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States of Hemoglobin in Solution†

Ronald T. Ogata‡ and Harden M. McConnell*

ABSTRACT: The equilibrium concentrations of partially oxygenated molecules of hemoglobin A, hemoglobin Chesapeake, and hemoglobin Kempsey can be calculated from the parameters of a two-state generalized concerted transition model, where the parameters are determined from a study of the binding of spin label triphosphates to partially liganded hemoglobin solutions. In this model it is essential to use different parameters for the α- and β-heme groups.

Since the discovery by Muirhead and Perutz (1963) that oxyhemoglobin and ligand-free hemoglobin have significantly different molecular structures, there have been many efforts to understand the role played by these two structures in cooperative oxygen binding. In 1965, Monod, Wyman, and Changeux introduced the allosteric transition model to account for cooperativity in hemoglobin and other oligomeric proteins. In this model an oxygen-induced transition between the ligand-free structure (T) and the oxy structure (R) is essential for the cooperative mechanism in hemoglobin, since the hemes in the T and R structures are assumed to have low and high oxygen affinities, respectively. Perutz (1970) has recently given a penetrating discussion of the extent to which the crystallographic studies of hemoglobin are consistent with the allosteric model. This discussion has been amplified in a later article by Perutz and TenEyck (1971). There have also been many attempts to test the validity of the MWC[1] model

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[1] Abbreviations used are: MWC model, Monod–Wyman–Changeux allosteric transition model; GCT, generalized concerted transition model; HbA, hemoglobin A; DPG, 2,3-diphosphoglycerate; ATP, adenosine 5′-triphosphate; IHP, inositol hexaphosphate.

from studies of the physical and chemical properties of hemoglobin solutions. Some of these have been summarized in a review by Antonini and Brunori (1970).

Virtually all of the experimental studies that have been cited as providing evidence *for* the validity of the MWC model can be criticized for one or both of two reasons. (1) Spectroscopic methods that detect only two signals—assumed due to molecules in the R and T states—may not have sufficient sensitivity to detect signals from intermediate states. (2) Conformity or lack of conformity of kinetic or equilibrium data to the original MWC model has questionable significance, since for some properties of hemoglobin the functional roles of α and β hemes are very different; this difference between α and β hemes can be even more pronounced in hemoglobin mutants (see the following discussion). With respect to the first point, Ogawa and Shulman (1971) have interpreted the nuclear resonance spectra of partially liganded hemoglobin solutions in terms of an equilibrium between only two structures, R and T. On the other hand, Ogawa *et al.* (1968) have shown that there are at least three distinct signals in the paramagnetic resonance of β-93 spin-labeled hemoglobin during oxygenation. With respect to the second point, nuclear resonance (Ogawa and Shulman, 1971; Davis *et al.*, 1970; Cassoly *et al.*, 1971) and kinetic studies (Olson and Gibson, 1970), the heme spin label studies of Asakura and Drott (1971), and the phosphate spin label studies discussed here and earlier (Ogata and McConnell, 1971, 1972; Ogata *et al.*, 1972) all indicate a strong asymmetry in the effect of α- *vs.* β-heme ligation. Studies of hemoglobin in solution that can be used to test critically the MWC model require the highest possible sensitivity, and must necessarily relax the restraint that the α subunits are equivalent to the β subunits.

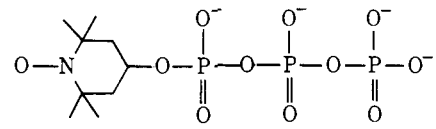
A number of spin labels have been used to test the validity of the MWC model (McConnell, 1971). Recent studies supporting the validity of this model for nonequivalent α- and β-heme groups have used two triphosphate spin labels which closely mimic the hemoglobin binding properties of 2,3-diphosphoglycerate (DPG) (Ogata and McConnell, 1971 and 1972). For brevity we refer here and elsewhere to the MWC model with nonequivalent α and β subunits as the "generalized concerted transition" (GCT) model.[2] The structural implications of the GCT model and the derived parameters appear to be in complete accord with the stereochemical mechanism proposed by Perutz (1970). On the other hand, the model is as yet quite general and the derived parameters do not provide a test for various features of the Perutz mechanism, such as the role of the salt bridges, and the Bohr effect. Recently Szabo and Karplus (1972) have attempted to express the detailed Perutz stereochemical mechanism in thermodynamic terms. A comparison of the Szabo–Karplus type calculation with the present GCT model indicates essential agreement except with respect to the relative oxygen affinities of the α and β subunits. This disagreement does not in any sense contradict the Perutz mechanism, since it is due to differences in the set of parameters chosen.

[2] In some respects there is no substantial difference between the "GCT model" discussed here and the original MWC model. Unfortunately, extensive usage of the term "MWC allosteric model" has led to a number of ambiguities as to what this term means. For example, some authors have assumed a narrow interpretation of the MWC model that requires conservation of symmetry even for partially liganded molecules. This is only one of the possibilities considered by MWC and, in fact, their model explicitly includes the possibility of symmetry-breaking structures in partially liganded molecules. To avoid this ambiguity, we use the term "GCT model."

In the present paper, we present (a) a brief, critical discussion of the range of validity of the GCT model, (b) an enumeration of the states of hemoglobin in solution and their relative populations under equilibrium conditions (as calculated by the model), and (c) an interpretation of the properties of mutant hemoglobins.

Materials and Methods

The spin labels used here are 1-oxyl-2,2,6,6-tetramethylpiperidine 4-triphosphate (I) and N^6-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)adenosine triphosphate (II).



