OXYGEN–ORGANOPHOSPHATE LINKAGE IN HEMOGLOBIN A

The Double Hump Effect

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ABSTRACT At low concentrations of chloride ions, and in the presence of nonsaturating concentrations of organophosphates, the oxygen equilibrium curves (OEC) for solutions of human adult hemoglobin exhibit a biphasic shape conveniently revealed by graphical analysis of the first derivative of the Hill equation with a characteristic form that we call "the double hump effect." This shape, observed for sub-saturating concentrations of organophosphates, stands in marked contrast to the simple lateral shifts of the OEC represented largely by scaling factors when pH or chloride are varied. In the case of protons or chloride, there is a self-buffering effect due to the presence of a large reservoir of proton or chloride binding sites not necessarily linked to oxygen, whereas such sites do not exist in the case of organophosphates. In addition, in the former case, we are dealing with curves measured at constant activity of the effector, while in the latter, at constant concentration.

In the presence of saturating concentrations of inositol hexaphosphate (IHP), at low chloride concentration, the entire OEC is shifted to the right, including both its upper and lower asymptotes, indicating a decrease in the intrinsic oxygen affinities of both the T and R states. Theoretical considerations leading to a successful modeling of OEC obtained under nonsaturating and saturating concentrations of IHP required an expanded two-state allosteric model in which IHP-dependent variations in the oxygen association constants for both the T and R conformations are taken into account.

INTRODUCTION

A classical example of linkage as a source of regulation and control at a macromolecular level is provided by the effect of organophosphates on the oxygen equilibrium curves (OEC)[1] of human adult hemoglobin A (HbA). This effect, first reported independently by Benesch and Benesch (1967) and by Chanutin and Curnish (1967), and later studied in much greater detail (Benesch and Benesch, 1974; Herzfeld and Stanley, 1974; Szabo and Karplus, 1976; Ackers, 1979; Ackers et al., 1982; Imai, 1982) may be considered as a special kind of Bohr effect in which a proton is replaced by inositol hexaphosphate (IHP) or 2,3-bis phosphoglycerate (DPG) as the effector. In the presence of an excess of organophosphates the OEC is shifted far to the right without any marked change of shape. This shift brings it into a region that is more favorable for the exchange of oxygen in the course of the respiratory cycle. Thus, if the curve were too far to the left, the circulatory blood would remain mostly saturated with respect to oxygen (or if too far to the right, mostly unsaturated), and the efficiency of oxygen transport in vivo would be greatly reduced. A somewhat surprising and obviously significant feature of the picture, first noticed by Benesch and Benesch (1974), is that in the presence of lower, nonsaturating amounts of IHP or DPG, the OEC becomes biphasic. In view of the major physiological importance of these phenomena and their relevance to an understanding of the underlying mechanism of linkage, it was decided to reexamine the reactions making use of recently developed methods of high precision for studying gaseous equilibria in Hb solutions.

MATERIALS AND METHODS

Hemoglobin was prepared from fresh blood of non-smoking donors. The red cells were washed three times in isotonic 40 mM Hepes buffer, pH 7.4, centrifuged at 4°C to remove plasma and the white cells, and then lysed with bidistilled water (ratio 3:1). After removal of the membrane debris by centrifugation, the hemolysate was extensively dialyzed against 50 mM Tris-HCl buffer pH 8.0 for 36 h under continuous agitation; three exchanges of the buffer were performed. The lysate was then passed

[1] *Abbreviations used in this paper:* HbA, human adult hemoglobin A; MetHb, methemoglobin; DPG, 2,3-bis diphosphoglycerate; IHP, inositol hexaphosphate; P_{50}, pO_2 at half saturation; P_m, median pO_2; n_{50}, Hill coefficient at half saturation; MWC, Monod-Wyman-Changeux.

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through a DEAE-Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) column to separate HbA ($\alpha_2\beta_2$) from the minor Hb fractions HbA_1 and HbA_2 using a linear pH gradient of 50 mM Tris/HCl buffer from pH 7.9 to 6.9. After separation the purity of the isolated HbA was verified by isoelectrofocusing, which revealed a single band migrating at pI 6.95. The resulting HbA solution was freed of remaining small anions (phosphate and chloride) through a recirculating mixed-bed ion exchange resin column (Dowex AG501-X8; Bio-Rad Laboratories, Richmond, CA) that had been pre-washed with bidistilled water (Jelkmann and Bauer, 1976). Repeated tests for the presence of phosphate compounds demonstrated negligible amounts after this procedure. The pure stripped HbA solution was concentrated under gas pressure in a Diaflo Amicon cell (Amicon Corp., Danvers, MA), frozen in small droplets in liquid nitrogen, and used within 1 mo. Before recording the OEC, the thawed Hb stock solution was equilibrated with pure oxygen and exposed to intense light for 30 min in an ice-water bath under gentle agitation to remove any trace of carbon monoxide that might have bound to Hb during exposure to room air. We tested for the presence of methemoglobin (MetHb) in the sample by recording the electronic spectrum of an oxygenated diluted solution on a Cary 219 spectrophotometer (Varian Associates, Inc., Palo Alto, CA). From the ratio $A_{576.5}/A_{500}$ nm at pH 7.0, 25°C (Kilmartin et al., 1978), <1% MetHb was present in the stock solution used in this study. We have also checked for the presence of small amounts of cofactors remaining in the solution by measuring the oxygen affinity in pure distilled water. The P_{50} (pO_2 at half saturation) of the HbA solution under this condition (pure water, 25°C, pH 7.5) was found to be 0.7 mmHg, in good agreement with results reported by Berger et al. (1973).

