and NO^+ have biochemical precedence.

In the latter phases of the reaction, during which the rate of MbNO formation decreases relative to that for $HN_2O_3^-$ decomposition (Table I), one can consider whether the decrease is due to a change in the rate-determining step from $HN_2O_3^-$ breakdown (zero-order in $[Mb^+]$) to nitroxyl trapping (presumably first-order in $[Mb^+]$) or to an increasingly unfavorable partition between fast trapping and fast N_2O production. In the second case, the reaction should remain zero-order in $[Mb^+]$ throughout. The experiments of Figure 3 suggest that the second case is the more likely, because the reactions appear to remain zero-order in $[Mb^+]$ or $[Hb^+]$ (2-fold rate difference throughout) and do not tend to show a transition from a 2-fold to a 4-fold rate difference toward the end of the reaction. It is reasonable to expect nitroxyl trapping to be a fast reaction, because nitroxyl dimerization/dehydration in the gas phase is known to be a fast reaction[2] and because, in our systems, the reaction must compete with addition of an anionic ligand to Mb^+ or Hb^+ for which rate constants $\geq 10^4$ M^{-1} s^{-1} are not unreasonable.[25] The rate constant for dimerization/dehydration of nitroxyl in neutral aqueous solution is probably $\geq 10^9$ M^{-1} s^{-1}.[26]

The stoichiometric ratio of about 1.4:1 established in Figure 2 for 50 μM heme represents an average over the entire course of the reaction and includes the latter phases during which time nitroxyl capture by heme protein would be decreasingly competitive against N_2O formation. We believe that a better measure is the ratio A/B in Table I, which is about 1.25 for Mb^+. Thus, in the 50 μM range, Mb^+ can trap something in the order of 80% of the nitroxyl generated in $HN_2O_3^-$ breakdown. The rather high efficiency for trapping can be attributed to lack of a firmly bound sixth protein ligand to iron in Mb^+ and Hb^+, the dissociation of which might be expected to be slow, as is the case generally with cytochromes. This view is supported by the fact that certain concentrations of HCN can block the conversion of Mb^+CN^- to MbNO by $HN_2O_3^-$, whereas lower concentrations either cannot or are only partially effective. The observation suggests that HNO and HCN can compete with each other for the sixth ligand position of Mb^+ and Hb^+.

The sigmoidal progress curves observed with Hb^+ are likely due to a cooperative reactivity of the Hb^+ tetramer. Cooperativity in ligand binding or redox reactions of Hb^+ is unknown at present,[27] and reaction with nitroxyl may be the first example. Alternatively, the sigmoidicity may result from a high molecular weight inhibitor which is destroyed during the course of the reaction. This possibility seems very unlikely but has not been entirely ruled out.

Acknowledgment. This work was supported by grants from the National Science Foundation (PCM 82-18000) and Biomedical Research Support Grant S07 RR 07044 from the National Institute of Health. We thank Francis Bonner for useful discussions and information on the trioxodinitrate system and Joanne Goretski for having synthesized $Na_2N_2O_3$.

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On the Reaction of Trioxodinitrate(II) with Hemoglobin and Myoglobin

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Abstract: The reaction of hemoglobin (Hb) or myoglobin (Mb) with the monoanion ($HN_2O_3^-$, **1**) of sodium trioxodinitrate was studied in anaerobic 50 mM potassium phosphate buffer at pH 7.0, 25 °C. The reaction showed two kinetic phases, and the initial more rapid reaction is the reaction of interest. That reaction was first-order in **1** and zero-order in [Hb]. The rate of disappearance of Hb was nearly twice that of **1**, and in titrations, 1 mol of **1** consumed about 2 mol of Hb. The unimolecular rate-determining step in the disappearance of **1** in the presence of Hb is apparently identical with that in its absence. At 50-100 μM Hb, the products were methemoglobin (Hb^+) and nitrosylhemoglobin (HbNO) in approximately equimolar amounts. At 1 mM Hb, HbNO was the chief product. Similar results to above were obtained with Mb. Formation of Hb^+ and HbNO could suppress formation of both nitrite and N_2O, which are the normal decomposition products of **1** in the absence of Hb. The results are explained for the most part by rate-determining conversion of **1** to a reactive form (or to primary decomposition products), **1***, followed by reduction of **1*** by Hb or Mb. The composition of products would appear to depend on the efficiency with which newly formed Hb^+ traps nitrosyl hydride (nitroxyl, HNO), or related species, to form HbNO, relative to dimerization of HNO to form N_2O. The stoichiometry reported recently (Doyle, M. P.; Mahapatro, S. N. *J. Am. Chem. Soc.* **1984**, *106*, 3678-3679) for the reaction of Hb with **1** is apparently incorrect and the mechanism inferred therefrom in question.

