Session VII

BONDING IN BIOCHEMICAL AND METAL-ORGANIC SYSTEMS; MÖSSBAUER SCATTERING EXPERIMENTS

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FERRIHEMOPROTEIN HYDROXIDES: A CORRELATION BETWEEN MAGNETIC AND SPECTROSCOPIC PROPERTIES: *P. George, J. Beetlestone, and J. S. Griffith*

CONTRIBUTED PAPERS

Ferrihemoprotein Hydroxides: A Correlation Between Magnetic and Spectroscopic Properties

P. GEORGE, J. BEETLESTONE, and J. S. GRIFFITH

Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania

FOREWORD

This presentation[1] is an introduction to certain facets of hemoprotein chemistry, dealing especially with the theory that certain biologically important compounds may be considered as thermal equilibrium of high and low spin systems. The subsequent discussion includes a presentation of optical absorption spectra and magnetic moments from paramagnetic susceptibility measurements for three hemoproteins: hemoglobin, myoglobin, and peroxidase. These three compounds have in common one very important group of atoms. This is a planar assemblage called protoporphyrin. The basic structure of the plane is indicated in Fig. 1, where carbon atoms are located at every vertex and H = hydrogen; M = [$-CH_3$] (methyl group); V = [$-CH = CH_2$] (Vinyl group); and P = [$-CH_2 - CH_2 - COOH$] (propionic acid group).

In these three proteins that we are discussing and in many other compounds, the two hydrogens on the center nitrogens are replaced by one iron atom, as in Fig. 2. With the nitrogens occupying four of the six coordination positions of the iron atom, the two remaining are free to combine with many different groups.

Hemoglobin, in name at least, is probably familiar as being intimately connected with oxygen transport in the circulatory system. As a molecule, hemoglobin has molecular weight of 68 000 amu and is roughly spherical with a radius of about 70 Å. The molecule consists of four intertwined chains of amino acids; on the end of each chain is found the protoporphyrin group. The details of the amino acid chains vary from protein to protein, but this will not interest us here. Hemoglobin then has four protoporphyrins and thus four iron sites at which we may substitute different ligands.

Myoglobin is a compound consisting essentially of $\frac{1}{4}$ of a hemoglobin molecule. It is believed to function in the oxygen transport in muscles. It contains only

[1] Professor Philip George was invited to present this paper at the *Third International Conference on the Mössbauer Effect* because of the development of interest in this subject among workers with the Mössbauer Effect. The paper is reprinted from *Hematin Enzymes*, a volume of the symposium series for the International Union of Biochemistry published by Pergamon Press (New York). The editor of the Proceedings wishes to acknowledge his gratitude to Pergamon Press for permission to republish this material.

one protoporphyrin and thus one iron atom. The coordination of the iron allows the interchange of ligands in one position.

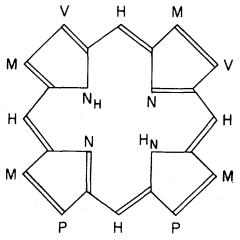
Peroxidase with molecular weight of 44 000 is an enzyme, that is, a biologically specific catalysis for the decomposition of hydrogen peroxide. The oxidation state of the iron is believed to be 3+, and there is only one such iron atom in its corresponding protoporphyrin.

I. INTRODUCTION

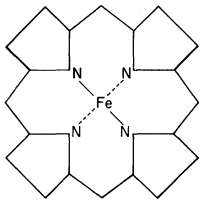
Ferrihemoglobin, ferrimyoglobin, ferriperoxidase, and ferricytochrome *c* all undergo ionizations in alkaline solution that are accompanied by significant changes in absorption spectra and magnetic susceptibility. These reactions can be represented simply as

$$\text{Acidic form} \rightleftharpoons \text{Alkaline form} + H^+ \qquad (1)$$

That of ferrihemoglobin, where the color changes from chocolate brown to wine red, is the most familiar, and has been known from the earliest days of spectroscopic observations on hemoglobin and hemin by Hoppe-Seylet, Stokes and Gamgee (Gamgee, 1868). The pK values which characterize these reactions are listed in Table I. Ferricatalase, which in complex formation with many ligands resembles the other ferrihemoproteins, is an exception for no such ionization has been observed. It may well be that the pK is so high that protein denaturation sets in before the ionization can take place.



It is generally accepted that these reactions involve the bonding of the hydroxyl ion to the ferriporphyrin iron atom, but the evidence is circumstantial. First, there are many ionizations of aquo-ions and simple coordination compounds in which the



bonding of OH^- is unequivocal (see Basolo and Pearson, 1958), for example,

$$Fe(H_2O)_6^{3+} \rightleftharpoons Fe(H_2O)_5OH^{++} + H^+, \quad pK = 2.2 \qquad (2)$$

$$Co(NH_3)_5H_2O^{3+} \rightleftharpoons Co(NH_3)_5OH^{++} + H^+, \quad pK = 5.7 \qquad (3)$$

$$\textit{trans}\ Co(en)_2NO_2\cdot H_2O^{++} \rightleftharpoons \textit{trans}\ Co(en)_2NO_2\cdot OH^+ + H^+, \quad pK = 6.4\,. \qquad (4)$$

Secondly, the changes in spectrum and magnetic susceptibility when the ferrihemoproteins ionize are similar to those which accompany the formation of complexes where other ligands such as F^-, CN^-, N_3^-, HS^-, etc., are bonded to the iron. Yet the remote possibility that these changes originate in the ionization of a group distant from the iron, but, for instance, connected to it via a conjugated system of double and single bonds, cannot be excluded.

Following the general practice it will be assumed that hydroxides are produced, although the correlation developed in Sec. IV between spectroscopic and magnetic properties is equally valid provided that the same structural feature is present in the alkaline forms of all the ferrihemoproteins upon which the calculations are based.

TABLE I. pK values for ferrihemoprotein ionization reactions.

Ferrihemoprotein	Source	pK	Ionic strength	Temp. °C	Reference
Myoglobin	Horse heart	9.04 ± 0.03	$I \rightarrow 0$	20	George and Hanania, 1952
Hemoglobin	Horse erythocytes	8.86 ± 0.02	$I \rightarrow 0$	20	George and Hanania, 1953
Hemoglobin	*Chironomus plumosus*	8.23	$I \rightarrow 0$	21	Scheler and Fischbach, 1958
Leghemoglobin	Soybean	8.25	0.10	22	Sternberg and Virtanen, 1952
Peroxidase	Japanese radish	9.57	0.10	18	Morita and Kameda, 1958
Peroxidase	Horseradish	10.9–11.3	...	20	Theorell, 1942
Cytochrome *c*	Horse heart	12.8[a]	...	22	Theorell and Åkesson, 1941

[a] In this case the value of n for the titration was found to be greater than 1, i.e., 1.64, which may be an indication of additional reactions occurring in the very alkaline solution.

II. THE INTERPRETATION OF THE MAGNETIC MOMENTS OF THE HYDROXIDES ACCORDING TO VARIOUS THEORIES OF THE ELECTRONIC STRUCTURE OF COORDINATION COMPLEXES

Coryell, Stitt, and Pauling (1937) were the first to measure the magnetic moment of one of these hydroxides, and obtained 4.47 Bohr magnetons (μ_B) for the ferrihemoglobin derivative. This value differed in a striking manner, not only from the values found for acidic ferrihemoglobin and the F^- complex, 5.80 and 5.92 μ_B, but also from those for the CN^- and SH^- complexes, 2.50 and 2.26 μ_B. These other moments were in close agreement with the theoretical values calculated from the contribution of five and one unpaired electrons, respectively. For five unpaired electrons the electronic configuration corresponds to ^6S of the free ion, and therefore one expects a magneton number very close to the free spin value of 5.92 (see Table II) as, for example, in $(NH_4)_3FeF_6$.

TABLE II. Spin magnetic moments for metal complexes containing from one to five unpaired electrons, $\mu = [n(n+2)]^{\frac{1}{2}}$

Unpaired electrons, n	1	2	3	4	5
μ, Bohr magneton	1.73	2.83	3.87	4.90	5.92

For one unpaired electron there is a spatial degeneracy of three, and associated with this a considerable orbital magnetic moment. The observed magneton number for $K_3Fe(CN)_6$ is 2.33 compared with the spin-only value of 1.73, and the large difference is due to the orbital moment combined with effects of the spin–orbit coupling (Howard, 1935; Kotani, 1949). According to the then current theory, Coryell *et al.* (1937) described the bonding of the iron as essentially ionic in the first group, and essentially covalent in the second group through $3d^24s4p^3$ orbital hybridization.

It was noted that the intermediate value for the hydroxide corresponded more nearly to the theoretical value for the spin contribution of three unpaired electrons, which would be anticipated for a square planar ferric complex with essentially covalent bonding through $3d4s4p^2$ orbital hybridization. But since the chemical structure of ferrihemoprotein complexes requires octahedral coordination, it was suggested that while the moment of the hydroxide results from the electronic structure with three unpaired electrons, the four covalent bonds resonate among the six coordinated groups.

This interpretation was widely accepted, and was not seriously questioned for many years. In an extensive review of coordination compounds, Taube (1952) proposed that the utilization of d orbitals with the next higher principal quantum number might occur in complexes having the high magnetic moments. For example, the bonding in the cyanide complex of ferrihemoglobin would be attributed to $3d^24s4p^3$ hybridization as before, but in the fluoride complex to $4d^24s4p^3$ hybridization, leaving the five unpaired electrons in the $3d$ orbitals unaffected. Taube commented that with certain metal ions a close balance might occur between the energies of these inner and outer orbital complexes, so that with some ligands the complexes would have high magnetic moments, and with others low magnetic moments, e.g., K_3CoF_6, which is paramagnetic in contrast to the cobaltic amine complexes which are diamagnetic. Coryell, Stitt, and Pauling's measurements on hemoglobin derivatives showed that Fe^{++} and Fe^{3+} porphyrin compounds come into the same category; and Taube went on to suggest that, since OH^- is intermediate in polarizability between H_2O and SH^-, an alternative explanation for the anomalous magnetic moment of the hydroxide is the presence of inner and outer orbital complexes in equilibrium.