OEC were determined at pH 7.2 and 25° ± 0.2°C using an automatic device (Hemox Analyzer; TCS-Medical Products Co., Huntingdon Valley, PA) as described previously (Asakura, 1979; Craescu et al., 1986; Edelstein et al., 1986) interfaced to a Hewlett-Packard 85 computer (Hewlett-Packard Co., Palo Alto, CA). Each recording was obtained by bubbling pure nitrogen into the solution after its initial equilibration with pure oxygen. OEC curves measured by reoxygenation were in good agreement with deoxygenation curves, implying equilibrium conditions were established. Measurements of the curves require ~45 min and involve the recording of at least 300 points of the OEC. The results were stored on cassette for further graphical representation and analysis. In each case catalase (20 μg/ml) was added to the solution to limit oxidation of the hemes, which was always found to be <5% at the end of the runs. Concentrations of hemoglobin were measured as cyanomethemoglobin (with the Cary 219). Buffers were prepared with ultra-pure water (MilliQ; Millipore Corp., Bedford, MA) and contained 10 mM Hepes. Stock solutions of IHP at pH 7.2 were prepared with Na-phytate (Sigma Chemical Co., St. Louis, MO). The pH was kept constant in this series at 7.2 and did not vary significantly between the start and the end of the experiments.

CALCULATIONS

The abnormal shape of the OEC was analyzed graphically in terms of the Hill coefficient, n, as a function of the fractional oxygen saturation. This function was calculated as the first derivative of the Hill equation by regression analysis for every $i + 3$ and $i - 3$ points of the curve. IHP concentrations are expressed in terms of the molar ratio of the effector to the concentration of tetrameric Hb. Experimental OEC were fitted to the present expanded two-state model (Eq. 2) using an iterative nonlinear least-squares regression program according to Bevington (1969). Initial estimates of log KR and log KT were obtained from Hill graphs by assuming a slope of unity for $\bar{Y} > 0.99$ and $\bar{Y} < 0.01$, respectively. Although the initial estimate for log KT is readily obtained from the experimental graphs whether IHP is present or not, log KR is ill-defined in the presence of the cofactor. According to the theoretical considerations developed in the next section, initial estimate of log KR values for the OEC in the presence of IHP were therefore calculated as $\log KR' = \log P_m - \frac{1}{4} \log L'$, where KR', P_m, and L' are defined below. The quality of the fits was estimated by statistical analyses to obtain the standard error per experimental point (σ), the percentage error for each parameter value of the model, and graphs of the standard residuals, i.e., $(\bar{Y}_{exp} - \bar{Y}_{calc})/\sigma$.

P_{50} and n_{50} (Hill coefficient at half saturation) values were computed from the experimental points in the range of 40–60% oxygen saturation by linear regression analysis.

THEORETICAL CONSIDERATIONS

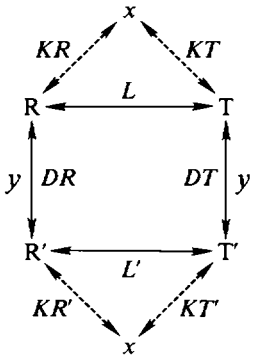
When both a ligand, x, which binds to Hb at four sites and an effector, y, which binds to one site are present, the general binding polynomial is (Wyman, 1964)

$$P_{(yx)} = \sum_{j=0}^{1} \sum_{i=0}^{4} \beta_{ji} x^i y^j \quad \text{with} \quad \beta_{00} = 1$$
$$= 1 + \beta_{01}x + \beta_{02}x^2 + \beta_{03}x^3 + \beta_{04}x^4 + \beta_{10}y$$
$$+ \beta_{11}xy + \beta_{12}x^2y + \beta_{13}x^3y + \beta_{14}x^4y, \quad (1)$$

where x and y refer to the activity of oxygen and organophosphate, respectively, and the β's are cumulative association constants. This general binding polynomial can also be expressed as the sum of two subpolynomials for x without y (P_{0x}) and for x with y (P_{1x}) (see Appendix A for details). In these equations y refers to the concentration of free organophosphate which is in the presence of Hb less than the total concentration of the effector present. Our experimental method does not distinguish between "free" and "bound" effector precluding therefore the determination of the five overall equilibrium association constants of the cofactor to Hb upon oxygen binding. To our knowledge there exists no simple method to measure independently these quantities as a function of oxygen saturation. MacQuarrie and Gibson (1972) have proposed the use of a fluorescent analogue of DPG as a probe of conformational changes in Hb upon ligand binding. Their method allows the determination of the free-analogue concentration at different levels of saturation, which is, at equilibrium, proportional and almost linearly related to ligand binding. However, it does not permit the accurate and physically meaningful determination of the equilibrium association constants of the fluorescent probe at the ith steps of oxygenation. We therefore analyzed the OEC as a function of the total concentration of the effector added. Only from data at sufficiently high concentrations of IHP can we determine the true binding polynomial. This led us to use the framework of the two-state allosteric formulation to provide a thermodynamic model that can explain the "double hump" effect.

In the analysis of the results described below we postulate that tertiary as well as quaternary changes are involved when a heterotropic cofactor like IHP is bound, i.e., that the organophosphate influences to varying extents the intrinsic affinities for oxygen of both the T and R tetramers.

The new expanded two-state allosteric model includes variation in the intrinsic affinities of the heme due to mass-law binding of IHP when the tetramers are either in the R or T quaternary states. In this model Hb may exist in four conformers, each with four identical and independent binding sites, and one organophosphate binding site according to the following scheme:



In terms of the allosteric parameters, Eq. 1 becomes

$$P_{(yx)} = (1 + \alpha)^4 + L(1 + c\alpha)^4 + y[(1 + \alpha')^4 DR + L(1 + c'\alpha')^4 DT]$$

or

$$= (1 + \alpha)^4 + L(1 + c\alpha)^4 + yDR[(1 + \alpha')^4 + L'(1 + c'\alpha')^4], \quad (2)$$

where $\alpha = x/KR, \alpha' = x/KR', c = KR/KT, c' = KR'/KT'$, $L = T_0/R_0$ and $L' = T_0'/R_0' = L(DR/DT)$. DT and DR are the association binding constants of IHP to the T and R states, respectively.

