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Conversion of Oxyhemoglobin to Methemoglobin by Organic and Inorganic Reductants†

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ABSTRACT: Human oxyhemoglobin is converted to methemoglobin by a wide array of organic and inorganic reductants. Depending upon the concentration and nature of the reductant, varying amounts of deoxyhemoglobin are produced. The general overall sequence is: $FeO_2 \overset{(1)}{\rightarrow} Fe^{III} \overset{(2)}{\rightarrow} Fe^{II}$. The intermediacy of methemoglobin can be demonstrated by direct spectral observation and by cyanide trapping. For organic reductants, the second-order rate constants for (1) vary from >300 (phenylhydroxylamine) to 1.4×10^{-4} M^{-1} s^{-1} (malononitrile). Generally the rates parallel the ease of hydrogen abstraction by iron-bound oxygen from the substrate, and simple hydrocarbons are reactive. Rates for these processes have been ascertained with recrystallized protein, lysed cells, and intact human erythrocytes. At room temperature oxyhemoglobin quantitatively converts benzaldehyde to benzoic acid and hydroquinone to benzoquinone. Rates for inorganic species (process 1) range from $>7 \times 10^3$ (chromous ion) to 0.015 M^{-1} s^{-1} (ferrocyanide). Ferrous ion rapidly deoxygenates oxyhemoglobin by direct attack on the oxy complex but methemoglobin *is not* an intermediate with this reagent. Taken together the results support the theoretical prediction that reductants should oxidize oxyhemoglobin, and they demonstrate at least some degree of radical character to the oxy complex.

A prediction of theory (Castro, 1971) is that oxyhemoglobin should be "oxidized", that is, converted to methemoglobin, by reducing agents. The prediction is a corollary to an explanation of the stability of the iron(II) complex to oxygen. Simply, the G conformation ascribed to the globins sterically prevents the attack of a second iron porphyrin upon the 1:1 oxy complex. These considerations were based primarily on the premise that the mechanism of oxidation of high spin iron(II) porphyrins by oxygen would parallel that of the oxidation of simple metal ions by unsaturated molecules (Castro and Stephens, 1964; Castro et al., 1966) and would entail an intermediate binuclear adduct:

$$M^{n+} + O_2 \rightleftharpoons MO_2^{n}$$
$$MO_2^{n+} + M^{n+} \longrightarrow MOOM^{2n+}$$
$$MOOM^{2n+} \xrightarrow{2H^+} 2M^{n+1+} + H_2O_2$$

While no direct kinetic measurements of the pure high spin systems have been reported, some indirect analysis and qualitative observations lend very strong support to this contention.

Arguments supporting this "axial metal addition" path for the oxidation of high spin iron(II) porphyrins by oxygen have been summarized recently (Castro, 1977), and only the most relevant observations are noted here. Under certain conditions, a term, second order in iron, is found in the rate expression for the oxidation of hemes in pyridine–benzene solutions (Cohen and Caughey, 1968), and solid μ-dioxo adducts were isolated from this reaction (Alben et al., 1968). The extreme sensitivity to oxygen characteristic of the high spin iron(II) complexes is exhibited by pentacoordinate imidazole adducts (the inner coordination sphere of hemoglobin) in amide solvents at room temperature (Castro, 1974). On the other hand, a variety of iron(II) porphyrins reversibly bind oxygen at low temperatures, and 1:1 oxygen complexes have been observed with N-substituted imidazole (Traylor and Chang, 1973; Almog et al., 1974), *N*-butyl mercaptide (Chang and Dolphin, 1976), or no

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added or affixed axial ligand at all (Almog et al., 1974; Basolo et al., 1975). Moreover, a one-sided "capped" *meso*-tetraphenylporphyrin complex will reversibly oxygenate at room temperature in pyridine (Almog et al., 1975). Finally, an iron oxy adduct of a sterically encumbered "picket fence" porphyrin maintains its integrity in the presence of a variety of trans axial bases, and crystalline 1:1 adducts with oxygen in this latter system have been fully characterized (Collman et al., 1975).

We summarize here our studies of the reaction of human oxyhemoglobin in solution, lysed cells and intact erythrocytes with organic and inorganic reductants. All reactions were conducted under the same conditions of pH and ionic strength. The results provide general validity for the above formulation, and they have a direct bearing upon the mechanism of drug-induced methemoglobinemia.

Experimental Procedures

Materials. The following inorganic salts were commercial analytical reagent grade and used without purification: $Fe(NH_4)_2(SO_4)\cdot 6H_2O$, $MnCl_2\cdot 4H_2O$, $CuCl_2$, $CoCl_2\cdot 6H_2O$, $NiCl_2\cdot 6H_2O$, $CaCl_2$, $Mg(C_2H_3O_2)_2\cdot 4H_2O$, $Fe(Cl)_3\cdot 6H_2O$, $CrCl_3\cdot 6H_2O$, and $Na_2S_2O_4$. Special care was taken with sodium dithionite in that the usual 10^{-1} M stock solution under argon in argon-purged water was prepared immediately before use. Chromous perchlorate (0.4 M) was prepared as previously described (Zurqiyah and Castro, 1969).