During the last seven years a more detailed understanding of the electronic structure of transition metal compounds has been arrived at using that combination of the molecular orbital method and the simple rigid crystal field method which has come to be known as ligand field theory (see Griffith and Orgel, 1957). Ligand field theory is in many ways more general than Pauling and Taube's schemes which really only discussed directed bond orbitals on the central ion formed from atomic orbitals which were assumed to be unchanged from those in the free ion. On the other hand, because of its close relationship to the simple crystal field model, it preserves to a considerable extent the possibility of making detailed interpretations of the electronic structure and semiquantitative calculations of experimental quantities.

We use here for convenience, but not necessity, the language of the crystal field theory. Briefly then, for the case of a regular octahedral complex, the five orbitals of the d shell, which have the same energy in the free ion, are split in the field imposed by six identical ligand groups into a lower set of three orbitals, denoted by t_{2g}, and an upper set of two orbitals, denoted by e_g, as shown in Figs. 1(a) and 1(b). The five d orbitals have different spatial orientations (regions of high electron density) characterized as far as their angular variation is concerned, by the sub-

scripts xy, xz, yz, $z^2-\frac{1}{3}r^2$, and x^2-y^2. For the first three, these regions lie midway between the axes x and y, x and z, and y and z, respectively, and thus point away from the ligand groups situated equidistantly on the x, y, and z axes (see Fig. 2). For the remaining two, i.e., $z^2-\frac{1}{3}r^2$ and x^2-y^2, these regions lie in the directions of the axes. Hence the energy of an electron in the e_g orbitals will be substantially increased by the mutual electrostatic repulsion between electron and ligand, and also by molecular orbital effects associated with the overlap (Griffith, 1956b), whereas the energy of an electron in the t_{2g} orbitals will be much less affected.

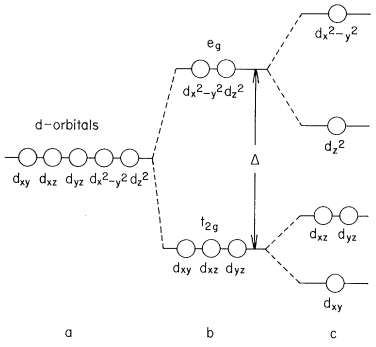


FIG. 1. Schematic illustration of the splitting of the energy level of the five d orbitals of the free transition metal ion (a), by a regular octahedral field (b), i.e., six identical ligands, and by an irregular octahedral field (c), i.e., four ligands of one kind equidistant on the x- and y-axes, and two other ligands farther away on the z-axis.

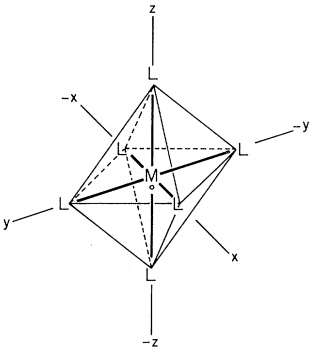


FIG. 2. An octahedral coordination complex, ML_6, showing the x-, y-, and z-axes with respect to which the orientation of the d orbitals are defined.

When transition metal ions with 4, 5, 6, and 7 d electrons form regular octahedral complexes, a choice of at least two electronic configurations thus arises. If the electrons distribute themselves between the t_{2g} and e_g orbitals the number of unpaired electrons remains the same, whereas if the lower t_{2g} orbitals are filled preferentially the number of unpaired electrons is necessarily reduced. For example, with ferrous and ferric complexes the former configuration gives 4 and 5 unpaired electrons, and the latter, zero and 1 unpaired electrons, respectively, as shown in Figs. 3(a) and 3(c). The former configuration will be favored if the energy separation Δ between the t_{2g} and e_g orbitals is small (i.e., a weak ligand field), and the latter by a large energy separation (i.e., a strong ligand field). In the latter case the gain in orbital energy, achieved by having the electrons together in the t_{2g} orbital, is to some extent offset by an increase in the Coulombic repulsion energy, and by a decrease in the quantum-mechanical exchange energy which affords extra stabilization for each pair of electrons with parallel spins. These two effects may be grouped together as "electron-pairing energy." Whether a particular complex of a given metal ion has the maximum or minimum number of unpaired electrons depends on the magnitude of this pairing energy and that of the energy separation, Δ. The magnetic moments of several complexes, notably those of the cobaltic ion, have been discussed from this point of view (Orgel, 1955; Griffith, 1956a), and, following the suggestion of Griffith and Orgel (1957), the two types will be referred to as "high-spin" and "low-spin" complexes, respectively.

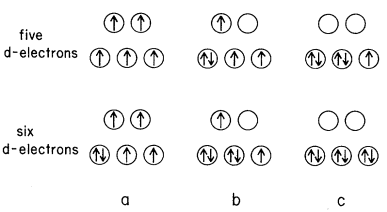


FIG. 3. Schematic illustration of the three ways in which the e_g and the t_{2g} orbitals can be occupied in regular octahedral complexes of metal ions containing five and six d electrons (e.g., ferric and ferrous complexes). (a) Maximum number of parallel spins: ferric 5, ferrous 4. (b) Intermediate number of parallel spins: ferric 3, ferrous 2. (c) Minimum number of parallel spins: ferric 1, ferrous 0.

The coordination in hemoprotein compounds is that of an irregular octahedron since the two groups bonded on the z axis differ from the four (identical) pyrrole nitrogen atoms bonded on the x and y axes. For such complexes the t_{2g} and e_g orbitals are split further, perhaps as shown in Fig. 1(c). The asymmetry in the field splits the e_g much more than the t_{2g} orbitals because the former but not the latter point towards the nearest neighbor atoms. It appears probable from electron resonance measurements that d_{xy} lies lowest (Gibson and Ingram, 1957; Griffith, 1957), $d_{x^2-y^2}$ is almost certainly at the top except possibly in some of the low-spin derivatives, but the position of d_{z^2} is less certain. Similar considerations to those

mentioned before determine whether electron pairing occurs.

For octahedral complexes of ions with five and six d electrons, a third electronic configuration in addition to the two characterizing the high- and low-spin complexes has to be considered. The passage of just one electron from the e_g orbital to the t_{2g} orbital results in a configuration with an intermediate number of unpaired electrons, as shown in Fig. 3(b). For a ferric complex this configuration with three unpaired electrons corresponds to that envisaged by Coryell, Stitt, and Pauling (1937) for ferrihemoglobin hydroxide. Theoretical treatment has shown, however, that a configuration of this kind is inherently unstable in the case of regular octahedral complexes. If spin pairing occurs to reduce the number of unpaired electrons from five to three, then further pairing is even more favored energetically, reducing the number from three to one (Griffith, 1956a, b). Because of the lower symmetry this is not necessarily true for hemoprotein derivatives. However a similar type of argument suggested that it is improbable that there should exist a hemoprotein which possesses derivatives of all the three kinds, high spin, low spin, and intermediate spin (Griffith, 1956c). Because of the definite existence of the first two, this casts doubt on the suggestion that ferrihemoglobin hydroxide has three unpaired electrons, and again raised the possibility that it is a thermal mixture of high- and low-spin forms.

Against the background of these developments in electronic theory certain other experimental observations take on added significance.

(1) Taube's comment, that the existence of high- and low-spin derivatives of hemoglobin indicates a close balance between the energies of the two forms in the case of iron porphyrin compounds, is further borne out by the fact that with the same ligand, namely, the azide ion, high- or low-spin complexes are formed depending on the particular hemoprotein. The magnetic moment of ferricatalase azide is 5.36 μ_B, compared to 2.84 for the ferrihemoglobin derivative (Deutsch and Ehrenberg, 1952; Coryell, Stitt, and Pauling, 1937). A fairly close balance between the energies would be a prerequisite for the two forms to exist in thermal equilibrium.

(2) While the magnetic moment of ferrihemoglobin hydroxide is 4.47 μ_B, just a little in excess of the theoretical value for the spin contribution of three unpaired electrons, the moments of the other ferrihemoprotein hydroxides are very substantially different, as shown in Table III. Ferriperoxidase and ferricytochrome c hydroxides come into the category of low-spin complexes, with moments comparable to those of the CN^- and SH^- derivatives; whereas the magnetic moment of ferrimyoglobin hydroxide approaches that of a high-spin complex, the value of 5.11 μ_B being even greater than the theoretical value for the spin contribution of four unpaired electrons. Hence, although the three unpaired electron configuration could still be invoked for ferrimyoglobin, by assuming a large orbital contribution, it is clearly impossible in the case of ferriperoxidase and ferricytochrome c.

(3) For a given ligand, the spectra of ferrihemoprotein complexes usually have absorption bands at approximately the same wavelengths with comparable extinction coefficients, independent of the particular hemoprotein. There are a few exceptions, and among them the hydroxides provide the most striking examples. These spectra show marked variations, exhibiting a regular trend from ferrimyoglobin at one extreme, through ferrihemoglobin, to ferriperoxidase and ferricytochrome c at the other.

In the discussions of electronic structure little use has been made of these observations, and in the following sections it will be shown, first, that the trend in spectroscopic properties parallels the trend in magnetic moments, and second, that the data are quantitatively consistent with the view that the hydroxides are thermal mixtures of high- and low-spin forms.

III. QUALITATIVE CORRELATIONS BETWEEN THE MAGNETIC MOMENTS AND THE SPECTRA OF FERRIHEMOPROTEIN DERIVATIVES

The close resemblance, which has long been recognized, between spectra of the same derivative of dif-

TABLE III. Magnetic moments of ferrihemoprotein hydroxides.

