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Investigation of the Electronic Term Scheme of Deoxygenated Human Haemoglobin by a Least Squares Fit Procedure Using Simultaneously Magnetic Susceptibility and Mössbauer Data

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Summary. The calculation of the magnetic susceptibility from a published term scheme for the ferrous iron in deoxygenated human haemoglobin is discussed and a procedure for the simultaneous least squares fit of susceptibility and Mössbauer data is presented. The application of this procedure to the appropriate measurements on human haemoglobin leads to a rearrangement of the low lying electronic levels of the iron. The term schemes received as results of two different sets of susceptibility data used in combination with one set of Mössbauer data overlap with their error bars. The obtained level scheme of the Fe is correlated with the distance of the iron atom from the haem plane and the distance Fe-HIS F8, and some biological implications of these correlations are discussed.

Key words: Haemoglobin – Mössbauer – Susceptibility – Least squares fit.

Introduction

In preceding papers (Eicher et al., 1974, 1976) the term schemes of the electronic structures of the ferrous iron in human haemoglobin and sperm whale myoglobin were presented. These term schemes were determined by a least squares fit procedure. The variable parameters of this fit are the energy of the lowest electronic levels, the rhombic distortion of the next neighbor symmetry of the iron, the covalency factor α^2, the spin orbit coupling constant λ, and the lattice contribution to the electric field gradient. These parameters were varied until an optimal agreement with the temperature dependence of the quadrupole splitting of the 14.4 keV state of the ^{57}Fe nucleus, as measured by Mössbauer spectroscopy, was obtained.

Other physical properties of the ferrous iron may then be calculated easily from this electronic level scheme. Recently magnetic susceptibility data of deoxygenated human haemoglobin (Nakano et al., 1971) were compared with values calculated from the electronic level scheme which had been extracted from the Mössbauer data as indicated (Bade et al., 1975). The comparison showed, that there is basic agreement between the two sets of data. It was clear, however, that an additional adjustment of the parameters of the electronic level scheme should yield a better agreement.

Since the susceptibility data contain highly useful information on the electronic term scheme, particularly at low temperatures, the development of a method which makes direct use of this additional information within the least squares fitting procedure seemed desirable. Such a method is described in this paper. The fact, that the Mössbauer data as well as the susceptibility data may be calculated starting from the same electronic term scheme, makes it possible to apply a least squares fit procedure simultaneously to the two different sets of experimental data.

Theory

A theoretical approach for the determination of the structure of the ferrous iron from the temperature dependence of the quadrupole splitting is described by Eicher and Trautwein (1969). On the basis of this theory a term scheme of the ferrous iron in myoglobin and haemoglobin was determined, which allows to understand not only the temperature dependence of the quadrupole splitting, but also Mössbauer experiments on single crystals of sperm whale myoglobin (Eicher et al., 1974, 1976). We now add further terms to the theory, which enable us to calculate the magnetic susceptibility from this term scheme. We believe the presentation of some details to be necessary, because the approximative degeneracy of the lowest singlets of the term scheme needs some special treatment, when the magnetic susceptibility is calculated. A straightforward calculation would yield wrong results.

The Hamiltonian describing the electronic structure of Fe^{+2} in haemoglobin is given in the first approximation by

$$\hat{\mathscr{H}} = \hat{\mathscr{H}}_0 + \hat{\mathscr{H}}_{\mathrm{Coul}} + \hat{\mathscr{H}}_{C_{4v}} \tag{1}$$

$\hat{\mathscr{H}}_0$ contains the kinetic and potential energies of the electrons and determines the absolute ground state of the system. It has no influence on the following discussion and can be neglected. $\hat{\mathscr{H}}_{\mathrm{Coul}}$ gives the Coulomb repulsion of the electrons and $\hat{\mathscr{H}}_{C_{4v}}$ their interaction with ligands in C_{4v} symmetry. In this symmetry the 5-fold degeneracy of the $3d$-levels is partially lifted. This influence of the ligand field can be described by the three energy parameters ε_1, ε_2, and ε_3. They are the separation in energy of the $d_{xz,\,yz}$, d_{z^2}, and $d_{x^2-y^2}$ orbitals relative to the d_{xy} orbital. It was shown (Eicher et al., 1969) that for realistic values of ε_1 to ε_3 the terms ${}^1A_1$, ${}^3E$, ${}^5E$, and ${}^5B_2$ are the lowest lying energy levels, if a simultaneous diagonalization of $\hat{\mathscr{H}}_{\mathrm{Coul}} + \hat{\mathscr{H}}_{C_{4v}}$ is carried out. The arrangement in energy of these terms of the $3d^6$ configuration is governed by the parameters