Organic substrates had physical constants that checked the literature, or they were purified prior to use. Benzaldehyde and acetylacetone were freshly distilled, and hydroquinone was recrystallized and sublimed.

Oxyhemoglobin. A concentrated solution of recrystallized hemoglobin (Sigma type IV) was treated with solid sodium dithionite (1 mg/mL) and immediately poured through a freshly prepared 1 × 20 cm column of Bio-Rad AG-1-X8 gel to remove dithionite and other salts. The oxy complex emanating from the column (5×10^{-3} to 2×10^{-2} M) was stored in air at 2 °C. An aliquot of this stock solution was added to the requisite amount of sodium phosphate–saline buffer (argon purged) so that a final solution of the oxy complex resulted that was 5×10^{-5} to 2×10^{-4} M in FeO_2 at pH 7.4 and contained 0.11 to 0.14 M NaCl or KCl and 0.01 M buffer. This medium was employed throughout for all reactions. The slightly higher ionic strength (0.14 M NaCl) was employed with intact erythrocytes and lysed cells. A change from 0.1 M KCl to 0.14 M NaCl did not significantly alter the rates of reaction of the recrystallized protein. The final solution was stored in a three-necked flask under argon. The flask was equipped with a magnetic stirring bar, inlet and outlet stopcocks, and a serum capped stopcock. Samples for kinetic analysis were transferred to serum capped cuvettes under argon by pressuring the solution through a no. 18 stainless steel hypodermic tube. The dilute solution was stable for about 24 h. An alternate apparatus for product work consisted of a 50-mL tube type flask similar to the above but possessing an addition funnel that could be separately sealed and flushed and a bottom stopcock fitted with a small ground glass joint such that cuvettes (containing inlet and outlet stopcocks) could be mounted directly to it. This system could be successively purged and evacuated, and the cuvettes could be filled rapidly with the flask contents under argon. Human erythrocytes were washed with the above saline buffer and centrifuged. This operation was repeated three times at 5 °C. The washed cells were suspended in the saline buffer in a three-necked flask in the fashion described above for recrystallized hemoglobin. The concentration of stock oxy complex suspension (assuming a homogeneous solution) was 5×10^{-5} to 2×10^{-4} M. Lysed cells were obtained from an aliquot of the above suspension by freezing in a CO_2–2-propanol bath and allowing to thaw to room temperature. The oxyhemoglobin content was not changed during this operation.

TABLE I: Extinction Coefficients for Human Hemoglobin.

	λ (nm)		$\epsilon \times 10^{-3}$ for[c] Fe^{II}	FeO_2	Fe^{III}
(a)	577	Whole or lysed cells[a,b]	9.50	15.0	3.80
(b)	560		12.0	8.80	3.48
(c)	542		10.6	14.0	6.0
(a)	575	Recryst	9.90	15.0	4.75
(b)	558		13.1	9.17	4.80
(c)	540		11.2	14.4	6.75

[a] For whole cells ε's calculated after subtraction of absorbance at 800 nm. [b] Treatment of oxygenated cells with $NaNO_2$ + KCN gave an ε of 11.1×10^3 at 540 nm for the $Fe^{III}CN$. [c] Note all ε's are reported per heme of the Hb tetramer, i.e., $4(Hb^+) = (Fe^{III})$.

Optical Properties. While close, a variety of extinction coefficients are reported for hemoglobin derivatives in the literature (Antonini and Brunori, 1971; Lemberg and Legge, 1949). To be internally consistent, authentic spectra and extinction coefficients were all calibrated to that of the iron(III) cyanide adduct at 540 nm. Values for hemeproteins (Antonini and Brunori, 1971; Lemberg and Legge, 1949; Drabkin, 1961) and iron porphyrins (Drabkin, 1961; Falk, 1964) are fairly consistent at this wavelength. An extinction coefficient of 11.5×10^3 was taken as correct. Employing aliquots of the same stock HbO_2 solution of recrystallized hemoglobin obtained as outlined above, the spectrum was recorded as a standard "HbO_2" in air. Another aliquot was treated with an approximate tenfold excess of $K_3Fe(CN)_6$. The spectrum was recorded as the standard Hb^+ spectrum. Solid KCN was added to the same Fe^{III} solution to result in standard $Fe^{III}CN$. Extinction coefficients were then calculated for the HbO_2 and Hb^+ solutions employing the concentration deduced from the $Fe^{III}CN$. Another portion of the stock HbO_2 solution was degassed by repetitive argon sweeping and evacuation while gently stirring. A sample transferred to a cuvette under argon did not alter its spectrum upon addition of sodium dithionite. This spectrum was taken as standard deoxyhemoglobin at a concentration equal to that of the stock HbO_2. Readdition of oxygen verified this experimentally and a conversion with ferricyanide/KCN resulted in the standard Hb^+CN^- spectrum. All measurements were taken with a Cary 118C spectrophotometer that was equipped with the capacity to handle light scattered samples. Heterogeneous cell suspensions were placed close to the photomultiplier. Absorbance by whole cell suspensions was corrected for turbidity by subtracting the small optical density at 800 nm from any absorbance. Lysed and whole cells showed identical spectra and extinction coefficients after this correction. Their absorbance was slightly different than that of the recrystallized protein complex. The extinction coefficients and the wavelengths used for kinetic analysis are given in Table I. For whole and lysed cells the extinction coefficient for the deoxy complex (555 nm) (Antonini and Brunori, 1971) was taken as absolute. Other values relative to this were determined in the manner outlined above. The values in the table correspond to the wavelengths of the α band (575 nm), trough (558), and β band (540) of the oxy complex. The largest differences in absorption between the three iron species occur at these wavelengths. Employing three simultaneous equations, a set of equations was developed from the data in

