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THE NITRITE METHEMOGLOBIN COMPLEX—ITS SIGNIFICANCE IN METHEMOGLOBIN ANALYSES AND ITS POSSIBLE ROLE IN METHEMOGLOBINEMIA

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Abstract—Evidence consistent with the formation of a reversible complex between excess free nitrite ion and the ferric heme groups of methemoglobin is presented. The existence of this complex in nitrited red cells or in lysates in the presence of appropriate concentrations of free nitrite can lead to gross underestimation of methemoglobin levels by a common spectrophotometric method. Complex formation is manifested by a decrease in the absorption of methemoglobin solutions in the range of 600–650 mμ. This source of error can be eliminated in red cell suspensions simply by washing. Similarly, lysate solutions may be dialyzed or diluted to effect dissociation of the complex. Attempts were made to estimate the dissociation constant of this complex at 25°, pH 7·4, and physiologic ionic strength. Although unknown side reactions between methemoglobin and nitrite interfere in the spectrophotometric determination of this constant, its value is probably not greater than 3 mM. This estimate indicates that the complex must exist transiently *in vivo* when mice receive nitrite doses adequate to convert more than one-third of the circulating blood pigment to methemoglobin. Thus, complex formation may contribute to the unusually sustained methemoglobinemia produced in mice by nitrite.

IT HAS always seemed anomalous that most, if not all, variations of the Evelyn and Malloy[1] spectrophometric method for the determination of methemoglobin in blood are standardized with methemoglobin prepared by treating solutions of oxyhemoglobin with potassium ferricyanide. Of the wide variety of chemicals capable of directly or indirectly oxidizing the ferrous iron of hemoglobin, ferricyanide appears to be unique in its inability to penetrate the red cell membrane.[2] It is, therefore, incapable of generating methemoglobin *in vivo* or even in red cell suspensions. As a consequence, one is always comparing an abnormal blood pigment produced *in vivo* with an artificial standard. Several chemical differences are already recognized among methemoglobins prepared in different ways. With respect to each of these differences ferricyanide-generated methemoglobin seems to be the exception. It contains fewer sulfhydryl groups than auto-oxidized or nitrite-treated hemoglobin.[3, 4] It is probably the only kind of methemoglobin containing iron bound to its globin moiety in a form resistant to dialysis.[5, 6] It appears to be devoid of extra-heme binding sites for hydrosulfide, a characteristic possessed by methemoglobins generated in mice by nitrite, hydroxylamine, and *p*-aminopropiophenone.[4, 7]

Because the above differences relate partially or exclusively to the globin moiety, it is possible that they exert no significant effect in the Evelyn–Malloy assay procedure for methemoglobin.[1] Unhappily, these authors neglected to include in their paper a

detailed discussion of the method for standardizing this assay to make the procedure quantitative. Since little or no reference to this point occurs in the literature, one wonders if the now standardized use of ferricyanide[8, 9] was an empirical development. Recent work[10] confirming older impressions of Jung and Remmer[11] indicates that under some circumstances, however, sodium nitrite is an inappropriate reagent with which to standardize methemoglobin assays. This finding, which is remarkable in that it appears to have escaped attention for so many years, is re-examined here. Large potential sources of error indeed exist if methemoglobin analyses are standardized with blood pigments in the presence of free nitrite ion or if determinations of methemoglobin in blood are made in the presence of an excess of nitrite.

The source of this error has been attributed to reversible complex formation between the ferric heme groups of methemoglobin and excess nitrite ion.[10, 11] The demonstration of this complex *in vitro* suggests the possibility that it may play a role in nitrite-induced methemoglobinemia *in vivo*. When various chemical agents were evaluated as methemoglobin producers in mice, the response to nitrite was noted to be atypically slow and persistent.[12] This phenomenon cannot be related to analytical errors in methemoglobin determination in highly diluted specimens of mouse blood, but it may be at least partially related to nitrite–methemoglobin complex formation *in vivo*.

