New NO-Donors with Antithrombotic and Vasodilating Activities, IV:

Chemical Reactivity of Nitrosimines and its Implications for their Pharmacologic Properties

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Received August 20, 1993

Nitrososydnone-5-imines and Thiazole-2-nitrosimines are susceptible to photolytic cleavage of the =N-NO bond. This can be achieved with a tungsten lamp. In water the corresponding syndnone imine salts are formed in 90% yield at 37°C. Only at higher temp. (70°C) ring opening is observed. In methanol about 25% of sydnones are obtained. On the other hand NO˙ and N_2O were detected in the head space of the reaction vials when oxygen was excluded. The formation of N_2O from nitrososydnone imine was increased up to elevenfold by glutathione while the amount of NO˙ was decreased. In the presence of light and thiols soluble guanylate cyclase (s-GC) was stimulated. The results suggest that the nitroxylate anion NO^- plays an important role in the stimulation of s-GC.

Neue NO-Pharmaka mit antithrombotischen und gefäßerweiternden Eigenschaften, 4. Mitt.:
Chemische Reaktivität von Nitrosiminen und ihr Einfluß auf pharmakologische Eigenschaften

Die =N-NO-Bindung von Nitrososydnon-5-iminen and Thiazol-2-nitrosiminen wird in Lösung durch Licht gespalten. Hierzu reicht eine normale Glühlampe aus. In Wasser bei 37°C werden die entspr. Sydnoniminsalze in 90proz. Ausb. erhalten. Nur bei höheren Temp. (70°C) wird auch Ringöffnung beobachtet. Werden methanolische Lösungen bestrahlt, so entstehen auch 25% Sydnone. Unter O_2-Ausschluß wurden in der Gasphase der Reaktionsgefäße NO˙ und N_2O gefunden. In Gegenwart von Glutathion wird bis zu elfmal mehr Lachgas gebildet, während die Menge an NO˙ abnimmt. In Gegenwart von Licht und Thiol wird die lösliche Guanylatyclase (s-GC) aktiviert. Die Ergebnisse sprechen für eine bedeutende Rolle des Nitroxylations NO^- bei der Stimulation der s-GC.

Recently we reported on the ability of several groups of nitrososydnone imines to inhibit the aggregation of blood platelets[1-3]. This effect *in vivo* led to an inhibition of the thrombus formation[1], so that these compounds can be classified as antithrombotics. This effect is accompanied by vasodilating properties. Therefore, a therapeutical application in the prophylaxis of thrombosis in people with risk factor hypertension could be envisaged. During our *in vitro* platelet studies we recognized that the *in vitro* effect can only be shown when the platelet rich plasma is irradiated with visible light *i.e.* simply by the lamp of the Elvi 840 aggregometer. We speculated that the active metabolite formed thereby should be a NO species. We, therefore, started a thourough investigation to clarify the structure of the decomposition products.

The photolytical decay of type **1** nitroso sydnonimines can be followed easily by electron spectroscopy. The intensive absorption at 318 nm (log ε = 4.29) is used. The results in HEPES buffer are shown in Fig. 1.

After 1 h of irradiation 14% of **1** have decomposed. If the results are plotted in a semi logarithmic scale it turns out that in this range of concentration the kinetic is roughly first order with a half live of ~ 210 min. This changes, however, when higher concentrations (40 mmol/L) are used for the structure elucidation of the metabolites (*v.i.*). When the probes are kept in the dark no degradation occurs at pH = 6.3.

