# Nitrosylhemoglobin Wood: Effects of Inositol Hexaphosphate on Thiol Reactivity and Electron Paramagnetic Resonance Spectrum†

Fumito Taketa,* William E. Antholine,‡ A. Grant Mauk, and Joseph A. Libnoch

ABSTRACT: Properties of Hb Wood ($\beta$97(FG4)His→Leu), a high oxygen affinity hemoglobin with reduced heme–heme interaction, were examined in its nitric oxide liganded form. The reactivity of the $\beta$-93 thiol groups and the electron paramagnetic resonance (EPR) spectrum were examined to determine what effect the amino acid substitution, which occurs at the $\alpha_1\beta_2$ interface, would have on inositol hexaphosphate induced transition of this form of the tetramer. Binding of inositol hexaphosphate (IHP) in a 1:1 stoichiometry was demonstrated. In spite of apparently normal interaction with IHP, there was little or no change in the reactivity of the $\beta$-93 thiol groups and in the electron paramagnetic resonance (EPR) spectrum as contrasted with the marked changes characteristic of normal hemoglobin (HbA). In contrast with NO-HbA, there was also no development of the EPR hyperfine structure in NO-Hb Wood with increased protonation of the protein at pH below 7.0. Taken together with the observations of Henry and Banerjee ((1973), *J. Mol. Biol. 73,* 469) on the development of NO-Hb EPR hyperfine structure and of Perutz et al. ((1974a), *Biochemistry 13,* 2174) on changes in thiol reactivity with the R→T transition, the results suggest that IHP or $H^+$ cannot switch NO-Hb Wood to the T conformation. Since the atomic structures of met- and deoxyhemoglobin offer no indication that His-97 plays any special part in the allosteric mechanism (M. E. Perutz, personal communication), it appears that the replacement of His-97 by Leu reduces the stability of the T structure relative to that of R.

A number of abnormal hemoglobins with high oxygen affinity and weak heme–heme interaction are known in which single amino acid substitutions occur at $\alpha_1\beta_2$ contact sites of the tetramer (Morimoto et al., 1971; Nagel and Bookchin, 1974). They remain predominantly in the high oxygen affinity R form even when deoxygenated (Olson and Gibson, 1972; Bunn and McDonough, 1974; Perutz et al., 1974a,c), apparently because the amino acid substitutions alter the interactions at the $\alpha_1\beta_2$ interface (Muirhead et al., 1967; Perutz and Ten Eyck, 1971) and shift the allosteric equilibrium between the R and T structures of the protein. Organic phosphates, particularly IHP,[1] can be used to switch some of these hemoglobins from R to T (Olson and Gibson, 1972; Ogawa et al., 1972; Bonaventura et al., 1972; Ho et al., 1973; Lindstrom et al., 1973) to study the way in which changes in quaternary globin structure can affect the state of the heme. Such pertubations in the heme environment have recently been examined by analysis of electronic and nuclear magnetic resonance spectra (Perutz et al., 1974a–c; Wiechelman et al., 1974).

Another convenient way to study how changes in globin structure affect the state of the heme is by electron paramagnetic resonance (EPR) spectroscopy of nitric oxide liganded hemoglobin (NO-Hb). In the presence of organic phosphates (2,3-DPG, IHP, ATP) and $H^+$ ions in buffers of appropriate pH, the EPR spectra of NO-Hb exhibit a hyperfine triplet centered about $g = 2.009$ (Rein et al., 1972; Trittelvitz et al., 1972; Antholine et al., 1973). Henry and Banerjee (1973) have suggested that this triplet is due to a particular electronic environment surrounding the $\alpha$-chain heme and that it is dependent upon the spin state of the heme carried by the neighboring $\beta$ chains.

