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Chemicals Reacting with Various Forms of Hemoglobin: Biological Significance, Mechanisms, and Determination

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ABSTRACT: The clinical chemistry of various forms of hemoglobin occupies a large fraction of the total activities of hospital and forensic science laboratories. Some of the potential pitfalls are reviewed here along with an account of the accidental discovery of two novel chemical forms. Human or mouse red blood cells were exposed to excess sodium nitrite to convert the intracellular pigment to methemoglobin. When these were subsequently incubated in Krebs-Ringer-phosphate-glucose medium, pH 7.4 at 37°C under nitrogen and in the presence of various concentrations of methylene blue, a blood pigment was generated in high yield which had unique properties. In lysates, the pigment was stable in air, and it could be maintained in liquid nitrogen for as long as a year without deterioration. The pigment had properties different from those of oxyhemoglobin, deoxyhemoglobin, methemoglobin, or carboxyhemoglobin. After separation by isoelectric focusing, the pigment gave a strong signal on electron paramagnetic resonance (EPR) spectroscopy. The other forms of hemoglobin given above are EPR-silent. The pigment was eventually identified as the nitrosylated valency hybrid species, $(\alpha^{2+}\beta^{3+})_2(NO)_2$. The corresponding species, $(\alpha^{3+}\beta^{2+})_2(NO)_2$, has similar properties. These species apparently owe their unusual stability in air to the presence of the oxidized subunits in the same tetramer.

KEYWORDS: toxicology, hemoglobin, valency hybrids and nitrosylated species, valency hybrids of hemoglobin, nitric oxide liganded to hemoglobin

Simple laboratory methods for the determination of various forms of hemoglobin fascinated early workers, and some have been used for many years. The wide acceptance of the infallibility of many of these techniques belies potential pitfalls for the unwary. Some of these are reviewed here, together with precautions for avoiding them.

Spectrophotometry of Carboxyhemoglobin and Methemoglobin

Two simple spectrophotometric assays performed on whole blood, namely analyses for carboxyhemoglobin and for methemoglobin, have much in common [*1,2*]. Both are based on absorbance readings taken before and after the addition of a chemical, so that it is immediately obvious whether the test is positive or negative. The assay for carboxyhem-

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oglobin is based on preliminary treatment of a highly diluted and lysed blood sample with sodium dithionite. Under these conditions carboxyhemoglobin is unaffected, but oxyhemoglobin is quickly converted to deoxyhemoglobin. When potassium ferricyanide is added subsequently, the deoxyhemoglobin is immediately converted to methemoglobin, and an optical density reading is taken at 635 nm. Some minutes later, the carboxyhemoglobin, which is more stable toward ferricyanide, is also oxidized to methemoglobin, and a second absorbance reading is taken. The difference, $(D_2 - D_1)$, is proportional to the concentration of carboxyhemoglobin present at the start. The original method was calibrated to give results in terms of volume percentage of carbon monoxide (CO). In order to convert that to the more familiar and convenient parameter of percentage of hemoglobin saturation, it was necessary to perform a separate total hemoglobin determination by the Drabkin spectrophotometric method, which involves conversion of the total pigment to cyanmethemoglobin by potassium ferricyanide and potassium cyanide and measuring the optical density at 540 nm.

The separate determination of total hemoglobin in the method is, in fact, superfluous [3]. The absorbance reading, D_2, is proportional to the total hemoglobin in the form of methemoglobin. Therefore, the expression $K(D_2 - D_1)/D_2$ should be proportional to the percentage of the total hemoglobin present originally as carboxyhemoglobin, or the percentage of carbon monoxide saturation. All that needs to be done to derive a value for the calibration constant, K, is to saturate a blood sample with CO, and run through the analysis, in which case $K = D_2/(D_2 - D_1)$. The assay and its calibration exhibit a satisfying symmetry in that the same reagents are used for both, and the one is procedurally the reciprocal of the other [3].

A similar thing can be done with the analysis for methemoglobin by the classical technique of Evelyn and Malloy [2], which dates back to 1938 but is still widely used today. Here an initial reading, D_1, is taken and then excess sodium cyanide is added and a second reading, D_2, is made. If methemoglobin had been present originally in the sample, it would combine with cyanide ion to form cyanmethemoglobin. Thus, the difference, $D_1 - D_2$, is proportional to the methemoglobin concentration. Again, a separate analysis for total hemoglobin allowed calculation of the methemoglobin percentage of the total blood pigment. However, since excess cyanide was present at the time of the D_2 reading, it is only necessary at that point to add excess ferricyanide, dilute it, and change wavelengths to have the equivalent of a Drabkin's assay or D_3 [4]. The expression $K(D_1 - D_2)/D_3$ is proportional to the percentage of methemoglobin; the calibration constant, K, can be derived from a blood sample that is totally in the form of methemoglobin, using the reciprocal relationship and all the same reagents, $K = D_3/(D_1 - D_2)$. These same chemical principles used for methemoglobin analysis have been exploited also in one approach to the management of cyanide poisoning in human patients [5] in that a deliberately induced methemoglobinemia avidly traps free cyanide as the stable complex, cyanmethemoglobin.

