*SPIN-LABEL STUDY OF HEMOGLOBIN CONFORMATIONS IN SOLUTION**

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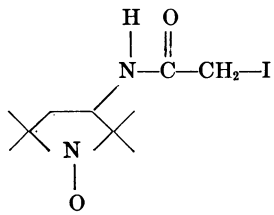
There is now a great deal of physical and chemical evidence that horse and human hemoglobins undergo a change of protein conformation on oxygenation.[1-9] The molecular mechanism whereby these hemoglobins show cooperative or sigmoidal oxygen binding has remained obscure, however. In order to understand this "heme-heme" interaction, one must obtain structural information on partially oxygenated hemoglobin molecules in solution. The present paper is a preliminary report of our study of oxygen-linked conformational changes in hemoglobin, using the spin-label technique. We also discuss the relation between conformational changes and allosteric interactions such as the cooperative binding of oxygen to hemoglobin.

Materials and Methods.—Horse and human hemoglobins were prepared according to the method of Benesch and Benesch[10] from fresh defibrinated horse blood (W. T. Bennett Ranch Laboratory) and from samples obtained through the courtesy of Dr. Marcus Krupp, Palo Alto Medical Clinic.

The spin label, N-(1-oxyl-2,2,5,5-tetramethyl-3-pyrrolidinyl)iodoacetamide (I),



(I)

was prepared by exchanging iodide with the reaction product of bromoacetylbromide and 2,2,5,5-tetramethyl-3-amino pyrrolidine-1-oxyl. Yellow needlelike crystals were obtained from toluene solution, mp, 150°C (uncorrected). *Analysis:* Calculated for $C_{10}H_{18}N_2O_2I$: C, 36.9; H, 5.6; N, 8.6; I, 39.0%. Found: C, 37.1; H, 5.6; N, 8.6; I, 39.1%.

The following conditions were used to optimize the preferential reaction of the label I with the two reactive SH groups in horse hemoglobin. A 10% solution of oxyhemoglobin at pH 7.8 was saturated with I at 2°C and allowed to stand for 28 hr. The extent of reaction with the reactive SH groups was monitored by the characteristic resonance spectrum of label I attached to this group. Complete reaction of I with the reactive SH groups was established by amino acid analysis. When these SH groups are first blocked with p-chloromercuribenzoate, iodoacetamide, or N-ethylmaleimide, it is found that I does react to a small extent under the above conditions to yield mostly weakly immobilized spectra that are not affected by oxygenation and deoxygenation of hemoglobin.

The oxygen binding to the labeled hemoglobin was determined by an optical method using a Cary model 15 spectrophotometer and a tonometer with an optical path of 0.6 mm that was multiply connected to a reservoir that could be evacuated. Oxygen was added with a Hamilton gas-tight syringe. The optical spectra of spin-labeled oxy- and deoxyhemoglobin between 500–700 mμ were identical with those of the unlabeled hemoglobin. Optical absorptions at four wavelengths were used to measure the degree of oxygen binding. Paramagnetic resonance spectra were obtained with a Varian X-band spectrometer.

Isolated spin-labeled β-subunits of human hemoglobin were prepared from labeled human hemoglobin A_1 by a simple modification of the method described by Bucci *et al.*[11] Oxyhemoglobin labeled with I at the reactive SH groups (β93) was treated with p-chloromercuribenzoate at pH

5.5 and then chromatographed on carboxymethylcellulose. The labeled β-chains were eluted with pH 6.7 phosphate buffer. Neither hemoglobin A nor the separated α-chains move on the CM cellulose column with this buffer. The eluted β-chains were treated with thioglycolate to remove the p-mercuribenzoate.

