Regiospecific Nitrosation of N-terminal-blocked Tryptophan Derivatives by N2O3 at Physiological pH*

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Nitric oxide (NO) is involved in a great variety of physiological and pathophysiological processes, including the regulation of vascular tone, immune response, and neurotransmission (1). In mammals the basal level of NO (in the aqueous phase) is in the nanomolar range (2). However, the local NO concentrations can be increased substantially in areas with high NO synthase activity and/or in hydrophobic regions (3, 4), thereby facilitating the formation of dinitrogen trioxide (N2O3) (Equations 1 (5) and 2 (6)).

\[ 2 \text{NO} + \text{O}_2 \rightarrow 2 \text{NO}_2 \quad k_1 = 2.9 \times 10^5 \text{M}^{-2} \text{s}^{-1} \quad (\text{Eq. 1}) \]

\[ \text{NO} + \text{NO}_3 \rightleftharpoons \text{N}_2\text{O}_3 \quad k_{-1} = 1.1 \times 10^5 \text{M}^{-1} \text{s}^{-1} \quad (\text{Eq. 2}) \]

Because N2O3 is highly effective in nitrosating thiol groups (7) (Equations 3 (8) and 4 (2)),

\[ \text{RSH} + \text{N}_2\text{O}_3 \rightarrow \text{RSNO} + (\text{H}^+) + \text{NO}_2 \quad k_3 = 6.4 \times 10^9 \text{M}^{-1} \text{s}^{-1} \quad (\text{Eq. 3}) \]

\[ \text{RS}^- + \text{N}_2\text{O}_3 \rightarrow \text{RSNO} + \text{NO}_2^- \quad k_4 = 1.8 \times 10^6 \text{M}^{-1} \text{s}^{-1} \quad (\text{Eq. 4}) \]

Further, S-nitrosation of thiols is postulated to induce several functional protein modifications in vivo (13–18). In addition to thiols, secondary amines also react rapidly with N2O3 (7). Consequently, the question arose whether (protein-bound) tryptophan could be nitrosated at the nitrogen atom of the indole ring at physiological pH 7.4. It has been demonstrated recently that melatonin (N-acetyl-5-methoxytryptamine) is N-nitrosated by NaNO2/HCl as well as by NO/O2 at pH 7.4. However, the reported rate constant for the latter reaction is rather low (k = 5.0 \times 10^2 s^{-1}) (19). Keeping the low physiological concentrations of melatonin in mind (e.g. the maximal concentration in human serum is \( < 75 \text{pg/ml} \)), this reaction therefore should not proceed to a significant extent in vivo. A similar reaction has not been established thus far for tryptophan at pH 7.4. However, there are indications that the secondary amine of tryptophan (N\textsubscript{indole}) can be nitrosated when the primary amine function (N\textsubscript{aliline}) is N-blocked. Consequently, albumin (20–23) or, more simply, N-acetyltryp- 

\[ \text{RSNO} + \text{ascorbate/Cu}^{2+} \rightarrow \text{RSNO} + \text{products} + \text{Cu}^{+} \rightarrow \text{NO} + \text{RS}^- + \text{Cu}^{2+} \quad (\text{Eq. 5}) \]

\[ \text{N}_2\text{O}_3 \rightarrow \text{N}_2\text{O}_2 \text{NO} \]

The putative formation of S-nitrosothiols is now generally believed to be of high physiological importance in vivo (Ref. 9 and references therein). For instance, S-nitrosothiols can operate as nitric oxide-donating compounds in vitro (Equation 5 (10)), and they are believed to induce NO-like biological activities in vivo, probably by releasing NO (11, 12).
\[ \text{N}-\text{nitrosotryptophan is expected to exhibit similar NO-donating capabilities as S-nitroscysteine.} \]

**MATERIALS AND METHODS**

**Chemicals**—\( \alpha \)-Tryptophan, \( N \)-acetyltryptophan, melatonin, lysine-tryptophan-lysine, \( \text{L}-\text{cysteine}, \text{morpholine, pipierazine and } \text{N}^2 \)-labeled sodium nitrite were obtained from Sigma. MAMA NONOate and spermine NONOate were purchased from Situs (Düsseldorf, Germany).

