CREVICE STRUCTURES IN HEMOPROTEIN REACTIONS

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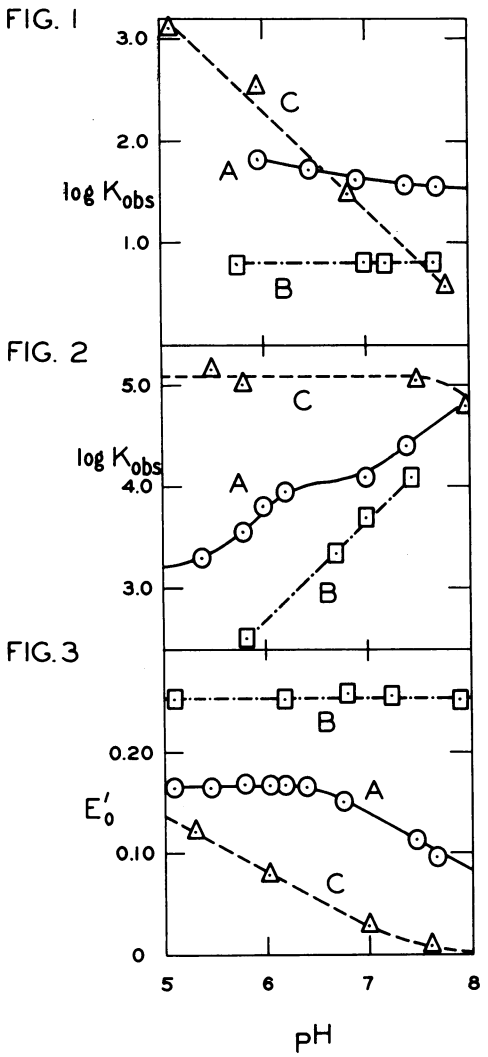
The hemoproteins are such versatile oxidation-reduction catalysts and such remarkable oxygen-binding pigments that there has always been a lively interest in the structural factors that determine the reactivity of the heme iron atom. The high specificity which characterizes many of their reactions, which is shown in only a rudimentary manner by heme itself, would appear to rule out structures with no direct Fe-protein bonding; so, in general, the binding of the heme can be considered in terms of one or two Fe-protein bonds, with additional bonding through the vinyl and propionic acid side chains. Furthermore, the configuration of the heme is also an important factor. A hemoprotein with the heme bound flat on the surface of the protein would be expected to show differences in reactivity, especially in the case of complex formation with ligands, from one in which the heme is buried in a crevice or fold in the polypeptide chain.

In the past the need to consider the influence of structural factors on reactivity, notably crevice structures, has arisen only in studies on cytochrome-c, hemoglobin, and myoglobin. However. in the present paper it will be shown that certain features of the formation of ferriperoxidase and ferricatalase complexes, which set them apart from the other three hemoproteins, can be explained by the simple hypothesis that a crevice structure with two Fe-protein bonds exists in the parent compounds, and, when a complex formation occurs, one of the bonds is broken. liberating a group with a high proton affinity.[1]

A. STATEMENT OF THE PROBLEM

The fact that ferrohemoproteins form complexes preferentially with neutral ligands such as O_2, CO, NO, and isocyanides, whereas ferrihemoproteins react preferentially with "fluoride," "cyanide," "azide," etc., is very suggestive that in the ferric complexes the anions F^-, CN^-, and N_3^- are bonded to the iron and not the neutral, i.e., uncharged, conjugate acid species HF, HCN, and HN_3.[2] Moreover, this distinction would be in accord with many other reactions in co-ordination chemistry in which fluoride, cyanide, and azide react with transition metal ions.[3] It is not surprising, therefore, that there are great similarities between the magnetic susceptibilities and the visible and near-ultraviolet spectra of the corresponding complexes of ferrihemoglobin, ferrimyoglobin, and ferriperoxidase with fluoride, cyanide, and azide—similarities which extend further to the fluoride and cyanide complexes of ferricatalase.[4, 5] The assumption has accordingly been made and generally accepted that these particular complexes all contain the anions bonded to the iron.

But, in spite of these similarities, the pH variations of the equilibrium constants for complex formation are very different. With relatively strong acid ligand systems, the equilibrium constants for ferrimyoglobin and ferrihemoglobin are approximately independent of $[H^+]$ in the pH range 5–8 and 5–7, whereas those for ferriperoxidase and ferricatalase are directly proportional to $[H^+]$ (see Fig. 1, *A* and



FIGS. 1–3.—Typical pH variation of log K_{obs} for complex formation by the ferrihemoproteins, and of E_0' for the ferrihemoprotein–ferrohemoprotein couples. For convenience in plotting the data, 1.0 has been subtracted from all the original log K_{obs} values in Fig. 2, A, and 0.3 volts added to all the original E_0' values in Fig. 3, C. FIG. 1: Relatively strong acid ligand systems. *A*, ferrimyoglobin + fluoride,[6] $pK_{HF} = 3.2$; *B*, ferricytochrome-c + Azide,[11] $pK_{HN_3} = 4.7$ *C*, ferriperoxidase + Fluoride.[8] FIG. 2: Very weak ligand systems. *A*, Ferrimyoglobin + cyanide,[6] $pK_{HCN} = 9.5$; *B*, ferricytochrome-c + cyanide,[12] *C*, ferricatalase + cyanide.[10] FIG. 3: E_0' values with respect to the standard hydrogen electrode. *A*, hemoglobin;[14] *B*, cytochrome-c;[15] *C*, peroxidase.[16] Experimental details—ionic strengths, temperatures, etc.—may be found in the references.

C); with very weak acid ligand systems, over the same pH ranges, the equilibrium constants are approximately inversely proportional to $[H^+]$ and independent of $[H^+]$, respectively (see Fig. 2, *A* and *C*).[6–10] The variations for ferricytochrome-c follow those for ferrimyoglobin and ferrihemoglobin with both types of ligand systems (see Figs. 1, *B*, and 2, *B*).[11, 12] These data indicate that a proton participates as a reactant in the ferriperoxidase and ferricatalase equilibria, but not in those of the other hemoproteins over the same pH range.

The pH variations of the oxidation-reduction potentials can be considered in the same context, because a one-equivalent reduction, like complex formation with an anionic ligand, reduces the charge on a metal ion complex by one unit. These pH variations also fall into two classes (see Fig. 3, *A*, *B*, and *C*). Those for myoglobin, hemoglobin, and cytochrome-c are all approximately independent of $[H^+]$ in the pH range 5–7, and correspond to that expected for single-electron transfer,[13–15] i.e.,

$$Fe^{III} + e^- \longleftrightarrow Fe^{II}$$

But the variation for peroxidase involves a direct proportionality to $[H^+]$, indicating that a proton again participates,[16] i.e.,

$$Fe^{III} + e^- + [H^+] \longleftrightarrow Fe^{II}$$

Oxidation reduction between the ferrous and the ferric states and the formation of the ferric complexes thus show entirely consistent behavior.

The following conclusions can be drawn: First, there is some fundamental difference in the composition and structure of either the parent compounds or their complexes, whereby, if anions are bonded to the heme

iron, a proton is bonded elsewhere in the complexes of ferriperoxidase and ferricatalase but not in those of ferrimyoglobin, ferrihemoglobin, and ferricytochrome-c. Second, since the oxidation-reduction reactions show the same behavior, the difference originates in the structure of the common reactant, namely, the parent ferrihemoprotein. The problem is thus to explain how the structure of a ferrihemoprotein can influence the pH dependence of its reactions, and this will first be discussed in general terms.

