Catecholamine-induced release of nitric oxide from *N*-nitrosotryptophan derivatives: A non-enzymatic method for catecholamine oxidation

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In recent years, interest in the physiological functions of *S*-nitrosothiols has strongly increased owing to the potential of these compounds to release nitric oxide. In contrast, little is known about similar functions of *N*-nitrosated (*N*-terminal-blocked) tryptophan derivatives, which can be also formed at physiological pH. Utilizing *N*-acetyl-*N*-nitrosotryptophan (NANT) and *N*-nitrosomelatonin (NOMela) as model compounds, we have studied their reaction with catechol and catecholamines such as epinephrine and dopamine. In these reactions, NANT was quantitatively converted to *N*-acetyltryptophan (NAT), and nitric oxide was identified as a volatile product. During this process, *ortho*-semiquinone-type radical anions deriving from catechol and dopamine, were detected by ESR spectrometry. The catechol radical concentration was about eight times higher under normoxia than under hypoxia and a similar relationship was found for the decay rates of NANT under these conditions. An epinephrine-derived oxidation product, namely adrenochrome, but not a catechol-derived one, was identified. These observations strongly indicate that *N*-nitrosotryptophan derivatives transfer their nitroso-function to an oxygen atom of the catecholamines, and that the so-formed intermediary aryl nitrite may decompose homolytically with release of nitric oxide, in addition to a competing hydrolysis reaction to yield nitrite and the corresponding catechol. These conclusions were supported by quantum chemical calculations performed at the CBS-QB3 level of theory. Since nitric oxide is non-enzymatically released from *N*-nitrosotryptophan derivatives on reaction with catecholamines, there might be a possibility for the development of epinephrine-antagonizing drugs in illnesses like hypertension and pheochromocytoma.

Introduction

Catecholamines are known to be oxidized *in vivo* mainly enzymatically by monoaminoxidase and catechol-*O*-methyltransferase.[1] Although catecholamines are well known for their vasoconstrictor activity, there are only a few reports about their involvement in endogenous nitric oxide production. Girgin *et al.*[2] hypothesized an indirect way of enhancement of nitric oxide production in the presence of elevated catecholamine levels, since an increased monoaminoxidase activity would increase endogenous L-arginine concentrations. Rigobello *et al.*[3] reported on the release of nitric oxide during reaction of *S*-nitrosoglutathione with epinephrine in the presence of copper ions, pointing out that the latter would be absolutely necessary for nitric oxide formation.

Since *N*-nitrosotryptophan derivatives are known for both their vasodilatative activity[4] and their nitric oxide releasing potency,[5,6] the question arises whether catecholamines can release nitric oxide from *N*-nitrosotryptophan derivatives in the absence of copper ions. In this paper we report on the highly complex nitric oxide yielding mechanism of the reaction between catecholamines and *N*-nitrosotryptophan derivatives, such as *N*-acetyl-*N*-nitrosotryptophan (NANT) and *N*-nitrosomelatonin (NOMela) (Scheme 1). These surprising reactions can be explained satisfactorily by proposing the intermediacy of short-lived aryl nitrites.

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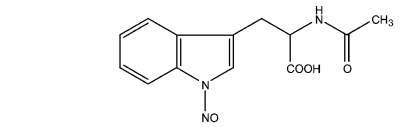
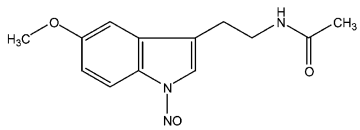
Results

Decomposition of NANT

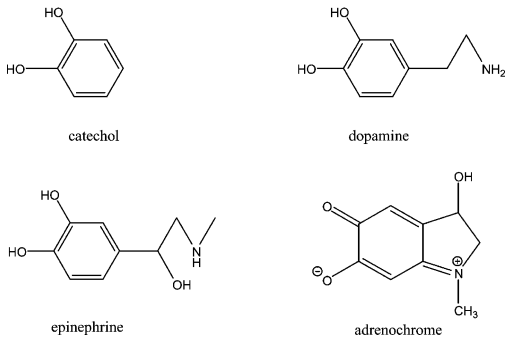
At pH 7.4, NANT is not stable in aqueous solution and decays hydrolytically with an apparent first-order rate constant of $k_{\text{hydrolysis}} = (6.6 \pm 0.1) \times 10^{-5}$ s^{-1} to produce stochiometric amounts of *N*-acetyltryptophan (NAT) and NO_2^- (Fig. 1).[7] This is confirmed by the ^{15}N NMR spectrum (Fig. 2A), showing partial hydrolysis of 100 mM ^{15}N-NANT in phosphate buffer within 24 hours at 20 °C (nitrite/ammonium sulfate ratio 40 : 60). The presence of either catechol or epinephrine (100 μM each) accelerated the decay of NANT (100 μM) with regard to the hydrolysis reaction (Fig. 1). The decomposition of NANT in the presence of catechol follows a clean first-order rate law with $k_{\text{catechol}} = (1.9 \pm 0.2) \times 10^{-4}$ s^{-1}. When catechol was used in large excess (100 mM catechol and 100 μM NANT, data not shown), the decay of NANT followed further a clean pseudo-first-order rate law with an apparent rate constant of 7.6×10^{-4} s^{-1}, *i.e.*, the rate constant was increased only about four-fold by increasing the catechol concentration 1000-fold. Since initially a complete conversion of NANT should have occured at this high excess of catechol, and because a first-order rate law was additionally found at equimolar concentrations, a



N-acetyl-*N*-nitrosotryptophan (NANT)



N-nitroso-melatonin (NOMela)



Scheme 1

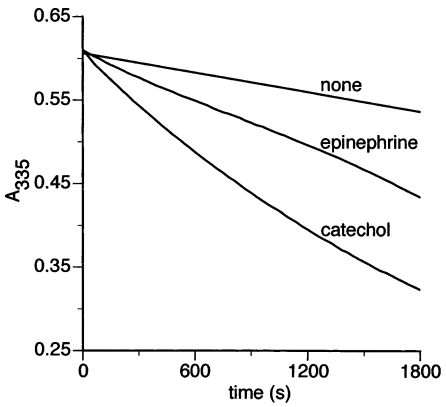
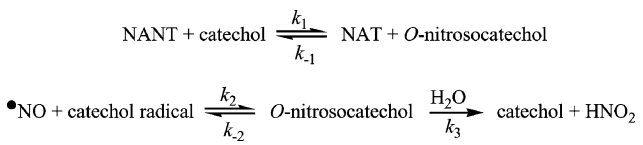


Fig. 1 The decay of 100 μM NANT in phosphate buffer (50 mM, pH 7.4, 37 °C), monitored spectrophotometrically at 335 nm in the absence and in the presence of either 100 μM catechol or 100 μM epinephrine. Each curve represents the mean of at least three experiments.

complex mechanism with a pre-equilibrium should operate, where the rate constant k_{-1} of the backward reaction is much faster than the rate constants k_2 and k_3 of the formation of products (Scheme 2). An even more complicated situation was evident in the presence of epinephrine, because the decay of NANT follows an unusual "wavy" time profile. Such behavior can be explained by the occurrence of an intermediate having a rather strong absorptivity compared to NANT. Only at the final stage of the reaction (1100