This representation for fitting the experimental data involves only seven parameters which can be further reduced to five (L, c, DR, DT, c') if one is interested only in the shape of the curve.

The calculation of the free effector concentration is obtained as described in Eq. A5 given in Appendix A, with

$$X = (1 + \alpha)^4 + L(1 + c\alpha)^4$$

and $$Z = [(1 + \alpha')^4 + L'(1 + c'\alpha')^4]DR.$$

An important parameter may be calculated from this model: the median pO_2 (P_m), given in Appendix B.

A clear understanding of the double hump effect in Hb also requires calculation of the variations of the fractional amounts of the four postulated conformers R, R', T, and T'. These were obtained from the following equations:

$$\bar{T} = \frac{L(1 + c\alpha)^4}{P_{(yx)}}, \quad \bar{T}' = \frac{L(1 + c'\alpha')^4 DTy}{P_{(yx)}},$$

$$\bar{R} = \frac{(1 + \alpha)^4}{P_{(yx)}}, \quad \bar{R}' = \frac{(1 + \alpha')^4 DRy}{P_{(yx)}}, \quad (3)$$

where $P_{(yx)}$ is defined by Eq. 2.

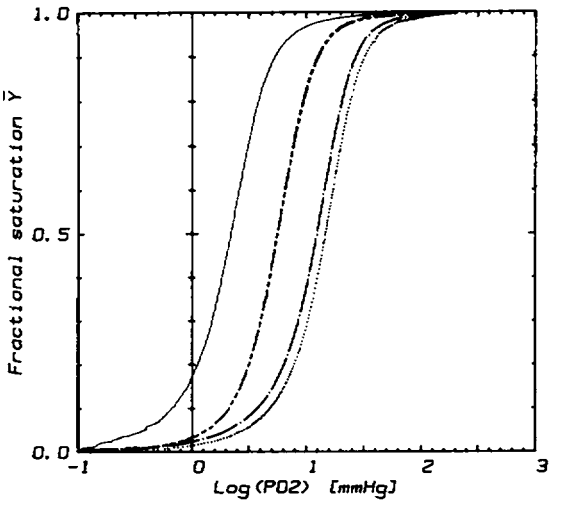


FIGURE 1 Effect of varying chloride concentrations on the OEC for dilute Hb solutions. From left to right: [Cl^-] = 5, 100, 400, 600 mM. Conditions: 10 mM Hepes buffer, pH 7.2, T 25°C, 180–200 μM [Heme].

RESULTS

Titration of hemoglobin solutions with chloride at any given pH leads to a shift of the oxygen binding curve to the

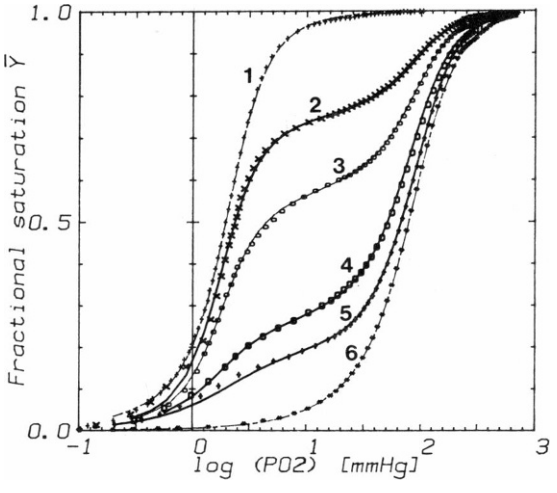


FIGURE 2 Effect of varying concentrations of IHP on the OEC for dilute Hb solutions. Experimental curves, from left to right, the [IHP]/[Hb_4] ratio was: (*1*) 0; (*2*) 0.25; (*3*) 0.44; (*4*) 0.75; (*5*) 0.82; (*6*) 2.0. Other conditions as in Fig. 1 at 5 mM chloride. Symbols are one of every six experimental data points. Lines are model curves calculated with Eq. 2 according to the procedure described under calculations.

right without appreciable change of shape (Fig. 1). For all these curves, the Hill coefficient n_{50}, always close to n_{max}, remains unchanged at ~3. A different pattern is observed when IHP is present at nonsaturating concentrations (Fig. 2). In this case the curves become biphasic with inflexion points depending on the phosphate/tetramer ratio. When nonsaturating levels of IHP are used (Fig. 3 *A*) and the first derivative of the Hill equation is examined (Fig. 3 *B*), two clearly distinct phases are seen. This pattern is what we call the double hump effect.

