Interactions between the Quaternary Structure of the Globin and the Spin State of the Heme in Ferric Mixed Spin Derivatives of Hemoglobin†

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ABSTRACT: We have studied the effect of the P_6-inositol (IHP)-induced change from the quaternary oxy (R) to the deoxy (T) structure in derivatives of human, trout IV, and carp methemoglobins. Addition of IHP to human fluoro- and aquomethemoglobin leads to the appearance of the slowly exchanging proton resonance at about −10 ppm from HDO diagnostic of the T structure. This experiment, and the crystallization of aquomethemoglobin + IHP by G. Fermi & M. F. Perutz ((1977) *J. Mol. Biol. 114,* 421) confirmed that the spectral change in the UV which IHP induces in these compounds can be used as a reliable indicator of the R→T transition. Judged by this spectral change, IHP converts all derivatives of *carp* hemoglobin from the R to the T structure. The pH at which the midpoint of the IHP-induced transition occurs increases with rising spin, being lowest in cyano, intermediate in azido, and highest in thiocyanate and aquomethemoglobin of carp. Conversely the replacement of water by fluoride or thiocyanate as the sixth ligand is unaffected by IHP because all three derivatives are predominantly high spin, but the affinity of azide for carp aquomethemoglobin is reduced 2.7-fold and that of cyanide 3.3-fold by IHP, corresponding to changes in the free energy of binding of 600 and 700 cal/mol heme. Conversion to the T structure of all carp methemoglobin derivatives except the cyanide one is accompanied by large changes in the visible absorption spectra, the most spectacular being that of the nitrite derivative whose color is changed from red to brown. IHP converts all *human* methemoglobin derivatives except the azide and cyanide ones from the R to the T structure. The conversion is accompanied by similar, but smaller, spectral changes than those in the corresponding carp derivatives. The change in paramagnetic susceptibility on addition of IHP to some of the derivatives was measured by NMR. Human aquo- and cyanate methemoglobins showed no changes. Human thiocyanate methemoglobin showed an 11%, imidazole methemoglobin a 5%, and hydroxymethemoglobin approximately 45% rise. The discovery of a 2.5-fold rise in azide methemoglobin of trout IV led to the detailed study of the magnetic properties of the closely related carp hemoglobin reported in the accompanying paper (Messana C., et al. (1978) *Biochemistry 17* (following paper in this issue)). The high- and low-spin components of azide methemoglobin show distinct N_3^- stretching frequencies in the infrared. We have used the relative optical density of the two infrared bands to study the spin equilibria of human and carp azide methemoglobins. Addition of IHP to those derivatives which are converted from the R to the T structure caused a change in the equilibrium corresponding to a free-energy change of about 1 kcal/mol heme; in derivatives in which the quaternary structure remains unchanged on addition of IHP, the free-energy change amounts to only 0.1–0.2 kcal/mol. We have also tried to measure the relaxation time of the spin equilibrium in the azide derivatives and found it to be 260 ns for the human but faster than 100 ns for the carp derivative and for myoglobin.

Heme–heme interaction arises from an equilibrium between states which differ in the tertiary structure of the α and β subunits and in their quaternary structure in the tetramer. This equilibrium is linked to the stereochemistry at the heme. In deoxyhemoglobin where the tense (T) quaternary structure is dominant, the heme irons are five coordinated and high spin. They are displaced by 0.6 (± 0.1) Å from the mean plane of the porphyrin atoms toward the proximal histidines, and the N_ϵ's of these histidines are displaced by an average distance of l = 2.7 (± 0.16) Å from the porphyrin plane (Fermi, 1975). These displacements are close to those observed in the synthetic analogue 2-methylimidazole-Fe(II) tetraphenylporphine (Hoard & Scheidt, 1973; unpublished, quoted by Hoard, 1975). In oxyhemoglobin where the relaxed (R) quaternary structure is dominant, the heme irons are six coordinated and low spin. Their stereochemistry has not been determined but can be inferred from the structure of the "picket fence" oxygen adduct in which the iron lies in the porphyrin plane and l = 2.07 Å (Collman et al., 1974). The reduction in l by 0.6 Å is a consequence of the average Fe–N bond length in this compound being 0.08–0.09 Å shorter than in the deoxyhemoglobin analogue mentioned above and of the transition from 6 to 5 coordinated geometry. The contraction in iron–ligand bond length on going from high to low spin is a general property of iron porphyrins and all other iron complexes (Table I). Especially striking are the crystallographic results on the dithiocarbamates, some of which show temperature-dependent spin equilibria, while the spin states of others are independent of temperature. For instance, in $Fe[S_2CN(CH_3)_2]_3$ and

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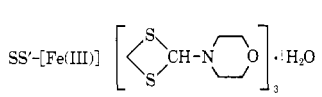
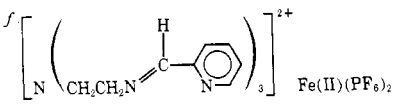
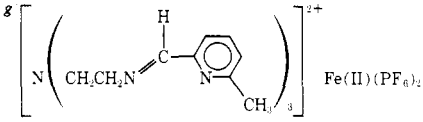
TABLE I: Iron-Ligand Distances in High- and Low-Spin Complexes.

complex	$Fe-N_{porphyrin}$ (Å)		reference
	high spin		
Fe(II)TpivPP[a]	2.069		Jameson et al., 1978a
2-methylimidazole Fe(II) TPP[b]	2.086		Hoard, 1975
(SCN^-) Fe(III) TPP	2.065		Hoard, 1975
Cl^- Fe(III) TPP	2.065		Hoard, 1975
	low spin		
1-methylimidazole Fe(II) TpivPP(O_2)[c]	1.979		Jameson et al., 1978b
(piperidine)$_2$Fe(II) TPP	2.004		Hoard, 1975
(imidazole)$_2$[Fe(III) TPP]$^+$ Cl^-·CH_3OH	1.989		Hoard, 1975
pyridine[Fe(III) TPP]$^+$ (N_3^-)	1.990		Hoard, 1975
	⟨Fe-S⟩		
[Fe(III)(mcd)$_3$]·H_2O; high spin[e]	2.443		Butcher et al., 1976
[Fe(III)(mcd)$_3$]·C_6H_6; low spin[e]	2.318		Butcher et al., 1976
Fe(III)[tris(*N*,*N*-diethyldithiocarbamate)]; 297 K; μ_e = 5.6 μ_β[d]	2.306		Leipold & Coppens, 1973
Fe(III)[tris(*N*,*N*-diethyldithiocarbamate)]; 79 K; μ_e = 2.8 μ_β[d]	2.306		
	Fe-N(pyridine)	Fe-N(imine)	
[Fe(II)(6-Me-Pyr)$_3$tren]$^{2+}$$PF_6^-$; high spin[f]	2.282	2.143	Hoselton et al., 1975
[Fe(II)(Pyr)$_3$tren]$^{2+}$$PF_6^-$; low spin[g]	1.966	1.942	Hoselton et al., 1975
	⟨Fe-N⟩	⟨Fe-O⟩	
[Fe(III)(acac)$_2$trien]PF_6; high spin[h]	2.136	1.928	Sim et al., 1978
[Fe(III)(sal)$_2$trien]Cl·$2H_2O$; low spin[i]	1.968	1.884	Sim et al., 1978

[a] An iron(II) "picket fence" derivative of tetraphenylporphin in which the fifth ligand of the iron is an amide oxygen. [b] TPP, tetraphenylporphine. [c] Oxygen adduct of the "picket fence" derivative of tetraphenylporphine. [d] Fe(III)(S_2CH·N-ethyl$_2$)$_3$. [e] mcd, morpholinocarbodithioato:

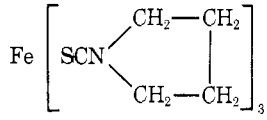
SS'-[Fe(III)]  ·H_2O

[f]  $Fe(II)(PF_6)_2$

[g]  $Fe(II)(PF_6)_2$

[h,i] Six-coordinated complexes of Fe(III) derived from triethylenetetramine (trien) and acetylacetone (acac) or salicylaldehyde (sal). In both compounds the iron is coordinated octahedrally to four nitrogens and two oxygens.

$Fe[S_2CN(C_4H_4OH)_2]_3$ the effective magnetic moment μ_{eff} decreases from 4.2 to 2.2 μ_B on going from 295 to 150 K. This reduction in paramagnetic susceptibility is accompanied by a contraction of the Fe-S bond length by 0.058 and 0.062 Å, respectively. On the other hand, in



where μ_{eff} remains constant over the same temperature range, the contraction in Fe-S bond length amounts to only 0.008 Å (Albertsson & Oskarsson, 1978). In summary, the table shows that the iron-ligand bond lengths in the fully low-spin compounds are shorter by about 0.1 Å than in the fully high-spin compounds. This property is fundamental to an understanding of heme-heme interaction and forms the basis for the arguments presented in this and the following paper.

In the absence of organic phosphates, the R structure is dominant in all hemoglobin derivatives in which the irons are six coordinated, but closer examination shows that the allosteric equilibrium between the two structures varies with the spin state of the iron. Regardless of the exact stereochemistry of the sixth ligand, the equilibrium constant $L = [T]/[R]$ is larger in high than in low-spin derivatives, presumably because their larger Fe-N bond distances raise the value of l (Perutz et al., 1974b; Banerjee et al., 1973; Henry & Banerjee, 1973; Nagai, 1977).

