Analysis of the Interaction of Organic Phosphates with Hemoglobin†

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ABSTRACT: The interaction of organic phosphates with hemoglobin is studied by use of a simple thermodynamic approach. A model-independent analysis is employed to evaluate the accuracy of Adair constants determined in the presence of 2,3-diphosphoglycerate (DPG). The change of oxygen affinity in the presence of phosphates is related to the macroscopic phosphate binding constants of oxy- and deoxyhemoglobin and used to extract such binding constants from oxygen equilibrium measurements. The change of the Bohr effect in the presence of phosphates and the competitive binding of carbon dioxide and DPG are treated quantitatively. The binding of organic phosphates is incorporated into an allosteric model, in which the effect of phosphate on both tertiary and quaternary structure changes is included. By use of this model, the factors which can be responsible for the increased functional heterogeneity of α and β chains in the presence of phosphates are clarified.

Organic polyphosphates such as DPG[1] (2,3-diphosphoglycerate) and IHP (inositol hexaphosphate) can reduce the oxygen affinity of hemoglobin by more than an order of magnitude. The qualitative explanation of this effect lies in the preferential binding of organic phosphates to deoxyhemoglobin. The x-ray studies of Arnone (1972) and of Arnone and Perutz (1974) have shown that both DPG and IHP are bound to deoxyhemoglobin at a site between the two β chains by means of primarily electrostatic interactions between the negatively charged groups of the phosphate and a cluster of protonated amino acids of the β chains. The fact that these residues have pK_a values in the physiological pH range accounts for the experimentally observed (Benesch et al., 1969) pH dependence of the phosphate binding constants.

In this paper we analyze first the implications of thermodynamic arguments on the interaction of organic phosphates with hemoglobin. It is important to know the results which can be obtained by thermodynamics alone, since they are independent of the choice of model for hemoglobin. Conversely, it must be noted that agreement between experiment and the results found by such arguments cannot be used as a test of a specific model even though one may have been used in the calculations. The thermodynamic approach to the effect of organic phosphates is developed in section I. By means of the appropriate generating function, the effect on the oxygen equilibrium of binding one or more phosphates to hemoglobin is determined. A consistency test is developed that permits one to ascertain the accuracy of values of the Adair constants measured in the presence of phosphates. A formula is derived for the determination of the phosphate binding constants of unliganded and fully liganded hemoglobin from the variation of $p_{1/2}$ with phosphate concentration. In section II the change of the Bohr effect in the presence of phosphates is quantitatively related to the pH dependence of the phosphate binding constants of oxy- and deoxyhemoglobin. Section III analyzes the competition between carbon dioxide and phosphate binding to hemoglobin.

In section IV, the incorporation of the effects of organic phosphates into the allosteric model for hemoglobin is discussed. A model in which phosphate binding depends not only on the quaternary structure of the tetramer but also on the tertiary structure of the β chains is formulated and used to analyze the factors responsible for the increased α and β chain heterogeneity observed by Johnson and Ho (1974). It is demonstrated that such a model predicts that hemoglobin frozen in a given quaternary structure would not bind oxygens independently in the presence of phosphates. This implies that it is not valid to incorporate the effect of phosphates into a Monod–Wyman–Changeux model by simply altering the allosteric constants L and c in the presence of organic phosphates. We summarize the conclusions in section V.

I. Thermodynamic Description of the Effect of Organic Phosphates

The effect of organic phosphates on the functional properties of hemoglobin is conveniently described by use of a generating function (Wyman, 1967; Szabo and Karplus, 1972, 1975). For a macromolecule M with N binding sites in equilibrium with ligand X (e.g., O_2) at concentration λ, the generating function is defined as:

$$\Xi(\lambda) = \sum_{i=0}^{N} A_i \lambda^i; A_0 = 1 \tag{1}$$

where A_i is proportional to the macroscopic equilibrium constant for binding i ligands

$$M + iX \rightleftarrows MX_i \tag{2}$$

Since each term $A_i\lambda^i$, $i = 0,1, \ldots N$, is proportional to the probability that i ligands are bound, the fractional saturation, $\langle y \rangle$, of the macromolecule with ligand is given by

$$\langle y \rangle = \frac{1}{N} \frac{\sum_{i=0}^{N} i A_i \lambda^i}{\Xi(\lambda)} = \frac{\lambda}{N} \frac{\partial[\log \Xi(\lambda)]}{\partial \lambda} \tag{3}$$

To incorporate the effect of phosphates we assume that the

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[1] Abbreviations used are: DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate.

macromolecule with i ligands of X bound can also bind one molecule of an organic phosphate with an equilibrium constant P_i. In the presence of a phosphate at concentration v, the generating function becomes

$$\Xi(\lambda,v) = \sum_{i=0}^{N} A_i(1 + vP_i)\,\lambda^i = \sum_{i=0}^{N} A_i^{(p)}\lambda^i \qquad (4)$$

where $A_i^{(p)} = A_i(1 + vP_i)$. If the total phosphate and hemoglobin concentrations are v_0 and h_0, respectively, v is obtained from

$$v_0 = v + h_0\,\langle y_v\rangle \qquad (5)$$

where $\langle y_v\rangle$, the fractional saturation with respect to phosphate, is given by

$$\langle y_v\rangle = \frac{v\partial[\log \Xi(\lambda,v)]}{\partial v} \qquad (6)$$

If the total phosphate concentration is much greater than the total hemoglobin concentration (i.e., $v_0 \gg h_0$) we can replace v by v_0 in eq. 4. At the other limit ($v_0 \lesssim h_0$), the free concentration of phosphates changes significantly during the course of oxygenation and the binding curves have a pronounced biphasic character (Imai and Tyuma, 1973; Herzfeld and Stanley, 1974).

The Adair constants, K_i, which are equilibrium constants for the reaction

$$MX_{i-1} + X \rightleftarrows MX_i$$

can be expressed in terms of the A_i's by use of the relation

$$K_i = \frac{A_i}{A_{i-1}}, \quad i = 1, 2, \ldots N \qquad (7)$$

In the presence of phosphates, the Adair constants $K_i^{(p)}$ are related to the Adair constants of the "stripped" macromolecule by

$$K_i^{(p)} = \frac{A_i^{(p)}}{A_{i-1}^{(p)}} = K_i\frac{(1 + vP_i)}{(1 + vP_{i-1})} \qquad (8)$$

The above results can be generalized to the case where more than one molecule of phosphate is bound. For example, if a maximum of two phosphates can be bound, eq 4 becomes

$$\Xi(\lambda,v) = \sum_{i=0}^{N} A_i(1 + vP_i^{(1)} + v^2P_i^{(2)})\lambda^i = \sum_{i=0}^{N} A_i^{(P)}\lambda^i \qquad (4a)$$

with $A_i^{(P)} = A_i(1 + vP_i^{(1)} + v^2P_i^{(2)})$.

If only one molecule of phosphate can bind to the macromolecule, as is assumed in eq 4 and 8, a complete thermodynamic description of oxygen binding at any phosphate concentration can be given in terms of $2N + 1$ constants; these are the N Adair constants, K_i, for the stripped macromolecule and the $N + 1$ phosphate binding constants, P_i, to the species MX_i, $i = 0, 1, \ldots N$. This observation provides an important consistency test for the accuracy of Adair constants (see below).