TABLE II: Relative Rates of Oxidation of Oxyhemoglobin by Organic Molecules at 25 °C.[g]

Substrate	Reactivity[a]	k_2 (L mol^{-1} s^{-1})
Phenylhydroxylamine	Very fast[b]	
Phenylhydrazine	Fast[c]	45
sym-Diphenylhydrazine	Fast[c]	
1,4-Cyclohexadiene	Moderate[d]	0.9
Hydroquinone	Moderate[d]	8.4×10^{-2}
Benzhydrol	Moderate[d]	7.9×10^{-2}
Methylhydrazine	Moderate[d]	
2-Methyl-1,3-cyclopentanedione	Moderate[d]	2.6×10^{-2}
Acetylacetone	Slow[e]	2.8×10^{-3}
Isopropyl alcohol	Slow[e]	
Benzaldehyde	Slow[e]	2.1×10^{-3}
Malononitrile	Slow[e]	1.4×10^{-4}
Piperidine, imidazole	Inert[f]	
Methylcyclohexane	Inert[f]	

[a] Initial scan estimate. [b] $k_2 > 300$ L mol^{-1} s^{-1}. [c] $k_2 \sim 40$. [d] $k_2 \sim 0.3$–0.01. [e] $k_2 \sim 10^{-3}$ to 10^{-4}. [f] No reaction in 5 h. [g] In the presence of KCN, pH 7.4, 0.01 M phosphate buffer, 0.1 M KCl.

Table I that gives the concentration of all three species in a spectrum that is a composite of the three. For recrystallized human hemoglobin they are:

$$(Fe^{II}) = [3.90D_a + 17.9D_b - 15.5D_c] \times 10^{-5}$$

$$(FeO_2) = [18.8D_a - 7.46D_b - 7.78D_c] \times 10^{-5}$$

$$(Fe^{III}) = [-46.4D_a - 13.7D_b + 57.3D_c] \times 10^{-5}$$

wherein D_a, D_b, and D_c correspond to the optical densities at 575, 558, and 540 nm, respectively. Experimental checks from repeated interconversion suggest these equations are valid to ±2%.

Kinetics. Reactions were initiated in two ways. Usually 2–5 μL of a 10^{-2} to 0.1 M stock solution of substrate under argon was injected directly into a solution of the oxy complex that was in a serum capped spectrophotometric cell under argon. After tipping, the cell was returned to the spectrophotometer cavity. Alternatively, the reactor described above for scaled up runs was employed. With it, reactants were mixed before filling the cells. Generally, reactions were slow enough such that repeat scans of the spectrum from 700 to 475 nm could be taken. Alternatively, rates were measured by following the disappearance of HbO_2 at 575 nm, or all three components by repeat runs at 575, 558, and 540 nm.

Scanning. Reactivity was initially ascertained with recrystallized oxyhemoglobin solutions in the presence of excess substrate and oxygen. A small aliquot of the oxyhemoglobin solution from the Bio-Rad gel was added to saline buffer in an open cuvette which was then capped with a serum cap. Argon was swept over the top of the solution. Initial concentrations were usually in the range of $\sim 10^{-5}$ M HbO_2 and $\sim 10^{-3}$ M substrate. Repeat scans of the spectrum showed an initial lag until the excess oxygen was consumed. Substances showing no change in spectrum after 5 h were considered inert.

Results

Organic Molecules. The relative rates of reaction of a series of organic molecules with human recrystallized oxyhemoglobin are given in Table II. The reactivity sequence is that deduced from the initial scan. Substrates from each of the reactivity groupings were selected for further kinetic analysis, and more accurate rate constants are reported for these by measuring the rates in the presence of KCN. For all cases a determination of the rate law from initial slopes of plots of the concentration