If a change from low- to high-spin iron contributes to the change in the conformational equilibrium in the globin, then it is to be expected that the structure of the globin will also influence the spin state of the iron. Changes in quaternary structure have no influence on the spin state in any of the derivatives in which the irons are either pure low or pure high spin or mixed spin, so that high- and low-spin states are equally populated independent of temperature in proportion to their spin degeneracies, but we suspected that they might affect the paramagnetic susceptibilities of methemoglobin derivatives which are in a thermal equilibrium between two spin states and therefore show reverse Curie behavior. Perutz et al. (1974a) reported such effects for aquomethemoglobin, but these later proved spurious (Gupta & Mildvan, 1975; Anusiem, 1978) because aquomethemoglobin at ambient temperature belongs to the mixed spin category just described and therefore shows normal Curie behavior (Messana et al., 1978).

We therefore decided to investigate other methemoglobin derivatives for which thermal spin equilibria have been reported. An initial survey of magnetic changes in solution by NMR showed that a transition from the R to the T structure produces a moderate rise in the paramagnetic susceptibility of human thiocyanate methemoglobin, and large rises in human hydroxy and trout IV azide methemoglobins. All these are mixed spin derivatives whose spin transition lies within the accessible temperature range of 0–30 °C.

We then asked ourselves whether the thermal equilibria

represent fast changes in electronic state with small change in geometry or slower oscillations between stereochemically distinct species, such as five- and six-coordinated hemes. W. S. Caughey pointed out to us that the former explanation was unlikely because the high- and low-spin components of azide metmyoglobin showed separate IR stretching frequencies (McCoy & Caughey, 1970; Alben & Fager, 1972). Stimulated by him we measured the effect of the R→T transition on the IR spectrum of carp azide methemoglobin and found a dramatic rise of the high-spin and fall of the low-spin azide stretching frequency, consistent with the magnetic effects we had observed in trout IV by NMR, and implying that the high- and low-spin components have lifetimes longer than the period of the IR radiation of 10^{-12} s. This encouraged us to study the relaxation of the thermal spin equilibrium by temperature-jump methods. The results showed vibrational free energies of activation of 9 kcal/mol or less. Such energies are large enough for the thermal spin equilibria to represent oscillations between conformationally distinct species, but probably too small for a rupture of the Fe–N_ϵ bond, suggesting that the high- and low-spin states might differ only by the lengths of the Fe–N bonds.

Magnetic changes are linked to changes in electronic absorption spectra. A rise in paramagnetic susceptibility of a methemoglobin compound always produces a blue shift of the Soret band, a rise in intensity of the "high-spin" bands at 500 and 630 nm and in the near-infrared, and a fall in intensity of the "low-spin" bands at 540 and 470 nm. However, the converse is not always true. We have found that a transition of the quaternary structure from R to T or a rise in temperature produces a rise of "high-spin" and a fall of "low-spin" bands, no matter whether the paramagnetic susceptibility rises, falls, or remains constant. Visible spectra by themselves therefore are poor guides to magnetic properties, and the nomenclature of high- and low-spin bands might well be abandoned.

Materials and Methods

Hemoglobin solutions were prepared and electronic difference spectra measured as described by Perutz et al. (1974a). Spectra were recorded with a Cary 17 spectrophotometer. In general we used heme concentrations between 60 and 160 μM in 0.05 M Bistris[1] and 0.1 M heme ligand at pH 6.5 in matched 1-cm cuvettes, but some experiments were done at heme concentrations of several mM using matched 0.2-mm pathlength cuvettes. The R→T transition was accomplished by addition of 2 mol of IHP/mol of hemoglobin tetramer. Of the methemoglobin derivatives studied only azide and thiocyanate were stable at room temperature; the others were investigated between 0 and 5 °C.

Equilibria of ligand binding to human methemoglobin were measured by difference spectroscopy at 20 °C in the same buffer as above for the R state and in the presence of 4 mol of IHP/mol of hemoglobin tetramer using 40 μM heme. The measurements of the equilibria of ligand binding to carp aquomethemoglobin were carried out at 20 °C in 0.05 M Bistris buffer at pH 6.0 for the R state and in 0.05 M Bistris and 1 mM IHP for the T state. Hemoglobin concentrations varied from 6 to 60 μM in heme equivalents. Equilibration times varied with the ligand under study, being overnight for cyanide, 2 h for azide, and 1 h for fluoride and thiocyanate. Fractional saturation or extent of replacement of liganded water was determined spectrophotometrically using a Cary 17 or Cary 14 spectrophotometer. Because of the similarities of the visible spectra of the aquo and thiocyanate derivatives of carp hemoglobin, the binding of thiocyanate was best measured in the Soret region of the spectrum. For the other ligands both the Soret and visible regions could be used. In Cambridge, transitions in quaternary structure were measured by difference spectroscopy in the UV between 260 and 360 nm with the Cary 17 spectrophotometer using 1-cm pathlength cuvettes and solutions that were 60 μM heme. The buffers used were 0.05 M sodium cacodylate or Bistris below and Tris above pH 7.0, plus chloride as required by the pH adjustment with HCl. The IHP concentration was 1 mM. In Pittsburgh, transitions in quaternary structure were judged by the appearance of the slowly exchangeable proton resonance at −10 ppm from H_2O as described by Fung & Ho (1975).

In Cambridge, magnetic susceptibilities were measured at 32 °C on a 100-MHz NMR machine in coaxial tubes with chloroform in the outer and the sample in the inner tube. In some experiments we held the tube stationary and used the method of Reilly et al. (1955). In other experiments we spun the tube, locked the machine on the water signal, and measured the displacement of the chloroform proton signal from the water signal. The displacements were calibrated with $CuSO_4$ solutions. The accuracy was ±1% in Hz separation from the water signal. Concentrated methemoglobin solutions were prepared as follows: To a 14 mM Fe solution of methemoglobin equilibrated against 0.1 M NaCl, 0.25 equiv of $K_3Fe(CN)_6$/heme Fe was added to oxidize any oxyhemoglobin which may have formed on standing. To 2 mL of this solution, 0.11 mL of a 2 M solution of the particular heme ligand were added. The solution was then divided into two parts. To 1 mL we added 50 μL 0.2 M IHP of pH 5.5; this produces only a small pH change because the low pH of the IHP solution compensates for the Bohr effect and the dilution effect produced on addition to the methemoglobin solution. To another 1 mL we added 50 μL of H_2O. The pH of the two solutions was then adjusted to the desired value by addition of either 4 M NaH_2PO_4 or 3 M Tris with an Agla syringe.

In Pittsburgh, the solutions of aquomethemoglobin were prepared by additions of 90 mg of $K_3Fe(CN)_6$ to 10 mL of a 6 mM Fe solution of human HbCO A. After stirring for half an hour, the solution was passed through a stripping column of coarse Sephadex G-25 equilibrated against 0.01 M Tris and 0.1 M NaCl of pH 7.6. The effluent was dialyzed overnight against deionized H_2O and concentrated to 9 mM Fe by ultrafiltration. The pH of the solution was adjusted by addition of 1 M Bistris followed by dialysis against 0.1 M Bistris of the same pH. To one portion of the sample we added 100 μL of 0.1 M IHP (pH 5.5) and to the other 100 μL of 1 M Bistris to maintain the same concentration and pH in the two portions. NMR measurements were done in three sets of 5–3 coaxial tubes of 5-mm o.d. in the outer and 3-mm o.d. in the inner tube. In one set, the outer tube was filled with methemoglobin and the inner with methemoglobin plus IHP. In the second set, the outer tube was filled with methemoglobin and the inner with water. In the third set, the outer tube was filled with methemoglobin plus IHP and the inner again with water. The proton resonances of water were recorded on the MPC-HF 250-MHz superconducting spectrometer with sample tubes in the spinning mode. The relative magnetic susceptibilities were obtained directly from the chemical shifts of the water absorption resonances. For example, tubes oriented parallel to the applied magnetic field as in our case, $\delta_A - \delta_B = (4\pi/3)(\chi_A - \chi_B)$, where δ's are the chemical shifts of the water resonances and χ's are the magnetic susceptibilities of the samples.

A sample of trout IV oxyhemoglobin was sent to Cambridge

[1] Abbreviations used: Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; NMR, nuclear magnetic resonance; IR, infrared; IHP, P_6-inositol; TPP, tetraphenylporphine; mcd, morpholinocarbodithioato-SS′; Hb, hemoglobin; μ_B, Bohr magneton.

from Rome by Professor M. Brunori. It was oxidized by the addition of 3 mol of $K_3Fe(CN)_6$/mol of heme Fe, equilibrated against 0.1 M NaCl in a Sephadex G-25 column and concentrated by prolonged ultracentrifugation, yielding 210 μL of a 14 mM Fe solution of methemoglobin. It was taken to Pittsburgh where we added 2 μL of 1 M $K_3Fe(CN)_6$ to oxidize any fraction of methemoglobin that might have reverted to HbO_2, plus 15 μL of 2 M NaN_3 and 8 μL of 4 M NaH_2PO_4 to bring the pH to 6.08. After the first NMR measurement the pH in the NMR tube was raised to 8.6 by addition of 24 μL of 3 M Tris and the NMR measurement was repeated. We used coaxial tubes in the spinning mode with the hemoglobin solution in the inner and chloroform in the outer tube and measured the separation of the water signal from the chloroform signal. Relative susceptibilities were checked by calibrating the system with solutions of $CuSO_4$. Solutions of human azide methemoglobin were made up and measured similarly to those of trout.