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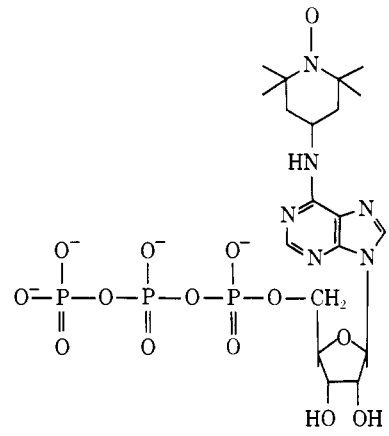

II

The synthesis and properties of these spin labels, the preparation and isolation of chemically modified and mutant hemoglobins, the derivation of model parameters, and model calculations have been described in detail previously (Ogata and McConnell, 1971, 1972; Ogata *et al.*, 1972; Ogata, 1971). Paramagnetic resonance spectra were obtained in 0.05 M bis-Tris buffer, pH 7.3, 0.1 M Cl^- at 13°.

Results and Discussion

The Model

The GCT model is identical with the MWC model in assuming the existence of two distinct thermodynamic states of hemoglobin, T and R. We assume that T and R refer to quaternary structure and identify these with the quaternary structures of ligand-free ("deoxy") and liganded hemoglobin, as determined by X-ray crystallography (Muirhead and Perutz, 1963).

In the GCT model a hemoglobin molecule in the T or the R isomeric state having m oxygen molecules bound need not be structurally identical with another molecule in the same isomeric state having n oxygen molecules bound, where $m \neq n$. Also, in the GCT model, ligation-induced structural changes within the T isomer or within the R isomer may occur and no restriction is placed on the symmetry of partially liganded molecules.[2] However, it is assumed that *ligand binding to a hemoglobin molecule in either the T state or the R state takes place with negligible cooperativity or anticooperativity.* The GCT model is generalized in the sense that the α and β subunits of hemoglobin are not assumed equivalent. In all other respects, the model is identical with the MWC model.[2]

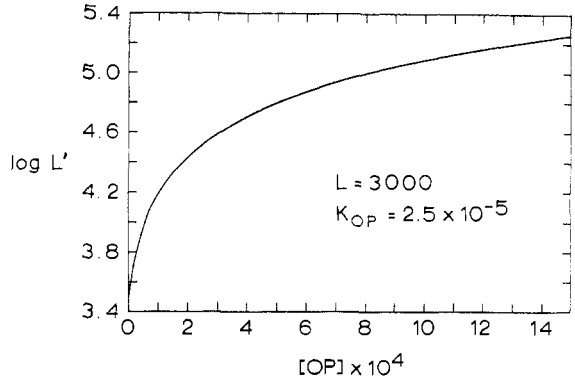

FIGURE 1: Dependence of the allosteric constant L' on the concentration of organic phosphate free in solution, calculated from eq 1 in the text.

TABLE 1: Parameters of the Generalized Concerted Transition Model.[a]

Hemoglobin	L	K_R^α	K_R^β	K_T^α	K_T^β
A	3000	0.36	0.18	24.2	32.8
$\alpha_2^{+CN}\beta_2$	3000	0.36	0.18	24.2	32.8
$\alpha_2\beta_2^{+CN}$	3000	0.36	0.18	24.2	32.8
Chesapeake	0.53	0.36	0.18	0.36	32.8
Kempsey	0.17	0.36	0.18	24.2	0.36

[a] For $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$, when the ferrous subunits are ligand free, T/R is equal to $L(K_R^\alpha/K_T^\alpha)^2$ and $L(K_R^\beta/K_T^\beta)^2$, respectively. L refers to phosphate-free hemoglobin solutions. These parameters apply only to the experimental conditions described in the text. K values in Torr.

Selection of Parameters. The GCT model contains the parameters K_R^α, K_R^β, K_T^α, and K_T^β, which are the dissociation constants describing the binding of oxygen to the α and β subunits of hemoglobin in the R and T isomeric states, and the parameter L, the allosteric constant describing the R $\rightleftharpoons$ T equilibrium in the absence of heme ligands. In selecting model parameters, we have assumed that (1) the values of K_R^α and K_R^β approximate closely the values of the dissociation constants describing oxygen binding to isolated α and β chains (Edelstein, 1971), and (2) the structures of the hybrids $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$ are accurate representations of the structures of the partially oxygenated hemoglobin molecules $\alpha_2^{O_2}\beta_2$ and $\alpha_2\beta_2^{O_2}$. Given the latter assumption, the affinities of the ferricyanide hybrids for spin label I, relative to that of HbA, are used to determine the ratios of K_R^α/K_T^α and K_R^β/K_T^β for different values of L (Ogata and McConnell, 1971). Although the GCT model has five independently adjustable parameters, the assumptions we have made place severe restrictions on the values these parameters can take. Indeed, with these restrictions, *L is the only adjustable parameter in the model.* In practice, however, partially because no data describing the oxygen equilibria of isolated α and β chains are available for our experimental conditions, we have allowed K_R^α and K_R^β to vary within the range of values reported for isolated chains (Ranney *et al.*, 1965; Antonini *et al.*, 1965a; Benesch *et al.*, 1968; Tyuma *et al.*, 1971b). This was done in order to achieve maximal agreement between experimental and calculated oxygen binding curves for HbA and the hybrids $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$ (Ogata and McConnell, 1972; Ogata, 1971). We believe that, within the limits defined by the assumptions made earlier, the parameters obtained for HbA are a unique set which best accounts for the properties of HbA and the two hybrids. The parameters selected are listed in Table I.

