THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 256, No. 23, Issue of December 10, pp. 12393-12398, 1981
Printed in U.S.A.

Kinetics and Mechanism of the Oxidation of Human Deoxyhemoglobin by Nitrites*

(Received for publication, April 9, 1981)

Michael P. Doyle‡, Ruth A. Pickering, Thomas M. DeWeert, James W. Hoekstra, and David Pater
From the Department of Chemistry, Hope College, Holland, Michigan 49423

The reactions of human hemoglobin with sodium nitrite and ethyl nitrite in deoxygenated media have been examined in kinetic detail. Nitrous acid has been identified from kinetic pH dependence studies as the principal oxidant of hemoglobin in reactions with sodium nitrite. Nitrosylhemoglobin is produced concurrently with methemoglobin as a result of reductive release of nitric oxide from nitrous acid. However, oxidation of hemoglobin by nitric oxide competes with association, and this process is proposed to arise from the action of the nitric oxide dimer on hemoglobin. Ethyl nitrite, which serves as a model for nitrous acid, reacts with hemoglobin at rates that are at least 10 times slower than those extrapolated for nitrous acid, and hydrolysis of the alkyl nitrite is not competitive with oxidation of hemoglobin. The composite experimental results are interpreted to describe alkyl nitrite, and, presumably, nitrous acid association with hemoglobin followed by rate-limiting electron transfer resulting in nitric oxide and alkoxide (or hydroxide) production. Proton transfer resulting in alcohol (or water) formation occurs subsequent to the rate-limiting step as do reactions of hemoglobin with nitric oxide.

The capability of sodium nitrite to oxidize hemoglobin and related hemoproteins has been recognized for more than 100 years (1). Nitrites are classified as direct causative agents for methemoglobinemia (2, 3) and lethal intoxications have been diagnosed, particularly in infants (4-6). However, despite the impressive amount of experimentation that has been accorded this complex transformation (2, 7-9), the nature of the oxidative process is not well understood.

In early studies, both methemoglobin and nitrosylhemoglobin were reported as products of the action of nitrite on oxyhemoglobin (7, 8); however, nitrosylhemoglobin has not been described in more recent studies (9), and the cause of its prior detection has not been explained. Furthermore, when hemoglobin solutions are deoxygenated, the formation of methemoglobin has been reported to proceed slowly, if at all (2), but the actual effect of nitrite on deoxyhemoglobin remains obscured by the absence of critical experimental detail for this transformation. In addition, inositol hexaphosphate (IHP) inhibits the rate of oxidation by nitrite (10, 11), which contrasts with the rate acceleration by inositol hexaphosphate that is observed in oxidations of hemoglobin by ferricyanide, hydroxylamine, chlorate, hydrogen peroxide, quinones, and in autooxidation (11). Since inositol hexaphosphate and related organic phosphates affect the oxygen-binding capability of hemoglobin (12-14), inhibition of hemoglobin oxidation in the presence of inositol hexaphosphate has been interpreted as a direct indication that nitrite oxidations of hemoglobin are favored in the state of hemoglobin having high oxygen affinity (10, 11). Thus, although nitrites have often been classified with a variety of substances whose oxidative action on hemoglobin is better understood, this classification is arbitrary and nitrite appears to be a complex, but unique, oxidant.

The complexity of nitrite oxidations may be considered to be a consequence of many factors, including divergent mechanistic pathways for oxidations of oxyhemoglobin and deoxyhemoglobin by nitrite ion or nitrous acid. Clearly, compartmentalization of these factors should allow a more precise understanding of the mechanism of nitrite oxidations. Furthermore, the action of alkyl nitrites on hemoglobin, although obscured by considerations that these esters rapidly hydrolyze to their constituent alcohols and nitrite ion (15, 16), should serve to portray the characteristic effects of nitrous acid. In order to assess the nature of hemoglobin oxidation by sodium nitrite, the reactions of deoxyhemoglobin with sodium nitrite and a representative nitrite ester, ethyl nitrite, have been investigated for the first time in kinetic detail. Since oxygen dependence is not a factor in these reactions, an understanding of the action of nitrites on deoxyhemoglobin should provide a basis for the mechanistic assessment of the complex nitrite oxidations of oxyhemoglobin.

MATERIALS AND METHODS

Human hemoglobin A (type IV), obtained from Sigma Chemical Co., was reduced with excess sodium dithionite and further purified by passing the resulting aqueous solution through a G-25 Sephadex column using 0.05 M phosphate buffer. Concentrated solutions of oxyhemoglobin (approximately 1.0 mM heme) were degassed under reduced pressure (less than 0.5 torr), and the resultant deoxyhemoglobin was maintained in a sealed air-tight flask at atmospheric pressure under nitrogen. Reagent grade sodium nitrite was employed. Ethyl and isopentyl nitrite was prepared by standard methods (17) and purified by distillation. Nitric oxide (99%), obtained from Union Carbide Corp., Linde Division, was purified by passing the gas over sodium hydroxide pellets to remove trace quantities of the higher oxides of nitrogen.

Phosphate buffers for the pH range of 6.0-8.0 were individually prepared from reagent grade chemicals, deoxygenated under reduced pressure (less than 0.5 torr) prior to their use, and maintained in sealed air-tight flasks at atmospheric pressure under nitrogen. Stock solutions of sodium nitrite and nitric oxide solutions were prepared in the appropriate deoxygenated buffer. Heme concentrations were calculated from the extinction coefficients of Banerjee *et al.* (18). Hemoglobin, ethyl nitrite, and nitric oxide solutions were prepared immediately prior to their use for product or kinetic determinations.