There exists kinetic and isotopic evidence[1-6] to suggest that the primary products of decomposition of the monoanion (**1**)[7] of sodium trioxodinitrate (Angeli's salt,[8] $Na_2N_2O_3$) in neutral aqueous solution are nitrosyl hydride (nitroxyl, HNO) from N(1) and nitrite from N(2) (eq 1).

Nitroxyl is thought then to dimerize and dehydrate rapidly (eq 2) to form N_2O as final product, just as is known to occur in the gas phase.[9] In support of nitroxyl formation in eq 1 are reactions which can compete with dimerization to trap nitroxyl nitrogen.

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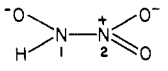


1

$$1 \xrightarrow{\text{r.d.s.}} HNO + NO_2^- \quad (1)$$

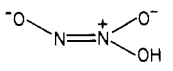
$$HNO \xrightarrow{\text{fast}} \tfrac{1}{2}N_2O + \tfrac{1}{2}H_2O \quad (2)$$

Among known trapping agents are tetracyanonickel(II) which is converted to tricyanonitrosylnickel,[3] NH_2OH which is converted to N_2 containing one N atom from NH_2OH and the other from N(1) of **1**,[2] and methemoglobin and metmyoglobin which are converted to nitrosylhemoglobin and nitrosylmyoglobin.[6]

Doyle and Mahapatro[10] concluded recently that the primary products in the decomposition of **1** in neutral aqueous solution are actually NO and $(HONO)^-$ (both being N^{2+} species) and that the final products, N_2O and NO_2^-, arise by way of a set of three subsequent rapid reactions, none of which involves nitroxyl. This was inferred from the kinetics and stoichiometry of the reaction between **1** and hemoglobin (Hb). The stoichiometry of this reaction was reported to be eq 3, where HbNO and Hb^+ are nitrosylhemoglobin and methemoglobin, respectively. Given the

$$2\mathbf{1} + 2Hb \rightarrow HbNO + Hb^+ + NO_2^- + N_2O + 2OH^- \quad (3)$$

structure of **1**, we were unable to imagine a likely mechanism for N–N bond cleavage that could yield NO and $HONO^-$ (or HNO^+ and ONO^{2-}) as primary products. Heterolytic cleavage with N(2) retaining the bonding electron pair would yield nitroxyl (N^+) and nitrite (N^{3+}); homolytic cleavage would yield N^0 and N^{4+} fragments; heterolytic cleavage with N(1) retaining the bonding electron pair would yield N^- and N^{5+} fragments. No way is evident to obtain primary fragments both of which are in the N^{2+} state. A tautomer of **1** with an N=N bond, **2**, would be unlikely to undergo N=N cleavage. But if it did so, the fragments from



2

the three cleavage modes described above would be in states N^{3+} and N^+, N^+ and N^{3+} and N^- and N^{5+} for N(1) and N(2), respectively. This difficulty prompted us to reexamine the reaction between **1** and Hb (and Mb), and we now report the results of the study.

Experimental Procedures

Deoxyhemoglobin (hemoglobin, Hb) was prepared by reducing adult human hemoglobin A, type IV (Sigma), with a 10-fold molar excess of dithionite relative to heme. Dithionite and bisulfite were separated from Hb by passing the solution through a G-25 Sephadex column (Pharmacia) using anaerobic 50 mM potassium phosphate buffer, pH 7.0, as the eluting buffer. Sperm whale deoxymyoglobin (Mb) (Mann) was prepared similarly. Hb and Mb solutions (1–2 mM) were stored in sealed flasks under H_2/N_2 and used within several hours. The above reduction and purification steps were performed in an anaerobic chamber under a H_2/N_2 atmosphere.