$$\begin{aligned} E_1 &= E({}^5E) - E({}^5B_2) \\ E_2 &= E({}^3E) - E({}^5B_2) \\ E_3 &= E({}^1A_1) - E({}^5B_2) \end{aligned} \tag{2}$$

which in turn are functions of the parameters ε_1, ε_2, and ε_3 [see Eq. (2) in Eicher et al. (1976)]. The addition of a Hamiltonian which contains the reduction to rhomboedric ligand symmetry and the spin orbit coupling

$$\hat{\mathscr{H}} = \hat{\mathscr{H}}_D + \hat{\mathscr{H}}_{LS} \tag{3}$$

to Equation (1) finally splits the terms ${}^1A_1$ to ${}^5B_2$ into 22 singlet states $|n\rangle$ with energies e_n.

In the calculation of magnetic susceptibility the Hamiltonian

$$\hat{\mathscr{H}}_M = \beta\vec{H}(\vec{L} + 2\,\vec{S}) \tag{4}$$

(where β is the Bohr magneton and $\vec{H}$ is the external magnetic field) must be added to Equations (1) and (3). Since the matrix elements of $\hat{\mathscr{H}}_M$ are believed not to exceed 1 cm^{-1}, one usually calculates the magnetic susceptibility by second order perturbation theory (Eicher and Trautwein, 1970; Huynh et al., 1974; Zimmermann, 1975). The 22 singlet states are then shifted by the interaction with the magnetic field:

$$(\Delta e_n'')_i = \beta^2\vec{H}^2 \sum_{\substack{m=1\\ m\neq n}}^{22} \frac{|\langle n|L_i + 2\,S_i|m\rangle|^2}{e_n - e_m}, \tag{5}$$

where $i = 1, 2, 3$, corresponds to x, y, z, and the magnetic field $\vec{H}$ is directed along the i-th axis in Equation (4). The induced magnetic moments are then

$$(\mu_n)_i = -\frac{\partial(\Delta e_n'')_i}{\partial H} \tag{6}$$

One obtains for the i-th component of the observed magnetic susceptibility per mole

$$\chi_i = \frac{\langle M\rangle_i}{H} = \frac{N}{H}\,\frac{\sum_{n=1}^{22} (\mu_n)_i \exp\left(\frac{e_n + (\Delta e_n'')_i}{k_B\,T}\right)}{\sum_{n=1}^{22} \exp\left(\frac{e_n + (\Delta e_n'')_i}{k_B\,T}\right)} \tag{7}$$

(where N is Avogadro's number and k_B is Boltzmann's constant). The molar susceptibility measured with a random sample is the average over all three components.

In Equation (7) the actual energies of the electronic states in the external magnetic field H are inserted in the exponents. In weak magnetic fields H the shifts $(\Delta e_n'')_i$ are small and may be omitted in the exponents. Then the components of the average magnetization $\langle M\rangle_i$ are exactly proportional to H and the observed magnetic susceptibility does not depend on H. Consequently the common definition of the magnetic susceptibility

$$\chi = \lim_{H\to 0} \frac{\langle M\rangle}{H} \tag{8}$$

becomes equivalent to

$$\chi_H = \frac{\langle M \rangle}{H}. \tag{9}$$

This is the quantity, which is actually measured as the magnetic susceptibility. Most authors, however, do not bother to carry out the extrapolation for $H \to 0$. They are usually satisfied, when the measured magnetic susceptibility does not depend on the magnetic field H.

In the term scheme for deoxygenated human haemoglobin (Eicher et al., 1976) one finds the lowest singlets to be separated by only 1.76 cm^{-1}. On the other hand, the matrix element $\langle 1|\hat{\mathscr{H}}_M|2\rangle$ can reach 2 cm^{-1} in magnetic fields up to 10 kOe. This means, that the lowest singlets will approximatively appear degenerated to the energy of the applied magnetic field. Thus they act effectively as a doublet. Then second order perturbation theory will not hold any longer, and the calculation of the averaged magnetization $\langle M \rangle$ must be modified. Furthermore the $(\Delta e_n'')_i$ are no longer neglegible compared to the e_n at least for the lowest states and one has to use Equation (7) in full. As a consequence the quotient $\frac{\langle M \rangle}{H}$ is not necessarily independent of the applied field H. We therefore always used data calculated by Equation (9) for comparison with experimental susceptibility data. We shall still call (somewhat inconsequently) χ_H the "magnetic susceptibility". It should be emphasized, that the use of Equation (7) and Equation (9) does not necessarily mean, that the measured χ_H has to depend openly on H. There may well be a compensation of the field dependences of the different terms in the sum over the 22 singlet states of Equation (7).