With only minor variations around the same theme, this technique can be used to determine oxyhemoglobin (HbO_2), or deoxyhemoglobin (Hb), or both. If after the D_1 reading, ferricyanide is added instead of cyanide and D_2 is taken, then the expression $K(D_1 - D_2)$ is proportional to the total concentration of reduced heme whether it is liganded with oxygen or not. Cyanide is then added for the D_3 reading as above. This method gives accurate results even in the presence of vital dyes such as methylene blue [6].

Methemoglobin and Hydrogen Sulfide

Stimulation of the carotid body chemoreceptors, the fulminating central respiratory arrest, and inhibitory effects on cytochrome oxidase produced by exposure to toxic

concentrations of hydrogen sulfide are all highly reminiscent of cyanide poisoning [5]. Many years ago Linus Pauling and his associates described a complex between methemoglobin and the sulfide (HS^-) anion [7]. Sulfmethemoglobin, as it was called, was exactly analagous to cyanmethemoglobin, but the relationship between sulfmethemoglobin and the so-called sulfhemoglobin, which was part of the Evelyn and Malloy [2] method for measuring oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood, is not at all clear.

It was eventually shown that the induction of methemoglobinemia with sodium nitrite was both protective and antidotal against acute sulfide poisoning in laboratory animals, including mice, rats, rabbits, and armadillos, because of the transient formation of sulfmethemoglobin. The procedure has been used subsequently in the successful resuscitation of several human victims of hydrogen sulfide poisoning [8–11]. Despite literature reports to the contrary, exposure of animals to lethal amounts of sulfide did not result in the formation of sulfhemoglobin or any other abnormal form of hemoglobin in blood. Hydrogen sulfide appears to be a cellular poison and not a blood one [12]. The term sulfhemoglobin appears to have been coined by the great physiological chemist Hoppe-Seyler in 1866 when he noted the formation of a greenish pigment as pure hydrogen sulfide was passed through a sample of shed blood [13]. This abnormal pigment was not reactive toward cyanide.

"Sulfhemoglobin"

It is possible that when Evelyn and Malloy were applying their method for methemoglobin to blood specimens from a variety of patients exposed occupationally to various xenobiotics or taking "oxidant" drugs such as acetanilid, acetaminophen, or sulfanilamide, they sometimes found an absorption maximum in the range of 620 nm which was not abolished by the addition of cyanide. This peak was in addition to the 635-nm peak for methemoglobin, and it was most clearly seen after the 635-nm band had been abolished by cyanide addition. Because of what in retrospect may have been only coincidences, Evelyn and Malloy may have ascribed this peak to the "sulfhemoglobin" of Hoppe-Seyler. It did not seem to bother them that many of the patients which appeared to have sulfhemoglobin in their blood were not known to have been exposed to an exogenous source of sulfur. Apparently they assumed that some endogenous sulfur donor could also participate in the reaction.

It is possible that this old and confusing literature arose from three different and unrelated phenomena. The first and the best defined is sulf*met*hemoglobin (above), a pigment in which sulfide anion (HS^-) is ionically bound to ferric heme groups. Its distinct absorption spectrum is seen immediately after adding sodium or hydrogen sulfide to a solution of methemoglobin at neutral pH. The second phenomenon might be called true sulfhemoglobin. Although it has not been generated in vivo, its formation requires exogenous sulfide or some other unknown form of sulfur, together with only distinctly unphysiologic chemicals and conditions. Such pigments have been generated in high yield, but only in vitro. It may be a single pigment in which sulfur has replaced nitrogen at one or more sites in the heme prophyrin. The third phenomenon, encountered by Evelyn and Malloy in vivo, may be a mixture of partially oxidized and denatured pigments that might better be called pseudo-sulfhemoglobin. This phenomenon occurs most consistently in connection with glucose-6-phosphate dehydrogenase deficiency after a triggering chemical exposure has initiated the full-blown triad of sulfhemoglobin, Heinz bodies, and hemolysis. Some of the globin probably exists as mixed disulfides with glutathione, and denatured hemoglobin eventually binds to the interior of the red cell membrane to form Heinz bodies. Much of the absorbance at 620 nm may be due simply to turbidity [14]. In any event, the application of the Evelyn and Malloy method for sulfhemoglobin [2] has no valid basis in cases of hydrogen sulfide poisoning.

