Formation of Bovine Nitrosylmyoglobin. I. *p*H 4.5–6.5*

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The rates of formation of nitrosylmyoglobin and nitrosylhemoglobin in systems with varying amounts of nitrite, ascorbate, and hydrogen ion have been studied. The rate of conversion of metmyoglobin to nitrosylmyoglobin has been found to be zero order with respect to the pigment over a wide range of varied reactants and has been found to vary linearly with nitrite concentration up to 5:1 nitrite-to-metmyoglobin ratios and as the one-third power of nitrite concentration above this ratio. The zero-order rate also varies as the one-half power of ascorbate concentration and the one and one-half power of hydrogen ion concentration. These results are explained by a reaction mechanism in which nitrous and ascorbic acids form an intermediate complex which decomposes slowly to yield nitric oxide. The nitric oxide is immediately complexed with metmyoglobin (or methemoglobin) to form nitrosylmetmyoglobin, which is subsequently reduced to nitrosylmyoglobin. Since the reaction involves the undissociated acid forms of nitrite and ascorbate, the over-all reaction rate increases sharply with decreasing *p*H.

The formation of nitrosylmyoglobin (nitrosylhemoglobin) is of great commercial importance, since these compounds have been shown to be the principal pigments in cured meats. The postulated mechanism (Watts and Lehmann, 1952) is one in which metmyoglobin is reduced to myoglobin, which then forms the nitrosylmyoglobin complex by combining directly with nitric oxide. The nitric oxide is generally described as being formed by either the reduction of nitrite or the termolecular dismutation of nitrous acid. Although it would be possible to determine from the kinetics of the over-all reaction which of these two reactions is the principal source of nitric oxide, there is no such kinetic information available. Some question may be raised concerning a sequence involving free nitric oxide and/or reduced myoglobin. Nitrite rapidly oxidizes reduced hemoglobin and myoglobin (Gamgee, 1898: Hartridge, 1920), and nitric oxide is rapidly oxidized by oxygen. In the presence of excess nitrite and oxygen, one would expect either intermediate to have a very short half-life and the quantities in solution to be very limited. It was felt that some studies should be performed to determine what quantities of myoglobin and nitric oxide are produced in various systems and whether the observed rates of nitrosylmyoglobin formation could be accounted for by these quantities.

Another consideration prompted an investigation of the reaction. Recent advances in the technology of meat curing have introduced new reagents and faster curing processes. Ascorbate and isoascorbate are added to speed the reduction processes; added phosphates and polyphosphates raise the *p*H. However, the development of undesirable color often occurs, which appears to be related to the relative proportions of added reagents as well as the *p*H of the finished product. A study of the effect of varying reagent concentrations and *p*H was inaugurated in model systems containing purified myoglobin and/or hemoglobin, nitrite, and ascorbate or cysteine. The over-all reaction was broken down into its component steps as far as possible, and each step was studied individually.

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EXPERIMENTAL

The myoglobin used in these experiments was extracted from beef hearts obtained immediately after slaughter. The hearts were trimmed of fat, ground, and mixed with an equal weight of water. The mixture was frozen at −3° overnight and then moved to a 7° room, where the container was tipped on its side and the partially frozen mix allowed to drip. The resulting extract was then concentrated to one third its original volume by freezing and partial thawing and separated into 0.4 and 1.0 saturation ammonium sulfate fractions at *p*H 7.0. The precipitate from the 0.4–1.0 saturation fraction was dialyzed against distilled water and the resulting solution pervaporated to about one fifth its original volume. The solution, containing myoglobin, hemoglobin, and some extraneous proteins, was mixed with triethanolamine buffer to give a final buffer concentration of 0.1 M, *p*H 7.0. An appropriate volume of this solution, containing about 2% protein, was chromatographed on a Sephadex G-75 column. The columns were 300 mm in height; higher columns tended to clog because of compression of the soft resin. The hemoglobin is too large for the pore sizes of the resin and passes first through the column along with most of the other non-heme-containing protein. A small nonheme protein peak closely precedes the myoglobin fraction. The ratio of the absorbancies of the solutions at 280 and 525 mμ was used as the criterion of purity. This ratio measures the relative amounts of protein and heme in the myoglobin preparations. Preparations with a ratio of 4.24 or less were found to contain less than 5% extraneous protein by ultracentrifugation and electrophoresis. Preparations with ratios less than 3.9 tended to be unstable and to precipitate out of solution on storage. All preparations used in this study had A_{280}/A_{525} ratios between 4.0 and 4.24.

