Influence of Globin Structure on the State of the Heme. I. Human Deoxyhemoglobin†

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ABSTRACT: Heme–heme interaction arises through a transition between two alternative quaternary structures of the hemoglobin tetramer: one, the oxy or R form, having a high oxygen affinity, and the other, the deoxy or T form, a low one. The fall in affinity must be related to the influence of the globin on the state of the heme, but this influence cannot normally be studied by itself, because it cannot be separated from that of the dissociation of ligands from the heme. We have exploited a system which allows us to study the effect on the heme of the transition between the two alternative quaternary structures of the globin in the absence of any chemical reaction at the heme. Two modified and one abnormal hemoglobin remain in a quaternary structure of the R type even when fully deoxygenated. Their electronic spectra, circular dichroism in the ultraviolet region, and their paramagnetically shifted proton resonances differ markedly from those of deoxyhemoglobin A, but resemble those given by the sum of the free deoxy α and β subunits. Combination of these hemoglobins with 1 mol of inositol hexaphosphate/mol of tetramer switches them to a form which has electronic and nuclear magnetic resonance spectra like those of native deoxyhemoglobin A. The transition is accompanied by changes in absorption and circular dichroism in the aromatic region of the ultraviolet spectrum that are probably due to the changed environments of a tryptophan (C2(37)β) and a tyrosine (C7(42)α) at the $\alpha_1\beta_2$ contact. They are characteristic for the change in quaternary structure of the globin and are independent of the state of ligation of the hemes or the changes in tertiary structure accompanying them. The changes in the electronic spectra of the heme include rises in the intensities of the Soret band and of shoulders at 320 and 590 nm, and blue shifts of all the absorption bands from the near-ultraviolet region to the near-infrared region. The significance of these blue shifts will be discussed in part III (Perutz *et al.* (1974b), *Biochemistry 13*, 2187).

Heme–heme interaction consists of a rise of the oxygen affinity of hemoglobin solutions with rising oxygen saturation. Experiments have shown that this rise occurs only when the reaction with oxygen is accompanied by a transition between two alternative quaternary structures of the globin (Kilmartin and Hewitt, 1971; Perutz and Ten Eyck, 1971), and this is confirmed by the results of Bradley, Bunn, and Gibson quoted below. In the oxy or R structure, the oxygen affinity is similar to that of free α and β subunits (Brunori *et al.*, 1966), but in the deoxy or T structure, the affinity is about 70 times lower. There is evidence that it becomes lower not simply because some of the heme pockets become too narrow to accommodate the oxygen molecules but also because the globin somehow lowers the ligand affinities of the hemes themselves. This effect of the globin on the heme lies at the heart of the problem of heme–heme interaction.

On the basis of preliminary results on methemoglobin and on valency hybrids, Perutz (1972) suggested that the low oxygen affinity of the T structure may be related to an increased tension at the heme which pulls the iron atom further away from the plane of the porphyrin ring and thus opposes its movement into the ring on reaction with oxygen. We have put that hypothesis to the test by studying the changes in electronic spectra, paramagnetically shifted proton resonances, and paramagnetic susceptibilities that accompany the R → T transition in ferrous and ferric hemoglobins.

The R and T structures are defined by the distances between the iron atoms and by the dovetailing of the $\alpha_1\beta_2$ contacts (Muirhead *et al.*, 1967; Perutz and Ten Eyck, 1971). They are stabilized by different sets of interactions within and between the subunits, including the salt bridges and hydrogen bonds listed in Table I. Modifications of residues involved in these interactions, or mutations, may shift the allosteric equilibrium between the two forms in varying degrees; in extreme cases they may even cause the fully liganded molecules to remain in the T state or the unliganded one to remain in the R state. Organic phosphates stabilize the T structure. Among these inositol hexaphosphate (IHP)[1] has a particularly powerful effect and can be used to switch the structure of certain hemoglobin derivatives from R to T (Olson and Gibson, 1972; Ogawa *et al.*, 1972; Bonaventura *et al.*, 1972; Ho *et al.*, 1973; Lindstrom *et al.*, 1973), thus providing a tool for studying the way changes in quaternary structure of the globin affect the state of the heme.

In this paper we investigate chemically modified hemoglobins and a mutant one which can be made to remain in the R state when fully deoxygenated and switched to the T state by the addition of 1 mol of IHP/mol of tetramer. This means that the effect of the R → T transition on the five-coordinated ferrous hemes can be studied in the absence of any chemical reaction actually occurring at the hemes.

NES-des-Arg-hemoglobin is a derivative in which the formation of the C-terminal salt bridges that normally stabilize the T structure is inhibited by the enzymatic removal of Arg-HC3(141)α and by the addition of *N*-ethylmaleimide to Cys-F9(93)β to form an *N*-ethylsuccinimide derivative which pushes His-HC3(146)β away from Asp-FG1(94)β and Lys-C6(40)α

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

TABLE I: Salt Bridges and Hydrogen Bonds Specifically Stabilizing the T or R Structures.

Interactions within Subunits	Interactions between Subunits	Loss of Interactions Sufficient to Tip Equil of Unliganded Hb Fully to R
	T Structure	
1. Tyr-140α-OH----OC-Val-93α	6. Lys-127α_1-$NH_3^+\cdots{}^-OOC$	1, 6, 7. Des-Arg-141α-Tyr-140α
2. Tyr-145β-OH----OC-Val-98β	Arg-141α_2	2, 4, 5. Stripped Hb Bethesda (Tyr-145$\beta\rightarrow$His)
3. His-146β-$Im^+\cdots{}^-OOC$-Asp-94β	7. Asp-126α_1-$COO^-\cdots{}^+$Gua	3, 6, 7, 8. NES-des-Arg-141α-des-Arg-141α-His-146β
4. $^+NH_3$-Val-1β	8. Lys-40α_1-$NH_3^+\cdots{}^-OOC$-His-146β_2	
	9. Val-1β-NH_3^+	13. Kempsey (Asp-99$\beta\rightarrow$Asn)
PO_4^{2-}		
5. $^+NH_3$-Lys-82β	10. Lys-82-NH_3^+ 2,3-DPG[a]	
	11. His-2-Im^+	
	12. His-143-Im^+	
	13. Tyr-42α_1-OH----OOC-Asp-99β_2	
	R Structure	
None detected so far	14. Lys-127α_1-$NH_3^+\cdots{}^-OOC$-Arg-141α_2[b]	Loss of interaction sufficient to tip allosteric equilibrium of liganded Hb fully to the T structure
	15. Val-1β_1-$NH_3^+\cdots{}^-OOC$-His-146β_2[b]	
	16. Asp-94α_1-COO^-----NH_2-Asn-102β_2	16. Kansas (Asn-102β-Thr)

[a] DPG = diphosphoglycerate. [b] Probably very weak salt bridges.

(see Table I). It exhibits high oxygen affinity, noncooperative ligand binding, and no Bohr effect, and it crystallizes in a form closely related to normal oxyhemoglobin, showing that it remains in a structure of the R type even when fully deoxygenated. Addition of IHP lowers the oxygen affinity and restores cooperativity and the Bohr effect. For instance, without phosphate in 0.05 M Bis-Tris and 0.1 M Cl^- p_{50} = 0.56 mm and n = 1.3. On addition of 1 mM IHP, p_{50} = 1.66 mm and n = 2.0 (J. C. Kilmartin, J. A. Hewitt, and J. F, Wootton, in preparation).

Hemoglobin Kempsey is a mutant in which Asp-99β is replaced by Asn, so that the hydrogen bond with Tyr-42α that normally stabilizes the $\alpha_1\beta_2$ contact in the T structure is lost (Reed *et al.*, 1968). It has a high oxygen affinity, low cooperativity, and a small Bohr effect (H. F. Bunn, T. B. Bradley, and R. C. Wohl, personal communication). Ogata and McConnell (1972) found evidence for its deoxy form being largely in the R state. We have crystallized deoxyhemoglobin Kempsey and found it to be isomorphous with deoxyhemoglobin NES-des-Arg, which shows that it does indeed have a structure of the R type. This is also borne out by its sulfhydryl reactivity which, without IHP, is similar to that of oxyhemoglobin A and, with IHP, similar to that of deoxyhemoglobin A. Its oxygen affinity and cooperativity are similarly affected. In 0.05 M Bis-Tris of pH 7.2 + 0.1 M NaCl and 20° *without IHP*, it is found that p_{50} = 0.23 mm and n = 1.1. On addition of 1 mM IHP it is found that p_{50} = 1.1 mm and n = 1.7 (T. B. Bradley, H. F. Bunn, and Q. H. Gibson, personal communication). Note that cooperativity in both NES-des-Arg and Kempsey is restored by IHP. It will be shown here that this happens because IHP switches both deoxyhemoglobins to the T state.

