Kinetics of Co-operative Ligand Binding in Proteins: The Effects of Organic Phosphates on Hemoglobin Oxygenation†

Rama Bansil, Judith Herzfeld‡ and H. Eugene Stanley

*Harvard–MIT Program in Health Sciences and Technology
and Department of Physics
Massachusetts Institute of Technology
Cambridge, Mass. 02139, U.S.A.*

A master equation approach to the kinetics of co-operative ligand binding reactions in proteins is developed. As a specific example, equations based on the Perutz description are applied to several reactions of hemoglobin, both in the absence and in the presence of organic phosphates.

The observed time dependence of O_2 binding to stripped hemoglobin is fitted with a total of four parameters, of which two are held fixed at values determined from the experimentally measured rate constants for the last step of ligand binding. An analysis of Gibson's data (1970a) for oxygen-binding kinetics of unstripped hemoglobin shows that phosphate binding not only stabilizes the deoxy quaternary conformation of hemoglobin but also increases *both* the strength of the quaternary conformational constraints and the kinetic time scaling parameter for the deoxy quaternary conformation.

A single set of five parameters is used to fit the deoxygenation data of Salhany *et al.* (1970) in the presence of varying concentrations of P_2Glyc§. The five parameters are determined by fitting the theory to the data in the absence of P_2Glyc and at a single concentration of P_2Glyc. The predictive ability of the model is then tested by using these same parameter values to calculate the curves for intermediate P_2Glyc concentrations, and then comparing the predicted curves with experimental data. Whereas deoxygenation data in the presence of P_2Glyc can be fitted with the subunits considered as equivalent, we find that α, β subunit inequivalence is particularly important in interpreting deoxygenation data in the presence of the inhibitor, IHP. Since the phosphate dependence of the reaction is explicitly included, the model can also describe biphasic deoxygenation, as observed by Gray & Gibson (1971) for the deoxygenation reaction in the presence of IHP.

In the deoxygenation reaction, the quaternary conformation is found to change from oxy to deoxy just prior to the release of the second oxygen molecule; the switch occurs somewhat earlier in the presence of organic phosphates. As a consequence of this switch, the release of oxygen lags behind the binding of organic phosphates; the lag is more pronounced for IHP than for P_2Glyc. The analogous result of MacQuarrie & Gibson (1972) concerning the lag between the release of HPT and binding of CO is well reproduced; the fractional saturation

---

of hemoglobin with CO and the fraction of HPT released are both fitted with a single set of five parameters.

The dependence of the Adair (1925a,b) rate constants (for $\alpha = \beta$) and Olson–Gibson (1972) rate constants (for $\alpha \neq \beta$) on the concentration of organic phosphate is calculated. It is found that (i) the experimentally observed increase in the overall deoxygenation rate with increasing organic phosphate concentration is primarily due to the increase in the rate of dissociation of the second oxygen molecule, (ii) the "on" rate constants most affected by organic phosphate are k_1' and k_2', the rate constants for the binding of the first and second oxygen molecules, (iii) the "off" rate constants, k_j, are affected more than the "on" rate constants, k_j', (iv) the rate constants for the reactions of the β subunits are affected more by IHP than those of the α subunits, and (v) the rate constants involving the intermediate species with two β subunits and one α subunit liganded are influenced the most by the binding of phosphates.

1. Introduction

The kinetics of co-operative ligand binding reactions in an oligomeric protein are sufficiently complex that an elaborate reaction scheme is required to analyze kinetic data. In the particular case of hemoglobin, most kinetic data have been analyzed using the Gibson–Roughton (1957) extension of the Adair formulation (Adair, 1925a,b). In this mass-action scheme, oxygenation takes place in four steps, each corresponding to the oxygenation of one heme group. Co-operativity is reflected in the variation of the rate constants at each step. Mass-action schemes do not provide any insight into the physical origin of co-operativity. Moreover, such schemes get prohibitively complex when one allows for inequivalent subunits (Olson & Gibson, 1972), different quaternary conformations of the molecule, and variations in the concentration of effectors.

In contrast to the Adair mass-action scheme, various molecular models have been developed in which co-operativity is attributed to specific intramolecular interactions. Basically, two different types of allosteric mechanisms have been used to account for the co-operativity. The first, due to Monod et $al.$ (1965), considers the protein to exist in two or more stable quaternary conformations which differ in their affinity for ligand ($preferential binding$). According to this model, in any quaternary conformation all the subunits are symmetrically arranged and subject to the same constraints—i.e. the conformational change is $concerted$. Because of this symmetry requirement, it follows that partial ligand binding can stabilize only that quaternary conformation which favors further ligand binding; therefore the Monod–Wyman–Changeux model cannot account for negative homotropic interactions. Hopfield et $al.$ (1971) have extended the Monod–Wyman–Changeux equilibrium model to the kinetics of hemoglobin reactions. Their analysis can explain the results of both stopped-flow experiments (Gibson, 1970a) and flash photolysis experiments (Gibson, 1956,1959a; Antonini et $al.$, 1967) at fixed effector concentrations. In particular, the existence of different quaternary conformations which react with ligand at different rates offers a direct explanation for the rapidly-reacting species seen in some flash photolysis experiments. However, their model does not provide a detailed quantitative analysis of the heterotropic co-operativity between oxygenation and organic phosphate binding.

In contrast to the Monod–Wyman–Changeux mechanism, Koshland et $al.$ (1966) consider an allosteric mechanism in which ligand binding of a subunit induces in it a tertiary conformational change ($induced$-$fit binding$). This induced tertiary conformational change in a subunit alters the probability of a neighboring subunit

being in a given tertiary conformation, and thereby affects the ligand binding affinity of the neighboring subunit. This model can account for both positive and negative homotropic interactions, depending on whether a pair of neighboring subunits is more stable in similar tertiary conformations (positive homotropic interactions) or in dissimilar tertiary conformations (negative homotropic interactions). The mechanism of Koshland *et al.* has been used by Bush & Thompson (1971*a,b*) to analyze the kinetics of hemoglobin oxygenation in the *absence* of effectors and by Chay & Brillhart (1974 *a,b*) to analyze the oxygenation kinetics of stripped and unstripped hemoglobin.

In recent years considerable experimental evidence has accumulated to suggest that in the case of hemoglobin, aspects of *both* allosteric mechanisms play an important role. This evidence comes from nuclear magnetic resonance (Shulman *et al.*, 1969,1970), electron spin resonance (Ogawa *et al.*, 1968), sulfhydryl reactivity studies (Antonini & Brunori, 1969), and X-ray crystal structure determinations (Perutz, 1970*a,b*). Based on these findings, Perutz suggested that the binding of O_2 to the individual subunits of hemoglobin is of the induced-fit type (Koshland–Némethy–Filmer mechanism). This induced-fit binding, however, does not lead directly to alterations in the tertiary equilibrium of the neighboring subunits. Rather, the change in tertiary conformation, induced by O_2 binding to a subunit, is coupled to the quaternary conformational equilibrium (Monod–Wyman–Changeux mechanism). Thus the co-operative interaction is provided by conformational constraints coupling the quaternary and tertiary equilibria. This qualitative description of Perutz (1970*a,b*) was developed into quantitative models by Szabo & Karplus (1972) and by Herzfeld & Stanley (1974).

Our purpose here is to provide a quantitative approach to the kinetics of hemoglobin oxygenation in the presence of effectors using the Perutz description. For the sake of generality (i.e. to allow future application to more complicated systems), we have developed our model so as to include different types of homotropic and heterotropic interactions, and the possibility of inequivalent subunits. The descriptions of Monod *et al.*, Koshland *et al.* and Perutz each correspond to a special case of this general formulation. This approach to kinetics is similar to that used in previous work on equilibrium aspects of co-operativity in hemoglobin (Herzfeld & Stanley, 1974) and glyceraldehyde-3-phosphate dehydrogenase (Herzfeld & Schlesinger, 1975). As an example of heterotropic interactions we have analyzed the effects of the organic phosphates 2,3-diphosphoglycerate (P_2Glyc) and inositol-hexaphosphate on the kinetics of the reactions of hemoglobin.

Our quantitative description of the kinetics uses the master equation approach of statistical mechanics (Oppenheim, 1969; Montroll, 1967; Stanley, 1971), as opposed to the conventional mass-action method of chemical kinetics. The master equation approach has the conceptual advantage that one can relate co-operative behavior to the various molecular states of the protein. The equilibrium constants for the various reactions can be expressed in terms of the energies of these states, and the reaction rates can be expressed as functions of both the energies of these states and the transition probabilities between various states.

Our approach also has the practical advantage that the number of parameters needed to describe a given set of data is reduced. Since the rate constants can be expressed as a product of various factors, each of which represents the effect of a specific type of interaction, it is possible to systematically assess the contribution of the various possible interactions to the overall rate constant. Our detailed formalism

is quite analogous to that used by Glauber (1963) to describe the time development of an Ising spin system. The Glauber formalism has previously been applied by Goel (1968) to explain the kinetics of denaturation of DNA and by Bush & Thompson (1971*a,b*) to extend the Koshland–Némethy–Filmer (Koshland *et al.*, 1966) model to the kinetics of hemoglobin oxygenation.

In sections 2 to 5 we describe the theoretical aspects of our approach to kinetics. The Glauber formalism is reviewed briefly in section 2. In section 3 we develop a general model for homotropic effects in hemoglobin kinetics. This is extended to include heterotropic interactions in section 4. Our approach is compared with mass-action schemes in section 5. In sections 6 and 7 we compare the calculations based on the Perutz description, with data on the kinetics of CO binding, O_2 binding and O_2 dissociation in the presence of organic phosphates (MacQuarrie & Gibson, 1972; Gibson, 1970*a*; Salhany *et al.*, 1970; Gray & Gibson, 1971). The dependence of the Adair rate constants on organic phosphate concentration is discussed in section 8. A concluding discussion is presented in section 9.

2. Formalism

In order to describe the equilibrium and kinetic properties of ligand binding to an oligomeric protein, we need to evaluate quantities such as the average number of occupied sites, the average number of molecules in a given quaternary or tertiary conformation, and so forth. In statistical mechanical terms, any observable quantity is given by an average of a molecular variable, v, over all the states of the protein molecule at time t

$$V(t) \equiv \langle v \rangle \equiv \sum_{\substack{\text{all} \\ \text{states}}} vP(A;t). \tag{1}$$

The basic quantity describing the system, $P(A;t)$, is the probability of finding a molecule in a given state, A, at time t.

In order to calculate $P(A;t)$, given the initial probability distribution $P(A;t=0)$, we need an expression for the rate of change of the probability, $\mathrm{d}P(A;t)/\mathrm{d}t$. The time derivative $\mathrm{d}P(A;t)/\mathrm{d}t$ consists of two sorts of terms. The first type contributes to the *increase* in $P(A;t)$, and arises from the possibility of transitions into state A from other states A'; hence it is given by the product of the probability of the molecule being in state A' and the transition probability per unit time $W(A' \to A)$. The second term contributes to the *decrease* in $P(A;t)$, and arises from the possibility of transitions out of state A; hence it is given by the product of the probability of the molecule being in state A and the transition probability per unit time $W(A \to A')$. Thus the full differential equation for $P(A,t)$ is

$$\frac{\mathrm{d}}{\mathrm{d}t} P(A;t) = \sum_{A'} [W(A' \to A)P(A';t) - W(A \to A')P(A;t)]. \tag{2}$$

Equation (2) is known as the master equation (Montroll, 1967; Oppenheim, 1969; Stanley, 1971). Several basic assumptions underlie this equation.

(i) The variables describing the state of the system are stochastic functions of time—i.e. each variable can take any one of a given set of values with a finite probability, and the variable can make transitions between these values randomly. These transitions take place because of the weak interactions between the protein molecule and its environment (which, in statistical mechanical terms, is a heat and ligand reservoir).

(ii) The system under consideration satisfies the Markoff property (Cox & Miller,

1965)—i.e. the probability of a transition occurring in a small time interval $(t, t + \delta t)$ depends *only* on the state occupied at time t and not on the states occupied *before* time t.

(iii) The probability of a transition occurring in a small time interval $(t, t + \delta t)$ is proportional to δt, with a transition probability per unit time, $W(A' \to A)$, which depends only upon the momentary values of the variables describing the states A and A' as well as upon the influence of the environment of the system.

As t approaches infinity, the system under consideration achieves equilibrium. Thus $\lim_{t \to \infty} P(A, t) = P_{eq}(A)$, where

$$P_{eq}(A) = \exp\{-\beta \mathscr{E}(A)\}/Z. \tag{3a}$$

Here

$$\mathscr{E}(A) = G(A) - n_A \mu_S \tag{3b}$$

and $\beta = 1/kT$, where $G(A)$ denotes the free energy of the molecule in the state A, n_A the number of bound substrate molecules, μ_S the chemical potential of the substrate, k the Boltzmann constant and T the temperature. The normalization constant, $Z = \sum_A [\exp\{-\beta \mathscr{E}(A)\}]$, is the partition function. A sufficient but not necessary condition for the achievement of this result is obtained by setting

$$\frac{W(A \to A')}{W(A' \to A)} = \frac{P_{eq}(A')}{P_{eq}(A)} = \exp\{\beta(\mathscr{E}(A) - \mathscr{E}(A'))\}. \tag{4}$$

Equation (4) is known as the principle of detailed balance.

We also assume that in the time interval $(t, t + \delta t)$ *only* those transitions which involve the change of a single variable occur. If $v_1, v_2, \ldots, v_n$ denote the n variables characterizing the state A, then this assumption requires that

$$W(\{v_1, v_2, \ldots, v_i, \ldots, v_j, \ldots, v_n\} \to \{v_1, \ldots, v_i', \ldots, v_j', \ldots, v_n\})$$

be negligibly small. This assumption implies, for example, that whereas the probability of binding one ligand molecule in the time interval $(t, t + \delta t)$ is proportional to δt, the probability of binding two ligand molecules in the same time interval is proportional to $(\delta t)^2$, and hence makes a negligible contribution to the reaction. However, if the variables v_i and v_j are coupled by an infinitely strong interaction, so that the value of v_j is completely determined by the value of v_i, and the two never change independently, then we include the simultaneous transition of v_i and v_j to v_i' and v_j' in equation (2). These concepts are illustrated schematically in Figure 1.

Given the state of the system at some time, say $t = 0$, and knowing the functional form for the transition probabilities $W(A \to A')$, one can, in principle, solve the master equation to determine the set of functions $P(A; t)$ at all times for all states. In practice, however, the total number of states is usually so large that such a solution would be impossible; moreover the functions $P(A; t)$ in their entirety contain vastly more information than we usually require. In general it suffices to know the time development of the various average quantities one usually measures.

In this work the variables v_i will typically represent such characteristics of the molecule as ligand binding, tertiary conformation, and quaternary conformation. In the case of those molecules for which each variable v_i can take on only two distinct values (e.g. liganded and unliganded), equation (2) assumes the form (Glauber, 1963)

$$\frac{d}{dt} P(v_1, \ldots, v_j, \ldots, v_n) = \sum_{j=1}^{n} [W(-v_j \to v_j)P(v_1, \ldots, -v_j, \ldots, v_n)$$
$$- W(v_j \to -v_j)P(v_1, \ldots, v_j, \ldots, v_n)], \tag{5}$$
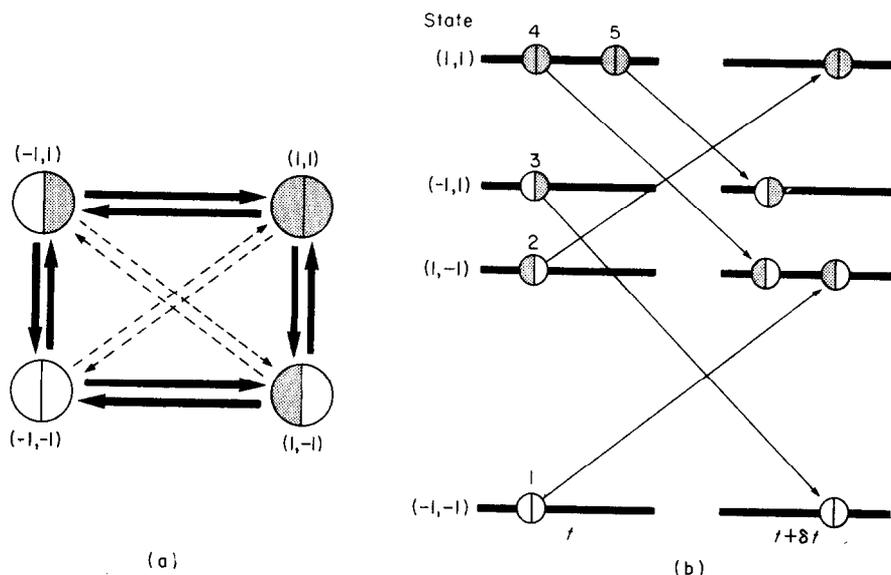
Fig. 1. (a) Schematic representation of the concept of allowed and forbidden transitions between the 4 possible states of a dimeric molecule with equivalent subunits (e.g. the $\alpha\beta$ dimer produced by dissociation of hemoglobin). The state of the molecule can be described by the two variables (s_1, s_2), where $s_j = +1$ if the jth site is occupied by ligand and -1 if empty. The shaded semicircle represents an occupied site and the open semicircle an empty site. The solid arrows indicate the *allowed* transitions and the broken arrows correspond to *forbidden* transitions. (b) One possible distribution of five dimeric molecules among the 4 possible states at time t and at time $t + \delta t$. The arrows indicate transitions which occurred in the interval δt. To illustrate the use of the master equation, eqn (2), consider the state $(1,-1)$. The increase of 1 in the number of molecules in this state during the time interval $(t, t + \delta t)$ is due to 2 transitions *into* this state (from $(-1, -1)$ and $(1,1)$) and 1 transition *out* of this state (to $(1,1)$).

where the two values of each variable v_j are denoted by $+1$ and -1. In writing equation (5) we have used the condensed notation of specifying only that variable which changes in a transition; thus $W(v_j \to -v_j)$ represents $W(\{v_1, v_2, \ldots, v_j, \ldots, v_n\} \to \{v_1, v_2, \ldots, -v_j, \ldots, v_n\})$.