Scheme 2

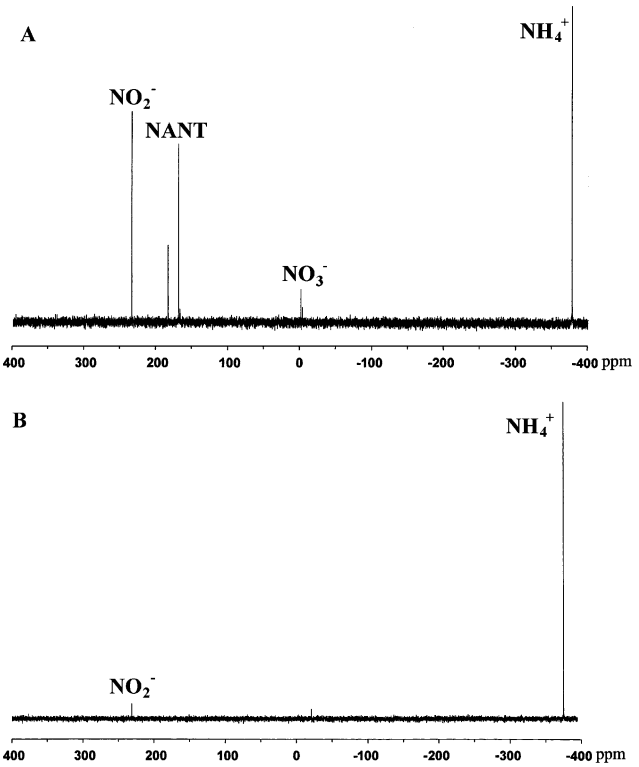


Fig. 2 ^{15}N NMR spectra. (A) The 24 h hydrolysis of 100 mM ^{15}N-NANT in 70 mM phosphate buffer yielded mainly $^{15}NO_2^-$ and some $^{15}NO_3$. (B) In contrast, the reaction of 100 mM catechol and 100 mM ^{15}N-NANT in 70 mM phosphate buffer yielded only small amounts of $^{15}NO_2^-$ after complete decomposition (3 h) when continuously purged with N_2. $(^{15}NH_4)_2SO_2$ was added as an internal standard after the above times. ^{15}N NMR data (phosphate buffer): ^{15}N-NANT: $\delta = 165.2/179.6$ ppm; $^{15}NO_2$: $\delta = 229.6$ ppm.

to 1800 s) did the decay trace seem to follow a first-order process, with a rate constant of $k = (4.3 \pm 0.9) \times 10^{-4}$ s^{-1}, *i.e.*, *ca.* 10 times higher than the first-order constant of the hydrolysis reaction of NANT. In addition, the decay of the optical density at 335 nm varied significantly with pH (Fig. 3). The apparent first-order behavior at the final stage of the reaction increased with decreasing

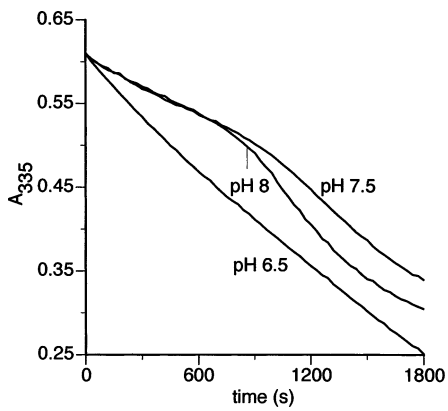


Fig. 3 pH Dependence of the reaction of 100 μM epinephrine with 100 μM NANT in phosphate buffer (50 mM, 37 °C, pH 6.5, pH 7.5, and pH 8). The decay of NANT was monitored by recording the optical density at 335 nm. Representative measurements for each pH are shown.

[H+] because it was most prominent at pH 8. At pH values lower than 6.5, the NANT hydrolysis reaction competed effectively with the bimolecular reaction and this limited the application of the acid pH value to 6.5.

The ^{15}N NMR spectrum shows products other than those produced from the hydrolysis reaction. Only small amounts of $^{15}NO_2^-$ (nitrite/ammonium sulfate ratio 5 : 95) and $^{15}NO_3^-$ (Fig. 2B) were formed. As the reaction mixture was continously purged with oxygen-free nitrogen during the course of the reaction, the formation of a volatile ^{15}N product, most probably nitric oxide, is indicated (see below). The low amounts of nitrite then represent the fraction of NANT hydrolysis within the 3 h reaction period. In line with these findings, under normoxic conditions the NANT-dependent production of nitrite, as determined by the Griess assay, corresponded to 100% and 70% in the absence and in the presence of 100 μM epinephrine, respectively. Thus, the catechol-dependent change in the product pattern clearly demonstrates the occurence of a direct reaction between NANT and catechol.

Notably, the reaction with catechol and catecholamines was specific for *N*-nitrosotryptophan derivatives, because under copper-free conditions GSNO did reacted neither with epinephrine nor catechol (data not shown) in agreement with the results of Rigobello *et al.*[3]

Formation of NAT

As the product pattern of the reaction between catechol and NANT differed significantly from that of the hydrolysis of NANT, we investigated whether NAT was formed quantitatively in the former reaction. Formation of NAT was monitored by following its fluorescence at 358 nm because NANT did not fluoresce at this wavelength (Fig. 4A). In fact, the increase in the fluorescence of NAT ($\lambda_{exc} = 270 \pm 5$ nm, $\lambda_{em} = 358 \pm 5$ nm) corresponded well with the decrease of the NANT concentration (Fig. 4B), demonstrating that NANT was quantitatively converted to NAT.

^1H, ^{13}C and ^{15}N NMR product studies

In order to identify the formation of other tryptophan- and/or catechol-derived reaction products, *e.g.*, nitrated or nitrosated intermediates and products, NMR spectra were performed with 100 mM catechol and 100 mM ^{15}N-NANT or NANT, respectively, in phosphate buffer. These spectra showed that catechol reacted with NANT with stoichiometric formation of NAT (Fig. 5), in line with the earlier experiments (Fig. 4B). Nitrosation and nitration products of NAT such as 1-NO_2-NAT, 6-NO_2-NAT, or other *C*-nitroso-NAT derivatives, as reported in ref. 8, were not detected in phosphate buffer, nor were any other products found at levels exceeding *ca.* 8%. Additionally, reaction of 10 mM ^{13}C-catechol with 10 mM ^{15}N-NANT in phosphate buffer was monitored by ^{13}C NMR in order to detect possible nitration of catechol in aqueous solution (data not shown). Again, these spectra did not show any significant formation of products deriving from catechol.

Adrenochrome formation

In contrast to the experiments performed with catechol, substantial formation of an epinephrine-derived product, namely adrenochrome, could easily be detected during reaction of epinephrine with NANT or *N*-nitrosomelatonin (NOMela). Reaction of 100 μM epinephrine with 100 μM NANT yielded adrenochrome concentrations of 12.1 ± 1.01 μM at 25 °C and of 26.9 ± 1.76 μM at 37 °C, respectively. Under the applied experimental conditions, the reaction periods were too short to allow autoxidation of epinephrine. A maximum concentration of 2.9 ± 0.99 μM adrenochrome was formed by autoxidation of 100 μM epinephrine after 30 minutes of incubation at 37 °C (Fig. 6).

Characteristic UV-vis spectra from the reaction between NANT and epinephrine at 37 °C are collected in Fig. 7. Since isosbestic points were identified (236 nm, 276 nm, 309 nm, 410 nm) during the reaction, the formation of an intermediate with a long lifetime at substantial concentrations can be safely excluded. Nevertheless, the formation of an intermediate, *i.e.*, a short-lived aryl nitrite, which either regenerates the reactants, or yields products according to Scheme 2 is still possible.

