Free Radical Research, Vol. 35, pp. 857–866
Reprints available directly from the publisher
Photocopying permitted by license only

1-Nitrosomelatonin is a Spontaneous NO-releasing Compound

BEATRICE BLANCHARD-FILLION, CLAUDINE SERVY and CLAIRE DUCROCQ*

Institut de Chimie des Substances Naturelles, CNRS SCUPR 2301, 91190 Gif-sur-Yvette, France

Accepted by Professor N. Taniguchi

(Received 1 December 2000; In revised form 26 March 2001)

Melatonin (*N*-acetyl-5-methoxytryptamin), the main hormone secreted by the pineal gland in mammals, is nitrosated by nitrite at acidic pH and by NO in the presence of oxygen under neutral conditions. Melatonin is also partly converted to 1-nitrosomelatonin by oxoperoxonitrate ($ONOO^-$, peroxynitrite) in phosphate-buffered solutions at pH 7–10 [Blanchard, B., *et al.* (2000) *Journal of Pineal Research* **29**, 184–192]. In the present report, we show that 1-nitrosomelatonin in turn behaves as an NO-donor regenerating melatonin. NO-release is evidenced by the formation of nitrite in phosphate-buffered solutions and oxidation of HbO_2. No peroxynitrite was formed during that decomposition because serotonin used as a probe was converted only to 4-nitroso-serotonin as expected for a true NO-donor [Blanchard, B., *et al.* (2001) *Free Radical Research*, 34, 177–188]. The spontaneous decay of 1-nitrosomelatonin is not affected by GSH and metallic ions but its decomposition is accelerated in acidic pH or in the presence of NADH or ascorbate. Furthermore, melatonin is partially or entirely recovered in the absence or presence of ascorbate, respectively. A homolytic cleavage of 1-nitrosomelatonin is strongly suggested and discussed. Formation of 1-nitrosomelatonin from melatonin and reactive nitrogen species (RNS) followed by its decay into NO demonstrates that melatonin could reduce these RNS to NO.

Keywords: 1-Nitrosomelatonin; NO-donors; reactive nitrogen species

INTRODUCTION

In the past decade, nitric oxide (NO) has been discovered to be a major signalling agent of great importance throughout the animal kingdom. Endogenous NO is generated from the oxidation of L-arginine by the catalytic activity of NO-synthases and also by redox-sensitive release from multiple forms of storage such as S-, Fe- and N-nitrosocompounds. 3-Substituted indoles occur widely in nature, such as tryptophan derivatives and plant growth hormones, indole-3-acetic acid and indole-3-acetonitrile. Melatonin (*N*-acetyl-5-methoxytryptamine) is present in most plants and is endogenously synthesized in

*Corresponding author. Tel.: +33-1-69-82-30-05. Fax: +33-1-69-07-72-47. E-mail: claire.ducrocq@icsn.cnrs-gif.fr

vertebrates especially in the pineal gland.[1–3] Its oral ingestion in large quantities is believed to promote the action of anti-aging and anti-infectious drugs or to complement anti-tumor treatment.[4] However, like other indole-containing compounds,[5] melatonin can be nitrosated on the pyrrolic nitrogen by various routes. The same nitrosation reaction seen with nitrous acid was found to occur with NO in the presence of O_2, or oxoperoxonitrate (currently named peroxynitrite, $ONOO^-$) in neutral and slightly basic aqueous solution.[6] Indeed, this transformation can take place in animal tissues during inflammation or in areas where superoxide anion and $ONOO^-$ are produced. Whereas $ONOO^-$ often appears as a strong oxidant at pH 7 in the presence of CO_2, some evidence suggests that it could also be oxidized to give rise to a milder oxidant, the $ONOO^{\bullet}$ radical,[7] which is able to nitrosate phenols, such as propofol or melatonin.[6,8]

From melatonin, the 1-nitrosoderivative produced by these various pathways was found to be unstable in aqueous solutions under physiological conditions and the aim of this work was to identify the products of this decomposition and the physicochemical conditions in order to investigate the mechanism involved. If 1-nitrosomelatonin decay produces NO as demonstrated here, whereas its synthesis involves reactive nitrogen species (RNS), such a cycle suggests that melatonin could play an antioxidant role by reducing RNS.

MATERIALS AND METHODS

Chemicals

Melatonin, 5-hydroxytryptamin hydrochloride, glutathione (GSH), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), chelating resin (iminodiacetic acid), chelex 100 were from Sigma (St. Louis, MO). Diethylamine–nitric oxide (DEA/NO) and dipropylenetriamine/NONOate (DPTA/NO) with a 2 min and 5 h, respectively, half-life at physiological pH and temperature, were from Research Biochemicals International (San Diego, CA). Ascorbate was from Fluka Chemie. Dimethylsulphoxide (DMSO), copper (II) sulfate, iron (II) sulfate, iron (III) chloride, sodium phosphates and tris(hydroxymethyl)aminomethane (TRIS) were from Prolabo (France). Melatonin was first dissolved in DMSO before dilution in 0.4 M sodium phosphate-buffered solution (pH 7.4) or in 50 mM TRIS–1 mM EDTA (pH 7.4) containing less than 1% DMSO.

