Product analyses were carried out on experiments run similarly but at higher concentrations of reagents. After reaction, solutions were neutralized with NaOH, metal hydroxides separated by centrifuging, and aliquots analyzed by flame ionization glc after addition of internal standards (dioxane, 1,4-butanediol, and 2,5-hexanediol). Most separations were done on 6 ft × 1/8 in. 10% Carbowax 20M or Polapak Q columns, and products were identified by retention time on two columns.

Ethyl Triflate Hydrolyses. A solution of 10 mmol of ethyl triflate in 10 ml of CH_2Cl_2 was added dropwise with stirring to 40 ml of 0.5 N $Fe(ClO_4)_2$–0.25 N $HClO_4$ solution. The system was bubbled with nitrogen and the effluent passed through a trap at −78°. Titration showed the formation of 0.44 mmol of Fe^{3+} and 5.88 mmol of acid ($\Delta Fe^{3+}/\Delta H^+ = 7.48\%$). Analysis of the trap content by glc showed traces of butane. In a second experiment, undiluted ethyl triflate was used, and 1 ml of n-hexane was placed in the trap to give more quantitative trapping of butane: yields, Fe^{3+}, 8.35%; C_4H_{10}, 3.9%. A blank experiment without Fe^{2+} gave no butane, and a similar hydrolysis in the presence of 1.5 M acrylonitrile gave a small precipitate of polymer.

Nuclear Magnetic Resonance Studies of Metal Porphyrins. I. Kinetics of Ligand Exchange and of Transitions between High- and Low-Spin States in the System Hemin–Pyridine–Water[1a]

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Abstract: Nuclear magnetic resonance studies of hemin in pyridine–water solutions yielded information on the structure of the complex formed. The proton magnetic resonance spectra of the coordinated pyridine molecules have been observed, and it was found that one water molecule and one pyridine molecule are coordinated to the hemin in solutions containing 5–20% by weight of water in pyridine. The temperature dependence of the relaxation times and chemical shifts of the hemin protons revealed transitions between high- and low-spin states of the ferric ion of the heme moiety. The concurrent phenomena of ligand exchange and transitions between high- and low-spin states required extension of the former theoretical treatment of exchange between two magnetic environments to this particular case of exchange between three magnetic environments. The kinetic and thermodynamic parameters for the ligand exchange rates were calculated from the transverse relaxation times of the water and pyridine protons and their temperature dependence. The kinetic and thermodynamic parameters for the transitions between the high- and low-spin states were calculated from the relaxation times of the ring hemin protons in the temperature region from −60 to +60°. The hyperfine coupling constant of the α protons of pyridine bound to hemin was directly determined for the low-spin species and calculated for the high-spin complex. The pseudocontact contribution to the hyperfine coupling constant of the heme protons was calculated from geometrical considerations, thereby allowing the determination of the isotropic part of the hyperfine coupling constant. The spin densities at the π orbitals of the peripheral carbon atoms have been calculated from the isotropic shifts. The correlation time for the nuclear relaxation of the methyl protons in the state of low spin was calculated from the experimental data and found to be governed by the electron relaxation time.

A number of important biological functions of the heme proteins and other derivatives of the metal porphyrins result from their ability to exchange ligands coordinated to the metal ions in the axial positions. Other functions originate in the ability of these compounds to participate in oxidation–reduction reactions. These phenomena are particularly amenable to magnetic resonance studies. Such studies may yield information regarding the kinetic and thermodynamic parameters for the exchange reaction between the coordinated and noncoordinated ligands and regarding the structure of the complexes. Water molecules are among the most important ligands that may bind reversibly to the iron in the ferric and ferrous porphyrin complexes. The particular importance that may be ascribed to the studies of water exchange in these particular systems is due to the role that water plays in the process of binding oxygen to hemoglobin.

The relaxation effects of some heme proteins on the water protons have been investigated previously by Davidson and Gold[2a] and by Kon and Davidson.[2b] The relaxation times of the water protons in the systems iron(III)-water, heme, hemin, hemoglobin, myoglobin, and methmyoglobin have been measured at room temperature, and from the experimental results the distance of the iron atoms from the protein surface has been calculated.[3,4] However, these

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(1) (a) Based on the thesis submitted by Hadassa Asman Degani to the Feinberg Graduate School, The Weizmann Institute of Science, Rehovoth, Israel, in partial fulfillment of the requirements toward the M.Sc. Degree, Mar 1969. Preliminary reports were presented at the Eleventh (H. A. Degani and D. Fiat, *Proc. Int. Conf. Coord. Chem.*, *1968*, 11, 474 (1968)) and Twelfth (H. A. Degani and D. Fiat, *ibid.*, *1969*, 12, 121 (1969)) International Conferences on Coordination Chemistry and at the Second International Symposium on Nuclear Magnetic Resonance (D. Fiat, H. A. Degani, J. Reuben, and A. M. Chmelnick, *Cienc. Cult.* (*Sao Paulo*), 20, 536 (1968)). (b) Argonne National Laboratory; address correspondence to this author at: the Weizmann Institute of Science, Rehovot, Israel.

(2) (a) N. Davidson and R. Gold, *Biochim. Biophys. Acta*, **26**, 370 (1957); (b) H. Kon and N. Davidson, *J. Mol. Biol.*, **1**, 190 (1959).

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investigators have not considered the effect of exchange between different magnetic environments,[5,6] or the effect of possible polymerization[7,8] of the hemin in alkaline solutions.

Pmr spectra of porphyrin in its un-ionized and ionized form have been studied extensively,[9-14] and interpreted in terms of ring current effects. The proton magnetic resonance spectra of ferricytochrome C, heme proteins, and hematoheminpyridine revealed large chemical shifts to both low and high fields.[15] These large shifts have been ascribed either to contact or pseudocontact interactions between the protons and the unpaired electrons of the paramagnetic ions. Recently, proton magnetic resonance shifts of some derivatives of hemin and methmyoglobin have been reported.[16,17] Chemical shifts to high and low fields as large as 70 ppm have been observed. Attempts to reach definite conclusions in regard to the origin of the chemical shift have been made, primarily by studying the temperature dependence of the chemical shift over a relatively narrow range.

We have been studying both ligand exchange reactions and magnetic properties of some metal porphyrins in order to gain better understanding of the magnetic behavior and electronic structure of these important substances and of their behavior in exchange reactions. Naturally, we made strong efforts to study these substances in aqueous solutions. However, difficulties have been encountered due to their low solubility and decomposition. Extensive studies have been made on the system hemin–pyridine–water because of the biological importance of both water and nitrogen bases as ligands.