**Experimental Conditions**—Because nitrosation reactions are sensitive to the presence of metal ions, solutions were exposed to chelating resin (Chelex 100) as described previously (27).

**Nitrosation by Preformed NO Gas**—Nitric oxide (\( \text{NO} \)) or \( \text{N}^2 \)-labeled nitric oxide (\( \text{NO}_2 \)) was prepared daily by the addition of glacial acetic acid to an aqueous solution of \( \text{K}_2[\text{Fe(CN)}_6] \) (0.7 M) containing either \( \text{NaNO}_2 \) or \( \text{Na}_2\text{NO}_2 \) (0.72 M) under oxygen-free conditions. The overall stoichiometry is presented by the equation,

\[ \text{K}_2[\text{Fe(CN)}_6] + \text{Na}_2\text{NO}_2 + 2\text{CH}_3\text{COOH} \rightarrow \text{K}_2[\text{Fe(CN)}_6] + \text{CH}_3\text{COOK} + \text{CH}_3\text{COONa} + \text{H}_2\text{O} + \text{NO} \quad \text{(Eq. 6)} \]

The reliability of this procedure was confirmed by detecting the release of nitric oxide polaro graphically with a graphite nitric oxide-sensing electrode (World Precision Instruments, Berlin, Germany).

\( N \)-blocked derivatives of tryptophan were nitrosated in oxygen-saturated phosphate/triglycine buffer (250/250 mM, pH 8.0) with the above described preformed NO gas by bubbling it gently into the solution through a needle until the pH value had dropped to 7.4.

**Nitrosation by NO Donor Compounds**—MAMA NONOate and spermine NONOate were prepared as 100-fold stock solutions in 10 mM NaOH at 4 °C and used immediately. A stock solution of nitratoscysteine was prepared by \( S \)-nitrosation of 100 mM cysteine with equimolar amounts of \( \text{NaNO}_2 \) in acidic solution (pH 2) at 0 °C (24). Nitrosation reactions were performed in 1 ml of potassium phosphate/NaHCO\(_3\)/CO\(_2\) buffer (50 mM/25 mM/5%, pH 7.4, 37 °C) in 35-mm dishes.

**Nitrosation by \( \text{NO}_2 \) under Acidic Conditions**—Substances were exposed to equimolar concentrations of sodium nitrite in \( \text{H}_2\text{O} \) equilibrated with glacial acetic acid to \( \text{pH} \) 3.5.

\( \text{\textsuperscript{15}} \text{N} \) NMR Identification of Nitrosated Products—Immediately after nitrosation, sample aliquots were supplemented with 10% \( \text{D}_2\text{O} \) and analyzed by \( \text{\textsuperscript{15}} \text{N} \) NMR spectrometry. The adducts were identified by 50.67 MHz \( \text{\textsuperscript{15}} \text{N} \) NMR spectroscopy on a Bruker AVANCE DIX 500 instrument. Chemical shifts (\( \delta \)) are given in ppm relative to neat nitromethane (\( \delta = 0 \)) as the external standard.

**Reactivity of \( N \)-Acetyltryptophan toward \( \text{NO}_2 \)—** Morpholine (0–100 mM) or pipierazine (0–20 mM) and \( \text{t} \)-tryptophan (2 mM) or its derivatives were incubated with 0.5 mM MAMA NONOate in potassium phosphate buffer (50 mM, pH 7.5, 37 °C) for 30 min. The reaction products were analyzed spectrophotometrically at 335 nm (or 346 nm for nitratoscysteine) with a SPECORD S 100 spectrophotometer from Analytik Jena (Jena, Germany). A similar experiment was carried out with pipierazine as the competitor. Control experiments demonstrated that transnitration reactions did not proceed at this pH between nitrosomorpholine + \( N \)-acetyltryptophan, nitratospiripiperazine + \( N \)-acetyltryptophan, morpholine + \( N \)-acyetylnitrosotryptophan, or pipierazine + \( N \)-acetylnitrosotryptophan, in line with observations reported by Meyer et al. (28).