Deoxygenated stock solutions of isopentyl nitrite in absolute ethanol were employed to produce ethyl nitrite for kinetic investigations, but the total amount of ethanol added to the reaction solution never exceeded 1%. Quantitative exchange of the nitrosyl group was observed within 2 min following mixing of isopentyl nitrite in ethanol, and spectral analyses validated the complete conversion of isopentyl

* This research was supported by United States Public Health Service Grant ES 01673. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom all reprint requests should be sent.

nitrite to ethyl nitrite. Ethanol compositions up to 5% of the reaction solution volume did not affect reaction rates or product distributions.

Kinetic Measurements and Analysis—Reactions were initiated with the injection, using a gas-tight syringe, of a concentrated nitrite solution into the deoxyhemglobin (usually 20–50 μM) contained in 0.05 M phosphate buffer. Rates for hemoglobin reactions with sodium nitrite were determined at 25.0 °C, and those with ethyl nitrite were determined at 10.0 °C by monitoring the decrease in absorbance at 552 nm with time using either a Pye Unicam SP8-200 or a GCA-McPherson EU-707D spectrophotometer. In all kinetic experiments, reactions were carried out under pseudo-first order conditions where nitrite concentrations were in 5- to 25-fold excess with respect to the total heme groups present. The resultant time courses were fitted to an integrated single exponential process from which the pseudo-first order rate constants were calculated. Typically, from 3–12 replicate time courses were obtained for each kinetic determination, and the averaged rate constants are reported.

Product Identifications—The UV-vis spectra of the products formed in reactions of deoxyhemoglobin with either sodium nitrite or ethyl nitrite were determined to be exact composites of the spectra of methemoglobin and nitrosylhemoglobin. Furthermore, addition of dithionite to the product mixture produced a mixture of deoxyhemoglobin and nitrosylhemoglobin which had, within experimental error, the same composition of nitrosylhemoglobin as had been determined to exist in the methemoglobin-nitrosylhemoglobin reaction mixture. Nitrosylhemoglobin was slowly converted to deoxyhemoglobin in the presence of dithionite (19), which required that these measurements be taken immediately following dithionite addition. The compositions of nitrosylhemoglobin and methemoglobin were calculated from the product absorbances observed at 542, 552, and 572 nm in each experiment, and the resulting composition determinations were averaged to obtain the reported values. Deviations in per cent HbNO from calculations at these three wavelengths were generally less than 1%, and deviations in per cent HbNO between individual reaction determinations amounted to ±2%.

Hydrolysis of Ethyl Nitrite—The rate of hydrolysis of ethyl nitrite was determined at 10.0 °C by monitoring the decrease in absorbance at 378 nm with time using a Pye Unicam SP8-200 spectrophotometer. Reactions were initiated with the injection of a freshly prepared alcohol solution of the nitrite ester into a 0.05 M phosphate-buffered solution (pH 7.0). First order kinetics was observed through greater than two half-lives over the concentration range of $2.3–12.5 \times 10^{-4}$ M ethyl nitrite in the buffered aqueous solutions that contained less than 2% ethanol, and the rate constant for hydrolysis of ethyl nitrite under these conditions was 0.87×10^{-4} s^{-1}.

RESULTS AND DISCUSSION

Reactions with Sodium Nitrite—Treatment of deoxyhemoglobin A with sodium nitrite produces a mixture composed of methemoglobin and nitrosylhemoglobin. Observation of both MetHb[1] and HbNO has previously been reported for reactions between hemoglobin and sodium nitrite (7, 8) even though, in view of the exceptional oxidative capability of nitric oxide toward bound dioxygen in hemoproteins (20), nitrosylhemoglobin should not have been formed from reactions performed in an aerobic environment. We are left to speculate that reducing agents such as dithionite were present in the hemoglobin samples employed for those early studies. Such reagents react readily with nitrites to form nitric oxide (7, 21), and their action explains the experimental discrepancies that characterize early studies of nitrite oxidations of hemoglobin.

The appearance of nitrosylhemoglobin is consistent with the known chemical behavior of sodium nitrite toward iron(II) in acidic media under an inert atmosphere (22). Nitrous acid causes the oxidation of iron in the ferrous state to the ferric ion with concomitant formation of nitric oxide. Thus, the production of nitrosylhemoglobin is consistent with initial oxidation of deoxyhemoglobin by the nitrite reagent to form nitric oxide which subsequently associates with deoxyhemoglobin. However, this analysis provides the prediction that equal amounts of MetHb and HbNO should be produced since association of Hb with NO is near diffusion controlled (23), when experimentally 72% MetHb and 28% HbNO are actually observed at pH 7.0. Both MetHb and HbNO are stable toward nitrite ion under the reaction conditions employed for this investigation.

Fig. 1 describes the spectral behavior observed in the transformation of Hb to MetHb and HbNO and demonstrates the uniformity of the production of these components. Isosbestic points are observed at 602, 526, and 452 nm throughout the time course of this transformation. Consequently, production of HbNO is directly related to the formation of MetHb, and the molar ratio of these two products remains constant as the reaction progresses to completion.

