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A Magnetochemical Study of Equilibria between High and Low Spin States of Metmyoglobin Complexes*

J. Beetlestone† and P. George

From the Department of Chemistry, University of Pennsylvania, Philadelphia

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Many methemoprotein complexes have magnetic susceptibilities between the values characteristic of five and one unpaired electrons. In this paper the temperature dependences of the magnetic susceptibilities and visual spectra of some complexes of metmyoglobin are investigated. It is shown that the temperature dependence of the magnetic susceptibilities of those complexes which have intermediate susceptibilities deviates markedly from the Curie law. The results are interpreted in terms of the theory that these complexes are thermal mixtures of high- and low-spin forms. Standard free energy, enthalpy, and entropy changes for the high- to low-spin transition are calculated. The temperature dependence of the magnetic susceptibility is correlated with the temperature dependence of the spectra, and with the results of electron spin resonance experiments with these complexes carried out by other authors.

The first detailed study of the magnetochemistry of hemoproteins and their complexes was made by Coryell *et al.* (1937). The complexes of methemoglobin were found to have paramagnetic susceptibilities corresponding to five unpaired electrons, characteristic of "ionic" bonding, or to one unpaired electron, characteristic of "covalent" bonding. The parent compound, methemoglobin, which was presumed to have a water molecule in the sixth coordination position of the central ferric ion, was shown to be a typical "ionic" complex. Replacement of the water molecule with a fluoride ion produced a complex with a slightly higher paramagnetic susceptibility, whereas cyanide and azide ions and imidazole produced complexes with susceptibilities characteristic of one unpaired electron. The hydroxide complex was found to be anomalous in that its susceptibility was characteristic of three unpaired electrons, and it was suggested that this could be accounted for by assuming that four covalent bonds resonate among six positions. Subsequently it was demonstrated that the hydroxide complex of metmyoglobin also has a susceptibility intermediate between that of the high- and low-spin forms, but appreciably higher than that of methemoglobin hydroxide (Theorell and Ehrenberg, 1951). Following more recent terminology, the terms high spin and low spin will be used throughout this paper to describe complexes with susceptibilities characteristic of five and one unpaired electrons, respectively. Extension of magnetochemical and spectroscopic measurements to other hemoproteins and other ligands has revealed that the hydroxide complexes of methemoglobin and metmyoglobin are not unique in having susceptibilities intermediate between those of high- and low-spin complexes (Hartree, 1946; Scheler *et al.*, 1957; Havemann and Haberditzl, 1958).

Taube (1952) suggested that the hydroxide complex might be a mixture of two forms, one high spin and the other low spin, and numerous authors (Williams, 1956; Scheler *et al.*, 1957; Havemann and Haberditzl, 1958) since then have made similar suggestions. Griffith (1956a) calculated that, in a regular octahedral complex, if spin pairing occurs to reduce the number of electrons from five to three, then further pairing is even more favored energetically, reducing the number from three to one. Although a similar quantitative treatment of ferrimyoglobin hydroxide is not yet

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† Now at Department of Chemistry, University of Ibadan, Nigeria.

TABLE I

EXPERIMENTAL CONDITIONS FOR THE DETERMINATION OF THE MAGNETIC SUSCEPTIBILITIES OF METMYOGLOBIN COMPLEXES

		Concentration of Complex (M)	pH	Concentration of Ligand (M)
Metmyoglobin fluoride	(MetMbF)	5.56×10^{-4}	7.8	1
Metmyoglobin	($MetMbOH_2$)	5.87×10^{-4}	6.5	
Metmyoglobin hydroxide	(MetMbOH)	7.05×10^{-4}	11.0	
Metmyoblobin azide	($MetMbN_3$)	6.09×10^{-4}	6.2	0.1
Metmyoglobin imidazole	(MetMbIm)	1.01×10^{-3}	8.5	0.5
Metmyoglobin cyanide	(MetMbCN)	1.71×10^{-3}	8.5	0.1

possible, this conclusion casts serious doubt on the hypothesis that this complex has a configuration of three unpaired electrons, and Griffith (1956b) again raised the question whether it is a thermal mixture of high- and low-spin forms. The indirect magnetochemical and spectroscopic observations which support this hypothesis have been discussed in detail by George *et al.* (1959). Direct experimental confirmation of the mixture hypothesis in experiments using a single hemoprotein was provided by the temperature dependence of the spectrum of the hydroxide complex of metmyoglobin. In addition, preliminary magnetochemical measurements at different temperatures demonstrated the possibility of calculating the spectra of the high- and low-spin forms of the hydroxide complex, and the enthalpy change associated with the high- to low-spin transition.

In this paper, the temperature dependence of the paramagnetic susceptibility and the visual spectra of metmyoglobin and its complexes with five ligands are described. To conform with previous terminology, the parent compound will be referred to throughout as metmyoglobin rather than metmyoglobin hydrate; it may be noted that Kendrew *et al.* (1961) have shown that a water molecule does indeed occupy the sixth coordination position as has been assumed in the past.

EXPERIMENTAL

Material.—Horse metmyoglobin was prepared according to the method of George and Hanania (1952). Analytical grade chemicals were used throughout.