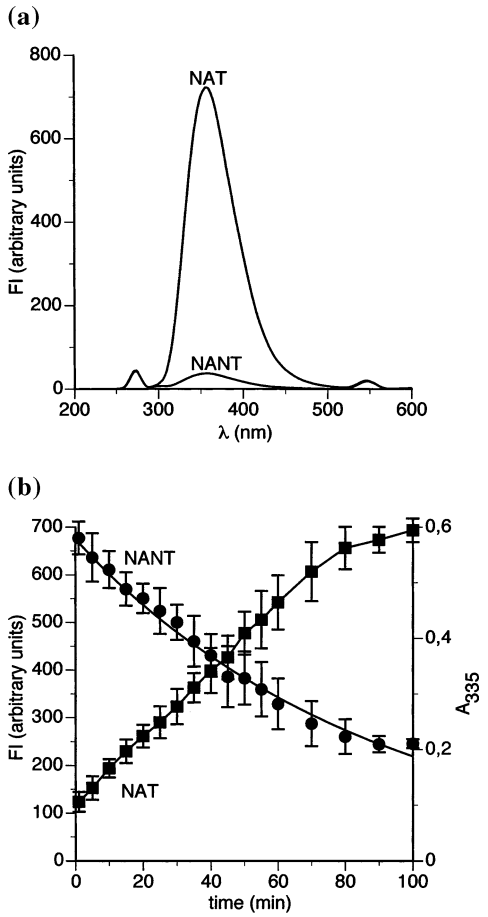


Fig. 4 (A) Fluorescence spectra of NAT and NANT ($\lambda_{exc} = 270 \pm 5$ nm, $\lambda_{em} = 358 \pm 5$ nm) in 50 mM phosphate buffer. (B) The formation of NAT from reaction of 200 μM NANT with 200 μM epinephrine was monitored spectrofluorophotometrically in phosphate buffer (50 mM, room temperature). The fluorescence intensity was determined from a 10-fold diluted aliquot of the reaction mixture. The decay of NANT was determined simultaneously by recording the absorption at 335 nm from a 2-fold diluted aliquot of the reaction mixture. Each value represents the mean of four experiments.

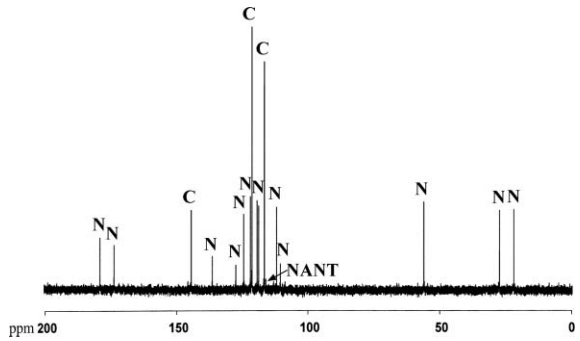


Fig. 5 ^{13}C NMR spectrum of the products from the reaction of 100 mM ^{15}N-NANT with 100 mM catechol in phosphate buffer pH 7.4, after four days. Signals of catechol and NAT are labelled "C" and "N", respectively. ^{13}C NMR data (phosphate buffer): NANT: $\delta = 22.06/22.1$ (CH_3), 27.6/27.4 (β-CH_2), 54.6/54.8 (α-CH), 113.5/111.3 (C-3), 115.7/119.1 (C-7), 120.2/121.8 (C-6), 125.6/125.56 (C-5), 126.3/122.6 (C-4), 126.8/127.8 (C-2), 129.7/129.5 (C-3a), 135.4/128.4 (C-7a), 173.5/173.4 (C=O), 177.6/177.8 (COOH) ppm. NAT: $\delta = 22.1$ (CH_3), 27.6 (β-CH_2), 56.1 (α-CH), 110.5 (C-3), 112.0 (C-7), 118.7 (C-5), 119.3 (C-6), 121.9 (C-4), 124.4 (C-2), 127.4 (C-3a), 136.3 (C-7a), 173.4 (C=O), 178.7 (COOH) ppm. Catechol: $\delta = 116.5$ (C-3, C-6), 121.2 (C-4, C-5), 144.3 (C-1, C-2) ppm.

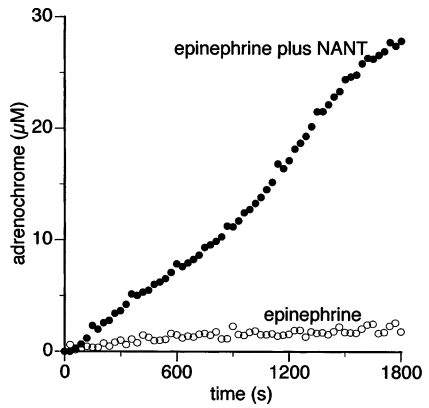


Fig. 6 Adrenochrome formation from 100 μM epinephrine in the presence and absence of 100 μM NANT, monitored spectrophotometrically at $\lambda_{max} = 487$ nm. Autoxidation of 100 μM epinephrine in 50 mM phosphate buffer (50 mM, pH 7.4, 37 °C) yielded *ca.* 3% adrenochrome. In the presence of 100 μM NANT, adrenochrome formation increased to *ca.* 27 μM. Each value represents the mean of at least three experiments.

Identification of hidden peaks

In order to evaluate whether the region at around 335 nm is influenced by adrenochrome, the UV-vis spectra were analyzed in detail. The deconvolution of the adrenochrome UV-vis spectra predicted in fact the existence of a UV-vis resonance at around 335 nm (Table 1), and this finding was confirmed by TD-DFT calculations. Since UV-vis spectra of both adrenochrome and NANT were accurately analyzed, peak-fitting software was used to re-analyze kinetic runs, an example of which is outlined in Fig. 7. The deconvolution of the UV-vis spectra predicted that the amount of NANT should fall within the reaction period of 30 min when 100 μM NANT is treated with 100 μM epinephrine at 37 °C (data not shown). After a reaction period of 17.5 min, the UV-vis resonances of adrenochrome, but not of any other intermediate, were clearly distinguishable. Thus, the optical density at 335 nm

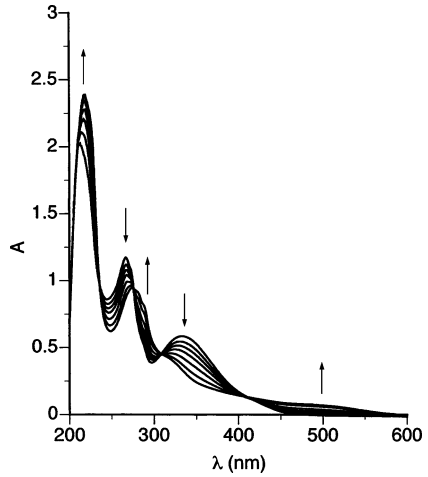


Fig. 7 UV-vis spectra from the reaction of 100 μM NANT with 100 μM epinephrine in phosphate buffer (50 mM, pH 7.4, 37 °C), taken every 5 min for an overall reaction period of 30 min. Data for a representative measurement are shown.

should be flawed by the adrenochrome formation at least for the last 12.5 min. This conclusion, however, is in striking contrast to the fact that during the reaction of NANT with epinephrine (Fig. 1) the decrease of the optical density at 335 nm was strongly enhanced during the last 12.5 min of reaction. This discrepancy can be only explained by the occurrence of an additional reaction (see below).

