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Reaction of Drugs with Sodium Nitroprusside as a Source of Nitrosamines

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Abstract □ Potentially dangerous nitrosamines have been shown to result from the reaction of sodium nitroprusside with several drugs under physiological conditions (pH 7.3 and 37℃). In each case the products were identical to those produced upon reaction with nitrous acid at much lower pH values. Reaction rates were shown to reflect a first order dependence on both amine and nitroprusside concentrations and to increase at higher pH values, approximately in proportion to concentrations of unprotonated amine. Fast reactions of sodium nitroprusside with reduced glutathione, cysteine, and ascorbate suppress but do not prevent the conversion of amines into N-nitrosamines. These results show sodium nitroprusside to be very potent nitrosating agent under physiological conditions and suggests nitrosamines may be formed during its normal pharmacological administration.

Keywords □ N-Nitrosation, secondary amines, carcinogen, blocking agents

Sodium nitroprusside, SNP, $[Na_2Fe(CN)_5NO \cdot 2H_2O]$, is a potent, fast acting, intravenous hypotensive agent. It is used to lower blood pressure during hypertensive and cardiac emergencies and for induction of "controlled hypotension" during various types of surgery[1-5]. It is administered as a dilute aqueous solution directly into the blood stream and is said to be the fastest acting and most dependable of all known hypotensive agents. Its hypotensive effects were first reported in 1887[6], but it was not widely used until 1974, when it became available in a convenient to administer form. Its use has been increased rapidly since that time.

Malz *et al.*[7] showed that SNP could be used to effect the nitrosation of amines under alkaline conditions. Casado *et al.*[8] have described evidence for intermediates in such reactions, formed by addition of the unprotonated amine to the coordinated nitrosyl moiety, as illustrated in Equation (1).

$$[(CN)_5FeNO]^{-2} + R_2NH + OH \longrightarrow [(CN)_5FeNONR_2]^{-3} \longrightarrow [(CN)_5FeOH]^{-4} + R_2NNO + H^+ \quad (1)$$

These short-lived adducts then undergo rapid ligand exchange to give nitrosamines as shown. The ability of SNP to react with several secondary amine drugs under physiological conditions and the hazard presented by the potentially carcinogenic nitrosamine products of those reactions, has been recently noted[9]. In this report we will present additional data on the susceptibility of drugs to nitrosation by SNP under mild conditions. We will also describe the influence of circulating thiols and ascorbate on some of those reactions.

EXPERIMENTAL METHODS

SNP and the amines emplolyed in this study, as listed in Table I, were obtained from Sigma Chemical Co. Phentolamine was obtained as Regitine methylate (a registered trademark of Ciba Phamaceutical Co. for phentolamine mesylate).

Reactions of SNP with drugs were conducted at 37℃ in the dark in 0.01 M phosphate buffer, pH 7.3, excepted as noted in the text. Reaction times and reactant concentrations are given in Table I. Yields

Table I. Products obtained from the reaction of SNP with secondary amine drugs

Amine	Products					
	m/z	IR[N=O], cm^{-1}	mp.	Yield (%)	Preussmann Test[g]	Name
Morpholine ·	116[a]	1460	–	91.3[c,e]	+	N-Nitrosomorpholine
Piperazine	115[a]	–	–	86.3[c,e]	+	N-Nitrosopiperazine
	144[a]	–	–	13.4[c,e]	+	N,N′-Dinitrosopiperazine
Ephedrine	195[b]	1450	90°	1.5[d,f]	+	N-Nitrosoephedrine
Phenylephrine	242[b]	1460	–	16.0[d,f]	+	N-Nitrosonitrophenylephrine
Synephrine	242[b]	1460	–	11.7[d,f]	+	N-Nitrosonitrosynephrine
Propranonol	289[b]	–	–	1.2[d,f]	+	N-Nitrosopropranonol
Phentolamine	311[b]	–	–	1.4[d,f]	+	N-Nitrosopentolamine

[a] Molecular ions in the electron impact mode or, [b] the chemical ionization mode.
[c] Yields determined from peak areas obtained by gas chromatography; [d] high-performance liquid chromatography.
[e] Reactions were conducted in the absence of light at 37℃ in 0.1 M phosphate buffer; pH 7.3 with 0.05 M amine and 0.1 M SNP for 2 hrs; [f] 0.2 M amine and 0.5 M SNP for 2 hrs. [g] Specific assay for nitrosamines and N-nitrosoamides.