We emphasize that the set of parameters determined for HbA apply only to our experimental conditions (0.05 M bis-Tris buffer, pH 7.3, 13°, and 0.1 M Cl^-). Given the assumptions we have made, K_R^α and K_R^β should depend only on temperature, since the oxygen affinities of isolated α and β chains are dependent only on temperature (Antonini and Brunori, 1970; Benesch and Benesch, 1969). However, K_T^α, K_T^β, and L probably depend on temperature, pH, ionic strength, and the buffer used. Therefore, to determine a suitable set of parameters for other experimental conditions, it may be necessary to repeat measurements of the type described earlier under the new set of conditions.

Effect of Organic Phosphates. In the present model, the binding of organic phosphates to hemoglobin and the effect of organic phosphates on the oxygen binding properties of hemoglobin are accounted for by assuming that organic phosphates bind exclusively to the T isomer and thereby stabilize this isomer. The result is an increase in the allosteric equilibrium constant, L, to a new value, L'. This is given by the following equation (Monod *et al.*, 1965)

$$L' = L(1 + [OP]/K_{OP}) \quad (1)$$

Here [OP] is the concentration of organic phosphate free in solution and K_{OP} is the dissociation constant of the [Hb(T)·OP] complex. Hb(T) refers to a hemoglobin molecule in the T isomeric state. The dependence of L' on [OP] when L = 3000 and $K_{OP} = 2.5 \times 10^{-5}$ M is given in Figure 1.

The present analysis applies to any organic phosphate that (1) binds to ligand-free hemoglobin but not to tetraliganded hemoglobin, and (2) binds to ligand-free hemoglobin with a stoichiometry of one organic phosphate molecule per hemoglobin tetramer. The value of K_{OP} used in eq 1 must be experimentally determined and will depend on the structure of the organic phosphate and on experimental conditions (*e.g.*, pH, ionic strength, and temperature). By substituting the appropriate value of K_{OP} into eq 1, the GCT model should account for the effect of organic phosphates such as DPG, ATP, triphosphate spin labels, and pyridoxal phosphate (Renthal *et al.*, 1970) on the oxygen-binding properties of hemoglobin. Equation 1 does not apply to the effect of inositol hexaphosphate (IHP) on oxygen binding to hemoglobin, since IHP binds to fully liganded hemoglobin (Gray and Gibson, 1971; Tyuma *et al.*, 1971a). Equation 1 also does not apply to the effect of organic phosphates in media of low ionic strength, where binding to fully liganded hemoglobin and binding stoichiometries greater than 1:1 are observed (Gray and Gibson, 1971; Garby *et al.*, 1969; Chanutin and Hermann, 1969; Benesch *et al.*, 1971).

Evidence for the Model. Most of the evidence supporting the validity of the GCT model has been presented previously (Ogata and McConnell, 1971, 1972; Ogata *et al.*, 1972). Here, we present some additional experimental evidence supporting the model and discuss the applicability of the model to the experimental observations of other workers.

STRUCTURES OF HEMOGLOBIN MOLECULES BINDING ORGANIC PHOSPHATE. We have previously reported (Ogata and McConnell, 1971) that the hybrids $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$ have affinities for label I which are 2.5 and 10 times lower than that of ligand-free HbA. In developing the GCT model, we have

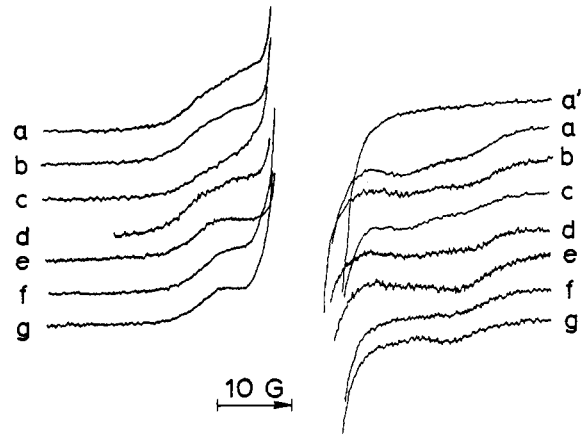

FIGURE 2: Extreme low- and high-field portions of the paramagnetic resonance spectra of label II bound to (a) ligand-free HbA, (b) $\alpha_2{}^{+CN}\beta_2$, (c) $\alpha_2\beta_2{}^{+CN}$, (d) ligand-free Hb Chesapeake, (3) ligand-free Hb Kempsey, (f) ligand-free β_4, and (g) fully liganded β_4. The spectrum labeled a′ is exhibited by label II in a solution of fully CO-liganded HbA. The fact that a′ shows no evidence of a resonance absorption in the high-field region of the spectrum indicates that II does not bind to fully liganded HbA. Conditions are described in the text.

