succinimide, was eluted from the column. By comparison with a calibration curve, yields of the sulfonamide were found to range from 60 to 95%, according to the NBS/substrate ratio and the rate of oxidation.

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Studies on the Oxidation-Reduction Potentials of Heme Proteins. IV. The Kinetics of Oxidation of Hemoglobin and Myoglobin by Ferricyanide*

Eraldo Antonini, Maurizio Brunori, and Jeffries Wyman

ABSTRACT: The kinetics of oxidation of horse myoglobin (Mb) and human hemoglobin (Hb) by ferricyanide have been studied in a stopped-flow apparatus. The oxidation of Mb corresponds to a bimolecular reaction; at 19° the second-order rate constant (o') is $\sim 2 \times 10^6$ M^{-1} sec^{-1} at *p*H 6.0 and $\sim 1.4 \times 10^6$ M^{-1} sec^{-1} at *p*H 9.2.

The results on the rate of the reaction of ferricyanide with MbO_2, measured as a function of O_2 concentration, are similar to those on the displacement of O_2 by CO and indicate a complex situation. The reaction of Hb with ferricyanide does not correspond to a simple bimolecular reaction; at alkaline *p*H the rate tends to increase as the reaction proceeds, while at acid *p*H values it tends to decrease. This phenomenon corresponds to the change of shape of the equilibrium curve with *p*H. The apparent initial second-order rate constant for the oxidation of Hb obtained by extrapolation is, at 19.5°, $\sim 7 \times 10^4$ M^{-1} sec^{-1} at *p*H 6.0 and $\sim 0.8 \times 10^4$ M^{-1} sec^{-1} at *p*H 9.2.

Since the introduction by Hartridge and Roughton (1923) of methods for studying fast reactions in solution, the kinetics of the reaction of hemoglobin and myoglobin[1] with ligands has been extensively investigated. The results obtained up to 1959 have been reviewed and discussed in detail by Gibson (1959); later work has been summarized in some recent reviews (Antonini, 1963, 1965; Rossi Fanelli *et al.*, 1964). As a first approximation, the reactions of ferromyoglobin with various ligands (O_2, CO, isocyanides, nitroso-aromatic com-

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[1] Abbreviations used in this work: Hb, ferrohemoglobin; HbO_2, oxyhemoglobin; Hb^+, ferrihemoglobin; Mb, ferromyoglobin; MbO_2, oxymyoglobin; Mb^+, ferrimyoglobin; k', rate constant for the combination with oxygen; k, rate constant for the dissociation of HbO_2; o', rate constant for the oxidation (by ferricyanide); l', rate constant for combination with carbon monoxide.

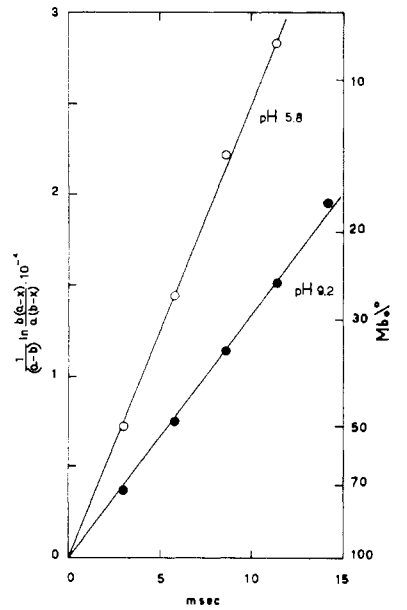

FIGURE 1: Time course of the reaction of Mb with ferricyanide. Ordinates (left): $1/(a - b)$ ln $[b(a - x)/a(b - x)]$, where a = [ferricyanide] = 1×10^{-4} M at $t = 0$; b = [Mb] = 0.2×10^{-4} M at $t = 0$; x = [Mb^+] at time t. Ordinates (right): the per cent of Mb remaining at various times. Abscissas: time in milliseconds. Buffers: 0.1 M phosphate, *p*H 5.8; 1% borate, *p*H 9.2. Temperature = 18.4°. Observations made at λ = 560 mμ.