Theoretical Introduction

Chemical exchange between a paramagnetic and nonparamagnetic environment may be described by simplified forms of McConnell's equation.[5] The simplification results from the relatively fast relaxation of the nuclei in the paramagnetic environment. Swift and Connick[6] have derived a simplified form of McConnell's equation for dilute solutions of paramagnetic ions. This treatment describes explicitly the relaxation and chemical-shift behavior of a system composed of three environments, a, b, and c, where only exchange between the a–b and a–c environments takes place. They have made use of this simplified form in their studies of the hydration of paramagnetic ions in dilute solutions under the conditions that two distinct magnetic environments may be defined: that of water molecules coordinated to the paramagnetic ions and that of the noncoordinated water molecules. Recently, we have extended the treatment to concentrated solutions,[18] and in the present study of the exchange of ligands between metal porphyrins and the solution we have found that we cannot use the approximation that only a–b and a–c exchange are allowed. Therefore, it has been found necessary to extend the treatment to the case where b–c exchange also exists. These transitions are of particular interest as they correspond to transitions between low- and high-spin species.

McConnell's equations,[5] describing chemical exchange between three magnetic environments, a, b, and c, under steady-state conditions are

$$\begin{aligned} -A_aG_a + \frac{G_b}{\tau_{ba}} + \frac{G_c}{\tau_{ca}} &= i\omega_1 M_0{}^a \\ \frac{G_a}{\tau_{ab}} - A_bG_b + \frac{G_c}{\tau_{cb}} &= i\omega_1 M_0{}^b \qquad (1) \\ \frac{G_a}{\tau_{ac}} + \frac{G_b}{\tau_{bc}} - A_cG_c &= i\omega_1 M_0{}^c \end{aligned}$$

where $A_i = 1/T_{2i} + 1/\tau_{ij} + 1/\tau_{ik} - i\Delta\omega_i = A_i' - i\Delta\omega_i$, $i = a, b, c$, $j = a, b, c$, $k = a, b, c$, $i \neq j \neq k$, $G_i = u_i + v_i$, u and v are the magnetization vectors in the plane perpendicular to the external field, τ_{ij} is the time interval between the entry of a nucleus to environment i and its transfer to j, T_{2i} is the transverse relaxation time of a nucleus in environment i, and $\Delta\omega_i$ is the difference between the resonance frequency in environment i and the transmitted external radiofrequency.

$$G_a = \frac{i\omega_1\left[M_0{}^a\left(A_bA_c - \frac{1}{\tau_{bc}\tau_{cb}}\right) + M_0{}^b\left(\frac{1}{\tau_{bc}\tau_{ca}} + \frac{A_c}{\tau_{ba}}\right) + M_0{}^c\left(\frac{1}{\tau_{ba}\tau_{cb}} + \frac{A_b}{\tau_{ca}}\right)\right]}{-A_aA_bA_c + \frac{1}{\tau_{ba}\tau_{cb}\tau_{ac}} + \frac{1}{\tau_{ab}\tau_{bc}\tau_{ca}} + \frac{A_b}{\tau_{bc}\tau_{cb}} + \frac{A_b}{\tau_{ac}\tau_{ca}} + \frac{A_c}{\tau_{ab}\tau_{ba}}} \qquad (2)$$

The solution for G_a is given here by eq 2. The solution for b and c may be obtained by cyclic permutation of the indices. For dilute solutions [a] ≫ [b], [c] and, therefore, $M_0{}^a \gg M_0{}^b$, $M_0{}^c$ and $1/\tau_{ab} \ll 1/\tau_{ca}$ and $1/\tau_{ac} \ll 1/\tau_{ca}$. G_a will thus be given to a good approximation by

$$G_a = \frac{i\omega_1 M_0{}^a\left(A_bA_c - \frac{1}{\tau_{bc}\tau_{cb}}\right)}{-A_aA_bA_c + \left[\frac{2}{\tau_{ab}\tau_{bc}\tau_{ca}} + \frac{A_a}{\tau_{bc}\tau_{cb}} + \frac{A_b}{\tau_{ac}\tau_{ca}} + \frac{A_c}{\tau_{ab}\tau_{ba}}\right]} \qquad (3)$$

If, in addition, the measurement is made near the resonance frequency of pure a, then to a good approximation[5] $G = G_a + G_b + G_c \approx G_a$ and therefore

$$G = \frac{-i\omega_1 M_0{}^a}{A_a - \left[\frac{2}{\tau_{ab}\tau_{bc}\tau_{ca}} + \frac{A_b}{\tau_{ac}\tau_{ca}} + \frac{A_c}{\tau_{ab}\tau_{ba}}\right] \times \frac{1}{A_bA_c - \frac{1}{\tau_{bc}\tau_{cb}}}} \qquad (4)$$

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v, the imaginary part of G, will be given by

$$v = \frac{-\omega_1 M_0{}^{a}\left[A_a' - \sum_{i=b}^{c}\sum_{j=b}^{c}{}' \tau_{ai}{}^{-1}\frac{\left[\left(\frac{A_j'}{\tau_{ia}} + \frac{1}{\tau_{ij}\tau_{ja}}\right)R + \frac{\Delta\omega_j}{\tau_{ia}}Q\right]}{R^2 + Q^2}\right]}{\left[A_a' - \sum_i\sum_j{}'(\tau_{ai})^{-1}\frac{\left(\frac{A_j'}{\tau_{ia}} + \frac{1}{\tau_{ij}\tau_{ja}}\right)R + \frac{\Delta\omega_j}{\tau_{ia}}Q}{R^2 + Q^2}\right]^2 + \left[-\Delta\omega_a - \sum_i\sum_j(\tau_{ai})^{-1}\frac{\frac{A_j'}{\tau_{ia}} + \frac{1}{\tau_{ij}\tau_{ja}}Q + \frac{\Delta\omega_j}{\tau_{ia}}R}{R^2 + Q^2}\right]^2} \tag{5}$$

where $R = A_b'A_c' - \Delta\omega_b\Delta\omega_c - 1/\tau_{bc}\tau_{cb}$ and $Q = A_b'\Delta\omega_c + A_c'\Delta\omega_b$.

The first term in the denominator and the term in brackets in the numerator are essentially independent of the frequency ω_a, inasmuch as $\Delta\omega_b$ and $\Delta\omega_c$ are either large and do not change much relative to other terms, or they are small and make a negligible contribution.