•NO-release

As NANT was almost quantitatively converted to NAT by catechol but no ^{15}N-labelled product could be detected in the ^{15}N NMR experiment (see above), nitric oxide was expected as the second product, similar to what has been observed from reaction of NANT with ascorbate.[5] Nitric oxide formation was monitored with an •NO-sensitive electrode at 25 °C and 37 °C. Epinephrine (500 μM) yielded a steady-state nitric oxide concentration of 2.4 ± 0.3 μM at 25 °C and 3.0 ± 0.8 μM at 37 °C. At the same concentrations, dopamine and catechol produced lower steady-state concentrations of nitric oxide, *viz.* 1.2 ± 0.1 μM from catechol at 25 °C and 2.7 ± 0.4 μM from dopamine at 37 °C. The steady-state nitric oxide concentration obtained from reaction of epinephrine with 100 μM NANT at 37 °C followed the epinephrine concentration in an exponential manner and leveled off at *ca.* 2.8 μM •NO at epinephrine concentrations >250 μM. The concentration-dependence of nitric oxide release from dopamine and catechol, respectively, was similar to the one found for epinephrine (data not shown).

Similar results were obtained when 100 μM NANT was replaced by 100 μM NOMela. Now nearly the same steady-state concentration of •NO was detected, *viz.* 2.4 ± 0.2 μM at 25 °C and 3.0 ± 0.5 μM •NO at 37 °C with 500 μM epinephrine. Thus, the epinephrine-stimulated release of •NO is not restricted to the model compound NANT.

The initial rates of nitric oxide release were evaluated from the initial slopes of the •NO-time profiles. The rates of •NO release from 100 μM NANT increased in the presence of epinephrine in a non-linear fashion with the epinephrine concentration (Fig. 8),

Table 1 Evaluation of peaks in the optical density UV-vis spectra of 100 μM adrenochrome and of 100 μM NANT in 50 mM phosphate buffer were evaluated with the PeakFit® program as well as with the TD-DFT: MPW1B95/6–311 + + (G,d,p)//O3LYP/6-311 + G(2d,p) density functional theory calculations

Adrenochrome					NANT						
								TD-DFT			
Exp.	Deconvol.		TD-DFT		Exp.	Deconvol.		*Z*-Conformer		*E*-Conformer	
λ/nm	λ/nm	Area	λ/nm	f^a	λ/nm	λ/nm	Area	λ/nm	f	λ/nm	f
218.9	219.4	69.9	223.9	0.004	201.0	199.7	65.9	212.8	0.015	210.8	0.013
			228.3	0.231				225.6	0.079	228.9	0.088
	271.9	8.5	278.3	0.021		236.0	17.0	236.7	0.013	235.7	0.010
303.8	303.5	35.5	296.7	0.305	265.7	264.8	26.0	241.0	0.399	240.7	0.368
	332.4	7.1	323.1	0.000		308.3	11.0	299.9	0.218	—	—
	368.7	4.9	—	—	334.8	335.5	15.7	—	—	320.5	0.203
	435.9	9.6	—	—		365.5	12.0	364.5	0.041	365.8	0.061
	483.8	10.3	—	—				429.0	0.001	432.3	0.001
486.6	487.5	10.4	492.8	0.074							
	523.2	8.2	513.9	0.000							
	577.6	2.6	—	—							

[a] f = Oscillator frequency.

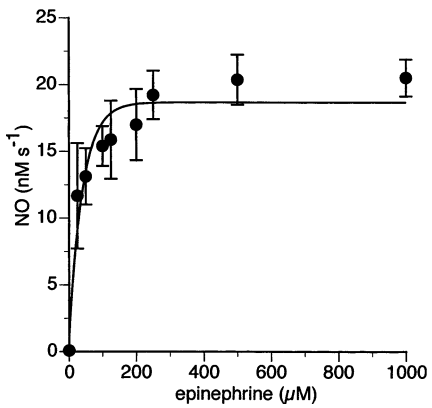


Fig. 8 The initial rates of •NO production from the reaction of 100 μM NANT with epinephrine, as a function of epinephrine concentration (25–1000 μM), determined in phosphate buffer (50 mM, pH 7.4, 25 °C). Each value represents the mean of at least four experiments.

to level off at about 21 nM s^{-1} (25 °C) and 27 nM s^{-1} (37 °C), respectively, at an epinephrine concentration of about 100 μM (data not shown). Epinephrine (100 μM) was somewhat faster (35 nM s^{-1}) in releasing nitric oxide from *N*-nitrosomelatonin at 37 °C than from NANT but the rate was lower at 25 °C (about 16 nM s^{-1}). Due to the leveling off in the rates of nitric oxide release at higher epinephrine concentrations, the experimental rates were evaluated only for epinephrine concentrations $\leq$100 μM. In addition, kinetic measurements were performed in order to evaluate the corresponding NANT decay rates.

Kinetic measurements

The initial decomposition of NANT or NOMela, respectively, was observed spectrophotometrically by monitoring the decay at 335 nm or 346 nm for the first 210 seconds of the reaction. The NANT decay rate was almost identical to the NOMela decay rate at equimolar conditions. When 100 μM epinephrine were added to 100 μM NANT at 25 °C, NANT decayed at 14.8 ± 4.1 nM s^{-1} and NOMela at 16.7 ± 4.2 nM s^{-1}. These decay rates of the *N*-nitrosotryptophan derivatives corresponded very well with the observed •NO-releasing rates shown in Fig. 8. In order to verify whether the reaction of NANT and catechol follows a second-order reaction, 700 μM epinephrine was added to 100 μM NANT at 25 °C (Table 2). The observed decay rate of 30.2 ± 3.1 nM s^{-1} was only about two times faster than the corresponding decay rate at equimolar concentrations, whereas a factor of 7 was expected for

Table 2 Decay rates of reactions between epinephrine and NANT. Varying amounts of NANT were added to varying amounts of epinephrine in 50 mM phosphate buffer, pH 7.4. The decay of NANT was determined spectrophotometrically at 335 nm by taking measurements every 30 s at 25 °C or 30 °C. The decay rate was evaluated from the first linear 210 s of data. Experiments were also performed in the presence of superoxide dismutase as well as under hypoxic conditions within an argon-flushed glove bag. Each data represents the mean of at least four experiments

Reactants	T/°C	Decay rate/nM s^{-1}
100 μM NANT + 100 μM epinephrine	25	14.8 ± 4.1
100 μM NANT + 100 μM epinephrine + 300 U ml^{-1} SOD	25	20.5 ± 2.9
100 μM NANT + 700 μM epinephrine	25	30.2 ± 3.1
100 μM NANT + 700 μM epinephrine + 300 U ml^{-1} SOD	25	32.1 ± 5.0
700 μM NANT + 100 μM epinephrine	25	53.5 ± 10.1
700 μM NANT + 100 μM epinephrine + 300 U ml^{-1} SOD	25	41.9 ± 5.1
700 μM NANT + 700 μM epinephrine	25	95.8 ± 4.7
100 μM NANT + 100 μM epinephrine	30	24.0 ± 2.5
100 μM NANT + 100 μM epinephrine under hypoxia	30	10.0 ± 1.6

a true second-order process. When 700 μM NANT was reacted with 100 μM epinephrine, a decay rate of 53.5 ± 10.1 nM s^{-1} was measured. The observed rates depended more strongly on the concentration of NANT than on the concentration of epinephrine. Employing 700 μM NANT and 700 μM epinephrine, the decay rate increased only 6.5-fold (95.8 ± 4.7 nM s^{-1} (Table 2)) whereas a 49-fold increase would be expected from a true second-order reaction. Further decay rates are collected in Table 2. These results clearly indicate that the reaction between epinephrine and NANT does not follow a simple second-order rate law.