The heme protein products from the reaction of Hb with **1** were HbNO and Hb^+. The concentrations of Hb, HbNO, and Hb^+ in reaction mixtures were determined by solution of a set of three simultaneous equations based on absorbance and molar extinction coefficients at 630, 572, and 555 nm. The extinction coefficients used were from spectra of reference compounds prepared in this laboratory[6] and were in close agreement with literature values.[11] The same procedure, but at slightly different wavelengths, was applied to mixtures of Mb, MbNO, and Mb^+. The error in the estimation is believed to be about ±10% of the value, except for mole fractions less than 0.1 for which an error of about ±20% applied. Mole fractions less than 0.03 were considered to be zero.

Preparation of spectrophotometric heme protein standards, synthesis of Angeli's salt, reaction conditions, and the analysis of NO, N_2O, and nitrite were as described in the preceding paper in this journal.[6]

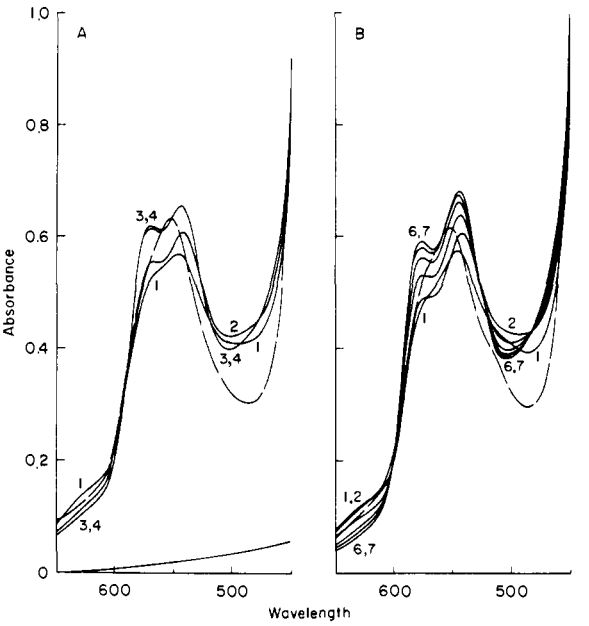


Figure 1. Spectral time course for the reactions of hemoglobin (A) and myoglobin (B) with trioxodinitrate monoanion at pH 7.0, 25 °C. Reaction was initiated by the injection of anaerobic $Na_2N_2O_3$ to 100 μM into 50 μM heme protein ([heme]) dissolved in anaerobic 50 mM potassium phosphate buffer, pH 7.0. Spectrum A1 was recorded 2 min after initiation of reaction, and subsequent spectra were recorded at 4-min intervals thereafter. Scan time was 2.0 min. Spectrum B1 was recorded 1 min after initiation, and subsequent spectra were recorded at 3-min intervals thereafter. Spectra of Hb and Mb are shown in A and B, respectively, as dashed lines. Buffer blank is shown at the bottom of A.

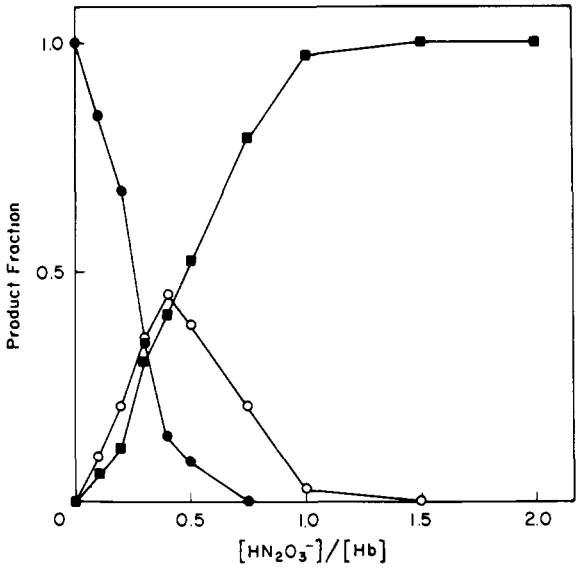


Figure 2. Titration of 50 μM hemoglobin with trioxodinitrate monoanion pH 7.0, 25 °C. The conditions and method of Figure 1 applied. Open circles, methemoglobin; closed circles, hemoglobin; closed squares, nitrosylhemoglobin. The amounts of these three heme protein species were estimated by way of the spectrum obtained at a time sufficient to have allowed about 95% of the original $HN_2O_3^-$ to decompose.