B. CREVICE STRUCTURES AND HEME REACTIVITY

Two types of crevice structure can be envisaged, depending on whether the iron atom is joined to the protein on one or both sides of the porphyrin ring, and, for brevity, they will be referred to as "one-bond" and "two-bond" crevice structures, respectively. The one-bond crevice structure can be regarded as one extreme (Fig. 4, *A*) in a whole series of structures ranging from another extreme in which the heme is held flat on the surface of the protein (Fig. 4, *B*). In these cases it has been

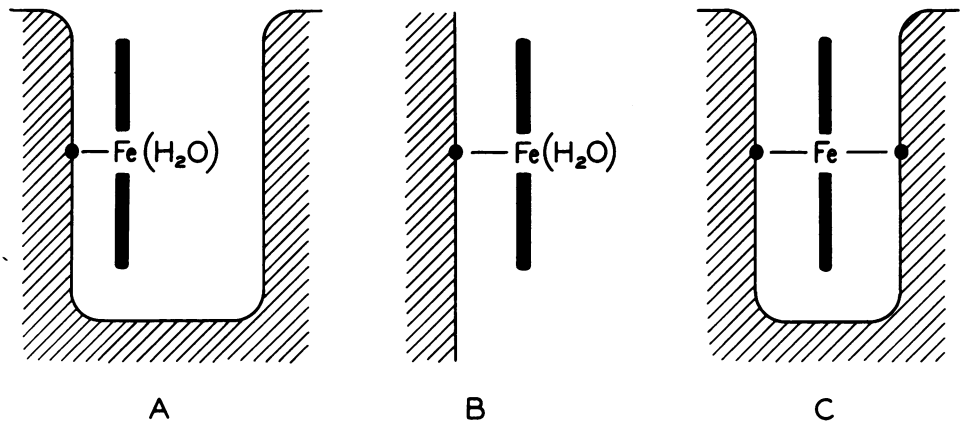


FIG. 4.—Hemoprotein structures. *A*, one-bond crevice; *B*, heme flat on surface; *C*, two-bond crevice. The plane of the porphyrin ring is perpendicular to the paper, and bonding via the side chains has been omitted.

customary to assume that the sixth co-ordination of the iron is occupied by a water molecule. Experiments on the drying of hemoglobin and its derivatives furnish some indirect evidence for the presence of a co-ordinated water molecule of this kind.[17, 18] If, however, the orientation of the heme in the crevice makes possible co-ordination with a second group in the protein to give an intramolecular complex, a two-bond crevice structure is obtained (Fig. 4, *C*).

The one-bond and two-bond crevice structures have an important property in common: the part of the protein which shields the heme may sterically hinder the entry of ligand molecules. It is this aspect of the influence of crevice structures on reactivity that has claimed most attention. Extensive kinetic and equilibrium data for the reaction of cyanide with ferricytochrome-c, which has a well-established two-bond crevice structure[19–22] have been discussed from this point of view.[12] On the basis of equilibrium constants alone, steric hindrance has been suspected in the combination of ferrohemoglobin and ferromyoglobin with *tert*-butyl isocyanide, because the constants are much less than those for the ethyl, *n*-propyl, and *iso*-

propyl derivatives, and, furthermore, no similar decrease is found in the corresponding reactions of ferroheme.[23, 24] A one-bond crevice structure for hemoglobin has also been advanced to explain heme-heme interaction in the oxygenation reaction,[25] and it has been suggested that one heme-linked ionizing group is a histidine residue shielding the heme but held in such a way that direct co-ordination with the iron cannot occur.[26] On the other hand, the very substantial differences between the thermodynamic data for corresponding reactions of ferrihemoglobin and ferrimyoglobin and those of ferricytochrome-c (amounting to 20 kcal/mole and 55 e.u. in ΔH^0 and ΔS^0, respectively) have been taken as evidence that the heme is either flat on the surface in both hemoglobin and myoglobin or else situated in a much shallower crevice than it is in cytochrome-c.[27] In the case of myoglobin in the crystalline state, this conclusion is now amply supported by recent X-ray studies.[28] The heme is held by links to at least four neighboring polypeptide chains, and on one side it is readily accessible from the environment for the attachment of small ligands like oxygen and large ligands such as *para*-iodo-phenylhydroxylamine.

Another important property of all hemoprotein structures and especially crevice structures is the proton affinity of the group that is replaced by the ligand when complex formation occurs. This has attracted little interest, although very significant differences are possible. For hemoproteins in which the sixth co-ordination position is occupied by a water molecule, including, therefore, those with a one-bond crevice structure, the addition of a ligand simply liberates a solvent water molecule. On the other hand, when a hemoprotein with a two-bond crevice structure forms a complex, the weaker Fe-protein bond is broken, and the group liberated may have a high proton affinity or a low proton affinity. In the latter case the reactions may be indistinguishable from those involving the replacement of a water molecule as will appear shortly.

C. HYDROGEN ION PARTICIPATION IN HEMOPROTEIN REACTIONS

The way hydrogen ions are *directly* involved in complex formation will be governed by the following factors, associated with the hemoprotein and with the ligand, respectively:

1. The pK of the group liberated by the ligand (i.e., its proton affinity) relative to the pH of the solution will determine to what extent the addition of a ligand to the iron is accompanied by the addition of a proton to this group.

2. If the ligand participates in an acid-base equilibrium, e.g., F^-, CN^-, etc., then the pK of its conjugate acid relative to the pK of the solution will determine the state of ionization of the uncombined ligand in equilibrium with the complex.

In the absence of other effects, the observed equilibrium constant, calculated in the usual manner from the equation

$$K_{obs} = \frac{[\text{Complex}]}{[\text{Uncombined hemoprotein}][\text{uncombined ligand}]} \quad (1)$$

will show a $[H^+]$ dependence determined solely by the interplay of these factors.

But, in addition, hydrogen ions may be *indirectly* involved through the ionization of heme-linked groups. The resulting effect, first observed in the hemoglobin-oxygen reaction and attributed to groups on the protein in close proximity to the heme, is to change K_{obs} by an amount equivalent to the difference between the free

energy of ionization of the groups in the parent hemoprotein and those in the complex.[29] The pH variation of K_{obs} is, as a consequence, somewhat modified over a limited pH range, but its general character remains unaltered.