Acetate, cacodylate, and phosphate buffers were used to study the *p*H range 4.5 to 6.5. They were chosen because they showed no color reactions with the heme pigment. Nitrogenous buffer compounds form weak complexes with the heme, causing difficulties in the interpretation of the observed spectral changes. When it was necessary to maintain a specific *p*H, the reductant and the nitrite were adjusted to the appropriate *p*H before addition to the reaction mixture. The concentrations of reactants shown in Table I were the standard conditions at which the rates of reaction of different myoglobin and hemoglobin preparations were tested. The figures in parentheses were the

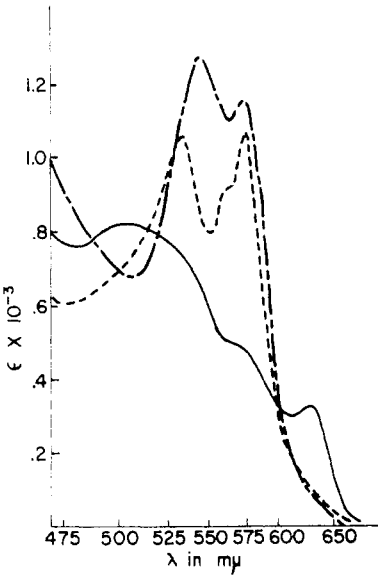

FIG. 1.—Spectra of metmyoglobin-nitrite (——), nitrosylmetmyoglobin (- - -), and nitrosylmyoglobin (— -).

TABLE I
CONCENTRATIONS OF REACTANTS

Reactant	Conc. (mM)
Buffer (Γ/2)	50
Myoglobin	0.05
Ascorbate or cysteine	5.0 (0.05–50.0)
Nitrite	5.0 (0.05–50.0)

range over which specific reactants were varied to study their effect on the reaction.

The molar absorptivities used in this study are given in Table II. The absorptivities of metmyoglobin were determined by measuring the spectra of a solution of crystalline metmyoglobin prepared according to a previously published method (Lewis and Schweigert, 1955). A measured volume of the solution was then lyophilized in a weighing bottle, an Abderhalden drying pistol being used as the vacuum chamber, with the bulb immersed in dry ice–acetone. The sample was then dried to constant weight at 100° *in vacuo* over phosphorus pentoxide in the drying pistol. These values were used to determine the initial metmyoglobin concentrations and to determine the absorptivities of the specific reaction product at the completion of the reaction. The values for the absorptivities of metmyoglobin are in close agreement with those reported by Bowen (1949) except that he reported the principal maximum at 500 mμ whereas it has been found to be at 505 mμ on instruments in this laboratory. The absorbance at 525 mμ was used for determining the initial concentration of heme pigment, as this was determined to be the isosbestic point between oxymyoglobin and metmyoglobin, the two forms present in the fresh preparations used.

All of the reactions were followed spectrophotometrically in a constant-temperature room at 20°. All solutions of reactants were at 20°, and little or no temperature shift was observed while the cuvets were in the instruments. Except where noted, the reactions were run by first mixing stock solutions of buffer, metmyoglobin, and nitrite. The volume was then made up to the appropriate level and the reaction started by addition of the ascorbate. Solutions of ascorbate were made up fresh each day, as the reductant oxidizes slowly in air. Ascorbate reacts with either nitrite or metmyoglobin alone and was therefore added last to start the reaction. Nitrite and metmyoglobin immediately form a complex which is stable in the absence of reductant. The complex formed (Fig. 1) is similar to the nitrite-methemoglobin complex reported by Marshall and Marshall (1945). They found that the height of the shoulders at 530–540 and 570–580 mμ in the methemoglobin absorbancy spectrum varied with the amount of nitrite added to methemoglobin and concluded that the compound formed was an ionic complex of nitrite and methemoglobin. The same observation has been made in this study for mixtures of metmyoglobin and nitrite. In those reactions where nitrite was varied, this meant that the initial absorptivity at 545 mμ of the heme pigments increased with increased nitrite. An initial absorption value for the mixture of metmyoglobin and metmyoglobin-nitrite was calculated for each concentration of nitrite, and this value was used in calculating the rate of pigment conversion from the change in optical absorption during the reaction. Since the relative proportions of the two forms of metmyoglobin changed during the reaction because of the shift in the equilibrium due to the removal of oxidized pigment, the calculations are not strictly accurate. However, no detectable error is introduced, especially since the data were calculated from the initial phases of the reaction.

