Influence of Globin Structure on the State of the Heme. III. Changes in Heme Spectra Accompanying Allosteric Transitions in Methemoglobin and Their Implications for Heme–Heme Interaction†

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ABSTRACT: Parts I and II (Perutz *et al.* (1974), *Biochemistry 13*, 2163, 2174) presented evidence indicating that inositol hexaphosphate (IHP) is capable of switching the quaternary structure of certain high-spin ferrous and ferric hemoglobins from the oxy (R) to the deoxy (T) state. Here we analyze the resulting changes in visible and near-infrared absorption spectra, paramagnetic susceptibility, and paramagnetically shifted proton and electron spin resonances. In aquo- and hydroxymethemoglobin where components of high and low spin coexist in thermal equilibrium, IHP causes changes in the absorption spectra and paramagnetically shifted proton resonances indicative of a shift of the equilibrium to higher spin. This is confirmed by a stoichiometric rise in the paramagnetic susceptibility of aquomethemoglobin solutions by up to 6.4% at concentrations of 1 mol of IHP/mol of tetramer. Other spectral changes produced by IHP include a rise in intensity of the Soret band and red shifts of all high-spin bands. The spectral changes are identical with those observed by Perutz ((1972), *Nature (London) 237*, 495) on dissociation of carbon monoxide from valency hybrids. In fluoromethemoglobin which is probably pure high spin, IHP causes red shifts of all bands, a rise in intensity of the Soret band and a fall in all others. In iron–porphyrin complexes which are in thermal equilibrium between two different spin states, a shift toward higher spin is stereochemically equivalent to a lengthening of the distances between the iron atom and its electronegative ligands. Such a lengthening would diminish electrostatic repulsion of electronic charge on the iron, thus lessening the energy needed to transfer charge from the porphyrin to the iron and raising the energy required for the transfer of charge from the iron to the porphyrin. Qualitatively this would account for the blue shifts of charge-transfer bands in deoxyhemoglobin (part I) and for their red shifts in high-spin methemoglobins. A mechanism explaining the observed intensity changes is also proposed. It was shown in part I that in the R structure the hemes appear to be in the same state as in free α and β subunits. The lengthening of the iron–nitrogen bond distances observed in the T structure implies that the globin exercises a tension on the heme which pulls the iron atoms further from the planes of the porphyrin rings. Such a tension would oppose the transition to the low-spin state that is needed for combination with oxygen and thereby lower the oxygen affinity. Since heme–heme interaction is observed only when reaction with ligands is accompanied by a change of quaternary structure, our results imply that it is coupled to a change of tension at the heme, transmitted by a change in quaternary structure of the globin.

Evidence presented in parts I and II (Perutz *et al.*, 1974a,b) indicated that inositol hexaphosphate (IHP)[1] is capable of converting high-spin hemoglobin derivatives from the quaternary R to the T structure, and that this transition is accompanied by marked spectral changes. IHP was also found to cause spectral changes in several low-spin derivatives, but these were weaker and did not appear to be associated with transitions between the two quaternary structures. In this paper we examine in detail the changes in electronic heme spectra, paramagnetic susceptibility, paramagnetically shifted proton resonances, and electron spin resonances produced in methemoglobin by IHP.

The derivatives we have examined include fluoromethemoglobin which is almost pure high spin, aquo-, hydroxy-, and azidomethemoglobin in which components of high and low spin coexist in thermal equilibria, and cyanomethemoglobin which is almost pure low spin. In fluoromethemoglobin IHP strengthens the Soret band and shifts it slightly to the red; it also causes weakening and red shifts of all absorption bands in the visible and near infrared regions. In azidomethemoglobin, which is mostly low spin and is not converted to the T structure, IHP slightly weakens the Soret band and shifts it to the blue; it also strengthens the high- and weakens the low-spin bands in the visible, but without causing any significant shifts. Aquo- and hydroxymethemoglobin exhibit mixtures of the two different effects seen in fluoro- and azidomethemoglobin. The Soret band is strengthened and shifts to the red or blue depending on pH. The low-spin bands are always weakened. The high-spin bands of hydroxymethemoglobin are always strengthened, but the intensities of the high-spin aquomet bands go either up or down, apparently depending on the dominance of either the fall associated with the red shifts as in fluoromet or the rise associated with the change in spin equilibrium referred to below. In cyanomethemoglobin IHP possibly causes as light red shift of the Soret band, but no significant changes in the visible spectrum.

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[1] Abbreviations used are: IHP, inositol hexaphosphate; NES, *N*-ethylsuccinimide.

TABLE I: Conditions Used for Determination of Difference Spectra.[a]

Derivative	pH	Spectral Region	Hb Concn (μM)/ Tetramer	Heme Ligand Concn (M)	IHP (mol/ Tetramer Methb	Buffer (M)
F-met	6.5	Soret	3.0	NaF (0.1)	200	Bis-Tris (0.1)
		Visible	38	NaF (0.1)		Bis-Tris (0.1)
		Ir	150	NaF (0.1)	2	Bis-Tris (0.1)
Aquomet	5.0	Soret	2.4		200	Acetate (0.1) + NaCl (0.1)
		Visible	38		1	Acetate (0.1) + NaCl (0.1)
		Ir	150; 300		1	Acetate (0.1) + NaCl (0.1)
Aquomet	6.0–7.2	Soret	1.5; 3.0		200	Bis-Tris (0.05) + NaCl (0.1) or Bis-Tris (0.2)
		Visible	38		1–10	
		Ir	150		1–4	
Aquomet	7.55	Visible	38		10	
N_3-met	6.5	Soret	3.0	Na N_3 (0.1)	300	Bis-Tris (0.05) + NaCl (0.1)
		Visible	33		1.7; 10	Bis-Tris (0.05) + NaCl (0.1)
CN-met	6.5	Soret	3.0	NaCN (30 μM)	200	Bis-Tris (0.05) + NaCl (0.1)
		Visible	15			Bis-Tris (0.05) + NaCl (0.1)

[a] Temperature, 20°.

Our optical studies suggested that IHP shifts the equilibrium between high- and low-spin components of aquo-, hydroxy-, and azidomethemoglobin to higher spin. This was confirmed when we measured the paramagnetic susceptibilities of these methemoglobins as a function of IHP concentration. Below pH 7.0 the susceptibility of aquomethemoglobin was found to rise linearly by up to 6.4% at a concentration of 1 mol of IHP/mol of tetramer. Addition of IHP to azidomethemoglobin also raises its (very low) paramagnetic susceptibility significantly.

In compounds containing a paramagnetic ion, the magnitudes of the paramagnetic shifts of the proton resonances are, to a first approximation, proportional to $S(S + 1)$, where S is the total electronic spin of the ion. The paramagnetically shifted proton resonances of aquomethemoglobin lie downfield from the HDO peak by between 30 and 100 ppm. As expected from the change in spin equilibrium, IHP was found to increase the magnitudes of the downfield shifts. We have also observed a small increase in the intensity of the electron resonance (esr) line of the high-spin aquomet component at $g = 6.0$, but this increase probably occurred because IHP inhibited the formation of hemichrome which normally takes place on cooling solutions of aquomethemoglobin to low temperatures (see Discussion).

In iron–porphyrin complexes which are in thermal equilibrium between two different spin states, a shift toward higher spin is stereochemically equivalent to a lengthening of the bonds from the iron atom to its electronegative ligands. This finding provides the key to the interpretation of the changes in absorption spectra and ligand affinities in other hemoglobin derivatives in which a single spin state predominates regardless of the quaternary structure of the globin. It will be shown that the lengthening of the iron–nitrogen bonds can account for the blue shifts of the visible and near-infrared absorption bands observed in the R → T transition of certain deoxyhemoglobins (part I; Perutz *et al.*, 1974b) and their red shifts in fluoromethemoglobin. It may also account for the strengthening of the Soret band and the weakening of the charge-transfer bands in these two hemoglobins. Finally, it contributes to the lowering of the oxygen affinity on transition from the R to the T state. The implications of these findings for heme–heme interaction will be discussed.

Methods

Human methemoglobin was prepared as described in part I (Perutz *et al.*, 1974b). Conditions for optical spectroscopy are listed in Table I. Using a glass electrode, solutions of the sodium salt of inositol hexaphosphate (Sigma) were titrated with HCl to the same pH as the methemoglobin solution under study before addition to the sample.