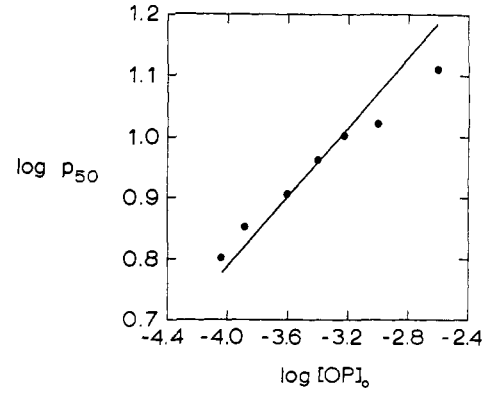

FIGURE 3: A comparison of the experimental (bis-Tris buffer, pH 7.3, 20°) and calculated (solid line) dependence of the oxygen affinity of hemoglobin (log p_{50}) on the total concentration of organic phosphates in solution (log $[OP]_0$). Here $K_{OP} = 1.5 \times 10^{-5}$ M and the total hemoglobin concentration is 6×10^{-5} M. Model parameters used are $K_R{}^{\alpha} = 0.75$, $K_R{}^{\beta} = 0.38$, $K_T{}^{\alpha} = 50$, and $K_T{}^{\beta} = 67$ Torr (oxygen), and $L = 3000$. The values of the oxygen affinities were obtained by simply multiplying the corresponding parameters for HbA given in Table I by 2.08. This was done to partially account for the effect of the higher temperature used here.

assumed that these differences are due solely to differences in the position of the $T \rightleftharpoons R$ equilibrium for each hemoglobin species. That is, we assume that these lowered affinities are due to decreases in the concentrations of phosphate-binding or T quaternary structures rather than to changes in the quaternary structures of phosphate-binding tetramers. This assumption implies that all HbA, $\alpha_2{}^{+CN}\beta_2$, and $\alpha_2\beta_2{}^{+CN}$ molecules which *do* bind I have *identical quaternary structures*.

The paramagnetic resonance spectra of spin labels attached to macromolecules such as hemoglobin are very sensitive to the structure of the macromolecule (McConnell and McFarland, 1970). As is the case for DPG, labels I and II doubtless bind at the central cavity of hemoglobin, along the dyad axis of symmetry (Benesch and Benesch, 1969). The structure of this central cavity is strongly dependent on the quaternary structure of hemoglobin and therefore, when bound to hemoglobin, these labels give resonance spectra which are believed to be sensitive to the quaternary structure of the tetramer. Figure 2 shows portions of the resonance spectra exhibited by II bound to various hemoglobin species. The marked similarity of the spectra due to II bound to ligand-free HbA, to $\alpha_2{}^{+CN}\beta_2$, and to $\alpha_2\beta_2{}^{+CN}$ demonstrates that the quaternary structures of these three hemoglobins are in fact very similar, if not identical, at least in the region of the binding site. Also, the resonance spectrum of II bound to ligand-free β_4 (hemoglobin H) is nearly identical with that of II bound to fully liganded β_4. Because it is known that β_4 has a ligand-free type quaternary structure and does not undergo a quaternary structural change on ligation (Perutz and Mazzarella, 1963), this similarity provides further evidence that label II is sensitive to the quaternary structures of hemoglobin molecules.

ORGANIC PHOSPHATES. Although the parameters of the GCT model have been deduced largely through studies of the binding of a triphosphate spin label to hemoglobin, these studies did not involve a determination of the effect of a large range of organic phosphate concentrations on the oxygen affinity of hemoglobin. Benesch *et al.* (1971) have studied the oxygen affinity of hemoglobin as a function of a large range of DPG concentrations. The experimental data obtained are compared with the dependence calculated by the GCT model in Figure 3. While the agreement between calculated and experimental results is reasonably good, it is not perfect. This may be due in part to the fact that the parameters used (see legend) do not apply to the experimental conditions used by Benesch *et al.*

The GCT model does not appear to be consistent with the results of Tyuma *et al.* (1971b), particularly at low oxygen saturation. That is, in the GCT model, the value of the Adair (1925) parameter, K_1, should be inversely proportional to $K_T{}^{\alpha}$ or $K_T{}^{\beta}$ (which differ by a factor of 1.35) and should be essentially independent of the organic phosphate concentration. Tyuma *et al.* find, however, that K_1 measured in the absence of organic phosphates is 6, 10, and 18 times greater than K_1 in the presence of high concentrations of ATP, DPG, and IHP, respectively. The experimental conditions used by these workers are somewhat different from those used in our experiments (primarily a difference in ionic strength) and this may account for some of the disagreement between these results and the predictions of the GCT model. It is possible that this discrepancy is due to an effect of phosphate on the oxygen affinities of the T isomer (*i.e.*, changes in $K_T{}^{\beta}$ or even $K_T{}^{\alpha}$). If this were the case, one might not expect the model to account for the binding data of Benesch *et al.* (1971) which it does reasonably well (Figure 3). The magnitude of an effect of this kind should depend strongly on the structure of the specific organic phosphate. However, the following observations argue against a large allosteric interaction between the binding of DPG and triphosphates and oxygen binding within the T isomer: (1) the resonance spectra of labels I and II bound to β_4 are insensitive to the presence of heme ligands; (2) the oxygen affinity of β_4 is the same in the absence and in the presence of DPG (Benesch and Benesch, 1969); and (3) the paramagnetic resonance spectra of label II bound to HbA and to $\alpha_2\beta_2{}^{+CN}$ are very similar (see Figure 2).

Properties of the GCT Model

As stated earlier, it is likely that within the restrictions which have been imposed, the GCT model parameters for HbA (given in Table I) constitute essentially a unique set.