of volatile nitrosamines were determined using a Varian Model 3300 gas chromatograph with a 6′×1/8″ i.d. column of 3% OV-17 on 100/120 mesh Gas-Chrom Q, with helium as carrier gas and a thermal conductivity detector. Yields of nonvolatile nitrosamines were determined using an LDC/Milton Roy high-performance liquid chromatograph (two Constametric IIIG dual piston pumps, Gradient Master, Dynamixer) with a 4.6×250 mm Brownless column of 10 μ Lichrosorb RP-18 and a linear gradient from 0.1% aqueous phosphoric acid to a 1:1 mixture of 0.1% aqueous phosphoric acid and acetonitrile, monitored at 215 nm with a Kratos Spectroflow 773 variable wavelength absorbance monitor.

Mass spectra were obtained with a Finnigan Model 4021 quadrupole GC-mass spectrometer with a combined electron impact/chemical ionization ion source and a 20 m×0.25 mm glass capillary column coated with either OV-17 or DB-1 and/or direct inlet mass spectrometry. Identification was corroborated, where appropriate, by NMR (Varian T60, $DCCl_3$ with TMS as an internal standard) and infra-red spectra (Beckman Model 4220 IR spectrophotometer with Nujol mulls) and melting points as compared to earlier reports[10-13]. The procedure of Preussmann *et al.*[14] was used to confirm the identification of each nitrosamine.

RESULTS AND DISCUSSION

N-Nitroso derivatives of the drugs listed in Table I were prepared by reaction with excess SNP at pH 7.3 and 37℃ as described. The resulting nitrosamines were identified by GC-mass spectrometry and/or by direct inlet-mass spectrometry and corroborated by NMR, infra-red spectra, and melting points as compared to earlier reports[10-13]. A positive Preussmann reaction was obtained in all cases.

In every case, the major product was the same as found upon reaction with nitrous acid. Under the conditions employed (i.e., 2 to 24 hrs at pH 7.3 and 37℃; see Table I) yields varied from 100% for the most reactive amine (i.e., piperazine) to about 1% for the least reactive amine. SNP thus reacts with each of the indicated compounds under conditions similar to those existing generally throughout the human body. Reactions of secondary amines with nitrous acid, on the other hand, occur only at low pH values like those found physiologically only in the stomach or as may exist at certain stages during the preparation of some foods.

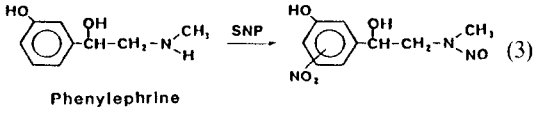
In the case of piperazine, the reaction with SNP gave both mono- and dinitroso- derivatives, with no detectable piperazine remaining under the conditions described in Table I. With shorter reaction

times, smaller amounts of both products and significant amounts of unreacted piperazine were detected. To demonstrated the sequence of reactions by which N,N-dinitrosopiperazine arose (Equation 2), we isolated N-nitrosopiperazine from an

$$\text{piperazine} \longrightarrow \text{mononitrosopiperazine} \longrightarrow \text{dinitrosopiperazine} \quad (2)$$

incomplete reaction and examined its future reaction with SNP. On the basis of gas chromatographic peak areas, approximately 50% conversion of 10 mM N-nitrosopiperazine to N,N-dinitrosopiperazine was observed after reaction with 20 mM SNP for 1 hr at pH 7.3 and 37℃. The conversion on mono- into dinitrosopiperazine thus appears to be only slightly slower than the conversion of piperazine into mononitrosopiperazine.

The sympathominetic drug, phenylephrine and its isomer, synephrine appear to undergo both rapid N-nitrosation and nitration of the aromatic ring upon reaction with excess SNP as illustrated in Equation (3).