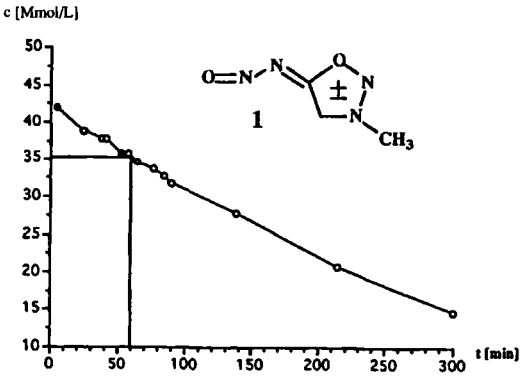

Fig. 1: Photolysis of the nitrosimine **1** with a 60 W tungsten lamp in HEPES buffer (pH 6.3) at 37°C (HEPES = 2-[4-(hydroxyethyl)-1-piperazino]-ethane sulfonic acid)

To get an impression what would happen to **1** when applied orally, we investigated the decay in 0.1 N HCl. In the absence of light we observe a half live of $t_{1/2}$ = 52 min indicating a mere hydrolytic degradation. The half-live is shortened by irradiation to 34 min.

To elucidate the structure of the products of the photolytical experiments a higher concentration (40 mmol/L) was used. Irradiation was performed with a 300 W Hg-lamp. The decay was controlled by tlc. After 65 h and 115 h the solvent was removed *in vacuo* at 37°C. The decomposition of the reaction mixture was assayed qualitatively and

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† Dedicated to *E. Böhme*, who died in June 1993

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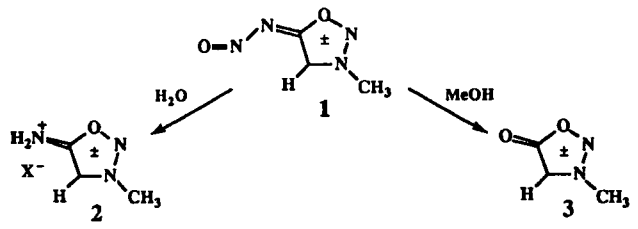
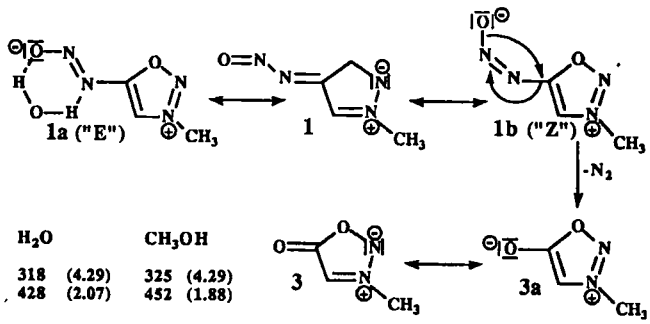


Solvent	H_2O	H_2O	H_2O	H_2O	MeOH	MeOH
atmosphere	air	air	N_2	N_2	air	air
time [h]	65	115	65	115	65	115
1 [%]	45	15	36	-	41	-
2 [%]	55	85	64	91	36	75
3 [%]	-	-	-	-	23	25
other [%]	-	-	-	NH_4^+/9	-	-

Scheme 1: Decomposition products of **1** when irradiated with a 300 W lamp at 37°C

quantitatively by ^{1}H-NMR spectroscopy. The decisive NMR data for **1-4** have been reported recently[2]. The results are compiled in Scheme 1.

It is obvious that in millimolar concentrations a longer time of irradiation for the decay is needed than in micromolar ones. Comparison of the values at 65 h and 115 h roughly suggests a zero order kinetic of the degradation. This is reasonable because of the limited power of the lamp. In water when oxygen is present the only product is the sydnone imine **2**. This shows its stability against ring opening reactions. This is in contrast to the behaviour of SIN 1, a metabolite of molsidomine. When the photolysis is performed under N_2 similar results are obtained. The reaction proceeds more quickly. After 115 h 9% of ring opening is observed.