TABLE II
MOLAR ABSORPTIVITIES OF SEVERAL BOVINE MYOGLOBIN DERIVATIVES
m.w. = 18,000

λ (mμ)	$\epsilon \times 10^{-3}$ (liter/mole-cm)				
			$MMbNO_2$		
	MMb^+	MbO_2	100:1	$MMbNO^+$	MbNO
505	9.7 (9.8)[a]				
522	8.1		8.4	8.7	8.4
525	7.7	7.7			
535	6.4			10.7 (10.2)[b]	
545	5.4		8.0		13.3 (12.2)[c]
575	2.97			10.8 (10.5)[b]	12.2
582	2.91	(15.1)[a]			

[a] Bowen (1949). [b] Ehrenberg and Szczepkowski (1960). [c] Walsh and Rose (1956).

The conversion of metmyoglobin to nitrosylmyoglobin was followed by measuring the increase in optical absorption at 545 mμ. The reduction of metmyoglobin to myoglobin was followed at 582 mμ, one of the absorption maxima of oxymyoglobin, rather than at 555 mμ, the maximum for myoglobin. These reactions were run in air, which resulted in the immediate formation of oxymyoglobin from the reduced pigment. Neither the conversion to nitrosylmyoglobin nor the reduction to myoglobin showed any differences in initial rate whether run in air or under nitrogen.

RESULTS

Within certain limits of nitrite and ascorbate concentrations, the conversion of metmyoglobin to nitrosylmyoglobin was zero order with respect to the pigment over the *p*H range 4.5–6.5. The increase in absorption at 545 mμ was linear until 70–80% of the pigment was converted. The zero-order rate was not constant with hydrogen ion concentration, but varied

as shown in Figure 2. Since one of the things we wished to know was whether the reduction of metmyoglobin to myoglobin was a part of the conversion of metmyoglobin to nitrosylmyoglobin, the rate of reduction of metmyoglobin was followed in the same system, without nitrite, over the same *p*H range. The reduction of metmyoglobin to myoglobin was found to be first order with respect to the heme pigment and to vary little, if at all, with *p*H (dashed line in Figure 2). Since both reactions contained the same initial concentration of heme pigment, the data for the reduction reaction were calculated as initial turnover rates by multiplying the first-order rate constant by the initial pigment concentration. Thus both curves in Figure 2 are directly comparable, being turnover rates at the same concentration.

The *p*H range shown in Figure 2 is the total range over which it was possible to follow the conversion of metmyoglobin to nitrosylmyoglobin. At the upper limit it was too slow to follow; at *p*H 7.0 there were no spectral changes over a 24-hour period. At the lower limit, below *p*H 4.5, the spectrum of the final product was no longer that of nitrosylmyoglobin. At *p*H 4.0 the final absorption spectrum was the same as the spectrum reported for nitrosylhemochrome (Hornsey, 1956). At *p*H values between 4.0 and 4.5 the solutions, after a few minutes, contained mixtures of the two nitric oxide heme pigments, but upon continued standing the spectra of these solutions changed to that of the *p*H 4.0 end-product. The spectral identity between the *p*H 4.0 end-product and the acetone-soluble nitrosylhemochrome suggests cleavage of the heme from the globin, although the pigment may well be a water-soluble denatured globin nitrosylhemochrome. At low *p*H, one other reaction was observed in the system with nitrite. If the pigment and nitrite were allowed to stand without the addition of ascorbate, a green heme pigment was formed which had an absorption maximum at 618 mμ. This pigment was also formed at higher *p*H values by concentrations of 15 mM nitrite and higher. The conversion of metmyoglobin to the green pigment defined the boundary conditions for the study of the formation of nitrosylmyoglobin, as the green heme compound would no longer react in the system.

The effect of varying the concentration of ascorbate in the system is shown in Figure 3. For this study the concentration of nitrite was held constant at 5 mM and that of ascorbate was varied from 0.05 to 15 mM (1:1 to 300:1 ascorbate-to-metmyoglobon ratios). Below 0.25 mM ascorbate the reaction rates were variable and unreliable, but from 0.50 mM to 5 mM ascorbate the zero-order rates for the formation of nitrosylmyoglobin were found to vary with the square root of the ascorbate concentration. For convenience in consideration of the data in Figure 3 values on the abscissa are given as the square roots of the ascorbate-metmyoglobin ratios. With ascorbate concentrations above 5 mM (higher than 100:1 ascorbate to pigment) the initial turnover rates continued to increase, but the curves became increasingly first order with respect to metmyoglobin. At 15 mM ascorbate the curves were very close to first order. Higher concentrations (above 15 mM ascorbate) could not be studied owing to the evolution of gas and resultant foaming of the solutions. Chromatography of the gas on 5 A molecular sieve identified it as nitric oxide by comparison with a known sample. Karrer and Bendas (1934) have also identified the gas produced by this reaction as nitric oxide.