The rates for reactions of deoxyhemoglobin with sodium nitrite have been investigated as a function of time at 25 °C and pH 7.0. Typical time courses for these reactions at different molar ratios of $[NaNO_2]/[Hb]$ are presented in Fig. 2. Pseudo-first order kinetics was observed for these reactions through greater than 80% of their time course, which established the direct first order relationship of the rate of reaction with the concentration of hemoglobin. Furthermore, the pseudo-first order rate constants obtained for reactions performed as a function of $[NaNO_2]$ describe a first order dependence of the rate on the nitrite concentration. Therefore, the reaction of deoxyhemoglobin with sodium nitrite follows a second order rate law with a second order rate constant, k_{obs}, of 2.69 $M^{-1}s^{-1}$ at 25 °C and pH 7.0.

The relatively simple kinetic behavior of this transformation contrasts with the complex autocatalytic kinetics observed for sodium nitrite oxidations of hemoglobin in oxygen-saturated media (9) and with the biphasic character of rate plots for the autooxidation of hemoglobin (24). The time courses for the reactions of nitrite with Hb appear distinctly

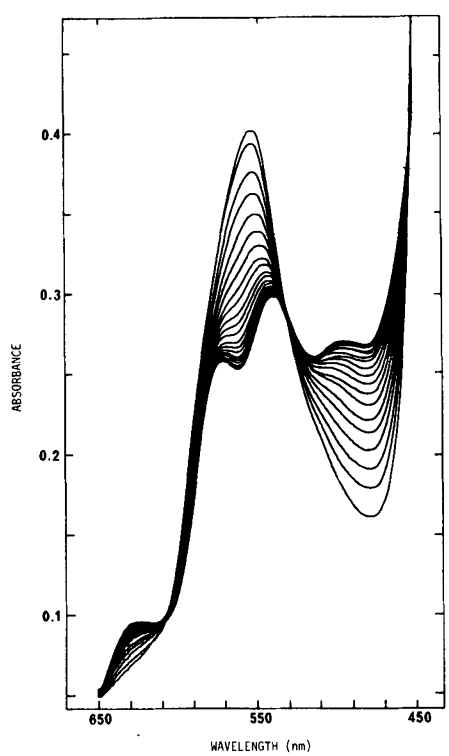

FIG. 1. **Spectral time course for the reaction of hemoglobin A with sodium nitrite.** Reaction was performed on the deoxygenated solution in 0.05 M phosphate buffer, pH 7.0, 25.0 °C: 2.92×10^{-5} M (heme), 2.86×10^{-4} M $NaNO_2$. Spectra were recorded at 4.00-min intervals. The initial spectrum exhibits a maximum at 560 nm and a corresponding minimum at 476 nm.

[1] The abbreviation used is: MetHb, methemoglobin.

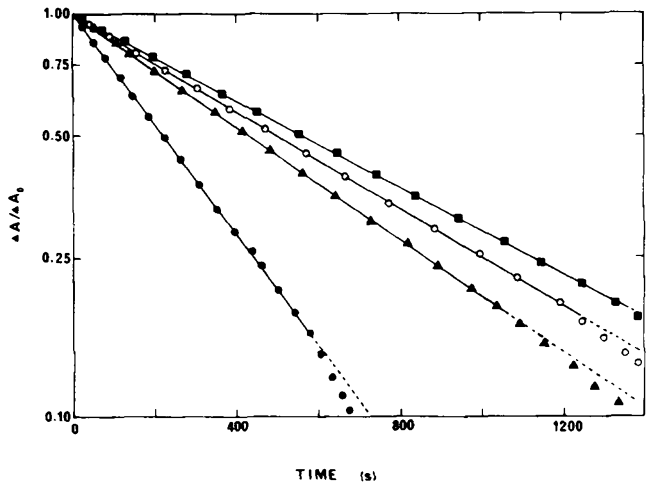

FIG. 2. **Typical time courses for reactions of deoxyhemoglobin with sodium nitrite.** Reactions were observed at 552 nm on deoxygenated solutions in 0.05 M phosphate buffer, pH 7.0, 25.0 °C. ■, 3.4×10^{-5} M (heme), 5.0×10^{-4} M $NaNO_2$; ○, 5.8×10^{-5} M (heme), 5.2×10^{-4} M $NaNO_2$; ▲, 3.7×10^{-5} M (heme), 5.8×10^{-4} M $NaNO_2$; ●, 5.1×10^{-5} M (heme), 12.7×10^{-4} M $NaNO_2$. When plotted against sodium nitrite concentration, the reaction rate constants from such time courses extending from $[NaNO_2]/[Hb]$ molar ratios of 5–25 describe a linear first order dependence on the concentration of nitrite.

monophasic, and their second order rate dependence is consistent with electron transfer from iron in the ferrous state to nitrite or nitrous acid. That deviations from linearity often occur after 80% of the time course of the reaction can be readily understood to result from coordination of the product methemoglobin with nitrite ion (9) and minor disruptions in the relative rates for formation of HbNO and MetHb.