Methemoglobinemia

Many known methemoglobin-generating chemicals have been tested and characterized as to their dose-response relationships and the time courses of their effects. The generally accepted view is that the chief mechanism for reversal and termination of a chemically acquired methemoglobinemia was through the activity of an endogenous, erythrocytic enzyme known first as diaphorase and then as methemoglobin reductase. Today the enzyme is known to be cytochrome b_5 (EC 1.6.2.2), and it requires nicotinamide adenine dinucleotide (NADH) as its cofactor.

There are some remarkable and as yet unexplained species differences in the activity of cytochrome b_5 in that the red cells of small laboratory rodents have extremely brisk rates of activity, whereas those of larger animals from cat on up, including man, have rather more sluggish systems [*12*]. The difference in rates of methemoglobin reduction between mice and humans is at least tenfold, if not more. When red cells are lysed, methemoglobin reductase activity virtually comes to a standstill. In lysates of mouse and human red cells, a chemical like sodium nitrite generates about equal levels of methemoglobin. It was previously thought that mice and rats were particularly resistant to chemicals that generate methemoglobin. The preceding observation indicates that human and mouse hemoglobins are about equally sensitive to oxidants and that the differences in vivo or in intact red cells with substrate may be ascribed instead to differences in rates of reductase activity. For reasons that are not entirely clear, rat hemoglobin is uniquely less water soluble than human or mouse hemoglobin; thus, it is difficult to prepare lysates or solutions of rat hemoglobin in concentrations that approximate total cellular hemoglobin in their blood [*15*].

The chemicals that generate methemoglobin (Fig. 1) can be classified in several ways

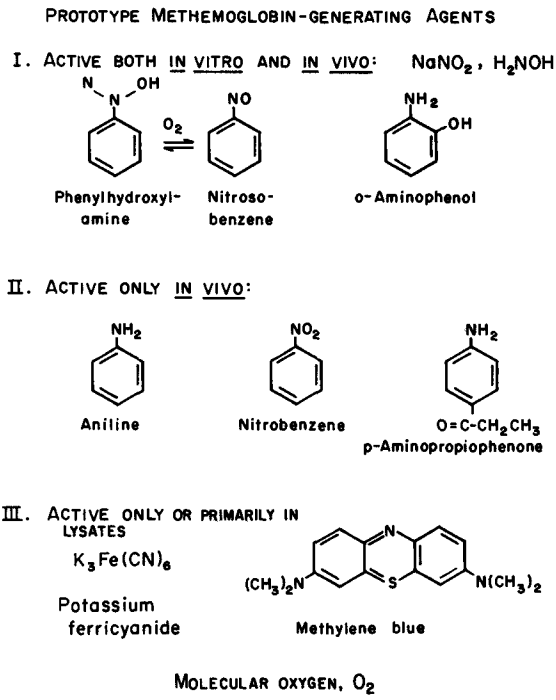

FIG. 1—*A classification scheme for methemoglobin-generating xenobiotics (adapted with permission from Ref* 16).

[16]. In terms of potency sodium nitrite, hydroxylamine, and aminophenols seemed to react with ferrous heme groups with a stoichiometry of 1:1 or less whether tested in intact cells or in lysates. In contrast, one mole of phenylhydroxylamine generated much more than one equivalent of oxidized heme in intact red cells provided with substrate, but in lysates or in substrate-starved cells, phenylhydroxylamine also reacted with a 1:1 stoichiometry or less. The obvious explanation is that an energy-consuming reaction is responsible for recycling phenylhydroxylamine to its active form in intact cells. The intermediate form is now believed to be nitrosobenzene, and the enzyme involved in the recycling is believed to require nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. The requirement for NADPH implicates the involvement of the pentose phosphate shunt as opposed to the glycolytic sequence which generates NADH.

Nitrite is doubly unique among all the chemicals in Fig. 1. First, it can form an ionic complex with ferric heme groups analogous to those formed by cyanide or sulfide (above), although it is considerably less stable. The nitrite-methemoglobin complex has no known physiological significance, but it can interfere in methemoglobin analyses. If, for example, excess nitrite is used to oxidize hemoglobin to methemoglobin, some of the excess will form a complex, resulting in an underestimation of methemoglobin levels [17]. Second, some evidence exists to suggest that nitrite or a product of the nitrite-hemoglobin reaction can inhibit cytochrome b_5, which could account for the fact that a nitrite-generated methemoglobinemia in some species is unusually prolonged, relative to that of any other type in vivo or in erythrocyte suspensions with substrate [18].