Magnetic Susceptibility Measurements.—Magnetic susceptibilities were determined on a sensitive Gouy balance composed of a Varian V4004 magnet and a Sartorius MPR-S II microbalance. The sample tube, which was suspended by silver wire (diameter 0.005) between 1-in. tapered pole pieces, consisted of two lengths of square-section thin-walled glass tubing cemented together with heat-setting "Araldite" across a thin glass diaphragm, the tubing having a wall thickness of 0.015 cm and a cross sectional area of 0.36 cm^2. The field strength in the pole gap was about 23,000 gauss, and the balance had a sensitivity of 10^{-6} g, giving a volume susceptibility sensitivity of 10^{-11} emu. That is to say, a 5×10^{-4} M solution of a compound with molar susceptibility of $14,000 \times 10^{-6}$ emu gave rise to an apparent increase in weight of 7.25×10^{-4} g when the magnetic field was turned on. The balance was calibrated with nickel chloride solution. The sample tube was enclosed in a thermally insulted, water-jacketed bronze box designed to allow the pole pieces to be brought within 3 mm of the tube; and when water was circulated through the jacket from a constant-temperature bath at 1.00° the temperature in the sample tube as measured by a thermistor probe thermometer was 1.04°. The constant-temperature bath was controlled to ± 0.01°, and a calibration curve allowed calculation of the sample-tube temperature for any given bath temperature.

The method used to determine the paramagnetic susceptibility of metmyoglobin derivatives is illustrated by the following description of an experiment with the fluoride complex. A 1.5 M potassium fluoride solution (3 ml) was added to 1.5 ml of stock metmyoglobin solution, and 6 ml of the potassium fluoride solution was added to 3 ml of water. The final concentrations of metmyoglobin and potassium fluoride were 4.98×10^{-4} M and 1 M, respectively. Both solutions were left in a constant-temperature bath at 25° for 1 hour, with occasional shaking to ensure that both solutions contained equal concentrations of oxygen. Errors arising from the assumption that the solubility of oxygen is the same in the potassium fluoride solution with and without added metmyoglobin may be shown to be insignificant. The sample tube was then filled on both sides with the potassium fluoride solution and attached to the balance. After allowing 30 minutes for the tube to come to thermal equilibrium with the thermostated jacket at about 20°, the difference in weight with the magnetic field off and on was measured twenty times and the average of these readings, Δw_1, was used in subsequent calculations. The solution in one half of the tube was replaced by the metmyoglobin fluoride solution and the above procedure was repeated to give Δw_2. The change in weight on applying the magnetic field was then measured at other temperatures between 0° and 30°. Δw_2 at each temperature was corrected for the change in molar concentration with temperature. To determine the diamagnetic correction due to the protein, the above procedure was followed using solutions of sodium hydrosulfite and carboxymyoglobin. From similar experiments with solutions of nickel chloride of known molar concentration, a cell constant, K, was calculated relating Δw_1 and Δw_2 with molar susceptibility:

$$\chi = \frac{K}{c}(\Delta w_2 - \Delta w_1)$$

where c is the molar concentration of a compound of molar susceptibility χ. For a typical sample tube and field strength, $K = 96 \times 10^{-4}$.

Spectrophotometric Measurements.—Spectra were taken on a Beckman DK1 spectrophotometer, and on a sensitive recording spectrophotometer specially constructed at the Johnson Foundation for Medical Physics.

RESULTS

Magnetic Susceptibilities.—The molar paramagnetic susceptibilities at several temperatures of six metmyoglobin complexes measured under the conditions given in Table I are listed in Table II. All values are subject to an error of 1.5% because of the uncertainty in the iron content of the stock metmyoglobin. Estimated

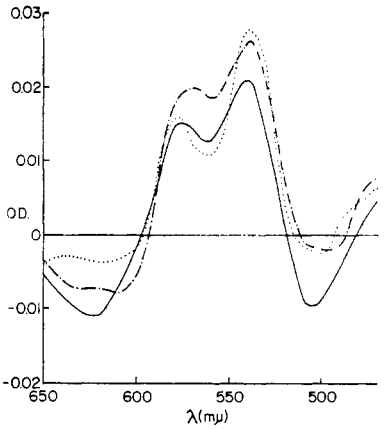

FIG. 1.—5°/35° difference spectra for metmyoglobin hydroxide —, metmyoglobin azide —·—·—, and metmyoglobin imidazole . . . . . Solution at 5° in the reference cuvet.

errors arising from the magnetic measurement are different for each complex, since the uncertainty in Δw remains about $\pm 3 \times 10^{-6}$ g, irrespective of the absolute value of Δw. Estimated maximum errors were $\pm 1\%$ for the fluoride, hydrate, and hydroxide complexes, $\pm 3\%$ for the cyanide and imidazole complexes, and $\pm 4\%$ for the azide complex. Comparison of any susceptibility with values in the literature involves the additional error caused by the uncertainty in the iron concentration, and the total estimated maximum error including this factor is $\pm 2.5\%$ for the fluoride complex and $\pm 5.5\%$ for the cyanide complex. Errors arising from incomplete formation of the complexes are not significant.