For maximum absorption $dv/d\omega_a = 0$, the second term in the denominator will vanish, and we obtain

$$\Delta\omega_a = -\sum_{i=b}^{c}\sum_{j=b}^{c}(\tau_{al})^{-1}\frac{\frac{A_j'}{\tau_{ia}} + \frac{1}{\tau_{ij}\tau_{ja}}Q - \frac{\Delta\omega_j}{\tau_{la}}R}{R^2 + Q^2} = -\sum_i\sum_j{}'(\tau_{al})^{-1}\frac{\Delta\omega_i\left(\frac{\Delta\omega_j{}^2 + A_j'^2}{\tau_{ia}} + \frac{A_j'}{\tau_{ij}\tau_{ja}}\right) + \Delta\omega_j\left(\frac{1}{\tau_{ij}\tau_{ji}\tau_{ja}} + \frac{A_i'}{\tau_{ij}\tau_{ja}}\right)}{\left(A_i'A_j' - \Delta\omega_l\Delta\omega_j - \frac{1}{\tau_{ij}\tau_{ji}}\right)^2 + (A_i'\Delta\omega_j + A_j'\Delta\omega_i)^2} \tag{6}$$

and the width at half-height will be given by

$$\frac{1}{T_2} = A_a' - \sum_i\sum_j{}'(\tau_{ai})^{-1}\frac{\left(\frac{A_j'}{\tau_{la}} + \frac{1}{\tau_{ij}\tau_{ja}}\right)R + \frac{\Delta\omega_j}{\tau_{ia}}Q}{R^2 + Q^2} =$$

$$\frac{1}{T_{2a}} + \sum_i\sum_j{}'(\tau_{al})^{-1}\frac{\left(A_l'A_j' - \Delta\omega_l\Delta\omega_j - \frac{1}{\tau_{ij}\tau_{ji}}\right)\left(\frac{A_j'}{T_{2l}} + \frac{1}{T_{2j}\tau_{lj}}\right) + (A_j'\Delta\omega_i + A_l'\Delta\omega_j)\left(A_j'\Delta\omega_i + A_i'\Delta\omega_j - \frac{\Delta\omega_j}{\tau_{ia}}\right)}{\left(A_i'A_j' - \Delta\omega_l\Delta\omega_j - \frac{1}{\tau_{ij}\tau_{ji}}\right)^2 + (A_l'\Delta\omega_j + A_j'\Delta\omega_i)^2} \tag{7}$$

Under certain limiting conditions these expressions have a greatly simplified form.

The limiting case is expressed by the inequality $\Delta\omega_i\Delta\omega_j \gg 1/\tau_{ij}\tau_{ji}$, $1/\tau_{la}\tau_{aj}$ which for i = j clearly corresponds to slow exchange.

Equation 7 is reduced to

$$\frac{1}{T_2} = \frac{1}{T_{2a}} + \sum_{j=b}^{c}\frac{1}{\tau_{aj}} \tag{8}$$

The other extreme, which for i = j clearly corresponds to fast exchange, is expressed by the inequality $\Delta\omega_i\Delta\omega_j \ll 1/\tau_{ij}\tau_{ji}$, $1/\tau_{ia}\tau_{aj}$.

The final simplified form of the equation will depend on the dominating mechanism of the nuclear transverse relaxation time. Under the circumstances that the dominating mechanism is the change of the precessional frequency in b and c, we obtain eq 9. Under

$$\frac{1}{T_2} = \frac{1}{T_{2a}} + \sum_{i=b}^{c}\sum_{j=b}^{c}{}'\tau_{ai}{}^{-1} \times \frac{\frac{\Delta\omega_j}{\tau_{ij}}\left(\frac{2\Delta\omega_i + \Delta\omega_j}{\tau_{ji}} + \frac{\Delta\omega_i}{\tau_{ja}} + \frac{\Delta\omega_j}{\tau_{ia}}\right) + \Delta\omega_i{}^2\left(\frac{1}{\tau_{ja}} + \frac{1}{\tau_{ji}}\right)^2}{\left(\frac{1}{\tau_{ia}\tau_{ja}} + \frac{1}{\tau_{ia}\tau_{ji}} + \frac{1}{\tau_{ij}\tau_{ja}}\right)^2} \tag{9}$$

the circumstances that exchange of nuclei between b and c environments does not occur, all the terms of $1/\tau_{ij}$ will vanish and eq 7 reduces to

$$\frac{1}{T_2} = \frac{1}{T_{2a}} + \sum_{j=b}^{c}\frac{\tau_{ja}{}^2}{\tau_{aj}}\Delta\omega_j{}^2 \tag{10}$$

Under the circumstances that the relaxation in environment a is dominated by the rate of relaxation of the nuclei in the b environment, the transverse relaxation time of the nuclei in the a environment will be given by

$$\frac{1}{T_2} = \frac{1}{T_{2a}} + \sum_i\sum_j{}'\tau_{al}{}^{-1}\frac{\frac{1}{T_{2i}\tau_{ja}} + \frac{1}{T_{2i}\tau_{ji}} + \frac{1}{T_{2j}\tau_{ij}}}{\frac{1}{\tau_{ia}\tau_{ja}} + \frac{1}{\tau_{ia}\tau_{jl}} + \frac{1}{\tau_{ij}\tau_{ja}}} \tag{11}$$

Under the circumstances that exchange between b and c does not occur, eq 11 reduces to

$$\frac{1}{T_2} = \frac{1}{T_{2a}} + \sum_{j=b}^{c}\frac{\tau_{ja}}{\tau_{aj}}\frac{1}{T_{2j}} \tag{12}$$

The chemical shift of the nuclei in the a environment in the region of slow chemical exchange is negligible. In the fast exchange region the chemical shift is given by

$$\Delta\omega_a = -\sum_{j=b}^{c}\Delta\omega_j\frac{\tau_{ja}}{\tau_{aj}} \tag{13}$$

We will define an average chemical shift $\Delta\omega_M$ that corresponds to the chemical shift of nuclei under conditions of fast exchange between environments b and c, ignoring the effect of exchange with environment a. The average chemical shift, $\Delta\omega_M$, will be given by

$$\overline{\Delta\omega_M} = \frac{n_b}{n_b + n_c}\Delta\omega_b + \frac{n_c}{n_b + n_c}\Delta\omega_c$$

Under conditions of equilibrium the following relation holds

$$\frac{n_a}{\tau_{aj}} = \frac{n_j}{\tau_{ja}}$$

where n_a and n_j are the molal concentrations of the nuclei at environments a and j. Therefore

$$\overline{\Delta\omega_M} = -\Delta\omega_a \frac{n_a}{n_b + n_c} \tag{14}$$

The difference, $\Delta\omega_P$, between the resonance frequency in pure a and the average resonance frequency of nuclei under fast exchange conditions between environments b and c will be given by

$$\Delta\omega_P = \Delta\omega_a - \overline{\Delta\omega_M} = \Delta\omega_a\left(1 + \frac{n_a}{n_b + n_c}\right) \tag{15}$$

The temperature dependence of the residence time in a particular environment will be assumed in the following calculation to follow Eyring's equation

$$\tau_{ja} = \frac{h}{kT}\exp\left(\frac{\Delta H^{\ddagger}}{RT} - \frac{\Delta S^{\ddagger}}{R}\right) \tag{16}$$

where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation for the exchange reactions.