If the total hemoglobin concentration is much less than the total phosphate concentration, a simple formula can be obtained which describes how the oxygen affinity varies with phosphate concentration. We define the median ligand affinity, p_m, for a macromolecule M in equilibrium with ligand X as the concentration of X for which the concentration of the unliganded and the fully liganded macromolecules are equal (i.e., $[M] = [MX_N]$). From the definition of the A_i's, it follows that

$$A_0 = A_N(p_m)^N \qquad (9)$$

A binding curve is said to be symmetric if at $p_{1/2}$, the ligand concentration for which $\langle y\rangle = \frac{1}{2}$, the concentration of macromolecules with i and $N - i$ ligands bound are equal (i.e., $[MX_i] = [MX_{N-i}]$, all i). Since $A_i(p_{1/2})^i$ is proportional to the probability that i ligands are bound (see above), a symmetric binding curve requires

$$A_i(p_{1/2})^i = A_{N-i}(p_{1/2})^{N-i}, \; i = 0, 1, \ldots N \qquad (10)$$

For $i = 0$, eq 9 and 10 are identical so that $p_m = p_{1/2}$ for a symmetric binding curve. For a macromolecule with only two binding sites ($N = 2$), such as the valency hybrids of hemoglobin (Szabo and Karplus, 1975), eq. 9 and 10 are identical and the binding curves must be symmetric. The argument used here is a simplified version of an analysis of Wyman (1967).

With eq 9 and the definition of $A_i^{(P)}$ for a single phosphate bound (eq 4), the median ligand affinity in the presence of organic phosphates is:

$$\log p_m = \frac{1}{N}\log\left(\frac{A_0^{(P)}}{A_N^{(P)}}\right) = \frac{1}{N}\log\left(\frac{A_0}{A_N}\right) + \frac{1}{N}\log\left(\frac{1 + vP_0}{1 + vP_N}\right) = (\log p_m)_{\text{stripped}} + \frac{1}{N}\log\left(\frac{1 + vP_0}{1 + vP_N}\right) \qquad (11)$$

where P_0 and P_N are the organic phosphate binding constants to the unliganded and fully liganded macromolecules respectively. We note that, as the phosphate concentration increases ($v \rightarrow v_0 \rightarrow \infty$), p_m also increases and reaches a maximum value given by

$$(\log p_m)_{max} = (\log p_m)_{\text{stripped}} + \frac{1}{N}\log\left(\frac{P_0}{P_N}\right) \qquad (12)$$

Thus, the oxygen affinity in the limit of large phosphate concentration is determined by the ratio of the phosphate binding constants to the unliganded and fully liganded macromolecules. Since this ratio depends on external conditions such as pH and the nature of the phosphate, the maximum effect of phosphates will also depend on these factors.

For the more general case (eq 4a) with more than one phosphate bound, the expression corresponding to eq 11 is

$$\log p_m = (\log p_m)_{\text{stripped}} + \frac{1}{N}\log\left(\frac{1 + vP_0^{(1)} + v^2P_0^{(2)}\ldots}{1 + vP_N^{(1)} + v^2P_N^{(2)}\ldots}\right) \qquad (11a)$$

If the same number of phosphates bind to the liganded and fully unliganded species, $\log p_m$ would reach a maximum analogous to that for the single phosphate case; e.g., for r phosphates bound, we have

$$(\log p_m)_{max} = (\log p_m)_{\text{stripped}} + \frac{1}{N}\log\left(\frac{P_0^{(r)}}{P_N^{(r)}}\right) \qquad (12a)$$

However, it is also possible that, as the phosphate concentration increases, the value of $\log p_m$ goes through a maximum and then decreases. This would be true if more phosphate molecules were able to bind to the fully liganded than to the unliganded species. We then have

$$\log p_m = (\log p_m)_{\text{stripped}} + \frac{1}{N}\log\left(\frac{1 + vP_0^{(1)}\ldots v^rP_0^{(r)}}{1 + vP_N^{(1)}\ldots v^sP_N^{(s)}}\right)$$

and

$$\lim_{v\to\infty} \log p_m = (\log p_m)_{\text{stripped}} + \frac{1}{N}\log v^{r-s}\left(\frac{P_0^{(r)}}{P_N^{(s)}}\right) \qquad s > r$$

which implies that there is no limiting value. In their recent work on polyvalent anion binding to hemoglobin, Desbois and Banerjee (1975) have found some binding curves (e.g., benzenehexacarboxylate) in accord with this formulation.

The thermodynamic results obtained above will now be used to provide a criterion for testing the accuracy of Adair constants determined in the presence of phosphates, to extract phosphate binding constants for deoxy- and oxyhemoglobin and cyanomet valency hybrids from measurements of the change of oxygen affinity with phosphate concentration, and to analyze the phosphate binding of fetal hemoglobin. In all cases the assumption is made that only a single phosphate binds so that eq 11 and 12 are applicable.

(*A*) *Accuracy of Adair Constants.* At pH 7.4 in 0.1 M NaCl, Tyuma et al. (1973) have determined the Adair constants for hemoglobin stripped of organic phosphates and in the presence of four different concentrations of DPG. The smallest DPG concentration used was more than an order of magnitude greater than the total hemoglobin concentration. Since the measurement technique that was employed appears to be the most accurate available at present and the numerical values of the Adair constants can provide a basis for differentiating various models for hemoglobin (see section IV), it is important to know the reliability of the constants determined by Tyuma et al. A model-independent test of the error limits of their Adair constants can be made under the assumptions (1) that the hemoglobin tetramers are not dissociating to a significant degree and (2) that in 0.1 M NaCl only one molecule of DPG binds/tetramer. As we have shown, the oxygenation curves in the presence of phosphates is then determined uniquely by the four Adair constants for stripped hemoglobin and the five phosphate binding constants to hemoglobin containing 0, 1, . . . 4 oxygen molecules, respectively. Any Adair constant in the presence of DPG can be expressed in terms of these constants and the free DPG concentration by use of eq 8. Thus, the 20 Adair constants determined by Tyuma et al. (1973) should be reproducible *within their given error limits* by nine constants. In the Appendix we show that even if the quoted error limits are multiplied by a factor of two, it is not possible to find nine such constants. Hence, their Adair constants cannot be as accurate as claimed if our two assumptions are applicable.

The reasons for the discrepancy, aside from trivial experimental errors such as the presence of impurities or inaccurate determination of the oxygen concentration, could be due to some combination of the following factors: (1) the tetramers are dissociating so that the Adair scheme does not offer a complete thermodynamic description of oxygen binding; (2) in 0.1 M NaCl, more than one molecule of DPG can bind to a tetramer; (3) the spectra of partially oxygenated species in the presence of phosphates are significantly different so that there is no isosbestic point; (4) the method of extraction of the Adair constants from the raw data and the assignment of error bars is invalid. Since the resolution of the problems raised by the inconsistency of the results of Tyuma et al. is important, it would be worthwhile to study the dependence of the binding curves on the hemoglobin concentration, the external salt concentration, and the choice of monitoring wavelengths. At present, all that one can say is that the error bars (some of which are greater than 50%) on the Adair constants of Tyuma et al. (1973) are too small and, hence, that conclusions based on the precise values of these constants must be regarded with caution.