Magnetic susceptibilities were measured on a Gouy balance as described by Beetlestone and George (1964). The balance was standardized with 1-4 mM solutions of $NiCl_2$. When determining the absolute values of susceptibilities, we must consider errors in the calibration of the balance, the corrections for the diamagnetism of the protein and the blank cell, the concentration of hemoglobin and the apparent change in weight upon turning on the magnetic field. Taking into account all these factors we estimate our standard error in χ_M to be ±1%. However, all except the last of these errors should remain constant within any one particular series of experiment measuring only the change of susceptibility as a function of IHP concentration, which reduces the standard error to 0.4%. Hemoglobin concentrations were determined from the optical density of cyanomethemoglobin at 541 nm using a molar extinction coefficient of 10.9 mM heme. IHP solutions were prepared as described above. The buffer compositions are shown in Table II. As a check we also determined susceptibilities by the nuclear magnetic resonance method of Reilly *et al.* (1955), as described in part I. For this purpose "stripped" solutions of methemoglobin were concentrated by pressure dialysis to above 4 mM tetramer. To a 1-ml sample of this solution, 1.25 mol equiv of IHP was added in the form of 50 μl of a 0.1 M solution of IHP titrated to pH 7.0. This raised the pH of the methemoglobin solution, due both to the Bohr effect accompanying the change in quaternary structure and the uptake of protons by the weakly basic amino acid side chains that combine with IHP (Kilmartin, 1973). The solution was then titrated to the desired pH with 4 M NaH_2PO_4

TABLE II: Composition of Solutions Used for Measuring Susceptibilities with the Gouy Balance.

	pH	[Hb heme]	Buffer	[N_3]
Hb^+OH_2	6.04	1.58×10^{-3}	Bis-Tris I = 0.05	
	6.65	1.428×10^{-3}	Bis-Tris I = 0.05	
	7.45	1.55×10^{-3}	Bis-Tris I = 0.05	
	NB composition of buffer:		Bis-Tris, ~0.008 M NaCl, ~0.042 M	
HbN_3	6.04	2.86×10^{-3}	Bis-Tris I = 0.05	0.02 M
	NB composition of buffer:		Bis-Tris, ~0.006 M NaCl, ~0.044 M	

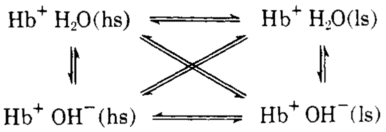
using a small glass electrode and a microburet. The sample without IHP was similarly titrated and an aliquot of water was added with a microburet to bring it to exactly the same volume as that of the first sample. To convert samples to azidomethemoglobin, 2 molar equiv of NaN_3/heme was added in the form of a 0.5 M solution of NaN_3 titrated to pH 7.0, before addition of IHP. The optical absorption spectra of the samples with and without IHP were recorded after the susceptibility measurements to check that they conformed to those previously observed in dilute methemoglobin solutions. No special pains were taken to find the absolute values of χ_M as we aimed merely at measuring the changes produced by IHP.

Nuclear magnetic resonance (nmr) spectra were obtained on the MPC-HF 250-MHz superconducting spectrometer in Pittsburgh, as described in part I. For electron spin resonance (esr) studies it became essential to add a reference standard to the sample. $CuSO_4$ was found to denature the hemoglobin, but when chelated to EDTA it had no adverse effect. We therefore dialyzed a "stripped" 3.5 mM (tetramer) solution of methemoglobin against 0.05 M Bis-Tris + 0.1 M NaCl + 0.8 mM $CuSO_4$ + 1.6 mM EDTA of pH 7.0. After dialysis we divided the sample into two. To 2.5 ml of methemoglobin solution we added 0.34 ml of a 50 mM solution of IHP titrated to pH 6.7. We then equalized the pH of the samples with and without IHP. Esr measurements were made on a conventional X-band 10-GHz spectrometer at 77°K, and the intensities of the lines were measured relative to the copper standard.

Spin States and Spectra of Methemoglobin Derivatives. The spectroscopic and magnetic properties of methemoglobin derivatives in solution or in the crystalline state indicate that they contain mixtures of components of high spin ($S = {}^5/_2$) and low spin ($S = {}^1/_2$) in thermal equilibria (for references see part II). There is evidence that the fraction of low-spin component in fluorometmyoglobin is less than 3% (Theorell and Ehrenberg, 1951; Scheler *et al.*, 1957; George *et al.*, 1961); since the paramagnetic susceptibility of fluoromethemoglobin is the same as that of myoglobin, the fraction of low-spin component should be equally low. At 20° the fraction of high-spin component in azidomethemoglobin is somewhat less than 10%. Cyanomethemoglobin is probably pure low spin.

Aquomethemoglobin is a more complex system, since the heme-linked water molecule ionizes to form a hydroxyl ion (pK = 8.05 at 20° and ionic strength = 0.2), and both the hydrate and the hydroxide contain high- and low-spin states in thermal equilibrium. The fraction of low-spin component in aquomethemoglobin at 20° and pH 6.5 is about 10% and that in hydroxyhemoglobin at pH 10.0 is about 50% (George *et al.*, 1961). As the temperature is lowered, the fraction of low-spin component rises in hydroxy- and azidomethemoglobin and falls in aquomethemoglobin, so that above 0° Curie's law is not obeyed in any of these derivatives (Beetlestone and George, 1964). Below about 120°K aquometmyoglobin begins to obey Curie's law for a pure high-spin d^5 ion, while azidometmyoglobin behaves as a pure low-spin d^5 ion (Iizuka and Kotani, 1969a). The same is true for azido- and hydroxymethemoglobin, but aquomethemoglobin exhibits more complex properties (Iizuka and Kotani, 1969b) which will be considered in the Discussion. The equilibria between the different forms of aquo- and hydroxymethemoglobin are pictured below. Their individual characteristics are mostly unknown. The high-spin forms of aquo- and hydroxyhemoglobin and the low-spin form of hydroxyhemoglobin (see Scheme I) each have a distinct spectrum (George *et al.*, 1961) but the spectrum of low-spin aquomethemoglobin is not known because its fractional concentration is too low to observe it.

SCHEME I[a]



[a] hs = high spin; ls = low spin.

Table III lists the absorption bands of the five derivatives examined here. The bands fall into two distinct classes: the α, β, and γ bands which are observed in metal-free porphyrins and are thought to be due to transitions from highest filled π orbitals to empty or partially filled π^* orbitals. The second class comprises all those absorption bands not present in metal-free porphyrins, which are believed to be partly or wholly charge-transfer transitions. These transitions would be from highest filled π orbitals of the porphyrin to the lowest partially filled $e_g(d^*\pi)$ orbitals of the ferric ion. Those bands that are close in energy to the very intense Soret band could also borrow intensity from it through mixing of the iron $e_g(d\pi)$ orbitals with the lowest empty porphyrin $e_g(\pi^*)$ orbitals. Conversely the absorption bands in the near-infrared region, which are furthest from the Soret band, are the ones most likely to be predominantly charge transfer, and their transition energy is likely to be governed mainly by the degree to which the negative charges of the heme ligands repel the transferred electron. This point will be crucial to the interpretation of the spectral changes to be described here. (See, for instance, Platt, 1956, Zerner *et al.*, 1966, Eaton and Hochstrasser, 1968, and Smith and Williams, 1970.)

Various other properties of the methemoglobin spectra are noteworthy. (1) The wavelength of the Soret or γ band of the various derivatives decreases with rising paramagnetic susceptibility. Eaton and Hochstrasser (1968) discovered that in aquomethemoglobin and other ferric derivatives of both high and low spin it is split into two components polarized in mutually perpendicular directions in the plane of the porphyrin. (2) The intensity of the β band rises with decreasing paramagnetic susceptibility. (3) The α band is always weak in high-spin compounds and of variable strength in low-spin ones. (4) The band near 500 nm generally becomes stronger and that near 630 nm weaker when red shifted (Smith and Williams, 1970). The polarization ratio of the band near 500 nm tends to be the same as that of the Soret band, while the changes in polarization ratio across its width follow that of the β band. Eaton and Hochstrasser (1968) therefore attribute it to vibrational splitting of the β band, while Smith and Williams (1970) consider it likely that it arises from configuration interaction

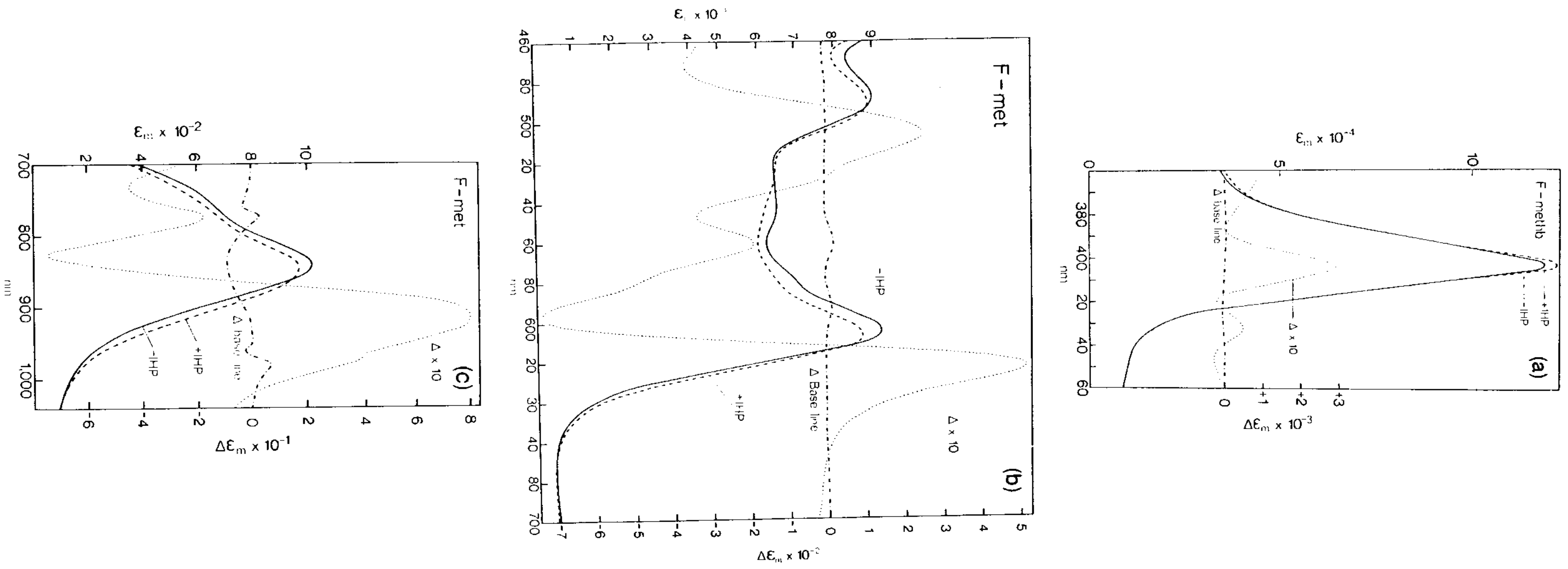


FIGURE 1: Effect of IHP on the electronic absorption spectrum of fluoromethemoglobin: (a) the Soret band; (b) the visible; (c) near infrared region. For concentrations of reagents in this and subsequent figures, see Table I. The Δ base line in all figures is 10× magnified, like the difference spectra. The latter were not corrected for variations in the base line but are reproduced as recorded. All values of ε in this and subsequent figures are in moles of heme.