We shall define an average residence time $\overline{\tau_M}$ of a nucleus by the expression

$$\frac{1}{\overline{\tau_M}} = \frac{n_b}{n_b + n_c}\frac{1}{\tau_{ba}} + \frac{n_c}{n_b + n_c}\frac{1}{\tau_{ca}}$$

Under conditions of slow exchange, therefore, we obtain

$$\frac{1}{T_{2p}} = \frac{\dfrac{n_b + n_c}{n_a}}{\overline{\tau_M}} \tag{17}$$

where

$$\frac{1}{T_{2p}} = \frac{1}{T_2} - \frac{1}{T_{2a}}$$

From the temperature dependence of T_{2p} and the independent determination of n_b and n_c as functions of temperature, it is thus possible to calculate the average residence time and the average enthalpy and entropy of activation for the exchange reaction as a function of temperature.

Under conditions of fast exchange between three magnetic environments, we have obtained eq 9 and 11, which simplify to eq 10 and 12 when b to c transitions are forbidden. The temperature dependence of T_{2p} will be determined essentially by the temperature dependence of the residence time of the nuclei in environments b and c, being therefore a sum of exponentials. The linear dependence of $\Delta\omega_b$ and $\Delta\omega_c$ may usually be neglected. The temperature dependence of the chemical shift, $\Delta\omega_a$, has been given by eq 13. For the model of two magnetic environments, $\Delta\omega_a$ describes a linear dependence on the reciprocal of the absolute temperature. However, when exchange exists between three magnetic environments the chemical shift will be proportional not only to $\Delta\omega_b$ and $\Delta\omega_c$, but also to the ratio of population in b and c. Therefore, although $\Delta\omega_b$ and $\Delta\omega_c$ will be linearly dependent on the reciprocal of the temperature, the relative populations will depend on an exponential Boltzmann factor. This explains deviations from the Curie law, as will be shown later.

The basic equations that describe isotropic nuclear resonance shifts in solution due to interactions between unpaired electrons and nuclei, and which are pertinent to the present studies, have been given by McConnell and Robertson.[19] It has been assumed that the hyperfine coupling constant may be considered as a sum of two terms, the contact, A_c, and pseudocontact, A_p

$$A = A_c + A_p \tag{18}$$

$$A_c = \frac{4\pi}{3h} g_e\beta_e g_n\beta_n\rho(\vec{r}_n) \tag{19a}$$

where

$$\rho(\vec{r}_n) = \int\psi\sum_k 2S_z\sigma(\vec{r}_k - \vec{r}_n)\psi d\tau \tag{19b}$$

Under the circumstance that the electron spin relaxation time T_{1e} is small compared to the correlation time for the tumbling motion τ

$$A_p = \frac{g_e\beta_e g_n\beta_n}{60r^3}(3\cos^2\chi - 1)(3g_{||} + 4g_{\perp})(g_{||} + g_{\perp}) \tag{20}$$

where χ is the angle between the symmetry axis of the molecule and the radius vector $\bar{r}$ at the position of the paramagnetic ion and pointing in the direction of the nucleus. All other symbols have their usual meaning.

The isotropic resonance shift will, therefore, be given by

$$\frac{\Delta\omega}{\omega} = -A\frac{S(S + 1)\gamma_e}{3kT\,\gamma_n} \tag{21}$$

where $\Delta\omega$ is the frequency shift, and ω is the Larmor frequency.

The dipolar contribution to the transverse relaxation times of the nuclei due to interactions between unpaired electrons and nuclei have been given by Fiat and Connick[20] for the case in which the relaxation is governed by the electron relaxation time

$$\left(\frac{1}{T_2}\right)_{DD} = \frac{1}{15}S(S + 1)\frac{\hbar^2\gamma_e\gamma_n}{r^6}\left(4\tau_1 + \frac{\tau_2}{1 + (\omega_n - \omega_e)^2\tau_2^2} + \frac{3\tau_1}{1 + \omega_n^2\tau_1^2} + \frac{6\tau_2}{1 + \omega_e^2\tau_2^2} + \frac{6\tau_2}{1 + (\omega_n + \omega_e)^2\tau_2^2}\right) \tag{22}$$

where τ_1 and τ_2 refer to the longitudinal and transverse electron relaxation times of the nuclei, respectively.

Under circumstances such that the correlation time governing the relaxation should be ascribed to the random tumbling motions of the molecule, $\tau_1 \equiv \tau_2$ and the equation is simplified to the well-known form given by Bloembergen.[21]

The hyperfine contribution to the nuclear relaxation will be given for the special case in which the correlation time may be identified with the electron relaxation time by[22]

$$\left(\frac{1}{T_2}\right)_{HF} = {}^1/_3 S(S + 1)\frac{A^2}{\hbar^2}\left(\tau_1 + \frac{\tau_2}{1 + (\omega_n - \omega_e)^2\tau_2^2}\right) \tag{23}$$

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[19] H. M. McConnell and R. E. Robertson, *J. Chem. Phys.*, **29**, 1361 (1958).

[20] R. E. Connick and D. Fiat, *ibid.*, **44**, 4103 (1966).

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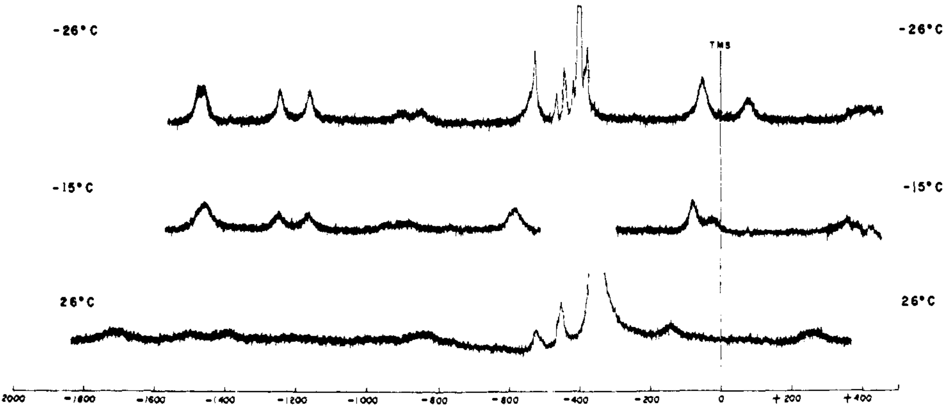

Figure 1. Pmr spectra of hemin dissolved in a mixture of pyridine-d_5 and D_2O at −26, −15, and 26°. Solution composition: 0.05 *m* hemin in pyridine-7 to 8% water. Positive shifts are upfield relative to TMS. (Measurements were done in the A-60 spectrometer.)

