The Active Center of Methemoglobin Hb(H₂O) Investigated by Mössbauer and Susceptibility Experiments

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Methemoglobin, Mössbauer, Susceptibility

A fast freezing technique using liquid propane was used to obtain frozen solutions of methemoglobin at pH 7. With this method it was possible to eliminate largely the presence of a low spin component which is usually found in slowly frozen solutions but not present in the sample at room temperature. The magnetic susceptibility and Mössbauer spectra of Hb(H₂O) in the temperature range 4.2 K ≤ T ≤ 250 K have been measured. The data of the high spin compound of Hb(H₂O) were evaluated with a Hamiltonian containing the Coulomb repulsion of the five 3d-electrons of the Fe³⁺ ion, a crystal electric field of C₅ᵥ symmetry and the spin orbit coupling. The term describing the crystal electric field depends on five energy parameters ε₁, ε₂, D, and E which are determined by least squares fits to the experimental data. The most relevant parameters ε₂ and ε₃ which equal the energies of the antibonding single 3d-electron orbitals 3dz² and 3dx²-y² with respect to the 3dx² orbital are compared with earlier results of these energies ε₂ and ε₃ of the high spin compound of Mb(H₂O). From this comparison conclusions regarding the different spatial arrangements of Fe³⁺ in Hb(H₂O) and Mb(H₂O) are drawn.

1. Introduction

Optical absorption spectra¹ and magnetic susceptibility measurements² of aqueous solutions of methemoglobin at pH 6 — 7 taken at room temperature show that the electronic ground state of the ferric iron in Hb(H₂O) is a high spin configuration. In contrast to these results the Mössbauer spectra of a frozen solution of Hb(H₂O) at T = 195 K showed a large quadrupole splitting of about 2 mm/s³ which is typical for low spin ferric compounds. The results of the temperature dependence of the magnetic susceptibility of Hb(H₂O) at low temperatures have also been at variance with the assumption of a high spin electronic ground state of the ferric iron.⁴

Careful investigations of frozen samples of Hb(H₂O) by means of EPR spectroscopy⁵,⁶ and magnetic susceptibility measurements⁷ showed clearly that frozen samples of Hb(H₂O) always contain a low spin compound in addition to the expected high spin compound. It could be shown, that the relative amount of sample material in the low spin state decreases when the freezing time of the sample becomes shorter.⁷ From these investigations one may conclude that at room temperature only the high spin component is present and that the low spin compound of Hb(H₂O) is formed during the freezing process. Therefore in order to study the properties of methemoglobin we were predominantly interested in the properties of the high spin compound of Hb(H₂O). In this paper susceptibility and Mössbauer experiments on frozen samples of methemoglobin Hb(H₂O) are described. The formation of a low spin component was largely avoided by freezing the sample as rapidly as possible. In the data analysis the low spin part was separated and treated as a contamination. Our susceptibility data were evaluated with a Hamiltonian H acting on the 3d⁵ configuration which takes into account the Coulomb repulsion (H_eb) of the five 3d-electrons of the ferric iron ions of Hb(H₂O), the interaction of a crystal electric field of C₅ᵥ-symmetry (Hₕₑᵥ⁴(ε₁, ε₂, ε₃) + Hₕₑᵥ₉οm(D,E)) and the spin orbit coupling (Hₗₛ).

\[ H = H_{eb} + H_{ₑᵥ⁴}(ε₁, ε₂, ε₃) + H_{ₑᵥ₉οm}(D,E) + H_{ₗₛ}. \] (1)