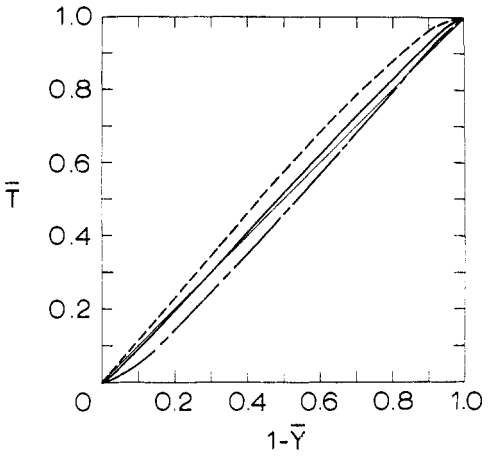

FIGURE 4: Calculated dependence of the fraction ($\bar{T}$) of hemoglobin molecules in the T isomeric state on the fraction of ligand-free hemes. Here, $K_{OP} = 2.5 \times 10^{-5}$ M, the total hemoglobin concentration is 3×10^{-4} M, and $[OP]_0$ is 0.0 (— -), 3×10^{-4} M (—), and 1.5×10^{-3} M (- - -).

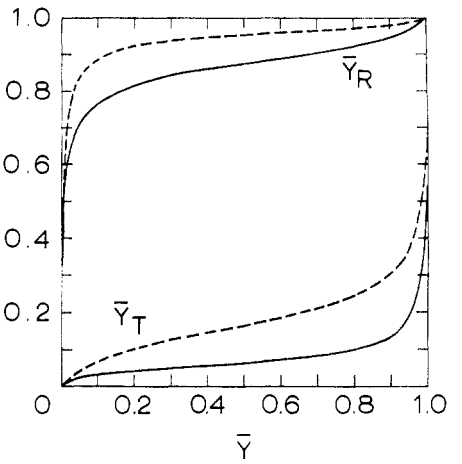

FIGURE 6: Dependence of the fractional saturation of the R isomer ($\bar{Y}_R$) and the T isomer ($\bar{Y}_T$) on the total fractional saturation of the hemes. The fractional saturation of each isomer is defined by $\bar{Y} = \bar{Y}_R\bar{R} + \bar{Y}_T\bar{T}$. Here $K_{OP} = 2.5 \times 10^{-5}$ M, the total hemoglobin concentration is 3×10^{-4} M, and $[OP]_0$ is 0.0 (—) and 1.5×10^{-3} M (- - -).

The uniqueness of this set of parameters warrants a discussion of its properties. These parameters lead to a number of predictions which can be used to test the validity of (1) the assumptions we have made in the selection of model parameters and (2) the GCT model itself.

Degree of T to R Conversion. Figure 4 shows the calculated dependence of $\bar{T}$, the fraction of tetramers in the T isomeric state, on $1 - \bar{Y}$, the fraction of ligand-free heme groups. This plot applies to HbA under the conditions of our experiments. Note that for certain concentrations of organic phosphate, this dependence is almost linear, *i.e.*, $\bar{T}$ is very nearly equal to $1 - \bar{Y}$ for all values of $1 - \bar{Y}$. However, at very high or very low concentrations of organic phosphate, $\bar{T}$ differs significantly from $1 - \bar{Y}$. Using triphosphate spin labels, $\bar{T}$ is calculated and measured to be very nearly equal to $1 - \bar{Y}$ (Ogata and McConnell, 1971, 1972). We therefore have no direct experimental evidence for the nonlinear dependence of $\bar{T}$ on $1 - \bar{Y}$ that is predicted for very high and very low concentrations of organic phosphate.

Figure 5 shows the calculated dependence of log (T/R) on the concentration of free organic phosphate for various partially oxygenated hemoglobin molecules. In the GCT model, the degree of conversion from T to R depends strongly on the concentration of organic phosphate for each of the partially oxygenated molecules. The three dioxygenated molecules pass through an equivalence point, where $T/R = 1$, at particular concentrations of organic phosphate. At low levels of organic phosphate, most hemoglobin molecules are in the R state after two oxygen molecules are bound, whereas at higher organic phosphate concentrations, the transition from T to R takes place primarily after three oxygen molecules are bound.

Figure 6 shows another illustrative plot calculated using the parameters of the GCT model. This figure shows the average degree of saturation of the R and T isomers of hemoglobin as a function of $\bar{Y}$, the fraction of saturated heme groups. Note that in general, the saturation of the T isomer is small and the saturation of the R isomer is large. Therefore, as required in a highly cooperative system, the predominant species in solution are tetraliganded and ligand-free hemoglobin.

Figure 7 gives the fractional concentrations of ligand-free, mono-, di-, tri-, and tetraoxygenated hemoglobin molecules as a function of $\bar{Y}$. These calculated results are given for phosphate-free and high-phosphate conditions. Although the relative amounts of each species are strongly dependent on the concentration of organic phosphates, the apparent cooperativity, as measured by Hill's constant, n, is essentially unchanged.

