# Extensions of the Allosteric Model for Hemoglobin. II. Consequences of Functional Nonequivalence of the $\alpha$ and $\beta$ Chains†

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ABSTRACT: Extensions of the allosteric model for hemoglobin (Edelstein, S. J. (1971), *Nature (London) 230,* 224) have been expanded to include distinct binding parameters for $\alpha$ and $\beta$ chains. Chain nonequivalence can appear in either of the two conformational states upon which the model is based, T and R, the low affinity and high affinity forms, respectively. The equilibrium between the two states is defined by $L$, where $L = (T)/(R)$. Simulated data generated by the model demonstrate that $\alpha$-$\beta$ nonequivalence will be detectable in equilibrium measurements during ligand binding only under discrete conditions. Nonequivalence in the T state can be detected only under conditions corresponding to high $L$ values, while nonequivalence in the R can be detected only under conditions corresponding to low $L$ values. This situation is related to the degree of saturation, $\bar{Y}'$, at which the midpoint of T → R transition occurs. At high $L$ values, which correspond to the physiological conditions for hemoglobin, $\bar{Y}'$ at the midpoint of the T-R transition approaches 0.75 and the fraction of molecules in the R state, $\bar{R}'$, is significantly below $\bar{Y}'$. Under these conditions equilibrium measurements for oxygen can be represented by preferentially binding to $\alpha$ chains in the T state. Measurements for $n$-butyl isocyanide can be represented by binding exclusively to $\beta$ chains in the T state for conditions ranging from very low to very high $L$ values. The nonequivalence is only apparent, however, at relatively high $L$ values. Although data on binding of $n$-butyl isocyanide to hemoglobin with an $^{19}F$ label at the $\beta$93 position were interpreted in terms of preferential binding to $\alpha$ chains, it appears to be more likely, on the basis of the analysis reported here, that these experiments monitored $\bar{R}$, not binding to $\beta$ chains, accounting for the discrepancy.

Many features of the cooperative binding of ligand by hemoglobin can be accommodated by the two-state model of Monod *et al.* (1965) when proper values of $L$, the equilibrium constant for the two states, are used, as demonstrated in the first paper of this series (Edelstein, 1971). The constant $L$ is defined by $L = (T)/(R)$ where T and R refer to states with low and high affinity for ligand, respectively. Appropriate values of $L$ can be deduced from values of $p_{1/2}$, the partial pressure of oxygen at half-saturation, by relating values of $p_{1/2}$ to the values for isolated chains, which appear to be functionally equivalent to the R state. On the basis of such linkage arguments a minimum value of $L = 3 \times 10^5$ was estimated for near physiological conditions (Edelstein, 1971). A similar value was obtained by estimating $L$ from the ratio of the tetramer-dimer dissociation constants for the T and R states (Thomas and Edelstein, 1972). Increases in pH, which result in increased affinity for oxygen (the Bohr effect) lower $L$, but cooperativity is largely unchanged since cooperativity (indicated by $n$, the Hill constant) is a bell-shaped function of log $L$ (Rubin and Changeux, 1966) and the range of values corresponding to the Bohr effect occurs at the top of the bell curve where $n$ is relatively invariant (Edelstein, 1971). Decreased cooperativity is observed for variants of hemoglobin with $L$ values either abnormally high or abnormally low.