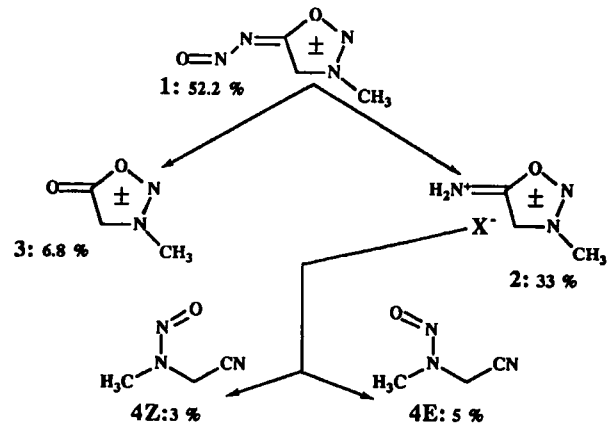


Scheme 2: Formation of the sydnone **3** in MeOH.
Spectral data (λmax, log ε) refer to compound **1** in the solvent stated

This can be seen by the formation of ammonium ions. - When the photolysis is carried out in methanol a remarkable ammount of the sydnone **3** is formed.

The reason for this difference is printed out in Scheme 2. The mesomeric forms of **1**, - **1a** and **1b** - suggest a partial double bond character of the N-N-bond in this nitrosimine. In water the true status of the molecule should correspond mainly to **1a**, the terminal oxygen being in an *E*-position. In methanol such a shift to **1a** does not seem favourable so that an electronic status of the molecule as depicted by **1b** becomes more probable and the elimination of molecular N_2 by an electrocyclic reaction *via* the intermediate formation of a diazaoxetane is observed. The evolution of N_2 often is regarded to be a thermal process. As compound **1** in methanol is stable at 37°C unless exposed to light it becomes evident that the activation energy can as well be supplied by radiation instead of temperature. Our interpretation is backed by the UV/VIS-spectrum of **1** in methanol or water. A remarkable hypsochromic shift is observed in water. This is quite consistent with the stabilization of **1a** in this solvent. The correlation between light and thermal energy was further investigated by performing the photolysis of **1** at 70°C, but only irradiating with a 60 W lamp. The results are compiled in Scheme 3. A considerable amount of **1** is retained showing the thermal stability of this compound. The behaviour of **2** when subjected to thermal stress is shown by its decomposition products **4Z** and **4E** which are formed by ring opening. This is a retro synthesis as **4Z** and **4E** can be cyclized to **2** in methanolic HCl.

The photolytic decomposition of nitrososydnone imines does not occur uniformly. When electron withdrawing substituents like an aromatic ring



Scheme 3: Photolysis of **1** (40 mmol/L) in water at 70°C with a 60 W tungsten lamp (120 h). The *Z/E* nomenclature was chosen because of the partial double bond character of the N-NO bond in nitrosamines

are present in position 4, an easier and much more drastic degradation is found. Obviously the stability of the primarily formed sydnone imine is decreased. This is in accordance with the observation that an electronegative atom as nitrogen in position 3 facilitates the ring opening reaction dramatically (cf. SIN 1).

On the other hand the photocleavage of nitrosimines is not restricted to the sydnone moiety. As shown in Fig. 2 quite similar observations can be made with thiazole-2-nitrosimines.

HEPES buffer was chosen as solvent because it was applied in the *Born*-test for determining the antiplatelet activity of **5**. An addition of DMSO was used because this solvent often is necessary to get compounds dissolved for this test, and we had observed an increase in the IC_{50} of **5** (3.5 µmol/L) when an 1:1 mixture of HEPES and DMSO was used as stock solution to prepare the dilutions for the test. In this case an IC_{50} = 120 µmol/L was observed which is quite near to the inhibiting effect of DMSO itself.

The reason for this observation can be deduced as well from Fig. 2. In DMSO a rapid degradation of **5** to the thiazolone **7** occurs by the expulsion of N_2.

