5-18.
Weber, G., Stubbs, M., and Morris, H. P. (1971), *Cancer Res. 31,* 2177-2183.
Williamson, D. H., Lund, P., and Krebs, H. A. (1967), *Biochem. J. 103,* 514-527.
Williamson, D. H., Mellanby, J., and Krebs, H. A. (1962), *Biochem. J. 82,* 90-96.
Wilson, D. F., Owen, C., Mela, L., and Weiner, L. (1973), *Biochem. Biophys. Res. Commun. 53,* 326-334.
Wilson, D. F., Stubbs, M., Veech, R. L., Erecińska, M., and Krebs, H. A. (1974), *Biochem. J. 140,* 57-64.
Wollenberger, A., Ristau, O., and Schoffa, G. (1960), *Pfluegers Arch. Gesamte Physiol. Menschen Tiere 270,* 399-406.

Mechanism of Cooperative Oxygen Binding to Hemoglobin: Kinetic Aspects†

Teresa Ree Chay* and David Brillhart

ABSTRACT: The theory of oxygen binding to hemoglobin formulated by T. R. Chay and D. Brillhart ((1974), *Biochemistry,* to be published) has been used to study the kinetics of ligand binding to hemoglobin. We find that the theory is in good agreement with Gibson's recent stopped-flow experiment on human hemoglobin at pH 7. Our model predicts that a large amount of DPG comes off from non-stripped hemoglobin at about the fourth oxygenation stage and that oxygen goes to the β chain initially, but the β chain does not retain oxygen for a long period of time.

About 4 years ago, Gibson (1970) measured the kinetics of the oxygenation process of hemoglobin (Hb)[1] by stopped-flow methods. Interpretations of these data have been made with the kinetic versions of the Adair equation by using eight Adair-rate parameters (Gibson, 1970) and of the allosteric transition model of Monod *et al.* (1965) using five parameters (Hopfield *et al.,* 1971). The basic assumption behind these interpretations is that the α and β chains in Hb are equivalent. A recent experiment by Gibson (1973), however, indicates very significant differences between the α and β chains. In particular, Gibson has found experimentally that, in the presence of phosphates, the association and dissociation rate constants of oxygen to the β chain are larger than those of the α chain in such a way that in equilibrium, the ligand affinity of the α chain is larger than that of the β chain. This means that the kinetic equivalences of the Adair equation (Gibson, 1970) and of the allosteric transition model (Hopfield *et al.,* 1971) are quite unsuited to represent the kinetics of the oxygen-Hb reaction (Gibson, 1973). A modification of the Adair equation, which accounts for the nonequivalent α and β chains, has been proposed by Gibson and his coworkers (Olson and Gibson, 1972,1973; Tan *et al.,* 1973; Cole and Gibson, 1973). These workers showed that in general 32 kinetic parameters are needed to describe the reaction. Since there are too many parameters, the modified Adair equation is very impractical. A simple theory is needed to study the kinetic mechanism of the oxygen binding process of Hb. The model of Chay and Brillhart (1974) has been shown to be capable of fitting the equilibrium oxygenation curves. In this paper, we use this model to see whether or not the kinetic data can be explained by our model. We also use the model to study the time courses of ligand binding to Hb, of the DPG effect, and of the inhomogeneity of the α and β chains.

Kinetic Equation for the Model

Very recently, we have developed a model (Chay and Brillhart, 1974) of the oxygen binding process of Hb on the basis that (i) in the absence of phosphate, the binding of oxygen to Hb follows the sequential theory of Koshland *et al.* (1966); (ii) 1 mol of organic phosphate (OP) can combine with 1 mole of Hb in any oxygenation stage in a reversible manner (Tyuma *et al.,* 1973); and (iii) in the presence of OP, the oxygenation of Hb follows the scheme presented by Figure 1. In Figure 1, the upper portion of the scheme shows the reaction between oxygen and OP-Hb complex, and the lower portion of the scheme shows the reaction between oxygen and OP-free Hb (*i.e.,* stripped Hb).

The equilibrium parameters associated with the lower portion of the scheme (*i.e.,* OP-free Hb) are Q and Z, which are defined respectively as (Chay and Ho, 1973)

$$Q = K_L K_t (K_{AA}/K_{BB})^{3/2} \quad (1)$$

and

$$Z = K_{AB}/(K_{AA}K_{BB})^{1/2} \quad (2)$$

where Q is a measure of the relative stability between the conformation A (unliganded subunit conformation) and B (liganded subunit conformation), and Z is a measure for K_{AB}, the strength of the subunit interaction between A and B, relative to K_{AA} and K_{BB}, which are defined as the strengths of the interactions between AA and BB conformations, respectively. In eq 1, $K_L = (BL)/(B)(L)$ is the ligand

† From the Department of Biophysics and Microbiology, University of Pittsburgh, Pittsburgh, Pennsylvania 15260. *Received May 24, 1974.* This research was supported by the Health Research and Services Foundation Grant P-67. T.R.C. is a recipient of the Career Development Award from the National Institutes of Health (5 K04 GM 70015-03).

[1] Abbreviations used are: Hb, hemoglobin; OP, organic phosphate; DPG, 2,3-diphosphoglycerate.