(*B*) *Determination of Phosphate Binding Constants.* For hemoglobin in the limit $v_0 \gg h_0$ with the assumption of a single phosphate/tetramer, the variation of the median oxygen affinity with phosphate concentration is given by eq 11 with $N = 4$ and $v = v_0$; that is,

$$\log p_m = (\log p_m)_{\text{stripped}} + \frac{1}{4}\log\left(\frac{1 + v_0 P_{\text{deoxy}}}{1 + v_0 P_{\text{oxy}}}\right) \quad (13)$$

where P_{deoxy} and P_{oxy} are the phosphate binding constants to unliganded and fully liganded hemoglobin. It is difficult to determine p_m accurately, but for systems with high cooperativity p_m is often very close to $p_{1/2}$. For hemoglobin, if the concentrations of the singly and triply oxygenated species were equal at $p_{1/2}$, p_m would be the same as $p_{1/2}$. In a highly cooperative system such as hemoglobin (Szabo and Karplus, 1972), the concentrations and hence the differences between the concentrations of partially oxygenated species are small so that $p_m \simeq p_{1/2}$. To a good approximation, eq 13 can then be replaced by

$$\log p_{1/2} \simeq (\log p_{1/2})_{\text{stripped}} + \frac{1}{4}\log\left(\frac{1 + v_0 P_{\text{deoxy}}}{1 + v_0 P_{\text{oxy}}}\right) \quad (14)$$

This formula is similar in form to the empirical expression used by Benesch et al. (1971); however, they replaced the factor $\frac{1}{4}$ by $1/n$, where n is the Hill constant, without any theoretical justification. Berger et al. used eq 14, along with the formula of Benesch et al. (1971) to extract binding constants to deoxy- and oxyhemoglobin from measurements of $p_{1/2}$ as a function of phosphate concentration. Bare et al. (1974) also used eq 14. In all cases, it appears to have been assumed that the factor $\frac{1}{4}$ is appropriate only if oxygen binding by hemoglobin is a concerted reaction and no intermediates exist. The present development makes clear the greater generality and validity of eq 11 and 14.

An important consequence of the applicability of eq 14, which was derived in a model-independent way, is that the *quantitative* description of the change of $p_{1/2}$ with phosphate concentration cannot be used to distinguish between models for hemoglobin cooperativity. Any model which assumes that unliganded and fully liganded tetramers have phosphate binding constants equal to P_{deoxy} and P_{oxy}, respectively, and whose parameters are chosen to produce a highly cooperative binding curve predicts that $p_{1/2}$ changes with phosphate concentration in accord with eq 14. It is to be expected, therefore, that models of the Monod-Wyman-Changeux type (Szabo and Karplus, 1972; Ogata and McConnell, 1972b; Herzfeld and Stanley, 1974) and of the Koshland-Nemethy-Filmer type (Chay and Billhart, 1974), which are based on very different physical mechanisms, describe the effect of phosphates on $p_{1/2}$ equally well.

Benesch et al. (1971) have measured the binding constant of DPG in 0.1 M NaCl and found $P_{\text{deoxy}} = 6.7 \times 10^4\ M^{-1}$. The binding constant to oxyhemoglobin was too small to measure directly. From their data of the variation of $p_{1/2}$ with DPG concentration for total DPG concentrations at least ten times greater than the total hemoglobin concentration, the values of P_{deoxy} and P_{oxy} can be obtained from eq 14. By a least-squares fit, we find $P_{\text{deoxy}} = 8.5 \times 10^4\ M^{-1}$, in excellent agreement with their independently determined value under the same conditions. Moreover, we find $P_{\text{oxy}} = 330\ M^{-1}$, which

demonstrates that oxyhemoglobin binds DPG about 200 times less readily than deoxyhemoglobin under the conditions of their experiment (Szabo and Karplus, 1972).

For IHP, it is possible to determine the binding constant to fully liganded hemoglobin, but the binding constant to unliganded hemoglobin is too large to measure directly. Gray and Gibson (1971) found at pH 7, 0.1 M NaCl, that $P_{oxy} \simeq 10^6$ M^{-1}. From the data of Bunn and Guidotti (1972), who measured the oxygen affinities of hemoglobin in 0.1 M NaCl in the absence and presence of 1×10^{-3} M IHP, we can estimate the ratio P_{deoxy}/P_{oxy}. If $P_{oxy} > 10^4$ M^{-1} and $P_{deoxy} > P_{oxy}$, we have $v_0 P_{deoxy} > v_0 P_{oxy} \gg 1$ for [IHP] = 1×10^{-3} M; eq 14 can be simplified to:

$$\frac{P_{deoxy}}{P_{oxy}} = \left[\frac{(p_{1/2})_{IHP}}{(p_{1/2})_{stripped}}\right]^4 \tag{15}$$

Using the data of Bunn and Guidotti (1972), we find $(P_{deoxy}/P_{oxy}) = 1.1 \times 10^4$ and 1.3×10^4 at pH 6.8 and 7.2 respectively. With the results of Gray and Gibson (1971), this yields $P_{deoxy} \simeq 10^{10}$ M^{-1} at pH 7 in 0.1 M NaCl.

The above procedure is particularly well suited for extracting phosphate binding constants of species, such as the valency hybrids of hemoglobin, which have only two binding sites so that p_m is identical to $p_{1/2}$. Unfortunately, data on the variation of $p_{1/2}$ with phosphate concentration in the presence of sufficient salt to ensure one-to-one binding is not available. Maeda et al. (1972) did determine the $p_{1/2}$ values for the two cyanomet valency hybrids in 0.1 M NaCl, pH 7.4, in the absence and presence of 2×10^{-3} M DPG. Although, as was pointed out previously (Szabo and Karplus, 1975), their data are not entirely satisfactory because the observed binding curves are not symmetric, we show how the ratio of the phosphate binding constants to unliganded $\alpha_2^+(CN^-)_2\beta_2$ and $\alpha_2\beta_2^+(CN^-)_2$ hybrids can be estimated. If we assume that the oxygenated forms of the two hybrids have the same binding constants and that at a DPG concentration of 2×10^{-3} M the unliganded forms are fully saturated with DPG ($v_0 P_{deoxy} \gg 1$), it follows from eq. 11 that:

$$\frac{P_{deoxy}(\alpha_2^+\beta_2)}{P_{deoxy}(\alpha_2\beta_2^+)} = \left[\left(\frac{p_{1/2}(\alpha_2^+\beta_2)}{p_{1/2}{}^S(\alpha_2^+\beta_2)}\right)\left(\frac{p_{1/2}{}^S(\alpha_2\beta_2^+)}{p_{1/2}(\alpha_2\beta_2^+)}\right)\right]^2$$

where $p_{1/2}{}^S$ and $p_{1/2}$ refer to results obtained in the absence (stripped) and presence of DPG. Using the data of Maeda et al. (1972) in 0.1 M NaCl, pH 7.4, we find that unliganded $\alpha_2^+\beta_2$ binds DPG more strongly than $\alpha_2\beta_2^+$ by a factor of four. This is in reasonable agreement with the results of Bauer et al. (1973) who find factors of 2.8 and 8 at pH 7.0 and 7.7, respectively. If it is assumed that, in the presence of 2×10^{-3} DPG in 0.1 M NaCl, the $p_{1/2}$ are maximally shifted, we can estimate the ratio of the binding constants to the liganded and unliganded forms of these hybrids by using eq 12 with $N = 2$. We find that $P_{deoxy}/P_{oxy} = 12$ for $\alpha_2^+\beta_2$ and only 3 for $\alpha_2\beta_2^+$. It is clear that more extensive data on the variation of $p_{1/2}$ with phosphate concentration for the valency hybrids are needed to determine quantitatively the phosphate binding constants of these species.

