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Formation of Nitrosamines from Sodium Nitroprusside and Physiological Amines

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Abstract□Several physiological components containing a secondary amino group were capable of reacting sodium nitroprusside to form potentially carcinogenic nitrosamines under physiological conditions (pH 7.3, 37 °C). In each case the products were identical to those produced upon reaction with nitrous acid at much lower pH values. Reaction rates measured with proline were shown to reflect a first order dependence on both amine and nitroprusside concentrations. The strong influences of pH on the reactions of sodium nitroprusside with amines were also observed. These results show sodium nitroprusside could be a very potent nitrosation agent under physiological conditions.

Keywords□Hypotensive drug, N-nitrosation, carcinogen.

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 cardic emergencies, for the clinical treatment of malignant hypertension and to lower cardiac back pressure during periods of cardiac insufficiency[1]. As the only agent available to effect rapid, short-term reduction of blood pressure, it is also used for the induction of "controlled hypotension" during many kinds of surgery[2,3] and for a variety of other purposes[4]. 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 know hypotensive agents. Although its hypotensive effects were first reported in 1887, it was not widely used until 1974, when it became available in a convenient to administer form. Its use has increased rapidly since that time.

SNP dissolves readily in water to give clear, bright orange solutions that are stable for at least six months at 25 °C in the dark. The nitroprusside ion, $[(CN)_5FeNO]^{2-}$ is, however very reactive and decomposes rapidly in alkaline solutions or upon exposure to light. It also reacts rapidly with many kinds of nucleophiles to give adducts having the general structure, $[(CN)_5FeNOX]$[3-5,6]. All of these reactions appear to involve addition of an anion, X^{-1}, to the coordinated NO moiety. In each case the nitrosyl ligand reacts, in effect, like a stabilized nitrosonium ion (*i.e.* NO^+).

Although N-nitrosation reactivity of SNP on the simple secondary amines under alkaline conditions has been reported by a few authors,[7,8] its potential reactivity under physiological conditions has not been considered seriously. The ability of SNP to react with several secondary amino drugs under physiological conditions and the hazard presented by products of those reactions have been recently noted[9]. In this report we will present additional data on the reactions of SNP with physiologically important secondary amines under mild conditions.

MATERIALS AND METHODS

Materials

SNP and the amines employed in this study were obtained from either Sigma Chemical Co. or Aldrich Chemicals. All the reagents were of reagent grade.

Nitrosation of amines with SNP

The nitrosation of proline (5 mmol) with SNP (20 mmol) was carried out in 50 m*l* of 0.3 M phosphate buffer, pH 7.3. After reacting for 24 hours at 37 °C, water was evaporated from the reaction mixture using a rotary evaporator and the residue was extracted with pure acetone. The acetone was removed by evaporation in the stream of nitrogen. The N-nitroso derivative of creatine was prepared by adding creatine hydrate (5 mmol) to 50 m*l* of buffer

containing SNP (25 mmol). After 24 hours incubation at 37 °C the reaction mixture was acidified and extracted three times with 20 m*l* portions of diethyl ether. The diethyl ether solution was evaporated and the oily residue was allowed to crystallize. Repeated crystallization from ethyl acetate gave a pale yellow sample. The N-nitroso derivative of sarcosine was prepared from the reaction of sarcosine (5 mmol) and SNP (25 mmol) in 50 m*l* of buffer. The reaction was carried out for 6 hours at 37 °C. The resulting mixture was acidified and extracted with diethyl ether. The reaction of creatinine (5 mmol) and SNP (20 mmol) was carried out in 50 m*l* buffer for 24 hours at 37 °C. The resulting solid was removed by filtration and washed with water. The product was recrystallized from hot water after treatment with charcoal.

Identification of N-nitrosamines

Mass spectra were obtained with a Finnigan Model 4021 quadrupole GC/MS with a combined EI/CI ion source and a 20 m × 0.25 mm glass capillary column coated with either OV-17 or DB-1 and/or by direct inlet mass spectrometry. Identification was corroborated, where appropriate, by NMR (Varian T60, $CDCl_3$ with TMS as an internal standard) and infra-red (IR) spectra (Beckman Model 4220 IR spectrophotometer with Nujol mulls).

Colorimetric method for the detection of N-nitrosamines

The colorimetric detection of N-nitroso compounds was carried out by the method described by Preussmann *et al.*[10] Prepared reagent was applied to the layer. Irradiation of the moist plate with UV light ($\lambda_{max} = 240$ nm) for few minutes produced blue to violet spots in the case of N-nitroso compounds.

RESULTS AND DISCUSSION

N-Nitroso derivatives of the four physiological amines listed in Table I were prepared by reaction with excess SNP at pH 7.3 and 37 °C as described. A positive Preussmann reaction was obtained in all but one case. In that case the reaction of creatinine with SNP gave rise to creatinine-5-oxime, as observed upon its reaction with nitrous acid[11]. N-nitrosamines show three relatively intense bands in the IR region at 1470-1350, 1316-1163 and 1093-1047 cm^{-1}. The first two have been assigned to the vibrations of the N = O and the last to the vibrations of the N-N[12]. Especially, the band at 1470-1350 cm^{-1} was used additional evidence for the N-nitrosamine detection.

The natural occurrence of amino acids which contain the secondary amine structure such as free proline and sarcosine in blood constitute a potential source of nitrosamines via interaction with SNP. Products induced from the reaction of SNP with proline and sarcosine was identified as N-nitrosoproline and N-nitrososarcosine, respectively.