In order to assess the potential for the involvement of nitrous acid in these reactions, the pH dependence of the second order rate constant, k_{obs}, was determined. Nitrous acid is a relatively weak acid, $pK_a = 3.148$ at 25 °C (25), which at a solution pH of 7.0 exists almost entirely as the nitrite ion ($[NO_2^-]/[HONO] = 9500$). In the pH range between 6 and 8, nitrite is not known to react with amino acids or with porphyrins and hemes (26, 27) and, consequently, this pH region was chosen for kinetic investigation. The results of this study, presented in Fig. 3, describe a reaction process that is linearly dependent on the hydrogen ion concentration in the pH range of 6.0–8.0. The slope of the line, which is the order of rate dependence on $[H^+]$, is 0.88. The deviation of this value from the ideal 1.0, which would have corresponded to a first order dependence of the rate on $[H^+]$ and thus implicated nitrous acid as the probable sole reactant in nitrite oxidations of deoxyhemoglobin, may reflect conformational changes in hemoglobin. In correspondence with this explanation, the reaction rates for ligand replacement on human adult hemoglobin are recognized to be pH dependent (28) although no simple relationship between these rates and pH exists. However, an alternate explanation that nitrite ion itself is capable of oxidizing hemoglobin cannot be dismissed.

The composite experimental results are consistent with a mechanism for nitrite activity that principally involves rate-limiting oxidative interaction of nitrous acid with hemoglobin to produce nitric oxide and methemoglobin, followed by rapid association of nitric oxide with hemoglobin:

$$H^+ + NO_2^- \overset{K}{\rightleftharpoons} HONO \quad (1)$$

$$Hb + HONO \xrightarrow{k_o} MetHb + NO + OH^- \quad (2)$$

$$Hb + NO \xrightarrow{k_a} HbNO \quad (3)$$

$$Hb + NO_2^- \xrightarrow{k'} MetHb + \text{"}NO_2^{2-}\text{"} \quad (4)$$

The nitrite reduction product formulated in equation 4 is anticipated to form nitric oxide and water in a subsequent reaction. In the absence of any other process involving nitrite or nitric oxide, the rate equation predicted by this mechanism is the two-term equation

$$\frac{-d[Hb]}{dt} = [Hb][NO_2^-]\left[\left(\frac{k_o[H^+]}{K_a + [H^+]}\right) + k'\right] \quad (5)$$

where $k_o = 12.3 \times 10^3$ $M^{-1}s^{-1}$ and $k' = 0.10$ $M^{-1}s^{-1}$. However, this mechanism predicts that oxidation of deoxyhemoglobin will produce equivalent amounts of MetHb and HbNO, and this is not observed. Instead, a relatively constant but not equivalent ratio of these two products is formed, and the relative rates for their formation are relatively independent of the solution pH. Fig. 4 describes the per cent yield of HbNO formed over the pH range employed for the kinetic study. A relatively insignificant change in the percentage of MetHb is observed as the pH is changed from 8.0 to 6.0.

The cause of the enhanced yield of methemoglobin beyond the expected equivalency with HbNO may be attributed either to oxidation of hemoglobin by nitric oxide or to the formation of nitrous oxide from nitric oxide by a process that does not directly involve methemoglobin production. Hemoglobin is known to undergo oxidation in the presence of low concentrations of nitric oxide (29). In addition, the production of nitrous oxide has been reported from reactions of nitrous acid with ferrous ion (22), and we speculate that this product is probably formed through oxidative oxygen transfer (30) from the nitric oxide dimer.

Reactions with Nitric Oxide—Oxidation of deoxyhemoglobin by nitric oxide is disconcerting in view of the relative ease

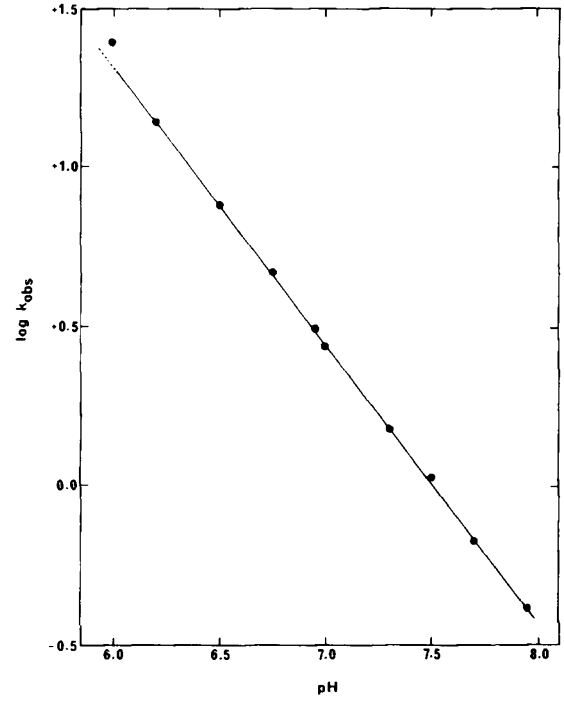

FIG. 3. **Dependence of the rates for sodium nitrite reactions with hemoglobin on solution pH.** All reactions were observed at 552 nm on deoxygenated solutions in 0.05 M phosphate buffer at 25.0 °C. Deviation of the point at pH 6.0 from linearity is paralleled by similar behavior in reactions of ethyl nitrite with hemoglobin (Fig. 6).