Since under our experimental conditions epinephrine might yield small amounts of superoxide, which is well-known to react with *S*-nitrosothiols,[9–11] one may suspect that our kinetic experiments might be somewhat flawed by the intermediacy of this radical. However, additional experiments with 300 units ml^{-1} superoxide dismutase in the presence of varying concentrations of NANT and epinephrine at 25 °C revealed that the decay rates did not depend significantly on the presence of SOD (Table 2). Thus, superoxide is obviously not involved in the reaction between epinephrine and NANT. On the contrary, the decay rates are influenced by molecular oxygen. Under normoxia, a decay rate of 24.0 ± 2.5 nM s^{-1} was found from reaction of 100 μM epinephrine with 100 μM NANT, which was about 2.4 times faster than under hypoxic conditions, as outlined in Table 2.

Adrenochrome–NANT reaction

The "wavy" time profile (Fig. 1) of the NANT–epinephrine reaction not only derived from the interference with the absorption of adrenochrome at 335 nm, but also was affected by a reaction of adrenochrome with NANT. In order to simulate the conditions achieved during reaction of epinephrine and NANT, in which 100 μM NANT and 100 μM epinephrine yielded about 27 μM adrenochrome at 37 °C, the reaction of 25 μM adrenochrome with 100 μM NANT was observed in phosphate buffer (Fig. 9). At 37 °C, a decay at 335 nm as well as at 487 nm was observed spectrophotometrically. Since the optical density at 335 nm is flawed by adrenochrome, the NANT concentration cannot be deduced spectrophotometrically, but it can be safely assumed that *ca.* 4.93 ± 0.32 μM adrenochrome was consumed during this reaction (data not shown) by monitoring the decay of the absorption at 487 nm. The reaction of NANT with adrenochrome yielded NAT, in line with the reaction between NANT and epinephrine. In contrast to the latter reaction, nitric oxide was not detected with the •NO-sensitive electrode (data not shown), so that the reaction mechanism between NANT and adrenochrome is different from the reaction of NANT with epinephrine.

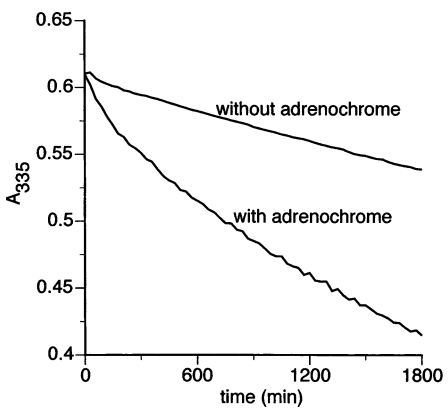


Fig. 9 The decay of 100 μM NANT in the presence and absence of 25 μM adrenochrome, followed by monitoring the optical density at 335 nm in phosphate buffer (50 mM, pH 7.4, room temperature). Each value represents the mean of at least three experiments.

Catechol radical anion and dopamine radical anion

The release of the radical nitric oxide *via* homolysis of an epinephrine-derived aryl nitrite necessarily implicates the formation of a second radical. Since the solubility of epinephrine was too low for the ESR experiments, this compound was replaced by dopamine. In fact, after mixing 7.5 mM NANT and 7.5 mM dopamine in phosphate buffer at pH 7.4, the characteristic ESR signal of the dopamine radical anion was immediately detected (Fig. 10A). The ESR signal decayed, in correspondence with the foregoing kinetic experiments. The catechol radical anion was detected by ESR on mixing NANT with catechol under similar conditions. In order to exclude the possibility that the catechol-type radicals were exclusively formed from reaction of catechol with nitrogen dioxide, which may be rapidly formed by autoxidation of •NO,[12] additional experiments were performed under strict oxygen-free conditions. Although the signal intensity was about eight times lower than under normoxic conditions, the generation

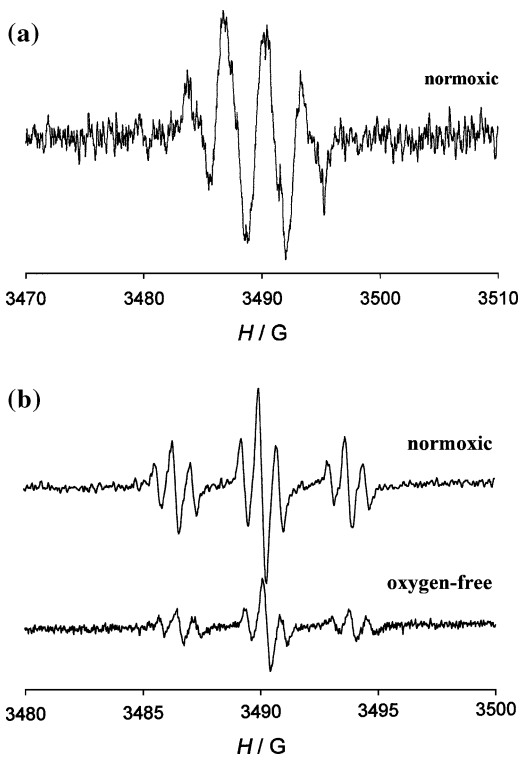


Fig. 10 (A) ESR spectrum of the dopamine radical anion recorded after rapid mixing of 7.5 mM dopamine and 7.5 mM NANT in phosphate buffer (50 mM, pH 7.4, room temperature). (B) Under similar conditions, the ESR spectrum of the catechol radical anion was recorded from 7.5 mM catechol and 7.5 mM NANT under normoxic and hypoxic conditions . The mixtures were supplemented by 100 μM DTPA to avoid any influence of metal ions.

of the radicals in the absence of oxygen was unequivocally demonstrated (Fig. 10B). This observation points clearly to the intermediacy of the short-lived aryl nitrite. A possible influence of metal ions was excluded by supplemention of 100 μM DTPA to the reaction mixture, after which no significant effect on the steady-state concentration of catechol radical ion was observed (data not shown).

