Perutz, M. F., Heidner, E. J., Ladner, J. E., Beetlestone, J. G., Ho, C., & Slade, E. F. (1974c) *Biochemistry 13,* 2187.
Pörschke, D. (1976) *Rev. Sci. Instrum. 47,* 1363.
Reilly, C. A., McConnell, H. M., & Meisenheimer, R. G. (1955) *Phys. Rev. 98,* 264.
Salhany, J. M. (1974) *FEBS Lett. 49,* 84.
Scheidt, R. W., Brinegar, A. C., Ferro, E. B., & Kirner, J. F. (1977) *99,* 7315.
Scheler, W., Schoffa, G., & Jung, F. (1957) *Biochem. Z. 329,* 232.
Sinn, E., Sim, G., Dose, E. V., Tweedle, M. F., & Wilson, L. J. (1978) *J. Am. Chem. Soc. 100,* 3375.
Stryer, L., Kendrew, J. C., & Watson, H. C. (1964) *J. Mol. Biol. 8,* 96.
Tan, A. L., & Noble, R. W. (1973) *J. Biol. Chem. 248,* 7412.
Tan, A. L., Noble, R. W., & Gibson, Q. H. (1973) *J. Biol. Chem. 248,* 2880.
Tweedle, M. F., & Wilson, L. J. (1976) *J. Am. Chem. Soc. 98,* 4824.

Influence of Quaternary Structure of the Globin on Thermal Spin Equilibria in Different Methemoglobin Derivatives†

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ABSTRACT: We have measured the paramagnetic susceptibilities of sperm whale azide metmyoglobin and of carp azide, thiocyanate, and nitrite methemoglobin in the quaternary oxy (R) and deoxy (T) structures between about 300 and 90 K, using a new sensitive superconducting magnetometer. We have also measured the pressure dependence of the high- and low-spin optical absorption bands of azide metmyoglobin and of carp azide methemoglobin in the R and T structures between 1 and 2000–4000 atmospheres. At low temperatures all the derivatives show normal Curie behavior, but above 200–250 K this is reversed, so that a thermal spin equilibrium is set up and the paramagnetic susceptibilities rise steeply with rising temperature. At all temperatures the effective magnetic moments in the T structure are higher than in the R structure. The magnetic data for azide methemoglobin have been subjected to detailed analysis. Below 250 K the magnetic moment in the R structure is 1.98 μ_B, characteristic of pure low spin, but that in the T structure is 2.80 μ_B, suggestive of a random mixture of high- and low-spin centers which have become frozen in by the immobility of the surrounding protein. Comparison of the thermal spin equilibria above 250 K shows that in the T structure the equilibrium is biased toward higher spin by the equivalent of about 1 kcal/mol relative to the R structure. Hydrostatic pressure reduces the optical density of the high-spin band at 630 nm and increases that of the low-spin bands at 541 and 573 nm. We have calibrated the optical density of the band at 630 nm against the measured paramagnetic susceptibilities of sperm whale azide metmyoglobin and carp azide methemoglobin in the R and T structures and have used this calibration to determine the dependence of the spin equilibria on hydrostatic pressure; this has allowed us to calculate the volume contraction associated with the transition from the fully high to the fully low-spin state. This amounts to −6.7 and −13.3 mL/mol heme for carp azide methemoglobins in the R and T structures, respectively, and to −12.5 mL/mol heme for azide metmyoglobin. These volume contractions are larger than those of about −4 mL/mol Fe found in synthetic iron chelates. Apparently stereochemical changes of the globin surrounding the heme also contribute to the volume changes; these must be larger in the T than in the R structure. The significance of these observations for the mechanism of heme–heme interaction is discussed.

The spin state of many transition metal ions depends on the symmetry and strength of the field created by the ligands surrounding them (Orgel, 1960; Figgis, 1966). The two important spin states of the ferrous ion are illustrated by deoxyhemoglobin ($S = 2$; high spin) and carbonmonoxyhemoglobin ($S = 0$; low spin). The effect of the spin change on uptake of oxygen cannot be examined separately from the accompanying change from the tense (T) to the relaxed (R) quaternary structure. In this paper we take advantage of thermal spin equilibria between high- and low-spin states present in some methemoglobin derivatives, and of the ability of P_6-inositol (IHP)[1] to switch these derivatives from the R to the T structure, to study the effect of the R → T transition on the spin equilibrium.

high spin, $S = 5/2$, R ⇌ low spin, $S = 1/2$, R
$-\uparrow\downarrow$ + IHP $-\uparrow\downarrow$ + IHP
high spin, $S = 5/2$, T ⇌ low spin, $S = 1/2$, T

In the preceding paper (Perutz et al., 1978) we have shown that this change in quaternary structure is sometimes accompanied by an increase in magnetic susceptibility, but the measurements were made at a single temperature on a relative scale, so that the full influence of the change in structure on

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[1] Abbreviations used; μ_B, Bohr magneton; IR, infrared; emu, electromagnetic units; IHP, P_6-inositol.

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the spin equilibrium was unclear. We have now extended the measurements over a wide range of temperature and put them on an absolute scale, using a new, highly sensitive, superconducting magnetometer (Cerdonio et al., 1976a). We have concentrated mainly on carp methemoglobin derivatives because they can all be switched to the T structure by IHP and have obtained useful results with the azide, thiocyanate, and nitrite, showing marked shifts in equilibrium toward higher spin on addition of IHP. The results on the thiocyanate and nitrite derivatives are difficult to interpret quantitatively because spin transitions may be accompanied by changes in the nature of the ligand atom which bonds to the iron. This cannot happen with the azide derivative which was therefore singled out for further theoretical and experimental work.

We regard the higher spin in the T structure as an expression of the tension or restraint that opposes the shortening of the Fe–N distances associated with a transition to the low-spin state. For purposes of understanding heme–heme interaction it is important to know how much of that tension is actually stored at the heme. We have attempted to answer that question by fitting the observed temperature dependencies of the paramagnetic susceptibilities of the R and T structures to theoretical curves calculated with variable values for the enthalpy and entropy of the spin equilibrium. We have also plotted directly the change in the spin equilibrium constant as a function of temperature and derived the free energy equivalent of the difference between the equilibrium constants in the R and T structures.

A change to higher spin is expected to involve a lengthening of the Fe–N distances (see Table I of Perutz et al., 1978) and should therefore be accompanied by an expansion in molecular volume. Conversely, a volume contraction produced by hydrostatic pressure should bring about a transition to lower spin. The pressure dependence of the spin equilibrium should allow determination of the volume change accompanying the spin transition.

Thermal spin equilibria in methemoglobin derivatives have been studied extensively (for a review, see Iizuka & Yonetani, 1970). Such equilibria have also been observed in other iron complexes (for a review, see Barefield et al., 1968; for a comprehensive list of recent references, see Sim et al., 1978). Most ferric iron complexes remain in the same spin state over a wide temperature range and thus obey the Curie–Weiss Law, $\chi \equiv 1/(T - \theta)$, where T is the absolute temperature and θ is a constant, but in some complexes the separation in energy between the low- ($^2T_2$) and high- ($^6A_1$) spin states is so small that it approaches the thermal energy (Figure 1). Such compounds exhibit a temperature-dependent spin equilibrium. If the low-spin state is the ground state, they show a region at low-temperature where they are pure low spin ($S = 1/2$) and obey the Curie–Weiss law, then a transitional region where their paramagnetic susceptibility rises with rising temperature until the high- ($S = 5/2$) and low-spin states are equally populated in proportion to their spin degeneracies, and finally a region of mixed spin where the Curie–Weiss law is again obeyed (Figure 1). This state of mixed spin may be contrasted with the true intermediate spin of $S = 3/2$ which has been observed in only a few synthetic compounds. One example is [Fe(III)-octaethylporphin](ClO_4) in which the coordination about the iron is square planar because the ClO_4^- is bound to the iron only very weakly (Dolphin et al., 1977). The state of $S = 3/2$ is best distinguished from a spin equilibrium by its temperature-dependent magnetic susceptibility and Mössbauer spectrum.

The pressure dependencies of the visible absorption spectra of hemoglobin and myoglobin derivatives have also been studied extensively (Fabry & Hunt, 1968; Gibson & Carey, 1975; Zipp et al., 1972; Ogunmola et al., 1977). High-spin methemoglobins in which the sixth ligand is weakly bound are reversibly denatured, at pressures as low as 1 kbar at some pHs, to a low spin form in which the distal imidazole probably becomes the sixth ligand. Low-spin derivatives such as cyanomethemoglobin can withstand pressures of up to 8 kbar without denaturation. There have also been studies of the effect of pressure on the electronic and Mössbauer spectra of ferric iron porphyrins which showed the appearance of mixed or intermediate spin states and a reduction of the iron to the ferrous state (Grenoble et al., 1971). However, the pressures used in these experiments ranged from 20 to 140 kbars, whereas those in our experiments did not exceed 4 kbars.