Infrared spectra were recorded with a Digilab FTS-14 Michelson-type interferometer with a KBr-supported germanium beam splitter for use in the region from 4000–400 cm^{-1}, giving a resolution of 4 cm^{-1}. A 4K Nova 1200 minicomputer and 128K disc were incorporated for Fourier transformation. Approximately 3 mM Fe hemoglobin solutions were filled into CaF_2 cells with a pathlength of 25 μm. Spectra were collected at room temperature (~20 °C) in a single beam mode and ratioed against a previously stored background spectrum of hemoglobin solution. Each spectrum resulted from the addition of 100 interferograms. The azide bands had sufficient signal-to-noise with 100 scans to make longer accumulations unnecessary. The azide stretching frequencies occur at a "window" in the water and protein absorption, so that it was possible to examine spectra directly rather than as difference spectra.

Temperature-jump experiments were carried out in a Messanlagen "T. J. Transient Spectrophotometer", type SBA 7, using a mercury lamp and a 1-mL microcell, thermostated at 18.0 ± 0.5 °C. The temperature jump was 4 °C, so that the final temperature was 22.0 ± 0.5 °C. The output of the T jump spectrophotometer was stored on a digital transient recorder (Biomation Model 802). The recorded punched tape was analyzed by a small computer.

Relaxation measurements in the nanosecond range were performed using a cable temperature jump apparatus (Hoffman, 1971; Pörschke, 1976). In this apparatus a sample volume of 100 μL is heated by a cable discharge. In the present experiments the temperature was increased from 0 to 4 °C and the relaxation toward the new equilibrium was observed at the 546-nm mercury line. For human methemoglobin the signal-to-noise ratio was increased by adjusting the time constant of the detection system to 280 ns and evaluating the relaxation time by an analogue deconvolution technique.

Results

Slowly Exchangeable Proton Resonances. In solutions of all ferric human hemoglobins A, the R structure is normally predominant, but addition of IHP switches some of these derivatives to a structure which has SH reactivities and UV electronic absorption and circular dichroism spectral characteristics of the T structure (Perutz et al., 1974b). The diagnostic value of these properties has been confirmed by Fermi & Perutz (1977) who crystallized human fluoromethemoglobin + IHP from an aqueous solution of poly(ethylene glycol) and found, by x-ray analysis, that it does indeed have the T structure, while crystallization without IHP produced the R structure. Human aquomethemoglobin + IHP has so far

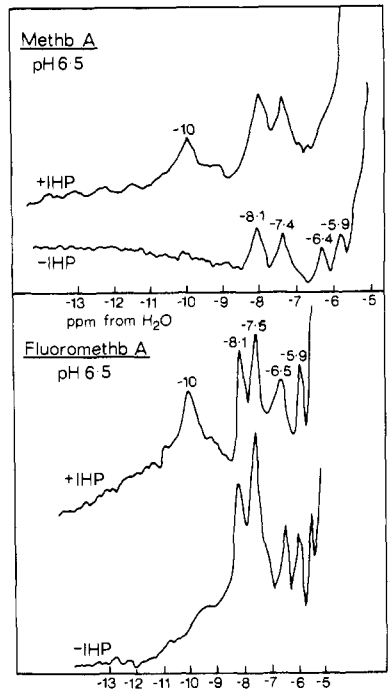


FIGURE 1: Slowly exchanging proton resonances in H_2O of methemoglobin A and fluoromethemoglobin A in 0.05 M Bistris of pH 6.5, with and without IHP.

failed to crystallize. In order to see whether it has the same quaternary structure as fluoromethemoglobin + IHP we have compared the slowly exchangeable proton resonances of the two derivatives by NMR. We found that they both show the same resonances, including the diagnostic one at −10 ppm from H_2O due to the hydrogen bond between Tyr-42α and Asp-99β at the $\alpha_1\beta_2$ contact which forms only in the T structure (Figure 1) (Fung & Ho, 1975). These results prove that in solution both fluoro- and aquomethemoglobin + IHP have the quaternary T structure.

UV Difference Spectra. In this work we have used the UV difference spectrum near 290 nm, shown in Figure 4 of Perutz et al. (1974b), as our criterion for changes in quaternary structure. By this criterion all derivatives of human methemoglobin explored by Scheler et al. (1957), except the cyano and azide derivatives, are switched from R to the T structure at pH 6.5 by IHP. These include the aquo, fluoro, cyanate, thiocyanate, selenocyanate, nitrite, formate, acetate, and imidazole derivatives. Following Brunori's (1975) demonstration that carbonmonoxyhemoglobin of trout IV can be switched from the R to the T structure by lowering the pH, trout IV azide methemoglobin in phosphate buffer was switched to the T structure by lowering the pH from 8.5 to 6.0. Addition of IHP to the solution at pH 6.0 did not produce further significant spectral changes. In carp azide methemoglobin, on the other hand, complete conversion to the T structure required low pH as well as inorganic phosphate or polyphosphates such as ATP or IHP (Tan & Noble, 1973; Tan et al., 1973).

Figure 2 shows a typical IHP-induced difference spectrum for carp azide methemoglobin. The shift of the UV difference spectrum relative to the baseline is the result of a large change in the δ band of this derivative which tails into the UV region. The magnitude of the absorbance change between 293 and 287 nm was taken as a measure of the IHP-induced conversion of the molecule from the R to the T structure. In Figure 3 the magnitude of this IHP-induced spectral band is plotted as a function of pH for four derivatives of carp methemoglobin. In three cases the extent of the R→T transition produced by IHP

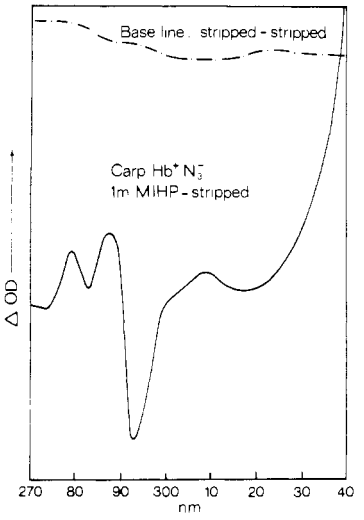


FIGURE 2: UV difference spectrum of a 60 μM Fe solution of carp methemoglobin in 0.05 M sodium cacodylate and 0.1 M NaN_3 (pH 6.0) + 1 mM IHP-stripped. The vertical scale is arbitrary.

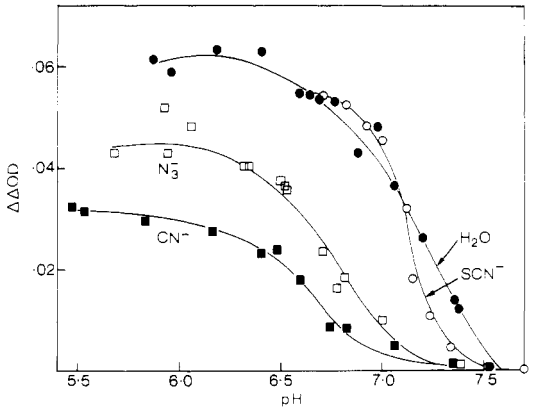


FIGURE 3: Magnitude of IHP-induced absorbance change between 293 and 287 nm plotted against pH for carp aquo (●), thiocyanate (○), azide (□), and cyanomethemoglobin (■). The thiocyanate curve is bell shaped and comes to a minimum at $\Delta OD = 0.1$ and pH 6.0.

addition decreases with increasing pH. However, this pH dependence is different for the three derivatives. For aquomethemoglobin the conversion to the T state persists at higher pH values than it does for the azide or cyanide derivatives. This is consistent with the proposition that high-spin ligands favor the transition to the T state more than low-spin ligands. The small difference seen between the azide and cyanide derivatives is consistent with our IR evidence that in the T state the azide derivative is approximately half high spin (see below), while the cyanide derivative is known to remain pure low spin. In the experiments reported in Figure 2 the azide and cyanide concentrations were both 10 mM. It was found that increasing these concentrations to 100 mM altered the pH dependence of the R→T transition slightly, probably because of the interaction of these ions with the polyphosphate binding site. The behavior of the carp thiocyanate derivative is different. The magnitudes of its IHP-induced UV or visible difference spectra follow bell-shaped curves, with a maximum at pH 6.7. The visible spectra and the magnetic properties reported by Messana et al. (1978) indicate that in the absence of IHP at pH $\geq$6.7 the R structure is dominant, while at pH $\leq$6.7 an increasing fraction of the molecules take up the T structure, so that IHP produces no further spectral changes. Another point to be noted is that the magnitude of the spectral change

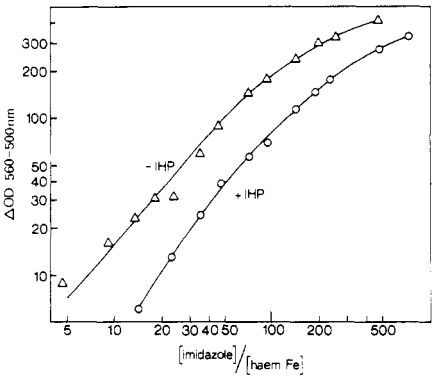


FIGURE 4: Binding of imidazole to a 60 μM Fe solution of human methemoglobin in the presence and absence of IHP at pH 6.6 and 20 °C.

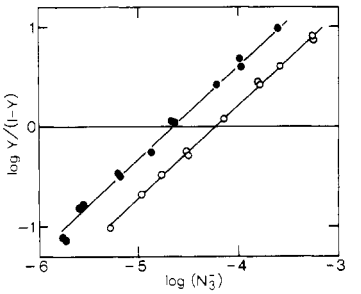


FIGURE 5: Hill plot of the binding of azide ion to carp methemoglobin in the presence (○) and absence (●) of 1 mM IHP in 0.05 M Bistris, pH 7.0, and 20 °C.

at 287 nm is dependent on both quaternary and tertiary structure, being much larger for the high- than the low-spin compounds.