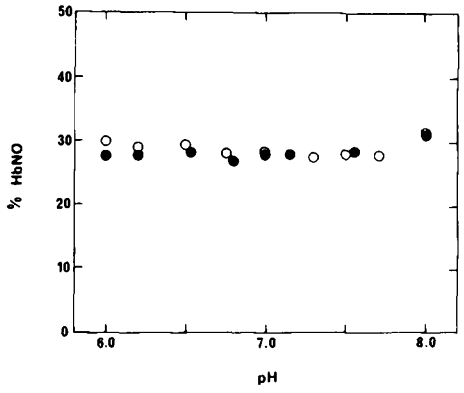

FIG. 4. **Percentage of total hemoglobin converted to nitrosylhemoglobin.** ○, Reaction of deoxyhemoglobin with sodium nitrite at 25 °C under conditions defined in Fig. 3; ●, reaction of deoxyhemoglobin with ethyl nitrite at 10 °C under conditions defined in Fig. 6. The resultant hemoglobin product is methemoglobin.

with which nitric oxide associates with hemoglobin to form the stable nitrosylhemoglobin complex. The rates for the exceptionally rapid nitric oxide association have been established, and the absence of cooperativity in association with the tetrameric hemoglobin has been reported (23). In contrast, the oxidation of hemoglobin by nitric oxide is rarely reported and, consequently, the defining features of this process remain undetermined. We have, therefore, examined the products formed after treatment of deoxyhemoglobin with nitric oxide under various conditions in order to quantitate the extent to which oxidation occurs.

When nitric oxide dissolved in aqueous deoxygenated phosphate buffer is injected directly into a deoxygenated solution of hemoglobin in the phosphate buffer at pH 7.0, HbNO and MetHb are observed in a molar ratio of 86:14 when a 1:1 molar ratio based on NO/heme iron is employed. Nearly identical results are obtained when nitric oxide is injected into the nitrogen atmosphere directly above the solution containing an equivalent amount of deoxyhemoglobin and the system is immediately thoroughly mixed (HbNO/MetHb = 87:13). However, if nitric oxide is allowed to slowly diffuse into the undisturbed aqueous medium containing deoxyhemoglobin, substantially greater amounts of MetHb are produced (up to 50% of initial Hb), and similar results are obtained for NO/heme iron molar ratios of between 1:1 and 10:1. Since nitric oxide was rigorously purified to remove trace quantities of the higher oxides of nitrogen and reproducible results were obtained for reactions between deoxyhemoglobin and nitric oxide, oxygen contamination cannot be regarded as the cause of these oxidations.

That monomeric nitric oxide is solely responsible for both association and oxidation upon exposure of hemoglobin to equivalent amounts of nitric oxide is inconsistent with the behavior of Hb toward NO under different conditions of mixing. Furthermore, transfer of nitric oxide to hemoglobin by cobalt nitrosyls yields only HbNO without any observable evidence for the production of MetHb (31) even when stoichiometric amounts of the cobalt nitrosyl transfer reagent are employed. These observations suggest that a nitric oxide oxidant other than monomeric NO is responsible for the oxidation of Hb to MetHb, and we propose that the dimeric form of nitric oxide $(NO)_2$ is that oxidant:

$$2NO \rightleftharpoons (NO)_2 \quad (6)$$

$$2H^+ + (NO)_2 + 2Hb \rightarrow 2MetHb + N_2O + H_2O \quad (7)$$

The compositions of gaseous products from reactions of soluble ferrous salts with nitrous acid in aqueous acidic media with and without solution agitation (22) correspond to those predicted by the above equation to have been formed from the oxidations of hemoglobin. The absence of a pH effect on the formation of HbNO and corresponding MetHb in reactions of hemoglobin with the nitrite ion (Fig. 4) is also consistent with this interpretation.

An alternate explanation for the enhanced production of MetHb in reactions of Hb with nitrite ion, that nitric oxide is converted to nitrous oxide independent of hemoglobin oxidation, is inconsistent with the kinetic and product data that have been obtained. Such loss of nitric oxide would be dependent on the rate of nitric oxide production, and neither the effect of pH on per cent MetHb nor the kinetic results obtained at widely differing concentrations of hemoglobin or nitrite ion supports this explanation.

The physical and structural properties of dimeric nitric oxide have been established (32–34). Although it is a minor constituent of nitric oxide composition in the gas phase, $(NO)_2$ is the dominant species in the liquid and solid phases. The formulation of transition metal dinitrosyl complexes as dinitrogen dioxide complexes for their reactions with carbon monoxide and nitric oxide has been proposed (35), and numerous examples of oxidative transformations involving electron transfer to coordinated $(NO)_2$ are now known (36). However, since the composition of nitric oxide in aqueous solutions has not been determined, only indirect evidence can be drawn from this study for the involvement of $(NO)_2$ in reactions with deoxyhemoglobin.

The proposed involvement of $(NO)_2$ in these reactions implies that dimeric association of nitric oxide is near diffusion controlled and that the rate for oxidation of Hb by $(NO)_2$ is competitive with the rate for association of Hb with NO. Oxidation of deoxyhemoglobin by $(NO)_2$ explains the normal oxidative instability of HbNO, and interaction of $(NO)_2$ with MetHb could be responsible for the relative ease by which this iron(III) metalloprotein is converted to HbNO (37). Further investigations of the nature of nitric oxide in solution, particularly as they relate to processes involving metalloproteins, are clearly warranted.

