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THIOL-MEDIATED GENERATION OF NITRIC OXIDE ACCOUNTS FOR THE VASODILATOR ACTION OF FUROXANS

MARTIN FEELISCH,* KARL SCHÖNAFINGER† and EIKE NOACK

Institute of Pharmacology, Heinrich-Heine-University, Düsseldorf; and †Department of Medicinal Chemistry, Cassella AG, Frankfurt am Main, Federal Republic of Germany

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Abstract—Furoxans (1,2,5-oxadiazole-2-oxides) are widely used in organic chemistry as intermediate compounds for the synthesis of various heterocycles. Despite the fact that some furoxans have been found to possess remarkable biological activities, up to now no systematic study on their mode of action has been reported. The aim of the present study was to investigate the molecular mode of the vasodilator action of furoxans. Furoxans, but not the corresponding furazans, concentration-dependently increased coronary flow in an isolated working rat heart preparation. This effect was blunted upon coinfusion with methylene blue. All tested furoxans were demonstrated to increase potently the activity of soluble guanylate cyclase. Enzyme stimulation was found to be mediated by the generation of nitric oxide (NO) following chemical reaction of the furoxans with sulfhydryl groups of low molecular weight thiols and proteins. Furoxans are thus prodrugs which increase the level of cyclic GMP via formation of NO and may therefore be classified as nitrovasodilators. Along with the generation of NO, nitrite and nitrate ions and *S*-nitrosothiols were formed. The rates of formation of these metabolites, however, did not appear to be related to enzyme stimulation. A tentative reaction scheme that fits the obtained experimental data is proposed. Recently reported cytotoxic, mutagenic, immunosuppressive and anticancer effects of furoxans are discussed in the light of their ability to release NO upon reaction with thiols.

Within recent years, furoxans (1,2,5-oxadiazole-2-oxides, furazan-*N*-oxides) have increasingly attracted the interest of organic chemists due to their usefulness as nitrile oxide precursors for the synthesis of various heterocycles and the modification of polymers (for review see Refs 1, 2). Moreover, furoxans can be easily reduced to the corresponding furazan compounds, some of which are difficult to obtain by other routes. Apart from the chemical and commercial interest in this class of compounds some furoxans have been shown to possess remarkable biological activities. Antibacterial, antihelmintic and fungicidal activities have been described for a number of furoxan compounds [3–8]. Some phenyl-substituted furoxans appear to have central muscle relaxant and anticonvulsant activity [9]. Furoxanobenzofuroxan was reported to enhance serotonin levels in the brain via inhibition of monoamine oxidase activity while blocking its effect in the periphery [10]. Nitrobenzofuroxans were found to inhibit nucleic acid and protein biosynthesis in various types of animal cells with particular effectiveness in leukocytes [11–13]. Along with these findings, some compounds were reported to exert distinct cardiovascular effects. *In vitro* vasodilator action was demonstrated in an isolated rabbit ear artery preparation [14]. Oral and intravenous administration of 3,4-disubstituted furoxans produced vasodilatation of the liver sinusoid in mice [15] and exerted potent depressor effects in cats, dogs and spontaneously hypertensive rats [14, 16, 17]. Moreover, some furoxans were found to effectively inhibit platelet aggregation *in vitro* and to possess potent anti-anginal oral activity (Bohn H and Just M, unpublished). The aim of the present study was to investigate the molecular mode of the vasodilator action of furoxans. Since some compounds of this chemical class were known to react with mercaptans [3, 11, 13, 18–20] experiments were performed in the presence and absence of different aliphatic thiols. We here report for the first time that furoxans are prodrugs which mimic the endogenous formation of NO following chemical reaction with sulfhydryl groups of low molecular weight thiols and proteins.

* Corresponding author at present address: Dr Martin Feelisch, Department of Pharmacology, Schwarz Pharma AG, Alfred Nobel Str. 10, D-4019 Monheim, F.R.G. Tel. (49) 2173-481739; FAX (49) 2173-481574.