The approximative degeneracy of the states $|1\rangle$ and $|2\rangle$ must first be removed by a diagonalization procedure as described by Davydow (1965) and then second order perturbation theory can be applied again. The condition for the validity of pure second order perturbation theory in this case is (Davydow, 1965)

$$|\langle 1|\hat{\mathscr{H}}_M|2\rangle| \ll e_2 - e_1. \tag{10}$$

To remove the approximative degeneracy of the states $|1\rangle$ and $|2\rangle$ first the matrix

$$\begin{pmatrix} 0 & \langle 1|\hat{\mathscr{H}}_M|2\rangle \\ \langle 2|\hat{\mathscr{H}}_M|1\rangle & \Delta \end{pmatrix} \tag{11}$$

with $\Delta = e_2 - e_1$ is diagonalized, giving the new eigenvalues

$$\begin{aligned} (e_1')_i &= \frac{\Delta}{2} - \frac{1}{2}\sqrt{\Delta^2 + 4|\langle 1|\hat{\mathscr{H}}_M|2\rangle|^2} \\ (e_2')_i &= \frac{\Delta}{2} + \frac{1}{2}\sqrt{\Delta^2 + 4|\langle 1|\hat{\mathscr{H}}_M|2\rangle|^2} \end{aligned} \tag{12}$$

and corresponding eigenvectors $|1'\rangle$ and $|2'\rangle$. It is easily seen, that the eigenvalues $|e_1'\rangle$ and $|e_2'\rangle$ are no longer proportional to H^2, and the eigenvectors can become complex, when $\langle 1|\hat{\mathscr{H}}_M|2\rangle$ is imaginary. This fact must be considered, when now the second order contributions $(\Delta e_n'')_i$ are calculated with the substitution

$$
\begin{aligned}
&|1\rangle \rightarrow |1'\rangle \\
&|2\rangle \rightarrow |2'\rangle \\
&e_1 \rightarrow (e_1')_i \\
&e_2 \rightarrow (e_2')_i
\end{aligned}
\tag{13}
$$

in Equation (5). The total energy shifts for the states $|1\rangle$ and $|2\rangle$ become now

$$
\begin{aligned}
(\Delta e_1^{\mathrm{tot}})_i &= (e_1')_i + (\Delta e_1'')_i \\
(\Delta e_2^{\mathrm{tot}})_i &= (e_2')_i + (\Delta e_2'')_i.
\end{aligned}
\tag{14}
$$

This leads to the induced magnetic moments

$$
\begin{aligned}
(\mu_1)_i &= -\frac{\partial(\Delta e_1^{\mathrm{tot}})_i}{\partial H} \\
(\mu_2)_i &= -\frac{\partial(\Delta e_2^{\mathrm{tot}})_i}{\partial H}
\end{aligned}
\tag{15}
$$

for the states $|1\rangle$ and $|2\rangle$. The calculation is then continued using Equation (6) (for $n \geq 3$) and Equation (7). For $n = 1, 2$ the total energy shifts from Equation (14) must be inserted in the exponents of Equation (7). Finally the averaging over the three components is to be carried out in the usual manner.

It should be mentioned that the most satisfying way for the calculation of the magnetic susceptibility would require the full diagonalisation of the Hamiltonian including the term of Equation (4). As in the special case of the diagonalisation of the lowest singlet states, all the wavefunctions become complex, because a diagonalisation of a complex 22×22-matrix is necessary. Such a procedure was carried out once. From this calculation we know, that in the particular case discussed here the combined procedure as described is a very good approach for magnetic fields up to 20 kOe. The complete diagonalisation of the 22×22-matrix, however, needs much more time than the calculation described above and therefore cannot be applied in the fit procedure.

Fit Procedure and Results

The mathematical background for the nonlinear least squares fit procedure used for our problem is described by Marquardt (1963). The fit procedure needs as an input (i) a theory function $Y^c(p_1, p_2, \ldots p_k; x)$ dependent on the k adjustable parameters p_k and the independent variable x, (ii) the experimental data $(Y_i^{\mathrm{exp}}, x_i)$ with an estimated experimental error ΔY_i, and (iii) the derivatives of the theory function Y^c with respect to the parameters p_k.