Order of Oxygenation. In discussing the mechanism of cooperative oxygen binding to hemoglobin, a question frequently asked is "What is the order of oxygenation?" On the basis of the parameters given in Table I, we expect that in the T isomer, the α subunits have a slightly greater oxygen affinity than the β subunits, and in the R isomer, the β subunits have an affinity for oxygen which is twice that of the α subunits. Consequently, the GCT model predicts that at low oxygen saturation levels, oxygenation of the α subunits is

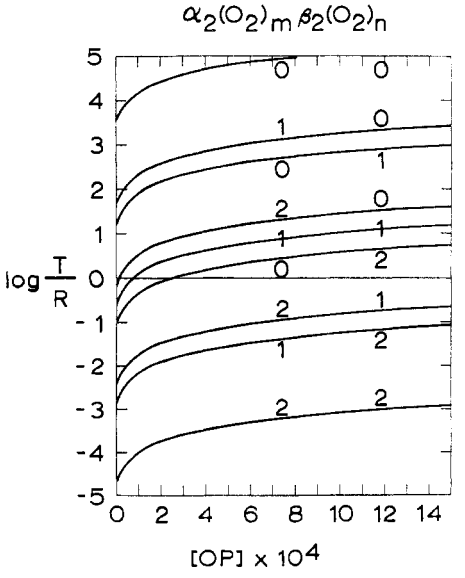

FIGURE 5: Dependence of T/R on the concentration of organic phosphate free in solution for HbA species at various stages of oxygenation. Here, T and R refer to the concentration of tetramers in the T and R isomeric states for each species. The parameters used are given in Table I. Here $K_{OP} = 2.5 \times 10^{-5}$ M.

preferred slightly, and at high oxygen saturation levels, there is a slightly greater tendency to oxygenate the β subunits. The former effect decreases and the latter increases slightly as the concentration of organic phosphate is decreased. However, both effects are quite small over a wide range of organic phosphate concentrations and therefore the α and β subunits are predicted to oxygenate with almost equal probabilities for most values of $\bar{Y}$ (Ogata and McConnell, 1972). This prediction is consistent with the nuclear resonance studies of Davis *et al.* (1971), who find essentially no difference in the ligand saturation levels of the α and β subunits as a function of total heme ligation. In recent work, Lindstrom and Ho (1972) have found that in the presence of large concentrations of DPG, the α chains of HbA oxygenate before the β chains. The preferential oxygenation of the α chains observed by Lindstrom and Ho is consistent with the preferential oxygenation estimated using the parameters in Table I.

The Bohr Effect. At pH values above 6.0, the binding of oxygen to hemoglobin is accompanied by a release of protons. This is known as the alkaline Bohr effect. Within the framework of the GCT model, the release of Bohr protons may depend on: (a) ligation-induced tertiary structural changes which take place independently of the quaternary structure of the hemoglobin molecule; (b) the T → R quaternary structural change alone; or (c) a combination of tertiary and quaternary structural changes.

If the release of Bohr protons is dependent on a above, then the fractional release of protons ($\bar{H}$) should be perfectly linear with the fractional heme ligation ($\bar{Y}$), providing that (1) the degree of ligation of the α and the β subunits is the same at all stages of oxygenation and (2) each subunit contributes the same number of Bohr protons. The dependence of $\bar{H}$ on $\bar{Y}$ expected for case b is given in Figure 4, where $\bar{H}$ is equivalent to $1 - \bar{T}$. For case c, the dependence of $\bar{H}$ on $\bar{Y}$ depends strongly on the specific assumptions made and can be either linear or nonlinear. For example, let us assume that half of the Bohr protons are released when the β subunits are oxygenated, independent of quaternary structure, and that the rest of the Bohr protons are released either by the T → R transition, or by oxygenation of the α subunits in the T state. In the GCT model, these assumptions lead to the following expression for the overall release of Bohr protons

$$\bar{H} = 0.5\bar{Y}_\beta + 0.5\bar{R} + 0.5(1 - \bar{R})\bar{Y}_\alpha{}^{T} \qquad (2)$$

Here $\bar{Y}_\beta$ is the fractional oxygen saturation of the β subunits [$\bar{Y} = (\bar{Y}_\alpha + \bar{Y}_\beta)/2$], $\bar{R}$ is the fraction of tetramers in the R state, and $\bar{Y}_\alpha{}^{T}$ is the fraction of oxygenated α subunits in the T state [$\bar{Y}_\alpha = (1 - \bar{R})\bar{Y}_\alpha{}^{T} + \bar{R}\bar{Y}_\alpha{}^{R}$]. Equation 2 leads to a plot of $\bar{H}$ *vs.* $\bar{Y}$ in which $\bar{H}$ is greater than $\bar{Y}$ for all values of $\bar{Y}$ and is greater than $\bar{Y}$ by ~8% at very low organic phosphate concentrations and by ~2% at high concentrations of organic phosphate.

The experimental evidence available suggests that $\bar{H}$ is nearly linear with $\bar{Y}$ (Antonini *et al.*, 1963, 1965b; Gray, 1970). On the basis of our calculations and the available experimental data, none of the above cases can be rigorously excluded. With further experiments, it may be possible to test case b. As shown in Figure 4, the dependence expected for case b is strongly dependent on the concentration of organic phosphate. However, the experimental results were obtained in 0.3 M NaCl. Benesch *et al.* (1969) have shown that chloride ions have an effect on hemoglobin which is analogous to that of organic phosphates. Therefore, the relatively high NaCl concentrations used in the above-mentioned measurements must correspond, in our calculations, to a moderate concentration of organic phosphate. As Figure 4 shows, at intermediate concentrations of organic phosphate, $1 - \bar{Y}(\bar{H})$ is very close to being linear with $\bar{Y}$. In fact, the small deviation of $\bar{H}$ from $\bar{Y}$ reported by Antonini *et al.* (1963) is strikingly similar to the deviation of $1 - \bar{T}$ from $\bar{Y}$ for intermediate concentrations of organic phosphate. (A plot of $1 - \bar{T}$ *vs.* $\bar{Y}$ can be seen by reference to the solid curve in Figure 4, when this figure is viewed upside down.) By measuring $\bar{H}$ at different concentrations of NaCl, it may be possible to test whether the release of Bohr protons is dependent only on quaternary structural changes.

