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NITRIC OXIDE GENERATION FROM NITROPRUSSIDE BY VASCULAR TISSUE

EVIDENCE THAT REDUCTION OF THE NITROPRUSSIDE ANION AND CYANIDE LOSS ARE REQUIRED

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Abstract—Nitric oxide (NO) was produced from sodium nitroprusside in the presence of vascular tissue but was not released spontaneously from the nitroprusside anion. In the absence of tissue in the dark nitroprusside did not release NO. When solutions of nitroprusside alone were irradiated with visible light, nitric oxide was released at rates linearly proportional to nitroprusside concentration and light intensity. Nitric oxide was produced from solutions of nitroprusside in the dark after the addition of vascular tissue, including lengths of rabbit aorta, subcellular fractions of aorta, and human plasma. NO was also released from nitroprusside after reaction with various reducing agents including cysteine and other thiols, ascorbic acid, sodium dithionite, ferrous chloride, hemoglobin, myoglobin, and partially purified cytochrome P450 with an NADPH-regenerating system. HCN was simultaneously produced in these solutions, and addition of KCN blocked NO release. Iodine oxidized intermediate cyanoferrates and blocked nitric oxide release. KCN or iodine also blocked NO production by tissue, but had no effect upon photochemical NO release. These results show that, apart from photolysis which makes no physiological contribution, release of nitric oxide from nitroprusside, in simple solutions and in biological tissue, occurs after nitroprusside has undergone reduction and lost cyanide.

Sodium nitroprusside (SNP‖) is a potent vasodilator that causes relaxation by releasing nitric oxide (NO) which activates the cytosolic isozyme of guanylate cyclase (EC 4.6.1.2) [1–4]. The mechanism by which the nitroprusside anion, a coordination complex of ferrous ion (Fe^{2+}) with five cyanide anions (CN^-) and a nitrosonium (NO^+) ion, releases NO is unknown, but a leading theory is that it occurs spontaneously [5, 6]. The spontaneous unimolecular release of an uncharged molecule of NO implies an internal transfer of an electron to NO^+ from Fe^{2+} or CN^-, and is supported by experiments which detect nitric oxide released from simple solutions of SNP [5, 6]. However, the only documented chemical reactions leading to the release of NO from pure solutions of SNP are photochemical reactions [7, 8], and prior reports of spontaneous release of NO from sodium nitroprusside have not excluded a contribution of photolysis.

An alternate mechanism of NO release from sodium nitroprusside is that an electron is transferred to the NO^+ ligand from another molecule as the nitroprusside anion is reduced. The concept that reduction of sodium nitroprusside leads to NO release is supported by studies showing a stimulatory effect of reducing agents such as thiols or hemoproteins on the decomposition of SNP or its activity on guanylate cyclase [9–18]. It is known that nitroprusside reacts with thiols at alkaline pH to form purple pigments that further decompose to other cyanoferrates and cyanide [19]; however, nitric oxide release in these reactions has not been documented. Thiols have been shown to augment the ability of sodium nitroprusside to activate partially purified guanylate cyclase [14–18, 20]. Based on this observation, several investigators have proposed that thiols reduce SNP to intermediates that release NO [13, 14, 20], but have not correlated reduction with NO release. In fact, in two studies [5, 6] thiols were found to paradoxically inhibit NO release from sodium nitroprusside. Thus, the role of thiols in the release of NO from sodium nitroprusside remains controversial.

Like thiols, hemoproteins have been proposed to act as reducing agents contributing to the release of NO from sodium nitroprusside. Smith and Kruszyna [11] showed that hemoglobin would reduce sodium nitroprusside causing the release of cyanide, but in the absence of other strong reducing agents NO could not be detected.

If nitric oxide is the active metabolite of nitroprusside, then NO should be detectable in biological preparations containing SNP under conditions where it is active. In this report the release of nitric oxide from nitroprusside was studied

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‖ Abbreviations: SNP, sodium nitroprusside; NO, nitric oxide; NADPH, nicotinamide adenine dinucleotide phosphate (reduced); and NADP, nicotaminde adenine dinucleotide phosphate (oxidized).

by the direct detection of free NO using a chemiluminescence technique. In an effort to clarify the mechanisms by which SNP acts in biological systems, the present study was designed to test the hypothesis that sodium nitroprusside releases NO in biological tissue after nitroprusside is reduced. We examined the capacity for sodium nitroprusside to spontaneously release NO, to release NO upon exposure to light, and to release NO in a variety of reducing and non-reducing environments.

MATERIALS AND METHODS

Measurement of NO. Nitric oxide liberated from nitroprusside was measured by a chemiluminescence technique. The apparatus consisted of a closed borosilicate glass tube containing 5 mL of freshly prepared sodium nitroprusside at various concentrations in 0.1 M sodium phosphate buffer (pH 7.5), or other buffer as indicated. Other reagents were added via needle puncture of a rubber septum. Temperature was regulated by a thermostatically controlled water bath around the reaction tube. A vacuum pump drew gas from the headspace of the reaction vessel through a nitric oxide analyzer (model 2105, Dasibi Corp., Glendale, CA) that detected nitric oxide by monitoring the chemiluminescence of the reaction of nitric oxide with ozone. The system is specific for nitric oxide and can detect 1 part per billion nitric oxide in the gas stream. The gas drawn from the headspace was replaced continuously by argon that entered at the bottom of the tube and bubbled through the solution. The gas flow rate was 250 cc/min and the usual vessel pressure was 510–700 torr.