(C) Phosphate Binding to Fetal Hemoglobin. As a further illustration of the thermodynamic approach, we consider the effect of mutations (e.g., fetal hemoglobin) on the interaction of hemoglobin with organic phosphates. For any mutant hemoglobin an equation corresponding to eq 11 applies; with the superscript M designating the constants for the mutant species, we have:

$$\log p_m{}^M = (\log p_m{}^M)_{stripped} + \frac{1}{4}\log\left(\frac{1 + vP_{deoxy}{}^M}{1 + vP_{oxy}{}^M}\right)$$

Depending on how the binding constants for organic phosphates are affected by the modification, the affinity of the mutant hemoglobin can be more or less sensitive to phosphates than is HbA. In fetal hemoglobin His-143 is replaced by serine, thus eliminating one of the interactions responsible for the binding of DPG. Tyuma and Shimizu (1970) found that the affinity of stripped fetal hemoglobin is lower than the affinity of HbA ($(p_{1/2})_{HbF} = 3.6$; $(p_{1/2})_{HbA} = 1.9$ at pH 7.4), indicating that the mutation destabilizes the liganded quaternary structure relative to the unliganded structure. However, since the binding constant of DPG is decreased, the effect of DPG is smaller for HbF than for HbA. Therefore, for sufficiently large phosphate concentrations, the affinity of HbF is expected to be higher than the affinity of HbA. Tyuma and Shimizu found for $v_0 = 2 \times 10^{-3}$ M, $(p_{1/2})_{HbF} = 9.3$ and $(p_{1/2})_{HbA} = 12.6$.

II. Organic Phosphates and the Bohr Effect

Since organic phosphates are bound to hemoglobin primarily by means of electrostatic forces between their ionized groups and certain protonated nitrogens of the β chains, the degree of phosphates binding depends on pH (Benesch et al., 1969). A consequence of the pH dependence of the binding constants is that the Bohr effect is altered by the presence of phosphates. If the concentration of organic phosphates, v_0, is significantly greater than the total hemoglobin concentration, the difference, Δ, in the Bohr effect in the presence and absence of phosphates can be obtained directly by differentiating eq 13 with respect to pH; We find

$$\Delta = \frac{\partial(\log p_m)}{\partial\,\mathrm{pH}} - \left(\frac{\partial(\log p_m)}{\partial\,\mathrm{pH}}\right)_{stripped}$$
$$= \frac{1}{4}\left[\frac{v_0 P_{deoxy}}{1 + v_0 P_{deoxy}}\left(\frac{\partial(\log P_{deoxy})}{\partial\,\mathrm{pH}}\right) - \frac{v_0 P_{oxy}}{1 + v_0 P_{oxy}}\left(\frac{\partial(\log P_{oxy})}{\partial\,\mathrm{pH}}\right)\right] \tag{16}$$

We note that, if P_{deoxy} and/or P_{oxy} are pH dependent, the Bohr effect is expected to vary with phosphate concentration. If the phosphate concentration is not sufficiently large for eq 13 to be valid, eq 11 has to be used and additional terms appear due to the change of v with pH.

Recently de Bruin et al. (1973, 1974) and Kilmartin (1974) have measured the number of protons released when an excess of DPG is mixed with deoxy- and oxyhemoglobin as a function of pH. To determine this quantity we use the result of Wyman (1964) that relates the release of protons and the change of median oxygen affinity with pH; that is:

$$-\frac{(\Delta H^+)}{4} = \frac{\partial \log p_m}{\partial\,\mathrm{pH}} \tag{17}$$

where $-(\Delta H^+)/4$ is the number of protons released/subunit in going from $\langle y\rangle = 0$ to $\langle y\rangle = 1$; eq 17 is valid for $v_0 \gg h_0$. By corresponding arguments, the number of protons released by unliganded hemoglobin on binding DPG is

$$-\left[\frac{(\Delta H^+)}{4}\right]_{deoxy} = \frac{1}{4}\left[\frac{v_0 P_{deoxy}}{1 + v_0 P_{deoxy}}\left(\frac{\partial \log P_{deoxy}}{\partial\,\mathrm{pH}}\right)\right] \tag{18}$$

with a similar expression for liganded hemoglobin. Combining eq 16–18 we find that the number of protons/subunit released upon oxygenation in the presence of an excess of phosphate is

given by

$$\frac{(\Delta H^+)}{4} = \left[\frac{(\Delta H^+)}{4}\right]_{\text{stripped}} + \left[\frac{(\Delta H^+)}{4}\right]_{\text{deoxy}} - \left[\frac{(\Delta H^+)}{4}\right]_{\text{oxy}} \quad (19)$$

Although eq 19 is rigorously valid only when the total phosphate concentration is much greater than the total hemoglobin concentration, Kilmartin measured all four quantities in this equation as a function of pH under conditions that $v_0 \simeq 1.5h_0$ and found that the equation is satisfied. Moreover, he showed that the number of protons released by deoxy- and oxyhemoglobin as a function of pH is different. This demonstrates that the DPG binding constants to oxy- and deoxyhemoglobin must have a different pH dependence.

Benesch et al. (1969) determined the alkaline Bohr effect from measurement of $(\partial[\log p_{1/2}]/\partial\text{pH})$ as a function of DPG concentration at pH 7.3. They found that at small DPG concentrations the Bohr effect was increased; however, at higher DPG concentrations, the Bohr effect decreased, reaching the stripped value when $v_0 = 2 \times 10^{-3}$ M. These results were analyzed by Riggs (1971), who used a model which did not explicitly include oxygen binding. The analysis was criticized by Kilmartin and Rossi-Bernardi (1973) because of the implicit assumption that the DPG binding constants to deoxy- and oxyhemoglobin have an identical pH dependence. A thermodynamic treatment of this problem is provided by eq 16. In the limit of large phosphate concentration ($v_0 \to \infty$), the change in the Bohr effect is given by:

$$\Delta_\infty = \frac{1}{4}\left[\frac{\partial[\log P_{\text{deoxy}}]}{\partial\,\text{pH}} - \frac{\partial[\log P_{\text{oxy}}]}{\partial\,\text{pH}}\right] \quad (20)$$

Only when the pH dependence of P_{deoxy} and P_{oxy} is identical does Δ_∞ vanish for all pH. If P_{deoxy} and P_{oxy} vary differently with pH, Δ_∞ can be either positive or negative depending on the pH; that is, the Bohr effect of hemoglobin at large phosphate concentration could be larger or smaller than the Bohr effect of stripped hemoglobin. This has been confirmed by de Bruin et al. (1974) who measured the number of protons released by hemoglobin at different pH's as a function of phosphate concentration. They find that at pH ~7.3, $\Delta_\infty = 0$, in agreement with Benesch et al. (1969), but that $\Delta_\infty > 0$ for higher pH and $\Delta_\infty < 0$ for lower pH. These results imply that the change with pH of the log of the binding constants of DPG to liganded and unliganded hemoglobin is about the same at pH 7.3 but $\partial \log P_{\text{deoxy}}/\partial$ pH is greater (smaller) than $\partial \log P_{\text{oxy}}/\partial$ pH at higher (lower) pH.

