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EFFECTS OF TEMPERATURE, OXYGEN, HEME LIGANDS AND SULFHYDRYL ALKYLATION ON THE REACTIONS OF NITROPRUSSIDE AND NITROGLYCERIN WITH HEMOGLOBIN

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Abstract—Nitrovasodilators react with hemoglobin (Hb) to form heme(III) and nitric oxide (NO)Hb. These reactions can be exploited as models for events that take place at the cellular level leading to the biological effects of the prodrugs. Sodium nitroprusside (SNP) is known to undergo a one-electron reduction in its reaction with heme(II), resulting in the labilization of the cyanide ligand *trans* to the NO ligand. This reduced form is here called "penta." Upon dissociation of the *trans*-cyanide, the resulting species is here called "tetra." Dissociation of the *trans*-cyanide is obligatory for transfer of the NO to a heme(II) group. NO release from penta is blocked by excess free cyanide in solution, which prevents the formation of tetra. As reported here, both penta and tetra had unique EPR signals when frozen at −196°, but only tetra gave an EPR signal at 22°. NOHb also has a unique EPR signal, but it could not be detected when SNP was incubated with Hb in air or 10 or 5% oxygen. NOHb was detected in similar incubations under 1% oxygen, but the levels were 3- to 10-fold lower than those found under 100% nitrogen. The concentration of tetra was also much lower under 1% oxygen and penta was not detectable, suggesting that oxygen may either shift the penta–tetra equilibrium towards tetra or that penta may be susceptible to oxidation by molecular oxygen. Nitroglycerin (GTN) also generated much less NOHb but more heme(III) under 1% oxygen than under nitrogen. Carbon monoxide (CO), which binds to heme(II), completely blocked the reactions of SNP and GTN with Hb, whereas *N*-ethylmaleimide (NEM) alkylation of globin sulfhydryl groups increased both NOHb and heme(III) formation. ^{13}C NMR studies on uniformly ^{13}C-labeled SNP suggested that oxygen had little effect on the concentrations of the NMR-detectable species in the reaction. In summary, the most oxygen-sensitive step in the nitrosylation of Hb by SNP was probably the transfer of NO to heme(II). However, the penta–tetra equilibrium was affected by oxygen, temperature and cyanide. No evidence was found for the involvement of the globin sulfhydryl groups in either the GTN or the SNP reaction with Hb.

Sodium nitroprusside ($Na_2[Fe(CN)_5NO]$, SNP‡) is a vasodilator by virtue of its ability to release nitric oxide (NO) on contact with the vascular wall. Acute overdoses of SNP in laboratory animals, however, result in death by cyanide poisoning [1]. Under aerobic and anaerobic conditions, SNP can undergo a one-electron exchange reaction with hemoglobin (Hb) heme(II), which produces methemoglobin heme(III) and cyanide, either free or complexed with heme(III). This one-electron reduction of SNP is required for the release of the NO and its transfer to other heme(II) groups either on the same Hb molecule or on adjacent molecules [1, 2]; all five cyanides are released as well.

Like all agents that oxidize heme(II) to heme(III), Hb solutions partially oxidized by SNP are presumed to contain valency hybrid species in which either the α- or the β-subunits are oxidized and the paired subunits are reduced [3]. Thus, NO can bind to reduced subunits on the same tetramer on which SNP has oxidized a heme(II). These valency hybrid species cannot be separated by isoelectric focusing of crude reaction mixtures because the excess cyanide liberated from SNP binds tenaciously to the heme(III) and abolishes the charge differential essential for their separation [4]. When such solutions are dialyzed to remove cyanide, the pigments can be separated, but this may have been incorrectly ascribed to an effect of nitric oxide on amino acid residues in the globin chain [5].

The reaction between SNP and Hb occurs both in solution and in intact red blood cells. We have used the reaction of SNP with Hb as a model for the biotransformation and release of NO from SNP in biological systems, but *in vivo* it is likely that SNP vasodilation involves a reaction at the level of the blood vessel wall. Also, the reaction with the blood vessel wall likely accounts for most of the total cyanide released [6, 7].