Spectra.—The temperature dependence of the spectra of the parent metmyoglobin and the cyanide and fluoride complexes was measured by directly observing the difference spectrum between a reference cuvet containing the complexes at 5° and a sample cuvet at 35°. No optical density differences greater than 0.001 were observed. However, the spectra of the imidazole, azide, and hydroxide complexes did change with temperature and these changes are shown as difference spectra in Figure 1. The hydroxide difference spectrum has been previously reported (George *et al.*, 1959) and is included for comparison.

The differences between the spectra of metmyoglobin, the azide, cyanide, and hydroxide complexes in D_2O and H_2O were measured by the difference-spectra technique. The spectra of the cyanide and azide complexes were unaffected by the use of D_2O as solvent, whereas the hydroxide complex gave an H_2O versus D_2O difference spectrum similar in shape and in magnitude to the 5° versus 35° spectrum, D_2O favoring the low-spin form. The metmyoglobin spectrum was found to be only slightly affected when the solvent was D_2O.

Interpretation of Results in Terms of an Equilibrium between High- and Low-Spin States.—It has been the custom in the literature on the magnetochemistry of hemoproteins (Hartree, 1946) to assume that the temperature dependence of the susceptibility follows the Curie law,

$$\mu_{eff} = 2.84\sqrt{\chi T} \qquad (1)$$

Thus a plot of $1/\chi$ versus T should be linear with a slope proportional to μ^2_{eff}. Figure 2 shows the data for the metmyoglobin complexes plotted in this manner, and it is seen that for the azide and imidazole complexes μ^2_{eff} calculated from the slope of these plots is apparently negative, dramatically demonstrating that to assume the Curie law is invalid. The evidence supporting the hypothesis that the hydroxide complex is a mixture of high- and low-spin forms in thermal equilibrium has been discussed by George *et al.* (1959), and it is suggested here that the extension of this hypothesis to the other derivatives successfully accounts for these extraordinary values of μ^2_{eff}.

Specifically, the hypothesis states that for any metmyoglobin complex the molar magnetic susceptibility at a temperature T°K, χ^T, is the sum of the susceptibilities of low-spin and high-spin fractions.

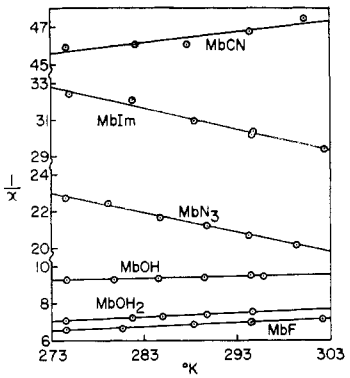

FIG. 2.—The variation of the molar magnetic susceptibility, χ, with temperature for metmyoglobin complexes in the temperature range of 0–25°.

TABLE II
THE MOLAR MAGNETIC SUSCEPTIBILITIES OF METMYOGLOBIN COMPLEXES AT VARIOUS TEMPERATURES

Complex	Temperature (°C)	$10^6\times$ Molar Susceptibility
MetMbF	1.60	15,280
	7.66	15,010
	15.44	14,660
	21.52	14,330
	21.60	14,350
	29.20	14,050
$MetMbOH_2$	1.64	14,180
	8.80	13,880
	12.02	13,760
	16.80	13,560
	21.70	13,340
MetMbOH	1.74	10,780
	6.84	10,770
	11.60	10,730
	16.64	10,660
	21.46	10,560
	22.90	10,000
$MetMbN_3$	1.58	4,405
	6.24	4,460
	11.78	4,625
	16.44	4,720
	21.32	4,835
	26.50	4,975
MetMbIm	1.96	2,995
	8.80	3,125
	15.52	3,235
	21.70	3,315
	21.78	3,300
	29.50	3,410
MetMbCN	1.50	2,140
	1.68	2,180
	9.06	2,175
	14.70	2,175
	21.48	2,140
	27.32	2,110

TABLE III

VALUES OF χ AT 0° AND 20° FOR EACH OF THE METMYOGLOBIN COMPLEXES OBTAINED FROM THE LEAST SQUARES ANALYSIS OF THE DATA IN TABLE II

	$10^6\chi^{0°}$	$10^6\chi^{20°}$
MetMbF	15,360 ± 60	14,430 ± 60
$MetMbOH_2$	14,270 ± 80	13,420 ± 80
MetMbOH	10,830 ± 30	10,620 ± 30
$MetMbN_3$	4,355 ± 15	4,805 ± 15
MetMbIm	3,050 ± 20	3,280 ± 20
MetMbCN	2,195 ± 25	2,145 ± 25

If α is the fraction of low-spin form, then $(1 - \alpha)$ is the fraction of the high-spin form and

$$\chi^T = \alpha\chi_L^T + (1 - \alpha)\chi_H^T \qquad (2)$$

Further, we may define an equilibrium constant K for the equilibrium

$$\text{MetMbX}_H \rightleftharpoons \text{MetMbX}_L \qquad \text{(i)}$$

where MetMbX_H and MetMbX_L are the high- and low-spin forms, respectively, of the X complex of metmyoglobin and

$$K = \frac{[\text{MetMbX}_L]}{[\text{MetMbX}_H]} = \frac{\alpha}{1 - \alpha}$$

The susceptibility of any complex will depend on K for that complex, and the temperature dependence of the susceptibility will depend on the temperature dependence of K. Therefore, depending on the sign of the enthalpy change for reaction (i), the susceptibility of a complex may increase or decrease with temperature. It is obvious that this hypothesis readily accounts qualitatively for the observed deviations from the Curie law, and for those susceptibilities intermediate between the expected values for one and five unpaired electrons. For any complex we can calculate α, and hence K, at any temperature if we know the susceptibility of the complex at that temperature together with the susceptibilities of the high- and low-spin forms.