Typical reactions of hemoglobin, myoglobin, cytochrome-c, peroxidase, and catalase will now be examined in turn to see how the participation of hydrogen ions, revealed by the pH variations illustrated in Figures 1, 2, and 3, can be accounted for.

a) Hemoglobin and Myoglobin.—On the assumption that ferrihemoglobin and ferrimyoglobin have a structure in which the sixth co-ordination position is occupied by a water molecule, the fluoride and cyanide reactions in the pH range 5–7 can be represented thus:

$$\text{Pr-Fe}^{+}(\text{H}_2\text{O}) + \text{F}^{-} \longleftrightarrow \text{Pr-FeF} + \text{H}_2\text{O} \ldots \quad (2)$$

$$\text{Pr-Fe}^{+}(\text{H}_2\text{O}) + \text{HCN} \longleftrightarrow \text{Pr-FeCN} + \text{H}_2\text{O} + \text{H}^{+} \ldots \quad (3)$$

where Pr stands for the protein moiety and Fe^{+} the protoporphyrin iron atom carrying the net charge of +1.[6] The reacting ligands are written as F^{-} and HCN because, with the pK values for HF and HCN of 3.2 and 9.5, respectively, these are the species that predominate in the acid-base equilibria in the pH range 5–7 (see factor 2 above). Inspection shows that equilibrium constants for reactions (2) and (3), calculated using equation (1), will be independent of $[H^{+}]$ and inversely proportional to $[H^{+}]$, respectively, in accord with the main trend of the experimental values (Figs. 1, *A*, and 2, *A*).

In increasingly alkaline solution there is a progressive decrease in the affinity (lower values of K_{obs}) for both ligands. However, the parent hemoproteins show marked changes in color and absorption spectra in these solutions, which have been attributed to the ionization of a proton from the water molecule,

$$\text{Pr-Fe}^{+}(\text{H}_2\text{O}) \overset{K_{Fe}}{\longleftrightarrow} \text{Pr-FeOH} + \text{H}^{+} \ldots \quad (4)$$

with pK_{Fe} having values 8.86 and 9.04 at 20° for ferrihemoglobin and ferrimyoglobin, respectively.[30, 31] This ionization is equivalent to the formation of the hydroxide complex, i.e.,

$$\text{Pr-Fe}^{+}(\text{H}_2\text{O}) + \text{OH}^{-} \longleftrightarrow \text{Pr-FeOH} + \text{H}_2\text{O} \ldots \quad (5)$$

so the decrease in ligand affinity finds a ready explanation in terms of competition between the ligand and OH^{-}. This can be expressed in a slightly different way. In alkaline solution, pH > 10, the ferrihemoproteins are present in the form Pr-FeOH, and reactions (2) and (3) become

$$\text{Pr-FeOH} + \text{F}^{-} + \text{H}^{+} \longleftrightarrow \text{Pr-FeF} + \text{H}_2\text{O} \ldots \quad (6)$$

$$\text{Pr-FeOH} + \text{CN}^{-} + \text{H}^{+} \longleftrightarrow \text{Pr-FeCN} + \text{H}_2\text{O} \ldots \quad (7)$$

On account of its very high proton affinity, the OH^{-} group liberated combines with a proton (see factor 1 above) and, as can be seen from reactions (6) and (7), K_{obs} will be directly proportional to $[H^{+}]$, i.e., it will decrease with increasing pH.

Detailed analysis of the ferrimyoglobin reactions has shown that good quantitative agreement over the whole pH range is obtained if the small, but additional, pH variation is attributed to one heme-linked group.[6] The cyanide reaction (Fig. 2, *A*) shows the effect particularly well. The theoretical equation for the variation

of K_{obs} with $[H^+]$, in terms of the various fundamental constants of the reacting species, is

$$K_{obs} = K_L \cdot \frac{K_{HL}}{(K_{HL} + [H^+])} \cdot \frac{[H^+]}{(K_{Fe} + [H^+])} \cdot \frac{K_r}{(K_r + [H^+])} \cdot \frac{(K_p + [H^+])}{K_p} \cdots \quad (8)$$

where K_{HL}, K_{Fe}, K_r, and K_p are the ionization constants of the ligand conjugate acid HL, and co-ordinated water molecule (reaction [4]), and the heme-linked group in reactant and product, respectively. K_L is the equilibrium constant for the addition of L^- when the heme-linked group is dissociated in both reactant and product, i.e., the parent hemoprotein and the complex.

In ferrohemoglobin and ferromyoglobin it is reasonable to assume that the sixth co-ordination position is also occupied by a water molecule, and the variation of E_0' with pH can be explained in the same way. The theoretical equation, based on the single electron-transfer reaction,

$$\text{Pr-Fe}^+(H_2O) + e^- \longleftrightarrow \text{Pr-Fe}(H_2O) \cdots \quad (9)$$

is analogous to equation (8) with the omission of the term containing K_{HL}. In the case of hemoglobin the onset of the direct proportionality to $[H^+]$ at pH 6.5 (see Fig. 3, *A*) has been attributed to K_{Fe} and K_p having very similar values.[26]

b) Cytochrome-c.—From the time of its discovery, the observation that the absorption spectra of the ferrous and ferric forms of cytochrome-c resemble so closely those of hemochromogens and parahematins left little doubt that the heme is held in a two-bond crevice structure with Fe-N bonds.[19] Later, following on the discussion of heme-linked ionizations in hemoglobin in terms of histidine residues with one joined directly to the iron,[26] the changes in magnetic susceptibility and absorption spectra of ferricytochrome-c over a wide pH range were attributed to the presence of two histidine groups joined directly to the iron.[32] The structure is now known in some detail from amino acid sequence studies on the peptides produced by trypsin digestion.[20-21] It is established that one Fe-histidine bond could be formed within the hemopeptide fragment, which also contains thio-ether linkages binding the heme through α-addition of cysteine SH groups to the vinyl side chains, but the identity of the second group joined to the iron in the native hemoprotein is still an open question.

The pH variations for the ferricytochrome-c reactions throw some light on this problem. Since in this respect ferricytochrome-c resembles ferrihemoglobin and ferrimyoglobin (see Figs. 1, *A* and *B*, 2, *A* and *B*), the reactions must also involve simple replacement:

$$\text{pH} > 6 < 8: \quad \overline{\text{Pr-Fe}^+\text{-X}} + N_3^- \longleftrightarrow \overline{\text{Pr-FeN}_3\text{ X}} \cdots \quad (10)$$

$$\overline{\text{Pr-Fe}^+\text{-X}} + \text{HCN} \longleftrightarrow \overline{\text{Pr-FeCN X}} + H^+ \cdots \quad (11)$$

where X stands for one of the nitrogenous base groups. It can be seen that the equilibrium constants calculated using equation (1) will be independent of $[H^+]$ and inversely proportional to $[H^+]$, respectively, in accord with experiment. However, if the reactions are to occur in this way, the group X must have a low proton

affinity such that the pH range employed for the equilibrium-constant measurements, beginning at pH 6, lies on the alkaline side of its pK value. Otherwise the addition of a ligand to the iron would be accompanied by the addition of a proton to X. This criterion would appear to exclude all nitrogenous base groups with the exception of the glyoxalinium nitrogen atom of histidine, which, in the free amino acid, has a pK of 6.0.[33] Hence, if the inference based on spectroscopic similarities is correct, one of the groups can be identified as histidine. However, there is no evidence at present to indicate whether it is the one located in the hemopeptide fragment or is a second histidine group situated in a more remote part of the polypeptide chain of the native hemoprotein.