between a charge transfer and a π^* state. They regard both the bands at 500 and 630 as mixed, the former acquiring more and the latter less $\pi \rightarrow \pi^*$ character (and intensity) as they

TABLE III: Heme Absorption Bands of Human Methemoglobin Derivatives.[a]

Probable Assignments	Mostly Protein	Tyr	Shoulder on Soret band	γ = Soret Mostly $\pi \rightarrow \pi^*$	$\pi \rightarrow \pi^*$ and/or CT	β Mostly $\pi \rightarrow \pi^*$	α Mostly $\pi \rightarrow \pi^*$	CT and $\pi \rightarrow \pi^*$	Mostly CT		
Fluoro	274 (28)	290 s (21)		403.7 (119)	486 (8.5)	543 (6.5)	579 s (6.7)	603 (8.5)	738 s (0.62)	840 (1.09)	1110 s (0.15)
Aquo pH 6.5	275 (26)	290 s (21)		404.9 (161)	500.2 (9.04)	540 s (5.8)	580 (3.4)	630.5 (4.25)		1000 (0.86)	
Hydroxy pH 10.1	272 (34)	290 s (25)		412.4 (95)	486 s (8.2)	540 s (9.8)	576.5 (8.9)	600 s (5.9)	816 (0.52)	980 (0.19)	
Azide	275 (33)	290 s (27)	353 s (29)	417.7 (118)		542 (11.4)	573 (8.9)	628 s (1.9)		960 s (0.17)	1263 (0.24)
Cyanide	274 (30)	290 s (23)	356 s (30.5)	420 (112)		541 (11.0)				1060 s (0.09)	1280 (0.20)

[a] CT = charge transfer; s = shoulders. Figures in parentheses indicate ϵ_{mM} (heme). They are based on the internationally accepted standard of ϵ_M (541 nm) = 11.0 for human cyanomethemoglobin (van Kampen and Zijlstra, 1961; van Assendelft, 1970) and have been determined with a Cary 14 spectrophotometer under the conditions listed in Table I. The spectrum of hydroxymethemoglobin was determined in 0.1 M borate buffer of pH 10.1.

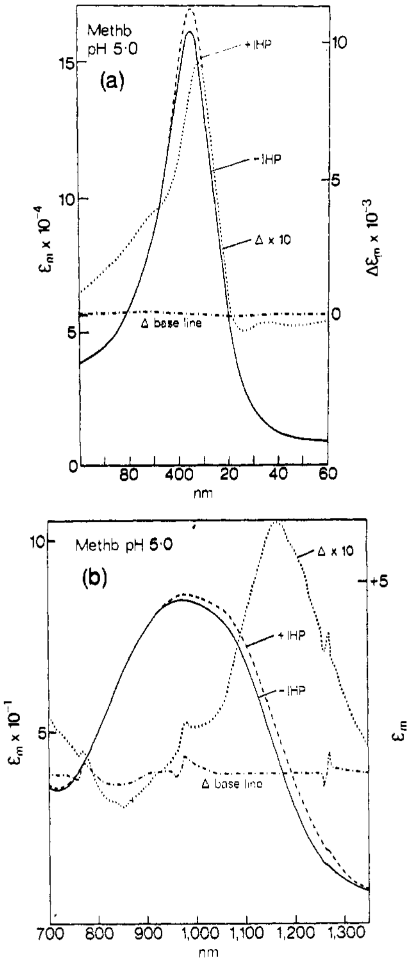


FIGURE 2: Effect of IHP on the spectrum of aquomethemoglobin at pH 5.0: (a) Soret band; (b) near-infrared spectrum.

are red shifted. We shall see that these regularities do not always apply in the transitions studied here. (5) All bands are polarized in the plane of the porphyrin, with the possible exception of an out-of-plane polarized component underlying some of the in-plane polarized visible bands of azidomethemoglobin (Eaton and Hochstrasser, 1968).

Results

Changes in the Soret Band and the Visible and Near-Infrared Absorption Spectra. The changes produced by IHP in the electronic heme spectra of different methemoglobin derivatives are shown in Figures 1–6 and listed in Table IV. Addition of IHP to fluoromethemoglobin raises the intensity of the Soret band and shifts it slightly to the red. It also lowers the intensity and causes marked red shifts of all absorption bands in the visible and near-infrared region. In azidomethemoglobin IHP slightly lowers the intensity of the Soret band and shifts it to the blue. It also lowers the intensity of the α and the β bands and raises that of the band at 630 nm. Finally, it gives rise to the appearance of a new band at 500 nm and possibly a shoulder at 600 nm. According to the relationships between spectra and magnetic susceptibility described earlier, all the changes in azidomethemoglobin are indicative of a shift of the thermal equilibrium to higher spin. The magnitude of the shift is estimated in Table V. It could be argued that the same spectral effects might have been produced by a slight dissociation of the azide complex in the presence of IHP, but this possibility is excluded by the observation that IHP produced the same difference spectrum in the presence of a 670-fold excess of azide as it did in the presence of an only 10-fold excess.

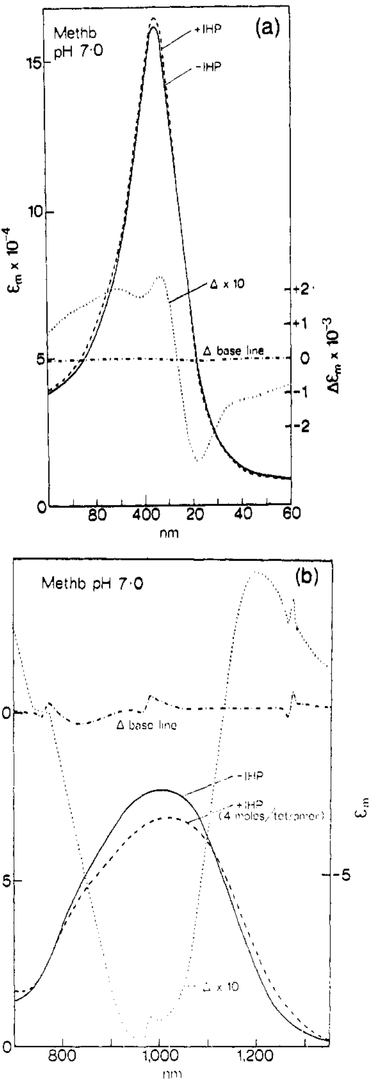


FIGURE 3: Effect of IHP on the spectrum of aquomethemoglobin at pH 7.0: (a) Soret band; (b) near-infrared spectrum.

We shall now consider the changes in aquo- and hydroxymethemoglobin at different pH's, bearing in mind that the affinity for IHP drops sharply above pH 7.0. We have been able to cover the range from pH 5.0 to 7.55, corresponding to fractional concentrations of hydroxyhemoglobin of between 0.1 and 22%. At pH 5.0, IHP raises the intensities of the Soret band and of the bands at 500, 630, and 1000 nm, and lowers the intensities of the α and β bands (Figures 2 and 4). The difference spectra also show peaks on the long-wavelength side of the bands at 500, 630, and 1000 nm which could be indicative either of red shifts or shoulders. However, the absorption bands themselves show no signs of any new shoulders after addition of IHP, unlike the band of deoxyhemoglobin at 555 nm where a clear shoulder develops (Figure 4, part I). We are therefore inclined to interpret the changes as red shifts.

The effect of rising pH on the difference spectra in the visible is shown in Figures 4 and 5a. At pH 6.0 and 6.5 the difference spectra look similar to that at pH 5.0, except that a shoulder at 600 nm has grown into a peak. The spectra themselves continue to show a rise and red shift of the band at 500 nm, but now a fall and red shift of the band at 630 nm. As the pH rises, first to 7.2 and then to 7.55, the difference spectra change