Nevertheless, the SNP–Hb reaction has been exploited to elucidate the mechanism of the reductive decomposition of SNP which is obligatory for release of the NO and its biological effects. For example, work with this model system has provided an explanation for cyanide reversal of the biological

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‡ Abbreviations: GTN, nitroglycerin; Hb, hemoglobin; NEM, *N*-ethylmaleimide; and SNP, sodium nitroprusside.

effects of SNP. The nitroprusside anion is not detectable by EPR spectroscopy in solution, but the one-electron reduced product, "penta" (because it still contains five cyanides, $Fe(CN)_5NO^{-3}$) has a distinctive EPR signal. The cyanide *trans* to the NO is labile in penta, and its dissociation results in "tetra" (because it contains only the four equatorial cyanides, $Fe(CN)_4NO^{-2}$), which also has a distinctive EPR signal different from that of penta. Loss of this *trans*-cyanide is required for dissociation of the NO. In the presence of excess free cyanide, however, the equilibrium between penta and tetra is shifted toward penta, and the NO-group cannot be transferred [2, 8]. Thus, excess cyanide prevents the transfer of NO from SNP to Hb by preventing the formation of tetra, which is obligatory for NO transfer. Cyanide is thought to block or reverse the biological effects of SNP on platelets, aortic strips and other smooth muscles by the same mechanism [9–11]. In the absence of added cyanide, NO is released for binding to an effector site; the other products of the complete disintegration of SNP are iron and the remaining four equivalents of cyanide.

Recently, Rao *et al.* [12] confirmed a reductive pathway for SNP decomposition in rat hepatocytes and human erythrocytes, but they reported only one EPR-detectable species as a reaction product. Earlier, Butler *et al.* [13] had concluded, on the basis of NMR studies with ^{13}C-labeled SNP in blood, that no chemical reaction occurred between SNP and Hb over 14 hr. We are now able to offer some explanations for these contradictory observations, and further clarify the redox reaction between SNP and Hb. Finally, since reactions of SNP with both heme(II) and sulfhydryl groups are said to liberate NO [14], experiments were performed to evaluate the relative importance of these two groups as they are found on Hb.

MATERIALS AND METHODS

The *in vitro* experiments involving incubations with *N*-ethylmaleimide (NEM)-treated Hb, carbon monoxide (CO)Hb and Hb at various ambient oxygen tensions were all carried out in the same manner. Washed red blood cells (RBC) from pooled routine hematology specimens were lysed by dilution with distilled water and freezing and thawing twice with a dry ice–acetone bath. The NEM-treated Hb was prepared by the method of Guidotti and Konigsberg [15] with slight modifications. NEM (Sigma, St. Louis, MO) was dissolved in 0.1 M phosphate buffer, pH 7.4. The lysate, which was approximately 6 mM in heme, was incubated at 37° for 3 hr with NEM in a 4-fold molar excess to the Hb. Excess, unreacted NEM was removed by passing the solution through a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer. The eluate was then passed through an affinity column containing immobilized *p*-chloromercuribenzoate (Pierce, Rockford, IL) to remove any Hb with free globin sulfhydryl groups. The method of Ellman [16] was used to confirm that alkylation of the sulfhydryl groups in the final eluate was complete. The eluate was reconcentrated to 4–6 mM in heme in miniconcentrators (Amicon, Beverly, MA).

In the experiments with COHb, lysates were placed in a cylindrical tonometer with a stopcock on each end and a middle side arm capped with a rubber septum and exposed to pure CO; the shift in the Soret maximum for oxyHb (415 nm) to that for COHb (419 nm) [17] was followed until saturation was complete. Solutions of nitroglycerin (GTN, LyphoMed, Inc., Melrose Park, IL, 50 mg/10 mL) or SNP (cert. ACS grade, Fisher, Fair Lawn, NJ) were added by injection through the rubber septum on the side arm. Other appropriately treated lysates were diluted with phosphate buffer to give a final concentration of 1.3 mM in heme, and placed in the tonometer at 22°. The tonometer was purged either with 100% nitrogen or with various mixtures of oxygen in nitrogen. Either GTN or SNP was added anaerobically to a final concentration of 1.6 mM, and the tonometer was sealed and rotated to ensure complete equilibration; aliquots for analysis were removed anaerobically at periodic intervals. In all experiments, untreated lysate was run in tandem as a control.

In some experiments, it was necessary to determine the concentration of heme(III) in the presence of cyanide. This was accomplished after the addition of excess sodium cyanide (cert. ACS grade, Fisher) to blood samples to convert all heme(III) to cyanmethemoglobin. The heme(II) fraction was then determined by the difference in absorption at 576 nm before and after the addition of excess potassium ferricyanide (Fisher) to convert all heme(II) to heme(III). The heme(II) fraction was then subtracted from the total heme, determined as cyanmethemoglobin at 540 nm, to obtain indirectly the heme(III) originally present.

The EPR technique for quantifying NOHb and paramagnetic intermediates of SNP has been described previously [2]. The method for quantifying ^{13}C-labeled cyanide and SNP was also described previously [8, 13]. The EPR spectra of SNP reduction products were obtained by incubating 8.2 mM SNP with 0.3 mM sodium borohydride (Sigma, St. Louis, MO) and collecting samples in 1-mm capillary tubes capped immediately with rubber septa. The capillary tubes were either placed directly into the EPR cavity at room temperature (22°) or frozen in liquid nitrogen (−196°) in a Wilmad EPR Dewar that was placed in the EPR cavity. EPR spectra were obtained on a Bruker ESP300 X-band spectrometer operating at 10 mW microwave power, 100 kHz modulation frequency, and 9.92 G modulation amplitude. The reported anisotropic g values for the frozen specimens are effective ones taken from the experimental spectra.