Results.—The paramagnetic resonance spectra of horse hemoglobin labeled with I are shown in Figure 1 for three degrees of oxygenation. These large spectral changes are completely reversible. Since it is known that the spectra of spin labels attached to large molecules depend on the local conformation that affects the motion of the labels,[12] the observed spectra are then a measure of conformational changes in hemoglobin that are associated with oxygenation. As seen in Figure 1, the resonance spectra have clear isosbestic points, showing that there is only one conformational change associated with oxygenation that affects the resonance spectra. It is well known that the optical spectra show isosbestic points associated with oxygenation. The sigmoidal oxygenation equilibrium curve of the labeled hemoglobin is shown in Figure 2. The Hill constant n is a measure of the cooperativity of oxygen binding, and is found to be $n = 2.3$ for the labeled hemoglobin, as compared to $n \sim 3$ for native hemoglobin.[7, 8]

At one atmosphere of oxygen pressure, hemoglobin is fully oxygenated; at the lowest pressures used in these experiments, hemoglobin is completely deoxygenated. A plot of the fractional change S in the spin-label spectra versus fractional change in the optical spectra is given in Figure 3. The fractional change in optical spectra is assumed equal to the fraction of the heme groups that are oxygenated, $\bar{Y}$. It is seen that $S = \bar{Y}$ to within the experimental error.

Horse methemoglobin derivatives labeled with I were also obtained. Low-spin derivatives (met HbCN and met HbN_3) showed almost the same spectra as oxyhemoglobin, but high-spin derivatives (acid met Hb and met HbF) showed label resonance spectra somewhat similar to deoxyhemoglobin.[13] These results raise an interesting problem since Perutz and co-workers[1-3, 14] have shown that methemo-

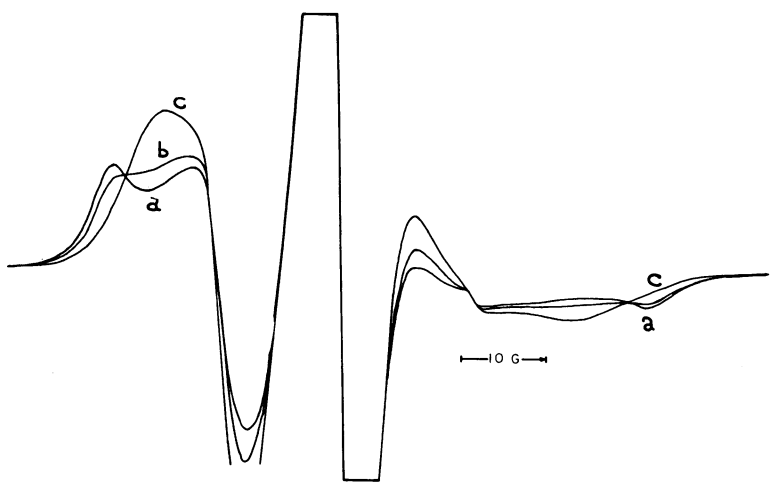
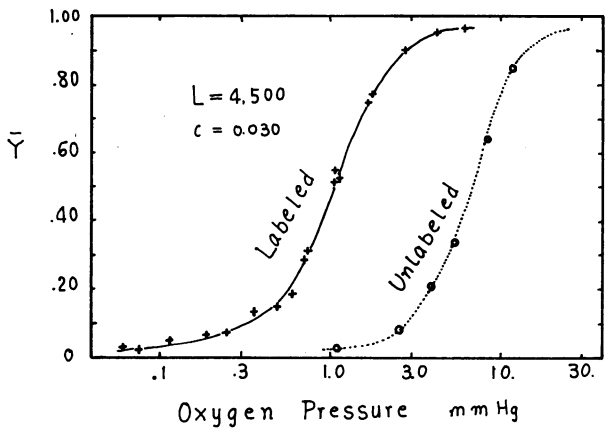
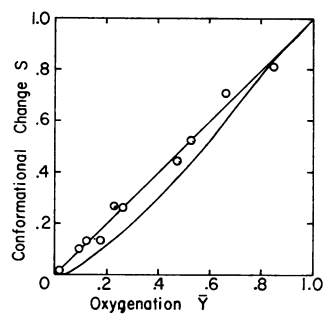


FIG. 1.—The paramagnetic resonance spectra of spin-labeled horse hemoglobin: (*a*) completely oxygenated, $S = 1.0$; (*b*) partially oxygenated, $S = 0.71$; (*c*) completely deoxygenated, $S = 0.0$.