Binding Studies. In binding studies with methemoglobin, one measures the partition coefficient between ligand and water, but the results are not represented as such because of the difficulty of defining the activity of the free ligand and free water. We shall refer here to *apparent* dissociation constants. In human methemoglobin A, IHP affects this constant even if the quaternary structure remains R. Thus Perutz et al. (1974b) found that the apparent dissociation constant of cyanide is 1.4 times larger and that of azide 1.6 times larger in the presence of IHP than in its absence, an effect which they attributed to an as yet unknown modification of the R structure by IHP. We have compared the apparent dissociation constants of human methemoglobin A from thiocyanate, a mixed spin ligand, in the absence and presence of IHP and found them to be the same. On the other hand, the apparent affinity for imidazole, a low-spin ligand, is affected, as shown in Figure 4. It was not possible to saturate the hemoglobin with imidazole even in solutions of 6 mM heme, so that no dissociation constants could be derived, but the lower affinity in the T structure is evident.

At pH 6.0 the equilibrium constant of the reaction of carp methemoglobin with fluoride, a high-spin ligand, is 9 mM and with thiocyanate, a mixed spin ligand, is 3.9 mM, independent of IHP. When one measures the effect of IHP on the replacement of water by a strong field, predominantly low-spin ligand, a different pattern emerges. Figure 5 presents the Hill plots for the binding of azide to carp methemoglobin. In the absence of IHP the apparent dissociation constant for azide is 2.1×10^{-5} M. In the presence of 1 mM IHP this is increased to 5.6×10^{-5} M to give a 2.7-fold decrease in azide affinity. The effect of quaternary state on the binding of cyanide ion, a pure

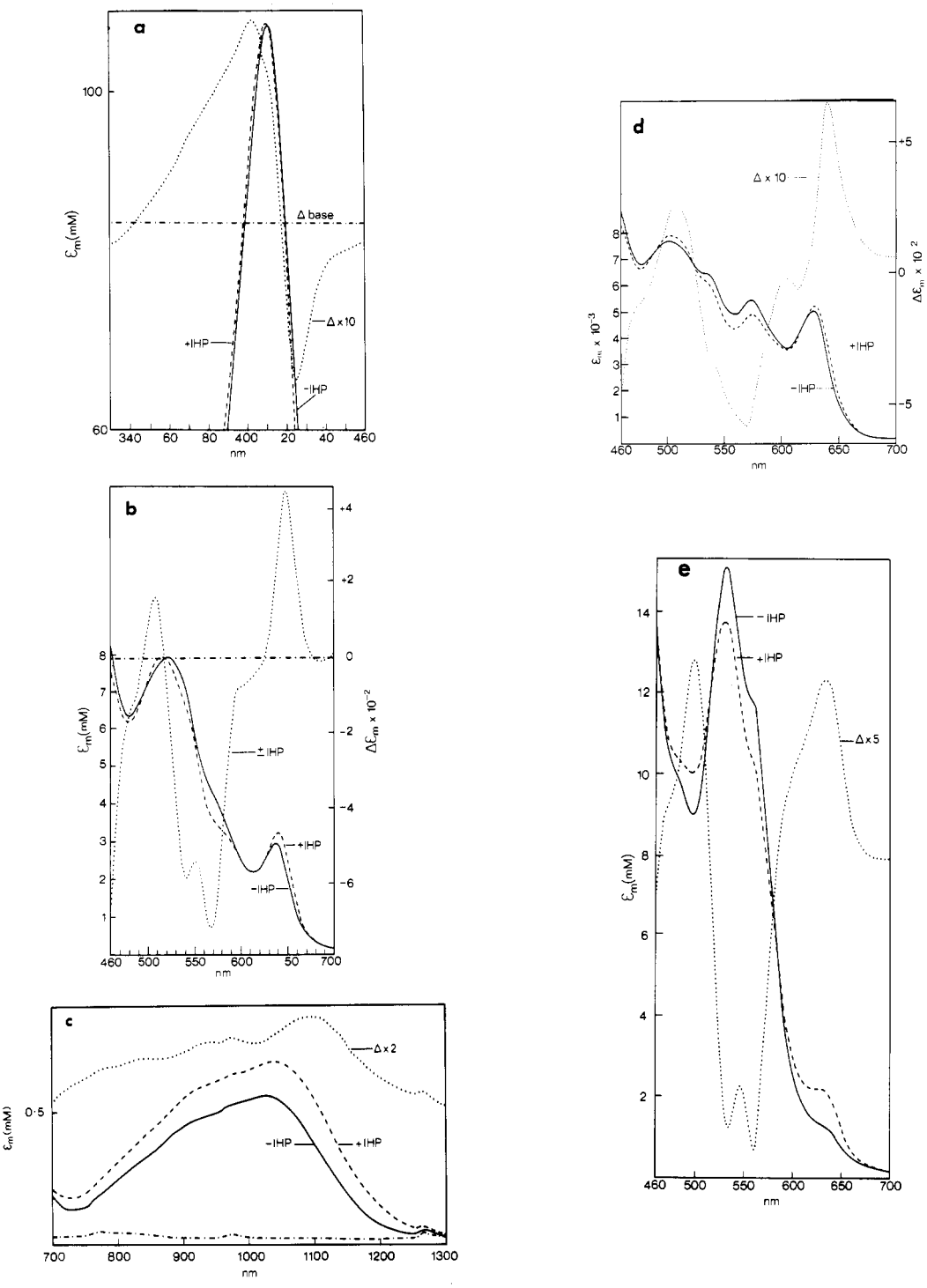


FIGURE 6: Difference spectra of various derivatives of human methemoglobin A ± IHP. (a) With 27 μM Fe methemoglobin in 0.05 M Bistris + 0.1 NaSCN (pH 6.0) ± 32 μM IHP at 0 °C; (b and c) 136 μM Fe methemoglobin in 0.05 M Bistris and 0.1 M NaSCN (pH 6.0) ± 160 μM IHP at 0 °C; (d) 160 μM Fe methemoglobin in 0.05 M Bistris + 0.1 M NaOCN (pH 6.5) ± 150 M IHP at 3 °C; (e) 140 μM Fe methemoglobin in 0.1 M imidazole (pH 6.6) ± 125 μM IHP at 3 °C.

low-spin ligand, is somewhat greater. Assuming a pK for cyanide of 9.31, the apparent dissociation constant for cyanide ion at pH 6.0 in the absence of IHP is 1.4×10^{-7} M. In the presence of 1 mM IHP at the same pH, the affinity is decreased 3.3-fold to give an apparent dissociation constant of 4.6×10^{-7} M.

The Hill coefficients, n, for the binding isotherms of the various ligands with carp methemoglobin vary considerably, from a value near unity for cyanide to one of 0.68 for thiocyanate. The low n values are probably due to subunit heterogeneity. It has been suggested that the R to T transition in hemoglobin may have unequal effects on the two subunit types, inducing a heterogeneity or modifying any heterogeneity intrinsic to the R state. No evidence for such disproportionate effects is observed in the binding data presented here. In no case is a significant difference in the n values for the two quaternary states observed. For azide binding the n values both in the presence and absence of IHP are 0.93. For thiocyanate binding the two sets of data have slightly different Hill coefficients, but these differences are below the range of significance.