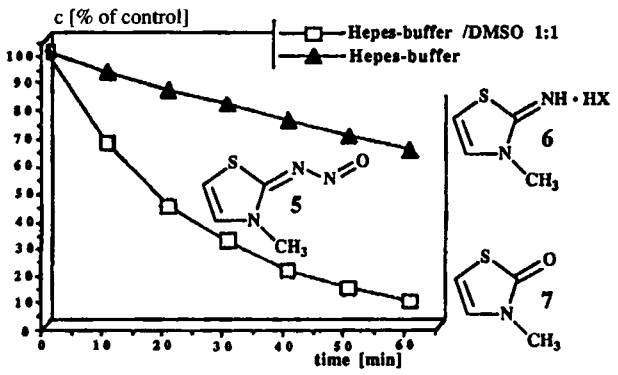


Fig. 2: Photodegradation of **5** (37°C, 60 Watt, C_o = 0.4 mmol/L), Observation wavelength 328 [□] or 334 [▲] nm

In HEPES buffer a much slower decomposition takes place which leads to the thiazolimine **6**. As both compounds **6** and **7** are inactive in the *Born*-test it becomes clear that only in mere aqueous solution the active metabolite of **5** is released by photolysis. Compound **5** itself was as well inactive when an aggregometer with a wavelength of 700 nm was used (IC_{50} > 250 µm). These results prompted us to have a closer look on this metabolite. When these experiments were carried out it had just become probable that EDRF (endothelial derived relaxing factor) were gaseous nitric oxide or at least a derivative of it. We, therefore, investigated the release of NO· from **1** during irradiation. The device for the chemiluminescence measurements is shown in Fig. 3.

When 37.5 µmol **1** were dissolved in 15 ml buffer (phosphate DAB 9, pH 7) and irradiated with a 100 W lamp for 10 min ~ 668 ± 22 nmol NO· were determined in the analyzer. This means that approximately 2% of **1** were cleaved in 10 min. This corresponds fairly well to the half live of 210 min obtained from the spectral measurement described above. As the formation of nitrosothiols from nitric oxide as transport from of EDRF is still in discussion, we carried out the same experiment in the presence of a threefold excess of glutathione (GSH). In this case the amount of nitric oxide was decreased by 30% indicating a reaction with GSH, presumably forming a "nitrosothiol". Another cause could be the reduction of NO· (*v.i.*).

As there have been reported anionic properties for EDRF[4,5] we took into account, that the nitroxylate ion (NO^-) might be involved into the EDRF mediated activation of soluble guanylate cyclase (s-GC). We, therefore, looked for the formation of this species during the light induced decay of nitrosimines. In aqueous solution NO^- stems from nitrosohydrogen (HNO), which is a weak acid (pK_S = 4.7)[6]. At neutral pH it is therefore dissociated > 99% to NO^-. The MO-theory suggest that two of its electrons occupy antibinding orbitals either in a singlett or triplett state[20]. This accounts for the low stability of NO^- which dimerizes to the dianion of hyponitrous acid (pK_{S1} = 7, pK_{S2} = 11[7]). At physiological pH in aqueous solution therefore a nearly equimolar mixture of the monoanion and hyponitrous acid should be present.

The monoanion is less stable than the acid and degrades to dinitrogen oxide (N_2O) and OH^- ($t_{1/2}$ = 97 min at pH 6.64, 25°C [8]). The detection and determination of NO^- can therefore be performed indirectly by an N_2O assay. As in the presence of oxygen NO^- forms peroxynitrite which rearranges to the nitrate ion, all experiments have to be carried out in an inert atmosphere (N_2, Ar). As N_2O strongly absorbs infrared light at 2200-2250 cm^{-1} it can be detected with the device shown in Fig. 4.

When 5 mmol **1** in 50 ml water were irradiated with a 60 W tungsten lamp for 24 h, a concentration of 50 ppm N_2O could be detected from the headspace by the analyzer. As