Photochemical release of NO. Experiments on photochemical decomposition were performed in a darkened room and the sample was illuminated by an incandescent light controlled by a rheostat to adjust light intensity. Light intensity was measured using a cadmium sulfide light meter (Sekonic model 246) placed adjacent to the sample and in the plane of the front surface of the reaction tube. Experiments on chemical decomposition were performed in opaque or brown glass vessels that were demonstrated to allow no photolysis of SNP.

Tissue. Hepatic microsomes were obtained from male Sprague–Dawley rats (190–210 g) which were purchased from Amitech Inc. (Omaha, NE) and fed Teklad rat chow. Animals were killed with CO_2, and microsomes were prepared by differential centrifugation [21]. The microsomes contained 3 mg/mL total protein [22] and 5.28 μM cytochrome P450 as determined by reduced carbon monoxide difference spectroscopy [23]. Microsomes were diluted 10-fold in the final reaction vessel. An NADPH-regenerating system consisting of 0.8 units of glucose-6-phosphate dehydrogenase, 4.6 μmol glucose-6-phosphate, and 1.8 μmol NADP was added to the reaction vessel. Thoracic aorta segments were obtained from male New Zealand White rabbits killed by exsanguination under anesthesia. The vessels were cleaned of adventitia, cut into 5-cm lengths, and incised lengthwise to open the lumen. The vessels were handled in a manner to preserve endothelium, but were not further tested for the presence of endothelium. Some vessel samples were cut into fine pieces, partially homogenized with a mortar and pestle, subjected to two cycles of freeze–thaw lysis in phosphate buffer by liquid nitrogen, and separated by centrifugation at 10,000 *g* for 20 min into particulate and soluble fractions. Fresh human heparin-anticoagulated blood from multiple donors was obtained from the discarded portion of samples from an arterial blood gas laboratory.

Cyanide measurements. Cyanide, flushed from the reaction chamber as HCN gas, was measured in selected experiments with a cyanide-specific electrode (Orion). A trap containing 50 mL of 10 N NaOH was inserted in the gas circuit between the sample and the NO analyzer so that all effluent argon from the sample bubbled through the NaOH before entering the NO analyzer. A cyanide electrode and a reference electrode were inserted through gas-tight ports into the NaOH solution and cyanide concentration was measured continuously. The response time of the electrode (<4 min from baseline to a stable reading) was fast compared to the rate of cyanide increase, and electrode readings did not continue to change after abrupt interruptions of the HCN-containing gas flow.

Chemicals. L-Cysteine-HCl, *N*-acetyl-L-cysteine, DL-penicillamine, glutathione, and ferrous chloride (from Sigma, St. Louis, MO) and L-ascorbic acid and sodium nitroprusside dihydrate (from Fisher, Pittsburgh, PA) were used without further purification. Sodium dithionite (from EM Science, Cherry Hill, NJ) was prepared as a 0.1 M solution in distilled water that had been flushed with nitrogen and was kept in tubes sealed with a gas-tight rubber stopper. Aliquots were taken via needle puncture of the stopper with an argon-filled syringe so that the removed volume was replaced by argon. Samples were periodically titrated against iodine ($Na_2S_2O_4 + 3I_2 + 4H_2O \rightarrow 2NaHSO_4 + 6HI$) and were discarded after more than a 5–10% loss of dithionite was detected. All other chemicals were reagent grade. Human hemoglobin and sperm whale myoglobin (Sigma) were reduced with dithionite and separated from unreacted dithionite by chromatography on Sephadex G-10 resin equilibrated in 0.1 M sodium phosphate, pH 7.5. Methemoglobin was prepared by oxidation of hemoglobin by sodium nitrite prior to chromatography. In experiments where iodine was employed as an oxidizing agent, aliquots of 0.5 M I_2 in ethanol were added to the reaction mixture.

Statistics. Results are expressed as means ± SEM of multiple determinations as indicated. Comparisons were made using ANOVA and analysis of covariance with $P < 0.05$ considered significant.