The pH independence of the oxidation-reduction potential follows if the two-bond crevice structure is preserved over the same pH range in ferrocytochrome-c. There is good spectroscopic evidence that this is the case. Reduction then involves single electron transfer:

$$\text{pH 5–7:}\quad \underset{\text{(Ferricytochrome-c)}}{\overline{\text{Pr-Fe}^{+}\text{-X}}} + e^{-} \longleftrightarrow \underset{\text{(Ferrocytochrome-c)}}{\overline{\text{Pr-Fe-X}}}\ldots \qquad (12)$$

It may be noted in passing, however, that data for the cyanide reaction and for oxidation-reduction in more alkaline solution cannot be explained by these reactions alone, and the participation of heme-linked ionizing groups has been suggested.[12, 15]

c) Peroxidase and Catalase.—In the general discussion of crevice structures and heme reactivity, it was envisaged that with two-bond crevice structures the bond broken when complex formation occurs can be to a group having either a low or a high proton affinity; and the structure of cytochrome-c has accordingly been shown to come into the former category. However, the pH variations for the reactions of ferriperoxidase and ferricatalase indicate that these equilibria differ in that a proton participates as a reactant.

This can be accounted for very simply by the hypothesis that ferriperoxidase and ferricatalase have the other kind of two-bond crevice structure where the group liberated has a high proton affinity.[1] If the pH range of equilibrium-constant measurements lies on the acidic side of its pK, addition of the ligand to the iron will be accompanied by the addition of a proton to this group. The fluoride and cyanide reactions can be represented thus:

$$\text{pH 5–7:}\quad \overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{F}^{-} + \text{H}^{+} \longleftrightarrow \overline{\text{Pr-FeF YH}^{+}}\ldots \qquad (13)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HCN} \longleftrightarrow \overline{\text{Pr-FeCN YH}^{+}}\ldots \qquad (14)$$

where the group Y may be either neutral or negatively charged. Inspection shows that the equilibrium constants for reactions (13) and (14), calculated using equation (1), will be directly proportional to $[H^+]$ and independent of $[H^+]$, as found experimentally (Figs. 1, *C*, and 2, *C*). In the pH range 4–7 the variation of E_0' with pH for ferriperoxidase (Fig. 3, *C*) can be explained in a similar manner by a single equivalent reduction reaction which entails proton addition as well as electron transfer:

$$\text{pH 4–7:}\quad \underset{\text{Ferriperoxidase)}}{\overline{\text{Pr-Fe}^{+}\text{-Y}}} + e^{-} + \text{H}^{+} + \text{H}_2\text{O} \longleftrightarrow \underset{\text{(Ferroperoxidase)}}{\overline{\text{Pr-Fe(H}_2\text{O) YH}^{+}}} \qquad (15)$$

But, as indicated in reaction (15), the further assumption has to be made that, unlike ferrocytochrome-c, the crevice structure is no longer present in ferroperoxidase.

D. EQUATIONS FOR COMPLEX FORMATION WITH TWO-BOND CREVICE STRUCTURES

It will be apparent that the difference between two-bond crevice structures where the groups liberated have high and low proton affinities, respectively, is only one of degree, and the actual pH variation observed might well depend on the pH range of the equilibrium measurements. However, the role which the hemoprotein structure can play is a little more complicated than that described in Section C under factor 1, because a two-bond crevice structure will be stable only over a certain pH range determined by the pK of the group concerned, the strength of the bond to the iron, and the affinity of the iron for OH^-.

In acidic solution, provided that the protein is not denatured and the strength of the crevice bond and the pK of $-YH^+$ have appropriate magnitudes, the crevice will be opened:[34]

$$\overline{\text{Pr-Fe}^+\text{-Y}} + H^+ + H_2O \longleftrightarrow \overline{\text{Pr-Fe}^+(H_2O)\,YH^+}\ldots \qquad (16)$$

On the other hand, in alkaline solution, depending on the hydroxyl ion affinity of the iron and, again, on the pK of $-YH^+$, the crevice will be opened:

$$\overline{\text{Pr-Fe}^+\text{-Y}} + H_2O \longleftrightarrow \overline{\text{Pr-FeOH}\ Y} + H^+\ldots \qquad (17)$$

This is the reaction that corresponds to the ionization of the co-ordinated water molecule with the other type of hemoprotein structure (e.g., ferrihemoglobin and ferrimyoglobin), and it can be formulated as the direct formation of the hydroxide complex in a similar way:

$$\overline{\text{Pr-Fe}^+\text{-Y}} + OH^- \longleftrightarrow \overline{\text{Pr-FeOH}\ Y}\ldots \qquad (18)$$

Summing up, the crevice structure for the parent hemoprotein will, in principle, be maintained between a pH value in acidic solution, such that $[H^+]$ is no longer high enough for H^+ to compete effectively with Fe^+ for the group Y (reaction [16]), and a pH value in alkaline solution, such that $[H^+]$ is so low that OH^- competes effectively with Y for the Fe^+ (reaction [18]).

Typical reactions representing fluoride and cyanide complex formation and the resulting $[H^+]$ dependence of the equilibrium constants can now be listed for the entire range, from acidic to alkaline solution.

(i) *Acidic Solution.*—Crevice open: uncombined ligands present as HF and HCN:

$$\overline{\text{Pr-Fe}^+(H_2O)\ YH^+} + HF \longleftrightarrow \overline{\text{Pr-FeF}\ YH^+} + H_2O + H^+\ldots \qquad (19)$$

$$\overline{\text{Pr-Fe}^+(H_2O)\ YH^+} + HCN \longleftrightarrow \overline{\text{Pr-FeCN}\ YH^+} + H_2O + H^+\ldots \qquad (20)$$

Equilibrium constants for both reactions inversely proportional to $[H^+]$.

(ii) *Less Acidic Solution.*—Crevice intact: uncombined ligands present as HF and HCN:

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HF} \longleftrightarrow \overline{\text{Pr-FeF YH}^{+}}\ldots \quad (21)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HCN} \longleftrightarrow \overline{\text{Pr-FeCN YH}^{+}}\ldots \quad (14)$$

Equilibrium constants for both reactions independent of $[H^+]$.

(iii) *Weakly Acidic Solution.*—Crevice intact: uncombined ligands present as F^- and HCN:

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{F}^- + \text{H}^+ \longleftrightarrow \overline{\text{Pr-FeF YH}^{+}}\ldots \quad (13)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HCN} \longleftrightarrow \overline{\text{Pr-FeCN YH}^{+}}\ldots \quad (14)$$

Equilibrium constants directly proportional to $[H^+]$ and independent of $[H^+]$, respectively.

(iv) *Weakly Alkaline Solution, but pH > pK_{HY}.*—Crevice intact: uncombined ligands present as F^- and HCN:

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{F}^- \longleftrightarrow \overline{\text{Pr-FeF Y}}\ldots \quad (22)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{HCN} \longleftrightarrow \overline{\text{Pr-FeCN Y}} + \text{H}^+\ldots \quad (23)$$

Equilibrium constants independent of $[H^+]$ and inversely proportional to $[H^+]$, respectively.

(v) *More Alkaline Solution.*—Crevice intact: uncombined ligands present as F^- and CN^-:

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{F}^- \longleftrightarrow \overline{\text{Pr-FeF Y}}\ldots \quad (24)$$

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{CN}^- \longleftrightarrow \overline{\text{Pr-FeCN Y}}\ldots \quad (25)$$

Equilibrium constants for both reactions independent of $[H^+]$.