^{13}C-Labeled SNP was synthesized by the method of Butler *et al.* [18], and confirmed with elemental analysis by Galbraith Laboratories, Knoxville, TN. Calculated: C 21.4%, H 1.3%, N 27.7% and Fe 18.4%. Found: C 21.4%, H 1.4%, N 27.4% and Fe 18.2% based on two moles of water of hydration.

RESULTS

Temperature equilibrium of SNP reduction products. Figure 1 shows a representative EPR spectrum for borohydride-reduced SNP at 22° (Fig.

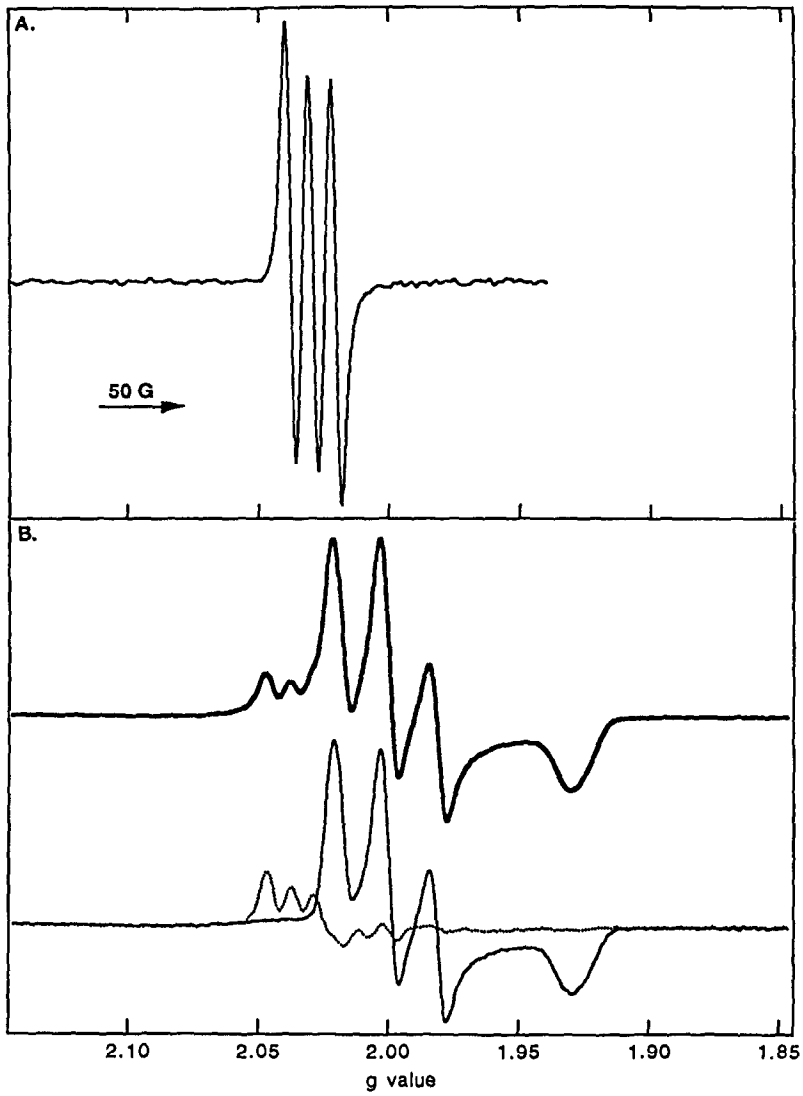


Fig. 1. EPR spectra for borohydride-reduced SNP at two different temperatures. (A) At 22° (——) which is characteristic of the reduced species, tetra. (B) At −196° (——) which is the sum of the signals for tetra (·····) and penta (——).

1A) and at −196° (Fig. 1B) The anisotropic frozen solution spectrum (Fig. 1B) found at −196° was a combination of the EPR signals of both reduced species, penta ($g_{\|} = 1.930$; $g_{\perp} = 1.998$; $a_{\perp}(^{14}N) = 30$ G) and tetra ($g_{\|} = 2.008$; $a_{\|}$ (^{14}N) = 16 G; $g_{\perp} = 2.038$; $a_{\perp}(^{14}N) = 14.5$ G), whereas the isotropic solution spectrum obtained at 22° (Fig. 1A) was due solely to the reduced species, tetra ($g_{iso} = 2.028$; $A_{iso} = 15.7$ G) [2]; penta would have a signal with $g_{iso} = 1.975$. The same 22° and −196° EPR spectra could be obtained repeatedly and reproducibly by freezing and thawing a borohydride-reduced SNP sample in a single capillary tube. This suggests that there is a temperature-dependent equilibrium between two species which favors tetra at higher temperatures. Since it is known that excess cyanide also shifts the equilibrium between penta and tetra [2], we attempted to reduce SNP by borohydride in the presence of an equimolar concentration of cyanide. No EPR signals were observed at either temperature in that reaction mixture. However, if borohydride was added first, followed within 1 min by cyanide, the frozen solution EPR spectrum was identical to that of penta, but no EPR signal was observed in the 22° solution under conditions identical to those above.