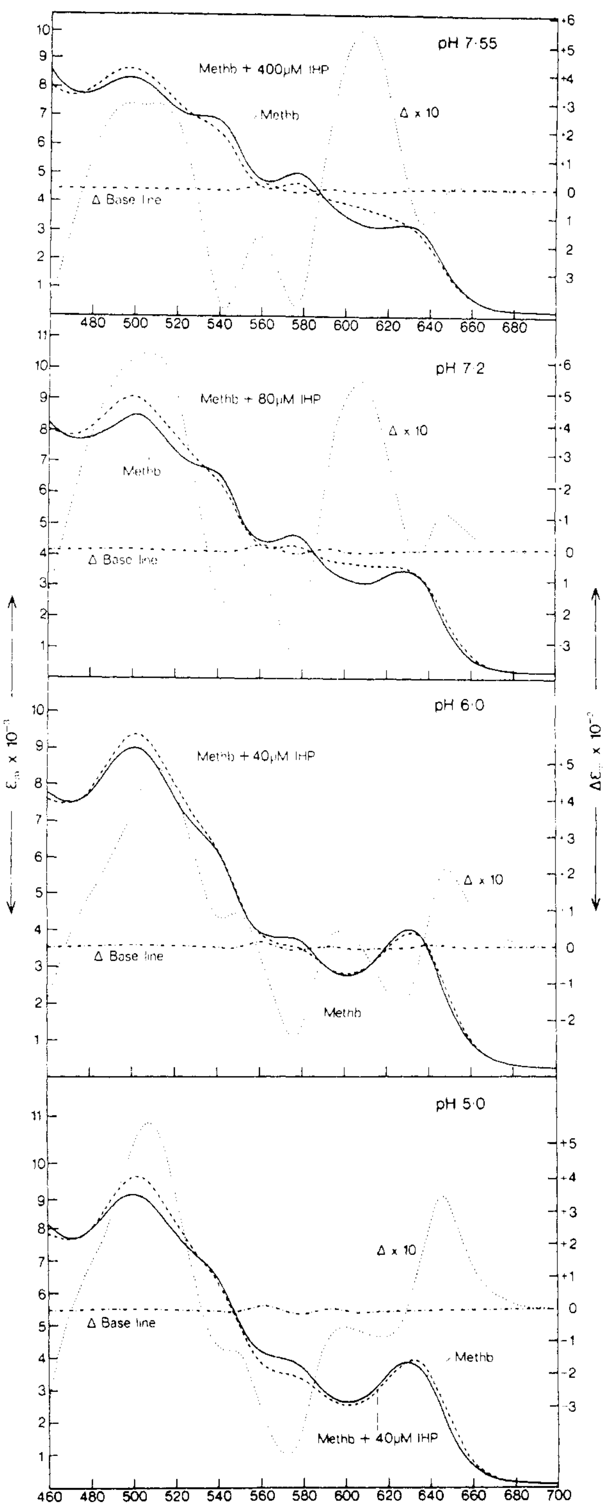


FIGURE 4: Effect of IHP on the visible absorption spectrum of 38 μM aquomethemoglobin at different pH's: (a) 5.0; (b) 6.0; (c) 7.2; (d) 7.55. The molar proportion of IHP to methemoglobin tetramer was raised from 1:1 at pH 5.0 and 6.0 to 2:1 at pH 7.2 and 10:1 at pH 7.55, so as to compensate for the increase in the dissociation constant with rising pH (see part II).

markedly. The peak at 600 nm, now shifted to 609 nm, becomes dominant and a new, high peak arises at 490 nm. Very strong negative peaks appear at the positions of both the α and β bands. The infrared band at 1000 nm continues to be red shifted by IHP at both pH 6.5 and 7.0, but as the pH rises that red shift is associated with a larger and larger fall in intensity (Figures 3b and 5b). The Soret band continues to increase in intensity, but at pH 7.0 is blue rather than red shifted by IHP.

At all pH's the difference spectra show falls in the α and β bands, indicative of falls in the fractional concentration of the low-spin components. At pH 5.0 this is associated with a rise of all the high-spin bands of aquomethemoglobin. The two bands at 495 and 609 nm which come to dominate the difference spectrum at pH 7.55 appear to be slightly red-shifted versions of the absorption bands of the high-spin form of hydroxymethemoglobin at 490 and 600 nm. Table V shows rough estimates of the changes in spin equilibria derived from these spectral changes, and of the ones in azidomethemoglobin.

In cyanomethemoglobin IHP produced a very slight red shift of the Soret band, but no significant changes in the band at 541 nm.

Paramagnetic Susceptibilities (χ_M). Solutions, 1.5 mM (tetramer), of aquomethemoglobin were titrated with IHP as described under Methods. The results are shown in Figure 7. At pH 6.06 and 6.64, χ_M rises linearly with IHP concentration reaching saturation at a ratio of 1 mol of IHP/mol of tetramer, just like the changes in visible absorption and sulfhydryl reactivity described in part II (Perutz *et al.*, 1974a). From the slight curvature in the upper parts of the plots the dissociation constant can be estimated to be on the order of 10^{-5}–10^{-6} M, in agreement with the value of 1.4 μM derived from spectrophotometric titration (part II). From the curve at pH 7.45 one can calculate a dissociation constant of 1.6 ($\pm$0.2) $\times$ 10^{-4}, confirming the large rise in the constant above pH 7.0.

Taken by itself, the rise in χ_M could be due either to a shift of the thermal equilibrium toward higher spin or to an increased orbital contribution. However, the latter effect is not likely to cause the fall in intensity of the low-spin bands shown in Figures 4, 5a, and 6b. Taken together with these spectroscopic changes the rise in χ_M is probably consistent only with a transition to higher spin. Table VI shows the drop in the fraction of low spin component and the free energy changes of the spin equilibrium. They are calculated as follows. α, the fraction of low-spin component, is given by $\alpha = (\chi_{hs} - \chi_{Hb^+H_2O})/(\chi_{hs} - \chi_{ls})$, where χ_{hs} and χ_{ls} are the molar susceptibilities of the high- and low-spin forms, respectively. For χ_{hs} we used the value for methemoglobin fluoride ($\mu_M^{20°}$ = 14,170) and for χ_{ls} the χ_M for cyanomethemoglobin ($\chi_M^{20°}$ = 2220) which is not affected by IHP. The standard free energy of the spin state change is given by

$$\Delta G°_S = -RT \ln [\alpha/(1 - \alpha)]$$

The differences in $\Delta G°_S$ in the presence and absence of IHP amount to 630 cal at pH 6.06 and 890 at pH 6.64. It was not possible to reach an end point at pH 7.45.

We have also measured the effect of IHP on the susceptibility of azidomethemoglobin. At pH 6.04 addition of 1 mol of IHP/mol of tetramer increased χ_M from 2575 $\times$ 10^{-6} to 3365 $\times$ 10^{-6}. The increase in the fraction of high-spin component is from 2 to 8% corresponding to an increase in $\Delta G°_S$ of 800 $\pm$ 100 cal per mol. The large error on this figure arises from the uncertainty in the correct value of χ_{ls} to use.

Table VII shows the results of susceptibility measurements by the nuclear magnetic resonance (nmr) method of Reilly *et al.* (1955). Its accuracy proved insufficient for magnetometric titrations but adequate for estimating the total percentage increase in susceptibility on saturation with IHP, which amount to 5% for aquomethemoglobin at pH 6.0 and 6.95, and to 40% for azidomethemoglobin at pH 6.5, compared to the increases of over 6 and 32%, respectively, measured

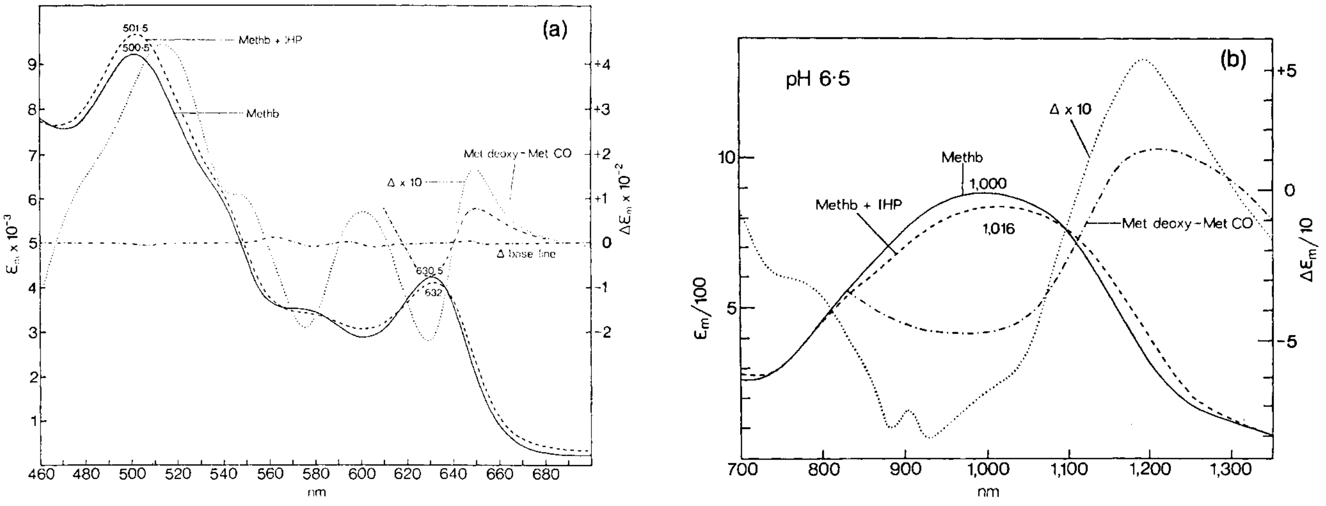


FIGURE 5: Effect of IHP on (a) the visible and (b) the near-infrared absorption spectrum of aquomethemoglobin at pH 6.5. The dot-dashed lines in (a) and (b) represent the difference spectrum observed on dissociation of CO from valency hybrids.

with the Gouy balance. The absolute values of the susceptibilities measured by nmr are lower than those measured with the Gouy balance, but since we were interested only in the increases brought about by IHP, this was not further investigated.

Proton Magnetic Resonance Spectra. The paramagnetically shifted resonances of the ferric hemes arise from contact coupling between the unpaired electrons of the iron and the protons of the porphyrin, or from pseudo-contact interactions with the protons of the porphyrin and the surrounding globin. The magnitude of the paramagnetic shifts are, to a first approximation, proportional to $S(S + 1)$, where S is the total electronic spin (for a review of theory and results, see Wüthrich, 1970). Consequently a transition to higher spin should magnify the hyperfine shifts. Figure 8 shows the paramagnetically shifted resonances of aquomethemoglobin with and without IHP. The line at 64.6 ppm is shifted to 69.5, that at 51.4 to 54.9, and that at 46.6 to 50.5 ppm. In fact, the entire block of resonances between 28.9 and 51.4 ppm appears to be shifted further downfield, as expected.