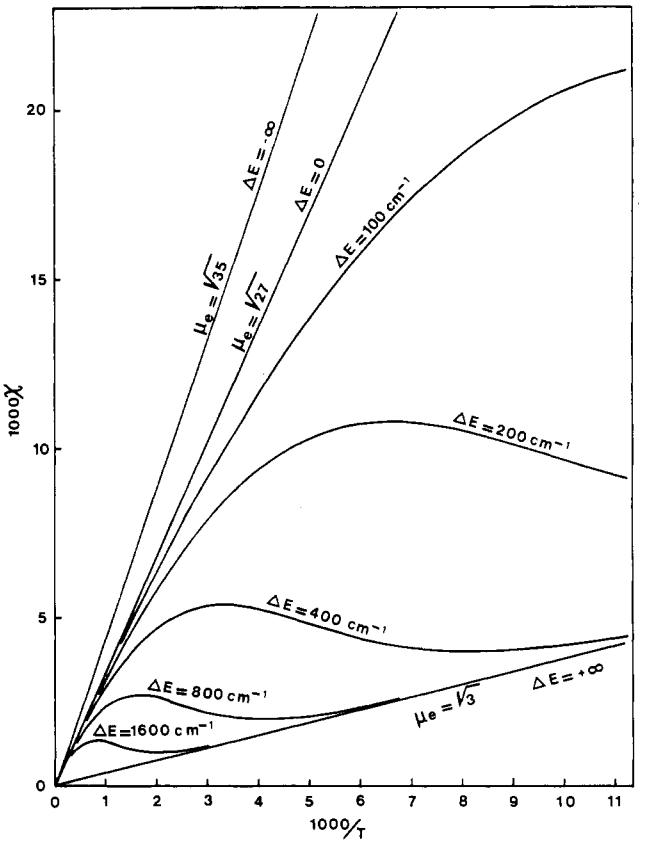


FIGURE 1: Temperature dependence of paramagnetic susceptibility calculated from $[35(3e^{-\Delta E/kT}) + 3]/(3e^{-\Delta E/kT} + 1)$, where k is the Boltzman constant, T the temperature in K, and E the difference in energy between the high-spin $^6A_1$ and the low-spin $^2T_2$ states. The effective magnetic moment $\mu_e = 2.828\sqrt{\chi_A T}\,\mu_B$. The top line $\Delta E = -\infty$ represents a pure high-spin compound, the next $\Delta E = 0$ a mixed-spin compound, and the lowest line $\Delta E = +\infty$ a pure low-spin compound. The curves represent thermal spin equilibria for various values of E. Note that at high temperature these all converge onto the line of $\Delta E = 0$. The low-spin case represents $\mu_e = 2\sqrt{S(S+1)} = \sqrt{3}\mu_B$. Due to spin orbit, coupling values of $\mu_e \leqslant \sqrt{5}\,\mu_B$ are known to occur.

Methods

Stripped human or carp methemoglobin was prepared by oxidation of solutions of oxyhemoglobin with a threefold molar excess of ferricyanide. After 30 min at 4 °C the solutions were passed through a Sephadex G-25 column to remove ferricyanide and NaCl. They were then passed slowly through a Dintzis column to deionize them completely and concentrated to 10–20 mM heme by ultrafiltration in an Amicon cell. For magnetic work 10 volumes of such hemoglobin solutions were mixed with 1 volume of 1 M heme ligand (NaN_3, $NaNO_2$, NaSCN) in an ice bath. The pH was adjusted to 6.5 for human and 6.0 for

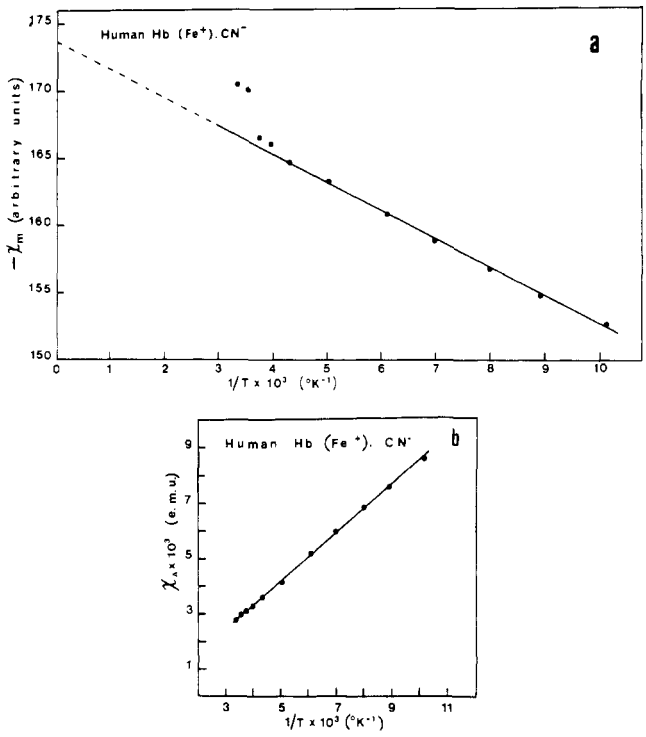


FIGURE 2: (a) Raw values of the magnetic susceptibility of human cyanometmyoglobin as a function of temperature. (b) The same data after correction for the diamagnetic contribution of the solvent.

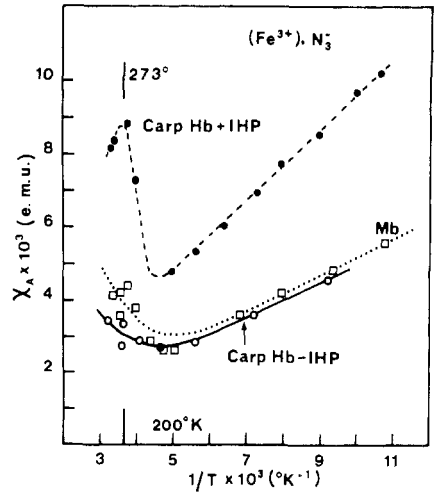


FIGURE 3: Magnetic susceptibilities of the azide derivatives of sperm whale metmyoglobin and of carp methemoglobin with and without IHP. The dotted curve represents an attempt to fit the myoglobin data to a curve calculated from eq 4 with the exponential terms multiplied by a factor $\gamma = 10$, and with $\Delta E \approx 1000$ cm^{-1}. Note that the fit is poor. The other two curves have been drawn to fit the data.

carp hemoglobin with Bistris or NaH_2PO_4, keeping the amount of buffer to a minimum so as not to weaken IHP binding. IHP was added in the form of a 0.2 M stock solution of pH 5.3, so that its addition caused little pH change, to a concentration of 1.5–2.0 M per hemoglobin tetramer. All solutions except the azide derivative were kept below 5 °C to avoid side reactions. Sperm whale metmyoglobin for the magnetic work was given to us as a lyophilized powder by Dr. T. Takano. It was dissolved in water to make up a 12 mM solution. Nine volumes of this solution was mixed with 1 volume of 0.1 M NaCN or 1 M NaN_3 of pH 6.5. Sperm whale myoglobin for the high-pressure work was obtained from Sigma Chemical Co. and used without purification.

Magnetic susceptibilities were measured between 300 and 90 K with the high resolution superconducting magnetometer (Cerdonio et al., 1976a), using a modified sample holder (Cerdonio et al., 1976b) which effectively nulls the contribution from the holder and allows molar susceptibilities to be measured at comparatively low magnetic fields, typically 60 oersteds. The sample volume was 0.15 cm^3. To check for possible stabilization effects in our samples (Yonetani et al., 1972), the temperature range was scanned at least twice, starting from room temperature and back. The diamagnetic contribution to the total molar susceptibility of the sample was subtracted by the following procedure: at low temperature all the susceptibilities obeyed a simple Curie law; this linear portion of the curve was extrapolated to infinite temperature. Its intercept with the $1/T = 0$ axis represents the diamagnetic contribution A which was then subtracted from the raw data, so that $\chi_m = \alpha(\chi_{au} - A)$, where χ_{au} is the total susceptibility in arbitrary units and α is a constant which includes the calibration constant of the instrument, the magnetic field and the mass of the sample. Above 220 K we also corrected for the temperature variation of the diamagnetism of ice near the freezing point and the jump in its diamagnetism at the freezing point (Cabrera, 1941; Cerdonio et al., 1977) by collecting blank data from solutions of other proteins of similar concentration and buffer conditions which follow a Curie law, such as carbonic anhydrase, lysozyme, alcohol dehydrogenase, and plastocyanine. Heme concentrations were determined by atomic absorption spectroscopy. The method was tested with human cyanomethemoglobin which is known to have a spin of $S = 1/2$ and to obey the Curie law. Figure 2 shows plots of the raw and of the corrected data. The value of $\mu_e = 2.59\ \mu_B$ agrees with that of 2.49 μ_B of Coryell et al. (1937) but is higher than that of 2.23 μ_B found by Iizuka and Kotani (1969b). As a further check we measured the temperature dependence of the paramagnetic susceptibility of sperm whale azide metmyoglobin. The slope of our curve in the linear range corresponds to $\mu_e^2 = 4.1\ \mu_B^2$ compared with 4.7 μ_B^2 of Iizuka & Kotani (1969a) (Figure 3).

The high-pressure optical bomb used was designed by Dr. W. B. Daniels and has been described elsewhere (Zipp, 1973). At each increment of pressure the system was allowed 5 min to regain temperature equilibrium. Measurements were made at both rising and falling pressures. For each test, the reversibility of the spectrum was checked by measurement of absorbance of the sample at 1 atm pressure (100 kPa) after it had gone through a cycle of pressure changes. The experiments were carried out at 21 ± 0.05 °C (Ogunmola et al., 1977).