RESULTS

Photochemical decomposition. Solutions of nitroprusside in phosphate buffer exposed to light produced nitric oxide at rates proportional to light intensity (Fig. 1). Steady-state rates of photochemical nitric oxide production were determined over ranges of temperature (2–37°), nitroprusside concentration (0.05–10 mM), and light intensity (0–10,000 lux). There was a pronounced dependence of nitric oxide

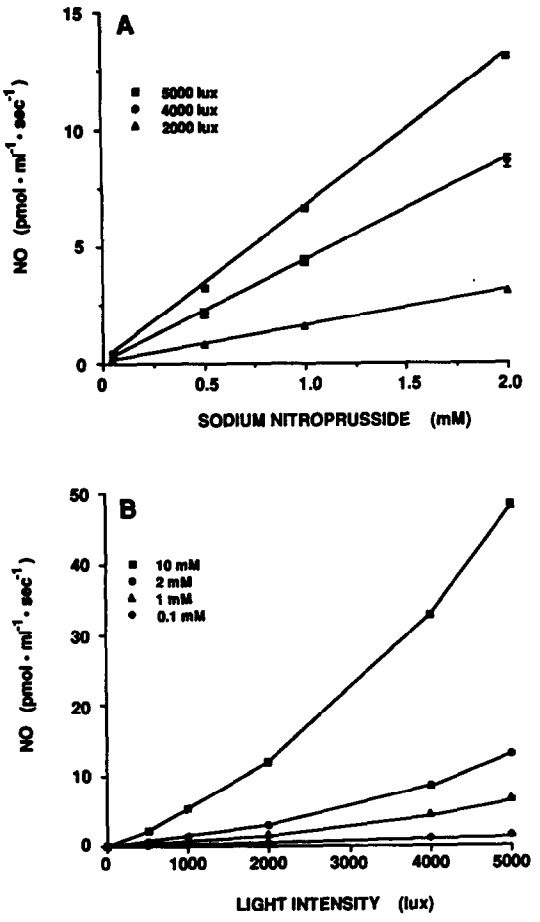

Fig. 1. Photochemical release of nitric oxide from nitroprusside. Data points represent the mean of triplicate determinations ± SEM performed in 0.1 M sodium phosphate, pH 7.5, at 25°. For most data points the symbol size is greater than the SEM. (A) NO release as a function of nitroprusside concentration at indicated light intensities. (B) NO release as a function of light intensity at indicated nitroprusside concentrations. Light intensity measurements were made at the plane of the front surface of the reaction vessel. Adjustment of the lamp intensity caused a simultaneous small spectral shift in accordance with Planck's radiation law as the filament temperature changed, and this spectral blue shift at higher lamp intensities may have contributed to the slight upward curvature of nitric oxide versus light intensity [8].

Table 1. Release of NO from nitroprusside by tissue*

Rectants	NO (pmol/mL/sec)
SNP	0
SNP + 10% whole blood	0
SNP + plasma	43.3 ± 2.40
SNP + 5 cm rabbit aorta	9.45 ± 2.93
SNP + cytosol from 5 cm aorta†	17.45 ± 2.48
SNP + precipitate from 5 cm aorta†	31.8 ± 14.76
SNP + 5 cm rabbit aorta + 5 mM KCN	0
SNP + 5 cm rabbit aorta + 10 mM iodine	0

* SNP and the indicated tissue were mixed in 5 mL of buffered saline (20 mM sodium phosphate, pH 7.5, in 0.9% sodium chloride) at 37° in a reaction vessel, or SNP was added to 3 mL of undiluted fresh human plasma at 37°. The vessel was continuously purged with 250 cc/min argon that was subsequently directed to a nitric oxide analyzer. SNP concentration in the reaction vessel was 1.0 mM. NO measurements are expressed as rate of generation of NO in the reaction solution. Values are means ± SEM from 3–6 determinations each

† Cytosol and precipitate from rabbit aorta were prepared by freeze–thaw lysis and centrifuged as described.

Table 2. Release of NO from nitroprusside by reducing agents*

Reactants	NO (pmol/mL/sec)
SNP	0
SNP + cysteine	50.49 ± 2.11
SNP + penicillamine	10.83 ± 1.01
SNP + *N*-acetylcysteine	7.31 ± 0.53
SNP + glutathione	28.42 ± 1.26
SNP + ascorbate	8.21 ± 0.76
SNP + $FeCl_2$	6.42 ± 0.81
SNP + $NaHSO_3$	0
SNP + KCN	0
SNP + KCN + cysteine	0
SNP + iodine + cysteine	0

* Reagents were mixed in 5 mL of 0.1 M sodium phosphate, pH 7.5, at 37°. SNP concentration was 1.0 mM; thiols, ascorbate, and $FeCl_2$ were 0.2 mM; KCN, iodine and $NaHSO_3$ were 10 mM. Samples were purged with 250 cc/min argon that was subsequently directed to a nitric oxide analyzer. NO measurements are expressed as rate of generation of NO in the reaction solution. Values are means ± SEM from 3–6 determinations each.

production on both nitroprusside concentration and light intensity (Fig. 1). In the absence of light there was no detectable nitric oxide production at any concentration of sodium nitroprusside.

To eliminate any contribution by photolysis, all experiments not specifically measuring photochemical generation of NO were performed with samples protected from light.

Decomposition of nitroprusside by tissue. The addition of plasma to solutions of nitroprusside resulted in the liberation of nitric oxide (Table 1). Similarly, when 5-cm lengths of rabbit aorta were added to solutions of nitroprusside, nitric oxide was released. Fractionation of the vessel wall by freeze–thaw lysis and centrifugation showed that activity was associated with both the soluble and particulate fractions of the vessel. In contrast, the addition of whole blood or red cell suspensions, with their high concentrations of hemoglobin, which avidly binds free NO, produced no detectable nitric oxide.