Electron Spin Resonance (Esr) Spectra. Rein *et al.* (1971) reported a change in the relative intensities of the high- and low-spin esr signals on addition of ATP to solutions of human methemoglobin. Addition of 40 mol of ATP/mol of stripped methemoglobin tetramer caused a doubling in the strength of the high-spin signal at $g = 6.0$ and a weakening of several low-spin signals. We found that addition of 1.5 mol of IHP/mol of tetramer produced only a 10% increase in the high-spin signal relative to that of the copper standard. Gray and Slade (1972) therefore investigated the temperature variations of the optical absorption spectra of some methemoglobin derivatives. In aquomethemoglobin + IHP and in fluoromethemoglobin variations of the temperature between 97 and 243°K produced no differences in the ratio of the absorption bands at 540 and 630 nm, indicating a single spin state in each of the two derivatives. In aquomethemoglobin without IHP, on the other hand, there was a large temperature variation as would be expected from the coexistence of more than one spin state. The chemical nature of the low-spin component seen spectroscopically will be discussed below.

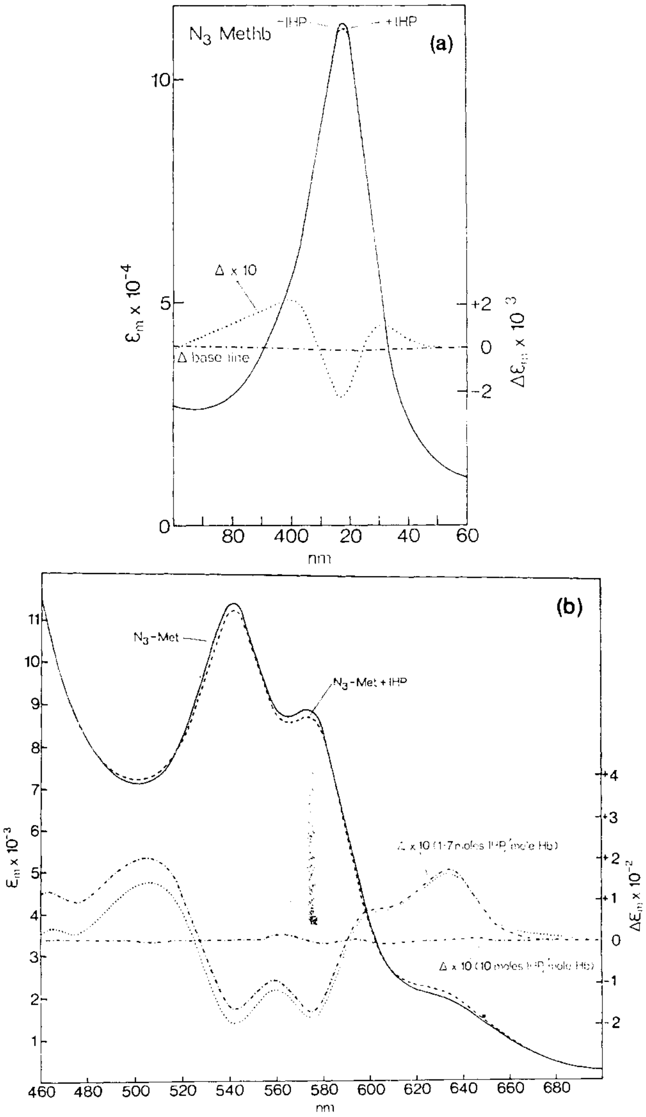


FIGURE 6: Effect of IHP on (a) the Soret band and (b) the visible absorption spectrum of azidomethemoglobin.

Discussion

Comparison of Changes in Electronic, Nmr, and Esr Spectra Produced by IHP with Those Observed in Valency Hybrids. If the spectral changes found here in fluoro-, aquo-, and hydroxymethemoglobin are due to a transition in quaternary structure induced by IHP, then similar spectral changes should

TABLE IV: Effect of IHP on Electronic Spectra of Methemoglobin Derivatives.

Derivative	pH	Absorption Band (nm)	Intensity Change (%)	Wavelength Shift (nm)	Figure
Fmet	6.5	403.7	+3.0	+<0.5	1a
		486	−1.4	+2	1b
		543	−5.2	+	1b
		603	−5.5	+2	1b
		738	−	+	1c
		840	−4	+9	1c
H_2O-Met (0.1% OH^-)	5.0	405	+5	Possible shoulder or red shift	2a
		500	+4	+2	4a
		540	−	None observable	4a
		572	−10	None observable	4a
		630	+2	+3	2b
		1000	+2	Shoulder at 1163	
H_2O-MetHb (1% OH^-)	6.0	500	+5	+2	4b
		540	−	None observable	4b
		580	−6	None observable	4b
		600	+	None observable	4b
		630.5	−3.5	+2	4b
H_2O-MetHb (~10% OH^-)	7.0	405	+2.5	None observable	3a
		1004	−12	Shoulder at 1200	3b
H_2O-MetHb (~12% OH^-)	7.2	(495)		Shoulder or red shift of band at 490?	4c
		500	+7	−2.5	4c
		540	−	None observable	4c
		580	−7.5	None observable	4c
		(609)	+15	Shoulder or red shift of band at 600 nm	4c
		630.5	0	+	4c
H_2O-MetHb (~22% OH^-)	7.55	(495)		Shoulder or red shift of band at 490 nm?	4d
		513		Shoulder or red shift of band at 500 nm?	4d
		540	−6	None observable	4d
		580	−8.5	None observable	4d
		(609)	+18	Shoulder or red shift of band at 600 nm?	4d
		630.5	−	None observable	4d
N_3-Met	6.5	418	−1.5	−	6a
		505	+2.5	None observable	6b
		542	−1.7	None observable	6b
		573	−5	None observable	6b
		628	+4	None observable	6b

occur in ferric hemes of valency hybrids on dissociation of ligands from the ferrous hemes. This is indeed the case. Methemoglobin M Milwaukee is a natural valency hybrid in which the α hemes are ferrous and the β heme high-spin ferric, because their iron atoms are linked to the carboxyl groups of glutamate as the sixth ligands. X-Ray studies have shown the hybrids ($\alpha_2\beta_2{}^{+\mathrm{Mil}}$) and ($\alpha_2{}^{O_2}\beta_2{}^{+\mathrm{Mil}}$) to have the same structures as deoxy- and oxyhemoglobin A, respectively, thus proving that binding of ligands to the ferrous α chains converts the quaternary structure of the tetramer from T to R (Perutz *et al.*, 1972). The close isomorphism of the two different valency hybrids to deoxy- and oxyhemoglobin A also implied that the tertiary structure of the ferric β subunits must be adapting itself to the changes in quaternary structure, being close to that of deoxy in one and oxy in the other hybrid. The only observable part of the ferric spectrum in the hybrids was a line at 620 nm. On dissociation of ligands from the ferrous hemes at pH 6.5 this was found to decrease in intensity and shift to the red just as the equivalent lines in aquo- and fluoro methemoglobin do on addition of IHP. Nmr studies showed that the magnitudes of the downfield shifts of the hyperfine shifted proton resonances of the ferric hemes were increased on dissociation of ligands from the ferrous ones, one resonanc moving from −51 to −54 ppm and another from −43 to −47 ppm (Lindstrom *et al.*, 1972). The allosteric equilibrium of hemoglobin M Milwaukee is shifted so far toward the T structure that it can be held in that structure by IHP or by 2,3-diphosphoglycerate even when the ferrous α subunits have taken up ligand. When this is done, dissociation of ligand from the ferrous hemes produces no changes in the optical or nmr spectra of the ferric ones. The absence of spectroscopic interaction between the hemes without a change of quaternary structure was consistent with the results of functional studie which had shown that 2,3-diphosphoglycerate lowered Hill'

TABLE V: Alterations in Spin Equilibrium Estimated Very Approximately from Spectral Changes on Addition of IHP to Aquo-, Hydroxy-, and Azidomethemoglobin.

Derivative	pH	Molar or % Changes in Absorp			Estimated Rise in Fraction of High-Spin Component (%)
H_2O	5.0		572 (−6 μM)		6[a]
OH^-	7.2			600 + (5.6 μM)	83[b]
N_3^-	6.5	540 (−2%)	570 (−2.5%)	630 (+26%)	26[c]

[a] The sample was 109 μM in heme. For calculating the percentage rise, the fractional concentration of high-spin component in the absence of IHP was assumed to be 0.1. [b] The sample was 109 μM in heme. At pH 7.2, 12% or 13 μM would be hydroxymethemoglobin. About half of this, or 6.5 μM, is thought to be high spin (George *et al.*, 1961). These authors give $\epsilon_M(600)$ = 11 mM. The observed increase in OD at 609 nm equals 0.061. 0.061/11 × 10^3 = 5.6 μM or 83% of the total hydroxy concentration without IHP. [c] The absorption coefficient of the high-spin component of azidomethemoglobin at 630 nm in unknown. Therefore the best we could do was to estimate the difference between the height of the observed peak at 630 nm and a hypothetical curve representing the density of a smoothly decreasing α band. The difference between the two curves increased by about 26% on addition of IHP. Judging by the fall in absorption of the low-spin bands, this makes the percentage high-spin component in the absence and presence of IHP about 8 and 10%, respectively.

coefficient in the reaction with oxygen from about 1.5 to unity, evidently because it prevents the T → R transition (Udem *et al.*, 1970).

Valency hybrids of hemoglobin A were prepared by partial reduction of aquomethemoglobin solutions with sodium dithionite. Dissociation of ligand from the ferrous hemes was found to decrease the intensities and cause red shifts of the absorption bands at 630 and 1000 nm (Perutz, 1972). In Figure 5a,b the difference spectra found in the valency hybrids are superimposed on the ones observed here on addition of IHP. They are seen to be identical. In valency hybrids of chemically modified hemoglobins which fail to undergo the R → T transition, such as BME or NES-des-Arg-hemoglobin, dissociation of ligands from the ferrous hemes failed to induce any spectral changes in the ferric ones.