**Decay Kinetic Experiments**—The decay kinetics of \( N \)-acyetyl-N-nitrosotryptophan (100 \( \mu \)M) at various temperatures (15–45 °C) were determined in air-tight quartz cuvettes by rapid scan monitoring the UV-visible absorption at \( \lambda_{\text{max}} = 335 \text{ nm} \) (250–600 °C) at 24°C. Between recordings the samples were protected from light. The temperature was maintained at ± 0.1 °C.

**RESULTS**

**Formation of \( N \)-Nitrosotryptophan in Phosphate Buffer, pH 7.4**—Evidence exists that under acidic conditions similar to those in the stomach the \( \text{NaNO}_2 \)-dependent nitrosation of human serum albumin, bovine serum albumin, or the dipetide glycine-tryptophan yields \( N \)-nitrosotryptophan (21, 23). The main nitrosating species in this reaction is postulated to be dinitrogen trioxide (\( \text{N}_2\text{O}_3 \)) formed from \( \text{HNO}_2 \) dehydration (7, 29). Provided that this is indeed the case, \( \text{N}_2\text{O}_3 \) formed from \( \text{NO} \) in the presence of oxygen at pH 7.4 should also nitrosate \( N \)-terminal-blocked tryptophan derivatives, like \( N \)-acetyltryptophan and peptide-associated tryptophan (lysine-tryptophan-lysine).

To verify the nitrosation of \( N \)-blocked tryptophan derivatives by \( \text{N}_2\text{O}_3 \) at pH 7.4, we selected various \( \text{N}_2\text{O}_3 \)-generating systems. We employed preformed nitric oxide as well as \textit{in situ} generation of NO from \( S \)-nitroscysteine and spermine NONOate, respectively. In these systems, \( \text{N}_2\text{O}_3 \) is formed from oxidation of nitric oxide (\( \text{NO} \) autoxidation) according to Equations 1 and 2 (see the Introduction). Because the formation of \( N \)-acetyl-N-nitrosotryptophan from the reaction of \( N \)-acyetyltryptophan with \( \text{NaNO}_2 \) at pH 3.5 has been described in detail (24, 30), we used this reaction as a reference system. In analogy to the data of Bonnett and Holleyhead (24) we recorded the UV-visible spectrum of \( N \)-acyetyl-N-nitrosotryptophan with an absorption maximum at 335 nm (Fig. 1A, trace b). Interestingly, an almost identical UV-visible spectrum was observed when \( N \)-acyetyltryptophan was allowed to react with preformed nitric oxide in the presence of oxygen at physiological pH 7.4 (Fig. 1A, trace c). This finding indicated very strongly that \( \text{N}_2\text{O}_3 \) can indeed nitrosate the secondary amine of the indole system in tryptophan. Likewise, the UV-visible spectrum of \( N \)-acetyl-N-nitrosotryptophan was also detected during reaction of \( N \)-acyetyltryptophan with the above mentioned nitric oxide-donating compounds in the presence of air (Fig. 1A, traces c and d).

Because \textit{in vivo} \( N \)-terminal-blocked tryptophan is present primarily in proteins, the \( \text{N}_2\text{O}_3 \)-mediated nitrosation of the nitrogen atom of the indole ring was also investigated for peptide-bound tryptophan by employing lysine-tryptophan-lysine. In full agreement with the experiments performed with \( N \)-acyetyltryptophan (see above), all applied \( \text{N}_2\text{O}_3 \)-generating systems were able to nitrosate peptide-bound tryptophan effectively at the selected \( \text{pH} \) values (Fig. 1B). In contrast, the nitrosation of \( t \)-tryptophan by the \( \text{N}_2\text{O}_3 \)-generating systems was much less effective (data not shown), which is in line with data from the literature (28, 30).CLUSIVELY, only \( N \)-terminal-blocked tryptophan but not \( t \)-tryptophan is a relevant target for \( \text{N}_2\text{O}_3 \) at physiological pH.

