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The Effect of Heme-linked Ionizable Groups on Cyanide Binding to Methemoglobin*

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The pH dependence of the kinetics of the binding of cyanide ion to methemoglobins A and S and to guinea pig and pigeon methemoglobins appears to be not directly correlated with the net charges on the proteins. The kinetics can, however, be adequately explained in terms of three *sets* of heme-linked ionizable groups with pK_1 ranging between 4.9 and 5.3, pK_2 between 6.2 and 7.9, and pK_3 between 8.0 and 8.5 at 20 °C. pK_1 is assigned to carboxylic acid groups, pK_2 to histidines and terminal amino groups, and pK_3 to the acid-alkaline methemoglobin transition. Kinetic second order rate constants have also been determined for the binding of cyanide ion by the four sets of methemoglobin species present in solution. The pK_i values and the rate constants of methemoglobin S are strikingly different from those of methemoglobin A. This result is explained in terms of different electrostatic contributions to the free energy of heme linkage arising from differences in the environments of ionizable groups at the surfaces of the two molecules.

The binding of ligands to the iron atoms of heme proteins is influenced to a considerable degree by the presence of ionizable groups on the protein. In the case of ferric heme proteins kinetic studies of ligand binding as a function of pH give information on the number and nature of the so-called heme-linked groups. Although considerable work of this nature has been done on the single subunit heme proteins metmyoglobin and ferric soybean leghemoglobin (1–5), there is no report of a comprehensive kinetic study of the pH dependence of ligand binding to methemoglobin, a multiple subunit heme protein. This probably arises from the fact that methemoglobin α and β subunits react at different rates with most ligands, and an analysis of such a heterogeneous system would be quite complex. It has been demonstrated, however, that cyanide ion reacts with methemoglobin without heterogeneity (6). This ligand, therefore, presents an attractive tool for a study of the nature of the heme-linked groups in methemoglobin, since the α and β subunits display the same kinetic characteristics toward this ligand.

The pH dependence of the kinetics of the binding of ligands to metmyoglobin (3) and to ferric soybean leghemoglobin (5) has been accounted for in terms of three heme-linked ionizable groups. In similar studies with cyanide as ligand only two heme-linked groups were required to fit the data (4, 5). A study of the pH dependence of the affinity of sperm whale metmyoglobin for azide ion has shown that not just a few, but nearly all, ionizable groups on the protein are heme linked (7).

The heme-linked ionizable groups of hemoglobin may be classed into three sets (8). The first set consists of groups with pK_i values in the range 2.2–4.9 and includes amino acids with carboxyl functions, together with the heme propionic acids. The second set contains ionizable groups with pK_i values mostly in the range 6.3–7.7 and consists of histidines and terminal amino groups. The third set contains cysteines, tyrosines, lysines, and arginines with p$K_i > 10$. An additional ionization is found in ferric hemoglobin with p$K_i \sim 8$ which can be attributed solely to the acid-alkaline methemoglobin transition.

We have carried out a kinetic study of the binding of cyanide ion to methemoglobin. Our data are adequately accounted for in terms of the effect of three *sets* of heme-linked ionizable groups. There appears to be no correlation between the kinetics and the net charges on four selected methemoglobins. The rates of cyanide binding are, therefore, more decisively influenced by the charges on the heme-linked groups than by the net charge on the protein. We find that the rate constants for the binding of cyanide, as well as the pK values of ionizable heme-linked groups, are strikingly different in methemoglobins A and S, two methemoglobins that differ by only a single point amino acid substitution.

MATERIALS AND METHODS

The kinetics were studied under pseudo-first order conditions with a Unicam SP 30UV spectrophotometer equipped with a thermostated cell compartment. Solutions of methemoglobin (2 μM heme) were prepared in phosphate buffers (pH 5.4–8.0) or borate buffers (pH 8–9), each of total ionic strength 0.05 M. These solutions were allowed to temperature equilibrate in a thermostat at 20 °C. For each solution an aliquot was pipetted into a spectrophotometric cell, and the cell was thereafter placed in the thermostated cell compartment of the spectrophotometer. After allowing for temperature equilibration the absorbance of the solution in the cell was recorded. A few microliters of a cyanide solution was then added with a glass rod (one end of which was shaped in the form of a shallow spoon) which served as the stirrer; the final cyanide concentration in the cuvette ranged between 33 and 133 μM. The absorbance of the mixture was recorded at intervals of time at 405 nm.

Reactions were followed for at least 1.5 half-lives before a few crystals of KCN were added to determine the absorbance at the completion of the reaction. Linear plots were analyzed by linear least squares regression.