(*Left*) FIG. 2.—Oxygen binding to spin-labeled (15°C) and native (17°C) horse oxyhemoglobin at pH 7.1, 0.1 *M* phosphate buffer.

(*Right*) FIG. 3.—Conformation change as a function of oxygenation, $\bar{Y}$, at 15°C. The circles are experimental points for fractional change in spin-label spectra (= fractional conformational change) vs. fractional change in optical spectra (= heme group oxygenation, $\bar{Y}$). Straight line of 45° slope corresponds to $S = \bar{Y}$. Curved line gives R ⇆ T conformation change vs. oxygenation $\bar{Y}$ predicted by fitting the data on the oxygenation of labeled hemoglobin in Fig. 2 to the allosteric model of Monod, Wyman, and Changeux.

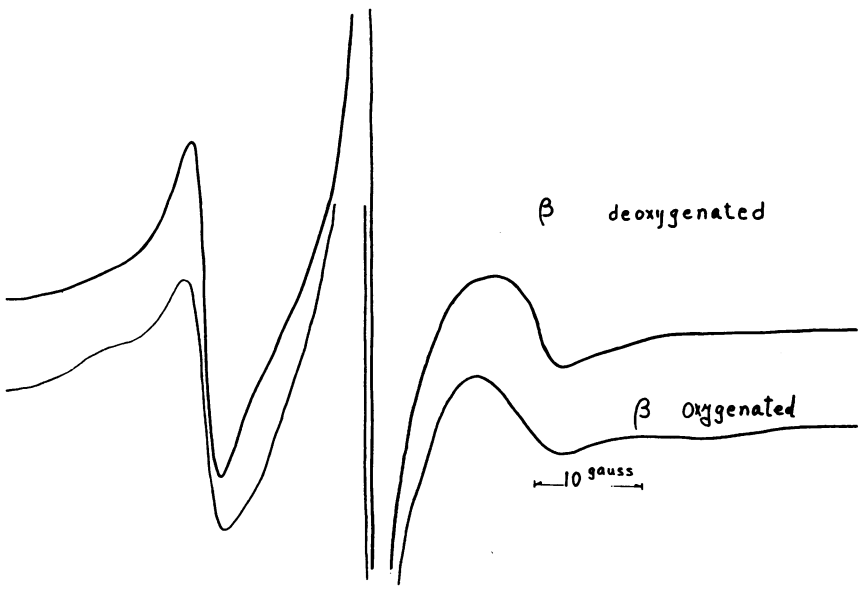


FIG. 4.—Paramagnetic resonance of oxy and deoxy β-chains of human hemoglobin spin-labeled at β93 SH with label I.

globin, oxyhemoglobin and azide methemoglobin must have very similar secondary and tertiary structures, and identical quaternary structures, whereas the structure of deoxyhemoglobin differs markedly from these three.

The large change in label resonance spectra induced by deoxygenation is not due to magnetic or exchange spin-spin interactions between the iron spin (in deoxy-

hemoglobin) and the label spins. First, the spectrum of deoxyhemoglobin seen in Figure 1 is typical of spin-label spectra with "intermediate immobilization," and shows no evidence of significant spin-spin line broadening. Second, the label spectrum of oxyhemoglobin is essentially unchanged when the magnetic state of the iron is changed by conversion from oxyhemoglobin ($S = 0$) to methemoglobin azide or cyanide ($S = 1/2$). Even though the label resonance of deoxyhemoglobin is somewhat similar to that found for paramagnetic methemoglobin fluoride, the paramagnetic spin lattice relaxation times of these two iron spin states must be so different that this similarity in spectrum could hardly arise from spin-spin interactions between iron and label spins. Finally, examination of atomic models of horse oxyhemoglobin to which models of label I have been attached further indicates that the label spins are well removed from the iron spins (by distances greater than 15–20 Å).[15]

Isolated labeled β-chains from human hemoglobin show resonance spectra (Fig. 4) that are quite different from those of labeled hemoglobin A_1. When the labeled β-chains were mixed with the equivalent amount of α-chains, the original labeled HbA_1 spectrum was obtained. The sedimentation pattern of spin-labeled β-chains was broad compared with labeled HbA_1, which was essentially identical with unlabeled HbA_1. The labeled β-chains showed a sedimentation velocity $s_{20,w}$ of about 3.5, indicating the solution to contain an equilibrium of several polymeric species β_n, $n \sim 2$–4.[11]

The resonance spectra of the isolated spin-labeled β-chains change on deoxygenation, as seen in Figure 4. Met β derivatives show resonance spectra analogous to those reported for the met Hb derivatives. The acid and fluoride derivatives of β show spectra similar to the deoxy β spectrum, but the cyanide derivative showed a resonance spectrum similar to oxy β.