Preparation of the 1-nitrosomelatonin

1-Nitrosomelatonin was obtained from the reaction between melatonin (5 mM) and NO (Air Liquide, France) in an aerated melatonin solution in 0.4 M phosphate buffer (pH 7.4).[6]

Authentic crystalline 1-nitrosomelatonin was prepared following the procedure used for the synthesis of 1-nitrosoindole acetic acid.[5] Yellow crystals (mp 138°C) were dissolved in DMSO and diluted in phosphate-buffered solution at pH 8 up to 1% DMSO. Identity was confirmed by spectrophotometry showing the characteristic absorbance at 346 nm ($\varepsilon = 8300\,M^{-1}\,cm^{-1}$), HPLC analysis, ^1H NMR, mass spectrometry by chemical ionization and centesimal elementary analysis.[6]

HPLC System

The analytical system consisted of a Waters injector and a gradient pump linked to a Shandon (C18 Si, 5 μm) Hypersyl column (46 × 250 mm). The column was eluted using a 10–50% gradient of acetonitrile with 0.05% trifluoroacetic acid (TFA) for 60 min at a flow rate of 1 ml min^{-1}. Compounds were detected by measuring UV absorbance at 215 and 350 nm. HPLC analysis of the products formed in the reaction mixture showed peaks eluting at 35 and 55 min corresponding to melatonin and 1-nitrosomelatonin, respectively. HPLC combined to mass

spectrometry by electrospray ionization analysis results in protonated $[M + H]^+$ peak corresponding to m/z 233 for melatonin and melatonyl radical (m/z 232) for 1-nitrosomelatonin.

Decomposition in Aqueous Solutions

Kinetics of 1-nitrosomelatonin decomposition was followed at 25°C by spectrophotometry at 346 nm ($\varepsilon = 8300\,M^{-1}\,cm^{-1}$) in phosphate (pH 7.4) or in TRIS–EDTA (pH 7.5) containing less than 1% DMSO. In the presence of NADH or NADPH, kinetics was followed at 400 nm ($\varepsilon = 2000\,M^{-1}\,cm^{-1}$).

Nitrite Measurement

When formed in aerated neutral solutions, NO is quantitatively converted into nitrite. Nitrite concentration was measured by the Griess reaction only after complete decomposition of 1-nitrosomelatonin.

Detection of NO-release from 1-nitrosomelatonin by Oxidation of Oxyhaemoglobin (oxyHb) and by IsoNO Electrode

OxyHb (Sigma) was previously reduced by sodium dithionite treatment and purified on Sephadex G25.[9] Differential spectra of a solution containing 0.05 mM oxyHb and 0.01 mM 1-nitrosomelatonin against 0.05 mM oxyHb in phosphate-buffered solutions at pH 7.4 were recorded every 3 min and superimposed in the first 30 min of decay. An identical assay was performed with a NONOate, DPTA/NO. DPTA/NO with oxyHb showed the same peaks and isobestic points as obtained with 1-nitrosomelatonin.

An NO-output signal was obtained through an electrode sensor ISO-NO immersed in a freshly prepared 1-nitrosomelatonin solution, and was collected using a Duo 18 data acquisition system (World Precision Instruments, Sarasota, FL). Electrodes were calibrated with aliquots of a saturated NO-solution injected in the neutral phosphate-buffered solutions or with added diethylaminodiazenium diolate (DEA/NO) NONOate, which has a shorter half-life time. However, this method which unambiguously reveals the formation of NO in solution, cannot give a quantitative yield of NO released from slow NO-donors, such as 1-nitrosomelatonin or DPTA/NO.

Measurement of NO-release from 1-nitrosomelatonin by Reaction with Serotonin in Aqueous Neutral Solution

1-Nitrosomelatonin, first dissolved in DMSO, was then diluted (2.5 mM) in a 0.4 M sodium phosphate-buffered (pH 7.4) solution containing serotonin (0.5 mM). The reaction was monitored by HPLC analysis. Using an elution system with a gradient of 5–35% acetonitrile in 0.05% TFA in water for 50 min, serotonin (5-HT), 4-nitroso-5-HT and 4-nitro-5-HT were eluted at 20, 26 and 33 min, respectively, and were quantified using external standards.[10]

RESULTS

When melatonin was treated with NO in the presence of oxygen or with peroxynitrite in neutral aqueous buffered solutions, analysis of the medium revealed that a part of the melatonin was converted to 1-nitrosomelatonin, which slowly disappeared, and the level of melatonin seemed to rise again. Similar observations were made using No-solution in methanol (Fig. 1). The identity of the compound identified by HPLC as melatonin was checked by mass spectrometry and spectrophotometry. In the same manner, synthesized crystallized 1-nitrosomelatonin decomposed spontaneously when dissolved in aqueous or organic medium: for instance, 80% of 1 mM 1-nitrosomelatonin decomposed for 6 h in phosphate-buffered solutions at pH 7.4 (Fig. 2). Following the decrease in absorbance at 350 nm, pseudo-first-order rates of degradation were