Reactions with Ethyl Nitrite—Alkyl nitrites have traditionally been considered to be hydrolytic sources of the nitrite ion, and their activities have thus been attributed to the nitrite ion and constituent alcohol (38). Similarly, drug nitrates such as glyceryl trinitrate and erythrityl tetranitrate are presumed to be reduced in the body to their corresponding nitrites which then rapidly hydrolyze to furnish nitrite ion (15, 16). However, the rates of hydrolysis for alkyl nitrites under physiological conditions have not been determined and, consequently, experimental evidence to support this hydrolytic conjecture is not evident.

We have presumed that, to a first approximation, alkyl nitrites would represent ideal models for nitrous acid in reactions with hemoglobin. Unlike nitrous acid, which as a weak acid is subject to the concentration constraints of its acid dissociation equilibrium, the action of alkyl nitrites should be direct and related to the actual concentration of these nitrite reagents. Since results obtained for sodium nitrite oxidations of deoxyhemoglobin strongly suggested nitrous acid to be the principal oxidant of hemoglobin, alkyl nitrites were anticipated to exhibit exceptional oxidative activity toward hemoglobin.

Treatment of deoxyhemoglobin A with ethyl nitrite produces a mixture composed of MetHb and HbNO. The per cent compositions of MetHb exhibited a marked independence on the molar ratio of ethyl nitrite to deoxyhemoglobin (72 ± 2% MetHb for [ethyl nitrite]/[Hb] ratios between 1.0 and 10.0), which is consistent with the previously advanced mech-

anism for the action of nitrous acid on hemoglobin. Only when the alkyl nitrite concentration exceeded the heme concentration by factors greater than 10 did the MetHb yield increase from this limiting value. At [ethyl nitrite]/[Hb] equal to 100, for example, MetHb accounted for 80% of the product heme. Such an increase in the relative yield of MetHb is indicative of alkyl nitrite association with deoxyhemoglobin that inhibits competitive nitric oxide association or oxidation of Hb by the dimer of nitric oxide. If, as might be anticipated from the known coordination of nitrosobenzene to Hb (39), reversible alkyl nitrite association with hemoglobin precedes oxidation, the approximate value of the association equilibrium constant calculated from the product data for ethyl nitrite at 10 °C is 4×10^{-3} M^{-1}.

The rates for reactions of deoxyhemoglobin with ethyl nitrite have been investigated as a function of time at 10 °C and pH 7.0. Typical time courses for these reactions at different molar ratios of [ethyl nitrite]/[Hb] are presented in Fig. 5. Pseudo-first order kinetics was observed for these reactions through greater than 95% of their time course, which established the direct first order relationship of the rate of reaction with the concentration of hemoglobin. In addition, the pseudo-first order rate constants obtained for reactions performed as a function of [ethyl nitrite] describe a first order dependence of the rate on the alkyl nitrite concentration. Thus, as was expected from similar observations with sodium nitrite, the reaction of deoxyhemoglobin with ethyl nitrite follows a second order rate law and is characterized by a second order rate constant, k_{obs}, of 11.2×10^2 M^{-1}s^{-1} at 10 °C and pH 7.0.

The rate for hydrolysis of ethyl nitrite was determined from separate experiments performed under reaction conditions similar to those employed for reactions with hemoglobin (pH 7.0, 10 °C) to be first order in ethyl nitrite. The half-life for hydrolysis was 2.2 h which corresponded to a 1500-fold slower rate for hydrolysis than that observed for reactions with hemoglobin at comparable nitrite concentrations when the molar ratio of ethyl nitrite to hemoglobin was 10.0. Since the rate of reaction of hemoglobin with ethyl nitrite is at least 400 times faster than that with sodium nitrite at pH 7.0 and the rate of reaction of ethyl nitrite with hemoglobin is more than

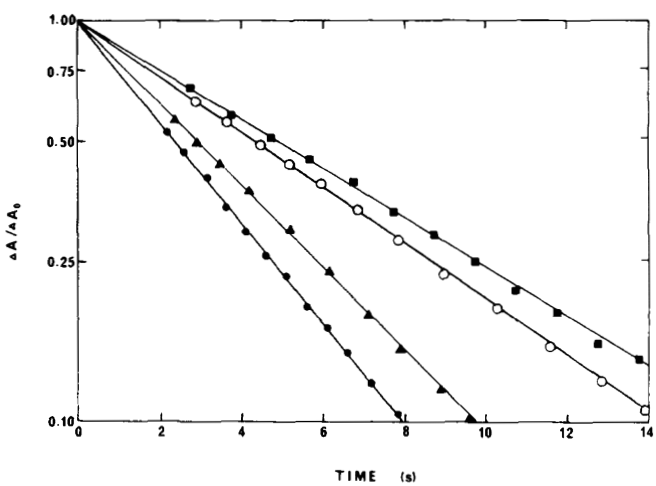

FIG. 5. **Typical time courses for reactions of deoxyhemoglobin with ethyl nitrite.** Reactions were observed at 552 nm on deoxygenated solutions in 0.05 M phosphate buffer, pH 7.0, 10.0 °C. ■, 2.5×10^{-5} M (heme), 1.3×10^{-4} M ethyl nitrite; ○, 2.8×10^{-5} M (heme), 1.5×10^{-4} M ethyl nitrite; ▲, 2.0×10^{-5} M (heme), 1.9×10^{-4} M ethyl nitrite; ●, 2.5×10^{-5} M (heme), 2.6×10^{-4} M ethyl nitrite. When plotted against ethyl nitrite concentration, the reaction rate constants from such time courses extending from [ethyl nitrite]/[Hb] molar ratios of 5–10 describe a linear first order dependence on the concentration of alkyl nitrite.

