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REDUCTION OF HUMAN FERRIHEMOGLOBIN A IN THE PRESENCE OF CYSTEINE AND THE EFFECT OF CARBON MONOXIDE

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SUMMARY

The rate of reduction of human ferrihemoglobin A in the presence of cysteine is studied as a function of hemoglobin level, cysteine level, temperature, pH, ionic strength, stereospecificity, atmospheric composition and method of isolation of the hemoglobin. A mechanism for the cysteine reduction of ferrihemoglobin is suggested.

CO inhibits the reduction. In the absence of other reductants ferrihemoglobin is reduced in the presence of CO alone. The rate of CO reduction is accelerated by ultraviolet light. Evidence is presented for the formation of a ferrihemoglobin–ammonia complex in aqueous solution. Extinction coefficients are given for several hemoglobin compounds at various wavelengths.

PART I

The reduction of ferrihemoglobin in the presence of cysteine

INTRODUCTION

In studies of subunit transfer in mixtures of ferrihemoglobin and carbonmonoxyferrohemoglobin, charge-transfer reactions and reduction by residual amounts of glutathione in the preparations complicate the interpretation of results. SINGER AND ITANO[1] and ITANO AND ROBINSON[2] assumed the charge-transfer reaction to be of minor importance. In unpublished work in this laboratory it was observed both spectrophotometrically and chromatographically that human ferrihemoglobin C invariably was partially reduced to carbonmonoxyferrohemoglobin C when acidified and reneutralized in a carbon monoxide atmosphere. As part of an effort to understand these results it was decided to investigate the quantitative aspects of the reduction of ferrihemoglobin in the presence of cysteine and carbon monoxide.

Abbreviations: RSH, un-ionized cysteine; RS^-, ionized cysteine; C_T, total initial RSH + RS^-; $Hb^{3+}OH_2$, ferrihemoglobin ≡ methemoglobin (mol. wt., 66 000); $Hb^{3+}OH^-$, ferrihemoglobin hydroxide; Hb^{2+}, ferrohemoglobin ≡ reduced hemoglobin; H_T, total hemoglobin concentration; k, pseudo first-order rate constant defined below (units: min^{-1}).

* Contribution No. 2810.

CREMER[3] showed that cysteine could reduce hemin. MORRISON AND WILLIAMS[4] investigated the reduction of human ferrihemoglobin in the presence of cysteine in air, H_2, CO, and *in vacuo*, and stated that reduction was complete between approx. 0 and 24 h depending upon the cysteine level. KIESE[5] studied horse ferrihemoglobin reduction quantitatively using a variety of reductants, including cysteine. He found the reaction to be approximately linear with respect to ferrihemoglobin, non-linear in cysteine, and he gave the pH dependence of the initial reaction rate. In light of the present paper KIESE's work is subject to revaluation because he followed the reaction by manometrically determining CO uptake. We have found CO to be an inhibitor of the cysteine reduction of ferrihemoglobin. Therefore KIESE's rate constants can be expected to be low. The CO inhibition may explain his reported deviations from the first-order dependence on the ferrihemoglobin concentration.

EXPERIMENTAL

Materials

Inorganic chemicals were in all cases reagent-grade quality. The L-cysteine free base was purchased from Nutritional Biochemicals Corporation. The D-cysteine hydrochloride was purchased from California Corporation for Biochemical Research. L-Cysteine was used in all experiments except one; in the latter, D-cysteine was used to determine the stereospecificity of the reaction. Commercial CO from the Matheson Co., Inc., and Linde high-purity N_2 were used in the experiments requiring different atmospheres. All reagents were used as purchased, without additional purification.

Dialyzed, "unpurified" ferrihemoglobin A was prepared by washing freshly drawn blood four times with NKM (7.61 g NaCl, 0.39 g KCl, 1.52 g $MgCl_2 \cdot 6H_2O$ in 1 l), lysing with toluene and centrifuging at 20000 rev./min in a Spinco preparative centrifuge. The fresh lysate was treated with a three-fold molar excess of 0.018 F ($K_3Fe(CN)_6$), in potassium phosphate buffer (pH 6.94; $I = 0.01$) for 1 h at 0° to convert the oxyhemoglobin to ferrihemoglobin. The latter was then dialyzed against 0.1 F NaCl for 24–30 h with constant stirring and with a change of the NaCl solution every 8–12 h. The resulting product was in all cases $\geqslant$ 99.0 % ferrihemoglobin and this figure remained constant to within 1 % for at least a month when the sample was stored under nitrogen at 4°.

"Purified" ferrihemoglobin A was prepared by dialyzing approx. 500 mg of carbonmonoxyhemoglobin lysate against chromatographic developer 3A (10.65 g Na_2HPO_4, 6.90 g $NaH_2PO_4 \cdot H_2O$, 3.02 g $H_3ASO_4 \cdot \frac{1}{2}H_2O$, 2.24 g KOH in 4 l) for 48 h at 4°. This developer is a cyanide-free modification of developer No. 3 used by ALLEN, SCHROEDER AND BALOG[6]. Developer 3A has the same sodium and potassium ion concentration, the same pH, and approximately the same total ionic strength as developer 3. The arsenate was chosen as the substitute for cyanide in the hope that the buffer could be stored at room temperature without the growth of microorganisms. Over a period of a week no growth was observed. However, in operating columns after several days a blue-green growth was observed at the buffer–resin (GC-50) interface. This growth was always removed before beginning a chromatographic run. The dialyzed lysate was pipetted in a total volume of 5 ml on to the top of a GC-50, 70 × 1 cm chromatographic column which had been equilibrated with developer

3A at 6°. A fast fraction with a maximum at 47 ml (flow rate 15 ml/h) and a minimum at 106 ml was discarded. This comprised approx. 10% of the total hemoglobin. At 106 ml the column was warmed to 28° and the eluate collected in 3-ml portions. The central tubes containing approx. three-quarters of the main fraction were concentrated by centrifugation and rechromatographed. Fig. 1 shows the absence of any fast fraction. The material between 230 and 248 ml was reconcentrated and twice crystallized by the method of DRABKIN[7,8]. The conversion to ferrihemoglobin was the same as for the dialyzed "unpurified" material.

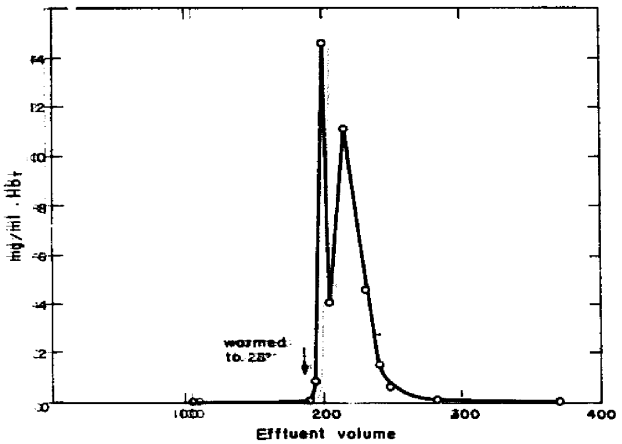


Fig. 1. Chromatogram of the major chromatographic fraction of human carbonmonoxyhemoglobin. Developer 3A. GC-50 resin at 6°. 13.8 ml/h.