Results

As illustrated in Figure 1, the reaction of Hb (Figure 1A) or Mb (Figure 1B) with a 2-fold excess of **1** is a two-phase reaction. In the initial rapid phase, Hb^+ (band at 630 nm) and HbNO (band at 543 nm) are formed from Hb. In the latter phase, Hb^+ is converted to HbNO, and this reaction, which is described in ref 6, shows isosbestic points at about 597, 524, and 480 nm. Mb behaves analogously, except that the isosbestic points occur at

(10) Doyle, M. P.; Mahapatro, S. N. *J. Am. Chem. Soc.* **1984**, *106*, 3678–3679.

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Table I. Products of the Reaction of Trioxodinitrate Monoanion with 1 mM Hemoglobin at pH 7.0, 25 °C

reactants, nmol		products,[a] ng-atom of N					
$HN_2O_3^-$	Hb	Hb^+, nmol	HbNO	free NO	nitrite	N_2O	total N
660	0			0	650 (693)	670 (660)	1320 (1353)
1320	0			5	1380 (1366)	1260 (1320)	2645 (2772)
760	2000	450	1060	0	250 (38)	120 (450)	1430 (1558)
1520	2000	180	1620	15	900 (596)	500 (700)	3035 (3116)

[a] The values in parentheses are expected values based on the stoichiometries of eq 5–8 and include an estimated 2.5% contamination of $NaNO_2$ nitrogen in $Na_2N_2O_3$ nitrogen. Approximate errors: Hb^+, HbNO, nitrite, and N_2O, ±10%; free NO, ±50%.

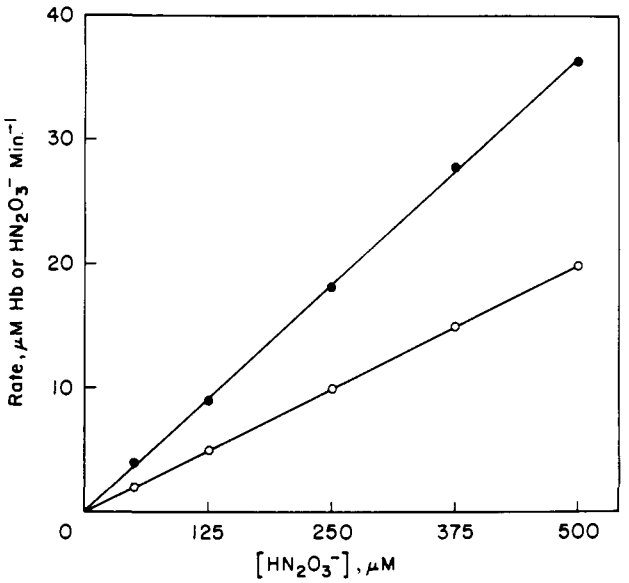

Figure 3. Initial rates of reaction of hemoglobin (●) and trioxodinitrate monoanion (○) at pH 7.0, 25 °C. The conditions and method of Figure 1 applied. The reaction mixtures contained $HN_2O_3^-$ as indicated and 50 μM Hb. Hb disappearance was followed at 480 nm (an isosbestic point for $Hb^+ \rightarrow$ HbNO), and the rate of disappearance of $HN_2O_3^-$ was calculated from its first-order decay constant of 6.6×10^{-4} s^{-1}.