In unliganded hemoglobin, the organic phosphates interact with certain positively charged residues of the $\beta$ chains in the central cavity of the tetramer (Perutz, 1972; Arnone, 1972; Arnone and Perutz, 1974) and stabilize the deoxy or T conformation (Perutz, 1972). The interaction of organic phosphates with NO-Hb apparently occurs at these same $\beta$-chain sites (Antholine et al., 1973). The EPR hyperfine splitting that results presumably reflects changes in the interactions at the $\alpha_1\beta_2$ contacts that lead to stabilization of a T type structure. Thus the development of hyperfine lines in the EPR spectra of the NO derivatives of hemoglobin with amino acid substitutions at the $\alpha_1\beta_2$ interface can be used to assess the role of specific residues in communicating the effects of organic phosphates from the $\beta$ to the $\alpha$ chains.

Hb Wood ($\beta$97(FG4)His→Leu), a high oxygen affinity hemoglobin with weak heme–heme interaction, was recently discovered and characterized in this laboratory (F. Taketa et al., submitted for publication). The substitution occurs in the same position as in Hb Malmö ($\beta$97(FG4)His→Gln) (Lorkin et al., 1970; Boyer et al., 1972) and in the homologous position of Hb Chesapeake ($\alpha$92(FG4)Arg→Leu) (Clegg et al., 1966) and HbJ Capetown ($\alpha$92-(FG4)Arg→Gln) (Botha et al., 1966). Residues FG4$\alpha$ and FG4$\beta$ are found at $\alpha_1\beta_2$ contacts (Muirhead et al., 1967; Perutz and Ten Eyck, 1971) and all four of these hemoglobins demonstrate high oxygen affinity and reduced heme–

† From the Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53233 (F.T. and A.G.M.), and the Department of Medicine, Veterans Administration Center, Wood, Wisconsin 53193 (J.A.L.). *Received February 14, 1975.* This work was supported by Grant AM-15770 from the National Institutes of Health (to F.T.) and Project No. 3778-01 from the Veterans Administration (to J.A.L.).

‡ Present address: Department of Radiology, Medical College of Wisconsin.

[1] Abbreviations used are: Hb, hemoglobin; IHP, inositol hexaphosphate; 2,3-DPG, 2,3-diphosphoglycerate; EDTA, etheylenediaminetetraacetic acid; PDS, 4,4′-dipyridyl disulfide; bis-tris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

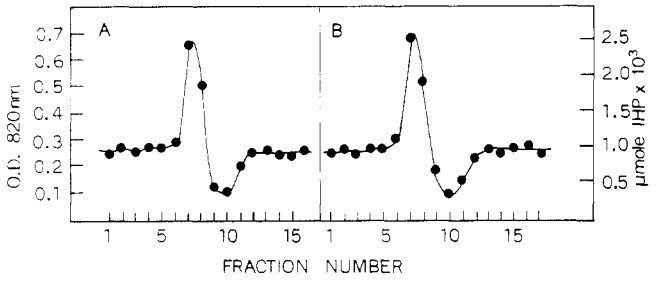



FIGURE 1: Sephadex G-25 gel filtration of nitric oxide hemoglobins in the presence of IHP; 0.5-ml solutions of $2 \times 10^{-3}$ *M* NO-Hb in 0.05*M* bis-tris buffer (pH 7.0) containing 0.1 *M* NaCl and 0.4 m*M* IHP were loaded on a 1 × 30 cm column equilibrated with the same buffer. Elution was carried out with the same buffer. The entire operation was conducted in an argon filled glove box; 0.4-ml fractions were collected and analyzed for phosphate and hemoglobin as described in the text. Data on phosphate elution are shown in the figure. Elution of hemoglobins was coincident with the phosphate peaks. The IHP concentration was calculated from the phosphate content. (A) NO-Hb Wood; (B) NO-HbA.