The time derivatives of the expectation values (averages at time t) $\langle v_j \rangle$, $\langle v_j v_k \rangle$, $\langle v_j v_k v_l \rangle$, . . . can be expressed directly in terms of averages involving the transition probabilities (Glauber, 1963). Straightforward algebra gives the following results:

$$\frac{\mathrm{d}}{\mathrm{d}t} \langle v_k \rangle = -2 \langle v_k W(v_k \to -v_k) \rangle$$

$$\frac{\mathrm{d}}{\mathrm{d}t} \langle v_j v_k \rangle = -2 \langle v_j v_k [W(v_j \to -v_j) + W(v_k \to -v_k)] \rangle \qquad (6)$$

$$\frac{\mathrm{d}}{\mathrm{d}t} \langle v_j v_k v_l \rangle = -2 \langle v_j v_k v_l [W(v_j \to -v_j) + W(v_k \to -v_k) + W(v_l \to -v_l)] \rangle.$$

The above equations are quite general; details of the molecular model are introduced by specifying the energies of the various states in equation (3).

3. Homotropic Co-operativity

In this section we apply the general formalism of section 2 to the specific case of a protein in the *absence* of effector molecules. For the sake of simplicity we treat the subunits as equivalent; the modifications necessary if the subunits are inequivalent are discussed in section 5. Although the methods of this section apply to ligand binding in any co-operative protein, our specific calculations will deal with hemoglobin. We can describe the state of the hemoglobin tetramer by the variables s_i, t_i, and q, where the index i varies from 1 to N, and N is the number of subunits in the molecule.

(i) s_i represents the association of substrate with the substrate binding site on the ith subunit. Here $s_i = +1$ if the ith site is occupied by substrate and -1 otherwise.

(ii) t_i represents the tertiary conformation of the ith subunit. For hemoglobin we consider only two tertiary conformations for each subunit, which we denote by $t_i = +1$ for the "oxy" tertiary conformation and $t_i = -1$ for the "deoxy" tertiary conformation.

(iii) q represents the quaternary conformation of the molecule. For hemoglobin we consider only two quaternary conformations, the oxy and deoxy quaternary conformations corresponding to $q = +1$ and -1, respectively.

Since N, the number of subunits, is 4 for hemoglobin, we have a total of $2^4 \times 2^4 \times 2 = 2^9$ possible states. In principle all of these states can occur; however, some of them may occur with negligible probability.

Homotropic co-operativity arises from coupling among these variables. This may occur in a number of ways.

(i) The substrate binding equilibria may be coupled to the conformational equilibria by preferential substrate binding to certain conformations (Monod *et al.*, 1965). An extreme case, in which the protein is in one conformation when substrate is bound and in another when substrate is not bound, is called induced-fit binding (Koshland *et al.*, 1966).

(ii) The tertiary conformational equilibrium of a subunit may be coupled to that of a neighboring subunit by nearest-neighbor constraints (Koshland *et al.*, 1966).

(iii) The tertiary conformational equilibrium of a subunit may be coupled to the quaternary conformational equilibrium of the molecule by molecular constraints, i.e. the dependence on quaternary conformation of the constraints placed on one subunit by the other subunits (Monod *et al.*, 1965).

Thus, for hemoglobin the quantity $\mathscr{E}$, defined in equation (3b), is given by (Herzfeld & Stanley, 1974):

$$\mathscr{E} = \tfrac{1}{2}G_Q q + \sum_{i=1}^{N} \left[\left(\frac{G_T}{2} - \frac{G_{TT}}{2}(t_{i+1} + t_{i-1}) - G_{QT}q \right) t_i \right.$$
$$\left. + \left(-\mu_S + G_S + t_i G_{ST} \right) \left(\frac{1 + s_i}{2} \right) \right]. \quad (7)$$

Here G_Q is the difference in free energy between the two quaternary conformations of the molecule, $(G_T - 2qG_{QT} - (t_{i+1} + t_{i-1})G_{TT})$ is the difference in energy between the two tertiary conformations of a given subunit when the molecule is in the q quaternary conformation and the two neighboring subunits are in the t_{i+1} and t_{i-1}

tertiary conformations†, and $(G_S + t_i G_{ST})$ is the energy of substrate binding to a subunit in the t_i tertiary conformation. Note that if $G_{ST} = 0$ substrate binding is not preferential, if $G_{TT} = 0$ the tertiary conformational equilibria of neighboring subunits are not coupled, and if $G_{QT} = 0$ the tertiary conformational equilibria of individual subunits are not coupled to the quaternary conformational equilibrium of the molecule.

The expression for $\mathscr{E}$ simplifies considerably in limiting cases (Herzfeld & Stanley, 1974). In particular, the allosteric models of Monod et al. (1965) and Koshland et al. (1966), and the Perutz (1970a,b) description of co-operativity in hemoglobin are three such simple limiting cases. In this paper we will consider the kinetics of hemoglobin within the framework of the Perutz model. All the following discussion is restricted to this model. The method can be generalized to consider any other model of co-operative interaction.

According to Perutz there are no nearest-neighbor constraints in hemoglobin ($G_{TT} = 0$). However, the quaternary conformational constraints are finite and non-zero ($0 < G_{QT} < \infty$). Also, Perutz has suggested that oxygen binding to hemoglobin is induced-fit ($G_T \to \infty, G_{ST} \to -\infty$, $G_T + G_{ST}$ finite). Hence, according to this picture, the state of the hemoglobin molecule can be described by the 5 variables $(s_i \equiv t_i, q)$ (with $i = 1, \ldots, 4$) and the expression $\mathscr{E}$ of equation (7) reduces to

$$\mathscr{E} = \tfrac{1}{2} G_Q q + \sum_{i=1}^{4} \left[(-\mu_S + G_S + G_{ST}) \left(\frac{1 + s_i}{2} \right) + \left(\frac{G_T}{2} - G_{QT} q \right) s_i \right]. \qquad (8)$$

Since, in the case being considered, each of the variables takes on only two possible values, $+1$ or -1, the master equation describing the time development can be written in the simplified form of equation (5)‡:

$$\frac{d}{dt} P(\{s_i\}; q) = \sum_{i=1}^{N} [W_{ST}(-s_i \to s_i) P(-s_i, \ldots) - W_{ST}(s_i \to -s_i) P(s_i, \ldots)]$$
$$+ [W_Q(-q \to q) P(\ldots, -q) - W_Q(q \to -q) P(\ldots, q)]. \qquad (9)$$

The induced-fit binding assumption is incorporated in the transition probability per unit time, $W_{ST}(s_i \to -s_i)$, for the simultaneous change of the substrate occupancy variable and the tertiary conformational variable of the ith subunit. Recent experiments (Alpert et al., 1974) show that the tertiary conformational change occurs on a time scale of microseconds, whereas the ligand binding occurs in a time scale of milliseconds, so that it is reasonable to assume that the tertiary conformation of a subunit changes almost instantaneously upon changing the state of ligation of that subunit.

The principle of detailed balance can be used to express the transition probabilities in terms of the parameters appearing in the expression for $\mathscr{E}$ (equation (8)). The most general form of the transition probability consistent with equations (4) and (8) is shown in the Appendix to be

$$W_{ST}(s_i \to -s_i) = k(1 + \alpha q)(1 + \theta_{ST} s_i)(1 - \theta_{QT} s_i q). \qquad (10)$$

† For the sake of simplicity we consider G_{TT} to be independent of q; if G_{TT} is dependent on the quaternary conformation, then we can write $G_{TT}(q) = G'_{TT} + q G''_{TT}$ and the rest of the analysis follows analogously. Note that we are considering here only cases where each subunit interacts with just two others (i.e. a square model for a molecule with four subunits). However, the formalism can be generalized to include other patterns of interaction.

‡ The general master equation, equation (2), can be used in those cases where it is necessary to consider more than two distinct quaternary or tertiary conformations.

Here

$$\theta_{ST} \equiv \tanh\{\tfrac{1}{2}\beta(G_S + G_{ST} + G_T - \mu_S)\} \tag{11a}$$

and

$$\theta_{QT} \equiv \tanh\{\beta G_{QT}\} \tag{11b}$$

are related to equilibrium properties, while the parameters k and α have no equilibrium analogs. Note that $k(1 + \alpha q)$ is a time scaling factor (k has the dimension s^{-1} while α is dimensionless) and it depends on the temperature of the system. It should be mentioned that $W_{ST}(s_i \to -s_i)$ is independent of $s_j(j \neq i)$, the substrate occupancy states of the other subunits, because in the Perutz model there is no direct coupling between events at one subunit and its neighbors (i.e. no tertiary–tertiary interactions).

Notice from equation (11a) that when $s_i = -1$, $(1 + s_i\theta_{ST})/(1 + \theta_{ST})$ is proportional to $\exp(\beta\mu_S)$; hence this factor is proportional to the substrate concentration for $s_i = -1$ and is independent of substrate concentration for $s_i = +1$. Thus, if we write equation (10) in the form

$$W_{ST}(s_i \to -s_i) = \omega_{ST}(1 - \theta_{QT}s_i q)(1 + \theta_{ST}s_i)/(1 + \theta_{ST}), \tag{12}$$

where

$$\omega_{ST} \equiv k(1 + \alpha q)(1 + \theta_{ST}), \tag{13}$$

then it becomes immediately apparent that the rate of binding of a substrate molecule ($s_i = -1$) is directly proportional to the substrate concentration, whereas the rate of dissociation ($s_i = +1$) is independent of substrate concentration. Hence ω_{ST} is equal to the rate of substrate dissociation from a molecule in which the quaternary conformation provides no constraints on the tertiary equilibrium (i.e. $G_{QT} = 0$, $\theta_{QT} = 0$). If α is zero, then ω_{ST} has the same value for both quaternary conformations. A non-zero value of α implies that the quaternary conformation affects the *rates* of tertiary conformational changes, even if it does not affect the tertiary *equilibria* (i.e. the forward and backward rates are affected equally and the ratio remains unaffected). It should be emphasized that the above statements can be made only because a microscopic picture has been used to derive the kinetics of the system.

As was mentioned above, the master equation formalism has been used to analyze the kinetics of DNA denaturation (Goel, 1968), oxygenation kinetics in the Koshland–Nemethy–Filmer model (Bush & Thompson, 1971a,b), and the time dependence of the Ising model in magnetism (Glauber, 1963; Suzuki & Kubo, 1968). In all these applications, the transition probability had a form corresponding to the case $\alpha = 0$ in equation (10). However, in this work the effect of non-zero α will be considered when fitting experimental data.

Equation (10) can be used to obtain expressions for the rate constants for substrate binding and substrate dissociation when the molecule is in either the oxy or the deoxy quaternary conformation. On considering all four possibilities, we have

$$W_{ST}(s_i = -1 \to s_i = +1 \mid q = oxy) = k(1 + \alpha)(1 + \theta_{QT})K_{ST}[S]_{free} \tag{14a}$$

$$W_{ST}(s_i = -1 \to s_i = +1 \mid q = deoxy) = k(1 - \alpha)(1 - \theta_{QT})K_{ST}[S]_{free} \tag{14b}$$

$$W_{ST}(s_i = +1 \to s_i = -1 \mid q = oxy) = k(1 + \alpha)(1 - \theta_{QT}) \tag{14c}$$

$$W_{ST}(s_i = +1 \to s_i = -1 \mid q = deoxy) = k(1 - \alpha)(1 + \theta_{QT}). \tag{14d}$$

Here we have used the fact that the concentration of free substrate molecules $[S]_{\text{free}}$ is directly proportional to $\exp(\beta\mu_S)$ to define the equilibrium constant K_{ST},

$$K_{ST}[S]_{\text{free}} = \frac{1 - \theta_{ST}}{1 + \theta_{ST}} = \exp(\beta\mu_S)\exp\{-\beta(G_S + G_T + G_{ST})\}. \tag{15}$$

For positive quaternary–tertiary interactions $(G_{QT} > 0,\ \theta_{QT} > 0)$, note that if $\alpha > 0$, the substrate binding to a subunit is faster when the molecule is in the oxy quaternary conformation than when it is in the deoxy quaternary conformation. The reverse holds for the dissociation rates provided $\alpha < \theta_{QT}$. For the case $0 \leq \alpha < \theta_{QT}$, this result can be physically interpreted to mean that transitions in which the final state has compatible quaternary and tertiary conformations $(qt = 1)$ are more probable than those for which the final state will have $qt = -1$.

An analogous choice for $W_Q(q \to -q)$, the transition probability for the quaternary conformational change, is

$$W_Q(q \to -q) = \omega_Q(1 + q\theta_Q) \prod_{i=1}^{4} (1 - s_i q\theta_{QT}), \tag{16a}$$

where ω_Q is the time scaling parameter for quaternary conformational change, and

$$\theta_Q \equiv \tanh(\tfrac{1}{2}\beta G_Q). \tag{16b}$$

As was discussed in the preceding paragraph, ω_Q can depend on the values $\{t_i\}$, where $t_i = s_i$ for induced-fit binding. By substituting the expressions for the transitions probabilities (eqns (10) and (16)) into eqn (6) we obtain rate equations for the various averages (e.g. $\langle q \rangle$, $\sum_i \langle s_i \rangle$, $\sum_i \langle q s_i \rangle$, $\sum_{i \neq j} \langle s_i s_j \rangle$, ...) (Bansil, 1975). The resulting set of nine coupled first-order differential equations can be solved numerically, with appropriate initial conditions, to obtain the kinetics of the reaction under consideration. The kinetic equations of the Perutz model are mathematically equivalent to those obtained with the Monod–Wyman–Changeux model; the two descriptions differ in the physical interpretation of the model parameters.

4. Heterotropic Co-operativity

The formalism developed above is adequate to describe the reaction of hemoglobin with ligands at a *single fixed* concentration of each effector. Organic phosphates have been shown to bind preferentially to the deoxy quaternary conformation of hemoglobin (Perutz, 1970a,b; Benesch et al., 1969; Benesch & Benesch, 1974) and thereby to shift the quaternary equilibrium. In addition, the strength of the quaternary conformational constraints may vary with the binding of organic phosphate (Arnone, 1972; Herzfeld & Stanley, 1974). These ideas can be included in a quantitative manner if we extend the expression for $\mathscr{E}$ (equation (8)) to include the increase in free energy, $\delta\mathscr{E}$, due to the binding of the effector,

$$\delta\mathscr{E} = \left[-\mu_E + G_E + qG_{QE} - \sum_{i=1}^{4} qt_i G_{QT,E}\right] \left(\frac{1 + s_E}{2}\right), \tag{17}$$

where $t_i \equiv s_i$ for induced-fit binding of substrate. Here μ_E is the chemical potential of the quaternary effector, $(G_E + qG_{QE})$ is the energy of effector binding to a molecule in the q quaternary conformation and s_E is the variable representing occupancy of the effector binding site ($s_E = +1$ if effector is bound, -1 if not bound to the molecule). The effect of the last term in equation (17) is to increase the strength of the

quaternary conformational constraints G_{QT} by the amount $G_{QT,E}$ when the effector is bound.

The master equation, equation (9), now becomes

$$\frac{\mathrm{d}}{\mathrm{d}t} P(s_i = t_i, q, s_E; t) =$$

$$\sum_{i=1}^{N} [W_{ST}(-s_i \to s_i)P(-s_i, \ldots; t) - W_{ST}(s_i \to -s_i)P(\ldots, s_i; t)]$$

$$+ [W_Q(-q \to q)P(\ldots, -q; t) - W_Q(q \to -q)P(\ldots, q; t)]$$

$$+ [W_E(-s_E \to s_E)P(\ldots, -s_E; t) - W_E(s_E \to -s_E)P(\ldots, s_E; t)], \quad (18)$$

where the last term represents the transition involving the change in the effector occupancy variable, s_E. As before, a choice for the transition probability $W_E(s_E \to -s_E)$ consistent with the principle of detailed balance, equation (4), and of the same form as W_{ST} is

$$W_E(+s_E \to -s_E) = \omega_E \left(\frac{1 + \theta_E s_E}{1 + \theta_E}\right)(1 + \theta_{QE}qs_E) \prod_{i=1}^{4} (1 - \theta_{QT,E}q^{s_i}s_E). \quad (19)$$

Here

$$\theta_E = \tanh[\tfrac{1}{2}\beta(G_E - \mu_E)] \quad (20a)$$

$$\theta_{QE} = \tanh(\tfrac{1}{2}\beta G_{QE}), \quad (20b)$$

$$\theta_{QT,E} = \tanh(\tfrac{1}{2}\beta G_{QT,E}), \quad (20c)$$

and ω_E is the time scaling parameter for the transition $s_E \to -s_E$. The transition probability W_Q, equation (16), must be modified to include the coupling between the effector binding equilibrium and the quaternary conformational equilibrium and is given by

$$W_Q(q \to -q) = \omega_Q(1 + \theta_Q q)\left\{ \prod_{i=1}^{N} (1 - \theta_{QT}(\tilde{n})qs_i)\right\} (1 + q\tilde{n}\tanh(\beta G_{QE})), \quad (21)$$

where

$$\theta_{QT}(\tilde{n}) = \tanh\{\beta(G_{QT} + \tilde{n}G_{QT,E})\} \quad (22a)$$

and

$$\tilde{n} = (1 + s_E)/2. \quad (22b)$$

The transition probability W_{ST} remains unchanged in form provided θ_{QT} is replaced by $\theta_{QT}(\tilde{n})$. The time scaling parameter ω_{ST} of equation (13) may now depend on the presence of organic phosphates, in addition to its dependence on the quaternary conformation. The time scaling parameter ω_Q could depend on the presence of organic phosphates, and ω_E could depend on the quaternary conformation and on the number of bound substrates.