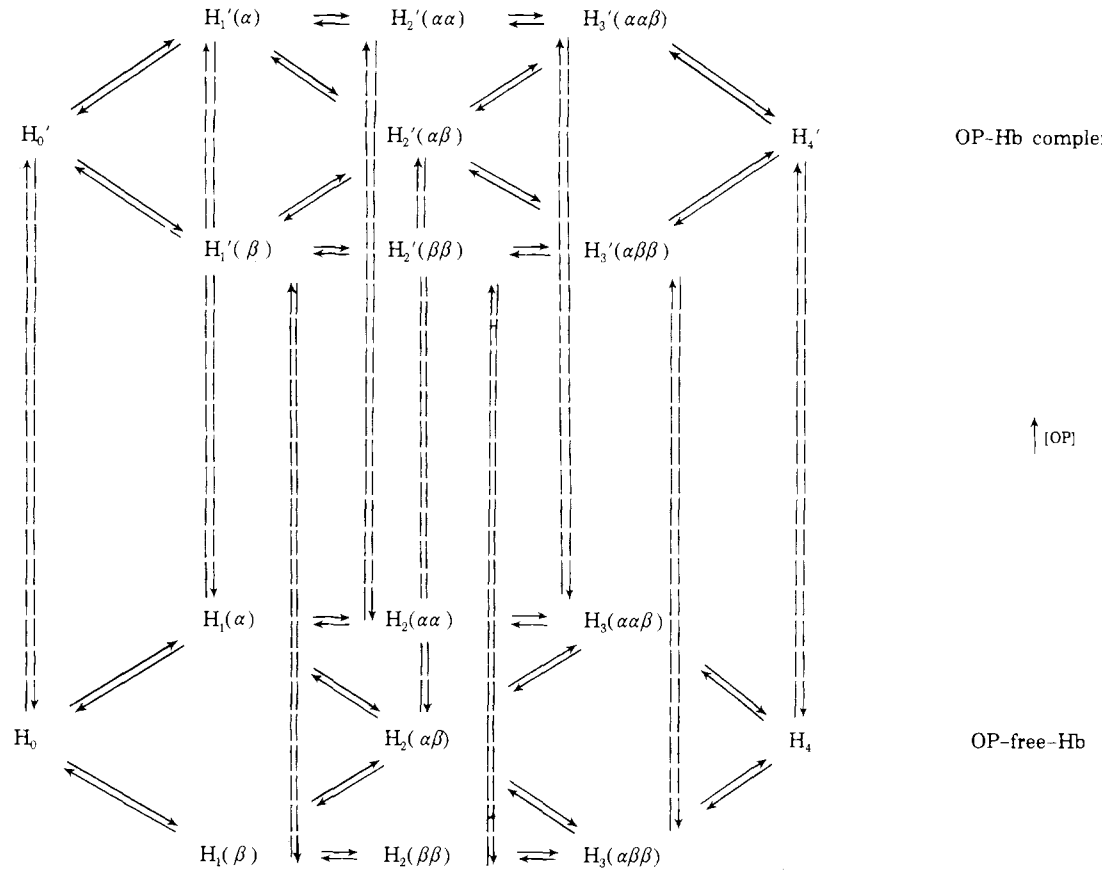

FIGURE 1: Generalized mass action scheme for the reaction of stripped (see the lower portion) and nonstripped hemoglobin. The association and dissociation rate constants for this scheme are given in Table I.

binding constant, and $K_t = (B)/(A)$ is the equilibrium constant for changing the A conformation to the B conformation. The equilibrium parameters associated with the upper portion of Figure 1 are Q_α and Q_β, which measure the ligand affinities of the α and β chains in OP-Hb complex, respectively. The strength of the subunit interactions of OP-Hb complex, Z, is assumed to take the same value as that of OP-free Hb for simplicity.

The association and dissociation rate constants for successive oxygenation steps in Figure 1 are given in columns 2-4 of Table I, and the binding association and dissociation rates of OP are given in the last three columns of the same table. The rate constants for oxygenations are assigned according to the symmetric scheme proposed by Hill and his coworkers (Hill and Chen, 1971; Paul and Hill, 1972) in connection with the study on K^+ transport across the nerve membrane. In Table I, the kinetic parameter k is the time scaling factor for stripped Hb, and the other kinetic parameters, k_α and k_β, are the parameters relating to the ligand association rate constants of the α and β chains, respectively. The parameter γ is the OP dissociation rate constant of deoxy-Hb.

The time behavior of 18 species shown in Figure 1 may be calculated by setting up 18 coupled kinetic equations; *e.g.*

$$\frac{dH_0'}{dt} = -[2Q_\alpha Z^{3/2}\lambda(t)k_\alpha + 2Q_\beta Z^{3/2}\lambda(t)k_\beta + \gamma]H_0' + k_{op}[OP]\gamma H_0 + Z^{-3/2}k_\alpha H_1'(\alpha) + Z^{-3/2}k_\beta H_1'(\beta). \quad (3)$$

Here, $\lambda(t)$ is the free oxygen concentration at time t and is related to $\lambda(0)$, the total (initial) concentration of oxygen, and Hb(0), the total concentration of hemoglobin, by

$$\lambda(t) = \lambda(0) - 4Y(t)Hb(0) \quad (4)$$

where $Y(t)$ is the fractional oxygen saturation at time t

$$Y(t) = \frac{1}{4}\sum_{i=1}^{4} i[H_i' + H_i] \quad (5)$$

The 18 differential equations are changed to the difference form and are solved numerically with the initial conditions

$$H_0' = k_{op}[OP]/(1 + k_{op}[OP]) \quad (6a)$$

$$H_0 = 1/(1 + k_{op}[OP]) \quad (6b)$$

and given Hb(0) and $\lambda(0)$.

Results and Discussion

In Figure 2, we compare the theoretical and experimental oxygenation reaction of stripped human deoxy-Hb (53 μM) with 62, 31, 15.5, and 7.8 μM O_2. The data points are obtained from the eight kinetic Adair-rate parameters given by Gibson (1970), and the solid curves are calculated from the theory with the time scaling factor k, which is found to be 28.5 sec^{-1}. The equilibrium parameters used are $Q = 0.325$ and $Z = 0.573$, which have been obtained previously by Chay and Brillhart (1974). We note that there is an excellent agreement between the theory and experiment with no adjustable parameter.