pounds) correspond to a simple bi- or monomolecular process; on the other hand, in the case of hemoglobin the kinetics are much more complex, reflecting the heme-heme interaction, the Bohr effect, and the other characteristic aspects of the equilibria. Even for myoglobin recent studies indicate that the kinetics are far from simple (E. Antonini, Q. H. Gibson, and J. Wyman, paper in preparation), and for hemoglobin the problem of the quantitative treatment of the kinetics is at present beyond our reach, although great progress on the experimental side has been made thanks to Gibson and Roughton (1955; Gibson, 1959).

Ferricyanide reacts with the ferrous derivatives of myoglobin and hemoglobin, oxidizing the heme iron to the ferric form. One equivalent of ferricyanide oxidizes one equivalent of the protein. The reaction is commonly used in potentiometric studies of the oxidation-reduction equilibrium of heme proteins (Clark, 1960). In the course of studies on the oxidation-reduction potentials of heme proteins (Antonini *et al.*, 1964; Brunori *et al.*, 1964), it seemed interesting to study the kinetics of the oxidation of ferromyoglobin and ferrohemoglobin by ferricyanide. In spite of the limitations imposed by the difficulty of interpreting in detail the kinetics of the reactions of hemoglobin and even of myoglobin, it was thought that valuable information could be obtained in this way. In particular it seemed desirable to find out how far the large difference of the potentials of these two proteins at half oxidation ($E_{1/2}$), as well as other characteristic differences in the equilibrium curves, are reflected in the kinetics of the oxidation process; also to what extent these kinetics resemble those of reactions with other ligands. Such studies are reported in the present paper.

Materials and Methods

Crystalline horse myoglobin was prepared by the method of Rossi Fanelli (1949) and stored in the cold under saturated ammonium sulfate. Ferrimyoglobin was enzymically reduced to the ferro form (Rossi Fanelli and Antonini, 1958) and stored in a tonometer under argon as deoxymyoglobin for no more than 10 days.

Human hemoglobin was prepared from freshly drawn blood according to the standard procedure in use in our laboratory (Rossi Fanelli *et al.*, 1961). Potassium ferricyanide (Carlo Erba) solutions were freshly prepared before the experiments.

Kinetic measurements were performed with a Gibson stopped-flow apparatus (Gibson, 1964) equipped with a 2-cm observation tube. The band width employed was less than 4 mμ.

Concentrations of hemoglobin and myoglobin were determined spectrophotometrically using recent values for the extinction coefficients (Antonini, 1965). For both pigments the concentration is given in heme equivalents.

Solutions containing varying amounts of oxygen were prepared by dilution of solutions equilibrated with 1 atm of oxygen or with air (1 atm of O_2 at 20° gives a concentration of gas in water of 1.34×10^{-3} M).

Results

Reaction of Ferromyoglobin with Ferricyanide. The time course of the oxidation of ferromyoglobin by ferricyanide is shown in Figure 1. It is evident from the graph that the reaction is a second-order one and is described accurately by the equation:

$$o' = \frac{1}{(a - b)} \ln \frac{b(a - x)}{a(b - x)} \times \frac{1}{t} \qquad (1)$$

where a = concentration of ferricyanide at $t = 0$, b = concentration of Mb at $t = 0$, x = concentration of Mb^+ at time t, and o' is the combination velocity constant.

The values of the second-order rate constant calculated according to equation (1) from measurements made under different conditions are reported in Table I.

On the basis of equation (2)

$$\Delta H^{\ddagger} = \frac{RT^2 \, d \ln o'}{dT} \qquad (2)$$

the results at *p*H 6 yield an activation energy ($\Delta H^{\ddagger}$) of +4 kcal; those at *p*H 9.2, 0 kcal. However in view of

TABLE I: Reaction of Ferromyoglobin (Mb) with Ferricyanide.