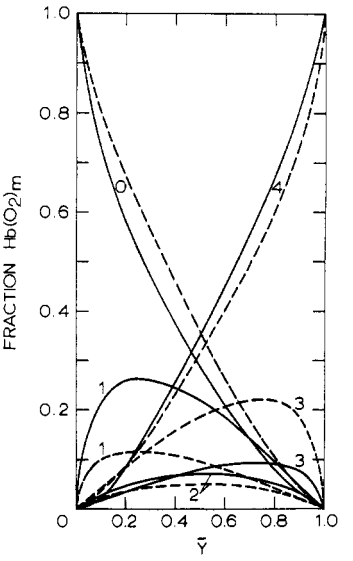

FIGURE 7: Calculated dependence of the fractional concentration of hemoglobin molecules having 0–4 oxygen molecules bound on the total heme saturation. Here the total hemoglobin concentration is 3×10^{-4} M and $[OP]_0$ is 0.0 (- - -) and 1.5×10^{-3} M (—). Hill's constant for the phosphate-free case is 2.9 and is 2.7 for the high-phosphate case.

Mutant Hemoglobins

In treating the α and β subunits of hemoglobin as nonequivalent, the GCT model offers the possibility of realistically accounting for the properties of mutant hemoglobins. A distinction between the α and β subunits is likely to be necessary in any model attempting to account for the properties of mutant hemoglobins because most mutations involve the replacement of a single amino acid residue in either the α or the β subunits, and because, in the tetramer, amino acid substitutions in one pair of subunits do not cause equivalent structural alterations in both the mutated and the normal subunits (Greer, 1971a,b). Indeed, in many cases these structural perturbations are confined primarily to the mutated subunits (Davis *et al.*, 1971; Greer, 1971c; Winterhalter and Wüthrich, 1972). Therefore, it is very likely that for a given hemoglobin mutant (excluding those with only one kind of subunit, such as HbH), the functional properties of the two mutated subunits (in each tetramer) will be more greatly perturbed than those of the normal subunits. Obviously, a model treating all subunits equally cannot accommodate changes of this type. The GCT model, on the other hand, may be capable of accounting for the properties of many mutant and modified hemoglobins. However, the determination of model parameters for each abnormal hemoglobin (by methods analogous to

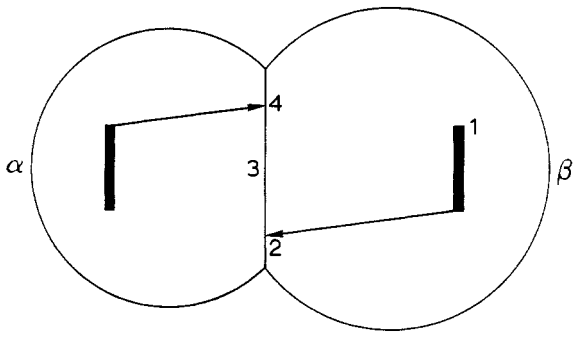

FIGURE 8: Schematic representation of the oxygenation-linked intersubunit interactions in hemoglobin. An arrow leaving a heme group indicates an oxygenation-induced conformation change (or strain) that breaks (or weakens) an α–β intersubunit bond in the T state. The two conformation changes (or strains) shown here are assumed to be noninteracting. Numbers 1, 2, 3, and 4 refer to different sites in the molecule where mutations have potentially different effects on the parameters of the generalized concerted transition model. See discussion of mutant hemoglobins.

those outlined earlier [Ogata and McConnell, 1971, 1972]) may prove difficult experimentally in some cases.

We have recently studied two mutant hemoglobins which have high oxygen affinities and bind oxygen with little cooperativity (Ogata and McConnell, 1972; Ogata *et al.*, 1972). One is an α-subunit mutant, Hb Chesapeake (α-92 Arg→Leu), and the other is a β-subunit mutant, Hb Kempsey (β-99 Asp→Asn). The model parameters used to fit the properties of Hb Chesapeake and Hb Kempsey are given in Table I. In selecting these parameters, we have tried to fit all experimental data as closely as possible while making a minimum number of physically plausible changes in the model parameters determined for HbA. Thus, for each mutant, we have varied only the allosteric constant, L, and the oxygen affinity of the mutated subunit in the T isomeric state (K_T^α or K_T^β). The values of L given are deduced from the experimentally determined affinities of each mutant for label I in the absence of heme ligands. The value of K_T^α (or K_T^β) is deduced from the binding of label I to these mutants measured as a function of heme ligation.

The parameters given in Table I for Hb Chesapeake and Hb Kempsey have the following functional significance. In the case of Hb Chesapeake, $K_R^\alpha = K_T^\alpha = K_R^\alpha$(HbA) signifies that (a) the α-heme groups have a high oxygen affinity in both the R and T states of the tetramer and (b) oxygenation of the α-heme groups does not affect the R $\leftrightarrows$ T equilibrium. In the case of Hb Kempsey, $K_T^\beta \sim K_R^\beta = K_R^\beta$(HbA) signifies that the β-heme groups have a high oxygen affinity in both the R and T states of the tetramer, and that oxygenation of the β-heme groups does not greatly affect the R $\leftrightarrows$ T equilibrium. These parameters account *quantitatively* for the preferential oxygenation of the α hemes in Hb Chesapeake (Davis *et al.*, 1971) and at least qualitatively for the preferential oxygenation of the β hemes in Hb Kempsey (Ho, 1972),[3] as observed by nuclear magnetic resonance. The parameters also account for triphosphate release on oxygenation, for the oxygen binding curves, for the effect of organic phosphates on oxygen binding, and for the observed affinity of ligand-free Hb Chesapeake and Hb Kempsey for triphosphate (Ogata and McConnell, 1971, 1972; Ogata *et al.*, 1972).