Note Added after Submission of This Paper. Recently Benesch and Rubin (1975) argued theoretically and presented experimental evidence to show that the two measures of the Bohr effect (i.e., $\partial \log P_m/\partial$ pH and the number of protons released by hemoglobin, $-(\Delta H^+)$) are not the same. While it is true that the two measures of the Bohr effect are not rigorously identical for *all* phosphate concentrations, eq 17 holds when the concentration of phosphates is independent of oxygenation. This is clearly the case in the two limits; that is, when no phosphate is present and when the total phosphate concentration is much greater than the total hemoglobin concentration. Experimentally, they find at pH 7.3 for $v_0 = 1.5 \times 10^{-3}$ M and $h_0 = 5 \times 10^{-5}$ M ($v_0 = 30h_0$) that there is a difference between the two measures of the Bohr effect; this disagrees with the results of de Bruin et al. (1974) who worked at higher hemoglobin concentrations ($h_0 = 2.5 \times 10^{-4}$ M). In light of the above and the fact that the number of protons released upon oxygenation is very difficult to measure under the conditions (i.e., very low h_0 and very high v_0) of the Benesch and Rubin experiment because of the large buffering capacity of DPG, a carefully reinvestigation of this phenomenon appears to be warranted.

III. Competitive Binding of CO_2 and Organic Phosphates

Carbon dioxide binds to the N-terminal amino groups of both the α and β chains of hemoglobin (Kilmartin and Rossi-Bernardi (1973)). An x-ray study of the CO_2 binding site has been made by Arnone (1974). Since DPG also interacts with the α-amino groups of the β chains, the binding of these two species is expected to be competitive. Related experiments involving pyridoxal compounds that also bind specifically to the α or β N-terminal amino groups have been performed by Benesch et al. (1973).

We consider first the effect of CO_2 on the oxygen binding curves of hemoglobin, and then analyze the competition between CO_2 and phosphates. If $C^{\alpha(\beta)}{}_{\text{deoxy}}$ and $C^{\alpha(\beta)}{}_{\text{oxy}}$ are the binding constants of CO_2 to the $\alpha(\beta)$ chains in deoxy- and oxyhemoglobin, respectively, and γ is the CO_2 concentration, the change in the median oxygen affinity in the presence of CO_2 is given by

$$\log p_m = \log p_m{}^0 + \frac{1}{2}\log\left[\frac{1+\gamma C^{\alpha}{}_{\text{deoxy}}}{1+\gamma C^{\alpha}{}_{\text{oxy}}}\right] + \frac{1}{2}\log\left[\frac{1+\gamma C^{\beta}{}_{\text{deoxy}}}{1+\gamma C^{\beta}{}_{\text{oxy}}}\right] \quad (21)$$

where $p_m{}^0$ corresponds to hemoglobin in the absence of CO_2. Equation 21 assumes that the amino groups bind CO_2 independently. Kilmartin and Rossi-Bernardi (1969) have reacted the N-terminal amino groups of horse hemoglobin with cyanate and obtained the following carbamylated derivatives: $\alpha_2{}^c\beta_2{}^c$, $\alpha_2\beta_2{}^c$, $\alpha_2{}^c\beta_2$. Since a blocked amino group cannot bind CO_2, the oxygen affinity of the fully carbamylated molecule does not change with carbon dioxide concentration. If we assume that blocking the β chains does not affect the CO_2 binding of the α chains and vice versa, the oxygen affinities of the carbamylated hybrids are given by

$$(\log p_m)_{\alpha_2\beta_2{}^c} = (\log p_m{}^0)_{\alpha_2\beta_2{}^c} + \frac{1}{2}\log\left(\frac{1+\gamma C^{\alpha}{}_{\text{deoxy}}}{1+\gamma C^{\alpha}{}_{\text{oxy}}}\right) \quad (22a)$$

and

$$(\log p_m)_{\alpha_2{}^c\beta_2} = (\log p_m{}^0)_{\alpha_2{}^c\beta_2} + \frac{1}{2}\log\left(\frac{1+\gamma C^{\beta}{}_{\text{deoxy}}}{1+\gamma C^{\beta}{}_{\text{oxy}}}\right) \quad (22b)$$

We see from eq 22a and 22b that the assumptions used in deriving them require that the change of the median oxygen affinity in the presence of CO_2 is additive; that is, for $\Delta \log p_m = \log p_m - \log p_m{}^0$,

$$(\Delta \log p_m)_{\alpha_2\beta_2} = (\Delta \log p_m)_{\alpha_2\beta_2{}^c} + (\Delta \log p_m)_{\alpha_2{}^c\beta_2}$$

This additivity has been confirmed recently for $p_{1/2}$ by Kilmartin et al. (1973).

In analogy with the earlier discussion of phosphate binding (section I) eq 22a and 22b provide a simple way of determining the CO_2 binding constants to the N-terminal amino groups of the α and β chains. From measurements of the oxygen affinity of the two carbamylated hybrids as a function of CO_2 concentration (in the limit that the total CO_2 concentration is much greater than hemoglobin concentration), the CO_2 binding constants to unliganded and liganded hemoglobin can

be obtained. Further, from a series of such measurements as a function of pH, it is possible to determine both K_z, the ionization constant of the amino group, and K_c, the association constant for carbamino CO_2 binding, for the N-terminal amino groups of the α and β chains of both liganded and unliganded hemoglobin. This can be done, as pointed out by Kilmartin and Rossi-Bernardi (1973), for example, by measuring C as a function of pH and using eq 23

$$C^{\alpha,\beta}{}_{\text{oxy,deoxy}} = \frac{(K_c)^{\alpha,\beta}{}_{\text{oxy,deoxy}}(K_z)^{\alpha,\beta}{}_{\text{oxy,deoxy}}}{(K_z)^{\alpha,\beta}{}_{\text{oxy,deoxy}}h + h^2} \quad (23)$$

where h is the hydrogen ion concentration, to determine K_c and K_z by a least-squares fitting procedure. An alternate way to obtain information on K_z would be to measure the number of protons released by the tetramer when CO_2 is added to both the liganded and unliganded forms of the two carbamylated hybrids. In analogy with the organic phosphate results (e.g., eq 17), the number of protons/subunit released when CO_2 is added to each of these four species is obtained by differentiating eq 22 and 23 with respect to pH; we have

$$-\frac{(\Delta H^+)}{4} = \frac{\gamma C}{2(1+\gamma C)}\frac{\partial \log C}{\partial \text{pH}} = \frac{\gamma C(K_z + 2h)}{2(1+\gamma C)(K_z + h)} \quad (24)$$

where the labels (α,β; oxy,deoxy) on the equilibrium constants have been dropped for compactness.