For the diagonalization of H we used the five multi-electron wave functions⁶AJ, ⁴A₂, ⁵E, ⁴B₂ and ⁴E as a base which will be split into 12 Kramers doublets by (Hₑᵥ₉οm(D,E) + Hₑᵥ₉ₗₛ). Within a certain range the relative energies of the split multi-electron wave functions (i.e. neglecting Hₑᵥ₉οm(D,E) + Hₑᵥ₉ₗₛ) are linear dependent on the parameters ε₁, ε₂, ε₃ which determine the strength of the crystal electric field. The parameters ε₁, ε₂, ε₃ equal the splitting energies of the five antibonding single 3d-electron orbitals dₓᵧ, dₓz and dᵧz, dₓ²-y², dₚ² (see...
Fig. 6) as caused by the crystal electric field of $C_{4v}$-Symmetry. The rhombic distortion $H_{\text{rh}}(D, E)$ then splits the still degenerated orbitals $d_{xz}$ and $d_{yz}$ by $2 \cdot (D + E)$. This theoretical treatment was developed by different authors $^{8-10}$ and has already successfully been used for the analysis of experiments performed on different high spin and low spin compounds of metmyoglobin $^{11}$. The calculation of the temperature dependence of the induced paramagnetic magnetization $\langle M \rangle_T$ of the Fe$^{3+}$-ion and of the $g$-factors $g(\theta, \varphi)$ of the lowest Kramers doublet can be done in a straightforward manner and has been described in our previous work $^{11}$ in some detail. It is obvious that $\langle M \rangle_T$ and $g(\theta, \varphi)$ are dependent on the electronic structure of the iron, and thus they are functions of the parameters $\varepsilon_1$, $\varepsilon_2$, $\varepsilon_3$, $D$ and $E$. These parameters in principle can be determined by a least squares fit to the susceptibility data. In addition the experimental $g$-factors of the lowest Kramers doublet $^{12}$ are part of our interpretation. Especially from the parameters $\varepsilon_2$ and $\varepsilon_3$ one may draw information on the spatial arrangement of the iron $\text{Fe}^{3+}$ in the active centers of Hb(H$_2$O) with respect to their nearest neighbours. As these parameters have already been determined in several myoglobin compounds $^{11}$ a comparison between the relative positions of the iron in metmyoglobin Mb(H$_2$O) and methemoglobin Hb(H$_2$O) can now be undertaken.

## 2. Sample Preparation and Experimental Techniques

The magnetization measurements at temperatures between $T = 4.2$ K and $T = 120$ K were performed using a commercial vibrating sample magnetometer of “Princeton Applied Research Corporation”. The Mössbauer experiments at different temperatures and small longitudinally applied magnetic fields were carried out in a liquid He-cryostat equipped with a superconducting solenoid delivered by “Siemens AG”. In order to obtain Mössbauer data at temperatures below 4.2 K the original construction of the cryostat had to be changed. A more detailed description of the magnetometer and of the Mössbauer equipment has already been given in a previous report $^{11}$. The Mössbauer spectra were measured by means of a $^{57}$Co in Rh source and an electromechanical doppler drive coupled to a 400 channel analyser operated in time mode.

The Mössbauer sample was a solution of methemoglobin Hb(H$_2$O) prepared in a 0.66 M Kaliun-Natriumphosphate buffer at pH 6.0. The preparation was done according to the procedures given by Perutz $^{13}$. In order to get an appreciable absorption strength the natural iron of the Hb(H$_2$O) molecules was chemically exchanged by $^{57}$Fe. The enrichment of $^{57}$Fe $^{14}$ amounted to 80%. The concentration of hemoglobin was about 70 mg/ml, the volume of our sample holder 1 ml.

The samples used for the susceptibility measurements contained exclusively crystalline material, in order to get a higher concentration. The preparation and crystallization of Hb(H$_2$O) again was done in a Kaliun-Natriumphosphate buffer at pH 6.7 $^{13}$. The volumes of our sample holders were roughly 50 mm$^3$, and thus the sample contained $(0.2 - 0.3) \times 10^{17}$ molecules of Hb(H$_2$O). The exact number of molecules in the samples was determined from the intensities of the optical absorption lines at $\lambda = 540$ nm and $\lambda = 557.5$ nm of the reduced pyridine hemochromogen complex $^{15}$ to which the hemoglobin had been transformed after the susceptibility measurements. By this method we achieved an accuracy of $\pm 1.5\%$ in the determination of the number of molecules in our samples. The single crystals of Hb(H$_2$O) were broken into small pieces by a glass rod before freezing the samples in order to avoid an influence on the data by anisotropy effects.