Ferrihemoprotein	Source	μ, Bohr magnetons	Reference
Myoglobin	Horse heart	5.11	Theorell and Ehrenberg, 1951
Hemoglobin	Horse erthrocytes	4.47	Coryell, Stitt, and Pauling, 1937
Hemoglobin	*Chironomus plumosus*	4.45	Scheler, Schoffa, and Jung, 1957
Peroxidase	Horseradish	2.66	Theorell, 1942
Cytochrome c	Horse heart	2.14	Theorell, 1941

ferent hemoproteins, is illustrated in Figs. 4 to 6 in the case of the visible spectra of acidic ferrihemoproteins and their fluoride and cyanide complexes. Furthermore, all the high-spin ferric complexes have visible spectra like the acidic ferrihemoproteins and

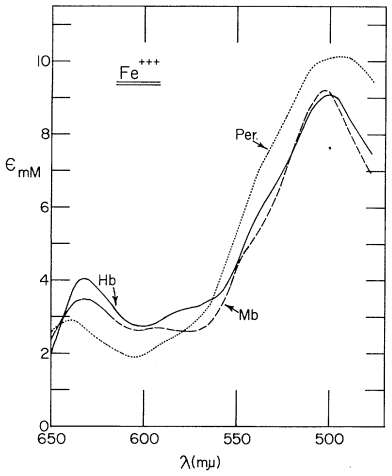


FIG. 4. Visible spectra of acidic ferrimyoglobin, ferrihemoglobin, and ferriperoxidase (Keilin and Hartree, 1951; Hanania, 1953).

fluoride derivatives, with an absorption band between 600 and 640 mμ and a second band at about 500 mμ; all the low-spin ferric complexes have spectra like the cyanide derivatives, with a very pronounced absorption band at about 540 mμ and a shoulder, or second band, at about 580 mμ (Theorell, 1942). The

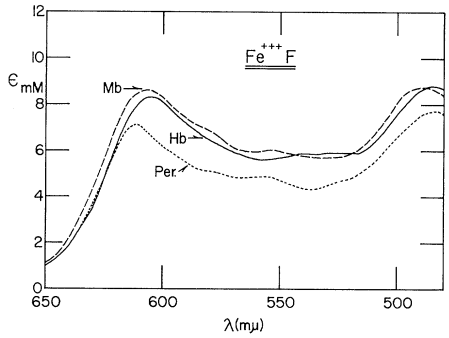


FIG. 5. Visible spectra for ferrimyoglobin, ferrihemoglobin, and ferriperoxidase fluoride (Keilin and Hartree, 1951; Hanania, 1953).

high-spin complexes have additional minor bands of lower intensity, at about 580 and 540 mμ, but for the present it is the positions of the major bands which differentiate the two types of complex that are important.

Similar contrasting features appear in other regions of the absorption spectrum. In the near infrared the fluoride complex has a well-defined absorption band at about 850 mμ with a shoulder at about 750 mμ, whereas the cyanide complex has remarkably low absorption throughout the whole range 700 to 950 mμ as shown in Fig. 7 (George and Hanania, 1955). In the ultraviolet, from 280 to 450 mμ, there are three regions to consider. The very intense Soret band lies between 405 and 410 mμ for the acidic ferrihemoproteins and the fluoride complexes, the latter

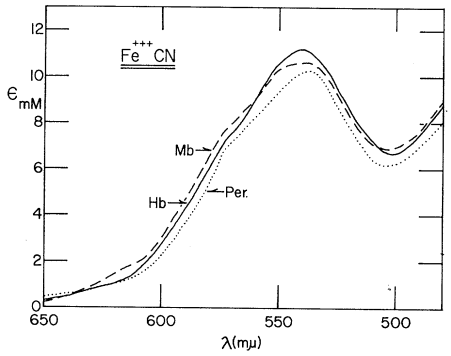


FIG. 6. Visible spectra of ferrimyoglobin, ferrihemoglobin, and ferriperoxidase cyanide (Keilin and Hartree, 1951; Hanania, 1953).

having lower absorption in the case of myoglobin and hemoglobin but higher in the case of peroxidase. On the other hand, the low-spin derivatives have the band shifted towards the red in the neighborhood of 418 to 425 mμ (see Fig. 8). Minor bands occur at about 350 mμ. These are unresolved in the case of the acidic ferrihemoproteins and the fluoride complexes, but two distinct bands at about 345 and 360 mμ can be distinguished in the case of the cyanide complex. At shorter wavelengths, from 260 to 300 mμ, ab-

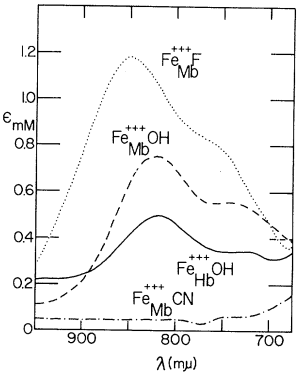


FIG. 7. Near infrared spectra of ferrimyoglobin fluoride, hydroxide, and cyanide, and ferrihemoglobin hydroxide (Hanania, 1953).

sorption due to both the ferriporphyrin prosthetic group and tyrosine and tryptophane residues in the protein occurs, as evidenced by the greater absorption of the ferrihemoproteins as compared to their apo-proteins. As shown in Fig. 9 the low-spin cyanide

derivative has greater absorption than the high-spin fluoride derivative throughout this region, although the band at 290 mμ is less well resolved.

While the spectra of the high- and low-spin derivatives exhibit these characteristic distinguishing features, which as far as can be judged are common

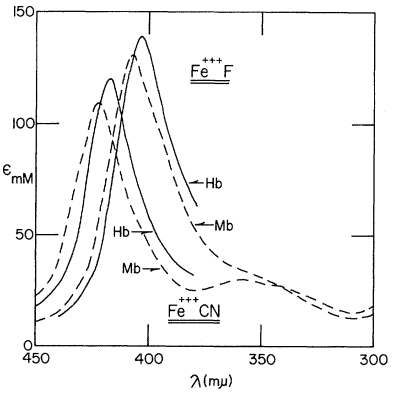


FIG. 8. Ultraviolet spectra of ferrimoglobin and ferrihemoglobin cyanide and fluoride (Keilin and Hartree, 1951; Hanania, 1953).

to myoglobin, hemoglobin, and peroxidase, the spectra of the hydroxides vary a great deal as shown in Figs. 7, 10, 11, and 14. Moreover, these variations are not haphazard, but appear to be related to the change in magnetic moment, i.e.,

$$\text{FerriMb} \rightarrow \text{FerriHb} \rightarrow \text{FerriPer}. \qquad (5)$$
$$5.11\ \mu_B \qquad 4.45\ \mu_B \qquad 2.66\ \mu_B$$

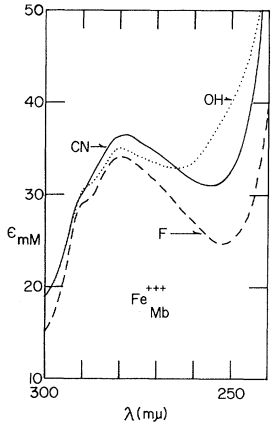


FIG. 9. Ultraviolet spectra of ferrimyoglobin fluoride, hydroxide, and cyanide in the region of tyrosine and tryptophane absorption (Hanania, 1953).

To take but one example, in the region of 600 mμ, the extinction coefficients follow the order

$$\epsilon_{Mb} > \epsilon_{Hb} > \epsilon_{Per}, \qquad (6)$$

as shown in Fig. 10.

Now the regular and systematic differences between the spectra of high- and low-spin complexes suggest very strongly that if the hydroxides are mixtures of high- and low-spin forms their spectra and magnetic moments should conform to a certain pattern.

(a) For the same hemoprotein, the extinction coefficients for the hydroxide should be intermediate in value between those for typical high- and low-spin complexes in the regions where the major absorption bands occur.

(b) For a series of hydroxides, there should be a regular trend in the extinction coefficients in the region of the major absorption bands, such that the higher magnetic moment hydroxides resemble more closely the high-spin complexes, and the lower magnetic moment hydroxides resemble more closely the low-spin complexes.

In the case of ferrimyoglobin, the only hemoprotein for which complete data are available at pres-

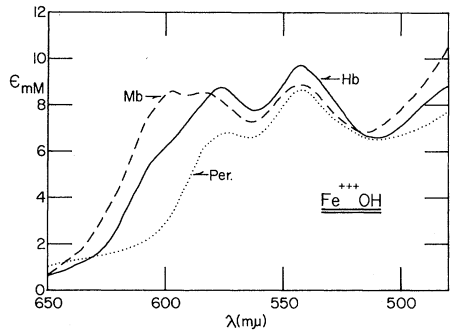


FIG. 10. Visible spectra of ferrimyoglobin, ferrihemoglobin, and ferriperoxidase hydroxide (Keilin and Hartree, 1951; Hanania, 1953).

ent, the first criterion is found to hold throughout the entire range of wavelength, 250 to 950 mμ. For the visible region the myoglobin curve in Fig. 10 is to be compared with those in Figs. 5 and 6; Fig. 7 covers the region 700 to 950 mμ; Figs. 8 and 11 give the Soret bands, and the smaller bands in the region 330 to 370 mμ; and Fig. 9 covers the region of composite absorption, 250 to 300 mμ. The second criterion is borne out by a comparison of the spectra of ferrimyoglobin and ferrihemoglobin hydroxides in Figs. 7 and 10, where the extinction coefficients follow the sequence

$$\text{Fluoride Complex} \rightarrow \text{FerriMb Hydroxide}$$
$$\text{high spin} \qquad 5.11\ \mu_B$$
$$\rightarrow \text{FerriHb Hydroxide} \rightarrow \text{Cyanide Complex} \qquad (7)$$
$$4.47\ \mu_B \qquad \text{low spin}$$

in order either of increasing or decreasing magnitudes,

depending on the particular wavelength. In the ultraviolet region, 330 to 450 mμ, the trend is not so clear-cut, but, as will be shown in the next section, this can be attributed to the small but significant shift of all the ferrimyoglobin band maxima relative

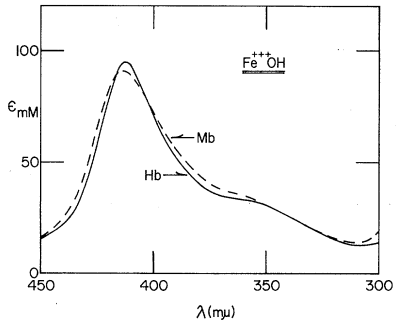


FIG. 11. Ultraviolet spectra of ferrimyoglobin and ferrihemoglobin hydroxide (Hanania, 1953).

to those for ferrihemoglobin, together with systematically lower extinction coefficients (see Fig. 8). In the region 250 to 300 mμ no strict evaluation is possible because myoglobin and hemoglobin are not alike in tyrosine and tryptophane content. Nevertheless, it is interesting that the curve for ferrimyoglobin hydroxide, which has higher moment, in contrast to that for ferrihemoglobin with the lower moment, has a well-defined shoulder at 290 mμ like the high-spin fluoride complex (see Figs. 9 and 12).