Limited changes in model parameters such as those described above can at best only be approximations. For example, it is likely that the amino acid substitution changes the affinity of the mutated subunit in the R state as well as in the T state. The isolated α-chain affinities suggest that this is the case for Hb Chesapeake (Bunn, 1970). Mutations may also affect the properties of the normal subunits. For Hb Chesapeake and Hb Kempsey, only changes in the model parameters describing the oxygen affinities of the normal subunits in the R state will greatly influence properties such as the binding of oxygen. This is due to the very low values of L found for these mutants. Some mutations may cause changes in the structures of the organic phosphate binding sites which are large enough to render invalid our method of determining L. As we have discussed previously, this possibility is considered unlikely for Hb Chesapeake and Hb Kempsey due to the similarity in the resonance spectra of spin label II bound to ligand-free HbA, Hb Chesapeake, and Hb Kempsey.

The possible effects of various types of mutations on model parameters can be seen by reference to Figure 8. In this figure, an arrow leaving one heme group in one subunit represents one or more subunit–subunit bonds (*e.g.*, salt links) that are broken on oxygenation of the heme group at the origin of the arrow. For simplicity, only two subunits are shown. According to the stereochemical model of Perutz (1970), binding of oxygen reduces the number of subunit–subunit bonds in the T isomer, and thus increases the thermodynamic probability of the T → R transition. *A priori*, we may conceive of at least four types of changes in model parameters due to mutations. For example, a mutation in the β-heme region (1 in Figure 8) might affect only K_R^β and/or K_T^β. A mutation in the contact region (2 in Figure 8) might affect K_R^α and/or K_R^β as well as L. A mutation in the contact region (3 in Figure 8) might affect L only. Finally, a mutation might in principle affect all the model parameters.

In general one can anticipate that mutations that affect the equilibrium constant, L, may also affect the heme-group oxygen affinities, since the affinities are closely coupled to the T → R transition. Even so, the model parameters for Hb Chesapeake and Hb Kempsey are remarkably simple in two respects. First, in each case mutations in the α–β contact region affect primarily the oxygen affinity of the mutated subunit. Second, the changes in the oxygen affinities of the mutated subunits bear a simple quantitative relation to the change in the allosteric equilibrium constant, L. The structural implications of each of these results are discussed below.

The molecular basis of the allosteric mechanism presumabiy involves ligand-induced conformation *changes* or *strains* that break or weaken subunit–subunit bonds. These ligand-induced changes do not interact with one another directly within the T state (Perutz, 1970), but only indirectly by increasing the thermodynamic probability of the T → R transition. (This is illustrated schematically in Figure 8 where the ligand-induced changes are indicated by arrows and do not overlap.) Therefore, it is entirely plausible that a mutation in the α–β contact region may affect the equilibrium constant L and only the affinities of either the α- or β-heme groups. This is evidently the case for Hb Chesapeake and Hb Kempsey. (By the same argument, it is possible that a β-chain mutation in an α–β contact region may affect primarily the oxygen affinity of the α heme. For example, a β-chain mutation at site 4 in Figure 8 could give rise to an increase in the α-chain heme affinity.)

The second interesting point concerning the parameters for Hb Chesapeake and Hb Kempsey is the quantitative relationship that exists between the effect of the mutation on L, and

[3] C. Ho, private communication.

n the α-heme affinity (Hb Chesapeake) and the β-heme ffinity (Hb Kempsey). As noted before (Ogata and Mconnell, 1972; Ogata *et al.*, 1972), the experimental values of for Hb Chesapeake and Kempsey (0.53, 0.17) are close to ıe experimental ratio of T/R for $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$ (0.67, 09) (Ogata and McConnell, 1971). These results indicate ıat in these mutants there is a quantitative or at least semiıantitative relation between the effect of the mutation on L ıd on the heme group affinities. This relation can be deribed as follows.

Assume as above that oxygenation of a given subunit in emoglobin in the T state breaks or weakens one or more of ıe intersubunit bonds that inhibit the T → R transition. A ıutation may also break or weaken the same intersubunit ɔnds. In this case the free energy of the T → R transition ay be decreased by just the same amount as the free energy oxygenation of the α (or β) heme group is decreased. This ıuality leads to the following relationship between L, $\bar{L}$, r, and $\bar{K}_T$, where the bars refer to the mutant parameters

$$\bar{L} = L(\bar{K}_T/K_T)^2 \quad (3)$$

or example, in the case of Hb Chesapeake the experimenlly determined $\bar{L}$ is 0.53 and the value of $L(\bar{K}_T^{\alpha}/K_T^{\alpha})^2$ is 0.67. milarly, for the case of Hb Kempsey, the experimentally termined $\bar{L}$ is 0.17, and the value of $L(\bar{K}_T^{\beta}/K_T^{\beta})^2$ is 0.36. quation 3 can be readily generalized to include the change L due to mutation that affects both oxygen affinities K_T^{α} ıd K_T^{β}.) It is possible that the functional and structural operties of other hemoglobin mutants have an equally mple interpretation.

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