METHODS

A modification[13] of the Evelyn–Malloy procedure[1] for methemoglobin determinations was used throughout. Appropriate aliquots of blood samples were lyzed with saponin in 0·07 M phosphate buffer, pH 6·6, and absorbance readings were made at 635 mμ before (D_1) and after (D_2), the addition of two drops of 10% sodium cyanide solution. Two drops of 10% potassium ferricyanide and an additional drop of cyanide are then added, and the solution is diluted 8-fold with buffer for a reading at 540 mμ (D_3). In some experiments with sodium nitrite, saponin was omitted until the cells had been washed and the reaction mixture reconstituted to its original volume. The method is standardized by starting with blood samples in the presence of excess ferricyanide and making D_1, D_2, and D_3 readings as described above. From these absorbance values a calibration constant can be derived which permits direct calculation of experimental results as the per cent methemoglobin of the total blood pigment.[13, 14] Analyses were performed in 0·5-in. cuvettes with a Bausch & Lomb Spectronic 20. Complete absorption spectra were traced in 1-cm cells with a Bausch & Lomb Spectronic 505 recording instrument.

Human blood used in these experiments consisted of Versenated specimens drawn for routine blood counts. Specimens were selected at random and used singly in some experiments (Tables 1 and 3) or pooled in lots of a dozen for others. In incubation experiments the cells were washed three times in saline, suspended in Krebs–Ringer phosphate with glucose, and exposed to various concentrations of sodium nitrite.[15] Commercially obtained, twice-crystallized, human hemoglobin was employed in some experiments.

Spectrophotometric titrations of various methemoglobins with sodium nitrite were performed in 1-cm cells at 560 mμ in a Zeiss Q II spectrophotometer. Methemoglobin in 0·1 M phosphate buffer, pH 7·4, was titrated at 25° with either 0·1 or 1·0 M solutions of sodium nitrite in buffer. Titrant was added in μl aliquots with a digital burette.

Corrected estimates of the dissociation constant of the nitrite–methemoglobin complex were derived from these data as previously described for the corresponding hydrosulfide complex.[4]

RESULTS

It was noted by accident that a procedure for the spectrophotometric determination of methemoglobin standardized with potassium ferricyanide and dilute hemolyzed blood specimens[13, 14] gave bizarre results when applied to blood specimens exposed to an excess of nitrite. Even in the presence of large multiples of the amount of nitrite calculated to effect complete conversion of the hemoglobin present to methemoglobin, the ferricyanide-based determination yielded results as low as 33 per cent apparent conversion to methemoglobin.

Inasmuch as ferricyanide standardization seemed inappropriate for the determination of nitrite-generated methemoglobin, an attempt was made to restandardize the assay procedure based on nitrite-treated hemoglobin. The results of this experiment are summarized in Table 1. The value of the calibration constant derived from methemoglobin oxidized by ferricyanide is obviously quite different from that derived from methemoglobin oxidized by nitrite ($P < 0.001$).

TABLE 1. OPTICAL DENSITY VALUES AND CALCULATED CALIBRATION CONSTANTS FROM FERRICYANIDE AND NITRITE-TREATED HUMAN RED CELL LYSATES AT 635 Mμ*

Tube No.	D_1	D_2	D_3	Mean calibration constant ± S.D.
Ferricyanide treatment				
1	0·35	0·04	0·14	
2	0·54	0·08	0·21	
3	0·60	0·08	0·25	
4	0·52	0·07	0·23	47·1 ± 5·6
5	0·62	0·08	0·25	
6	0·66	0·09	0·32	
Nitrite treatment				
1	0·26	0·05	0·14	
2	0·38	0·07	0·22	
3	0·45	0·10	0·24	
4	0·38	0·07	0·23	73·7 ± 5·5
5	0·46	0·08	0·25	
6	0·50	0·09	0·31	

* Each tube Nos 1–6 represents a blood specimen from a different patient. See Methods section for explanation of column headings.

These two different calibration constants were then employed to calculate the methemoglobin concentrations as a percentage of the total pigment in a series of reaction mixtures of washed human erythrocytes (5·0 mM in heme) exposed to various concentrations of sodium nitrite.[15] Two hours after the addition of nitrite, aliquots were removed from each reaction mixture, the cells were washed three times with saline, and analyzed for methemoglobin. The results of this experiment are summarized in Table 2 where the percent methemoglobin of each reaction mixture has been calculated twice by using the two constants derived in Table 1.