Effects of oxygen on SNP reduction products. The above experiments were conducted under anaerobic conditions from the start of the reaction to the EPR analysis because one or more reaction products are unstable in the presence of oxygen [4]. Recently, we have shown that the stability of NOHb is very temperature sensitive when exposed to air [19], but the stability of penta and tetra in air has not been studied previously. Reactions of SNP and Hb conducted under 10 or 5% oxygen in nitrogen yielded

Table 1. Effect of oxygen on the SNP–Hb reaction*

Min	Penta (N_2)	Penta (O_2)	Tetra (N_2)	Tetra (O_2)	NOHb (N_2)	NOHb (O_2)
2	32	0	34	10	35	11
8	32	0	34	9	59	18
20	32	0	49	8	74	29
60	0	0	14	0	178	31
180	0	0	7	0	282	29

* Incubations were carried out at 22°, pH 7.4, under 1% oxygen in nitrogen (O_2) or under 100% nitrogen (N_2). The concentrations of EPR-detectable species were quantified (μM), as previously described [2], in aliquots removed at the indicated times and frozen for EPR analysis.

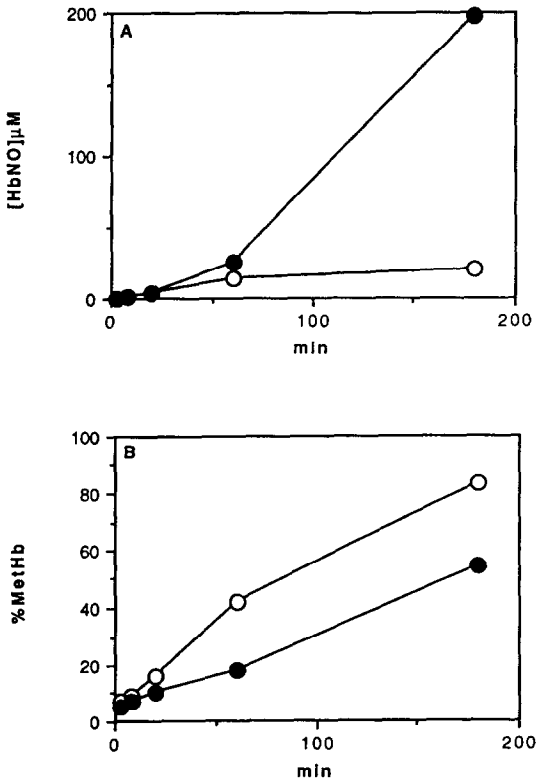


Fig. 2. (A) Formation of NOHb in GTN–Hb mixtures under 100% nitrogen (●) and under 1% oxygen in nitrogen (○). (B) Formation of heme(III) in GTN–Hb mixtures under 100% nitrogen (●) and under 1% oxygen in nitrogen (○). Results are from single representative experiments; see Fig. 3B for typical magnitude of analytical errors.

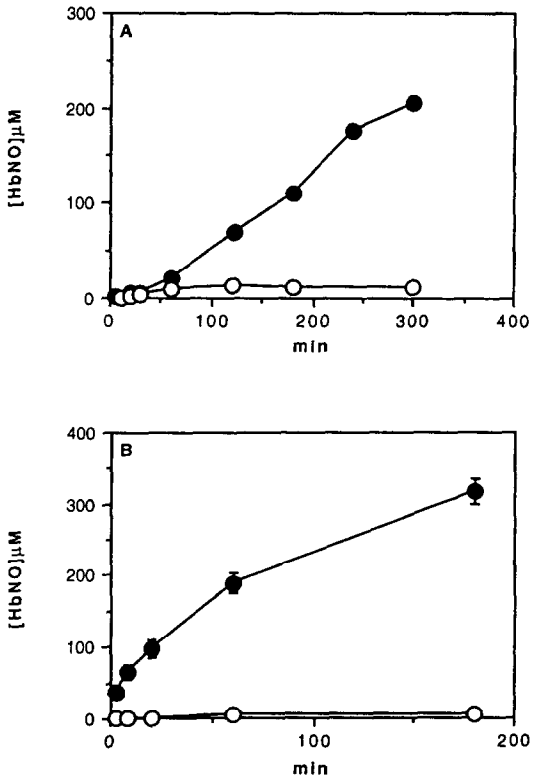


Fig. 3. (A) Formation of NOHb in GTN–Hb (●) and in GTN–COHb (○) mixtures under nitrogen. (B) Formation of NOHb in SNP–Hb (●) and in SNP–COHb (○) mixtures under nitrogen. Results are from single representative experiments except in panel B, which shows SE bars for N = 5.

essentially the same results as under ambient air, namely, little or no formation of NOHb.