For other kinds of effectors, the form of $\delta\mathscr{E}$ is different from that given in equation (17) and the expressions for the transition probabilities, W, differ accordingly. However, the procedure outlined above is quite generally applicable to all types of effectors.

5. Relation to Mass-action Schemes

(a) General mass-action scheme for equivalent subunits

Ligand binding to hemoglobin, in the presence of varying concentrations of organic phosphates, may also be described by a mass-action scheme, as shown in Figure 2. From the master equation (eqn 18) one can obtain rate equations for average quantities such as $\sum_{i=1}^{4} \langle s_i \rangle$, $\sum_{i=1}^{4} \langle qs_i \rangle$, $\sum_i \sum_{j \neq i} \langle s_i s_j \rangle$, etc. These averages are linearly

(a)

(b)   (c)
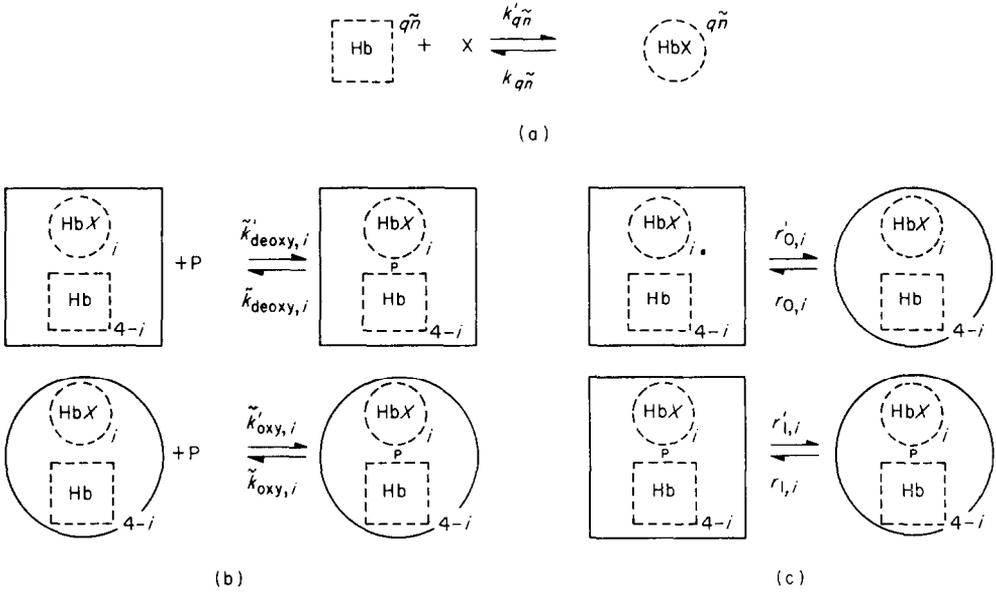
Fig. 2. Reaction of hemoglobin with substrate in the presence of organic phosphates, assuming equivalent subunits. The broken circles and squares denote, respectively, the oxy and deoxy tertiary conformations of a subunit, while the solid circles and squares denote the oxy and deoxy quaternary conformations of the hemoglobin tetramer. The assumption of induced-fit binding implies that the liganded subunits are in the oxy tertiary conformation and the unliganded subunits are in the deoxy tertiary conformation.

(a) The reaction of substrate, X, with an individual subunit of a hemoglobin molecule which is in the q quaternary conformation and has $\tilde{n}(\tilde{n} = 0,1)$ molecules of organic phosphate bound; $k'_{q,\tilde{n}}$ and $k_{q,\tilde{n}}$ denote, respectively, the on and off rate constants. (b) The reaction of organic phosphate, P, with the Hb tetramer which is in the q quarternary conformation and has i liganded subunits ($i = 0, 1, ..., 4$). $\tilde{k}'_{qi}$ and $\tilde{k}_{qi}$ denote, respectively, the on and off rate constants for the organic phosphate binding reaction. (c) The quaternary conformational transformation between the oxy and deoxy conformations of the hemoglobin tetramer with i-subunits liganded and $\tilde{n}$ bound molecules of organic phosphate; $r_{\tilde{n},i}$ and $r'_{\tilde{n},i}$ denote the rates for these transformations.

related to the concentrations of the variously liganded species produced in the reaction (Bansil, 1975). The 48 rate constants of the mass-action scheme may be given in term of our parameters, as follows:

(i) The rate constants for the ligand binding reaction are

$$k'_{q\tilde{n}} = \omega_{ST} K_{ST}(1 + q\theta_{QT}(\tilde{n})) \tag{23a}$$

$$k_{q\tilde{n}} = \omega_{ST}(1 - q\theta_{QT}(\tilde{n})), \tag{23b}$$

where

$$K_{ST}[S]_{free} = (1 - \theta_{ST})/(1 + \theta_{ST}). \tag{23c}$$

(ii) The effector binding reaction has the rate constants

$$\tilde{k}'_{qi} = \omega_E K_E(1 - q\theta_{QE})(1 + q\theta_{QT,E})^i(1 - q\theta_{QT,E})^{4-i} \tag{24a}$$

$$\tilde{k}_{qi} = \omega_E(1 + q\theta_{QE})(1 - q\theta_{QT,E})^i(1 + q\theta_{QT,E})^{4-i}, \tag{24b}$$

where

$$K_E[E]_{free} = (1 - \theta_E)/(1 + \theta_E). \tag{24c}$$

(iii) The quaternary conformational transition occurs with the rate constants

$$r_{\tilde{n}i} = \omega_{\mathbf{Q}}(1 + \theta_{\mathbf{Q}})(1 - \theta_{\mathbf{QT}}(\tilde{n}))^i(1 + \theta_{\mathbf{QT}}(\tilde{n}))^{4-i}(1 + \tilde{n}\tanh(\beta G_{\mathbf{QE}})) \tag{25a}$$

$$r'_{\tilde{n}i} = \omega_{\mathbf{Q}}(1 - \theta_{\mathbf{Q}})(1 + \theta_{\mathbf{QT}}(\tilde{n}))^i(1 - \theta_{\mathbf{QT}}(\tilde{n}))^{4-i}(1 - \tilde{n}\tanh(\beta G_{\mathbf{QE}})) \tag{25b}$$

where $\tilde{n}$ denotes the number of organic molecules bound to the hemoglobin tetramer ($\tilde{n} = 0$ or 1).

For a pure quaternary effector ($\theta_{\mathbf{QT,E}} = 0$) the only dependence of the rates for the effector binding reaction on the number of substrate molecules bound is due to the implicit dependence of $\omega_{\mathbf{E}}$ on i. We also note that if $\omega_{\mathbf{ST}}$ is independent of the binding of the effector then rates for substrate binding to an individual subunit (eqn 23) are not affected by the binding of the effector.

If the binding of the effector strengthens the quaternary constraints ($G_{\mathbf{QT,E}} > 0$ and $\theta_{\mathbf{QT}}(\tilde{n} = 1) > \theta_{\mathbf{QT}}(\tilde{n} = 0)$) but does not affect $\omega_{\mathbf{ST}}$, we note from equations (23a) and (23b) that the rate constant for substrate binding to an individual subunit of a molecule in the deoxy conformation decreases and the rate constant for substrate dissociation increases with increasing effector concentration. The reverse holds for a subunit of a molecule in the oxy quaternary conformation.

(b) *Adair scheme*

The reactions shown in Figure 2 reduce to an Adair scheme if *both* the quaternary conformational equilibria and the phosphate binding equilibria are established very rapidly as compared to the substrate binding equilibria. For this special case the Adair rate constants (defined on a per heme basis) are given by

$$k'_i = \sum_q \sum_{\tilde{n}} f_i^{q\tilde{n}} {}_1 k'_{q\tilde{n}} \qquad \text{and} \qquad k_i = \sum_q \sum_{\tilde{n}} f_i^{q\tilde{n}} k_{q\tilde{n}}. \tag{26}$$

Here $f_i^{q\tilde{n}}$ denotes the fraction of molecules with i-ligands bound which are in the q-quaternary conformation and have $\tilde{n}$ organic phosphate molecules bound.

The fractional concentrations are determined by the normalization requirement,

$$\sum_q \sum_{\tilde{n}} f_i^{q\tilde{n}} = 1, \tag{27}$$

by the requirement that the organic phosphate binding be in equilibrium,

$$f_i^{q1}/f_i^{q0} = [E]_{\text{free}} \,(\tilde{k}'_{qi}/\tilde{k}_{qi})$$
$$= [E]_{\text{free}} \,\tilde{K}_q[\exp(2\beta q G_{\mathbf{QT,E}})]^i, \tag{28}$$

and by the requirement that the quaternary conformational transition be in equilibrium,

$$f_i^{\text{deoxy}, \tilde{n}}/f_i^{\text{oxy}, \tilde{n}} = (r_{\tilde{n},i}/r'_{\tilde{n},i})$$
$$= K_{\mathbf{Q}}[\exp\{-4\beta(G_{\mathbf{QT}} + \tilde{n}G_{\mathbf{QT,E}})\}]^i\left(\frac{\tilde{K}_{\text{deoxy}}}{\tilde{K}_{\text{oxy}}}\right)^{\tilde{n}}. \tag{29}$$

Here

$$\tilde{K}_q = K_{\mathbf{E}}[\exp\{-q\beta(G_{\mathbf{QE}} + 4G_{\mathbf{QT,E}})\}] \tag{30}$$

is the equilibrium constant for organic phosphate binding to completely unliganded hemoglobin in the q quaternary conformation and

$$K_{\mathbf{Q}} = \exp\{\beta(G_{\mathbf{Q}} + 8G_{\mathbf{QT}})\} \tag{31}$$

is the quaternary conformational equilibrium constant for hemoglobin with no substrate or effector bound.

From equations (26) to (31) and (23) it can be seen that whereas the Adair scheme requires an *independent* set of 8 rate constants for *each* concentration of organic phosphate, our use of the master equation approach and the Perutz model allows us to describe the reaction kinetics at *all* concentrations of organic phosphate with *one* set of parameters; the total number of parameters ranges from a minimum of 6 (ω_{ST}, K_{ST}, G_{QT}, K_Q, $\tilde{K}_{oxy}$, $\tilde{K}_{deoxy}$) to a maximum of 10 ($\omega_{ST}(oxy, \tilde{n} = 0)$, $\omega_{ST}(oxy, \tilde{n} = 1)$, $\omega_{ST}(deoxy, \tilde{n} = 0)$, $\omega_{ST}(deoxy, \tilde{n} = 1)$, K_{ST}, G_{QT}, $G_{QT,E}$, K_Q, $\tilde{K}_{oxy}$, $\tilde{K}_{deoxy}$). In principle, the parameters $\omega_{ST}(oxy, \tilde{n} = 0)$, $\omega_{ST}(deoxy, \tilde{n} = 0)$, K_{ST}, G_{QT} and K_Q can be determined from kinetic data in the *absence* of organic phosphates. The five remaining parameters can be determined from the analysis of kinetic data at a *single* concentration of organic phosphate, all other conditions being the same as that for the experiment in the absence of organic phosphate. The complete set of parameters, thus determined, can be used to calculate the kinetics at *any other* concentration of organic phosphate.

(c) *Olson–Gibson scheme*

If the subunits are not all equivalent, as in the case of hemoglobin, which has two α and two β subunits, then the model parameters G_{QT}, $G_{QT,E}$ and ω_{ST}, and the mass-action parameters $k'_{q\tilde{n}}$ and $k_{q\tilde{n}}$, may be considered different for the two types of subunits.

Thus for reactions involving the α subunits, the Olson–Gibson (1972) rate constants are given by

$$k'^{\alpha}_{ij} = \sum_q \sum_{\tilde{n}} f^{q\tilde{n}}_{i-1,j} k'^{\alpha}_{q\tilde{n}} \tag{32a}$$

$$k^{\alpha}_{ij} = \sum_q \sum_{\tilde{n}} f^{qn}_{ij} k^{\alpha}_{q\tilde{n}}. \tag{32b}$$

Similarly for the β-subunits,

$$k'^{\beta}_{ij} = \sum_q \sum_{\tilde{n}} f^{q\tilde{n}}_{i,j-1} k'^{\beta}_{q\tilde{n}} \tag{33a}$$

$$k^{\beta}_{ij} = \sum_q \sum_{\tilde{n}} f^{q\tilde{n}}_{ij} \quad k^{\beta}_{q\tilde{n}}. \tag{33b}$$

Note that the k_{ij} have been defined on a per heme basis. To obtain the actual rate constant for a given step we have to multiply by the statistical factors (Olson & Gibson, 1972) corresponding to the number of hemes of a given type (α or β) available for oxygenation or deoxygenation.

In equation (32) the individual subunit rate constants $k'^{\alpha}_{q\tilde{n}}$ and $k^{\alpha}_{q\tilde{n}}$ appropriate to the α subunits can be obtained from equations (23a) and (23b) with the replacement of ω_{ST}, K_{ST}, $\theta_{QT}(\tilde{n})$ by ω^{α}_{ST}, K^{α}_{ST}, $\theta^{\alpha}_{QT}(\tilde{n})$, respectively. The $f^{q\tilde{n}}_{ij}$ now denote the fraction of molecules with i α- and j β-subunits liganded which are in the q-quaternary conformation and have $\tilde{n}$ organic phosphate molecules bound. These fractional concentrations can be determined in a manner exactly analogous to that used in section 5(b). Equations (28) and (29) can be applied to the present case if we recognize that there are two types of liganded subunits, so that we must replace iG_{QT} by the weighted average $(iG^{\alpha}_{QT} + jG^{\beta}_{QT})$ and $iG_{QT,E}$ by the corresponding quantity $(iG^{\alpha}_{QT,E} + jG^{\beta}_{QT,E})$. The equilibrium constants for the unliganded species are given by equations (30) and

(31) with G_{QT} and $G_{QT,E}$ replaced by the corresponding averages over the two kinds of subunits, namely $G_{QT} = (G_{QT}^\alpha + G_{QT}^\beta)/2$, $G_{QT,E} = (G_{QT,E}^\alpha + G_{QT,E}^\beta)2$.

Notice that whereas the Olson–Gibson mass-action scheme requires an *independent* set of 32 rate constants for each concentration of organic phosphate, our use of the master equation approach and the Perutz model allows us to describe the reaction kinetics at *all concentrations* of *organic phosphate with a single set of parameters*; the *total number of parameters ranging from a minimum of 9* (ω_{ST}^β, ω_{ST}^β, K_{ST}^α, K_{ST}^β, G_{QT}^α, G_{QT}^β, K_Q, $\tilde{K}_{oxy}$, $\tilde{K}_{deoxy}$) *to a maximum of 17* $\omega_{ST}^\alpha(\text{deoxy}, \tilde{n} = 0)$, $\omega_{ST}^\alpha(\text{deoxy}, \tilde{n} = 1)$, $\omega_{ST}^\alpha(\text{oxy}, \tilde{n} = 0)$, $\omega_{ST}^\alpha(\text{oxy}, \tilde{n} = 1)$, $\omega_{ST}^\beta(\text{deoxy}, \tilde{n} = 0)$, $\omega_{ST}^\beta(\text{deoxy}, \tilde{n} = 1)$, $\omega_{ST}^\beta(\text{oxy}, \tilde{n} = 0)$, $\omega_{ST}^\beta(\text{oxy}, \tilde{n} = 1)$, K_{ST}^α, K_{ST}^β, G_{QT}^α, G_{QT}^β, $G_{QT,E}^\alpha$, $G_{QT,E}^\beta$, K_Q, $\tilde{K}_{oxy}$, $\tilde{K}_{deoxy}$). Also note that in principle the parameters $\omega_{ST}^\alpha(q, \tilde{n} = 0)$, $\omega_{ST}^\beta(q, \tilde{n} = 0)$, K_{ST}^α, K_{ST}^β, G_{QT}^α, G_{QT}^β and K_Q can be determined from an analysis of ligand binding reactions in the absence of organic phosphates, and the remaining phosphate-related parameters $\omega_{ST}^\alpha(q, \tilde{n} = 1)$, $\omega_{ST}^\beta(q, \tilde{n} = 1)$, $G_{QT,E}^\alpha$, $G_{QT,E}^\beta$, $\tilde{K}_{oxy}$ and $\tilde{K}_{deoxy}$ can be determined from an analysis of the same reaction at a single concentration of organic phosphate.

In the following sections we will compare the results of our calculations with experimental data. We will show that in most cases the theoretical results obtained by using the minimal set of parameters are quite adequate to explain the data. For example, using only five parameters (considerably less than required in the Adair scheme) we can explain the deoxygenation kinetics over a wide range of P$_2$Glyc† concentrations and with only seven parameters (again considerably less than in the Olson–Gibson scheme) we can account for the dependence of the deoxygenation reaction over a wide range of IHP concentrations.

6. Ligand Binding to Deoxygenated Hemoglobin

In this section we apply the approach developed above to the stopped-flow kinetic data of (a) the reaction of hemoglobin with CO in the presence of HPT, a fluorescent analog of P$_2$Glyc and (b) the reaction of stripped and unstripped hemoglobin with O$_2$.

Here we assume that *both* the quaternary conformational equilibria and organic phosphate binding equilibria are established rapidly compared to the ligand binding equilibria.