Another way of subdividing methemoglobin-generating chemicals (Fig. 1) is in terms of whether they are active only in vivo or equally active in vivo and in vitro [16]. Obviously, those that are active only in vivo must undergo biotransformation to active forms. Presumably, these reactions are mediated via hepatic mixed-function oxidases. The prototype chemicals in this group are aniline and nitrobenzene, but almost any aromatic amino- or nitro-compound must be suspect as a potential methemoglobin former. The active metabolites may be either phenyl-*N*-hydroxylamines or aminophenols, which exhibit prominent potency differences, as indicated above. The alleged sensitivity of the cat to methemoglobin-generating chemicals is due both to a sluggish reductase system and a relative inability to detoxify aromatic amines.

There are three exceptional chemicals (Fig. 1) that are primarily active in lysates, but for different reasons [16]. The spectrophotometric method for methemoglobin analyses is usually calibrated with ferricyanide, which is totally excluded from intact red cells because of its large molecular diameter and high degree of charge. Ferricyanide is also unusual in that it is the only methemoglobin-generating chemical known in which the oxidation in solution results in a quantitative release of the heme oxygen [16]. With all other oxidants tested, part or nearly all of the heme oxygen is consumed during the reaction. Two other chemicals readily penetrate the red cell membrane, but are still much more active in lysates–namely, molecular oxygen (autoxidation) and methylene blue. Both of these act so slowly in intact cells that even the most sluggish reductase can maintain the equilibrium far in the direction of reduced hemoglobin. In lysates, however, methemoglobin slowly accumulates, and the reaction can go all the way to completion with either agent. Even the method of preparing lysates may be critical. Repeated freezing and thawing is probably safe, but some detergents have been shown to catalyze the rate of autoxidation of hemoglobin [19].

Methylene Blue

The above reaction also makes methylene blue doubly interesting since it is well known as an antidote in cases of acquired methemoglobinemia. Methylene blue accumulates in intact normal red cells provided with substrate against high-concentration gradients. It

is reduced by an intraerythrocytic enzyme which has no known physiological function but which requires NADPH as a cofactor. Very rare patients who lack this enzyme seem to have no physiological compromise. In its reduced, leuco-form the dye is colorless, and its redox potential is favorable for the nonenzymatic reduction of methemoglobin. The oxidized dye is then available for recycling by the reductase. In the absence of methemoglobin, the leuco-dye will react with molecular oxygen to increase greatly the oxygen consumption of red cells. In human cases of acquired methemoglobinemia, the intravenous injection of an appropriate dose of methylene blue can accelerate rates of methemoglobin reduction as much as tenfold. Thus, in intact cells, the methemoglobin-generating activity of methylene blue is masked because the reaction equilibrium is far in the direction of reduced hemoglobin [*12*]. The equilibrium, however, is set a little more in the direction of methemoglobin with methylene blue present than in its absence, and because the rate of hemoglobin-methemoglobin turnover is greatly increased, there is more methemoglobin present at any one point in time than in cells not exposed to the dye. This phenomenon is believed to account for the weak anticyanide activity of methylene blue, an effect that has been confirmed many times [*20*].

The absolute requirement for NADPH is a contraindication for the use of methylene blue for any purpose in patients with glucose-6-phosphate dehydrogenase deficiency. In such patients, methylene blue is only another of the scores of offending chemicals which can initiate the triad of "sulfhemoglobin," Heinz bodies, and hemolysis [*12*]. A systematic investigation of the factors that favored methemoglobin oxidation by methylene blue showed that the major factor was hemolysis, but it was also favored by anaerobic conditions, and there was a small photochemical component. Conversely, methemoglobin reduction by methylene blue required intact cells but was also favored by anaerobic conditions, and it proceeded a little more rapidly in the dark [*20*].

"Hemoglobin X"

Human red cells in which all the hemoglobin had been converted to methemoglobin by exposure to excess sodium nitrite were incubated in Krebs-Ringer-phosphate-glucose, pH 7.4 in 37°C, with or without methylene blue, and under either nitrogen or oxygen (Table 1). Total hemoglobin was estimated by the conventional method of Drabkin and methemoglobin by its spectrophotometric method [*4*]. Oxyhemoglobin and deoxyhe-

TABLE 1—*Composition of the blood pigment in incubation mixtures of human red cells exposed to sodium nitrite, washed, and resuspended with or without methylene blue, under oxygen or under nitrogen.*[a]

Methylene Blue, m*M*	Gas Phase		Composition, %		
		HbO_2	Hb	MetHb	HbX
0	O_2	31 ± 2	0	76 ± 2	0
	N_2	0	0.5 ± 0.8	76 ± 7	23.1
10	O_2	90 ± 9	0	8 ± 4	1.5
	N_2	0	3 ± 2	5 ± 3	92
100	O_2	52 ± 36	12 ± 11	8 ± 2	28
	N_2	0	1 ± 1	13 ± 3	86