Des-Arg(141)β-hemoglobin binds ligands cooperatively at low pH, where the T state is stabilized by the salt bridges of His-146β, but noncooperatively at high pH, where these bridges are broken. In des-Arg-Tyr the removal of the two C-terminal residues of the α chains has done away with the C-terminal salt bridges of Arg-141α as well as the hydrogen bonds of the phenolic hydroxyls of the tyrosines-140α to the carbonyls of Val-FG5(93)α. Its ligand binding properties are similar to those of NES-des-Arg, suggesting that it too remains in a structure of the R type, but this could not be proved crystallographically because its crystals were not isomorphous with any known structure.

In all these hemoglobins addition of IHP is accompanied by characteristic changes in ultraviolet circular dichroism, electronic spectra, and hyperfine shifted proton resonances. In the R state the electronic and nuclear magnetic resonance (nmr) spectra resemble those that are given by the sum of the free α and β subunits, but on addition of IHP they become closely similar to those given by deoxyhemoglobin A. The principal change in the electronic absorption bands of the heme in the visible and near-infrared regions consists of blue shifts. It will be shown in part III that these blue shifts are consistent with an increased displacement of the iron atom from the plane of the porphyrin ring. There is also a rise in intensity of the Soret band and of shoulders at 320 and 590 nm.

Perutz (1972) suggested that the different degrees of displacement of the iron atom from the plane of the porphyrin in the R and T states of deoxyhemoglobin should affect the spin state of the heme, and that there might be a reciprocal relationship between the oxygen affinity of a hemoglobin and its spin state in the deoxy form. We have tested this hypothesis by comparing the paramagnetic susceptibilities of deoxyhemoglobin which differ widely in their oxygen affinities and in their allosteric equilibria, but found their susceptibilities to be the same within our limits of error, corresponding to a spin value of S = 2.

Methods

Preparation, Optical, and X-Ray Studies. NES-des-Arg- and des-Arg-Tyr-hemoglobin were prepared as described by Kilmartin ank Hewitt (1971). To purify hemoglobin Kempsey, hemolysate in the carbonmonoxy form containing 2 g of mixed Kempsey and A was passed through a Sephadex G-25 (fine) column equilibrated with 0.05 M sodium phosphate buffer of

pH 7.2 containing 3.02 g of Na_2HPO_4 and 1.42 g of $NaH_2PO_4 \cdot 2H_2O$ per l. It was then loaded on a 3 × 50 cm column of Bio-Rex 70 (Bio-Rad Laboratories) equilibrated with the same buffer at 4°. The purified hemoglobin Kempsey was retarded relative to hemoglobin A and was eluted by 0.1 M sodium phosphate of pH 7.2. For spectroscopic work the eluate was "stripped" according to Berman *et al.* (1971) and CO was replaced by oxygen as described by Kilmartin and Rossi-Bernardi (1971). Free α and β chains were prepared according to Kilmartin *et al.* (1973). Stock solutions of the three hemoglobins were diluted with 0.05 M Bis-Tris of pH 7.0 + 0.1 M NaCl to tetramer concentration of 380 μM for the near-infrared spectrum, 15 μM for the visible and ultraviolet (uv) spectra, and 1.5 μM for the Soret band. They were reduced by addition of a 1.5 molar excess of a 30 mM solution of $Na_2S_2O_4$ and filled into matched and stoppered cuvets. To one of each pair of cuvets an IHP solution of pH 7.0 was added in the proportion of 2 mol/mol of tetramer for the three more concentrated hemoglobin solutions and 4 mol/mol of tetramer for the 1.5 μM hemoglobin solution. Dissolution of the $Na_2S_2O_4$, reduction of the hemoglobin solutions, and filling of the cuvets were done in a nitrogen-filled glove box. Spectra were recorded with a Cary 14 spectrophotometer. Crystallization of the deoxyhemoglobins NES-des-Arg and Kempsey was done by the standard methods described by Perutz (1968). Unit cell dimensions and space groups were determined by taking 9° precession photographs with Cu Kα radiation.

Circular dichroism was recorded on a Cary 60 spectropolarimeter with Model 6001 attachment, modified by repositioning an improved electrooptic modulator (Isomet Corp.) outside the sample compartment, immediately behind the polarizer. Data were collected and analyzed as described by Simon and Cantor (1969).

Nmr Spectra. Hemoglobin Kempsey samples used for nmr studies were isolated and purified from hemolysate according to Lindstrom *et al.* (1973). In order to reduce the intense proton signal from aqueous samples, the hemoglobin samples were exchanged with D_2O by repeated dilution with D_2O and concentration by ultrafiltration through an Amicon UM-20E membrane. Appropriate amounts of 1.0 M deuterated potassium phosphate buffer or 1.0 M Bis-Tris buffer were added to the hemoglobin solutions after the final concentration so that the hemoglobin solutions were buffered at the desired pD with the appropriate buffer. Sodium inositol hexaphosphate (Sigma Type V) was dissolved in D_2O and titrated to pH 6.7 with NaOH and then repeatedly lyophilized and redissolved in D_2O to make the stock solution. Appropriate amounts of IHP stock solution were added to the hemoglobin solutions. The pD of solutions was determined by adding 0.4 pH unit (Glasoe and Long, 1960) to the value read from a Radiometer Model 26 pH meter equipped with a Beckman Model 39030 combination electrode. To prepare deoxy samples, the CO ligand was replaced by O_2 by flushing the hemoglobin CO solution in a rotatory evaporator in an ice–water bath under a Sylvania 150-W flood lamp. Oxygen was then removed by flushing the solution with prepurified nitrogen. The hemoglobin concentration in our nmr studies was about 10%.

Nmr spectra were obtained on a Bruker HFX 90-MHz spectrometer. Probe temperature of the spectrometer was 29°. Proton chemical shifts are referenced with respect to the residual water (HDO) in each sample and the resonances which are downfield from HDO signal are noted by a negative sign. The accuracy of the hyperfine shifted resonances in deoxyhemoglobin samples is ±0.1 ppm. The chemical shift of HDO is −4.83 ppm from the proton resonance of a standard, 2,2-dimethyl-2-silapentane-5-sulfonate at 29°. The signal to noise ratios were improved by signal averaging with a Fabri-Tek Model 1074 computer.

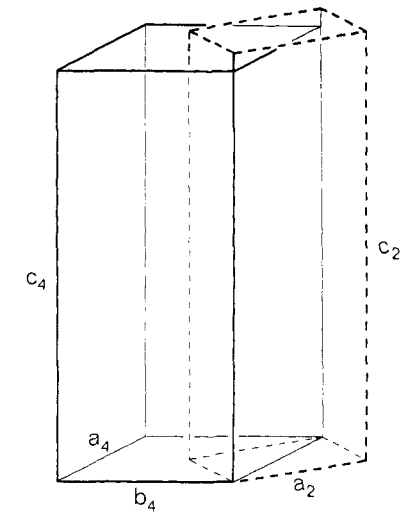


FIGURE 1: Relation of pseudo-face-centred orthorhombic unit cell of human deoxyhemoglobins NES-des-Arg and Kempsey (a_4, b_4, c_4) to tetragonal cell of normal human oxyhemoglobin. The former, if transformed to a primitive cell, has the dimensions $a_4' = 56.0$ Å, $c_4 = 186.0$ Å; the latter $a_2 = 53.7$ Å, $c_2 = 193.5$ Å. The intensity distribution of the *hhl* reflexions of the orthorhombic crystals is similar to that of the *h0l* reflexions of the tetragonal ones.