Reduction of nitroprusside. Nitric oxide was produced in solutions of nitroprusside and various reducing agents (Table 2). Nitric oxide was also

Table 3. Release of NO from nitroprusside by hemoproteins*

Reactants	NO (pmol/mL/sec)
SNP	0
SNP + hemoglobin, reduced	4.24 ± 0.20
SNP + myoglobin , reduced	5.66 ± 1.13
SNP + microsomes† (no NADPH)	2.92 ± 2.96
SNP + NADPH regenerating system‡	1.33 ± 0.96
SNP + microsomes† + NADPH regenerating system‡	53.52 ± 6.41
SNP + methemoglobin	0
SNP + hemoglobin, reduced + KCN	0

* Conditions were as in Table 2. Protein concentrations were hemoglobin, 4.12 μM; myoglobin, 21 μM; and methemoglobin, 8 μM. KCN concentration was 5 mM. Values are means ± SEM from 3–6 determinations each.

† Microsomes contained 0.528 μM cytochrome P450, and 0.3 mg/mL protein after dilution in the reaction vessel.

‡ NADPH-regenerating system consisted of 0.8 units of glucose-6-phosphate dehydrogenase, 4.6 μmol glucose-6-phosphate, and 1.8 μmol NADP.

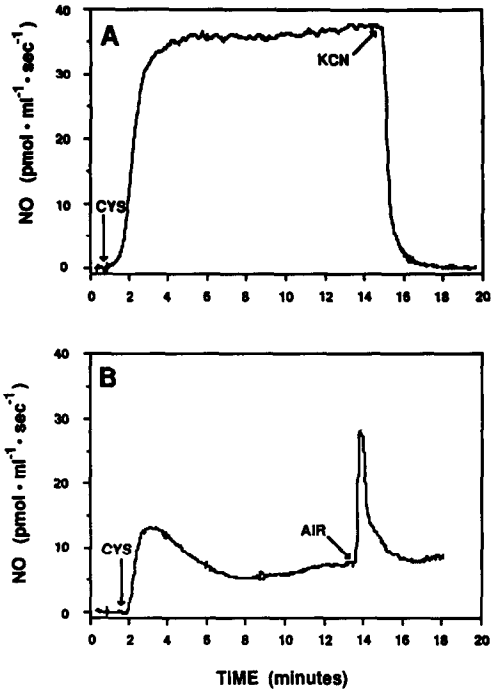

Fig. 2. Two types of kinetics of NO production from SNP and thiols. The figure shows NO signal recordings from 1 mM SNP in 0.1 M sodium phosphate, pH 7.5, at 37°. Cysteine and KCN (10 mM) were added to the reaction vessel at the times indicated. The cysteine concentration was 80 μM in (A) and 1 mM in (B). At the point labeled "AIR" the argon purge stream was replaced by air for 10 sec, resulting in approximately 40 cc of air passing through the sample.

produced by the reaction of nitroprusside and several reduced deoxygenated hemoproteins (Table 3). Low concentrations of hemoglobin and myoglobin caused nitroprusside decomposition with nitric oxide release, and the resulting oxidized hemoproteins did not interfere with nitric oxide detection. Higher concentrations of hemoglobin and myoglobin had diminished or absent effects because of nitric oxide absorption by remaining reduced hemoproteins. Oxidized hemoproteins (methemoglobin) had no effect on nitroprusside. Microsomes containing cytochrome P450 and NADPH were active producers of nitric oxide. Microsomes without an NADPH-regenerating system, and an NADPH-regenerating system without microsomes each caused nitric oxide production, but much less than the combination.

Biphasic effect of reducing agents on NO *release.* Under conditions employed in this study, reducing agents fell into two categories based on kinetics of NO release. At low concentrations, all agents liberated NO from sodium nitroprusside with relatively simple kinetics (Fig. 2A) involving an initial rapid rise in NO production followed by a slowly rising plateau phase. A few agents (e.g. *N*-acetylcysteine and glutathione) retained these simple kinetics at concentrations up to 10 mM. For most agents, however, the kinetics of NO release became more complex at higher concentrations (Fig. 2B), and the maximum rates of NO production were lower. When NO release from a fixed (1 mM) concentration of nitroprusside was studied as a function of cysteine concentration (Fig. 3), the rate and quantity of NO release increased with cysteine concentrations up to 0.2 mM and then decreased progressively with higher concentrations. At the higher cysteine concentrations, but not at low cysteine concentrations, if samples of the reaction in progress were bubbled with air for 5–10 sec, an immediate but transient marked rise in NO production occurred, consistent with NO release from the products of this reaction by an oxidative mechanism (Fig. 2B). Only the higher concentrations of cysteine or other reducing agents appeared to