The problem now arises as to what values should be adopted for χ_H and χ_L, the susceptibilities of the high- and low-spin forms, respectively. The fluoride complex has a susceptibility close to the theoretical limit for five unpaired electrons and is assumed to be 100% high-spin form. The validity of this assumption is discussed later. In view of the high-ligand field strength of cyanide ion (Shimura and Tsuchida, 1956) compared with the other ligands used in these experiments, it is assumed that the cyanide complex is 100% low-spin form, the deviation of its susceptibility from the theoretical "spin-only" value for one unpaired electron being attributed to orbital contribution to the magnetic moment. It is also assumed that the orbital contribution is the same for all low-spin forms irrespective of the nature of the ligand.

In order to calculate K, $\Delta F°$, $\Delta H°$ and $\Delta S°$ for reaction (i) for all the derivatives, it is necessary to know the susceptibility of all the derivatives at two temperatures. Zero and 20° were chosen and values of χ at these temperatures were calculated from the least squares analysis of the data in Figure 2 and are given in Table III. The hypothesis that the complexes are a mixture of high- and low-spin forms does not imply a linear dependence of $1/\chi$ on T, but the deviation from linearity to be expected over the small range of temperature used in these experiments is less than experimental error, and for convenience this method was chosen to calculate χ at 0° and 20° from the experimental data.

From the value of K at 20° we can calculate the standard free energy change for the transition. Assuming the Van't Hoff isochore, we may calculate $\Delta H°$, and hence $\Delta S°$ from the values of K at 0° and 20°. Values for all the complexes of α, $\Delta F°$, $T\Delta S°$ and $\Delta S°$ at 20°, together with $\Delta H°$ obtained from K at 0° and 20°, are given in Table IV. Although the experiments were carried out at large concentrations of ligands, the standard free energies may be calculated from the observed equilibrium constants since it has been shown that the position of equilibrium is independent of the ionic strength in the range 0.05–2 M.

Several features of the data in Table IV call for detailed comment. First, it is seen that the enthalpy change favors the low-spin form in all the complexes, and becomes steadily more negative as one passes from the predominantly high-spin metmyoglobin to the imidazole complex which is predominantly low-spin. That the high-spin form occurs at all is because of the unfavorable entropy change associated with the high- to low-spin transition. The ligands used in this study may be arranged in order of ligand field strength according to the spectrochemical series of Shimura and Tsuchida (1956) to give the sequence

$$F^- < OH_2 < OH^- < N_3^- < \text{Im} < CN^-$$

The same sequence is obtained if the ligands are placed in order of decreasing $\Delta H°$ for reaction (i), indicating that this enthalpy change is primarily a measure of the difference between the electronic energy of the high- and low-spin forms, a decrease in $\Delta H°$ corresponding to a greater ligand field-splitting of the d orbitals of the iron atom.

The constancy of the $T\Delta S°$ term and consequently of $\Delta S°$ is in contrast to the change in $\Delta H°$ with change in the ligand. The $\Delta S°$ values are slightly more negative for the complexes predominantly in the low-spin form, but the trend is within the standard deviation of the individual entropy values. It is of interest to examine the possible origin of the entropy change. Evidently, it is not associated specifically with the ligand since such widely differing ligands as the hydroxyl ion and imidazole give complexes for which the entropy changes, for the high- to low-spin transition, are about the same. Similarly, the entropy change is about the same for the charged hydroxyl ion and uncharged water molecule. The change of multiplicity involved in the transition accounts for at most 2.2 eu, and this factor has been fully discussed by George *et al.* (1959). The remaining 2.2–4.8 eu must arise either from changes in unspecific solvent interactions, or from specific structural effects in the protein brought about by the change in the symmetry of the electrons in the d orbitals arising from the spin change. The general solvent effect will be discussed later in connection with the effect of D_2O on the spectrum of the hydroxide and azide complexes.

A possible objection to the method of calculating the thermodynamic functions is that the susceptibility of the fluoride complex is not that of the 100% high-spin form. However, the values of the susceptibility of the fluoride complex obtained by Theorell and Ehrenberg (1951), and by Scheler *et al.* (1957), together with the value reported in this paper, are all close to the theoretical limit for a d^5 ion in an S state. In addition, the difference between the theoretical limit and the experimental value is smaller than is initially apparent if the correction factor suggested by Griffith is introduced. Griffith (1958a) calculated that about 300×10^{-6} emu should be added to the susceptibilities of all the complexes owing to the underestimate of the diamagnetism of the carboxymyoglobin as a result

TABLE IV

VALUES OF α, $\Delta F°$, $T\,\Delta S°$, AND $\Delta S°$ AT 20°, AND $\Delta H°$ FOR THE TEMPERATURE RANGE 0–20°[a]