It is obvious that the result obtained at 6·75 mM nitrite in Table 2 with the nitrite calibration factor cannot be correct. Even though an analysis standardized with nitrite-treated hemoglobin was employed to estimate methemoglobin concentrations in red cells exposed to nitrite, the system apparently overestimated methemoglobin

TABLE 2. A COMPARISON OF THE METHEMOGLOBIN LEVELS GENERATED WHEN HUMAN RED CELL SUSPENSIONS IN KREBS–RINGER PHOSPHATE GLUCOSE WERE EXPOSED FOR 2 HR TO THE INDICATED CONCENTRATIONS OF SODIUM NITRITE*

Nitrite conc. (mM)	Per cent methemoglobin	
	Ferricyanide calibration	Nitrite calibration
0	1·4	2·2
0·25	7·1	11·0
0·75	22·8	35·7
2·25	53·8	84·2
6·75	92·0	144·0

* The results were computed by the two different calibration constants derived in Table 1.

concentrations by 55–57 per cent. The nitrite calibration constant must, therefore, be aberrantly large. If this constant is too large, it follows from an examination of Table 1 that the D_1 readings for nitrite-treated hemoglobin are too low. The D_2 and D_3 values in Table 1 agree well irrespective of which oxidant was employed to generate methemoglobin.

The experiment of Table 1 was then repeated with two modifications: (1) D_1 and D_2 readings were taken at 620 mμ as well as at 635 mμ; and (2) after the initial D_1 readings, nitrite was added to the tubes that originally contained ferricyanide and vice versa. The D_1 readings were repeated at both wavelengths, as were the subsequent D_2 readings. The addition of nitrite to tubes that originally contained ferricyanide depressed D_1 values at both wavelengths to absorbances equivalent to those of reaction mixtures containing only nitrite. The addition of ferricyanide to mixtures that originally contained nitrite had no significant effect on the D_1 absorbance values. The ultimately derived calibration constant at 635 mμ was not significantly different from the nitrite-based constant of Table 1. The derived constant at 620 mμ was also aberrantly large relative to a ferricyanide constant at this wavelength.

A significant difference between the determination of the nitrite calibration constant (Table 1) and the incubation experiments of Table 2 is that in the latter the cells were washed prior to hemolysis. A calibration constant for nitrite-treated hemoglobin, which is not significantly different from that of ferricyanide-treated lysates, can be derived as shown in Table 3 if red cells exposed to an excess of nitrite are simply washed prior to hemolysis. Even a single washing under these conditions appears adequate to eliminate the source of error.

Fig. 1 shows the effect of sodium nitrite on the absorption spectrum of ferricyanide-generated methemoglobin. The absorbance values of the latter mixture are depressed by nitrite in the 600–650 mμ range, and the difference between the two preparations is at a maximum at about 630–635 mμ. The absorbances of known

solutions of potassium ferrocyanide and potassium ferricyanide in comparable concentration ranges were negligible at these wavelengths. The absorption spectrum of a dialysate of the methemoglobin–ferricyanide reaction mixture was indistinguishable from that of known ferricyanide.

TABLE 3. THE EFFECT OF WASHING NITRITED RED CELL SUSPENSIONS ON THE DERIVED VALUE OF THE CALIBRATION CONSTANT*

Preparation	Mean calibration constant ± S.D.
Ferricyanide-treated lysates	45·3 ± 2·6
Nitrited red cells after 1 wash	46·5 ± 1·6 ($P > 0·1$)
Nitrited red cells after 3 washes	42·7 ± 1·9 ($0·05 < P < 0·1$)

* Each constant was derived as in Table 1 from data on blood samples from 6 different patients. Saponin was omitted from nitrite-treated red cells until after the final washing.