Discussion

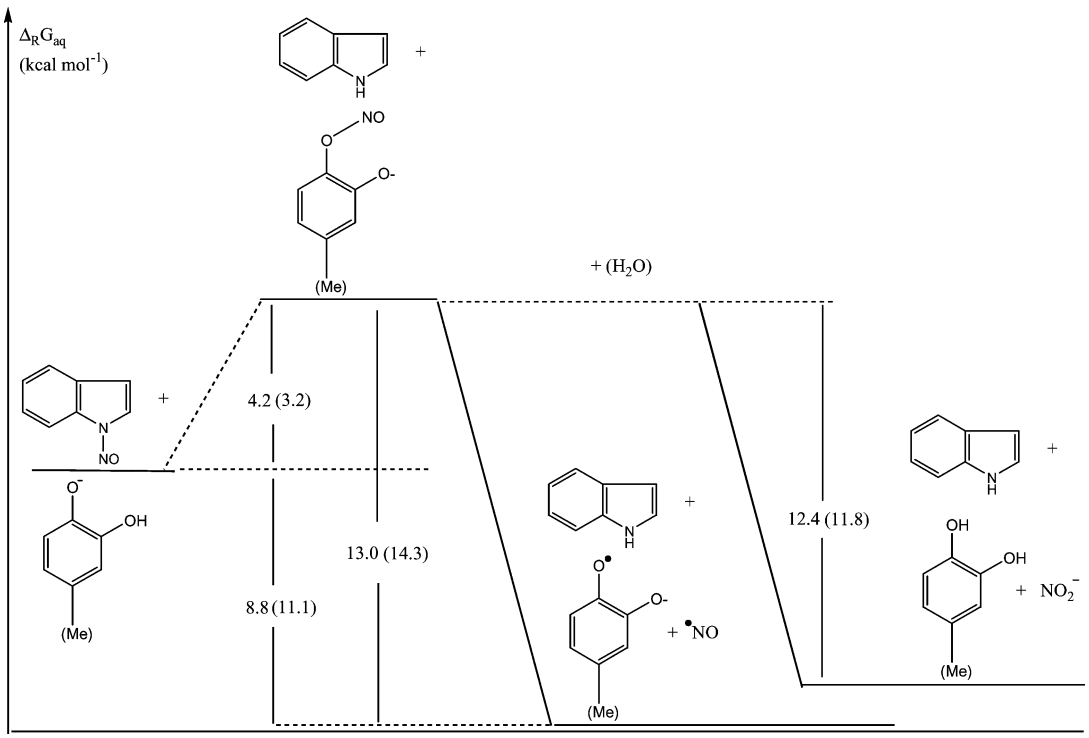
Our findings demonstrate for the first time the release of nitric oxide on reaction of catechols (*ortho*-hydroxyphenols) and catecholamines with *N*-nitrosoindoles like NANT or NOMela. Product formation proceeds either by initial electron transfer or *via* an intermediate aryl nitrite. Since catechol has an oxidation potential of $E_{ox} = 0.3$ V[13] it is unlikely to reduce *N*-nitrosotryptophan derivatives to the corresponding *N*-nitrosoindole radical anions ($E_{red} = -0.6$ V).[14] Therefore, initial electron transfer can be safely excluded. *N*-Nitrosotryptophan derivatives are known to nitrosate butanol,[15] thiols,[6] H_2O_2,[16] primary aromatic amines,[17] and tryptophan-derivatives;[18] hence, a similar transnitrosation reaction between NANT and the catechol/catecholamines appears feasible. In line with this, Darbeau *et al.*[19] reported on a transfer of the nitroso function from *N*-nitrosoamides to alcohols. Although our NMR and UV-vis spectrometric experiments failed to identify intermediary formation of *O*-nitrosocatechols, they should be the key intermediates. The formation of n-butyl nitrite from the reaction of NANT with butanol, and regeneration of NAT was described in 1977.[15,20] Likewise, NANT effectively *O*-nitrosates hydrogen peroxide.[16] Aryl nitrites are unstable towards rearrangement and transnitrosation[21] and *O*-nitrosophenols are known for their short lifetimes, as they are in equilibrium with $^{\bullet}$NO and the corresponding phenoxyl-type radical.[22–24] High-level quantum mechanical calculations performed with the multi-level method CBS-QB3, using *N*-nitrosoindole as placeholder for NANT and 4-methylcatechol as placeholder for epinephrine, indeed predict that the formation of *O*-nitrosocatechol and 4-methyl-*O*-nitrosocatechol are moderately endergonic processes (Table 3, entries 1–3). In aqueous solution, the aryl nitrite of catechol is predicted not to be a stable compound because the dissociation into nitric oxide and the corresponding catechol radical is calculated to be slightly endergonic by 0.8 kcal mol^{-1} for *O*-nitrosocatechol and slightly exergonic by −0.4 kcal mol^{-1} for 4-methyl-*O*-nitrosocatechol (Table 3, entries 4 and 5), in line with the experimental observations.[22–24] The hydrolysis of *O*-nitrosocatechol is expected to compete with the homolysis reaction, as it is calculated to be exergonic for both *O*-nitrosocatechol (−4.3 kcal mol^{-1}, Table 3, entry 6) and for 4-methyl-*O*-nitrosocatechol (−4.0 kcal mol^{-1}, Table 3, entry 7). The alternative mechanism, direct homolysis of NANT to give nitric oxide and the NAT aminyl radical (Table 3, entry 8), can be excluded because of its endergonic character, in line with the data of Zhu *et al.*[14] Noticeably, reaction of *N*-nitrosoindole compounds with catechol anions to yield the corresponding *O*-nitrosocatechols seems to be thermochemically favored (Scheme 3). Although the formation of the *O*-nitrosocatechol is predicted to be slightly endergonic (3–4 kcal mol^{-1}), the homolysis (−11 to −14 kcal mol^{-1}) as well as the hydrolysis (*ca.* −12 kcal mol^{-1}) of these aryl nitrites are calculated to be clearly exergonic, so that the reaction between *N*-nitrosoindole compounds and catechol anions appears feasible. The CBS-QB3 calculations further predict that the homolysis reaction (−14.3 kcal mol^{-1}) is thermodynamically slightly favored over the hydrolysis reaction (−11.8 kcal mol^{-1}) for 4-methyl-*O*-nitrosocatechol anion but not for *O*-nitrosocatechol anion (Scheme 3).

These data suggest that hydrolysis and homolytic fragmentation represent competitive pathways with a slight preference for hydrolysis of *O*-nitrosocatechol, but enhanced formation of $^{\bullet}$NO and the epinephrine-derived phenoxyl radical from *O*-nitrosoepinephrine. The additional release of catechol-type radicals is further predicted to enforce the decay of NANT (Table 3, entries 9 and 10). Since under aerobic conditions a higher NANT concentration leads to an enhanced release of nitric oxide, and thus of nitrogen dioxide,[12] the catechol radicals are additionally produced by reaction of catechol with nitrogen dioxide,[25] in line with an increased ESR-signal intensity of the catechol radical (Fig. 10B) and a faster decay of NANT (Table 2) under normoxic conditions. Hence, the reaction of NANT with catechol compounds cannot follow a simple second-order process, as was shown above. In the case of epinephrine the system is further complicated by the fact that the formed adrenochrome (Fig. 7) also enhances significantly the decay of NANT, albeit slowly. Since during this reaction nitric oxide release could not be detected by the $^{\bullet}$NO-sensitive electrode, we assume that adrenochrome supports the hydrolysis of NANT. As adrenochrome is mainly formed at alkaline pH,[26] this would explain the apparent first-order behaviour of NANT decay

Table 3 Quantum chemical calculations. Complete basis set (CBS-QB3) computations were carried out with Gaussian 03 (revision AM64L-G03RevC0.2) suite of programs. Gibbs free energies of solvation for water were estimated for the optimized gas-phase geometries with the CPCM-UAHF procedure incorporated in Gaussian 03

Entry	Reactant	$\Delta_R G$/kcal mol^{-1}	$\Delta_R E_{solv}$/kcal mol^{-1}	$\Delta_R G_{solv}$/kcal mol^{-1}
1	*E*-NO-indole + cat → indole + NO-cat	6.2	0.9	7.1
2	*Z*-NO-indole + cat → indole + NO-cat	6.2	1.0	7.2
3	*E*-NO-indole + mecat → indole + NO-mecat	5.8	1.0	6.8
4	NO-cat → $^{\bullet}$NO + cat-radical	0.4	0.4	0.8
5	NO-mecat → $^{\bullet}$NO + mecat-radical	−0.8	−0.4	−0.4
6	NO-cat + H_2O → *trans*-HNO_2 + cat	1.2	−5.5	−4.3
7	NO-mecat + H_2O → *trans*-HNO_2 + mecat	1.6	−5.6	−4.0
8	*E*-NO-indole → indole-radical + $^{\bullet}$NO	15.5	−1.8	13.7
9	*E*-NO-indole + cat-radical → indole + $^{\bullet}$NO + cat ox	−1.7	−11.9	−13.6
10	*E*-NO-indole + mecat-radical → indole + $^{\bullet}$NO + mecat ox	−1.8	−11.7	−13.5

cat = catechol, mecat = methylcatechol, NO-cat = *O*-nitrosocatechol, NO-mecat = 4-methyl-*O*-nitrosocatechol, cat-radical = catechol radical, mecat-radical = methylcatechol radical, cat ox = oxidized catechol (catechol dichinon), mecat ox = oxidized methylcatechol (quinone of 4-methylcatechol).