**Detection of \( N \)-Nitrosotryptophan by \textit{\textsuperscript{15}} \text{N} \) NMR Spectrometry**—Because UV-visible absorption spectra cannot provide unambiguous evidence that the secondary amine function of the indole ring was truly nitrosated, we used \( \textit{\textsuperscript{15}} \text{N} \) NMR spectrometry as a more reliable analytical tool. In 1986, Dorie et al. (31) recorded the \( \textit{\textsuperscript{15}} \text{N} \) NMR spectrum of \( \textit{\textsuperscript{15}} \text{N} \)-labeled \( N \)-acyetyl-\( \textit{\textsuperscript{15}} \text{N} \)nitrosotryptophan from the reaction of \( N \)-acyetyltryptophan with \( \text{Na}_2\text{NO}_2 \) at pH 4. As commonly observed for \( N \)-nitroso compounds (32) the \( \textit{\textsuperscript{15}} \text{N} \) NMR spectrum exhibited two resonances, at 184.6 and 169.6 ppm relative to neat nitromethane, of the \( Z \)- and \( E \)-conformer of \( N \)-acyetyl-N-nitrosotryptophan, respectively. Fig. 2A shows the \( \textit{\textsuperscript{15}} \text{N} \) NMR spectrum of preformed (authentic) \( N \)-acyetyl-\( \textit{\textsuperscript{15}} \text{N} \)-nitrosotryptophan at pH 7.4, exhibiting two resonance lines at 180.8 and 166.3 ppm, respectively. The small shift difference of ~3 ppm compared with the data of Dorie et al. (31) may be explained by differ-

\[ \text{Scheme 1} \]
Fig. 1. UV-visible absorption spectra of N-nitroso-tryptophan residues generated from the reactions of both N-acetyltryptophan and lysine-tryptophan-lysine with various NO sources. A, spectra of N-acetyltryptophan (trace a) and authentic N-acetyl-N-nitrosotryptophan (trace b) in air-saturated potassium phosphate buffer/NaHCO3 at pH 7.4, and spectra observed after reaction of N-acetyltryptophan (1 mM) with spermine NONOate (2 mM) (trace c), S-nitrosocysteine (3.4 mM) (trace d, dilution 1:4) in the same buffer solution, or preformed NO gas in oxygenated 250 mM potassium phosphate buffer, pH 8.0, 25 mM triglycine (trace e, dilution 1:10). B, spectrum of lysine-tryptophan-lysine (5 mM) (trace a) in air-saturated potassium phosphate/NaHCO3/CO2 buffer (50 mM/25 mM/5%, pH 7.4, 37°C); spectrum observed after reaction of lysine-tryptophan-lysine with NaN3O3 (5 mM each) at pH 7.5 (trace b); and spectra observed after reaction of lysine-tryptophan-lysine (5 mM) with S-nitrosocysteine (3.4 mM) (trace c, dilution 1:2), spermine NONOate (2 mM) (trace d) in air-saturated potassium phosphate/NaHCO3/CO2 buffer (50 mM/25 mM/5%, pH 7.4, 37°C), or preformed NO gas in oxygenated potassium phosphate/triglycine buffer (250/25 mM, pH 8.0, 37°C) (trace e, dilution 1:10). In the latter buffer, the pH decreased to 7.4 during the experiment.

Fig. 2. 15N NMR spectra of 15N-labeled N-nitrosotryptophan derivatives. A, 15N-labeled N-acetyl-N-nitrosotryptophan prepared from reaction of N-acetyltryptophan with NaN3O3 (20 mM each) in acidified aqueous solution at pH 3.5 (after completion of the reaction, the reaction mixture was adjusted to pH 7.4 by the addition of 1 N NaOH). 15N-Labeled N-acetyl-N-nitrosotryptophan (B) and 15N-labeled lysine-15N-nitrosotryptophan-lysine (C) were produced from the reaction of either 100 mM N-acetyltryptophan or 200 mM lysine-tryptophan-lysine with preformed 15NO in oxygenated potassium phosphate/triglycine buffer (250 mM/25 mM, pH 8.0, 37°C). Under the conditions in B and C, the pH decreased to 7.4 during the experiment.

ences in the recording conditions, e.g. different pH values as well as improvements of the 15N NMR spectrometric techniques during the past years. Nevertheless, nitrosation of the nitrogen atom at the indole ring is proven by this characteristic 15N NMR spectrum. When N-acetyltryptophan was reacted with authentic 15NO in the presence of oxygen, the two new 15N NMR resonances at 180.1 and 165.6 ppm were detected, consistent with the formation of N-acetyl-N-nitrosotryptophan (Fig. 2B). To the best of our knowledge, this is the first direct proof that a tryptophan derivative can be nitrosated by NzO3 at the nitrogen atom of the indole system at physiological pH.