Buffer	*p*H	*t* (°C)	λ (mμ)	[Mb] (M × 10^4)	[Ferricyanide] (M × 10^4)	*o'* (M^{-1} sec^{-1} × 10^{-6})
Phosphate,	6.0	19	560	0.34	0.34	1.8
0.1 M	6.0	12	560	0.34	0.34	1.5
	5.75	18.5	560	0.20	1.0	2.5
	5.75	11.5	560	0.20	1.0	2.1
Borate,	9.2	19	560	0.34	0.34	1.4
1%	9.2	12	560	0.34	0.34	1.5
	9.2	18.5	560	0.20	1.0	1.4
	9.2	18.5	630	0.20	1.0	1.4

the very small temperature interval involved (7°) and the errors of the individual measurements (estimated as ±10%), these figures should not be taken too literally.

Reaction of Oxymyoglobin with Ferricyanide. It may be assumed that the reaction of MbO_2 with ferricyanide occurs according to the following scheme:

$$MbO_2 \underset{k}{\overset{k'}{\rightleftharpoons}} Mb + O_2$$

$$Mb + \text{ferricyanide} \xrightarrow{o'} Mb^+ + \text{ferrocyanide} \quad (3)$$

The rate of formation of Mb^+ should therefore depend on the rate of dissociation of O_2 and on the rates of reaction of Mb with O_2 or ferrocyanide. As in the analogous case of the displacement of O_2 by CO (Gibson, 1959), it can be shown that if the rate of oxidation (r) is followed at different O_2 concentrations, keeping the [ferricyanide] constant, the plot of $1/r$ versus O_2 concentration should be linear. The intercept should give $1/k$, k being the rate of dissociation of O_2, and the slope should be $k'/(k \cdot o')$[ferricyanide]. Knowing the velocity of combination of O_2 with myoglobin (k') (E. Antonini, Q. H. Gibson, and J. Wyman, paper in preparation) this slope should allow an independent estimation of the velocity constant for the reaction of unliganded myoglobin with ferricyanide.[2]

The rates of formation of Mb^+ from MbO_2 and ferricyanide in the presence of different O_2 concentrations are reported in Table II and a plot of their reciprocals against oxygen concentration is shown in Figure 2. The plot is linear at *p*H 6, but shows a marked downward curvature at *p*H 9.2. As will be discussed later, this curvature indicates a departure from the simple scheme of equation (3), such as has also been found in the analogous case of displacement of oxygen by CO (E. Antonini, Q. H. Gibson, and J. Wyman, paper in preparation).

The intercept is the same for the data at *p*H 6.0 and 9.2 and gives a value for the dissociation constant of

[2] The conditions implied here are given by Gibson (1959). They were satisfied in the present experiments.

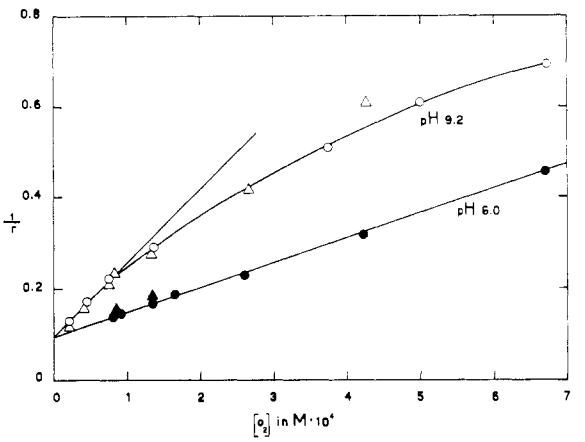

FIGURE 2: Reaction on MbO_2 with ferricyanide. Ordinates give $1/r$ where r is the rate of replacement. Abscissas give O_2 concentration. Different symbols (circles and triangles) indicate different experiments. Buffers 0.1 M phosphate, *p*H 6.0, and 1% borate, *p*H 9.2. Temperature = 19.1°.