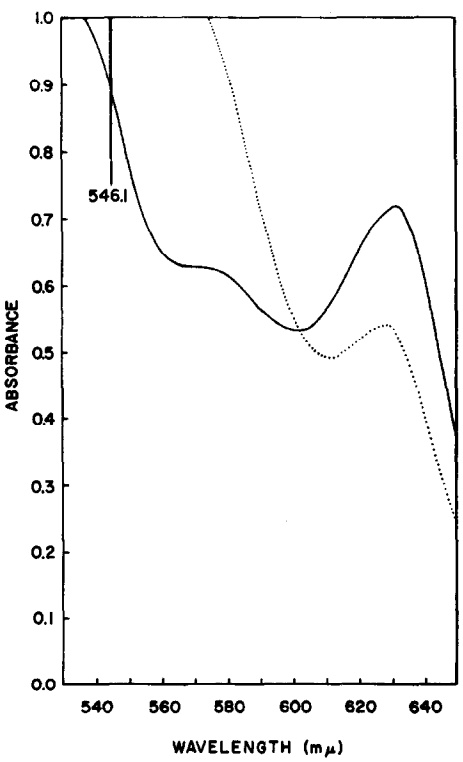

FIG. 1. The absorption spectra of ferricyanide–methemoglobin in 0·07 M phosphate buffer, pH 6·6, before —— and after ..... the addition of excess sodium nitrite.

As shown above, nitrite has the ability to suppress the absorbance of solutions of ferricyanide-treated hemoglobin at wavelengths critical to methemoglobin analyses. Qualitatively similar shifts in absorption spectra are often associated with the binding of ligands to the ferric heme groups of methemoglobin. This potential source of error in spectrophometric analyses of methemoglobin in the presence of nitrite, as already

adduced by Van Assendelft and Ziljstra,[10] probably represents reversible complex formation between excess nitrite ion and ferric heme groups.

An alternative explanation that is still possible, however, is that the nitrite–hemoglobin reaction proceeds differently in solution than in intact red cells. A strong case for this interpretation could be made if a weak methemoglobin complex produced in red cells could still be demonstrated after washing. As shown in Table 1, methemoglobin in cyanide solutions is indifferent to the presence of excess nitrite, but cyanide is bound very tightly to ferric heme groups and cannot be displaced by washing or reasonable dilution. The fluoride ion is bound much less tightly than cyanide by ferric heme. If red cells are incubated in the presence of both nitrite and fluoride (the latter in a 10-fold excess), the absorption spectrum of the lysate after a wash

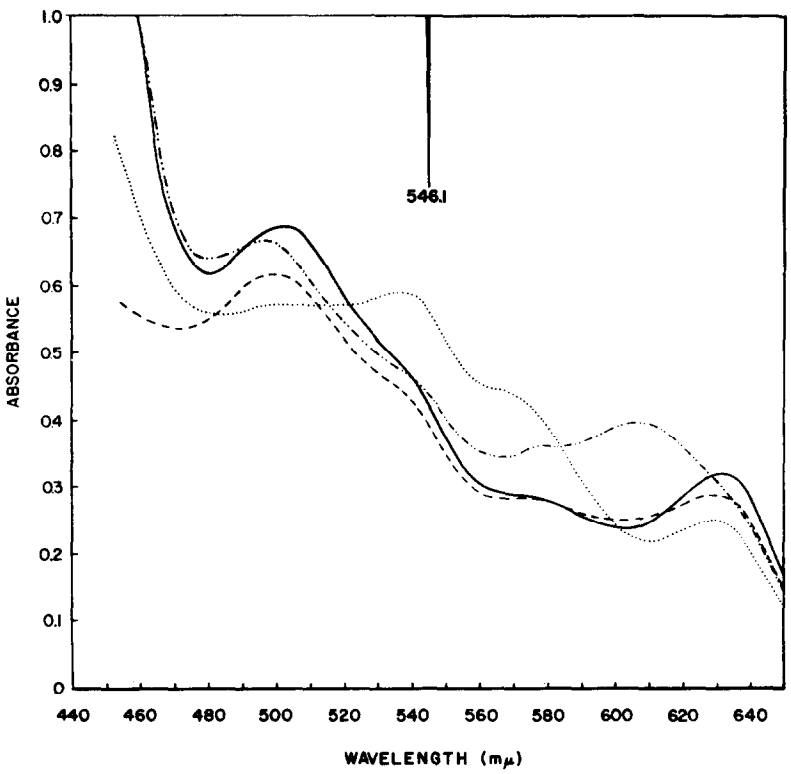

FIG. 2. The absorption spectrum of ferricyanide–methemoglobin in 0·07 M phosphate buffer, pH 6·6 ——. The addition of sodium nitrite to 60 mM produced changes indicated . . . . . The addition of sodium fluoride alone to 60 mM produced changes indicated —·· —·· —. When a red cell suspension was exposed to both 60 mM nitrite and 600 mM fluoride followed by a single wash and lysis, the spectrum indicated - - - - - was produced.