The existence of a functionally significant T ↔ R equilibrium has now been established for many forms of hemoglobin, including (a) high affinity variants obtained by removal of C-terminal residues with carboxypeptidase (Bonaventura *et al.*, 1972, 1974; Hewitt and Gibson, 1973; Moffat *et al.*, 1973); (b) naturally occurring mutant forms of hemoglobin (Olson *et al.*, 1972; Olson and Gibson, 1972a; Ogawa and Shulman, 1972); and (c) valence hybrids (Ogawa and Shulman, 1971, 1972; Cassoly *et al.*, 1971; Cassoly and Gibson, 1972). Efforts at extending the two-state model to kinetic data have been attempted by Hopfield *et al.* (1971), with good results for CO binding data since chain nonequivalence does not appear to be a major factor for CO binding. While oxygen binding data were also apparently accommodated (Hopfield *et al.*, 1971) more recent experiments of Gibson (1973a) indicate a marked nonequivalence in oxygen binding behavior of the $\alpha$ and $\beta$ chains. Oxygen binds more rapidly to and dissociates more rapidly from $\beta$ chains than $\alpha$ chains. Nonequivalent rates of reaction for $\alpha$ and $\beta$ chains have also been observed for BIC,[1] CO and NO, particularly in the presence of organic phosphates (Olson and Gibson, 1971, 1972b, 1973; Gray and Gibson, 1971; Henry and Cassoly, 1973). The chain nonequivalence extends to equilibrium effects as revealed by nuclear magnetic resonance measurements on partially saturated solutions (Lindstrom *et al.*, 1971; Lindstrom and Ho, 1972) and oxygen binding experiments in conjunction with spin-label studies (Ogata and McConnell, 1971, 1972). Chain nonequivalence in the redox reaction has also been detected (MacQuarrie and Gibson, 1971; Edelstein and Gibson, 1974).

Since there is now extensive evidence of $\alpha$-$\beta$ nonequivalence, an examination of the consequences of distinct bind-

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[1] Abbreviations used are: BIC, $n$-butyl isocyanide; IHP, inositol hexaphosphate; DPG, diphosphoglycerate.

ing parameters for the $\alpha$ and $\beta$ chains is warranted. An extension of the allosteric model is presented here which incorporates $\alpha$-$\beta$ nonequivalence. The formulation is similar to an earlier effort by Ogata and McConnell (1971) but differs in that separate $\alpha$ and $\beta$ binding functions are derived so that the consequences of nonequivalence in either the T or R states can be more readily evaluated under different conditions. The formulation presented here is identical with the treatment used in the analysis of $\alpha$-$\beta$ nonequivalence in the redox reaction of hemoglobin (Edelstein and Gibson, 1974) but extended to include consideration of the state function and applications to ligand binding. Among the applications examined in this report are the apparent inconsistencies in relating nmr data which indicate preferential binding of BIC to $\beta$ chains (Lindstrom *et al.*, 1971) and preferential binding of $O_2$ to $\alpha$ chains (Lindstrom and Ho, 1972), even though combination velocities are similar in that both bind more rapidly to $\beta$ chains (Olson and Gibson, 1972b; Gibson, 1973a). Further inconsistencies arise from the data of Huestis and Raftery (1972) which has been interpreted in terms of preferential binding of BIC to $\alpha$ chains. However, a quantitative consideration of the two-state model with chain nonequivalence permits a reconciliation of these seemingly disparate observations and leads to a clearer understanding of the general consequences of nonequivalent binding sites.

## Theory

The two-state model (Monod *et al.*, 1965) as applied to hemoglobin leads to an expression for fractional saturation ($\bar{Y}$) of the form

$$\bar{Y} = \frac{\alpha(1 + \alpha)^3 + Lc\alpha(1 + c\alpha)^3}{(1 + \alpha)^4 + Lc\alpha(1 + c\alpha)^4} \quad (1)$$

where $\alpha$ is the concentration of ligand, (X), normalized to the intrinsic dissociation constant of the R state, $K_R$ ($\alpha = (X)/K_R$); $L$ is the equilibrium constant for the two states, T and R ($L = (T)/(R)$); and $c$ is the ratio of intrinsic dissociation constants for the T and R states ($c = K_R/K_T$). Cooperative behavior occurs for the condition $L > 1$ and $c < 1$. A distinct feature of the two-state model is that the formation of molecules in the R state, $\bar{R}$, is not necessarily equivalent to the fraction of sites occupied, $\bar{Y}$. The expression for $\bar{R}$ is given by