In our case the theory function is the procedure for the calculation of the quadrupole splitting $\frac{1}{2}\, e^2qQ = E_Q$ and the susceptibility $\frac{\langle M(H)\rangle}{H} = \chi_H$, which both have the parameters E_1, E_2, E_3, and D, and as independent variable the temperature. D

describes the rhombic distortion of the C_{4v}-symmetry as defined by Eicher et al. (1976). The experimental data are $(\chi_{Hj}^{\mathrm{exp}}, T_j)$ and $(E_{Qi}^{\mathrm{exp}}, T_i)$. The derivatives of the theory function with respect to the term scheme parameters were replaced by the corresponding divided differences, for example $\frac{\partial E_Q}{\partial D}$ was replaced by $\frac{\Delta E_Q}{\Delta D}$ for $\Delta D \approx 0.05\ D$. This is necessary, because the derivations of the theory functions with respect to the term scheme parameters are not available in an analytical form.

In the concrete example the fit procedure then minimizes the sum Z depending simultaneously on Mössbauer and susceptibility data

$$
\begin{aligned}
Z = w_{EQ} \sum_i \frac{1}{(\Delta E_{Qi}^{\mathrm{exp}})^2} (E_{Qi}^{c}(E_1, E_2, E_3, D;\ T_i) - E_{Qi}^{\mathrm{exp}}(T_i))^2 \\
+ w_{xH} \sum_j \frac{1}{(\Delta \chi_{Hj}^{\mathrm{exp}})^2} (\chi_{Hj}^{c}(E_1, E_2, E_3, D;\ T_j) - \chi_{Hi}^{\mathrm{exp}}(T_j))^2.
\end{aligned}
\tag{16}
$$

The summation index i runs over all temperatures, at which the quadrupole splitting was measured, and the summation index j over all temperatures, at which the susceptibility was measured. As the errors in the temperature measurement are not included in the fit procedure, this fact must be compensated by an corresponding enlargement of the errors ΔE_{Qi} and $\Delta \chi_{Hj}$. The weights w_{EQ} and w_{xH} make it possible to adjust the relative importance of the Mössbauer data and the susceptibility data, respectively. The relative weight of w_{EQ} to w_{xH} introduces some arbitrariness into the interpretation of the data and must be chosen from physical considerations.

From a first view there seems to be no reason for a different weight of the experimental data. The quadrupole splitting as well as the magnetic susceptibility are calculated from a Boltzmann average over the whole term scheme. Each singlet contributes an induced magnetic moment as well as an electric field gradient to the quantity, which is measured. The induced magnetic moments for neighboring singlets, however, have often the same magnitude but are opposite in sign. Therefore the magnetic susceptibility is only large when there are strong differences in the thermal population of these singlets, because of the Boltzmann weights. At higher temperatures the susceptibility decreases rapidly proportional to the inverse temperature. The proportionality factor, however, depends mainly on the relative energetical distance of neighboring singlets, but not on the energetical distance of these singlets to the ground state. Therefore the magnetic susceptibility is mainly determined by the relative distances of the lowest singlets and it cannot give much information about the position of the upper levels of the term scheme.

In the case of the quadrupole splitting the different contributions to the EFG from the different singlets are practically the same, when the singlets arise from the same term. Therefore these contributions from neighboring singlets usually do not change systematically in sign and compensation effects appear in a larger temperature range. For this reason the quadrupole splitting is not only sensitive in the low temperature range, where the population differences make the susceptibility important, but also at higher temperatures.

In our special case the lowest singlets of the term scheme descend mainly from the ${}^5E$ term. As these singlets give practically the same contribution to the EFG, the quadrupole splitting in the low temperature range is not very sensitive to relative