Results

Temperature Dependence of Paramagnetic Susceptibility. At the lowest temperatures all the plots of χ vs. $1/T$ are linear; some are linear over the entire range (Figures 3–5). From the linear parts of the curves the effective magnetic moments μ_e can be derived:

$$\mu_e = \sqrt{\frac{3\chi kT}{N\mu_B{}^2}} = 2.828\sqrt{\chi T} \qquad (1)$$

where N is Avogadro's number and μ_B the Bohr magneton. The magnetic moments are listed in Table I. For all but one of the hemoglobins in the R structure and for myoglobin the moments have values characteristic for low-spin heme complexes, but for all carp hemoglobins in the T structure and for the thiocyanate derivative in the R structure their values are intermediate between low and high spin. In all instances the effective magnetic moment for the frozen carp hemoglobins in the

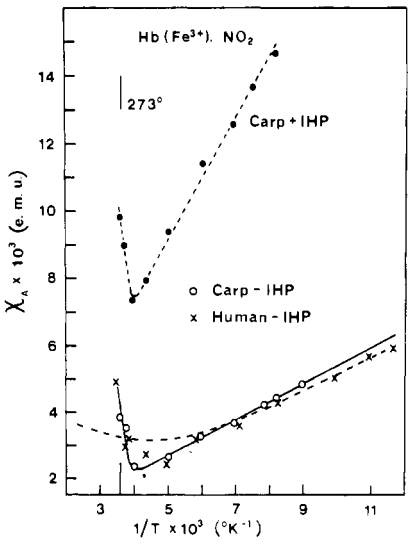


FIGURE 4: Magnetic susceptibilities of the nitrite derivatives of human and carp methemoglobin. Note that without IHP the susceptibilities of the two species are the same. The broken curve represents an attempt to fit the data to a curve calculated from eq 4. Note that it is much flatter than the observed curves.

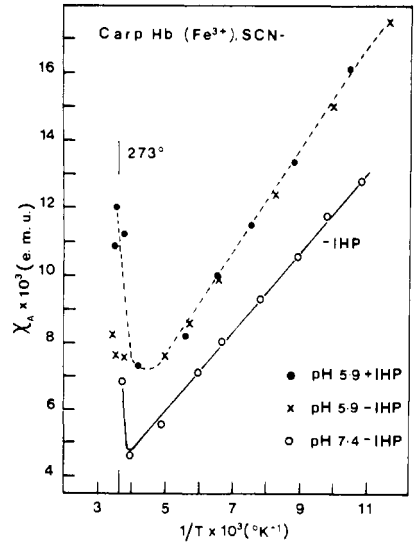


FIGURE 5: Magnetic susceptibilities of carp thiocyanate methemoglobin. Note that at low temperature the susceptibility at pH 5.9 is the same with or without IHP; only at high temperature does IHP raise the susceptibility at that pH. These data are in accord with Figure 3 of Perutz et al. (1978) which showed that carp thiocyanate methemoglobin at pH 5.9 is mostly in the T structure even in the absence of IHP.

T structure is larger than in the R structure. In solution, several derivatives show reverse Curie behavior characteristic of compounds in which there exists a thermal equilibrium between two spin states. This behavior persists after freezing down to temperatures of between 250 and 200 K. In all the derivatives of carp methemoglobin examined here the magnetic moment in solution in the T structure is larger than in the R structure (Table II); the rise just above the freezing point varies from 1.26-fold in thiocyanate to 1.84-fold in the nitrite derivative. The 1.52-fold rise in carp azide methemoglobin at room temperature corresponds to a 2.4-fold rise in magnetic susceptibility which agrees with the 2.5-fold increase found in the closely related trout IV azide methemoglobin by NMR and also with the increase calculated from the change in the relative intensities of the high- and low-spin infrared azide stretching

TABLE I: Effective Magnetic Moments of Hemoglobin and Myoglobin Derivatives in the Curie Range at Low Temperature.

derivative	species	IHP	$\mu_e \pm 0.05\ \mu_B$	α^a (high-spin fraction)
$Hb^+NO_2^-$	carp	+	3.80	0.34
$Hb^+NO_2^-$	carp	−	2.07	0
$Hb^+N_3^-$	carp	+	2.80	0.12
$Hb^+N_3^-$	carp	−	1.98	0
Hb^+SCN^-	carp	+	3.49	0.26
Hb^+SCN^-	carp	−	3.09	0.18
Hb^+CN^-	human	−	2.59[b] (2.23)[c]	0
Hb^+H_2O	human (0–30 °C)	−	5.3 (±0.1)	0.78
$Mb^+N_3^-$	sperm whale		2.03 (2.17)[c]	0

[a] α is calculated as in Table II. [b] The higher value of μ_e for human Hb^+CN^- than for the derivatives of carp hemoglobin − IHP must be attributed to a larger orbital contribution since all these derivatives are low spin, $S = 1/2$. [c] The values in brackets are those found by Iizuka & Kotani (1969b).

TABLE II: Rise in Effective Magnetic Moment μ_e on Addition of IHP in Solution.

derivative	T (K)	− IHP μ_e	− IHP α	+ IHP μ_e	+ IHP α^a
N_3^-	275	2.6	0.09	4.4	0.50
	300	2.9	0.14	4.4	0.50
SCN^-	275	3.9	0.36	4.9	0.64
NO_2	275	2.5	0.07	4.6	0.56

[a] α is the fraction high spin, obtained by assuming $\mu_{LS}^2 = 4.0$ and $\mu_{HS}^2 = 35$. $\alpha = (\mu_e^2 - 4)/31$.

frequencies of carp azide methemoglobin (see Perutz et al., 1978).

Carp azide methemoglobin in the T structure shows another interesting feature. While χ drops steeply with $1/T$ between 273 and 250 K, χ rises with $1/T$ from 303 to 273 K; in this range the high- and low-spin states are equally populated as shown by the magnetic moments in Table II. This is consistent with the IR spectra reported in the preceding paper which show the N_3^- stretching frequencies for the high- and low-spin components to be equal in intensity at 293 K.

We have also tried to get comparable curves for human hemoglobin but we encountered several difficulties. Human azide methemoglobin is not converted to the T structure by IHP. We have tried to measure the effect of IHP on human thiocyanate methemoglobin which is converted to the T structure and is also relatively stable, but obtained discontinuities in the χ vs. $1/T$ curve which we could not interpret. NMR studies have shown its paramagnetic susceptibility to rise by only 12% at 32 °C (see preceding paper). This result, and some preliminary measurements with human nitrite methemoglobin, indicated that the transition from the R to the T structure has a smaller effect on the heme in human than in carp hemoglobin.

Human aquomethemoglobin between 0 and 30 °C has $\mu_e = 5.3\ \mu_B$ and over this short temperature range obeys the Curie law. This susceptibility corresponds to a state of mixed spin in which the two spin states of $S = 1/2$ and $S = 5/2$ are equally populated independent of temperature according to their spin degeneracies, because the thermal energy is greater than E, the difference in energy between the two states ($\chi = (1/$

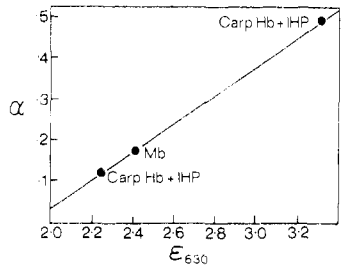


FIGURE 6: Calibration of α, the fraction of high-spin component, vs. ϵ_m, the extinction coefficient at 630 nm for the azide met derivatives of sperm whale myoglobin (Mb) and carp hemoglobin ± IHP at 20 °C.

$8T)\cdot[(2 \times 3) + (6 \times 35)]/(2 + 6)$. This explains why no further rise in susceptibility occurs on transition of aquomethemoglobin from the R to the T structure (Gupta & Mildvan, 1975; Perutz et al., 1978).

Pressure Dependence of the Paramagnetic Susceptibility. Our pressure bomb was designed to record optical rather than magnetic changes. We therefore concentrated on the azide derivatives, because the relationship between their optical absorption and paramagnetic susceptibilities can be calibrated on the basis of both magnetic measurements and IR spectra. Asuming values of $\chi = 0.014$ and 0.0022 emu for the high- and low-spin components, respectively, it can be shown that the absorption coefficient ϵ_m (630 nm) is related to α, the fraction of high-spin component, by the equation $\alpha = 0.032 + 0.348\cdot(\epsilon_{630} - 2)$ (Figure 6). The changes in ϵ_m with hydrostatic pressure of myoglobin and of carp hemoglobin with and without IHP are shown in Figure 7a. Absorption at the "high-spin band" at 630 nm rises and at the "low-spin bands" at 541 and 573 nm falls with rising pressure. These spectral changes were reversible and took place at lower pressures than the gross spectral changes which characterize denaturation (Zipp & Kauzmann, 1973). From points in Figures 6 and 7a we can calculate the spin equilibrium $K = [^6A_1]/[^2T_2]$ as a function of pressure (Figure 7b). Thermodynamics gives

$$(\partial(\ln K)/\partial P)_T = -\Delta V/RT \qquad (2)$$

where ΔV is the change in volume which occurs when protein containing 1 mol of heme is converted from the high- to the low-spin form. The volume changes derived from eq 2 and Figure 7b are as follows:

	$-\Delta V$ (mL/mol heme)
sperm whale azide metmyoglobin	12.5 ± 1.7
carp azide methemoglobin − IHP	6.7 ± 1.3
carp azide methemoglobin + IHP	13.3 ± 1.0

This shows that the transition from the high- to low-spin state is accompanied by a volume contraction, as in other iron chelates, and that in hemoglobin in the T structure this contraction is twice as large as in the R structure. The contraction in myoglobin is near to that in the T structure of hemoglobin.