(i) The justification for a rapid oxy $\rightarrow$ deoxy conformational transition comes from flash photolysis experiments. For example Gibson (1959*a*) finds the average time required for the oxy $\rightarrow$ deoxy transition is less than 1 ms at 20°C. McCray (1972) reports the conformational transition occurs in less than a few μs. All of these estimates are considerably faster than the effective time scale of the CO binding reaction ($\simeq$100ms; Gibson, 1959*b*) as well as the time scale for the O$_2$ binding reaction ($\simeq$10 ms; Gibson, 1970*a*).

(ii) The justification of rapid phosphate binding comes from the experimental observation that exactly the same kinetics are observed when P$_2$Glyc, IHP or HPT is mixed with hemoglobin in the stopped-flow apparatus as when hemoglobin is preincubated with the organic phosphate (Gibson, 1970*b*; Gray & Gibson, 1971; MacQuarrie & Gibson, 1972). This result implies that the reaction of these organic phosphates with both oxy and deoxy Hb must occur within the dead time of the stopped-flow apparatus (2 ms).

† See footnote to p. 89.

As a result of assumptions (i) and (ii), the reaction of Hb with O_2 (or CO) in the presence of organic phosphate can be described by an Adair scheme (if the subunits are considered equivalent) with the rate constants given by equations (23) and (26) to (31) or by the Olson–Gibson (1972) modified Adair scheme (if the subunits are considered inequivalent) with the rate constants given by equations (32) and (33).

(a) CO binding reaction in the presence of 8-hydroxy-1,3,6-pyrenetrisulfonate

Figure 3(a) shows the data of MacQuarrie & Gibson (1972) for the simultaneous measurement of Y_{CO}, the fractional saturation of Hb with CO (determined by the change in absorbance) and f_{HPT}, the fraction of HPT released (determined by the change in fluorescence). Thus

$$Y_{CO}(t) = \{C_1(t) + 2C_2(t) + 3C_3(t) + 4C_4(t)\}/4[Hb_4]_{total} \tag{34a}$$

$$f_{HPT}(t) = \{[HPT]_{free,t} - [HPT]_{free,t=0}\}/\{[HPT]_{free,t\to\infty} - [HPT]_{free,t=0}\} \tag{34b}$$

and $[HPT]_{free,t}$ is given by

$$[HPT]_{free,t} + \sum_{i=0}^{4} \{(f_i^{oxy,1} + f_i^{deoxy,1})C_i(t)\} = [HPT]_{total}. \tag{34c}$$
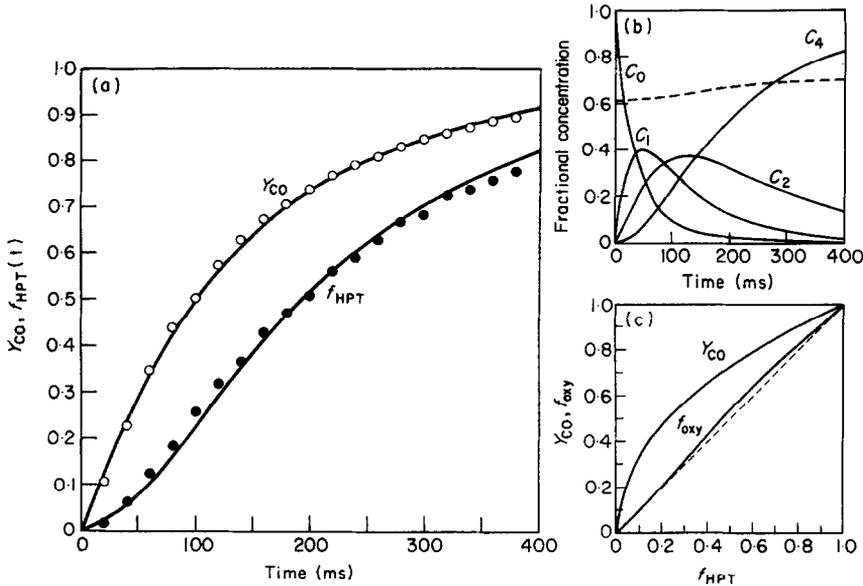
FIG. 3. (a) Comparison of calculated curves with experimental data (MacQuarrie & Gibson, 1972) for the kinetics of CO binding and HPT release from hemoglobin at pH 6·0 (50 mM-bis-Tris buffer), 20°C; [Hb] = 29·2 μM (by heme), [CO]$_{tot}$ = 46·0 μM and [HPT] = 1·0 μM. (○), Y_{CO}, the fractional saturation of Hb with CO; (●), f_{HPT}, the fraction of HPT released; (————) the best fit of the thereotical expressions obtained by solving the rate equations corresponding to the reaction scheme of section 5(b) with equivalent subunits ($\alpha = \beta$). The 5 parameters used are $(\omega_{ST}K_{ST})$ = 9·8 μM^{-1} s^{-1}, θ_{QT} = 0·9848, K_Q = 2·71×10^{12}, $\bar{K}_{oxy}$ = 52·6 mM^{-1} and $\bar{K}_{deoxy}$ = 91 mM^{-1}.

(b) Predicted time dependence of the concentrations of the various species produced in the reaction of (a). (————) Time dependence of C_i, the fraction of molecules with i CO molecules bound (i = 0, ..., 4) (C_i = [Hb$_4$(CO)$_i$]/[Hb$_4$]$_{total}$). (- - - -) [HPT]$_{free,\,t}$/[HPT]$_{total}$ calculated using the parameters of (a). [Hb$_4$]$_{total}$ = 7·3 μM and [HPT]$_{total}$ = 1·0 μM. Note that the fractional concentration, C_3, is less than 10^{-2} and cannot be displayed on the chosen scale.

(c) Relation of Y_{CO}, fractional saturation of Hb with CO, and f_{oxy}, fraction of molecules in the oxy quaternary conformation, with the fraction of HPT released. The calculated curves are based on the parameter values of (a).

Here $C_i(t)$ denotes the concentration of the species $Hb_4(CO)_i$ at time t and f_i^{q1} can be obtained from equations (28) and (29). Since in the reaction of CO with Hb the dissociation rates k_i can be neglected (Gibson & Roughton, 1957) the best least-squares fit to Y_{CO} and f_{HPT} simultaneously is achieved by varying a single set of five parameters ($\omega_{ST} K_{ST}$, θ_{QT}, K_Q, $\tilde{K}_{oxy}$, $\tilde{K}_{deoxy}$). The results are shown in Figure 3(a). The average accuracy of the fit is better than 1%. The values of the HPT binding constants $\tilde{K}_{oxy}$ and $\tilde{K}_{deoxy}$ for the best fit are in very good agreement with those obtained by MacQuarrie & Gibson (1972).

Figure 3(b) shows the time dependence of the concentrations $C_i (i = 0, \ldots 4)$ and the concentration of free HPT. The species $Hb_4(CO)_3$ is produced in very small quantities. Our calculation predicts that the species with two or less CO molecules bound are predominantly in the deoxy quaternary conformation both in the presence and absence of HPT.

Figure 3(c) shows Y_{CO} and f_{oxy}, the fraction of molecules in the oxy quaternary conformation, as a function of f_{HPT}. Notice that the release of HPT lags behind the binding of CO, and that the fraction of HPT released is almost equal to the fraction of molecules in the oxy quaternary conformation. Our calculation shows that $f_{oxy} = \{0.63[Hb_4(CO)_3] + [Hb_4(CO)_4]\}/[Hb_4]_{tot}$. Thus we see that HPT is released mainly from the molecules in the oxy quaternary conformation, and the switch to this conformation occurs at about the binding of the third CO molecule, giving rise to the observed lag between CO binding and HPT release.

For these data we find no significant improvement in fit on allowing for (i) dependence of ω_{ST} on q and $\tilde{n}$ (8 parameters), (ii) effects of HPT on the conformational constraints ($G_{QT,E} \neq 0$) (6 parameters), and (iii) inequivalence of subunits (7 parameters). However, it is possible that any dependence of ω_{ST} on $\tilde{n}$ and any effect of HPT binding on the conformational constraints would not be apparent because the data analyzed here are only for a *single* concentration of HPT. The result that the fit is unaffected by allowing for inequivalent subunits is consistent with the experimental finding (MacQuarrie & Gibson, 1972) that—so far as CO binding is concerned—the α and β subunits are functionally similar.

(b) O_2 binding reaction for stripped and unstripped hemoglobin

Figure 4(a) shows stopped-flow data of Gibson (1970a) for the reaction of O_2 with stripped hemoglobin. The theoretical curves were calculated by solving the differential equations corresponding to the Adair scheme for equivalent subunits under the assumption (i) of rapid conformational equilibria. The Adair rate constants are expressed in terms of the 4 parameters: ω_{ST}, K_{ST}, θ_{QT}, and K_Q, according to equations (23) and (26) to (31) with $[P]_{free} = 0$ and $\tilde{n} = 0$. For the calculation shown in Figure 4(a) the parameters ω_{ST} and K_{ST} were specified by requiring that the individual subunit rate constants in the oxy quaternary conformation, $k'_{oxy} = \omega_{ST} K_{ST}(1 + \theta_{QT})$ and $k_{oxy} = \omega_{ST}(1 - \theta_{QT})$, are fixed at the values $33.0 \ \mu M^{-1} s^{-1}$ and $12.5 \ s^{-1}$, respec-

at pH 7.0 (Gibson, 1970a). The solid curves are calculated using the parameters of (a) for *stripped* Hb and the parameters of (c) for *unstripped* Hb.

Note that the data points shown were generated using the rate constants and equilibrium constants reported by Gibson (1970a). Since the original data are not available, this procedure was adopted to eliminate the extrapolation to zero time for the stopped-flow data as well as to avoid the uncertainty associated with reading data from published curves. Since the calculation of Gibson agrees with the actual data to an accuracy of $\pm 0.78\%$ saturation, this procedure of comparing theoretical curves with calculated data points seems well justified.
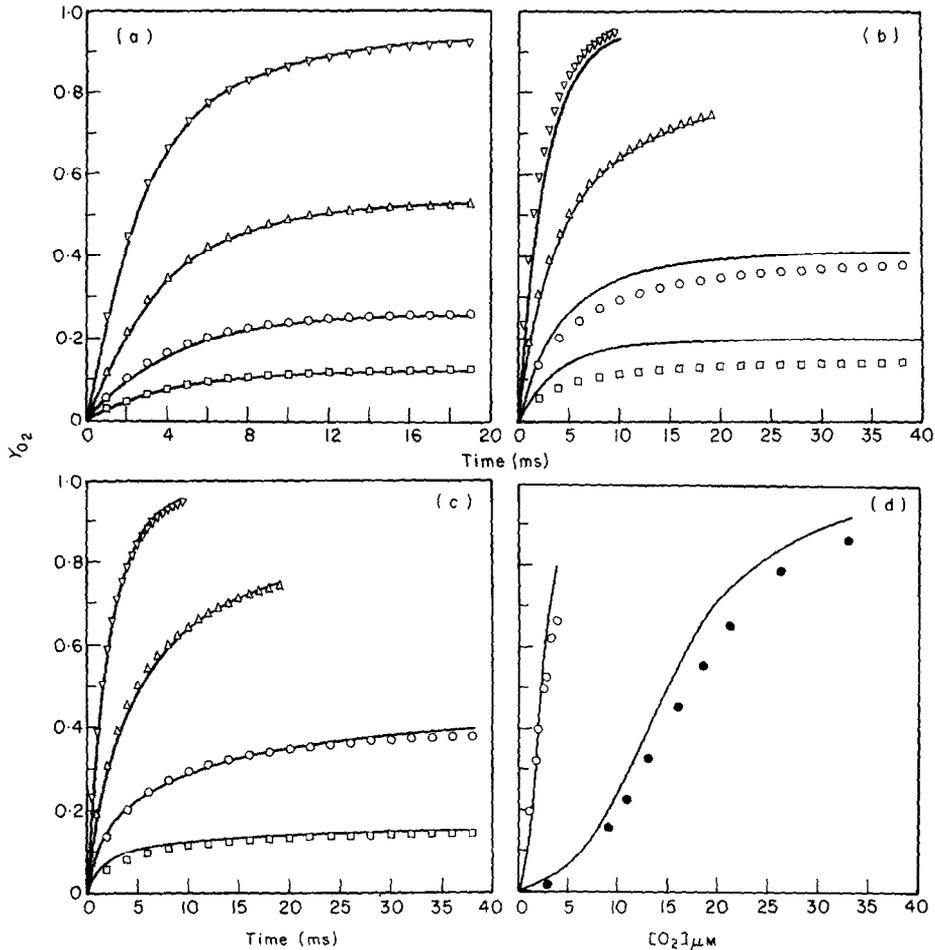
FIG. 4. The reaction of O_2 with stripped and unstripped hemoglobin (Gibson, 1970a) at pH 7·0, 20°C.

The data shown in (a) are for the reaction of *stripped* hemoglobin (53 μM in heme) with 62 μM-O_2 (—▽—▽—), 31 μM-O_2 (—△—△—), 15·5 μM-O_2 (—◯—◯—) and 7·8 μM-O_2 (—□—□—) in 0·05 M-bis-Tris buffer. The solid curves are obtained as discussed in section 6(b) by assuming identical subunits with the parameter values $\omega_{ST} = 60\cdot0$ s^{-1}, $K_{ST} = 0\cdot307$ μM^{-1}, $\theta_{QT} = 0\cdot793$ and $K_Q = 1\cdot469 \times 10^3$.

The data points of (b) and (c) are for the reaction of *unstripped* hemoglobin (41·5 μM in heme) with 124 μM-O_2 (—▽—▽—), 62 μM-O_2 (—△—△—), 31 μM-O_2 (—◯—◯—) and 15·5 μM-O_2 (—□—□—), in 0·1 M-phosphate buffer. The solid curves of (b) are calculated by considering the phosphates (P$_2$Glyc and inorganic phosphate) as a quaternary effector which also alters the strength of the conformational constraints (see text for discussion). The values of the parameters ω_{ST}, K_{ST}, $\theta_{QT}(\bar{n} = 0)$ and K_Q are the same values used in the calculation for stripped hemoglobin shown in (a). The three phosphate-related parameters are $\theta_{QT}(\bar{n} = 1) = 0\cdot8151$, $\tilde{K}_{oxy}$ [P]$_{total} = 0\cdot466 \times 10^{-2}$, $\tilde{K}_{deoxy}$ [P]$_{total} = 1\cdot359 \times 10^3$. The solid curves of (c) were calculated on the assumption that the binding of phosphates alters *both* the conformational constraints and the time scaling parameter, ω_{ST}(deoxy). The values of the parameters ω_{ST}(oxy) $= \omega_{ST}$(deoxy, $\bar{n} = 0$), K_{ST}, θ_{QT} ($\bar{n} = 0$) and K_Q are the same as those used in the calculation of (a). The four phosphate-related parameters are ω_{ST}(deoxy, $\bar{n} = 1$) $= 205\cdot8$ s^{-1}, $\theta_{QT}(\bar{n} = 1) = 0\cdot9319$, $\tilde{K}_{oxy}$[P]$_{total} = 0\cdot13 \times 10^{-3}$, and $\tilde{K}_{deoxy}$ [P]$_{total} = 1\cdot305 \times 10^3$.

The equilibrium oxygenation data in (d) are for *stripped* (◯) and unstripped (●) hemoglobin

tively (Gibson, 1959b,1970a); these numbers correspond to the "on" and "off" rate constants for the binding of the fourth O_2 molecule (expressed on a per subunit basis). Thus for the fit shown in Figure 4(a) there are *only two* variable parameters, θ_{QT} and K_Q.

For these data we find that allowing for a possible dependence of ω_{ST} on the quaternary conformation does not produce any further improvement in the fit. We note from equation (23) that if ω_{ST} were independent of the quaternary conformation then $k'_{oxy}/k'_{deoxy} = k_{deoxy}/k_{oxy}$. Since the completely unliganded species and the species with one ligand bound are almost exclusively in the deoxy quaternary conformation, the on and off rate constants for the first step of ligand binding (expressed on a per subunit basis) can be regarded as estimates of k'_{deoxy} and k_{deoxy}, respectively. Similarly, the species with 3 and 4 ligand molecules bound are predominantly in the oxy quaternary conformation and thus the on and off rate constants for the binding of the fourth O_2 molecule can be regarded as estimates of k'_{oxy} and k_{oxy}. Using the Adair rate constants determined by Gibson (1970a) one finds $k_{deoxy}/k_{oxy} = 10.9$ and $k'_{oxy}/k'_{deoxy} = 8.9$. The similarity of these two values supports our conclusion that ω_{ST} is independent of the quaternary conformation, at least to the experimental accuracy of the presently available data.