	α	$\Delta F°$ (cal/mole)	$\Delta H°$ (cal/mole)	$T\,\Delta S°$ (cal/mole)	$\Delta S°$ (eu)
$MetMbOH_2$	0.08 ± 0.01	+1220 ± 110	−60 ± 2130	−1290 ± 2170	−4.4 ± 7.4
MetMbOH	0.31 ± 0.01	+465 ± 17	−1230 ± 450	−1760 ± 470	−5.8 ± 1.6
$MetMbN_3$	0.785 ± 0.005	−747 ± 15	−2740 ± 400	−2050 ± 290	−6.8 ± 1.4
MetMbIm	0.945 ± 0.005	−1341 ± 40	−3040 ± 840	−1700 ± 880	−5.8 ± 3.0

[a] Errors on α were calculated from the standard deviations of experimental points from the least squares line. Errors in $\Delta F°$, $\Delta H°$, and $\Delta S°$ were obtained by using maximum and minimum values of α.

of induced paramagnetism. Addition of this correction to the observed susceptibility of the fluoride complex gives $\chi^{20°} = 14{,}730 \times 10^{-6}$ emu, which is very close indeed to the theoretical limit of 14,820 $\times 10^{-6}$. Thus, if the 100% high-spin form has a susceptibility equal to the theoretical limit at 20°, the fluoride complex consists of 0.7% of the low-spin form. This is sufficiently small to render insignificant any changes in $\Delta F°$ for the other complexes arising from the use of the susceptibility of the fluoride complex instead of the theoretical limit for the susceptibility of the 100% high-spin form. However, as the following calculations show, the difference between the susceptibility of the fluoride complex and the theoretical limit is consistent with electron spin resonance experiments on this complex if it is assumed that it is entirely in the high-spin form.

Griffith[1] has shown that the temperature dependence of the effective magnetic moment of a high-spin metmyoglobin complex is given by

$$\mu^2_{eff} = \frac{19 + 16x^{-1} + e^{-2x}(9 - 11x^{-1}) + e^{-6x}(25 - 5x^{-1})}{1 + e^{-2x} + e^{-6x}} \qquad (3)$$

where μ_{eff} is defined by equation (1) and

$$x = \frac{D}{kT} \qquad (4)$$

where D is the zero-field splitting parameter. In deriving formula (3) he assumed a form DS^2_z for the fine-structure part of the spin-Hamiltonian.[1] This means that the $S_z = \pm 3/2$ and $S_z = \pm 5/2$ levels lie, respectively, $2D$ and $6D$ above the $S_z = \pm 1/2$ levels. Using equation (3) and the data for the fluoride complex given in Table III it is possible to calculate D. μ^2_{eff} as a function of x in the range $x = 0$ to 0.5 is shown in Figure 3. μ^2_{eff} at 20° for the fluoride complex calculated from $\chi^{20°} = 14{,}730$ is 34.8 with an estimated error of ±0.4. Inspection of Figure 3 shows that when $\mu^2_{eff} = 34.8$, $x = 0.135$. Substitution in equation (4) gives $D/k = 40°$ corresponding to $D = 28\ cm^{-1}$. The lower limit of μ^2_{eff} corresponds to $D = 51\ cm^{-1}$. The interpretation of the anomalous g values of metmyoglobin fluoride (Griffith, 1961a) require that $D > 5\ cm^{-1}$, for this complex. Thus the results given in this paper together with the electron spin resonance experiments indicate that $5 < D < 51\ cm^{-1}$ with a most probable value of $28\ cm^{-1}$. Using these values of D we can calculate from equation (3) the expected temperature dependence of χ. It is found that $(\chi^{0°} - \chi^{20°})_{calc} = 1082$, 1066, and 1026 $\times 10^{-6}$ emu for $D = 5$, 27, and 51 cm^{-1}, respectively. Inspection of Table III shows that $(\chi^{0°} - \chi^{20°})_{exp} = 930 \pm 120 \times 10^{-6}$ emu, indicating that the temperature variation of the susceptibility of the fluoride

[1] J. S. Griffith, private communication, 1961

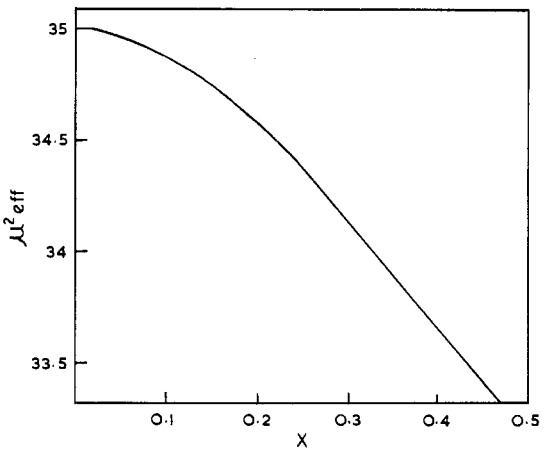

FIG. 3.—The dependence of μ^2_{eff} on x for a high-spin metmyoglobin complex as given by equation (3).

complex is consistent with its absolute value at 20°, assuming that it consists of 100% high-spin form. Thus it is unnecessary to postulate a small fraction of low spin in the fluoride complex to account for its magnetic susceptibility at 20°.