1500 times that of its hydrolysis, the results observed for reactions of hemoglobin with ethyl nitrite can only be attributed to ethyl nitrite. The hydrolysis of ethyl nitrite is negligible and its hydrolytic product, sodium nitrite, does not contribute to its activity toward hemoglobin.

The oxidative action of nitrites on hemoglobin can be considered to occur by either of two pathways. Reaction of hemoglobin in the ferrous state can be expected to produce MetHb, nitric oxide, and alkoxide (or hydroxide), followed by subsequent rapid proton transfer:

$$\text{RONO} + \text{Fe(II)} \rightarrow \text{Fe(III)} + \text{NO} + \text{RO}^- \quad (8)$$

$$\text{RO}^- + \text{H}^+ \rightarrow \text{ROH} \quad (9)$$

Alternatively, protonation of the alkyl nitrite (or nitrous acid) could be anticipated to occur prior to its interaction with hemoglobin:

$$\text{RONO} + \text{H}^+ \rightleftharpoons \overset{+}{\underset{\text{H}}{\text{RONO}}} \quad (10)$$

$$\overset{+}{\underset{\text{H}}{\text{RONO}}} + \text{Fe(II)} \rightarrow \text{Fe(III)} + \text{NO} + \text{ROH} \quad (11)$$

Although, by analogy, the rate dependence on pH for the reaction of hemoglobin with sodium nitrite appears to indicate only first order rate dependence on the hydrogen ion concentration and, thus, suggests the former pathway, the first order rate dependence could be expected to mask contributions from the latter pathway. This would, of course, be particularly true if the oxidative transformation involved proton transfer from an internal proton donor located near the site of electron transfer.

The pH dependence of the rates for reaction of ethyl nitrite with deoxyhemoglobin was determined over the pH range of 6.0–7.5. Erratic results were obtained at pH values between 7.6 and 8.0 and, since alkyl nitrite hydrolysis is accelerated in basic media, the extension of this pH profile to pH 8.0 was not further pursued. The results of this study, presented in Fig. 6, describe little or no pH dependence on the rate of oxidation by ethyl nitrite, which indicates the reaction pathway described by equations 8 and 9.

As could now be anticipated from prior discussions of the product distributions from nitrite reactions with hemoglobin, the per cent yield of HbNO from reactions of ethyl nitrite with Hb is relatively independent of pH (Fig. 4) and corresponds to similar data obtained for the sodium nitrite reac-

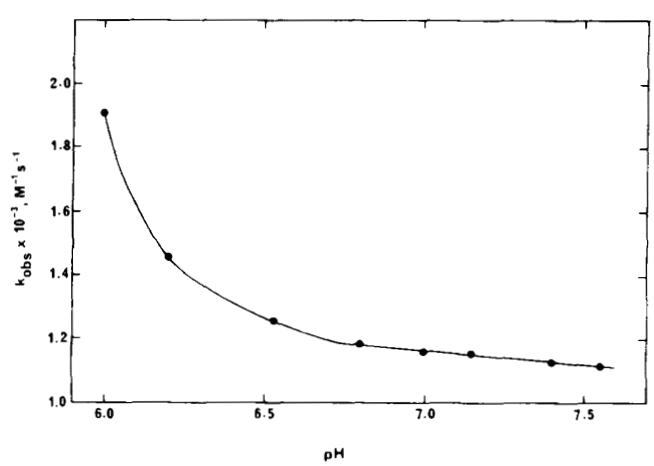

FIG. 6. **Dependence of the rates for ethyl nitrite reactions with hemoglobin on solution pH.** All reactions were observed at 552 nm on deoxygenated solutions in 0.05 M phosphate buffer at 10.0 °C.

tions. Thus, product distributions are apparently independent of the rates for nitric oxide formation and suggest that nitric oxide dimer formation may indeed be an exceedingly rapid process.

The composite experimental results obtained for nitrite oxidations of Hb describe a series of molecular events commencing with alkyl nitrite, and, presumably, nitrous acid association with hemoglobin followed by rate-limiting electron transfer resulting in nitric oxide and alkoxide (or hydroxide) production. Nitric oxide is proposed to exist in equilibrium with its dimer, and oxidation of Hb by $(NO)_2$ is competitive with nitric oxide association with hemoglobin:

$$Hb + RONO \rightleftharpoons Hb(RONO) \quad (12)$$

$$Hb(RONO) \rightarrow MetHb + NO + RO^- \quad (13)$$

$$2NO \rightleftharpoons (NO)_2 \quad (14)$$

$$NO + Hb \rightarrow HbNO \quad (15)$$

$$(NO)_2 + 2Hb + 2H^+ \rightarrow 2MetHb + N_2O + H_2O \quad (16)$$

$$RO^- + H^+ \rightarrow ROH \quad (17)$$

From the extrapolated data obtained for nitrite oxidations, nitrous acid is at least 10 times more reactive than ethyl nitrite toward hemoglobin, which is indicative of the steric barrier for association (40) as well as electronic influences from the nitrite. Although the results that we have obtained do not directly portray the chemical nature of the electron transfer process, intimate association of iron(II) with the nitrosyl nitrogen of the alkyl nitrite or nitrous acid offers a distinctively attractive pathway for electron transfer from iron(II) to the nitrosyl group. The minor role played by the nitrite anion, as opposed to nitrous acid, in oxidations by sodium nitrite suggests that an alternative pathway exists for nitrite oxidations, but this pathway becomes important only in solutions of pH greater than 8.0.