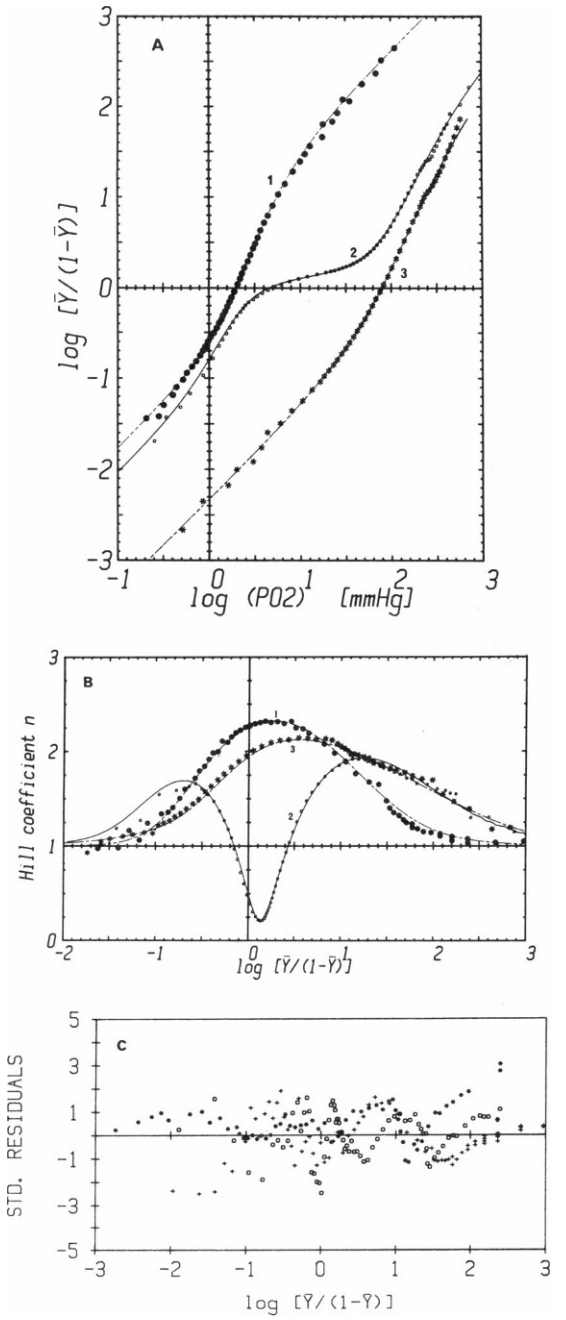


FIGURE 3 Effect of constant, nonsaturating and saturating concentrations of IHP on the shape of the OEC for dilute Hb solutions. Symbols are one of every six experimental data points. Lines are the best fits calculated from the theoretical model described in the text (Eq. 2). *1* [IHP] = 0 (●); *2*, [IHP]/[Hb_4] = 0.44 (o); *3*, [IHP]/[Hb_4] = 2 (•). (*A*) Hill plots; (*B*) cooperativity curves; (*C*) standard residuals [= $(\bar{Y}_{obs} - \bar{Y}_{calc})/\sigma$] for each curve after fitting to Eq. 2. Other conditions as in the legend of Fig. 2.

Figs. 2 and 3 reveal another important aspect of the effects of IHP on the OEC. Compared with the control curve, addition of varying concentrations of IHP leads to a shift to the right of the entire OEC including both its lower and upper portions. This effect indicates that addition of organophosphate results in a change in the intrinsic oxygen affinity of the heme whether the tetramers are in the R or T states.

Table I gives the values of the oxygen binding parameters calculated from the expanded two-state model described above. Model curves shown in Figs. 2 and 3 satisfactorily fit the experimental data points. These curves were calculated with parameter values given in Tables I and II (line 1).

The graph representing the standard residuals as a function of log $[\bar{Y}/(1 - \bar{Y})]$ for these curves is shown in Fig. 3 *C* and indicates that no systematic errors were present due to the experimental procedure. Fig. 2 presents a family of curves calculated with these same parameters for varying concentrations of IHP that correspond closely to the experimental curves.

Lacking an independent experimental estimate for the values of the parameters *DT* and *DR* we have checked for the possibility of several satisfactory solutions with different sets of values capable of giving fits of the quality shown in Figs. 2 and 3. This was done by fixing all the parameters given in Table II except *DR* or *DT* and initial

TABLE I
OXYGEN BINDING PARAMETERS FOR DILUTE Hb SOLUTIONS WITH VARYING IHP OR CHLORIDE CONCENTRATIONS

	[Cl]	[IHP]/[Hb_4]	P_{50}	n_{50}	P^*_m	$L^‡$	c	P_m/P_{50}	$\sigma^§$
	mM		*mm Hg*						$\times 10^3$
A	Effect of varying IHP concentrations								
1	5	0	1.8	2.3	1.8	6.6×10^2	0.051	1.0	3.1
2	5	0.25	2.3	1.8	4.2	6.9×10^2	0.113	1.8	5.8
3	5	0.44	4.0	0.5	8.3	1.0×10^4	0.069	2.1	4.3
4	5	0.75	49.4	1.2	23.8	7.1×10^5	0.056	0.5	4.9
5	5	0.82	62.3	1.5	34.6	3.1×10^6	0.047	0.6	9.8
6	5	2.0	77.2	2.0	67.3	4.5×10^7	0.006	0.9	4.6
B	Effect of varying chloride concentrations with nonsaturating concentrations of IHP								
7	100	0.44	9.2	1.0	11.2	6.5×10^4	0.028	1.2	4.6
8	400	0.50	13.1	2.7	13.1	1.9×10^5	0.006	1.0	3.0

Conditions were pH 7.2, 10 mM Hepes buffer, 25°C, [heme] = 160–200 μM.

*Calculated according to Eq. B7 (Appendix B) with the expanded two-state model.

‡In the absence of IHP, $L = (P^o_m/KR)^4$; in the presence of IHP, $L = (P_m/KR')^4$.

§Standard error per point calculated after an iterative nonlinear least-square fitting procedure with Eq. 2 given in the text.