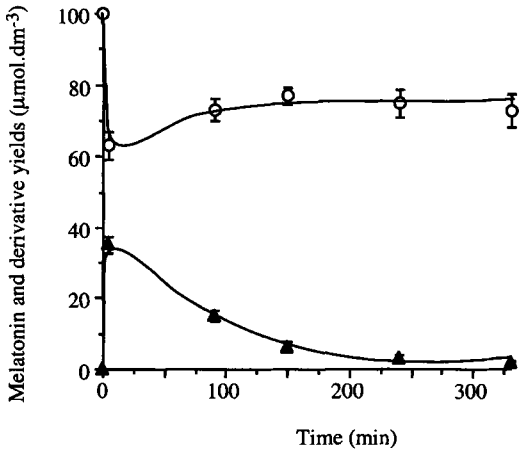

FIGURE 1 Time-course of the formation of 1-nitrosomelatonin (▲) from 1 mM melatonin (○) and 5 mM NO in the presence of oxygen and the following decomposition in methanol.

observed with a pH-dependent kinetic rate constant: $k_{obs} = 7.0 \pm 0.5 \times 10^{-5}\,s^{-1}$ at 25°C and pH 7.4. The decomposition was accelerated with decreasing pH (Fig. 3) in proportion to a logarithmic scale of the kinetic rate constant, suggesting that proton concentration has an effect in inducing decomposition.

We investigated the nature of the nitroso group released from 1-nitrosomelatonin and whether free NO was liberated. First, in aqueous solution buffered at pH 6, a quantitative yield of nitrite was measured after complete 1-nitrosomelatonin decay; this yield dropped to 75% at pH 7.4 and did not vary in the presence of reducing agents like ascorbate, NADH, NADPH, GSH or albumin. It is noteworthy that transnitrosation towards free thiols did not occur since GSNO is stable under these conditions.

During the 1-nitrosomelatonin decomposition in the presence of oxyhaemoglobin (oxyHb), oxyHb was converted into methaemoglobin (Fig. 4). Figure 4 shows the 1-nitrosomelatonin decomposition in terms of decreasing absorbance at 250, 270 and 346 nm, in the same time than the conversion of oxyhaemoglobin (oxyHb) at 420 nm and formation of methaemoglobin at 402 nm with a characteristic isobestic point at 411 nm. Similar profiles were obtained with DTPA/NO (known to produce 1 mol of NO per mole in 5 h at physiological pH and temperature).

We have previously described a method of measuring the NO and $ONOO^-$ formed in the course of NO-donor decay, using serotonin (5-HT) as a probe.[10] Briefly, this method is based on the measurement by quantitative HPLC analysis of the 60 μM of 4-nitrososerotonin (4-NO-5-HT) produced from 1 mM NO and 1 mM 5-HT in phosphate-buffered solution at pH 7.4. In the presence of 1 mM serotonin (5-HT), the 1-nitrosomelatonin decay paralleled the formation of 4-NO-5-HT (Fig. 5), without detection of any 4-nitro-serotonin. This result showed that no $ONOO^-$ was formed during 1-nitrosomelatonin decay. Since 10% 4-NO-5-HT was obtained from 2.5 mM 1-nitrosomelatonin, 1.5 mM NO has to be released from 1-nitrosomelatonin, corresponding to 60% release of NO when 85% 1-nitrosomelatonin has decomposed. This yield correlates well with the 75% nitrite yield previously described. All these results point out a free NO-release.

In terms of the indole ring, 50% of melatonin was recovered following 1-nitrosomelatonin decay in phosphate-buffered solutions (Fig. 2). HPLC analysis with the usual 215 nm detection did not allow us to observe what becomes of the other half of melatonin but detection at 350 nm (Fig. 6) showed that several compounds were formed corresponding to oxidized derivatives of melatonin according to mass spectrometric analysis of each HPLC peak. If M is the molecular mass of melatonin, m/z 248, 265 and 280 could correspond to $[M + O + H]^+$, $[M + 2O + H]^+$, $[M^{\cdot} + 3O + H^+]$, respectively. Electrospray MS was able to detect protonated radicals. However, the 250 mass has not been interpreted yet. Furthermore, when argon was bubbled through the solution of 1-nitrosomelatonin for 30 min to eliminate oxygen, before the addition of peroxynitrite, the amounts of all these oxidized derivatives decreased significantly (data not shown). This is in agreement with the transient formation