Table 1

	(a)	(b)	(c)	(d)
E_1 (cm^{-1})	18 ± 50	-30 ± 25	-234 ± 30	-303 ± 150
E_2 (cm^{-1})	1916 ± 550	2035^{+700}_{-230}	1933^{+400}_{-170}	1537 ± 800
E_3 (cm^{-1})	-194 ± 35	-224 ± 10	-341 ± 21	-415 ± 120
D (cm^{-1})	-327 ± 35	-299 ± 15	-215 ± 8	-246 ± 130
ε_1 (cm^{-1})	18 ± 50	-30 ± 25	-234 ± 30	-303 ± 150
ε_2 (cm^{-1})	9345 ± 555	9459^{+700}_{-240}	9275^{+400}_{-175}	8850 ± 820
ε_3 (cm^{-1})	15950 ± 550	15776^{+235}_{-700}	15669^{+173}_{-400}	16015 ± 810

shifts of these singlets. On the other hand the susceptibility depends strongly on the spin of these lowest singlets, which have mainly an $S = 2$ character. By the application of the magnetic field these singlets get a magnetic moment, which depends strongly on their relative distance in energy. Therefore the magnetic susceptibility is much more sensitive to the relative position of the low lying singlets than the quadrupole splitting.

The specifical information of one set of experimental data may be easily found out, when in a first step the two sets of data are used separately in the fit procedure. In addition to the Mössbauer data (Eicher et al., 1976) we had two different sets of magnetic susceptibility data available as input for our fit procedure. The magnetic susceptibility of deoxygenated human haemoglobin has been investigated by Nakano et al. (1971) down to $T = 2.0$ K. Furthermore new susceptibility measurements on deoxygenated human haemoglobin have recently been performed by Alpert and Banerjee (1975), who obtained different results in the low temperature range from 10 K to 4.6 K. These two sets of susceptibility data were combined separately with the Mössbauer data. For the fits described in the following text we assumed the relative errors $(\Delta E_{Qi}^{\mathrm{exp}}/E_{Qi}^{\mathrm{exp}}) = 0.01$ and $(\Delta\chi_{HJ}^{\mathrm{exp}}/\chi_{HJ}^{\mathrm{exp}}) = 0.03$. The calculations were carried out with fixed values for the spin orbit coupling constant $\lambda = 69$ cm^{-1} and the covalency factor $\alpha^2 = 0.89$. These are average values found from a 7 parameters least squares fit to Mössbauer data of a series of different haem proteins investigated in our laboratory.

The least squares fit to the Mössbauer data separately as given by Eicher et al. (1976) corresponds to $w_{EQ} = 1$ and $w_{xH} = 0$. The obtained term scheme parameters are given in Table 1 (a) and the corresponding curves for the magnetic susceptibility and the quadrupole splitting are shown in Figures 1, 2, and 3, plot (a). As clearly seen, the parameters obtained from the Mössbauer data can describe fairly well the susceptibility measurements.

We then went to the opposite case and used the magnetic susceptibility data of Nakano et al. (1971) separately, which means $w_{EQ} = 0$ and $w_{xH} = 1$. The result is shown in Figure 3, plot (d). The quadrupole splitting calculated from the corresponding term scheme (d) in Table 1 is in clear disagreement with the measurements [compare plot (d) in Fig. 1].

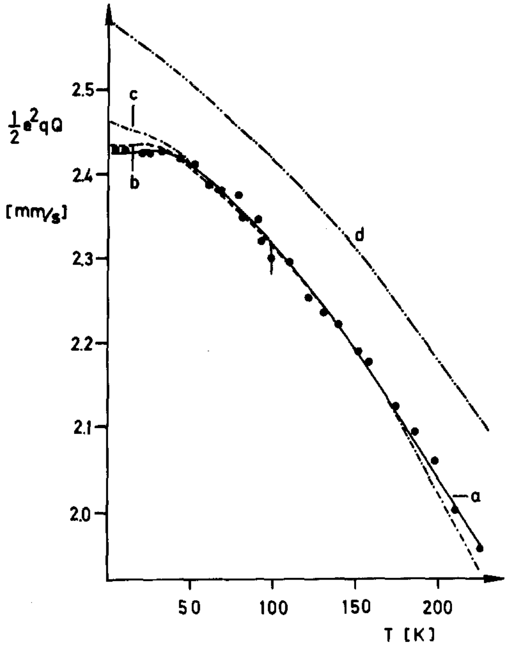

Fig. 1. Temperature dependent quadrupole splitting curves compared with the experimental data from Eicher et al. (1976) for deoxygenated human haemoglobin. (a) fit to Mössbauer data separately, (b) fit to Mössbauer data in combination with susceptibility data of Alpert et al. (1976), (c) fit to Mössbauer data in combination with susceptibility data of Nakano et al. (1971), (d) curve calculated from a term scheme resulting from a separate fit to the susceptibility data of Nakano et al. (1971). Lattice contribution $\kappa = 1.55 \times 10^{-5}$ cm [for definition of κ see Eicher et al. (1976)]