Scheme 3

at pH 8 at later stages of the reaction (Fig. 3). Adrenochrome formation can be explained by the intermediate formation of the corresponding quinone of epinephrine. The full reaction mechanism is shown in Scheme 4.

Zhang *et al.*[4] observed a vasorelaxation of aorta rings from male New Zealand white rabbits when treated with the nitrosated dipeptide *N*-nitroso-Gly-Trp. In that study the vessel rings were prepared in the presence of 1 μM phenylepinephrine. Our above results now provide an explanation for the observed vasorelaxation because, similarly to NANT, *N*-nitroso-Gly-Trp is expected to react with phenylepinephrine in the aortic tissues with the release of nitric oxide.

The reaction between *N*-nitrosated tryptophan derivatives and catechols might offer a starting point for the development of a new class of drugs for the treatment of diseases in which high catecholamine levels were found, as for instance in pheochromocytoma. This neuroendocrine tumor exhibits catecholamine concentrations up to 200 nmol l^{-1} in the corresponding adrenal vein,[27] and the general plasma catecholamine levels are 10 times higher than in the control group.[28] Due to these high catecholamine concentrations, pheochromocytoma patients can develop life-threatening hypertensive crisis.[29] It is conceivable that the application of a drug based on the reaction mechanism presented might be a useful therapeutical intervention in these illnesses because the catecholamines can be non-enzymatically destroyed with simultaneous release of nitric oxide (see Scheme 4).

On the other hand, Venitt *et al.*[30] reported on the mutagenicity of *N*-acetyl-*N*-nitrosotryptophan at high concentrations (>120 μM) in bacteria which would counteract pharmacological use. However, one should keep in mind that a variety of drugs are harmful at high concentrations, *e.g.* the drug sodium nitroprusside,[31,32] but that lower doses can sucessfully be applied.

In order to avoid any misunderstandings, we do not suggest the application of NANT to patients with hypertensive diseases. Nevertheless, one might speculate that in the future an optimized, yet unavailable *N*-nitrosotryptophan derivative may be found which will be both an effective vasodilatative and epinephrine-decreasing drug.

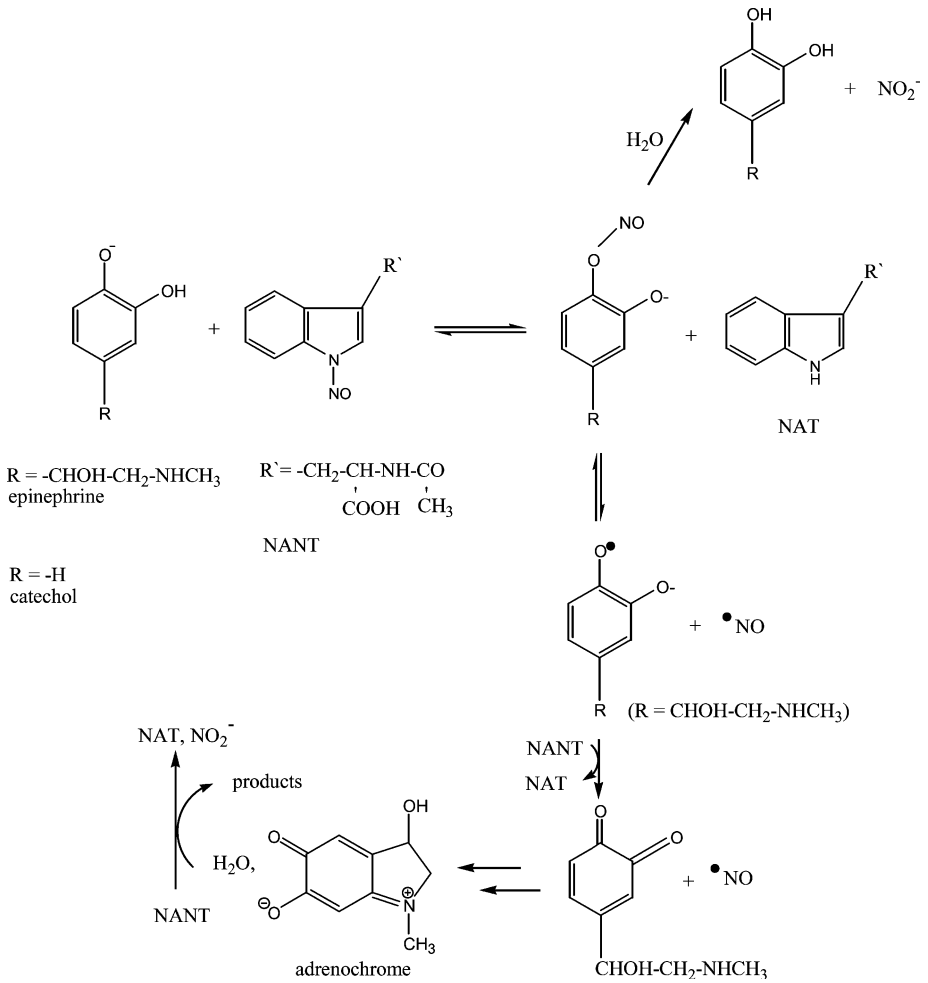
Experimental

Chemicals and solutions

N-Acetyl-DL-tryptophan, melatonin, catechol, epinephrine, adrenochrome, dopamine, and chelex 100 were obtained from Sigma (Taufkirchen, Germany). $^{13}C_6$-Catechol (99%) was purchased from Cambridge Isotope Laboratories Inc. (Andover, USA). Superoxide dismutase from bovine erythrocytes was from Roche (Mannheim, Germany). *N*-Acetyl-*N*-nitroso-DL-tryptophan (NANT), ^{15}N-NANT, *N*-nitrosomelatonin, and *S*-nitrosoglutathione were prepared as described in ref. 18. Stock solutions were prepared daily and their concentrations were spectrophotometrically determined at 335 nm and 346 nm, respectively, and checked at least every 6 h. All other chemicals were of the highest purity commercially available.

Experimental conditions

Since nitrosation reactions are highly sensitive to the presence of metal ions, phosphate buffer solutions (50 mM) were treated with the heavy metal scavenger resin Chelex 100 (0.5 g in 15 ml). After gently shaking, the solution was stored overnight and was then carefully decanted from the resin. Afterwards, the pH of all



Scheme 4

solutions was readjusted to pH 7.4 ± 0.1 by the addition of 50 mM H_3PO_4 or 50 mM K_3PO_4.

Determination of nitric oxide with an •NO sensitive electrode

Nitric oxide formation was determined using an •NO sensitive electrode (ISO-NO; World Precision Instruments, Sarasota, Florida) as described in ref. 33. The reaction mixtures were continously stirred throughout the measurements, and the temperature was kept at 25 ± 1 °C or 37 ± 1 °C. The electrode was calibrated daily, and •NO production was quantified according to the manufacturer's instructions, employing potassium iodide (100 mM) in H_2SO_4 (0.1 M) as a calibration solution to which varying amounts of $NaNO_2$ (0.5 mM) were added. A standard concentration of 100 μM of the nitrosated tryptophan derivative was applied to varying concentrations of catechols.