TABLE II: Reaction of Oxymyoglobin with Ferricyanide.[a]

*p*H 6.0		*p*H 9.2	
O_2 Concentration (M × 10^4)	r (sec^{-1})	O_2 Concentration (M × 10^4)	r (sec^{-1})
6.7	2.25	6.7	1.45
4.2	3.14	5.0	1.65
2.6	4.38	3.75	1.95
1.65	5.48	1.35	3.44
1.35	5.86	0.75	4.55
0.95	7.0	0.45	5.8
0.81	7.43	0.22	7.8

[a] Determinations were carried out at 19.1°. [Mb] = 0.10 × 10^{-4} *M* at *p*H 6.0 and 0.15 × 10^{-4} M at *p*H 9.2; [ferricyanide] = 5 × 10^{-4} M; observations made at λ = 580 mμ.

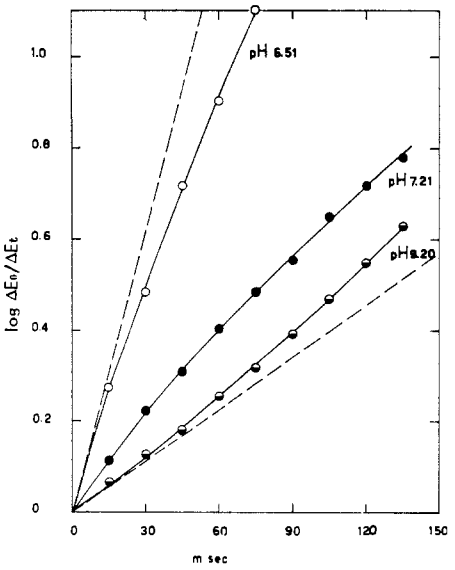

FIGURE 3: Time course of the reaction of Hb with ferricyanide at different *p*H values. Ordinates give log $\Delta E_0/\Delta E_t$ where ΔE_t is the extinction change at time t; ΔE_0 extinction change at time 0. Abscissas give time in milliseconds. The dashed lines show the predicted behavior for a bimolecular reaction calculated from the initial rate constants. Buffers: 0.1 M phosphate, *p*H 6.51 and 7.21; 1% borate, *p*H 9.2. Temperature = 18.5°. [Hb] = 0.17×10^{-4} M; [ferricyanide] = 1×10^{-3} M.

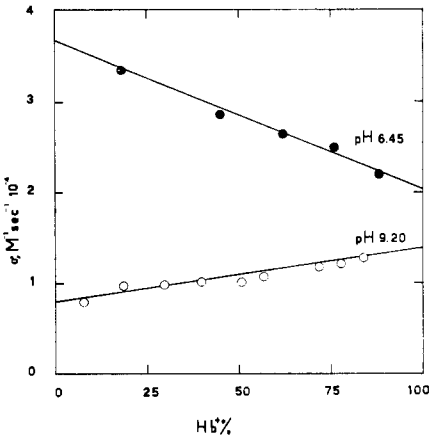

FIGURE 4: Reaction of Hb with ferricyanide. Plot of the apparent second-order rate constant (o'), obtained over successive time intervals, versus the extent of the reaction (% Hb^+). The initial (o'_{in}) and final (o'_{fin}) velocity constants are obtained by extrapolation. Buffers: 0.1 M phosphate, *p*H 6.45, and 1% borate, *p*H 9.2. Temperature 18.5°.

oxygen (k) of 10.4 sec^{-1} at 19.1°; this value is very near to that obtained by other methods. Taking a value of $k' = 1.44 \times 10^7$ M^{-1} sec^{-1} for the combination velocity constant of oxygen, the value of the velocity constant for the reaction of ferricyanide with myoglobin (o'), calculated from the initial slope, is equal to about 1.7×10^6 M^{-1} sec^{-1} at *p*H 9.2 and 5.0×10^6 M^{-1} sec^{-1} at *p*H 6. These are to be compared with directly determined values of $\sim 1.4 \times 10^6$ M^{-1} sec^{-1} at *p*H 9.2 and $\sim 2 \times 10^6$ M^{-1} sec^{-1} at *p*H 6.0 (see Table I).