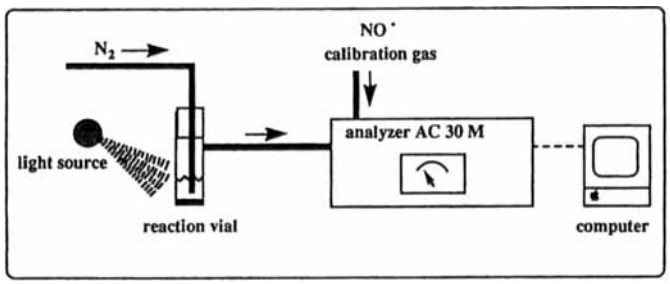


Fig. 3: Determination of nitric oxide released from nitrosimines by irradiation with visible light by chemiluminescence

this method is not suitable for the determination of small amounts of N_2O we further used a gc method. Hereby less than 1 nmol N_2O can be determined when an electron capture detector (ECD) is used. The reactions were carried out in head space vials (Experimental Part). The results obtained with two nitrosimines (**1**,**5**), two standards ($NO^{\cdot}$, SNP) and an HNO releaser ("Angeli's" salt) are compiled in table 1.

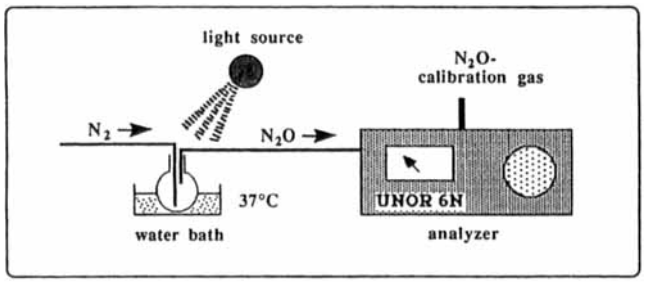


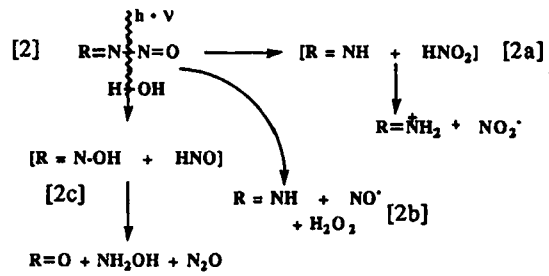
Fig. 4: Device for the detection of N_2O by IR-spectroscopy

The experiment with SNP is mere buffer not unexpectedly shows, that no nitroxylate is released (exp. 1). This changes when GSH is added (2). Now N_2O is formed suggesting the appearance of NO^- as an intermediate. It remains open whether HNO is displaced directly from SNP or whether NO^+ is reduced by GSH during or after dissociation from the complex. The following experiment with $NO^{\cdot}$ gas (5) shows that at least the second step really occurs at physiological pH values. The addition of GSH^* (3) which in principle could catalyze the reaction [1] i.e. the reaction of glutathione with electrophilic centers was without effect. On the other hand NO^+ at physiological conditions (water, pH = 7.4) is NO_2^-. This might indicate that a reduction of NO^+ during the release from SNP is more probable.

[1] $R{-}NO + GSH \xrightarrow{GSH^*} RSG + HNO$

Experiment 6 again shows that the nitrosimine **1** is completely stable provided visible light is carefully excluded. When irradiated the results established by IR-analysis are confirmed: 9 nmol of nitrous oxide are formed.

Regarding the mechanism of the imine formation already stated above, the following reactions are suggested [2].



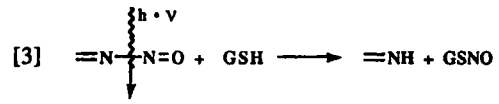
Reaction [**2a**] accounts for the overwhelming formation of sydnone imine salt stated by NMR-spectroscopy. If gaseous $NO^{\cdot}$ escapes into the headspace it can be detected by chemilumineszence. The sydnone imine and H_2O_2 possibly are byproducts. When $NO^{\cdot}$ recombines with $^{\cdot}H$ (instead of $OH^{\cdot}$) in a cross over reaction HNO, NO^- and finally N_2O are formed. Byproduct might be the sydnone and hydroxylamine. The latter is able to form N_2O and ammonia *via* disproportionation or reaction with $NO^{\cdot}$. This might account at least partially for the formation of the ammonium ions stated in Fig. 1. Furthermore the reaction between NH_2OH and H_2O_2 could give rise to NO^- or $NO^{\cdot}$.