The effect of varying the nitrite concentration on the formation of nitrosylmyoglobin is shown in Figure 4. In order to encompass the entire range of nitrite concentration over which the reaction was zero order with respect to pigment, the curve is shown as a log-log plot. Below 5:1 nitrite-to-metmyoglobin ratios, the zero-order rate of conversion was directly proportional

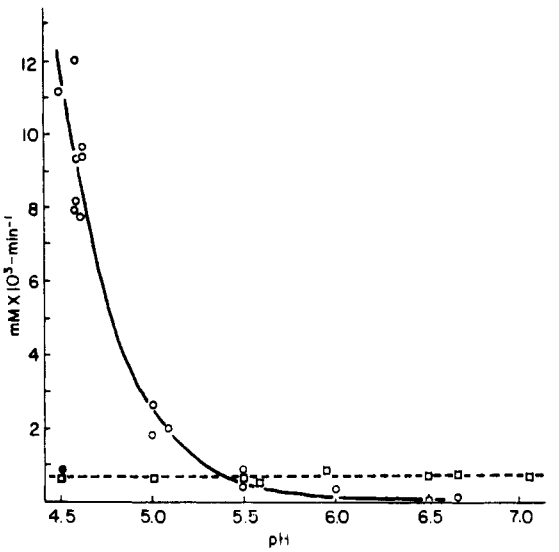

FIG. 2.—Zero order rates of formation of nitrosylmyoglobin and initial turnover rates for formation of oxymyoglobin. NOMb (○), O_2Mb (□), NOMb using cysteine as reductant (●). Solid curve for nitrosylmyoglobin formation was calculated as described in the text.

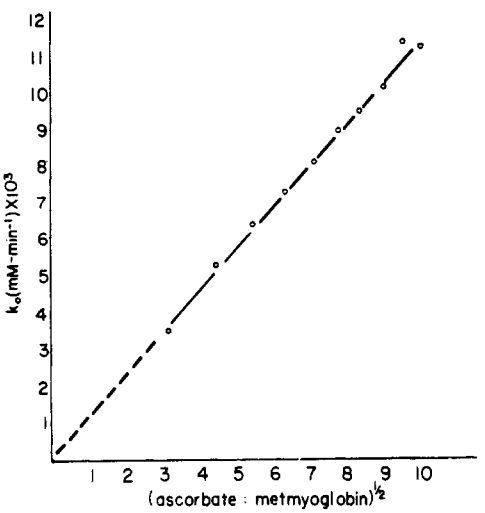

FIG. 3.—Zero order rate of formation of nitrosylmyoglobin versus square root of the ascorbate: metmyoglobin ratio.

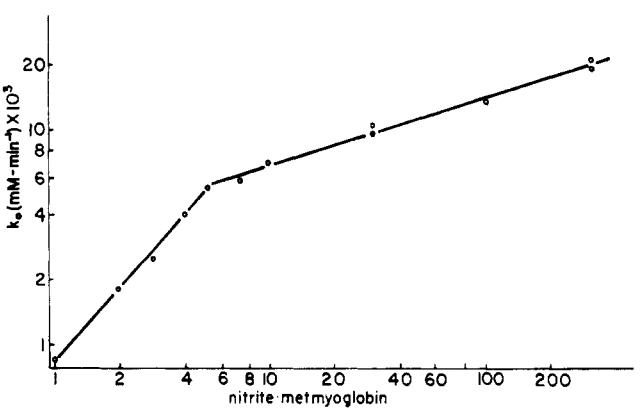

FIG. 4.—Zero order rate of formation of nitrosylmyoglobin versus nitrite to metmyoglobin ratio. Log-log plot.

to the nitrite concentration. Above 5:1 nitrite-to-metmyoglobin ratios the rate of conversion was zero order and varied as the one-third power of the nitrite concentration. The sharp break at this 5:1 ratio has no apparent significance and may be an artifact of the log-log plot. Above 300:1 nitrite-to-metmyoglobin ratios, the conversion of metmyoglobin to the green pigment predominated and accurate data could not be obtained for the conversion of metmyoglobin to nitrosylmyoglobin.