Phenylephrine (3)

Isobutane-chemical ionization mass spectra of the major product in each case gave the same protonated molecular ion, $(M+H)^+$ at *m/z* 242, corresponding to $C_9H_{12}N_3O_5^+$, and a prominent fragment ion, $[(M+H)\text{-}NO]^+$, at *m/z* 212. No simple N-nitroso derivative (i.e., $C_9H_{13}N_2O_3^+$; *m/z* 197) was observed in either case. Both products revealed a strong band at 1460 cm^{-1}, characteristic of the (N=O) group[15], and both gave a positive Prussmann test. The unexpected nitro groups in both cases are, presumably, substituents on the respective aromatic rings. Nitration of the similar phenolic ring of tyrosine by nitrous acid has been observed and is thought to involve two sequential steps, the introduction of a nitro group followed by its oxidation to a nitro group by excess nitrous acid[16].

Catechol has been reported to give a green color upon reaction with SNP under alkaline conditions [17]. At pH 7.3 and 37℃, however, catechol, epinephrine an norepinephrine all gave rise to very similar, slightly viscous, black solutions. The black polymer-like substance(s) plus unreacted nitroprusside ion and any other colored compounds that may have been present, were removed by passage through a 1.5×5 cm column of Bio-Rad AG 2×8 and the clear, colorless, eluents were subjected to analysis by HPLC. After the reaction of 10 mM epinephrine for 8 hrs with 40 mM SNP, two symmetrical UV absorbing (220 nm) peaks were revealed. The larger one eluted at the position of epinephrine (~4.5 m*l*) and gave a negative Preussmann test. The smaller, which amounted to about 15% of the initial epinephrine, based on their respective 220 nm absorbance, eluted at ~6.4 m*l* and gave a positive Preussmann test. Mass spectra of this material were ambiguous but, on the basis of its positive Preussmann test, we believe it was probably the N-nitroso derivative of epinephrine.

Casado *et al.*[8] studied the reactions of SNP with several secondary amines under alkaline conditions and observed complex rate equations, including both first and second order terms in respect to amine concentration. At low concentrations of amine, like those existing during the pharmacological use of SNP, only the first order term should be significant. The reaction of 5 mM ephedrine with excess SNP, thus, appears to be first order in both reactants (Fig. 1).

A similar first order dependence was observed for each of the four other secondary amines listed in Table II. The large differences between the determined rate constants, presumably, reflect differences in basicity, nucleophilicity and steric constraints. As upon reaction with nitrous acid at significantly lower pH values[18], the less basic amines were the most reactive under the present conditions (i.e. pH 7.3 and 37℃). The 4-fold greater reactivity of piperazine ($pK_{a1}=5.8$, $pK_{a2}=9.1$[19]) as compared to morpholine, $pK_a=8.5$[19], thus, appears to reflect its less complete protonation under those conditions. The lower reactivities of proline and the other amines listed in Table I appear to reflect both greater basicity (i.e. more extensive protonation under the conditions employed) and greater steric inhibition. Additional studies on a series of sterically similar amines of differing basicity will be needed to more clearly evaluate the importance of these factors on reactivities. As with nitrous acid, the less basic amines are, generally, the most reactive and there-

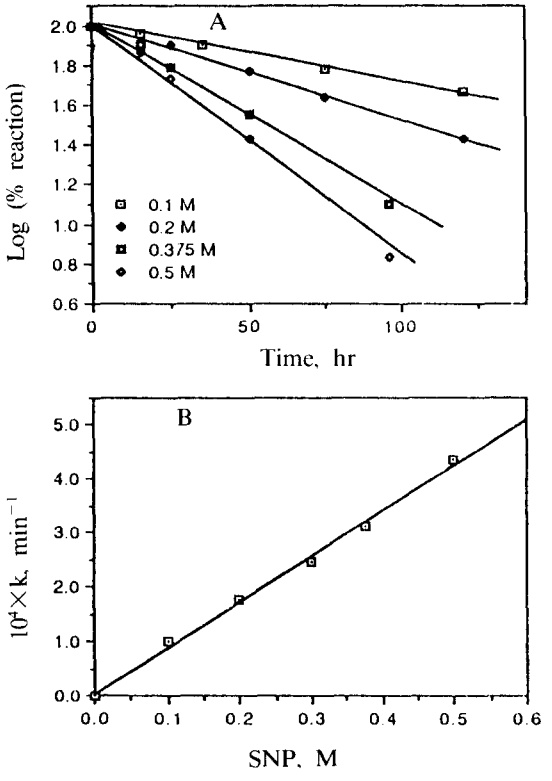


Fig. 1. (A) First-order plots for the nitrosation of 5 mM ephedrine with 0.1-0.5 M SNP in 0.1 M borate buffer, pH 10 and 37°C and (B) a plot of the pseudo-first order rate constants versus the SNP concentration.