As shown in the next chapter we measured the magnetic susceptibility of two different samples of Hb(H$_2$O), the results of which differ considerably. While the sample used for the Mössbauer experiments and the sample I of Hb(H$_2$O) used for the susceptibility measurements were frozen simply by putting these samples into liquid nitrogen, the sample II of Hb(H$_2$O) was frozen in liquid propane at a temperature of $T = 90$ K. As the boiling point of propane $T_b = 231$ K lies far above its melting point $T_m = 83.7$ K no boiling of the liquid propane occurs if the sample is put into liquid propane at $T = 90$ K. Thereby the freezing time of the sample II of Hb(H$_2$O) is considerably shorter than the one of the sample I of Hb(H$_2$O) which was frozen in liquid nitrogen.

## 3. Results and Data Analysis

### 3.1. Mössbauer experiments

In Fig. 1 the Mössbauer spectra of a frozen solution of Hb(H$_2$O) at pH 6.0 measured in the temperature range 4.2 K $\leq T$ $\leq 30$ K and at small longitudinal magnetizing fields $H_{\text{ext}} \approx 1$ kOe are shown. In addition Fig. 2 shows spectra of the same sample at more elevated temperature without an external field applied. In contrast to the results given
by Lang and Marshall\textsuperscript{3} one clearly recognizes in the spectra at $T = 4.2$ K (Fig. 1) and at $T = 190$ K (Fig. 2) that the sample contains at least two different species of hemoglobin. Based on results of EPR measurements\textsuperscript{5,6} only two different compounds of methemoglobin, namely a high spin and a low spin compound were considered in the analysis of the Mössbauer spectra. Similar to the case of Mb(H\textsubscript{2}O)\textsuperscript{11} it was not possible to least squares fit the Mössbauer spectra of Hb(H\textsubscript{2}O) at intermediate temperatures $4.2$ K $< T \leq 160$ K since an appropriate relaxation theory of paramagnetic spins is not available at present. In addition the detailed shape of the spectra of the low spin compound at these temperatures are not known, rendering the separation into the two components practically impossible.

In the spectrum of Hb(H\textsubscript{2}O) at $T = 4.2$ K (Fig. 1) the typical hyperfine pattern of a ferric high spin compound of heme proteins like Mb(H\textsubscript{2}O) and MbF\textsuperscript{11} can be seen. Beside this pattern no resolved resonance lines of the low spin compound of Hb(H\textsubscript{2}O) are recognizable. Therefore we attempted by a large number of simulations to find a simple analytical function, which is able to represent the broad and unresolved spectral shape arising from the low spin compound. We finally used for a phenomenological description of the low spin absorption spectrum a function proportional to $\exp[-(v/4.0)^8]$, where $v$ is the Doppler velocity in mm/s. The resulting spectral shape is indicated as a dashed line in Fig. 1. The fully drawn curve is the sum of the resonance spectra of both the high and the low spin compound, where the theoretical calculation of the spectrum of the high spin compound of Hb(H\textsubscript{2}O) was performed based on the considerations used earlier in the cases of Mb(H\textsubscript{2}O) and MbF\textsuperscript{11}. In order to determine the energies of the different resonance lines the Hamiltonians $H_{\text{NGR}}$ and $H_{\text{NGR}}$ describing the interaction of the electronic shell with the nuclear ground state and the 14.4 keV nuclear excited state of the $^{57}$Fe ion, respectively, was diagonalized. For example $H_{\text{NGR}}$ is given by:

$$H_{\text{NGR}} = \beta H_{\text{ext}}(\mathcal{L} + g_s S) + g_n^* A_0 S J^*$$

where $\beta$ is the Bohr magneton

- $H_{\text{ext}}$ is the external magnetizing field;
- $g_s = 2.0023$;
- $g_n^*$ is the magnetic hyperfine interaction constant;
- $Q$ is the nuclear quadrupole moment and
- $V_{zz}$ is the electric field gradient at the nucleus assumed to be rotational symmetric.