In attempting to understand the anomalously large value of ΔV associated with the spin transition of the T state of hemoglobin, it occurred to us that the quaternary structure itself might be changing under pressure. That is, we might be observing the process:

$$T\text{-}Hb(^6A_1) \rightarrow R\text{-}Hb(^2T_2)$$

rather than

$$T\text{-}Hb(^6A_1) \rightarrow T\text{-}Hb(^2T_2)$$

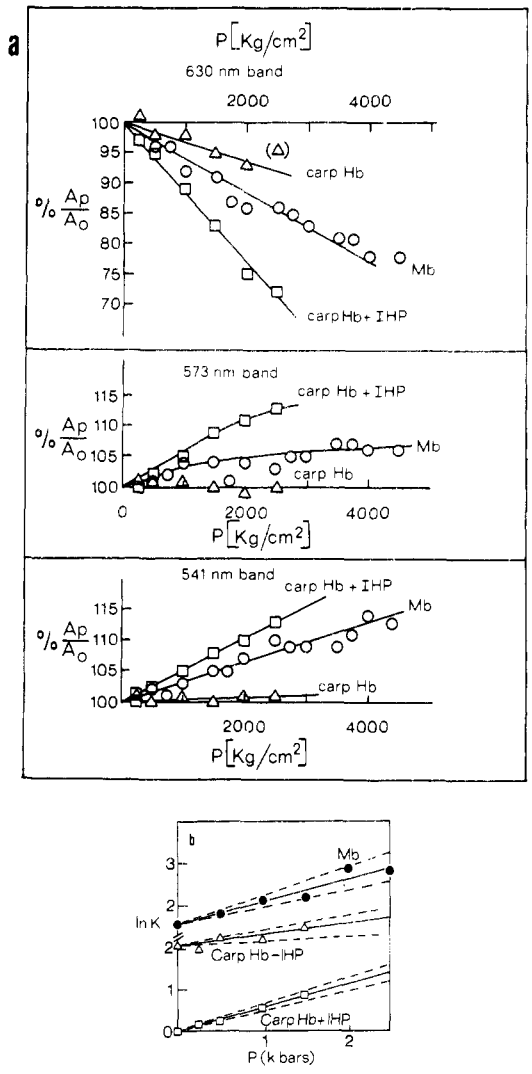


FIGURE 7: (a) Effect of hydrostatic pressure P on the optical densities of the visible absorption bands of myoglobin (Mb) and carp hemoglobin ± IHP. A_p = optical density at pressure P; A_0 = optical density at atmospheric pressure. All bands have been corrected for compression of water. (b) Spin equilibria (K) calculated from Figures 6 and 7a (top) as a function of hydrostatic pressure (P). The dotted lines indicate the error limits.

We feel that this is unlikely since no volume change has been observed in the binding of ferrous hemoglobin to dioxygen (Johnson & Schlegel, 1948). Thus, an anomalously large negative ΔV cannot be explained by this process. Another possibility we considered is that there might be a contribution to ΔV due to the dissociation of IHP; however, the larger than 50-fold excess of IHP used (1 mM), together with its high binding constant, would seem to ensure saturation at all pressures.

Analysis of Magnetic Results. If a transition metal compound exhibits two alternative spin states in thermal equilibrium, their populations should be proportional to

$$W_L \exp -\frac{E_L}{kT} \text{ and } W_H \exp -\frac{E_H}{kT} \qquad (3)$$

where W_L and W_H are proportional to the spin degeneracies $(2S_L + 1)$ and $(2S_H + 1)$, and E_L and E_H are the energies of the low- and high-spin states. If $\Delta E = E_H - E_L$, and μ_L and μ_H are the magnetic moments of the low- and high-spin states, respectively, the paramagnetic susceptibility χ_{Fe} should follow the equation

$$\chi_{Fe} = \frac{1}{8T}\frac{\frac{2S_H+1}{2S_L+1}e^{-\Delta E/kT}\mu_H^2 + \mu_L^2}{\frac{2S_H+1}{2S_L+1}e^{-\Delta E/kT} + 1} \quad (4a)$$

$$\chi_{Fe} = \frac{1}{8T}\frac{K\mu_H^2 + \mu_L^2}{K+1}, \quad (4b)$$

where $K = [HS]/[LS]$, i.e., the equilibrium constant between the high- and low-spin states.

However, Iizuka & Kotani (1969a,b) found that this was not true of any of their metmyoglobin or methemoglobin derivatives; the initial fall of χ with $1/T$ was always steeper than predicted by the simple theory. Our results confirm their findings. The broken line at the bottom of Figure 4 shows an example of a curve calculated from eq 3 in an attempt to fit the experimental results to those expected for a Boltzmann distribution of the two spin states; it shows that even in the R structure the observed fall in χ with $1/T$ is much steeper than the theoretically predicted one. In the azide derivatives, that fall becomes steeper as we go from the R structure of carp hemoglobin to myoglobin and to the T structure of carp hemoglobin.

Iizuka & Kotani (1969a) were able to fit their data by allowing the equilibrium constant K in eq 4 to have the more general form

$$K = \exp(-\Delta G/RT) = \exp\left(-\frac{\Delta H - T\Delta S}{RT}\right) \quad (5)$$

with constant, empirically chosen values for ΔH and ΔS. This more general form reduces to eq 4a when $\Delta H/R = \Delta E/k$ and $\Delta S = R \ln (2S_H + 1)/(2S_L + 1)$ ($= R \ln 3$ for $S_H = 5/2$ and $S_L = 1/2$), which is the entropy difference resulting from spin degeneracy alone. The value of μ_H^2 in eq 4 was taken to be 35 μ_B^2, the theoretical value for the $S = 5/2$ state, but the value of μ_L^2 was determined empirically from the linear (low temperature) portion of the χ vs. $1/T$ plots; because of spin-orbit coupling, the value of μ_L^2 can vary upwards by a factor of up to about two from the theoretical value $\mu_L^2 = 3$ for $S = 1/2$ (Ewald et al., 1964).

Even in inorganic ferric complexes anomalously steep falls in χ with $1/T$ are found. In a study of several such complexes, Ewald et al. (1964) found that curves of χ vs. $1/T$ could be fitted only by inclusion of an entropic factor exceeding that expected from spin-degeneracy alone by about 1.6 cm^{-1} deg^{-1} (4.6 cal mol^{-1} deg^{-1}); the extra entropic factor was required even when the theory took proper account of the moderate temperature dependence of μ_L^2 due to spin-orbit coupling. The physical basis of the required entropic factor is not fully understood (Hall & Hendrickson, 1976). In ferric heme proteins one may expect additional entropic and enthalpic effects on the spin-state equilibria resulting from structural changes in the protein induced by contraction of the iron–ligand bond lengths in going from the high- to the low-spin state.

A difficulty in applying the procedure of Iizuka & Kotani to fit our data by means of eq 4 and 5 is that the empirical values of μ_L^2 (as determined from the slope of the χ vs. $1/T$ plots at low temperature and listed in Table I) for the T state hemoglobins are much higher than the maximum plausible value of about 6 μ_B^2 expected for the low spin $S = 1/2$ state. The possibility of a ground state with $S = 3/2$ can be excluded at least for T state azide hemoglobin, for its experimental value of $\mu_L^2 = 7.84$ μ_B^2 (Table I) is well below the value $\mu_L^2 = 15$ μ_B^2 expected for the $S = 3/2$ state. A plausible hypothesis to account for this anomaly is to suppose that, below some temperature, T_0, the globin and surrounding ice become too rigid

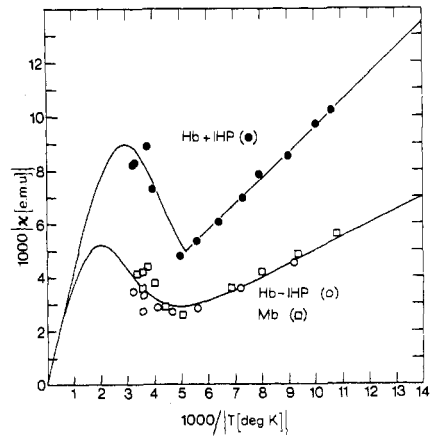


FIGURE 8: Comparison of observed temperature dependence of paramagnetic susceptibility of azide met derivatives of myoglobin (Mb) and carp hemoglobin with curves calculated from eq 4b and 5. Values used were $\mu_H^2 = 35$ μ_B^2, $\mu_L^2 = 4.0$ μ_B^2 for all derivatives; $\Delta H = 2.6$ kcal mol^{-1}, $\Delta S = 5.7$ cal mol^{-1} deg^{-1} for Mb and Hb – IHP; $\Delta H \mp 2.4$ kcal mol^{-1}, $\Delta S = 8.3$ cal mol^{-1} deg^{-1} for Hb + IHP; in the latter case the equilibrium constant $K = K(T_0)$ for $T < T_0 = 193$ K.

to accommodate the change in iron bond lengths that accompany spin changes, so that a random mixture of high- and low-spin hemes becomes frozen in and thermal equilibrium cannot be attained at temperatures below T_0; this hypothesis may be incorporated into eq 5 by assuming that for temperatures below T_0 the equilibrium constant K has the fixed value $K = K(T_0)$. Such freezing-in of spin equilibria has been observed by Mössbauer spectroscopy in cyanate methemoglobin by Winter et al. (1972) and in $[Fe(II)(2\text{-aminomethylpyridine})_3]^{2+}I_2$ by Renovitch & Baker (1967).