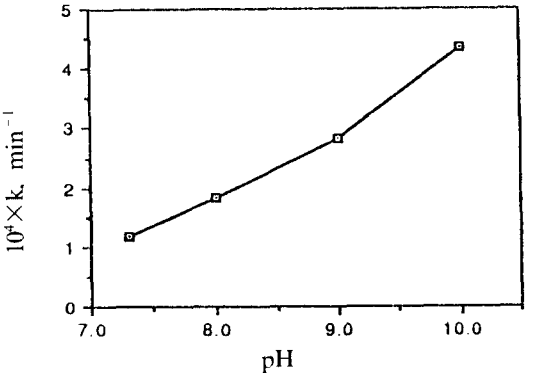


Fig. 2. The pH dependence for the reactions of SNP with ephedrine at 37°C in 0.1 M phosphate (pH 7.3), Tris (pH 8.4) and borate buffers (pH 9.0 and 10.0).

Table II. Pseudo-first order rate constants for the Nitrosation of secondary amine drugs by SNP[a]

Durg	[SNP], M	$10^2 \times k$ (min^{-1})
Piperazine	0.1	16.1[b]
Morpholine	0.1	2.2[b]
Ephedrine	0.5	0.012[c]
Propranonol	0.5	0.009[c]
Phentolamine	0.5	0.013[c]

[a] At 37°C in 0.1 M phosphate buffer, pH 7.3.
[b] Amounts of these nitrosamines were determined by gas chromatography as described in the text.
[c] Amounts of these nitrosamines were determined by HPLC as described in the text.

fore potentially the most dangerous under "physiological" conditions.

The strong influence of pH on the reaction of SNP with ephedrine is shown in Fig. 2. The reaction rate increased at higher pH values in proportion, approximately, with the increased concentration of the basic form of the amine [RR'NH]. The already high reactivity of SNP with amines under "physiological" conditions is, thus, increased further at higher pH values. By comparison, reactions of nitrous acid with amines are usually optimal at about pH 3 to 3.4 and decreased rapidly with increasing pH values[18]. Significant reaction of nitrous acid with amines has not been observed under "normal physiological" conditions.

The reaction of SNP with secondary amines in the blood stream ought to be suppressed by low concentrations of circulating glutathione, cysteine and other thiols with which it reacts very rapidly[20]. Thus, as shown in Table III, the *in vitro* nitrosation of piperazine is suppressed but not eliminated by 20 mM glutathione, cysteine or ascorbate. The relatively modest suppression in each case appears to reflect the fact that, although the reaction with thiols is very fast, it is rapidly reversible and the equilibrium is not very favorable under physiological conditions[20]. Furthermore, as concentrations of glutathione and cysteine in serum are still normally, much lower, they would be expected to have an even smaller influence under pharmacological conditions.

SNP is a potent nitrosating agent under normal physiological conditions. The product of its reactions with secondary amines are nitrosamines, the same as obtained at low pH values with nitrous

Table III. Effects of glutathione, cysteine and ascorbate on the reaction of SNP with piperazine[a]

[Piperazine] (mM)	[SNP] (mM)	[Glutathione] (mM)	[Cysteine] (mM)	[Ascorbate] (mM)	Yield[b] (%)
10	10	0	0	0	60.5
10	10	10	0	0	20.6
10	10	20	0	0	10.3
10	10	0	20	0	9.7
10	10	0	0	20	7.3

[a] Reactions were conducted in 0.1 M phosphate buffer at pH 7.3 for 20 min at 37℃.
[b] Yields based on the peak areas obtained for N-nitrosopiperazine and piperazine by gas chromatography.

acid (see Table I). It should be noted that many of the drugs used to treat cardiac and hypotensive conditions, some of which are listed in Table I, are secondary amines and that they are often administered shortly before or simultaneously with SNP. As many nitrosamines are carcinogenic, the pharmacological administration of SNP must pose long-term hearth risks. Until those risks are further clarified, it might be wise to limit the use of SNP to life threating situations where other hypotensive agents would be significantly less effective.

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