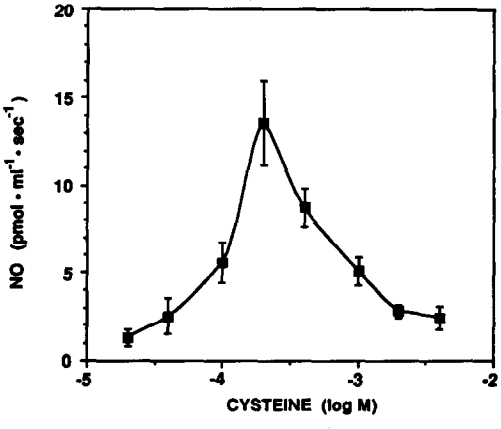

Fig. 3. Nitric oxide release as a function of cysteine concentration. Steady-state rates of NO release at 25° were measured from mixtures of cysteine and 1.0×10^{-3} M SNP in 75 mM sodium phosphate, pH 7.5. Data points represent the mean of triplicate determinations ±SEM.

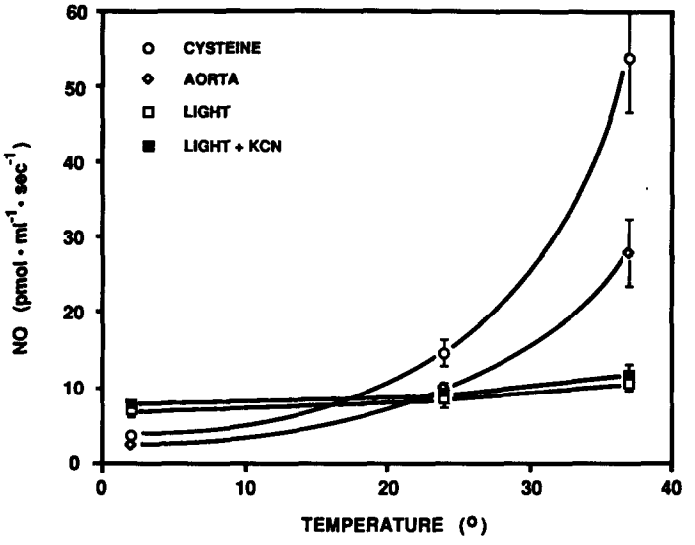

Fig. 4. Effect of temperature on NO release. Solutions contained 1 mM SNP in 0.1 M sodium phosphate, pH 7.5. Samples labeled "LIGHT" were exposed to 5000 lux from an incandescent lamp. Samples labeled "LIGHT + KCN" were identical except that they also contained 5 mM KCN. "CYSTEINE" samples contained 1 mM SNP + 0.2 mM cysteine in the dark. "AORTA" samples contained 1 mM SNP + 1 cm rabbit thoracic aorta (homogenized) in the dark. Temperature was regulated by a circulating water bath around the reaction vessel. Data points represent the mean of triplicate determinations ± SEM.

produce chemical species that would release NO after brief oxidation by air.

Effects of oxidizing agents. In solutions of nitroprusside and reducing agent or biological tissue that were producing NO, the addition of 5–10 mM I_2, an oxidizing agent which does not interact with NO, completely halted nitric oxide production (Tables 1 and 2). In solutions containing high concentrations of reducing agents, the inhibition was sometimes preceded by a short period of increased NO release corresponding to the effect of air on these same solutions (Fig. 2B), but in all cases the final effect was complete cessation of NO production. Other oxidizing agents such as sodium hypochlorite, ammonium persulfate, or oxygen also blocked nitric oxide production from nitroprusside, but these agents were not studied further because they also directly oxidized NO, confusing interpretation of whether these agents prevented NO release or simply oxidized free NO before it was measured. NO gas, injected into solutions containing 10 mM I_2, was not oxidized. Iodine did not block photochemical release of NO.

Temperature dependence. Photochemical production of nitric oxide from nitroprusside showed minimal temperature dependence (Fig. 4). Curves of NO production versus light intensity performed at different temperatures were not statistically different (analysis of covariance). In contrast, nitric oxide production by reduction of nitroprusside showed a more pronounced temperature dependence (Fig. 4), as did NO production by tissue.

Effect of cyanide. Reduction of nitroprusside resulted in the production of both nitric oxide and cyanide, the latter measured as HCN gas purged from the reaction vessel. Measurements of HCN and NO from reactions of nitroprusside and cysteine (Fig. 5) established that CN^- was released in greater amounts than NO, but such studies cannot determine

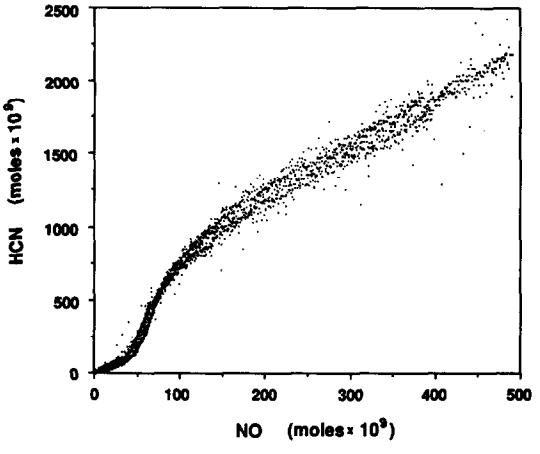

Fig. 5. Relationship of cumulative release of HCN and NO. Simultaneous measurements were made of NO and HCN released from samples of 5 mL of 1.0 mM nitroprusside and 1.0 mM cysteine at 25°. Data shown are the combined data from three experiments which were not significantly different as determined by analysis of covariance. Each data point represents measurements made over an individual sequential 5-sec interval and is expressed as cumulative HCN release versus cumulative NO release at the end of that interval.