$$\bar{R} = \frac{(1 + \alpha)^4}{(1 + \alpha)^4 + L(1 + c\alpha)^4} \quad (2)$$

The distinct sites of the $\alpha$ and $\beta$ chains of hemoglobin can be taken into account by defining new binding parameters. Binding to $\alpha$ chains in the R state is defined by $a$, where $a = (X)/K_R^{\alpha}$ and binding to $\beta$ chains in the R state is defined by $b$, where $b = (X)/K_R^{\beta}$. The binding parameters for the T state are then given by $c_\alpha$ and $c_\beta$, where $c_\alpha = K_R^{\alpha}/K_T^{\alpha}$ and $c_\beta = K_R^{\beta}/K_T^{\beta}$. For each state binding of 0, 1, or 2 molecules of ligand to one class of chains can occur on molecules for which the other class of chains are occupied by 0, 1, or 2 ligand molecules. Therefore, for each state nine distinct species can be identified at varying degrees of ligand binding (Edelstein and Gibson, 1974). When all states are taken into account, the expression for fractional saturation of $\alpha$ chains, $\bar{Y}_\alpha$, takes the form

$$\bar{Y}_\alpha = \frac{a(1 + a)(1 + b)^2 + Lc_\alpha a(1 + c_\alpha a)(1 + c_\beta b)^2}{(1 + a)^2(1 + b)^2 + L(1 + c_\alpha a)^2(1 + c_\beta b)^2} \quad (3)$$

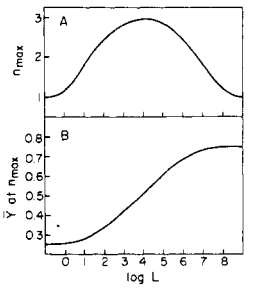


FIGURE 1: The maximum $n$ value, $n_{max}$ (A) and the value of $\bar{Y}$ at $n_{max}$ (B) as a function of the logarithm of $L$. Data compiled with the simple two-state model, with no chain nonequivalence (eq 1) using a value of $c = 0.01$.

and the expression for fractional saturation of $\beta$ chains, $\bar{Y}_\beta$, takes the form

$$\bar{Y}_\beta = \frac{b(1 + b)(1 + a)^2 + Lc_\beta b(1 + c_\beta b)(1 + c_\alpha a)^2}{(1 + a)^2(1 + b)^2 + L(1 + c_\alpha a)^2(1 + c_\beta b)^2} \quad (4)$$

The expression for binding to both chains, $\bar{Y}'$, is given by

$$\bar{Y}' = \frac{1}{2}(\bar{Y}_\alpha + \bar{Y}_\beta) \quad (5)$$

The ligand concentration normalized to R state affinities is now given by $\bar{\alpha}$, where $\bar{\alpha} = (a + b)/2$. The state function in the presence of nonequivalent $\alpha$ and $\beta$ chains takes the form

$$\bar{R}' = \frac{(1 + a)^2(1 + b)^2}{(1 + a)^2(1 + b)^2 + L(1 + a)^2(1 + b)^2} \quad (6)$$