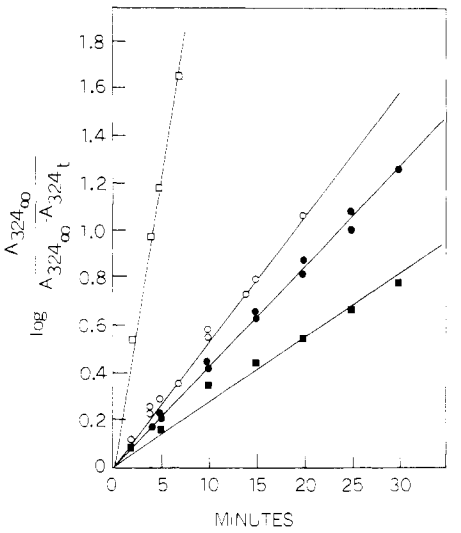



FIGURE 2: Pseudo-first-order rates of reaction of NO-HbA and NO-Hb Wood with the thiol reagent, PDS. Concentrations of reactants were $1.2 \times 10^{-5}$ *M* Hb, $1.2 \times 10^{-4}$ *M* IHP, and $1.6 \times 10^{-4}$ *M* PDS, respectively. (□) NO-HbA; (○) NO-Hb Wood; (■) NO-HbA + IHP; (●) NO-Hb Wood + IHP.

heme interactions. Hb Wood has provided the opportunity to study the effects of the β97His→Leu amino acid substitution on the generation of the IHP- and $H^+$-induced EPR hyperfine structure in the NO derivative. In addition, the effect of the substitution on the reactivity of the β93(F9) thiol group was analyzed to assess changes in tertiary and quaternary structure.

## Materials and Methods

*Hemoglobin Solutions.* Hemolysates were prepared from washed erythrocytes as previously described (Taketa and Morell, 1969). Hb Wood was isolated by CM-Sephadex column chromatography using 0.05 *M* phosphate buffer (pH 6.7) at 4° (F. Taketa et al., submitted for publication). Hemoglobin solutions were "stripped" by passing them through a Sephadex G-25 column as described by Berman et al. (1971) and then concentrated in an Amicon ultrafilter using a PM-10 membrane. Removal of phosphate by this procedure was verified by total phosphate analysis (Ames and Dubin, 1960). Hemoglobin concentration was determined by the cyanmethemoglobin method (Sunderman, 1964). Appropriate dilutions were then made in 0.05 *M* bis-tris buffers.

Hemoglobin solutions were deoxygenated by thorough flushing with argon in specially constructed tonometer cuvets. After verification of complete conversion to deoxyhemoglobin by spectral analysis, the solution was flushed with nitric oxide gas (Matheson Gas Products) passed through a column (1 × 20 cm) of solid KOH. Conversion to NO-hemoglobin was verified by spectral analysis, and excess NO was removed by flushing the solution with argon. The tonometer was then placed in an argon-filled glove box for subsequent transfer operations.

*Inositol Hexaphosphate Solutions.* The sodium salt of IHP (Sigma) was dissolved in deionized water and neutralized to pH 7.0 with HCl.

*IHP Binding.* Binding of IHP to NO-hemoglobin was evaluated by the gel filtration procedure of Hummel and Dreyer (1962) by placing 0.5 ml of Hb solution (0.2 m*M*) containing 0.1 *M* NaCl, 0.05 *M* bis-tris, 1 m*M* EDTA, and 0.05 m*M* IHP at pH 7.0. The column was run with a pump (Gilson minipuls) at a flow rate of 4.8 ml/hr, and 0.4-ml fractions were collected for phosphate analysis by the procedure of Ames and Dubin (1960). The entire operation was conducted in an argon-filled glove box. Dithionite was added to the deoxygenated buffers to assure complete deoxygenation.

*Sulfhydryl Reactivity.* Transfer of NO-hemoglobin and reagents into Thunberg type cuvets was carried out under argon in a glove box using automatic pipets (Oxford). Solutions of 4,4′-dipyridyl disulfide (PDS) were deoxygenated by flushing with argon and evacuation. Dithionite could not be used since it interferes with the assay. Reaction was initiated by tipping 0.2 ml of the PDS solution (0.44 mg/ml) from the side arm into the cuvet compartment containing 2.0 ml of 0.1% Hb solution (in 0.05 *M* bis-tris, 0.1 *M* NaCl, and 0.1 m*M* EDTA (pH 7.0)) and 0.3 ml of the buffer with or without IHP. The reaction was followed at 324 nm with a Gilford Model 240 recording spectrophotometer as previously described (Taketa and Morell, 1969).