We have determined the parameters of eq 4 and 5 required to fit our data for the azide derivatives by means of a least-squares procedure in which the values $\mu_H^2 = 35$ μ_B^2 and $\mu_L^2 = 4.0$ μ_B^2 were assumed. The data for azide metmyoglobin and hemoglobin – IHP were fitted together with a single pair of values ΔH, ΔS; in fitting the data for azide hemoglobin + IHP the additional parameter T_0 was included to represent the temperature below which spin equilibrium is assumed to be frozen in. A reasonably good fit was obtained, as is indicated in Figure 8, where eq 4b is plotted for the values $\Delta H = 2.6$ kcal mol^{-1} and $\Delta S = 5.7$ cal mol^{-1} deg^{-1} for myoglobin and hemoglobin – IHP, and with $\Delta H = 2.4$ kcal mol^{-1}, $\Delta S = 8.3$ cal mol^{-1} deg^{-1} and freeze in temperature $T_0 = 193$ K for hemoglobin + IHP. However, the values of ΔH and ΔS are subject to considerable uncertainty, especially in the case of hemoglobin + IHP, because the two parameters are highly correlated ($C = -0.99$) in the fitting procedure. This situation can be illustrated graphically by replotting the data in terms of the apparent equilibrium constant K', between the high- and low-spin states defined as

$$K' = \frac{8\chi T - \mu_L^2}{\mu_H^2 - 8\chi T} \quad (6)$$

The right-hand side of eq 6 is the solution of eq 4b for K; hence, if the data fit eq 4b and 5, then $K' = K$ and $-\ln K' = \Delta G/RT = (\Delta H/R)(1/T) - (\Delta S/R)$, so that ΔH and ΔS can be determined from the slope and intercept, respectively, of a plot of $-\ln K'$ against $1/T$. Such a plot is illustrated in Figure 9 for our data for the azide derivatives; the straight lines are drawn to correspond to the values of ΔH and ΔS corresponding to the curves in Figure 8.

It is seen that the data fit the linear approximation only in the region $1/T < 5 \times 10^{-3}$ deg^{-1}, i.e., only outside the region

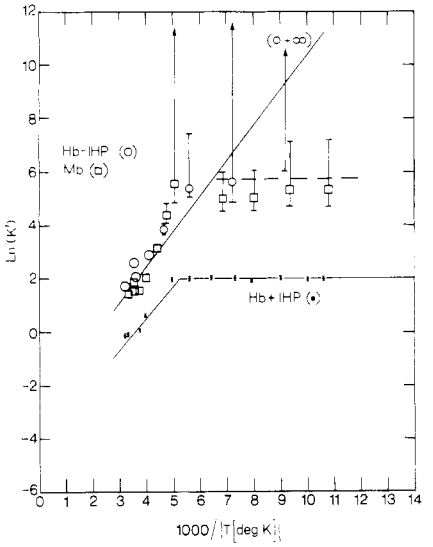


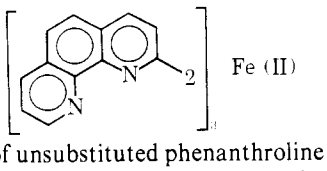
FIGURE 9: Data and theoretical curves of Figure 8 replotted as the negative logarithm of the apparent equilibrium constant, K', calculated from eq 6 with $\mu_H^2 = 35\ \mu_B^2$ and $\mu_L^2 = 4.0\ \mu_B^2$. The dashed line indicates the low-temperature asymptote of $-\ln K'$ expected for the myoglobin data resulting from the discrepancy between the assumed value $\mu_1^2 = 4.0\ \mu_B^2$ and the experimental value $\mu_L^2 = 4.1\ \mu_B^2$ (Table I) for the myoglobin data; values of $-\ln K'$ for $1/T$ less than about 5×10^{-3} deg^{-1} are not appreciably affected by this discrepancy. Error bars are drawn for an arbitrarily assumed error of ±3% in χ for all data and are included primarily to indicate that the low-temperature data are not useful for determining ΔH and ΔS. Error bars have been omitted where they would lie within the data point symbols.

of low-temperature Curie behavior. In the case of the myoglobin and hemoglobin − IHP derivatives this is simply because in the Curie region $8\chi T$ approaches μ_L^2 (see eq 1 and Table I), and eq 6 reveals that K' then fluctuates near zero owing to experimental scatter, so that $-\ln K'$ may be very large or even undefined ($K < 0$); this is indicated by the large error bars on the points corresponding to $1/T > 5 \times 10^{-3}$ deg^{-1}, which have been drawn for a constant 3% error in χ. In the case of hemoglobin + IHP the values of $-\ln K'$ flatten out at low temperature because the constant value of the slope of χ vs. $1/T$ in the Curie region is much greater than that corresponding to $\mu_L^2 = 4.0$. As previously suggested, this is probably due to a freezing in of the equilibrium at temperatures below $T_0 \approx 200$ K, so that K' is constant below that temperature. Thus it is only the data in the narrow range $1/T = 3$ to 5×10^{-3} deg^{-1} that can be used to determine the value of ΔH and ΔS and these data are too meager to determine separately the slope and intercept of the fitting lines. However, if it is accepted that a simple spin equilibrium does obtain within this temperature range, then we can estimate the free energy difference $\Delta(\Delta G) = \Delta G(-\text{IHP}) - \Delta G(+\text{IHP})$ that stabilizes the high-spin state in hemoglobin + IHP relative to its stability in hemoglobin − IHP. Since the vertical scale in Figure 9 is $\Delta G/RT$, the separation in height of the data points ± IHP gives $\Delta(\Delta G/RT)$. Thus at 300 K the difference in $-\ln K'$ is about 1.5 to 2.0, so $\Delta(\Delta G) = (1.5 \text{ to } 2.0) \times 1.98$ cal mol^{-1} deg^{-1} × 300 deg = 0.9 to 1.2 kcal, in agreement with the value derived from the IR measurements reported in the previous paper. At 250 K the difference is 1.3 to 2.3, so $\Delta(\Delta G) = 0.6$ to 1.1 kcal.

How does the stabilization of the $^6A_1$ state in hemoglobin + IHP relative to hemoglobin − IHP compare with the difference in oxygen affinity? The data of Tan et al. (1972) show that the mean affinities ($\log p_{50}$) of the R and T structures of carp hemoglobin equal −0.20 and +2.10, respectively, which correspond to a difference in binding energy of 3.1 kcal/mol heme, or about three times the difference in stabilization energy of the $^6A_1$ state of the azide met derivative (but see Note Added in Proof at end of paper).

Comparison of Hemoglobin with Synthetic Iron Chelates. A survey of the literature on the magnetic properties of synthetic iron chelates shows that there is nothing unique about the properties of hemoglobin. We have already mentioned that the freezing in of a thermal spin equilibrium to a mixture of low- and high-spin states in constant proportions has been observed in $[Fe(II)(2\text{-aminomethylpyridine})_3]^{2+}I_2$ (Renovitch & Baker, 1967). If the rise in χ with T in the non-Curie portions of the hemoglobin curves is steeper than predicted from Boltzmann statistics with weighting based on spin degeneracy alone, this is also true of the majority of synthetic iron chelates. The only compound we could find that does follow Boltzmann statistics based on such weighting is crystalline $[Fe(II)(5R\text{-phenanthroline})_2(SCN)_2]\cdot H_2O$, where R is either Cl or NO_2 (Cunningham et al., 1972). Most magnetic work has been done on crystalline powders which often show very sharp transitions from low to high spin at some critical temperature, probably because of cooperative effects in the crystal lattices, but even iron complexes in solutions show steeper transitions than predicted by the simple theory (Ewald et al., 1964; Beattie et al., 1973; Hoselton et al., 1975; Tweedle & Wilson, 1976). In crystalline samples, plots of the spin equilibrium constant against $1/T$ are often nonlinear, showing that there is not a simple intramolecular equilibrium; in solution such plots are mostly linear. If the spin equilibrium constant is defined as in eq 4 and the low-spin state is the ground state, then ΔH and ΔS are always positive, but ΔS is always larger than the value of 2.2 kcal/mol expected from the change in spin degeneracy alone. The excess ΔS has been attributed to the stereochemical changes accompanying the spin changes. The values of ΔH and ΔS found for azide metmyoglobin and hemoglobin by Iizuka & Kotani (1969a,b) are also positive and ΔS is several times larger than 2.2. Our results for myoglobin and for hemoglobin − IHP are consistent with Iizuka & Kotani's, though our analysis is not precise enough to decide whether the changes in free energy of the spin equilibrium are enthalpic or entropic in origin. Otsuka (1970) suggested that these large entropy changes might be due to temperature variations in the position of the porphyrin ring relative to the iron atom, brought about by the cooperative making or breaking of van der Waal contacts between the porphyrin and the globin.