TABLE II
OXYGEN AND IHP BINDING PARAMETERS USED IN THE EXPANDED TWO-STATE MODEL FOR HbA SOLUTIONS WITH VARYING CHLORIDE CONCENTRATIONS

[Cl]	DT*	DR*	L‡	c‡	KR‡	KR/KR'	c/c'	DT/DR
	I	II	III	IV	V	VI	VII	
mM	M^{-1}	M^{-1}			*mm Hg*			
1 5	2.5×10^{11}	2.1×10^{6}	6.6×10^{2}	0.051	0.34	0.41	10.1	119,000
2 100	5.5×10^{8}	1.2×10^{6}	3.7×10^{4}	0.019	0.34	0.49	2.7	458
3 400	6×10^{3}	3×10^{3}	2×10^{5}	0.006	0.63	1.0	1.0	2

Experimental conditions: pH 7.2, 10 mM Hepes buffer, 25°C, [heme] = 160–200 μM. For each condition of chloride concentration one can fit all the OEC for varying IHP concentrations with one set of seven parameters, labeled I to VII, defined in Eq. 2. The percentage of error (SE × 100/parameter value) for the parameters was 20–40% for DT and DR, and 5–15% for parameters III to VII.

*Association binding constants for IHP to deoxyHb(DT) and oxyHb (DR).

‡Oxygen binding parameters for OEC in the absence of IHP.

estimates differing by plus or minus one order of magnitude from the values used in the former calculations. We found that within a small range of error (±20%) the values of DT and DR given in Table II correspond to the best quality of the fits. One should stress also that the values of the association constants of IHP to the T or R states are within the range of reported values under similar experimental conditions of Hb and chloride concentrations (Hamasaki and Rose, 1974; Edalji et al., 1976; Goodford et al., 1977, 1978; Imaizumi et al., 1979; Imai, 1982).

Using Eq. B7 (Appendix B), it was possible to calculate the P_m values corresponding to the model curves (Table III). These values were compared with the calculated concentrations of the free cofactor at P_m (Eq. B8). The maximal slope of the regression line of log P_m versus log (IHP_{free}) at P_m is 0.25 M oxygen-linked IHP bound per heme indicating a stoichiometric ratio of unity for IHP binding to Hb tetramers.

TABLE III
THE FREE-IHP CONCENTRATION DEPENDENCE OF Log P_m, CALCULATED WITH THE EXPANDED TWO-STATE MODEL

[Cl]	$\frac{[IHP]}{[Hb_4]}$	log IHP_{total}	log IHP_{free} at P_m*	log p_m‡
mM				
5	0	—	—	0.233
5	0.25	−4.90	−9.83	0.626
5	0.50	−4.60	−8.28	1.012
5	0.75	−4.43	−6.79	1.383
5	1	−4.30	−5.36	1.717
5	2	−4.00	−4.64	1.828
5	20	−3.00	−3.39	1.883
5	200	−2.00	−2.36	1.887
5	1,000	−1.30	−1.66	1.887
100	0.5	−4.60	−7.25	1.051
200	0.5	−4.60	−6.00	1.070
400	0.5	−4.60	−4.70	1.118

[IHP] expressed in M, P_m in mm Hg. Calculations were made assuming 200 μM heme and using the parameters given in Table II.

*Calculated with Eq. B8.

‡Calculated with Eq. B7.

The values of KR in the presence of IHP cannot be estimated accurately by graphical analyses. The best initial estimate of KR' before fitting was therefore calculated from the P_m value at saturating amounts of IHP and the value of $L' = L(DT/DR)$ according to the square model described above. Taking a value for KR' of 0.82 mmHg at low chloride concentration (Table II) as a minimal estimate, the upper portion of the experimental curves and the resulting high value of their graphical asymptote may be interpreted as resulting from the presence of R state tetramers with and without IHP bound (R and R' states) and of oxygenated T' state tetramers with a much lower oxygen affinity. Fig. 4 shows curves for the variations of the percentage of the four conformations $\overline{R}$, $\overline{R}'$, $\overline{T}$, and $\overline{T}'$ upon oxygenation in the presence of varying concentrations of IHP (Eq. 3). These graphs indicate that, under condi-

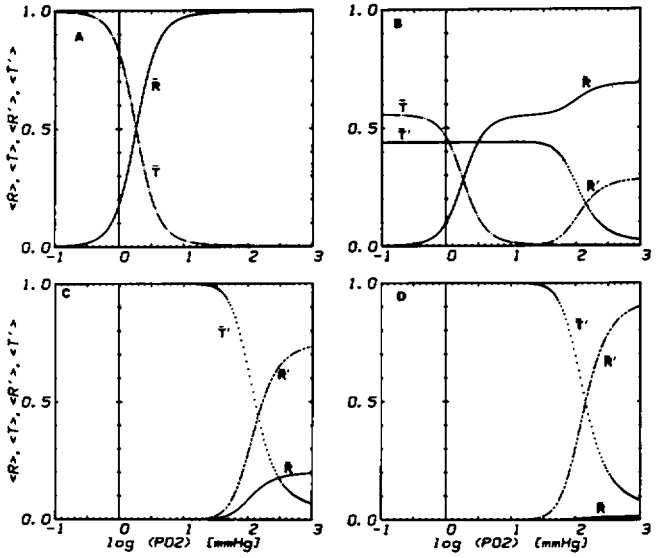


FIGURE 4 Model curves for the variation of the four conformers versus log pO_2 corresponding to the theoretical model described in the text, Eq. 3. (*A*) [IHP] = 0; (*B*) [IHP]/[Hb_4] = 0.5; (*C*) [IHP]/[Hb_4] = 2; (*D*) [IHP]/[Hb_4] = 20. The corresponding OEC are those shown in Fig. 2. Parameter values used for these calculations were those given in Table II at 5 mM chloride concentration.

tions where IHP is present in excess over tetramer concentration, a small fraction of T' state tetramers are indeed present under conditions of full oxygenation.