MATERIALS AND METHODS

Working heart. The effects on coronary hemodynamics were determined in a pressure-controlled working heart preparation [21]. Briefly, heparinized (2500 U heparin, i.p.) guinea pigs of either sex, weighing 250–350 g (heart weight 1.34 ± 0.04 g, N = 72), were killed by a blow on the neck and exsanguination. After opening of the chest, the aorta was cannulated *in situ* and the heart was perfused in a retrograde manner with ice-cold oxygenated Krebs–Henseleit buffer by means of a roller pump. The heart was excised within 60 sec, quickly fixed to an all-glass system and allowed to beat spontaneously at a constant temperature of 32 ± 0.2°. After 10 min the retrograde perfusion mode was switched to orthograde conditions with the heart working against

an afterload of approx. 45 mm Hg. Left ventricular pressure and heart rate were monitored continuously by means of a micro tip catheter (3F, Millar®) which was inserted into the left ventricle via the aortic valve. Cardiac influx and coronary flow were measured continuously using electromagnetic flow probes. All parameters were documented on a multichannel recorder. The first addition of any test compound to the perfusion medium was performed after an initial equilibration period of 30 min. Basal values obtained at this time point were as follows: cardiac influx 26.1 ± 0.4 mL/min, coronary flow 3.22 ± 0.08 mL/min, left ventricular pressure 63.9 ± 0.5 mm Hg, heart rate 160 ± 1 beats per minute (N = 72). All compounds were tested under non-recirculating conditions and each heart was challenged with two drug concentrations only. The vasodilator potency of the respective test compounds was compared to that of glyceryl trinitrate (GTN*) after construction of complete concentration–response curves for the increase in coronary flow.

Guanylate cyclase stimulation. The effect of furoxans on the activity of soluble guanylate cyclase was determined using an enzyme preparation from rat liver as described previously [22]. All incubation runs were performed in triplicate in 50 mM phosphate buffer pH 7.40 containing 4 mM $MgCl_2$ and 1 mM GTP. The reaction was started by addition of 30 μg protein and was terminated after 15 min incubation at 37 ± 0.05° by addition of ice-cold Na_2EDTA buffer. Produced cyclic GMP was determined using a commercially available radioimmunoassay kit (Amersham).

Nitric oxide formation. The formation of nitric oxide (NO) was measured by means of a difference-spectrophotometric technique which is based on the NO-induced oxidation of oxyhemoglobin (HbO_2) to methemoglobin (MetHb) [22]. According to the 1:1 stoechiometry of the underlying reaction (HbO_2 + NO → MetHb + NO_3^-) the time-dependent increase in the concentration of methemoglobin mirrors the rate of NO formation. This indirect method is highly sensitive, specific for NO and not prone to interference with dissolved oxygen or generated nitrite. All incubations were performed in 50 mM phosphate buffer pH 7.70 at 37 ± 0.05° in the presence and absence of thiol compounds. NO formation rates were expressed as initial kinetics calculated from the slope of the absorbance readings obtained within the first 10 min of incubation.

Nitrite/nitrate formation. Nitrite (NO_2^-) and nitrate (NO_3^-) ions were simultaneously determined by means of ion exchange HPLC as described previously [22]. Briefly, anion separation was achieved without prior sample processing using an amino phase (LichroSorb NH_2, 5 μm, 125 × 4 mm, Merck, Darmstadt, F.R.G.) with an acidic aqueous phosphate buffer as eluent running at 2.2 mL/min (10 g/L KH_2PO_4, adjusted to pH 3.15 with H_3PO_4). Typical retention times were 1.9 and 2.6 min for nitrite and nitrate, respectively. For the sake of direct comparison all incubation runs were carried out under the same conditions as described for the measurement of NO formation. Reported values reflect initial formation rates. One single kinetic run consisted of at least seven determination points separated by time intervals of 4 min.

S-Nitrosothiol formation. The formation of *S*-nitrosothiols was determined by means of reversed phase HPLC [23]. Briefly, separation was carried out on a Supersher RP-18 column (5 μm, 12.5 × 0.4 cm, Merck) which was maintained at 20 ± 0.1° and eluted with a mixture of 12% methanol and 88% 50 mM phosphate buffer pH 2.45 at a flow rate of 1.0 mL/min. Detection wavelength was set at 332 nm. Peak identity was verified by comparison with freshly prepared aqueous solutions of crystalline *S*-nitrosothiols. Calibration was performed with aqueous solutions comprised of 50 μM thiol and 50 μM NO_2^-, preincubated in citrate buffer pH 2.0 at 4° after completeness of *S*-nitrosothiol formation had been verified photometrically. Initial kinetics were derived from the slope of the linear concentration increase observed within the first 20 min of incubation. Each single run consisted of at least five determination points.