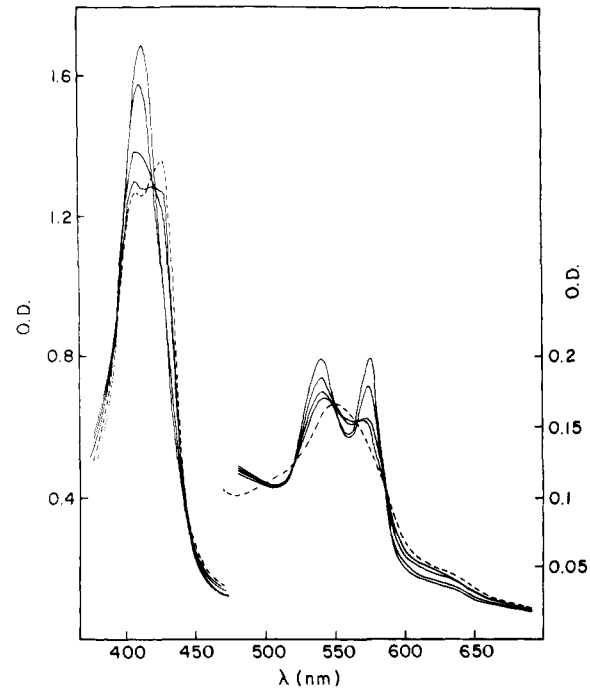


FIGURE 1: Spectral changes accompanying the reaction of benzaldehyde with oxyhemoglobin, pH 7.4, 0.1 M KCl, 25 °C. $(PhCHO)_0 = 2.6 \times 10^{-4}$ M; $(HbO_2)_0 = 3.3 \times 10^{-6}$ M; scans at 0, 2.5, 5.25, 10.65, and 22.65 (- - -) h.

of HbO_2 vs. time yielded the expression: rate $= -d(FeO_2)/dt = k(FeO_2)$ (reductant). In addition to the substances in the table, the hydrocarbons fluorene, triphenylmethane, cycloheptatriene, and 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane all oxidize the oxy complex. Relative rates for these latter are not reported because their solubilities (and the homogeneity of the reaction solutions) were not certain. The reactivity toward oxyhemoglobin roughly parallels carbon–hydrogen, nitrogen–hydrogen, or oxygen–hydrogen bond dissociation energy. The favorable influence of neighboring phenyl, amino, and nitrile substituents reflects the capacity for delocalization of the incipient radical. All of these substances convert oxyhemoglobin directly to methemoglobin, and we formulate this process as a hydrogen abstraction from the substrate by the iron oxy complex.

$$HbO_2 + RH \rightarrow Hb^+ + OOH^- + R\cdot \qquad (1)$$

However, except for hydroquinone, with an excess of substrate, there is an overall net conversion to the deoxy iron(II) complex (eq 2).

$$Hb^+ + \text{intermediates} \rightarrow Hb \qquad (2)$$

The spectral changes accompanying the reaction of benzaldehyde (Figure 1) are illustrative. Thus the drop, spread, split, and left-right shift in the soret band is typical of the sequence: $FeO_2 \rightarrow Fe^{III} \rightarrow Fe^{II}$. It was observed for all net $HbO_2 \rightarrow Hb$ conversions noted herein except the reaction of ferrous ion. As a reference standard, spectra of the hemoglobin complexes are given in Figure 2.

In contrast to the general character of the benzaldehyde reaction, the presence of even a modest excess of fast reacting substrate like phenylhydrazine results apparently in a direct conversion of iron (oxy) to iron(II) hemoglobin (eq 3a). Yet in the presence of cyanide, the reaction yields the cyano iron(III) adduct (eq 3b) exclusively.

$$FeO_2 + PhNHNH_2 \xrightarrow{a} Fe^{II} \qquad (3)$$

$$Fe^{II} \xrightarrow[b]{CN^-} Fe^{III}CN$$

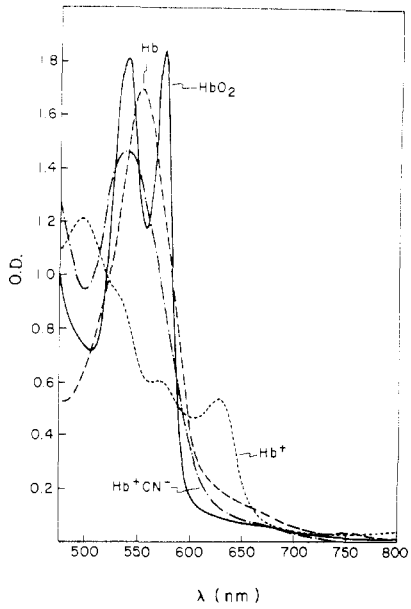


FIGURE 2: Visible spectra of hemoglobin derivatives 0.1 M phosphate buffer, pH 7.4, 0.1 M KCl, 25 °C.