An alternative to such steric effects might be inductive effects due to changes in the positions of electron-donating or withdrawing groups relative to the hemes or to changes in the orientation of its carboxylate and vinyl side chains. If synthetic complexes are any guide, they suggest that steric effects generally dominate over inductive ones. This is illustrated by the effect of substituting methyl for hydrogen in position 2 of $[Fe(II)(1,10\text{-phenanthroline})_3]$.



Ferrous salts of unsubstituted phenanthroline are diamagnetic, whereas those of the 2-methylphenanthroline show a thermal spin equilibrium (Goodwin & Sylva, 1968). The inductive effect of a methyl group in position 2 increases the electron density at, and hence the basicity of, the neighboring nitrogen; this is shown by the methyl-substituted phenanthroline being a stronger base than the unsubstituted one. In terms of the inductive effect alone, therefore, the former is

likely to bind more strongly to the iron than the latter. However, the inductive effect of the methyl group is offset by its steric effect in preventing the iron atom approaching the nitrogen as closely as in its absence. In a related series of complexes with hexadentate ligands, the paramagnetism increases with the number of methyl substituents (Hoselton et al., 1975), again showing the dominance of steric effects.

In heme proteins inductive effects may differ where there are marked differences between the amino acid residues in the heme pocket, such as exist between hemoglobin and myoglobin, but in mammalian hemoglobins these residues hardly vary. With the exception of the proximal and distal histidines all the amino acid side chains surrounding the hemes are nonpolar. On going from the R to the T quaternary structure their positions relative to the heme change only slightly, nor are there any significant changes in the orientation of the porphyrin side chains (Fermi, 1975; Ladner et al., 1977). For all these reasons inductive effects are unlikely to play any significant part in the phenomena discussed here.

Finally, one might envisage that an intermediate spin state of $S = 3/2$ might play a part in some of the phenomena observed here, but this has been found only in porphyrins where axial bonds are either absent or extremely weak, such as the [Fe(III)octaethylporphin](ClO_4) structure already mentioned (Dolphin et al., 1977). It is unlikely, therefore, that this spin state exists in any of the compounds studied here, but the question can be answered with certainty only by Mössbauer spectroscopy. A pure $S = 3/2$ state is ruled out for azide methemoglobin of carp in the T structure because its squared magnetic moment is far below 15 μ_B^2.

Influence of Quaternary Structure on the Spin Equilibrium. We have tried to present a tentative structural interpretation of the experimental findings in the form of a schematic diagram (Figure 10). This shows the heme pocket with the heme clamped between helices E and F. The proximal and distal histidines have been omitted. We have assumed that the Fe-N bonds in the low-spin complex are too strong to be affected by the globin and have therefore drawn this complex with the same geometry in the R and T structures. In the R structure, both the complex and the globin are relaxed and the free energy of the $^2T_2$ state is therefore low. In the T structure, on the other hand, an octahedrally coordinated heme complex with the iron atom in the porphyrin plane is a misfit and the complex is therefore under stress, so that the free energy of the $^2T_2$ state is raised. However, the resulting strain is transferred to the subunit boundaries and is indicated in Figure 2c by the stretched spring.

We now come to the high-spin $^6A_1$ complex. Synthetic iron porphyrin complexes showing the same N_3^- stretching frequency as high-spin azide hemoglobin are five coordinated with the iron displaced from the porphyrin plane toward the azide ion (Hoard, 1975). We therefore assume that this is the form with the lowest free energy. The R quaternary structure imposes regular octahedral coordination. We would therefore expect it to impose a strain on the complex and on the quaternary structure. We do not know how this strain affects the complex, but have indicated its presence by the "bent" Fe-N_{porph} bonds and the slight distortion of the heme pocket in Figure 10b, and by the high level of the $^6A_1$ state in the energy level diagram between Figures 10a and 10b.

The T structure stabilizes five-coordinated or pseudo-five-coordinated complexes; if presented with a six-coordinated complex it tends to stretch or break the bond from the iron to either the sixth ligand or N_ϵ of the proximal histidine (Pulsinelli et al., 1972; Perutz et al., 1976; Fermi & Perutz, 1977). Since we expect the Fe^+-N_3^- bond to be the stronger of the two

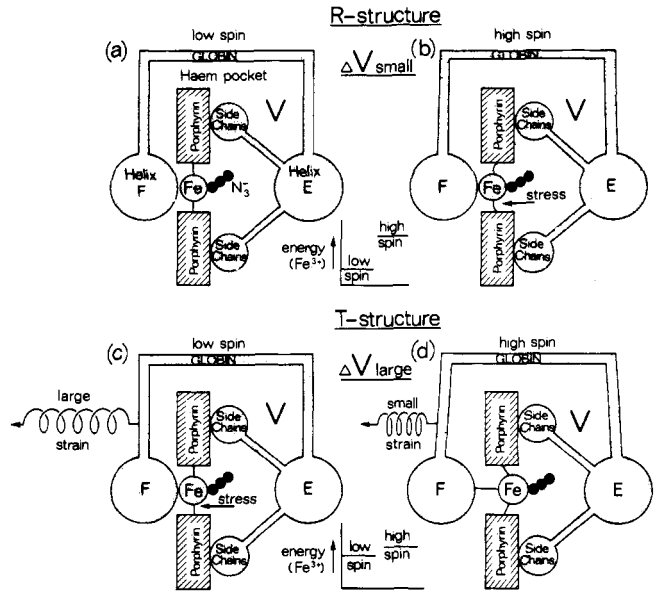


FIGURE 10: Tentative diagram illustrating possible structural changes accompanying the spin transitions in the two alternative quaternary structures. (a) The $^2T_2$ iron nitrogen complex is relaxed, as is the globin; the volume of the heme pocket (V) is at a minimum. (b) The $^6A_1$ iron is trying to move out of the porphyrin plane toward the azide ion, but is held back by the proximal histidine. Fe-N bonds are weakened by occupation of the E_g orbitals. V is slightly enlarged. (c) The octahedral $^2T_2$ iron nitrogen complex is under stress, but its bonds are too strong to be stretched so that the strain is transferred to the protein. (d) The protein stabilizes the pseudo-five-coordinated Fe-N complex with a stretched bond to N_ϵ of the proximal histidine; V is much increased. The widening of the heme pocket in b and d is drawn grossly exaggerated and may in fact not happen. One could equally well imagine that the extra volume changes associated with the spin transitions take place at the subunit contacts, and that the Fe-N_3 bond is stretched rather than the Fe-N_ϵ bond.

bonds, we have drawn the high-spin complex in the T structure with the Fe-N_ϵ bond stretched and the iron displaced from the porphyrin plane toward the azide ion. Stretching of this bond has also been observed in model compounds. In the unhindered [(1-methylimidazole)-$\alpha,\beta,\gamma,\delta$-tetraphenylporphinatocobalt(II)], the length of the Co-N_ϵ bond is 2.157 Å, but, in the corresponding bis(piperidine) complex in which the approach of the piperidine nitrogens to the cobalt atom is sterically hindered, the Co-N_ϵ distance is stretched to 2.436 Å (Scheidt, 1974). Alternatively the Fe-N_3 bond might be stretched, a possibility favoured by the high spin azide stretching frequency being close to that of the free azide ion. We have assumed that either one or the other of these conformations lowers the free energy of the high-spin complex without having to pay a large price in terms of strain at the subunit contacts. The spring in Figure 10d is therefore shown more relaxed than in Figure 10c. We have also drawn the heme pocket exaggeratedly widened, to conform with the larger volume increase associated with the $^2T_2 \rightarrow {}^6A_1$ transition in the T structure. The energy level diagram between Figures 10c and 10d shows the difference in energy of the two states to have diminished, conforming with the magnetic moments in Table II and with the IR measurements reported in the preceding paper which show the two states to have become equally populated.

We have tried to test our ideas concerning the nature of the high-spin azide complex in the T structure by x-ray analysis, but failed to crystallize it. We did obtain very small crystals of deoxyhemoglobin of carp with which it should be isomorphous. These had the unit cell dimensions $a = 63.2$ Å, $b = 170$ Å, $c = 73.7$ Å, $\beta = 107°$ and the space group $P2_1$ with four molecules in the unit cell. With two hemoglobin tetramers per

asymmetric unit, this structure would be difficult to solve, even if single crystals of the azide derivative could be obtained.