## Applications and Discussion

*Conditions for Appearance of Chain Nonequivalence.* In equilibrium measurements, probes that can distinguish binding of ligand to the $\alpha$ or $\beta$ chains will register preferential binding to one of the chains in either the T or R state only if the appropriate state is present in varying degrees of saturation. For example, normal conditions for ligand binding to hemoglobin correspond to $L$ values in the range $10^5$-$10^6$ (Edelstein, 1971). The transition from a predominance of T state to a predominance of R state will have its midpoint at a value of $\bar{Y} = -\log L/4 \log c$. This relationship arises from the fact that at the transition point, $[T_x] = [R_x]$, where the subscript $x$ gives the number of ligand molecules bound. Since $L_x = [T_x]/[R_x] = Lc^x = 1$ at the transition point, $x = -\log L/\log c$ and the fractional saturation ($\bar{Y}$) is given by $x/4$. The T $\rightarrow$ R transition for hemoglobin, with $L \sim 5 \times 10^5$ and $c \sim 10^{-2}$ will occur at a value of $\bar{Y} \sim 0.7$, a relatively high degree of saturation. The midpoint of the transition is related to the point of maximum cooperativity in the binding curve which can vary from $\bar{Y} = 0.25$ for very low values of $L$ to $\bar{Y} = 0.75$ for very high values of $L$ in the range of the bell curve of cooperativity *vs.* $\log L$ (Figure 1). Therefore ligand binding to hemoglobin with $L \sim 5 \times 10^5$ proceeds with a predominance of T forms in varying degrees of ligand binding with up to three molecules of ligand bound. For molecules with three molecules of ligand bound the R state is roughly equal to the T state in energy. The binding sequence can thus be described to a good first approximation as $T_0 \rightarrow T_1 \rightarrow T_2 \rightarrow T_3 \rightarrow R_3 \rightarrow R_4$ where subscripts refer to molecules of ligand bound.

Since the T state is present in multiple degrees of saturation, the possibility exists for expression of $\alpha$-$\beta$ differences in binding affinity for ligands in the T state; in contrast $\alpha$-$\beta$

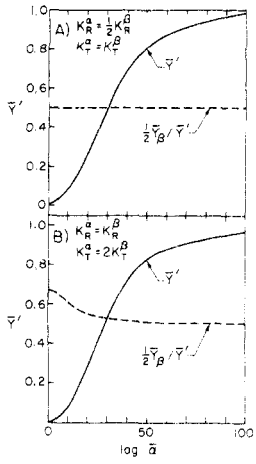

FIGURE 2: Simulated ligand binding curves of $\bar{Y}'$ *vs.* $\bar{\alpha}$ at a high $L$ value. (A) Chain nonequivalence in the R state; (B) chain nonequivalence in the T state. Data compiled with eq 5 using the values $L = 5 \times 10^5$, $c_\alpha = 0.005$, $c_\beta = 0.01$, and $K_R{}^\alpha = K_R{}^\beta/2$ for A and $K_R{}^\alpha = K_R{}^\beta$ for B.

differences in the R state cannot be expressed since fully saturated molecules predominate in which differences in affinities of the chains are obscured. This point is illustrated with sample binding curves in Figures 2 and 3. With a high value of $L$ ($5 \times 10^5$) only nonequivalence in T is revealed. A twofold difference in the affinity of the $\alpha$ and $\beta$ chains of the R state for ligand gives no measurable divergence from 50% in the saturation to either chain compared to the total (Figure 2A). However, a twofold difference in the affinity of the $\alpha$ and $\beta$ chains of the T state for ligand ($K_T{}^\beta < K_T{}^\alpha$) gives a significant divergence from 50% in the degree of saturation of the $\beta$ chains (Figure 2B). At a low $L$ value (10) the opposite behavior is seen. Expression of chain differences occurs only in the R state (Figure 3).

*Chain Nonequivalence in Oxygen Binding.* Preferential binding of oxygen to $\alpha$ chains of hemoglobin is observed in the presence of IHP or DPG (Lindstrom and Ho, 1972). Since IHP binds preferentially to the T state of hemoglobin (Benesch *et al.*, 1971), the value of $L$ is increased and a value of $L = 5 \times 10^6$ has been estimated for hemoglobin in the presence of IHP (Edelstein and Gibson, 1974) from linkage arguments based on the effects of IHP (Bunn and Guidotti, 1972; Kilmartin, 1973). The effect of IHP in revealing chain nonequivalence could have two origins: (1) by increasing the value of $L$, addition of IHP could expose a relatively slight chain nonequivalence present in the T state, but not revealed at lower $L$ values; (2) IHP binding could give rise to a specific chain nonequivalence in the T state. As will be demonstrated for the BIC binding properties of hemoglobin (see below) the first explanation appears to be the dominant factor. However, for oxygen binding, either explanation could account for the observed behavior and the preferential binding to $\alpha$ chains can be represented in simulated binding curves with $K_T{}^\alpha < K_T{}^\beta$ and $K_R{}^\alpha = K_R{}^\beta$, as seen in Figure 4a. In this simulation all of the binding parameters have their usual values with the exception of $K_T{}^\beta$ which is increased fivefold. In accord with the observations of Lindstrom and Ho (1972) at 40% saturation or below, the degree of occupancy of $\alpha$ chains is approximately twice that of $\beta$ chains.