Discussion.—Two simple and plausible assumptions account for the experimental results: (*a*) The resonance spectrum of a label attached to a given β-chain depends only on whether that β-heme group is oxygenated, and not on whether the other α- and β-heme groups are oxygenated. (*b*) The heme groups in the α- and labeled β-chains are equivalent to one another in oxygen affinity. That is, when a molecule of oxygen binds to a molecule of deoxyhemoglobin, it has an equal probability of going to any one of the four heme groups. Assumptions (*a*) and (*b*) are based directly on the linearity of the data in Figure 3, the slope of 45°, and the sharp isosbestic points in the paramagnetic resonance spectra.

We think it unlikely that the conformation change seen in our spectra can be the $R \rightleftharpoons T$ conformational change introduced in the model of Monod, Wyman, and Changeux (MWC)[16] for allosteric interactions such as the cooperative binding of oxygen to hemoglobin. As seen in Figure 2, our measured equilibrium binding of oxygen to labeled hemoglobin can be accounted for very well with the model of MWC using the following parameters of their theory: $L = 4{,}500$, $c = 0.03$. These parameters then lead to a predicted $R \rightleftharpoons T$ conformation change versus oxygen binding ($\bar{Y}$) curve that is in substantial disagreement with our linear curve, as seen in Figure 3. The present results also make it very unlikely that the labeled globin portion of the hemoglobin molecule can have a twofold symmetry axis when an odd number of oxygen molecules is bound.

The linear plot in Figure 3 *is* compatible with the "symmetry-breaking" model of

Koshland, Némethy, and Filmer (KNF),[17] provided the conformation changes detected here propagate to the region of subunit interactions. In this model our resonance spectra are direct indicators of the conformation change giving rise to the heme-heme interaction. It is interesting to recall that the first successful experimental effort to distinguish between symmetry-breaking and symmetry-conserving spin states[18] of the four iron atoms in hemoglobin was made by Coryell, Pauling, and Dodson.[19]

The existence of a conformation change involving a label attached to $\beta 93$ is not surprising in view of the model of Perutz[1-3] and the related structure of myoglobin determined by Kendrew and co-workers.[20, 21] That is, the label is directly bonded to the sulfur atom of cysteine $\beta 93$ (helix position F9); this amino acid is next to proximal histidine $\beta 92$ (F8) whose imidazole ring is directly linked to the iron atom of the heme group. Bonding of oxygen to the iron atom must result in some motion of the proximal imidazole ring, either through a change in the imidazole nitrogen-iron bond distance, or a distortion of the heme group, or a motion of the iron relative to the heme plane, or some combination of these events.[22] This motion of the imidazole could then be propagated directly to the region of the label, with a concomitant effect on the label resonance spectrum. The apparent correlation between the spin-label conformation change and the (high-spin)-(low-spin) state of the iron mentioned above is certainly compatible with a mechanism of this type. One should *not* imagine a conformation change of this type to be so localized to the vicinity of the heme group as to be irrelevant to the question of the heme-heme interaction. On the contrary, there is a good argument that this particular conformation change does propagate to the "surface" of the β-subunit. First, we note that in preliminary studies of the paramagnetic resonance of labeled single crystals of horse methemoglobin fluoride, and horse hemoglobin nitric oxide, no dipolar interaction between the heme spin and the label spin has been detected. This means that the paramagnetic nitroxide group is extended far away from the iron atom, by $\sim$15–20 Å.[23] (Recall that all the iron-iron distances in horse oxyhemoglobin are only 25–36 Å.[1-3]) This conclusion is consistent with likely modes of attachment of a model of I to the model of horse oxyhemoglobin at Medical Research Council, Cambridge. We are indebted to Dr. Max Perutz for this demonstration. All of this points to the sterically protected nitroxide group being on the "surface" of the β-subunit to which the label is attached, along with the amino acid side chains. Thus, the conformation change which the resonance spectra show involves the paramagnetic portion of the label which is at the surface of the β-subunit, and it is entirely reasonable that neighboring amino acid side chains are similarly involved. Second, the label resonance spectrum is affected by α-β-subunit interactions as evidenced by the effect of adding α-chains to labeled β-chains (compare Figs. 1 and 4). If the α-β interaction affects the label resonance, and oxygenation affects the label resonance, it is certainly most plausible that oxygenation affects the α-β interaction.