However, when the correlation time is determined by the chemical exchange process, τ_1 becomes identical with τ_2 and corresponds to the nucleus residence time in the paramagnetic environment.

Experimental Section

The Varian A-60 high-resolution and the DP-60 spectrometers operating at 60 and 56.4 MHz have been used for proton magnetic resonance studies. The temperature of the sample was controlled to better than 1° by means of the commercially available Varian controllers. The temperature either was measured directly with a copper constantan thermocouple or it was calculated from the spectrum of methanol at temperatures below 25° and from that of ethylene glycol above 25°.

Materials. The following materials have been used: pyridine of analytical grade containing not more than 0.2% water, deuterated pyridine containing 99 atom % deuterium, and water enriched to 99.7 atom % D_2O. Recrystallized hemin was obtained from Mann Research Laboratories, Inc. Deuterohemin was prepared by the method of Fisher and Orth,[23a] and was characterized spectrophotometrically in the visible and the Soret regions.

Solutions of hemin were prepared by dissolving it in mixtures of pyridine and water prior to the nmr measurements. Only freshly prepared solutions were used. Tetramethylsilane and cyclohexane were used as internal references for chemical-shift studies of proton magnetic resonance spectra.

Results

The proton magnetic resonance spectra of hemin dissolved in a mixture of deuterated pyridine and D_2O have been studied as a function of temperature in the temperature region −26 to +26° (Figure 1). The chemical shifts relative to TMS (as internal reference) are given in Table I. The considerations leading to the assignment of the chemical shift to particular functional groups are given in detail in the Discussion.

Table I. Proton Chemical Shift[a] from TMS of Hemin and Deuterohemin Dissolved in Mixtures of Pyridine and Water

	Deuterohemin		Hemin	
	−35°	25°	−26°	25°
1,3,5,8-Methyl protons	−26.7	−31.0	−24.7	−29.1
	−23.5	−27.4	−20.8	−25.6
	−21.0	−24.6	−19.5	−23.9
2,4-Vinyl protons			−15.0	
			−14.2	
6,7-Methylene protons		−15.7	−9.0	−14.0
	1.0		−1.0	−2.9
$\alpha,\beta,\gamma,\delta$-Methene protons	0.9		1.3	
	2.5		6.7	4.2

[a] In parts per million.

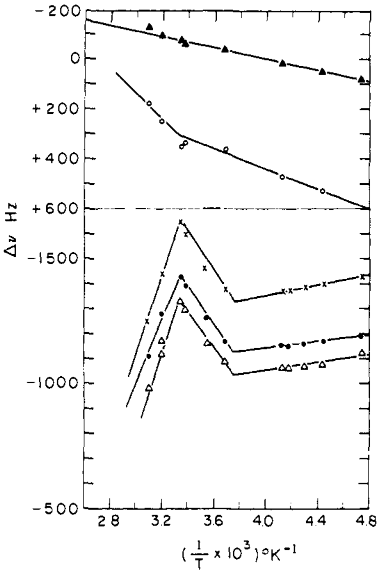

Figure 2. Temperature dependence of the chemical shifts of the porphyrin protons of hemin. Composition as in Figure 1. Positive shifts are upfield relative to cyclohexane.

The temperature dependence of the chemical shifts and the relaxation times of the hemin and deuterohemin protons revealed anomalous behavior. The chemical shifts did not obey the Curie law (Figure 2), and the temperature dependence of the relaxation time (Figure 3) gave evidence of exchange between two magnetic states of the hemin. Susceptibility studies[23b] (Figure 4) indicate that an equilibrium exists between high- and low-spin states.

The nuclear magnetic resonance and susceptibility measurements led to the conclusion that at temperatures higher than room temperature a relatively fast decomposition occurs. In the hemin-pyridine-water system, the exchange of the pyridine and water between the axial positions of the hemin and the solvent has also been studied.

The broadening of the lines of pyridine and water in the presence of hemin as a solute reveals that in the solution, pyridine and water coordinate to the hemin.

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(23) (a) H. Fisher and H. Orth, "Die Chemie des Pyroles," Vol. 2, Part 1, Akademie-Verlag Gesselshaft, Leipzig, 1937, p 209. (b) The susceptibility studies were kindly carried out by Dr. S. M. Nelson, Belfast University, N. Ireland.

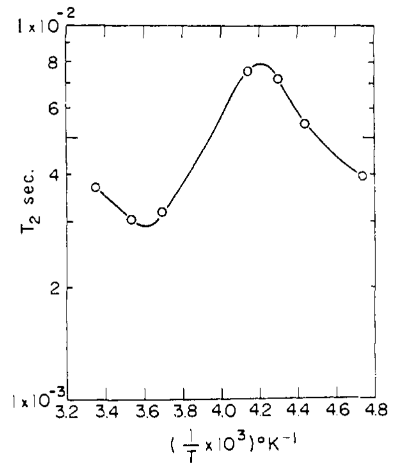

Figure 3. Temperature dependence of the transverse relaxation time of the methyl protons. Composition as in Figure 1.

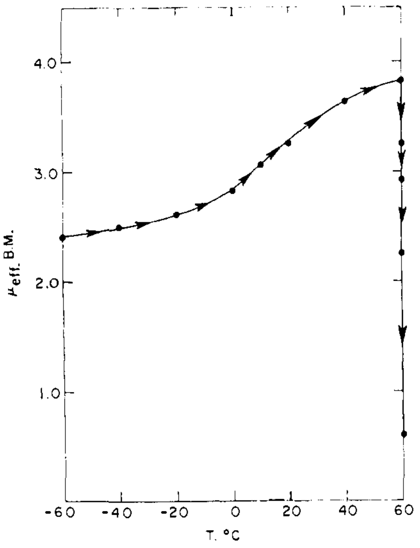

Figure 4. Temperature dependence of the effective magnetic moment of hemin in pyridine + water. Solution composition: 0.2226 g of hemin in a 5-ml solution containing 14% water (by weight). Arrows indicate sequence of measurements.