Figure 4(b) and (c) shows the stopped-flow data of Gibson (1970a) for the reaction of O_2 with *unstripped* hemoglobin in 0.1 M-phosphate buffer. Using the parameters ω_{ST}, K_{ST}, θ_{QT} and K_Q determined from the calculation for the reaction with stripped hemoglobin, we attempted to fit these unstripped data using the phosphate related parameters $\tilde{K}_{oxy}[P]$, $\theta_{QT}(\tilde{n}=1)$ and $\omega_{ST}(\tilde{n}=1)$. Here [P] denotes the free concentration of all phosphate (P_2Glyc and inorganic phosphate). Since this quantity is not exactly known for the data under consideration, we have to consider $\tilde{K}_{deoxy}[P]$ and $\tilde{K}_{oxy}[P]$ as the variable parameters in this case. Several calculations corresponding to different types of effects of phosphates—with different numbers of parameters—were performed. (i) The phosphates may be considered to alter only the quarternary equilibrium, (i.e. $G_{QT,E} = 0$ and $\omega_{ST}(\tilde{n}=1) = \omega_{ST}(\tilde{n}=0)$). This corresponds to the case of a pure quaternary effector and requires the *two* effector-related parameters, $\tilde{K}_{oxy}[P]$ and $\tilde{K}_{deoxy}[P]$. Since such an effector changes the parameter K_Q to $K_Q(1 + \tilde{K}_{deoxy}[P])/(1 + \tilde{K}_{oxy}[P])$ (Herzfeld & Stanley, 1974), for a single value of [P], we effectively need only *one* additional parameter, $(1 + \tilde{K}_{deoxy}[P])/(1 + \tilde{K}_{oxy}[P])$†. However, we could not achieve a satisfactory fit to the data for unstripped Hb by varying just this one additional parameter. We next re-fitted the stripped data simultaneously with the unstripped data by varying the three parameters K_Q, θ_{QT} and $(1 + \tilde{K}_{oxy}[P])/(1 + \tilde{K}_{deoxy}[P])$. ($\omega_{ST}$, K_{ST} are fixed as in the calculation of Figure 4(a).) This procedure also does not result in a satisfactory fit. (ii) The phosphates were considered to alter the conformational constraints, in addition to shifting the quaternary equilibrium, but are assumed not to affect the time scaling parameter, ω_{ST}. ($G_{QT,E} > 0$, $\omega_{ST}(\tilde{n}=1) = \omega_{ST}(\tilde{n}=0)$). Since in this case the conformational equilibria of the differently ligated species are shifted by different amounts (eqn 28), we need three effector-related parameters $\tilde{K}_{oxy}[P]$, $\tilde{K}_{deoxy}[P]$ and $\theta_{QT}(1)$. The results of this calculation for O_2 binding to unstripped hemoglobin are shown by the solid curves in Figure 4(b). We see that, whereas curves corresponding to high total O_2 concentrations agree reasonably well with the data, the curves corresponding to low

† Note that a single parameter can only be used if $[P]_{total}$ is high enough so that we can regard $[P]_{free}$ as constant in time. For the data under consideration this condition should hold.

O_2 concentrations do not. (iii) The phosphates were considered to affect the time scaling parameter for the deoxy quaternary conformation ω_{ST}(deoxy) but not alter either ω_{ST}(oxy) or the quaternary conformational constraints. $\{G_{QT,E} = 0, \omega_{ST}$(deoxy, $\tilde{n} = 1) \neq \omega_{ST}$(deoxy, $\tilde{n} = 0$), ω_{ST}(oxy, $\tilde{n}) = \omega_{ST}$(deoxy, $\tilde{n} = 0$)$\}$ again requiring three effector-related parameters $\tilde{K}_{oxy}[P]$, $\tilde{K}_{deoxy}[P]$ and ω_{ST}(deoxy, $\tilde{n} = 1$). This calculation, whose predictions are similar to those of Figure 4(b), corresponds to the assumption that, whereas in the absence of phosphates ω_{ST} is independent of the quaternary conformation, the binding of phosphate alters ω_{ST} for the deoxy conformation, leaving ω_{ST} for the oxy conformation unaltered. Since very little phosphate is bound to hemoglobin molecules in the oxy quaternary conformation our results are essentially unchanged on allowing for an effect of phosphate binding on ω_{ST} in the oxy quaternary conformation.

(iv) Finally, we consider the case where the phosphate binding, in addition to altering the quaternary equilibrium, alters *both* the conformational constraints, and the time scaling parameter ω_{ST}(deoxy). In this calculation (Fig. 4(c)) four of the eight parameters used, K_{ST}, θ_{QT}, K_Q, ω_{ST}(oxy, $\tilde{n} = 0) = \omega_{ST}$(oxy, $\tilde{n} = 1) = \omega_{ST}$(deoxy, $\tilde{n} = 0$), are held constant at the values determined from the calculation of Figure 4(a) and the four effector-related parameters $\tilde{K}_{deoxy}[P]$, $\tilde{K}_{oxy}[P]$, $\theta_{QT}(1)$ and ω_{ST}(deoxy, $\tilde{n} = 1$) are varied to obtain the best fit to the kinetic data for unstripped hemoglobin.

A comparison of the calculated curves of Figure 4(b) and (c) indicates that the effects of altering conformational constraints and the time scaling parameter ω_{ST}(deoxy) show up in the fit to low O_2 concentration data and consequently these effects influence primarily the earlier steps of the ligand binding reaction. Figure 4(d) shows the predicted *equilibrium* oxygenation curve for both stripped and unstripped hemoglobin *using the parameters obtained by fitting the kinetic data* of Figure 4(a) and (c). The predicted curves are compared with equilibrium data for stripped and unstripped Hb (Gibson, 1970a; Roughton & Lyster, 1965). The agreement between theory and experiment is somewhat better for stripped hemoglobin than for unstripped hemoglobin.

In the calculations shown in Figure 4 we consider the α and β subunits to be equivalent. Gibson (1970a) and Hopfield et al. (1971) have also analyzed the same data assuming equivalent subunits. However, the Adair approach used by Gibson (1970a) requires two sets of eight rate constants, one set for the reaction of O_2 with stripped Hb and the other set for the reaction of O_2 with unstripped Hb. In Gibson's calculations, the two rate constants corresponding to the last step of ligand binding were fixed at previously determined values. The Monod–Wyman–Changeux model used by Hopfield et al. (1971) requires five parameters to describe the reaction with stripped hemoglobin and three additional parameters (i.e. 8 total) to treat the reaction with unstripped hemoglobin. However, Hopfield et al. neglect the phosphate binding to the oxy quaternary conformation. We find that although $\tilde{K}_{oxy}$ is considerably smaller than $\tilde{K}_{deoxy}$, it is significantly greater than zero. Note that if in our model we were to neglect phosphate binding to the oxy conformation, we would have seven adjustable parameters (one less than Hopfield et al. require). Moreover, if [P] were known for the data of Figure 4(b), the parameters determined by us could be used to calculate the kinetic curves at any other phosphate concentration. Such an extension is not possible for the methods used by Gibson and by Hopfield et al.—they require a new set of rate constants for each phosphate concentration.
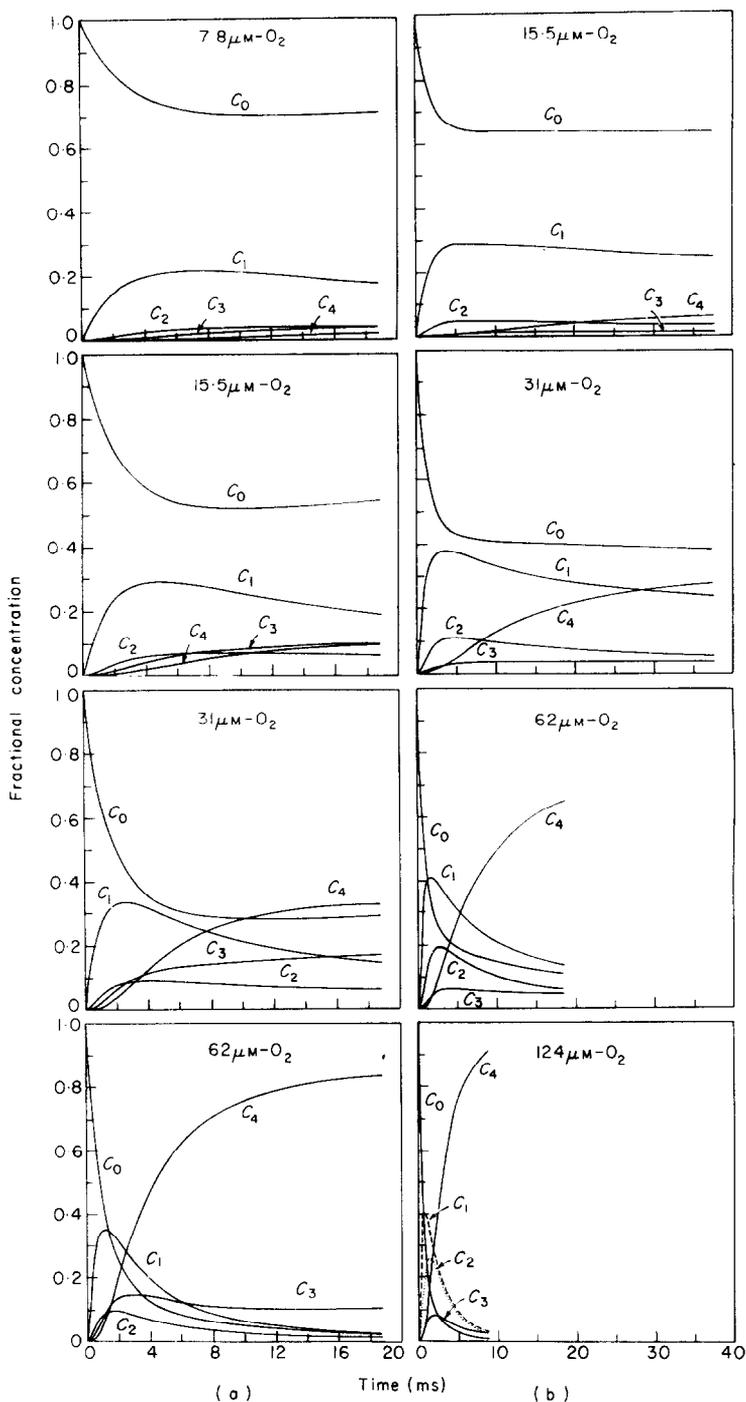
FIG. 5. The calculated time dependence of C_i, the fraction of molecules with i ligands bound ($i = 0, 1, ..., 4$), produced in the reaction of O_2 with stripped and unstripped hemoglobin, $C_i = [Hb_4(O_2)_i]/[Hb_4]_{total}$. The curves shown in (a) are calculated for the reaction of *stripped* Hb ($[Hb_4]_{total} = 13.25 \ \mu M$) with O_2 ($[O_2]$ is indicated in each panel) using the parameters of Fig. 4(a). The curves shown in (b) are calculated for the reaction of unstripped Hb ($[Hb_4]_{total} = 10.375 \ \mu M$) with O_2 using the parameters of Fig. 4(c).

Figure 5 shows the time dependence of the concentrations of the various species $Hb_4(O_2)_i$, $i = 0, 1, \ldots, 4$, produced in the reaction of stripped and unstripped hemo-globin with different concentrations of O_2. Notice that the contribution of the species with 0 and 1 molecules bound is much greater for the reaction at low O_2 concentration as compared to the reaction at high O_2 concentration. It is due to the relatively low contribution of these species to the reaction at high O_2 concentration that the oxygen-ation data at high O_2 concentrations can be reproduced well even if the combined effect of phosphates on the strength of the conformational constraints and the time scaling parameter $\omega_{ST}(\text{deoxy})$ is neglected (cf. Fig. 4(b)), since these effects primarily influence the rate constants k_1 and k_2.

7. Deoxygenation of Oxyhemoglobin in the Presence of Organic Phosphates

The data analyzed in this section are for deoxygenation of completely oxygenated hemoglobin by the addition of dithionite $(Na_2S_2O_4)$ in the presence of varying con-centrations of P_2Glyc and IHP. The observed rate of dissociation of oxygen is about 10 to 30 s^{-1} at 20°C (Gibson & Roughton, 1955; Salhany *et al.*, 1970; Gray & Gibson, 1971). Therefore, the quaternary conformational equilibrium may be assumed to be rapid compared to deoxygenation (as well as compared to oxygenation). The organic phosphate binding equilibria are also rapid (compared to deoxygenation) for P_2Glyc (Gibson, 1970b) and IHP (Gray & Gibson, 1971). In analyzing the dithion-ite deoxygenation experiments, we assume that dithionite reduces the effective con-centration of free O_2 to zero so rapidly that we need only consider the off rate con-stants k_i (k_{ij} if $\alpha \neq \beta$) as contributing to the reaction. When dithionite reacts with excess oxygen, the reaction rate is virtually independent of oxygen concentration (zero order in O_2 and first-order in $Na_2S_2O_4$; Lambeth & Palmer, 1973; Morello *et al.*, 1964). According to Lambeth & Palmer the zero order rate constant is 1·7 s^{-1} at pH 8·0 and 20°C, whereas Morello *et al.* obtain a value of 42 s^{-1} at 37°C and pH 13. Since the reaction of dithionite with oxygen is faster at pH 7·0 than at pH 8·0 (Hart-ridge & Roughton, 1923), we arrive at an approximate upper limit of 5 ms for the time during which O_2 is effectively reduced to zero for the data considered here.

(a) *Deoxygenation in the presence of 2,3-diphosphoglycerate*

Figure 6 shows the data of Salhany *et al.* (1970) for the deoxygenation of $Hb_4(O_2)_4$ by the addition of dithionite in the presence of varying concentrations of P_2Glyc. The data are plotted as the fractional saturation of hemoglobin with oxygen *versus* time. The solid curves represent the best least-squares fit to the data obtained by solving the rate equations corresponding to the reaction scheme of section 5(b) for equivalent subunits, with the rate constants given by equations (23) and (26) to (31). Since in the deoxygenation reaction we consider only the contribution of the off rate constants, the Adair rate constants at any concentration of P_2Glyc can be described in terms of only five parameters (K_Q, $\tilde{K}_{oxy}$, $\tilde{K}_{deoxy}$, ω_{ST}, θ_{QT}) if P_2Glyc is considered purely as a quaternary effector. If P_2Glyc affects the conformational constraints we need six parameters, because $\theta_{QT}(\bar{n} = 1) \neq \theta_{QT}(\bar{n} = 0)$. The solid curves shown in Figure 6(a) are calculated considering P_2Glyc to be a pure quaternary effector only. The five parameters are determined by fitting the theory to the data for the extreme curves, i.e. $[P_2Glyc] = 0$ and 0·5 mM. These *same* five parameters are then used to calculate the intermediate curves, $[P_2Glyc] = 0.05$ mM and 0·25 mM. In calculating
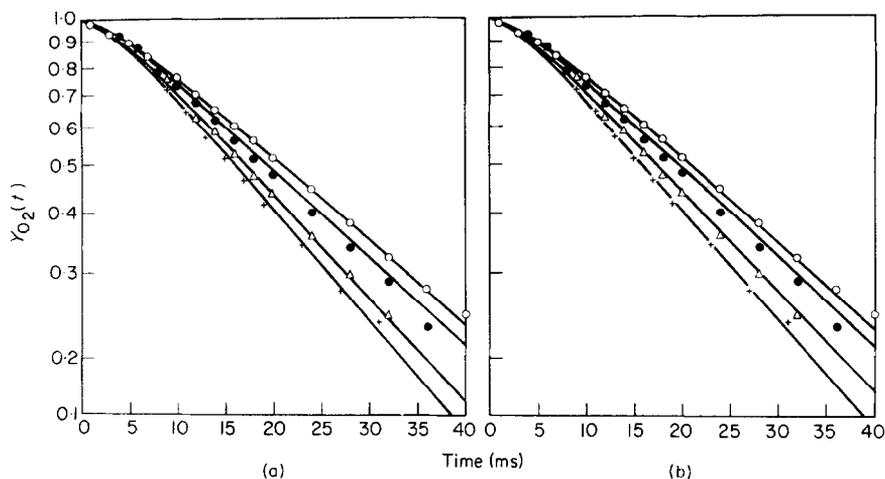
Fig. 6. A comparison of the calculated deoxygenation curves with the data of Salhany *et al.* (1970) for the deoxygenation of oxy Hb by dithionite in the presence of P_2Glyc at pH 7·0 (0·05 M-trishydroxymethylaminomethane·HCl buffer), 24°C, [Hb] $=$ 0·05 mM (by heme), $[Na_2S_2O_4]$ $=$ 0·1%, total $[P_2Glyc]$: (○) 0·0; (●) 0·05 mM; (△) 0·25 mM; and (+) 0·5 mM. The curves in (a) are calculated assuming identical subunits and considering P_2Glyc to be a quaternary effector only (see text) with $\omega_{ST} = 147·5$ s^{-1}, $\theta_{QT} = 0·9403$, $K_Q = 4·468 \times 10^9$, $\tilde{K}_{oxy} = 3·2$ mM^{-1}, $\tilde{K}_{deoxy}$ $= 21·3$ mM^{-1}.

The curves in (b) are calculated assuming identical subunits and considering P_2Glyc to alter *both* the quaternary conformational constraints and the kinetic parameter ω_{ST}(deoxy) in addition to being a quaternary effector. The 7 parameters are $\omega_{ST}(\tilde{n} = 0) = 137·0$ s^{-1}, ω_{ST}(deoxy, 1) $= 164·0$ s^{-1}, $\theta_{QT}(1) = 0·9420$, $\theta_{QT}(0) = 0·9305$, $K_Q = 8·684 \times 10^8$, $\tilde{K}_{oxy} = 0·753$ mM^{-1}, $\tilde{K}_{deoxy} = 32·28$ mM^{-1}.

the deoxygenation reaction with the intermediate concentrations of P_2Glyc we have taken into account the variation of $[P_2Glyc]_{free}$ with time. Thus the off rate constants decrease as the reaction proceeds. The agreement between the calculated curves and data is very good, giving us confidence in the predictive ability of our approach.

No improvement in the fit was obtained by considering that the binding of P_2Glyc, in addition to shifting the quaternary equilibrium, also alters the conformational constraints (6-parameter fit). Similarly no improvement in fit is achieved by considering the binding of P_2Glyc to affect the time scaling parameter, ω_{ST} (also a 6-parameter fit). or by considering the binding of P_2Glyc to affect both the conformational constraints and the parameter ω_{ST}(deoxy) (a 7-parameter fit). The latter fit is shown in Figure 6(b). Since the deoxygenation reaction is not very sensitive to the rates of dissociation of the last two O_2 molecules (i.e. to the rate constants k_1 and k_2 of the Adair scheme). and since the effects of altering the quaternary conformational constraints and varying ω_{ST}(deoxy) upon the binding of P_2Glyc are mainly exerted on k_1 and k_2, it is not surprising that the calculations shown in Figure 6(a) and (b) both agree equally well with the data.