Ogawa and Shulman (1972) prepared the cyanomet hybrids $\alpha_2^{+CN}\beta^{O_2}$ and $\alpha_2^{+O_2}\beta_2^{CN}$, and studied the changes in hyperfine-shifted proton resonances of the ferric hemes on dissociation of ligands from the ferrous ones. In these hybrids the allosteric equilibrium is shifted toward the R state so that the R → T transition fails to take place on dissociation of oxygen from $\alpha_2^{+CN}\beta_2^{O_2}$ except in the presence of either organic or inorganic phosphates, nor from $\alpha_2^{O_2}\beta_2^{+CN}$ except in the presence of IHP. Dissociation of ligand from the ferrous hemes produced striking changes in the resonances of the ferric ones, provided that this was accompanied by a transition in quaternary structure from R to T. Without that transition no changes occurred. Note that in hemoglobin M Milwaukee heme–heme interaction was observed only in the absence of diphosphoglycerate or IHP, whereas in the cyanomet hybrids it required their presence. It cannot therefore be argued that IHP has any influence on heme–heme interaction except in regulating the allosteric equilibrium.

TABLE VI: Free Energy of Spin State Change on Addition of IHP to Aquomethemoglobin at 20°.[a]

	pH	$10^6\chi_M$	α	$\Delta G°_S$ (kcal mol^{-1})
Hb OH_2	6.06	12,950 ± 50	0.102	+1.27 ± 0.02
IHP Hb OH_2	6.06	13,760 ± 50	0.037	+1.90 ± 0.07
Hb OH_2	6.64	13,170 ± 50	0.086	+1.38 ± 0.02
IHP Hb OH_2	6.64	13,970 ± 50	0.020	+2.27 ± 0.17
Hb N_3^-	6.04	2,575 ± 50	0.98	−2.27 ± 0.1
IHP Hb N_3^-	6.04	3,365 ± 50	0.92	+1.42 ± 0.1

[a] The values of χ_M for IHP Hb OH_2 are taken from the saturation values in Figure 7. The values of $\Delta G°_S$ for azidomethemoglobin are based on an assumed value of χ_{ls} = 2220 × 10^{-6}.

Henry and Banerjee (1973) examined the esr spectra of hybrids containing subunits ligated to nitric oxide which are low-spin ferrous. Their experiments were done in 0.1 M phosphate buffer of pH 6.95 at concentrations of 100 μM hemoglobin tetramer, at room temperature and 77°K. In hybrids of $\alpha_2\beta_2^{NO}$, the esr spectra of the β subunits were the same regardless of the spin state of the α subunits. In hybrids of $\alpha_2^{NO}\beta_2$, on the other hand, the esr spectra of the α subunits were of two different kinds: one for low-spin derivatives of the β subunits (β^{+CN^-}, $\beta^{+N_3^-}$, β^{O_2}, β^{CO}) and another for high-spin derivatives (β^{+H_2O}, β^{+F^-}, β^{deoxy}). The esr spectra of the latter show marked splitting. Our interpretation of these results is as follows. In hybrids of $\alpha_2\beta_2^{NO}$, the low-spin β sub-

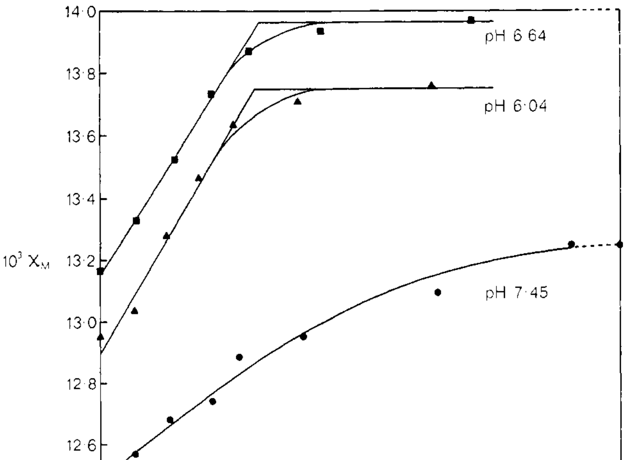


FIGURE 7: Effect of IHP on the paramagnetic susceptibility (χ_M) of aquomethemoglobin.

TABLE VII: Effect of IHP on Paramagnetic Susceptibilities of Methemoglobin at 35° Measured by the Nmr Method of Reilly *et al.* (1955).

Derivative	pH	IHP	Equiv $CuSO_4$ (mM)	Fe (mM)	χ_M cgs Units, $\times 10^3$	% Change
H_2O-met	6.0	−	106.0	15.2	11.2	
		+	111.5		11.8	+5
H_2O-met	6.95	−	120.0	16.7	11.6	
		+	126.0		12.2	+5
N_3-met	6.5	−	14.0	15.6	1.45	
		+	19.5		2.02	+40

[a] $\chi_M = 1.612 \times 10^{-3} \times ([CuSO_4]/[\text{heme Fe}])$.

units would have kept the quaternary structure in the R state regardless of the spin state of the α subunits. This behavior is in accord with that of cyanomet hybrids, where the allosteric constant L in $\alpha_2\beta_2{}^{+CN^-}$ is smaller than in $\alpha_2{}^{+CN^-}\beta_2$ (Ogawa and Shulman, 1972). The absence of a change in quaternary structure explains why the spin state of the α subunits has no effect on the esr spectra of the β^{NO} subunits. We predict that such an effect will be observed on addition of organic phosphates which will change the hybrid $\alpha_2{}^{deoxy}\beta_2{}^{NO}$, and possibly others, to the T structure. We now come to the hybrids $\alpha_2{}^{NO}\beta_2$. These probably have an R-type quaternary structure when the β subunits are low spin, and a T-type quaternary structure when they are high spin. The splitting of the esr signals in the T-type structure must be due to the tension on the hemes referred to below. We predict that the spectra of $\alpha_2{}^{NO}\beta_2{}^{+H_2O}$ or $\alpha_2{}^{NO}\beta_2{}^{+F^-}$ will revert to the R type if measurements are made under conditions favoring the R state, such as "stripping" of phosphates, or high pH or after digestion with carboxypeptidase A or B.

Rein *et al.* (1972) reported that the addition of IHP to NO-hemoglobin in the ratio of 1 mol of IHP/mol of tetramer produced a splitting of the esr spectrum into a fine structure. Comparison of this fine structure with the one observed by Henry and Banerjee in hybrids of α^{NO} with high-spin β subunits shows it to be the same. We suggest that both are due to conversion to the quaternary T structure. Ogawa and Shulman's nmr and our uv difference spectra are both consistent with this conclusion (see Perutz *et al.*, 1974a). Now IHP does not convert either CO or cyanomethemoglobin to the T structure. The new data therefore indicate that the allosteric constant $L' = [T]^{NO}/[R]^{NO}$ is larger than L' for either CO or cyanomethemoglobin. The stereochemical reason for this interesting property appears to lie in a transfer of part of the density of the unpaired electron from the NO nitrogen to the d_{z^2} orbital of the iron (Kon, 1968; Kon and Kataoka, 1969) which would lengthen the bond from the iron to N^ϵ of the proximal histidine.

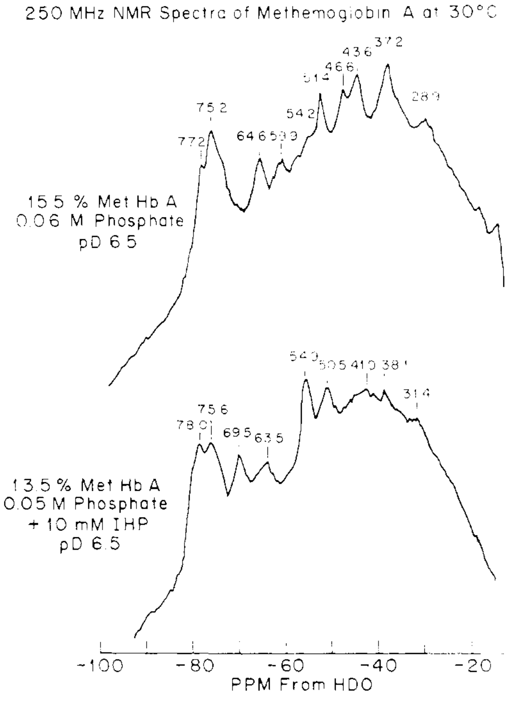


FIGURE 8: Hyperfine-shifted proton resonances of aquomethemoglobin without and with IHP.

Another intriguing example of the control of the allosteric equilibrium by the spin state of the heme is the behavior of ferrous subunits conbined with fluoro-, cyano-, or azidomet subunits (Banerjee *et al.*, 1973). The oxygen affinity of $\alpha_2\beta_2{}^+$ hybrids falls in the order of $N_3^- \rightarrow CN^- \rightarrow F^-$. At pH 6.0 the oxygen affinity of aquomet hybrid is the same as that of the fluoromet one, while at pH 9.0, where the β hemes would have been converted to the low-spin hydroxy form, the affinity of the α hemes rises to that of the azido- and cyanomet hybrids. Conversely, $\alpha_2\beta_2{}^{-H_2O}$ has a lower affinity for azide than $\alpha_2{}^{CO}\beta_2{}^{+H_2O}$, but a similar affinity for fluoride. The experiments illustrate how one pair of subunits in the high-spin state opposes the transition of the other pair to the low-spin state by biassing the allosteric equilibrium toward the T state, and *vice versa.*

Interpretation of Changes in Electronic Spectra. In the three derivatives in which components of high and low spin coexist in thermal equilibrium, IHP causes spectral changes indicative of a shift toward higher spin. This shift has been confirmed by our magnetic susceptibility studies and is consistent also with the changes that IHP produces in the nmr spectra. Stereochemically, it corresponds to a lengthening of the bond distances from the iron atom to its electronegative ligands and a consequent increase in the displacement of the iron atom from the plane of the porphyrin ring. This is due to increased occupancy of the $d_{x^2-y^2}$ and the d_{z^2} orbitals which point in the direction of the iron–nitrogen bonds and tend to repel the nitrogen atoms of the porphyrin and the heme-linked histidine, as well as the sixth ligand.