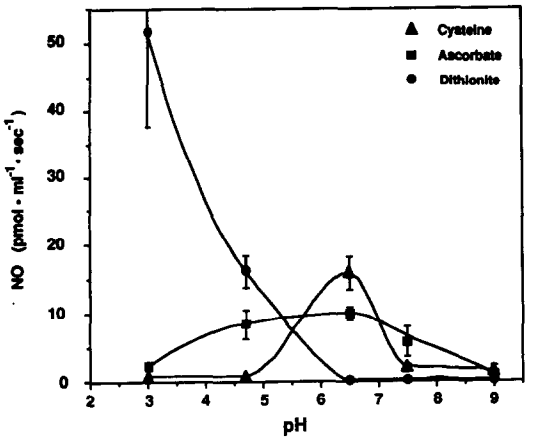

Fig. 6. Effect of pH on NO release. Solutions were prepared at 25° by addition of SNP and reducing agent to 0.1 M acetate, phosphate, or borate buffer, as appropriate to buffer at the indicated pH, to give final concentrations of 1.0 mM SNP and 10 mM reducing agent. NO release from 1.0 mM SNP was not seen at any pH in the absence of reducing agent. Data points represent the mean of triplicate determinations ± SEM.

the sequence of ligand release. In experiments to investigate the sequence of ligand release it was shown that in solutions of nitroprusside and reducing agent the addition of KCN brought a complete halt to nitric oxide production (Table 2, Fig. 2A). KCN also blocked NO release from SNP by vascular tissue, plasma (Table 1), and hemoproteins (Table 3). When solutions of SNP, reducing agent, and KCN were acidified to remove cyanide as volatile HCN, NO production was restored to levels observed under the same conditions in the absence of KCN. The inhibition of NO release by KCN was therefore reversible and dependent upon the continued presence of KCN. Photolysis also resulted in production of both NO and HCN, in agreement with other reports [7, 8], but exogenous KCN had no effect on the rate of photochemical release of NO (Fig. 4).

Effect of pH. The rate of NO release from nitroprusside and reducing agents was greatly affected by pH in a manner that depended upon the reducing agent used (Fig. 6). When the effect of pH on nitric oxide production from nitroprusside by cysteine, ascorbate, or dithionite was studied, all mixtures produced little nitric oxide at pH 9.0 and had increased rates of NO production at lower pH. Cysteine, with a thiol group that becomes progressively less ionized as pH decreases, increased NO production as pH was lowered from 9.0, but below pH 6.5 NO production decreased again. Ascorbate, with a lower pK, showed a similar pattern but with less suppression at slightly acidic pH. Sodium dithionite, a stronger reducing agent that remains ionized over the pH range studied, caused less nitric oxide production than did cysteine or ascorbate at high pH, but the rate increased progressively as the pH decreased.

DISCUSSION

In the present experiments, we directly examined the release of NO from solutions of sodium nitroprusside under a variety of conditions. The major new findings are: (1) In the absence of light, sodium nitroprusside did not spontaneously liberate nitric oxide. (2) Sodium nitroprusside was photochemically degraded to nitric oxide with rates directly proportional to light intensity and nitroprusside concentration. (3) Several tissues, including vascular tissues, promoted the release of nitric oxide from sodium nitroprusside. The effect of these tissues on sodium nitroprusside could be mimicked by reducing agents, providing an explanation for their effect. The kinetics of this process with regard to the concentration of reducing agent were complex and revealed more than one reaction. (4) NO release by reducing agents was accompanied by CN^- release and could be inhibited by the addition of exogenous KCN.

The significance of each of these findings will be considered in this discussion.

Spontaneous versus photochemical release of NO. Simple solutions of sodium nitroprusside without reducing agents only released NO photochemically. These findings expand upon earlier studies of photolysis by showing the absolute and linear dependence of these reactions on light and nitroprusside concentration [7, 8]. In the present experiments a 1 mM solution of sodium nitroprusside exposed to a light intensity of 1000 lux (a value similar to ambient laboratory light) produced about 0.7 pmol/mL/sec. This effect of light likely explains other reports of spontaneous NO release from sodium nitroprusside in experiments where light exposure was not controlled.

Photolysis clearly has no role in the *in vivo* pharmacology of SNP where light exposure is negligible. It is also unlikely that photolysis is a meaningful factor in most *in vitro* experiments that use low concentrations of nitroprusside in ambient light. Based on our present data, a 1 μM solution of sodium nitroprusside, which would normally cause marked vasodilation, would photochemically produce NO at a rate of about 10^{-12} M/sec at 1000 lux, a very low value when compared to the potency of NO ($EC_{50} > 10^{-9}$ M) and its short half-life (<30 sec) in biological buffers [24]. However, studies using higher concentrations of SNP or greater illumination might be affected by the contribution of photolysis.