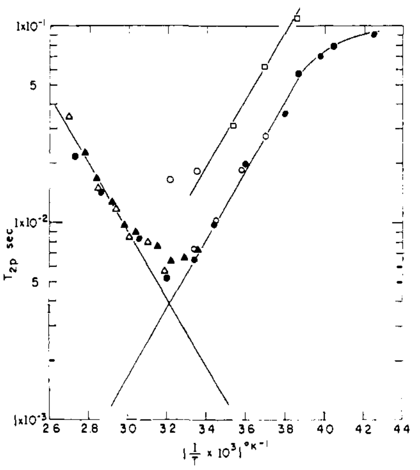

Figure 5. Temperature dependence of the transverse relaxation time of the bulk water. Each symbol corresponds to freshly prepared solution: □, 0.025 *m* deuterohemin in pyridine-7 to 8% water; ○, ●, △, ▲, composition as in Figure 1. Each symbol corresponds to a freshly prepared solution.

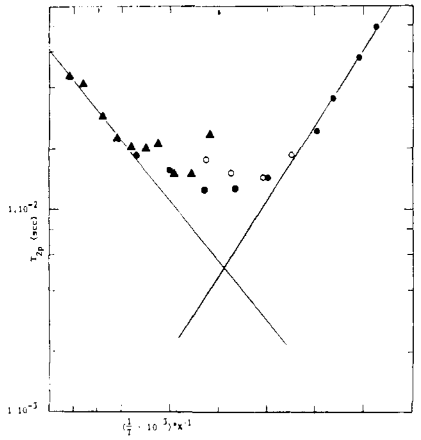

Figure 6. Temperature dependence of the transverse relaxation time of the α protons of noncoordinated pyridine in hemin–pyridine–water solution. Composition as in Figure 1. Each symbol corresponds to a freshly prepared solution.

The kinetic and thermodynamic parameters for the exchange of these ligands have been calculated from the temperature dependence of the transverse relaxation times of the noncoordinated ligands (Figures 5 and 6), according to eq 8 and 17, and are given in Table II. In this calculation it has been assumed that one water and one pyridine are coordinated to each hemin.

Table II. Kinetic and Thermodynamic Parameters for the Exchange of Water and Pyridine between the Molecules Coordinated to Hemin and the Noncoordinated Molecules

	Water	Pyridine
τ_M (25°)	$(1.9 \pm 0.5)10^{-4}$	$(3.6 \pm 0.7)10^{-5}$
$\Delta H^{\ddagger}$, kcal/mol (25°)	7.4 ± 0.8	8.4 ± 1.6
$\Delta S^{\ddagger}$, eu (25°)	-16 ± 2	-10 ± 2
$\Delta F^{\ddagger}$, kcal/mol (25°)	12.4 ± 1	11.4 ± 2

At low temperatures under conditions of slow exchange it was found possible to observe the proton magnetic resonance spectra of pyridine molecules coordinated to hemin (Figure 7), and thus it was possible to determine directly the number of coordinated pyridine molecules. The observed line was assigned to the α protons and the number of coordinated pyridine molecules was determined at three different relative concentrations of water to pyridine. The results are given in Table III.

Table III. Coordination of Pyridine to Hemin in Solutions of Hemin–Pyridine–Water

Water %	Pyridines coord/hemin
3.9	1.06
8.0	1.15
17.0	0.85

The nuclear magnetic resonance signal of the coordinated water molecules has not been observed. However, it may be concluded that one water molecule is also coordinated to each hemin, as the structure of the complex is presumably an octahedral one. It may also be concluded from chemical-shift studies of the

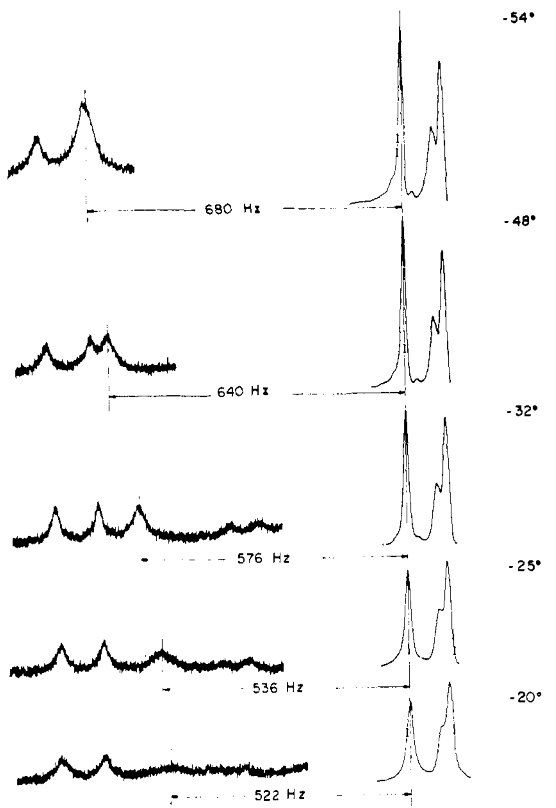

Figure 7. Resonance lines of coordinated and noncoordinated pyridine molecules. The resonance line of the coordinated pyridine is 320 times amplified with respect to the noncoordinated pyridine lines.

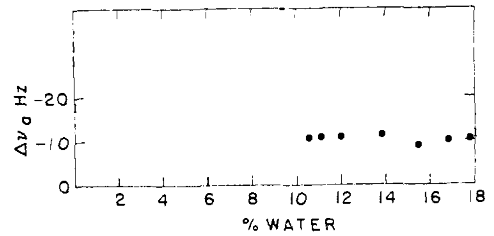

Figure 8. The chemical shift of the noncoordinated water molecules at 40° at various water per cents and constant hemin–water ratio.

noncoordinated water molecules under conditions of fast exchange (40°, Figures 8–10) that the coordination number of the water molecules does not depend on the relative amounts of water to pyridine in the concentration range studied (from 5 to 20% water).

The temperature dependence of the proton chemical shifts of the noncoordinated water molecules (Figure 11) allows the calculation of the average isotropic chemical shift between the coordinated and the noncoordinated water molecules (eq 14). This shift was found to be $-6300\ \mathrm{sec}^{-1}$ at 25°.

The chemical shifts of noncoordinated pyridine protons in the fast exchange region relative to that of the pure solvent have been found to be very small. The average isotropic chemical shift of the α protons was calculated from the shift of the noncoordinated pyridine molecules under conditions of fast exchange, and was found to be $+4900\ \mathrm{sec}^{-1}$ at 39°. The isotropic shift of the α protons of the pyridine coordinated to hemin in the low-spin state was determined directly (Figure 7) and was found to be $-4300\ \mathrm{sec}^{-1}$ at $-54°$ and $-3000\ \mathrm{sec}^{-1}$ at 39°.