RESULTS AND DISCUSSION

The homogeneous reaction between methemoglobin and cyanide ion may be written simply as,

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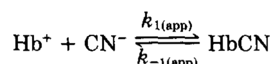


$$Hb^+ + CN^- \underset{k_{-1(app)}}{\overset{k_{1(app)}}{\rightleftharpoons}} HbCN$$

where $k_{1(app)}$ is the apparent recombination rate constant and $k_{-1(app)}$ is the apparent dissociation rate constant. Under pseudo-first order conditions, the observed rate constant is given by,

$$k_{obs} = k_{1(app)}[CN] + k_{-1(app)}. \quad (1)$$

Values of k_{obs} at fixed cyanide concentrations were calculated from the slopes of plots of $\ln(E_t - E_\infty)$ against time (Fig. 1). E_t is the absorbance at time t, and E_∞ is the absorbance at the completion of the reaction. In all cases straight lines were obtained, demonstrating that the reaction adhered to pseudo-first order kinetics and that the α and β chains reacted at the same rate. Plots of k_{obs} *versus* cyanide concentration at constant pH (Fig. 2) gave straight lines, in accordance with Equation 1. From the slopes the values of $k_{1(app)}$ were obtained. The emphasis in these experiments was to obtain good values

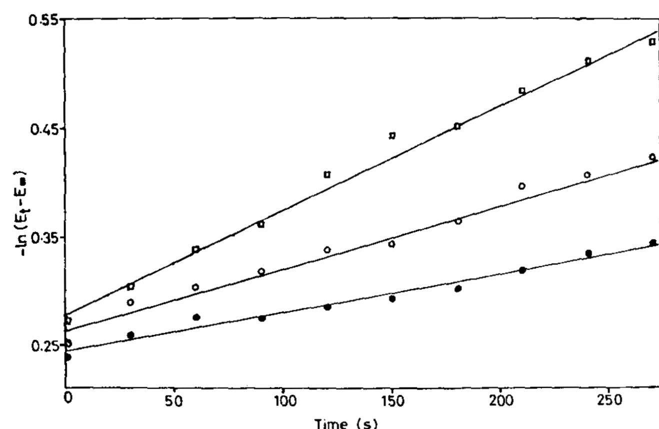


FIG. 1. **Pseudo-first order rate plots for the binding of cyanide ion to methemoglobin S.** Conditions: phosphate buffer, pH 7.7; ionic strength, 0.05 M (added salt, NaCl); 20 °C; methemoglobin concentration, 2 μM heme. The KCN concentrations are 40 μM (*filled circles*), 60 μM (*open circles*), and 80 μM (*squares*). E_t is the absorbance of the mixture at time t and E_∞ is the absorbance at the completion of the reaction.

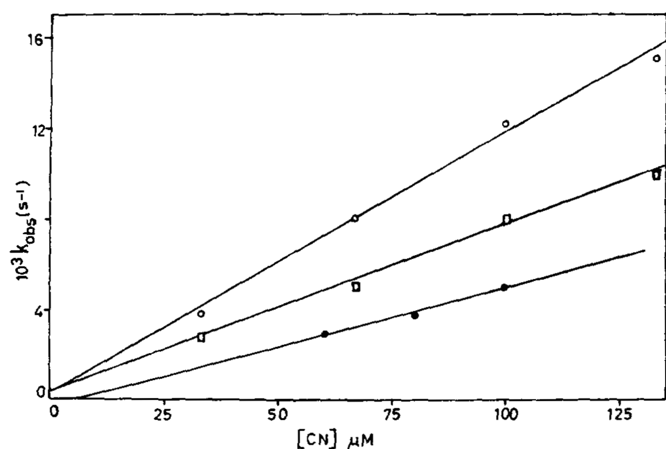


FIG. 2. **Dependence of observed rate constant, k_{obs}, on cyanide concentration at 20 °C for various methemoglobins.** *Open circles*, guinea pig at pH 6.9; $k_{1(app)} = 111$ M^{-1} s^{-1}. *Squares*, pigeon at pH 5.7; $k_{1(app)} = 73$ M^{-1} s^{-1}. *Filled circles*, human S at pH 8.9; $k_{1(app)} =$ 52 M^{-1} s^{-1}. Other conditions as in Fig. 1. The errors involved in the determination of k_{obs} do not exceed $\pm 5 \times 10^{-4}$ s^{-1} as determined from the least squares slopes of plots such as those in Fig. 1. For reasons of clarity, the data for human A are not included.