The ammount of N_2O is amplified up to elevenfold when GSH is added (exp. 8,9). The yield strongly depends on the ratio between **1** and GSH. Maximum N_2O is obtained when

Tab. 1: N_2O formation from several compounds in phosphate buffer (pH 7.0, DAB 9) at 37°C. SNP = sodium nitroprusside; GSH = glutathione; DTT = dithiothreitol; GSH^* = transferase added; $Na_2N_2O_3$ = "Angeli's" salt; irradiation by halogen light 100 W

No. of exp.	Compound	amount [µmol]	additives	amount [µmol]	light [h]	N_2O [nmol]
1	SNP	10	-	-	-	0
2	SNP	10	GSH	30	-	13
3	SNP	10	GSH*	30	-	14
4	$NO^{\cdot}$	saturated	-	-	-	0
5	$NO^{\cdot}$	saturated	GSH	30	-	100
6	1	10	-	-	-	0
7	1	10	-	-	24	9
8	1	10	GSH	4	24	33
9	1	10	GSH	8	24	110
10	1	10	GSH	8	-	0
11	1	10	GSH	30	24	27
12	5	10	-	-	-	0
13	5	10	-	-	21	12
14	5	10	GSH	20	21	68
15	$Na_2N_2O_3$	20	-	-	-	69
16	$Na_2N_2O_3$	20	GSH	20	-	1
17	$Na_2N_2O_3$	20	DTT	20	-	20
18	$Na_2N_2O_3$ (PRP)	20	-	-	-	40

a 1:1 ratio is used. This again shows that nitric oxide radical is reduced to NO^-. When a threefold excess of GSH is used the formation of N_2O drops down to 27 nmol (11). This could be due either to a further reduction *versus* hydroxylamine or - more probably - by scavenging NO˙as a nitrosothiol by equation [3].



[3] $=N-N=O + GSH \longrightarrow =NH + GSNO$ (h • ν)

Similar results were obtained with the nitrosimine **5**. In the absence of light **5** is stable at pH 7 (12). When irradiated a similar amount of N_2O as with **1** is determined (13). It is raised again by addition of GSH (14). Regarding the formation of N_2O quantitatively it is obvious that at best 2% of **1** are converted to NO^- (see exp. 9). This prompted us to investigate what happens with "Angeli's salt" ($Na_2N_2O_3$), which is a releaser of HNO or NO^-, respectively. In aqueous solution it decomposes rapidly. This can be observed at 237 nm ($t_{1/2}$ ~ 3 min). This is accompanied by a rapid loss of its antiplatelet activity in the *Born*-test. If a freshly prepared solution (age: 30 s) is used an IC_{50} = 0.15 µmol/L is measured. If the solution is allowed to stand for 4 min we get an IC_{50} = 3.5 µmol/L. The mechanism of the decay of Angeli's salt is controversial. Undoubtedly in a closed system the only end products are N_2O and NO_2^-. If the gaseous products are removed from the solution physically up to 0.5 mol NO˙/mol $HN_2O_3^-$ can be determined by chemiluminescence[9]. This corresponds to the results of several authors who were able to scavenge NO˙ by reaction with hemoproteins[10-12] *e.g.* in equation [4] with myoglobin[12] (Mb).

[4] $MbO_2 + NO^\bullet \longrightarrow Mb^+ + NO_3^-$

These results were explained as follows[13]:

[5] $HN_2O_3^- \longrightarrow NO^\bullet + HNO_2^{\bullet-}$

[6] $NO^\bullet + HNO_2^{\bullet-} + HN_2O_3^- \longrightarrow [ONNOHNO_2]^{2-} + NO_2^- + H^+$

[7] $[ONNOHNO_2]^{2-} \longrightarrow ONNOH^- + NO_2^-$

[8] $ONNOH^- \longrightarrow N_2O + OH^-$

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[9] $2HN_2O_3^- \longrightarrow N_2O + H_2O + 2NO_2^-$

According to equation [5] NO˙ is formed. If not removed or scavenged, reaction with excess $HN_2O_3^-$ occurs forming firstly $[ONNOHNO_2]^{2-}$ and secondly hyponitrite $ONNOH^-$. The latter slowly decomposes to N_2O.