Figure 3 shows the reaction of nonstripped deoxy-Hb (41.5 μM) with 124, 62, 31, and 15.5 μM O_2. The data points are obtained from the eight kinetic Adair-rate pa-

TABLE I: The Association and Dissociation Constants for the Hemoglobin–Oxygen Reaction Shown in Figure 1.[a]

	$H_0' \rightleftarrows H_1'(\alpha)$	$H_1'(\alpha) \rightleftarrows H_2'(\alpha\alpha)$	$H_0' \rightleftarrows H_0$	$H_1'(\alpha) \rightleftarrows H_1(\alpha)$	$H_2'(\alpha\alpha) \rightleftarrows H_2(\alpha\alpha)$
	$2Q_\alpha Z^{1/2}\lambda(t)k_\alpha$	$Q_\alpha Z^{1/2}\lambda(t)k_\alpha$	γ	$(Q/Q_\alpha)^{1/2}\gamma$	$(Q/Q_\alpha)\gamma$
$\uparrow\downarrow$	$Z^{-1/2}k_\alpha$	$2Z^{-1/2}k_\alpha$	$k_{op}[OP]\gamma$	$(Q_\alpha/Q)^{1/2}k_{op}[OP]\gamma$	$(Q_\alpha/Q)k_{op}[OP]\gamma$
	$H_0' \rightleftarrows H_1'(\beta)$	$H_1'(\alpha) \rightleftarrows H_2'(\alpha\beta)$	$H_4' \rightleftarrows H_4$	$H_1'(\beta) \rightleftarrows H_1(\beta)$	$H_2'(\alpha\beta) \rightleftarrows H_2(\alpha\beta)$
	$2Q_\beta Z^{1/2}\lambda(t)k_\beta$	$Q_\beta Z^{-1/2}\lambda(t)k_\beta$	$(Q/Q_\alpha)(Q/Q_\beta)\gamma$	$(Q/Q_\beta)^{1/2}\gamma$	$(Q/Q_\alpha)^{1/2}(Q/Q_\beta)^{1/2}\gamma$
$\uparrow\downarrow$	$Z^{-1/2}k_\beta$	$2Z^{1/2}k_\alpha$	$(Q_\alpha/Q)(Q_\beta/Q)k_{op}[OP]\gamma$	$(Q_\beta/Q)^{1/2}k_{op}[OP]\gamma$	$(Q_\alpha/Q)^{1/2}(Q_\beta/Q)^{1/2}k_{op}[OP]\gamma$
	$H_3'(\alpha\alpha\beta) \rightleftarrows H_4$	$H_2'(\alpha\beta) \rightleftarrows H_3'(\alpha\beta)$		$H_3'(\alpha\alpha\beta) \rightleftarrows H_3(\alpha\alpha\beta)$	$H_2'(\beta\beta) \rightleftarrows H_2(\beta\beta)$
	$Q_\beta Z^{-1/2}\lambda(t)k_\beta$	$Q_\alpha Z^{1/2}\lambda(t)k_\alpha$		$(Q/Q_\alpha)(Q/Q_\beta)^{1/2}\gamma$	$(Q/Q_\beta)\gamma$
$\uparrow\downarrow$	$2Z^{1/2}k_\beta$	$Z^{-1/2}k_\alpha$		$(Q_\alpha/Q)(Q_\beta/Q)^{1/2}k_{op}[OP]\gamma$	$(Q_\beta/Q)k_{op}[OP]\gamma$
	$H_3'(\alpha\beta\beta) \rightleftarrows H_4$	$H_2'(\beta) \rightleftarrows H_2'(\beta\beta)$		$H_3'(\alpha\beta\beta) \rightleftarrows H_3(\alpha\beta\beta)$	
	$Q_\alpha Z^{-1/2}\lambda(t)k_\alpha$	$Q_\beta Z^{1/2}\lambda(t)k_\beta$		$(Q/Q_\alpha)^{1/2}(Q/Q_\beta)\gamma$	
$\uparrow\downarrow$	$2Z^{1/2}k_\alpha$	$2Z^{-1/2}k_\beta$		$(Q_\alpha/Q)^{1/2}(Q/Q_\beta)k_{op}[OP]\gamma$	

[a] The association and dissociation constants of oxygen for OP– free Hb are the same as those for OP–Hb complex except that Q_α, Q_β, k_α, and k_β are replaced by Q, Q, k, and k, respectively.

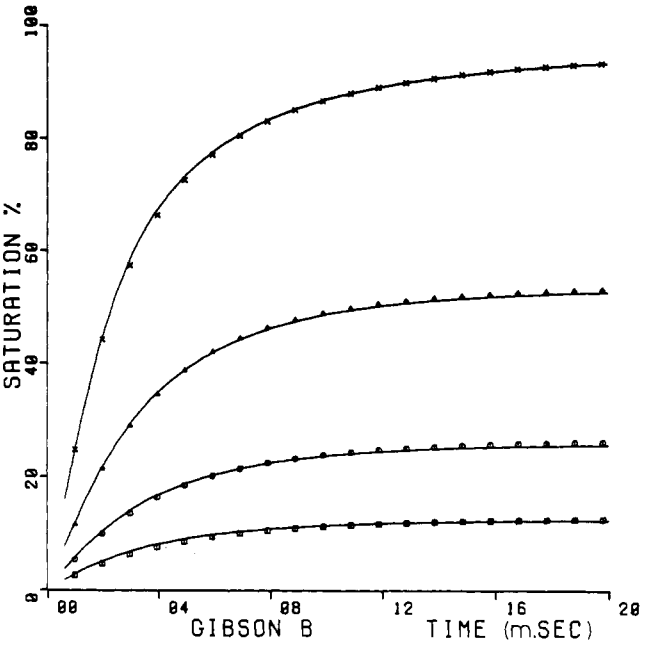

FIGURE 2: The reaction of stripped deoxyhemoglobin (53 μM) with 62 μM (x), 31 μM (△), 15.5 μM (○), and 7.8 μM (□) of oxygen. The points are observed (Gibson, 1970), and the lines are computed using the scheme in Figure 1 with the value $k = 28.5$ sec^{-1}.