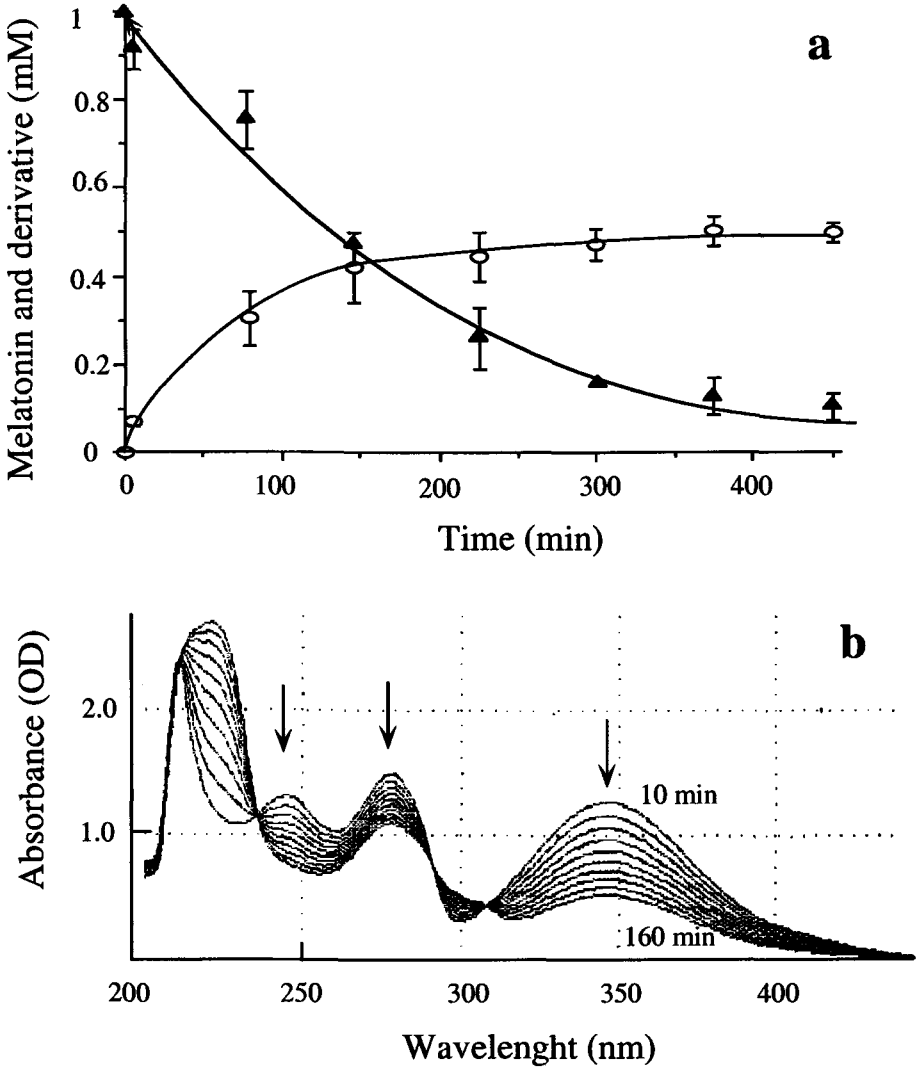

FIGURE 2 Time-course of the decomposition of 1-nitrosomelatonin in 0.4 M phosphate-buffered solutions at pH 7.4. (a) Degradation of 1 mM 1-nitrosomelatonin (▲) and formation of melatonin (○) evaluated by HPLC analysis of aliquots, using external standards. (b) Spectrophotometric recordings of 0.15 mM 1-nitrosomelatonin every 10 min.

of a melatoninyl radical able to react easily with oxygen leading to various oxides and peroxides.[6,11]

Effects of Metallic Ion

Degradation of *S*-nitrosothiols was extensively studied and some factors were shown to interfere, such as chelating compounds and metallic cations. Considering that *S*-nitrosothiols bear the same nitroso group as 1-nitrosomelatonin, the effect of metallic ions was considered: chelex treatment of buffered solutions as well as addition of equimolar concentrations of EDTA, DTPA or various metallic ions (Fe^{2+}, Fe^{3+}, Cu^{+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}) were assayed but did not affect 1-nitrosomelatonin decay.

Effects of Reducing Agents

We considered free thiols such as glutathione (GSH) and albumin, ascorbate, NADH and

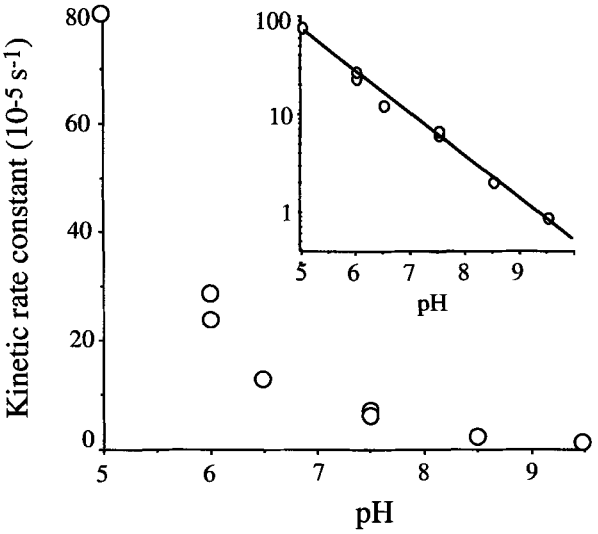

FIGURE 3 Effects of pH on the observed first-order kinetic rate constant of 1-nitrosomelatonin decay in phosphate-buffered solutions.

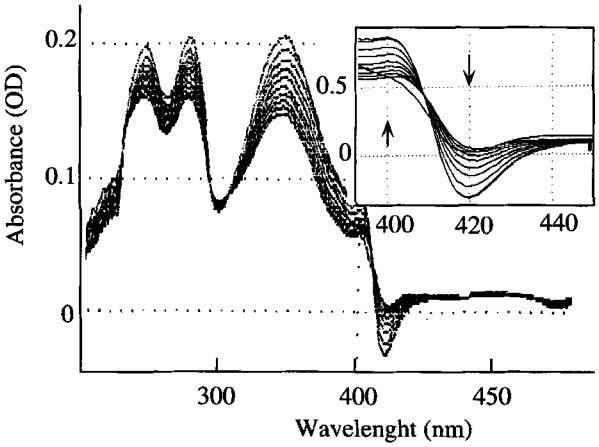

FIGURE 4 Conversion of oxyhaemoglobin (oxyHb) to methaemoglobin in the time of 1-nitrosomelatonin degradation. The difference spectra of a mixture containing 0.05 mM oxyHb and 0.01 mM 1-nitrosomelatonin versus 0.05 mM oxyHb in phosphate-buffered solutions at pH 7.4 were recorded every 3 min and superimposed.