might exhibit features of the fluoride complex. That this was not the case is shown in Fig. 2. The absorption spectrum of the lysate appears intermediate between that of ferricyanide–methemoglobin and nitrite–methemoglobin. The effect of washing nitrited red cells is, therefore, consistent with a dilution effect that allows the intraerythrocytic complex to dissociate. That the absorption spectrum of the lysate more

closely resembled the nitrite–methemoglobin complex than the fluoride–methemoglobin complex, despite an initial 10-fold excess of fluoride, implies that nitrite is bound more tightly to ferric heme groups than is fluoride.

Since the nitrite–methemoglobin complex is apparently formed in red cells as well as in simple solution, it appears probable that this complex plays a role in nitrite-induced methemoglobinemia in man or experimental animals. Central to this question are the relative concentrations of the reactants and the dissociation constant of the complex. Several estimates of the dissociation constant of the nitrite–methemoglobin

TABLE 4. ESTIMATES OF THE APPARENT DISSOCIATION CONSTANT OF THE NITRITE–METHEMOGLOBIN COMPLEX IN 0·1 M PHOSPHATE BUFFER, pH 7·4 AND 25°

Preparation	K′ (mM)
Twice-crystallized human hemoglobin	2·8
Twice-crystallized human hemoglobin	2·8
Dialyzed human lysates*	3·9
Dialyzed human lysates exposed to 0·5 M nitrite*	2·6
Dialyzed human lysates exposed to 0·5 M nitrite*	3·4

* Red cells washed 3 times in saline, lysed with saponin, and dialyzed against phosphate buffer. In some cases the lysates were exposed to nitrite for 20 min at room temperature before dialysis.

complex using methemoglobins prepared in different ways are summarized in Table 4. Although these values appear in relatively good agreement and are consistent with an older estimate,[11] there is much evidence to suggest that they are all too large. There are many possibilities for chemical reactions between nitrite and the methemoglobin molecule other than reversible complex formation with ferric heme binding sites. It was noted that absorbance values of freshly prepared mixtures of methemoglobin and excess nitrite were always lower than apparent titration endpoints when the titration process was prolonged for periods of an hour or more. The absorbance values of freshly prepared mixtures of nitrite and methemoglobin also tended to increase slowly with time. Since the association of nitrite ion with ferric heme binding sites proceeds almost instantaneously, other chromogenic reactions which proceed more slowly must have interfered in these experiments. For this reason all titrations were conducted rapidly, but the possibility of side reactions cannot be excluded despite this precaution.

Also troublesome was the circumstance that the above chromogenic side reactions prevented the addition of large excesses of nitrite at the end of a titration to assure saturation of all heme binding sites. This phenomenon necessitated the employment of an artificial endpoint, namely the absorbance value of freshly prepared solutions of methemoglobin in nitrite concentration ranges of 0·1 and 0·5 M. Preparations of this type agreed well in their initial absorbance values suggesting saturation of heme sites, although they all tended to increase slowly with time. A further attempt to surmount this difficulty is illustrated in the last two titrations of Table 4. Here methemoglobin was exposed for about 20 min at room temperature to a large excess of nitrite (0·5 M) followed by exhaustive dialysis against distilled water. It was hoped that by this procedure side reactions could be forced to completion, whereas

nitrite bound to ferric heme sites would be completely removed by dialysis. Indeed, after dialysis for 48 hr, this pigment when re-exposed to 0·1 M nitrite showed an increased stability in its absorbance (0·01 unit increase in a 45-min period). This treatment carries an unknown risk of altering the binding properties of the ferric heme site, but the apparent dissociation constants derived by titrations were in accord with those of the other preparations.