Materials. All furoxan and furazan compounds were kindly donated by Cassella AG (Frankfurt, F.R.G.) and were stored dry, cool and protected from light. Stock solutions were prepared in dimethyl sulfoxide and diluted with buffer to the desired concentration. Final dimethyl sulfoxide concentration in all assay systems was < 1%. GTN was a kind gift from Schwarz Pharma AG (Monheim, F.R.G.) and was diluted with double distilled water from a commercial formulation in 5% aqueous glucose solution (Perlinganit®). *S*-Nitrosothiols were prepared by acid-catalysed S-nitrosation of the respective thiol compounds with sodium nitrite. Separation of solid compounds was achieved by aceton/ether precipitation at 4° and drying of the collected crystals in a stream of argon in the dark [24]. Identity was confirmed by elementar analysis, UV/VIS spectroscopy, mass spectrometry (M^+-30) and HPLC. All other chemicals were purchased from Sigma (Taufkirchen, F.R.G.) and were of the highest purity available. Aqueous solutions of all compounds were used within 30 min of preparation.

Data analysis. If not otherwise indicated data are presented as mean values ± SEM from three independent observations. Using Student's two-tailed *t*-test for paired variables differences were regarded as statistically significant when $P < 0.05$. Calculation of the concentrations at which guanylate cyclase stimulation was half-maximal (EC_{50} values) was assessed using the logit transformation [25].

RESULTS

Coronary vasodilator effects of furoxans

All tested furoxan compounds revealed marked dilator activity in the coronary circulation of isolated working hearts. The most pronounced effects were seen with C80-1324 (ipramidil) which was therefore selected for further in-depth investigation. The vasodilator action of ipramidil on coronary vessels was more potent than that of GTN and appeared to be biphasic (Fig. 1). The increase in coronary flow

* Abbreviations: GTN, glyceryl trinitrate; HbO_2, oxyhemoglobin; MetHb, methemoglobin; RS^-, thiolate anions.

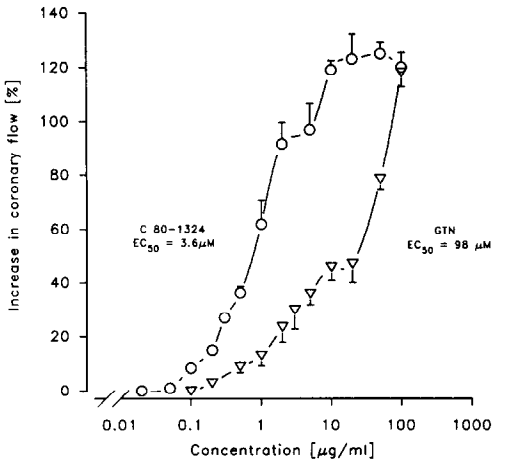


Fig. 1. Comparative concentration–response curves for the vasodilating effects of ipramidil and GTN on the isolated working guinea pig heart (N = 3).

caused by furoxans as well as by GTN was blunted upon coinfusion of the guanylate cyclase inhibitor methylene blue (data not shown). No sign of tachyphylaxis of the vasodilator response was seen upon the repeated or continuous (60 min) application of ipramidil at a concentration of 1 μg/mL, which induced an increase in coronary flow of 67 ± 9% (N = 5). Moreover, the vasodilator effect of ipramidil was not different from control when the compound was given after a 60 min preinfusion of 10 μg/mL GTN, i.e. under conditions of nitrate tolerance (P = 0.493, N = 3). However, when GTN was applied after a 30 min infusion of ipramidil, its dilator response was significantly diminished (P = 0.006, N = 3). Unlike GTN, ipramidil concentration independently increased the spontaneous beating rate of the hearts by 10–30 beats per minute and appeared to have a weak positive inotropic effect. The two furazans investigated, C81-1512 and C81-1525, did not reveal vasodilator activity toward the isolated working heart, but produced a weak contraction of coronary vessels instead (N = 2).

Guanylate cyclase stimulation by furoxans

Unlike organic nitrates furoxans stimulated soluble guanylate cyclase even in the absence of sulfhydryl-containing compounds. The addition of thiols markedly enhanced enzyme stimulation (Table 1). The individual susceptibility of furoxans to the presence of thiols appeared to differ substantially (data not shown). The chemical structure of the added thiol did not appear to play a crucial role since L-cysteine, L-*N*-acetylcysteine, D,L-homocysteine, L-glutathione, cysteamine, thioglycolic acid and 2-mercaptoethanol all induced a significant leftward-shift of the concentration–response curve for a given furoxan. The extent of potentiation was concentration-dependent and appeared to be related to the pK_{SH} of the respective mercapto group ($r = 0.94$, N = 7, data not shown). Guanylate cyclase stimulation was inhibitable by methylene blue and was completely abolished in the presence of oxyhemoglobin (Fig. 2) suggesting that enzyme activation by furoxans was mediated via the generation of NO. In neither the absence nor the presence of thiols could any guanylate cyclase stimulation be detected with the corresponding reduced counterparts of C80-1206 and C80-1324, the furazan compounds C81-1512 and C81-1525, at concentrations of up to 10 mM (N = 2).