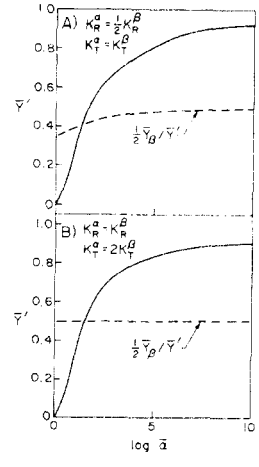

FIGURE 3: Simulated ligand binding curves of $\bar{Y}'$ *vs.* $\alpha$ at a low $L$ value. (A) Chain nonequivalence in the R state; (B) chain nonequivalence in the T state. Data compiled with $L = 10$; other details as in Figure 2.

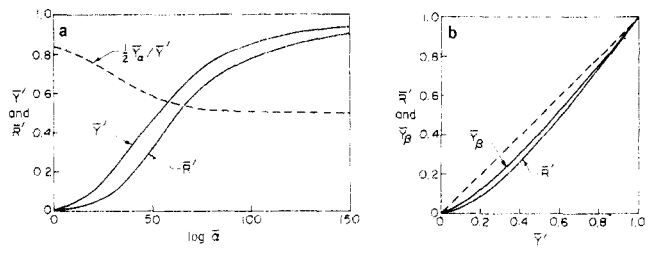

FIGURE 4: (A) Simulated oxygen binding curve. Data compiled with eq 5 using values of $L = 5 \times 10^6$, $K_R{}^\alpha = K_R{}^\beta$ and $c_\alpha = 10^{-2}$, $c_\beta = 2 \times 10^{-3}$. (B) Cross plot of $\bar{Y}_\beta$ and $\bar{R}'$ *vs.* $\bar{Y}'$.

Figure 4a also includes the state function, $\bar{R}'$, for these conditions. This behavior of the state function is a corollary of the properties of the two-state model described in Figure 1. At low $L$ values, the maximum cooperativity occurs at $\bar{Y} < 0.5$, and under these conditions the value of the state function is always greater than the saturation function. At $L \sim c^{-2}$, maximum cooperativity occurs at $\bar{Y} \sim 0.5$ and the state and saturation functions have effectively identical values. At values of $L > c^{-2}$, the state function will have lower values than the saturation function, $\bar{Y}'$, as seen in Figure 4a. Thus the capacity for chain nonequivalence in either the T or R state to be expressed, which depends on the $L$ value as described above, is closely correlated with the extent to which the state function is unequal to the saturation function. At high $L$ values, the expression of chain nonequivalence in the T state is a consequence of the fact that $\bar{R}' < \bar{Y}'$ throughout the binding curve indicating the presence of molecules in the T state in varying degrees of saturation. The relatedness of the state function and chain nonequivalence is demonstrated in Figure 4b in which the data of Figure 4a is replotted to give $\bar{Y}_\beta$ and $\bar{R}'$ *vs.* $\bar{Y}'$. The curve indicates that $\bar{R}'$ and $\bar{Y}_\beta$ both deviate from $\bar{Y}'$ (since nondeviation is indicated by the dashed line) and furthermore, $\bar{R}'$ and $\bar{Y}_\beta$ vary similarly as a function of $\bar{Y}'$. Thus, binding to $\beta$ chains occurs predominantly in the R state, so that appearance of the R state and disappearance of free $\beta$ chain sites follow a similar course. Hence, extreme caution must be given to interpretations of preferential binding to $\alpha$ or $\beta$ chains based on probes which may be a monitor not of saturation of a particular chain but of the T $\rightarrow$ R transition. This difficulty is illustrated in the analysis of data for BIC binding to hemoglobin.