Samples which were purified by chromatography only and which had been twice crystallized but not chromatographed were also prepared to determine the effect of each treatment separately.

Analysis of the extent of the reaction

The progress of the reaction was followed on a Beckman Model-DU spectrophotometer at wavelengths of 505, 538, 568, and 630 mμ. The extinction coefficients used were determined by standardizing the appropriate hemoglobin on a Cary

TABLE I

EXTINCTION COEFFICIENTS FOR SEVERAL HUMAN HEMOGLOBIN COMPLEXES

Phosphate buffer (pH 6.86; $I = 1.00$).

(mμ)	$E^{1\%}_{1\,cm}$		
	Hb^{Met}	$HbCO$	HbO_2
505	4.89	4.89	3.47
520.5	4.71	5.52	3.88
538	3.92	8.99	8.55
568	2.35	9.11	6.85
576	2.32	6.78	9.53
630	2.44	0.17	0.11

recording spectrophotometer against cyanoferrihemoglobin at 541.5 mμ using the NAS–NRC[9] value for $E^{1\%}_{1\,cm}$ at this wavelength of 6.90 as the primary reference point. Extinction coefficients determined in this manner are listed in Table I for several hemoglobin complexes. The amounts of ferrihemoglobin and reduced hemoglobin (as carbonmonoxyhemoglobin) determined by absorbancy ratios at the wavelength pairs 538, 518; 568, 518; 630, 568 were in agreement to within two absolute percentage units (*i.e.* 73 ± 2%, etc.) down to 30% ferrihemoglobin. Below 30% the absorption at 630 mμ was too weak to give reliable values. The other two pairs of ratios were still in agreement.

Apparatus

The experimental apparatus is shown in Fig. 2. 1 ml of ferrihemoglobin solution was placed in the test tube previously flushed with nitrogen, swept with nitrogen (unless otherwise stated, all experiments were done under nitrogen), stoppered, and lowered into a thermostatically controlled water bath at 25 ± 0.1°. Cysteine, dis-

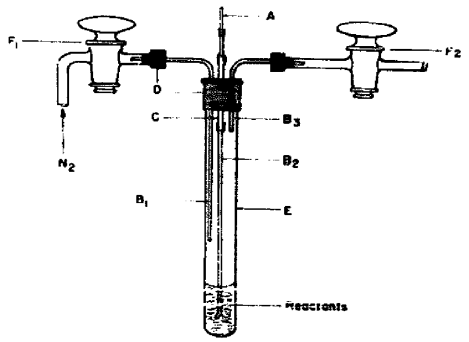


Fig. 2. Apparatus for kinetic studies in controlled atmospheres. A, 100-λ micropipet, B_1, 40-mil intramedic polyethylene tubing; C, stainless-steel tubing; D, rubber serum-bottle stopper; E, 1 cm × 10 cm test tube; F_1, ground glass stopcocks.

solved in the appropriate nitrogen-bubbled buffer, was placed in a 10-ml volumetric flask in the same water bath. After allowing 1–2 h for thermal equilibrium to be established 1 ml of the cysteine solution was transferred by a pipet into the test tube containing the ferrihemoglobin. The reactants were vigorously hand-stirred with a glass rod for 1 min, swept with nitrogen, and sealed with a tight-fitting rubber serum-bottle stopper through which three 40-mil intramedic polyethylene tubes were passed. A short section of tube B_2 which passed through the stopper was of stainless steel so that it could be raised and lowered easily. When it was desired to withdraw a sample, tube B_2 was lowered into the solution with stopcocks F_1 and F_2 closed; a 100-λ micropipet was placed inside the tube B_2 and the aliquot forced into the pipet with N_2 by opening F_1. The flow of sample was controlled by finger pressure on the end of the pipet. When the pipet was full, F_1 was closed, F_2 opened, and the 100-λ aliquot diluted into 3 ml of CO-saturated phosphate buffer at 4°, (pH 6.82; $I = 1.00$). The percentage reduced hemoglobin was read spectrophotometrically. After an aliquot had been taken F_2 was closed, and tube B_2 was withdrawn from the reactants and cleared by opening F_1. This flushed the system with nitrogen. All tubes were then closed until the next aliquot was taken. All volumetric apparatus was calibrated.

RESULTS

Effect of atmosphere on reaction rate

The first experiments were done under an atmosphere of CO in the expectation that if ferrohemoglobin were one of the reaction products its removal by CO would prevent possible inhibition by its accumulation. We were surprised, therefore, to find that the presence of CO has the opposite effect: *i.e.*, under nitrogen no inhibition is observed, while the presence of CO appreciably retards the reaction. There was also evidence that the reaction proceeds more slowly in air than in nitrogen, though here the accuracy of the data is insufficient to make a clear decision (Fig. 3). As a result of this finding all subsequent experiments were done under nitrogen.

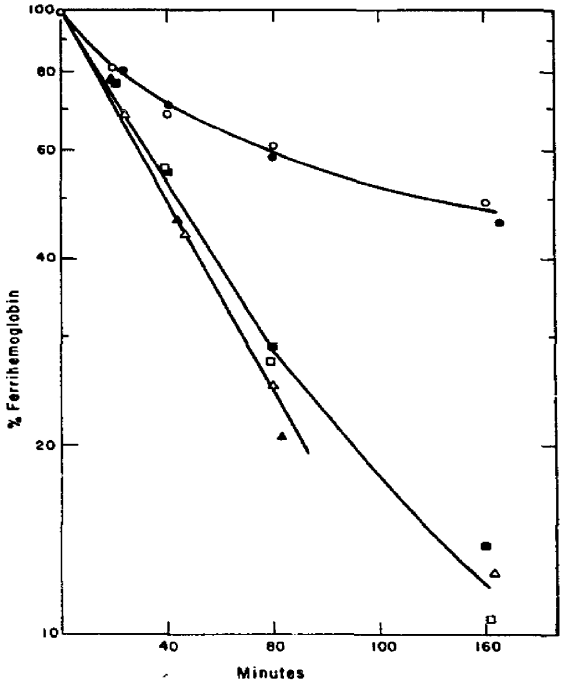


Fig. 3. Effect of gas composition on the reduction of ferrihemoglobin in the presence of cysteine. $C_T = 411 \cdot 10^{-4}$ M, $H_T = 3.57 \cdot 10^{-4}$ M, pH 6.79, $I_{NaCl} = 0.05$, $I_{PO_4} = 0.23$, EDTA, $88 \cdot 10^{-4}$ M. CO, ○, ●; air, □, ■; N_2, △, ▲. Each of the symbols refers to a single experiment.

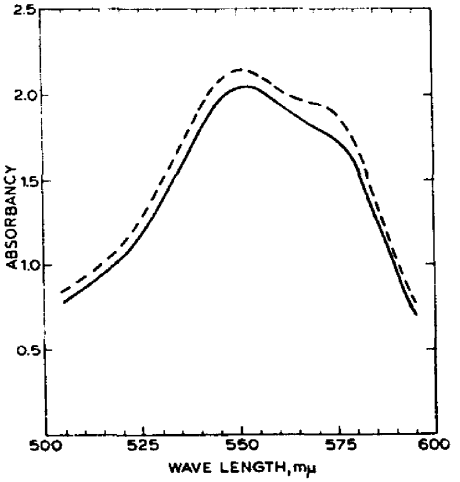


Fig. 4. Spectra of ferrihemoglobin (———) reduced in the presence of cysteine and vacuum-deoxygenated oxyferrohemoglobin (– – – –). These spectra have been corrected to eliminate the contributions of 9.4 and 18.9% ferrihemoglobin respectively.