The data for ferriperoxidase are not quite sufficient for it to be included in the sequence in Eq. (7), although there are ample indications that it would fit into the pattern and come between ferrihemoglobin hydroxide and the low-spin cyanide complex. The magnetic moment has been determined for horseradish peroxidase, 2.66 μ_B (Theorell, 1942), but the absorption spectrum, recorded by Keilin and Hartree (1951) and reproduced in Fig. 10, refers to a pH of 11.4, which, judging from the pK of 10.9–11.3, would give only about 60–75% hydroxide formation. It is already evident from Fig. 10, however, that the hydroxide has a pronounced peak at about 540 mμ with a second peak at about 575 mμ, and no peaks either in the region 600 to 640 mμ or at about 500 mμ. Spectroscopically, as well as magnetically, it can safely be classified as a low-spin complex. The spectroscopic type is fully substantiated by the corresponding spectrum for Japense root peroxidase (Morita and Kameda, 1958), which has absorption bands at 548 and 578 mμ, with ϵ_{mM} = 12.3 and 10.1, respectively, together with relatively lower absorption in the region 620 to 650 mμ, compared to horseradish peroxidase in Fig. 10. But its magnetic moment has not yet been measured.

The data for two other hemoglobins may be considered at this point. The first, Chironomus hemoglobin (Scheler and Fischbach, 1958), presents some anomalous features. The visible spectrum of the hydroxide is most like that of ferrimyoglobin, and the shape of the curve in the region of 600 mμ suggests that it should come between ferrimyoglobin and ferrihaemoglobin in the sequence in Eq. (7), but on a quantitative scale nearer the former. However its magnetic moment is 4.45 μ_B, a little less than that of ferrihemoglobin hydroxide (Scheler, Schoffa, and Jung, 1957). No explanation can be offered for this discrepancy, although it is to be noted that the magnetic moment of the acidic ferrihemoglobin is appreciably lower than the values determined for ferrimyoglobin and erythrocyte ferrihemoglobin, namely, 5.68 and 5.80 μ_B, respectively (Theorell and Ehrenberg, 1951; Coryell, Stitt, and Pauling, 1937).

The second, root nodule hemoglobin (leghemoglobin), is particularly interesting. The visible spectrum of the hydroxide, reproduced in Fig. 16 (Sternberg and Virtanen, 1952), is almost the same as that of Japense root peroxidase, which indicates that it is a low-spin complex. Furthermore, preliminary spectroscopic observations by George, Hanania, and Thorogood (1959) in the near infrared have shown it to have significantly lower absorption in the region 700 to 900 mμ than the myoglobin and hemoglobin derivatives, which is in keeping with the trend in extinction coefficients from high- to low-spin complexes (see Fig. 7). The magnetic moment however still remains to be determined. Hence, provided it is appropriate to regard leghemoglobin as a true hemoglobin,[2] the hemoglobins themselves, without recourse to peroxidase, furnish a series of hydroxides covering almost the whole range of spectroscopic characteristics.

There is thus a substantial body of evidence to suggest that the hydroxides, especially of ferrimyoglobin and ferrihemoglobin, are mixtures of high- and low-spin forms, and in the next section this hypothesis will be put to a quantitative test.

IV. QUANTITATIVE CORRELATION BETWEEN THE MAGNETIC MOMENTS AND THE SPECTRA OF FERRIHEMOPROTEIN HYDROXIDES

Making the assumption that the hydroxides are mixtures of high- and low-spin forms, the magnetic

[2] This classification is based on the ability of ferroleghemoglobin to form an oxygen complex, and it is further substantiated by the reaction of ferrileghemoglobin with hydrogen peroxide. An intermediate compound is formed with absorption bands at 550 and 575 mμ, resembling the ferrimyoglobin and ferrihemoglobin derivatives, in contrast to ferriperoxidase and ferricatalase, which give two such compounds neither having bands at these wavelengths.

moments and extinction coefficients at each wavelength should be interrelated in the following way. Denoting the moments of the high- and low-spin forms by μ_h and μ_l, the moments of, say, ferrimyoglobin and ferrihaemoglobin hydroxide, μ_{Mb} and μ_{Hb}, are determined by the equations

$$\mu^2_{Mb} = \mu^2_l\alpha_{Mb} + \mu^2_h(1 - \alpha_{Mb}) , \quad (8)$$

$$\mu^2_{Hb} = \mu^2_l\alpha_{Hb} + \mu^2_h(1 - \alpha_{Hb}) , \quad (9)$$

where α_{Mb} and α_{Hb} are the fractions of the low-spin form present in ferrimyoglobin and ferrihemoglobin hydroxide, respectively. The reason why the terms contain μ^2 rather than μ is that the additive magnetic property is the molar susceptibility, χ_M, which is related to μ through the expression $\mu = 2.84(\chi_M T)^{\frac{1}{2}}$. At a given wavelength the extinction coefficients of the two hydroxides, ϵ_{Mb} and ϵ_{Hb}, are also determined by the fractions α_{Mb} and α_{Hb} according to the equations

$$\epsilon_{Mb} = \alpha_{Mb}\epsilon_l + (1 - \alpha_{Mb})\epsilon_h , \quad (10)$$

$$\epsilon_{Hb} = \alpha_{Hb}\epsilon_l + (1 - \alpha_{Hb})\epsilon_h , \quad (11)$$

where ϵ_h and ϵ_l are the extinction coefficients of the high- and low-spin forms at this particular wavelength. Since μ_{Mb}, μ_{Hb}, ϵ_{Mb}, and ϵ_{Hb} are known experimental quantities, and since reasonable values can be adopted for μ_h and μ_l, these equations can best be used to evaluate ϵ_h and ϵ_l, and thus obtain the absorption spectra of the high- and low-spin components.

However, there are several reasons why calculations of this kind can only be approximate. First, although the high-spin hydroxide can be assigned a magnetic moment of 5.92 μ_B, the theoretical spin contribution of five unpaired electrons, the value for the low-spin hydroxide is a matter of choice. Some contribution of orbital angular momentum must be taken into account in view of the values in excess of the spin contribution of one unpaired electron obtained for the cyanide complexes (see below), where the very strong ligand field makes it extremely unlikely that thermal mixtures exist. Secondly, although a value of 5.92 μ_B is equally valid for the high-spin form of both ferrimyoglobin and ferrihemoglobin hydroxide, the moment of the low-spin form could very well differ by up to 0.5 μ_B, since the values obtained for cyanide complexes are 2.35, 2.50, 2.66, and 2.29 μ_B in the case of ferrimyoglobin, ferrihemoglobin, ferriperoxidase, and ferricatalase, respectively (Theorell and Ehrenberg, 1951; Coryell, Stitt, and Pauling, 1937; Thoerell, 1942; Deutsch and Ehrenberg, 1952). In practice, slightly different values of μ_l could be used in Eqs. (8) and (9); but, in the absence of independent evidence, the same value will be used for the present calculations. Thirdly, the band maxima for corresponding derivatives of myoglobin and hemoglobin do not occur at exactly the same wavelengths, nor are the extinction coefficients identical, hence small variations would also be anticipated between the high-spin forms, and between the low-spin forms, of both hemoproteins. In those regions where the spectra of both fluoride and cyanide derivatives do differ in this way, correction factors can be introduced by adjusting the wavelength scale and/or all extinction coefficients by the appropriate amount. But even if this is done, the method of calculation leads unavoidably to extinction coefficients, ϵ_h and ϵ_l, that represent a kind of average of the true values for the two individual hemoproteins. Because of these limitations, reliance can only be placed on predominant features in the calculated absorption spectra, i.e., the positions and extinction coefficients of well-defined absorption bands. If the assumption of a thermal mixture is correct, then these would resemble those for the fluoride and cyanide derivatives; moreover, in no region of the spectrum should the extinction coefficients assume substantially negative values.

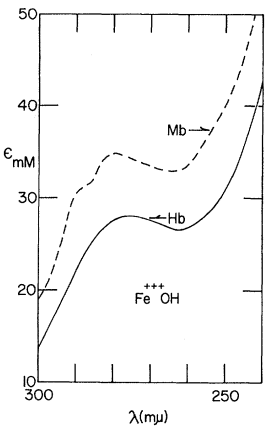


FIG. 12. Ultraviolet spectra of ferrimyoglobin and ferrihemoglobin hydroxide in the region of tyrosine and tryptophane absorption (Hanania, 1953).

In all but one of the sets of calculations, the value of 2.24 μ_B has been adopted for μ_l. This is about the minimum obtained for ferric complexes of myoglobin, hemoglobin, peroxidase, and catalase, and it has the advantage that μ^2_l is a whole number, so that, on substituting for μ_{Mb} and μ_{Hb}, Eqs. (8) and (9) are simply

$$26 = 5\alpha_{Mb} + 35(1 - \alpha_{Mb}) , \quad (8a)$$

$$20 = 5\alpha_{Hb} + 35(1 - \alpha_{Hb}) . \quad (9a)$$

The fractions of the low-spin forms (α) in ferrimyo-

globin and ferrihemoglobin hydroxide are thus 0.3 and 0.5, respectively. To check how sensitive the results are to the value chosen for μ_l, $2.84\mu_B$ has been used in one set of calculations. This gives $\mu_l^2 = 8$, $\alpha_{Mb} = 0.33$, and $\alpha_{Hb} = 0.55$: but as will be seen, this change of about 10% in α does not affect the type of absorption spectra obtained.

In view of the approximate nature of the present calculations, a small correction that should be made to the experimental values of μ has been neglected. This amounts to about 0.13 for $\mu = 2.70$, and 0.06 for $\mu = 5.92$, when the values are obtained in the usual way by taking a hemoprotein derivative that has no unpaired electrons, e.g., carbonmonoxy–ferrohemoglobin, as the reference compound to allow for the diamagnetism of the protein. This method entails the assumption that the paired d electrons of the iron atom make no contribution to the magnetic moment, but recent calculations have shown that an orbital paramagnetism, induced by the applied magnetic field, necessitates corrections of the magnitude quoted above (Griffith, 1958).