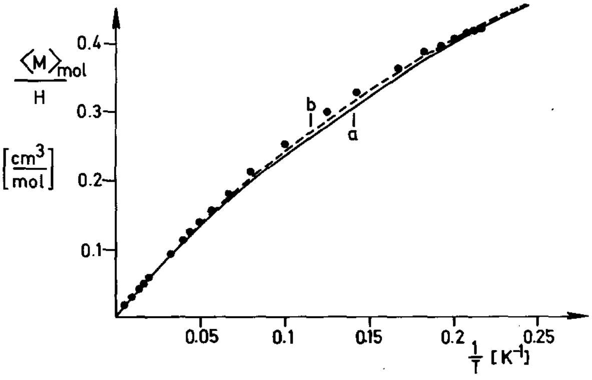

Fig. 2. Magnetic susceptibility curves compared with the experimental data of Alpert et al. (1975). (a) calculated from the result of the fit to Mössbauer data separately, (b) resulting from the simultaneous fit to the Mössbauer data and the susceptibility data of Alpert et al. (1975). Calculations are performed with $H = 10$ kOe

As mentioned above this may be simply caused by the fact, that the magnetic susceptibility cannot appoint the upper levels of the term scheme. As a consequence of these both extreme cases it is obvious, that in a simultaneous fit the weight factor for the Mössbauer data should be larger than for the susceptibility data. The best results were obtained with $w_{EQ} = 0.7$ and $w_{xH} = 0.3$. We want to point out, that a small change of the weights does not influence the qualitative result of the simultaneous fit: Clearly the particular choice of the weights will always bring the results

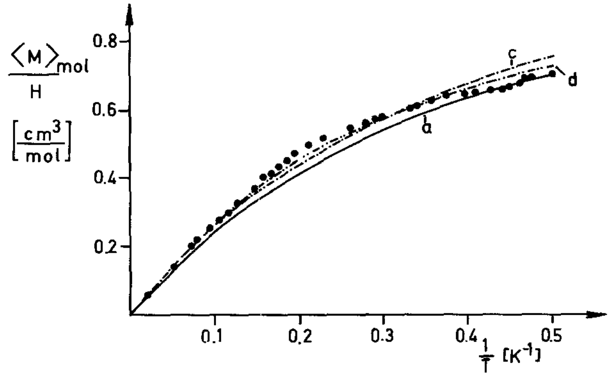

Fig. 3. Magnetic susceptibility curves compared with the experimental data of Nakano et al. (1971). (a) calculated from the fit to the Mössbauer data separately, (c) resulting from the simultaneous fit to the Mössbauer data and the susceptibility data of Nakano et al. (1971), (d) fit to the susceptibility data separately. Calculations are performed with $H = 8$ kOe

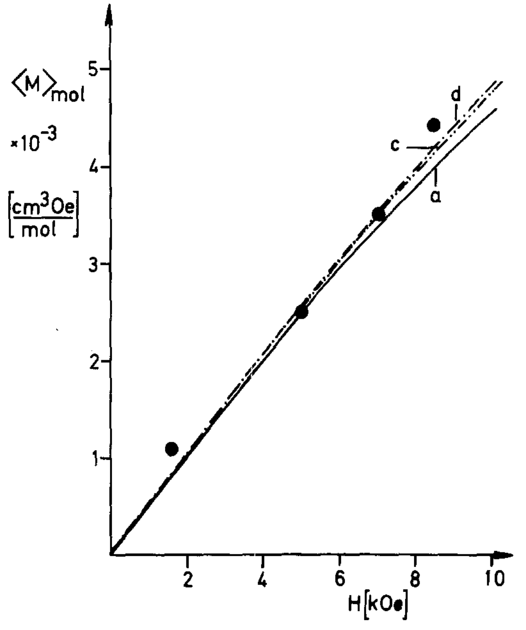

Fig. 4. Field dependent average molar magnetization $\langle M(H) \rangle_{mol}$ at $T = 4.2$ K calculated from a term scheme resulting (a) from the fit to the Mössbauer data separately, (c) from the fit to the Mössbauer data simultaneously with the susceptibility data of Nakano et al. (1971), (d) from a fit to the susceptibility data of Nakano et al. (1971) separately compared with experimental values of Nakano et al. (1971)

closer to the separate fit of the susceptibility or the quadrupole splitting data, respectively. The progress of the simultaneous fit can be seen in the fact, that the error of the relevant parameters decreases, compared to a separate fit with only one set of data.