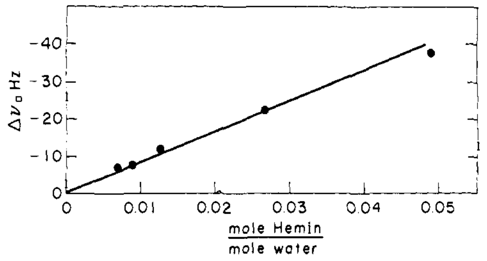

Figure 9. The chemical shift of the noncoordinated water molecules at 40° *vs.* hemin–water ratio, at constant hemin–pyridine ratio.

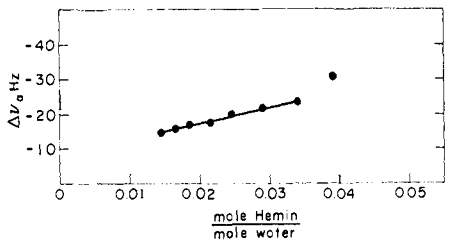

Figure 10. The chemical shift of the noncoordinated water molecules at 40° *vs.* hemin–water ratio, at constant water-pyridine ratio.

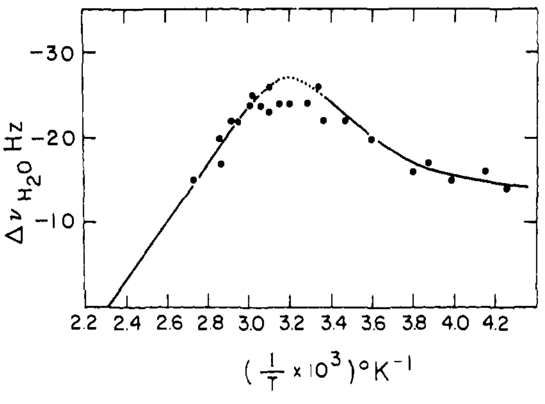

Figure 11. Temperature dependence of the chemical shift of the noncoordinated water molecules. Composition as in Figure 1.

The nuclear relaxation time of the methyl protons was calculated from the line widths of these protons at $-26°$, a temperature at which the hemin is in a low-spin state. The correlation time characterizing this relaxation must originate in the interruption of the electron nucleus interaction and can be shown (see Discussion) to be governed by the relaxation of the electron. Assuming $T_{1e} = T_{2e}$, $\tau = (7.4 \pm 0.7)10^{-12}$ sec and assuming $T_{1e} \gg T_{2e}$, $\tau = (1.7 \pm 0.2)10^{-11}$ sec.

Discussion

The broadening of the resonance lines of the water and pyridine in a solution of hemin in pyridine and water, and the distinctive difference between the spectra of the hemin protons when dissolved in pure pyridine (Figure 12) and when dissolved in a solution of pyridine and water (Figure 1) indicate that the structure of the complex formed in the pyridine solution differs from that formed in the pyridine–water solution, and

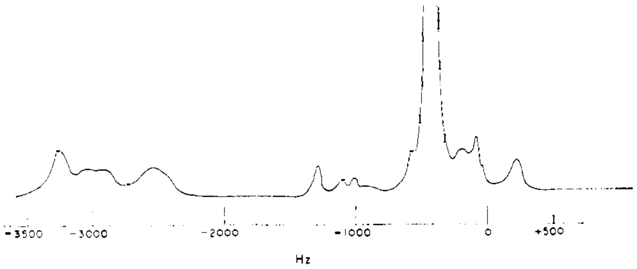

Figure 12. Pmr spectrum of the porphyrin protons in a solution of hemin dissolved in pyridine-d_5 at 22°. Solution composition: 0.05 *m* hemin in pyridine (measurements done with the DP-60 spectrometer).

that the origin of the difference is in the coordination of the water to the complex.

The composition of the complex formed in the solution of hemin in pyridine and water was studied.[1,24] However, the structure of the complex was not definitely determined.

Electron spin resonance measurements at liquid air temperatures of hemin in solutions of pyridine and water (at concentrations between 5 and 10% water) have shown that pyridine molecules are coordinated to hemin in the axial position, and, therefore, presumably the other axial position is occupied by the water molecule.[24]

The nuclear magnetic resonance measurements reported here allow the identification of the proton resonance line of the pyridine molecules coordinated to the hemin (Figure 7). This line has been assigned to the pyridine uniquely by comparing the spectra to that of systems differing only in that they contain deuterated pyridine (Figure 1) in place of normal pyridine. Making the assumption that the observed line corresponds to either the α or β proton yields a coordination number of one, whereas assuming that the line corresponds to the γ protons yields a coordination number of two. A coordination number of two would mean that water molecules do not coordinate to the hemin. We may reject this possibility however, inasmuch as the lines of the noncoordinated water molecules have been found to broaden significantly.

Our failure to observe the absorption line of the γ and presumably also that of the β protons is probably due to their overlap with the α protons of the noncoordinated pyridine molecules.

On the basis of these considerations and the other sources of information mentioned above, we conclude that the coordination number of pyridine is one. No evidence for a change of the coordination number with temperature was observed, and in the following calculations we assumed that the coordination number with respect to pyridine and water remains constant.

The fact that the composition of the complex does not vary with the relative change in the amounts of water and pyridine is demonstrated in the constancy of the coordination number with respect to pyridine at three different concentrations of water (Table III) and from the measurements of the chemical shifts of the noncoordinated water molecules relative to the pure solvent (Figures 8–10). From eq 13 and 14 it can be seen that $\Delta\omega_a$ of the noncoordinated water molecules will remain constant as long as the ratio of the coordinated to the noncoordinated water molecules remains constant (at the same temperature), and $\Delta\omega_a$ will depend linearly on this ratio. The observations that the chemical shift of water is independent of the ratio of water to pyridine, if the ratio of water to hemin remains constant, and the linear dependence of the chemical shift on the concentration ratio of hemin to water show that the coordination number with respect to water molecules is constant. The kinetic and thermodynamic parameters for the exchange of water and pyridine have been determined from the temperature dependence of the relaxation time of the protons of water and pyridine.

It should, however, be remembered that the values of the parameters are an average over the values of the two magnetic species (Table II).