Analogous to N-acetyltryptophan the tripeptide lysine-tryptophan-lysine was nitrosated at pH 7.4 by preformed 15NO as proven by the 15N NMR resonance lines at 181.1 and 166.7 ppm, respectively (Fig. 2C).

Reactivity of N-blocked Tryptophan Derivatives Toward NzO3—For a discussion of the putative significance of tryptophan nitrosation in vivo it is necessary to determine the rate constants of the reaction of N-acetyltryptophan or its derivat
NONOate-induced nitrosation of N-acetyltryptophan was half-maximally inhibited at a piperezine concentration of 2.7 mM. The observation that the corrected IC\textsubscript{50} value of morpholine is lower than the experimental IC\textsubscript{50} value of piperezine (Table I) is in agreement with the report that N\textsubscript{2}O\textsubscript{3} reacts faster with morpholine than with piperezine (7). Not unexpectedly, the reactivity of other N-terminal-blocked tryptophan derivatives toward N\textsubscript{2}O\textsubscript{3} was found to be similar (Table I). To compare the rate constant of these nitrosation reactions with other nitrosation reactions reported in the literature, one experiment with NONOate-induced nitrosation of N2O3 reacted faster with N\textsubscript{2}O\textsubscript{3} (see the Discussion).

Decomposition of N-Acetyl-N-nitrosotryptophan—In contrast to common, stable nitrosamines, protein-bound N-nitrosotryptophan has been reported to undergo slow decay at pH 2 (23). To quantify this capability at pH 7.4, we analyzed the decomposition of N-acetyl-N-nitrosotryptophan by monitoring its absorption at 335 nm. At 37 °C N-acetyl-N-nitrosotryptophan decayed in a first-order manner (Fig. 4A). Similarly, first-order decay kinetics were also observed at other temperatures (15–45 °C, data not shown). From the excellent Arrhenius plot of the rate data (Fig. 4B) an activation barrier of \( E_a = 13.2 \pm 0.1 \) kcal mol\(^{-1}\) and an \( A\)-factor of \( 1.7 \pm 0.3 \times 10^5 \) s\(^{-1}\) were extracted. This \( A\)-factor appears to be extremely low for a simple first-order (homolysis) decomposition. From the Arrhenius parameters the half-life of N-acetyl-N-nitrosotryptophan at physiological conditions (\( T = 37 °C, pH 7.4 \)) can be calculated to 140 min. Thus, the N-nitrosamines of N-blocked tryptophan derivatives are rather long-lived intermediates.

Because the half-life of peptide-bound N-nitrosotryptophan was found to be similar to that of N-acetyl-N-nitrosotryptophan (data not shown), one may ask whether it would make any sense for physiological functions to nitrosate tryptophan \textit{in vivo}. Recently, Harohalli et al. (23) reported that N-nitrosotryptophan very slowly releases \( 'NO \) at pH 2, and Blanchard-Fillion et al. (40) observed that nitrosomelatonin spontaneously decays at pH 7.4, thereby releasing nitric oxide at a yield of 71%. In our hands, however, authentic nitrosomelatonin released only negligible amounts of nitric oxide on decomposition in Hepes-free buffer solution (data not shown). Noticeably neither Harohalli et al. (23) nor Blanchard-Fillion et al. (40) directly detected \( 'NO \), e.g. with a NO electrode.

As nitrosothiols release nitric oxide in the presence of vitamin C (10), we compared the potential of ascorbate to induce \( 'NO \) release from S-nitrosoglutathione \textit{versus} N-acetyl-N-nitrosotryptophan (Fig. 5). In the absence of copper ions, the release of \( 'NO \) from N-acetyl-N-nitrosotryptophan after the addition of ascorbate was about 5-fold higher than from S-nitrosoglutathione. In contrast, a "simple" N-nitrosamine like N-nitroso-morpholine did not liberate nitric oxide under similar conditions.