Creatinine, the end product of creatine metabolism, is found together with creatine in muscle tissues, milk and blood. In view of the abundance of creatinine and creatine in muscle tissues and blood, the intravenous administration may cause possible hazard in terms of formation of N-nitroso and related compounds. The nitrosated product of creatine by SNP was a pale yellow sample with m.p. 66-67 °C, and characterized as N-nitrososarcosine. A possible mechanism for the nitrosation of creatine by SNP could be similar to the mechanism proposed on the reaction of nitrous acid with creatine[11]. Reaction of creatine with SNP to yield N-carbamyl-N-methylglycine, and then this product can react with more SNP to yield N-carboxy-

Table I. Products obtained from the reaction of SNP with amines

Amines	Product				
	m/z(CI)	m.p.(°C)	IR(N = O), cm^{-1}	Name	Yield(%)*
Proline	145	99-100	1430	N-Nitrosoproline	1.2
Sarcosine	119	66-67	1455	N-Nitrososarcosine	11.3
Creatine	119	66-67	1455	N-Nitrososarcosine	4.1
Creatinine	143	254-255	—	Creatinine-5-oxime	13.4

Reactions were carried out at pH 7.3, 37 °C as described in text.
* Yields determined from either peak areas obtained by HPLC or the weight of isolated products. HPLC conditions were as follows: column, RP-8 (25 × 0.46 cm, 5μm); mobile phase, a linear gradient from 0.1% phosphoric acid to 0.1% phosphoric acid containing 50% acetonitrile over 20 minutes; flow rate, 1 m*l*/min.

N-methylglycine, which gives N-methylglycine (sarcosine) as a decarboxylated product. Because sarcosine contains secondary amino group it can be converted to N-nitrososarcosine by SNP. The reaction of creatinine with SNP yielded a white solid, m.p. 254-255 °C, which gave a negative result in a Preussmann test for a N-nitroso group. In the isobutane CI mass spectrum, a quasi-molecular ion at m/z 143 as a base peak, and IR spectrum confirmed product as creatinine-5-oxime. The mechanism of the reaction to produce creatinine-5-oxime from creatinine and SNP remains obscure, though the imino group in creatinine and SNP remains obscure, though the imino group in creatinine does seem implicated in its reactivity, and the secondary amino group in creatinine appears to be unreactive. Although the toxicity of creatinine-5-oxime is as yet unknown, oximes and their derivatives have often been reported to be toxic. Any toxicity of oximes has been due to the oximino group (= N-OH) which can be transformed into hydroxylamine.

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, only the first order term should be significant. Reactions of 5 mM proline with excess amounts of SNP appeared to be first order. Pseudo-first order rate constant for the nitrosation of 5 mM proline with 100 mM SNP was $8.2 \times 10^{-5} min^{-1}$.

The strong influence of pH on the reactions of SNP with creatine and creatinine are shown in Fig. 1. In each case, yields were increased at higher pH values in proportion, approximately, with the increased concentrations of the basic form of the amine [RR'NH]. The already high reactivity of SNP with amines under physiological pH is, thus, enhanced further at higher pH values. Although formaldehyde catalyzes the conversion of various secondary amines to nitrosamines by nitrite in neutral and basic medium under drastic conditions[13], it is generally assumed that potentially hazardous quantities of carcinogenic N-nitroso compounds are mainly produced by reaction of nitrite with amines under mild conditions in acidic medium, usually optimal at about pH 3.0 to 3.4. Unlike nitrite, SNP preferently acts as a nitrosating agent in neutral and basic media. This fact holds important implications for the nitrosation mechanism of SNP. For the nitrite reaction, investigations on mechanism have suggested that "the protonation of nitrous acid appears necessary for initiating all

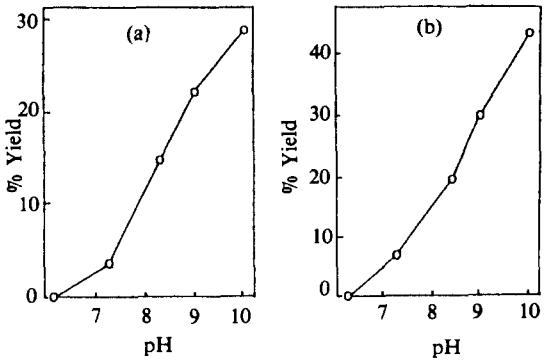

Fig. 1. The pH dependences for the reactions of SNP (10 mmol) with (a) creatine (2 mmol) and (b) creatinine (2 mmol) at 37 °C in 50 ml of 0.1 M phosphate (pH 6.2 and 7.3), Tris (pH 8.4) and borate buffers (pH 9.0 and 10.0).

Reactions were carried out for 24 hours for creatine and 12 hours for creatinine.

nitrosation reaction"[14,15]. Despite a qualitative parallel between the reaction of amines with nitrite and SNP, it can be assumed that the reactive form of SNP as a nitrosating agent is different from that of nitrite. Because of the nitrosation reactivity of SNP at physiological pH, it can act as a nitrosating agent in various organs, tissues and blood whereas nitrite could be an effective nitrosating agent only in a stomach.

Although SNP and amine concentrations for this study are quite high, it should be possible to predict that even very low concentrations of amines and SNP might produce enough N-nitrosamines to cause concern since the nitrosation reaction follows first order kinetics. It is now the consensus of opinion that N-nitrosamines should be considered as potential health hazard at concentrations of part per billion (ppb, $\mu g/kg$). In conclusion, SNP is a potent nitrosating agent under physiological conditions. Because even small amounts of nitrosamines are carcinogenic, the pharmacological administration of SNP may pose long-term health risks.

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