DISCUSSION

The conclusion of Van Assendelft and Ziljstra[10] that nitrite ion can form a reversible complex with ferric heme groups such as those of methemoglobin fits data presented here. This interaction results in a decrease in the absorbance of methemoglobin solutions in the range of 600–650 mμ, which can lead to a gross spectrophotometric underestimation of methemoglobin concentrations in the presence of excess free nitrite. This potential source of error is easily eliminated by washing nitrited red cell suspensions or by dialyzing or diluting lysates to allow dissociation of the complex. Cyanide is bound more tightly to ferric heme groups than nitrite[4] (2×10^{-8} vs. about 3×10^{-3} as reported here), and thus it can totally displace nitrite even at low cyanide concentrations. The absorbances of methemoglobin–hemoglobin mixtures with added cyanide are, therefore, independent of the presence of an excess of free nitrite in the mixture (cf. D_2 values in Table 1). Similarly, the total blood pigment is quantitatively converted to cyanmethemoglobin just as ferricyanide-generated methemoglobin is converted under the same conditions.

Inadequate washing of nitrite erythrocytes has also been suggested as a potential source of error in the determination of red cell concentrations of reduced glutathione.[16] With the demonstration that nitrite does not seem to have a propensity for directly oxidizing free sulfhydryl groups,[4, 17] this error probably relates to an interference by nitrite in the nitroprusside color reaction, as also suggested by Harley and Robin.[16]

In addition to complex formation with ferric heme groups, however, many additional possibilities must exist for interaction between nitrite and methemoglobin. Nitrite concentrations *in vivo* that convert only a third of the circulating blood pigment of mice to methemoglobin are known to produce other alterations in the molecule associated with the detoxication of injected sodium sulfide.[4, 7] Although these alterations do not appear to be chromogenic,[4] higher concentrations of nitrite must result in side reactions associated with color changes, as evidenced by difficulties encountered in the experiments of Table 4. Fox and Thomson[18] have characterized one such reaction occurring at low pH as a nitrosylation of the heme group.

The results of Fig. 2 are consistent with the hypothesis that the nitrite–methemoglobin complex is formed intra-erythrocytically as well as in simple solution. Indeed there appear to be no valid reasons why this should not be the case. It follows therefore that the formation of this complex may play a role in nitrite-induced methemoglobinemia *in vivo* as well as *in vitro*. Central to the question of evaluating the role of this complex *in vivo* is an estimate of its dissociation constant. Perhaps, for reasons discussed above, this parameter can never be measured precisely, but the results of Table 4 indicate that it is probably not greater than 3 mM. This value agrees well with an estimate by Jung and Remmer[11] of $2{\cdot}5 \times 10^{-3}$ M. Moreover, the results of Fig. 2

indicate that nitrite is bound to ferric heme groups more tightly than fluoride. The dissociation constant of the fluoride–methemoglobin complex at 25°, pH 7·0, and ionic strength of unity has been determined as 25 mM.[19] Although fluoride is bound only weakly by methemoglobin in comparison to cyanide, hydrosulfide or azide,[4] a biological effect has been attributed to its formation *in vivo*. Methemoglobinemia in mice significantly prolongs survival time after lethal doses of fluoride, although mortality is not decreased.[20]

Almost certainly, high doses of nitrite must lead to temporary formation of the nitrite–methemoglobin complex *in vivo*. Indeed, Jung and Remmer[11] have detected this complex in rats acutely poisoned with a large dose of sodium nitrite. Its role at nitrite doses that are tolerated by animals is perhaps open to question. A dose of 1·1 m-mole/kg of sodium nitrite in mice produces a methemoglobinemia that reaches peak values of about a third of the total circulating blood pigment.[12, 20] Although data on free nitrite concentrations under these conditions are not available, neglecting that fraction of injected nitrite which reacts during the first 20 min and assuming that the total dose is distributed in 500 ml/kg of total body water, the resulting concentration would be about 2 mM. Similarly, *in vitro*, a one-third conversion of the pigment in washed mouse red cells to methemoglobin requires an added nitrite concentration of 2·25 mM.[15] Conversion of a third of the total blood pigment of mice to methemoglobin is equivalent to a ferric heme group concentration in blood of between 2 and 3 mM. Therefore, reactant concentrations would appear to be present in ranges favorable to complex formation. Obviously, the demonstration of the existence of this complex *in vivo* at such doses of nitrite would be extremely difficult if not impossible. The dilution of blood necessary to demonstrate the existence of this complex spectrophotometrically would probably be adequate to effect its dissociation.