Since the binding of CO_2 and organic phosphates is competitive, the amount of CO_2 bound will decrease in the presence of organic phosphates. The effect of DPG on CO_2 binding has been considered by Brenna et al. (1972) under the assumption that the binding of DPG and CO_2 are mutually exclusive; i.e., if either one or two molecules of CO_2 are bound to the β chains then DPG cannot bind. Since the results obtained for the CO_2 binding curve in the presence of phosphates by use of this simplified model are not correct, we present the appropriate expressions here. We consider the binding of CO_2 to unliganded hemoglobin; corresponding formulae apply to liganded hemoglobin. Since DPG is not expected to interfere with the binding of CO_2 to the α chains, the generating function is

$$\Xi(\upsilon,\gamma) = (1+\gamma C^\alpha)^2[(1+\upsilon P^{(0)}) + 2\gamma C^\beta(1+\upsilon P^{(1)}) + (\gamma C^\beta)^2(1+\upsilon P^{(2)})] \quad (25)$$

where C^α and C^β are the CO_2 binding constants to the α and β chains, respectively, and $P^{(i)}$ is the DPG binding constant to deoxyhemoglobin with i molecules of CO_2 bound to the β chains; as above, γ is the concentration of CO_2 and υ that of phosphate. The subscript "deoxy" is omitted from all the binding constants for compactness. The average number of CO_2 molecules bound per tetramer is given by:

$$\langle y_\gamma \rangle = \gamma\frac{\partial}{\partial\gamma}(\log \Xi(\upsilon,\gamma)) = 2\left[\frac{\gamma C^\alpha}{1+\gamma C^\alpha} + \frac{\gamma C^\beta(1+\upsilon P^{(1)}) + (\gamma C^\beta)^2(1+\upsilon P^{(2)})}{1+\upsilon P^{(0)} + 2\gamma C^\beta(1+\upsilon P^{(1)}) + (\gamma C^\beta)^2(1+\upsilon P^{(2)})}\right] \quad (26a)$$

where the free DPG concentration, υ, can be obtained from the total hemoglobin (h_0) and phosphate (υ_0) concentrations by use of the equation

$$\upsilon_0 = \upsilon + h_0\langle y_\upsilon\rangle = \upsilon + h_0\frac{\upsilon[\log \Xi(\upsilon,\gamma)]}{\partial\upsilon} = \upsilon + \frac{h_0\upsilon[P^{(0)} + 2\gamma C^\beta P^{(1)} + (\gamma C^\beta)^2 P^{(2)}]}{1+\upsilon P^{(0)} + 2\gamma C^\beta(1+\upsilon P^{(1)}) + (\gamma C^\beta)^2(1+\upsilon P^{(2)})} \quad (26b)$$

In the limit of exclusive binding considered by Brenna et al. (1972), the constants $P^{(1)}$ and $P^{(2)}$ equal zero and eq 26a and 26b reduce to

$$\langle y_\gamma\rangle = 2\left[\frac{\gamma C^\alpha}{1+\gamma C^\alpha} + \frac{\gamma C^\beta}{1+\gamma C^\beta + \dfrac{\upsilon P^{(0)}}{1+\gamma C^\beta}}\right] \quad (27a)$$

and

$$\upsilon_0 = \upsilon + \frac{h_0\upsilon P^{(0)}}{(1+\gamma C^\beta)^2 + \upsilon P^{(0)}} \quad (27b)$$

which are not identical with the expression found by Brenna et al. (1972). The use of eq 27, instead of those of Brenna et al. (1972), leads to a considerably different value for the DPG binding constant; Perrella et al. (1975) have used eq 26 and 27 to extract DPG binding constants from carbon dioxide binding data.

IV. Organic Phosphates and the Allosteric Model

Perutz's stereochemical mechanism for cooperative ligand binding by hemoglobin can be described by a generalization of the model of Monod, Wyman and Changeux (1965) to the situation in which the α and β chains are functionally inequivalent (Perutz, 1970, 1972; Ogata and McConnell, 1972a,b; Szabo and Karplus, 1972; Edelstein, 1974; Szabo and Karplus, manuscript to be published). As we have shown elsewhere (Szabo and Karplus, 1972) Perutz's proposals allows one to relate the phenomenological parameters of a Monod-Wyman-Changeux (MWC) type model to structurally determined interactions. From this interpretation of the parameters, it is possible to make estimates of how they change when external conditions are altered or when the molecule itself is modified. In particular, we have shown how such an approach can be used to analyze the differences between the functional properties of high- and low-spin valency hybrids, the oxidation-reduction equilibrium of hemoglobin, and the ligand replacement reactions of methemoglobin (Szabo and Karplus, 1975). In this section, we consider how the effect of organic phosphates on the functional properties of hemoglobin can be described by means of the same allosteric model.

Organic phosphates are known to bind preferentially to deoxyhemoglobin. X-Ray crystallographic work (Arnone, 1972; Arnone and Perutz, 1974) has shown that both DPG and IHP bind to deoxyhemoglobin at a site between the two β chains. The binding site to oxyhemoglobin is not known. The *simplest* way to incorporate the effects of phosphates into the allosteric model is to assume that the phosphate binding constant is independent of the tertiary structure of the chains and depends solely on the quaternary structure of the tetramer (Szabo and Karplus, 1972; Ogata and McConnell, 1972b; Herzfeld and Stanley, 1974). With this assumption, if the phosphate binding constants to the deoxy and oxy quaternary structures are P_D and P_O, respectively, the appropriate generating function for one molecule of phosphate/tetramer is:

$$\Xi_{\text{MWC}}(\lambda,\upsilon) = L_0(1+\upsilon P_D)(1+cK\lambda)^4 + (1+\upsilon P_O)(1+\lambda K)^4 \quad (28)$$

where L_0 is the allosteric constant for stripped hemoglobin. In the present argument we have ignored chain heterogeneity; it is considered subsequently.

If the phosphate binding depends *both* on the quaternary structure of the tetramer and the tertiary structure of the subunits, a more general generating function is required. This

possibility has been discussed recently by Arnone and Perutz (1974) in structural terms. If $P_D^{(i)}$ and $P_O^{(i)}$ are the phosphate binding constants to the deoxy and oxy quaternary structures with i subunits liganded, the generating function in the presence of phosphates is given by

$$\Xi(\lambda,\upsilon) = L_0 \sum_{i=0}^{4} \binom{4}{i}(cK\lambda)^i(1 + \upsilon P_D^{(i)}) + \sum_{i=0}^{4} \binom{4}{i}(K\lambda)^i(1 + \upsilon P_O^{(i)}) \quad (29)$$

where the binomial coefficient, $\binom{4}{i}$, is the number of ways i ligands can be distributed among 4 sites. We note that for $P_D^{(i)} = P_D$ and $P_O^{(i)} = P_O$ for all i, eq 28 and 29 are identical. An immediate consequence of eq 29 is that, if phosphate binding is linked to both tertiary and quaternary structure, the binding of ligand to the subunits of the tetramer in the presence of phosphate is cooperative (i.e., nonindependent) even in the absence of a quaternary structure change. Thus, it is not possible in this case to describe hemoglobin in terms of the MWC model with both L and c changing with phosphate concentration, as has been done by Imai (1973).