The negative value of the entropy and the small and positive value of the enthalpy of activation seem to indicate that an SN2 mechanism predominates for the exchange both of pyridine and of water molecules. The formation of a heptacoordinated activated complex is accompanied by a decrease in the translational degrees of freedom and a negative entropy of activation, while involving only a relatively small change in the enthalpy of activation. The formation of a pentacoordinated complex by an SN1 mechanism would manifest itself in a high enthalpy of activation and a positive entropy of activation.[25]

The temperature dependence of the chemical shift of the noncoordinated water protons does not follow the Curie law (Figure 11). This is probably due to the fact that an equilibrium exists between the high- and low-spin states in addition to the exchange of water and pyridine with the hemin. The chemical shift is proportional both to the populations in the high- and low-spin states and to the inverse of the absolute temperature (eq 13 and 14). Therefore, the chemical shift will be proportional to an exponent of *T*, due to the exponential dependence of the relative populations, and it will be inversely proportional to *T*, due to the Curie law that each species follows.

The chemical shifts of the protons of the noncoordinated pyridine molecules were relatively very small. For the α protons of the pyridine, at 39°, the chemical shift relative to the α protons in the pure solvent was found to be 7 Hz. The isotropic chemical shift of the α protons in the low-spin state was calculated from the low-temperature results and the isotropic chemical shift of the high-spin state was calculated from the average shift and the relative populations. The results are: $(A/h)_{HS} = 7.2 \times 10^4$ Hz; $(A/h)_{LS} = 3.2 \times 10^5$ Hz.

The isotropic chemical shifts of the porphyrin protons of the hemin–pyridine–water complex were determined at various temperatures. The low-temperature results showed that at low temperatures the complex is in a low-spin state.

The unpaired electron of a complex in a low-spin state occupies a t_{2g} orbital. The symmetry of a t_{2g} orbital allows the formation of a π bond with the porphyrin. McConnell has given the following equation for a system of π radicals[5]

$$A_H = Q\rho_C^{\pi} \qquad (24)$$

where A_H is the hyperfine coupling constant of the pro-

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(24) H. Rein, O. Ristau, and F. Jung, *Z. Phys. Chem.*, **228**, 102 (1965).

(25) D. Fiat and R. E. Connick, *J. Amer. Chem. Soc.*, **90**, 608 (1968).

Table IV. The Pseudocontact and Contact Shifts and the Hyperfine and Spin Densities of Hemin in a Pyridine–Water Solution

	Pseudocontact shift, −26°, ppm	Contact shift, −26°, ppm	A/h ×10^{-5}, Hz	ρ_C^π, %
1,3,5,8-Methyl protons	0.81	−21.7	6.8	0.91
		−17.8	5.6	0.75
		−16.5	5.2	0.69
2,4-Vinyl protons	0.88	−9.5	3.0	−0.48
		−8.7	2.7	−0.43
$\alpha,\beta,\gamma,\delta$-Methene protons	2.08	14.4	−4.5	0.71
		9.1	−2.9	0.46
α protons of pyridine	−1.32	−10.2	3.2	−0.51

tons that are bound to an aromatic carbon atom, ρ_C^π is the spin density at the π orbital of the carbon atom, and Q is a semiempirical constant with a value of −22.5 G.[26] For a methyl group that is bound to an aromatic carbon atom the same equation holds but $Q = 27$ G.[27]

Comparison of the spectra of hemin and deuterohemin allowed us to make the assignment of the absorption lines of the vinyl group protons of hemin at positions 2 and 4. The methyl and methene proton lines were assigned by comparing their relative intensities and the observation that their positions are similar in the two spectra. It is reasonable to assume that the spin densities of the aromatic porphyrin carbons will obey McConnell's equation and will have a positive sign. These imply that the four ring methyl protons will be shifted to a low field and the bridge methene protons to a high field, which is consistent with the analysis given. (The 2,4-ring protons of the deuterohemin probably fall in a high field and could not be observed because of the limitations of the field in the A-60 apparatus.)

The chemical shifts due to the pseudocontact interaction have also been calculated according to eq 20. The crystallographic data are the literature values for hemin chloride.[28]

The hyperfine interactions and the spin densities on the carbon atoms have been calculated from the chemical shifts (Table IV).

The relaxation time of the methyl protons in the low-spin state (at −26°) has been determined from the line width. The correlation time governing the relaxation may be identified with the electron relaxation time. The tumbling time of the complex is much longer ($\tau_R > 10^{-10}$ sec).

The anomalous temperature dependence of the chemical shifts and the relaxation times of the hemin protons have led to the conclusion that the phenomena should be explained by the presence of transitions between high- and low-spin species. Susceptibility measurements confirm these results. Similar temperature dependences of the isotropic chemical shifts and susceptibility have been found for complexes of iron dithiocarbamates[29,30] and for methmyoglobin hydroxide;[31] in the latter case only susceptibility measurements were made.[31]

The population ratio between the high- and low-spin species has been calculated from the experimentally determined values of susceptibility, assuming that the magnetic moments are $\mu = 2.24$ BM and $\mu = 5.92$ BM for the low- and high-spin states, respectively.[31] The equilibrium constants have been determined as a function of temperature. The calculated entropy, enthalpy, and free energy for the process are: $\Delta H°$ (25°), 4.5 ± 0.5 kcal/mol; $\Delta F°$ (25°), 0.8 ± 1 kcal/mol; $\Delta S°$ (25°), 12.5 ± 2 eu, respectively.

The calculated values reveal that the low-spin state has lower entropy and enthalpy than the high-spin state. The amount of the high-spin species will therefore increase with increasing temperature, due to the increasing contribution of the entropy term to the free-energy term. Similar values have been obtained for the equilibrium between high- and low-spin methmyoglobin hydroxide.[31]

The rates of transitions between the low- and high-spin states have been calculated from the broadening of the resonance of the methyl protons. The kinetic and thermodynamic parameters for the transition between these states have been calculated in the temperature range for which the reciprocal of the rate is smaller than the two states: $\tau_{LS \rightarrow HS}$ (25°), $(1.7 \pm 5)10^{-3}$ sec; $\Delta H^{\ddagger}$ (25°), 4.5 ± 0.5 kcal/mol; $\Delta F^{\ddagger}$ (25°), 13.6 ± 1 kcal/mol; $\Delta S^{\ddagger}$, −30 ± 3 eu.

Comparison of the thermodynamic and kinetic parameters given shows that the enthalpy change of the transition is about the same as the enthalpy of activation, whereas the entropy change is much greater. Hence there is appreciable gain in entropy in going from the transition state to the high-spin state. The steep decrease in the chemical shift at temperatures higher than 25° is probably due to decomposition of the complex at high temperatures to yield a diamagnetic product. The measurements of susceptibility as a function of temperature confirm these results, and Figure 4 demonstrates the decomposition with time of the paramagnetic complex at 60°.

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