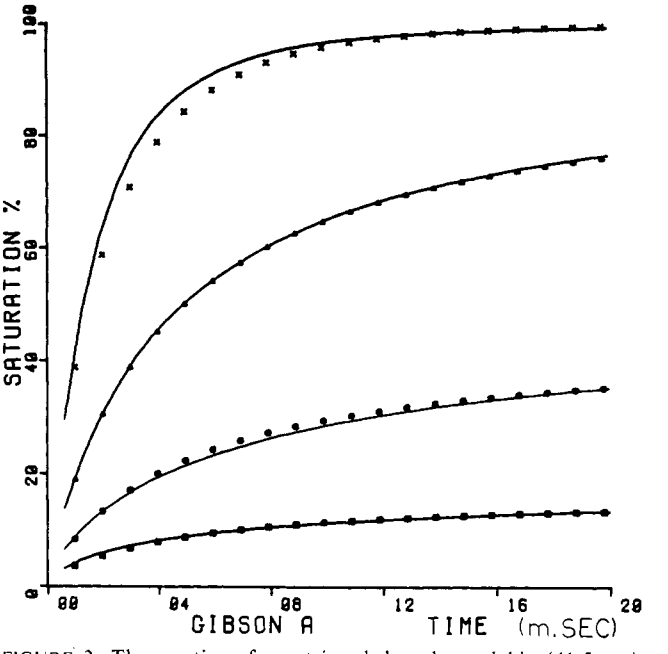

FIGURE 3: The reaction of nonstripped deoxyhemoglobin (41.5 μM) with 124 μM (x), 62 μM (▲), 31 μM (○), and 15.5 μM (□) of oxygen. The points are observed (Gibson, 1970), and the lines are computed using the scheme in Figure 1 with the values $k_\alpha = 3.732k$, $k_\beta = 28.35k$, and $\gamma = 4.390$ sec^{-1} (where $k = 28.5$ sec^{-1}).

rameters given by Gibson[2] (1970), and the solid curves are calculated from the theory with $k_\alpha/k = 3.732 \pm 0.070$, $k_\beta/k = 28.35 \pm 2.13$, $\gamma = 4.390 \pm 0.50$ sec, which are obtained by simultaneously fitting the experimental data points for 15.5, 31, and 62 μM O_2 by using a non-linear least-squares method (Bevington, 1969). The equilibrium constants used are $Q_\alpha = 0.157Q$ and $Q_\beta = 0.0628Q$, and

[2] We fit to these calculated curves instead of the raw data because Dr. Gibson suggested that this procedure was sufficiently accurate and the original data were not made available.