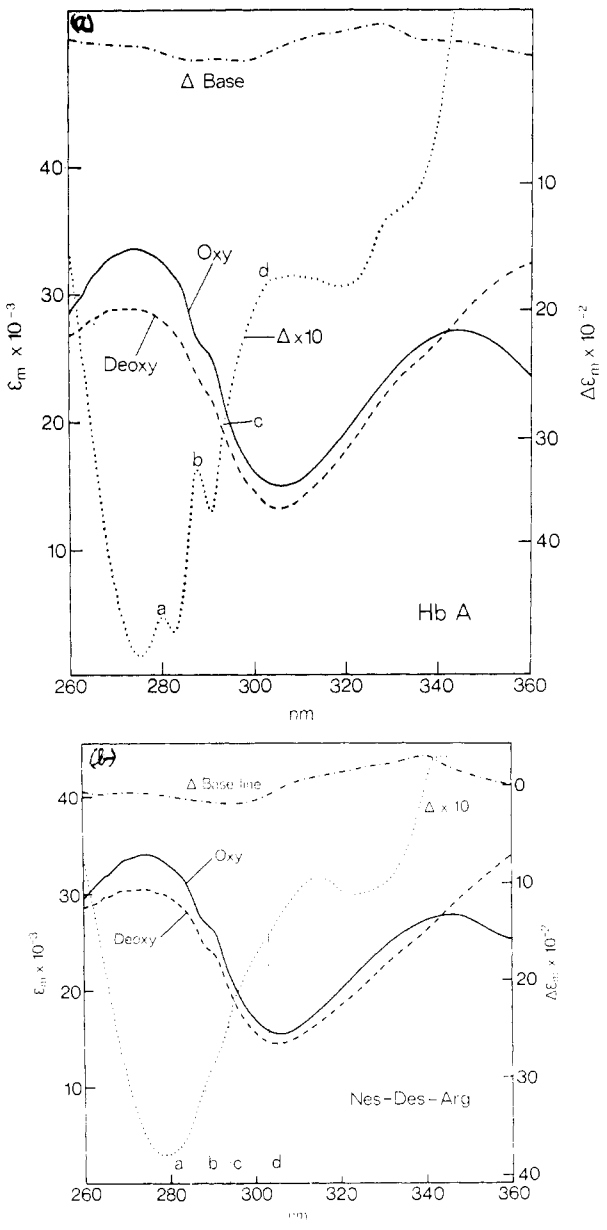
Magnetic Susceptibilities. The magnetic susceptibilities were measured by the method described by Reilly *et al.* (1955). A Varian T60 nmr spectrometer was used in the nonspin configuration with coaxial 5- and 3-mm diameter sample tubes. Benzene was used in the outer tube and the hemoglobin sample was placed in the inner tube. The tubes were standardized with aqueous $CuSO_4$ solutions in the inner tube.

Samples of hemoglobin were prepared and the sample tubes were filled inside a nitrogen box as described for the optical measurements. All susceptibility measurements were made at 35°.

The values of the molar susceptibilities were calculated using a mass susceptibility of 10.1×10^{-6} cgs unit for $CuSO_4$ (Amiel, 1941). The relative error between the measured susceptibilities is less than 0.5×10^{-3} cgs unit. The nmr spectra observed are quite broad and consequently measurements of the frequency differences between resonances are difficult. The spectra are, however, quite capable of detecting any substantial changes, and more subtle changes in the susceptibilities could be measured by using a higher frequency nmr spectrometer. The accuracy of the value of the computed molar susceptibilities is dependent upon the accuracy with which the solution concentrations are known and upon the value used for the magnetic susceptibility of $CuSO_4$.

Results

X-Ray Analysis. Crystals of deoxyhemoglobin Kempsey grown from ammonium sulfate solution buffered with P_i to pH 6.5, without IHP, have the cell dimensions $a = 78.5$ Å, $b = 80.0$ Å, $c = 186$ Å and the space group $P2_12_12_1$. These are identical with the cell dimensions and space group of NES-des-Arg-deoxyhemoglobin (see Table IV of Perutz and Ten Eyck, 1971). The unit cell is pseudo-*c*-face centered and the dimensions of the primitive cell derived from it are almost identical with the ones of the normal tetragonal form of human oxyhemoglobin (Figure 1).



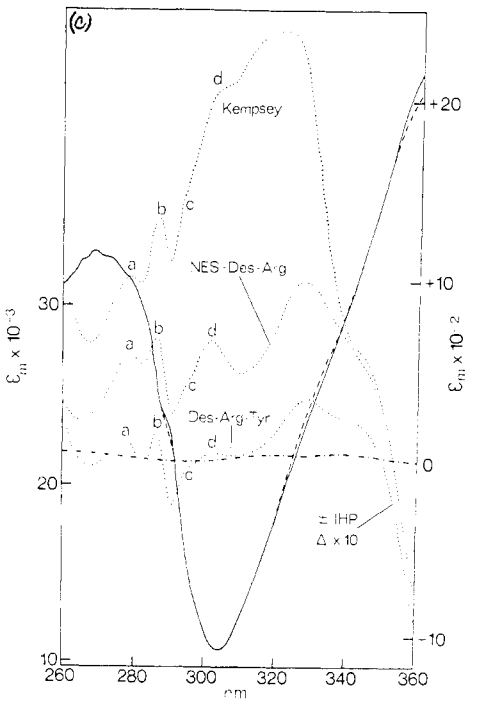


FIGURE 2: (a) Full curve, uv absorption spectrum of a 6.5 μM (tetramer) solution of human oxyhemoglobin A in 0.05 M Na_2HPO_4 + 0.1 mM IHP; broken curve, same deoxygenated; dotted curve, difference spectrum 10× magnified. The oxyhemoglobin solution was first deoxygenated by blowing nitrogen over it for 5 hr and was then filled into two matched cuvets. Oxygen was then blown through one cuvet. The deoxygenated solution showed an extinction coefficient at 540–560 nm of 0.85 which corresponds to 85% deoxygenation (Heilmeyer, 1943). Methemoglobin was not detectable in either solution. The spectra were recorded against pure buffer in the reference beam. The Δ base line in this and subsequent figures is 10× magnified. All values of ε in this and subsequent figures are in moles of heme. (b) Full curve, uv absorption spectrum of a 5 μM (tetramer) solution of human NES-des-Arg-oxyhemoglobin in 0.05 M Na_2HPO_4; broken curve, same solution deoxygenated; dotted curve, difference spectrum of deoxy-oxy 10× magnified. Other particulars are as for Figure 2a. (c) Full curve, uv absorption spectrum of a 15 μM (tetramer) solution of des-Arg(141α)-des-Tyr(140)-α-deoxyhemoglobin in 0.05 M Bis-Tris + 0.1 M NaCl of pH 7.0 without IHP. The curves for NES-des-Arg and Kempsey are closely similar. Broken curve, same with 30 μM IHP; except for a slightly diminished absorption in the tyrosine region, this curve is identical with curves obtained from deoxyhemoglobin A. Both spectra were recorded against pure buffer solution in the reference beam. Dotted curves, difference absorption spectra of 15 μM (tetramer) solutions of deoxyhemoglobins des-Arg-Tyr, NES-des-Arg, and Kempsey, ±IHP. All spectra were recorded at room temperature (20–25°).

Electronic Spectra. We shall first consider the aromatic region of the uv spectrum. The aromatic amino acids tryptophan, tyrosine, and phenylalanine have absorption maxima at 280, 275, and 258 nm, respectively, and their absorption coefficients have the ratios Trp:Tyr:Phe (27:7:1), so that the contribution of phenylalanine is small compared to those of the other two. When these amino acid residues form part of a native protein, their absorption maxima are shifted to longer wavelength (Haurowitz, 1963). The absorption spectrum of hemoglobin exhibits a prominent, broad, peak at 274 nm to which the aromatic amino acids of the globin and the porphyrin both contribute. We have compared the changes in absorption that occur in the aromatic region on deoxygenation of oxyhemoglobin A, which alters its quaternary structure from R to T, with those that occur on deoxygenation of oxyhemoglobin NES-des-Arg which fails to do so (Figure 2a,b). In both the native and modified hemoglobin the peak at 275 nm becomes less intense and shifts to shorter wavelength, giving rise to a large difference peak with a minimum at 279 nm. Superimposed on this the difference spectrum of deoxy- minus oxyhemoglobin A contains several smaller features which are absent or vanishingly small in the difference spectrum of NES-des-Arg-hemoglobin. These features consist of peaks at 279 nm (a), 287 nm (b), 302 nm (d), and a shoulder at 294 nm (c). We have found that these difference peaks can be produced in the spectrum of NES-des-Arg-deoxyhemoglobin and of deoxyhemoglobin Kempsey on addition of IHP, in the absence of any ligand at the heme (Figure 2c).