The point symmetry of the Fe\textsuperscript{3+} ions in Hb(H\textsubscript{2}O) was assumed to be $C_{4v}$. This should be a good approximation, because the experimental $g$-factors $g_x$ and $g_y$ of the lowest Kramers doublet\textsuperscript{12} differ only slightly (see Table IV). The form of the Hamiltonian $H_{\text{NGR}}$ is quite analogous, only the electric quadrupole interaction vanishes, because of $I = 1/2$. A more detailed description of the basis vectors used and of the calculation of the intensities of the different resonance lines was given in a previous publication\textsuperscript{11}.

Fitting the Mössbauer spectrum at $T = 4.2$ K and $H_{\text{ext}} = 1$ kOe the hyperfine parameters $A_0/\beta_n$ ($\beta_n$-
Fig. 2. Mössbauer spectra of the same sample as used in Fig. 1 at higher temperatures and without an external field applied. Only the spectrum at 190 K is least squares fitted with a pure quadrupole interaction.

nuclear magneton) and $\frac{1}{2} e Q V_{zz}$ as well as the isomer shift which appears as an additional parameter in the Mössbauer spectrum have been determined for the high spin compound of Hb(H$_2$O). From the ratio of the areas under the spectra of the high spin and the low spin compound one obtains the relative amount $\alpha$ of the low spin compound with respect to the total amount of Hb(H$_2$O). The numbers obtained from the fit are summarized in Table I.

At $T = 190$ K the relaxation times of the spin fluctuations are so short with respect to the period of the hyperfine interaction frequency that a paramagnetic hyperfine splitting can no longer be seen. We thus fitted the Mössbauer spectrum at $T = 190$ K with two independent quadrupole doublets. As can be seen from Fig. 3 a these two quadrupole pairs are not resolved. A least squares fit of such a spectrum with two independent quadrupole pairs involves nine independent parameters and therefore is unable to a unique result. It is necessary first to get additional information on the parameters of the two

Table I. The magnetic hyperfine interaction constant $A_0/\beta_n$, the quadrupole splitting $\frac{1}{2} e Q V_{zz}$, the isomer shift $S$ of the high spin compound of Hb(H$_2$O) and the relative amount of the low spin compound $\alpha$ determined by the least squares fit of the Mössbauer spectrum of Hb(H$_2$O) at $T = 4.2$ K and $H = 1$ kOe (in Fig. 1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_0/\beta_n$ [kOe]</td>
<td>201.3 ± 0.7</td>
</tr>
<tr>
<td>$\frac{1}{2} e Q V_{zz}$ [mm/s]</td>
<td>1.27 ± 0.10</td>
</tr>
<tr>
<td>$S$ [mm/s]</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>$\alpha$ [%]</td>
<td>43 ± 5</td>
</tr>
</tbody>
</table>
quadrupole interactions. This in turn will allow to keep some of the parameters constrained at reasonable values. From susceptibility experiments it is known that the relative amount \( \alpha \) of the low spin compound in a frozen sample of Hb(H\(_2\)O) will increase, if the temperature is raised into the range between \( T = 225 \) K and \( T = 250 \) K, but will no longer be changed by a new cool-down of the sample. This change in the relative amount of the low spin compound is clearly to be seen from the comparison of the Mössbauer spectra a) and c) in Fig. 3. The spectrum a) at \( T = 190 \) K was measured before warming up to sample to \( T = 250 \) K, whereas the spectrum c) was measured after the sample has been warmed up. The least squares fit of the spectrum c) in Fig. 3 could determine the Mössbauer parameters \( Q_{zz} \) and \( S \) of the low spin compound of Hb(H\(_2\)O) to a relative high accuracy, because \( \alpha \) had risen to more than 90%. The result of this fit is given in Table II. Keeping \( S \) and \( Q_{zz} \) of the low spin compound fixed at these values, it was possible to fit the spectrum a) in Fig. 3. The isomer shift, the quadrupole splitting and the relative amount \( \alpha \) at \( T = 190 \) K before warming up the sample to \( T = 250 \) K could thus be determined. The result is also given in Table III.