Inspection of Figure 3 shows that for a fixed temperature an increase in D produces a decrease in μ^2_{eff}. Thus the difference at 20° between the susceptibility of metmyoglobin and its fluoride complex could be due, at least in part, to the increase of D resulting from the increase in ligand field strength on replacing the fluoride ion by a water molecule.

The temperature dependence of the spectrum of the hydroxide complex has been discussed in detail previously (George *et al.*, 1959). The difference spectra of the azide and the imidazole complexes, as can be seen in Figure 1, closely resemble that of the hydroxide complex, but the measurements are not sufficiently accurate to allow a precise calculation of the spectra of the high- and low-spin forms. Nevertheless, trial calculations do show that like the other high-spin derivatives, the spectra of the high-spin forms of the complexes have bands or shoulders in the regions of 500 mμ and 600 mμ.

It can now be appreciated that the spectra of metmyoglobin and the cyanide complex are temperature independent for two different reasons. No effect is observed with metmyoglobin because, as is seen in Table IV, $\Delta H° = -60$ cal, and thus K changes by a negligible amount in the temperature range considered. By contrast, $\Delta H°$ is probably large and negative for the cyanide complex, but the percentage of high-spin form is very small; and even if the percentage of high-spin form changes from 1.5% at 20° to 1.0% at 0°, a change corresponding to a $\Delta H°$ of 4500 cal, the corresponding optical density change is about 0.0005, which would not be detected by the method used to determine the difference spectrum. If indeed the fluoride complex contains a very small percentage

of low-spin form, no change of spectrum with temperature would be detectable, because both $\Delta H°$ and the percentage of low spin are small.

In this connection it is interesting to note that Steinhardt *et al.* (1963) have recently suggested, on the basis of changes in stability at low *p*H, that the fluoride compound of horse methemoglobin is partly in the low-spin form at 0°, whereas at 20° it is entirely high spin. A direct comparison with the results of the present study is not possible due to the widely different *p*H values used, but the following calculations can be made.

From an analysis of the spectrum of horse methemoglobin hydroxide, George *et al.* (1959) estimated that this compound contains 55% of the low-spin form, corresponding to a standard free energy change for the equivalent of reaction (i) of −117 cal/mole; that is to say 587 cal/mole more negative than the corresponding metmyoglobin transition. If we now assume that the standard free energy changes for the high-to-low transition for each of the compounds of methemoglobin is 587 cal/mole more negative than for the corresponding metmyoglobin compounds and that metmyoglobin fluoride is 0.1% low spin, corresponding to $\Delta F° = +2680$ cal/mole for the equivalent of reaction (i), then $\Delta F°$ for methemoglobin fluoride would be +2090 cal/mole, corresponding to 4% low-spin form at 0°. This sort of value would be consistent with Steinhardt's conclusion.

However at 20° the presence of the low-spin form is apparently undetectable according to their criteria; so, taking 0.1% as the upper limit and using 4% as the value at 0°, we obtain $\Delta H° = -11{,}400$ cal/mole for the high- to low-spin transition of methemoglobin fluoride. Even higher upper limits for 20° which are still acceptable are bound to give substantial negative values for $\Delta H°$. Such a value seems improbable in view of the magnitude and trend of the values for the hydrate, hydroxide, azide, and imidazole complexes of metmyoglobin listed in Table IV, and we conclude that because of the difference in *p*H no really valid comparison can be made.

Bearing of the Present Results on Electron Spin Resonance Measurements.—The bearing of the work discussed in this paper on electron spin resonance experiments must be evaluated. Such experiments are carried out at much lower temperatures than the magnetic susceptibility measurements, and it would be of interest to calculate the percentage of high- and low-spin derivatives in each of the complexes at low temperatures. This cannot be done due to the change in $\Delta H°$ with temperature. However, the relatively high enthalpy changes associated with the high-low spin transition in the cyanide, azide, and imidazole complexes will ensure that at low temperatures only a very small amount of the high-spin form will remain. Thus the electron spin resonance signals will be those characteristic of the low-spin form as has been demonstrated experimentally by Gibson *et al.* (1959) for the azide complex of methemoglobin. The hydroxide complex might behave similarly, but the system is complicated by the fact that the ionization of metmyoglobin to form the hydroxide complex is endothermic to the extent of 5.8 kcal. Thus, while at *p*H 11 at room temperature about 99% of the metmyoglobin is in the form of the hydroxide complex, at lower temperatures considerably higher *p*H values would be required to produce the same degree of ionization. Inspection of Table III shows that it is likely that $\Delta H°$ is positive for the high-to-low transition in the fluoride complex, and therefore as the temperature is lowered the percentage of low-spin form decreases. Electron spin resonance signals for the fluoride complex will therefore be that of the high-spin form (Gibson *et al.* 1959). Of the derivatives studied, only metmyoglobin itself might be expected to be a mixture at very low temperatures. However, it must be emphasized that the errors on $\Delta H°$ for metmyoglobin allow the possibility that $\Delta H°$ is slightly positive, in which case, as the temperature is decreased, the fraction of high-spin component will increase. The work of Gibson *et al.* (1959) shows that at 20 °K the metmyoglobin derivative is homogeneous and in a high-spin state suggesting that $\Delta H°$ is indeed positive.