Formation of nitric oxide, nitrite and nitrate from furoxans

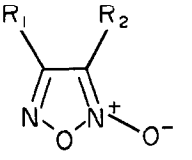
The investigated furoxans were found to be stable in aqueous solution at pH 7.4 for at least 2 hr as assessed by HPLC. There was no spontaneous formation of NO, nitrite or nitrate. Upon coincubation with reduced thiols, however, the furoxan compounds decomposed with the concomittant release of NO. Substitution of the furoxan ring in positions 3 and 4 obviously has a pronounced influence on the reactivity toward thiols and thus on the rate of NO release from the individual compounds (Table 2). Interestingly, the two methyl-substituted isomers C79-1030 and C79-1014 differed markedly in their ability to release NO, with the respective a-form (methyl-substitution in position 3) being more than 25-fold weaker than the b-form. The initial rates of NO release from a series of closely related furoxans correlated well with their individual potency of enzyme stimulation (data not shown), i.e. the higher the initial rates of NO formation from a given compound the lower was the respective concentration needed to stimulate soluble guanylate cyclase half-maximally (EC_{50} value). Virtually no formation of NO was detectable upon incubation of the furazan compounds C81-1512 and C81-1525 in the presence or absence of thiols. In accordance with the enzyme data, the extent of NO liberation from furoxans appeared to depend on the amount of free thiolate anions (RS^-) since (i) NO formation increased with increasing pH (tested for ipramidil and glutathione between pH 7.4 and 9; N = 4) and (ii) NO formation rates correlated with the pK_{SH} of the given thiol ($r = 0.905$, N = 5, data not shown).

The concentration dependence of NO formation was investigated in more detail with ipramidil and cysteine. At a given cysteine concentration of 1 mM the formation of NO was linear dependent on the concentration of ipramidil (0.01–1 mM; $r = 0.999$, N = 8). When the concentration of ipramidil was kept constant (0.2 mM) the rate of NO formation was linear dependent on the concentration of added cysteine in the range of 0.1–2 mM and plateaued at a concentration ratio of thiol : furoxan greater than 50 : 1 suggesting the accumulation of an intermediate reaction product, the decomposition of which becomes rate-limiting for NO production.

Along with the formation of NO a pronounced generation of nitrite and nitrate was observed when ipramidil was incubated with different thiols (Table 3). Nitrite : nitrate ratios for each of the five tested thiol compounds varied between 1.5 and 6.6 suggesting a rather complex stoicheiometry of the underlying reaction. When incubations were carried out in the presence of oxyhemoglobin thiol-mediated anion formation was shifted from nitrite to nitrate (due to cooxidation of scavenged NO to NO_3^- by HbO_2, see Materials and Methods), indicating

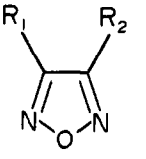
Table 1. Extent of soluble guanylate cyclase stimulation by 1,2,5-oxadiazole derivatives in the presence and absence of 5 mM L-cysteine

Furoxans



	R_1	R_2	EC_{50} (μM) + L-Cysteine	EC_{50} (μM) − L-Cysteine
C80-1324	CONH-*i*-propyl	CONH-*i*-propyl	2 ± 0.2	15 ± 1.3
C80-1206	CONH-methyl	CONH-methyl	5 ± 0.4	32 ± 3.9
C79-1030	$CONH_2$	methyl	185 ± 21	1750 ± 205
C79-1014	methyl	$CONH_2$	850 ± 54	4900 ± 570

Furazans



	R_1	R_2		
C81-1512	CONH-methyl	CONH-methyl	—	—
C81-1525	CONH-*i*-propyl	CONH-*i*-propyl	—	—

Listed EC_{50} values were derived from 2–4 complete concentration–response curves each obtained with a separate enzyme preparation.
Values are means ± SEM.

that nitrite partly originated from the hydrolytic decomposition of generated NO. In addition to the reaction with low molecular weight thiols, ipramidil was found to release NO upon incubation with freshly obtained human, porcine, dog and rat plasma, which might explain, at least in part, its antiaggregatory activity. Formation of NO was still detectable after removal of low molecular weight proteins by ultrafiltration of the plasma samples and appeared to correlate with total sulfhydryl concentration ($r = 0.938$, N = 6, data not shown). When plasma samples were preincubated with *N*-ethylmaleimide for 10 min NO formation was concentration-dependently diminished reaching complete inhibition at 3×10^{-4} M.