We may now consider the origin of these peaks. It will be shown in part II (Perutz *et al.*, 1974a) that peaks c and d vanish on replacement of Trp-C3(37) by serine in hemoglobin

Hirose. This tryptophan lies at the $\alpha_1\beta_2$ boundary, and its environment changes markedly during the R → T transition. Peaks a and b cannot be due to tyrosines-HC2 since their removal does not affect the peaks, nor to tyrosines-H8β or tryptophans-A12, since their environments hardly change during the R → T transition. They are probably due to Tyr-C7(42)α which also lies at the $\alpha_1\beta_2$ boundary and interacts closely with the guanidinium group of Arg-C6(40)β in the R, but not in the T state (see Discussion).

At the long-wavelength end the uv spectrum shows a steep rise which is part of the Soret band of the heme at 428 nm. The difference spectra show peaks at 315 and 320–325 nm, and shoulders at 345 nm. For reasons which are not clear, the magnitude of the peak at 320 nm varies widely in the three derivatives.

Figure 3 shows the difference spectrum of NES-des-Arg in the Soret band and compares it with the kinetic difference spectrum between deoxyhemoglobin A and the sum of the free deoxy α and β subunits discovered by Brunori *et al.* (1968). Except for some minor differences on the uv side of the Soret band, the two difference spectra are identical, and they show that on addition of IHP or on assembly of the subunits into a tetramer the Soret band becomes more intense and is shifted to slightly shorter wavelength.

Figure 4 shows the optical spectra of deoxyhemoglobins NES-des-Arg, des-Arg-Tyr, and Kempsey with and without IHP, together with the kinetic difference spectrum between the sum of the free deoxy subunits and deoxyhemoglobin A recorded by Brunori *et al.* (1968). They are all closely similar. Beginning at the short wave side there is a steep fall which is part of the negative difference peak at 442 nm shown in Figure 3. This is followed by a positive and negative pair centered at a shoulder of the main peak at 510 nm. Then comes a positive and negative pair centered on the main peak at 556 nm. Next a positive peak which coincides with the shoulder on the main peak at 590 nm. These difference peaks indicate that, on addition of IHP or on assembly of the free subunits into a tetramer, the shoulder at 510 nm and the main peak at 556 nm are both shifted towards the blue, the latter by 2 nm, and that the shoulder at 590 rises in intensity. Finally, there is a small continuous negative peak which stretches right into the near infrared. To see whether this feature is genuine, the difference spectrum was recorded with a five times stronger solution of NES-des-Arg-deoxyhemoglobin; this is represented by the dotted line marked $\Delta \times 50$ in Figure 4a and confirms the presence of a negative peak stretching toward the near-infrared region.

The near-infrared spectrum, shown in Figure 5, consists of two weak bands which both undergo blue shifts on addition of IHP, one by about 2 and the other by about 8 nm. Superimposed on these shifts is the continuation of the general fall in intensity whose beginning was shown in the curve marked $\Delta \times 50$ on the right of Figure 4a.

IHP does not affect any of the electronic spectra of deoxyhemoglobin A, nor the visible spectrum of carbonmonoxyhemoglobin A. Its effect on the visible spectrum of oxyhemoglobin A is still under study. IHP does affect the uv spectrum of both oxy- and carbonmonoxyhemoglobin, producing a sharp peak at 287 nm similar to peak b in Figure 2; there is also a trace of peak a. It will be shown in part II (Perutz *et al.*, 1974a) that IHP has similar effects on the uv spectra of low-spin ferric derivatives.

Circular Dichroism Spectra. Figure 6a shows the uv circular dichroism (CD) spectra of oxy- and deoxyhemoglobin A. Deoxygenation is accompanied by a large fall in CD near

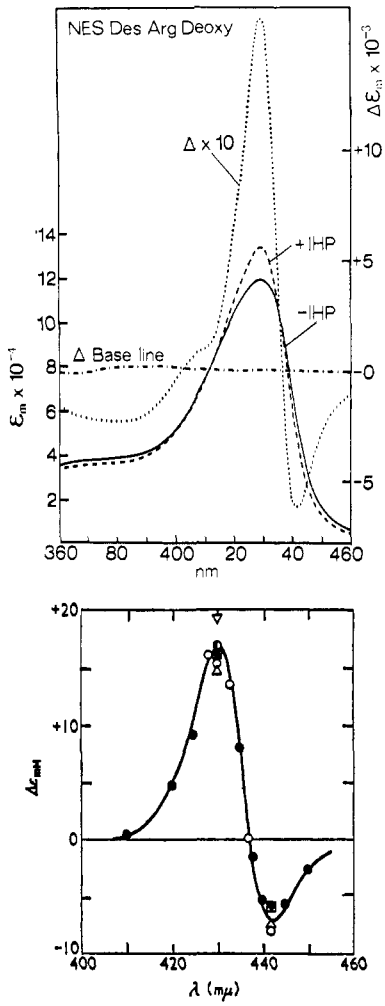


FIGURE 3: (a, top) Full curve, Soret band of a 1.5 μM (tetramer) solution of NES-des-Arg-deoxyhemoglobin in 0.1 M phosphate buffer of pH 7.0 without IHP; broken curve, same with 3 μM IHP. The dotted curve shows the difference spectrum ±IHP 10× enlarged. The same spectra and difference spectra were also obtained from deoxyhemoglobin Kempsey. (b, bottom) Kinetic difference spectrum of a solution containing a mixture of α and β deoxy subunits minus the sum of the absorption of two solutions containing the α and β subunits separately. Reproduced, by permission, from Brunori *et al.* (1968).

260 nm which is due to the heme, and by the development of a negative peak at 287 nm which lies in the aromatic region of the globin. Figure 6b shows the CD spectra of oxyhemoglobins NES-des-Arg and Kempsey and of their deoxy derivatives without and with IHP. The spectra of oxyhemoglobin Kempsey and of deoxyhemoglobin Kempsey with IHP are closely similar to the spectra of oxy- and deoxyhemoglobin A. The corresponding spectra of NES-des-Arg differ somewhat from those of hemoglobin A at the longer wavelength, presumably due to the presence of the succinimide ring in the chemically modified derivative. The spectra of the two deoxyhemoglobins without IHP lack the prominent negative peak at 287 nm which is so characteristic of deoxyhemoglobin A. Figure 7 shows the difference spectra of the two deoxyhemoglobins with and without IHP along with the difference spectrum of oxy- *vs.* deoxyhemoglobin A. Even though there are differences between the spectra of NES-des-Arg and Kempsey, their difference spectra are very similar. Moreover, they contain the same peak at 287 nm as the main peak in the difference spectrum of oxy- *vs.* deoxyhemoglobin A. The prominence of