Identification of the reaction products

A phosphate-buffered solution of hemoglobin and cysteine, H_T, $3.23 \cdot 10^{-4}$ M; C_T, $409 \cdot 10^{-4}$M; I_{PO_4}, 0.25; I_{NaCl}, 0.05; pH, 6.82, was allowed to stand at room temperature (25 ± 3°) for 2.5 h. The product was diluted with phosphate buffer and the spectrum determined between 2000 and 6750 Å on the Cary spectrophotometer (Fig. 4). The reacting sample was at all times under a positive nitrogen pressure. Transfers were accomplished with the aid of syringes and 23-gauge needles forced through rubber caps so as to avoid air. The spectrum is clearly that of ferrohemoglobin prepared by deoxygenation in vacuum. The other products of the reaction were not identified.

Order of the reaction with respect to ferrihemoglobin

From Fig. 5 and Table II it is seen that conversion of ferri- to ferrohemoglobin is first order with respect to ferrihemoglobin:

$$\frac{d[Hb^{2+}]}{dt} = k[Hb^{3+}OH_2]$$

where k is a function of the cysteine concentration (C_T) but is independent of the hemoglobin concentration for a fixed pH.

Occasionally at low values of pH when the reaction had proceeded to better than 80 % completion the rate would decrease slightly (Figs. 3 and 5). Near the pH optimum an increase in reaction rate was sometimes observed as the reaction progressed. The

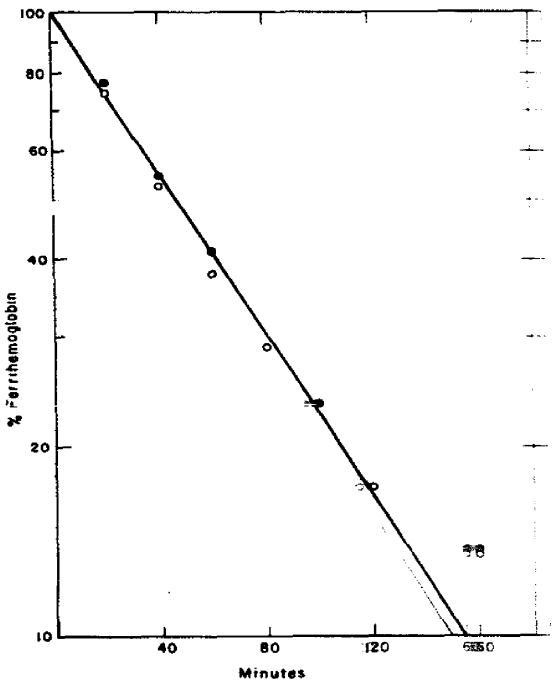


Fig. 5. The reduction of ferrihemoglobin in the presence of cysteine under N_2. $C_T = 81.0 \cdot 10^{-4}$ M, $H_T = 3.71 \cdot 10^{-4}$ M, pH 6.86, $I_{NaCl} = 0.05$, $I_{PO_4} = 0.25$, temp. 25.0°. Expt. 1, ○; Expt. 2, ●.

TABLE II

DEPENDENCE OF THE FIRST-ORDER RATE CONSTANT ON INITIAL FERRIHEMOGLOBIN CONCENTRATION

$I_{NaCl} = 0.05$; $I_{PO_4} = 0.50$; pH 6.83; temp., 24.9°; cysteine, $2.86 \cdot 10^{-4}$ M.

$H_T \times 10^4$ (M)	k (min^{-1})
0.638	0.0111
1.29	0.0108
1.95	0.0124
2.56	0.0135
3.19	0.0115

independence of the first-order rate constant with respect to the initial ferrihemoglobin concentration demonstrates that the reaction products do not affect the rate of reaction over the 5-fold level studied.

Dependence of the reaction rate on the cysteine level

The dependence of k on cysteine concentration is shown in Figs. 6a and 6b. Fig. 6b is discussed later The shape of the curve is similar to that found manometrically by KIESE[5]. At pH 6.78 saturation occurs when the cysteine level is approx. $80 \cdot 10^{-4}$ M a 6-fold excess over the heme concentration. Experiments in which the total [RS^-] was kept constant by reducing the cysteine concentration from $205 \cdot 10^{-4}$ M to $21.9 \cdot 10^{-4}$ M and by simultaneously raising the pH from 6.78 to 7.81 gave the same rate constant ($pK_{RSH} = 8.53$)[10,16]. This result and the occurrence of a pH optimum (Fig. 9) suggest RS^- is the reacting species. At cysteine levels below $13 \cdot 10^{-4}$ M we were unable to obtain closely reproducible results and no values are reported for

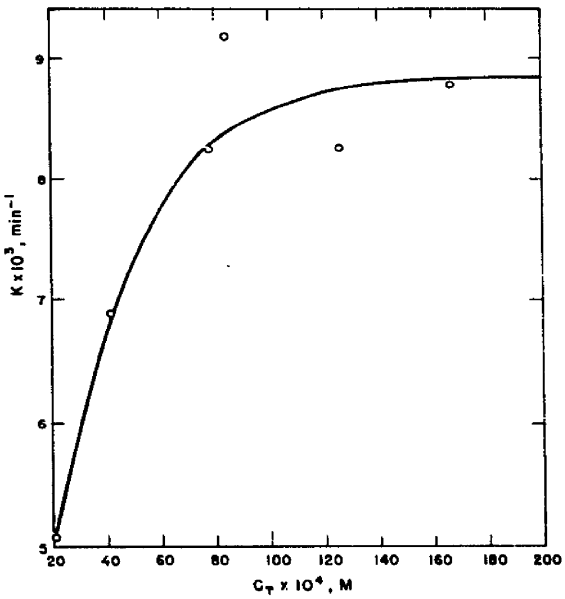


Fig. 6a. Dependence of the first-order rate constant on initial cysteine concentration. $H_T = 2.54 \cdot 10^{-4}$ M, pH 6.78, $I_{NaCl} = 0.05$, $I_{PO_4} = 0.50$, temp. = 24.9°.

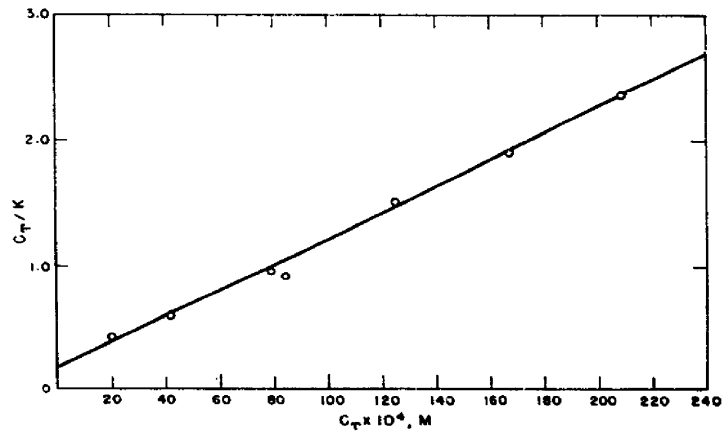


Fig. 6b. Data in Fig. 6a replotted in accordance with equations Ia and IIc1, see text.

this region. At cysteine level above $400 \cdot 10^{-4}$ M, 0.5–3.0 h after the addition of cysteine to the ferrihemoglobin a greenish precipitate and surface scum were observed in the reaction vessel.