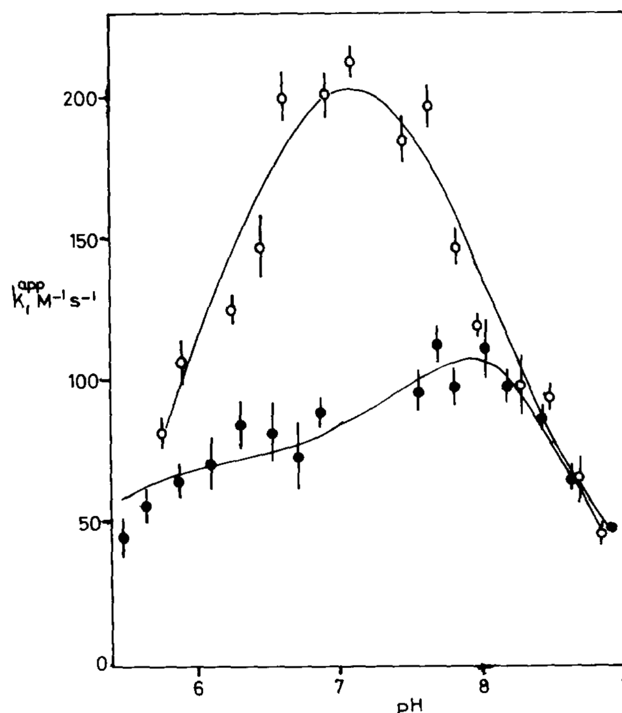


FIG. 3. **Dependence of the apparent second-order rate constant for cyanide binding to methemoglobin, $k_{1(app)}$, on pH at 20 °C.** Conditions: phosphate buffers (pH 5.4–8.0) and borate buffers (pH > 8.0); total ionic strength, 0.05 M; added salt, NaCl. The theoretical lines have been calculated from Equation 2 of the text with the parameters in Table II. *Open circles*, methemoglobin A; *filled circles*, methemoglobin S.

of $k_{1(app)}$ because it is known from the results of Job *et al.* (5) that $k_{-1(app)}$ values are very small and their determination from the intercepts of plots according to Equation 1 would be subject to a great deal of uncertainty. No attempt was therefore made to evaluate $k_{-1(app)}$.

Fig. 3 shows the variation of $k_{1(app)}$ with pH for metHbA[1] and metHbS, and Fig. 4 shows the corresponding results for guinea pig and pigeon. The two sets of results were separated for clarity.

Table I compares the net charge as a function of pH for the methemoglobins at 20 °C (9, 10). The pH dependence of the net charge of metHbS is not known. However, metHbS differs from metHbA by the replacement of a negatively charged glutamic acid residue, β6Glu A3, by a neutral valine on each β subunit. MetHbS must therefore carry a higher net positive charge at any pH than metHbA. On the basis of a consideration of net charges only, it may be predicted that 1) metHbS would react faster with a negatively charged cyanide ion than metHbA at all pH values; 2) guinea pig methemoglobin would react faster than metHbA below pH 7; 3) pigeon methemoglobin would react faster than guinea pig methemoglobin at pH $\geq$ 7 and more slowly at pH < 7. Figs. 3 and 4 show that none of these predictions is borne out. In particular, metHbS reacts more slowly than metHbA throughout most of the pH range studied, except over the narrow range pH > 8.2 where the reactivities of the two methemoglobins become similar. These results indicate that the kinetics of cyanide binding cannot be predicted on the basis of net charge.

The kinetics of ligand binding to ferric heme proteins have been analyzed in terms of the effect of heme-linked ionizable groups (2–5). In these analyses it was assumed that only a

[1] The abbreviations used are: MetHbA, human methemoglobin A; MetHbS, human methemoglobin S.