Table 1 compares the results obtained in reaction mixtures incubated under 1% oxygen in nitrogen or under 100% nitrogen. Under pure nitrogen the NOHb concentration increased steadily over the 3-hr incubation to a final value of 282 μM, whereas under 1% oxygen the values plateaued by 20 min at 29 μM. The concentration of tetra, which transfers NO to Hb [2], peaked at 2 min under 1% oxygen and was no longer detectable by 60 min. Under nitrogen, the intensity of the tetra signal peaked at 20 min and steadily declined thereafter although it was still detectable at 3 hr. No penta was detected at any time under 1% oxygen, whereas under nitrogen it had reached a maximum concentration by 2.5 min and was undetectable after 60 min. These results suggest that penta is the more oxygen-sensitive species, and that it may be reoxidized back to nitroprusside anion.

Effects of oxygen on the GTN–Hb reaction. The only EPR-detectable species generated in the

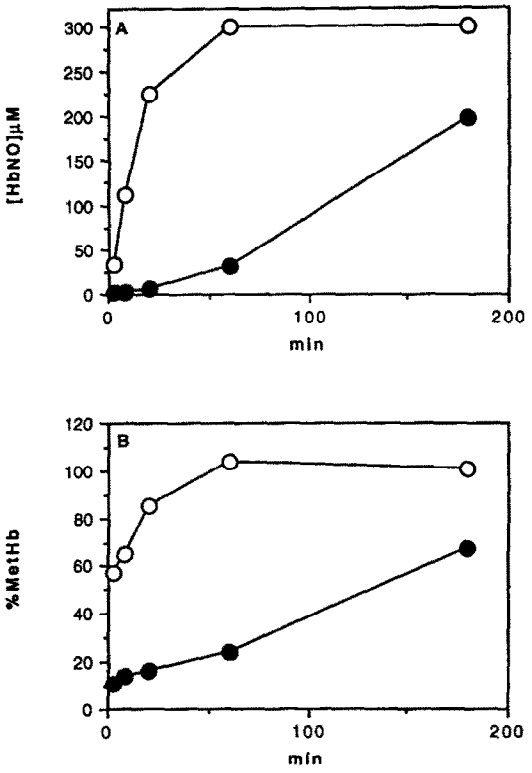


Fig. 4. (A) Formation of NOHb in GTN–Hb (●) and in GTN–NEMHb (○) mixtures under nitrogen. (B) Formation of heme(III) in GTN–Hb (●) and in GTN–NEMHb (○) mixtures under nitrogen. Results are from single representative experiments; see Fig. 3B for typical magnitude of analytical errors.

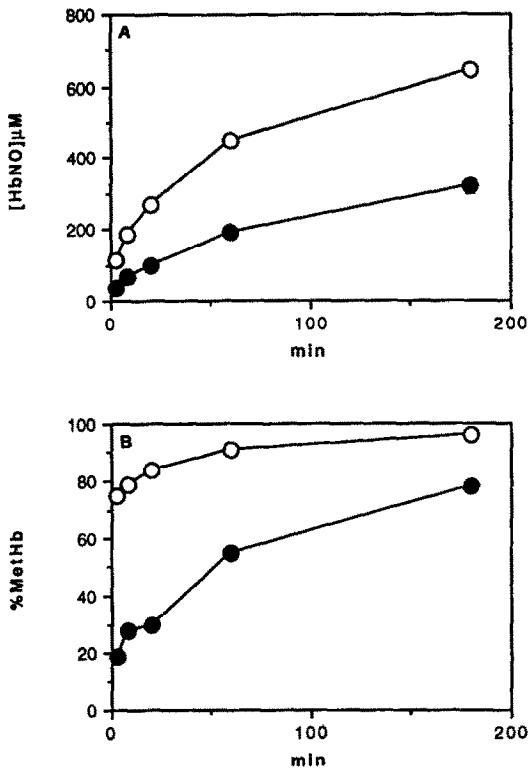


Fig. 5. (A) Formation of NOHb in mixtures of SNP–Hb (●) and in mixtures of SNP–NEMHb (○) under nitrogen. (B) Formation of heme(III) in mixtures of SNP–Hb (●) and in mixtures of SNP–NEMHb (○) under nitrogen. Results are from single representative experiments; see Fig. 3B for typical magnitude of analytical errors.