For these data we find that allowing for inequivalent subunits (7 parameters) also does not lead to any further improvement in the fit. This feature will be further discussed in section (b), below.

(b) *Deoxygenation in the presence of inositol hexaphosphate*

Figure 7 shows the data of Gray & Gibson (1971) for the deoxygenation of $Hb_4(O_2)_4$ by dithionite in the presence of varying amounts of IHP. These data are also plotted
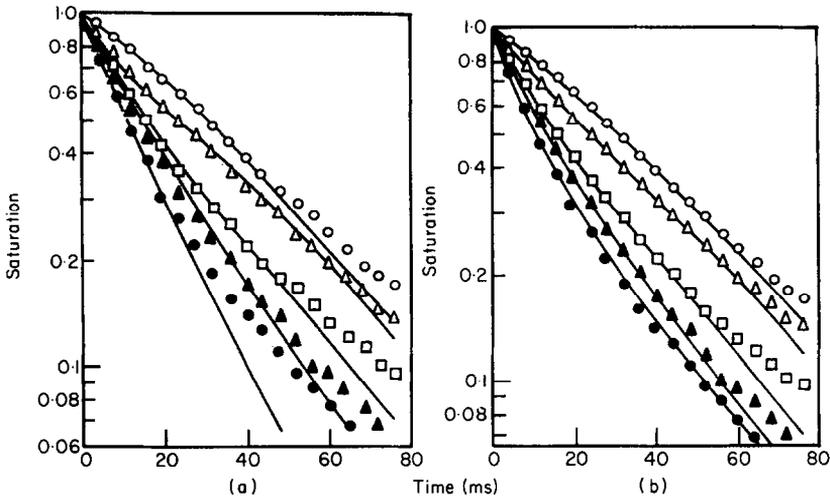
FIG. 7. Experimental data of Gray & Gibson (1971) for the deoxygenation of oxy Hb by dithionite in the presence of IHP at pH 7·0 (0·05 M-bis-Tris buffer), 22°C compared with the theoretical kinetic curves. The concentration of Hb is 25 μM, and that of dithionite 0·2%. Total [IHP]: ($\bigcirc$) 0·0; ($\triangle$) 3·0 μM; ($\square$) 6·0 μM; ($\blacktriangle$) 9·0 μM; ($\bullet$) 24·0 μM. The theoretical curves shown in (a) are calculated by considering equivalent subunits ($\alpha = \beta$) with the parameters $\omega_{ST} = 556\cdot0$ s^{-1}, $\theta_{QT} = 0\cdot9866$, $K_Q = 3\cdot08 \times 10^{11}$, $\tilde{K}_{oxy} = 0\cdot094$ μM^{-1}, $\tilde{K}_{deoxy} = 726\cdot1$ μM^{-1}.

The calculation shown in (b) allows for inequivalent subunits ($\alpha \neq \beta$). The theoretical parameters (see section 5(c) for discussion of parameters) are $\omega_{ST}^{\alpha} = 18$ s^{-1}, $\omega_{ST}^{\beta} = 1384$ s^{-1}, $\theta_{QT}^{\alpha} = 0\cdot9496$, $\theta_{QT}^{\beta} = 0\cdot9873$, $K_Q = 5\cdot327 \times 10^{10}$, $\tilde{K}_{oxy} = 0\cdot269$ μM^{-1}, $\tilde{K}_{deoxy} = 183\cdot1$ μM^{-1}.

as the fractional oxygenation of hemoglobin *versus* time. The calculated curves for the case of equivalent subunits ($\alpha = \beta$) are obtained by solving the rate equations corresponding to the reaction scheme of section 5(b), with the rate constants given by equations (23) and (26) to (31). When the subunits are considered to be inequivalent ($\alpha \neq \beta$) the appropriate equations are obtained from the reaction scheme of section 5 using equations (32) and (33).

Figure 7(a) compares the calculated curves for the case $\alpha = \beta$, where IHP is considered to be a pure quaternary effector, with the data. The five parameters ω_{ST}, K_Q, $\tilde{K}_{oxy}$, $\tilde{K}_{deoxy}$ and θ_{QT} were varied to achieve the best least-squares fit to the data. The agreement with the data is not very good and only slight improvement is obtained upon including the possibility that (i) IHP binding alters the conformational constraints (6 parameters), (ii) IHP binding alters the time scaling parameters, ω_{ST} (6 parameters), (iii) IHP binding alters both the conformational constraints and the time scaling parameters, ω_{ST} (7 parameters).

However, when we consider the possibility of inequivalent subunits ($\alpha \neq \beta$) a significant improvement is obtained (Fig. 7(b)). Here IHP is treated as a quaternary effector only. The seven parameters ω_{ST}^{α}, ω_{ST}^{β}, θ_{QT}^{α}, θ_{QT}^{β}, K_Q, $\tilde{K}_{oxy}$, $\tilde{K}_{deoxy}$ were determined by simultaneously fitting the deoxygenation data for all five concentrations of IHP. No further improvement of any significance is obtained by considering the effects of IHP binding on the quaternary conformational constraints and on the time scaling parameter, ω_{ST}.

These results suggest that the α and β subunits may be kinetically inequivalent with respect to the reaction with O_2 in the presence of IHP. That this inequivalence

TABLE 1

Summary of the parameters which are used to calculate the curves of Figures 3(a), 4(a), 4(c), 6 and 7(b)

Type of experiment / Reference	CO binding MacQuarrie & Gibson (1972)		O₂ binding Gibson (1970a)	O₂ dissociation Salhany et al. (1970)		O₂ dissociation Gray & Gibson (1971)
[Hb] μM (heme)	29.2	53.0	41.5	50		25
Organic phosphate	HPT	None	P₂Glyc + inorganic phosphate	P₂Glyc		IHP
Buffer	0.05 M-bis-Tris	0.05 M-bis-Tris	0.1 M-phosphate	0.05 M-THAM-HCl†		0.05 M-bis-Tris
pH	6.0	7.0	7.0	7.0		7.0
Temperature (°C)	20	20	20	24		22
Figure	3(a)	4(a)	4(c)	6(a)	6(b)	7(b)
Number of parameters	5	2 fixed, 2 varied	4 fixed, 4 varied	5	7	7
K_Q	2.7107×10^{12}	1.469×10^{3}	1.469×10^{3}	4.468×10^{9}	8.684×10^{8}	5.327×10^{10}
$\bar{K}_{oxy}$	52.6 mM^{-1}	—	$\bar{K}_{oxy}[P]_{total} = 0.13 \times 10^{-3}$	3.2 mM^{-1}	0.75 mM^{-1}	0.27 μM^{-1}
$\bar{K}_{deoxy}$	91.0 mM^{-1}	—	$\bar{K}_{deoxy}[P]_{total} = 1305.6$	21.3 mM^{-1}	32.3 mM^{-1}	183.0 μM^{-1}
θ_{QT}	0.9848	0.7929	$\theta_{QT}(0)$ 0.7929 / $\theta_{QT}(1)$ 0.9319	0.9403	$\theta_{QT}(0)$ 0.9305 / $\theta_{QT}(1)$ 0.9420	α 0.9496 / β 0.9873
K_{ST} (μM^{-1}) [$\omega_{ST}K_{ST} = 9.8$ μM^{-1} s^{-1}]	0.307 μM^{-1}	0.307 μM^{-1}	0.307 μM^{-1}	—	—	—
ω_{ST} (s^{-1})	60.0	60.0	$\omega_{ST}(oxy)$ 60.0 / $\omega_{ST}(deoxy, \tilde{n}=1)$ 205.8	147.5	$\omega_{ST}(oxy)$ 137.0 / $\omega_{ST}(deoxy, \tilde{n}=1)$ 164.1	18.27 / 1384.0
Root-mean-square error	0.0108 in Y_{CO} / 0.0173 in f_{HPT}	0.0057	0.0116	0.0105	0.0114	0.0133

Since these parameters are determined by fitting the theory to the data in general they are not a unique set. The stability of the parameter values was tested by different sets of "initial guesses" to start the optimization process. These calculations indicate that, whereas the parameters ω_{ST}, θ_{QT} and $\bar{K}_{deoxy}$ are well determined in each case, K_Q and $\bar{K}_{oxy}$ are not that well determined in general; however, for IHP and HPT the value of $\bar{K}_{oxy}$ is fairly stable.

For the calculation shown in Fig. 4 the parameters ω_{ST} and K_{ST} were held fixed by requiring that $k'_{oxy,0}$ and $k_{oxy,0}$ given by eqn (23), equal 33.0 μM^{-1} s^{-1} and 12.5 s^{-1}, respectively. Thus, for the calculation shown in Fig. 4(a), the variable parameters ω_{ST}, K_{ST}, $\theta_{QT}(oxy)$, K_{ST}, $\theta_{QT}(0)$ and K_Q were taken as θ_{QT} and K_Q. For the calculation of Fig. 4(b), $\omega_{ST}(oxy)$, K_{ST}, $\theta_{QT}(0)$ and K_Q were held constant at the values (determined by the calculation of Fig. 4(a)) and the 4 phosphate-related parameters were varied.

The root-mean-square (r.m.s.) error is defined as

$$\left\{ \sum_{i=1}^{n} |Y_i \text{(experimental)} - Y_i \text{(calculated)}|^2/n \right\}^{\frac{1}{2}},$$

where n denotes the total number of data points and Y_i the *fractional* oxygenation for the *i*th point. For the calculation of Fig. 3(a) the r.m.s. error in Y_{CO} and f_{HPT} is given separately although the parameters were determined by least-squares fit to both Y_{CO} and f_{HPT} simultaneously.

† THAM.HCl, trishydroxymethylaminomethane-HCl.

does not appear with P_2Glyc may be due to the fact that, whereas IHP affects the limiting rate constant in the deoxygenation reaction (i.e. $k_4 = (k_{22}^\alpha + k_{22}^\beta)/2$, the rate of dissociation of the first O_2 molecule from $Hb_4(O_2)_4$) P_2Glyc does not. In this analysis we have identified the subunit with the greater value of k_{22} as the β subunit, in accordance with the results of Olson *et al.* (1971).

The variation of $[IHP]_{free}$ during the course of the reaction is taken into account in the above calculations. The biphasic deoxygenation, seen when $[IHP]_{total}$ is comparable to $[Hb_4]_{total}$, occurs due to the fact that $[IHP]_{free}$ decreases as the reaction proceeds, causing the rate constants k_{ij} to decrease with time.
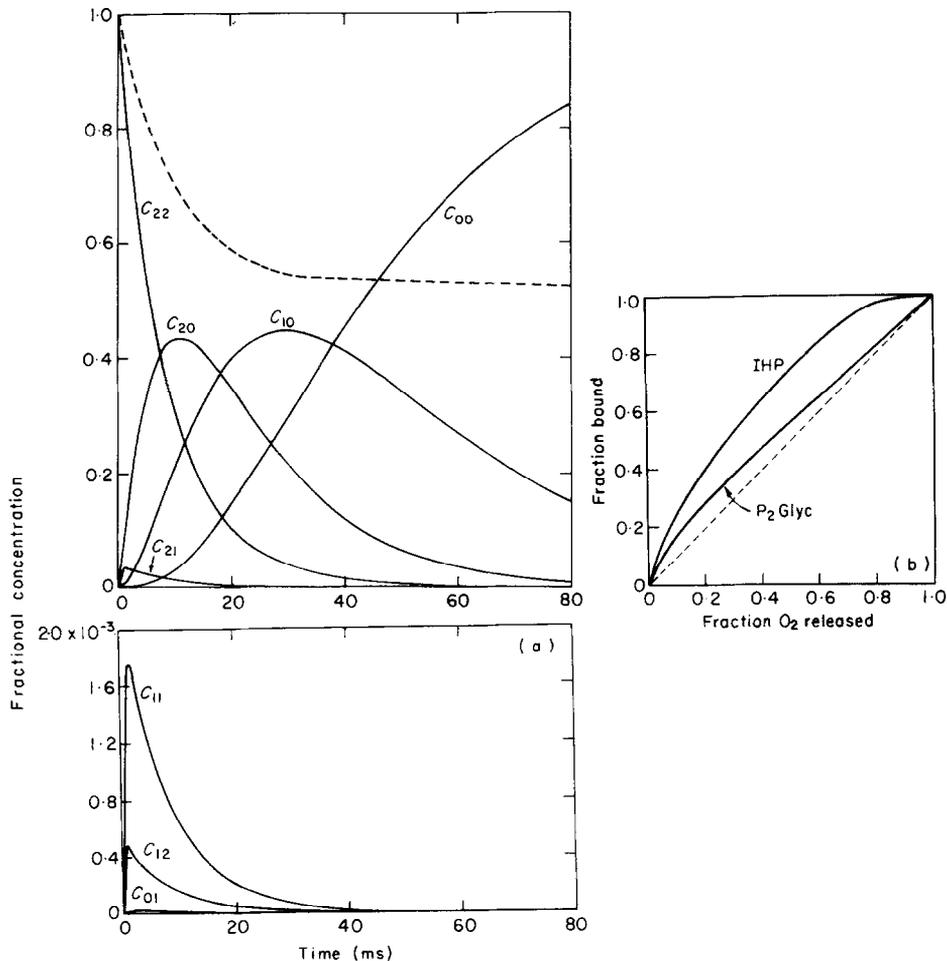
Fig. 8. (a) The calculated time dependence of C_{ij}, the fraction of molecules with i α-subunits and j β-subunits liganded, produced in the deoxygenation of oxy Hb ($[Hb_4]_{total} = 6 \cdot 25$ μM) by dithionite in the presence of $9 \cdot 0$ μM-IHP ($C_{ij} = [(\alpha X)_i \, (\beta X)_j]/[Hb_4]_{total}$). The parameter values used in this calculation are the same as those used in Fig. 7(b). (— — — —) Shows the variation of $[IHP]_{free}/([IHP]_{free \, at \, t=0})$ with time. C_{02} is less than 10^{-5} and cannot be displayed on either scale.

(b) The relation between the fraction of IHP bound, f_{IHP}, and the fraction of O_2 released, $(1 - Y_{O_2})$, calculated using the parameters of Fig. 7(b). Also shown is the fraction of P_2Glyc bound *versus* the fraction of O_2 released for the deoxygenation data shown in Fig. 6. The calculation is based on the parameters of Fig. 6(a).

TABLE 2

Fraction of molecules with i-liganded subunits which are in the deoxy quaternary conformation, f_i^{deoxy}, calculated using the parameters of Figures 3(a), 4(a) and (c), 6(a) and (b)

Figure	$[P]_{free}$	f_0^{deoxy}	f_1^{deoxy}	f_2^{deoxy}	f_3^{deoxy}	f_4^{deoxy}
3(a)	HPT = 0.70 μM	1.0000	1.0000	0.9999	0.3630	0.34×10^{-4}
4(a)	None	0.9993	0.9514	0.2072	0.0035	0.46×10^{-4}
4(c)	Total phosphate concentration not known, *unstripped* Hb in 0.1 M-phosphate buffer	1.0000	0.9999	0.9696	0.1162	0.56×10^{-3}
6(a)	$[P_2Glyc]_{free}$ = 0.0 mM	1.0000	1.0000	0.9993	0.6533	0.24×10^{-2}
6(a)	0.05 mM	1.0000	1.0000	0.9996	0.7723	0.40×10^{-2}
6(a)	0.25 mM	1.0000	1.0000	0.9999	0.8924	0.91×10^{-2}
6(a)	0.50 mM	1.0000	1.0000	0.9999	0.9263	1.36×10^{-2}

Figure	$[P]_{free}$	f_{00}	f_{01}	f_{02}	f_{10}	f_{11}	f_{12}	f_{20}	f_{21}	f_{22}
7(b)	$[IHP]_{free}$ = 0.0 μM	1.0000	1.0000	0.9889	1.0000	0.9993	0.0561	1.0000	0.4928	0.39×10^{-4}
7(b)	3.1 μM	1.0000	1.0000	1.0000	1.0000	1.0000	0.9486	1.0000	0.9967	0.12×10^{-1}
7(b)	5.3 μM	1.0000	1.0000	1.0000	1.0000	1.0000	0.9596	1.0000	0.9974	0.15×10^{-1}
7(b)	18.0 μM	1.0000	1.0000	1.0000	1.0000	1.0000	0.9710	1.0000	0.9982	0.22×10^{-1}

In the presence of phosphates, $f_i^{deoxy} = f_i^{deoxy,\,\bar{n}=0} + f_1^{deoxy,\,\bar{n}=1}$, where $f_i^{deoxy,\,\bar{n}}$ can be obtained from equations (27) to (29). Note that f_i^{deoxy} depends on $[P]_{free}$. If the subunits are considered inequivalent ($\alpha \neq \beta$) then the appropriate quantity, f_{ij}^{deoxy}, where i and j denote, respectively, the number of liganded α and β subunits (i,j = 0,1,2), can be obtained as outlined in section 5(c). Except for the data of Fig. 4, hemoglobin molecules with 2 or less hemes liganded are predominantly in the deoxy quaternary conformation, whereas the completely liganded molecules are primarily in the oxy quaternary conformation. Increasing the concentration of organic phosphate significantly increases f_3^{deoxy}. The value of f_2^{deoxy} for the data of Fig. 4(a) (for the reaction of O_2 with *stripped* hemoglobin) suggests an earlier point of cross-over to the oxy quaternary conformation.

The binding of IHP to the oxy conformation is significantly greater than that of P_2Glyc. The value of the binding constant $\tilde{K}_{oxy}$ obtained from the analysis of the deoxygenation data in the presence of IHP agrees with the experimental measurement of Gray & Gibson (1971), and the value of $\tilde{K}_{deoxy}$ is within the range of their estimate. The parameters of the theoretical curves shown in Figures 3(a), 4(a) and (c), 6(a) and (b), and 7(b) are summarized and discussed further in Table 1.