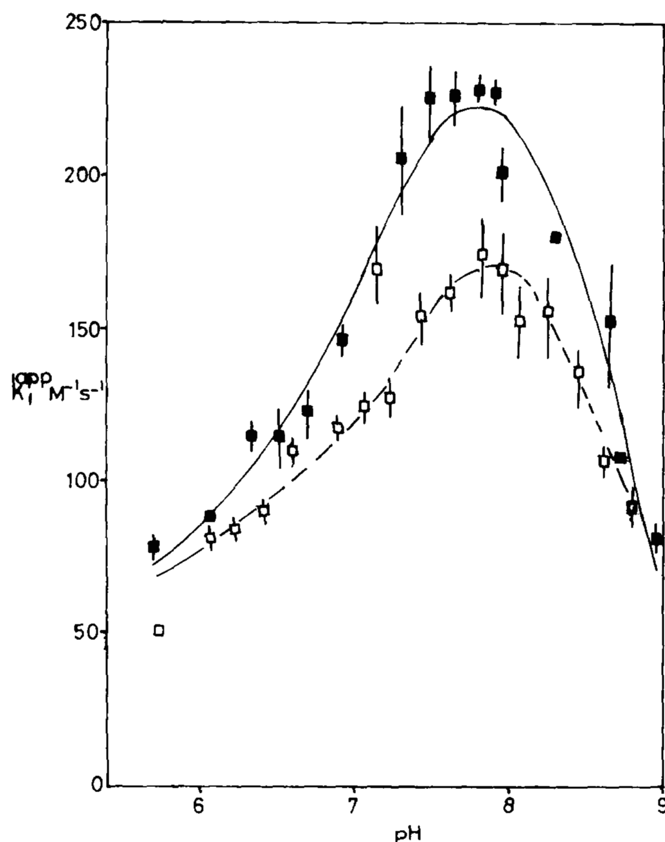


FIG. 4. **Dependence of the apparent second-order rate constant for cyanide binding to methemoglobin, $k_{1(app)}$, on pH at 20 °C.** Conditions as in Fig. 1. The theoretical lines have been calculated from Equation 2 of the text with the parameters in Table II. *Open squares*, guinea pig methemoglobin; *filled squares*, pigeon methemoglobin.

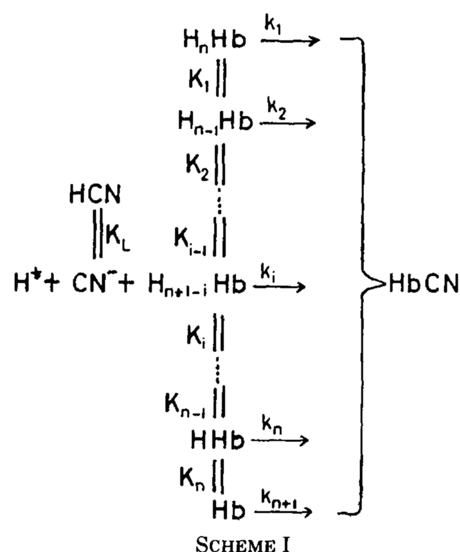
TABLE I

Net charge on methemoglobins as a function of pH

Conditions: 20 °C; ionic strength, 0.05 M. The metHbA data are from Ref. 9 and the pigeon and guinea pig data are from Ref. 10.

pH	6.0	6.5	7.0	7.5	8.0	8.5	9.0
Methemoglobin A	17.2	11.6	5.8	0.1	−4.7	−8.6	−11.5
Guinea pig	17.7	12.3	5.1	−0.2	−.65	−11.0	−14.5
Pigeon	15.7	11.8	7.8	2.4	−1.10	−4.7	−8.2

few ionizable groups close to the heme have any influence on the binding of ligand. However, in a study of the pH dependence of the affinity of sperm whale metmyoglobin for azide ion (7) it was demonstrated that nearly all ionizable groups on the molecule are heme linked. Therefore, any reaction scheme based on the effect of heme-linked ionizable groups must take account of a large number, n, of heme-linked groups. We therefore formulate Scheme I for the binding of cyanide ion to methemoglobin. It can be shown that for this scheme the proton equilibria are fast relative to cyanide ion binding under almost all our experimental conditions.[2]



SCHEME I

Assuming that, of the two species HCN and CN^-, CN^- binds exclusively to methemoglobin (5), it is readily shown that for Scheme I the expression for $k_{1(app)}$ (see Equation 1) is given by:

$$k_{1(app)} = \frac{K_L}{K_L + [H^+]} \cdot \frac{k_1[H^+]^n + \sum_{i=2}^{n+1} k_i [H^+]^{n+1-i} \prod_{j=1}^{i-1} K_j}{[H^+]^n + \sum_{i=2}^{n+1} [H^+]^{n+1-i} \prod_{j=1}^{i-1} K_j} \quad (2)$$

Job *et al.* (5) assumed that the binding of cyanide ion to leghemoglobins is influenced by only two heme-linked ionizable groups. Their scheme and fitting equation are generated by putting $n = 2$ in Scheme I and Equation 2, respectively. In view of the results of Friend *et al.* (7) it is clear that the data of Job *et al.* (5) may be accounted for not as the effect of two heme-linked groups but of two *sets* of heme-linked groups, each set containing a large number of ionizable groups of closely similar pK (compare with Ref. 8). Following Job *et al.*

[2] The kinetic model of Scheme I assumes that the protolytic equilibria corresponding to pK_L, pK_1, pK_2, and pK_3 (Scheme I) are established much faster than the binding of cyanide to methemoglobin. The equilibria corresponding to pK_L, pK_1, and pK_2 are known to have second order rate constants of the order of 10^{10} M^{-1} s^{-1} (23–26). This value is about 4 orders of magnitude higher than k_1 (Table II) and about 6 (8) orders of magnitude higher than $k_2(k_3)$. The equilibrium corresponding to pK_3 is the acid-alkaline methemoglobin transition. It is known that this transition occurs in the time range of 1–10 μs and is too fast to be followed with a conventional temperature-jump apparatus (27). These results (23–27) justify the assumption that the protolytic steps of Scheme I are very fast compared to the binding of cyanide ion.