The Visible Region, 480 to 650 mμ

Using the extinction coefficients for ferrimyoglobin and ferrihemoglobin hydroxide given in Fig. 10, and without applying any corrections, the absorption curves in Fig. 13 are obtained. The remarkable extent to which these spectra show the predominant features expected of high- and low-spin complexes is immediately apparent.

Reference to the spectra for the fluoride and cyanide derivatives in Figs. 5 and 6 shows that the extinction coefficients are so very similar for myoglobin and hemoglobin that no correction on this account need be considered. On the other hand, the absorption bands of the ferrimyoglobin derivatives are all displaced toward longer wavelengths by a few millimicrons relative to hemoglobin. Calculations allowing for a 2, 3, and 5 mμ displacement have been carried out with the following results. The main absorption bands are little affected either in position or in intensity, as shown by comparing the data in lines (b), (c), and (d) with those in line (a) of Table IV. However, minor improvements in the spectra are obtained. The extinction coefficients for the low-spin form take on small positive values, $\epsilon_{mM} \approx 1$, in the range 600 to 650 mμ, and the values are also increased a little in the region 480 to 510 mμ. Further calculations using $\mu_l = 2.84\ \mu_B$ gave almost identical spectra; the absorption bands occur at the same wavelengths, and the extinction coefficients change by only about 7% [see lines (d) and (e) of Table IV].

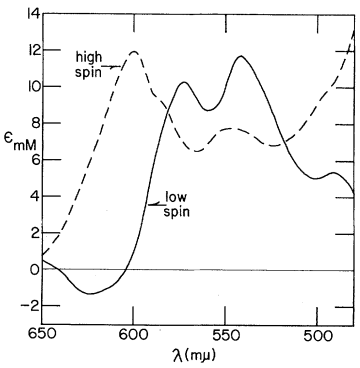


FIG. 13. Visible spectra for the high and low-spin hydroxides calculated from the data for ferrimyoglobin and ferrihemoglobin hydroxides. No λ or ϵ_{mM} corrections: $\mu_l = 2.24$, $\mu_h = 5.92$.

The absorption curves for the hydroxides of Japanese root peroxidase and leghemoglobin can be utilized in similar calculations, although at present assumptions have to be made as to the values of their magnetic moments. Adopting 2.66 μ_B for the Japanese root peroxidase derivative, like that for horseradish peroxidase, α_{Per}, the fraction of the low-spin form, calculated from an equation corresponding to (8) or (9) is found to be 0.93. The fraction of the high-spin form is thus 0.07. Absorption spectra

TABLE IV. Calculated band maxima and millimolar extinction coefficients (given in brackets) for the high- and low-spin hydroxides in the wavelength range 480 to 650 mμ.[a]

Spectra used	Values adopted for μ_l and μ_B	Details of calculations	High-spin hydroxide[b]		Low-spin hydroxide	
(a) Hb and Mb[c]	2.24, 5.92	No λ or ϵ_{mM} corrections	600(12.0)	548(7.8)	573(10.2)	542(11.7)
(b) Hb and Mb	2.24, 5.92	λ_{Mb} decreased by 2 mμ	598(11.5)	538(7.8)	574 (9.9)	544(11.8)
(c) Hb and Mb	2.24, 5.92	λ_{Mb} decreased by 3 mμ	597(11.4)	535(8.1)	575 (9.7)	544(11.8)
(d) Hb and Mb	2.24, 5.92	λ_{Mb} decreased by 5 mμ	595(11.1)	532(8.4)	575 (9.4)	545(12.3)
(e) Hb and Mb	2.84, 5.92	λ_{Mb} decreased by 5 mμ	595(11.4)	532(8.5)	575 (9.1)	545(11.6)
(f) Per and Mb	2.24, 5.92	No λ or ϵ_{mM} corrections	600(10.3)	538(7.8)	578(10.4)	548(12.6)
(g) Leg Hb and Mb	2.24, 5.92	No λ or ϵ_{mM} corrections	600(10.5)	545(7.5)	572(10.6)	543(12.0)
(h) Leg Hb and Hb	2.24, 5.92	No λ or ϵ_{mM} corrections	595 (9.0)	542(7.3)	572(10.6)	543(12.0)

[a] $\mu_{MB} = 5.11$: $\mu_{HB} = 4.47$: $\mu_{Per} = 2.66$.
[b] Band at about 490 mμ not fully resolved.
[c] Abbreviations: Hb, hemoglobin; Mb, myoglobin; Per, peroxidase; Leg Hb, leghemoglobin.

for the high- and low-spin forms based on the extinction coefficients of ferrimyoglobin hydroxide and this peroxidase hydroxide are plotted in Fig. 14 where it can be seen that they are very similar to those in Fig. 13. The spectrum of the low-spin form follows that of the root peroxidase derivative very closely, as would be expected with α_{Per} having a value

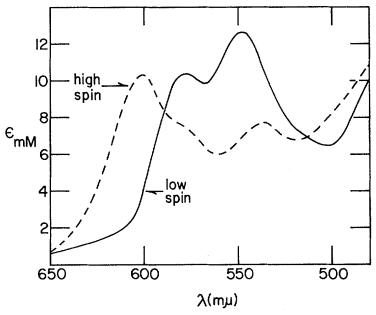


FIG. 14. Visible spectra for the high and low-spin hydroxides calculated from the data for ferrimyoglobin and Japanese root ferriperoxidase hydroxide (Morita and Kameda, 1958).

so near that of unity. In this case the extinction coefficients in the region 600 to 650 mμ have small positive values, with no corrections being applied. The band maxima occur at almost the same wavelengths, with extinction coefficients very similar to those obtained before, as shown in line (f) of Table IV.

With the previous value of $\alpha_{Hb} = 0.5$, these absorption curves can be used to calculate the spectrum of ferrihemoglobin hydroxide, with the result shown in Fig. 15. The agreement is quite satisfactory, the

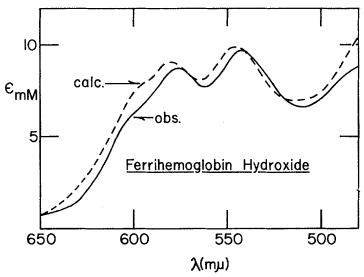


FIG. 15. The observed visible spectrum of ferrihemoglobin hydroxide, and that calculated from the spectra of the high- and low-spin forms illustrated in Fig. 14.

shoulder at about 600 mμ being reproduced very well.

The absorption curve for ferrileghemoglobin hydroxide can be used in a slightly different way. Making the assumption that this is entirely the low-spin form, the spectrum of the high-spin form can be obtained from either the ferrimyoglobin or the ferrihemoglobin data as shown in Fig. 16. These spectra compare very favorably with those for the high-spin form in Figs. 13 and 14. The slight shift in the wavelengths for maximum absorption, and the small variations in extinction coefficients [see lines (g) and (h) in Table IV], are to be expected in view of the systematic displacement of the bands of ferrimyoglobin derivatives relative to those of ferrihemoglobin noted previously. The spectra in Figs. 14 and 16 show very clearly that the main absorption bands are not very dependent on the low-spin values chosen for the magnetic moments of the root peroxidase and leghemoglobin hydroxides.

The Ultraviolet Region, 300 to 480 mμ

Inspection of the curves for the fluoride and cyanide derivatives in Fig. 8 shows that those for ferrimyoglobin are displaced by 4 to 5 mμ towards the red and have lower extinction coefficients throughout the Soret band region, 380 to 440 mμ. The ratios of Soret band maxima, $\epsilon_{Hb}/\epsilon_{Mb}$, are 1.06 and 1.10 for the fluoride and cyanide derivatives, respectively. It is not surprising therefore that calculations using the

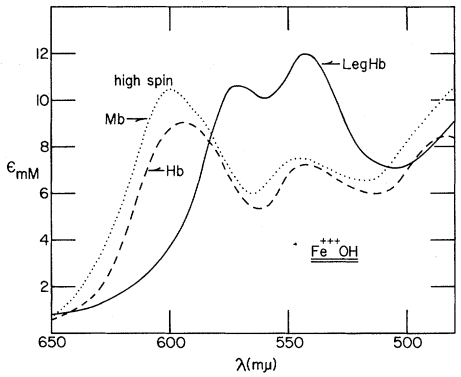


FIG. 16. Visible spectra of the high-spin hydroxide calculated from the data for ferrimyoglobin, ferrihemoglobin, and ferrileghemoglobin hydroxide (Sternberg and Virtanen, 1952), assuming that the latter is 100% low-spin. No λ or ϵ_{mM} corrections: $\mu_l = 2.24$, $\mu_h = 5.92$.

extinction coefficients for the two hydroxides taken from Fig. 11, with no corrections to allow for these systematic differences, give absorption spectra for the high- and low-spin forms which show none of the expected features. A narrow band is obtained for the low-spin form having a maximum at 412 mμ with $\epsilon_{mM} = 112$, in contrast to a very wide band for the high-spin form, having a maximum at 418 mμ with $\epsilon_{mM} = 88$ broadening out to a shoulder at 400 mμ with $\epsilon_{mM} = 75$.

Repeating the calculation, but correcting for the wavelength displacement by a factor of 5 mμ, and for the differences in intensity by multiplying the extinction coefficients for ferrimyoglobin hydroxide by the average of the values given above, namely, 1.08, gives the curves shown in Fig. 17. These are now seen to exhibit the characteristic features of high- and low-spin complexes. The Soret bands are at

405 mμ with ϵ_{mM} = 116, and 417 mμ with ϵ_{mM} = 104, respectively. These positions and relative intensities compare very favorably with those for the fluoride and cyanide derivatives. Furthermore, in the region 330 to 370 mμ, the low-spin form has two distinct bands at about 340 and 360 mμ, whereas the high-spin form has an unresolved shoulder at about 350 mμ, like the cyanide and fluoride derivatives, respectively (see Fig. 8). It is to be noted, however, that the band width of the high-spin form is larger than usual, and that of the low-spin form smaller, which results in the high-spin form having relatively higher extinction and the low-spin form relatively lower extinction between 350 and 400 mμ.