Three immediate conclusions may be drawn concerning the reaction mechanisms under study. First, since a sequence of reactions cannot be faster than the slowest reaction in the sequence, it is clear that below *p*H 5.4 the reduction of metmyoglobin to myoglobin is not an intermediate step in the principal reaction sequence whereby nitrosylmyoglobin is formed. Above *p*H 5.4, the reduction of metmyoglobin may be part of the sequence, but it is not the rate-limiting reaction. Moreover, above *p*H 5.4, no spectral evidence of accumulation of either myoglobin or oxymyoglobin was observed. Whenever a spectral change occurred, analysis of the absorption spectrum showed the product to be nitrosylmyoglobin. These observations support the objection made to the postulation of free myoglobin in a system containing nitrite.

Second, from the observed dependences of the rate of pigment conversion on the three reactants—pigment, zero order; nitrite, first order; ascorbate, half order—it is clear that the rate-limiting steps involve nitrite and ascorbate, but not the heme pigment. The sequence of reactions must therefore be such that a reaction or series of reactions between nitrite and ascorbate produce an intermediate compound at a limiting rate. The heme pigment reacts immediately with this intermediate in a reaction or series of reactions that are not rate limiting. Although the oxidized pigment exists in the solutions in two forms, metmyoglobin and metmyoglobin-nitrite, for the purposes of the latter reactions they are essentially equivalent since they are in equilibrium with each other.

The third conclusion concerns the reaction(s) of ascorbate in this system. The observed zero-order rate of metmyoglobin conversion to nitrosylmyoglobin was dependent upon the square root of the ascorbate concentration. Such a dependence would be observed if the ascorbate formed a reactive intermediate, which would then undergo a bimolecular reaction in the backward direction to re-form ascorbate or some other compound that will react in the forward direction. It is the backward reaction that introduces the square root dependence. Evidence from previous studies (Dahn and Loewe, 1958, 1960) indicates that such an intermediate does exist and is the nitrosylated derivative of ascorbic acid or ascorbate. Assuming that this intermediate would undergo a bimolecular dismutation in a backward direction, and with the foregoing conclusions and limitations in mind, the following reaction sequence was written:[1]

$$2\ HNO_2 \overset{K_1}{\rightleftharpoons} N_2O_3 + H_2O \quad \text{(equilibrium)} \quad (1)$$

$$N_2O_3 + AH_2 \xrightarrow{k_2} AHNO + HNO_2 \quad \text{(fast)} \quad (2)$$

$$2\ AHNO + H_2O \xrightarrow{k_3} 2\ AH_2 + N_2O_3 \quad \text{(fast)} \quad (3)$$

[1] The following abbreviations are used in the equations: AH_2, ascorbic acid, undissociated; AHNO, the nitrosoascorbic acid intermediate; AH·, ascorbic acid radical; MMb, metmyoglobin; NOMMb, nitrosylmetmyoglobin; and NOMb, nitrosylmyoglobin.

$$AHNO \xrightarrow{k_4} AH\cdot + NO \quad \text{(slow)} \quad (4)$$

$$NO + MMb \xrightarrow{k_5} NOMMb \quad \text{(fast)} \quad (5)$$

$$NOMMb + \text{reductant} \xrightarrow{k_6} NOMb \quad \text{(fast)} \quad (6)$$

In order to solve this series of reactions for the rate expression the steady state assumption was made, that is, it was assumed that the concentrations of all the reaction intermediates remained constant during the course of the reaction and therefore the rate of change in concentration of each was equal to zero.

$$\frac{d[AHNO]}{dt} = k_2[N_2O_3][AH_2] - k_3[AHNO]^2[H_2O] - k_4[AHNO] = 0 \quad (7)$$

$$\frac{d[NO]}{dt} = k_4[AHNO] - k_5[NO][MMb] = 0 \quad (8)$$

$$\frac{d[NOMMb]}{dt} = k_5[NO][MMb] - k_6[NOMMb][\text{reductant}] = 0 \quad (9)$$

For this derivation the concentration of N_2O_3 is considered to be dependent upon the equilibrium expression for equation (1). Since reaction (4) is postulated to be the slow reaction, the last term in equation (7) is small in comparison with the first two terms and was neglected in the calculations. Solving for the concentration of the nitrosoascorbic acid intermediate in equation (7), and substituting the equilibrium expression for the concentration of N_2O_3, gives equation (10).