Reaction of Hemoglobin with Ferricyanide. The time course of the oxidation of hemoglobin by ferricyanide is shown in Figure 3. In these experiments the reaction has been treated as a pseudo-first-order one since the ferricyanide concentration largely exceeded the Hb concentration.

It is evident that the shape of the kinetic curves does not correspond to a simple bimolecular reaction. At alkaline *p*H the rate increases with the progress of the reaction; as the *p*H decreases the shape of the curve gradually changes and at *p*H values below 8 the rate tends to slow down as the reaction proceeds. As in the case of myoglobin (see Table I) the same rate is obtained when the reaction is followed at different wavelengths.

Figure 4 shows a plot of the apparent second-order rate constant (o') versus the extent of reaction (% Hb^+) at *p*H 6.45 and 9.2. The rate constant seems to change linearly with the extent of the reaction; the initial (o'_{in}) and final (o'_{fin}) values, which may be obtained by extrapolation to 0 and 100% of the reaction, have been empirically used to characterize the kinetics of the reaction under the various conditions and are reported in Table III.

It may be seen from Table III that the values of o'_{in} and o'_{fin} are independent of the FeCy concentration both at high and low *p*H. The initial velocity constant (o'_{in}) decreases largely with lowered *p*H, passing from about 0.8×10^4 M^{-1} sec^{-1} at *p*H 9.2 to about 7.0×10^4 M^{-1} sec^{-1} at *p*H 6. It may be noted that these values are much lower ($\sim$0.01) than those found for myoglobin under similar conditions.

It might well be questioned whether the rates just presented apply exclusively to the oxidation of the hemes or whether they involve the effect of side reactions, such as the oxidation of SH groups, as well. Special experiments were undertaken to settle this point. In the first place a series of spectrophotometric titrations of oxy- and deoxyhemoglobin were made at *p*H 7.0 and 9.2. In all cases the spectroscopic end point occurred, within less than 5%, at a 1:1 ratio between heme iron and ferricyanide added. (These experiments will be described in detail in another connection later.) In the second place three additional sets of kinetic experiments reported in Table IV were carried out. In one set the concentration of the ferricyanide was reduced to, or lower than, that of the heme iron. Calculated initial and final values of the velocity constant remained the same even under these conditions and thus do not change over a 60-fold range of ferricyanide concentration. In a second set of experiments EDTA was added to the reaction mixture. It is known that this reagent inhibits the oxidation of the SH groups by ferricyanide (Antonini *et al.*, 1963). In the presence of

TABLE III: Reaction of Ferrohemoglobin (Hb) with Ferricyanide.

Buffer	[Hb] = 0.175 × 10^{-4} M			[Ferricyanide] (M × 10^{3})	o'_{in} (M^{-1} sec^{-1} × 10^{-4})	o'_{fin} (M^{-1} sec^{-1} × 10^{-4})	o'_{fin}/o'_{in}
	*p*H	*t* (°C)	λ (mμ)				
Phosphate, 0.1 M	5.99	19.5	560	1.0	7.4	4.9	0.66
	6.45	20	500, 540, 560, 630	0.5	3.7	2.0	0.54
	6.51	18.5	500, 560, 630	0.25	5.0	2.7	0.54
	6.51	18.5	560	0.5	4.7	2.3	0.49
	6.51	18.5	560	1.0	4.9	2.4	0.49
	6.70	19.5	560	1.0	2.7	1.4	0.52
	6.82	18.5	560	1.0	2.5	1.2	0.48
	7.21	18.5	560	1.0	2.0	0.96	0.45
	7.31	19.5	560	1.0	1.6	0.95	0.60
	8.20	19.5	560	1.0	1.05	1.25	1.19
	8.54	18.5	560	1.0	1.1	1.3	1.18
Borate, 1%	8.82	19.5	560	1.0	1.0	1.1	1.10
	9.01	19.5	560	1.0	0.85	1.2	1.41
	9.17	20	560, 595	0.5	0.8	1.3	1.63
	9.18	18.5	500, 560, 620	1.0	0.8	1.3	1.63
	9.18	18.5	560	0.5	0.8	1.3	1.63
	9.18	18.5	500, 560, 620	0.25	0.85	1.4	1.65
	9.25	19.5	560	1.0	0.75	1.1	1.47