Mechanism of Cooperative Oxygen Binding.—Changes of protein conformation are presumably responsible for most,[16, 17] if not all,[24] allosteric interactions, including the cooperative oxygen binding to hemoglobin. The studies of Perutz and coworkers leave no doubt that on oxygen binding, a very substantial conformation change does take place.[1, 2] However, these crystal structure studies do not differentiate between, for example, the MWC and KNF models for cooperative oxygen

binding. When one looks carefully into the question of distinguishing experimentally between models such as those of KNF and MWC, it becomes clear that this is quite difficult when one includes symmetry-breaking generalizations of the MWC theory. Perhaps the most crucial question is the *range* of conformational changes produced by *partial* ligand binding. If this range were large, comparable to or greater than the subunit sizes, then there would be no evident physical need to introduce an *ad hoc* two-state hypothesis. If this range were much less, then a two-state protein of the MWC type would be necessary to account for allosteric interactions.

In the preceding section we have given arguments that with partial ligand binding, conformational changes do propagate to the region of subunit contacts. Since, however, the conformational changes we detect are seemingly independent of one another, it is helpful to consider from a theoretical point of view how such conformational changes can interact with one another to give allosteric effects.

Let F be the free energy associated with the conformational change of a protein due to ligand binding. Let q_i be a convenient set of variables describing the protein structure, and Δq_i the change of these variables due to structural changes associated with ligand binding. We represent F as a series expansion:

$$F = F_0 + \sum_i \left(\frac{\partial F}{\partial q_i}\right) \Delta q_i + \frac{1}{2} \sum_{i,j} \left(\frac{\partial^2 F}{\partial q_i \partial q_j}\right) \Delta q_i \Delta q_j + \cdots . \quad (1)$$

Here F_0 is the structural free energy of the protein in the absence of ligand. If we now compare the structural free energy $F(A, B)$ associated with the binding of two ligands, A and B, with the free energies of single ligand bindings, $F(A)$ and $F(B)$, it is clear that one can define an allosteric interaction term,

$$\Delta F(A,B) = F(A,B) - F(A) - F(B). \quad (2)$$

This quantity can be thought of as an interaction of sequential conformational changes produced by sequential ligand bindings. For simplicity of discussion, we assume that variables q_i are so chosen that the conformational changes are simply additive. Thus, $\Delta q_i = \Delta q_i^A$ when only ligand A is bound, $\Delta q_i = \Delta q_i^B$ when only ligand B is bound, $\Delta q_i = \Delta q_i^A + \Delta q_i^B$ when both A and B are bound. For conformational changes that are additive in this sense,

$$\Delta F(A,B) = \sum_{i,j} \left(\frac{\partial^2 F}{\partial q_i \partial q_j}\right) \Delta q_i^A \Delta q_j^B. \quad (3)$$

The model calculations of KNF of course utilize sequential interactions of sequential conformational changes. In a sense the MWC model also involves an interaction of conformation changes but in a quite different way. In each of their two exclusive protein conformations, MWC neglect the coupling term $\Delta F(A,B)$.[16] The allosteric conformation changes they consider are not sequential with sequential ligand binding, but are rather completely concerted.