$$[AHNO] = \left[\frac{K_1k_2}{k_3}\right]^{1/2}[HNO_2][AH_2]^{1/2} \quad (10)$$

From equations (8) and (9) it is seen that $k_4[AHNO]$ is equal to $k_6[NOMMb]$ [reductant]. The latter term is the desired expression for the production of nitrosylmyoglobin, v_{NOMb}. Setting these rate expressions equal, and substituting the expression for the concentration of the nitrosoascorbic acid intermediate from equation (10), the rate expression in equation (11) is

$$v_{NOMb} = k_4\left[\frac{K_1k_2}{k_3}\right]^{1/2}[HNO_2][AH_2]^{1/2} \quad (11)$$

obtained. Finally, in order to explain the *p*H dependence it was assumed that the reaction takes place with the un-ionized forms of nitric acid and ascorbic acid. Rewriting equation (11) with the equilibrium expressions for the acid dissociations gives equation (12), where *Y* and *Z* refer to the sums of the concentrations of all species of nitrite and ascorbate, respectively. To test equation (12), a value of $k_4' = 0.250$ was

$$v_{NOMb} = k_4'\left[\frac{Y[H^+]}{K_{aY} + [H^+]}\right]\left[\frac{Z[H^+]}{K_{aZ} + [H^+]}\right]^{1/2} \quad (12)$$

calculated from the experimental data at *p*H 5.0. This value was used in equation (12) to calculate the solid curve in Figure 2, using values of $pK_{aY} = 3.4$ and $pK_{aZ} = 4.17$. As a further test other powers of the hydrogen ion concentrations were tried, but none fit the data as well as equation (12).

Discussion

The Relative Rates of Reactions (1) and (2).—If reaction (1) were not an equilibrium expression, then the rate expression for the over-all reaction would be determined solely by the rate expression of reaction

(1) as a result of the steady state assumption. Since the concentration of the reductant does influence the over-all reaction, reaction (1) must be at equilibrium. The rate of formation is therefore dependent upon the concentration of reductant and might reasonably be expected to be dependent on the kind of reductant. This has been found to be true. Brooks (1937) used dithionite to form nitrosylhemoglobin in the *p*H range 5.15–5.66 and reported a rate approximately 50 times as fast as the rate found in this study with ascorbate. Because of the rapid oxidation of dithionite, it is difficult to quantitate the reaction, but when tested in the *p*H 4.5 system the rate of nitrosylmyoglobin formation was 50–60 times as fast with dithionite as with ascorbate. By comparison, cysteine reduces metmyoglobin and nitrite to nitrosylmyoglobin at about one twelfth the rate of ascorbate. All three reductants show the same *p*H dependence (increasing rate with decreasing *p*H), and the reaction with cysteine was found to be zero order with respect to the pigment. The zero order rate was linear with cysteine concentration, suggesting that the semistable intermediate complex formation is peculiar to ascorbate. These results confirm the relative rate assumption and, since the reaction was zero order with respect to pigment with both ascorbate and cysteine, they confirm the conclusion that the rate-limiting expressions involve only nitrite and reductant.

The Backward Reaction (Reaction 3).—Neither Dahn and Loewe (1958, 1960) nor the present authors have found any direct evidence for the nitroso-ascorbic acid intermediate. Its existence is postulated solely to explain a given set of kinetic observations. In this study, the intermediate is postulated on the basis of the dependence of the rate of nitrosylmyoglobin formation on the square root of the ascorbate concentration. Such a dependence is explained by a bimolecular reaction of an intermediate, produced from ascorbate, which proceeds in a backward direction. A similar backward reaction has been postulated by Yamazaki (1962) for an ascorbic acid radical. He found that the rate of reduction of cytochrome *c* with ascorbate and ascorbic acid oxidase depended on the square root of the ascorbate concentration.

The Formation of Nitric Oxide.—Reaction (4), the rate-limiting step, was formulated as a monomolecular decomposition of the nitroso-ascorbic acid intermediate. Since it is the rate-limiting step, interaction of the intermediate with other reactive species would introduce a higher order of molecularity in that particular species, which was not observed. For example, if the intermediate were assumed to decompose by reaction with a nitrous acid molecule, the molecularity in nitrous acid would be two in the final rate formulation. As mentioned previously, higher orders of the various reactants were tried, but did not fit the data. The formation of the ascorbic acid radical, shown as AH·, may be justified on the basis of Yamazaki's finding that semistable ascorbic acid radical intermediate may be formed in solution. The ascorbic acid radical may undergo further reactions, but apparently none of them influences the formation of nitrosylmyoglobin.