NO release from nitroprusside by vascular tissue and by reducing agents. Since spontaneous formation of nitric oxide from nitroprusside does not occur, a chemical transformation of the molecule must be necessary. In the present experiments rabbit aorta, as intact tissue or soluble or particulate fractions of tissue homogenates, caused release of nitric oxide from sodium nitroprusside. Plasma also had a similar effect showing that intravenously infused sodium nitroprusside is degraded by both blood and blood vessel wall to yield nitric oxide. However, because of the rapid removal of NO from whole blood by hemoglobin (Table 1), it is likely that only nitric oxide produced at the vessel wall is pharmacologically active.

In experiments examining the mechanism by which vascular tissue and plasma yield nitric oxide from sodium nitroprusside, it was observed that iodine, an oxidizing agent, completely inhibited the release of NO from solutions of sodium nitroprusside in aortic homogenates. This suggests that vascular homogenates act as reducing agents.

The effect of several reducing agents on the release of NO from sodium nitroprusside further supports that hypothesis. While simple solutions of SNP in buffer did not release NO, the addition of a single agent capable of reducing SNP [25–29] resulted in the generation of large amounts of NO. This property was shared by a variety of biochemical and inorganic compounds (Tables 2 and 3), similar only in their ability to act as reducing agents, and was unique to such compounds. Reaction of SNP with sodium bisulfite ($NaHSO_3$), a compound which combines with SNP to form a complex similar to the SNP–thiol complex, but which does not reduce the nitroprusside anion [19], did not result in NO release (Table 2).

Hemoglobin and other hemoproteins also caused nitric oxide to be released from nitroprusside, but the effect of hemoglobin on SNP was a balance of two opposing processes. Hemoglobin can reduce nitroprusside leading to nitric oxide release, and hemoglobin avidly binds NO inhibiting its action. In the present experiments (Table 3), hemoglobin was used in much lower concentrations than nitroprusside, with the result that most of the hemoglobin was oxidized by nitroprusside to methemoglobin which binds NO poorly. At higher concentrations of hemoglobin, as found in blood (Table 1), the binding of NO by excess reduced hemoglobin obscured any effect on NO release.

As was the case for vascular tissue, the addition of oxidizing agents completely inhibited the effect of reducing agents. Likewise, thiols such as cysteine, which reduce nitroprusside in a pH-dependent reaction where only the thiolate (RS^-) anion reacts by way of the intermediate $([CN]_5FeNOSR)^{3-}$ to give reduced analogs of nitroprusside [19, 25, 27, 28], did not cause nitric oxide release at low pH (Fig. 6). In contrast, sodium dithionite, which is able to reduce nitroprusside over the entire pH range studied, showed no decrease in nitric oxide release at low pH. Reduction was clearly a prerequisite to NO release by these agents, and biological tissue appeared to produce NO by a similar reductive process.

In experiments with cysteine and nitroprusside, we found that at lower concentrations (<0.2 mM) cysteine enhanced the release of NO, while at higher concentrations cysteine was inhibitory (Fig. 3). All but a few reducing agents, such as *N*-acetylcysteine, had a similar inhibitory effect on NO release at higher concentrations. This observation, which may explain discrepancies in previous literature [5, 6, 14, 20] where thiols have been reported to both promote and inhibit NO release from sodium nitroprusside, suggests a process where cysteine first produces, and then at higher concentrations, destroys the intermediates responsible for NO production. Nitroprusside is known to be reversibly reduced in two steps [19, 30, 31] and our present data support

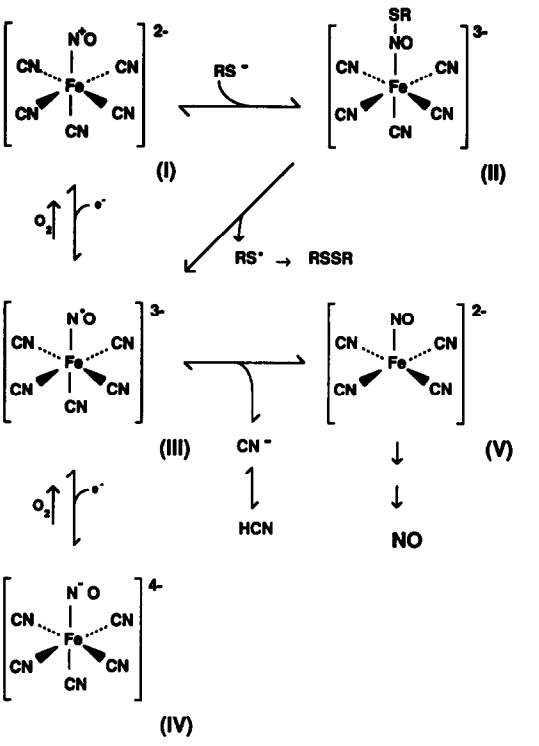

Fig. 7. Decomposition of nitroprusside to NO. Nitroprusside (I) is reduced directly to (III) by hemoproteins, etc., or indirectly via (II) by thiols. The pathway leading to NO release is (I)→(III)→(V)→→NO. Product (IV) is further reduced and does not release NO.