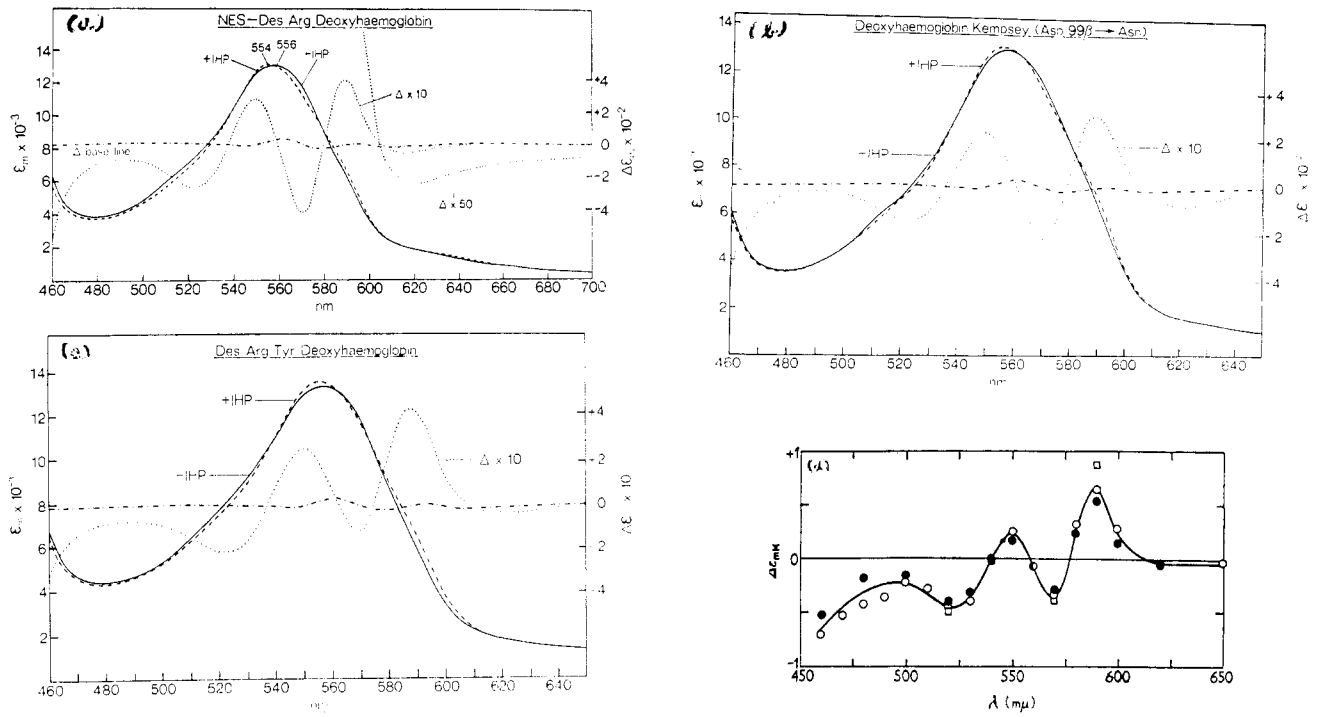


FIGURE 4: (a–c) Visible absorption spectra of 15 μM solutions of NES-des-Arg-, Kempsey, and des-Arg-Tyr-deoxyhemoglobins in 0.1 M phosphate buffer of pH 7.0 without IHP. Broken curves, same with 30 μM IHP; dotted curves, difference spectra ±IHP 10× enlarged. The dotted curve on part a marked ×50 was obtained from a pair of 75 μM solutions. (d) Kinetic difference spectrum of a solution containing a mixture of α and β deoxy subunits minus the sum of the absorption of two solutions containing the α and β subunits separately. Reproduced, by permission, from Brunori *et al.* (1968).

the band at 287 nm in the latter probably reflects the additional contribution of the change in state of the heme. A similar difference spectrum is also shown by des-Arg-deoxyhemoglobin at pH 9.0 *vs.* pH 7.0.

Nmr Spectra. Figure 8 shows the paramagnetically shifted proton resonances in the downfield region of deoxyhemoglobins NES-des-Arg and Kempsey in 0.1 M phosphate with and without IHP at pD 7.0, and of deoxyhemoglobin A. The latter shows three prominent paramagnetically shifted proton resonances which normally appear at ~−7.4, ~−12 and ~−17.5 ppm from HDO, and will be referred to respectively as α_1, α_2, and β, because the first two resonances are due to the α and the third to the β heme (Davis *et al.*, 1971; Lindstrom *et al.*, 1972). In the presence of 0.1 M phosphate, IHP has no significant effect on the paramagnetically shifted resonances in deoxyhemoglobin A, but it has a strong effect on the resonances of the modified hemoglobins. In the absence of IHP, in both deoxyhemoglobins NES-des-Arg and Kempsey the β-heme resonance at −1.75 is shifted to ~−15.4 ppm. The α_2 peak at −12.0 ppm is split into two resonances in deoxyhemoglobin NES-des-Arg, lying at −12.0 and −10.7 ppm, and is replaced by a single resonance, in a position midway between these, in deoxyhemoglobin Kempsey. The α_1 resonance which normally appears at −7.4 ppm is shifted to −7.6 ppm in deoxyhemoglobin Kempsey, but its exact position is difficult to ascertain in deoxyhemoglobin NES-des-Arg due to a change in the base line. In the presence of IHP, the α_2- and β-heme resonances of deoxyhemoglobins NES-des-Arg and Kempsey take up positions close to those of deoxyhemoglobin A. The position of α_1 in NES-des-Arg is hard to ascertain for the same reason as above.

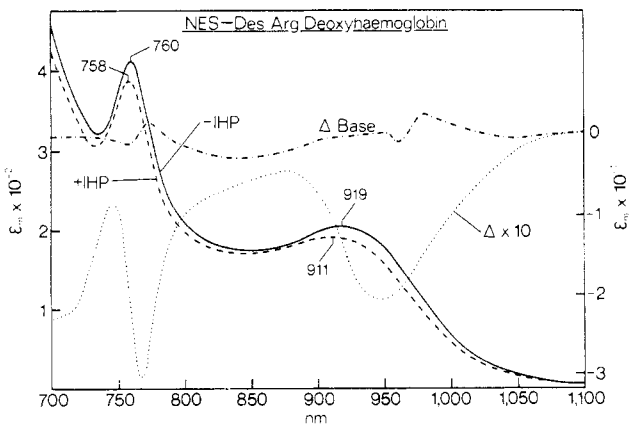


FIGURE 5: Near-infrared spectrum of a 380 μM (tetramer) solution of NES-des-Arg-deoxyhemoglobin in 0.1 M phosphate buffer of pH 6.5 without IHP. Broken curve, same with 380 μM IHP; dotted curve, difference spectrum ±IHP 10× enlarged.

It was shown above that the electronic spectra of deoxyhemoglobins NES-des-Arg and Kempsey without IHP correspond to those given by the sum of free deoxy α and β chains, which led us to suspect that the same might be true of the nmr spectra. Figure 9 presents the spectra of the isolated deoxy α and β chains, the spectrum resulting from the superposition of the different chains, and the spectrum of a sample obtained by mixing the chains in a 1:1 ratio in 0.1 M phosphate at pD 7.0. The latter is similar to the spectrum of deoxyhemoglobin A, as was to be expected from the results of Brunori *et al.* (1968). The superimposed spectra of the isolated α and β chains, if added, would produce peaks at about −14.3, −10.6, and −8.1 ppm, compared to the peaks in deoxyhemoglobin Kempsey–IHP at −15.3, −11.4, and −7.6 ppm. Thus the paramagnetically shifted resonances of deoxyhemoglobin in the R state are similar to those of the sum of the free deoxy chains, as was to be expected from the similarity of the electronic spectra, but there are slight differences in the magnitudes of the chemical shifts.

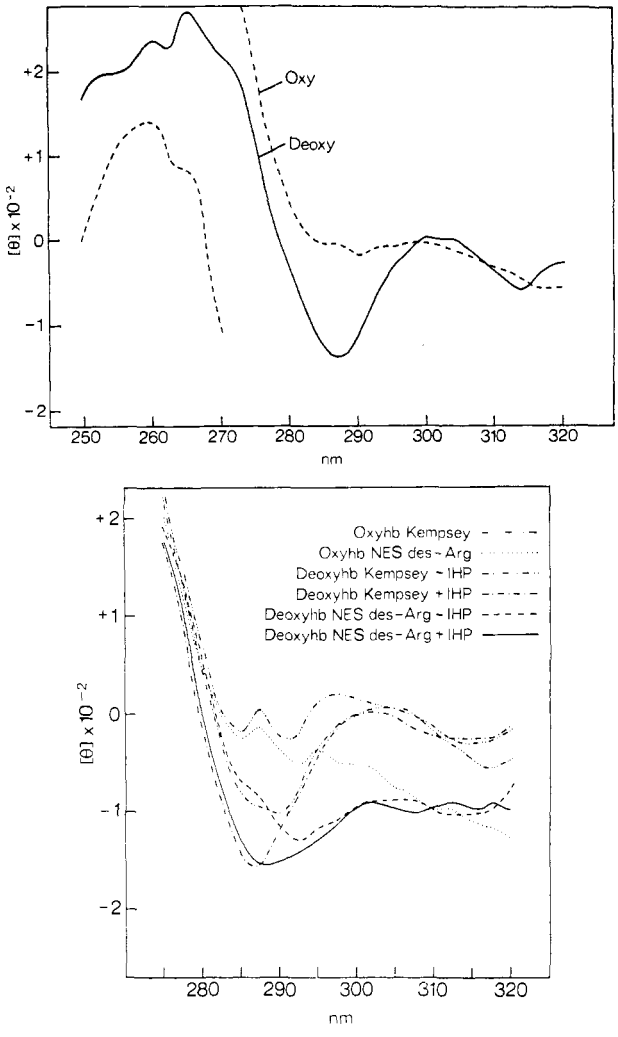


FIGURE 6: CD spectra studies. (a, top) Of oxyhemoglobin A (- - - - -) and deoxyhemoglobin A (—) in 0.1 M Bis-Tris of pH 7.0. All hemoglobin solutions were 2.5×10^{-4} in heme. Molar ellipticities expressed in terms of single chains. (b, bottom) Of 62 μM (tetramer) solutions of deoxyhemoglobins NES-des-Arg and Kempsey in 0.1 M Bis-Tris of pH 7.0. Molar ellipticities expressed in terms of single chains.