Soret, Visible, and Near-Infrared Difference Spectra. Of

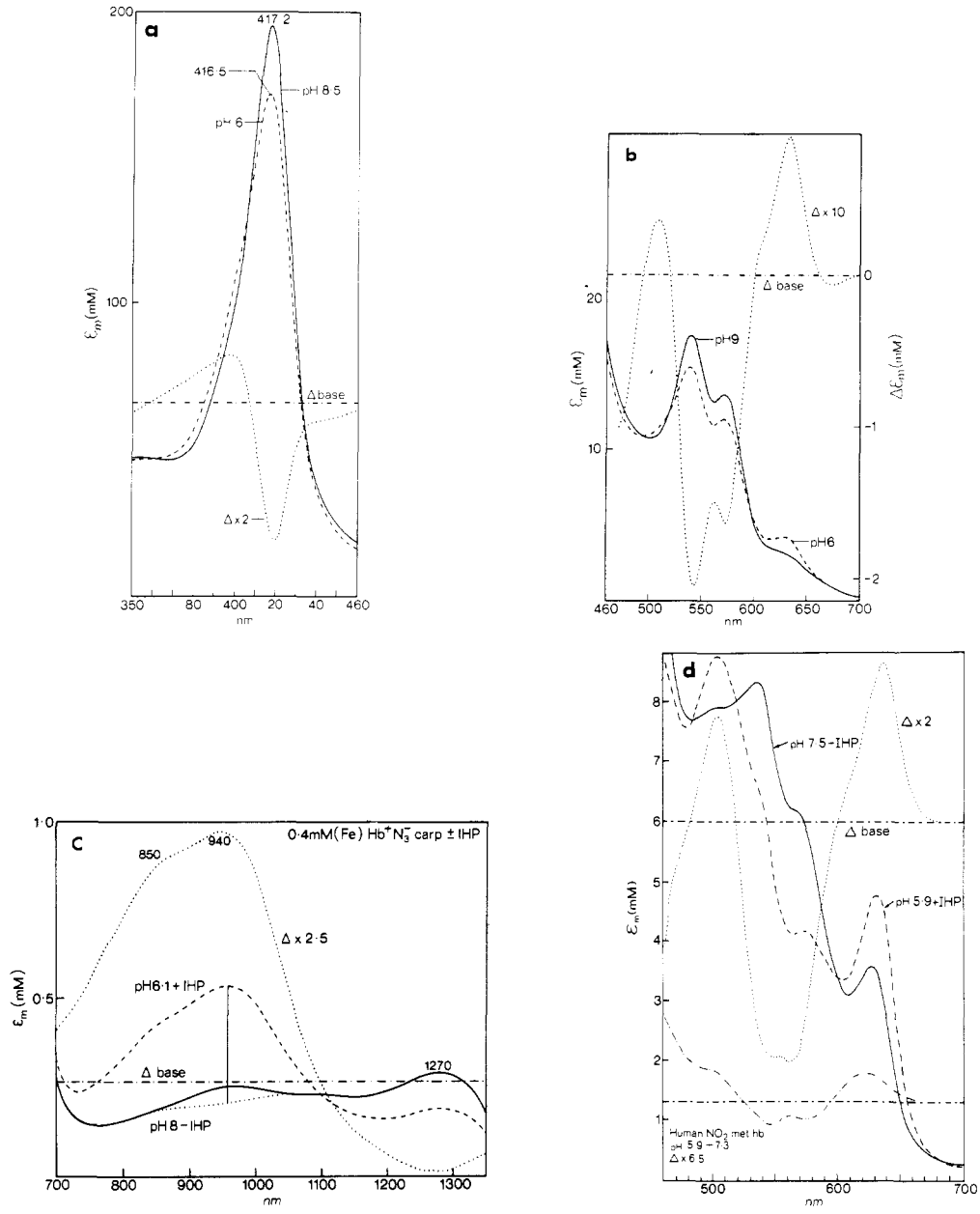


FIGURE 7: Difference spectra of azide methemoglobins of trout and carp. (a and b) With 48 μM Fe methemoglobin of trout IV in 0.1 M phosphate + 0.1 M NaN_3 (pH 6.0–8.5) at 20 °C; (c) 0.4 mM Fe methemoglobin of carp in 0.05 M sodium cacodylate + 0.01 M NaN_3 + 1 mM IHP (pH 6.1) minus 0.05 M Tris + 0.01 M NaN_3 (pH 8.0) at 20 °C; (d) 0.16 mM Fe methemoglobin of carp in 0.05 M sodium cacodylate + 0.1 M $NaNO_2$ + 0.32 mM IHP minus 0.05 M Bistris + 0.1 M $NaNO_2$. Same for the human form but without the IHP.

the methemoglobin derivatives studied by Scheler et al. (1957), formate and acetate had low affinities for human methemoglobin and denatured it rapidly even at 0 °C. They were therefore abandoned. This left cyanate, thiocyanate, selenocyanate, nitrite, and imidazole. As already mentioned, addition of IHP to all these derivatives produced the UV difference spectrum diagnostic for the R→T transition. In all except the cyanate derivative it produced small blue shifts of the Soret band. All the derivatives showed decreases in the intensities of the low-spin bands at 540 and 570 nm and increases in the high-spin bands at 500 and 630 nm (Figure 6). Except in imidazole hemoglobin, these changes were the same at low (60 μM Fe) and high (3 mM Fe) hemoglobin concentrations, showing that they were not caused by dissociation of heme ligand.

Addition of IHP to human azide methemoglobin produces only a weak difference spectrum, because there is no switch of quaternary structure (Perutz et al., 1974c); a change of pH has no effect on the spectrum of azide methemoglobin and little on that of thiocyanate or nitrite methemoglobin. On the other hand, trout IV and carp azide, thiocyanate, and nitrite methemoglobins show striking IHP and pH-dependent difference spectra (Figure 7); these include blue shifts and reduction in intensity of the Soret bands, very marked decreases of the low-spin bands and increases of the high-spin bands in the visible and great enhancements of the near-IR bands. If these near-IR bands are charge-transfer bands characteristic of a high-spin form, then the concentration of this form should have increased markedly on lowering the pH and adding IHP. Thiocyanate and nitrite methemoglobins of carp showed difference spectra similar to, though more intense than, those of the human forms. The nitrite derivative of carp is red in the R state (pH 7.5) and brown in the T state (pH 5.9 + IHP). This is the most striking effect of change in quaternary structure of the globin on the state of the heme yet found.

Figure 8 shows temperature-dependent difference spectra

TABLE II: Changes in Paramagnetic Susceptibilities on Switching Various Methemoglobin Derivatives from the Quaternary R to the T Structure.

derivative	machine (MHz)	pH	T (°C)	% change in chemical shift[a]	fraction hydroxy, F	calcd chem shift[f] $F \times 0.45$
human thiocyanate	100	6.5	32	+11[b]		
	100	6.5	32	+12[c]		
human metHb	250	7.85	12	+15[d]	0.32	+14.4
	250	7.65	24	+9	0.20	+9
	100	7.65	32	+9		
	100	7.1	32	+3	0.06	+3
	250	6.95	24	0	0.04	+2
	100	6.7	32	+2	0.02	+1
	250	6.05	24	0	<0.01	0
	100	5.95	32	−1	<0.01	0
trout IV azide metHb	250	6.1 → 8.5	12	−64[e]		

[a] For details of measurements, see Materials and Methods section. [b] Spinning mode of coaxial tube. [c] Stationary mode of coaxial tube. [d] Corrected for presence of 2.4 mM $K_3Fe(CN)_6$. [e] Corrected for presence of 8.7 and 7.9 mM $K_3Fe(CN)_6$ at pH 6.1 and 8.5, respectively. Some fraction of the ferricyanide would have been reduced so that the percentage changes marked *d* and *e* are probably overcorrected; i.e., they may be slightly too large. [f] Calculated on the assumption that the fraction hydroxymethemoglobin present undergoes a 45% rise in paramagnetic susceptibility.

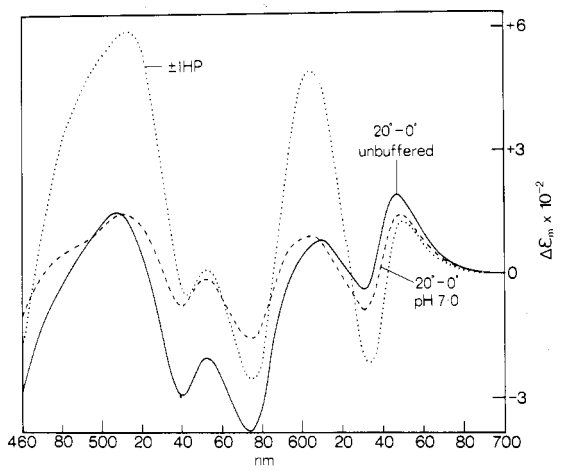


FIGURE 8: Comparison of temperature-dependent difference spectra of human aquomethemoglobin A with IHP-dependent difference spectrum; 160 μM Fe.

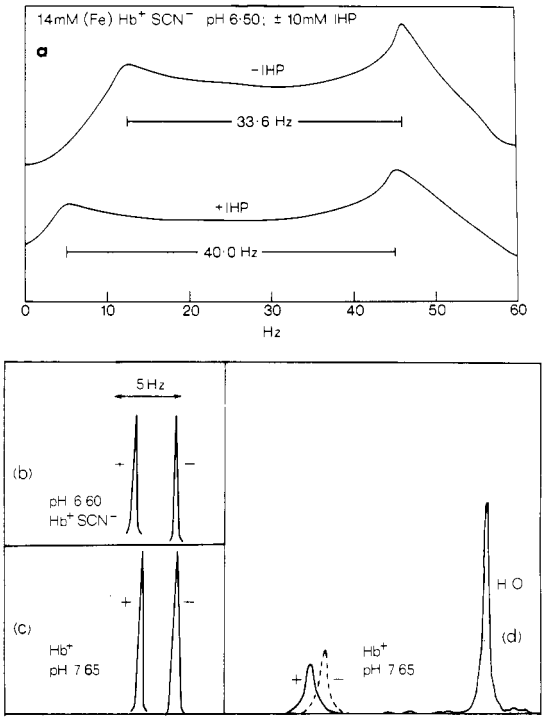


FIGURE 9: Effect of IHP on paramagnetic susceptibilities of solutions of human thiocyanate and hydroxymethemoglobins. (a) Splitting of $HCCl_3$ proton resonance in outer tube by thiocyanate methemoglobin in inner tube in stationary mode. (b) Changes in chemical shift of $HCCl_3$ proton resonance in outer tube by thiocyanate methemoglobin in inner tube in spinning mode. Both a and b were 14 mM Fe at pH 6.5 ± 10 mM IHP. (c) Changes in chemical shift of $HCCl_3$ proton resonance in outer tube by 14 mM Fe solution of methemoglobin (pH 7.65) ± 10 mM IHP; spinning mode. (d) Changes in chemical shift of H_2O resonance by 9 mM methemoglobin solution at pH 7.65 in outer tube relative to that of pure H_2O in inner tube; spinning mode. a, b, and c were done at Cambridge at 32 °C; d was done at Pittsburgh at 24 °C.

of aquomethemoglobin, superimposed on an IHP-dependent spectrum at the same pH, to demonstrate their similarity. The same similarity was found in azide methemoglobin of carp and man, even though in aquomethemoglobin the paramagnetic susceptibility falls while in azide methemoglobin it rises on going from 0 to 20 °C, and IHP has no effect on the paramagnetic susceptibility of aquomethemoglobin, but raises that of azide methemoglobin.