Search for a ferrihemoglobin–cysteine complex

With respect to the reaction mechanism (see DISCUSSION) it is of interest to know whether a complex exists between ferrihemoglobin and cysteine. The kinetics of the oxidized reacting mixture (Fig. 7) in a 0.1-cm path cell were followed with the Beckman DK-2 spectrophotometer. For a two-component $Hb^{3+}OH_2$–Hb^{2+} system (*i.e.* no absorbing complex present) the following equation should hold:

$$\ln | A - \beta | = | \alpha | - kt$$

where α and β are calculated from the known hemoglobin concentration and the extinction coefficients[17–19] of $Hb^{3+}OH_2$ and Hb^{2+}. This equation was used to extrapolate the spectrophotometric data to zero time. At $t = 0$ both cysteine and $Hb^{3+}OH_2$ and therefore the complex should be at their maximum concentrations. Relatively large errors in α and β can be tolerated without affecting the extrapolation. Each extrapolation was constructed from five absorbancy measurements spaced at 4-min intervals at a given wavelength; the linearity of all plots was excellent. The kinetic data extrapolated to $t = 0$ is compared with the spectra for pure ferrihemoglobin in Fig. 7.

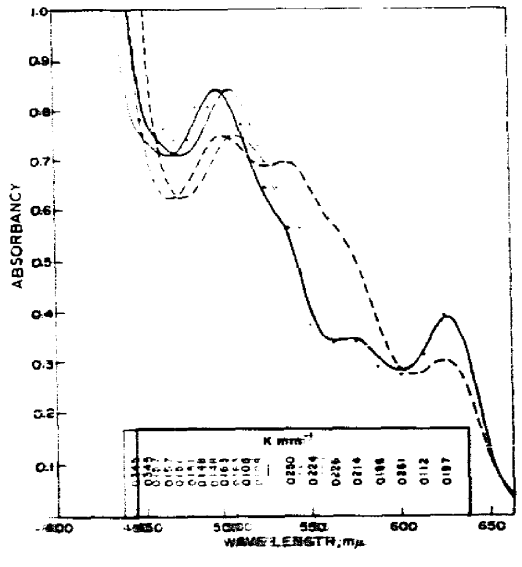


Fig. 7. Spectral absence of a ferrihemoglobin–cysteine complex. ———, 100% $Hb^{3+}OH_2$, direct measurement; . . . . , $Hb^{3+}OH_2$–cysteine system extrapolated to $t = 0$; – – – –, $Hb^{3+}OH_2$–cysteine system between 18.35 and 21.68 min; $H_T = 221.5 \cdot 10^{-4}$ M, $C_T = 180 \cdot 10^{-4}$ M, pH 6.78, $I = 0.555$, temp. = 20.5°.

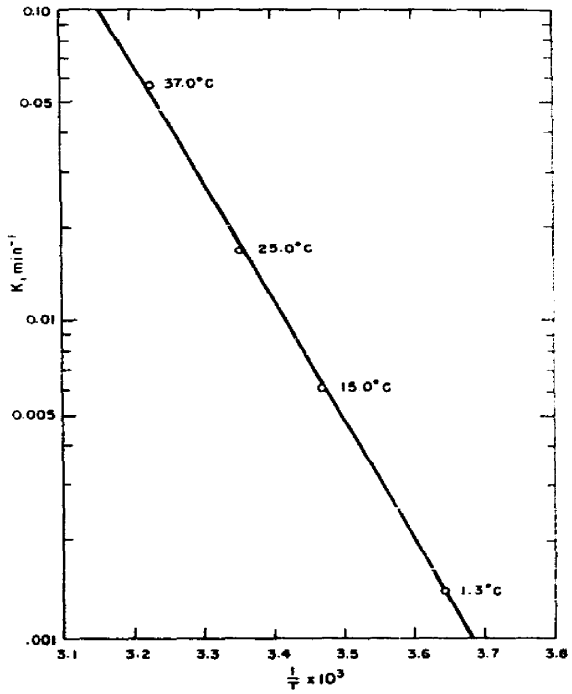


Fig. 8. Temperature dependence of the first-order rate constant for the reduction of ferrihemoglobin in the presence of cysteine under N_2. $C_T = 205 \cdot 10^{-4}$ M, $H_T = 3.73 \cdot 10^{-4}$ M, pH 6.86, $I_{NaCl} = 0.05$, $I_{PO_4} = 0.25$.

The spectra between 18.35 (700 mμ) and 21.68 min (400 mμ) is included to indicate the extent of the reaction. The results show no evidence for an absorbing complex in this wavelength region.

The values of the first-order rate constant k are shown in Fig. 7 at the corresponding wavelength. The difference in k values below and above 500 mμ suggests that different processes are being measured in the two regions. The value of k at 612.5 mμ appears so abruptly out of line with the remaining data in this region that its reliability is suspect, although a careful check of the data and calculations revealed no reason to reject it.

Temperature dependence of the reaction rate

If the reaction proceeds *via*

$$eX^{-} + Fe^{3+} \leftrightarrows (eXFe)^{2+} \quad \text{(a)}$$

$$(eXFe)^{2+} \xrightarrow{k_p} Fe^{2+} + X \quad \text{(b)}$$

where X is an electron carrier and reaction (b) is the rate-determining step, then from the Arrhenius activation energy of 18.58 kcal calculated from Fig. 8 a standard enthalpy, free energy, and entropy of reaction (a) above of 17.99, 22.32 kcal/mole Fe^{3+}, and −14.51 cal/deg mole Fe^{3+} is calculated at 25°. ΔH, ΔS, (eX^{-}), and the transmission coefficient γ are assumed to be independent of temperature in these calcu-

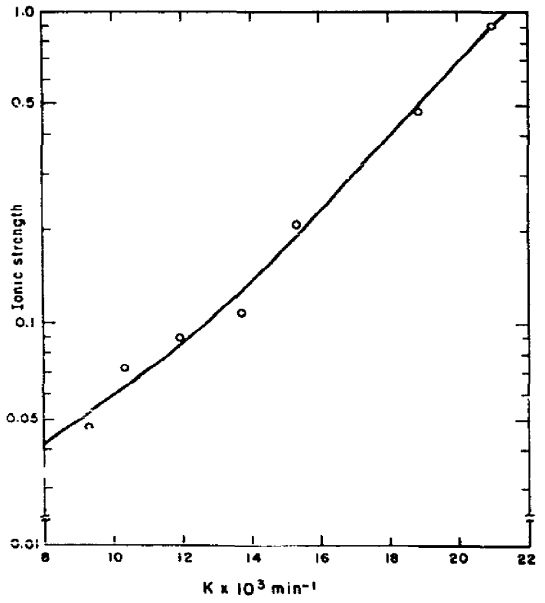


Fig. 9. Ionic strength dependence of the first-order rate constant for the reduction of ferrihemoglobin in the presence of cysteine under N_2. $C_T = 205 \cdot 10^{-4}$ M, $H_T = 2.90 \cdot 10^{-4}$ M, pH 6.67 ± 0.12, temp. = 24.9°.