Chloride is another anionic cofactor of tetrametric Hb that binds preferentially to deoxy Hb, but with much lower binding constants than organophosphates (Haire and Hedlund, 1977). Its binding occurs on both α- and β-chains sites, the latter being close or identical in those for IHP or DPG (Chiancone et al., 1975). Therefore chloride competes with organophosphate binding. Experiments described in Fig. 5 show that addition of varying concentrations of chloride to solutions containing nonsaturating amounts of IHP shifts the upper portion of the OEC to the left and its lower portion far to the right. At sufficiently high concentrations of chloride (400 mM) the double hump effect vanishes and the shape of the OEC becomes monophasic. The curves shown in Fig. 5 were analyzed within the framework of the present expanded model. Satisfactory fits were obtained with parameters values given in Table II. These include both a decrease in the absolute values of DT and DR and of the DT/DR ratio which becomes equal to 2 at the highest chloride concentration. In this case the model reduces to a simple two-state model as KR/KR' and c/c' are also unity.

DISCUSSION

The most interesting and challenging feature of the observations reported here in complete agreement with earlier work (Imai and Tyuma, 1973; Benesch and Benesch, 1974; Ackers, 1979), is the transitory change of shape of the oxygen binding curve in the presence of nonsaturating amounts of organophosphate effectors and the way this change is affected by addition of chloride ion. In this respect, the organophosphates stand in marked contrast to

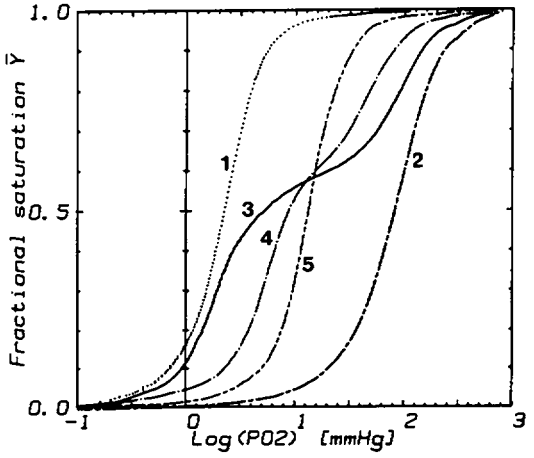


FIGURE 5 Effect of chloride on the shape of OEC for dilute Hb solutions in the presence of constant, nonsaturating concentrations of IHP. Curves *1* and *2* are the reference curves determined in the absence and in the presence of a saturating concentration of IHP. Curves *3–5* are OEC determined at [IHP]/[Hb_4] ratio of ~0.5 in the presence of 5, 100, and 400 mM chloride, respectively.

protons as allosteric effectors at least for oxygen binding by hemoglobin A where the oxygen binding curves shift without any such change in shape as pH is varied. Direct comparison, however, is complicated by the fact that in one case we are concerned with curves measured at constant concentration, and in the other, at constant activity of the effector. Moreover, in the case of the organophosphates there is no self-buffering, as occurs for protons due to the presence of a large reservoir of proton binding sites unlinked to oxygen. The result of this difference is that a very slight change in the concentration of free organophosphate will produce a large change in its activity. When this effect is taken into account there is no reason for postulating any fundamental difference of behavior between the two effectors.

A phenomenological interpretation of the double hump effect was rationalized by Imai and Tyuma (1973) and Ackers (1979) who developed a model with nine parameters for each individual curve whose values cannot be independently determined experimentally. Their approach has been successful in reproducing a "double hump" effect and for estimating the variations of the free cofactor concentration upon oxygen binding. However the resulting association binding constants for the phosphate to Hb are of doubtful physical meaning. Furthermore their explanation does not take into account the variations of the intrinsic oxygen affinity at the ith step of ligand binding in the presence of the cofactor. More clearly in this analysis the constants a_i are equal to a_i' in Eq. B2 (see Appendix B).

Any attempt to analyze the biphasic shape of the OEC for nonsaturating concentratons of organophosphates using the simple two-state model (Monod et al., 1965) was unsuccessful (Fig. 6). Failure of the original Monod-Wyman-Changeux (MWC) model to reproduce complex heterotropic effects in Hb has been recognized by several authors (Herzfeld and Stanley, 1974; Goodford et al., 1977, 1978; Szabo and Karplus, 1976; Ackers, 1979; Ackers et al., 1982; Lee and Karplus, 1983; Lee et al., 1987), because this model does not take into account variations in the intrinsic affinity for ligand of both the T and R states. The present results show that, in addition to the double hump effect, such variations do occur upon addition of potent cofactors such as IHP.

This led us to consider an expansion of the original MWC model. Minton and Imai (1974) had developed earlier a three-state model taking into account the obvious variation of KT in the presence of heterotropic cofactors. The equation for $\bar{Y}$ in the three-state model is the same as Eq. 2 except that $\alpha' = \alpha$. It is shown in Fig. 6 that this model does not fit accurately the upper portion of the curve. This discrepancy between a three-state model and the present expanded two-state model may be explained by the presence of 100 mM chloride in the experiments reported by Minton and Imai (1974). Indeed, in the presence of 100 mM chloride the intrinsic oxygen affinity