Paramagnetic Susceptibilities. The heme-linked water molecule in methemoglobin has a pK of 8.05 at 20 °C. Both aquo- and hydroxymethemoglobin are mixed spin compounds, but while aquomet is mostly high spin and obeys the Curie law at ambient temperatures, hydroxymet is mostly low spin and its paramagnetic susceptibility rises between 0 and 30 °C. The same is true of azide and thiocyanate methemoglobin. Imidazole methemoglobin is pure low spin (Beetlestone & George, 1964; Iizuka & Morishima, 1974). Table II shows the relative changes in paramagnetic susceptibility observed on addition of IHP to various methemoglobin derivatives. In agreement with Mildvan and Gupta (1975) we found the susceptibility of human aquomethemoglobin at pH 6.0 to be unaffected by IHP, but we found marked rises above pH 7.0 (Figure 9), indicating that the R→T transition produces no change in the susceptibility of aquomethemoglobin, but that it does raise the paramagnetic susceptibility of hydroxymethemoglobin. It is not possible to convert methemoglobin at high pH to the T structure because its affinity for IHP drops steeply above pH 7.0. Failing this, we have tried to see if the change in suscep-

TABLE III: Ratios of Low Spin to High Infrared Bands in Azide Methemoglobin.

species	pH	±IHP	quaternary structure	$K_s = A_{LS}/A_{HS}$	χ_M(calcd) $\times 10^{-6}$ (cgs units)	ΔF (±IHP)
carp	8.3	−	R	7.2 ± 1.0	3600 (±200)	
	5.9	−	R	9.1 ± 1.3	3400 (±200)	
	5.9	+	T	1.2 ± 0.2	7500 (±600)	1.0–1.2
human A	6.5	−	R	11.7 ± 1.5	3100 (±200)	
	6.5	+	R	7.9 ± 1.0	3400 (±200)	0.2
M Iwate	6.5	−	T	3.3 ± 0.4	5000 (±300)	
	6.5	+	T	2.8 ± 0.3	5300 (±300)	0.1
M Milwaukee	6.5	−	R	⩾15	⩽2900	
	6.5	+	T	3.7 ± 0.4	4700 (±300)	>0.8

[a] A_{LS} and A_{HS} are the optical densities of the low- and high-spin bands, respectively. χ_M (calcd) is the molar susceptibility calculated on the assumption that the susceptibilities of the low- and high-spin components are 2200 and 14 000 × 10^6 cgs units, respectively, and that their absorption coefficients are equal. $\Delta F(\pm IHP) = 1.34 \log [K_s(+IHP)]/[K_s(-IHP)]$.

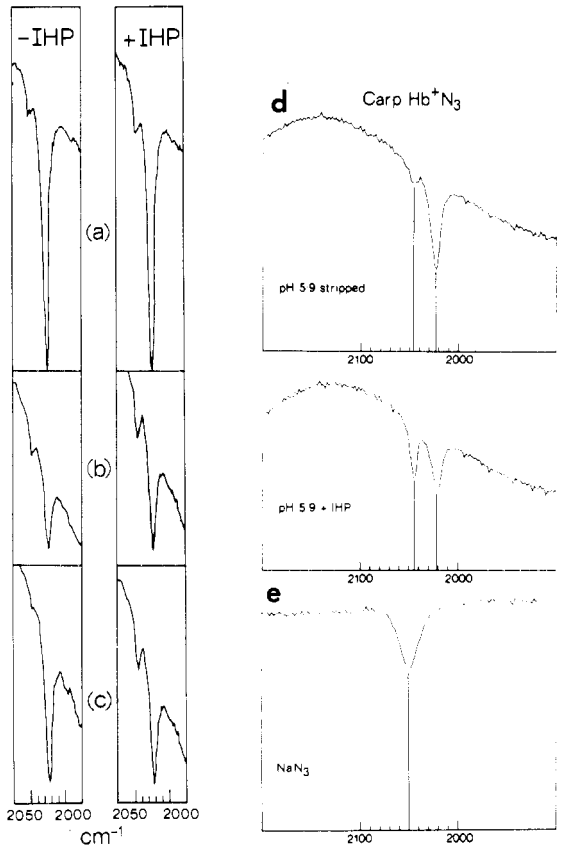


FIGURE 10: Effect of IHP on infrared azide stretching frequencies of human and carp azide methemoglobin at 20 °C. Other details are given in Materials and Methods section. (a) Human $Hb^+N_3^-$. (b) Hb M Iwate ($\alpha_2^+\beta_2^+N_3^-$). (c) Hb M Milwaukee ($\alpha_2^{+N_3^-}\beta_2^+$). (d) Carp $Hb^+N_3^-$. (e) 3 mM NaN_3.

tibility varies approximately with the fraction of hydroxymethemoglobin present in the samples. These fractions are listed in the sixth column of Table II; the last column shows rises in paramagnetic susceptibility calculated by assuming that the rise at pH 7.65 is due to the hydroxy component alone. The 9% rise in paramagnetic susceptibility at pH 7.65, if divided by the fraction of hydroxymethemoglobin present (0.2), corresponds to a 45% rise in the susceptibility of hydroxymethemoglobin on transition from R to T. The changes at other pHs do seem to follow the exponential change in the concentration of the hydroxymet component.

Human thiocyanate methemoglobin shows a rise of 11%, imidazole one of 5% and cyanate methemoglobin none at all. This last result suprised us because the difference spectrum of cyanate methemoglobin had shown a rise of the high-spin and fall of the low-spin bands on addition of IHP similar to that found in thiocyanate methemoglobin. However, the NMR results are conclusive, because our instrument could have detected an increase in the chemical shift of the water proton resonance in the hemoglobin solution relative to the resonance of pure water of 1 Hz, which corresponds to 57 μM Fe of a high-spin component of methemoglobin at a total hemoglobin concentration of 11 mM Fe, or a high-spin content of 0.5%. The small rise in imidazole methemoglobin, which is also pure low spin, must be due to dissociation of imidazole. The largest change was found in azide methemoglobin of trout IV. On changing its quaternary structure from T to R by raising the pH of our 12.8 mM Fe solution from 6.1 to 8.5, the $CuSO_4$ equivalent of the chemical shift of the chloroform proton dropped by a factor of 0.4. A similar change of pH had no effect on the paramagnetic susceptibility of human azide methemoglobin which remains in the R structure.

Infrared Spectra. McCoy & Caughey (1970) found that azide metmyoglobin and hemoglobin give two IR stretching frequencies, at 2023 and 2046 cm^{-1}, which they assigned to the low- and high-spin components, respectively. This assignment was confirmed by Alben & Fager (1972) who showed that the relative amplitudes of the two bands vary with temperature as would be expected from a thermal spin equilibrium with a reverse Curie law. From the spectra of human azide methemoglobin measured at 8 and 34 °C, they estimated an equilibrium constant $k^{25°C} = N_3^{lowspin}/N_3^{highspin} = 18$. We have measured the ratios of the two bands with and without IHP in carp hemoglobin, in human hemoglobin A, and in the abnormal human hemoglobins M Milwaukee (Val-67β→Glu) and M Iwate (His-87α→Tyr). The high-spin band at 2046 cm^{-1} overlaps the band of free azide ion at 2048 cm^{-1}, so that its amplitude cannot be measured without knowing the concentration of free azide. We have calculated the contribution of free azide to the peaks at 2046 cm^{-1} from the binding curves in Figure 4 for carp hemoglobin, the data of Perutz et al. (1974b) for human hemoglobin A, and the height of the IR peak given by a solution of 3 mM NaN_3. To minimize the amount of free azide present, the azide derivatives of both human hemoglobin A and carp hemoglobin were prepared by adding sufficient azide to saturate only 90% of the heme groups. In order to assure that azide bound to only the normal subunits of hemoglobins M Milwaukee and M Iwate, sufficient azide was added to saturate less than 25% of the heme groups and no correction for free azide was made.

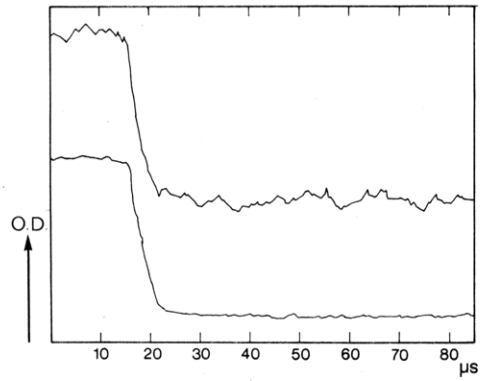


FIGURE 11: T-jump experiments. The upper trace was recorded with 100 μM human methemoglobin in 0.2 M sodium azide, 50 mM Bistris buffer (pH 6.5). λ of observation was 546 nm. The lower trace was obtained with 0.05 mM chlorophenol red in the same ionic mixture, and λ = 578 nm. The initial temperature was 18 °C and ΔT = 4 °C. The horizontal scale is 10 μs per division; the vertical scale is in arbitrary units. In the upper trace the total drop of the fast step corresponds to a 0.25% drop in optical density.

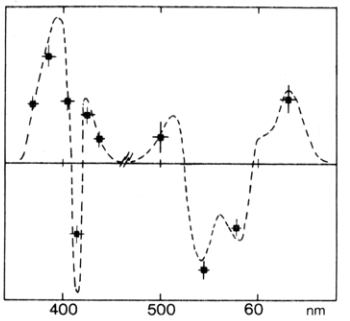


FIGURE 12: T-jump experiments. Spectral changes of the fast step measured at different wavelengths are compared with the difference spectrum produced by addition of IHP. The left part of the curves (λ < 450 nm) is multiplied by 0.2.