### Table II. The isomer shift \( S \) and the quadrupole splitting \( Q_{zz} \) of the low spin compound of Hb(H\(_2\)O) at \( T = 190 \) K as obtained from spectrum c) of Fig. 3.

<table>
<thead>
<tr>
<th>( Q_{zz} ) [mm/s]</th>
<th>( S ) [mm/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.16 ± 0.10</td>
<td>0.05 ± 0.03</td>
</tr>
</tbody>
</table>

### Table III. The isomer shift \( S \) and the quadrupole splitting \( Q_{zz} \) of the high spin compound of Hb(H\(_2\)O) at \( T = 190 \) K together with the relative amount of the low spin compound \( \alpha \) as obtained from the Mössbauer spectrum a) of Fig. 3.

<table>
<thead>
<tr>
<th>( Q_{zz} ) [mm/s]</th>
<th>( S ) [mm/s]</th>
<th>( \alpha ) [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.44 ± 0.15</td>
<td>0.16 ± 0.05</td>
<td>47 ± 5</td>
</tr>
</tbody>
</table>

#### 3.2. Susceptibility measurements

Since a frozen sample of methemoglobin Hb(H\(_2\)O) prepared under normal conditions always contains the high spin (HS) and the low spin (LS) compound of Hb(H\(_2\)O), the experimentally determined paramagnetic magnetizations \( M_{\text{exp}} \) of such a sample are composed of two independent compounds \( M_{\text{exp,HS}} \) and \( M_{\text{exp,LS}} \). The relative amount of the low spin compound of the sample again shall be denoted by \( \alpha \). The value of \( \alpha \) has to be determined by the experiment. In general we fitted the experimental data \( M_{\text{exp}} \) at different temperatures to the following expression:

\[
\frac{M_{\text{exp}}}{H} = \frac{M_{\text{exp,HS}}}{H} + \frac{M_{\text{exp,LS}}}{H} = (1 - \alpha) \cdot N \cdot \langle M \rangle_{T,\text{HS}} + \alpha \cdot N \cdot \langle M \rangle_{T,\text{LS}} + M_{\text{dia}}
\]

where \( H \) is the strength of the applied magnetizing field, \( N \) is the total number of Fe\(^{3+} \) ions in the sample and \( M_{\text{dia}} \) is the total diamagnetic contribution of the sample including the sample holder. \( M_{\text{dia}} \) was treated as an additional fitting parameter. \( \langle M \rangle_{T,\text{HS}} \) or \( \langle M \rangle_{T,\text{LS}} \) are the calculated mean values of the magnetizations of the irons in the high spin (HS) state or low spin state, respectively, at a given temperature. As already mentioned above \( \langle M \rangle_T \) is a function of the fitting parameters \( \epsilon_1, \epsilon_2, \epsilon_3, D \), and \( E \). Because of their mutual linear dependence it is possible to use either the energies \( \epsilon_1, \epsilon_2, \epsilon_3 \) or the relative energy separations of the
multi-electron wave-functions \((E(^4A_2), E(^2E), E(^2B_2))\) from the \(^6A_1\) state as fitting parameters.

It is obvious that it is impossible to infer electronic structures of the HS and LS compound of Hb\((H_2O)\) simultaneously by fitting the temperature dependence of \(M_{exp}/H\) via Eqn (3), because of the large number of free parameters. Therefore, if \(\alpha \neq 0\) one has to use further independent information in order to reduce the number of parameters.

The evaluations of the data of MbCN\(^{11}\) which is a low spin compound showed that the \(g\)-factors of the lowest Kramers doublet and the temperature dependence of the magnetic susceptibility of MbCN are mainly determined by the energy difference \(E(^2B_2) - E(^2E)\) or \(\varepsilon_1\), respectively, and by the rhombic distortion energy \(D\). (In all considerations the parameter \(E\) has been fixed to \(E = 0\) cm\(^{-1}\)).