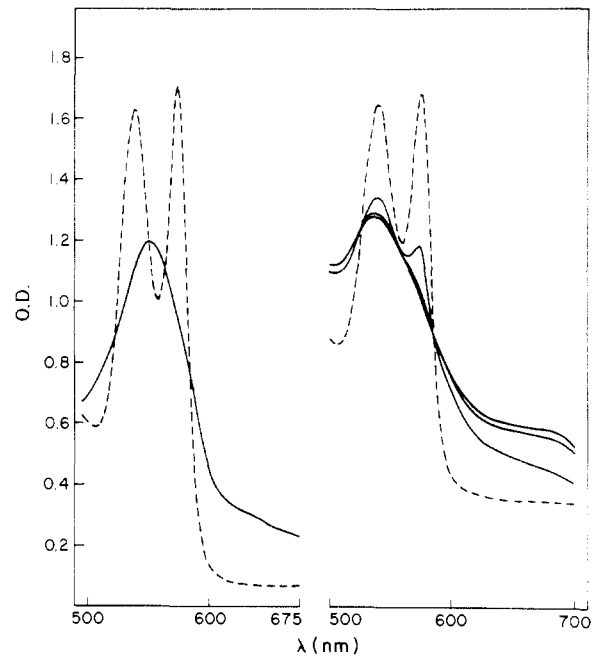


FIGURE 3: The reaction of oxyhemoglobin with phenylhydrazine. (Left) With lysed cells in the absence of potassium cyanide at 0 (- - -) and 5 min after mixing; (right) with whole cells in the presence of potassium cyanide, 0, 1, 4, and 7 min after mixing. Initial concentrations: (Left) $(FeO_2)_0$ = 1.1×10^{-4} M; $(PhNHNH_2)_0 = 3.3 \times 10^{-3}$ M; (right) $(FeO_2)_0 = 1.0 \times 10^{-4}$ M; $(PhNHNH_2)_0 = 6.7 \times 10^{-4}$ M; $(KCN)_0 = 1.6 \times 10^{-3}$ M.

We have chosen to illustrate this reaction in Figure 3 with whole cell suspensions, and lysed cells, but the same phenomena occur with oxyhemoglobin in any environmental state. The reaction characterizes the behavior of faster reacting substrates. Rates for reaction (eq 3) in the presence and absence of cyanide are given in Table III. It will be noted from the table that while phenylhydrazine will reduce iron(III) to iron(II) hemoglobin the rate is too slow to account for the iron(II) product emanating from reaction with the oxy complex. Moreover, while the product changes dramatically in the presence of cyanide the rate is unaltered. Thus, the "cyanide trapping" experiments demonstrate that iron(III) hemoglobin is an intermediate in eq 3a, and we formulate the general path of reaction with this substrate in eq 4.

$$HbO_2 + PhNHNH_2 \xrightarrow[\text{slow}]{k} Hb^+ \xrightarrow[\text{fast}]{\text{intermediates}} Hb \qquad (4)$$
$$\text{fast} \downarrow CN^-$$
$$Hb^+CN^-$$

The conversion to iron(III) is rate limiting. A variety of species may be responsible for the fast follow up conversion of met- to deoxyhemoglobin (cf. Discussion).

The heme protein product observable from these transformations is a function of the initial concentration of substrate and the relative rates of reactions 1 and 2. Hydroquinone is a case in which $k_1 \gg k_2$ ($k_2 = 0$) and a conversion to iron(III) hemoglobin only is observed. On the other hand with phenylhydrazine $k_2 \gg k_1$ and consequently an excess of this reductant results only in iron(II) hemoglobin.

The reactivity pattern exhibited by oxyhemoglobin in intact erythrocytes is the same as that for the purified protein in solution, but reactions can be much slower. Approximate rates from the initial scan are presented in Table IV.

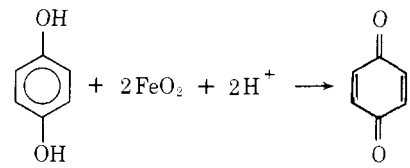
We presume that the wide variation between the relative rates of reaction in solution and in whole cells reflects differences in permeation by these substances. Indeed all of the substances in Table I were scanned with whole cells. Those not listed in Table IV were unreactive in 4 h.

Organic Products. The overall stoichiometry for hydroquinone and benzaldehyde is:

TABLE III: Rates of Reaction of Phenylhydrazine with Hemoglobin Derivatives.

Hemoglobin	State milieu	Product	k_2 (L mol^{-1} s^{-1})
Iron(III)	Human, recryst[a]	Fe^{II} [c]	0.33
Iron-O_2	Human, recryst[a]	Fe^{II}	44
Iron-O_2 + KCN[b]	Human, recryst[a]	$Fe^{III}CN$	44
Iron-O_2	Intact erythrocytes in saline	Fe^{II}	25
Iron-O_2 + KCN	Intact erythrocytes in saline	$Fe^{III}CN$	25
Iron-O_2	Lysed cells	Fe^{II}	25
Iron-O_2 + KCN	Lysed cells	$Fe^{III}CN$	25

[a] With 0.01 M phosphate buffer, pH 7.4, 0.1 M KCl. [b] $(KCN)_0 = 1.67 \times 10^{-3}$ M. [c] Predominant product, see text.



$$+ 2FeO_2 + 2H^+ \longrightarrow + 2Fe^{III} + 2H_2O_2 \qquad (5)$$

$$2PhCHO + FeO_2 \longrightarrow 2PhCO_2H + Fe^{II} \qquad (6)$$

Reaction 6 followed by reoxygenation of deoxyhemoglobin results in a catalytic cycle.

Inorganics. The results of the scan of a variety of inorganics with human recrystallized oxyhemoglobin in solution are summarized in Table V. Reactants are listed in a decreasing order of reactivity.

As noted above with the organic reductants, the character

TABLE IV: Oxidation of Oxyhemoglobin in Intact Erythrocytes at 25 °C.

Substrate	Initial scan[a] ($L\ mol^{-1}\ s^{-1}$)	$k_{purified}/k_{cells}$
Phenylhydroxylamine	>300	?
Phenylhydrazine	25	2
sym-Diphenylhydrazine	0.1	400
Hydrazine	0.006	50
Benzaldehyde	0.001	2

[a] pH 7.4, 0.14 M NaCl, 0.01 M phosphate buffer.