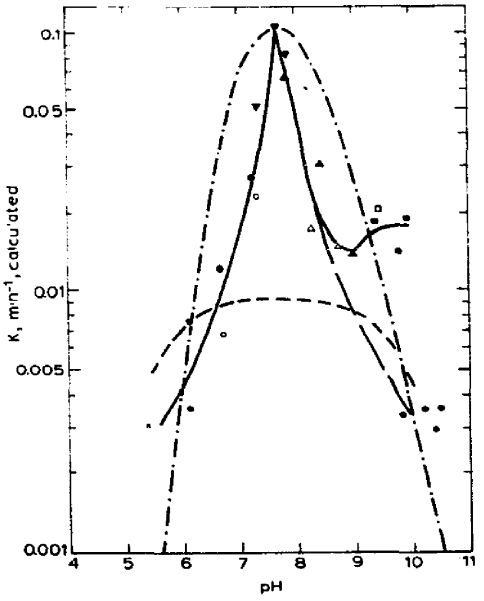


Fig. 10. pH dependence of first-order rate constant. $C_T = 205 \cdot 10^{-4}$ M, $H_T = 2.54$–$3.13 \cdot 10^{-4}$ M. $I_{NaCl} = 0.05$, $I_{buffer} = 0.476$, temp. = 24.9°. Acetate, ×; phosphate, ○, ●; Tris, △, ▼, ▲; NH_3, □, ■; carbonate, ◒. Each of the symbols refers to a single donor. Experimental, ———; calculated from simple model Eqn. Ia, – – – –; calculated from improved model Eqn. IIa1, –.–.–.–.

lations, and in calculating ΔS, ΔF, K_{SM}, and k_p a transmission coefficient of unity is assumed. From $\Delta F^\circ = -RT \ln K_{SM}$, where K_{SM} is the association constant for $(eXFe)^{2+}$ in reaction (a), $1/K_{SM}$ is calculated to be $2.28 \cdot 10^{16}$ moles/l at 25°. The significance of this small value of K_{SM} is discussed later with respect to the reaction mechanism. Under the assumption that $\gamma = 1$, $k_p = \gamma kT/h = 3.77 \cdot 10^{14}\ min^{-1}$.

Dependence of k on ionic strength

The ionic-strength experiments were performed in unbuffered aqueous solutions in which the ionic strength was adjusted by the addition of NaCl to the cysteine solution before addition to the reaction vessel. The pH was measured at $t = 0$ and at the end of the experiment. The pH decreased by approx. 0.2 pH unit between $t = 0$ and t_{final}. Fig. 9 shows that the rate of reaction increases with increasing ionic strength.

TABLE III

COMPOSITION OF BUFFER SOLUTIONS

The amounts of HAc, HCl, NH_3 are given in millilitres of commercial reagent-grade solution. Solids are given in grams.

pH*	NaAc	HAc	
4.82	7.82	3.63	
5.38	7.82	1.00	
	$K_2HPO_4 \cdot 3H_2O$	KH_2PO_4	
6.10	3.72	6.31	
6.69	5.45	3.12	
7.29	6.62	1.12	
	Tris	HCl	NaCl
7.39	13.89	8.22	
7.78	17.71	8.21	
8.27	35.71	8.21	
8.72	29.60	2.76	3.71
	NH_4Cl	NH_3	
9.42	5.10	5.99	
9.82	5.10	17.70	
10.25	5.10	22.91	
	$NaHCO_3$	Na_2CO_3	
9.89	1.27	2.84	
10.39	0.45	3.18	
10.51	0.35	3.22	
10.88	0.12	3.46	
11.31	0	3.54	$I = 1.00$

* When diluted to 100 ml, $I = 0.953$, except for the pH-11.31 buffer.

Stereospecificity of k

The reaction rate constant was the same for both L- and D-cysteine. In these experiments at pH 6.49, and $C_T = 208 \cdot 10^{-4}$ M, k was 0.0552 min^{-1}.

pH dependence of k

The buffers used in these experiments are given in Table III. The ionic strength of all buffers was 0.953 before addition to the ferrihemoglobin. The final ionic strength taking into account the NaCl from the dialysis was 0.53. The pH values plotted in Fig. 10 are not those of the buffers in Table III, but are the measured values after

TABLE IV

DEPENDENCE OF THE FIRST-ORDER RATE CONSTANT ON THE METHOD OF ISOLATION OF THE CARBONMONOXYHEMOGLOBIN

Method of isolation	pH*	
	7.71	6.79
	k (min^{-1})	
Twice crystallized	0.296	—
Twice chromatographed	0.295	—
$R_e + R_x$**	0.413	0.0213
Dialyzed lysate	0.0816	0.0099

* Buffer compositions are given in Table III.
** Twice crystallized and twice chromatographed.

the addition of 0.05 g cysteine to a water–buffer mixture (1:1). Three features are of interest in Fig. 10: (a) the sharp maximum at pH 7.68, (b) the 34-fold change in rate constant between pH 5.5 and 7.68, and (c) the catalytic effect of ammonia.

Aqueous ammonia has a marked catalytic effect on the reduction. At the same pH the rate is 5–6 times more rapid in NH_3 buffers than in carbonate buffers. Spectral evidence (Fig. 11) clearly shows that ferrihemoglobin hydroxide is the major species in carbonate buffers. In NH_3 buffers the hemoglobin appears to have complexed

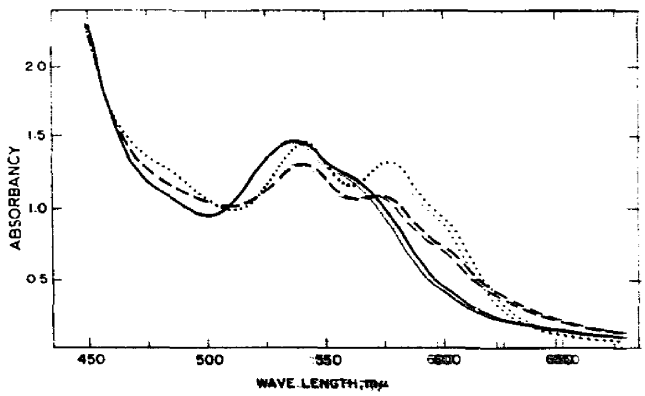


Fig. 11. Spectra of hemoglobin complexes at high pH. $Hb_T = 2.2$ mg/ml. NH_3 (pH 9.84), ———; sodium carbonate (pH 9.90) – – – –; titrated with KOH (pH 9.91), ... In daylight the ammonia complex appears red. The other solutions appear brown.

with ammonia. This complex is spectrally similar to the hydroxylamine–ferrihemoglobin complex reported by BANASCHAK AND JUNG[11]. The spectra differ in the positions of the maxima. These are 538 and 564 mμ for the ferrihemoglobin–ammonia complex and 543 and 575 mμ for the hydroxylamine complex.

Dependence of k on method of preparing ferrihemoglobin

The rate constant determined on lysates from different individuals can be seen from Fig. 10 to vary by as much as a factor of two. Often the difference is considerably less. To avoid confusion from this source, for any given series of experiments the same ferrihemoglobin was used throughout that series. When this was done the variability in the rate constant was typically less than 10% (Figs. 3 and 5).