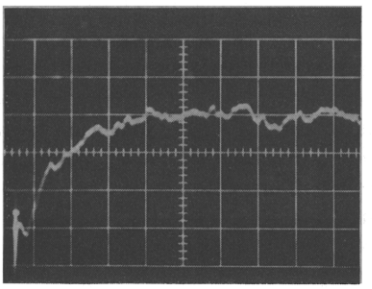


FIGURE 13: Change in transmission at 564 nm of human methemoglobin observed after a cable temperature jump from 0 to 4 °C in 0.1 M sodium azide, 0.1 M sodium phosphate (pH 6.5). The horizontal scale marks indicate intervals of 100 ns.

The results are shown in Figure 10 and Table III. In carp hemoglobin which changes its quaternary structure from R to T on addition of IHP, A_{LS}/A_{HS} drops from 9.1 to 1.2 (for definition see Table III). In human hemoglobin A, where the structure remains R, it merely drops from 12 to 8. In hemoglobin M Iwate only the β hemes react with azide and the molecule remains in the T structure regardless of the state of ligation of the β hemes (Hayashi et al., 1969; Greer, 1971). Note that A_{LS}/A_{HS} is lower than in hemoglobin A and that it drops from 3.3 to 2.8 even though IHP does not change the quaternary structure. Hemoglobin M Milwaukee without IHP has the normal R structure and with IHP the normal T structure. Its β-hemes are blocked by the carboxyl group of Glu-67 so that their affinity for azide is very low ($K_{eq} \sim 0.1$) and only the α-hemes react with azide (Hayashi et al., 1969; Perutz et al., 1972). The results show that in the R state the α-hemes are almost pure low spin, and that conversion to the T structure lowers A_{LS}/A_{HS} to about 4. These values should be representative for the state of the α-hemes also in azide methemoglobin A. Note that in the T state with IHP the fractions of high-spin component in the α-heme of the hemoglobin M Milwaukee and in the β-hemes of hemoglobin M Iwate are approximately equal.

Temperature-Jump Experiments. The magnetic and infrared measurements of carp azide methemoglobin show a striking increase in the high-spin fraction on transition to the T structure, but they provide no information about the chemical structure of the high-spin form. McCoy & Caughey (1970) drew attention to the similarity between the stretching frequency of high spin azide in hemoglobin and in the five-coordinated azidoprotoporphyrin IX dimethyl ester iron(III), and suggested that the Fe–N_ϵ bond might break on transition from the low- to the high-spin azide complex. If that were so, a large activation energy should be needed for the transition, which should manifest itself in a slow transition time. In nitrosylhemoglobin A, for instance, the transition from the six- to the five-coordinated heme takes about 0.5 s (Cassoly, 1974; Salhany, 1974). In collaboration with Dr. A. R. Fersht, we examined the kinetics of the spectral change accompanying the reaction of carp azide methemoglobin with IHP with a stopped-flow machine, but found that it occurred within the dead time of the instrument. We therefore turned to temperature-jump measurements to measure the relaxation time of thermal spin transitions that occur without change of quaternary structure.

Figure 11 shows the oscilloscope trace of a temperature-jump experiment with carp azide methemoglobin in the absence of IHP. The sudden rise in temperature is seen to be accompanied by a fall in the absorbance at 546 nm which is as fast as the spectral change in the very fast reacting dye chlorophenol red; both lie within the dead time of the instrument, whence the relaxation time $\tau_0 \leq 2$ μs at a confidence level of 90%. Figure 12 shows the wavelength dependence of the amplitudes of the effect observed in carp or human azide methemoglobin superimposed on the IHP-dependent difference spectrum of human azide methemoglobin; they agree well. In the absence of IHP no further changes in absorbance follow the temperature jump even at the slowest time we can observe, i.e., to an interval of 1 s. In the presence of 2.0 to 20 equiv of IHP/tetramer additional, slower spectral changes are observed in both human and carp hemoglobins. Their time constants varied in different derivatives and different species and their interpretation is unclear. Experiments with the cable temperature-jump apparatus showed that the fast relaxation time of azide methemoglobin in 0.1 M NaN_3 and 0.01 M sodium phosphate of pH 6.5 for a jump from 0 to 4 °C is faster than 100 ns for carp and is 260 ns for the human form. It is also faster than 100 ns in azide metmyoglobin of carp.

Discussion

Our results show that the change in quaternary structure from R to T is associated with transitions to higher spin in solutions of those methemoglobin derivatives which show a thermal spin equilibrium with a reverse Curie behavior at ambient temperatures. From changes in IR spectra small increases in paramagnetic susceptibility are inferred also on combination of IHP with human azide methemoglobin A which remains in the R structure and with azide methemoglobin M Iwate which remains in the T structure. The free-energy equivalents of these small changes in spin equilibria are

only 0.1–0.2 kcal/mol of heme, while those associated with R→T transitions are of the order of 1 kcal/mol of heme (Table III). This shows that the allosteric effector influences the state of the heme even in the absence of a change of quaternary structure, but that influence is weaker by about an order of magnitude than in the presence of a quaternary change.

Spin State and Bond Lengths in Azide Derivatives. What structural change in metal–ligand bonding is expected to accompany the changes from low to high spin in azide methemoglobins? The exact stereochemistry of the low-spin form can be inferred from the structure of (pyridine)[Fe(III) tetraphenylporphin]$^+$(N_3^-) in which the iron is displaced by 0.031 Å from the plane of the porphyrin nitrogens toward the azide group. The linear azido ligand is coordinated end-on and makes a Fe–N–N angle of 125°. Fe–N_{porph} = 1.990 Å and Fe–N_{az} = 1.926 Å (K. Adams, P. G. Rasmussen, and W. R. Scheidt, unpublished; quoted by Hoard, 1975). In azide metmyoglobin, the Fe–N–N angle is 111°, but the resolution was insufficient to derive accurate interatomic distances (Stryer et al., 1964). The N_3 ion points toward the methene bridge between pyrroles I and II in the notation of Perutz (1969) or β-methene in the Fischer notation (Hoard, 1975). No high-spin six-coordinated iron N_3^- porphyrins are known; the structure of a high-spin five-coordinated one, (N_3^-)[Fe(III) (tetraphenylporphine)]$^+$ shows a Fe–N_{porph} bond length of 2.065 Å, much longer than that of the low-spin one, and an Fe–N_{az} distance of 1.91 Å, slightly shorter than that of the low-spin one, probably because the iron lies further out of the plane of the porphyrin (0.45 Å) and is therefore more accessible to the azide ion (Hoard, 1975).

In azide metmyoglobin and hemoglobin one would therefore expect the displacement of the iron from the porphyrin plane to be correlated with the spin equilibrium, but in the absence of exact structural information on these derivatives themselves, we may now ask whether there is a correlation between the displacement of the iron from the mean plane of the porphyrin ring in various aquomet derivatives whose structures are accurately known and the spin equilibrium of the corresponding azide derivatives. That displacement is 0.40 Å in sperm whale aquometmyoglobin, 0.07 Å in the α hemes, and 0.21 Å in the β-hemes of horse aquomethemoglobin. We may assume that the displacements in human aquomethemoglobin are the same as in horse. At 25 °C the equilibrium constants [$N_3^{lowspin}$]/[$N_3^{highspin}$] derived from IR spectroscopy by Alben & Fager (1972) are 8.1 for sperm whale azide metmyoglobin and 18 for human azide methemoglobin in the R structure. Our IR spectra show that in the R structure of azide methemoglobin M Milwaukee the α hemes are almost pure low spin. Since the R structure of hemoglobin M Milwaukee is the same as that of hemoglobin A, this result implies the α-hemes of azide methemoglobin A are also almost pure low spin and that most, if not all, the high-spin component of azide methemoglobin A is therefore in the β-hemes, i.e., the ones where the displacement of the iron from the porphyrin plane in aquomethemoglobin is larger. The spin equilibrium of azide methemoglobin or myoglobins therefore appears to be correlated with the displacement of the iron from the plane of the porphyrin in the corresponding aquo derivatives, the greater that displacement, the higher the spin. The magnitude of the displacement must be imposed on the heme by the structure of the globin.

Stereochemical Changes Accompanying Spin Changes in Synthetic Iron Complexes. As will be shown below, the short relaxation time of the thermal spin equilibrium suggests that in high-spin azide methemoglobin the iron atoms remain six-coordinated. We must therefore ask whether there is any precedence for a spin change without change of coordination, and, if so, what stereochemical and other changes could be expected to accompany it. Thermal spin equilibria have been observed in a great variety of ferrous and ferric octahedral complexes in which the iron atoms are chelated to either nitrogen, oxygen, or sulfur, and in many instances the iron–ligand distances have been determined by x-ray analysis. In Fe(III)$(mcd)_3$ the iron is coordinated to six sulfur atoms (for the formula of this and other complexes quoted here see Table I). If this complex is crystallized from water or other hydrogen-bonding solvents, its ground state is $S = 3/2$ with ⟨Fe–S⟩ = 2.443 Å. If the identical complex is crystallized from benzene its ground state is $S = 1/2$ with ⟨Fe–S⟩ = 2.318 Å (Butcher et al., 1976; Butcher & Sinn, 1976). The difference is due to the occlusion of solvent molecules in the crystal lattice; hydrogen bonding solvent molecules may withdraw electrons from the sulfur atoms, thus weakening the Fe–S bonds and favoring higher spin. The complex [Fe(II)(6-Me-Pyr)$_3$tren]$^{2+}$ in solution is high spin, while the complex [Fe(II)(Pyr)$_3$tren]$^{2+}$ in solution is low spin; solutions of complexes in which either one or two of the three pyridines carry methyl groups in position 6 show thermal spin equilibria. The iron atoms are octahedrally coordinated to the three pyridine and the three imino nitrogens. On transition from the high to the low spin form, the Fe–N_{pyr} bond lengths shorten by 0.31 Å and the Fe–N_{im} bond lengths by 0.20 Å. Note that in the high-spin form these bonds are unusually long; this is believed to be due to overcrowding of the complex by the six methyl groups which prevent as close an approach of the iron atoms to the nitrogens as would be needed in a low-spin complex (Hoselton et al., 1975). A similar phenomenon had been found in [tris(2-methyl-1,10-phenanthrolin)Fe(II)] by Goodwin & Sylva (1968).