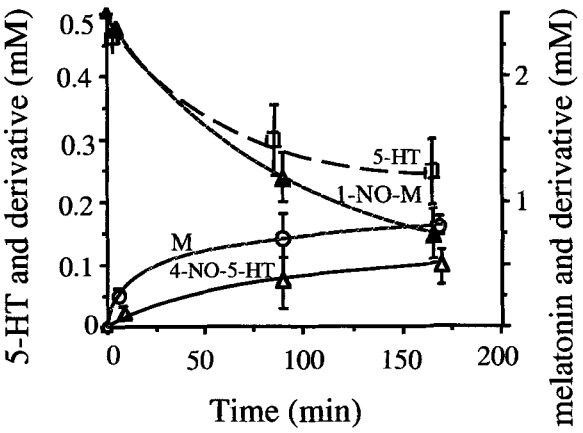

FIGURE 5 Time-course of simultaneous conversions of 1-nitrosomelatonin (▲) to melatonin (○) and serotonin (5-HT) (□) to 4-nitroso-5-HT (△). Mixture of 2.5 mM 1-nitrosomelatonin and 1 mM 5-HT in 1 ml sodium phosphate-buffered solution (pH 7.4) was left at room temperature and analyzed by HPLC using external standards detected at 215 nm. No trace of 4-nitro-5-HT was detected showing no peroxynitrite formation.

NADPH. Thiols (1–10 mM) did not modify the rate constant of 1-nitrosomelatonin decomposition. Ascorbate, NADH and NADPH, on the other hand, enhanced the rate of decomposition of 1-nitrosomelatonin. Thus, at 25°C and pH 7.4 first-order rate constants of $12.7 \pm 0.5 \times 10^{-5}$ and $20 \pm 1 \times 10^{-5}\ s^{-1}$ were measured in the presence of NADH (0.5/0.25 mM 1-nitrosomelatonin) and ascorbate (2 mM ascorbate/0.2 mM 1-nitrosomelatonin), respectively. These rates were not affected by the concentrations of NADH, NADPH and ascorbate. This increase in decay rate could be due to the rapid reaction with melatoninyl radical, preventing the reversibility of the homolytic rupture of 1-nitrosomelatonin (Fig. 7). Moreover, analysis revealed a decrease of oxidized melatonin derivatives in the presence of any one of these reducing agents. In particular, no oxidized products were formed on addition of ascorbate (from 1 to 10 equiv. ascorbate/1-nitrosomelatonin) and recovery of melatonin was complete.

DISCUSSION

The spontaneous degradation of 1-nitrosomelatonin occurred in aqueous solution at pH 7.4, giving melatonin in 50 or 100% yield in the absence or presence of ascorbate, respectively.