REFERENCES

1. Gamgee, A. (1868) *Phil. Trans. Royal Soc. London* **158,** 589-592
2. Kiese, M. (1966) *Pharmacol. Rev.* **18,** 1091-1161
3. Swann, P. F. (1975) *J. Sci. Fd. Agric.* **26,** 1761-1770
4. Bartos, H. R., Desforges, J. F., and Clark, N. (1966) *Pediatrics* **37,** 991-993
5. Bodansky, O. (1951) *Pharmacol. Rev.* **3,** 144-196
6. Sherman, J. M., and Smith, K. (1979) *Am. J. Dis. Child* **133,** 439
7. Marshall, W., and Marshall, C. R. (1945) *J. Biol. Chem.* **158,** 187-208
8. Austin, J. H., and Drabkin, D. L. (1935) *J. Biol. Chem.* **112,** 67-88
9. Rodkey, F. L. (1976) *Clin. Chem.* **22,** 1986-1990
10. Tomoda, A., Matsukawa, S., Takeshita, M., and Yoneyama, Y. (1977) *Biochem. Biophys. Res. Commun.* **74,** 1469-1474
11. Tomoda, A., and Yoneyama, Y. (1979) *Experientia* **35,** 15-16
12. Perutz, M. F., Ladner, J. E., Simon, S. R., and Ho, C. (1974) *Biochemistry* **13,** 2163-2173
13. Cassoly, R. (1975) *J. Mol. Biol.* **98,** 581-595
14. Olson, J. S., and Gibson, Q. H. (1972) *J. Biol. Chem.* **247,** 3662-3670
15. Heppel, L. A., and Hilmoe, R. J. (1950) *J. Biol. Chem.* **183,** 129-138
16. DiCarlo, F. J., and Melgar, M. D. (1970) *Biochem. Pharmacol.* **19,** 1371-1379
17. Noyes, W. A. (1943) in *Organic Syntheses*, Collective Vol. II, pp. 108-109, Wiley, New York
18. Banerjee, R., Alpert, Y., Leterrier, F., and Williams, R. J. P. (1969) *Biochemistry* **8,** 2862-2867
19. Moore, E. G., and Gibson, Q. H. (1976) *J. Biol. Chem.* **251,** 2788-2794
20. Doyle, M. P., and Hoekstra, J. W. (1981) *J. Inorg. Biochem.* **14,** 351-358
21. Mulvey, D., and Waters, W. A. (1974) *J. Chem. Soc., Perkin II* 772-773
22. Chalamet, A. (1973) *Ann. Chim.* **8,** 353-358
23. Cassoly, R., and Gibson, Q. H. (1975) *J. Mol. Biol.* **91,** 301-313
24. Mansouri, A., and Winterhalter, K. H. (1974) *Biochemistry* **13,** 3311-3314
25. Lumme, P., Lahermo, P., and Tummavuori, J. (1965) *Acta Chem. Scand.* **19,** 2175-2188
26. Bonnett, R., Charalambides, A. A., Martin, R. A., Sales, K. D., and Fitzsimmons, B. W. (1975) *J. Chem. Soc. Chem. Commun.* 884-885
27. Bonnett, R., and Nicolaidow, P. (1977) *Heterocycles* **7,** 637-659
28. McDonald, M. J., and Noble, R. W. (1972) *J. Biol. Chem.* **247,** 4282-4287
29. Hille, R., Palmer, G., and Olson, J. S. (1977) *J. Biol. Chem.* **252,** 403-405
30. Doyle, M. P., Maciejko, J. J., and Busman, S. C. (1973) *J. Am. Chem. Soc.* **95,** 952-953
31. Doyle, M. P., Van Doornik, F. J., and Funckes, C. L. (1980) *Inorg. Chim. Acta* **46,** L111-L113
32. Williams, J. E., and Murrell, J. N. (1971) *J. Am. Chem. Soc.* **93,** 7149-7155
33. Buschmann, H. W. (1974) *Z. Phys. Chem.* **92,** 11-22
34. Ohlsen, J. R., and Laane, J. (1978) *J. Am. Chem. Soc.* **100,** 6948-6955
35. Haymore, B. L., and Ibers, J. A. (1974) *J. Am. Chem. Soc.* **96,** 3325-3327
36. McCleverty, J. A. (1979) *Chem. Rev.* **79,** 53-76
37. Chien, J. C. W. (1969) *J. Am. Chem. Soc.* **91,** 2166-2168
38. LeMaistre, J. W. (1972) in *Study Syst., Coronary Myocardial Effects of Nitrates, Proceedings Symposium, 1970*, pp. 251-257
39. Holecek, V., Kopecky, J., and Skramovsky, S. (1979) *Coll. Czech. Chem. Commun.* **44,** 981-985
40. Reisberg, P. I., and Olson, J. S. (1980) *J. Biol. Chem.* **255,** 4151-4158