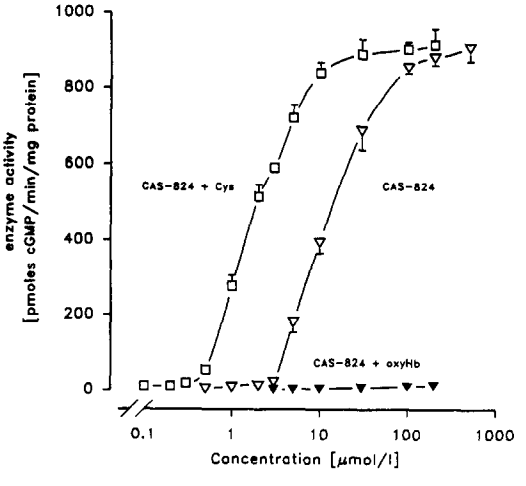


Fig. 2. Concentration–response curve for the stimulation of guanylate cyclase by ipramidil in the presence and absence of cysteine. oxyHb, oxyhemoglobin 10 μM; L-cysteine 5 mM, pH 7.40, 37°, N = 3.

S-*Nitrosothiol formation with furoxans*

Upon incubation of furoxans with different thiols a rapid and marked formation of *S*-nitrosothiols was measured. With ipramidil the highest formation rates were measured with cysteine and its *N*-acetyl analogue, followed by glutathione, cysteamine and homocysteine (Table 3). Initial rates of nitrosothiol formation linearly increased with increasing thiol concentration (N = 4, data not shown). The pH dependence of this reaction was investigated in more detail for ipramidil and glutathione. When the pH

Table 2. Initial rates of NO formation from several furoxans in the presence of cysteine

Compound	NO formation (μM/min)
C80-1324	19.91 ± 0.68
C80-1206	17.95 ± 0.83
C79-1030	0.81 ± 0.02
C79-1014	0.03 ± 0.01

Furoxans 1 mM; L-cysteine 5 mM, pH 7.70, 37°, N = 3–5.
Values are means ± SEM.

Table 3. Initial rates of formation of nitric oxide, nitrite, nitrate and *S*-nitrosothiol upon incubation of ipramidil with different thiols

Compounds	Formation rate (μM/min) NO	NO_2^-	NO_3^-	*S*-Nitrosothiol
Cysteamine	1.74 ± 0.44	1.65 ± 0.14	0.35 ± 0.02	0.13 ± 0.03
Glutathione	1.31 ± 0.18	1.16 ± 0.04	0.47 ± 0.03	0.24 ± 0.02
Cysteine	1.05 ± 0.05	2.05 ± 0.33	0.31 ± 0.02	0.54 ± 0.04
N-Acetylcysteine	0.51 ± 0.06	0.43 ± 0.05	0.28 ± 0.02	0.29 ± 0.02
Homocysteine	0.45 ± 0.04	0.86 ± 0.02	0.42 ± 0.03	0.09 ± 0.01

Ipramidil 0.2 mM, thiols 1 mM, 37°, pH 7.70, N = 4–6. Rates of nitrosothiol formation were not corrected for decomposition of the individual compounds under the conditions applied. Values are means ± SEM.

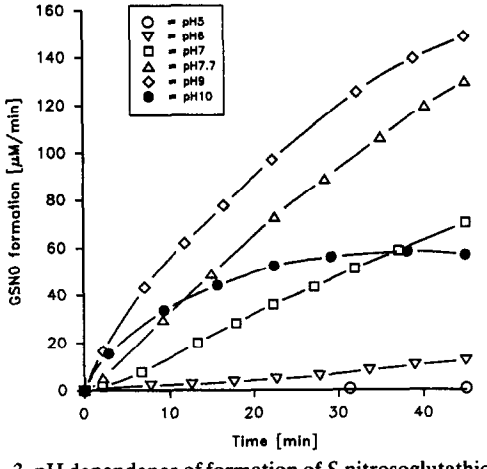


Fig. 3. pH dependence of formation of *S*-nitrosoglutathione (GSNO) upon incubation of ipramidil with reduced glutathione. Reaction conditions: C80-1324 1 mM, glutathione 5 mM, 37°, N = 2.