**Table I**  
*Rate data for the reaction of tryptophan derivatives with N\textsubscript{2}O\textsubscript{3}*

| N\textsubscript{2}O\textsubscript{3} target | Competitor | \( T \) °C | IC\textsubscript{50} (apparent) | IC\textsubscript{50} (corrected) | \( k \times 10^7 \) (apparent) |
|----------------------------------------|------------|-----------|-------------------------------|-------------------------------|-----------------------------|
| 2 mM                                   | N-Acetyltryptophan | 37        | 13.4                          | 2.1                           | 6.4                         |
|                                       | N-Acetyltryptophan | 37        | 13.4                          | 2.1                           | 6.4                         |
|                                       | Tripeptide    | 37        | 14.8                          | 2.7                           | 6.4                         |
|                                       | N-Acetyltryptophan | 25        | 15.0                          | 2.7                           | 6.4                         |
|                                       | Melatonin     | 37        | 23.4                          | 2.7                           | 6.4                         |

\( ^{a} \) Corrected for the concentration of the operating amine.

\( ^{b} \) Not calculable.
The results presented above clearly show that N\textsubscript{2}O\textsubscript{3} nitrosates the secondary amine function at the indole ring of N-blocked tryptophan with high reactivity at physiological pH values. It is known that indole and indole derivatives are easily attacked by N\textsubscript{2}O\textsubscript{3} under acidic conditions, however, with exclusive C-nitrosation, thus yielding 3-nitroso products (30, 41). Such products were not observed for tryptophan because here the C-3 position is blocked by the alanine residue, rendering nitrosation at position N-1 (N\textsubscript{indole}) more feasible. Nitrosation at C-2 is possible only for derivatives carrying powerful electron donors at the C-3 position (30). Our data indicate that N-acetyltryptophan, melatonin, and the tripeptide lysine-tryptophan-lysine are nitrosated directly by N\textsubscript{2}O\textsubscript{3} because these nitrosation reactions could be inhibited effectively by the N\textsubscript{2}O\textsubscript{3} scavengers morpholine and piperazine, respectively. In contrast, Turjanski et al. (42) reported that melatonin is nitrosated mainly by a combined attack of the radicals NO\textsubscript{2} and NO. It should be noted that such a mechanism has never been proven for ordinary amines and that a variety of side products, i.e. N-nitro, C-nitro, and C-nitrosoglutathione, had to be produced by an operating radical mechanism. Noticeably, the \textsuperscript{15}N NMR data demonstrated that reaction of \textsuperscript{15}N\textsubscript{2}O\textsubscript{3} with N-blocked tryptophan exclusively yields \textsuperscript{15}N-nitrosotryptophan.