*Chain Nonequivalence in BIC Binding.* In contrast to the results with oxygen, a preferential binding of BIC to the $\beta$ chains of hemoglobin has been observed in spectral and nmr measurements (Olson and Gibson, 1971; Lindstrom *et al.*,

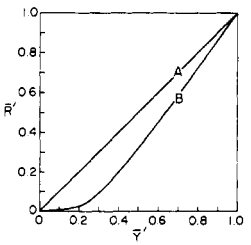


FIGURE 5: Cross plot of $\bar{R}'$ and $\bar{Y}'$ for data corresponding to BIC binding to hemoglobin. Data calculated with eq 5 using the values of $K_R^{\alpha} = K_T^{\alpha}$, $c_{\alpha} = 10^{-4}$, and $c_{\beta} = 0.04$, and $L = 10^3$ for A, $L = 5 \times 10^5$ for B.

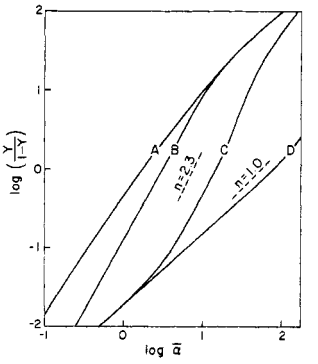


FIGURE 6: Hill plots corresponding to the binding of BIC to hemoglobin. Parameters as in Figure 5 with $L$ values varied for the curves using 64 for A, $4 \times 10^3$ for B, $6 \times 10^4$ for C, and $8 \times 10^6$ for D.

1971). However, Huestis and Raftery (1972) reported preferential BIC binding to $\alpha$ chains in a derivative of hemoglobin with $^{19}F$ trifluoroacetone at the $\beta$93 SH. The TFA-Hb shows a change in the $^{19}F$ nmr spectrum upon addition of ligands. At pH 6.75 in 0.1 M NaCl the nmr changes are linear with ligand binding, but in the presence of DPG the nmr change lags behind the binding function. This nonlinearity was interpreted by Huestis and Raftery (1972) as preferential binding of BIC to $\alpha$ chains, based on the assumption that the TFA signal reflects occupancy of the $\beta$ chains. However, in the *p*-mercuribenzoate reaction at the same position, Gibson (1973b) has demonstrated that the reaction rate does not monitor ligand binding, but rather appears to follow the T $\rightarrow$ R transition. Therefore, the possibility must be considered that the TFA signal is an indicator of the state function, $\bar{R}$, not binding of ligands to $\beta$ chains. This view is supported by simulated ligand binding curves generated with binding only to $\beta$ chains in the T state. Parameters were adjusted to give a maximum Hill constant of $n = 2.3$ (Olson and Gibson, 1972b). With an $L$ value corresponding to the presence of DPG the curve for $\bar{R}'$ *vs.* $\bar{Y}'$ (Figure 5) is markedly nonlinear and is in close accord with the data obtained by Huestis and Raftery (1972). In addition, lowering the value of $L$ to correspond to the properties of hemoglobin in the absence of DPG yields a linear dependence of $\bar{R}'$ on $\bar{Y}'$ (Figure 5), although under these conditions kinetic effects of chain nonequivalence are retained (Olson and Gibson, 1973). Thus, even though the trifluoroacetone group resides on the $\beta$ chains and might be expected to respond to ligand binding to the $\beta$ heme site, it appears to respond to conformational state as in the case of other indicators of this site (Gibson, 1973b).