Differences in "purified" and "unpurified" lysates are shown in Table IV. The 2–5-fold rate increase upon purification may be due to the removal of an inhibitor or to a sensitization to reduction caused by a change in the hemoglobin molecule during the purification process.

PART II

The reduction of ferrihemoglobin in the presense of carbon monoxide

The results of the atmospheric composition experiments in PART I led us to investigate the effect of CO on ferrihemoglobin reduction in the absence of other reductants. The buffered hemoglobin was placed in a test tube which had been wrapped with black electrical tape to exclude all light. The solution was then flushed with CO, tightly stoppered, and placed in a dark cabinet at room temperature (25 ± 3°). A duplicate experiment was performed in a quartz tube 12 cm in front of a 85-W G.E. H85-3C mercury arc lamp. The light was collimated but unfiltered. SCHUBERTH[12] found that ferrihemoglobin was rapidly reduced ($\tau_{1/2} = 10$ min) by CO in ultraviolet light. The results are given in Table V. A slow reduction of ferrihemoglobin by CO occurs in the dark. Ultraviolet light increases the reaction rate slightly. The quantitative

TABLE V

REDUCTION OF FERRIHEMOGLOBIN IN THE PRESENCE OF CO

$I = 0.53$; temp., 25 ± 3°.

Method of isolation	*pH**	*h*	*Illumination*	*Atmosphere*	$[H_T \times 10^4]$	$Hb^{3+}OH_2$ (%)	*Appearance*
Dialyzed lysate	7.81	59.75	dark	CO	2.68	82.5	clear
Dialyzed lysate	6.78	59.75	dark	CO	2.73	82.0	clear
$R_e + R_x$**	6.78	59.75	dark	CO	1.49	79.9	clear
Dialyzed lysate	5.38	59.75	dark	CO	2.62	83.0	moderate precipitate
Dialyzed lysate	6.78	59.75	dark	N_2	1.54	98.2	clear
Dialyzed lysate	6.78	1.00	visible light	CO	2.63	98.6	clear
Dialyzed lysate	6.78	1.00	ultraviolet	CO	2.54	93.0	clear

* Buffer compositions are given in Table III.
** Twice crystallized and twice chromatographed.

difference between the results given in Table V and those of SCHUBERTH may be due to the 5-fold higher hemoglobin concentration in our experiments or to a difference in light sources.

The reduction by CO, unlike that by cysteine, is pH independent.

PART III

Discussion

General

Although the pH and cysteine dependence described in PART I suggest that RS^-, not RSH, is a reacting species, the result of the ionic-strength experiment is opposite to that expected from charge considerations if the reaction is between RS^- and $Hb^{3+}OH_2$. It should be noted that a similar effect is found for the ionization of ferrihemoglobinhydroxide. In this case the pK_1 increases with ionic strength, a direction opposite to that expected from the increased electrical shielding at higher ionic strengths[13].

If one assumes that the dissociation constant of the $:NH_3$ complex with ferrihemoglobin is of the same order of magnitude as that found for the hydroxylamine complex, then at pH 9.9 the mole ratio of the ammonia complex to ferrihemoglobinhydroxide is approx. 18000. This result implies that the spectrum in Fig. 11 is that of the complex free of ferrihemoglobinhydroxide.

The increase in rate on purification is consistent with the hypothesis that the reaction is not enzymically controlled. The nonstereospecificity for D- or L-cysteine also supports the nonenzymic character of the electron transfer from cysteine to the primary electron acceptor. From the work of HUENNEKENS *et al.*[14] participation by methemoglobin reductase in the reaction is ruled out since they found no reductase activity after crystallization. Whether the increase in rate on purification is due to the removal of an inhibitor or to some other variable in the method of preparation is not known. If it is due to the latter, then a method of preparation which leaves the hemoglobin molecule unchanged must be found before it will be possible to measure unique rate constants.

A comparison of the pH dependence of human and horse hemoglobin is available through the work of KIESE[5]. For horse hemoglobin the pH optimum occurs at pH 8.1; for human it appears at pH 7.68. The pH curve for human hemoglobin also appears considerably more symmetrical and convex than does the curve for horse hemoglobin reported by KIESE. Indeed, it is quite similar both in its convexity and sharpness to the curve shown by KIESE for the iron-catalyzed oxidation of cysteine by oxygen at various pH values. In the case of human hemoglobin a 34-fold increase in rate is observed between pH 5.50 and 7.68. For horse hemoglobin or the iron-catalyzed oxidation of cysteine the increase is only 4–5 fold.

The results on the reduction of ferrihemoglobin in the presence of both cysteine and CO show that it is, in this case at least, not valid to study the kinetics manometrically by CO uptake, since CO is not inactive but inhibits the reaction.

The pH and cysteine dependence of ferrihemoglobin reduction

To explain the observed reaction kinetics quantitatively, the following reactions (1–11) are considered. It is assumed that the electron carrier (eX^-), *cf.* PART I, is some

form of ionized cysteine which we represent as RS^-. Eqns. 1–11 immediately following will be referred to in the subsequent development. The k's above the arrows are rate constants.

$$Fe^{3+}OH_2 \rightleftharpoons Fe^{3+}OH^- + H^+ \qquad \text{equilibrium constant, } K_H \qquad (1)$$

$$imH^+ \rightleftharpoons im + H^+ \qquad \text{equilibrium constant, } K_m \qquad (2)$$

$$RSH \rightleftharpoons RS^- + H^+ \qquad \text{equilibrium constant, } K_S \qquad (3)$$

$$RS^- \xrightarrow{k^*} RS^{-*} \qquad (4)$$

$$RS^{-*} \xrightarrow{k^*} RS^- \qquad (5)$$

$$RS^{-*} + RSH \xrightarrow{k_{01}} RS^- + RSH \qquad (6)$$

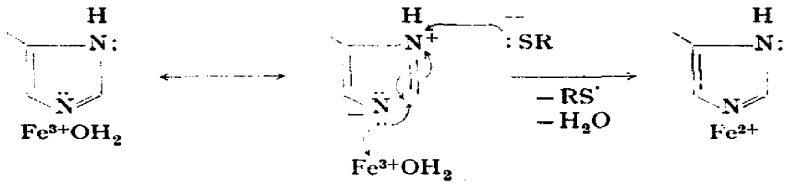
$$RS^{-*} + RS^- \xrightarrow{k_{02}} RS^- + RS^- \qquad (7)$$

$$RS^{-*} + Hb \xrightarrow{k_h} RS^- + Hb \qquad (8)$$

$$RS^{-*} + imFe^{3+}OH_2 \underset{k_c^-}{\overset{k_c}{\rightleftharpoons}} (RS^-imFe^{3+}OH_2)^* \qquad \text{equilibrium constant} = K_{SM} = \frac{k_c}{k_c^-} \qquad (9)$$

$$(RS^-imFe^{3+}OH_2)^* \xrightarrow{k_p'} RS^\cdot + imFe^{2+} + H_2O \qquad (10)$$

Explicitly Eqns. 9 and 10 are hypothesized as follows:



It should be noted that processes 9 and 10 together are kinetically indistinguishable from:

$$RS^{-*} + imFe^{3+}OH_2 \xrightarrow{k_p} RS^\cdot + imFe^{2+} + H_2O \qquad (11)$$

where $k_p = k'_p K_{SM}$.