The validity of this assumption at high pH, where the hydrogen ion concentration is low, may be questioned. At high pH the H_3Hb species (Scheme I) ceases to exist, and only H_2Hb and HHb need be considered. We may make a rough comparison of the velocities of reaction of cyanide ion and hydrogen ion, respectively, with H_2Hb. At pH 9, our highest experimental pH, the hydrogen ion concentration is ~10^{-9} M. When this is multiplied by the second order rate constant of ~10^{10} M^{-1} s^{-1} observed for the protolytic steps (23–26) one obtains a velocity of ~10 s^{-1}. The highest cyanide concentration that we used was ~10^{-4} M. The product of this concentration and the highest k_2 value of ~10^5 M^{-1} s^{-1} (that of metHbA, Table II) gives a velocity of ~10 s^{-1}, the same as for the protolytic steps. Thus, at the highest experimental pH and cyanide concentration, the assumption that the protolytic steps of Scheme I equilibrate much faster than the cyanide-binding reaction may not be valid for metHbA. It will, however, be valid for the other methemoglobins we have studied, because the product of their k_2 (~10^4 M^{-1} s^{-1}, Table II) and the highest experimental cyanide concentration (~10^{-4} M) gives a velocity of ~1 s^{-1} which is roughly an order of magnitude slower than the velocity of the protolytic steps, ~10 s^{-1}. At about pH 8 the assumption of fast protolytic steps would be valid, even for metHbA, because the velocity of the protolytic steps, now ~10^2 s^{-1}, would be 10-fold higher than the velocity (~10 s^{-1}) of cyanide binding at the highest experimental cyanide concentration. The assumption of fast proteolytic steps is of course valid at low cyanide concentration (~10^{-5} M) under all our experimental conditions. We conclude that, except for metHbA over a very narrow pH range close to pH 9 and at the highest experimental cyanide concentration, the assumption of fast protolytic equilibration inherent in Scheme I is valid for all the methemoglobins we studied.

(5), we attempted to fit the data of Figs. 3 and 4 with two *sets* of heme-linked groups but obtained unsatisfactory fits to all the data. In particular, the pK values of the acid-alkaline methemoglobin transition were required to be about 6.5 to give anything close to a satisfactory fit, compared to the experimental value of about 8.0 (11, 12). For this reason, we fitted the data with three *sets* of heme-linked groups, that is with $n = 3$ in Scheme I and Equation 2. Satisfactory fits were obtained to all the data. The *lines* in Figs. 3 and 4 are theoretical lines calculated from Equation 2 with $n = 3$. The fitting parameters are shown in Table II.

The curve fitting was treated as a constrained nonlinear optimization problem of the unknown parameters, the reaction rate constants k_i and the equilibrium constants K_i. The objective function was χ_r^2. The user-friendly program, developed for the nonspecialist user of curve-fitting algorithms, permits the separation of parameters which appear linearly in the expressions to be fitted (13, 14) (in the present case the reaction rate constants k_i) in such a way that the optimization search is carried out in the reduced hyperspace of the nonseparable parameters which, in the present case, are the equilibrium constants K_i. Besides, a non-negativity constraint was imposed on the equilibrium constants by using pK_i rather than K_i values. Kaufman and Pereyra (14) developed separation algorithms for the case of equality constraints. By using penalty functions (15) we have developed an algorithm which can be used with inequality constraints.

Friend *et al.* (7) analyzed the pH dependence of the equilibrium binding constant of azide ion to sperm whale metmyoglobin in terms of a modified discrete charge electrostatic theory (16). An important aspect of their analysis is the introduction of the concept of a burial parameter for a heme-linked ionizable group. Therefore, depending on the size of the functional electrostatic domain of a given heme protein, the extent of heme linkage of a given ionizable group is a function of that group's effective charge, its accessibility to solvent, denoted by its static solvent accessibility, and its distance from the ferric heme iron. Thus, the fact that a given ionizable group is relatively distant from the heme iron is not necessarily a good indication of weak heme linkage of that group. An ionizable group that is far from the heme iron may be significantly heme linked if its static solvent accessibility is low and if its effective charge is high. For this reason it would not be correct to assign every one of the pK_i in Table II exclusively to individual ionizable groups near the heme. The pK_i can, however, be assigned to sets of ionizable groups having similar functional groups. Three such sets of ionizable groups are present in hemoglobin, as shown by calculations of acid-base titration curves (8) which are based on the modified discrete charge electrostatic model (16). On the basis of these results (8) the following assignment of the pK_i in Table II are made. pK_1 is assigned to the set of ionizable groups with carboxylic acid function; pK_2 is assigned to histidines and terminal amino groups; and pK_3 is assigned to the acid-alkaline methemoglobin transition.