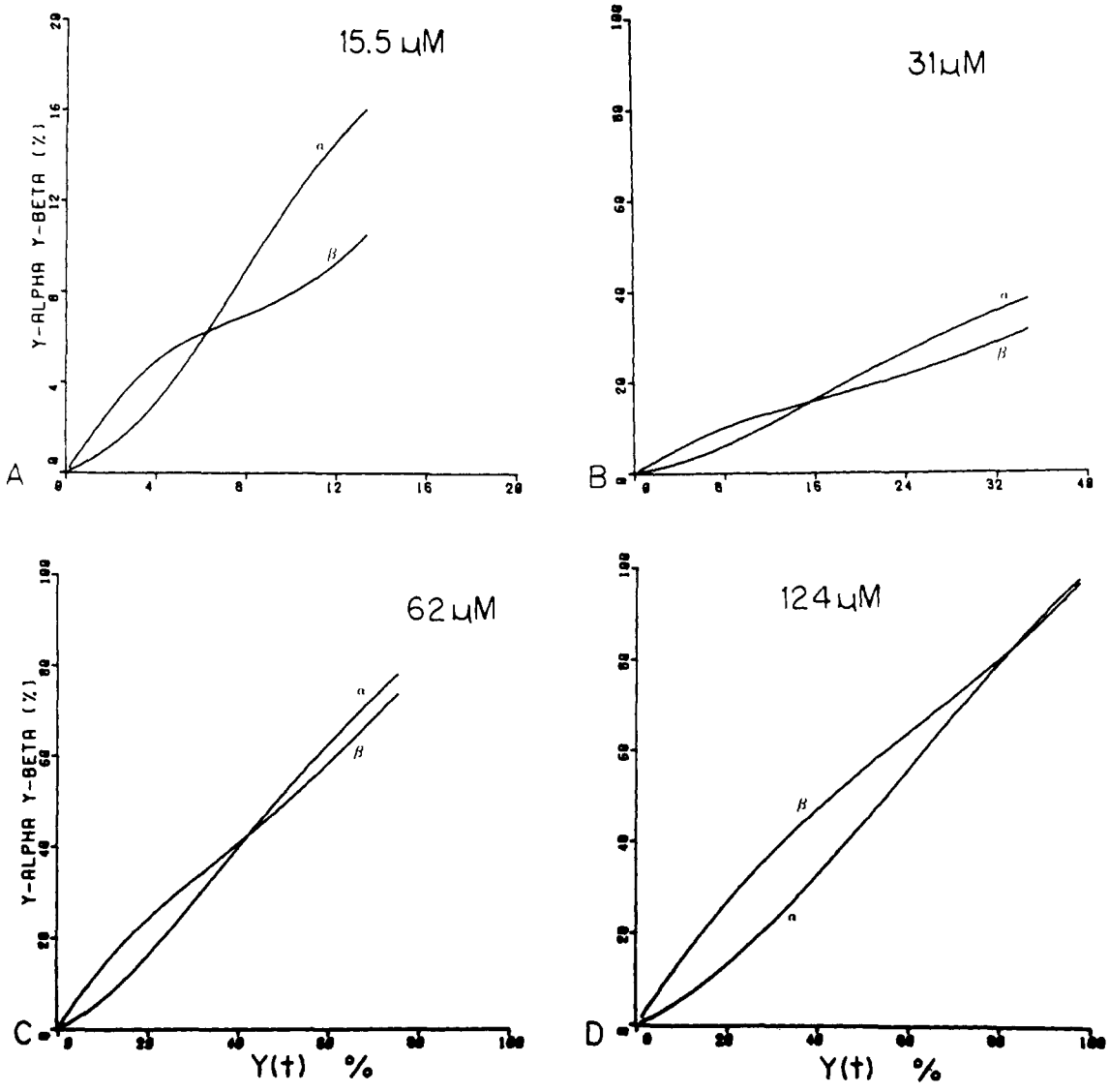

FIGURE 4: The time dependent fractional α and β subunit saturations, $Y_\alpha(t)$ and $Y_\beta(t)$, as a function of the oxygen saturation at various oxygen concentrations.

k_{op}[OP] = 1010, which have been obtained previously by Chay and Brillhart (1974). We note that an agreement between the theory and experiment is very good, except the one for 124 μM O_2. The parameters which fit the data points for 124 μM O_2 best are k_α/k = 3.2, k_β/k = 26.6, and γ = 5.9, which are not too different from the parameters used for the calculations.

Figure 4 shows the fractional saturations of oxygen in the α and β chains, $Y_\alpha(t)$ and $Y_\beta(t)$, as a function of the total oxygen saturation $Y(t)$ at various oxygen concentrations. As shown in this figure, $Y_\beta(t)$ is greater than $Y_\alpha(t)$ at first, but the role is reversed as equilibrium is approached. This means that a large amount of oxygen goes to the β chain first, but the β chain does not retain oxygen for a long period of time. Hence, in equilibrium the α chain has the higher ligand affinity than the β chain. This is consistent with the experimental findings of Gibson (1973) and of Ho and his collaborators (Lindstrom and Ho, 1972; Johnson and Ho, 1974).

Figure 5 shows the relation between the DPG saturation and oxygen saturation at various oxygen concentrations. Note that when 124 μM O_2 is reacted with 41.5 μM nonstripped Hb, nearly all DPG are bound to Hb until about 60% of oxygen saturation has occurred. On the other hand, the equilibrium curve (see Figure 6 of Chay and Brillhart, 1974) shows that there is almost linear relationship between the DPG and oxygen saturations.

Figures 6 and 7 illustrate the fraction of five species [$Hb(O_2)_n$] as a function of oxygen saturation at various oxygen concentration for both stripped and nonstripped Hb. By comparing the concentrations of stripped and nonstripped Hb, we find that, at the same total oxygen concentration and saturation, the concentrations of deoxy- and fully-liganded species of nonstripped Hb are always greater than those of stripped Hb and that one-liganded species of nonstripped Hb is always smaller than that of stripped Hb. The concentrations of two- and three-liganded species of nonstripped Hb are greater than those of stripped Hb at low oxygen saturation, but these concentrations become smaller as the oxygen saturation increases.

In Table II, we list the concentrations of each species, *i.e.*, DPG–Hb complexes and DPG-free species, in each oxygenation stages, when 41.5 μM Hb reacted with 124 μM O_2. It is clear from this table that a main path for oxygenation of hemoglobin is in the following order: first the β, α, β, and α chains of DPG–Hb complex are successively oxy-

TABLE II: Concentrations of Liganded and Unliganded Species when 41.5 μM Hb is Reacted with 124 μM O_2.[a]