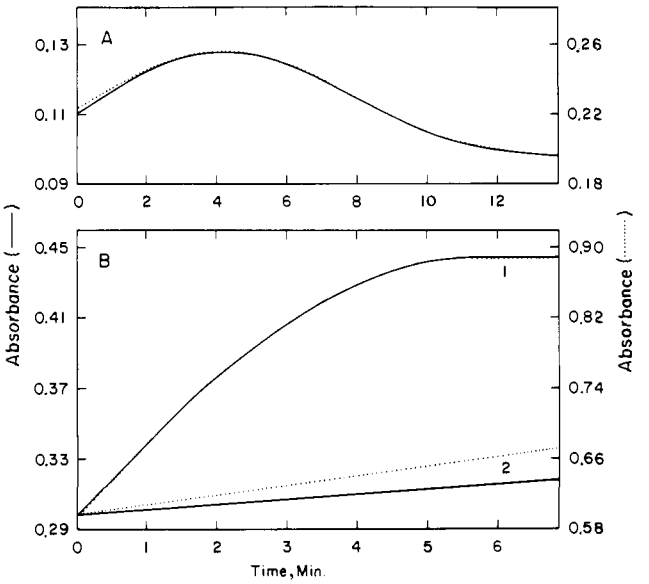

Figure 4. Progress curves for the reaction of hemoglobin with trioxodinitrate monoanion or nitrite at pH 7.0, 25 °C. The conditions and method of Figure 1 applied. A and B1 (solid lines), 50 μM Hb plus 125 μM $HN_2O_3^-$; A and B1 (dotted lines), 100 μM Hb plus 250 μM $HN_2O_3^-$; B_2 solid line, 50 μM Hb and 125 μM nitrite; B2 dotted line, 100 μM Hb and 250 μM nitrite. Reactions were followed at 627 nm (A) and 480 nm (B).

about 600, 526, and 484 nm. If there is an insufficient amount of **1**, Hb^+ (or Mb^+) formed in the first phase is not converted to HbNO (or MbNO). This can be seen in Figure 2 which represents a titration of Hb with **1**. The end point for the first phase, during which the Hb^+/HbNO product ratio appears from numerous experiments to be 1.2 ± 0.2, occurs at a **1**/Hb ratio of 0.5 ± 0.1. Hb is very nearly exhausted at this ratio. The end point for the second phase, in which Hb^+ is converted to HbNO, occurs by extrapolation at a **1**/Hb ratio of 1.1 ± 0.1. Very nearly 2 mol of Hb is consumed per mole of **1** in the first phase, whereas about 0.8 mol of Hb^+ is consumed per mole of **1** in the second.

Figure 3 is a comparison of the initial rates of Hb consumption as monitored at 480 nm (an isosbestic point for the $Hb^+ \rightarrow$ HbNO reaction) and the rates of spontaneous decomposition of **1** at the indicated concentrations, based on a first-order rate constant of 6.6×10^{-4} s^{-1}.[5,6] The ratio of the two slopes is 1.85 and confirms that the stoichiometry in the initial phase is Hb/**1** ≃ 2. Figure 3 also shows that the initial reaction is first-order in [**1**]. The second phase is approximately first-order in [**1**].[6] Figure 4 shows that a doubling of both [Hb] and [**1**] provides progress curves (Figure 4A and curves 1 of Figure 4B) that are superimposable throughout when compared on absorbance scales that differ by a factor of 2. This establishes that both phases of the reaction are zero-order in [Hb]. Progress curves for reactions in which only the [Hb] was changed (not shown) confirm that the initial phase is zero-order in [Hb].

Note in Figure 4B that the initial rate of reaction of Hb with nitrite is 15 times slower than that for the reaction of Hb with the same concentration of **1**. In addition, the reaction of Hb with nitrite is clearly first-order in [Hb] and $[NO_2^-]$.[12] Although the sample of Angeli's salt used in these experiments probably contained traces of $NaNO_2$,[6] it is clear that any contribution of nitrite to the kinetics of the reactions at hand must be small.

When reactions were run with 1 mM Hb (Table I), as opposed to 50–100 μM (Figures 1–4), the product mixture during the initial phase of reaction shifted in favor of HbNO. At a **1**/Hb ratio of 0.38, the Hb^+/HbNO product ratio was about 0.4 (Table I) rather than about 1. It is clear in Table I that production of both nitrite and N_2O are suppressed in the presence of Hb. Within the error of the method, all the nitrogen of **1** can be accounted for as nitrosyl N, nitrite, N_2O, and traces of free NO. If we take the reactant stoichiometry, **1**/Hb, as 0.5, the reaction of 760 nmol of **1** with 2000 nmol of Hb (Table I) could maximally generate 1520 nmol of Hb^+ plus HbNO. The amount realized is similar to this figure.