In the pair of complexes [Fe(III)$(acac)_2$trien]PF_6 and [Fe(III)$(sal)_2$trien]$Cl_2 \cdot H_2O$ the iron atoms are coordinated octahedrally to four atoms of nitrogen and two of oxygen; yet the former is pure high spin and the latter pure low spin (Sim et al., 1978). This difference is brought about, apparently, through the solvent water molecules in the latter which act as proton acceptors in hydrogen bonds from NH groups coordinated to the iron (H_2O···HN·Fe). These hydrogen bonds weaken the N–H bonds and thereby strengthen the N–Fe bonds, so that the complex becomes low spin, with a reduction of 0.12 Å in the average iron–ligand bond distance. Finally, Leipold & Coppens (1973) have determined the crystal structure of the complex Fe(III)-[tris(N^1,N-diethyldithiocarbamate)] at 297 K, where it is mostly high spin and at 79 K where it is mixed spin, and found no change in coordination, but a contraction of the average Fe–S bond lengths by 0.051 Å. Clearly then, a change from low to high spin can occur without change in coordination, but it is accompanied by marked increases in the lengths of Fe–ligand bonds.

The analogies between the magnetic and other properties of azide methemoglobin and synthetic iron complexes which remain six-coordinated in both high- and low-spin states suggest that azide methemoglobin may also remain six coordinated. An alternative to rupture of the Fe–N_ϵ bond would be an extension, due to tension of the heme and to its weakening by occupation of the d_{z^2} antibonding orbital. This is not unlikely since an Fe–$N_{piperidine}$ bond as long as 2.47 Å has been observed in the sterically hindered (nitrosyl-α_1,β_1,γ_1,δ_1-tetraphenylporphinato(4-methyl)piperidine)Fe(II), in which the d_{z^2} antibonding orbital is occupied by the unpaired NO electron (Scheidt et al., 1978). This example shows that the Fe–N_ϵ is capable of considerable stretching.

Comparison of Vibrational Spectra and Relaxation Times with Those of Synthetic Iron Complexes. The azide stretching frequency in high-spin azide methemoglobin (2046 cm^{-1}) is

slightly lower than in the free azide ion (2048 cm^{-1}) and higher than in the low-spin form (2023 cm^{-1}). Effects of spin change on vibrational spectra have also been observed in comparable synthetic complexes. For example, in [Fe(III)$(mcd)_3$] the Fe–S stretching frequencies of the two difference spin states differ by 37 cm^{-1} (Butcher et al., 1976). In [Fe(II)(phenanthrolin)$_2(NCS)_2$] and in [Fe(II)(6-Me-Pyr)$_3$tren] the CN stretching frequencies of the high- and low-spin forms differ by $\leq$50 cm^{-1} (Tweedle & Wilson, 1976).

The relaxation time in carp azide methemoglobin (<100 ns) is more than 10^7 times shorter than the time constant of the spectral change associated with the transition from six- to five-coordinated heme in nitrosylhemoglobin (Cassoly, 1974; Salhany, 1974), and may be comparable with the relaxation time of 15 ns in hydroxymetmyoglobin (Dose et al., 1977) and with the relaxation times of synthetic iron complexes *in solution*, which are 32 ns in [Fe(II)[hydrotris(pyrazolyl)borate] (Beattie et al., 1973) and 10–100 ns in [Fe(II) $(Pyr)_3tren^{2+}$] (Hoselton et al., 1975). This means that the vibrational free energy of activation of the thermal spin equilibrium in carp azide methemoglobin must be less than 8 kcal/mol and makes it unlikely that it involves the rupture of any chemical bond.

Optical Spectra and Magnetic Changes. Optical absorption spectra and their changes with temperature or quaternary structure have proved reliable guides to changes in quaternary structure, but unreliable guides to spin states. IHP produces qualitatively similar difference spectra in several derivatives such as azide, cyanate, and aquomethemoglobin, no matter whether their paramagnetic susceptibilities rise or remain unchanged. Azide methemoglobin and aquomethemoglobin both show a fall in the "low-spin" bands and a rise in the "high-spin" bands as the temperature rises from 0 to 20 °C; yet in azide methemoglobin the paramagnetic susceptibility rises, while in aquomethemoglobin it falls. Clearly the interpretation of these bands needs to be reconsidered, and the names "high" and "low" spin bands should be dropped. Inconsistencies between optical spectra and paramagnetic susceptibilities of synthetic iron porphyrins have been found also by Brault & Rougee (1974).

Spin States and Ligand Affinities. It is interesting to note that spin state appears to be the main parameter controlling the relative affinities of water and other ligands for the R and T states of the hemoglobin molecule. Thus the ease of replacing water by the predominantly high-spin ligands, fluoride and thiocyanate, is insensitive to quaternary structure of the molecule, even though thiocyanate is a bulkier ligand than fluoride. The replacement of water by either azide or cyanide is sensitive to quaternary state, being inhibited by conversion to the T structure upon addition of IHP. The effect is somewhat larger for cyanide than for azide, consistent with the fact that the cyanide derivative is entirely low spin in both the R and T states, while the azide derivative is partially high spin in the R structure and becomes more high spin in the T conformation.

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References

Alben, J. O., & Fager, L. Y. (1972) *Biochemistry 11,* 842.

Albertsson, J., & Oskarsson, Å. (1978) *Acta Crystallogr., Sect. B 33,* 1871.

Anusiem, A. C. I. (1978) *J. West Afr. Sci. Assoc. 21* (in press).

Banerjee, R., Stetzkowski, F., & Henry, Y. (1973) *J. Mol. Biol. 73,* 455.

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

Beetlestone, J. G., & George, P. (1964) *Biochemistry 3,* 707.

Brault, D., & Rougee, M. (1974) *Biochemistry 13,* 4598.

Brunori, M. (1975) *Curr. Top. Cell. Regul. 9,* 1.

Butcher, R. J., & Sinn, E. (1976) *J. Am. Chem. Soc. 98,* 5159.

Butcher, R. J., Ferraro, J. R., & Sinn, E. (1976) *J. Chem. Soc., Chem. Commun.,* 910

Cassoly, R. (1974) *C. R. Hebd. Seances Acad. Sci., Ser. D 278,* 1417.

Cassoly, R., & Gibson, Q. H. (1972) *J. Biol. Chem. 247,* 7332.

Collman, J. P., Gagne, R. R., Reed, C. A., Robinson, W. T., & Rodley, G. A. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 1326.

Dose, E. V., Tweedle, M. F., Wilson, L. J., & Sutin, N. (1977) *J. Am. Chem. Soc. 99,* 3886.

Fermi, G. (1975) *J. Mol. Biol. 97,* 247.

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

Fung, L. W.-M., & Ho, C. (1975) *Biochemistry 14,* 2526.

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

Greer, J. (1971) *J. Mol. Biol. 29,* 107.

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

Hayashi, A., Suzuki, T., Imai, K., Morimoto, H., & Watari, H. (1969) *Biochim. Biophys. Acta 194,* 6.

Henry, Y., & Banerjee, R. (1973) *J. Mol. Biol. 73,* 469.

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

Hoard, J. L., & Scheidt, R. W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70,* 3919.

Hoffman, G. W. (1971) *Rev. Sci. Instrum. 42,* 1643.

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

Iizuka, T., & Morishima, I. (1974) *Biochim. Biophys. Acta 371,* 1.

Jameson, G. B., Robinson, W. T., Collman, J. P., & Sorrell, T. N. (1978a) *Inorg. Chem. 17,* 858.

Jameson, G. B., Rodley, G. A., Robinson, W. T., Gagne, R. Reed, C. A., & Collman, J. P. (1978b) *Inorg. Chem. 17,* 850.

Leipold, J. G., & Coppins, P. (1973) *Inorg. Chem. 12,* 2269.

McCoy, S., & Caughey, W. S. (1970) *Biochemistry 9,* 2387.

Messana, C., Cerdonio, M., Shenkin, P., Noble, R. W., Fermi, G., Perutz, R. N., & Perutz, M. F. (1978) *Biochemistry 17* (following paper in this issue).

Nagai, K. (1977) *J. Mol. Biol. 111,* 41.

Perutz, M. F. (1969) *Proc. R. Soc. London, Ser. B 173,* 113.

Perutz, M. F., Pulsinelli, P. D., & Ranney, H. M. (1972) *Nature (London), New Biol. 237,* 259.

Perutz, M. F., Ladner, J. E., Simon, S. R., & Ho, C. (1974a) *Biochemistry 13,* 2163.

Perutz, M. F., Fersht, A. R., Simon, S. R., & Roberts, G. C. K. (1974b) *Biochemistry 13,* 2174.

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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