*EPR Measurements.* NO-hemoglobin samples were placed in 4 mm (i.d.) precision bore quartz tubes and frozen in liquid nitrogen for measurement as previously described (Antholine et al., 1973). Spectra were recorded at 77°K using a liquid nitrogen finger dewar at X-band with a Varian E-9 spectrometer. Peak height intensities of triplet hyperfine structures were calculated from averages of the second derivative spectra.

## Results

The Sephadex G-25 elution profile for the NO derivatives of normal HbA and Hb Wood shown in Figure 1 indicate that both proteins can bind IHP. It appears that under the conditions used here, IHP eluted more rapidly on Sephadex than would be expected on the basis of its molecular weight alone. Berman et al. (1971) have reported that at pH 7 and below, 2,3-DPG behaves similarly. Failure to separate the phosphate peak and trough has apparently plagued previous studies of this kind with hemoglobin (Janig et al., 1971; Luque et al., 1969). Such problems frequently arise from overloading the column with protein, but smaller amounts of hemoglobin produced similar elution patterns. Despite these difficulties, the profile in Figure 1 and others not shown for deoxy and CO derivatives indicate that both HbA and Hb Wood bind IHP with a 1:1 stoichiometry.

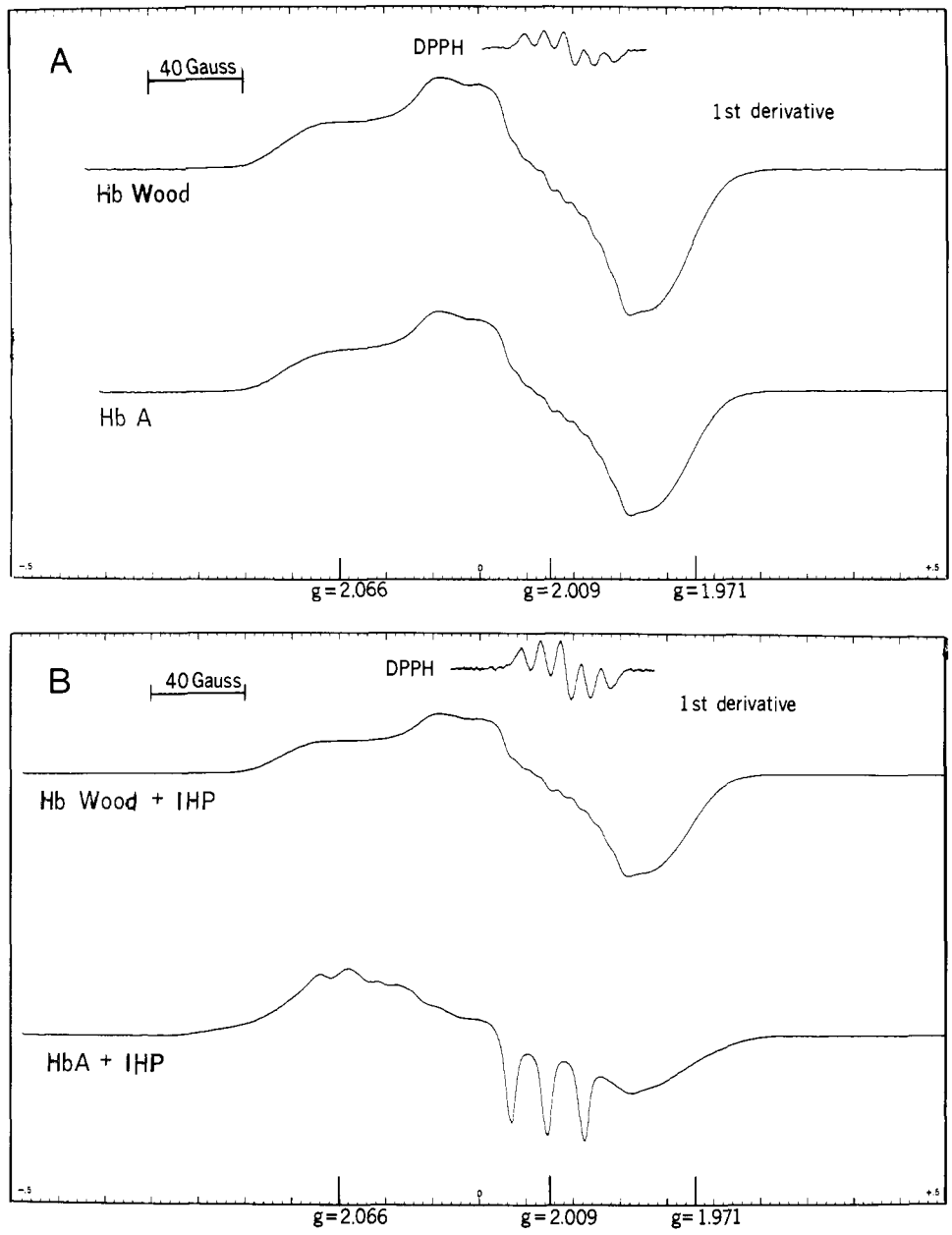



FIGURE 3: First derivative X-band EPR spectra of 0.1 m*M* NO-Hb Wood and NO-HbA at pH 7.0 in 0.05 *M* bis-tris, and 0.4 m*M* heme. (A) Without added IHP; (B) with 0.2 m*M* IHP.

The reactivity of $\beta$-93 cysteine in the absence of IHP is greater for NO-HbA than for NO-Hb Wood (Figure 2). It is also clear from Figure 2 that with the addition of IHP, the reactivity of -SH groups in NO-HbA is markedly decreased while that of NO-Hb Wood is only slightly reduced. Perutz