EDTA the rate constants remained unaltered. In a third set of experiments ferrocyanide was added with the ferricyanide to the reaction mixture. No effect was observed. Thus it would seem that the oxidation of the hemes takes precedence over any side reactions which may or may not come later.

Reaction of Oxyhemoglobin with Ferricyanide. The rate of oxidation of oxyhemoglobin by ferricyanide was measured in a few experiments. As in the case of myoglobin it may be assumed that oxygen and ferricyanide compete for the same site. According to Gibson and Roughton (1955), the rate of displacement of one ligand by another in hemoglobin is determined by the velocity constants corresponding to the fourth step of the sequence of reactions involved in the Adair scheme, i.e.,

$$Hb_4X_3 + X \underset{k_4}{\overset{k_4'}{\rightleftharpoons}} Hb_4X_4$$

$$K_4 = \frac{k_4'}{k_4} \qquad (4)$$

Therefore, by considerations similar to those mentioned above for myoglobin in the case of oxidation of oxyhemoglobin by ferricyanide, the plot of the reciprocal of the displacement rate (r) versus the oxygen concentration, when ferricyanide is held constant, should be linear; the intercept of such a plot should correspond to $4/k_4$ and the slope to $4k_4'/k_4o'_4$[ferricyanide].

The results of experiments of this kind are reported in Table V. The plots of $1/r$ versus O_2 concentration, not shown, were very steep, so that the intercept could not be determined with accuracy. The value of $4k'_4/k_4o'_4$ which, according to the treatment of Gibson and Roughton (1955), may be obtained by multiplying the slope by the FeCy concentration, is found to be about 7 at *p*H 6.1 and about 190 at *p*H 9.1. It may be noted that in the case of myoglobin the values of k'/ko', obtained by the same procedure, were 0.27 at *p*H 6 and 0.90 at *p*H 9.2.

It is worthwhile to note that the progress of the displacement reaction followed a simple course, the rate being independent of the extent of the reaction (of course the validity of this is limited to cases in which the change in O_2 concentration during the reaction can be neglected).

Discussion

Although, as mentioned in the introduction, the reaction of ferrohemoglobin and ferromyoglobin with ferricyanide seems to be specific for the oxidation of the heme iron, additional effects may be present. Thus, after treatment of hemoglobin with ferricyanide, the protein appears to contain strongly bound nonheminic iron which is very difficult to remove by dialysis (Steinhard, 1958). Moreover, the properties of ferrihemoglobin obtained by oxidation with ferricyanide differ in some minor details from those of the same derivative prepared by other methods (Antonini *et al.*, 1964). However, the oxidation of hemoglobin and myoglobin by ferricyanide is potentiometrically an entirely reversible process and most of the data on the oxidation-