	H_i											
$Y(t)$	H_0'	$H_1'(\alpha)$	$H_1'(\beta)$	$H_2'(\alpha\alpha)$	$H_2'(\alpha\beta)$	$H_2'(\beta\beta)$	H_3'-($\alpha\alpha\beta$)	H_3'-($\alpha\beta\beta$)	H_3-($\alpha\alpha\beta$)	H_3-($\alpha\beta\beta$)	H_4'	H_4
0.05	0.82	0.04	0.12	0.00	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00
0.10	0.66	0.07	0.19	0.00	0.03	0.02	0.00	0.01	0.00	0.00	0.00	0.00
0.15	0.57	0.08	0.22	0.00	0.06	0.04	0.01	0.02	0.00	0.00	0.00	0.00
0.20	0.48	0.09	0.22	0.01	0.08	0.06	0.01	0.04	0.00	0.00	0.01	0.00
0.25	0.41	0.09	0.22	0.01	0.09	0.07	0.02	0.06	0.00	0.00	0.03	0.00
0.30	0.35	0.09	0.20	0.01	0.10	0.07	0.02	0.08	0.00	0.00	0.05	0.01
0.35	0.31	0.09	0.19	0.01	0.11	0.07	0.03	0.10	0.00	0.00	0.07	0.01
0.40	0.27	0.08	0.17	0.01	0.12	0.07	0.03	0.11	0.00	0.01	0.10	0.02
0.45	0.24	0.08	0.15	0.01	0.12	0.06	0.04	0.12	0.00	0.01	0.13	0.03
0.50	0.20	0.08	0.13	0.01	0.11	0.06	0.05	0.13	0.00	0.01	0.16	0.05
0.55	0.17	0.07	0.11	0.01	0.11	0.05	0.05	0.13	0.00	0.01	0.19	0.07
0.60	0.15	0.06	0.10	0.01	0.10	0.04	0.06	0.12	0.00	0.01	0.22	0.10
0.65	0.12	0.06	0.08	0.01	0.09	0.03	0.06	0.11	0.00	0.01	0.25	0.15
0.70	0.10	0.05	0.06	0.01	0.08	0.03	0.07	0.10	0.01	0.01	0.27	0.20
0.75	0.08	0.04	0.05	0.01	0.07	0.02	0.07	0.09	0.01	0.01	0.28	0.26
0.80	0.06	0.04	0.04	0.01	0.06	0.02	0.07	0.07	0.01	0.01	0.27	0.34
0.85	0.04	0.03	0.02	0.01	0.04	0.01	0.06	0.05	0.01	0.01	0.25	0.44
0.90	0.02	0.02	0.01	0.01	0.03	0.01	0.05	0.04	0.01	0.01	0.21	0.57
0.95	0.01	0.01	0.01	0.01	0.01	0.00	0.03	0.02	0.01	0.01	0.14	0.73
1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.01	0.01	0.08	0.85

[a] Here, H_0, $H_1(\alpha)$, $H_1(\beta)$, $H_2(\alpha\alpha)$, $H_2(\alpha\beta)$, and $H_2(\beta\beta)$ are always less than 0.005.

genated; second DPG will be expelled from fully liganded complex; and finally DPG-free oxy-Hb will be formed. This result is in contradiction to the stereochemical mechanism proposed by Perutz (1970). Perutz, from his X-ray diffraction studies, has suggested that only after two α chains in DPG–Hb complex are oxygenated, DPG will be expelled and then the two β chains will be oxygenated. This is not quite consistent with a theoretical work of Herzfeld and Stanley (1974), who found that the quaternary conformation changes from the deoxy to the oxy form at about the third oxygenation, depending only slightly on DPG concentration.

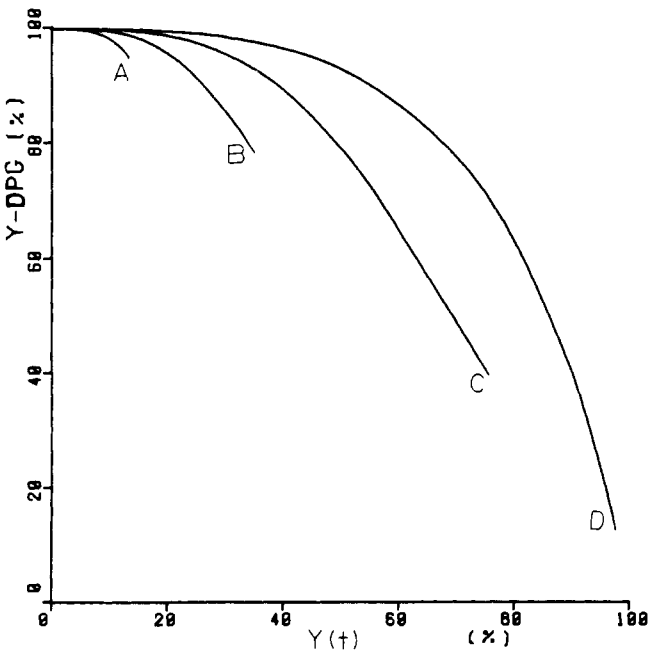

FIGURE 5: Relation between the organic phosphate saturation and the oxygen saturation for various oxygen concentrations. Curves A, B, C, and D correspond to 15.5, 31, 62, and 124 μM O_2.

Our assignment of the association and dissociation rate constants of OP (see the last three columns in Table I) accounts for the observation made by Gray and Gibson (1971) that the effect of OP appears primarily as an increase in the dissociation rate constants k_n with the exception of k_4, the dissociation rate constant of O_2 from fully liganded hemoglobins. This is because the dissociation of oxygen from a fully oxygenated H_4 has the dissociation constant of

$$k_4 = k_{op}[OP](Q_\alpha/Q)(Q_\beta/Q) + 4Z^{3/2}k$$

where [OP] independent term is much larger than [OP] dependent term because of a small magnitude of Q_β. For k_n where $n \leq 3$, we can easily see why the dissociation rates are dependent of [OP] from Figure 1 and Table I.

It should be pointed out that our conclusions are based on the values of the three kinetic parameters, $k_\alpha = 3.732k$, $k_\beta = 28.35k$ (where $k = 28.5$ sec^{-1}), and $\gamma = 4.390$, which we have obtained by fitting Gibson's nonstripped Hb data (Gibson, 1970). Although these parameters give the best fit to the data, there are other parameters which may fit the data with somewhat larger deviations;[3] *e.g.*, the parame-

[3] As in all non-linear least-square procedures, one must be aware of possible multi-minimum in χ^2 space for complex models. Our program uses two search algorithms (GRIDLS and GRADLS, Bevington 1969) and variable step sizes and starting points to ensure that fits found are at the absolute minimum in χ^2 space. The second fit reported here is at an auxiliary minimum but has a reasonably small χ^2 and is physically interesting.