et al. (1974b) have recently provided evidence that the large change in -SH reactivity that is observed when human hemoglobin is deoxygenated depends upon quaternary transition from the R to the T structure and that a relatively small change in -SH reactivity is found on conversion to the tertiary deoxy structure alone. If the same interpretation is applied here, IHP binds to NO-Hb Wood and thereby induces changes in the tertiary structure of the $\beta$ chains, but an accompanying transition to the quaternary T structure does not occur as in NO-HbA. Evidence for IHP induced transition from R to T structure in ferri- and deoxyhemoglobin A has been presented by Perutz et al. (1974a-c).

Since IHP interacts with NO-Hb Wood and alters the conformation of the $\beta$ chain its effects on the generation of the EPR hyperfine spectra were examined. Figure 3 shows that at pH 7.0 and in the absence of IHP, the EPR spectra of the NO derivatives of HbA and Hb Wood are indistinguishable. When saturating concentrations of IHP are added, however, a clear difference in the spectra of these nitrosylhemoglobins is noted. Whereas NO-HbA responds to the effector with development of a hyperfine triplet structure, NO-Hb Wood demonstrates no change whatsoever in its spectrum. The hyperfine structure fails to develop in EPR spectra of Hb Wood even when higher concentrations of IHP are added.

Rein et al. (1972) and Trittelvitz et al. (1972) have reported that the same hyperfine triplet can also be observed below pH 7.0 in NO-human and NO-horse hemoglobins even in the absence of organic phosphates. Since the p*K* for this effect was about 5.3, the latter group of workers suggested that the hyperfine structure may be related in some way to the acid Bohr effect; that is the protonation of cer-