TABLE IV: Reaction of Ferrohemoglobin (Hb) with Ferricyanide under Varying Experimental Conditions.[a]

pH	[Hb] (M × 10^4)	[Ferricyanide] (M × 10^4)	Total ΔOD	o'_{in}[b] (M^{-1} sec^{-1} × 10^{-4})	o'_{fin}[b]	o'_{fin}/o'_{in}
7.0	0.30	2.60	0.420	2.1	1.2	0.57
7.0	0.30	1.32	0.432	2.4	1.5	0.62
7.0	0.30	0.67	0.450	2.7	1.5	0.56
7.0	0.30	0.34	0.450	3.1	1.8	0.58
7.0	0.30	0.174	0.305	2.4	1.5	0.62
9.2	0.28	2.60	0.230	0.80	1.3	1.6
9.2	0.28	1.32	0.227	0.85	1.2	1.4
9.2	0.28	0.67	0.231	0.75	1.2	1.6
9.2	0.28	0.34	0.235	0.88	1.2	1.4
9.2	0.28	0.174	0.168	1.0	1.6	1.6
7.0	0.16	2.00	0.231	2.6	1.2	0.46
9.2	0.16	2.00	0.120	1.0		
7.0	0.16	1.8 + EDTA[c]	0.240	2.9	1.2	0.42
9.2	0.16	1.8 + EDTA[c]	0.121	1.1		
7.0	0.16	1.6 + ferrocyanide[d]	0.227	2.5	1.3	0.52
9.2	0.16	1.6 + ferrocyanide[d]	0.128	0.87	1.1	1.25

[a] Determinations were carried out at 19° in 0.1 M phosphate buffer (*p*H 7.0) and 1% borate (*p*H 9.2). Observations were made at λ = 560 mμ. [b] Apparent second-order rate constants (initial and final) have been calculated according to equation (1). [c] Final concentration = 5 × 10^{-4} M. [d] Final concentration = 0.32 × 10^{-4} M.

TABLE V: Reaction of Oxyhemoglobin with Ferricyanide.[a]

*p*H 6.1		*p*H 9.1	
O_2 Concentration (M × 10^4)	r (sec^{-1})	O_2 Concentration (M × 10^4)	r (sec^{-1})
6.7	0.98		
5.0	1.49	1.35	0.175
3.35	2.07	0.81	0.36
1.35	4.9	0.27	0.92
0.675	9.1		

[a] Determinations were carried out at 18.8. [Hb] = 0.1 × 10^{-4} M; [ferricyanide] = 50 × 10^{-4} M. Observations made at λ = 577 mμ.

reduction equilibrium of these proteins were obtained by titration with this reagent. In the present experiments no evidence of side effects or of reactions other than the oxidation of the heme iron was ever obtained, even in special experiments designed to detect it; moreover the rates were found to be the same when measured at different wavelengths and they were proportional to the free ferricyanide concentration when the ratio of ferricyanide to Hb was varied from 1 to 60.

The time course of oxidation of Mb by ferricyanide corresponds, as would be expected, to a simple bimolecular reaction, similar to the reactions of myoglobin with gaseous and nongaseous heme ligands (Gibson, 1959; E. Antonini, Q. H. Gibson, and J. Wyman, in preparation). The velocity constant ($o' \sim 2 \times 10^6$ M^{-1} sec^{-1}) is greater than that for the reaction of myoglobin with carbon monoxide ($l' = 5 \times 10^5$ M^{-1} sec^{-1}). It is slightly higher at *p*H 6 than at *p*H 9.2; this may reflect changes in the relative midpoint potentials of the reagents at the two *p*H values, since no *p*H effects are evident in other Mb reactions.