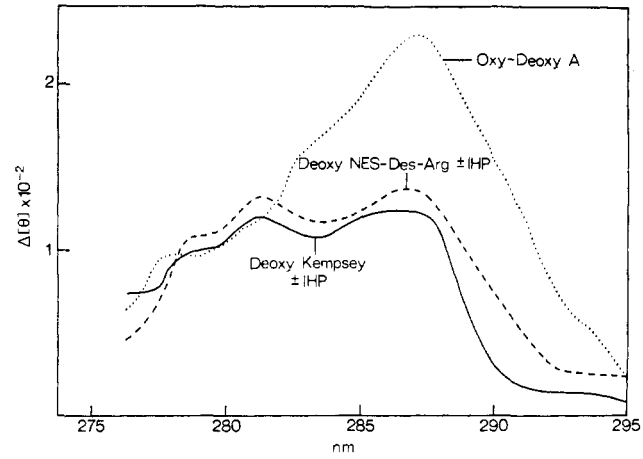


FIGURE 7: Difference CD spectra of solutions deoxyhemoglobins without IHP minus solutions with IHP and of oxy- minus deoxyhemoglobin A: deoxyhemoglobin Kempsey (—), deoxyhemoglobin NES-des-Arg (— —), oxyhemoglobin A minus deoxhemoglobin A (- - - - -).

NES-deoxyhemoglobin crystallizes isomorphously with deoxyhemoglobin A and contains no structural disturbances visible in the difference electron density maps at 5.5-Å resolution other than the displacement of His-146β by the *N*-ethylsuccinimide residue. Its paramagnetically shifted nmr spectra are similar to those of deoxyhemoglobin A in both the presence and absence of IHP in 0.1 M phosphate at pD 7.0.

Human des-Arg-deoxyhemoglobin also crystallizes isomorphously with deoxyhemoglobin A, but in solution it is known to contain an equilibrium mixture of molecules in the T and R states; the allosteric equilibrium shifts toward R with rising pH (Kilmartin and Hewitt, 1971; Hewitt *et al.*, 1972). Its nmr spectra are shown in Figure 10. At pD ~9.0, the paramagnetically shifted proton resonances of deoxy-des-Arg in 0.09 M Bis-Tris are −14.8 (low intensity), −10.4, and −7.0 ppm. These resonances are similar to those of deoxyhemoglobin Kempsey without IHP at pD 7.0, *i.e.*, corresponding to the R-type structure of hemoglobin. Owing to the high ligand affinity of des-Arg-deoxyhemoglobin at pD 9.0, it is difficult to prepare a completely deoxygenated sample at 10% protein concentration. The reduced intensity of the resonance at −14.8 ppm suggests that O_2 or CO binds to the β chain in preference to the α chain as in deoxyhemoglobins Kempsey and Yakima (Ho and Lindstrom, 1972; Lindstrom *et al.*, 1973). It should be noted that the paramagnetically shifted resonances of deoxyhemoglobin A in 0.08 M Bis-Tris at pD 9.0 are at −16.9, −11.6, and −7.6 ppm, similar to those of deoxyhemoglobin A at pH 7.0 and very different from those of des-Arg-deoxyhemoglobin at pD 9.0. On the other hand, the paramagnetically shifted resonances of des-Arg-deoxyhemoglobin at lower pD values (Figure 10a,c) are more complex with prominent peaks at −11.4 and −16.5 and shoulders at −15.4 and −17.4 ppm. These spectra suggest a mixture of the two different states in equilibrium in solution. The nmr spectra of des-Arg-deoxyhemoglobin in Bis-Tris buffer in the presence of IHP were not investigated. Figure 11 shows the nmr spectra of des-Arg-deoxyhemoglobin in 0.1 M phosphate with and without IHP at pD 7.0. The paramagnetically shifted resonances of des-Arg-deoxyhemoglobin are different from those of hemoglobin A, especially the line assigned to α_1 which is shifted from −7.4 to −6.1 ppm. The addition of 12 mM IHP to des-Arg-deoxyhemoglobin in 0.1 M phosphate at pD 7.0 has little effect on the paramagnetically shifted resonances.

Paramagnetic Susceptibilities. The paramagnetic susceptibilities of deoxyhemoglobins A and des-Arg at different pH values are shown in Table II. There is a 40-fold difference in oxygen affinity between deoxyhemoglobin A at pH 7.0 and des-Arg at pH 9.2; moreover, the former is in the T state while the latter is in the R state. The table shows that within our rather large limits of error, which were mainly due to difficulties in measuring the concentrations of our very concentrated solutions accurately, these differences have no

TABLE II: Paramagnetic Susceptibilities of Deoxyhemoglobin Derivatives at ~35°.

Derivative	pH	Fe (mM)	mM $CuSO_4$ Equiv	$10^3 \times \chi_M$ cgs Units	Log $pO_2(50)$
DeoxyHb A	7.0	11.0	78	11.4	1.1
	8.6	11.0	76	11.1	0.3
Des-Arg	7.2	9.3	70	12.2	0
	9.2	9.2	66	11.9	−0.5

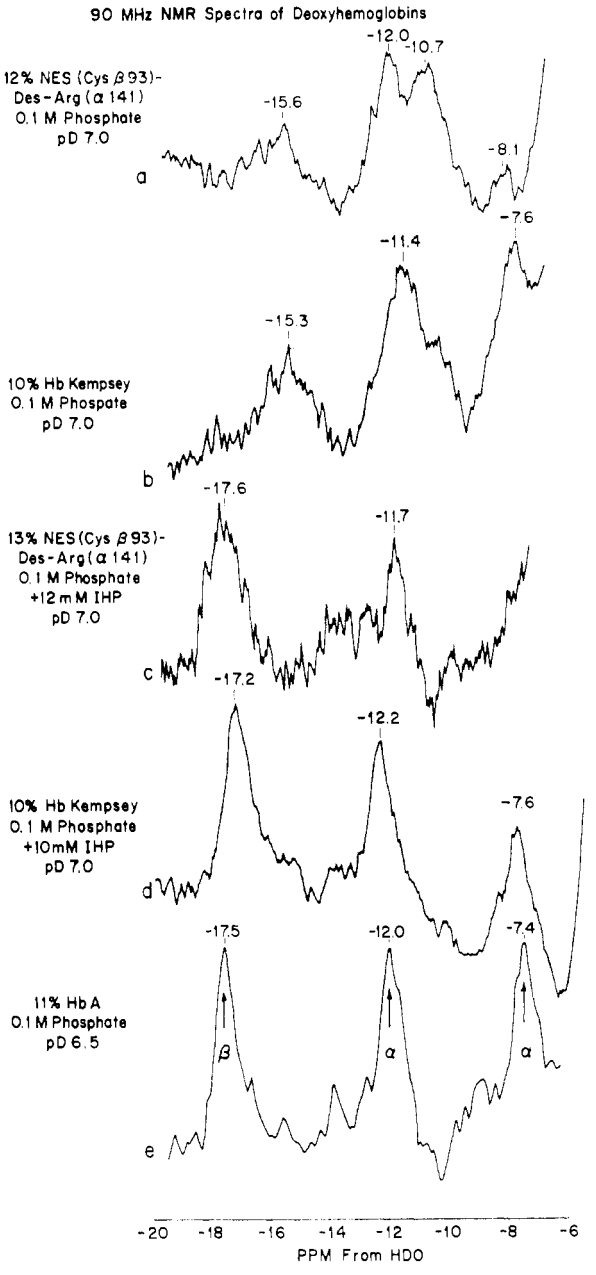


FIGURE 8: Paramagnetically shifted proton resonances of deoxyhemoglobins NES-des-Arg and Kempsey with and without IHP, compared to those of deoxyhemoglobin A.