The Near Infrared Region, 700 to 950 mμ

In the absence of spectroscopic data for ferrihemoglobin fluoride and cyanide in this region, it is impossible to judge at present whether any correction factors should be applied. However, without correction, calculations based on the extinction coefficients of ferrimyoglobin and ferrihemoglobin hydroxide in Fig. 7 give the spectra for the high- and low-spin forms shown in Fig. 18, which can be seen to have the expected features. The high-spin form has well-defined bands at about 740 mμ with ϵ_{mM} = 0.9 and at 830 mμ with ϵ_{mM} = 1.15, which correspond closely to the bands for the fluoride derivative. The low-spin form has scarcely any absorption throughout this region like the cyanide derivative (see Fig. 7). The small negative extinction coefficients actually obtained for the low-spin form can be attributed to the uncertainties in the calculation procedure when the correction factors are unknown, and also to experimental error. Precise extinction coefficients are very difficult to determine in this region because the magnitudes are so small, and errors introduced by extraneous background absorption, which is hard to remove completely, become significant.

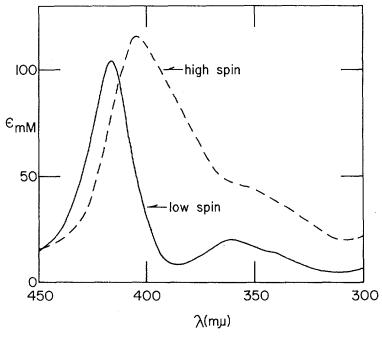


FIG. 17. Ultraviolet spectra of the high- and low-spin hydroxides calculated from the data for ferrimyoglobin and ferrihemoglobin hydroxides. The absorption curve of ferrimyoglobin hydroxide has been corrected by a 5 mμ displacement toward the red, and all extinction coefficients multiplied by 1.08: μ_l = 2.24, μ_h = 5.92.

Summary

Assuming that the ferrihemoprotein hydroxides are thermal mixtures, and adopting μ_h = 5.92 and μ_l = 2.24 as the magnetic moments of the high- and low-spin forms, calculations give the following percentages for the various hemoproteins:

Myoglobin: High—70%, Low—30%;

Hemoglobin: High—50%, Low—50%;

Peroxidase: High— 7%, Low—93%.

From the extinction coefficients of the hydroxides the spectra of the high- and low-spin forms have been obtained over the range 250 to 950 mμ. Major absorption bands, or shoulders, occur at about the following wavelengths, with extinction coefficients having the approximate values given in brackets:

High-spin form: 830 (1.2), 740 (0.9), 600 (11), 540 (8), 490 unresolved band (10), 405 (116), 350 shoulder (44).

Low-spin form: 575 (10), 545 (12), 417 (104), 360 (20), 340 (14).

The calculation procedure has certain inherent limitations, nevertheless these absorption bands correspond so closely to those which distinguish high- from low-spin derivatives that the assumption of a thermal mixture can be regarded as entirely consistent with the spectroscopic and magnetic data.

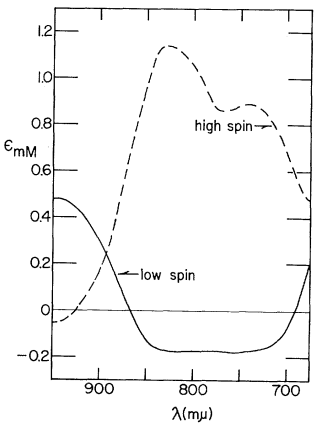


FIG. 18. Near infrared spectra of the high- and low-spin hydroxides calculated from the data for ferrimyoglobin and ferrihemoglobin hydroxide. No λ or ϵ_{mM} corrections: μ_l = 2.24, μ_h = 5.92.

V. THE EFFECT OF TEMPERATURE ON THE SPECTRUM AND ON THE MAGNETIC MOMENT OF FERRIMYOGLOBIN HYDROXIDE

As suggested previously, if the ferrihemoprotein hydroxides are thermal mixtures of high- and low-spin forms, then changing the temperature would be expected to influence the equilibrium,

$$\text{High-spin hydroxide} \overset{K_e}{\rightleftarrows} \text{Low-spin hydroxide} \qquad (12)$$

and a change should therefore be observable in the magnetic moment and in the absorption spectrum. The magnitude of the change would depend on the value of ΔH for reaction (12), since this determines the variation of equilibrium constant with temperature according to the van't Hoff Isochore. But, since a close balance between the energies of the two forms is to be anticipated, ΔH is likely to be small, and, as a consequence, K_e to have a small temperature dependence resulting in only slight changes in magnetic moment and absorption spectrum. This is borne out by the observation that there were no noticeable variations in the optical densities of ferrimyoglobin or ferrihemoglobin hydroxide solutions at 582 and 578 mμ, respectively, over the temperature range 7.5 to 37°C in experiments carried out to obtain thermodynamic data for the ionization reactions (George and Hanania, 1952, 1953).

However, further experiments have now been made using a sensitive recording spectrophotometer, and a temperature effect has been detected. The absorption spectrum of a concentrated solution of ferrimyoglobin hydroxide at pH 11.0 and 5°C was recorded from 470 to 650 mμ, the optical density at 540 mμ being 0.7411. The reference cuvette, which previously contained buffer, was then filled with more of the hydroxide, and a baseline was recorded over the wavelength range with both solutions at 5°C. The solution in one cuvette was then rapidly warmed to 35°C, and maintained at this temperature, by the insertion of a specially constructed hollow metal heating unit through which water from a thermostat was circulated. A difference spectrum was recorded, from which the curve illustrated in Fig. 19 was obtained after correction for the baseline. As can be seen, the effect of a 30° alteration in temperature is rather small. The change in optical density is at the most 2.9% at 542 mμ, while at 582 mμ it has dropped to 1.6%, which accounts for the effect escaping notice in the earlier investigations. Control experiments using the cyanide and fluoride derivatives showed no similar effect.

The negative regions from 480 to 520 mμ and above 600 mμ, together with the positive region in between which shows two well-defined bands, are qualitatively consistent with an increase in the fraction of the high-spin form as the temperature is increased. Some indication of its magnitude can be obtained from the difference between the extinction coefficients of the high- and low-spin forms calculated in Sec. IV. At 540 mμ the difference in ϵ_{mM} is about 4, which gives an increase of between 0.05 and 0.07,

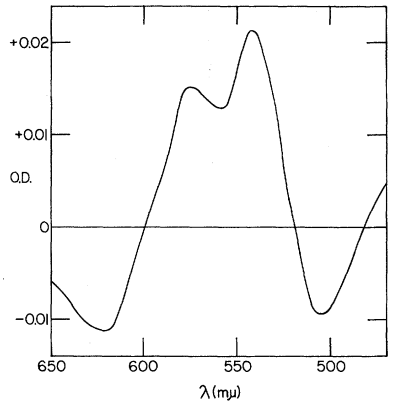


FIG. 19. The difference spectrum in the visible region for 8.4 $\times$ 10^{-5} M ferrimyoglobin hydroxide at 5° and 35°, plotted as $(d_5^\circ - d_{35}^\circ)$.

i.e., between 5 and 7%. However, in order to obtain the individual spectra of the high- and low-spin forms from the difference spectrum, an independent determination of the fractions present at the two temperatures is required. This can be seen from the equations,

$$\epsilon_5 = \alpha_5\epsilon_l + (1 - \alpha_5)\epsilon_h , \qquad (13)$$

$$\epsilon_{35} = \alpha_{35}\epsilon_l + (1 - \alpha_{35})\epsilon_h , \qquad (14)$$

where ϵ_5 and ϵ_{35} are the extinction coefficients of the hydroxide at 5° and 35°, α_5 and α_{35} are the fractions of the low-spin form at the two temperatures, and ϵ_h and ϵ_l are the extinction coefficients of the high- and low-spin forms. Since ϵ_5 is known and ϵ_{35} can be obtained from the difference spectrum, provided α_5 and α_{35} can be determined, μ_h and μ_l can be evaluated from the equations rearranged in the forms,

$$\epsilon_h = \frac{\epsilon_{35}\alpha_5 - \epsilon_5\alpha_{35}}{\alpha_5 - \alpha_{35}} , \qquad (15)$$

$$\epsilon_l = \frac{\epsilon_5(1 - \alpha_{35}) - \epsilon_{35}(1 - \alpha_5)}{\alpha_5 - \alpha_{35}} . \qquad (16)$$

The variation of α with temperature has been obtained in the following way. Using a sensitive Gouy balance, constructed from a Varian electromagnet V4004 and a Sartorius Microbalance MPR 5 II, and equipped with a coaxial glass thermostat surrounding the sample tube and suspension fiber, the change

in Δw was measured as a function of temperature over the range 1 to 30°C for the fluoride, cyanide, and hydroxide derivatives of ferrimyoglobin. Calibration with nickel chloride solution enabled these changes in Δw to be converted into changes in molar susceptibility, π_M. The value of χ_M obtained by Theorell and Ehrenberg (1951) for the three derivatives at 20°C were adopted, namely, 14 790, 2340, and 11 040 $\times 10^{-6}$ cgs units, respectively, and hence values of χ_M over the temperature range were obtained. The variation of χ_M for the fluoride and cyanide derivatives was found to follow very closely the simple Curie law, $\chi = \text{const}/T$. The magnitude of the change is illustrated by the following data: From 20° to 1°, χ_M for the fluoride and cyanide derivatives increases by 1046 $\times 10^{-6}$ and 82 $\times 10^{-6}$ cgs units, respectively. On the other hand, χ_M for the hydroxide, although it has a high value approaching that of the fluoride, only increases by 145 $\times 10^{-6}$ cgs units for the same decrease in temperature. As a consequence, the values of χ_M do not follow the Curie Law, and the type of deviation is just what would be expected if, on lowering the temperature, the fraction of the high-spin form decreases.