(vi) *Alkaline Solution.*—Crevice open: uncombined ligands present as F^- and CN^-:

$$\overline{\text{Pr-FeOH Y}} + \text{F}^- + \text{H}^+ \longleftrightarrow \overline{\text{Pr-FeF Y}} + \text{H}_2\text{O}\ldots \quad (26)$$

$$\overline{\text{Pr-FeOH Y}} + \text{CN}^- + \text{H}^+ \longleftrightarrow \overline{\text{Pr-FeCN Y}} + \text{H}_2\text{C}\ldots \quad (27)$$

Equilibrium constants for both reactions directly proportional to $[H^+]$.

For reactions (i), (iv), (v), and (vi) the equilibrium constants show the same $[H^+]$ dependence as would a hemoprotein with the co-ordinated water molecule structure, but, due to the addition of the proton to Y, the dependence is characteristically different for (ii) and (iii). Other sequences of changes can be obtained, for example, if HF is ionized before $[H^+]$ falls low enough for the crevice to form or if $pK_{HY} > pK_{HCN}$ or if the crevice is opened in alkaline solution at a pH less than pK_{HCN}.

In working out theoretical equations for the variation of K_{obs} with $[H^+]$ over the entire pH range, to cover all these cases, it is necessary to consider, first of all, the equilibrium existing in the absence of any ligand between the intact crevice struc-

ture and an open structure containing the elements of water. This equilibrium will assume one of two forms, depending on whether -YH^+ is a stronger or a weaker acid than the co-ordinated water molecule that would otherwise be attached to the iron in the absence of the group Y, i.e.,

$$K_{HY} > K_{Fe}: \quad \overline{\text{Pr-Fe}^+\text{-Y}} + H_2O \underset{K_{cr}}{\longleftarrow\!\!\longrightarrow} \overline{\text{Pr-Fe}^+(H_2O)\ \text{Y}} \ldots \tag{28}$$

$$K_{HY} < K_{Fe}: \quad \overline{\text{Pr-Fe}^+\text{-Y}} + H_2O \underset{K_{cr}'}{\longleftarrow\!\!\longrightarrow} \overline{\text{Pr-FeOH}\ \text{YH}^+} \ldots \tag{29}$$

For the hemoprotein to exist predominantly in the form $\overline{\text{Pr-Fe}^+\text{-Y}}$ the equilibrium constant for the formation of the crevice bond Fe^+-Y, K_{cr} in reaction (28) or K_{cr}' in reaction (29) must be greater than unity. To evaluate equilibrium constants for complex formation with an added ligand, one particular reacting species now has to be chosen for a reference reaction, and, to facilitate comparison with ferrihemoglobin and ferrimyoglobin,[6] the co-ordinated water molecule structure will again be adopted. As a first approximation it may be assumed that the equilibrium constant K_L is identical for both $\overline{\text{Pr-Fe}^+(H_2O)\text{Y}}$ and $\overline{\text{Pr-Fe}^+(H_2O)\text{YH}^+}$, and on this basis the two following equations are obtained:

If $K_{HY} > K_{Fe}$,

$$K_{obs} = K_L \times \frac{K_{HL}}{(K_{HL} + [H^+])} \times \frac{[H^+]([H^+] + K_{HY})}{([H^+]^2 + K_{HY}K_{cr}[H^+] + K_{HY}K_{Fe})} \ldots (30)$$

While, if $K_{Fe} > K_{HY}$,

$$K_{obs} = K_L \times \frac{K_{HL}}{(K_{HL} + [H^+])} \times \frac{[H^+]([H^+] + K_{HY})}{([H^+]^2 + K_{Fe}K_{cr}'[H^+] + K_{HY}K_{Fe})} \ldots \tag{31}$$

K_{HL} is the ionization constant of the ligand conjugate acid as before (see eq. [8]).

The change in the $[H^+]$ dependence in acidic solution which is brought about by the closing of the crevice, i.e., from (i) to (ii) in the above sequence of reactions, corresponds to the condition that

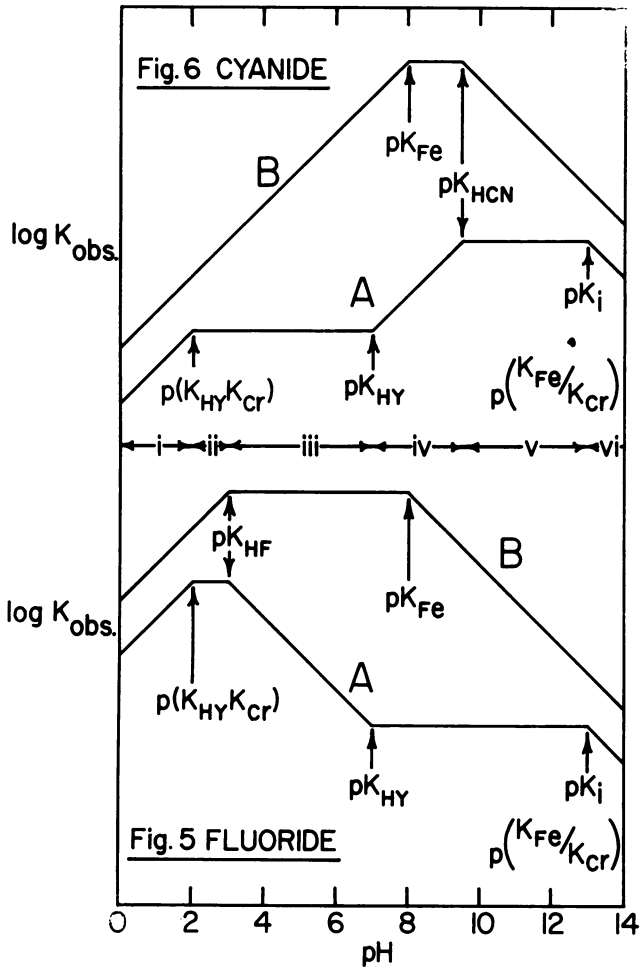
$$[H^+]^2 < K_{HY}K_{cr}[H^+] > K_{HY}K_{Fe}$$

or

$$[H^+]^2 < K_{Fe}K_{cr}'[H^+] > K_{HY}K_{Fe}$$

in the denominator of equation (30) or (31), respectively, while $[H^+]$ remains greater than K_{HY} in the numerator. The change from (iii) to (iv) follows when $[H^+] < K_{HY}$ in the numerator of either equation; and the hemoprotein with the two-bond crevice structure then reacts just like one having only the co-ordinated water molecule. This is illustrated in Figures 5 and 6, where K_{obs} for the formation of fluoride and cyanide complexes of both types of hemoprotein is plotted as a function of pH over the entire range (i)–(vi) according to equations (30) and (8) (omitting any contribution from heme-linked ionizations).