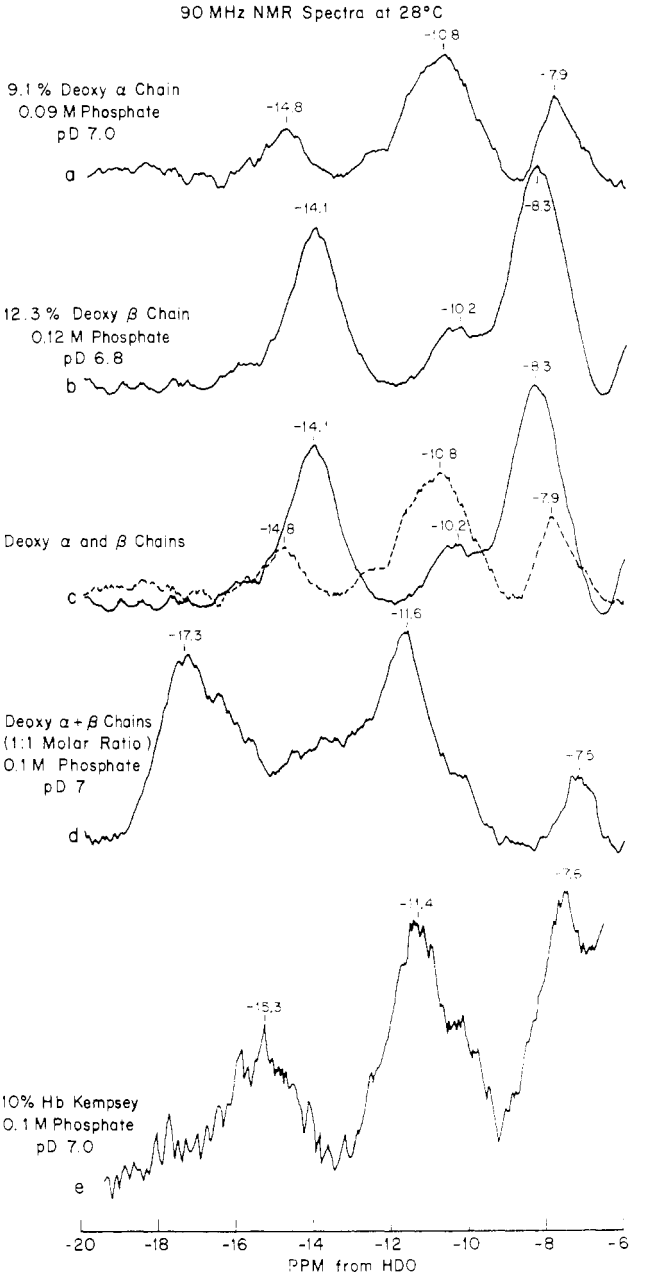


FIGURE 9: (a and b) Resonances of free deoxy α and β subunits. Similar results were obtained by Ogawa *et al.* (1971); (c) superposition of a and b; (d) mixture of α and β subunits forming deoxyhemoglobin A; (e) deoxyhemoglobin Kempsey; note the similarity with c above,

significant effect on the paramagnetic susceptibilities. Moreover, the paramagnetic susceptibility of NES-des-Arg-deoxyhemoblogin is the same with and without IHP (Y. Alpert, private communication). This was to be expected, since Taylor (1939) had shown that there is no difference in paramagnetic susceptibility between deoxyhemoglobin and deoxymyoglobin.

Discussion

Evidence for the two forms of deoxyhemoglobin described here was first discovered by Gibson (1959) when he observed that flash photolysis of carbonmonoxy hemoglobin resulted in a slowly and a fast-reacting phase (Hb and Hb*) which could be distinguished by the strength of their absorption at 430 nm. Olson and Gibson (1972) have since discovered that hemoglobin Bethesda (Tyr-145β-His) shows a weakened absorption at 430 nm similar to that of Hb* which rises in intensity to that of hemoglobin on addition of IHP. They suggested that the spectrum of Hb* corresponds to the ligated (R) form and that of Hb to the T form (Olson and Gibson, 1972). The spectrum of Hb* has also been observed in deoxygenated $\alpha\beta$ dimers (Kellett and Gutfreund, 1970; Anderson *et al.*, 1971; Hewitt *et al.*, 1972). However, this is the first time that it has been identified with two deoxyhemoglobins which crystallize in the R type of structure, and that the complete electronic and nmr spectra have been described.

Our results and those of Olson and Gibson and his colleagues show that the allosteric equilibrium of deoxyhemoglobin can be tipped to the R type of structure by the loss of several alternative pairs of bonds or a combination of them, and that it can be restored to the T type of structure by IHP. According to Table I, the loss of one pair of strong hydrogen bonds between a carboxyl and a phenolic OH, located in a position in the $\alpha_1\beta_2$ contact from which water is excluded, suffices to tip

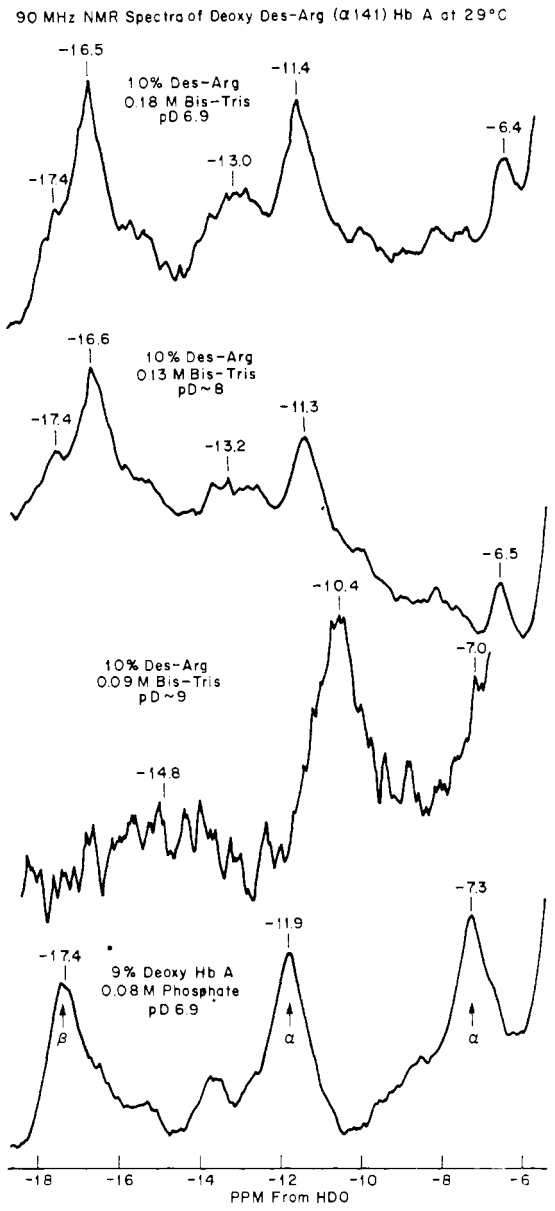


FIGURE 10: (a–c) Resonances of des-Arg(141)-α-deoxyhemoglobin and of deoxyhemoglobin A as a function of pH in Bis-Tris buffer.

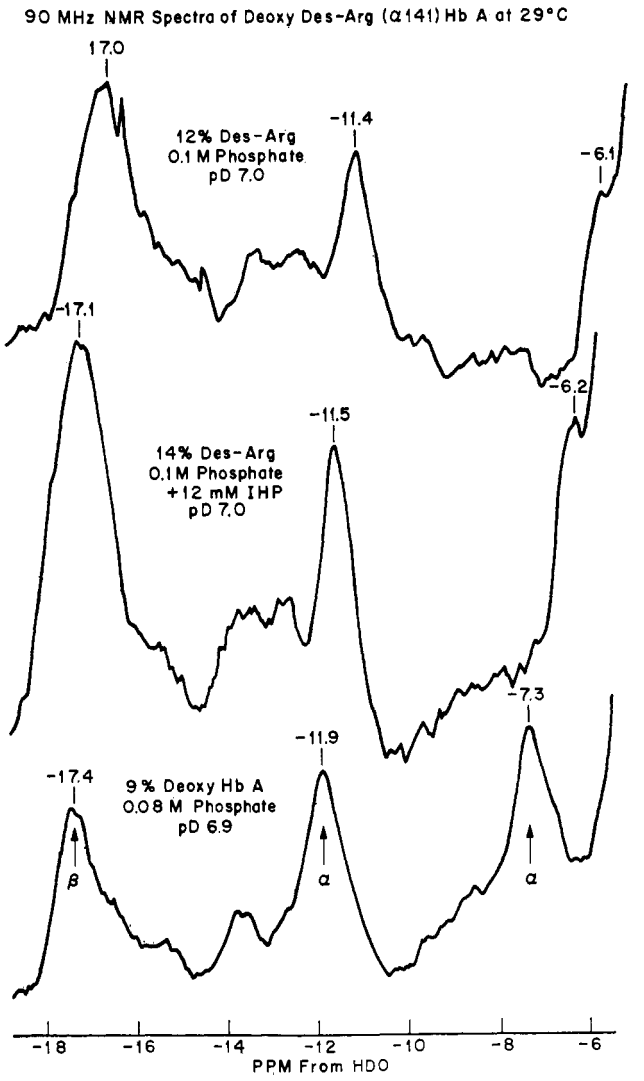


FIGURE 11: Resonances of des-Arg(141)-α-deoxyhemoglobin in phosphate buffer of pH 7.0 with and without IHP.

the equilibrium to the R type of structure (hemoglobin Kempsey). Alternatively, the same effect can be produced by the loss of two, somewhat weaker, hydrogen bonds between phenolic OH and main-chain carbonyl groups, together with the loss of two pairs of salt bridges from phosphates to amino groups (stripped hemoglobin Bethesda). Yet another method consists of the inhibition of the C-terminal salt bridges (NES-des-Arg). It would be interesting to estimate the energies of the bonds involved, but so far we do not have the data that would enable us to do this.