reaction of GTN with Hb was NOHb. Figure 2A shows that the reaction generated large amounts of NOHb under nitrogen, whereas only low levels were generated under 1% oxygen in nitrogen. In Fig. 2B it can be seen that the rate of heme(III) generation was about the same under pure nitrogen as under 1% oxygen, except for a greater rate of formation during the first hr in the presence of oxygen. By the end of the 3-hr incubation period more than 80% of the heme was oxidized in the presence of 1% oxygen, whereas only a little over half of the total heme was oxidized under nitrogen.

Effect of CO on the GTN- and SNP–Hb reactions. Figure 3 shows the results obtained when GTN (Fig. 3A) or SNP (Fig. 3B) was incubated with Hb under nitrogen in the absence and in the presence of sufficient CO to saturate all heme(II) sites. CO completely blocked or greatly obtunded the formation of NOHb by both GTN and SNP.

Effect of sulfhydryl alkylation on the GTN- and SNP–Hb reactions. Figure 4 summarizes the results obtained when NEM-treated Hb was incubated with GTN under nitrogen. Figure 4A shows that the formation of NOHb was much more rapid when the free sulfhydryls on the globin chain were blocked. The reaction had gone to completion in about 1 hr, whereas the reaction with normal Hb was still proceeding at 3 hr. Figure 4B shows that the formation of heme(III) also proceeded more rapidly with NEM-treated Hb. These results suggest that heme(II) oxidation is linked to NOHb formation by GTN.

Figure 5 shows the results obtained when NEM-treated Hb was incubated with SNP under nitrogen. The results paralleled those for GTN, above. Figure 5A shows that the formation of NOHb by SNP under nitrogen was favored in NEM-Hb over normal Hb. Figure 5B shows that the formation of heme(III) by SNP under nitrogen was also favored in NEM-Hb over normal Hb. Indeed, in each case, the approach to 100% heme(III) formation limited the amount of NOHb generated.

NMR studies on the ^{13}*C-labeled SNP–Hb reaction.* The ^{13}C NMR spectra of our ^{13}C-labeled SNP under both air and nitrogen showed ^{13}C NMR peaks for the *trans*- and the equatorial-cyanides of ^{13}C-labeled SNP at 133.7 and 135.8 ppm, respectively, and in the expected ratio of 1:4 [18]. No changes in the intensity of these peaks were noted after 5 hr of incubation with whole blood either under air or under nitrogen. However, after 23 hr of incubation under both conditions the 133.7 peak had disappeared, indicating that the *trans*-cyanide of nitroprusside had dissociated or had a dramatically altered

exchange rate (data not shown). The peak at 135.8 for the equatorial-cyanides was unchanged at this time. In both cases a new peak had appeared at 178 ppm, which was somewhat more prominent under anaerobic conditions. This peak was due to the formation of ferrocyanide, $Fe(CN)_6^{-4}$, which may have arisen from the decomposition of SNP or cyanide binding to adventitious iron in the sample. Ferricyanide, $Fe(CN)_6^{-3}$, like penta and tetra, is paramagnetic and not visible by ^{13}C NMR. These results confirm our earlier studies using ^{13}C-labeled sodium cyanide, SNP and hemoglobin or blood in air [8].

Additional ^{13}C-labeled SNP was used in an EPR study of its reaction with Hb, both to show that it behaved chemically like unenriched SNP [2] and to examine the reaction under aerobic conditions. In the Hb-mediated reduction under aerobic conditions, only the tetra form could be detected in frozen and room temperature samples over the first hr. No EPR-detectable species remained at 20 hr under aerobic conditions in either room temperature or frozen samples. In the Hb-mediated reduction under anaerobic conditions, the frozen samples showed that both penta and tetra were present initially and they decreased with time whereas the HbNO signal increased with time. The room temperature samples were similar to the frozen samples except that penta was not detected as in the case of unlabeled material (data not shown). The patterns for the appearance and disappearance of reduced ^{13}C-labeled SNP species under anaerobic conditions were essentially like those already reported above (Table 1) for unenriched SNP, confirming that the ^{13}C-label played no role in the reaction. Although the ^{13}C-labeled SNP behaved chemically like the unlabeled material and the EPR spectra of ^{13}C-labeled penta and tetra had g values identical to those of the unlabeled samples, the ^{13}C-labeled species exhibited a more complicated hyperfine pattern attributed to ^{13}C ($I = 1/2$) splitting of the EPR signal.