was varied between 5 and 10 the rates of nitrosothiol formation markedly increased upon alkalinization up to pH 9 (Fig. 3). From pH 8 onward the formation rate progressively decreased during the course of the incubation. The somewhat lower rate at pH 10 may be due either to a decreased stability of N_2O_3 as SH-nitrosating agent or of the formed nitrosoglutathione under these conditions, or may alternatively reflect the increasing participation of hydroxyl (OH^-) rather than thiolate anions in the reaction with furoxans. Moreover, above pH 8 the formation of three new metabolites of unknown structure was noted, which may be either products of alkaline hydrolysis of the furoxan itself or be due to the accumulation of reaction intermediates under these conditions.

The formation of *S*-nitrosothiols was abolished completely in the presence of 10 μM HbO_2 (verified for ipramidil and glutathione, and C80-1206 and *N*-acetylcysteine; N = 2 each). The same concentration of HbO_2 had no effect on the stability of either *S*-nitrosothiol but almost completely blocked S-nitrosation of both thiols by authentic NO under the experimental conditions applied (data not shown).

Identification of reaction products

Nitrite and nitrate ions were identified as stable end products in the incubation mixtures of furoxans and thiols by HPLC. Final NO_2^-:NO_3^- ratios were subject to considerable variation with different thiol compounds as reported for the respective initial rates of formation. No other stable ionic species were detected. The identity of formed *S*-nitrosothiols was validated by comparison with freshly synthesized crystalline *S*-nitrosothiols as described in Materials and Methods. No attempts were made to identify further intermediate compounds which were transiently formed during the reaction of furoxans with thiols due to their obvious instability. The main end products of the reaction between ipramidil and L-cysteine in phosphate buffer pH 7.4 at 37° were found to be an α,β-dioxime, the chemical structure of which was assigned to correspond to compound **13** in Fig. 4 (mp 228–9°, yield 34%), and the disulfide L-cystine **11**.

DISCUSSION

The present investigation demonstrates that furoxans are prodrugs which exert their vasodilator action via the NO/cGMP pathway. They may thus be classified as nitrovasodilators. The generation of NO from these compounds requires the preceeding chemical reaction with sulfhydryl groups. In order to gain insight into structure–activity relationships, chemical decomposition of and enzyme activation by a series of closely related furoxan compounds were investigated. For each compound the extent of guanylate cyclase stimulation correlated with the respective rate of cysteine induced NO formation (M. Feelisch *et al.*, manuscript in preparation). Parallel measurement of the formation of nitrite, nitrate and *S*-nitrosothiols following addition of different thiol compounds revealed a kinetic pattern distinct from that of NO formation suggesting that these metabolites arise from competing reactions unrelated to enzyme activation. The measured rates for the formation of nitrosothiols were remarkably lower than those for the formation of NO. Although we cannot entirely exclude that *S*-nitrosothiols might be either the precursors of NO generation or by-products of a competing reaction between furoxans and thiols, the results with HbO_2 strongly suggest that their formation is the result of S-nitrosation by NO following oxidation to N_2O_3 and N_2O_4. The measured rates of nitrosothiol formation may thus

reflect the net effect of individual trapping efficacy of the different thiols for NO and their respective stability under the experimental conditions applied. The finding that the rate of NO release appeared to be related to the pK_{SH} of the added thiol compound and increased upon alkalinization is in accordance with a nucleophilic reaction mechanism. We would like to propose the following reaction sequence which fits the experimentally obtained data (Fig. 4): delocalization of the positive charge on N-2 of the furoxan ring confers electrophilic character on C-3 and N-5. A nucleophilic attack of RS^- on furoxans **1** is thus conceivable to occur at positions 3, 4 and 5 of the heterocycle. As depicted in the first reaction sequence the attack of RS^- at position 3 or 4 leads to the formation of the intermediate compounds **2** or **5**, respectively. Both intermediates undergo ring opening to the nitroso derivatives **3** and **6**, respectively, from which NO is formed by oxidation of eliminated nitrosyl anions (NO^-). The reaction of **6** with further thiol may yield the *S*-nitrosothiol **8** which decomposes to NO via radical cleavage. In this speculative mechanism the nitroso compounds **4** and **7** are either end products or may undergo further hydrolytic or thiolytic reaction. The generated NO reacts with molecular oxygen to form NO_2 and N_2O_3 which besides hydrolysis to nitrite and nitrate anions may nitrosate thiols to form *S*-nitrosothiols and nitrite.