[a]Red cells exposed to excess sodium nitrite (90 to 100% MetHb) were washed with fresh media, resuspended with or without methylene blue and incubated for 4 h at 37°C, pH 7.4 under nitrogen or under oxygen. The MetHb was determined spectrophotometrically. Deoxy- and oxy-hemoglobin were determined with an oxygen electrode before and after aeration of an anaerobic sample. HbX represents the difference between the sum of the three determined pigments above and the total hemoglobin as determined spectrophotometrically.

moglobin were determined by several different methods, but all gave essentially the same results. Oxyhemoglobin was sometimes determined by the manometric method of Van Slyke, by a variation of that technique in which the oxygen liberated by ferricyanide was measured polarographically, or by a spectrophotometric method which involved oxidation of the pigment to methemoglobin, as alluded to previously [*6*]. In each case, any difference in the assay results between an anaerobic sample and an aerated one was ascribed to deoxyhemoglobin. All of these techniques are widely accepted as valid, and some have been in use for more than half a century.

The sums of the various components of the mixture were determined after incubation for 4 h, and these were subtracted from the total hemoglobin as determined by Drabkin's assay. The totally unexpected finding was that, under certain conditions, a large fraction of the total pigment could not be accounted for as any of the known forms of hemoglobin (Table 1). There was no reason to think that carboxyhemoglobin would be generated under these conditions, but even if it had been, it would have been measured as part of the oxyhemoglobin by two of the three methods used. This unknown fraction was dubbed "hemoglobin X," and it was generated in highest yield in the presence of methylene blue under anaerobic conditions (Table 1).

To put this observation in some perspective, hemoglobin is the most intensively studied protein of all time. It has attracted the meticulous attention of some of the best scientific minds in the world. It seemed highly unlikely that a previously unrecognized and chemically unknown form of hemoglobin should suddenly come to light. So, hemoglobin X began a covert existence that lasted nearly 20 years. Its discovery was alluded to in only one short paper and one abstract [*21,22*]. After numerous failed experiments, drawers full of uninterpretable absorption spectra, and many unsuccessful attempts at electrophoretic separation, its existence was confirmed over and over again, but its chemical nature remained unknown.

Valency Hybrid Species

Then in the late 1970s and early 1980s a series of papers by a Japanese group headed by Dr. A. Tomoda began to appear (see, for example, Refs *23* and *24*). Working with mixtures of partially oxidized hemoglobin and partially reduced methemoglobin, Tomoda and his co-workers had proven that these reactions proceeded by two pathways (Fig. 2) through intermediate forms which were valency hybrids, $(\alpha^{2+}\beta^{3+})_2$ and $(\alpha^{3+}\beta^{2+})_2$. Although it was possible to force the reactions to completion in either direction to the fully oxidized or fully reduced tetramers, at the halfway point the valency hybrids (for convenience designated Types I and II) predominated in the mixture. There were certain similarities between this phenomenon and that of hemoglobin X which seemed to go beyond mere coincidence. For example, methylene blue generated the hybrid species when the starting material was methemoglobin in red cells, and the generation of Type I was favored over the generation of Type II under anaerobic conditions.

Unfortunately, there was also some rather compelling evidence against the valency hybrids being related to hemoglobin X. The Japanese group had shown that the oxidized subunits on both species were measured as methemoglobin, along with any totally oxidized tetramer present. Moreover, the hybrid species had been artificially prepared some years previously by separating the α and the β subunits, and then oxidizing one or the other, followed by reconstitution of the valency hybrid tetramers. In those cases, the reduced subunits of both tetramers clearly bound molecular oxygen and, therefore, would be measured as part of the total oxyhemoglobin [*25*]. Since the respective subunits of each tetramer were measured by standard techniques as part of the total pigment, it did not seem possible that the hybrid species had anything to do with hemoglobin X. Hemoglobin X could only be explained by a pigment that was not measured by the standard techniques.