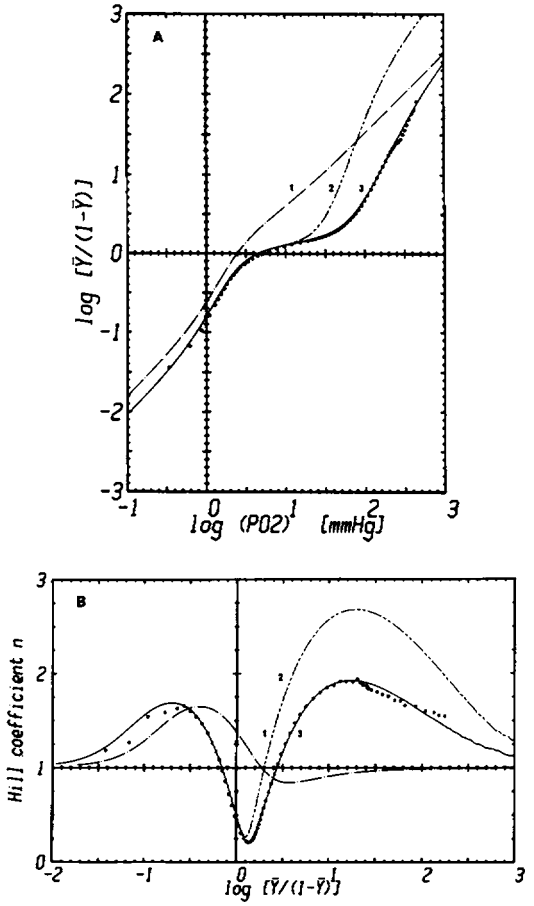


FIGURE 6 Model curves (Eq. 2) calculated with: (*1*) the simple two-state allosteric model with $c/c' = 1$ and $KR/KR' = 1$; (*2*) the three-state allosteric model with $c/c' = 24.5$ and $KR/KR' = 1$; (*3*) the expanded two-state model with $c/c' = 10.1$ and $KR/KR' = 0.41$. (*A*) Hill plots; (*B*) cooperativity curves. Symbols are one of every six experimental data points.

for the R state is modified by IHP to a lower extent due to the competition between the two anions. Another question raised by the three-state model is that it postulates the existence of a third stable quaternary super T state, the S state, which, in the formulation of the authors is not "a degenerated T state." Actually there are several upper limits for the variations of *KT* which depend upon the association constant of the cofactor present, and also, upon temperature and pH (MacQuarrie and Gibson, 1972; Benesch and Benesch, 1974; Edalji et al., 1976; Imai, 1982).

Variations for *KR* have been more difficult to demonstrate, mainly for experimental reasons, and have been usually considered as negligible. However, a pH-dependence of *KR* has been reported recently from both equilibrium and kinetic experiments (De Young et al., 1976; Schweitzer-Stenner et al., 1984, 1986; Lee et al., 1987). Changes in the R state intrinsic affinity of the hemes in the presence of IHP at low chloride concentrations are therefore likely to occur, as suggested from early kinetic studies by Gibson and Gray (1970) and also by Desbois and Banerjee (1975). Recently Bellelli et al. (1987) have shown that HbA cross-linked to pyridoxal phosphate derivative at the polyphosphate binding site has a "perturbated *KR*." Spectrophotometric evidence was also reported (Perutz et al., 1976; Maxwell and Caughey, 1976) showing modifications of stereochemistry of the hemes in nitrosyl Hb in the presence of IHP, indicating tertiary changes in the subunits in the liganded tetramers. If these variations are taken into account we are led to the present expanded two-state model which permits effector-dependent changes in *KR* and *KT* while keeping the basic concerted mechanism for the binding of oxygen.

The present model also allows a clear explanation of the double hump effect. Fig. 7 shows that the effect is related to large variations in the concentration of the free cofactor. We assume here that activity and concentration are identical. A one- to twofold molar excess of IHP over tetramer concentration is sufficient to render the OEC monophasic. For nonsaturating concentrations of IHP, addition of chloride inhibits to a large extent the variation of free IHP, contributing therefore to making the OEC progressively more symmetrical (Table IB).

Calculations of the variations of the percentage of the four conformers postulated in the present model give specific insight in the origin of the double hump effect. This aspect is shown in Fig. 4. When no IHP or saturating amounts of IHP are present only two quaternary conformers are present whose transition T ⇄ R or T′ ⇄ R′ upon oxygenation will contribute to one single peak of *n* value versus saturation, hence to monophasic curves. At nonsaturation concentrations of the cofactor, calculated curves in Fig. 4 *B* indicate that the two peaks of *n* values (Fig. 3 *B*) occur at oxygenation levels where the T ⇄ R or T′ ⇄ R′ are close to equilibrium. The trough between the two humps is characteristic of a condition where no variation of

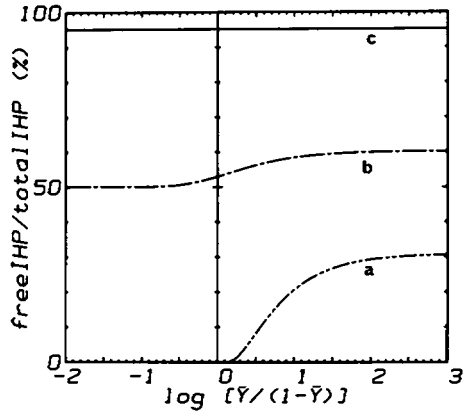


FIGURE 7 Calculated variations of the free IHP concentrations versus log $[\bar{Y}/(1 - \bar{Y})]$ (Eq. A5 given in Appendix A) at varying total IHP concentrations. (*A*) $[IHP]/[Hb_4] = 0.5$; (*B*) $[IHP]/[Hb_4] = 2$; (*C*) $[IHP]/[Hb_4] = 20$. Parameter values are those given in Table II at 5 mM chloride concentration.

the quaternary states T′ and R occurs over a wide range of pO_2 values. In conclusion the present model is presumably an oversimplification of the complex interactions that occur between hemoglobin and its effectors. It is the advantage but also the limit of any model to focus on particular experimental aspects and to neglect others. In the present report the interactions have been treated purely between tetrameric hemoglobin and its cofactor IHP neglecting the existence of dimers in the R state since IHP reduces the tetramer dissociation into dimers to negligible amount (Hensley et al., 1975; Benesch et al., 1986), the inequivalence between α and β hemes (Edelstein, 1975) and the possible influence of other solution conditions (Wyman, 1984).