The simplest method by which the fractions of the high- and low-spin forms can be calculated, throughout the temperature range, is to use the experimental values of χ_M for the fluoride and cyanide derivatives at various temperatures as the values appropriate to the high- and low-spin forms, and substitute in the equation,

$$\chi_{M(\text{hydroxide})} = \alpha\chi_{M(\text{cyanide})} + (1 - \alpha)\chi_{M(\text{fluoride})}. \quad (17)$$

In practice, this is equivalent to taking $\mu_l = 2.34\ \mu_B$, i.e., the value for the cyanide derivative, instead of 2.24 μ_B, as in the majority of calculations in Sec. IV. Such a slight change in μ_l only affects the value of α to a negligible extent, i.e., from 0.300 to 0.304. Values of α and $(1 - \alpha)$, obtained in this way, are listed in Table V for temperatures from 0° to 30°, together with values for K_e, the equilibrium constant for the conversion reaction (12).

TABLE V. The fractions of the low-spin and high-spin forms of ferrimyoglobin hydroxide, α and $(1 - \alpha)$, respectively, at different temperatures, calculated from the temperature variation of χ_M for ferrimyoglobin hydroxide, fluoride, and cyanide. K_e is the equilibrium constant for the conversion high-spin form $\rightleftharpoons$ low-spin form and is given by $\alpha/(1 - \alpha)$.

T(°C)	α	$(1 - \alpha)$	K_e
0	0.34	0.66	0.52
10	0.32	0.68	0.47
20	0.30	0.70	0.43
30	0.28_5	0.71_5	0.40

Interpolation and extrapolation for the temperature interval 5° to 35° gives 0.055 for the corresponding change in α. The spectra of the high- and low-spin forms of ferrimyoglobin hydroxide were then obtained by calculating ϵ_h and ϵ_l throughout the wavelength range according to Eqs. (15) and (16).

The similarity between these spectra, shown in Fig. 20, and those in Figs. 13, 14, and 16 is very gratifying. But it must be remembered that the previous spectra, calculated from data for pairs of hemoproteins, are approximations, consisting of average values of the extinction coefficient appropriate to the two individual high-spin forms and the two individual low-spin forms. The new spectra in Fig. 20, based entirely on data for one hemoprotein, are therefore more valid.

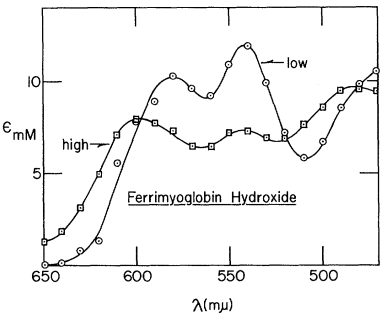


FIG. 20. The visible spectra of the high- and low-spin hydroxides calculated from the difference spectrum in Fig. 19, and the corresponding change in the fraction of the low-spin form, 0.055, as obtained from the variation of magnetic susceptibility with temperature.

VI. THE SPECTRA OF FERRIMYOGLOBIN DERIVATIVES IN HEAVY WATER

The interplay of structural and electronic factors necessary for a ferrimyoglobin derivative to exist as a mixture of high- and low-spin forms is evidently so critical that the ligands most closely related to the hydroxyl group in chemical type give predominantly, or entirely, high-spin or low-spin complexes. On the basis of spectroscopic data, or magnetic data, or both, it is clear that the complexes with phenol, and presumably ethanol, i.e., Fe_{MB}^{3+}—OC_6H_5 and Fe_{MB}^{3+}—OC_2H_5 come in the former category; whereas the complexes with the sulphur analogs, hydrogen sulphide, ethyl mercaptan, and thiophenol, i.e., Fe_{MB}^{3+}—SH, Fe_{MB}^{3+}—SC_2H_5 and Fe_{MB}^{3+}—SC_6H_5, come in the latter (George, Lyster, and Bettlestone, 1958; Coryell and Stitt, 1940; Keilin, 1933; Heussenstam and Coryell, 1954). The least drastic of all substitutions that can be achieved, with the exception of employing H_2O^{18}, is the replacement of hydrogen

by deuterium, and the spectrum of the alkaline form in heavy water, which should accordingly have the structure Fe_{MB}^{3+}—OD, has therefore been studied. In the preliminary experiments, reported below, the highest mole ratio of D_2O to H_2O that could be attained was 134:1. Hence, although the affinities of the iron atom for OH^- and OD^- also have to be taken into consideration because they determine the relative amounts of Fe_{MB}^{3+}—OH and Fe_{MB}^{3+}—OD formed, it is unlikely that in pure D_2O the effect observed would be very much enhanced.

A very concentrated solution of acidic ferrimyoglobin in ordinary water was used, so that only 0.02 ml in a total of 3 ml was required to give optical density values of about 0.7 at the band maxima in the visible region. Solutions of the alkaline form were prepared in the following way. Tiny quantities of caustic soda solution were added to acidic ferrimyoglobin (0.02 ml stock solution +2.98 ml ordinary water) from a microsyringe until the pH was 11.0. The same volume of caustic soda was added to a corresponding solution of acidic ferrimyoglobin made up in heavy water. Difference spectra were then recorded with the heavy water solution in the reference cuvette for the alkaline form, and, as controls, for the acidic form and the cyanide derivative, which was prepared by adding a little solid KCN.

With the cyanide derivative no difference could be detected and with the acidic form there was scarcely any change. But with the alkaline form a well-defined difference spectrum was obtained, very similar to that in Fig. 20, and the optical density differences were about the same in magnitude. The simplest interpretation of this result is that for the Fe_{MB}^{3+}—OD formed in heavy water the fraction of the low-spin form is about 6% higher than the fraction for Fe_{MB}^{3+}—OH under similar conditions. Using the data given in Table V for the hydroxide, an approximate value of the equilibrium constant for the reaction

$$\text{High-spin deuteroxide} \rightleftharpoons \text{Low-spin deuteroxide} \quad (18)$$

is found to be 0.55 at 25°C, compared to 0.41 for the hydroxide. Substitution of hydrogen by deuterium thus favours the conversion by about 0.2 kcal/mole in units of free energy. It is probable that the bulk of this difference arises *via* the water of solvation and not from any effect on the ligand field. The change of mass of the hydrogen nucleus affects the free energy of "crystallization" around the iron ion directly but the ligand field only very indirectly through the effect of the change of vibrational amplitudes for the OH group on the mean ligand field.

VII. GENERAL REMARKS

The experiments with heavy water offer particularly direct evidence for the existence of a thermal mixture in ferrimyoglobin hydroxide. Further, because the fundamental difference between the high- and low-spin form lies in the electronic structure of the iron ion, they show that water molecules (or those protons which are exchangeable with those of water) play an essential part in determining the free energy change. We would naturally guess that the water molecules which are "crystallized" around the iron ion (and also the hydrogen of the OH^- group) are the ones concerned here. Although this is far from direct evidence for the existence of the hydroxide structure it is at least thoroughly consistent with it.

The hypothesis of a thermal mixture is also fully borne out in the case of ferrimyoglobin hydroxide by the temperature variation of its spectrum and magnetic moment as described in Sec. V; and, in view of the self-consistent results of the calculations using magnetic and spectroscopic data in Sec. IV, it can be concluded that ferrihemoglobin hydroxide is also a mixture of high- and low-spin forms. This accounts equally well for its apparently anomalous magnetic moment as the explanation in terms of the electronic configuration with three unpaired electrons, which was shown to be unlikely on theoretical grounds (see Sec. II).

Thermodynamic data for the conversion of the high-spin to the low-spin form can be obtained from the values of K_e for ferrimyoglobin in Table V. A plot of $\ln K_e$ against $1/T$ gives $\Delta H = -1.5 \pm 0.2$ kcal/mole, and from the equation $\Delta G^\circ = \Delta H - T\Delta S^\circ$, with ΔG° equal to 0.5 kcal/mole at 25°C, ΔS° is found to be -6.7 ± 0.7 eu. The conversion is thus favored by the enthalpy change, but is appreciably hindered by the entropy change to such an extent that the resulting free energy change has a small positive value. In other words, with respect to their heats of formation the low-spin form is the more stable, whereas in terms of their entropies the high-spin form is the more stable.

The favorable value of ΔH may be regarded as purely fortuitous, because, although the conversion to the low-spin form implies an increase in the value of Δ, and hence extra stabilization, pairing energies have also to be taken into consideration and in addition solvent interaction effects may be important (see below). In order to discuss the entropy change accompanying the conversion, it is convenient to distinguish the contribution arising from the degeneracy of the electronic state of the iron in the two forms from the remainder. The following estimate shows

that this contribution is unlikely to be more than about -2 eu.

In the high-spin form the ferric ion has a ground term which is spatially nondegenerate but has a six-fold degeneracy due to the spin $S = \frac{5}{2}$. The ligand field combined with the spin–orbit coupling lifts the degeneracy into three Kramers doublets. If this splitting is large compared to kT, only one Kramers doublet is occupied and the effective degeneracy of the ferric ion is 2. If it is small then the degeneracy is 6. This means that the entropy associated with the degeneracy of the electronic state of the iron in the high-spin form lies between the two limits of $R \ln 2 = 1.38$ eu and $R \ln 6 = 3.56$ eu. The actual magnitude of the splitting is unknown. If we assume that it may be represented in a spin Hamiltonian for the ground term with $S = \frac{5}{2}$ by the quadratic expression

$$D(S_z^2 - \tfrac{35}{12})$$

then electron resonance measurements show that D can hardly be less than 4°K (Bennett and Ingram, 1956; Griffith, 1956c). With D in these units, the partition function Z is given by the equation,

$$Z = 2e^{-10D/3T} + 2e^{2D/3T} + 2e^{8D/3T} \qquad (19)$$

from which the entropy follows from the formula $S = \partial(RT \ln Z)/\partial T$. For D/T small we deduce $S = 3.56 - (28D^2R/9T^2)$. The second term is inappreciable (<0.01) at room temperature for $D < 12$°K, i.e., an over-all splitting of 48 cm^{-1}. Therefore it seems likely, although not certain, that at room temperature this contribution to S is close to 3.56 eu.