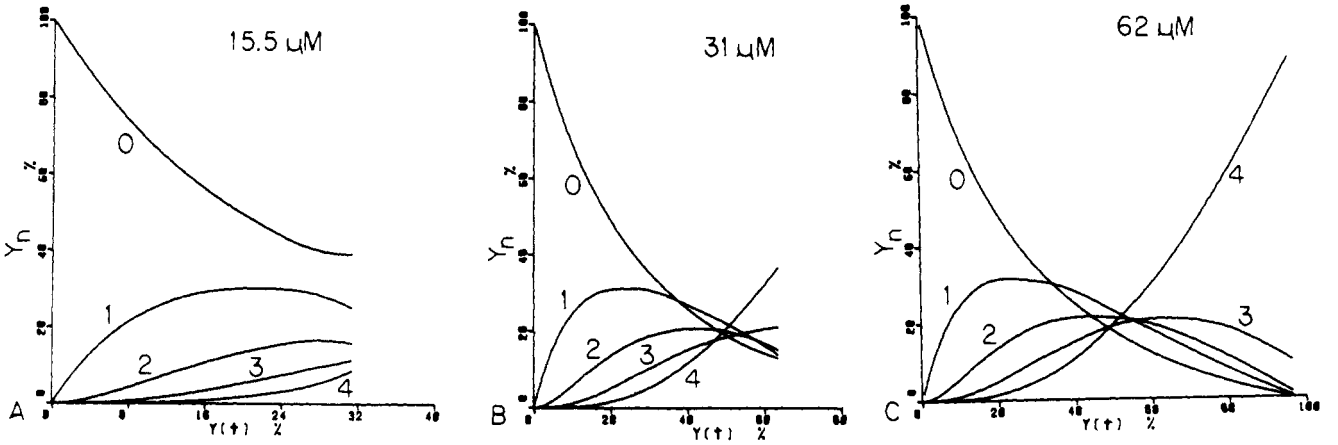

FIGURE 6: Fractional contribution of species $Hb(O_2)_n$ ($n = 0,1 \ldots 4$) as a function of the oxygen saturation for stripped hemoglobin.

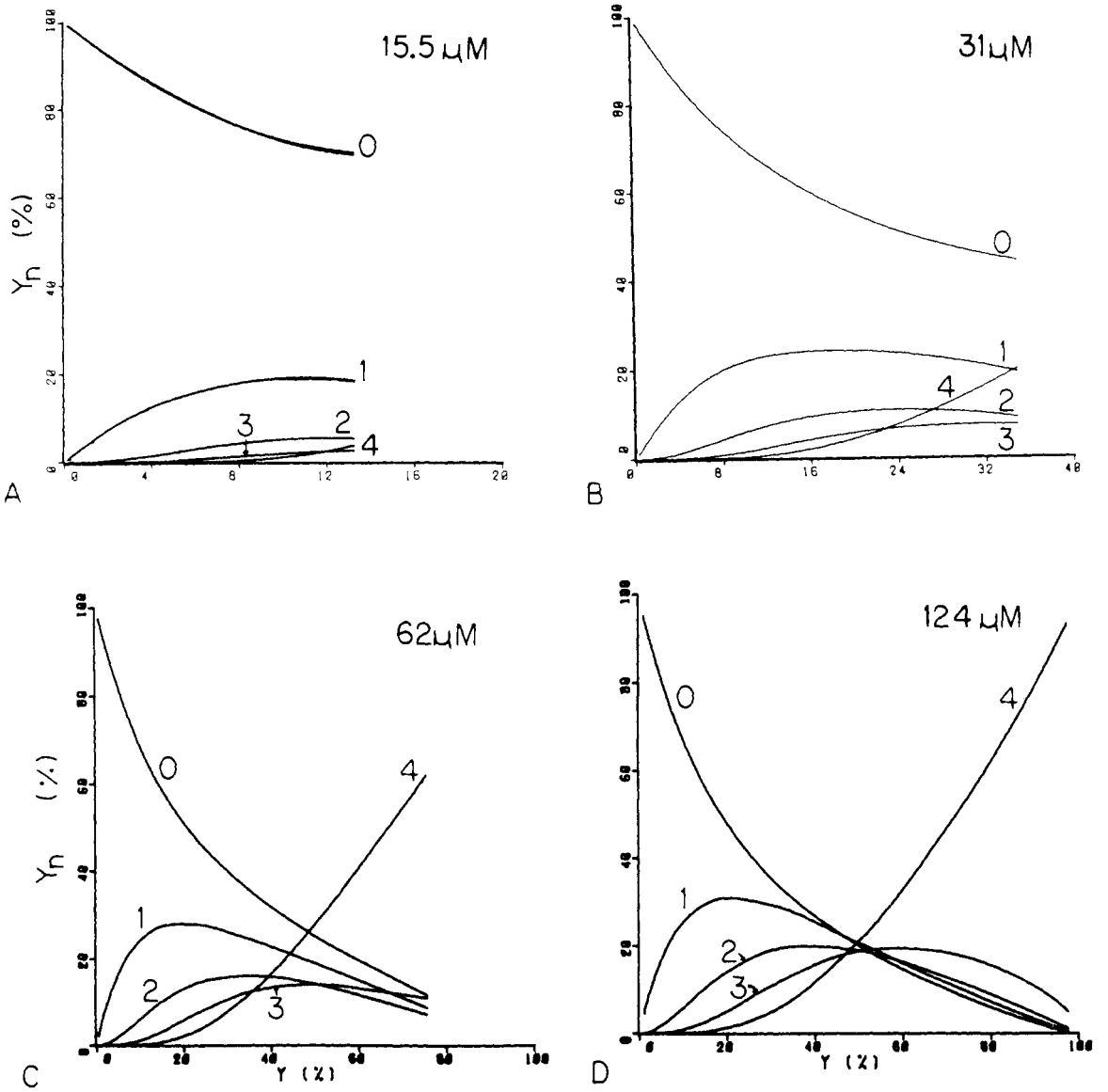

FIGURE 7: Fractional contributions of five species in terms of the oxygen saturation for nonstripped hemoglobin.