For different values of these energies we calculated the \(g\)-factors and compared them with the experimental ones of Hb\((H_2O)_{LS}\) \((g_x = 1.7, g_y = 2.2, g_z = 2.8)\) as determined by\(^6\). The best agreement was achieved for:

\[
E(^2B_2) - E(^2E) = 160 \text{ cm}^{-1} \\
D = 35 \text{ cm}^{-1} \\
E(^4A_2) - E(^2E) \geq 1000 \text{ cm}^{-1} \\
E(^6A_2) - E(^2E) \geq 2000 \text{ cm}^{-1}.
\]

Using these numbers we calculated \((M)_{TLS}\) which should represent the induced paramagnetic moment of one Fe\(^{3+}\) ion of the LS compound Hb\((H_2O)\). Fitting the experimental magnetizations \(M_{exp}/H\) measured with sample I of Hb\((H_2O)\) and taking further into consideration the \(g\)-factors of the HS compound of methemoglobin\(^{12}\) as additional experimental data we tried to determine the remaining free parameters of Eqn (3). These are \(\alpha, M_{dia}\) and the energies \(E(^4A_2), E(^2E)\) and \(D\) of the HS compound. Like in the case of the high spin compounds Mb\((H_2O)\) and MbF\(^{11}\) we kept again the energies \(E(^2B_2) - E(^2E) = 500 \text{ cm}^{-1}\) and \(E = 0 \text{ cm}^{-1}\) of Hb\((H_2O)_{HS}\) fixed in the present evaluations. Because \(\alpha\) is a free fitting parameter the accuracy of the results is rather poor. From sample I of Hb\((H_2O)\) we obtained the following results by the manner just described:

\[
\alpha = 44 \pm 8\% \\
E(^4A_2) = 700 \pm 400 \text{ cm}^{-1}.
\]

The energy \(E(^2E)\) should always exceed \(E(^4A_2)\). A reliable value of \(D\) could not be determined. For

**Table IV.** The energies of the unsplit low lying multiplets \(^4A_2, ^2E, ^2B_2\) and \(^2E\) relative to the \(^6A_1\) level of the Fe\(^{3+}\) ion for the high spin compound of methemoglobin Hb\((H_2O)\). The definitions of the energies \(\varepsilon_1\) and \(\varepsilon_3\) can be seen in Fig. 6. \(\varepsilon_1\) and \(\varepsilon_2\) are the splitting energies of the three Kramers doublets of the \(^6A_1\) level caused by the spin orbit coupling. Compare with Figs 5 and 6.

<table>
<thead>
<tr>
<th></th>
<th>(\text{Hb}(H_2O)_{HS}) pH 6.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E(^6A_1))</td>
<td>cm(^{-1})</td>
</tr>
<tr>
<td>(E(^4A_2))</td>
<td>cm(^{-1})</td>
</tr>
<tr>
<td>(E(^2E))</td>
<td>cm(^{-1})</td>
</tr>
<tr>
<td>(E(^2B_2))</td>
<td>cm(^{-1})</td>
</tr>
<tr>
<td>(E(^4E))</td>
<td>cm(^{-1})</td>
</tr>
<tr>
<td>(\varepsilon_2)</td>
<td>cm(^{-1})</td>
</tr>
<tr>
<td>(\varepsilon_3)</td>
<td>cm(^{-1})</td>
</tr>
<tr>
<td>(\varepsilon_1)</td>
<td>cm(^{-1})</td>
</tr>
<tr>
<td>(g_{xy} = g_x - g_y)</td>
<td>* 5.87</td>
</tr>
<tr>
<td>(g_{xy} = g_x - g_y)</td>
<td>0.14</td>
</tr>
<tr>
<td>(D)</td>
<td>cm(^{-1})</td>
</tr>
</tbody>
</table>

* The \(g\)-factors of the lowest Kramers doublet are taken from Gray and Buckmaster\(^{12}\).

Fig. 4. The temperature dependence of the induced paramagnetic moment of two samples (I and II) methemoglobin measured with an applied magnetizing field of \(H = 20.0 \pm 0.04\) kOe. Sample II was frozen with the use of liquid propane whereas for the freezing of sample I liquid nitrogen was used. The solid curves represent the least squares fit.