A close examination of Figure 7(b) shows that there is a systematic discrepancy between the calculated curves and the data near the end of the reaction. The data show the presence of a slow phase, which is not reproduced by the calculations For the hemoglobin concentration used in this experiment, approximately 10% of the oxyhemoglobin could be present as dimers in the absence of IHP (Gray & Gibson, 1971); the fraction of dimers would be less in the presence of IHP (Gray, 1974). Since we have not included the dissociation of hemoglobin, the observed discrepancy could be due to slow absorbance change associated with dimers (Kellett & Guttfreund, 1970). Although our model can be straightforwardly extended to include dissociation, good estimates of the parameters involved are not possible without data over a wide range of Hb concentrations.

Figure 8(a) shows the time dependence of $[IHP]_{free}$ and the concentrations of the variously liganded species produced in the deoxygenation reaction in the presence of IHP with the subunits considered inequivalent. Note that the concentrations of some of the intermediate species are very small compared to the fully liganded and fully unliganded species.

Figure 8(b) shows the fraction of IHP bound *versus* the fraction of O_2 released $(1 - Y_{O_2})$, calculated using the parameters of the fit shown in Figure 7(b). Also shown is the fraction of P_2Glyc bound *versus* $(1 - Y_{O_2})$ calculated using the parameters of Figure 6(a). Note that in the deoxygenation experiment O_2 release lags behind the binding of organic phosphate. The lag is more pronounced for IHP binding than for P_2Glyc binding. As discussed in section 6(a), most of the phosphate binding occurs *after* the switch to the deoxy quaternary conformation. Table 2 indicates that this switch occurs prior to the release of the second O_2 molecule, and occurs somewhat sooner in the presence of IHP than in the presence of P_2Glyc. Taken together these results indicate that the lag in the release of O_2 as compared with the binding of IHP or P_2Glyc is related to the switch to the deoxy quaternary conformation. Deoxygenation experiments using the fluorescent analog HPT could potentially verify this prediction.

8. Effect of Organic Phosphate on the Adair Rate Constants

The reaction scheme of section 5(a) reduces to the Adair (Gibson & Roughton, 1957) scheme if the conformational equilibria and the organic phosphate binding equilibria are rapid compared to the ligand binding equilibria. The eight rate constants of the Adair scheme at a given phosphate concentration, $[P]_{free}$, are given in terms of the parameters $\omega_{ST}(q, \tilde{n})$, K_{ST}, $\theta_{QT}(\tilde{n})$, K_Q, $\tilde{K}_{oxy}$, $\tilde{K}_{deoxy}$ and $[P]_{free}$ by equations (23) and (26) to (31). Similarly, as discussed in section 5(c), the 32 rate constants of the Olson–Gibson scheme at a given phosphate concentration can be expressed in terms of the parameters of the model and $[P]_{free}$ by equations (32) and (33).

Table 3 gives the Adair rate constants for the reaction of Hb with CO and for the

TABLE 3

Adair rate constants for the reaction of stripped and unstripped hemoglobin with O_2 and for the reaction of hemoglobin with CO in the presence of 8-hydroxy-1,3,6-pyrenetrisulfonate

Conditions	Ligand	$4k'_1$	$3k'_2$	$2k'_3$	k'_4	k_1	$2k_2$	$3k_3$	$4k_4$	Reference
			(μM^{-1} s^{-1})				(s^{-1})			
Stripped Hb pH 7·0	O_2	15·3	15·7	53·9	32·9	103	64·3	38·3	49·8	Fig. 4(a)
		14·7	35·2	15·8	33	136	15·7	138	50·0	Gibson (1970a)
Unstripped Hb in 0·1 M-phosphate pH 7·0	O_2	17·2	12·9	10·3	29·7	397	768	169	50·5	Fig. 4(c)
		17·7	33·2	4·9	33	1900	158	539	50·0	Gibson (1970a)
Stripped Hb + 1 μM-HPT pH 6·0	CO	0·600	0·450	0·304	12·5	—	—	—	—	Fig. 3(a)
		0·478 ±0·05	1·06 ±0·25	0·286 ±0·006	6·0	—	—	—	—	MacQuarrie & Gibson (1972)

Under the assumption of rapid conformational equilibria and rapid organic phosphate binding equilibria the reaction scheme shown in Fig. 2 reduces to the Adair scheme (section 5(b)). The Adair rate constants for the reactions of Figs 3 and 4 are calculated by substituting the parameters of Figs 3 and 4 in eqns (26) to (29) and (23). These values are compared with the previously published rate constants obtained by directly fitting the data to the Adair scheme.

reaction of O_2 with stripped and unstripped hemoglobin, calculated using the para-
meters of the fits shown in Figures 3(a), 4(a) and (c). We see from Table 3 that the
presence of phosphates decreases the on rate constants, increases the off rate con-
stants (and decreases the equilibrium constants), in agreement with previous results
(Gibson, 1970a; Tyuma et al., 1973). The rate constant k_4 is essentially unaltered
while k'_4 decreases slightly in the presence of phosphate. The largest effect, according
to our calculation, is on the rate constants k'_3, k_3 and k_2. k'_1 and k'_2 are only slightly
altered—actually k'_1 increases slightly in the presence of phosphates. We find that k_2
is affected the most, whereas Gibson (1970a) obtained the greatest effect on k_1. The
significant decrease of k'_3 and increase of k_2 is related to the fact that for these data
the binding of phosphates has the greatest effect on the conformational equilibrium
of the species with two ligands bound, as can be seen from Table 2.

Figure 9 shows the dependence of the dissociation rate constants, k_i, on $[P_2Glyc]_{free}$.
The calculation shown in Figure 9(a) uses the parameters obtained by treating P_2Glyc
purely as a quaternary effector; Figure 9(b) corresponds to the case where P_2Glyc
alters the conformational constraints and ω_{ST}(deoxy) in addition to shifting the
quaternary equilibrium. An examination of Figure 9(a) shows that for a quaternary
effector the rate constants k_1 and k_2 are essentially unaltered by increasing the
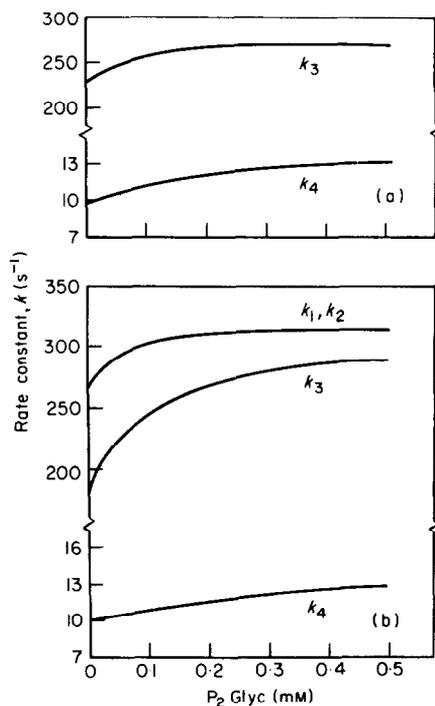
FIG. 9. Effect of P_2Glyc on the rate constants of the Adair scheme. Shown in (a) is the dependence
of the rate constants k_3 and k_4 of the Adair scheme on $[P_2Glyc]_{free}$ calculated on the assumption
that P_2Glyc is only a quaternary effector. The parameters used are those of Fig. 6(a). As discussed
in the text, the rate constants k_1 and k_2 are not significantly altered if P_2Glyc is considered purely
as a quaternary effector ($k_1 = k_2 = 286$ s^{-1}). Shown in (b) is the dependence of k_1, k_2, k_3 and k_4
on $[P_2Glyc]_{free}$ calculated using the parameters of Fig. 6(b). In this calculation the binding of
P_2Glyc, in addition to stabilizing the deoxy quaternary conformation, also strengthens the
quaternary conformational constraints ($\theta_{QT}(1) > \theta_{QT}(0)$) and increases the time-scaling parameter
ω_{ST}(deoxy). Note that all rate constants are expressed on a per heme basis.

concentration of P_2Glyc; k_3 is affected the most and k_4 increases slightly. Since $f^0_{\text{deoxy}} = f^1_{\text{deoxy}} = f^2_{\text{deoxy}} = 1$ (cf. Table 2), the binding of P_2Glyc cannot further increase the fractions of these species in the deoxy quaternary conformation and thus will not affect the rate constants associated with these species. However, when we consider P_2Glyc to alter the conformational constraints and ω_{ST}(deoxy) then the individual subunit dissociation rates $k_{q\tilde{n}}$ are themselves affected by P_2Glyc binding (eqn 23). In this case k_1 and k_2 also increase with increasing phosphate concentration, as shown in Figure 9(b). However, in both cases the dissociation rate constant most affected by P_2Glyc binding is k_3, the rate of dissociation of the second O_2 molecule. This result is related to the fact that the quaternary conformational equilibrium of the species with three ligands bound is affected the most by P_2Glyc.

Figure 10(a) shows the dependence of the rate constants k^α_{ij} and k^β_{ij} on $[\text{IHP}]_{\text{free}}$. Unlike P_2Glyc, IHP has a significant effect on k^β_{22}. The largest effect of IHP is on the off rate for the release of the second O_2 molecule from fully liganded Hb (i.e. k^α_{21}, k^α_{12}, k^β_{21}, k^β_{12}). In the case of IHP, with inequivalent subunits ($\alpha \neq \beta$), the increase is
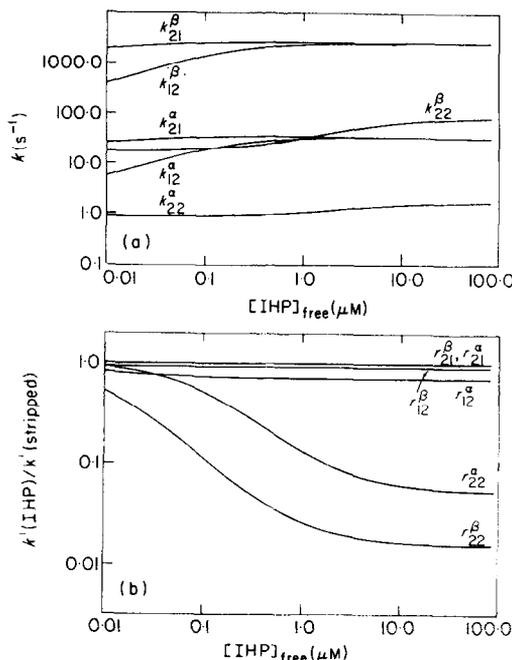
FIG. 10. Effect of IHP on the rate constants of the Olson–Gibson scheme with inequivalent subunits.

(a) The effect of increasing $[\text{IHP}]_{\text{free}}$ on the off rate constants k_{22}, k_{21}, and k_{12} for the α and β subunits calculated using the parameters of Fig. 7(b). The subunit with the greater value of k_{22} is identified as the β-subunit in accordance with the results of Olson et al. (1971). In this calculation IHP was considered purely as a quaternary effector and thus the off rates from species with at most 2 hemes liganded are not significantly affected by IHP; $k^\alpha_{10} = k^\alpha_{20} = k^\alpha_{11} = 35.6$ s^{-1}; $k^\beta_{01} = k^\beta_{02} = k^\beta_{11} = 2750$ s^{-1}. As discussed in the test the values of k_{ij} ($i + j \leq 2$) cannot be determined very accurately from an analysis of deoxygenation data.

(b) The calculated variation in the ratio $r^\alpha_{ij} \equiv k'^\alpha_{ij}(\text{IHP})/k'^\alpha_{ij}$ (IHP = 0), and $r^\beta_{ij} \equiv k'^\beta_{ij}(\text{IHP})/k'^\beta_{ij}$ (IHP = 0) with $[\text{IHP}]_{\text{free}}$, for $i + j \geq 3$. This prediction should be amenable to experimental verification. As in Fig. 9 all rate constants are expressed on a per subunit basis.

greatest for dissociation from the species with one α and two β subunits liganded (i.e. on k_{12}^{α} and k_{12}^{β}). It is the dependence of k_{22}, k_{21} and k_{12} on [IHP]$_{\text{free}}$ which causes biphasic deoxygenation when [IHP]$_{\text{free}}$ decreases significantly during the reaction. If IHP does not alter the quaternary conformational constraints and the kinetic parameter ω_{ST} then k_{10}^{α}, k_{01}^{β} would not be significantly altered by IHP. Hence dependence of these rate constants (and the equilibrium constants K_{10}^{α}, K_{01}^{β}) on IHP concentration would indicate an effect of IHP on the strength of the quaternary constraints and the parameter ω_{ST}. However, the deoxygenation experiment is not very sensitive to the rate constants k_{ij}, $i + j \leq 2$, since the species $(\alpha x)_i (\beta x)_j$ with $i + j \leq 2$ are produced in relatively small quantities, and these rate constants are at least an order of magnitude larger than k_{22}^{α} and k_{22}^{β}. Thus, whether or not there is an effect of IHP on the conformational constraints can only be determined from an analysis of O_2 binding data at low O_2 concentrations in the presence of varying amounts of IHP.

Figure 10(b) shows the predicted dependence of the ratio $r_{ij}^{\alpha} \equiv k_{ij}^{\prime \alpha}(\text{IHP})/k_{ij}^{\prime \alpha}$ (IHP $= 0$), (and r_{ij}^{β}) on [IHP]$_{\text{free}}$. This ratio is independent of the parameter K_{ST}^{α} (cf. eqn (23)) and can thus be calculated from the parameters obtained by an analysis of deoxygenation data even though the on rate constants do not contribute to this reaction. The decrease of the on rate constants in the presence of IHP agrees with the results of Gray & Gibson (1971) and Gibson (1970a). The effect of IHP on k_4' has been measured by Gray & Gibson (1971) and our predicted result agrees qualitatively. An analogous calculation was also done for the effect of P_2Glyc on the on rate constants using the parameters determined by fitting the deoxygenation data of Salhany et al. (1970) shown in Figure 6. We find that contrary to the prediction of Gibson (1970a), k_4' also decreases in the presence of P_2Glyc, although the decrease is less than in the presence of IHP. Our calculation shows that k_4' will be affected by P_2Glyc if the binding of P_2Glyc increases f_3^{deoxy}, the fraction of molecules which are in the deoxy quaternary conformation and have three ligands bound.

9. Concluding Discussion

We have seen that a kinetic model for hemoglobin based upon statistical mechanics and upon the qualitative description suggested by Perutz (1970a,b) agrees well with a wide variety of kinetic data (Gibson, 1970a; MacQuarrie & Gibson, 1972; Salhany et al., 1970; Gray & Gibson, 1971). Incorporation of specific homotropic and heterotropic interactions and use of the master equation formulation considerably reduce the number of independent parameters that enter into the model, and at the same time retain such important features of the Hb molecules as inequivalence of subunits and differences in tertiary and quaternary conformation.

Whereas the Adair scheme requires a different set of eight rate constants for each phosphate concentration, and the method of Tyuma et al. (1973), when extended to kinetics, would require 21 parameters, our approach describes kinetic data over a range of phosphate concentrations using a total of only six to eight parameters. For example, we fitted the data of Salhany et al. (1970) shown in Figure 6 with five adjustable parameters, whereas the Adair scheme requires 20 adjustable parameters (4 dissociation rate constants for each value of P_2Glyc). The five parameters were determined by fitting data at [P_2Glyc] $= 0$ and 0.5 mM. The fact that these same parameters, when used to calculate the curves at intermediate [P_2Glyc], are in good

agreement with the data, may be regarded as a test of the predictive ability of our approach. Allowing for inequivalent subunits increases the number of rate constants in the modified Adair scheme to 32 for *each* phosphate concentration, whereas our approach requires a total of 9 to 13 parameters (7 of which can be determined from data in the absence of phosphate).

In the absence of organic phosphates the Perutz model used here to describe the kinetics of Hb is mathematically equivalent to the Monod–Wyman–Changeux model used by Hopfield *et al.* (1971). In order to explain data for unstripped Hb, Hopfield *et al.* chose a different set of values for K_Q, k'_{deoxy} and k_{deoxy} (L, $^Tk'$ and ^Tk in their notation). Since the quaternary equilibrium depends on the free phosphate concentration the analysis of Hopfield *et al.* not only would require a different value of K_Q for each total phosphate concentration but also would be inapplicable in cases where $[P]|_{free}$ changes considerably during the course of the reaction (i.e. when $[P]_{total}$ is comparable to $[Hb_4]_{total}$). It would also not apply to those organic phosphates (e.g. IHP) which bind significantly to the oxy, as well as the deoxy, quaternary conformation. Furthermore, the Monod–Wyman–Changeux model as used by Hopfield *et al.* does not include the case where organic phosphate binding alters the quaternary conformational constraints; our analysis shows that the constraint effect is particularly important in analyzing kinetic data for unstripped Hb.

Chay & Brillhart (1974a,b) have analyzed equilibrium and kinetic data in terms of a model which presumes a P_2Glyc-induced inequivalence of the α and β subunits. This model has not been adequately tested since only a small and selected set of data was considered. Specifically, Chay & Brillhart only attempted to fit the kinetic data we show in Figure 4. This choice is particularly unfortunate because, in addition to being a limited choice of data, the data are only for stripped and unstripped hemoglobin (i.e. at zero phosphate concentration and at one non-zero unspecified value). It is crucial to test any model of P_2Glyc action against data taken over a wide range of P_2Glyc concentrations, especially including intermediate concentrations where $[P_2Glyc]_{free}$ changes significantly during the reaction (cf. Figs 5 and 6 of Herzfeld & Stanley (1974) and Figs 6 and 7 above). Another crucial test of their model would have been to fit the equilibrium data of Heustis & Raftery (1972) for the dependence of P_2Glyc binding on oxygen levels. Until the Chay–Brillhart model is tested against these existing data, it is impossible to judge the merit of the model.