Influence of Pressure and Temperature on the Spin Equilibrium. The volume changes associated with the $^6A_1 \rightarrow {}^2T_2$ transition in azide metmyoglobin and hemoglobin are in the same direction as those observed in simple iron chelates, but larger. For instance, the volume changes associated with the spin transitions of the octahedral FeS_6 complex in [Fe(III)-tris(dithiocarbamate)] were between 3.7 and 4.1 mL/mol, depending on the value assumed for $\mu_e(^2T_2)$. A molecular model showed that the solvent would have relatively free access to the FeS_6 core, and the authors therefore felt justified in assuming the entire volume change to be due to a change in radius of that core. Taking Fe-S = 2.75 Å, this gave a contraction in Fe-S bond length of 0.07 Å (Ewald et al., 1964). In azide methemoglobin, the maximum likely contraction in average Fe-N bond length would be from 2.10 to 1.98 Å (Hoard, 1975). If the solvent had access to the Fe-N core, this would produce a volume change of 3.7 mL/mol, close to the value found in the iron dithiocarbamate. In fact, the volume changes observed in azide methemoglobin are 6.7 mL/mol in the R and 13.3 mL/mol in the T structure. This means that there must be stereochemical changes in the protein associated with the spin transition and these must be larger in the T than in the R structure, presumably because the changes in Fe-N distances themselves are larger. We have tried to represent this in Figure 10 by drawing the high-spin iron six-coordinated in the R structure, but pseudo-five-coordinated in the T structure, and by making the changes in tertiary structure of the heme pocket also greater in the T than in the R structure.

In going from 300 to 200–250 K the magnetic moments of all our derivatives drop much more steeply than can be accounted for by the difference in spin degeneracy of the $^2T_2$ and $^6A_1$ states. Again, this must be due to stereochemical changes in the surrounding protein. We do not know what these are, but the simplest proposition would be that they consist mainly of changes in the width of the heme pocket. A rough calculation shows that such thermal changes might in fact influence the spin equilibrium. The pocket is lined with hydrocarbons. We may therefore expect its linear coefficient of thermal expansion to be of the same order as that of solid paraffin which is about 10^{-4} deg^{-1} at 273 K. The width of the pocket is defined by the distance apart of helices E and F which is about 20 Å. Cooling from 300 to 250 K would thus result in a contraction of about $20 \times 50 \times 10^{-4}$ Å = 0.1 Å. The observed movement of the ferric iron atom relative to the porphyrin plane in going from high- to low-spin complexes varies between 0.4 Å in myoglobin and 0.2 Å in the α chain of hemoglobin (Takano, 1977; Ladner et al., 1977). This shows that thermal contraction of the heme pocket over this temperature range may be of the same order as the movement of the iron atom relative to the porphyrin plane, and could therefore magnify the influence of temperature on the *spin equilibrium*. On the other hand, the freezing in of the spin equilibrium at 250° – 200°K implies that *spin transitions* cannot take place without structural changes in the protein which require a thermal activation energy greater than $\Delta E(^2T_2 - {}^6A_1)$.

Nitrite and Thiocyanate Methemoglobin. It was shown in the preceding paper that nitrite methemoglobin of carp exhibits very large spectral changes on addition of IHP and that its color actually changes from red to brown. These spectral changes are matched by large changes in magnetic susceptibility. Thiocyanate methemoglobin also shows marked spectral and susceptibility changes. Each of these ligands can combine with transition metals in two alternative ways: M-ONO or M-NO_2; M-SCN or M-NCS. In the first mode of ligation they lie nearer the high spin and in the second nearer the low-spin end of the spectrochemical series. The first example of an M-ONO $\rightleftharpoons$ M-NO_2 isomerization was discovered by Jørgensen in $[Co(NH_3)_5NO_2]Cl_2$ in 1894; it is sterically possible because NO_2 is not linear: the angle O-N-O = 134°. There is no reason why such isomerization should not take place in these hemoglobin derivatives on transition from the R to the T structure; inversion of the ligand on transition from low to high spin would weaken the Fe-ligand bond so that the steric requirements of the T structure could be satisfied by stretching it.

Sperm Whale Myoglobin. The magnetic behavior of sperm whale metmyoglobin resembles that of carp azide methemoglobin in the R structure, but the volume change associated with the spin transition is close to that of carp azide methemoglobin in the T structure. We have no explanation for this inconsistency, except the different construction of the heme pocket in the two proteins. They are lined with different residues, and the myoglobin pocket has a cavity on the proximal side of the heme which is large enough to bind xenon. This is absent in hemoglobin (Schoenborn et al., 1965; Schoenborn, 1965). The stereochemical rearrangements in the globin associated with the spin transition may therefore be quite different in the two proteins.

Implications for Mechanism of Heme-Heme Interaction. The free energy of heme-heme interaction is defined as $\Delta F = -RT \ln K_4/K_1$, where K_1 and K_4 are the association constants of the first and last molecules of oxygen to be taken up by hemoglobin. In terms of allosteric theory, these correspond respectively to K_T and K_R, the equilibrium constants of the quaternary deoxy and oxy structures. K_R is similar to the mean of the association constants of the free α and β subunits, but K_T is lower by the equivalent of more than 3 kcal/mol heme. Perutz (1972) suggested that this low affinity might be due to a tension exercised by the globin on the heme which opposes the movement of the iron atom from its position out-of-the plane of the porphyrin in deoxy to its in-plane position in oxyhemoglobin. He and his associates then attempted to measure this tension in deoxyhemoglobin from the shift of the near-infrared absorption bands that accompanies the R $\rightarrow$ T transition and found that the free energy equivalent amounted to only 300 cal (Perutz et al., 1974). Fluorescent x-ray absorption studies showed that the Fe-N bond distances of deoxyhemoglobin in the R and T structures differ by less than 0.02 Å (Eisenberger et al., 1976).

These results proved that the tension proposed by Perutz is small in the deoxy state and suggested that instead it may build up on ligation as the heme tries to take up its low-spin conformation (Warshel, 1977), so that it might be more appropriate to speak of a restraint rather than a tension (Collman, 1977). The present study was begun with the aim of finding a system in which that restraint could be measured. For this purpose it was necessary to find a derivative that could be switched from R to the T structure without dissociation of either the heme ligand or the proximal histidine and one which exhibits a measurable equilibrium between two thermodynamically distinct states of the heme in both these structures. Azide methemoglobin of carp has proved the ideal test compound because it is stable and exhibits a thermal spin equilibrium which can be measured over a wide temperature range. Transition from the R to the T structure leads to large changes in its electronic absorption spectra and relative intensities of the high- and low-spin infrared azide stretching bands. From either of these it can be inferred that the fraction of high-spin component rises five- to sixfold on transition from the R to the T structure. These estimates were confirmed, first by mea-

surement of the relative paramagnetic susceptibilities in the closely related trout IV hemoglobin by NMR, and finally by the direct magnetometer measurements of the absolute susceptibilities reported here. All these measurements point to the same answer, namely, that in the T structure ΔE, the free energy gap between the six-coordinate high- and low-spin states is about 1 kcal/mol heme lower than in the R structure. This answers the question asked at the outset as to the energy equivalent of the restraint that opposes transition to the low-spin state in the T structure, when the only chemical difference between the heme complexes in the two alternative quaternary structures consists of a change of spin. It amounts to about one-third of the energy equivalent of the difference in oxygen affinity between the R and T structures of carp hemoglobin. However, in the reaction with oxygen the change in spin is accompanied by a change in coordination number, so that the contribution made by the spin change cannot be separated experimentally from that due to the steric effects of the oxygen. Furthermore, the change of spin occurs in the ferrous form where the difference in energy between the high- and low-spin states may be larger than in the ferric form.

Can mechanical restraint as envisaged in our theory lead to a reduced oxygen affinity? Collman & his collaborators (1978a) have tried to answer this question by studies on synthetic models. They measured the thermodynamic constants of oxygen binding to cobalt and iron "picket fence" complexes with either *N*-methylimidazole or 1,2-methylimidazole as the fifth ligand. The former combines with the cobalt or iron atom without steric hindrance, while the latter is restrained by close contact of the 2-methyl group with the porphyrin, so that it opposes the movement of the metal atom into the plane of the porphyrin and the shortening of the Fe–N_ϵ bond on ligation with oxygen in the same way as Perutz imagined the globin does in the T structure of hemoglobin. The results show that the mean oxygen affinity (p_{50}) for the unhindered *N*-methyl cobalt complex is 150 Torr compared with 960 Torr in the hindered 1,2-methylimidazole cobalt complex. In the iron complexes the corresponding values are 0.59 and 38 Torr, corresponding to a difference in free energy of oxygen binding of 2.5 kcal/mol, comparable to the free energy of heme–heme interaction in hemoglobin (Collman et al., 1978b). The answer to the question asked at the beginning of this paragraph is therefore in the affirmative.

Note Added in Proof

W. A. Saffran and Q. H. Gibson kindly showed us their recent work on the kinetics of CO binding to the hemoglobin of menhagen, a fish hemoglobin with ligand binding properties similar to those of carp. In the T structure, the CO affinities of the two pairs of subunits are markedly different; in the R structure they are equal. In consequence the free energies of heme–heme interaction of the two pairs of subunits are also different, amounting to 3.6 and 1.95 kcal/mol. This result bears on ours because it suggests that the large spin changes are associated mainly with one pair of subunits, in which case the associated mainly with one pair of subunits, in which case the difference in the free energy of spin equilibrium in the R and T structures would amount to 2 kcal/mol heme for that pair. So far Saffran and Gibson have not been able to assign the different kinetic constant to the α and β subunits, but if human hemoglobin is any guide, the tension at the heme should be greater in the α subunits (Perutz et al., 1976).

Acknowledgments

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References

Barefield, E. K., Bush, D. H., & Nelson, S. M. (1968) *Q. Rev. Chem. Soc. 22,* 457.

Beattie, J. K., Sutin, N., Turner, D. H., & Flynn, G. W. (1973) *J. Am. Chem. Soc. 95,* 2052.