TABLE V: Rates of Reactions of Inorganics with Oxyhemoglobin at 25 °C.

Reductant	Reactivity	Product	k_2 ($L\ mol^{-1}\ s^{-1}$)[c]
Cr^{2+}	Fast	Fe^{III} then Fe^{II}	$>7 \times 10^3$
Fe^{2+}	Fast	Fe^{II} only	33 and 8[a]
H_2NNH_2	Moderate	Fe^{III} then Fe^{II}	0.3
$Fe(CN)_6^{4-}$	Slow	Fe^{III} only	0.015
Mn^{2+}	Slow	Fe^{III} then Fe^{II}	
Cu^{2+}	Denaturation	Fe^{III}	
Co^{2+}, Ni^{2+}	Inert[b]		
Ca^{2+}, Mg^{2+}	Inert[b]		
Fe^{3+}, Cr^{3+}	Inert[b]		

[a] The reaction is biphasic, see text. [b] Inert = no reaction in 24 h. [c] With 0.01 M phosphate buffer, pH 7.4, 0.1 M KCl.

of the reactions is a function of both the nature and concentration of the inorganic species. Denaturation of the protein and precipitation from solution were problems with all of these reactions, but it was extremely serious with copper. The rest of the information in the table was gathered over a time period in which denaturation was not observed.

Metal Ions. The reactivity of chromous, ferrous, and manganous and the inertness of other divalent transition and nontransition metal ions as well as that of the higher valent species Fe^{3+} and Cr^{3+} qualitatively show the reaction to be the result of an attack of the low valent metal ion on the oxyhemoglobin complex. The reaction of a denatured protein cannot explain the results. The reaction with Cr^{2+} is very similar in general character to the reactions observed for most of the organics. Hb^+ can be seen as an intermediate, and, depending upon starting concentration, it can be the major product. The rate of reaction with the oxy complex is faster ($k > 7 \times 10^3$ L $mol^{-1}\ s^{-1}$) than the rate of reduction of methemoglobin[1] ($\sim 4 \times 10^3$). With Fe^{2+} the only product of the reaction is the deoxy complex. There is no evidence for the intermediacy of methemoglobin with this ion. The rates for both chromous and ferrous ions are independent of the concentration of excess deoxyhemoglobin, and the data in Table VI are illustrative. Thus: rate = $k_2(M^{n+})(FeO_2)$.

It will be noted that the rate of reaction of oxyhemoglobin and methemoglobin with ferrous ion is the same. Pseudo-first-order plots of the data reveal the same initial rates but show these processes to be biphasic for both reactions of the oxy complex and methemoglobin. Figure 4 is typical for both hemoglobin species, and the same constants are obtained from both rate processes. Based upon these observations we consider

TABLE VI: Rates of Reaction of Ferrous Ion with Hemoglobin Derivatives.

$(HbO_2)_0$	$(Hb)_0$	$(Fe^{2+})_0$	k_2 ($L\ mol^{-1}\ s^{-1}$)
6.2×10^{-6}	0	2.45×10^{-4}	36[a]
3.12×10^{-6}	0	3.12×10^{-4}	32
5.7×10^{-6}	0	1.15×10^{-4}	32
3.12×10^{-6}	3.0×10^{-6}	2.45×10^{-4}	33
$(Hb^+)_0$		$(Fe^{2+})_0$	k_2
6.08×10^{-5}		1.22×10^{-4}	35[a]
2.45×10^{-4}		6.12×10^{-6}	36
6.45×10^{-6}		2.58×10^{-4}	32

[a] From initial slopes the reaction is biphasic.

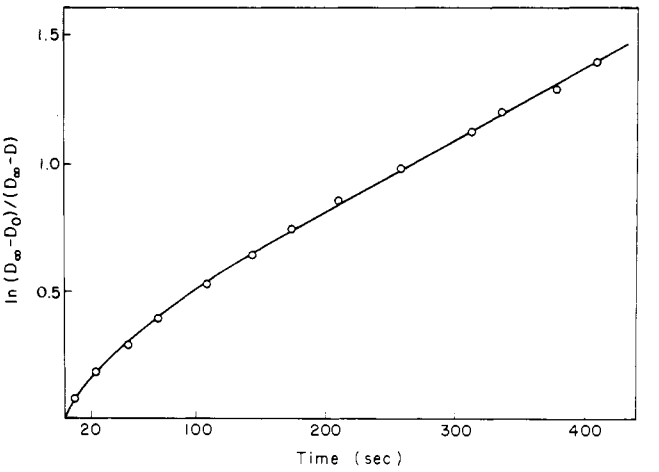


FIGURE 4: A pseudo-first-order plot for the reaction of ferrous ion with oxyhemoglobin at 25 °C; $(FeSO_4)_0 = 2.45 \times 10^{-4}$ M; $(FeO_2)_0 = 6.12 \times 10^{-6}$ M.

the ferrous reactions to represent a direct attack of the metal ion upon the ligand bonded to iron.[2]

Anions. In contrast to ferrous ion, ferrocyanide does effect a major conversion to methemoglobin, albeit relatively slowly. This anion, like hydroquinone, does not reduce iron(III) to iron(II) hemoglobin, but clearly it is able to react with the oxy complex.