The use of the master equation formulation introduces a major simplification by factoring the rate constants into a kinetic factor and a purely equilibrium factor. This allows one to assess separately the kinetic and equilibrium aspects of any effect. Since the parameters are related to the molecular properties of the protein, one can utilize data taken under a limited range of conditions to predict the behavior over a wide range of conditions.

In sections 6 and 7 we have analyzed the effect of the organic phosphates P_2Glyc and IHP (and the fluorescent analog, HPT) on Hb kinetics. These calculations show that the primary cause of the observed decrease in the overall combination rate and increase in the overall deoxygenation rate is the preferential binding of the organic phosphates to the deoxy quaternary conformation of the Hb molecule. The preferential binding of organic phosphate to the deoxy quaternary conformation is a well established fact (Benesch & Benesch, 1974, and references therein). In addition we find that binding of P_2Glyc strengthens the quaternary conformational constraints and also increases the time scaling parameter $\omega_{ST}(deoxy)$. Whereas the strengthening

of the conformational constraints has previously been found from an anlaysis of equilibrium data (Herzfeld & Stanley, 1974), the increase in the time scaling parameter $\omega_{ST}(\text{deoxy})$ is a purely kinetic effect. Both of these features primarily influence the rate constant for the earlier steps in the ligand binding reaction and are thus important in analyzing reactions at low oxygen concentrations. We suggest that experiments designed specifically to measure the rate constants k_1', k_1 (and the equilibrium constant K_1) of the Adair scheme, in the presence of varying concentrations of organic phosphates, will bear directly on the question of the strengthening of conformational constraints and increase of $\omega_{ST}(\text{deoxy})$. Our results suggest that the inequivalence of subunits ($\alpha \neq \beta$) is particularly important in analyzing kinetic data in the presence of IHP. Since we had explicitly included the dependence of the rate constants on $[\text{P}]_{\text{free}}$ our calculations can also account for the biphasic deoxygenation observed in the presence of IHP when $[\text{IHP}]_{\text{total}}$ is comparable to $[\text{Hb}_4]_{\text{total}}$.

The lag observed by MacQuarrie & Gibson (1972) between the binding of CO and release of HPT agrees very well with the predictions of our model (cf. section 6(a)). This lag—and also the analogous effects predicted by the analysis of deoxygenation data in the presence of P_2Glyc and IHP (cf. Fig. 8(b))—is consistent with the possibility that the switch from the deoxy to oxy quaternary conformation occurs approximately when the *third* ligand molecule binds (Heustis & Raftery, 1972; Gibson & Parkhurst, 1968; Hopfield *et al.*, 1971; Herzfeld & Stanley, 1974). The change in the quaternary conformation occurs somewhat later in the presence of organic phosphates. It is this increase in the fraction of molecules which have three ligands bound and are in the deoxy quaternary conformation which gives rise to the marked increase in k_3 in the presence of organic phosphates. However, the exact point of crossover can occur at a different state of ligation, depending on the experimental conditions (Hopfield *et al.*, 1971); for example, the O_2 binding data analyzed in section 6(b) suggests that for stripped hemoglobin there exists a substantial fraction of molecules in the oxy quaternary conformation with only two ligands bound.

The dependence of the Adair (Olson–Gibson for $\alpha \neq \beta$) rate constants on $[\text{P}]_{\text{free}}$ has been calculated for both P_2Glyc and IHP. Our results confirm the prediction of Gray & Gibson (1971) that IHP affects the rate constant for the dissociation of the first oxygen molecule, k_4, while P_2Glyc does not. The presently available experimental methods can only measure the effect of phosphates on k_4 and on the overall rate of deoxygenation. Our analysis predicts that both P_2Glyc and IHP have their strongest effect on the rate of dissociation of the second O_2 molecule, k_3. In the case of IHP with $\alpha \neq \beta$, we find that the rates for dissociation from β subunits are increased somewhat more than those for dissociation from the α subunits. The calculated values of k_4 and k_{22}^β agree well with those determined by Gibson (1970a) and Olson *et al.* (1971). We feel that, whereas k_4 and k_3 (k_{22}, k_{21} and k_{12} for $\alpha \neq \beta$) are well determined from our analysis of deoxygenation data, the values for the rate constants k_1, k_2 (k_{ij}, $i + j \leq 2$) can only be regarded as estimates, since the deoxygenation reaction is not very sensitive to these rate constants. Using the parameters obtained from the analysis of the deoxygenation experiments, we have calculated the variation of the ratio k_i' (organic phosphates)/k_i' (stripped) with increasing phosphate concentration; this ratio decreases, in agreement with the results of Gibson (1970a). The experimental observation of Gray & Gibson (1971) that k_4' is affected by IHP is borne out by the calculation. However, contrary to the results of Gibson (1970a) we also find a slight decrease in k_4' with increasing P_2Glyc concentration. To test these predictions, as

well as the constraint effect, an analysis of a series of O_2 binding reactions, similar to those measured by Gibson (1970a) for stripped and unstripped hemoglobin, at different organic phosphate concentrations, would be very useful—particularly because the O_2 binding reaction is potentially capable of yielding more information and is also free from any complications due to the side effects of dithionite.

The calculated values of the organic phosphate binding constants $\tilde{K}_{oxy}$ and $\tilde{K}_{deoxy}$ for HPT and IHP are in good agreement with those determined by MacQuarrie & Gibson (1972) and Gray & Gibson (1971). The value of $\tilde{K}_{deoxy}$ for P_2Glyc agrees well with that obtained from the analysis of equilibrium data by Tyuma et al. (1973). Although we have not performed extensive calculations to test the sensitivity of the model parameters, our comparison of parameter values obtained by using different initial values to start the process of minimization suggest that the parameters θ_{QT}, $\tilde{K}_{deoxy}$, ω_{ST} and K_{ST} are better determined than K_Q and $\tilde{K}_{oxy}$. (However, for IHP and HPT, $\tilde{K}_{oxy}$ was fairly well determined.)

The chief merit of this statistical mechanical approach lies in the systematic manner in which the detailed model can be modified to include other types of interaction. In this work we have considered the effect of organic phosphates in considerable detail. An analogous study of the kinetics of the Bohr effect (Bohr et al., 1904) could be performed based on the formalism developed in section 4.

APPENDIX

General Form of the Transition Probability

The principle of detailed balance (eqn (4)) can be used to obtain a general solution for the transition probability per unit time. For the transition $s_i \to -s_i$, involving the change in the substrate occupancy variable of the ith site, we have

$$\frac{W_{ST}(s_i \to -s_i)}{W_{ST}(-s_i \to s_i)} = \frac{P_{eq}(s_1, s_2, \ldots, -s_i, \ldots, s_N; q)}{P_{eq}(s_1, s_2, \ldots, s_i, \ldots, s_N; q)}. \tag{A1}$$

The above equation implies that if $W_{ST}(s_i \to -s_i)$ is expressed as a function of the variable s_i, $f(s_i)$, then $W_{ST}(-s_i \to s_i)$ must be the same function of $-s_i$, i.e. $f(-s_i)$ (Glauber, 1963). Using the expression for P_{eq} for the Perutz model (eqns (3) and (8)), and cancelling common factors, we obtain

$$\frac{W_{ST}(s_i \to -s_i)}{W_{ST}(-s_i \to s_i)} = \frac{\exp\{\frac{1}{2}\beta(G_S + G_T + G_{ST} - \mu_s)s_i\}\exp(-\beta G_{QT}qs_i)}{\exp\{-\frac{1}{2}\beta(G_S + G_T + G_{ST} - \mu_s)s_i\}\exp(\beta G_{QT}qs_i)}. \tag{A2}$$

Since s_i and qs_i can only take the values $+1$ or -1 we can use the Euler identity,

$$\exp uX = \cosh X \, (1 + u \tanh X), \quad \text{where } u = +1 \text{ or } -1, \tag{A3}$$

to express each of the exponentials appearing in equation (A2) in a form linear in the variables. Thus,

$$\frac{W_{ST}(s_i \to -s_i)}{W_{ST}(-s_i \to s_i)} = \frac{(1 + s_i\theta_{ST})(1 - qs_i\theta_{QT})}{(1 - s_i\theta_{ST})(1 + qs_i\theta_{QT})}. \tag{A4}$$

Here θ_{ST} and θ_{QT} are the hyperbolic tangents defined in equation (11). From equation (A4) we see that $W_{ST}(s_i \to -s_i)$ is a function of the variables s_i, q and qs_i. Since s_i, q and qs_i can each take only the two values $+1$ or -1, all functions which can be expanded in a power series can be expressed in the general form $f(u) = a(1 + bu)$ where u may be $+1$ or -1. This is true because $u^{2n} = 1$ and $u^{2n+1} = u$ where n is any integer. Hence $W_{ST}(s_i \to -s_i)$ is most generally written as a linear combination of the variables s_i, q and qs_i,

$$W_{ST}(s_i \to -s_i) = d(1 + as_i + bs_iq + cq), \tag{A5}$$

where d, a, b and c are constants. Notice that the transition probability $W_{ST}(s_i \to -s_i)$ does not depend on $s_j(j \neq i)$ because in the Perutz model there is no direct coupling between events at one subunit and its neighbors (i.e. no tertiary–tertiary interactions).

The general expression (eqn (A5)) must satisfy the detailed balance condition, (eqn (A4)). Substituting from (A5) into (A4)

$$\frac{(1 + as_i + bs_iq + cq)}{(1 - as_i - bs_iq + cq)} = \frac{(1 + \theta_{ST}s_i)(1 - \theta_{QT}s_iq)}{(1 - \theta_{ST}s_i)(1 + \theta_{QT}s_iq)}. \tag{A6}$$

The above equation can be simplified by cross-multiplication to give

$$s_i(a - \theta_{ST} + c\theta_{QT} - b\theta_{ST}\theta_{QT}) + s_iq(b + \theta_{QT} - c\theta_{ST} - a\theta_{ST}\theta_{QT}) = 0. \tag{A7}$$

Since equation (A7) is true for all values of s_i and q; we obtain two independent equations corresponding to $q = +1$ and $q = -1$, respectively. These are

$$(a + b)(1 - \theta_{ST}\theta_{QT}) + (c - 1)(\theta_{QT} - \theta_{ST}) = 0 \tag{A8a}$$

and

$$(a - b)(1 + \theta_{ST}\theta_{QT}) + (c - 1)(\theta_{QT} + \theta_{ST}) = 0. \tag{A8b}$$

Solving these equations for a and b in terms of c, θ_{ST} and θ_{QT} we obtain

$$\begin{aligned} a &= \theta_{ST} - c'\theta_{QT}(1 - \theta_{ST}^2)/(1 - \theta_{ST}^2\theta_{QT}^2) \\ b &= -\theta_{QT} + c'\theta_{ST}(1 - \theta_{QT}^2)/(1 - \theta_{ST}^2\theta_{QT}^2), \end{aligned} \tag{A9}$$

where $c' \equiv c + \theta_{ST}\theta_{QT}$.

Substituting from equation (A9) into equation (A5) we obtain

$$W_{ST}(s_i \to -s_i) = k(1 + \alpha q)(1 + s_i\theta_{ST})(1 - s_iq\theta_{QT}), \tag{A10}$$

where

$$k \equiv d(1 - \theta_{ST}^2\theta_{QT}^2 + c'\theta_{ST}\theta_{QT})/(1 - \theta_{ST}^2\theta_{QT}^2) \tag{A11a}$$

and

$$\alpha \equiv c'/(1 - \theta_{ST}^2\theta_{QT}^2 + c'\theta_{ST}\theta_{QT}). \tag{A11b}$$

REFERENCES

Adair, G. S. (1925a). *Proc. Roy. Soc. ser. A*, **109**, 292–300.

Adair, G. S. (1925b). *J. Biol. Chem.* **63**, 529–545.

Alpert, B., Banerjee, R. & Lindqvist, L. (1974). *Proc. Nat. Acad. Sci., U.S.A.* **71**, 558–562.

Antonini, E. & Brunori, M. (1969). *J. Biol. Chem.* **244**, 3909–3912.

Antonini, E., Chiancone, E. & Brunori, M. (1967). *J. Biol. Chem.* **242**, 4360–4366.

Arnone, A. (1972). *Nature (London)*, **237**, 146–149.

Bansil, R. (1975). Thesis, University of Rochester, Rochester, N.Y.

Bansil, R., Herzfeld, J. & Stanley, H. E. (1974). *Science*, **186**, 929–932.

Benesch, R. E. & Benesch, R. (1974). *Advan. Protein Chem.* **28**, 211–237.

Benesch, R. E., Benesch, R. & Yu, C. Y. (1969). *Biochemistry*, **8**, 2567–2571.

Bohr, C., Hasselbach, K. & Krogh, A. (1904). *Skand. Arch. Physiol.* **16**, 402.

Bush, R. T. & Thompson, C. J. (1971a). *Biopolymers*, **10**, 961–972.

Bush, R. T. & Thompson, C. J. (1971b). *Biopolymers*, **10**, 1331–1349.

Chay, T. R. & Brillhart, D. (1974a). *Biochemistry*, **13**, 4579–4585.

Chay, T. R. & Brillhart, D. (1974b). *Biochemistry*, **13**, 5311–5317.

Cox, D. R. & Miller, H. D. (1965). *The Theory of Stochastic Processes*, Wiley, New York.

Gibson, Q. H. (1956). *J. Physiol.* **134**, 123–134.

Gibson, Q. H. (1959a). *Biochem. J.* **71**, 293–303.

Gibson, Q. H. (1959b). *Progr. Biophys.* **9**, 1 53.

Gibson, Q. H. (1970a). *J. Biol. Chem.* **245**, 3285–3288.

Gibson, Q. H. (1970b). *Biochem. Biophys. Res. Commun.* **40**, 1319–1324.

Gibson, Q. H. & Parkhurst, L. J. (1968). *J. Biol. Chem.* **243**, 5521–5524.

Gibson, Q. H. & Roughton, F. J. W. (1955). *Proc. Roy. Soc. ser. B*, **143**, 310–334.

Gibson, Q. H. & Roughton, F. J. W. (1957). *Proc. Roy. Soc. ser. B*, **146**, 206–224.

Glauber, R. J. (1963). *J. Math. Phys.* **4**, 294–307.

Goel, N. S. (1968). *Biopolymers*, **6**, 55–72.

Gray, R. D. (1974). *J. Biol. Chem.* **249**, 2879–2885.

Gray, R. D. & Gibson, Q. H. (1971). *J. Biol. Chem.* **246**, 7168–7174.

Hartridge, H. & Roughton, F. J. W. (1923). *Proc. Roy. Soc. ser. A*, **104**, 395–430.

Herzfeld, J. & Schlesinger, P. A. (1975). *J. Mol. Biol.* **97**, 483–517.

Herzfeld, J. & Stanley, H. E. (1974). *J. Mol. Biol.* **82**, 231–265.

Heustis, W. H. & Raftery, M. A. (1972). *Biochem. Biophys. Res. Commun.* **49**, 428–433.

Hopfield, J. J., Shulman, R. G. & Ogawa, S. (1971). *J. Mol. Biol.* **61**, 425–443.

Kellett, G. L. & Guttfreund, H. (1970). *Nature (London)*, **227**, 921–926.

Koshland, D. E., Némethy, G. & Filmer, D. (1966). *Biochemistry*, **5**, 365–385.

Lambeth, D. O. & Palmer, G. (1973). *J. Biol. Chem.* **248**, 6095–6103.

MacQuarrie, R. & Gibson, Q. H. (1972). *J. Biol. Chem.* **247**, 5686–5694.

McCray, J. A. (1972). *Biochem. Biophys. Res. Commun.* **47**, 187–193.

Monod, J., Wyman, J. & Changeux, J. P. (1965). *J. Mol. Biol.* **12**, 88–118.

Montroll, E. W. (1967). *Energetics in Metallurgical Phenomena*, vol. 3, Gordon & Breach, New York.

Morello, J. A., Craw, M. R., Constantine, H. P. & Forster, R. E. (1964). *J. Appl. Physiol.* **19**, 522–525.

Ogawa, S., McConnell, H. M. & Horowitz, A. (1968). *Proc. Nat. Acad. Sci., U.S.A.* **61**, 401–406.

Olson, J. S. & Gibson, Q. H. (1972). *J. Biol. Chem.* **247**, 1713–1726.

Olson, J. S., Anderson, M. E. & Gibson, Q. H. (1971). *J. Biol. Chem.* **246**, 5919–5923.

Oppenheim, I. (1969). *Advan. Chem. Phys.* **15**, 1–10.

Perutz, M. F. (1970a). *Nature (London)*, **228**, 726–734.

Perutz, M. F. (1970b). *Nature (London)*, **228**, 734–739.

Roughton, F. J. W. & Lyster, R. L. J. (1965). *Hvalrådets Skrifter*, **48**, 185–198.

Salhany, J. M., Eliot, R. S. & Mizukami, H. (1970). *Biochem. Biophys. Res. Commun.* **39**, 1052–1057.

Shulman, R. G., Ogawa, S., Wuthrich, K., Yamane, T., Peisach, J. & Blumber, W. E. (1969). *Science*, **165**, 251–257.

Shulman, R. G., Wuthrich, K., Yamane, T., Patel, D. J. & Blumber, W. E. (1970). *J. Mol. Biol.* **53**, 143–157.

Stanley, H. E. (1971). *Introduction to Phase Transitions and Critical Phenomena*, Oxford Univ. Press, London.

Szabo, A. & Karplus, M. (1972). *J. Mol. Biol.* **72**, 163–197.

Suzuki, M. & Kubo, R. (1968). *J. Phys. Soc. Japan*, **24**, 51–60.

Tyuma, I., Imai, K. & Shimizu, K. (1973). *Biochemistry*, **12**, 1491–1498.