Cabrera, B. (1941) *J. Chem. Phys. 38,* 1.

Cerdonio, M., Cosmelli, C., Romani, G. L., Messana, C., & Gramaccioni, C. (1976a) *Rev. Sci. Instrum. 47,* 1.

Cerdonio, M., Messana, C., & Gramaccioni, C. (1976b) *Rev. Sci. Inst. 47,* 1551.

Cerdonio, M., Mogno, F., Romani, G. L., Messana, C., & Gramaccioni, C. (1977) *Rev. Sci. Instrum. 48,* 300.

Collman, J. P. (1977) *Acc. Chem. Res. 10,* 265.

Collman, J. P., Brauman, J. I., Doxsee, K. M., Halbert, T. R., Hayes, S. E., & Suslick, K. S. (1978a) *J. Am. Chem. Soc. 100,* 2761.

Collman, J. P., Brauman, J. I., Doxsee, K. M., Halbert, T. R., & Suslick, K. S. (1978b) *Proc. Natl. Acad. Sci. U.S.A. 75,* 564.

Coryell, C. D., Stitt, F., & Pauling, L. (1937) *J. Am. Chem. Soc. 59,* 633.

Cunningham, A. J., Ferguson, J. E., Powell, H. K. J., Sinn, E., & Wang, H. (1972) *J. Chem. Soc., Dalton Trans.,* 2155.

Dolphin, D. H., Sams, J. R., & Tsin, T. B. (1977) *Inorg. Chem. 16,* 711.

Eisenberger, P., Shulman, R. G., Brown, G. S., & Ogawa, S. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 491.

Ewald, A. H., Martin, R. L., Ross, I. G., & White, A. H. (1964) *Proc. R. Soc. London, Ser. A 280,* 235.

Fabry, T. L., & Hunt, J. W. (1968) *Arch. Biochem. Biophys. 123,* 428.

Fermi, G. (1975) *J. Mol. Biol. 97,* 237.

Fermi, G., & Perutz, M. F. (1977) *J. Mol. Biol. 114,* 421.

Figgis, B. H. (1966) *Introduction to Ligand Field Theory,* Interscience, New York, N.Y.

Gibson, Q. H., & Carey, F. G. (1975) *Biochem. Biophys. Res. Commun. 67,* 747.

Goodwin, H. A., & Sylva, R. N. (1968) *Aust. J. Chem. 21,* 83.

Grenoble, D. C., Frank, C. W., Bergeron, C. B., & Drickamer, H. G. (1971) *J. Chem. Phys. 55,* 1633.

Gupta, R. K., & Mildvan, A. S. (1975) *J. Biol. Chem. 250,* 246.

Hall, G. R., & Hendrickson, D. N. (1976) *Inorg. Chem. 15,* 607.

Hoard, J. L. (1975) in *Porphyrins and Metalloporphyrins* (Smith, K. M., Ed.) p 317, Elsevier, Amsterdam.

Hoselton, M. A., Wilson, L. G., & Drago, R. S. (1975) *J. Am. Chem. Soc. 97,* 1722.

Iizuka, T., & Kotani, M. (1969a) *Biochim. Biophys. Acta 181,* 275.

Iizuka, T., & Kotani, M. (1969b) *Biochim. Biophys. Acta 194,* 351.

Iizuka, T., & Yonetani, T. (1970) *Adv. Biophys. 1,* 157.

Johnson, F. H., & Schlegel, F. M. (1948) *J. Cell Comp. Physiol. 31,* 421.

Ladner, R. C., Heidner, E. J., & Perutz, M. F. (1977) *J. Mol. Biol. 114,* 385.

Ogunmola, G. B., Zipp, A., Chen, F., & Kauzmann, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 1.

Orgel, L. E. (1960) *An Introduction to Transition Metal Chemistry Ligand Field Theory,* Methuen & Co. Ltd., London.

Otsuka, J. (1970) *Biochim. Biophys. Acta 214,* 233.
Perutz, M. F. (1972) *Nature (London) 237,* 495.
Perutz, M. F., Heidner, E. J., Ladner, J. E., Beetlestone, J. G., Ho, C., & Slade, E. F. (1974) *Biochemistry 13,* 2187.
Perutz, M. F., Kilmartin, J. V., Nagai, K., Szabo, A., & Simon, S. R. (1976) *Biochemistry 15,* 378.
Perutz, M. F., Sanders, J. K. M., Chenery, D. H., Noble, R. W., Pennelly, R. R., Fung, L. W.-M., Ho, C., Giannini, I., Pörschke, D., & Winkler, H. (1978) *Biochemistry 17* (preceding paper in this issue).
Pulsinelli, P. D., Perutz, M. F., & Nagel, R. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 70,* 3870.
Renovitch, G. A., & Baker, W. A. (1967) *J. Am. Chem. Soc. 89,* 6377.
Scheidt, W. R. (1974) *J. Am. Chem. Soc. 96,* 84.
Schoenborn, B. P. (1965) *Nature (London) 208,* 760.
Schoenborn, B. P., Watson, H. C., & Kendrew, J. C. (1965) *Nature (London) 207,* 28.
Sinn, E., Sim, G., Dose, E. V., Tweedle, M. F., & Wilson, L. J. (1978) *J. Am. Chem. Soc. 100,* 3375.
Takano, T. (1977) *J. Mol. Biol. 110,* 537.
Tan, A. L., De Young, A., & Noble, R. W. (1972) *J. Biol. Chem. 247,* 2493.
Tweedle, M. F., & Wilson, L. J. (1976) *J. Am. Chem. Soc. 98,* 4824.
Warshel, A. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 1789.
Winter, M. R. C., Johnson, C. E., Lang, G., & Williams, R. J. P. (1972) *Biochim. Biophys. Acta 263,* 515.
Yonetani, T., Iizuka, T., Asakura, T., Otsuka, J., & Kotani, M. (1972) *J. Biol. Chem. 247,* 863.
Zipp, A. (1973) Ph.D. Dissertation, Princeton University.
Zipp, A., & Kauzmann, W. (1973) *Biochemistry 12,* 4217.
Zipp, A., Ogunmola, G. B., Newman, R. G., & Kauzmann, W. (1972) *J. Am. Chem. Soc. 94,* 2541.

Conformation Examination of Uridine Diphosphoglucose Using Lanthanide–Nitrilotriacetate Chelates as Shift Probes†

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ABSTRACT: A ^{13}C NMR study utilizing the lanthanide-induced shift (LIS) technique was carried out on uridine diphosphoglucose (UDPG) in order to determine the solution conformation. The neutral, water soluble nitrilotriacetate chelates, Ln(NTA), were used in preference to the bare ions due to the potency of the latter in hydrolyzing the UDPG. In addition to the primary binding site at the pyrophosphate moiety, secondary binding to the uracil carbonyl groups was observed and found to be a function of the size of the lanthanide ion; the largest ions showed the greatest relative affinity for the carbonyl groups. This effect was overcome by extrapolating the observed shift ratios to zero Ln(NTA)/[UDPG]. The extrapolated shift ratios were relatively constant for a series of five lanthanides indicating the solution conformation of UDPG is insensitive to metal ion size. The conformation of the UDPG was found to be extended so that the uracil carbons as well as carbons 1′, 2′, and 3′ of the ribose are outside of the pseudo-contact shift cone with the remaining carbons inside the cone.

Analysis of ^1H–^1H and ^1H–^{31}P coupling constants and chemical shift data has provided detailed information on the carbohydrate conformation in several nucleoside diphosphohexoses (Sarma et al., 1973; Lee & Sarma, 1976). Based on the absence of significant hexose shifts relative to the hexose 1-phosphates, indicating negligible ring current interactions in adenosine diphosphoglucose (ADPG),[1] it was concluded that the nucleotide and hexose moieties are relatively distant. This conclusion is somewhat weaker for uridine diphosphoglucose (UDPG) in which ring current shifts due to the uracil are smaller than those produced by adenine. Thus, the solution conformational structures of UDPG and, more importantly in terms of glycosyl transferase cofactors, its metal ion complexes are uncertain.

Lanthanide-induced shift (LIS) studies have become increasingly popular in probing solution structures of biologically important molecules (Dobson & Levine, 1976). Specific binding of these paramagnetic ions to a small molecule results in magnetic perturbations in NMR active nuclei which depend upon the average solution geometry of the metal–substrate complex. Solution structures have been derived for relatively nonflexible molecules such as 5′-adenosine monophosphate (Barry et al., 1971), cyclic adenosine 3′,5′-monophosphate (Lavallee & Zeltman, 1974), indole-3-acetate (Levine et al., 1974), and L-alanine (Sherry & Pascual, 1977) as well as for conformationally flexible molecules such as dinucleoside phosphates (Barry et al., 1972), phospholipids (Bystrov, 1971), adenosine triphosphate (Transwell et al., 1975), and simple peptides (Levine & Williams, 1975). In the present study, several lanthanide nitrilotriacetate (NTA) chelates have been used to obtain overall conformational information on UDPG. The use of a chelate was required due to the potency of the bare lanthanide ions in hydrolyzing the nucleotide diphosphohexoses

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[1] Abbreviations used: ADPG, adenosine diphosphoglucose; UDPG, uridine diphosphoglucose; LIS, lanthanide-induced shift; NTA, nitrilotriacetate.

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