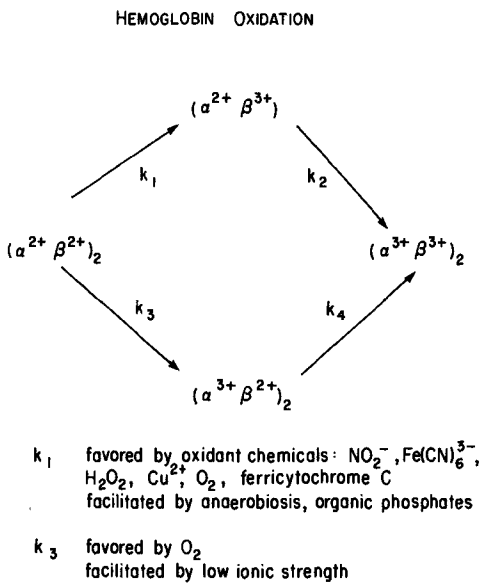

FIG. 2—*Pathways for hemoglobin oxidation in going from the fully reduced to the fully oxidized tetramer. It has been established that all oxidized subunits are determined as part of the total methemoglobin and that all reduced subunits bind oxygen and can be measured as part of the total oxyhemoglobin. The reverse pathways are utilized in the reduction of the fully oxidized to the fully reduced tetramer. In that case, the reverse reaction for k_4 is exclusive for ascorbate and favored by both red cell reductase systems, anaerobiosis and organophosphates* [23,24].

Nevertheless, the Japanese group had discovered that all four valency forms could be separated by isolectric focusing across a pH gradient and all could be determined at the same time by passing the focused gel through a densitometer. In applying this technique, it was found that, under conditions which led to the generation of high concentrations of hemoglobin X, the mixture also contained high concentrations of what appeared to be the valency hybrid species Type I. This analytical method was then scaled up to preparative isolectric focusing in order to obtain enough of each of the four species to subject them to standard methods of analysis for the various forms of hemoglobin. Astonishingly, the species which appeared in the same position as that of the hybrid species Type I did not bind molecular oxygen, even though it clearly contained reduced α subunits. Another very interesting finding was that when hemoglobin X was in solution instead of in red cells, with or without methylene blue, it was extremely stable in air. Frozen samples have been preserved for many months without deterioration.

Nitrosylated Species

Unfortunately, there was still no adequate explanation for why the reduced subunits failed to bind oxygen. Such a pigment had never been described before. If indeed the α-subunits were reduced, one possible reason why they could not bind molecular oxygen might be that those sites were already occupied by another ligand which was bound much more tightly. Carbon monoxide meets those criteria, but there was no reason to think that CO was generated under those conditions. Moreover, as indicated above, carboxyhemoglobin should have been measured by at least some of the techniques used for oxyhemoglobin. Another ligand that is bound thousands of time more tightly than oxygen and hundreds of times more tightly than carbon monoxide is nitric oxide (NO). It was

known that NO could be generated from sodium nitrite under certain conditions. Unfortunately, it was also known that nitrosyl hemoglobin was unstable in air where it is rapidly oxidized to methemoglobin.

One of the best ways to test for NO-hemoglobin is by a technique known as electron paramagnetic resonance spectroscopy. When a sample was at last analyzed by this technique, it was to prove (Fig. 3) absolutely that hemoglobin X was the $(\alpha^{2+}\beta^{3+})_2$ or valency hybrid Type I with NO liganded to the reduced α subunits [*26*]. Since then, the nitrosylated version of the other valency hybrid, Type II, in which NO is liganded to the reduced β subunits, has also been prepared. Both pigments are reasonably stable in air when in solution, but not in intact red cells provided with substrate and especially not in the continued presence of methylene blue. Since, under those conditions, the respective oxidized subunits are reduced either by cytochrome b_5 or by the methylene blue-activated system, it appears that the unusual stability of the nitrosylated forms is due to the presence of the oxidized subunits.

The technique has since been used to show that a wide variety of chemical structures that have come to be known as the nitric oxide vasodilators can in fact undergo biotransformation in red cell suspensions or in intact mice to yield NO [*27,28*], including hydralazine, nitroglycerin, nitroprusside, hydroxylamine, and azide. In an ironic footnote, it was subsequently discovered that hemoglobin X had actually been prepared and studied some years earlier by a laborious synthetic procedure [*29*]. Nothing was said in that communication about its stability in air because the investigator had taken great pains to keep it always under nitrogen.