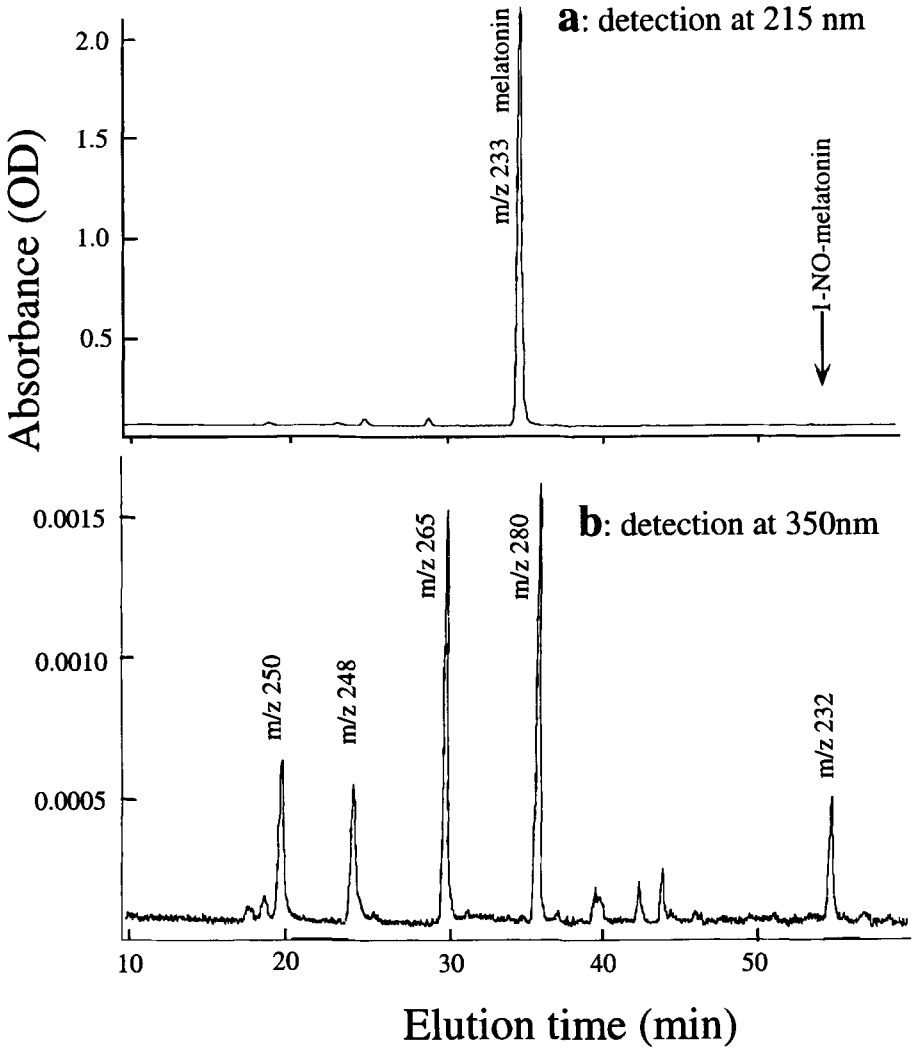

FIGURE 6 Typical HPLC analysis of the phosphate-buffered solution at pH 7.4 which contained initially the1-nitrosomelatonin in an open tube. Only melatonin was detected at 215 nm but other derivatives appeared at 350 nm. Mass spectrometric analysis was performed on all detected peaks: for the peak corresponding to 1-nitrosomelatonin, electrospray mass spectrometry gave m/z 232 corresponding to the protonated melatonyl radical. After 1-nitrosomelatonin decay, mass spectrometry gives the masses of oxygenated derivatives of melatonin (M) m/z 248 $[M + O] + H^+$, m/z 265 $[M + 2O] + H^+$ and m/z 280 which could be interpreted to be $[M^{\cdot} + 3O] + H^+$.

The formation of NO from 1-nitrosomelatonin was experimentally demonstrated directly and indirectly in aqueous buffered solutions: formation of nitrite and the conversion of oxyHb into methaemoglobin are in good agreement with NO-release. Indeed, in aqueous neutral buffered solutions, NO reacts with O_2 leading to N_2O_3 which could be trapped by either water or serotonin leading to nitrite and 4-nitrososerotonin, respectively. In both cases, the amounts of nitrosation products revealed the level of NO-release. Thus, using serotonin as a probe, we have previously described a method of NO or peroxynitrite evaluation from known NO-donors.[10] In the case of 1-nitrosomelatonin, we observed that no peroxynitrite was formed in the

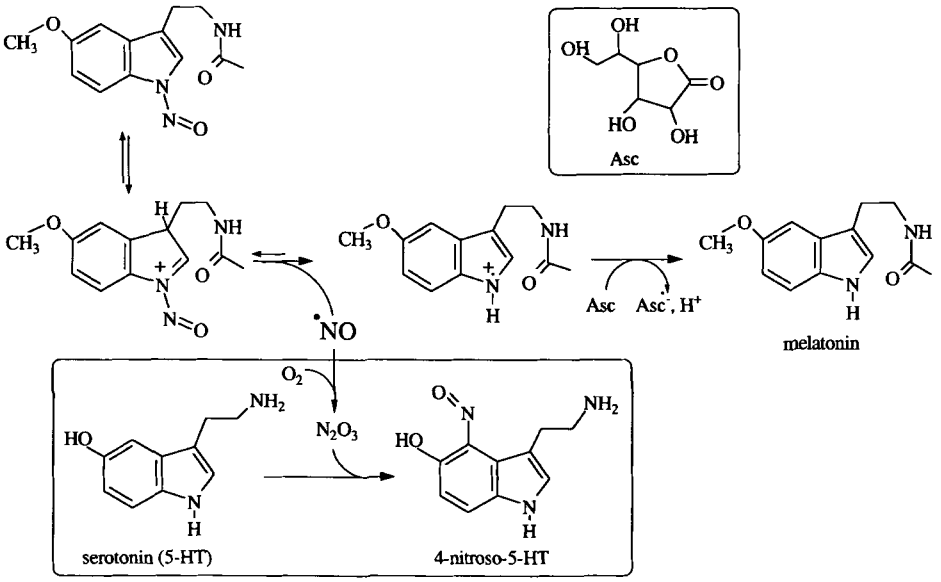

FIGURE 7 Scheme of 1-nitrosomelatonin decay in the presence of ascorbate. Formation of NO and subsequent nitrosation of serotonin (5-HT) is presented.

course of 1-nitrosomelatonin degradation while SIN-1 and sodium nitroprusside produced it in identical aerated solutions.[12,13]

Furthermore, a typical and significant NO-signal was detected by the ISO-NO electrode during the 1-nitrosomelatonin decay (result not shown). A similar signal was obtained with a slow donor like DPTA/NO.