The least squares fit was then started with the term scheme (a) (see Table 1 and Figures 1—3) from Eicher et al. (1976). The result of the fit to the data of Alpert et al. (1975) in combination with the Mössbauer data is shown in Figures 1 and 2, plot

(b) and Table 1 (b). The best fit to the data of Nakano et al. (1971) combined with the Mössbauer data was found with the parameters (c) in Table 1 and is shown in Figures 1 and 3, plot (c).

We then additionally looked at the average magnetization $\langle M(H)\rangle_{\mathrm{mol}}$ dependent on the magnetic field H. The calculations of $\langle M(H)\rangle_{\mathrm{mol}}$ at $T = 4.2$ K resulted in plots (a), (c) and (d) of Figure 4 and refer to the term schemes (a), (c) and (d) of Table 1.

Discussion

As there are two different sets of susceptibility data used as input for the simultaneous fit procedure, it is not surprising that the result consists of two different sets of term scheme parameters. However these term schemes (b) and (c) practically overlap in the error bars of the relevant parameters ε_2 and ε_3 with the term scheme (a), found separately from the Mössbauer data. In the fit the parameters ε_1 and D are strongly correlated. Therefore we have to interprete these two parameters as a whole, concluding that the three orbitals d_{xy}, d_{xz} and d_{yz} are split by not more than 400 cm^{-1} in energy.

The common fit of the susceptibility data of Nakano et al. (1971) together with the Mössbauer data [compare Fig. 1 and 3, plot (c)] give reasonable good agreement of theoretically calculated curves and experimental data, although systematical deviations can be seen. The rearrangement of the low lying energy levels required by the magnetic susceptibility data causes also variations in the quadrupole splitting at low temperatures, which cannot be completely compensated by shifts of the upper energy levels. That is why the fit result (c) [see plot (c) in Fig. 1] shows deviations from the Mössbauer data not only in the low temperature region, but also in the upper temperature range.

A least squares fit of the susceptibility data of Nakano et al. 1971) separately yields parameters which cannot explain the temperature dependence of the quadrupole splitting [compare plot (d) in Fig. 1 and 3]. Since the susceptibility data calculated from the parameter set (c) and (d) differ only slightly in contrast to the calculated quadrupole splitting, the level scheme of the Fe should never be determined from susceptibility data separately.

The common fit procedure gives most satisfying results, when the data of Alpert et al. (1975) are combined with the Mössbauer data. Unfortunately these susceptibility data range only down to $T = 4.6$ K. So a fair comparison with the results from the data of Nakano et al. (1971) is impossible. The visible differences between the two sets of susceptibility data however indicate, that there was a real difference between the two samples used for the susceptibility measurements. The Mössbauer investigations support the quality of the data of Alpert et al. (1975).

Experimental magnetization data were only available from Nakano et al. (1971). So it is not surprising that the plots (c) and (d) in Figure 4 obtained from fits to the corresponding susceptibility data give the best agreement with the magnetization data. The values of $\langle M(H)\rangle_{\mathrm{mol}}$ calculated from the term scheme (b) in Table 1 coincide practically with plot (a) in Figure 4 and have therefore been omitted in Figure 4. The linearity of $\langle M(H)\rangle_{\mathrm{mol}}$ is fairly good for all three term schemes up to $H = 8$ kOe.

The term schemes obtained in this paper show the same arrangement of levels as determined by Huynh et al. (1974), but the energies of the term scheme levels are significantly different. By Huynh et al. (1974) theoretical curves for the quadrupole splitting and for the susceptibility at different temperatures have been calculated and compared with experimental data. Essentially the same theory as described by Eicher et al. (1969) has been used. In our opinion the discrepancies come from three facts:

First the Mössbauer experiments in Huynh et al. (1974) have been done with natural haemoglobin and not with ^{57}Fe-enriched samples. For that reason these measurements are significantly less accurate than the data published by Eicher et al. (1976) and used in this publication. Furthermore no Mössbauer data were available between 4.2 K and 77 K. Secondly the susceptibility calculations were done without taking into account the approximative degeneracy of the lowest two singlets, and without the magnetic field dependent shifts of the energies in the exponent of Equation (7). Thirdly a comparison of calculated curves with experimental data will always yield improved parameters, if a comparison is done by a least squares fit procedure instead by eye.