As already noted, the appearance of $\alpha$-$\beta$ chain nonequivalence in the presence of IHP may be the unmasking of intrinsic differences in $K_T^{\alpha}$ and $K_T^{\beta}$ that are only revealed at high $L$ values. The data for BIC binding appears to fall in this category. Although binding under low $L$ conditions (high pH, low salt) does not show any indications of chain nonequivalence (Olson and Gibson, 1973), the BIC binding data can be represented as a two-state model with no effective binding to $\alpha$ chains in the T state (Figure 6A). Although binding occurs almost exclusively to $\beta$ chains in the T state, this nonequivalence is obscured at low $L$ values by the fact that the transition to the R state occurs with a midpoint near $\bar{Y}' = 0.25$. Thus, after one molecule of BIC is bound (to $\beta$ chains in the T state) a cooperative transition to the R state occurs (giving rise to a Hill constant of 2.3) and the remainder of the course of binding is dominated by effects of the R state. Only at high $L$ values, corresponding to data in the presence of IHP, is cooperativity abolished (Figure 6D) since binding is largely restricted to $\beta$ chains in the T state.

## General Conclusions

The analysis presented here demonstrates that when the two-state model is extended to $\alpha$-$\beta$ nonequivalence, the appearance of functional differences in the affinity of chains depends on the $L$ value. At high $L$ values only differences in the affinity of chains for ligands within the T state are detectable in equilibrium binding measurements. Under these conditions the state function will always be less than the binding function and incorrect identification of a state function as either $\bar{Y}_{\alpha}$ or $\bar{Y}_{\beta}$ can lead to an incorrect assignment of preferential binding to one of the chains. This possibility appears to account for the identification of preferential BIC binding to $\alpha$ chains when all other criteria indicated $\beta$ chains. In fact, the simulations presented here indicate that the behavior of hemoglobin in BIC binding can be represented with no binding to $\alpha$ chains in the T state under any conditions, although the $\alpha$-$\beta$ nonequivalence is revealed only under conditions corresponding to a high $L$ value. At low values the T $\rightarrow$ R transition at early stages of ligand binding obscures the inability of $\alpha$ chains in the T state to bind ligand. This hypothesis of no binding to $\alpha$ chains in the T state is in agreement with recent experiments by M. J. MacDonald, B. M. Hoffman, and Q. H. Gibson (in preparation) with $\alpha^{Fe^{2+}}\beta^{Mn^{2+}}$ hybrids that appear to be locked in the T state (see Hoffman *et al.*, 1974) and bind BIC extremely slowly. In contrast the opposite type, $\alpha^{Mn^{2+}}\beta^{Fe^{2+}}$ binds BIC with the rate characteristic of the rapid component ($\beta$ chains) in normal hemoglobin. A twofold difference in affinity for BIC of the $\alpha$ and $\beta$ chains in the R state has also been reported (Olson and Gibson, 1971), but it contributes very little to $\alpha$-$\beta$ differences observed in equilibrium measurements, particularly in the presence of organic phosphates.

The analysis presented here also serves to illustrate the difficulties in attempting to deduce ligand binding preference for the $\alpha$ and $\beta$ chains from inspection of models derived from X-ray studies. Perutz (1970) predicted preferential binding of ligands to $\alpha$ chains in deoxyhemoglobin, because of hindrance at the entrance to the heme pocket of the $\beta$ chains caused by the $\gamma$-methyl group of valine-$\beta$67. While this situation has been confirmed for oxygen (Lindstrom and Ho, 1972), the opposite situation prevails for BIC (Olson and Gibson, 1971; Lindstrom *et al.*, 1971; Olson and Gibson, 1972b). Since BIC is much larger, it might reasonably have been expected to amplify discrimination by the $\beta$ chains, although this does not appear to be the case. Therefore, factors not readily apparent from the structural models must be playing a dominant role in properties

of the T state. Similarly, deductions based on the ease of reduction of the $\alpha$ and $\beta$ chains of methemoglobin were advanced by Perutz (1970) but these are likely to apply to the R state only and may bear little relation to properties of the T state. The issues of chain nonequivalence and their implications for various models of cooperative ligand binding by hemoglobin are discussed more fully in a recent review (Edelstein, 1975).

Acknowledgments

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