By what mechanism does IHP shift the equilibrium back to the T state? Arnone and Perutz (1974) have shown that it combines with deoxyhemoglobin at the same site as 2,3-diphosphoglycerate (Arnone, 1972). It has a stronger affinity for the T structure than, 2,3-diphosphoglycerate, probably due to the formation of one additional salt bridge (to both lysines-EF5-(82)β instead of only one of them) and to the larger number of water molecules released on binding to deoxyhemoglobin.

The static difference spectra between tetrameric deoxyhemoglobins in the R and T states described here are the same as the kinetic difference spectra between the sum of the isolated and recombined deoxy subunits measured by Brunori *et al.* (1968). Significantly, these authors found no spectral differences between the sum of isolated and recombined oxy or carbonmonoxy subunits, and Shiga *et al.* (1969) found the electron spin resonance (esr) spectrum of nitric oxide hemoglobin to correspond to the arithmetic mean of the spectra of the isolated nitric oxide subunits, all of which goes to show that in the R state the hemes are in the same state as in the free subunits. There is just one result in apparent conflict with this notion. Aquomethemoglobin in solution is predominantly in the R state (see part III, Perutz *et al.*, 1974b), yet Banerjee *et al.* (1969) found marked spectral changes on association of free aquomet β subunits with free α subunits to form tetrameric aquomethemoglobin. It will be shown in part III that this may have been an artifact due to the partial denaturation of the free β subunits to form hemichromes, and the reversal of that denaturation on recombination with free α subunits.

The spectral changes that occur on transition of deoxyhemoglobin from the R to the T state show that the hemes are modified in some way by the change of quaternary structure of the globin. This must be the modification which is related to their lowered oxygen affinity. It does not appear to depend on the presence of any one particular type of constraint in the globin such as the hydrogen bonds of the penulti-

mate tyrosines to the carbonyls of valine-FG5, or on the terminal salt bridges; this is also apparent from the results of Bonaventura *et al.* (1972) on des-His-146β-Tyr-145β-hemoglobin; the presence of the quaternary T structure, however stabilized, is sufficient to produce it. The nmr spectra are clearly more sensitive to small stereochemical changes in the heme environment than the electronic ones and show that the analogy between the structure of free chains and that of the tetramer in the R state must not be pushed too far. The nmr spectra also indicate observable differences in the heme environment of the various derivatives studied here.

Perutz (1972) has suggested that the transition from the R to the T structure results in an increased tension at the heme which leads to a lengthening of the iron–nitrogen bond distances. In parts II and III evidence will be presented that such a lengthening of the iron nitrogen bond distances does actually take place when IHP reacts with aquomethemoglobin and changes its quaternary structure to the T state. It will also be shown in part III that this lengthening can account for the blue shifts observed here provided that a transfer of negative charges from the iron to the porphyrin contributes to the energies of the absorption bands concerned. A direct test of Perutz' hypothesis will require an accurate determination of the distance from the heme-linked histidine to the plane of the porphyrin ring by X-ray analysis at high resolution in both the T and R states of deoxyhemoglobin.

Our results show that in deoxyhemoglobin the R $\rightarrow$ T transition also causes characteristic changes in the absorption spectra and circular dichroism in the aromatic region of the uv spectrum. The peaks in the difference absorption spectrum at 278, 287, 294, and 302 nm are the same as those that occur on deoxygenation of oxyhemoglobin A, but they are not seen when the modified hemoglobins are deoxygenated while remaining in the R state. We therefore take these peaks and the sharp negative peak in the CD spectrum at 287 nm to be characteristic of the change in quaternary structure of the globin, but independent of the state of ligation of the heme and the accompanying changes in tertiary structure of the globin chains. They are clearly caused by the changes in the environment of the aromatic residues at the $\alpha_1\beta_2$ contact where nearly all the movement between subunits takes place. These residues include Tyr-C7(42)α, Phe-C7(41)β, and Trp-C3(37)β. The contribution of the tryptophan has been established by the disappearance of the bands at 294 and 302 nm in hemoglobin Hirose, in which the tryptophan is replaced by a serine (see part II). Phenylalanine absorbs much less than the other two residues and is therefore not likely to contribute significantly. The environment of the tyrosine side chain changes drastically, from being in contact with the guanidinium group of Arg-C6(40)β and hydrogen bonded to the amide group of Asn-G4(97)α in the R structure, to being removed from the arginine and hydrogen bonded to aspartate-G1(99)β in the T structure. Imai (1973) also recorded the uv difference spectra that appear on oxygenation of hemoglobin A and of three chemically modified hemoglobins with diminished cooperativity, including des-His-146β-Tyr-145β. He noted that the "fine structure peaks" in the aromatic region decreased in magnitude as the cooperativity of the reaction with oxygen fell and that they vanished in des-His-Try. He interpreted the decrease as being due to smaller conformational changes accompanying the reaction with oxygen.

The results of Bradley *et al.* quoted earlier demonstrate that the R $\rightarrow$ T transition of deoxyhemoglobin Kempsey is also accompanied by a marked lowering of the sulfhydryl reactivity. There again it is found to be the change in quaternary structure produced by IHP and not the changes in tertiary structure that alone accompany deoxygenation in the absence of IHP which causes the drop in sulfhydryl reactivity. In part II the changes in absorption and CD in the uv and the lowering of the sulfhydryl reactivity will therefore be used as diagnostic criteria for the R $\rightarrow$ T transition in ferric hemoglobin derivatives.

It may be asked whether the difference spectra recorded here might not have arisen because the deoxyhemoglobins in the R state were dissociated into $\alpha\beta$ dimers in the absence of IHP, and associated into tetramers in its presence. The tetramer–dimer dissociation constant of deoxyhemoglobin in the R form is not known but may reasonably be assumed to be of the same order as that of oxyhemoglobin under comparable conditions. If 0.1 M phosphate buffer at pH 6.5 is taken to correspond in ionic strength approximately to 0.114 M NaCl, then K_{diss} would be about 6 μM (tetramer). The solutions used for our measurements had concentrations of 1.5 μM (Soret), 15 μM (uv and visible), 62 μM (CD), and 380 μM (tetramer) (ir). The corresponding percentages of dimer present would be 61, 27, 13, and 6. Therefore, dissociation could not have made more than small contributions to the electronic difference spectra in the infrared or to the CD difference spectra. To check if it contributed to the visible difference spectra, the concentration of NES-des-Arg-deoxyhemoglobin was varied between 15 and 120 μM tetramer, corresponding to concentrations of 27 and 9% dimers, respectively, but no significant change in the difference spectra was seen. This leaves the Soret band where absorption is too high to permit measurements at high concentration, but seeing that the effects in the other spectral regions are independent of hemoglobin concentration it seems unlikely that the Soret band would be strongly affected by it. In fact, deoxyhemoglobin dimers are known to exhibit absorption spectra which are the same as those now recorded for tetrameric deoxyhemoglobin in the R state (Gibson, 1959; Anderson *et al.*, 1971).

Acknowledgments

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Added in Proof

Further studies have confirmed that the tyrosine is responsible for the sharp peaks at 278 and 287 nm in the difference absorption spectrum, while the tyroptophan contributes to the band at 287 nm in the difference CD spectrum (M. F. Perutz and S. R. Simon, unpublished).

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