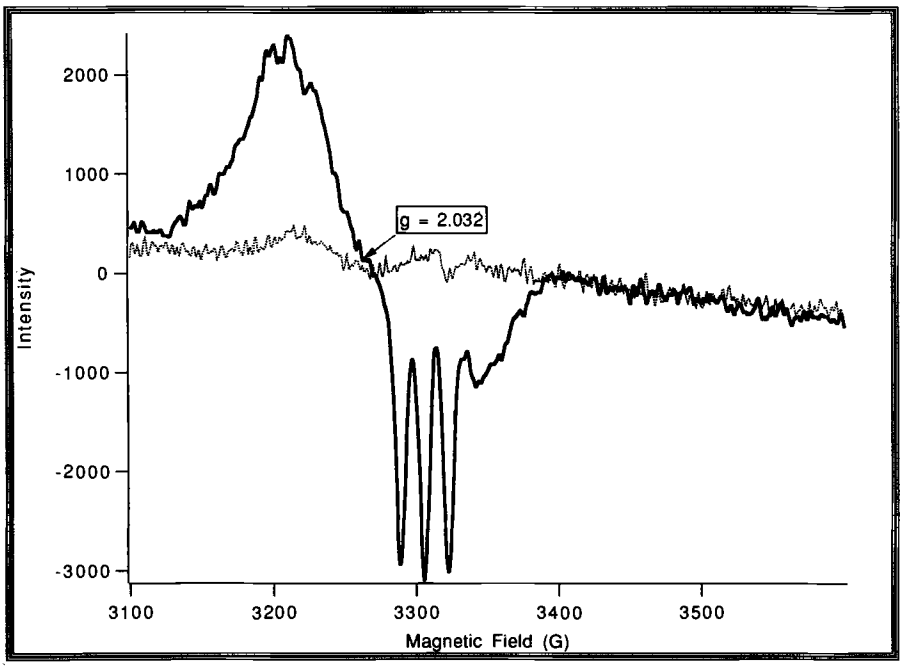

FIG. 3—*The frozen EPR spectra for hemoglobin X in the presence of 1mM inositol hexaphosphate* (solid line) *and for the Type II valency hybrid species* (dash-dot line) *(see text). DPPH is indicated for purposes of calibration. All blood pigments are 0.5 mM in heme in 0.1M bis-Tris buffer containing 0.1M NaCl (adapted with permission from Ref* 26).

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References

[1] Harper, P. V., "A New Spectrophotometric Method for the Determination of Carbon Monoxide in the Blood," *Journal of Laboratory and Clinical Medicine*, Vol. 40, No. 4, Oct. 1952, pp. 634–640.

[2] Evelyn, K. A. and Malloy, H. T., "Microdetermination of Oxyhemoglobin, Methemoglobin and Sulfhemoglobin in a Single Sample of Blood," *Journal of Biological Chemistry*, Vol. 126, No. 2, Dec. 1938, pp. 655–662.

[3] Berninger, H. and Smith, R., "A Modification of the Spectrophotometric Determination of Carbon Monoxide in Blood Enabling Direct Determination of Per Cent Hemoglobin Saturation," *Clinical Chemistry*, Vol. 5, No. 2, April 1959, pp. 127–134.

[4] Leahy, T. and Smith, R., "Notes on Methemoglobin Determination," *Clinical Chemistry*, Vol. 6, No. 2, April 1960, pp. 148–152 and "Erratum," *Clinical Chemistry*, Vol. 7, No. 2, April 1961, p. 148.

[5] Gosselin, R. E., Smith, R. P., and Hodge, H. C., *Clinical Toxicology of Commercial Products*, 5th ed., Williams and Wilkins, Baltimore, 1984.

[6] Smith, R. P., "Spectrophotometric Determination of Methemoglobin and Oxyhemoglobin in the Presence of Methylene Blue," *Clinical Toxicology*, Vol. 4, No. 2, June 1971, pp. 273–285.

[7] Coryell, C. D., Stitt, F., and Pauling, L., "The Magnetic Properties and Structure of Ferrihemoglobin (Methemoglobin) and Some of Its Compounds," *Journal of the American Chemical Society*, Vol. 59, No. 4, April 1937, pp. 633–642.

[8] Stine, R. J., Slosberg, B., and Beacham, B. E., "Hydrogen Sulfide Intoxication: A Case Report and Discussion of Treatment," *Annals of Internal Medicine*, Vol. 85, No. 6, Dec. 1976, pp. 756–758.

[9] Peters, J. W., "Hydrogen Sulfide Poisoning in a Hospital Setting," *Journal of the American Medical Association*, Vol. 246, No. 14, Dec. 1981, pp. 1588–1589.

[10] Hoidal, C. R., Hall, A. H., Robinson, M. D., Kulig, K., and Rumack, B. H., "Hydrogen Sulfide Poisoning from Toxic Inhalations of Roofing Asphalt Fumes," *Annals of Emergency Medicine*, Vol. 15, No. 7, July 1986, pp. 825–830.

[11] Huang, C.-C. and Chu, N.-S., "A Case of Acute Hydrogen Sulfide (H_2S) Intoxication Successfully Treated with Nitrites," *Journal of the Formosan Medical Association*, Vol. 86, No. 10, Oct. 1987, pp. 1018–1020.

[12] Smith, R. P., "Toxic Responses of the Blood" in *Casarett and Doull's Toxicology, The Basic Science of Poisons*, 3rd ed., Macmillan, New York, 1986.