The second order kinetic rate constants for cyanide binding to the various methemoglobin species are reported in Table II. The values of k_1 do not seem to vary much among the animal species we have studied. When these values are compared with the corresponding values for ferric soybean leghemoglobins a and c (5), it is seen that our values are about an order of magnitude lower. These results imply that when all the carboxylic acid groups in methemoglobin are protonated, the ligand-binding sites in metHbA, metHbS, guinea pig, and pigeon are equally accessible to cyanide ion but are less accessible than the binding sites in ferric soybean leghemoglobins. Values of k_3 for all the species (Scheme I, Table II) are of about the same magnitude. This would imply that when the heme-linked carboxyl and histidyl groups are fully deprotonated the doubly deprotonated aquomet form of the various animal species reacts with cyanide ion at about the same rate. In line with previous results (5) we obtain $k_4 \sim 0$ for all methemoglobins. This finding supports the conclusion that the alkaline methemoglobin species does not bind cyanide to any measurable extent.

A striking discrepancy is observed (Table II) between the value of k_2 for metHbA and those of the other methemoglobins; k_2 (metHbA) is almost 2 orders of magnitude greater than k_2 (metHbS) and is at least an order of magnitude greater than those of the other two animal species. The high reactivity of metHbA compared to metHbS and guinea pig (Figs. 3 and 4) can be accounted for mostly by this large value of k_2 (metHbA). This cannot, however, explain the fact that above about pH 7.2 $k_{1(\text{app})}$ for metHbA is lower than for pigeon.

Equation 2 shows that, in considering the contribution of each species (Scheme I) to the magnitude of $k_{1(\text{app})}$ at a given pH and cyanide concentration, the product of the rate constant k_i and the relative population P_i of each species i must be taken into account, not just k_i only. In Fig. 5 we compare the relative populations P_i of the reacting species (Scheme I) for metHbA and pigeon. The values of P_1 (corresponding to H_3Hb) are about the same for both methemoglobins and, since the k_1 values are about equal (Table II), species H_3Hb makes about the same contribution to $k_{1(\text{app})}$ for both methemoglobins. Although P_2 (metHbA) is lower than P_2 (pigeon) above pH 5.5, the much larger value of k_2 (metHbA) compared to k_2 (pigeon) (Table II) makes the product k_2P_2 (metHbA) greater than k_2P_2 (pigeon) below about pH 7.2. Therefore, up

TABLE II

Fitting parameters used to calculate the theoretical lines in Figs. 3 and 4 according to Equation 2 of the text (cf. Scheme I)

Parameter	$n = 3$				$n = 4$			
	metHbA	metHbS	Guinea pig	Pigeon	metHbA	metHbS	Guinea pig	Pigeon
$10^{-6}\ k_1\ M^{-1}\ s^{-1}$	0.6	2.3	1.2	1.0	0.8	0.8	0.5	1.0
$10^{-4}\ k_2\ M^{-1}\ s^{-1}$	29.1	0.5	1.2	3.4	22.6	0.4	0.9	3.8
$10^{-3}\ k_3\ M^{-1}\ s^{-1}$	0.7	0.5	1.5	0.5	0.6	0.6	1.3	0.7
$k_4\ M^{-1}\ s^{-1}$	0	0	0	0	0	0	0	0
$k_5\ M^{-1}\ s^{-1}$					0	0	0	0
pK_1	5.22	4.80	5.18	5.25	5.20	5.29	5.65	5.24
pK_2	6.23	7.91	7.70	7.25	6.35	7.89	7.85	7.17
pK_3	8.00	8.22	8.09	8.47	8.00	8.22	8.09	8.47
pK_4					11.5	11.5	11.5	11.5
pK_L	9.3	9.3	9.3	9.3	9.3	9.3	9.3	9.3