Discussion

All of the reactants encountered here react with oxyhemoglobin by initially transferring an electron or a hydrogen atom to the oxygen coordinated to iron, and as a concomitant the iron is oxidized. The general expressions (eq 7-9) accommodate all of our findings.

$$O_2 + Fe^{II} \rightleftarrows FeO_2 \quad (7)$$

$$FeO_2 + \text{reductant} \rightarrow Fe^{III} + HO_2^- \quad (8)$$

$$Fe^{III} + \text{reductant or intermediate} \rightarrow Fe^{II} \quad (9)$$

The results have a bearing upon synthetic and mechanistic chemistry as well as drug-induced methemoglobinemia. We comment briefly upon these.

Catalyzed Autoxidations. While the scope and practicality

[1] Both of these values are estimates from initial slopes. The value with the oxy complex is a minimal one. The rate with Hb^+ at pH 6 is $>7 \times 10^3$ in agreement with the reported value of 3.6×10^4 L $mol^{-1}\ s^{-1}$, at this pH (Huth et al., 1976).

[2] We construe that in both cases the measured rates reflect the steric accessibility of the iron bound ligand to ferrous ion. It should be emphasized here that a rapid conversion of HbO_2 to Hb^+ followed by the same slow rate of reduction for the latter is not consistent with these data. On this time scale any Hb^+ would be directly observable in the spectrum.

of the phenomena await exploration, the autoxidation of benzaldehyde in aqueous solutions represents a catalytic redox cycle that could be useful for the selective oxidation of organic molecules. Thus a partially deoxygenated, sealed, stirred solution of benzaldehyde in buffer at room temperature for 36 h afforded no benzoic acid. Benzaldehyde was not consumed. Alternatively, the same reaction solution that contained 4.5×10^{-6} M HbO_2 resulted in 8×10^{-6} mol of benzoic acid in the same time.

Electron Transfer to Iron Porphyrin Oxygen Complexes (Reaction 1). As indicated above, the simplest mechanism for organic molecules is a hydrogen abstraction by the oxygen coordinated to iron. The results do imply that the low spin iron–oxygen hemoglobin adduct does possess a significant degree of free radical character. Moreover, the oxygen coordinated to iron is more reactive than free oxygen in aqueous solution. In this sense, the oxy complex responds in the transition state at least as if it were an $Fe^{III}O_2^-$ adduct.

A variety of possible mechanisms for the oxidation of oxyhemoglobin have been recently discussed (Caughey et al., 1975). In addition to those upon which this work is based (cf. introductory section), the displacement of O_2^- by a strong nucleophile has been considered. We have no evidence for this reaction with CN^- or HO^-. Oxyhemoglobin solutions do not oxidize in the presence of 10^{-3} M potassium cyanide (cf., however, Wallace et al., 1974).

The Reduction of Methemoglobin to Deoxyhemoglobin (Reaction 2). With the exception of the hydrazines, none of the organics in Table I are capable of reducing Fe^{III} to Fe^{II} hemoglobin. The reaction with phenylhydrazine itself (Table IV) is not particularly rapid. The most likely "intermediates" capable of this process for the general case are the free radicals emanating from the initial hydrogen abstraction or the more oxidized molecules and fragments derived from them. Thus, for all cases, the reaction is feasible. A rapid axial inner sphere reduction of iron(III) porphyrins by alkyl radicals has been demonstrated as a general reaction of these species (Castro et al., 1974) and there is no reason why the accessible iron in the globin conformation should exclude it. The lack of reduction of Hb^+ with hydroquinone accords with the lethargy of oxy radicals (semiquinone) to engage in this process (Castro et al., 1974, 1977). Indeed a second hydrogen transfer to the oxy complex would seem the most reasonable occurrence in the system. Similar reactions can occur with the hydrazines. With phenylhydrazine as an example, following the initial scission (eq 11)