How do our results fit this scheme? Within the first 10 min during which Angeli's salt looses its antiplatelet activity, from 20 µmol $HN_2O_3^-$ there are formed 28 nmol N_2O. Within the next 4 h additional 40 nmol N_2O are yielded. This suggests that a direct decomposition according to equation [10] is possible.

[10] $HN_2O_3^- \longrightarrow NO^- + NO_2^- + H^+$

In parallel the vast amount of $HN_2O_3^-$ decomposes according to equations [5]-[8]. If the reactions [5] and [6] were quick while the reactions [7] and/or [8] were slow it is reasonable that additional N_2O is formed without biological active NO˙ or NO^- beeing present. As the half live of $HN_2O_2^-$ at pH 6.64 and 25°C [8] has been determined (spectroscopically at 248 nm) to be 97 min[8] we get an idea why so little N_2O is formed from $HN_2O_2^-$ in spite of beeing the only gaseous product[14-17]. On the other hand this suggests that a lot of NO^- might be stored at pH 7 as $HN_2O_2^-$ without forming N_2O immediately. So it seems possible that much more NO^- is formed from **1** and **5** than is determined by the nanomolar concentrations of N_2O. This would back the investigations of *Sies*[18] and *Fukuto*[19] who have postulated NO^- to be an important species in the activation of guanylate cyclase. When the decomposition of Angeli's salt is performed in the presence of GSH the N_2O formation is suppressed totally (exp. 16). This is in accordance with the results of *Doyle*[12] who found that at least thiophenol forms NH_2OH according to equation [11].

[11] $2\,C_6H_5SH + HN_2O_3^- \longrightarrow C_6H_5\text{-}S\text{-}S\text{-}C_6H_5 + NO_2^- + NH_2OH$

In the presence of dithiothreitol a reduced amount of N_2O is formed (exp. 17). When reacting degassed platelet rich plasma with $Na_2N_2O_3$ an intermediate amount on N_2O is found (exp. 18).

Finally we determined the stimulation of s-GC by SNP, **1**, **5** and Angeli's salt. For technical reasons these experiments could not be performed in the absence of O_2. Interestingly the enzyme is only active when reductive conditions are maintained by excess GSH or DDT. The results are compiled in tab. 2.

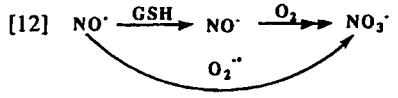
They show, that s-GC is stimulated by **1** and **5** in the presence of light. The presence of Mn^{2+} is more suitable than Mg^{2+} ions. The lower IC_{50} values for **1** and **5** compared to the K_M values only indicate a stronger release of the activator by the aggregometer lamp.

In summary our results show that during irradiation of nitrosimines NO˙ and NO^- are formed. In the presence of thiols the amount of NO^- is enhanced up to one order of magnitude. For the stimulation of s-GC the presence of thiols is a necessary requirement. One might speculate, therefore, that the nitroxylate ion is the endogenous stimulator of the s-GC. On the other hand it has to be taken into account

Tab. 2: Stimulation of s-GC by nitrosimines and SNP

Compound	day-light	K_M (Mg^{2+}) µmol/L	K_M (Mn^{2+}) µmol/L	IC_{50} µmol/L
1	+	280	10	2.4
1	-	670	370	2.4
5	+	647	72	3.5
SNP	+	2	0.4	1.5

that in the s-GC assay and in blood a certain concentration of O_2 is always present. So the reaction sequence [12] could easily explain why only nitrate is the physiological end product in the metabolism of NO·.