The production of nitric oxide from the preceding reactions is rate limiting in the over-all reaction producing nitrosylmyoglobin, and it remains to be shown that the subsequent steps involving the heme pigment are not rate limiting. Study of these last two steps showed them to be fast enough to account for the observed rates, and that nitric oxide and nitrosylmetmyoglobin were produced.

Nitrosylmetmyoglobin.—The evidence for the formation of this pigment is based on its spectral characteristics. Nitrosylmetmyoglobin was prepared by gassing a solution of the oxidized pigment with nitric oxide, and the absorption spectrum was recorded. The complex has two sharp absorption maxima at 535 and 575 mμ, a minimum at 552 mμ, and a shoulder at 560 mμ (Fig. 1). A similar absorption spectrum has been reported for nitrosylmethemoglobin by Ehrenberg and Szczepkowski (1960). Referring to Figure 1 it will be observed that metmyoglobin-nitrite and nitrosylmyoglobin are isosbestic at 522 mμ, but that the absorption of nitrosylmetmyoglobin is higher at this wave length. If metmyoglobin-nitrite and nitrosylmyoglobin were the only species present during the reaction, the absorption at 522 mμ would not change during the course of the reaction. If, however, nitrosylmetmyoglobin were produced, the absorption at 522 mμ would rise. To follow the reaction at *p*H 4.5 the recording spectrophotometer was set to scan at 522 mμ *versus* time. As soon as ascorbate was added to start the reaction, the absorption rose quickly about 0.008 units, remained steady until about 70–80% of the pigment was converted (the zero-order portion of the reaction curve), and then slowly dropped back to the initial absorption reading. This increase in absorption was slight, but could be duplicated readily. The magnitude of the rise was found to be associated with the rate of formation of nitrosylmyoglobin. At *p*H 6.5, where nitrosylmyoglobin was formed very slowly, no increase in the absorption at 522 mμ could be detected. An estimate of the amount of nitrosylmetmyoglobin formed at *p*H 4.5 represented by this increase in absorption indicates about 50% of the pigment was present in this form, which fits kinetically with the observed rates reported below.

If the spectra of the reacting solutions were scanned it was observed that the shorter wave length maximum was displaced from the 545 mμ maximum of nitrosylmyoglobin to 540–542 mμ. As the reaction went to completion the maximum shifted back to 545 mμ. Since the absorption maximum of nitrosylmetmyoglobin is at 535 mμ, a slight shift toward shorter wave lengths would be expected if nitrosylmetmyoglobin were found in appreciable amounts during the reaction.

If nitric oxide is produced by the slow reaction between nitrite and ascorbate, preincubation of these two reactants prior to introduction of metmyoglobin should result in the formation of nitrosylmetmyoglobin. Furthermore, increased nitrosylmetmyoglobin concentrations should be observed with increased preincubation times. As the amount of nitric oxide produced approaches saturation of the heme pigment, the reaction curves for the formation of nitrosylmyoglobin should shift from zero order to first order with respect to pigment concentration, and the initial turnover rate should increase accordingly. Nitrite and ascorbate were allowed to react for varying lengths of time at *p*H 4.5, and nitrosylmyoglobin formation was started by injecting metmyoglobin into the system. If ascorbate and metmyoglobin were injected simultaneously (zero time delay) the reaction was zero order with respect to pigment. As expected, the initial reaction rates increased as the preincubation period was lengthened, and the curves became increasingly first order. If nitrite and ascorbate were allowed to react for 4 or more minutes, the initial reaction rates were at a maximum and the reaction was first order with respect to the pigment until 90% of the pigment was converted to nitrosylmyoglobin. After 4 minutes of preincubation, the absorption spectrum of the pigment formed immediately upon the addition of metmyoglobin was that of nitrosylmetmyoglobin with the lower ab-

sorption maximum at 535 mμ. As the reaction proceeded, the maximum at 535 mμ shifted to 545 mμ, the result of the conversion of the oxidized to the reduced nitrosylheme pigment.