We shall now consider whether the increased distance between the iron atom and its electronegative ligands can account for the spectral changes produced by the R → T transition in fluoromet- and deoxyhemoglobin. One is ferric and the other ferrous and both are pure or almost pure high spin, so that we need not consider the effects of spin changes. In both derivatives the R → T transition strengthens the Soret band, shifting it slightly to the red in fluoromet and to the blue in deoxyhemoglobin. In fluoromethemoglobin it also causes red shifts and weakening of all visible and near-infrared bands; in deoxyhemoglobin it causes blue shifts of all absorption bands, with the possible exception of a shoulder at 590 nm which merely seems to rise in intensity. The intensity of the band at 555 nm remains unchanged but the near-infrared bands become weaker.

Consider first the shifts in wavelength. In the introductory section of Spin States and Spectra we mentioned that those bands that are not present in metal-free porphyrins, and especially the bands in the near-infrared region, are believed to be due mainly to charge-transfer transitions and that their energy tends to be dominated by the electrostatic term, *i.e.*, the repulsion of the transferred electron by the electronegative ligands coordinated to the iron. Williams (1955) pointed out that in ferric compounds the direction of charge transfer is likely to be from the porphyrin to the iron, and in ferrous compounds from the iron to the porphyrin. In that case increasing the distance from the iron to its electronegative ligands, which diminishes the energy of repulsion, would lower the energy of charge transfer in ferric compounds, causing the bands to be shifted to the red, and raise it in ferrous compounds, causing them to be shifted to the blue (Figure 9). This indeed is in accord with observation (see part I and Results section of this paper).

The change in free energy calculated from the shifts of the near-infrared bands in deoxy- and fluoromethemoglobin amounts to about 300 cal/mol of heme, which is no more than one-tenth of the free energy of heme–heme interaction. However, we do not know how large a fraction of the transition energy of the near-infrared bands really does come from charge transfer as opposed to covalent interactions, so we cannot tell whether this figure represents the full change of free energy at the heme. It is much lower than the values of 630–890 cal calculated from the changes in paramagnetic susceptibility.

The slight shifts of the Soret band in fluoromet- and deoxyhemoglobin follow the directions of those of the charge-transfer bands, which suggests that the shifts arise because some of the energy of the Soret band comes from mixing with the charge-transfer transitions. Alternatively the R → T transition could alter the balance of intensities between the two mutually perpendicular components polarized in the plane of the porphyrin, but it is hard to see why this balance should be tipped in opposite directions in ferrous and ferric derivatives.

Consider now the intensity changes which consist of a rise of the Soret band and a fall of all charge-transfer bands in both deoxy- and fluoromethemoglobin. In the introductory section on Spin States and Spectra we explained that charge-transfer bands can borrow intensity from the Soret band through mixing with $\pi \rightarrow \pi^*$ transitions of the porphyrin. On that basis there could be a seesaw—the rise in intensity of the Soret band being due directly to the same change in mixing of states which causes loss in intensity of the visible and near-infrared bands. The two phenomena would be symptoms of reduced mixing in the T state which could arise from increased displacement of the iron from the plane of the porphyrin.

Hopfield (1973) considered how tension at the heme could explain the sharpening of the Soret band observed on transition of deoxyhemoglobin from R to T. He assumed that a tension which removed the iron further from the plane of the porphyrin, increasing its z coordinates, would lessen the root-mean square of the thermal deviation of the z coordinate from its equilibrium value. This would reduce the energy spread of the spectral transition and thus sharpen the line. This may well be another of the factors involved and would be consistent with the accompanying rise in intensity of the α band.

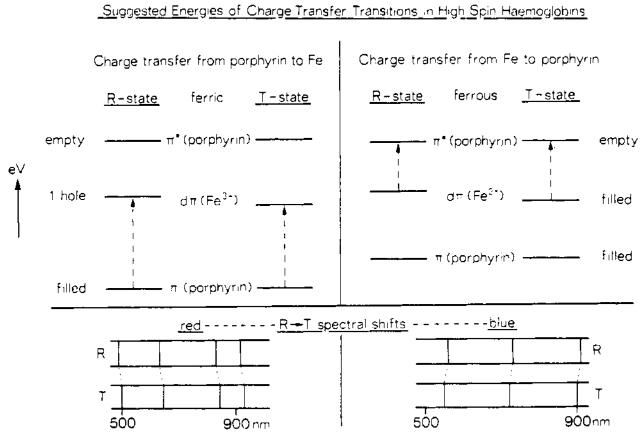


FIGURE 9: Effect of the R → T transition and the accompanying lengthening of the bonds from the iron to its electromagnetic ligands on the charge transfer bands in ferrous and ferric hemoglobins. As electrostatic repulsion is lessened, the energy of charge transfer rises in ferrous and falls in ferric derivatives.

We now come to aquo- and hydroxymethemoglobin. In these derivatives all charge-transfer bands are red shifted by IHP, as in fluoromethemoglobin, and all low-spin bands become weaker, but the intensity changes of the high-spin bands are more complex. The intensities of the high-spin bands of hydroxymethemoglobin always rise, as this derivative appears to be converted almost entirely from low to high spin. In aquomethemoglobin, on the other hand, where only a small fraction is low spin even in the absence of IHP, the intensity changes depend on pH. At pH 5.0 where the concentration of hydroxymethemoglobin is negligible, all the high-spin bands rise in intensity, but at higher pH's only the band at 500 nm rises, while the ones at 630 and 1000 nm fall. It is difficult to interpret these changes unambiguously because we do not know how the pK of the equilibrium between aquo- and hydroxymethemoglobin is affected by the transition to the T structure.

In conclusion, the transition to higher spin in derivatives with mixed spin on transition from the R to the T structure implies a lengthening of the distances between the iron atom and its electronegative ligands. In pure high-spin derivatives, this structural change is sufficient to account qualitatively for the observed wavelength shifts and may also account for most of the intensity changes. In aquomethemoglobin the wavelength shifts can also be accounted for, but the intensity changes at pH 6.0 and above are more difficult to interpret.

Naturally our interpretation of the spectral changes depends on the correctness of the band assignments upon which it is based. For instance, we have assumed the near-infrared bands of deoxyhemoglobin to be due to charge transfer from the metal to the porphyrin, but Dr R. J. P. Williams has pointed out to us that they might be due to d → d* transitions in the metal. Again, in high-spin methemoglobin derivatives we have assumed that those bands which do not coincide with the α, β, or γ bands of the porphyrin are predominantly due to charge transfer transitions from the porphyrin to the iron, but these charge-transfer bands are in fact the strongest ones in the visible spectrum, which makes it difficult to argue that most of their intensity is borrowed from the porphyrin bands. At this stage, more rigorous methods of spectral assignment would be most helpful and would give us more confidence in our interpretation of the spectral shifts.

Implications for Heme–Heme Interaction. Evidence was presented in part I that in hemoglobins which have the quaternary structure of the R type, the hemes are in the same state as they are in free α and β subunits. In the T structure, on the other hand, the state of heme is modified in a way which affects their spectra and lowers their ligand affinities. The analysis presented here allows us to diagnose that this modification includes a lengthening of the iron–nitrogen bond distances, leading to an increased displacement of the iron from the plane of the porphyrin ring. There may be other modifications not yet recognized, but it is encouraging that qualitatively at least, the lengthening of the bond distances is sufficient to account for the shifts in wavelength and possibly also the intensity changes accompanying the R → T transition.

If our interpretation is correct, then the displacement of the iron atom from the plane of the porphyrin in tetrameric deoxyhemoglobins in the T state should be greater than in monomeric ones which are not constrained and have higher oxygen affinities. There is evidence that this is so. From an electron density map of horse deoxyhemoglobin at 2.8-Å resolution, Bolton and Perutz (1970) estimated the displacement of the iron atom from the plane of the porphyrin as 0.75 Å. The same value has recently been obtained from an electron density map of human deoxyhemoglobin at 2.5-Å resolution (A. Arnone and L. F. Ten Eyck, unpublished data). In a difference electron density map of human met minus deoxyhemoglobin in the T state at 3.5-Å resolution, Anderson (1973) could actually see a movement of the iron atoms toward the plane of the porphyrin ring. On the other hand, in a difference map of deoxy minus met sperm-whale myoglobin at 2.8-Å resolution, Nobbs *et al.* (1966) failed to see any movement of the iron relative to the porphyrin ring; Huber *et al.* (1970) obtained the same negative results from a difference map of deoxy- minus meterythrocruorin of chironomus at 2.5-Å resolution. In both these proteins the displacement of the iron from the plane of the porphyrin in the met state is 0.3 Å. At the high resolutions employed for the difference maps movements of the iron relative to the plane of the porphyrin of 0.45 Å could not have escaped detection; failure to find them therefore indicates that the displacement of the iron atom in the deoxy derivatives of these monomeric hemoglobins is only very little larger than in the aquomet forms. This has recently been confirmed in a difference electron density map of sperm whale deoxyhemoglobin minus metmyoglobin at 2.0-Å resolution (T. Takano, unpublished). Another piece of relevant information comes from a crystal structure determination of the five-coordinated high-spin 2-methylimidazole-Fe^{2+}-$\alpha,\beta,\gamma,\delta$-tetraphenylporphine (L. J. Radonovich and J. L. Hoard, unpublished data but quoted by Hoard and Scheidt, 1973). The displacement of the iron atom from the mean plane of the porphyrin skeleton is only 0.55 Å, compared to 0.75 Å in human and horse deoxyhemoglobin; the distance of the imidazole nitrogen from the plane is 2.7 Å compared to 2.9 Å.