$$R\cdot + L\text{-}Fe^{III} \longrightarrow RL + Fe^{II} \qquad (10)$$

$$FeO_2 + PhNHNH_2 \rightarrow FeOOH + PhNNH_2 \qquad (11)$$

any of a series of species (Ph—N=NH, Ph—N=N·, Ph·) including the original radical itself may react with the oxy complex via H transfer or addition, or with Fe^{III} Hb to reduce it. All of these species are more potent reductants than phenylhydrazine itself. It should be emphasized here that studies of the reaction of phenylhydrazine with both low and high spin iron porphyrins demonstrate that, while this substance is a reductant for low spin iron(III) complexes, it is an *oxidant* for high spin iron(II) porphyrins (Ong and Castro, 1977). This latter process, however, is second order in iron(II) porphyrin. We conclude the appropriate mechanism is not operative with the high spin iron(II) hemoglobin complex because the "G" or globin conformation precludes attack by a second iron(II) porphyrin. Thus neither phenylhydrazine nor oxygen readily oxidizes iron(II) Hb for the same reason. The results with inorganics place some constraints upon the mechanism of methemoglobin reduction by these species. Clearly chromous and ferrous are capable of process 9. The rates can be equal to (ferrous) but are usually slower than the overall deoxygenation reaction (Table V). The ions capable of reducing methemoglobin reduce Fe^{III} cytochrome *c* at rates that are one or more orders of magnitude more rapid (Castro, 1977). The relative rates of reduction of iron(III) cytochrome *c* and iron(III) hemoglobin by ferrocyanide are at least $>10^6$, and this difference fits well with the disposition of the porphyrin in these proteins and the reactivity patterns assigned them (Castro, 1971, 1977). In short, with hemoglobin it is most reasonable that the reduction by inorganics parallels that of the organic molecules and proceeds by an axial inner sphere mechanism. In this case the reductant perhaps enters the inner coordination sphere ($Fe(CN)_6^{4-}$) or attacks the axial ligand be it oxygen or water.

Related processes can be envisioned for sulfoxylate, and this accords with recent studies of the reduction of metmyoglobin by this anion radical (Olivas et al., 1978).

Drug-Induced Methemoglobinemia. Recent and very old studies of a variety of "hemolytic" agents accord with the findings presented herein. Thus, the "oxidative denaturation" of the partially purified hemoglobin of a series of mammals by ascorbate and acetylphenylhydrazine has been assessed (Harvey and Kaneko, 1976). Both reductants undoubtedly directly attack the oxy complex as a first step in the process. The hydroxylation of aniline by oxyhemoglobin (Mieyal and Blumer, 1976) may very well entail an attack by aniline or other reductants upon the oxy adduct. In a related note, the reaction of a series of phenols with oxyhemoglobin (Wallace and Caughey, 1975) is reported to produce methemoglobin. The generation of hydrogen peroxide in erythrocytes by a variety of hemolytic agents including phenylhydrazine and hydroquinone has long been known (Cohen and Hochstein, 1964). In part the hemolytic action has been attributed to peroxide. We presume the hydrogen peroxide is produced by attack on the oxy complex. Most recently, superoxide anion is receiving increased attention as a hemolytic entity (Winterbourn et al., 1976; Goldberg and Stern, 1976). Its conversion of HbO_2 to Hb^+, however, appears quite slow (Goldberg and Stern, 1976; Lynch et al., 1976). Superoxide anion has been reported to result from the cupric ion catalyzed oxidation of phenylhydrazine. This reaction is also catalyzed by oxyhemoglobin (Misra and Fridovich, 1976). A reaction like eq 21 was inferred although it could not have been directly observed at these concentrations. Our results are in agreement with this assumption. While hydrogen peroxide and superoxide anion may play a role in hemolysis and drug-induced methemoglobinemia, it is clear from the present work that a variety of quite reactive radical intermediates can be generated in these processes, and the destruction of cellular integrity by them would be expected.

The reactions encountered herein suggest a multitude of transformations that may be effected through the interaction of a reductant or a paramagnetic species with small molecules coordinated to deoxyhemoglobin or myoglobin.

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Oxidation–Reduction Reactions of Hemoglobin A, Hemoglobin M Iwate, and Hemoglobin M Hyde Park†

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ABSTRACT: The kinetics and equilibrium of the redox reactions of hemoglobin A, hemoglobin M Iwate, and hemoglobin M Hyde Park using the iron(II) and iron(III) complexes of *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetate ($CDTA^{4-}$) as the reducing and oxidizing agents have been studied. With respect to the equilibrium it was found that hemoglobin M Iwate (where the β chains were reduced) was more readily reduced than hemoglobin M Hyde Park (where the α chains are reduced). This difference was shown to be a result of a difference in the rate constant for reduction but not oxidation. The observed rate constants for the reduction of all three hemoglobins were shown to decrease with increasing pH. This was attributed to a decrease in the [T]/[R] ratio. The observed rate constants for the oxidation reaction were shown to increase with increasing pH. Accompanying this increase was a change in the kinetic profile for hemoglobin A from pseudo first order to one in which the rate increased as the extent of reaction increased. Inositol hexaphosphate had no effect on the rate of oxidation of deoxyhemoglobin A. This was a result of binding of $FeCDTA^{2-}$ or $HCDTA^{3-}$ to the protein. However, in the presence of inositol hexaphosphate, the reduction of methemoglobin A exhibited biphasic kinetics. This result was interpreted in terms of the production of a small amount of a conformation which was more readily reduced.

The conformational changes involved in the oxidation of deoxyhemoglobin to methemoglobin are believed to be the same (T → R) as those involved in the oxygenation reaction. Nonetheless, in contrast to the oxygenation reaction, the "Hill" constant for the oxidation reaction varies with pH with increasing cooperativity being observed with increasing pH (Brunori et al., 1969; Antonini et al., 1964; Kilmartin, 1973). Two different mechanisms have been proposed for the variation of the "Hill" constant with pH. The first proposed by Perutz (1973) and Kilmartin (1973) is that the low value for the cooperativity is a result of the relatively high concentration of the T conformation present in methemoglobin at low pH, al-

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