[12] 

The enhancement of the action of NO· by superoxide dismutase would as well find its explanation in an augmented formation of NO^-, leaving GSH more time to form this NO-species.

Experimental part

Chemistry

Angeli's salt was prepared as described[14]. The synthesis of **1**[2] and **5**[22] has been reported. Structure elucidations of **2, 3, 4, 6,** and **7** were performed by NMR-spectroscopy. As indicators the following signals were used (ppm): **1** (s, 8.73); **2** (s, 7.95); **3** (s, 7.01); **4E** (s, 4.68; s, 3.86); **4Z** (s, 5.51; s, 3.07); **5** (s, 7.88); **6** (d, 7.41); **7** (s, 7.38).

Spectroscopical devices

NMR: Bruker AC 300 or WM 250

UV/VIS: Perkin-Elmer, Lambda 15 and Hewlett-Packard 8451 diode array spectrometer.

Irradiation experiments

For each probe 50 mg of compound **1** were solved in the declared solvents in test tubes. To evacuate O_2 the probes were flushed with N_2 for 15 min and hermetically sealed with stoppers. To protect the probes against light, the test tubes were wrapped in tin foil.

The probes were kept at 37°C on a water bath and irradiated with a 300 W Hg-lamp from a distance of 30 cm. After 65 h and 115 h the solvents were removed *in vacuo* at 37°C and the residues examined by tlc and ^{1}H-NMR in [D_6]-DMSO.

Chemiluminescence measurements

A $2.5 \cdot 10^{-3}$ M solution of compound **1** in 15 ml phosphate buffer pH 7.0 (DAB 9) was filled into the reaction vial. The whole apparatus (Fig. 6) was flushed with N_2 until there was reached a constant value (normally 0.0 ppm) at the scale. Thereafter the sample was irradiated 10 min with a lamp (100 W) from a distance of 15 cm. The gas formed in the reaction vial was transferred to the analyzer (ansyco AC 30 M) by a constant flow of N_2. The peak values (ppm) were observed on a screen whereas the quantitative determination was performed by computerized integration. To standardize the analyzer, a calibration gas with 1 ppm NO· was used.

Semi quantitative determination of N_2O by infrared spectroscopy

On a water bath 50 ml of a 0.1 M solution of compound **1** in phosphate buffer pH 7.0 (DAB 9) were kept at 37°C. The sample flask was linked with the analyzer (MAIHAK UNOR 6N). The device was sealed and flushed with N_2 for 15 min. After an irradiation of 24 h with a lamp (60 W) the gas formed in the reaction flask was transferred to the analyzer by N_2. The ppm values in the N_2/N_2O gas mixture of nitrous oxide could be observed on a scale. An unirradiated sample was used as control.

Quantitative determination of nitrous oxide by gas chromatography

In principle the method described by *Elkins*[23] was used. In short 2 ml of a $2.5 \cdot 10^{-3}$ M solution of the nitrosimine in phosphate buffer (DAB 9, pH 7) were placed in a head space vial (6 ml) and sealed tightly. By means of two thin cannulas argon or highly purified N_2 was bubbled through the solution. After 15 min the needles were removed from the septum and the vial irradiation in the device described above for the time given in Tab. 1.

Then the vial is inserted in a HS 6 head-space device which is connected with a Perkin-Elmer F 22 gas chromatograph. The vial is heated to 60°C so that about 97% of the N_2O is in the gas phase ($\alpha = 0.087$). An aliquot of the gas phase (flushing time 5 s) is transferred to a Porapak Q column (diameter 2 mm, length 12 feet, 80/100 mesh) and eluted with N_2 (10 ml/min) at 50°C. Nitrous oxide is detected with an electron capture detector at a retention time of 4.9 min. The detection limit is below 1 nmol. The calibration is performed with a series of dilutions starting from a saturated solution of nitrous oxide in water (20°C, $\alpha = 0.63$).

Stimulation of guanylate cyclase (s-GC)

The assays were carried out as described by *Böhme*[24]. Irradiation time was 10 min (60 W).

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