DISCUSSION

The failure of Rao *et al.* [12] and Glidewell and Johnson [20] to detect penta upon one-electron reduction of SNP may be ascribed to the fact that all their EPR studies were conducted at room temperature where, as shown here, only tetra is EPR-detectable. The penta form is shown here to be present upon reduction of SNP, but it is only EPR-detectable in frozen solutions ($T < -30°$) [21] The most obvious explanation is a temperature-dependent equilibrium between penta and tetra:

$$Fe(CN)_5NO^{-3} \rightleftarrows Fe(CN)_4NO^{-2} + CN^-$$

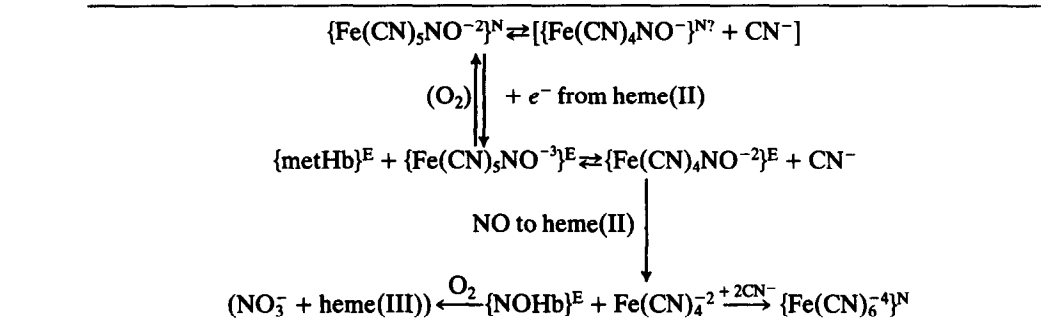
which favors tetra at higher temperatures. This is also supported by an obvious color change (blue to green) on freezing reduced SNP solutions and the reverse on thawing. However, the situation may be more complicated since the absence of a penta EPR signal at 22° may be due to different relaxation properties that render it EPR-invisible in solution. There was also a total absence of a solution EPR signal when attempts were made to reduce SNP with borohydride at room temperature in the presence of equimolar cyanide, which should shift the equilibrium toward penta [2]. We were also unable to reduce SNP electrolytically in the presence of excess cyanide (unpublished observation). In the case of borohydride, we suspect that a direct chemical reaction between cyanide and the reducing agent, as has been reported previously [22], was responsible for the failure, and in the case of electrolytic reduction we suspect cyanide "poisoning" of the electrodes. Cheney *et al.* [23] found the room temperature penta–tetra equilibrium constant to be 6.8 ± 10^{-5} at pH 6.7 and 7.9, which means that at 22° tetra is ten times more concentrated than penta in the absence of added cyanide. Thus, two factors, which depend on temperature, appear to contribute to the absence of a penta EPR signal in solution, namely, the penta–tetra equilibrium and the EPR relaxation properties of penta.

The aerobic stability of NOHb is also critically temperature dependent. The signal is essentially unchanged over 30 days at temperatures of −20° or colder, but the $T_{1/2}$ at 4° is 6.6 days, at 20° it is 3.5 hr and at 37° it is 40 min [19]. This may be due to a kinetic barrier in the irreversible reaction between NO and oxygen. In the experiments of Table 1 where incubations were carried out at 22°, the period of observation was approximately one half-life for NOHb in air. Under pure nitrogen the signal continued to grow throughout the incubation period, whereas under 1% oxygen it plateaued at 20 min and remained at that level thereafter. Clearly NOHb was being generated at a rate faster than its decomposition under nitrogen, and at a rate about equal to its decomposition under 1% oxygen.

As shown in Table 1, no penta could be detected under oxygen. However, penta was detected at least for the first 20 min under nitrogen. The tetra form, which transfers its NO to Hb, decayed steadily under both aerobic and anaerobic conditions although it reached a higher concentration under nitrogen, accounting for the much higher levels of NOHb formed. Therefore, both oxygen and high temperatures may shift the penta–tetra equilibrium in the direction of tetra, or penta may be susceptible to reoxidation to nitroprusside anion by molecular oxygen.

NMR is a much less sensitive quantitative method, but it detects diamagnetic species not observed by EPR. Thus, our ^{13}C NMR studies provided complementary insights about these reactions. The low sensitivity was overcome by using completely ^{13}C-enriched SNP [13], in contrast to our previous work that used only ^{13}C-enriched free sodium cyanide [8]. Two species, nitroprusside anion and ferrocyanide, were observed, and their concentrations monitored by NMR. In addition, a hypothetical species resulting from the loss of the *trans*-cyanide without one-electron reduction, i.e. $Fe(CN)_4NO^-$, may have been detected, but we cannot rule out an altered exchange rate for this cyanide as the reaction progressed. The presence of oxygen had only a minor effect on the relative concentrations of these NMR-detectable species, with the absence of oxygen resulting in somewhat more ferrocyanide production. The following scheme

indicates the different species obtained in the SNP–Hb reaction and their ^{13}C NMR$^{(N)}$ or EPR$^{(E)}$ detectability:



Only the initial SNP species, nitroprusside anion, and a final product, ferrocyanide, are detected by NMR. The intermediates, penta and tetra, and the Hb products, metHb and NOHb, are detected by EPR, but penta is only detected in frozen samples. The decreased ferrocyanide produced in the presence of oxygen reflects the decreased production of NOHb observed by EPR (Table 1) and the oxygen sensitivity of species, particularly penta, in this reaction scheme as discussed above.