In alkaline solution (vi) it can be seen that the affinity for the ligands again decreases with increasing pH due to competition with the OH^- ion. However, for



FIGS. 5–6.—Schematic plots for the variation of log $K_{obs.}$ with pH for the formation of fluoride and cyanide complexes. Figs. 5, *A*, and 6, *A:* A ferrihemoprotein with a two-bond crevice structure, values chosen for the constants $K_{HY} = 10^{-7}$ $K_{cr} = 10^{5}$, $K_{Fe} = 10^{-8}$. The over-all reactions occurring in the pH ranges (i) to (vi) are listed in Section D. Figs. 5, *B*, and 6, *B:* A ferrihemoprotein with a co-ordinated water molecule structure, value chosen for the constant $K_{Fe} = 10^{-8}$.

the two-bond crevice structure, the change from the "acidic" to the "alkaline" species, corresponding to the formation of the hydroxide complex, is no longer a simple ionization but also involves breaking the crevice (see reactions [17] and [18]). For the case where $K_{HY} > K_{Fe}$, the co-ordinated water molecule may be considered to ionize after reaction (28), i.e.,

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{H}_2\text{O} \underset{K_{cr}}{\longleftarrow\!\!\longrightarrow} \overline{\text{Pr-Fe}^{+}(\text{H}_2\text{O})\ \text{Y}}\ldots \qquad (28)$$

$$\overline{\text{Pr-Fe}^{+}(\text{H}_2\text{O})\ \text{Y}} \xrightleftharpoons{K_{\text{Fe}}} \overline{\text{Pr-FeOH Y}} + \text{H}^{+} \ldots \quad (32)$$

and for the case where $K_{\text{HY}} < K_{\text{Fe}}$, -YH^{+} may be considered to ionize after reaction (29), i.e.,

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{H}_2\text{O} \xrightleftharpoons[K_{\text{cr}}]{} \overline{\text{Pr-FeOH YH}^{+}} \ldots \quad (29)$$

$$\overline{\text{Pr-FeOH YH}^{+}} \xrightleftharpoons{K_{\text{HY}}} \overline{\text{Pr-FeOH Y}} + \text{H}^{+} \ldots \quad (33)$$

It follows that the constant for the over-all ionization in reaction (17) will be

$$K_i = \frac{K_{\text{Fe}}}{K_{\text{cr}}} \ldots \quad (34)$$

or

$$K_i' = \frac{K_{\text{HY}}}{K_{\text{cr}}'} \ldots \quad (35)$$

Substitution in equations (30) and (31) then gives, for $K_{\text{HY}} > K_{\text{Fe}}$,

$$K_{\text{obs}} = K_{\text{L}} \times \frac{K_{\text{HL}}}{(K_{\text{HL}} + [\text{H}^{+}])} \times \frac{[\text{H}^{+}]([\text{H}^{+}] + K_{\text{HY}})}{[\text{H}^{+}]^2 + K_{\text{HY}}K_{\text{cr}}([\text{H}^{+}] + K_i)} \ldots \quad (36)$$

and for $K_{\text{Fe}} > K_{\text{HY}}$,

$$K_{\text{obs}} = K_{\text{L}} \times \frac{K_{\text{HL}}}{(K_{\text{HL}} + [\text{H}^{+}])} \times \frac{[\text{H}^{+}]([\text{H}^{+}] + K_{\text{HY}})}{[\text{H}^{+}]^2 + K_{\text{Fe}}K_{\text{cr}}'([\text{H}^{+}] + K_i')} \ldots \quad (37)$$

Hence the final change in [H^{+}] dependence in alkaline solution from (v) to (vi) corresponds either to the condition that [H^{+}] $< K_i$, as shown in Figures 5, *A*, and 6, *A*, or that [H^{+}] $< K_i'$. By comparison, a more simple condition obtains with the hemoprotein having only the co-ordinated water molecule, namely, [H^{+}] $< K_{\text{Fe}}$, as can be seen from equation (8) and Figures 5, *B*, and 6, *B*.

E. FURTHER DISCUSSION OF A TWO-BOND CREVICE STRUCTURE FOR FERRIPEROXIDASE AND FERRICATALASE

There are insufficient data available at present to decide whether the pH variations for the formation of ferriperoxidase and ferricatalase complexes revert to those characteristic of ferrihemoglobin and ferrimyoglobin when measurements are made in rather alkaline solution. Such a change, from (iii) to (iv) in the sequence of reactions in Section D, would furnish substantial evidence for the participation of the bonding group -Y. Nevertheless, the ionization of ferriperoxidase between pH 10.5 and 11.5.[8] which is accompanied by marked changes in absorption spectra and magnetic susceptibility somewhat similar to those observed with ferrihemoglobin and ferrimyoglobin, is itself indicative of a fundamental alteration in the [H^{+}] dependence. An inspection of the detailed steps involved in the ionization of a two-bond crevice structure, given above in reactions (28) and (32) or (29) and (33), shows that the formation of a hydroxide complex can, in fact, occur only in solution alkaline to the pK of the group -YH^{+}. This can also be seen from the

following comparison. In relatively acidic solution the formation of the fluoride and cyanide complexes of ferriperoxidase show pH variations characteristic of the simultaneous addition of a proton, as in reactions (13) and (14). Now the equivalent reaction for the formation of a hydroxide complex, consistent with these pH variations, would be

$$\overline{\text{Pr-Fe}^{+}\text{-Y}} + \text{H}_2\text{O} \longleftrightarrow \overline{\text{Pr-FeOH YH}^{+}}\ldots \qquad \text{(i.e., 29)}$$

But this is not an ionization process, since no proton is liberated. The species formed, $\overline{\text{Pr-FeOH YH}^{+}}$, is simply one of the open structures that, by definition, can be present to only a small extent in equilibrium with the crevice structure, and its concentration is independent of pH; moreover, this reaction has already been taken into account as reaction (29) above. The complete formation of a species containing -FeOH is thus dependent upon the further ionization of $-\text{YH}^{+}$.

The relative merits of the present interpretation of ferriperoxidase and ferricatalase complex formation and three other explanations that have been advanced in the past will now be discussed.

First, if ferriperoxidase and ferricatalase differ structurally from ferrihemoglobin and ferrimyoglobin in having OH^{-} bonded to the iron instead of a water molecule, even in neutral solution, then complex formation would occur:[35]

$$\text{pH 5–8:} \quad \text{FeOH} + \text{F}^{-} + \text{H}^{+} \longleftrightarrow \text{FeF} + \text{H}_2\text{O}\ldots \qquad (38)$$

$$\text{FeOH} + \text{HCN} \longleftrightarrow \text{FeCN} + \text{H}_2\text{O}\ldots \qquad (39)$$

But this explanation fails to take into account the ionization of ferriperoxidase with pK$\sim$11, which, in view of the similarity with the ionizations that occur in ferrihemoglobin and ferrimyoglobin, is more naturally identified with the formation of the hydroxide complex.

Second, if the four parent ferrihemoproteins all have the co-ordinated water molecule structure but in the peroxidase and catalase complexes the conjugate acids are bonded to the iron instead of the anions, then complex formation would proceed:[10]

$$\text{pH 5–8:} \quad \text{Fe}^{+}(\text{H}_2\text{O}) + \text{F}^{-} + \text{H}^{+} \longleftrightarrow \text{Fe}^{+}(\text{HF}) + \text{H}_2\text{O}\ldots \qquad (40)$$

$$\text{Fe}^{+}(\text{H}_2\text{O}) + \text{HCN} \longleftrightarrow \text{Fe}^{+}(\text{HCN}) + \text{H}_2\text{O}\ldots \qquad (41)$$

This can be criticized on the grounds that the structures of the complexes differ from those of hemoglobin and myoglobin, which seems contrary to the many similarities in physical properties. Moreover, the protons bound in complexes of this kind would be expected to ionize at some point in the pH range from acidic to alkaline solution. For example, even with ferrocyanic acid, $\text{H}_4\text{Fe(CN)}_6$, the ionization of the last proton, from HFe(CN)_6^{3-}, which might well be regarded as unfavorable on account of the large negative charge, nevertheless occurs with a pK of about 4.[36] Furthermore, the conjugate acids of the simple complexes formed between transition metal ions and fluoride ions, e.g., H_3FeF_6, are strong acids already dissociated in solutions of very low pH.[3]