Discussion

The initial reaction between **1** and Hb results in the formation of Hb^+ and HbNO, with a stoichiometry of **1**/Hb ≃ 0.5. Hb^+ and HbNO are formed in about equal amounts at low concentrations (50–100 μM) of Hb, but HbNO can become the chief product at higher concentrations (1 mM). The initial reaction is first-order in [**1**] and zero-order in [Hb], and the rate of disappearance of Hb is almost twice the rate of disappearance of **1**. Similar results apply to Mb. The subsequent reaction between Hb^+ or Mb^+ and excess **1** proceeds as described in ref 6.

The initial reaction can be outlined by eq 4, wherein the reaction of Hb with an activated form of **1** or the primary breakdown products, **1***, is nearly quantitative relative to formation of the normal decomposition products.

$$\mathbf{1} \xrightarrow[6.6 \times 10^{-4}\ s^{-1}]{\text{r.d.s.}} \mathbf{1}^* \begin{cases} \xrightarrow{\text{minor}} \tfrac{1}{2}N_2O + NO_2^- \\ \xrightarrow[\text{major}]{Hb} Hb^+, \text{HbNO} \end{cases} \quad (4)$$

(12) Doyle, M. P.; Pickering, R. A.; DeWeert, T. M.; Hockstra, J. W.; Pater, D. *J. Biol. Chem.* **1981**, *256*, 12393–12398.

The simplest reactions consistent with most of the data are one- and two-electron reductions of **1*** by Hb. Consider a one-electron reaction following which HNO[6] and NO[13] are efficiently trapped (eq 5).

$$\mathrm{Hb} + \mathbf{1}^* + \mathrm{H}^+ \rightarrow \mathrm{Hb}^+ + \mathrm{HNO} + \mathrm{NO} + \mathrm{OH}^-$$
$$\mathrm{Hb}^+ + \mathrm{HNO} \rightarrow \mathrm{HbNO} + \mathrm{H}^+$$
$$\mathrm{Hb} + \mathrm{NO} \rightarrow \mathrm{HbNO}$$
$$\text{net:} \quad 2\mathrm{Hb} + \mathbf{1}^* \rightarrow 2\mathrm{HbNO} + \mathrm{OH}^- \qquad (5)$$

Consider a one-electron reduction following which NO is efficiently trapped[13] but HNO is not (eq 6).

$$\mathrm{Hb} + \mathbf{1}^* + \mathrm{H}^+ \rightarrow \mathrm{Hb}^+ + \mathrm{HNO} + \mathrm{NO} + \mathrm{OH}^-$$
$$\mathrm{Hb} + \mathrm{NO} \rightarrow \mathrm{HbNO}$$
$$\mathrm{HNO} \rightarrow {}^1/_2\mathrm{N_2O} + {}^1/_2\mathrm{HOH}$$
$$\text{net:} \quad 2\mathrm{Hb} + \mathbf{1}^* + \mathrm{H}^+ \rightarrow \mathrm{Hb}^+ + \mathrm{HbNO} + {}^1/_2\mathrm{N_2O} + {}^1/_2\mathrm{HOH} + \mathrm{OH}^- \qquad (6)$$

Consider a two-electron reduction following which HNO is efficiently trapped (eq 7).

$$2\mathrm{Hb} + \mathbf{1}^* + 2\mathrm{H}^+ \rightarrow 2\mathrm{Hb}^+ + 2\mathrm{HNO} + \mathrm{OH}^-$$
$$2\mathrm{Hb}^+ + 2\mathrm{HNO} \rightarrow 2\mathrm{HbNO} + 2\mathrm{H}^+$$
$$\text{net:} \quad 2\mathrm{Hb} + \mathbf{1}^* \rightarrow 2\mathrm{HbNO} + \mathrm{OH}^- \qquad (7)$$

Consider finally a two-electron reduction following which HNO is not trapped (eq 8).