The decomposition pathways of N-nitroso-compounds were compared to those of *S*-nitrosothiols which could serve as sources of NO. Even if heterolysis of some nitrosothiols may yield NO^+ or NO^-, there is much evidence for a homolysis pathway accounting for physiological roles such as vasodilatory effect and protection against ischaemic myocardium and angina pectoris.[14]

The effects of pH and Cu^+ and/or Cu^{2+} were compared taking account of the structural homology of 1-nitrosomelatonin with nitrosothiols (RS-NO) among other NO-donors.[15] 1-Nitrosomelatonin did not behave as RS-NO. Alkaline pH favored RS-NO decomposition probably because of nucleophilic attack by the RS^- released, whereas 1-nitrosomelatonin decay was accelerated under acidic conditions. Thus, decay must proceed via another pathway depending on the indole structure. It is known that protonation of indole in acid medium occurs on C3 to give an iminium.[16] The charge on the pyrrolic nitrogen induced the rupture of the N–N bond. Moreover, metallic cations intervene in the degradation of RS-NO by complexation on the sulfur: mercuric and cupric cations form $RS(Hg^{2+})NO$ and $RS(Cu^{2+})NO$ complexes which hydrolyze in water to nitrite, or nitrosate free thiols to *S*-nitrosothiols, or serotonin (5-HT) to 4-nitrososerotonin (4-NO-5-HT).[10] In the case of 1-nitrosomelatonin, on the other hand, metal cations probably do not complex the nitrogen atom linked to NO-group since no effect was observed.

L-ascorbate (HA-) is known to effect electron transfer in biological systems. Ascorbate promotes the release of NO from nitrosothiols and from nitroprusside.[17,18] In the case of GSNO, the reduction by ascorbate depends on the concentration of ascorbate suggesting that the reaction proceeds in one stage involving the reduction of the S–N bond followed by

NO-release.[18] A 1:1 molar ratio of HA- to 1-nitrosomelatonin is observed as to GSNO. However, in the case of 1-nitrosomelatonin decay, the reduction by ascorbate through an outer-sphere electron transfer mechanism as proposed for GSNO is unlikely. Probably, the main role of ascorbate is to reduce very rapidly the melatoninyl radical, which should be protonated in water to regenerate melatonin. Ascorbate would therefore be oxidized to give an ascorbyle radical anion and then dehydroascorbate acid as proposed in the literature. In the absence of ascorbate, the melatoninyl radical dismutated to produce 50% melatonin and various oxidized species. This proposed radical mechanism is in accordance with a homolytic rupture of 1-nitrosomelatonin leading to the melatoninyl and NO-radicals (Fig. 7).

Even if we cannot exclude minor NO^+ or NO^- formation, the quantitative recovery of melatonin in the presence of ascorbate is a good indication of a homolysis pathway to free NO-release.

CONCLUSION

1-Nitrosomelatonin which could be formed in the presence of NO or peroxynitrite may contribute to the pool of NO-releasing compounds. Formation and decomposition of 1-nitromelatonin demonstrate that melatonin could convert the RNS (NO_x, N_2O_3, $ONOO^-$) to NO. This raise the hypothesis that 1-nitrosomelatonin formed may be a detoxifying NO-derived species. This chemical scenario could explain the well-known antioxidant properties of melatonin.

On the other hand, 1-nitrosomelatonin is a model aromatic heterocyclic molecule able to carry NO and release it, in a suitable environment. However, nitrosoindoles whose biosynthesis from NO-derived nitrogen oxides has not yet demonstrated, have been shown to decompose by loss of the NO-group and/or transfer of the NO-group to a nearby receptor molecule such as phenol or DNA bases thus terminating a transnitrosation cycle.[19] Such mechanisms have not yet been described in particular with tryptophan while peroxynitrite is known to oxidize tryptophan by a one-electron pathway to tryptophan-centered radicals which were detected in plasma proteins.[20–22] Tryptophan could be nitrated by peroxynitrite with a $184\,M^{-1}\,s^{-1}$ second-order rate constant[23,24] and oxidized as shown various identified derivatives.[25] But these scarce data indicate that the mechanisms and reactions of peroxynitrite with free tryptophan or with tryptophan as a protein residue are not yet fully understood.

Acknowledgements

We thank Dr Yann Henry for his valuable advice and Dr Pierre Potier for encouragement. Financial support was from the Centre National de la Recherche Scientifique and Ministère de la Recherche et de la Technologie (France).

References

[1] Reiter, R.J. (1991) "Pineal melatonin: cell biology of its synthesis and of its physiological interactions, *Endocrine Reviews* **12**, 151–180.
[2] Reiter, R.J. (1993) "The melatonin rhythm: both a clock and a calendar", *Experientia* **49**, 654–664.
[3] Tan, D.X., Manchester, L.C., Reiter, R.J., Plummer, B.F., Hardies, L.J., Weintraub, S.T., Vijayalaxmi, S.T. and Shepherd, A.M.M. (1998) "A novel melatonin metabolite, cyclic 3-hydroxymelatonin: a biomarker of *in vivo* hydroxyl radical generation", *Biochemistry Biophysics Research Communications* **253**, 614–670.
[4] Reiter, R.J. (1998) "Oxidative damage in the central nervous system: protection by melatonin", *Progress in Neurobiology* **56**, 359–384.
[5] Bravo, C., Herves, P., Leis, J.R. and Pena, M.E. (1992) "Kinetic study of the nitrosation of 3-substituted indoles", *Journal of Chemical Society, Perkin Transactions* **2**, 185–189.
[6] Blanchard, B., Pompon, D. and Ducrocq, C. (2000) "Nitrosation of melatonin by nitric oxide and peroxynitrite", *Journal of Pineal Research* **29**, 184–192.
[7] Kissner, R., Nauser, T., Bugnon, P., Lye, P.G. and Koppenol, W.H. (1997) "Formation and properties of peroxynitrite as studied by laser flash photolysis, high pressure stopped flow technique, and pulse radiolysis", *Chemical Research Toxicology* **101**, 1285–1292.
[8] Cudic, M. and Ducrocq, C. (2000) "Transformations of 2,6-diisopropylphenol by NO-derived nitrogen oxides,