In the low-spin form we have a spatial degeneracy of three and a spin degeneracy of two. Here, however, it is probable that the three Kramers doublets have a separation large compared with kT at room temperature (Griffith, 1957) so that the contribution to S from the degeneracy is close to 1.38 eu. This means a contribution to ΔS for the conversion of the high-spin to the low-spin form of $1.38 - 3.56 = -2.18$ eu. If our assumptions are incorrect the numerical value of this contribution will almost certainly be lower.

It is much more difficult to obtain any *a priori* numerical estimate for the remainder of the entropy change, which, using the value obtained in the last paragraph for the degeneracy contribution, is seen to amount to about -5 eu.[3] We should expect it to be negative, however, for the following reason. In the high-spin form the over-all distribution of the five d electrons about the iron has nearly spherical symmetry, thus producing no orientating effect on the environment. On the other hand, in the low-spin form the five electrons are in the three orbitals away from the bond directions, thus imposing an extra rigidity on the environment of the iron. This would result partly in a more rigid ferrimyoglobin molecule, and partly in a more rigid arrangement of water molecules around the Fe—OH group.

Just as ΔH for the conversion is determined by other energy terms besides the electronic stabilization energy arising from the splitting of the d orbitals, so the values of ΔH for the formation of complexes with different ligands cannot be taken as an accurate indication of the variation in Δ. From one extreme to the other, however, a rough correlation would be expected, with the high-spin complexes having the less favorable values of ΔH. This trend, which has also been discussed by Havemann and Haberditzl (1958), is illustrated by the data in Table VI. The values of $\Delta S°$ become progressively more negative from fluoride to cyanide, but they are not amenable to any straightforward correlation because the entropies of the ligands themselves vary so much, with $\bar{S}°$ for F^-, OH^-, and CN^- having the values -2.5, -2.3, and $+28$ eu, respectively. Some allowance for this can nevertheless be made by comparing the differences in partial molal entropies of the complexes and the parent hemoprotein (George, 1956).

TABLE VI. ΔH and $\Delta S°$ values for the formation of ferrimyoglobin fluoride, hydroxide, and cyanide: and their magnetic moments—(George and Hanania, 1952, 1956: Theorell and Ehrenberg, 1951).

Ligand	ΔH kcal/mole	$\Delta S°$ eu	μ (μ_B)	Type of complex
F^-	-1.5	$+1.8$	5.75–5.92	high-spin
OH^-[a]	-7.65	-2.6	5.11	70% high, 30% low-spin
CN^-	-18.6	-24	2.35	low-spin

[a] See footnote to Table VII.

A more rigorous correlation can be sought, if, for the same ligand, data are available for closely related hemoproteins. But if the whole range from high- to low-spin complexes is to be covered, this would clearly be restricted to those ligands capable of giving thermal mixtures in some cases. For example, the data in Table VII for the various hemoglobin hydroxides show that the increase in the fraction of the low-spin form is accompanied by more negative

[3] The assumed additivity of entropies is equivalent to a factorization of the partition function, which is probably a good approximation here at room temperature or below.

(i.e., favorable) values of ΔH, which was to be anticipated from the over-all trend illustrated in Table VI. Furthermore, with a series of derivatives of this type, where the ligands are identical and the structure of the complex in the immediate neighborhood of the iron is presumably very similar, the values of ΔS can be taken as a true indication of a general trend paralleling the trend in ΔH. As the fraction of the low-spin form increases, ΔS assumes more negative (unfavorable) values, while ΔH assumes more negative (favorable) values. This trend in ΔS is entirely in accord with the entropy change obtained above for the conversion of the high-spin to the low-spin hydroxide in the case of ferrimyoglobin, and it can likewise be associated with a greater structural rigidity in the vicinity of the iron atom of the low-spin form.

TABLE VII. ΔH and $\Delta S°$ values for hydroxide formation.[a]

Hemoprotein	ΔH kcal/mole	$\Delta S°$ (eu)	Type of hydroxide
Ferrimyoglobin	−7.65	−2.6	70% high, 30% low-spin
Ferrihemoglobin	−9.5	−7.9	50% high, 50% low-spin
Ferrileghemoglobin	−11.0	−13.0	approaches 100% low-spin

[a] These values have been calculated from the corresponding data for the ionization reaction, and for the ionization of water (ΔH = +13.4 kcal/mole and $\Delta S°$ = −19.2 eu). The references for ferrimyoglobin and ferrihemoglobin are given in Table I; for ferrileghemoglobin, see George, Hanania, and Thorogood (1959).

The pK values for the ionization of ferriperoxidase and ferricytochrome c are so much higher than those for the hemoglobins (see Table I) that inevitably either the ΔH values, or the ΔS values, and very probably both, would show marked deviations from the correlation set out in Table VII. This is not unexpected because the acidic forms of these hemoproteins have different structures, and as a consequence the formation of the hydroxide is a different type of chemical reaction.

In the case of the hemoglobins, the reactions of the acidic form can be very adequately expressed by the hydrate structure, e.g., Prot.—$Fe_{Hb}^{3+}(H_2O)$, and the ionization is accordingly the simple dissociation of a proton,

$$\text{Prot.—}Fe_{Hb}^{3+}(H_2O) \rightleftarrows \text{Prot.—}Fe_{Hb}^{++}\text{—OH} + H^+ \qquad (20)$$

On the other hand, in the acidic form of ferricytochrome c the iron is bonded in an intricate crevice structure to nitrogenous base groups of the protein at both the fifth and sixth coordination positions. One of the crevice bonds must be broken if OH^- is to replace one of the groups, and the ionization reaction therefore takes the form,

$$\text{Prot.—}Fe^{3+}_{cyt.c}\text{—N(base)} + H_2O \rightleftarrows \text{Prot.—}Fe^{++}_{cyt.c}\text{—OH N(base)} + H^+ . \qquad (21)$$

With ferriperoxidase the nature of the reaction is rather more obscure, because in less alkaline solution the pH variations of the equilibrium constants for complex formation with cyanide, fluoride, azide, etc., differ systematically from the corresponding variations for ferrimyoglobin, ferrihemoglobin, and ferricytochrome c. The difference lies in the consumption of a proton accompanying the formation of the complex. George and Lyster (1958) have discussed various explanations that have been advanced, and suggested as a further possibility that acidic ferriperoxidase also has a crevice structure but with the labile bond, which is broken in complex formation and upon ionization, to some group other than a nitrogenous base, with a pK of about 10 in horseradish peroxidase. Whatever the true explanation may be, it is clear that no strict comparison can be made between thermodynamic data for the ionization of ferriperoxidase and ferricytochrome c and the corresponding data for the hemoglobins.

Finally, the question naturally arises as to whether any other hemoprotein derivatives are mixtures of high- and low-spin forms. Scheler, Schoffa, and Jung (1957) and Havemann and Haberditzl (1958) have suggested that this may be the case for several other derivatives where the magnetic moments differ appreciably from the usual high or low values. However, no quantitative correlation of magnetic and spectroscopic properties, of the kind used in Sec. IV to calculate the individual spectra of the high- and low-spin hydroxides, was considered. Since azide gives a high-spin complex with ferricatalase and a low-spin complex with ferrihemoglobin, it is quite likely that the ferrimyoglobin derivative would contain a significant fraction of the high-spin form. Preliminary calculations tend to confirm this. Nevertheless, until temperature variations of the magnetic moment and absorption spectra have furnished direct experimental evidence for the existence of a thermal mixture, as in the case of ferrimyoglobin hydroxide, it is perhaps better to leave it as an open question with regard to the other derivatives.

ADDENDUM

Additional experimental studies, not incorporated in the previous discussion, have led to a determination of the thermodynamic values for spin-state equilibria in ferrimyoglobin and ferrihemoglobin complexes. This work by Professor George, J. Beetlestone and J. Mullins is shown in Table VIII.

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TABLE VIII. Thermodynamic data for spin-state equilibria in ferrimyoglobin and ferrihemoglobin[a] complexes.
High Spin $\underset{(1-\alpha)}{\rightleftharpoons}$ Low Spin $\underset{\alpha}{}$, $K = \alpha/(1-\alpha)$.

Complex	$\alpha_{20°}$	$K_{20°}$	$\Delta F°_{20°}$ cal/mole	$\Delta H°$ cal/mole	$\Delta S°_{20°}$ cal/mole/deg
Ferrimyoglobin hydrate	0.08 ± 0.01	0.09	+1217 ± 109	−60 ± 2130	−4.4 ± 7.4
Ferrimyoglobin hydroxide	0.31 ± 0.01	0.45	+465 ± 17	−1227 ± 448	−5.8 ± 1.6
Ferrimyoglobin azide	0.785 ± 0.005	3.62	−675 ± 58	−2740 ± 400	−7.0 ± 1.0
Ferrimyoglobin imidazole	0.905 ± 0.005	9.84	−1341 ± 38	−3040 ± 840	−5.8 ± 3.0
Ferrihemoglobin thiocyanate	0.405	0.675	+228	−1400	−2.8
Ferrihemoglobin hydroxide	0.64	1.78	−335	−1900	−5.2
Ferrihemoglobin nitrite	0.715	2.49	−530	−2400	−6.4
Ferrihemoglobin azide	0.955	20.6	−1760	−9400	−26

[a] The values for the ferrihemoglobin complexes are provisional, however they serve to show that the trend in $\Delta H°$ is the same as that for the ferrimyoglobin complexes, i.e., as α increases, $\Delta H°$ assumes more favorable values.

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CONTRIBUTED PAPERS FOR SESSION VII

Mössbauer Effect in Hemoglobin Enriched in Fe^{57}

R. W. Grant and U. Gonser, *North American Aviation Science Center*
J. Kregzde, *Veterans Administration Hospital, Long Beach, California*

A number of iron-containing organic compounds, including the blood component hemin, have already been investigated by the Mössbauer technique. We have extended the experiments to the macromolecule oxyhemoglobin (molecular weight 68 000). Preliminary experiments were performed on natural oxyhemoglobin at liquid-nitrogen temperature indicating the necessity of using Fe^{57}-enriched material for further studies of high molecular weight blood components. This is, in part, due to the large competing electronic absorption process of the 14-keV γ ray in the