The third explanation requires the postulate of a heme-linked ionizing group in peroxidase and catalase that changes its acid strength when complex formation

occurs, so that, while it is in the conjugate base form in the parent compounds, it is in the conjugate acid form in the complexes over a pH range extending at least from pH 5 to 8.[37] Denoting the group by HZ-, complex formation can be represented thus:

$$\text{pH 5–8:} \quad Z^{-}\text{-Pr-Fe}^{+}(H_2O) + F^{-} + H^{+} \longrightarrow HZ\text{-Pr-FeF} + H_2O \ldots \quad (42)$$

$$Z^{-}\text{-Pr-Fe}^{+}(H_2O) + HCN \longrightarrow HZ\text{-Pr-FeCN} + H_2O \ldots \quad (43)$$

But the increment between the pK of HZ- in the parent compound and in the complex would need to be at least three pH units, far greater than the pK increments obtained for the heme-linked groups in hemoglobin and myoglobin derivatives which range from about 0.3 to 1.2 pH units.[6] The assumption that there are several groups of this kind in peroxidase and catalase with suitably overlapping pK ranges could meet this objection, but it seems very unlikely for structural reasons.

However, there is no very precise and exclusive definition of a heme-linked ionizing group. Sometimes it has been defined on a structural basis as one linked to the iron, and so the co-ordinated water molecule has been included in this category; at other times the "linked" thermodynamic behavior has been emphasized, namely, the way the ligand affinity of the iron depends upon the extent to which the group is ionized.[29] Hence the $-YH^{+}$ group in the reactions of the two-bond crevice structure could very well be regarded as a heme-linked group, for not only is it structurally linked to the iron when present on the conjugate base -Y over a certain pH range, but the ionization of $-YH^{+}$ has a profound effect on ligand affinity. Furthermore, reference to Figures 5, *A*, and 6, *A*, and equations (30) and (31) shows that the breaking of the bond to Y in acidic solution and the ionization of $-YH^{+}$ in more alkaline solution combine to give an apparent "pK increment" which has the value $\log K_{cr}$ if $K_{HY} > K_{Fe}$, or $\log (K_{cr}'K_{Fe}/K_{HY})$ if $K_{HY} < K_{Fe}$. In principle, this increment can be many pH units, since there is no restriction on the magnitudes of K_{cr}, etc., and the crevice-forming group can thus produce a "heme-linked" effect over a much bigger pH range than would seem possible with the group HZ- in reactions (42) and (43). But $-YH^{+}$, by virtue of the direct bonding of Y, cannot undergo a truly independent ionization in the parent hemoprotein, and it therefore differs from the type of heme-linked group believed to be present in hemoglobin and in myoglobin, which is capable of independent ionization in both the parent compound and the complex (see Sec. C, eq. [8], and reactions [42] and [43]). A clear distinction should therefore be made. For a similar reason the co-ordinated water molecule is a different kind of linked group. Although it can ionize in the parent compound, it cannot in the complex, since, having been replaced by the ligand, it is no longer an integral part of the hemoprotein structure. A more detailed comparison of these effects, on the basis of equations (8) and (30), has been given in a recent review.[38]

A major criticism of a two-bond crevice structure for ferriperoxidase and ferricatalase in relation to a co-ordinated water molecule structure for ferrimyoglobin and ferrihemoglobin is that the spectra of all four hemoproteins are very similar and differ significantly from that of ferricytochrome-c. While this is true, it does not necessarily rule out a crevice structure. Similar absorption spectra might arise because the two-bond crevice retains a co-ordinated water molecule, as suggested in another context for hemoglobin,[26] or because some atom other than nitrogen is

directly bonded to the iron. Several complexes are known where the spectra closely resemble that of the parent hemoprotein—for example, cyanate, thiocyanate, formate, acetate, and propionate.[39-41] Alcohols and phenols have been observed to combine with hemin, giving complexes with spectra like those of acidic ferrihemoglobin and ferrimyoglobin;[42] and in alkaline solution phenol, in the form of the phenoxide ion, forms a complex with ferrimyoglobin having a spectrum similar in some respects to that of catalase.[43] Of the amino acids commonly present in hemoproteins, serine, threonine, tyrosine, and possibly hydroxyproline have HO-groups in their side chains and can thus be regarded as potential crevice-forming groups. Moreover, a crevice could be closed, not by an ordinary amino acid side chain of the protein, but by some special group. For instance, in vitamin B_{12} there is the nucleotide co-ordinated to the Co^{III} via an amino-alcohol attached to the propionic acid side chain of the porphyrin-like ring.[44] If there were one special group per iron atom, it would be present in peroxidase and catalase in molecular weight units of 41,000 and 59,000, respectively, and in such low concentrations it might well be very difficult to detect in the protein hydrolyzates.

There is scarcely any evidence at present apart from the pH variation of the equilibrium constants to support the crevice structure; yet it may be significant that ferriperoxidase and ferricatalase are sometimes converted on storage into derivatives having the characteristic absorption spectra of parahematins.[5] This cannot be attributed to gross denaturation, since, on addition of the normal ligands, the familiar complexes are again formed. Similar behavior under such mild conditions has not been reported in the case of ferrihemoglobin and ferrimyoglobin, although with far more drastic treatment, in which the hemoproteins are dried and dehydrated to such an extent that the water-vapor pressure reaches about 12 mm., ferrohemoglobin yields a hemochromogen derivative and ferrihemoglobin a parahematin derivative.[17, 18] There is some evidence to suggest that intermolecular bonding occurs.[45] However, if the conversion of ferriperoxidase and ferricatalase is an intramolecular process, it is easy to visualize how a two-bond crevice structure present initially could facilitate the reaction. All that would be required would be a minor conformational change within the crevice that dislodges the normal group (-Y) and brings a nitrogenous base group into such a position that an Fe-N bond can form.

Although the nature of the Fe-protein bond in hemoglobin has often been discussed and bonding to histidine is regarded as the most likely,[2] there is scarcely any information on hemoglobin or its reactions that provides direct chemical evidence about even the type of group to which the iron is joined. However, it has recently been pointed out that the simplest interpretation of the experiments referred to above substantiates the hypothesis that an Fe-N bond is already present in the native hemoprotein, because, on drying, only the second Fe-N bond would then have to be formed.[38] For the same reason, the conversion of ferriperoxidase and ferricatalase into parahematins is a strong indication that one Fe-N bond is already present in these hemoproteins too. The structural environment of the heme iron atom can be represented thus:

Ferrihemoglobin and ferrimyoglobin: Protein(nitrogenous base)-$Fe^{+}(H_2O)$

Ferriperoxidase and ferricatalase: Protein(nitrogenous base)-Fe^{+}-Y

and its different reactivity would be attributable to the participation of the group Y and the possibility (or likelihood) that the nitrogenous base groups are not the same.