[13] Mitchell, C. W. and Davenport, S. J., "Hydrogen Sulfide Literature," *Public Health Reports*, Vol. 39, No. 1, 4 Jan. 1924, pp. 1–13.

[14] *Hydrogen Sulfide*, National Academy of Sciences, National Research Council, University Park Press, Baltimore, 1979.

[15] Smith, R. P. and Kruszyna, H., "Nitroprusside Produces Cyanide Poisoning via a Reaction with Hemoglobin," *Journal of Pharmacology and Experimental Therapeutics*, Vol. 191, No. 3, Dec. 1974, pp. 557–563.

[16] Smith, R. P. and Olson, M. V., "Drug-Induced Methemoglobinemia," *Seminars in Hematology*, Vol. 10, No. 3, July 1973, pp. 253–268.

[17] Smith, R. P., "The Nitrite-Methemoglobin Complex—Its Significance in Methemoglobin Analyses and Its Possible Role in Methemoglobinemia," *Biochemical Pharmacology*, Vol. 16, No. 9, Sept. 1967, pp. 1655–1664.

[18] Kruszyna, R., Kruszyna, H., and Smith, R. P., "Comparison of Hydroxylamine, 4-Dimethyl-

aminophenol and Nitrite Protection Against Cyanide Poisoning in Mice," *Archives of Toxicology*, Vol. 49, No. 3/4, March 1982, pp. 191–202.

[19] Kruszyna, H., Kruszyna, R., and Smith, R. P., "Detergent-catalyzed Autoxidation of Hemoglobin—Species Differences and Implications for Methemoglobin Analyses," *Archives of Toxicology*, Vol. 49, No. 1, Nov. 1981, pp. 43–48.

[20] Smith, R. P. and Thron, C. D., "Hemoglobin, Methylene Blue and Oxygen Interactions in Human Red Cells," *Journal of Pharmacology and Experimental Therapeutics*, Vol. 183, No. 3, Dec. 1972, pp. 549–558.

[21] Smith, R. P., "The Oxygen and Sulfide Binding Characteristics of Hemoglobins Generated from Methemoglobin by Two Erythrocytic Systems," *Molecular Pharmacology*, Vol. 3, No. 4, July 1967, pp. 378–385.

[22] Smith, R. P. and Russell, J. T., "A Hemoglobin Variant Induced by Methylene Blue and Low Oxygen Tensions," *Federation Proceedings*, Vol. 28, No. 2, March/April 1969, p. 479.

[23] Nagai, M., Tomoda, A., and Yoneyama, Y., "Reduction of Methemoglobin by Ferridoxin and Ferridoxin-NADP Reductase System," *Journal of Biological Chemistry*, Vol. 256, No. 17, 10 Sept. 1981, pp. 9195–9197.

[24] Tomoda, A. and Yoneyama, Y., "A Simple Method for Preparation of Valency Hybrid Hemoglobins, $(\alpha^{3+}\beta^{2+})_2$ and $(\alpha^{2+}\beta^{3+})_2$," *Analytical Biochemistry*, Vol. 110, No. 1, Jan. 1981, pp. 431–436.

[25] Banerjee, R. and Cassoly, R., "Oxygen Equilibria of Human Hemoglobin Valency Hybrids: Discussion on the Intrinsic Properties of α-and β-Chains in the Native Protein," *Journal of Molecular Biology*, Vol. 42, No. 3, 14 June 1969, pp. 351–361.

[26] Kruszyna, R., Kruszyna, H., Smith, R. P., Thron, C. D., and Wilcox, D. E., "Nitrite Conversion to Nitric Oxide in Red Cells and Its Stabilization as a Nitrosylated Valency Hybrid of Hemoglobin," *Journal of Pharmacology and Experimental Therapeutics*, Vol. 241, No. 1, April 1987, pp. 307–313.

[27] Kruszyna, H., Kruszyna, R., Smith, R. P., and Wilcox, D. E., "Red Blood Cells Generate Nitric Oxide from Directly Acting Nitrogenous Vasodilators," *Toxicology and Applied Pharmacology*, Vol. 91, No. 3, Dec. 1987, pp. 429–438.

[28] Kruszyna, R., Kruszyna, H., Smith, R. P., and Wilcox, D. E., "Generation of Valency Hybrids and Nitrosylated Species of Hemoglobin in Mice by Nitric Oxide Vasodilators," *Toxicology and Applied Pharmacology*, Vol. 94, No. 3, July 1988, pp. 458–465.

[29] Cassoly, R., "Relations Between Optical Spectrum and Structure in Nitrosyl Hemoglobins and Hybrids," *Journal of Molecular Biology*, Vol. 98, No. 3, 5 Nov. 1975, pp. 581–595.

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