ters, $k_\alpha = 7.21k$, $k_\beta = 8.46k$, and $\gamma = 8.2$, have χ^2 of 1.04 when fitted to the data points for the oxygen concentrations of 15.5, 31, and 62 μM. Of course, the conclusions drawn from this set of the parameters will be somewhat different from those in this paper. That is, these parameters predict that the α chain has a higher ligand association rate constant than the β chain such that first the two α chains will be oxygenated, then the two β chains, and then DPG will come off from the complex. The fact that the α chain has a higher ligand association rate constant is not consistent with

the experimental finding of Gibson (1973). For this reason and because of the fact that this set of the parameters does not give the best fit, we did not choose to use these parameters. One very significant result in this paper, though, is a surprisingly good agreement between the experimental data of stripped Hb and the theoretical value which is calculated without using any adjustable parameter. A comparison between a kinetic version of the allosteric transition model of Monod *et al.* (Hopfield *et al.*, 1971) and the experimental data, on the other hand, shows not very convincing agreement. Thus, we may conclude that the sequential theory of Koshland *et al.* (1966) is probably a correct model for representing the oxygenation process of stripped Hb.

Acknowledgment

We would like to thank Professor Chien Ho for very valuable comments during the progress of this research.

References

Bevington, P. R. (1969), Data Reduction and Error Analysis for the Physical Sciences, New York, N. Y., McGraw-Hill, Chapter 11.
Chay, T. R., and Brillhart, D. (1974), *Biochemistry,* to be published.
Chay, T. R., and Ho, C. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 3914.
Cole, F. X., and Gibson, Q. H. (1973), *J. Biol. Chem. 248,* 4998.
Gibson, Q. H. (1970), *J. Biol. Chem. 245,* 3285.
Gibson, Q. H. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1.
Gray, R. D., and Gibson, Q. H. (1971), *J. Biol. Chem. 246,* 7168.
Herzfeld, J., and Stanley, H. E. (1974), *J. Mol. Biol. 82,* 231.
Hill, T., and Chen, Y. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 1711.
Hopfield, J. J., Shulman, R. G., and Ogawa, S. (1971), *J. Mol. Biol. 61,* 425.
Johnson, M., and Ho. C. (1974), *Biochemistry, 13,* 3653.
Koshland, D. E., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5,* 365.
Lindstrom, T. R., and Ho, C. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 1707.
Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 881.
Olson, J. S., and Gibson, Q. H. (1972), *J. Biol. Chem. 247,* 1713.
Olson, J. S., and Gibson, Q. H. (1973), *J. Biol. Chem. 248,* 1623.
Paul, E., and Hill, T. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2246.
Perutz, M. F. (1970), *Nature (London) 228,* 726, 734.
Tan, A. L., Noble, R., and Gibson, Q. H. (1973), *J. Biol. Chem. 248,* 2880.
Tyuma, I., Imai, K., and Shimizu, K. (1973), *Biochemistry 12,* 1491.

Resonance Raman Spectra of Horseradish Peroxidase: Evidence for Anomalous Heme Structure†

Gopa Rakshit and Thomas G. Spiro*

ABSTRACT: Resonance Raman spectra are reported for native (Fe(III)) horseradish peroxidase (HRP) and its fluoro and cyano derivatives, and also for reduced (Fe(II)) HRP, and it carbonmonoxy and cyanide derivatives. As with other heme proteins, the main Raman bands are attributable to porphyrin vibrations and can be catalogued *via* their polarization properties. Several of the frequencies are sensitive to the structural concomitants of changes in spin and oxidation state of the heme group. Except in the case of native HRP itself, these frequencies classify as expected from the known spin states of the HRP derivatives. Although native HRP contains high-spin Fe(III), the Raman frequencies are less strongly shifted from the low-spin values than is the case for aquomethemoglobin. This finding suggests that doming of the porphyrin ring, which is characteristic of high-spin heme, is less pronounced in native HRP than in aquomethemoglobin. This interpretation is plausibly related to the mechanism of the peroxidase reaction, which appears to involve oxidation of the heme iron to Fe(IV). In a less domed high-spin heme, one electron would be in a relatively high energy orbital and might be subject to facile removal.

Horseradish peroxidase (HRP,[1] donor: H_2O_2 oxidoreductase EC 1.11.1.7) has been the object of extensive study both because of its similarities to and differences from myoglobin (Mb) and hemoglobin (Hb). HRP has been reviewed by Paul (1963), Saunders *et al.* (1964), and Brill (1966). Like the oxygen-carrying heme proteins, HRP contains iron-protoporphyrin IX as a noncovalently bound prosthetic group. The iron atom is accessible to binding by exogenous ligands in both the Fe(III) and Fe(II) oxidation states. These ligands produce the same sorts of alterations in spin state as in Mb and Hb. The constant axial (fifth) ligand is probably a histidine side chain, as evidenced by ultraviolet difference spectra (Brill and Sandberg, 1968), and nitrogen hyperfine splitting of the electron paramagnetic spectrum of the nitrosyl complex (Yonetani and Yamamoto, 1973). Despite these basic similarities, striking differences are also

† From the Department of Chemistry, Princeton University, Princeton, New Jersey 08540. *Received July 29, 1974.* This work was supported by U. S. Public Health Service Grants GM-13498 and HL 12526.

[1] Abbreviations used are: HRP, horseradish peroxidase; Mb, myoglobin; Hb, hemoglobin.