APPENDIX A

The nine β_{ij} (Eq. 1) can be expressed as the four Adair (1925) constants, A_i, corresponding to oxygen equilibrium association constant for $Hb(O_2)_i$:

$$A_i = \frac{\beta_{0i} + \beta_{1i}y}{1 + \beta_{10}y}, (i = 1 \text{ to } 4), \tag{A1}$$

permitting the general binding polynomial to be expressed as

$$P_{(yx)} = 1 + A_1x + A_2x^2 + A_3x^3 + A_4x^4. \tag{A2}$$

This general binding polynomial can also be expressed as the sum of the two sub-polynomials for x without y, $P_{(0x)}$, and for x with y, $P_{(1x)}$, such that

$$P_{(yx)} = P_{(0x)} + P_{(1x)} = X + Zy, \tag{A3}$$

where

$$X = \sum_{i=0}^{4} \beta_{0i}x^i \quad \text{and} \quad Z = \sum_{i=0}^{4} \beta_{1i}x^i.$$

The amount of free y can be calculated as the difference between its total amount and the amount bound to Hb by mass-law binding:

$$(y_{free}) = (y_{total}) - (\bar{Y}_{IHP})(Hb_4), \tag{A4}$$

where

$$(\bar{Y}_{IHP}) = \left(\frac{d \log P_{(yx)}}{d \log y}\right)_x$$

and (Hb_4) is the concentration of tetrameric Hb.

(y_{free}) is obtained as the positive root of the equation

$$Z(y_{free})^2 - \{[(y_{total}) - (Hb_4)]Z - X\}(y_{free}) - X(y_{total}) = 0. \tag{A5}$$

Application of these equations requires the knowledge of both the total concentration of tetrameric Hb and of the equilibrium association constants of the effector to hemoglobin.

APPENDIX B

When the OEC are asymmetrical, the median pO_2 (P_m) differs widely from the P_{50} value (Table I) and should be estimated accurately to obtain L. From the definition of the median pO_2 (Wyman, 1964),

$$P_m = (A_4)^{-1/4}. \tag{B1}$$

The fourth Adair coefficient A_4 of Eq. A1 (Appendix A) can be written as

$$A_4 = \frac{(a_4 + a'_4DRy)}{(1 + DTy)}, \tag{B2}$$

where

$$\beta_{04} = a_4, \quad \beta_{14} = a'_4DR, \quad \text{and} \quad \beta_{10} = DT.$$

We use two different coefficients for the individual equilibrium constants for the fourth oxygenation step without (a_4) and with (a'_4) y present to take into account the variation of the intrinsic affinity of the hemes on the fourth oxygen binding. Thus, we introduce the ratio G_4 as

$$A_4 = \frac{a_4(1 + G_4DRy)}{(1 + DTy)}. \tag{B3}$$

We calculated a_i and a'_i with use of the allosteric parameters of the present model (Edelstein, 1975):

$$a_i = \left(\frac{4!}{(4-i)!i!}\right)\frac{(1 + Lc^i)}{(1 + L)KR^i}, \; a'_i = \left(\frac{4!}{(4-i)!i!}\right)\frac{(1 + Lc'^i)}{(1 + L)KR'^i}$$

with $i = 1, 2, 3, 4$.

Then

$$G_4 = \frac{a'_4}{a_4} = \frac{(1 + Lc'^4)KR^4}{(1 + Lc^4)KR'^4}. \tag{B4}$$

At saturating concentrations of IHP and from the log form of Eq. B1, $\log P_m$ is obtained as

$$\log P_m = \log P^\circ_m + \frac{1}{4}\log\frac{(1 + DTy)}{(1 + DRG_4y)}, \tag{B5}$$

with $y_{free} = y_{total} = y$ and $P^\circ_m = (a_4)^{-1/4}$.

When the cofactor is present at nonsaturating concentrations and varies to a large extent upon oxygen binding its contribution to the equation for P_m must be considered. In this case the $(\bar{Y}_{IHP})$ integral between the limits y_0 ($x = 0$), y_∞ ($x = \infty$) should be expressed with Eq. A4 as

$$\int_{y_0}^{y_\infty} (\bar{Y}_{IHP})\, d \log y = \int_{y_0}^{y_\infty} \frac{y_{tot}}{(Hb_4)}\, d \log y - \int_{y_0}^{y_\infty} \frac{1}{(Hb_4)}\, dy$$

$$= \frac{y_{tot}}{(Hb_4)}\log\frac{y_\infty}{y_0} - \frac{(y_\infty - y_0)}{(Hb_4)}. \tag{B6}$$

Then

$$\log P_m = \log P^\circ_m + \frac{1}{4}\log\frac{(1 + DTy_0)}{(1 + DRG_4y_\infty)} + \frac{y_{tot}}{(Hb_4)}\log\frac{y_\infty}{y_0} - \frac{(y_\infty - y_0)}{(Hb_4)}, \tag{B7}$$

where y_0 and y_∞ are the concentrations of free organophosphate for the limits $x \to 0$ and $x \to \infty$, and are given as positive root of the Eq. A5 (Appendix A) with $X = 1$, $Z = DT$, and $X = 1$, $Z = DRG_4$, respectively.

From Eq. B5 and Eq. B7, it is possible to make an estimation of the concentration of (IHP_{free}) at the median pO_2 (Ackers, 1979):

$$(IHP_{free}) \text{ at } P_m = \frac{1 - \left(\frac{P_m}{P^\circ_m}\right)^4}{DRG_4\left(\frac{P_m}{P^\circ_m}\right)^4 - DT}. \tag{B8}$$

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