The determination of a term scheme of the iron in haemoglobin and similar compounds should give some additional insight into the mechanism of O_2 binding and the cooperative effect. With the described method we are of course limited to the neighbourhood of the Fe. For an understanding of the cooperative effect the distance of the iron from the haem plane is discussed by Perutz (1970), Perutz, Ladner, Simon, and Ho (1974), Perutz, Fersht, Simon, and Roberts (1974), Perutz, Heidner, Ladner, Beetlestone, and Ho (1974). In the "tensed structure" (as it is present in deoxygenated haemoglobin) the Fe atom is about 0.8 A out of plane[1], and in the "relaxed structure" (as exists for example in deoxygenated myoglobin) the iron atom is only about 0.3 A out of plane. Eicher et al. (1976) have shown, that the ε_1, ε_2, and ε_3 values obtained from the term scheme are related to both, the distance of the iron to the haemplane and to the N_ε atom of the proximal histidin. From Mössbauer data alone a distance of 0.5 A of the iron to the haemplane was calculated for deoxygenated haemoglobin. In an easy way one can correlate the ε_3-value with the distance of the iron from the haemplane. Since ε_3 gives the energy of the antibonding $d_{x^2-y^2}$-orbital with respect to the d_{xy}-orbital an increase in the value of ε_3 means a larger overlap of the $d_{x^2-y^2}$-orbital with the four N-atoms. Since these are the next neighbours to the Fe in the haemplane, a larger value of ε_3 is equivalent to a smaller distance between the iron and the haemplane. In Eicher et al. (1976) $\varepsilon_3 = 17269 \pm 154\ \text{cm}^{-1}$ was obtained for deoxygenated myoglobin. The present work gives $\varepsilon_3 = 15776\ {}^{+235}_{-700}\ \text{cm}^{-1}$ for deoxygenated haemoglobin. A comparison of these values shows, that the iron is closer to the haemplane in deoxygenated myoglobin than in deoxygenated haemoglobin in agreement with the model of Perutz.

In another model (see i.e. Braunitzer et al., 1974) the oxygen affinity of haemoglobin is correlated to the distance between the iron and the nitrogenatom N_ε on the 5th coordination place, which belongs to the proximal histidin HIS F8. The ε_2 value gives the energy of the antibonding d_{z^2}-orbital with respect to the d_{xy}-orbital. The d_{z^2}-orbital overlaps with the wavefunctions of the N_ε. An increase of the value ε_2

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[1] New investigations recently performed by Perutz yield about 0.6 A (Perutz, 1975)

gives an increase of the overlap and consequently a decrease of the distance Fe—N_ϵ. The comparison of the term schemes (b) to (d) resulting from these investigations with the term scheme for deoxygenated sperm whale myoglobin from Eicher et al. (1976) show besides the differences in the ϵ_3-value a significant larger ϵ_2-value for deoxygenated human haemoglobin than for deoxygenated sperm whale myoglobin. That means, that in deoxygenated haemoglobin (tensed structure) the distance Fe—N_ϵ is significantly smaller than in deoxygenated myoglobin (relaxed structure). This fits well into the model of Braunitzer et al. (1974). For a detailed discussion of the mechanism of O_2-affinities additional investigations are still necessary. Mössbauer and susceptibility measurements of Fe^{+3}-haemoglobins are reported by Thomanek et al. (1976).

The final differences between the experimental data and the fitted theoretical curves may be caused simply by the fact, that different samples were used. As well known (Antonini et al., 1962), the oxygen affinity of deoxygenated human haemoglobin depends sensitively on the surrounding medium, i.e. the p_H-value and the salt concentration. In this paper we compare three samples, which differ in p_H as well as in the salt concentration. The Mössbauer data were measured with a sample which contained practically no salt, $p_H = 7$. In Nakano et al. (1971) $p_H = 7.4$ and a 0.1 M phosphate buffer are used for the samples, whereas Alpert et al. (1975) made their experiments with $p_H = 6.5$—8.0 and an 0.1 M phosphate buffer. As already mentioned the distance of the ferrous iron to the haem plane as determined from X-ray structure analysis of crystals grown in a very concentrated salt solution seems to be slightly different from the corresponding value inferred from the Mössbauer data. We take this as a hint for the possibility, that the position of the iron is affected by the p_H-value or the salt concentration of the solution. For a final conclusion a more definite set of level scheme parameters should be expected, when Mössbauer and susceptibility data from one and the same sample are available. Such investigations on different haemproteins are presently in progress.

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