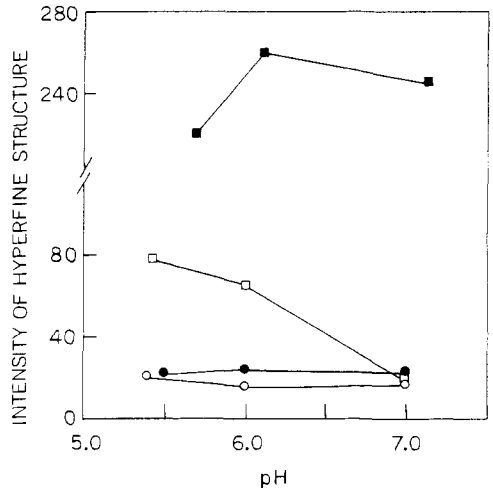



FIGURE 4: The effect of pH on the EPR hyperfine triplet intensity. EPR spectra were obtained for 0.1 m*M* NO-hemoglobins in 0.05*M* bis-tris buffers (0.1 *M* NaCl) in the presence and absence of 0.2 m*M* IHP. (○) NO-Hb Wood; (□) NO-HbA; (●) NO-Hb Wood + IHP; (■) NO-HbA + IHP.

tain carboxyl groups to produce an "acid" conformation. Although the relevant proton binding sites are not known, it was of interest to examine the spectra of NO-Hb Wood to determine whether or not the hyperfine structure is developed with increasing protonation as with NO-HbA and other animal NO-hemoglobins (F. Taketa et al., in preparation). Figure 4 compares the effect of pH on the relative peak heights of hyperfine structure in NO-HbA and NO-Hb Wood. As observed by others, lowering the pH from about 7.0 to 5.5 resulted in an increase in hyperfine structure intensity with NO-HbA, but such a change in pH failed to elicit any change in the EPR spectra of NO-Hb Wood. Even in the presence of equimolar concentration of IHP, no evidence for the development of hyperfine lines at pH 6.0 or 5.5 was found with NO-Hb Wood.

## Discussion

Henry and Banerjee (1973) demonstrated chain nonequivalence in NO-hemoglobin from studies on the EPR spectra of isolated NO-$\alpha$, NO-$\beta$, and mixed hybrid tetramers with various states of ligation in one or the other pair of subunits. They showed that the EPR hyperfine splitting in the spectrum of the hybrid, $\alpha_2^{NO}\beta_2$, is only observed when the neighboring $\beta$-chain heme is in the high spin state. Since no splitting was observed in the spectrum of the other hybrid, $\alpha_2\beta_2^{NO}$, it was concluded that the triplet was exclusively due to a particular environment surrounding the $\alpha$-chain NO-heme. According to this interpretation, the effects of changes in the tertiary structure of the $\beta$ chain are transmitted through the $\alpha_1\beta_2$ contacts to the neighboring $\alpha$ chains to induce an $\alpha$-chain NO-heme geometry that elicits the hyperfine triplet. Since it appears that organic phosphates interact with the $\beta$ chains of deoxy- as well as NO or ferrihemoglobins in a 1:1 stoichiometry (Benesch et al., 1968; Perutz, 1973; Antholine et al., 1973) the induction of the hyperfine triplet must then result from conformational transitions transmitted through the $\alpha_1\beta_2$ contact sites. Perutz et al. (1974b) have suggested an alternative interpretation for the observations of Henry and Banerjee. It was pointed out that the hyperfine splitting may be characteristic only of the T conformation and that it might be produced even with the $\alpha_2\beta_2^{NO}$ hybrid by the addition of IHP. Whatever may be the correct interpretation, it seems that the development of the EPR hyperfine splitting is associated with quaternary transition to a T type of structure.