Equilibrium between Spin States in Complexes of other Hemoproteins.—The demonstration that some complexes of metmyoglobin are mixtures of high- and low-spin forms raises the question of how widespread this phenomenon is. Magnetic susceptibility studies by several workers have shown that at 20° many complexes of hemoglobins, peroxidase, and catalase have susceptibilities intermediate between those expected for the high-spin and low-spin forms. A compilation of data from the literature is given in Table V (Hartree, 1946; Scheler *et al.*, 1957; Havemann and Haberditzl, 1958). The most striking value in the table is the susceptibility of the azide complex of catalase which approaches that of the high-spin form. In addition, the susceptibility of the cyanide and sulfide complexes of catalase are considerably higher than those expected for low-spin complexes. The susceptibilities of peroxidase and its hydroxide complex suggest that peroxidase complexes contain greater percentages of the low-spin form than do the corresponding metmyoglobin complexes. Complexes of both horse methemoglobin and *Chironomus* methemoglobin tend to contain higher percentages of the low-spin forms than the corresponding horse metmyoglobin complexes.

The origin of this variation could be 2-fold. First, the group on the fifth coordination position of the iron could differ between hemoproteins, giving rise to a difference in the average ligand field. Such a change would alter the enthalpy change and hence the equilibrium constant for the transition. Second, the entropy change for the transition might be different in various hemoproteins owing to changes in the environment of the iron atom arising from changes in the protein structure. Since both the energy levels of the iron atom and the specific configuration of the protein probably contribute to the specificity of hemoproteins, it would be of great interest to study quantitatively the high-low spin transition for different hemoproteins.

Spectra of Metmyoglobin Complexes in Heavy Water.—The difference between the spectrum of the hydroxide complex in water and in deuterium oxide indicates that the percentage of the low-spin form increases when deuterium is substituted for hydrogen. This effect has been discussed in detail by George *et al.* (1959), but it was stated there that the most probable origin of this effect was not the change in ligand field strength but the change in the free energy of "crystallization" of water molecules about the iron atom when hydrogen is replaced by deuterium. If this is correct, then the spectrum of metmyoglobin azide should be different in water and deuterium oxide. Within the limits of experimental detection this is not the case. In addition, the spectrum of the hydroxide complex is the same in solutions of ionic strength 0.05 and 1. A possible origin of this effect is suggested by the small effect of deuterium oxide on the spectrum of metmyoglobin. A specific property of a hydroxyl ion or a water molecule bound to the iron in a complex is the possibility of hydrogen bonding between the ligand

TABLE V

VALUES OF THE MOLAR MAGNETIC SUSCEPTIBILITY OF METHEMOPROTEIN DERIVATIVES REPORTED IN THE LITERATURE[a]

Complex	Metmyoglobin	Methemoglobin	*Chironomus* methemoglobin	Peroxidase[b]	Catalase[b]
F^-	14,210	14,610 (24°)	14,490	14,840	14,665
	14,430	14,180			
	14,240	14,300			
OH_2	14,200 (24°)	14,040 (24°)	11,820	12,560	14,665
	14,010	13,600			
	13,420	14,000			
	13,690				
$HCOO^-$	13,680	12,630	14,350		
OCN^-	8,270	12,750	11,200		
SCN^-	12,680	10,870	10,060		
		11,200			
OH^-	10,820	8,340 (24°)	8,220	2,800	
	10,620	9,290			
	11,040	8,340			
NO_2^-	11,180	7,220	10,060		
		7,300			
$SeCN^-$	10,100	6,400	10,020		
NH_3		3,700 (25°)			
N_3^-	4,610	3,330	5,170		14,500
	4,803	3,360 (25°)			
		2,380			
Im	2,510	2,940 (25°)	2,360		
	3,280	3,540			
SH^-		2,140 (24°)		2,440	7,290
CN^-	1,630	2,610 (24°)	2,290	2,970	6,830
	2,145	2,680			
	2,340	2,300			

[a] Hartree (1946), Scheler *et al.* (1957), Havemann and Haberditzl (1958), and this paper. Values for the fluoride, thiocyanate, cyanate, fulminate, azide, and ethyl mercaptide derivatives of (bovine) methemoglobin, obtained by Abers, Coryell, Heussenstamm, Lewis, and Vermeulen, are listed in an article by Coryell (1954). [b] A "hydrate" structure for these parent methemoproteins is in some doubt (see George and Lyster, 1958). A "crevice" structure, with bonds between the iron and the protein in both the fifth and sixth coordination positions, accounts much more satisfactorily for certain features of their reactions with ligands.

atom and protein side chains in close proximity to the heme ring. The work of Kendrew *et al.* (1961) shows the presence of a glutamic acid or a histidine residue close to the water molecule attached to the iron in the heme ring. The possibility of similar hydrogen bonding in the azide complex is absent. It is known that substitution of deuterium for hydrogen increases the length of hydrogen bonds (Ubbelohde and Gallagher, 1955). Such an increase in the length of a hydrogen bond joining the protein to the water or hydroxyl ion bound to the iron might change the magnitude of specific structural effects brought about by the spin change on the iron atom. These changes would be small, but the difference between the spectra of metmyoglobin hydroxide in water and deuterium oxide corresponds to a difference of only 200 cal/mole in the standard free energy charge for the high- to low-spin transition in the two solvents.