$$2\mathrm{Hb} + \mathbf{1}^* + 2\mathrm{H}^+ \rightarrow 2\mathrm{Hb}^+ + 2\mathrm{HNO} + \mathrm{OH}^-$$
$$2\mathrm{HNO} \rightarrow \mathrm{N_2O} + \mathrm{H_2O}$$
$$\text{net:} \quad 2\mathrm{Hb} + \mathbf{1}^* + 2\mathrm{H}^+ \rightarrow 2\mathrm{Hb}^+ + \mathrm{N_2O} + \mathrm{H_2O} + \mathrm{OH}^- \qquad (8)$$

Equations 5–8 must be viewed with qualification. Although they are written as if HNO or HNO plus NO were the products of the initial redox step, other products could also arise and, indeed, the N–N bond of **1** need not necessarily have been broken at this point. Thus, the relevance of eq 5–8 lies in the overall reactions and not in the details of the individual steps.

At 1 mM Hb, the data are consistent with a situation approximating that for eq 5 or 7. HbNO is the chief product, and production of N_2O and nitrite would be strongly suppressed. At 50–100 μM Hb, the results are more closely consistent with eq 6 than with eq 8. Hb^+ and HbNO are produced in nearly equal amounts and, as indicated by Doyle and Mahapatro,[10] production of nitrite is suppressed. We suggest therefore that the chief reactions are the one-electron reactions of eq 5 and 6, which are expected to vary in relative importance depending on [Hb].

Although eq 5–8 may explain much of the data, they cannot provide a complete explanation. They fail to predict, on the one hand, the formation of a small amount of nitrite (Table I) over and above that due to nitrite contamination and breakdown of **1** into N_2O and nitrite and, on the other, an equivalent deficiency in the amount of N_2O produced. This apparent conversion of a N_2O precursor into nitrite may be explained by traces of oxyhemoglobin (HbO_2) which, if present at or below the 5% level, would not have been detected in air leakage controls. If in the experiment of Table I 5% of the Hb had been HbO_2 (200 ng-atom of O), the yields of N_2O and nitrite could have been decreased and increased, respectively, by as much as 200 ng-atom of N. This is the magnitude roughly of the nitrite excess and N_2O deficiency actually observed in Table I and related experiments.

We have no information on the identity of **1***. It could be a tautomer or protonation isomer of **1** or it could represent primary breakdown products, one member of which is a reactive oxidant. The product pairs HNO/NO_2^- or NO^-/HNO_2 are unlikely candidates. Although HNO_2 can probably oxidize Hb 10^3–10^4 times more rapidly than does the nitrite anion,[12] it is unlikely that oxidation of Hb by HNO_2 could compete successfully against deprotonation of HNO_2 at pH 7. Involvement of nitrite was obviated by Figure 4.

Our results agree with those of Doyle and Mahapatro[10] in several regards: The reaction is zero-order in [Hb] and first order in [**1**]. The rate-determining step in decomposition of $HN_2O_3^-$ is the same as that in the **1**-Hb reaction. The rate constant for Hb disappearance is about twice that for decomposition of **1**. The product ratio, $HbNO/Hb^+$, equals about 1 at low concentrations of reactants. Excess **1** will eventually produce HbNO as the sole product by reaction with Hb^+ as an intermediate product. Nitrite can have no significant kinetic role. There is apparent disagreement, however, chiefly on the question of stoichiometry. Whereas we observe a reactant stoichiometry, **1**/Hb, of about 0.5 at low reactant concentrations, a stoichiometry of 1 is clearly stipulated in eq 4 of ref 10 (eq 3 herein). At 10–20-fold greater reactant concentrations, we observe that the reactant stoichiometry remains about 0.5, but the product ratio, $HbNO/Hb^+$, can exceed 1, and in fact values in excess of 2.3 were realized. In addition, under those conditions, production of both nitrite and N_2O was suppressed, relative to the system lacking Hb and not nitrite alone. Because the reaction mechanism offered by Doyle and Mahapatro (eq 5–8 of ref 10) is designed to rationalize a reactant stoichiometry of 1 rather than 0.5 and the suppression of nitrite production without suppression of N_2O production, that mechanism stands in question.

Acknowledgment. We thank Francis T. Bonner for helpful discussions and encouragement. This work was supported by a grant from the National Science Foundation (PCM 82-18000) and Biomedical Research Support Grant S07 RR 07044 from the National Institutes of Health.

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