Probably the most dramatic effect of oxygen is on the step in which NO is transferred to heme(II). This is due, in part, to the well known instability of NO in aerated solutions, but NO also reacts directly and even more rapidly with molecular oxygen bound to the heme(II) [24]. Actual measurements have shown that about 50% of the oxygen bound to heme is consumed in the SNP–oxyHb reaction [1]. Wennmalm *et al.* [25] similarly found that when arterial blood that was 94–99% saturated with oxygen was exposed to NO gas, there was a quantitative conversion to nitrate and heme(III), whereas in venous blood that was 36–85% oxygen saturated, NOHb was formed together with less nitrate.

We found no evidence for stable, EPR-detectable intermediates in the GTN–Hb reaction. Since NO only binds tightly to heme(II), the more rapid rate of oxidation to heme(III) in 1% oxygen could account in part for the smaller amount of NOHb generated under these conditions (Fig. 2). However, the lysate was 1.3 mM in heme so that at 80% oxidation, the heme(II) concentration was still 260 μM yet the NOHb concentration only reached 20 μM. At 50% oxidation under 100% nitrogen the heme(II) concentration would be 650 μM, and the NOHb reached 200 μM in 3 hr. An additional factor which may play a role is that at 1% oxygen tension the heme(II) saturation may still be on the order of 10% [26]. We have been unable to prepare NOHb in air–NO mixtures because of the rapid rate of formation of heme(III) due to the preferential reaction of NO with oxygen bound to heme [24]. Thus, both GTN and NO may contribute to the higher concentrations of heme(III) generated in the presence of 1% oxygen due to a reaction of NO with heme(II) oxygen to give heme(III), as in the case of SNP above. GTN undergoes a 3 e^- reduction, with the reducing equivalents likely provided by three heme(II) groups, to generate NO and GDN:

$$3H^+ + GTN + 3\,\text{heme(II)} \rightarrow 3\,\text{heme(III)} + NO + H_2O + GDN$$

The NO then binds to any free heme(II) on the same tetramer or on an adjacent molecule of Hb. Again, oxygen seems to play a more important role in the formation of NOHb than in the formation of heme(III) (Fig. 2).

The importance of the heme group to these reactions is shown in Fig. 3A. CO bound to the heme iron almost completely halted the production of NOHb by nitroglycerin. This observation suggests that a redox reaction involving the heme iron is critical for the GTN reaction since CO blocks heme(II) oxidation by ferricyanide, nitrite and hydroxylamine [e.g. Ref. 27]. CO also completely prevented the reaction of SNP with Hb (Fig. 3B). It has been established that if NO had been liberated by some other mechanism such as a reaction of SNP or GTN with globin sulfhydryl groups or reduced glutathione, it would compete successfully with CO for the heme(II) binding site [28, unpublished observations].

Bennett *et al.* [29] in an elegant study followed the disappearance of GTN in human red blood cell lysates under conditions similar to ours except that "a rapid stream of CO for 1 min" was passed through their lysates in covered Erlenmeyer flasks that had been exposed to air. They concluded that CO obtunded but did not totally block GTN biotransformation. This observation together with other evidence led them to suggest that GTN biotransformation in lysates is partly dependent on heme(II) and partly dependent on sulfhydryl groups. Since these results are not entirely consistent with Fig. 3, it may be that GTN undergoes biotransformation by mechanisms that do not yield NO, or that their Hb was not completely saturated with CO, or that the concentration of sulfhydryls in our lysates was in a range high enough to inhibit NO release as in the biphasic effect of sulfhydryls described by Bates *et al.* [14].

Finally, as shown in Fig. 4, both heme(II) oxidation and NOHb formation were actually accelerated in NEM-treated Hb, further supporting the key role played by heme(II) in GTN and SNP biotransformation. Only a minor, if any, role appears to be played by the free sulfhydryl groups on the globin moiety in the generation of NO. Some evidence suggests that NEM alkylation of the globin sulfhydryl groups lowers the reduction potential of the heme iron making it more susceptible to auto-oxidation or attack by oxidative agents [30, 31], such as GTN or SNP.

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