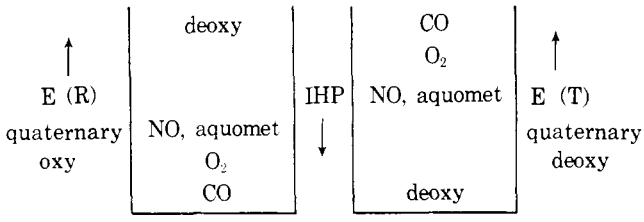
The fact that Hb Wood can interact with organic phosphates is clearly indicated by direct binding studies and by observations on changes in thiol activity, oxygen equilibrium, and peroxidase activity (F. Taketa et al., in preparation). In its deoxy form, the allosteric equilibrium of Hb Wood apparently can be shifted significantly toward the T state by IHP (F. Taketa et al., in preparation), not differing in this respect from HbA and the other high oxygen affinity mutants containing substitutions at $\alpha_1\beta_2$ contacts studied so far (Perutz et al., 1974a,c; Wiechelman et al., 1974). However, the stability of the T state is decreased in NO-Hb Wood even though binding of IHP occurs in this form as well. This is indicated by studies on thiol activity. In the absence of IHP the reactivity of $\beta$-93 thiol groups in NO-Hb Wood is somewhat lower than that of NO-HbA suggesting some difference in tertiary structure surrounding these groups. The striking feature, however, is their difference in response to IHP. Whereas a large decrease in reactivity is seen with NO-HbA, only a relatively small change is found with NO-Hb Wood. Perutz et al. (1974c) have correlated changes in $\beta$-93 thiol reactivity with shifts in tertiary and quaternary structure. Small changes in reactivity were associated with perturbations in tertiary structure and relatively large changes with transitions in quaternary structure. It appears from such evidence, therefore, that the R→T equilibrium of NO-HbA is shifted toward T by IHP but not to a detectable degree with NO-Hb Wood.

The lack of the organic phosphate as well as $H^+$ ion-induced EPR hyperfine triplet in NO-Hb Wood also indicates the important effect of the substitution at the $\beta$ chain FG4 position on the R→T equilibrium. Clearly, the allosteric effects of organic phosphates and $H^+$ ions on the heme–ligand loci are initiated at sites on the globin chains and are then transmitted by tertiary structural changes to the $\alpha_1\beta_2$ contacts. The resulting change in quaternary structure then leads to alterations in the NO-heme geometry. The NO derivatives of other human hemoglobins with substitutions at the $\alpha_1\beta_2$ contacts and reduced heme–heme interaction (Morimoto et al., 1971; Nagel and Bookchin, 1974) might be expected to demonstrate a similar lack of development of EPR hyperfine structure in the presence of organic phosphate effectors.

Perutz[2] has considered the data presented here and has offered the following comments and interpretation. They are included with his permission since they are consistent with the data and are useful in clarifying his concepts on how abnormal hemoglobins with high oxygen affinity work. "Ligands bias the equilibrium between the two allosteric forms of haemoglobin in varying degrees as shown purely qualitatively on the energy diagram below. IHP lowers the free energy of the T state so that certain derivatives such as NO and aquomet, which, in its absence, were more stable in the R state, become more stable in the T state. Some abnormal haemoglobins lack certain stereochemical interactions that normally stabilize the T state; characteristically, these show a low Adair contant $K_1$ (dissociation constant of oxygen in the T state) and an unchanged $K_4$ (dissociation constant of oxygen in the R state). Haemoglobin Chesapeake is an example (Imai, 1974) and Hb Wood seems to be another. In such haemoglobins the relative energy levels of the R

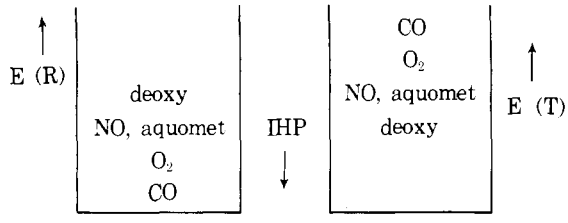
[2] M. F. Perutz, personal communication.

Hb A




and T states are shifted as shown below. This means that the additional free energy of stabilization provided for the T state by IHP is no longer capable of lowering the energy level for NO or aquomet Hb below that of the R state."

Hb Chesapeake or Wood




Acknowledgments

We thank Professor Max F. Perutz for this comments and suggestions in the preparation of this manuscript. We also thank Dr. Harold Swartz for generously allowing us the use of the EPR spectrometer in his laboratory.

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