The data of Mb\((H_2O)\) are taken from ref.\(^{11}\).
sample II of Hb(H₂O) the quick freezing method discussed earlier was applied. This made the parameter $\alpha$ smaller than 3% which means that this sample practically contains no Hb(H₂O) molecules in the low spin state. Therefore we set $\alpha = 0\%$ and extracted from this set of data the temperature dependence of $M_{\text{exp}}/H$ and the $g$-factors of the lowest Kramers doublet of Hb(H₂O)$_{HS}$. The results are given in Table IV. The possibility that $\alpha$ deviates somewhat from zero has been taken into consideration in determination of the error of the different parameters. Fig. 4 shows the experimental data $M_{\text{exp}}/H$ and reduced to 1 mol together with the theoretical temperature dependence of $\langle (M_T)/H_{\text{m}ol} \rangle$ as delivered by the least squares fitting procedure (solid curves). From the analysis of the data as described the relative positions of the low energy levels $^4A_1$, $^4A_2$, $^4E$, $^2B_2$, and $^2E$ and the positions of the antibonding single 3d-electron orbitals $d_{xz}$, $d_{yz}$, and $d_{xy}$, respectively, are obtained. They are shown in Figs 5 and 6 in an energy level diagram. For comparison we also show in these figures the data of Mb(H₂O) as obtained earlier.

4. Discussion

In agreement with the results of EPR spectroscopy and magnetic susceptibility measurements we also find two different species of ferric hemoglobin in our experiments at low temperatures. Especially our Mössbauer data clearly show the overlap of spectra of the high spin and the low spin compound of Hb(H₂O), in contrast to the Mössbauer spectra given by Lang et al. where only the quadrupole splitting characteristic for the low spin compound of Hb(H₂O) has been present. The appearance of a noticeable amount of the low spin compound in a sample can be avoided by freezing the solution of Hb(H₂O) very rapidly in liquid propane.

We are presently interested predominantly in the electronic properties of the ferric iron of the high spin compound of methemoglobin, because one believes that at room temperature only the high spin compound is present. This general belief is well-confirmed by our results of the susceptibility data. As we know the energies and the eigenvectors of the low lying electronic levels of the ferric iron from the evaluations of the experimental data, we are in the position to calculate the molar magnetic susceptibility of the high spin compound of Hb(H₂O) at any temperature. That means, it is possible to extrapolate our experimental data to room temperature. A comparison of the extrapolated value $(0.0141 \pm 0.0001 \text{ cm}^{-3}/\text{mol})$ with other experimental data collected directly at room temperature $(0.0140 \pm 0.00007 \text{ cm}^{-3}/\text{mol})$ shows very good agreement. For that reason we may first conclude that the electronic properties of the high spin compound of
Hb(H₂O) at low temperatures are identical with those of Hb(H₂O) at room temperature. This in turn proves secondly that in a solution of Hb(H₂O) at room temperature a low spin compound is not present. It is formed during the freezing of a sample of Hb(H₂O).

We proceed with a discussion of the differences between the electronic structures of the ferric iron in Hb(H₂O) and Mb(H₂O) and of the ferrous iron in deoxygenated Hb. For that purpose a comparison of the splitting energies $\varepsilon_2$ and $\varepsilon_3$ of the 3d-orbitals of the iron in these molecules is shown in Table V. In earlier publications $^{11,16}$ the dependence of the energies $\varepsilon_2$ and $\varepsilon_3$ on changes of the spatial arrangement of the iron ion relative to its nearest neighbours had been already discussed. $\varepsilon_2$ and $\varepsilon_3$ are the energies of the antibonding single 3d-electron orbitals $3d^2$ and $3d_{x^2-y^2}$ of the iron in methemoglobin, metmyoglobin and deoxygenated hemoglobin with respect to the $3d_{xy}$ orbital whose energy is taken to be zero.