A competing reaction is likely to occur via the attack of thiolate anions at N-5 of the furoxan ring. The corresponding reaction between ipramidil and L-cysteine is depicted in the lower part of Fig. 4. The proposed mechanism for this reduction is the attack of a cysteinyl anion at N-5 which is followed by an opening of the furoxan ring to form intermediates **9** and **10**. Further reaction with L-cysteine gives L-cystine **11** and the anion **12** which under physiological conditions is in equilibrium with the protonated dioxime **13**. At higher pH an increasing participation of hydroxyl anions (OH^-) may lead to the competing formation of the corresponding sulfenic acid **14** rather than the disulfide. This reaction mechanism is similar to the one proposed for the reaction between benzofuroxan and thiols [18].

Due to the high reactivity of furoxans with sulfhydryl groups, the prolonged incubation of tissues with such compounds may lead to a considerable depletion of endogenous thiol pools. This may explain the finding that the coronary vasodilator effect of GTN in this study was markedly diminished after preincubation with the furoxan ipramidil. In contrast to classical organic nitrates, the pharmacodynamic action of furoxans did not appear to depend on the presence of specific thiol compounds such as cysteine. This may explain why their pharmacological effect persisted in a working heart preparation that was rendered tolerant to organic nitrates by pretreatment with a high concentration of GTN. Preliminary experiments indicated that furoxans are metabolized to NO by cultured aortic smooth muscle cells and that this NO formation is accompanied by an increase in intracellular cGMP levels (M. Feelisch, unpublished observation). Since free thiol groups are abundant in the membrane as well as in the cytosolic fraction of virtually every cell, the bioactivation of furoxans to NO *in vivo* is unlikely to be restricted to certain tissues or organs and may not even depend on an enzymatic activation step. The bioactivation of furoxans *in vivo* is likely to be mediated mainly by non-enzymatic pathways involving the reaction with critical sulfhydryl groups. The total amount of free sulfhydryl groups and the prevailing pH of the respective microenvironment may be the major determinants for the extent of NO generation and thus the biological activity of furoxans *in vivo*. Whether or not the relatively high reactivity of furoxans with thiols will limit their therapeutic applicability and the question as to which critical factors will govern bioavailability and tissue selectivity of a given furoxan remain to be investigated.

The two furazans investigated in this study, which were considered as promising prodrugs of the respective furoxan compounds, did not show vasodilator activity *in vitro*. This is explained by the fact that these compounds did not release NO upon reaction with thiols and thus did not stimulate guanylate cyclase. The lack of vasodilator effect on the isolated working heart suggests that the conceivable N-oxidation of the furazans to the corresponding furoxans and the subsequent formation of NO either does not take place at all in the heart or is a reaction too slow to account for the overall biological action of these compounds *in vitro*.

Certain nuclear protein thiol groups appear to be intimately involved in gene control and neoplastic proliferation [26]. Compounds exerting an influence on the activity of such thiols may thus have potential anticancer effects. Some furoxans have been shown to possess antileukemic and immunosuppressive action [11, 12]. Here, we demonstrate that furoxans generate NO by interaction with sulfhydryl groups. These compounds may thus mimic the action of activated macrophages which are thought to exert their cytotoxic effect against tumor cells by an enhanced release of NO [27]. Whether the reported anticancer effects of furoxans [28–32] are brought about by the release of NO awaits further investigation. Very recent results from two independent groups furthermore suggest that high concentrations of NO may lead to partial deamination of DNA bases and DNA strand breaks [33, 34] which may explain, at least in part, the reported mutagenic or promutagenic potential of furoxans [35, 36].