All our evidence is consistent with the notion that the allosteric equilibrium of hemoglobin is governed primarily by the displacement of the iron and the proximal histidine from the plane of the porphyrin (Hoard, 1966; Perutz, 1970; Anderson 1973), though in the β subunits the steric effect of the ligand itself also plays a part (Perutz, 1970). Thus changes of tension at the hemes appear to alter the quaternary structure of the globin; Perutz (1972) proposed that the interaction between the heme and the globin is a reciprocal one, as indeed it must be on elementary principles of action and reaction, so that changes in quaternary structure of the globin in turn alter the tension at the hemes. Our present results confirm this idea and suggest that heme–heme interaction works in the following way. Starting with fully oxygenated hemoglobin in the R state, loss of oxygen from one or more subunits would change the quaternary structure to the T state. The resulting tension at the hemes and narrowing of the heme pocket in the β subunits would lower the oxygen affinity and facilitate the release of the remaining oxygen. Starting with fully deoxygenated hemoglobin in the T state, uptake of oxygen by some of the subunits would change the quaternary strudture to the R state, thereby releasing the tension on the hemes and opening out the heme pockets in the β subunits, and speeding full oxygenation. In summary, heme–heme interaction is coupled to a change of tension at the heme, transmitted by a change of quaternary structure of the globin. Hopfield (1973) has pointed out that in such a mechanism no more than a fraction of the free energy of heme–heme interaction need be stored in the form of strain energy at the heme itself and the rest could be distributed elsewhere in the molecule. Consistent with this theory, the free energy of the spin-state change we observed on transition from R to T amounts to no more than one-quarter to one-third of the total free energy of heme–heme interaction (Table VI). The rest of the energy could be stored at the subunit contacts, especially the salt bridges. The fraction of the total free energy of heme–heme interaction which is actually stored at the heme will depend on the force constants of the iron–nitrogen bonds in each particular compound. If we regard these bonds and the C-terminal salt bridges as two stretched springs coupled in series, with stiffnesses S_{H} and S_{S}, respectively, then the fraction of the strain energy at the heme can be shown to be equal to $(S_{\mathrm{S}}/S_{\mathrm{H}})/(1 + (S_{\mathrm{S}}/S_{\mathrm{H}}))$. Therefore if the force constant in some low-spin compound were ten times greater than in the high-spin ones considered here, the fraction of the strain energy at the heme would be 11 times smaller.

Transitions of Aquomethemoglobin at Low Temperatures. We shall now discuss the apparent inconsistency between the esr measurements of Rein *et al.* (1971) and our own, reported here. Rein *et al.* compared the esr spectra of solutions of "stripped" solutions of human aquomethemoglobin with those of solutions to which 40 mol of ATP/mol of tetramer had been added. At both 77 and 20°K, the solution without ATP showed several low-spin lines between $g = 1.83$ and 2.54, in addition to the prominent high-spin line at $g = 5.9$. In the solution with ATP, the low-spin lines were much weaker and the intensity of the high-spin line was doubled. At 77°K we did not observe the low-spin lines, and found only a 10% rise of the high-spin line on addition of IHP. However, on theoretical grounds we should have found no rise, since judging by the properties of metmyoglobin the thermal equilibrium at this low temperature should have been tipped to the pure high-spin form regardless of the presence or absence of IHP.

These inconsistencies can be resolved by considering several hitherto disconnected observations. Solutions of free α- or β-oxyhemoglobin subunits oxidized to the aquomet state by ferricyanide, if left to stand at 6°, show a new spectrum with a large peak at or near 558 nm and a smaller one at or near 528 nm. These peaks get stronger with time. A similar spectrum, with lines at 565 and 525 nm, is seen on addition of sodium salicylate or benzoate to aquomethemoglobin A or on leaving aquomethemoglobin A to stand at 4° for several months. The esr spectrum shows a weakening of the high-spin signal at $g = 6.0$ and the appearance of new signals at values of g between 2.91 and 1.66. These new lines in the electronic and esr spectra were identified as being due to hemichromes, six-coordinated low-spin ferriporphyrin compounds whose appearance in hemoglobins often heralds the first step toward denaturation of the globin. The formation of hemichromes can be prevented or even reversed by addition of strong heme ligands such as cyanide (Rachmilewitz, 1969; Rachmilewitz *et al.*, 1969, 1971).

Iizuka and Kotani (1969b) measured the paramagnetic susceptibility and the visible absorption spectrum of aquomethemoglobin solutions brought to pH 5.9 with H_3PO_4 between 293 and 77°K. The susceptibility showed a steep fall just below freezing point, a minimum at about 250°K, a maximum at about 200°K and then a gradual fall to 77°K. The exact behavior depended on the rate of freezing. The absorption spectrum showed two new bands, at 527 and 562 nm, which rose in intensity as the temperature fell, while the intensities of the high-spin lines at 500 and 630 nm dropped with falling temperature. Gray and Slade (1972) measured the visible absorption spectrum of aquomethemoglobin solutions buffered to pH 6.5 between 140 and 273°K. As the temperature fell, they observed a gradual rise of bands at 535 and 565 nm, accompanied by a fall of the bands at 500 and 630 nm. In the presence of IHP, on the other hand, the ratio of absorption at 540 and 630 nm remained constant over the entire temperature range, the normal methemoglobin spectrum being observed throughout.

In the light of the work of Rachmilewitz and his colleagues we can recognize the new lines observed in frozen solutions of aquomethemoglobin as characteristic for hemichromes. The nature of the stereochemical change involved is still unknown, but we suggest that the heme-linked water molecule may join the nearest ice crystal, and histidine E7 may take its place as the sixth ligand. By locking the molecules in the quaternary T structure with its much stronger bonds between the subunits, IHP evidently stabilizes the native conformation and inhibits the transition to hemichromes. ATP would be expected to have the same, but somewhat weaker, effect. What Rein *et al.* (1971) observed appears to have been the esr spectrum of a mixture of methemoglobin and hemichrome. The proportion of hemichrome was high in the absence, and low in the presence, of ATP. We may have minimized hemichrome formation by rapid cooling and did not see the low-spin lines in our esr spectra. All the same, it seems likely that at 77°K the 10% rise in the high-spin line at $g = 6.0$ solutions with IHP as compared to the ones without IHP was due to its prevention of hemichrome formation rather than to any effect of the quaternary structure on the spin state of the heme.

It might be asked whether the spectral changes and the rise in χ_M which IHP produce in solution of aquomethemoglobin at room temperature might not have been due to IHP converting small fractions of contaminating hemichromes to native methemoglobin. This possibility is excluded by the positions of the negative peaks at 542 and 575 in our IHP difference spectra, as compared to the absorption bands of hemichromes at around 530 and 562 nm, and by the stoichiometric effect of IHP, in the ratio of 1 mol/mol of tetramer, on the absorption spectrum, on χ_m, and on the sulfhydryl reactivity.

Other Artifacts Due to Hemichrome Formation. Lang and Marshall (1966) found a striking difference between the Mössbauer spectra of fluoro- and aquomethemoglobin at 1.2°K, the former being typically high spin and the latter similar to the low-spin spectrum of azidomethemoglobin. It seems clear from the preceding discussion that the spectrum of aquomethemoglobin published by Lang and Marshall is, in fact, that of hemichrome.

The work of Banerjee and Cassoly (1969) on free methemoglobin subunits has already been referred to in part I (Perutz *et al.*, 1974b). At pH 5.9, the spectrum of the α_{SH} subunits shows only a small increase in the fraction of low-spin component compared to native methemoglobin, but the spectrum of the β_{SH} subunits shows the prominent bands at about 535 and 565 nm characteristic for hemichromes, side by side with those of native methemoglobin. Oxidoreduction titrations showed the redox potential of the α subunits to be independent of pH, while that of the β subunits fell with rising pH, due to an oxidation-linked ionization with a pK near 7.0. In a later paper Banerjee *et al.* (1969) report spectral changes on reassociation of free aquomet α and β subunits to form tetramers. Their optical and esr spectra of the free β subunits again show evidence for the presence of a major proportion of hemichrome, but this largely disappears on association with the α subunits, apparently because incorporation into tetramers reverses the partial unfolding of the β chains that is necessary for hemichrome formation. The spectral changes observed on reassociation of the subunits have been used by the authors as a basis for a theory of heme–heme interaction, but in fact the phenomenon seems merely an example of reversible denaturation. Hemichrome formation also appears to be reversed on going to alkaline pH. This is in accord with the effect of other strong heme ligands such as azide and cyanide observed by Rachmilewitz *et al.* (1971). Henry and Banerjee (1970) reexamined the methods of preparing free β subunits and found that 1-dodecanethiol which had been used by Banerjee and Cassoly (1969) and Banerjee *et al.* (1969) had promoted the appearance of hemichrome bands in their esr spectra on frozen samples, but claim that it had not affected unfrozen samples. This can hardly be maintained in the face of the prominent hemichrome bands shown by the absorption spectra of their β_{SH} subunits in solution.

Gersonde and Wollmer (1970) reported a study of the pH dependence of the esr spectra of horse methemoglobin between pH 5.0 and 11.0. In acid solution low-spin bands appear at $g = 2.91$, 2.23, and 1.95, in addition to the usual high-spin bands at $g = 5.9$ and 2.0. The authors attribute the low-spin bands to the presence of a second, native, hemoglobin conformer, but clearly they are hemichrome bands. Significantly, these bands are absent when fluoride is added to the hemoglobin solution before freezing. Now aquo- and fluoromethemoglobin have practically identical high-spin structures, and the sole reason for the absence of low-spin bands in frozen solutions of fluoromethemoglobin, and their presence in aquomethemoglobin, can be that hemichrome forms on freezing only when the sixth ligand is a water molecule which prefers to join the surrounding ice.

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