Table V. The energies $\varepsilon_2$ and $\varepsilon_3$ of the antibonding single 3d-orbitals $3d^2$ and $3d_{x^2-y^2}$ of the iron in methemoglobin, metmyoglobin and deoxygenated hemoglobin with respect to the $3d_{xy}$ orbital whose energy is taken to be zero.

<table>
<thead>
<tr>
<th>Orbital</th>
<th>Hb(H₂O)</th>
<th>Mb(H₂O)</th>
<th>Hb₂O₂⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>$3d^2$</td>
<td>8300</td>
<td>10940</td>
<td>9440</td>
</tr>
<tr>
<td>$3d_{x^2-y^2}$</td>
<td>24000</td>
<td>23707</td>
<td>15880</td>
</tr>
</tbody>
</table>

* These values are taken from references $^{11,16}$.

of the energies $\varepsilon_2$ and $\varepsilon_3$ on changes of the spatial arrangement of the iron ion relative to its nearest neighbours had been already discussed. $\varepsilon_2$ and $\varepsilon_3$ are the energies of the antibonding $3d^2$ and $3d_{x^2-y^2}$ orbitals of the iron relative to the $3d_{xy}$ orbital, the energy of which is taken as zero. Because it is related to the antibonding character, the energy $\varepsilon_2$ has to increase, if the distance between the iron and one of its ligands lying in the z-direction decreases. Looking at the different values of $\varepsilon_2$ and $\varepsilon_3$ for Hb(H₂O)$_{HS}$ and Mb(H₂O) in Table V one finds that $\varepsilon_{3,Hb}$ is greater than $\varepsilon_{3,Mb}$, whereas $\varepsilon_{2,Hb}$ is smaller than $\varepsilon_{2,Mb}$. The difference in $\varepsilon_3$ infers that the ferric iron in methemoglobin lies closer to the heme plane than in metmyoglobin. Since two different ligands are bound to the iron the z-direction — i.e. a water molecule and the nitrogen atom $N_r$ of the proximal histidine — it is a priori not clear which of the ligands changes its distances with respect to the iron atom and thus causes the variation in $\varepsilon_2$. It is reasonable, however, to assume that the distance between the H₂O molecule and the ferric iron in Hb(H₂O) and in Mb(H₂O) remains nearly identical, because the water molecule is rather rigidly bound to the iron and only weakly bound (if at all) to the distal histidine. Therefore we believe the different $\varepsilon_2$ values of Hb(H₂O) and Mb(H₂O) being mainly due to a change of distances of the nitrogen $N_r$ relative to the ferric iron. That means that in methemoglobin the iron is placed nearer to the heme plane and the distance to the nitrogen atom $N_r$ is larger in comparison to metmyoglobin. The same type of correlation showing an increase of distance between Fe and $N_r$, when the iron is placed closer to the heme plane has already been apparent in a comparison of the electronic properties of the ferrous iron in different deoxygenated hemoglobins $^{16}$.

Upon comparing the spatial arrangement of the iron in Hb(H₂O) with the one in deoxygenated hemoglobin Hb one finds that the ferric iron lies much closer to the heme plane than the ferrous iron. The distance Fe–$N_r$ appears larger in methemoglobin because of its smaller $\varepsilon_2$ value. Taking into account the water molecule as an additional ligand in the z-direction in the Hb(H₂O) molecule the small $\varepsilon_2$ value then indicates a comparatively large increase in the distance Fe–$N_r$ in Hb(H₂O).

The fact that in methemoglobin the iron lies closer to the heme plane than in metmyoglobin also helps to understand the appearance of the low spin compound of Hb(H₂O) upon freezing the sample. It is known that in low spin compounds of heme proteins the iron lies nearly within the heme plane. Therefore, the geometrical structure of the heme group is much closer to a low spin conformation in Hb(H₂O) than in Mb(H₂O). Therefore, the mechanical forces generated by the expansion of the freezing solution are perhaps strong enough to change the structure of the hemoglobin molecule into a low spin conformation, a phenomenon which is not observed in Mb(H₂O) $^{11}$.

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