If this complex is formed transiently *in vivo*, it may influence the time course of a nitrite-induced methemoglobinemia. Two possible mechanisms may be operative, both of which would tend to sustain the methemoglobinemia: (1) the complex may be resistant to the action of methemoglobin reductases, or (2) the complex may serve as a temporary sink for nitrite ion until the local concentration falls enough to allow dissociation and the subsequent oxidation of other ferrous heme groups. In mice the methemoglobinemic response to nitrite has been noted to be atypically slow and persistent in comparison with a variety of other oxidants not recognized to bind to ferric heme sites.[12]. Transient formation of the nitrite–methemoglobin complex suggests itself as one possible factor contributing to this phenomenon. Other factors, such as an influence of nitrite on methemoglobin reductase and the kinetics of the nitrite–hemoglobin reaction, may also play important roles and are under investigation. It seems reasonable to speculate that, since nitrite forms complexes with ferric heme groups, part of its lethal potential may be related to cytochrome oxidase inhibition and its additional ability to form methemoglobin may serve as a self-protective mechanism against acute nitrite poisoning. Certainly, this phenomenon has relevance to any kinetic analyses of the reaction between nitrite and hemoglobin.

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REFERENCES

1. K. A. EVELYN and H. T. MALLOY, *J. biol. Chem.* **126**, 655 (1938).
2. D. L. DRABKIN and R. B. SINGER, *J. biol. Chem.* **129**, 739 (1939).
3. A. E. MIRSKY and M. L. ANSON, *J. gen. Physiol.* **19**, 439 (1936).
4. R. P. SMITH and R. E. GOSSELIN, *Toxicol. appl. Pharmac.* **8**, 159 (1966).
5. J. STEINHARD, in *Conference on Hemoglobin*, p. 154. Publication 157, National Academy of Sciences, National Research Council (1958).
6. R. P. SMITH, *Molec. Pharmac.* in press.
7. R. P. SMITH, *Fedn Proc.* **26**, 650 (1967).
8. A. HAINLINE, JR., in *Standard Methods of Clinical Chemistry* (Ed. S. MEITES), Vol. 5, p. 143. Academic Press, New York (1965).
9. E. J. VAN KAMPEN and W. G. ZILJSTRA, in *Advances in Clinical Chemistry* (Eds. H. SOBOTKA and C. P. STEWART), Vol. 8, p. 176. Academic Press, New York (1965).
10. O. W. VAN ASSENDELFT and W. G. ZILJSTRA, *Clinica chim. Acta* **11**, 571 (1965).
11. F. JUNG and H. REMMER, *Arch. exp. Path. Pharmak.* **206**, 459 (1949).
12. R. P. SMITH, A. A. ALKAITIS and P. R. SHAFER, *Biochem. Pharmac.* **16**, 317 (1967).
13. T. LEAHY and R. P. SMITH, *Clin. Chem.* **6**, 148 (1960).
14. T. LEAHY and R. P. SMITH, *Clin. Chem.* **7**, 148 (1961).
15. J. M. STOLK and R. P. SMITH, *Biochem. Pharmac.* **15**, 343 (1966).
16. J. D. HARLEY and H. ROBIN, *Blood* **20**, 710 (1962).
17. R. P. SMITH and R. A. ABBANAT, *Toxicol. appl. Pharmac.* **9**, 209 (1966).
18. J. B. FOX, JR. and J. S. THOMSON, *Biochemistry, N.Y.* **3**, 1323 (1964).
19. H. M. PERRY, JR., *Archs Biochem. Biophys.* **67**, 398 (1957).
20. R. A. ABBANAT and R. P. SMITH, *Toxicol. appl. Pharmac.* **6**, 576 (1964).