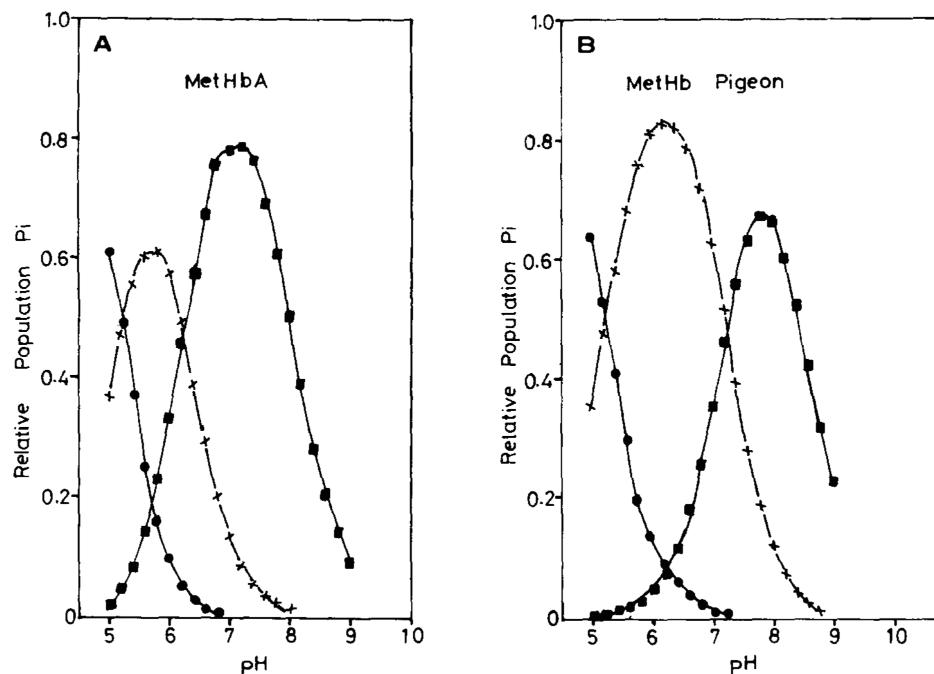


FIG. 5. *A* and *B*, dependence of the relative populations of reacting species on pH (*cf.* Scheme I and Equation 2 of the text). *Circles*, H_3Hb; *crosses*, H_2Hb; *squares*, HHb. The relative populations are referred to a total concentration of unity.

to about pH 7.2 $k_{1(app)}$ (metHbA) is greater than $k_{1(app)}$ (pigeon) due, largely, to the much higher reactivity of the species H_2Hb of metHbA compared to pigeon. Above pH 7.5, P_2 (pigeon) becomes very large compared to P_2 (metHbA) (see Fig. 5), and this difference in magnitude is just sufficient to cancel out the difference in k_2P_2 between the two methemoglobins, arising from a large value of k_2 (metHbA) compared to k_2 (pigeon). Additionally, above pH 7.5 the species HHb (Scheme I) becomes more highly populated in pigeon than in metHbA (see Fig. 5), that is P_3 (pigeon) is greater than P_3 (metHbA). Since k_3 is about the same for both methemoglobins (Table II), the relatively higher P_3 (pigeon) provides an explanation for the fact that $k_{1(app)}$ (pigeon) is higher than $k_{1(app)}$ (metHbA) at high pH. Similar detailed explanations suffice to show that $k_{1(app)}$ (metHbA) must be higher than the corresponding values for metHbS and guinea pig through most of the pH range studied.

A striking aspect of our results is that the rate constants and pK_i values of metHbS are considerably different from those of metHbA. Considering that both methemoglobins differ only by the single replacement of β6Glu A3 in metHbA by valine in metHbS the two methemoglobins would be expected to have similar k_i and pK_i values. In a study of the effect of organic phosphates on the sulfhydryl reactivities of oxyhemoglobins A and S and of methemoglobins A and S, we noted (17) that the two hemoglobins differ in structure in the vicinities of the reactive β93Cys F9 residues and at the organic phosphate-binding site; Curd *et al.* (18) have purified antibody fractions specific for oxyhemoglobin S, and their result suggests a possible modification of the amino-terminal region of the β chains of hemoglobin S compared to hemoglobin A. These results suggest gross structural differences between hemoglobins A and S. It is not likely that these structural differences will leave the heme-linked ionizable groups unaffected. In fact Elbaum *et al.* (19) have measured the surface tensions of hemoglobins A and S and have found that there are differences in their surface properties. Furthermore, Fung *et al.* (20) have observed by NMR that the resonances of some surface residues of hemoglobin S are altered compared to hemoglobin A. They conclude that these surface residues may be in a more hydrophobic environment in hemoglobin S compared to hemoglobin A. If the surface residues of metHbS are less accessible to solvent this would mean that their static solvent accessibilities are lower and that such surface residues would produce a greater electrostatic effect at the heme than the corresponding residues in metHbA. Moreover, the heme pocket has a low static solvent accessibility (21) and this increases its sensitivity to charge-site interactions (16, 22).