Finally, this kind of structure for ferriperoxidase and ferricatalase would provide a new basis for understanding why they behave differently from ferrihemoglobin and ferrimyoglobin when they react with peroxides and other strong oxidizing agents. The compounds formed, which play the role of "intermediate compounds" if an oxidizable substrate is present and catalytic oxidation ensues, have been shown to be higher oxidation states of the heme group.[46] While ferrimyoglobin and ferrihemoglobin give a single intermediate compound, one oxidation equivalent above the ferric state, ferriperoxidase and ferricatalase give two far more reactive intermediate compounds, one and two equivalents, respectively, above the ferric state. Furthermore, the absorption spectra of the one equivalent higher oxidation states are very different. With a crevice structure in the parent hemoprotein, there would be the possibility that it remained intact in one or in both of these higher oxidation states which are responsible for the highly specific catalytic activity.

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[1] A brief report of this work was presented before the meeting of the Federation of American Societies for Experimental Biology, Chicago, April, 1957 (*Federation Proc.*, **16**, 1051, 1957).

[2] See R. Lemberg and J. W. Legge, *Haematin Compounds and Bile Pigments* (New York and London: Interscience Publishers, 1949).

[3] See, *Stability Constants*, Part 2: *Inorganic Ligands*, compiled by J. Bjerrum, G. Schwarzenbach, and L. G. Sillén (London: Chemical Society, 1958).

[4] See E. F. Hartree, *Ann. Repts. Chem. Soc. London*, **43**, 287, 1947.

[5] D. Keilin and E. F. Hartree, *Biochem. J.*, **49**, 88, 1951.

[6] P. George and G. I. H. Hanania, *Faraday Soc. Disc.*, No. 20, p. 216, 1955.

[7] W. Scheler, F. Jung, and K. H. Hoffmann, *Biochem. Z.*, **325**, 401, 1954.

[8] H. Theorell, *Ark. Kemi, Mineral. Geol.*, **16A**, No. 3, 1, 1942.

[9] P. George, *Biochem. J.*, **55**, 220, 1953.

[10] B. Chance, *J. Biol. Chem.*, **194**, 483, 1952.

[11] B. L. Horecker and J. N. Stannard, *J. Biol. Chem.*, **172**, 589, 1948.

[12] P. George and C. L. Tsou, *Biochem. J.*, **50**, 440, 1952.

[13] J. F. Taylor and V. E. Morgan, *J. Biol. Chem.*, **144**, 15, 1942.

[14] J. F. Taylor and A. B. Hastings, *J. Biol. Chem.*, **131**, 649. 1939.

[15] F. L. Rodkey and E. C. Ball, *J. Biol. Chem.*, **182**, 17, 1950.

[16] H. A. Harbury, *J. Biol. Chem.*, **225**, 1009, 1957.

[17] F. Haurowitz, *J. Biol. Chem.*, **193**, 443, 1951.

[18] D. Keilin and E. F. Hartree, *Nature*, **170**, 161, 1952.

[19] D. Keilin, *Proc. Roy. Soc. London, B*, **98**, 312, 1925.

[20] H. Tuppy and G. Bodo, *Monatsh.*, **85**, 807, 1024, 1954.

[21] S. Paléus, A. Ehrenberg, and H. Tuppy, *Acta Chem. Scand.*, **9**, 365, 1955.

[22] E. Margoliash, *Nature*, **175**, 293, 1955.

[23] R. C. C. St. George and L. Pauling, *Science*, **114**, 629, 1951.

[24] A. Lein and L. Pauling, *Proc. Natl. Acad. Sci.*, **42**, 51, 1956.

[25] J. Wyman and D. W. Allen, *J. Polymer Sci.*, **7**, 499, 1951.

[26] C. D. Coryell and L. Pauling, *J. Biol. Chem.*, **132**, 769, 1940.

[27] P. George and G. I. H. Hanania, *Nature*, **175**, 1034, 1955.

[28] J. C. Kendrew, G. Bodo, H. M. Dintzis, R. G. Parrish, H. Wykoff, and D. C. Phillips, *Nature*, **181**, 660, 1958.

[29] See J. Wyman, Jr., *Advances in Protein Chem.*, **4**, 410, 1948.

[30] P. George and G. I. H. Hanania, *Biochem. J.*, **52**, 517, 1952.

[31] P. George and G. I. H. Hanania, *ibid.*, **55**, 236, 1953.

[32] H. Theorell, *J. Am. Chem. Soc.*, **63**, 1812, 1820, 1941.

[33] See J. T. Edsall and J. Wyman, Jr., *Biophysical Chemistry* (New York: Academic Press, Inc., 1958), **1**, 464.

[34] The formulas Pr-Fe$^+$-Y, etc., used previously to denote reactions where the group has a "high" proton affinity, will now be employed for the general case.

[35] H. Theorell and K. G. Paul, *Ark. Kemie, Mineral., Geol.*, **18A**, No. 12, 1, 1944.

[36] I. M. Kolthoff and W. J. Tomsicek, *J. Phys. Chem.*, **39**, 955, 1935.

[37] P. George and G. I. H. Hanania, *Nature*, **174**, 33, 1954.

[38] P. George and R. L. J. Lyster in *Conference on Hemoglobin, May 2–3, 1957* (Washington: National Academy of Sciences–National Research Council, 1957) (NAS-NRC Publication No. 557), 1958, p. 33.

[39] R. Havemann and W. Rausch, *Z. phys. Chem.*, **202**, 400, 1954.

[40] R. Havemann and P. Wolf, *Z. phys. Chem.*, **201**, 246, 1952.

[41] W. Scheler, A. Salewski, and F. Jung, *Biochem. Z.*, **326**, 288, 1955.

[42] P. George and A. Adler, unpublished observations.

[43] P. George, R. L. J. Lyster, and J. Beetlestone, *Nature*, **181**, 1534, 1958.

[44] D. Crowfoot Hodgkin, J. Pickworth, J. H. Robertson, K. N. Trueblood, R. J. Prosen, J. G. White, R. Bonnet, J. R. Cannon, A. W. Johnson, I. Sutherland, A. R. Todd, and E. Lester Smith, *Nature*, **176**, 325, 1955.

[45] F. Haurowitz, in *Hemoglobin-Barcroft Memorial Conference* (London: Butterworths Scientific Publications, 1949), p. 53.

[46] See P. George in *Currents in Biochemical Research*, 1956, ed. D. E. Green (New York and London: Interscience Publishers, 1956), p. 338.

THE SYNTHESIS OF HEMOGLOBIN IN A CELL-FREE SYSTEM

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The microsomal particles[1] have been implicated as the major sites of protein synthesis within the cells of a variety of tissues. The studies of Borsook, Zamecnik, Hultin, and others, with intact animals and various types of whole cell systems (see review by Askonas *et al.*[2]), as well as electron-microscope studies,[3] provided the original evidence for this conclusion. Zamecnik and Keller[4] have, in addition, developed cell-free systems which incorporate C^{14}-labeled amino acids into protein. These include microsomes as well as various soluble enzymes.[5] Further studies have partially defined the intermediate stages involved.[6] However, it has not yet been possible to equate incorporation of labeled amino acid into protein with actual protein synthesis (see review by Campbell[7]).

Kruh and Borsook[8] have demonstrated that rabbit reticulocytes synthesize hemoglobin in vitro and that approximately 85 per cent of the soluble protein made by such cells is of this one species. Studies with whole cells by Rabinovitz and Olson[9] have shown that microsomes participate in the synthesis of hemoglobin.