particularly peroxynitrite", *Nitric Oxide: Biology and Chemistry* **4**, 147–156.
[9] Feelisch, M. and Kubitzek, D. (1996) "The oxyhemoglobin assay", *Methods in Enzymology* **269**, 455–478.
[10] Blanchard, B., Servy, C. and Ducrocq, C. (2001) "Chemical evaluation of compounds as nitric oxide or peroxynitrite donors using the reactions with serotonin", *Free Radical Research*, **34**, 177–188.
[11] Shen, X., Lind, J., Eriksen, T.E. and Merenyi, G. (1990) "The reactions of indoles in aqueous solution initiated by one-electron oxidation", *Journal of Chemical Society, Perkin Transactions* **2**, 597–603.
[12] Singh, R.J., Hogg, N., Joseph, J., Konorev, E. and Kalyanaraman, B. (1999) "The peroxynitrite generator, SIN-1, becomes a nitric oxide donor in the presence of electron acceptors", *Archives of Biochemistry and Biophysics* **361**, 331–339.
[13] Aleryani, S., Milo, E. and Kostka, P. (1999) "Formation of peroxynitrite during thiol-mediated reduction of sodium nitroprusside", *Biochimica et Biophysica Acta* **1472**, 181–190.
[14] Ignarro, L.J., Lipton, H., Edwards, J.C., Baricos, W.H., Hyman, A.L., Kadowitz, P.J. and Gruetter, C.A. (1981) "Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of *S*-nitrosothiols as active intermediates", *Journal Pharmacology Experimental Therapeutics* **218**, 739–749.
[15] Singh, R.J., Hogg, N., Joseph, J. and Kalyanaraman, B. (1996) "Mechanisms of nitric oxide release from *S*-nitrosothiols", *Journal of Biological Chemistry* **271**, 18596–18603.
[16] Charlet-Fagnère, C., Laronze, J., Laronze, J.Y., Toupet, L., Vistelle, R., Lamiable, D., Mouchard, C., Renard, P. and Adam, G. (1996) "Etude de la dimérisation en milieu acide de la mélatonine et de quelques dérivés apparentés. Oxydation de 2-arylindolines en 2-arylindoles", *Bulletin de la Société Chimique Française* **133**, 39–50.
[17] Scorza, G., Pietraforte, D. and Minetti, M. (1997) "Role of ascorbate and protein thiols in the release of nitric oxide from *S*-nitroso-albumin and *S*-nitroso-glutathione in human plasma", *Free Radical Biology and Medicine* **22**, 633–642.
[18] Smith, J.N. and Dasgupta, T.P. (2000) "Kinetics and mechanism of the decomposition of *S*-nitrosoglutathione by L-ascorbic acid and copper ions in aqueous solution to produce nitric oxide", *Nitric oxide: Biology and Chemistry* **4**, 57–66.
[19] Lucas, L.T., Gatehous, D. and Shuker, D.E.G. (1999) "Efficient nitroso group transfer from *N*-nitrosoindoles to nucleotides and 2′-deoxyguanosine at physiological pH", *Journal of Biological Chemistry* **274**, 18319–18326.
[20] Pietraforte, D. and Minetti, M. (1997) "One-electron oxidation pathway of peroxynitrite decomposition in human blood plasma: evidence for the formation of protein tryptophan-centered radicals", *Biochemical Journal* **321**, 743–750.
[21] Pollet, E., Martinez, A., Metha, B., Watts, B.P. and Turrens, J.F. (1998) "Role of tryptophan oxidation in peroxynitrite-dependent protein chemiluminescence", *Archives of Biochemistry and Biophysics* **349**, 74–80.
[22] Zhang, H., Squadrito, G.I. and Pryor, W.A. (1998) "The reaction of melatonin with peroxynitrite: formation of melatonin radical cation and absence of stable nitrated products", *Biochemical and Biophysical Research Communications* **251**, 83–87.
[23] Padmaja, S., Ramezanian, M.S., Bounds, P.L. and Koppenol, W.H. (1996) "Reaction of peroxynitrite with L-tryptophan", *Redox Report* **2**, 173–177.
[24] Alvarez, B., Rubbo, H., Kirk, M., Barnes, S., Freeman, B.A. and Radi, R. (1996) "Peroxynitrite-dependent tryptophan nitration", *Chemical Research in Toxicology* **9**, 390–396.
[25] Kato, Y., Kawakishi, S., Aoki, T., Itakura, K. and Osawa, T. (1997) "Oxidative modification of tryptophan residues exposed to peroxynitrite", *Biochemical and Biophysical Research Communications* **234**, 82–84.