Decay kinetic experiments

The catechol- and catecholamine-induced decay of *N*-acetyl-*N*-nitrosotryptophan and *N*-nitrosomelatonin, respectively, was monitored on a SPECORD S 100 spectrophotometer from Analytic Jena (Jena, Germany) using the molar absorptivity ε_{487} = 2800 M^{-1} cm^{-1} for adrenochrome,[34] ε_{335} = 6100 M^{-1} cm^{-1} for NANT,[35] ε_{335} < 70 M^{-1} cm^{-1} for NAT, ε_{335} < 155 M^{-1} cm^{-1} for catechol, ε_{346} = 7070 M^{-1} cm^{-1} for *N*-nitrosomelatonin,[36] and ε_{336} = 770 M^{-1} cm^{-1} for GSNO.[37] The reaction between catechol/catecholamines and the nitrosated tryptophan derivatives (both 100 μM at pH 7.4) was monitored for 30 min by taking recordings every 30 s at 25 °C, 30 °C, and 37 °C. Experiments with epinephrine and NANT were performed also at pH 6.5, pH 7, pH 7.5, and pH 8. For the determination of the reaction order, the reaction between equimolar concentrations of catechol and NANT (100 μM and 700 μM) was monitored. Further, both compounds were reacted under pseudo-first-order conditions, *i.e.*, at 100 mM catechol and 100 μM NANT. In addition, 700 μM epinephrine was reacted with 100 μM NANT and *vice versa*. The possible influence of superoxide anions on this reaction was checked by adding 300 units ml^{-1} of superoxide dismutase (SOD) to the reaction mixture. Similar experiments (100 μM NANT and 100 μM epinephrine) were performed under hypoxic conditions in an argon-flushed glove box at 30 °C after the pO_2 of the buffer had dropped to <4.7 kPa (from 202–205 kPa at normoxia) as determined with a LICOX MCB® Oxygen Monitor (GMS, Kiel-Mielkendorf, Germany).[38]

Detection of NAT and NANT

The formation of NAT and the decrease of NANT were simultaneously monitored from a solution of 200 μM NANT and 200 μM

epinephrine in 50 mM phosphate buffer at room temperature for 100 min. The formation of NAT was determined by 10-fold dilution of the sample and recording of the fluorescence (λ_{exc} = 270 ± 5 nm, λ_{em} = 358 ± 5 nm) on a Shimadzu spectrofluorophotometer. The decomposition of NANT was spectrophotometrically determined by 1 : 1 dilution of the samples and reading the optical density at 335 nm.

Measurements of nitrite

The amounts of nitrite formed from both the reaction of 100 μM catechol or catecholamines with 100 μM NANT and the decomposition of NANT were quantified as follows: A 50 μl aliquot of the reaction mixture (NANT and catechol) and 250 μl phosphate buffer (pH 7.4) were added to 750 μl of the Griess reagent. The Griess reagent was prepared daily from 0.1% naphthylethylenediamine and 1% sulfanilamide (both from Sigma). After a reaction period of 10 min, the optical density at 542 nm was recorded and the nitrite concentration was evaluated by means of a calibration curve. All experiments were performed in quadruplicate 24 h after mixing the reactants.

ESR measurements

The catechol radical anion as well as the dopamine radical anion were identified by ESR spectrometry. ESR spectra were recorded at 18 °C on a Bruker ESP-300E X-band spectrometer (Bruker, Rheinstetten, Germany) equipped with a TM_{110} wide-bore cavity. Solutions were prepared from 1 ml of the buffer solution (pH 7.4) containing catechol and dopamine, respectively, and NANT (7.5 mM each). The reaction solutions were quickly transferred to a 0.4 mm aqueous solution quartz cell (Willmad, Buena, NY). Experiments with catechol were performed under normoxic and under hypoxic conditions. For the latter, a glove bag (Roth, Karlsruhe, Germany) flushed with argon was used. To check on the influence of metal ions, ESR spectra were also recorded in the presence of 100 μM DTPA (diethylenetriamine pentaacetic acid; Sigma). Recording conditions were as follows: microwave frequency, 9.79 GHz; modulation, 0.04 mT; signal gain, 5×10^5; sweep range, 10 mT; microwave power, 2 mW; sweep time, 2.8 min. Spectrum simulation was performed using the WinSim program.[39]

^{1}H, ^{13}C and ^{15}N NMR measurements

NMR experiments were performed on a Bruker AVANCE DRX 500 instrument (Bruker Biospin, Rheinstetten, Germany) at 500 MHz for ^{1}H NMR, 125.71 MHz for ^{13}C NMR, and 50.67 MHz for ^{15}N NMR spectra. ^{1}H and ^{13}C NMR chemical shifts (δ) are given in ppm relative to TMS ($\delta = 0$) as external standard. For ^{15}N NMR spectrometry, neat nitromethane ($\delta = 0$) was used as external standard. ^{15}N NMR measurements were performed on a mixture of 0.9 M catechol and 0.9 M ^{15}N NANT in a 9 : 1 DMSO/D_6-DMSO mixture. ^{13}C and ^{1}H NMR spectra were obtained from 1 M NANT and 1 M catechol in DMSO/D_6-DMSO 9 : 1 (v/v).

To elucidate the effect of water, similar measurements were performed with 100 mM catechol and 100 mM ^{15}N-NANT in 70 mM phosphate buffer solution at pH 7.2 in the presence of 10% D_2O as an internal lock. In addition, ^{13}C NMR spectra from 10 mM $^{13}C_6$-catechol and 10 mM ^{15}N-NANT were measured in phosphate buffer pH 7.45. In order to quantify the amount of nitrite formed during the decay of 100 mM ^{15}N-NANT in phosphate buffer as well as during the reaction of 100 mM ^{15}N-NANT with 100 mM catechol in ^{15}N NMR, 50 mM ^{15}N-ammonium sulfate (Sigma Aldrich) was added as an internal standard. To avoid the autoxidation of nitric oxide, the latter reaction mixture was continously flushed with nitrogen for 3 h.

Peak deconvolution software

In order to detect hidden peaks in the performed spectrophotometrical measurements, the PeakFit® program (SeaSolve Program Inc., Framingham, MA) was applied. Spectra from NANT, adrenochrome, and from the reaction of NANT with epinephrine (both 100 μM at 37 °C) were deconvoluted with the peak-fitting software.

Quantum-chemical calculations

Density functional theory (DFT) and complete basis set (CBS-QB3) computations were carried out with the Gaussian 03 (Rev C0.2) suite of programs.[40] Geometries were fully optimized to stationary points, using the O3LYP/6-311 + G(2d,p) level in the DFT calculations. Vertical excitation energies were computed on these optimized structures using the protocol of the time-dependent density functional theory[41] (TD-DFT) with MPW1B95/6-311 + + G(d,p) as model[42] for the first 10 excited states, in order to reproduce the UV-visible spectra of adrenochrome and *N*-nitrosoindole, respectively. To take into account solute–solvent-dependent spectral shifts in the UV-visible spectra, solvent effects were evaluated for all calculations of electronic spectra with the CPCM method. In addition, Gibbs free energies of solvation for water were estimated for the CBS-QB3 optimized gas-phase geometries with the CPCM-UAHF procedure incorporated in Gaussian 03. Both the CBS-QB3 and CPCM/(U)HF/6-31 + G(d) methodologies are known to provide thermodynamic estimates within "chemical accuracy" (±1 kcal mol^{-1}).[43,44]

Statistics

All experiments were repeated at least three times, except the ESR and NMR measurements which were performed twice. The results are expressed as means ± S.D.

Abbreviations

The abbreviations used are: •NO, nitric oxide; NOS, nitric oxide synthase; GSNO, *S*-nitrosoglutathione; NAT, *N*-acetyltryptophan; SOD, superoxide dismutase; NANT, *N*-acetyl-*N*-nitrosotryptophan; NOMela, *N*-nitrosomelatonin; DTPA, diethylenetriamine pentaacetic acid;

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