The kinetics of oxidation of oxymyoglobin shown in Figure 2 are essentially similar to those for the displacement of oxygen by carbon monoxide (E. Antonini, Q. H. Gibson, and J. Wyman, in preparation). In both cases the curvature of the $1/r$ versus O_2 concentration plot indicates a departure from the simple scheme embodied in equation (3), which seems to be a phenomenon of general occurrence in myoglobin reactions. It is, however, interesting to note that while this curvature is present in the displacement by ferricyanide only at *p*H 9.2, the displacement by CO is insensitive to *p*H (E. Antonini, Q. H. Gibson, and J. Wyman, in preparation). In spite of these complications, however, the velocity constant of the reaction of unliganded myoglobin with ferricyanide obtained from the displacement reaction is similar to that directly determined. This would appear to imply that scheme (3) represents a reasonably good approximation. This finding seems important in view of alternative mechanisms recently proposed for the same reaction, based on the hypothesis

that the oxygen-iron complex in hemoproteins has the structure of $Fe^{+}O_2^{-}$ (Weiss, 1964).

The results just discussed, which were obtained with myoglobin, may eventually be useful for the interpretation of the much more complex picture found in the reaction of hemoglobin with ferricyanide. In the case of hemoglobin the kinetic curves do not correspond to a simple bimolecular reaction and their shape changes with *p*H; this appears to be the kinetic aspect of the more complicated equilibrium behavior (Antonini *et al.*, 1964). At alkaline *p*H where a sigmoid equilibrium curve ($n \sim 2.5$) is present the rate of reaction gradually increases with time. This behavior duplicates what is found in the reaction of hemoglobin with other ligands (Gibson, 1959) and represents a kinetic expression of stabilizing heme-heme interactions The equilibrium curve for the oxidation-reduction reaction changes shape with *p*H, *n* falling to near 1.2 at *p*H about 6 (Antonini *et al.*, 1964). The behavior of the oxidation kinetics is parallel: the shape of the kinetic curves changes with *p*H and at *p*H below 8 the rate tends to slow down as the reaction proceeds. This latter phenomenon might be due to intramolecular heterogeneity as between α and β chains or to negative interactions between the hemes. It is interesting to note that such kinetic "heterogeneity" is found in the reaction of hemoglobins with oxygen or carbon monoxide when the value of *n* is low and the shape of the equilibrium curve changes with *p*H. This is the case of hemoglobin treated with some sulfhydryl reagents (Gibson, 1959; E. Antonini, J. F. Taylor, M. Brunori, and J. Wyman, in preparation), of acetylated hemoglobin (Bucci *et al.*, 1963), and of normal tuna hemoglobin (E. Antonini and M. Brunori, unpublished experiments). The significance of this phenomenological correlation, however, is not clear.

The initial rate constant of the reaction of deoxyhemoglobin with ferricyanide is much lower than that of deoxymyoglobin; the difference is about 100-fold and reflects the large difference in half potential ($E_{1/2}$) between the two proteins (Antonini *et al.*, 1964; Taylor and Morgan, 1942). It may be noted that in the case of autoxidation the velocity is also much greater for myoglobin than for hemoglobin (Lemberg and Legge, 1949).

The effect of *p*H on the value of the initial rate of reaction for hemoglobin is large; this rate constant increases about 10-fold in passing from *p*H 9.2 to *p*H 6. In the case of other combination velocity constants (e.g., that for O_2 or CO) there is a small *p*H effect in the opposite direction, which however varies from ligand to ligand and is, at least in part, the kinetic expression of the Bohr effect (Gibson, 1959). It is clear that in the case of the reaction of Hb with ferricyanide the situation is much more complicated and no easy interpretation of the *p*H effects is at hand.

A quantitative analysis of the kinetics of oxidation of HbO_2 is even more difficult than that of Hb owing to the even greater number of constants involved (see Gibson, 1959). Nevertheless it is worth pointing out that, within the limited range covered by the data, the rate corresponds to a simple first-order process and changes linearly, as far as can be judged, with O_2 concentration.

In conclusion we emphasize three aspects of these results: (1) The kinetics of the oxidation reactions of both myoglobin and hemoglobin are similar to those involving other ligands which combine with heme. (2) For all such ligand reactions there are large characteristic differences between Mb and Hb. (3) The specific features of the kinetics of the oxidation process of hemoglobin parallel the specific characteristics of the equilibrium behavior.

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