To make this discussion more concrete we introduce a simple model, also considered by Baldwin (1975), in which the binding of phosphate to the deoxy quaternary structure depends on the state of ligation of the β chains. In the absence of phosphates we assume that the generalized MWC model is applicable; i.e., the generating function (Szabo and Karplus, 1972) is:

$$\Xi(\lambda) = L_0(1 + c_\alpha K_\alpha\lambda)^2(1 + c_\beta K_\beta\lambda)^2 + (1 + K_\alpha\lambda)^2(1 + K_\beta\lambda)^2 \quad (30)$$

If the phosphate binding constants to the deoxy quaternary structure are P_D, P_D', P_D'' when none, one, and two of the β chains are liganded, and the binding constant to the oxy quaternary structure, P_O, is independent of tertiary structure, the appropriate generating function can be written:

$$\Xi(\lambda,\upsilon) = L_0(1 + \upsilon P_D)(1 + c_\alpha K_\alpha\lambda)^2 \times \left[1 + 2c_\beta K_\beta\lambda\left(\frac{1 + \upsilon P_D'}{1 + \upsilon P_D}\right) + (c_\beta K_\beta\lambda)^2\left(\frac{1 + \upsilon P_D''}{1 + \upsilon P_D}\right)\right] + (1 + \upsilon P_O)(1 + K_\alpha\lambda)^2(1 + K_\beta\lambda)^2 \quad (31)$$

We note that the generating function $\Xi(\lambda,\upsilon)$ does not have the MWC form. This implies that there is direct interaction between the oxygen binding sites in the deoxy quaternary structure. The resulting model, therefore, combines the effects considered in the formulations of Monod, Wyman, and Changeux (1965) and of Koshland, Nemethy, and Filmer (1966).

An important application of the above considerations can be made to the recent experiments of Johnson and Ho (1974). They find that the α and β chain inequivalence in oxygen binding is augmented by the presence of large concentrations of DPG and IHP, as determined by NMR measurements of the fractional saturations of the α and β chains. There are two factors which could be responsible for their results. These factors have been discussed recently by Edelstein (1974) in his consideration of the effects of chain inequivalence. The first possibility is that binding of phosphates merely *accentuates* the intrinsic chain heterogeneity which is quantitatively small under stripped conditions. It is clear that, if the α and β chains were functionally heterogeneous to *different* degrees in the oxy and deoxy quaternary structures, the presence of phosphates, which stabilize the deoxy quaternary structure, would produce relative chain affinities closer to those of the deoxy quaternary

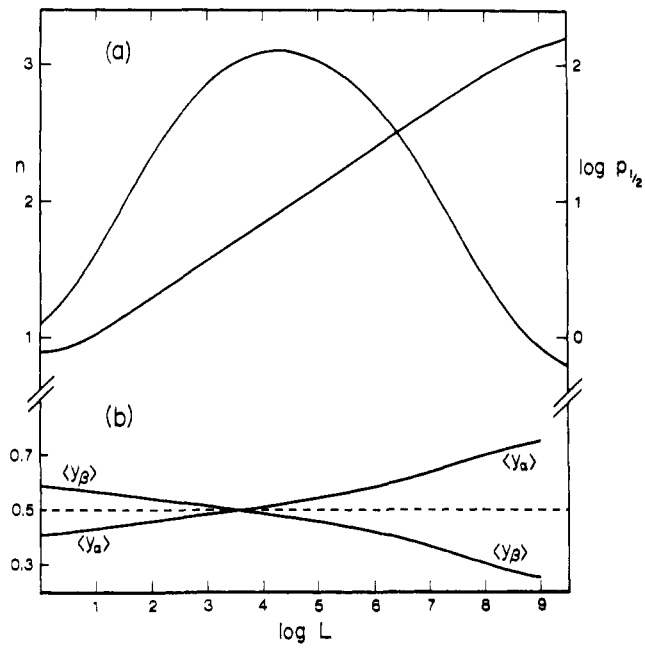

FIGURE 1: The variation of (a) n and log $p_{1/2}$ and (b) $\langle y_\alpha \rangle$ and $\langle y_\beta \rangle$ at 50% saturation with the log of the allosteric constant L. The curves were calculated using the generating function in eq 30 with $c_\alpha = 0.001$, $c_\beta = 0.02$, $K_\alpha = 1$, $K_\beta = 2$.

structure. We illustrate this effect in Figures 1a and 1b. In these figures, we assume that phosphates change only L in the allosteric model and plot n and log $p_{1/2}$, $\langle y_\alpha \rangle$ and $\langle y_\beta \rangle$ at 50% saturation against log L for the MWC model with $K_\alpha = 1$, $K_\beta = 2$, $c_\alpha = 0.02$ and $c_\beta = 0.001$. These parameters correspond to the β chains having twice the affinity of the α chains in the oxy quaternary structure, while in the deoxy quaternary structure the α chains bind oxygen more readily by a factor of ten. We note that under stripped conditions ($L \sim 10^3$–10^4) the chain heterogeneity is very small, whereas in the presence of phosphates ($L \sim 10^6$–10^7), it is significant.

The second possible effect is that phosphates *induce* heterogeneity by directly changing the affinities of the β chains. If in the deoxy quaternary structure, oxygenating a β chain reduces the phosphate binding constant to this structure, the oxygen affinity of the β chains would be smaller in the presence of phosphates than under stripped conditions. Quantitatively, this effect can be analyzed by use of eq 31. In the limit of a large excess of phosphate, which are the conditions of Johnson and Ho's experiment, eq 31 simplifies to

$$\Xi(\lambda,\infty) = L_0\frac{P_D}{P_O}(1 + c_\alpha K_\alpha\lambda)^2\left(1 + 2c_\beta K_\beta\lambda\frac{P_D'}{P_D} + (c_\beta K_\beta\lambda)^2\frac{P_D''}{P_D}\right) + (1 + K_\alpha\lambda)^2(1 + K_\beta\lambda)^2 \quad (32)$$

To clarify the physical content of the equation, we simplify it further by assuming that the phosphate binding constant decreases by the same factor d ($d < 1$) for each oxygen bound by the β chains (i.e., $P_D' = dP_D$ and $P_D'' = dP_D' = d^2P_D$) and obtain:

$$\Xi(\lambda,\infty) = L_0\frac{P_D}{P_O}(1 + c_\alpha K_\alpha\lambda)^2(1 + c_\beta dK_\beta\lambda)^2 + (1 + K_\alpha\lambda)^2(1 + K_\beta\lambda)^2 \quad (33)$$

We note from eq 33 that, even if in the absence of phosphates the α and β chains were functionally equivalent (i.e., $K_\alpha = K_\beta$, $c_\alpha = c_\beta$), the β chains would have lower affinity in the presence

of phosphates, since d is less than one. It should be pointed out that eq 33 retains the MWC form only because we made the simplifying assumption, for purposes of illustration, that the phosphate binding constants decrease by the same factor for each oxygen bound to the β chains. For this special case, and in the limit of large phosphate concentration, the effect of phosphate can be ascribed to an increase in L and a decrease in c; for the more general case corresponding to eq 31, such a description is not valid.

The available experimental data (Johnson and Ho, 1974) are not sufficient to distinguish between the two possible mechanisms for the increased chain heterogeneity observed in the presence of phosphates. It is possible, however, that the interplay between the two factors discussed could explain why for CO binding, in contrast to O_2 binding, there is very little heterogeneity observed even in the presence of IHP.