the scheme illustrated in Fig. 7, where only the first of these sequential reductions leads to NO production. With low concentrations of cysteine most of the thiol was consumed in the initial reduction of nitroprusside giving NO release that was proportional to cysteine concentration. At higher cysteine concentrations much of the reduced nitroprusside was reduced again by the remaining cysteine and NO release decreased. This concept is further supported by the observation that injections of air into the reaction mixture produced by higher concentrations of cysteine promptly increased the production of NO (Fig. 2B), suggesting that a metabolite was present which required oxidation to yield NO. The fact that both of the reduced forms of nitroprusside are easily oxidized by air [19, 30, 31] gives support to the pathway proposed in Fig. 7.

Relationship of cyanide release and NO release from sodium nitroprusside. In the present experiments, we found that CN^- was liberated from sodium nitroprusside in conjunction with NO release (Fig. 5). Further, the addition of KCN to solutions of reducing agents and sodium nitroprusside completely and promptly stopped the generation of NO (Fig. 2A). In contrast, the photochemical production of NO was also accompanied by cyanide release, but excess KCN had no effect on this reaction (Fig. 4). These findings strongly suggest that CN^- release was a prerequisite for NO production from sodium nitroprusside by reducing agents. The reversible

inhibition of NO release by an excess of the co-product CN^- is consistent with the sequence shown in Fig. 7 where KCN blocks, by mass action, the release of CN^- and formation of the intermediates necessary for NO release. The reaction where cyanide is lost from reduced nitroprusside has been studied carefully [32] and shown to be easily inhibited by CN^- ($K_d = 6.8 \times 10^{-5}$ M). The inhibition of NO release from reduced nitroprusside by high pH (Fig. 6) also coincides with the pH-dependent equilibrium of this reaction [32] where lower pH keeps CN^- concentrations low by conversion to HCN. Alternate mechanisms of inhibition by KCN are unlikely since five of the six ligand sites in nitroprusside are already filled with CN^-, and interactions of CN^- at the sixth site would lead to ferrocyanide formation which would not be readily reversible. Additionally, the failure of KCN to inhibit photochemical NO production argues against any inhibitory action of KCN directly on nitroprusside or nitric oxide.

The obligatory release of cyanide before NO means that cyanide production is an inescapable part of nitroprusside usage. Clinically nitroprusside is a sufficiently potent vasodilator that it can be used at doses resulting in cyanide production at rates that do not normally exceed the rate of metabolism of cyanide to thiocyanate [33]. If sodium nitroprusside is administered at rates that do lead to cyanide accumulation, the rising serum cyanide concentrations would have an inhibitory effect on NO release thereby weakening its vasodilator effects and compounding the problem by encouraging still larger doses of sodium nitroprusside.

Reduction of SNP by biological tissue. Release of NO from SNP in biological tissue appears to occur by the same chemistry that occurs between SNP and reducing agents. The kinetics, including temperature dependence, are similar; inhibition by oxidizers or KCN is the same; and many of the reducing agents tested here, such as thiols, hemoproteins, and possibly ascorbate, are abundant in most biological tissue. The reduction of SNP by a variety of reducing agents that are ubiquitous in biological tissue evidently is a prerequisite for nitric oxide release, but the nonspecific nature and widespread presence of these reducing agents are sufficient to give the appearance that nitric oxide release is spontaneous in many biological samples. Subcellular localization of NO release is not possible in these studies since arterial wall promotes SNP reduction and NO release that is associated with both intact cells and the soluble and particulate fractions of cell lysates.

The mechanism of NO release from SNP has implications beyond the action of nitroprusside itself. Other nitrovasodilators such as nitrosamines, organic nitrites, and nitrosothiols, also have NO-containing moieties with nitrogen in the same oxidation state as nitroprusside, and all apparently activate guanylate cyclase by generating free NO [1–4]. Release of NO from each of these compounds may occur by either spontaneous homolytic decomposition or by reduction [34]. Many of these compounds readily undergo homolytic cleavage with release of NO, yet even with the most unstable of these compounds, the nitrosothiols, there is evidence that spontaneous decomposition is not sufficient to explain the activity of these vasodilators [35]. Environments which promote NO release by reduction of nitroprusside would likely promote NO release by reduction of many of these other vasodilators as well.

In summary, the present studies show that nitroprusside readily released nitric oxide on contact with arterial vessel wall, as well as other biological tissue. This release was not due to spontaneous degradation since simple cleavage of NO from nitroprusside does not occur under physiological conditions. Reducing agents, including those present in biological tissue, will reduce nitroprusside and cause nitric oxide release. The one-electron reduction products of nitroprusside, but not more extensively reduced intermediates, were the source of nitric oxide. The release of nitric oxide was preceded by release of part or all of the bound cyanide. Further investigation is necessary to better characterize the intermediate reactions involved in NO release.

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