Numerical values of K_H, K_m, and K_S have been taken from refs. 13, 15, 10 and 16. These values are pK_H ($I = 0.55$) = 8.33, $pK_m = 6.65$, and $pK_S = 8.53$. $1/K_{SM}$ was estimated as $2.28 \cdot 10^{16}$ from the thermal data in Fig. 7.

The simplest model to come to mind is that based on Eqns. 1, 3, and

$$RS^- + Fe^{3+}OH_2 \underset{k_c^{-\prime}}{\overset{k_c^{*\prime}}{\rightleftharpoons}} (RS^-Fe^{3+}OH_2) \qquad \text{equilibrium constant} = K'_{SM} \qquad (9')$$

$$(RS^-Fe^{3+}OH_2) \xrightarrow{k_p^*} RS^\cdot + Fe^{2+} + H_2O \qquad (10')$$

In this model we must first decide whether Eqn. 9′ or Eqn. 10′ is rate-limiting. If Eqn. 9′ is rate-limiting, then an increase in cysteine concentration increases the rate of $(RS^-Fe^{3+}OH_2)$ formation proportionately; that is to say, there is no saturation at high cysteine levels. This contradicts the experimental fact. On the other hand if

Eqn. 9′ is rapid relative to Eqn. 10′ then the observed saturation at high cysteine levels is explained by the fact that the addition of more cysteine can have no effect when all the ferric iron is complexed. These qualitative observations can be made quantitative by using Eqns. 1, 3, 9′ and 10′ together with the conservation restrictions

$$C_T = (RS^-) + (RSH) + (RS^-Fe^{3+}OH_2)$$

$$fH_T = (Fe^{3+}OH_2) + (Fe^{3+}OH^-) + (RS^-Fe^{3+}OH_2)$$

Here f is the fraction unreduced iron present at time t, and H_T the total iron present. Assuming only that $H_T \ll C_T$ the pseudo first-order rate constant is given by

$$C_T/k = (1/K'_{SM}k''_p)(1 + H/K_S)(1 + K_H/H) + (1/k''_p)C_T \qquad (1a)$$

The rate constant k has a pH optimum at

$$H_{opt.} = (K_H K_S)^{1/2} \qquad (1b)$$

Obtaining $pH_{opt.} = 7.68$ from Fig. 10, pK_S is calculated to be 7.03 from Eqn. 1b. This value disagrees with the value of 8.53 given by BENESCH AND BENESCH[10]. $1/K'_{SM}$ ($5.38 \cdot 10^{-4}$) and k_p'' ($9.54 \cdot 10^{-3}$) are found from the slope and intercept of Fig. 6b using Eqn. 1a. These values are in serious disagreement with the thermal data of Fig. 8 which give $1/K'_{SM} = 2.28 \cdot 10^{16}$ and $k''_p = 3.77 \cdot 10^{14}$. Further, there is no spectral evidence for complex formation. The pH curve calculated from this simple model is shown as the short dashed line in Fig. 10. This curve is in obvious disagreement with the experimental curve. In view of these discordant results this simple model was abandoned.

Attempts to remedy these quantitative and qualitative defects led to the model represented by Eqns. 1–11.

Eqn. 2 shows the ionization of the heme-linked histidine residue. The inclusion of this ionization renders the proposed mechanism internally consistent. This point is discussed later. Since the RS^- anion is solvated in aqueous solution it is reasonable to propose Eqn. 4. This step includes the orientation of the cysteine molecule so that the cysteine sulfur is in the vicinity of the imidazole nitrogen and a removal of enough solvating water molecules shielding the sulfur electron pair to permit reaction with the histidine–iron system. At this point the activated anion can be deactivated in three ways: by collision with water and other ionic or molecular species whose concentration is constant (Eqn. 5), or cysteine (Eqns. 6 and 7), or hemoglobin (Eqn. 8) it can be resolvated or knocked out of position. If the RS^{-*} is not deactivated by one of the above processes it will form an intermediate with the histidine–heme system (Eqn. 9). The reduction is then accomplished by the transfer of one of the sulfur electrons to the heme iron (Eqn. 10). Alternatively the activated anion may react directly with the histidine–heme system (Eqn. 11). It has been explicitly assumed that of the four possible ionization states of the histidine–heme system the only one with which the activated cysteine anion can react to form reduced hemoglobin is that in which the iron is in the ferrihemoglobin (not ferrihemoglobinhydroxide) state and in which the imidazole nitrogen is in the $=NH$ (not $=NH_2^+$) state.

On the basis of this model saturation at high cysteine levels is the result not of complex formation but of the deactivating processes (Eqns. 6 and 7). Here, as in

the earlier model a pH optimum is expected due to the opposing processes of RS^- formation and $Fe^{3+}OH_2$ loss *via* $Fe^{3+}OH^-$, but in the present model the drop-off in rate is much sharper on either side of the optimum, again because of the additional deactivating steps (Eqns. 6 and 7). The ionization of the imidazole group makes this drop-off somewhat sharper on the acid side of the optimum than on the basic side.

Transposing these observations, in qualitative agreement with experiment, into quantitative formulas is more difficult than before because of the increased mathematical complexity resulting from the numerous equations. Therefore we make the following simplifying assumptions:

(a) Steady-state approximation:

$$D_t(RS^{-*}) = D_t(RS^- \, imFe^{3+}OH_2)^* = 0$$

(b) (RS^-), (RS^{-*}), $(RS^- \, imFe^{3+}OH_2)^* \ll RS^-$, RSH

(c) $(RS^- \, imFe^{3+}OH_2)^* \ll Fe^{3+}OH_2$, $Fe^{3+}OH^-$

Assuming the correctness of the model and the approximations, the experimental fact that the pseudo first-order rate constant is independent of the ferrihemoglobin level rigorously implies that $k_m = 0$. This is physically reasonable because any heme region oriented toward RS^{-*} would have a competitive steric advantage over non-oriented heme regions in other hemoglobin molecules. Also the large size of the hemoglobin molecule would shield RS^{-*}. The model then implies $k_c^- \gg k_p'$, or alternatively that $k_c = 0$ which is equivalent to replacing Eqn. 9 by Eqn. 11. For definiteness (and in view of the thermal data of Fig. 7) we shall assume no complex formation occurs and will use Eqn. 11 in place of Eqn. 9 in the following derivation. The pseudo first-order rate constant derived from Eqns. 1–11 is given below in three equivalent forms.

$$k = \frac{A}{\left(1 + \frac{H}{K_m}\right)\left(1 - \frac{K_H}{H_{opt.}10^{\delta}}\right)\left(1 + \frac{K_H}{H_{opt.}10^{-\delta}}\left(\frac{H_{opt.}}{H'_{opt.}}\right)^2\right)} \tag{IIa1}$$

where

$$H = \frac{k_p k^* C_T}{k_-^* + k_{02} C_T} \tag{IIa2}$$

and

$$H_{opt.} = (K_H x)^{1/2}$$

and δ is defined by $H = H_{opt.}10^{\delta}$ where $H_{opt.}$ is the experimental pH optimum. The equation

$$H^3_{opt.} + \tfrac{1}{2}(K_m - K_H + x)H^2_{opt.} - \tfrac{1}{2} K_m K_H x = 0$$

which results from setting $(dk/dH)_{H_{opt.}} = 0$ permits one to find x, which is defined as

$$x = K_S\left(\frac{k_-^* + k_{02} C_T}{k_-^* + k_{01} C_T}\right) \tag{IIa3}$$

Note that $H'_{opt.}$ is not the experimental pH optimum; but is an approximation thereto obtained by solving to zero-order terms the quadratic equation resulting from the above cubic equation when neglecting the term in H^3. Examination of equation IIa1 reveals that k is substantially symmetrical about the pH optimum.