As demonstrated by Friend *et al.* (7) the affinity of sperm whale metmyoglobin for azide ion decreases with increasing pH, and this result may be explained in terms of an increasingly unfavorable electrostatic contribution to the free energy of heme linkage for azide binding as pH increases from 4 to 6. Since our experiments were carried out at pH $\gtrsim$ 6 it is safe to assume that the same unfavorable electrostatic effect is operational in methemoglobin. Consequently, a higher unfavorable electrostatic contribution to heme linkage for anion binding would be sensed at the metHbS hemes than at the metHbA hemes, since the surface residues of metHbS are less accessible to solvent (20) and therefore have lower static solvent accessibilities. It is therefore to be expected that the ionization of the water molecules at the sixth coordination positions of the iron atoms of metHbS would be more difficult than for metHbA, since this ionization is formally analogous to the binding of an anionic ligand. This is indeed found to be the case (11), and pK_3 (metHbS) is greater than pK_3 (metHbA) (Table II).

Table II also shows the interesting finding that pK_2 (metHbS) is higher than pK_2 (metHbA) by as much as 1.7 pK units. The effective pK, pK_i, of an ionizable site i is given by (16),

$$\mathrm{p}K_i = (\mathrm{p}K_{int})_i - \frac{1}{2\cdot 303kT}\sum_{j\neq i}\frac{1}{Z_i}W_{ij}(1 - SA_j)$$

where W_{ij} is the free energy of interaction between a pair of unit point charges Z_i and Z_j, SA_j is the static solvent accessibility of the site j interacting with site i, and $(\mathrm{p}K_{int})_i$ is the pK value of a site i in a hypothetically discharged molecule. Table V of Ref. 8 shows that, except for His$^{\beta 143}$, histidine and terminal valine sites in deoxyhemoglobin A and oxyhemoglo-

bin A have pK_i values that are significantly higher than $(pK_{int})_i$. Thus the electrostatic interaction term in the above equation gives rise to an increase in pK over and above $(pK_{int})_i$ for these sites. The contribution of this term to the pK increase over and above $(pK_{int})_i$ is in inverse proportion to the magnitude of the static solvent accessibility, SA_j, of site j. Since SA_j values for metHbS are lower than those of metHbA it follows that pK_2 (metHbS) should be higher than pK_2 (metHbA), as has been observed (Table II). pK_2 is probably a very sensitive indicator of the difference between metHbA and metHbS because it is histidines that are titratable through most of the pH range 5.6–9 covered by our experiments.

The surprisingly low values of $k_{1(app)}$ for metHbS compared to metHbA are mirrored almost exclusively by k_2 (metHbS), as may be seen in Table II. This result may also be explained in terms of the electrostatic contribution to the activation energy for the binding of cyanide ion. It is likely that an unfavorable electrostatic contribution increases the activation energy barrier in species H_2Hb of metHbS compared to metHbA, thereby slowing down the reaction of metHbS with cyanide ion relative to metHbA.

From the above discussion it appears that the important species (Scheme I) giving rise to the surprising differences between metHbA and metHbS is H_2Hb (Scheme I with $n = 3$). H_2Hb is the species in which all the carboxylic acid residues of methemoglobin are fully deprotonated and therefore fully negatively charged. The extra negative charge on metHbA compared to metHbS (arising from the presence of β6Glu A3 which in metHbS is Val) does not appear to have produced the adverse effect on the binding of cyanide ion or on the acid-alkaline methemoglobin transition, expected for metHbA compared to metHbS. This emphasizes the role of the relatively more hydrophobic environment of the surface residues of metHbS in producing greater electrostatic effects at the heme than a single extra negative charge (on metHbA) which sticks out into the solvent, a high dielectric constant medium.

Since there are actually four sets of ionizable groups in methemoglobin (the three sets in ferrous hemoglobin (8) plus the acid-alkaline methemoglobin transition) we have also analyzed our data (Figs. 3 and 4) on the basis of four sets of ionizable heme-linked groups. In carrying out this analysis (Scheme I and Equation 2 with $n = 4$) we fixed pK_3 at the values obtained from the three-set analyses because they are close to the directly determined experimental values (11, 12). Since $k_4 = 0$ from the three-set analysis we quite reasonably set $k_5 = 0$; we also kept pK_L fixed at the value 9.3 (*cf.* Ref. 28) used for the three-set analysis. Cysteines, lysines, tyrosines, and arginines have $pK_i \approx 11.5$ (8). We therefore used this value for the four-set analysis. Table II compares the results of the three- and four-set analyses. The introduction of a fourth (high pK) set of heme-linked ionizable groups does not appear to have a significant effect on the relative values of pK_1 and pK_2 or on the k_i values. This may be because the groups in the fourth set do not titrate in the pH region of our experiments, pH $\leq$ 9. The theoretical curves obtained from the four-set analysis can hardly be distinguished from those obtained from the three-set analysis. We conclude that even though there are *four sets* of heme-linked ionizable groups in methemoglobin the difference between a three- and a four-set fit is not significant with respect to our data.

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