It has been demonstrated that nitrite reacts with olefins to form furoxans via a short-lived dinitrosoalkene intermediate [1, 37, 38] and a similar reaction has been described for α,β-unsaturated carbonyl compounds [39–41] such as *p*-hydroxycinnamic acid derivatives, which are natural constituents of vegetables. One may assume that furoxans might be formed *in vivo* by the reaction of nitrite with certain unsaturated fatty acids in cell membranes. Alternatively, NO generated by endothelial cells or activated macrophages might react with endogenous olefins to form furoxans following oxidation of NO to the nitrosating species N_2O_3 or N_2O_4. Although admittedly speculative, it is conceivable that endogenously formed furoxans

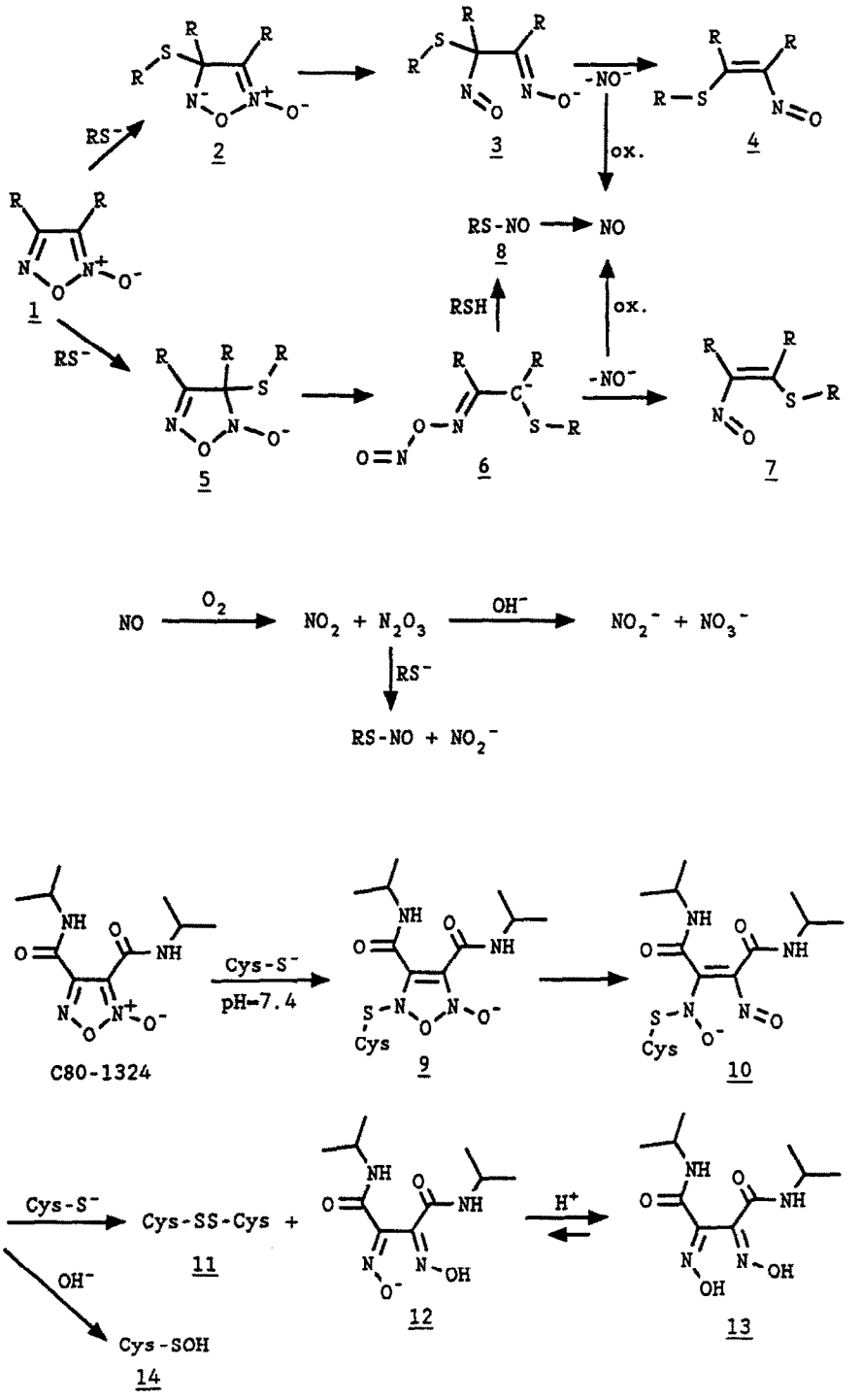


Fig. 4. Proposed mechanism of the chemical reaction between furoxans and thiols.

might be stored within distinct cellular compartments to give rise to NO when released via reaction with sulfhydryl groups of proteins or low molecular weight thiols such as glutathione. They would thus represent a form of intracellular storage for NO. Whether or not such a reaction does indeed occur *in vivo* will be the subject of future investigations.

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