The saturation at high cysteine levels is apparent from another form of equation IIa1:

$$k = \frac{k_p k^*}{\left(1 + \frac{H}{K_m}\right)\left(1 + \frac{K_H}{H}\right)\left(1 + \frac{H}{K_S}\right)k_-^* + \left(k_{01}\frac{H}{K_S} + k_{02}\right)C_T} \cdot C_T \qquad \text{(IIb)}$$

The equations

$$\frac{C_T}{k} = i + sC_T \qquad \text{(IIc1)}$$

where

$$i = \left(1 + \frac{K_H}{H}\right)\left(1 + \frac{H}{K_S}\right)\left(1 + \frac{H}{K_m}\right)\frac{k_-^*}{k_p k^*} \qquad \text{(IIc2)}$$

and

$$s = \frac{\left(\frac{H}{K_S}k_{01} + k_{02}\right)}{k_-^*\left(1 + \frac{H}{K_S}\right)}i \qquad \text{(IIc3)}$$

are useful for the evaluation of the various k_i from the linear plot of C_T/k *vs.* C_T.

Using $k = 0.105$ (Fig. 10) at pH 7.68 ($\delta = 0$), from Eqn. IIa1, A is found to be 0.156. The pH curve calculated from Eqn. IIa1 with this value of A is shown as a dotted curve in Fig. 10. This curve is in fair accord with the experimental curve. Substituting Eqn. IIa2 into Eqn. IIc2 we find $k_-^* = 2.48 \cdot 10^{-4} k_{02}$. When this value is substituted into Eqn. IIa3 we find $k_-^* = 7.70 \cdot 10^{-2} k_{01}$ and $k_{02} = 310\ k_{01}$. The ratio k_-^*/k_{01} can also be found from Eqn. IIc3; the result is $k_-^* = 6.10 \cdot 10^{-2} k_{01}$. This is a factor of 1.26 lower than the ratio found from Eqn. IIa3. If the "true" values of the rate constant at pH 6.78 were 17 % higher than those plotted in Fig. 6b the two ratios of k_-^*/k_{01} would agree identically being $4.28 \cdot 10^{-2}$ and then $k_{02} = 202\ k_{01}$. This factor of 17 % is well within the variability between individuals as well as within the variability found upon purification. The model is therefore internally consistent with respect to the ratio k_-^*/k_{01} when the latter is calculated by two independent methods. This consistency is impossible to obtain if Eqn. 2 and the assumptions stated explicitly above regarding the ionization states of the histidine–heme system are omitted from the mechanism.

The most obvious defect of the proposed mechanism is its failure to explain the convexity in the experimental pH dependence. This convexity must arise from factors other than those presented in Eqns. 1–11 and at present we do not know their nature. Nevertheless the proposed mechanism does give a reasonably quantitative explanation of the ferrihemoglobin, cysteine, and pH dependence of the reduction between pH 6 and 10.

If the model is a valid approximation to the physical situation, the fact that $k_{02} = 310\ k_{01}$ deserves comment. At first one might expect $k_{02} \ll k_{01}$ because of coulombic repulsion between RS^{-*} and RS^-. Apparently, however, the electrostatic field in the vicinity of the reduction is such that only species of charge opposite to that of the iron, and which are therefore attracted to it, are efficient deactivators of RS^{-*}. For the same reason H_2O should be a poor deactivator of RS^{-*}. This is borne out by the small value of the ratios k_-^*/k_{01} and k_-^*/k_{02} found above.

CONCLUSIONS

The rate of reduction of human ferrihemoglobin in the presence of cysteine is shown to be linear with respect to ferrihemoglobin and to depend in a nonlinear way on the cysteine level, becoming approximately zero order in cysteine at high cysteine levels. The temperature coefficient of the reaction was determined and shows that the reduction by cysteine does not proceed through a tightly associated heme–cysteine complex. The pH and ionic-strength dependence of the reaction rate was determined. The rate of reaction is the same for both D- and L-cysteine. The rate of reaction is shown to be 2–5 times more rapid upon purification. Evidence is presented for the formation of a ferrihemoglobin complex with aqueous ammonia which catalyzes the reduction. CO inhibits the reduction with cysteine and this fact must be taken into account when studying such systems manometrically. By itself, in the absence of other reductants, CO is able to reduce ferrihemoglobin slowly, and the rate of reduction is independent of pH in the region pH 5.38–7.81. Ultraviolet light is shown to increase the reduction of ferrihemoglobin by CO. Comparison is made between the results of this study on human hemoglobin and those found by KIESE for horse hemoglobin. Two detailed mechanism are discussed for the reduction with cysteine, one of which partially explains the observed facts.

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REFERENCES

1 S. J. SINGER AND H. A. ITANO, *Proc. Natl. Acad. Sci. U.S.*, 45 (1959) 174.
2 H. A. ITANO AND E. ROBINSON, *Biochim. Biophys. Acta*, 29 (1958) 545.
3 W. CREMER, *Biochem. Z.*, 192 (1928) 426.
4 D. B. MORRISON AND E. F. WILLIAMS, *Science*, 87 (1938) 15.
5 M. KIESE, *Biochem. Z.*, 316 (1943) 264.
6 D. W. ALLEN, W. A. SCHROEDER AND J. BALOG, *J. Am. Chem. Soc.*, 80 (1958) 1628.
7 D. L. DRABKIN, *J. Biol. Chem.*, 164 (1946) 703.
8 D. L. DRABKIN, *Arch. Biochem. Biophys.*, 21 (1949) 224.
9 Div. Med. Sciences, NAS–NRC, *Science*, 127 (1958) 1376.
10 R. E. BENESCH AND R. BENESCH, *J. Am. Chem. Soc.*, 77 (1955) 5877.
11 H. BANASCHAK AND F. JUNG, *Biochem. Z.*, 327 (1956) 515.
12 J. SCHUBERTH, *Acta Chem. Scand.*, 10 (1956) 338.
13 C. D. CORYELL, F. STITT AND L. PAULING, *J. Am. Chem. Soc.*, 59 (1937) 636.
14 F. M. HUENNEKENS, R. W. CAFFREY, R. E. BASFORD AND B. GABRIO, *J. Biol. Chem.*, 227 (1957) 261.
15 C. D. CORYELL AND L. PAULING, *J. Biol. Chem.*, 132 (1940) 769.
16 E. L. ELSON AND S. T. EDSALL, *Biochemistry*, 1 (1962) 1.
17 W. GRATZER, private communication.
18 W. D. HUTCHINSON, *Thesis*, California Institute of Technology, 1960.
19 W. R. HOLMQUIST AND J. VINOGRAD, unpublished data.