In conclusion, the relationship of this work to the magnetochemistry of simple transition metal ion complexes is considered. The ferric aquo ion is in a high-spin state, and has a susceptibility corresponding to five unpaired electrons. By contrast, the ferricyanide ion contains iron in the low-spin ferric state, and has a susceptibility corresponding to one unpaired electron. Presumably, as cyanide ions replace water molecules in the coordination sphere of the ferric ion, at some stage the high- to low-spin transition takes place, giving rise to the possibility that one of the ions $[Fe(OH_2)_n(CN^-)_{6-n}]^{n-3}$ is a mixture of high- and low-spin forms. Experimental demonstration of the existence of such a complex would be extremely difficult, as it would be in equilibrium with $[Fe(OH_2)_{n+1}(CN^-)_{5-n}]^{n-2}$ and $[Fe(OH_2)_{n-1}(CN^-)_{7-n}]^{n-4}$, the former a high-spin complex and the latter a low-spin complex. It has been pointed out by Pauling (1948) that a similar transition occurs when fluoride ions in the complex $[CoF_6]^{3-}$ are replaced by ammonia molecules. The study of a simple complex ion of intermediate susceptibility would be possible if the six ligand atoms of a sexadentate ligand produced an environment for the central ion in which the average ligand field strength was of such a magnitude as to give rise to a mixture of high- and low-spin forms. The ferrous ion complex with 3,6-dithia-1,8-(salicylideneamino)-octane is possibly a mixture of high- and low-spin forms because it has an effective moment of 3.22 corresponding to two unpaired electrons (Dwyer *et al.*, 1950). Griffith has suggested (1958b) that in ferrous phthalocyanine and ferrous porphyrin there may exist a thermal equilibrium between different spin states. It would be of particular interest to compare the entropy change for one of these high- to low-spin transitions with that for the hemoprotein transitions. Unfortunately the polydisperse nature of the solutions of porphyrins renders a significant measurement difficult.

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Kinetics of the Reversible Reaction of Sperm Whale Myoglobin with Zinc*

JOHN R. CANN

Contribution No. 224 from the Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver

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Kinetic measurements have been made on the suppression of Soret band intensity brought about by reaction of Zn ions with sperm whale ferrimyoglobin and on its reversal by diluting or sequestering the metal ion or by simply lowering the *p*H from 6.4 to a value less than 6. These measurements indicate a three-step process: (1) labilization of the protein structure by binding of Zn ions to sites on the surface of the macromolecule; (2) unfolding of the weakened structure, at least to a limited extent, concomitant with binding of a single Zn ion to a critical and otherwise inaccessible site, as the *rate-controlling* step; and (3) rapid polymerization of the Zn-reacted myoglobin such that interaction between the ferriheme moieties of the polymer suppresses and broadens the Soret band. The second step can be hastened by denaturing agents like alcohol. It is proposed that in the *rate-controlling* step the Zn ion ruptures the Fe^{3+}-F8 imidazole linkage and occupies the F8 imidazole group. Mutual binding of the Zn ion by the F8 and groups like the distal E7 imidazole group is not precluded. Sedimentation analyses of acid-denatured ferrimyoglobin show a large proportion of globin and a relatively small amount of protein aggregate. The latter fraction contains most, if not all, of the ferriheme. In this case, also, suppression and broadening of the Soret band is attributed to interaction between the ferriheme moieties of the aggregate.

Recently (Cann, 1963) it was shown that reaction of Zn ions with sperm whale ferrimyoglobin at *p*H 6.4 causes major changes in the ultraviolet and visible absorption spectra of the protein. It was concluded that Zn-reacted myoglobin is conformationally quite different from the unreacted protein. Reaction of the protein with Zn can be reversed to yield renatured ferrimyoglobin by one of the following three methods: (1) lowering the Zn concentration by dilution; (2) sequestering the Zn ions at *p*H 6.4 with EDTA[1] or citrate; or (3) lowering the *p*H of the reaction mixture to a value of 5.2. The renatured protein can be readily crystallized in the same crystal habit as ferrimyoglobin never exposed to Zn.

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[1] Abbreviations used in this work: EDTA, ethylenediaminetetraacetate; BAEE, benzoyl-L-arginine ethyl ester.

The most characteristic spectral change (Fig. 1) is a marked reduction in the Soret band intensity accompanied by a shift of the band from an absorption maximum of 408 mμ to one at 390 mμ. Whereas the former band is quite sharp, the latter is diffuse. Breslow and Gurd (1963) have described very similar spectral changes brought about by reaction of ferrimyoglobin with Cu^{2+} and have suggested that such changes are indicative of alterations in the ferriheme-protein linkage.

As shown by Figure 1, suppression of the Soret band by Zn proceeds at a measurable rate, and solutions of partially reacted protein apparently contain only two classes of absorbing species. The present communication describes kinetic measurements on the reaction and its reversal. These measurements indicate the three-step process that is summarized in the abstract.

The realization that binding of Zn ions by myoglobin mediates conformational changes has important implications, not only for methods of fractionation of biological materials, but also for the mechanisms of biochemical reactions such as the metal-activation and