In principle, the simplest way of determining whether phosphate binding is affected by tertiary structure changes is from accurate measurements of the first Adair constant for oxygenation in the presence and absence of organic phosphates. From eq 8 it follows that

$$(K_1)_{\text{phosphate}} = (K_1)_{\text{stripped}} \left(\frac{1 + \upsilon P_{\text{monoxy}}}{1 + \upsilon P_{\text{deoxy}}}\right) \qquad (34)$$

if it is assumed that only one molecule of phosphate can bind per tetramer. Thus, if K_1 changes significantly in the presence of phosphates, P_{monoxy} must be very different from P_{deoxy}. If we assume that hemoglobin with one oxygen bound exists primarily in the deoxy quaternary structure, it follows that the binding of phosphate depends on the tertiary structure of the subunits. On the basis of his measurements, Imai has suggested that K_1 is reduced in the presence of DPG. However, in light of the results of section II and the Appendix, we feel that the available Adair constants are not sufficiently accurate to provide an unambiguous answer to this important question.

V. Conclusions and Summary

In the first three sections, we considered the implications of thermodynamic, model-independent arguments on the interaction of organic phosphates with hemoglobin. The accuracy of the best available Adair constants in the presence of phosphates was questioned. A simple formula was derived and used to estimate phosphate binding constants to liganded and unliganded hemoglobin from the variation of $p_{1/2}$ with phosphate concentration. For the case of DPG, the binding constant to unliganded hemoglobin found by this formula is in excellent agreement with an independently measured value under the same experimental conditions. For IHP, the binding constant to unliganded hemoglobin was predicted. A quantitative expression relating the change of the Bohr effect in the presence of phosphates and the pH dependence of the phosphate binding constant was derived. A formula for the binding of carbon dioxide to hemoglobin in the presence of phosphates was obtained, and a method to determine CO_2 binding constants to the N-terminal amino groups of hemoglobin was suggested.

In section IV a model for hemoglobin which contained features of the allosteric model of Monod, Wyman, and Changeux (1965) and the sequential model of Koshland, Nemethy, and Filmer (1966) was used to analyze the factors reponsible for the increased chain heterogeneity observed in the presence of organic phosphates by Johnson and Ho (1974). It was pointed out that there are two different mechanisms that could be responsible, and that the available results do not determine to what extent each of these mechanisms contribute to the observed increase in functional heterogeneity of the α and β chains.

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Appendix

Tyuma et al. (1973) have determined the four Adair constants for oxygen binding to hemoglobin at five different concentrations of DPG (υ_0 = 0, 0.2, 0.5, 1.0, 2.0 mM DPG) at 25 °C, pH 7.4, in 0.1 M NaCl with the total tetramer concentration equal to 1.5×10^{-5} M. We note that the total DPG concentrations (except $\upsilon_0 = 0$, stripped hemoglobin) are greater at least by an order of magnitude than the total hemoglobin concentration. Consequently under the two assumptions discussed in the text, thermodynamic consistency requires that their 20 Adair constants $K_i(\upsilon_j)$, $i = 1, 2, \ldots 4, j = 1, 2, \ldots 5$ should be expressible within the quoted error bars $\pm\delta_i(\upsilon_j)$ in terms of the nine constants K_i, $i = 1, 2, \ldots 4$ and P_i, $i = 0, 1, \ldots 4$ by use of eq 8, i.e.,

$$K_i(\upsilon_j) \pm \delta_i(\upsilon_j) = K_i \left(\frac{1 + \upsilon_j P_i}{1 + \upsilon_j P_{i-1}}\right)$$

We constructed the function G given by:

$$G = \sum_{i=1}^{4} \sum_{j=1}^{5} \left[K_i(\upsilon_j) + \sin \theta_{ij} \delta_i(\upsilon_j) - K_i \left(\frac{1 + \upsilon_j P_i}{1 + \upsilon_j P_{i-1}}\right) \right]^2$$

and varied the constants θ_{ij}, K_i, and P_i to minimize G. The sine function was used because for any x, $-1 \leq \sin x \leq 1$. We found that even if the quoted error bars are increased by a factor of two, the minimum value of G was not equal to zero. It is not possible, therefore, to reproduce the 20 constants, within the given error limits, by the nine constants, as required by thermodynamic consistency.

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^{15}N Nuclear Magnetic Resonance of Flavins†

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ABSTRACT: Ninety-nine percent ^{15}N-enriched flavins were synthesized and their proton decoupled ^{15}N resonances were observed. The enriched compounds were [1,3-^{15}N]riboflavin, [1,3,5-^{15}N]riboflavin, [1,3-^{15}N]riboflavin 5′-phosphate, [1,3,5-^{15}N]riboflavin 5′-phosphate, [1,3,5-^{15}N]flavin adenine dinucleotide, [1,3,5-^{15}N]lumiflavin, and [1,3,5-^{15}N]lumichrome. By comparison of their spectra and from the nuclear Overhauser effect data each ^{15}N resonance peak could be assigned to each ^{15}N nucleus. The order of the chemical shifts well corresponds to that of the calculated π-electron densities. The N-3 nucleus gives the most intense inverted peak and the N-5 nucleus a small noninverted peak. By changing pH from neutral to alkaline, the chemical shift and the intensity of signal were mostly affected in the N-3 resonance of riboflavin 5′-phosphate. The N-5 signal of flavin adenine dinucleotide showed a fairly large downfield shift with the increase of temperature. These observations can be well interpreted by the chemical structure and the proposed conformation of riboflavin 5′-phosphate and flavin adenine dinucleotide.

Physicochemical properties of flavins such as their electronic spectra on their molecular basis have been amply discussed, but little information is available on the attitude of each atom or group of the isoalloxazine ring of flavins. To reveal the fine mechanism involved in the binding between flavin coenzymes and apoproteins and the oxidoreduction catalyzed by flavoproteins, such information is essential. For this purpose, nuclear magnetic resonance (NMR)[1] studies are quite promising. Accordingly, the structure of flavin coenzymes and their interaction with apoproteins have been studied by the use of proton magnetic resonance (Sarma et al., 1968; Kotowycz et al., 1969; Kainosho and Kyogoku, 1972; Crespi et al., 1972; Raszka and Kaplan, 1974). However, the parts which directly participate in redox reaction and the sites which interact with apoproteins are known to be mainly localized at the 1–5 positions in the isoalloxazine rings. Proton magnetic resonance cannot give us direct information on the positions, since protons are located only at the 6–9 positions. For this reason, carbon and nitrogen magnetic resonances seem to be more useful. ^{13}C resonance of FMN in natural abundance has already been reported (Breitmaier and Voelter, 1972), but some of the assignments were corrected by the ^{13}C spectra of ^{13}C-enriched riboflavin (Yagi et al., 1976). The interaction of riboflavin with egg white flavoprotein was studied using [^{13}C]riboflavin (Yagi et al., 1976). The present paper deals with the preparation of ^{15}N-enriched flavins and their ^{15}N magnetic resonance spectra. This is the first systematic report on the ^{15}N resonance spectra of flavins.

Materials and Methods

To obtain ^{15}N-enriched riboflavin, [^{15}N]urea (99.36 atom %, Prochem., England) and sodium [^{15}N]nitrite (99 atom %, Prochem., England) were used as starting materials. As shown in Figure 1, [1,3-^{15}N]riboflavin and [1,3,5-^{15}N]riboflavin were synthesized through barbituric acid and *N*-ribityl-3,4-dimethyl-6-phenylazoaniline. By phosphorylating ^{15}N-enriched

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[1] Abbreviations used are: FMN, riboflavin 5′-phosphate; FAD, flavin adenine dinucleotide; NOE, nuclear Overhauser effect; Me_2SO-d_6, dimethyl-d_6 sulfoxide; NMR, nuclear magnetic resonance.