Quantitative applications of the above equations to proteins are probably out of the question. However, as shown below, an oil drop in water can also exhibit allosteric interactions, and nicely illustrates this approach and the interaction of sequential conformational changes. Consider the surface free energy of an oil drop, $F = \gamma S$, where S is the surface area and γ is the interfacial tension. Consider a

single variable $q = V$, the volume of the drop. Let ΔV_A, ΔV_B be the volume changes produced by inserting ligands A and B into the oil drop. Then the free energy of the "allosteric interaction" $\Delta F(A,B)$ is

$$(\partial^2 F/\partial V^2)\Delta V^A \Delta V^B = -\,{}^2/_9\gamma S(\Delta V^A/V)(\Delta V^B/V). \quad (4)$$

If the oil drop has a volume roughly the same as that of hemoglobin, and an interfacial tension equal to that of water-toluene, then the allosteric interaction is $\sim$1000 cal/mole, of the same order as that observed for hemoglobin,[7] provided the volume changes ΔV^A, ΔV^B are of the order of 10 per cent of the total volume of the oil drop. Note that homotropic interactions in an oil drop are attractive, regardless of the sign of $\Delta V^A (= \Delta V^B)$, and heterotropic interactions are attractive if ΔV^A and ΔV^B have the same sign, and are repulsive if ΔV^A and ΔV^B have opposite signs.

Of course this oil drop effect tells us nothing about the mechanism of the allosteric interaction in hemoglobin, except to point out that the region of the molecule where the conformational changes do interact may be well removed from both ligand binding sites. It seems likely that in hemoglobin the region where the conformational changes interact most strongly is just simply the region of subunit contacts. A simple mechanism for the allosteric interaction, consistent with all our spin-label results, is the following. Consider the four "bonds" between the four subunits ($\alpha'\beta'$) ($\alpha''\beta''$) in the deoxyhemoglobin tetramer $\alpha_2\beta_2$. For *simplicity*, consider all these bonds strong, and equivalent. When oxygen binds to the heme group of one subunit, say α', then α' undergoes a conformation change that reduces the strength of the $\alpha'-\beta'$, $\alpha'-\alpha''$, and $\alpha'-\beta''$ bonds. This corresponds to an increase in the conformational free energy of the molecule. The addition of a second molecule of oxygen can be thought of as requiring a smaller increase of conformational free energy since this produces the weakening of two strong subunit bonds and one already weaker bond, and so forth. This sequential interaction of sequential conformation changes then provides the free energy terms for cooperative oxygen binding. The reduced positive free energy increase required for the weakening of an already weak bond is the type of effect we have attempted to represent in equation (3). However, the invariance of this equation to simultaneous sign reversals, $\Delta q_i \rightarrow -\Delta q_i$, $\Delta q_j \rightarrow -\Delta q_j$, means that bond strengths between oxygenated subunits could be considered to be the stronger.

It appears likely that the $\alpha' - \beta''$ subunit bonds ($\alpha_1 - \beta_2$ in the notation of Perutz[3]) are important for the heme-heme interaction.[3, 25] Since our spin label is thought to be close to this contact region, we conclude that the conformation change detected by the label is one of the sequential conformation changes responsible for the heme-heme interaction. In hemoglobin H (β_4) the absence of significant cooperativity may be due to the possibilities that (*i*) the β—β bonds are not made between regions of the β-chains with significant oxygen-linked conformation change, and/or (*ii*) the conformation change in the isolated β-chains may not be as large as this conformation change is in the β-chains in $\alpha_2\beta_2$, as suggested by the resonance spectra (compare Figs. 1 and 4).

Conclusions.—From studies of the paramagnetic resonance of spin-labeled horse and human hemoglobins we conclude that (*a*) each β-subunit of the tetramers $\alpha_2\beta_2$ as well as the human tetramer β_4 undergoes a substantial conformational change when that subunit binds a molecule of oxygen, and (*b*) this conformational change in

all likelihood propagates to the surface of the β-subunit, and to the region of subunit contacts in $\alpha_2\beta_2$. From these conclusions and from theoretical arguments that cooperative or allosteric binding of ligands to proteins can be due to an interaction of sequential conformational changes produced by sequential ligand binding, we conclude that (*c*) it is most plausible that this cooperative interaction takes place in the region of subunit contacts and corresponds to a modification of the subunit-subunit bond strengths.

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