONOates, appears very effective. The NONOate anions are very practical tools in generating $^{\cdot}NO$ spontaneously in physiological fluids or culture media where controlled and quantifiable exposure of $^{\cdot}NO$ is desired [7]. All other NO donors still used as drugs (SNP, molsidomine) are severely regulated by factors such as oxygen, reductants (e.g. 5-HT) and cupric or cuprous ions. For instance, in the presence of cupric ions, SIN-1 acts mainly as an $^{\cdot}NO$-donor and a poor peroxynitrite producer.

Furthermore, copper levels have been found to increase during the progression of atherogenesis, myocardial infarction and reperfusion injury. While cupric ions might contribute to these processes through deleterious reactions with membranes and lipoproteins, they could also play a protective role, enhancing the rate of NO-release from S-nitrosothiols [33] as well as from SNP or sydnonimines during treatment of cardiovascular diseases.

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