Rate of Reduction of Nitrosylmetmyoglobin.—An added reductant is required for the reduction of nitrosylmetmyoglobin to nitrosylmyoglobin. Solutions of nitrosylmetmyoglobin, prepared at *p*H 4.5, 5.5, and 6.5 by gassing metmyoglobin solutions with nitric oxide, did not produce detectable amounts of nitrosylmyoglobin. At *p*H 5.5 and 6.5 the nitrosylmetmyoglobin complex was stable for periods up to 1 hour at 20°. If the solutions were shaken with air, the pigment was converted to metmyoglobin nitrite. At *p*H 4.5, nitrosylmetmyoglobin decomposed to metmyoglobin nitrite in a few minutes. If either ascorbate or cysteine was added to the solutions, nitrosylmetmyoglobin was reduced to nitrosylmyoglobin. The reaction was first order with respect to the pigment and showed little variation in rate with *p*H. The first-order rate constant was 0.40 min.$^{-1}$ with 5 mM ascorbate and 1.26 min.$^{-1}$ with 15 mM ascorbate. The change in first-order rate constant was thus linear with respect to ascorbate concentration. These results confirm the conclusion that the dependence of the zero-order rate of the over-all reaction on the square root of the ascorbate concentration is not a function of the reduction of the nitrosylmetmyoglobin intermediate. The first-order rate constant calculated from the data obtained for the ascorbate-nitrite preincubation experiments was 0.42 min.$^{-1}$ (5 mM ascorbate). The rate at which nitrosylmetmyoglobin (at constant concentration) is reduced to nitrosylmyoglobin is the first-order rate constant times the nitrosylmetmyoglobin concentration. From the increase in absorption at 522 mμ it was previously estimated that about half the total pigment present during the reaction was nitrosylmetmyoglobin. Half the total pigment was 0.025 mM, which, multiplied by the first-order rate constant, gives a turnover rate of 10×10^{-3} mM pigment per minute. Since the estimate of the concentration of nitrosylmetmyoglobin is highly approximated, the agreement of this figure with the observed zero-order rate in the over-all reaction may be more gratifying than real. Nevertheless, it was clear that the reduction of nitrosylmetmyoglobin was not rate limiting.

The Effect of Nitrite.—The dependence of the rate of nitrosylmyoglobin formation on the nitrite concentration (Fig. 4) does not support the proposed mechanism except at low concentrations. At concentrations less than 0.25 mM nitrite (5:1 nitrite to metmyoglobin) the rate is linear with nitrite, but above this concentration the rate varies as the one-third power of nitrite concentration. From the *p*H studies, it was found that the increase in the zero-order rate constant was linear with nitrous acid concentration. Since the nitrous acid concentration is linear with total nitrite, at least at the low concentrations used in this study, the one-third power effect of nitrite is not due to nitrous acid and must therefore be due to nitrite ion. The fractional power dependence suggests a backward reaction, but the one-third power, if it is believed, means a termolecular reaction of a nitrite ion intermediate, for which no reasonable hypothesis has suggested itself. Examination of the data obtained to date has found no clues to the fractional order dependence. However, nitrite is highly reactive, not only with itself and other oxides of nitrogen but also with amino groups and the heme. Some possibilities along these lines are being investigated.

High Concentrations of Ascorbate.—At 15 mM ascorbate (300:1 ascorbate to metmyoglobin) the formation of nitrosylmyoglobin was found to be almost first order in the metmyoglobin-nitrite-ascorbate system. (There was a slight downward curve to the first-order plot, indicating that the actual reaction was somewhat less than first order.) Such a result is expected from the reaction sequence. At sufficiently high concentrations of ascorbate the rate of decomposition of the nitroso-ascorbic acid intermediate produces sufficient nitric oxide to saturate the metmyoglobin, and the reaction then depends on the rate of nitrosylmetmyoglobin reduction. The first-order rate constant was 0.93 min.$^{-1}$, compared to the value of 1.26 min.$^{-1}$ for the reduction of nitrosylmetmyglobin at 15 mM ascorbate. Higher concentrations of ascorbate would be expected to give an even closer approximation to first-order curves and correspondingly higher first-order rate constants. As mentioned previously, higher concentrations could not be studied because of the evolution of nitric oxide.

The Formation of Nitrosylhemoglobin.—The foregoing results were for the formation of nitrosylmyoglobin, but the study also included the formation of bovine nitrosylhemoglobin from methemoglobin, nitrite, and ascorbate. Methemoglobin reacted under all conditions at the same rate as metmyoglobin, the reaction being zero order with respect to the pigment. Again it is seen that the heme pigment was not involved in any rate-limiting step, in confirmation of previous conclusions.

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