Recently, the reaction between albumin and N\textsubscript{2}O\textsubscript{3} generated from NaNO\textsubscript{3} at low pH has been monitored by UV spectroscopy. On the basis of these measurements it was assumed that tryptophan would be nitrosated also (21, 23). However, this conclusion has not been generally accepted (22, 43). As there is presently no specific test for N-nitrosotryptophan available and because N-nitrosotryptophan derivatives and S-nitrosocysteine have almost identical UV-visible absorption spectra (\lambda\textsubscript{max} = 335 (24) and ~340 nm (30), respectively), the efficiency of nitrosation of both tryptophan and cysteine in proteins is hard to verify experimentally by UV-visible spectroscopy. On the other hand, the effectiveness of these reactions can reasonably be estimated on the basis of the experimental rate constants and the concentrations of both amino acids in proteins. At physiological pH values, tryptophan exists practically exclusively in the reactive nonprotonated form. Hence, the rate constant of N\textsubscript{2}O\textsubscript{3} with protein-bound tryptophan can be assumed to be k = 4.4 \times 10\textsuperscript{-7} M\textsuperscript{-1}s\textsuperscript{-1} (see "Results"). In contrast, thiols are only marginally deprotonated at pH 7.4. From the average pK\textsubscript{a} values of ~8.2 of thiolates in proteins (22), it can be deduced that only 13.5% of the cysteine residues are viable targets for N\textsubscript{2}O\textsubscript{3}. Thus, as the rate constants for reaction of thiolates (RS\textsubscript{−}) with N\textsubscript{2}O\textsubscript{3} is about k = 2 \times 10\textsuperscript{-8} M\textsuperscript{-1}s\textsuperscript{-1} (2), protein-bound cysteine should react at pH 7.4 with N\textsubscript{2}O\textsubscript{3} with a rate constant of k (cysteine + N\textsubscript{2}O\textsubscript{3}) = 2 \times 10\textsuperscript{-8} M\textsuperscript{-1}s\textsuperscript{-1} \times 0.135 = 2.7 \times 10\textsuperscript{-9} M\textsuperscript{-1}s\textsuperscript{-1}. Thus, the rate constant of the reaction of protein-bound tryptophan with N\textsubscript{2}O\textsubscript{3} is, somewhat unexpectedly, estimated to be about 63% higher than the rate constant of protein-bound cysteine with N\textsubscript{2}O\textsubscript{3}. To verify that this conclusion can be extended to the reaction rate, the amounts of both tryptophan and cysteine residues in proteins were verified by searching the RCSB Protein Data Bank (44) for those proteins that are believed to be S-nitrosated (Table II). From the data in Table II it can be deduced that in the selected proteins the total amount of cysteine is higher than the total amount of tryptophan. With regard to the differences in the rate constants (see above), one can now estimate that N\textsubscript{2}O\textsubscript{3} reacts primarily (40–90%) with tryptophan. Thus, we hypothesized that protein-bound tryptophan should be preferentially nitrosated under physiological conditions. To verify this prediction, we are currently developing highly sensitive protocols for the detection of both N-nitrosotryptophan and S-nitrosocysteine in proteins.

In this paper we have demonstrated additionally that N-nitrosotryptophan has the capability of releasing nitric oxide in the presence of ascorbate. This implies that N-nitrosotryptophan might operate as a nitric oxide carrier, a property that to date has been attributed more or less exclusively to S-nitrosocysteine (7, 11, 45, 46). However, as the chemistry of N-nitrosotryptophan derivatives in physiological environment is not very well developed, it is as yet too early to consider N-nitrosotryptophan derivatives as "harmless" compounds in biological sys-
tems. It might be assumed that potentially harmful reactive nitrogen species like peroxynitrite (40) or N₂O₃ are deactivated by their reaction with tryptophan residues and that this may give them some antioxidative functions. However, the observation of Venitt et al. (26) that N-acetyl-N-nitrosotryptophan at 5–15 ms induces mutagenicity in bacteria is, in our view, easily explained by its capability of releasing nitric oxide, which is known to induce such effects at unphysiologically high concentrations (47). In order not to be misunderstood, at present harmful effects of N-nitrosotryptophan derivatives cannot be ruled out. It should be remembered that harmful reactions are also known for S-nitrosothiols. For example, it has been reported that S-nitrosothiols react with thiols to yield nitroxyl (48), which generates hydroxyl radicals and/or peroxynitrite in the absence and presence of oxygen, respectively (49).

Because N-nitrosotryptophan derivatives are rather long-lived and yet not "indefinitely" stable compounds (the hydrolysis of N-acetyl-N-nitrosotryptophan is expected to yield the harmless products tryptophan and nitrite (28)), one may speculate that this could further decrease the putative mutagenic potential of N-nitrosotryptophan. On the other hand, the observed half-life (t₁/₂ > 2 h) is so long that N-nitrosotryptophan derivatives (N痫oehle,NO) may participate in physiological processes, e.g. in the transport and release of nitric oxide. In fact, Zhang et al. (21) observed that peptide-bound N-nitrosotryptophan induces vasorelaxation of rabbit aortic rings, a function that is typical for freely diffusing nitric oxide (45). In conclusion, we hypothesize that a putative physiological potential of N-nitrosotryptophan should be more important than its pathophysiological one. This feature remains to be clarified in the near future. In any case, we have identified, in addition to cysteine, a second major target for N₂O₃ in proteins. This fact will strongly influence the general understanding of protein nitrosation.

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