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Analysis of Oxygen Equilibrium of Hemoglobin and Control Mechanism of Organic Phosphates†

Itiro Tyuma,* Kiyohiro Imai, and Katsuhiko Shimizu

ABSTRACT: Oxygen equilibrium curves of hemoglobins precisely determined by the automatic recording apparatus have been analyzed according to Adair's (Adair, G. S. (1925), *J. Biol. Chem. 63*, 529) stepwise oxygenation theory, and four successive association constants (k values) for the binding of oxygen have been estimated by the least-squares method. 2,3-Diphosphoglycerate markedly reduces k_1, k_2, and k_3 for human adult hemoglobin without affecting k_4. In contrast, inositol hexaphosphate enormously reduces all four k values. The glycerate also shows a similar but weaker effect on human fetal hemoglobin. The effect of 0.1 M NaCl on the oxygen equilibrium parameters is qualitatively similar to that of the glycerate, except that the neutral salt increases k_4 slightly. Calculation of the hemoglobin fraction of various intermediate oxygenation stages reveals that the fraction of $Hb(O_2)_3$ is negligibly small in the presence of the organic phosphates and/or NaCl except at very high oxygen saturation. Analysis of k values in the presence of 0.1 M NaCl and various concentrations of 2,3-diphosphoglycerate indicates that the phosphates combine not only with the fully deoxygenated hemoglobin but also with the intermediates, $Hb(O_2)_1$ and $Hb(O_2)_2$, with considerable affinity. The binding constant of the phosphate for the fully deoxygenated human adult hemoglobin at pH 7.4 and 25° has been estimated as 3.1×10^4 M^{-1}, which agrees well with that obtained by the direct binding experiment. The overall free energy of interaction among oxygen combining sites is increased by 2 mM 2,3-diphosphoglycerate in the absence of added NaCl by about 5100 and 2700 cal/mol in the adult and fetal hemoglobins, respectively, which corresponds to the formation of four and two, respectively, additional salt bridges between the glycerate and the deoxyhemoglobins. These results are consistent with the model proposed by Perutz [Perutz, M. F. (1970), *Nature (London) 228*, 726] for the cooperative oxygen binding of hemoglobin.

The discovery of the tremendous effect of organic phosphates, especially 2,3-diphosphoglycerate (P_2glycerate)[1] and inositol hexaphosphate (IHP), in lowering the oxygen affinity of human adult hemoglobin (Hb A) (Benesch and Benesch, 1967; Chanutin and Curnish, 1967; Benesch *et al.*, 1968) has drawn much attention to the mechanism and physiological role of this heterotropic interaction. It has been shown that P_2glycerate binds with an overwhelming preference for deoxy Hb A, not for the fully oxygenated hemoglobin, and in a mole: mole ratio in a specified experimental condition (Benesch *et al.*, 1968, 1971). This will undoubtedly reduce the affinity of Hb A to the first oxygen molecule and increase the overall energy of interaction among oxygen binding sites. However, the effect of the phosphates on the oxygen affinity of Hb A in an intermediate stage of oxygenation as well as the overall free energy of interaction in the presence of the phosphates are still unknown. These questions are of critical importance to the understanding of the mechanism of action of the organic phosphates.

The present paper is an approach to these problems from an equilibrium point of view based on the successive oxygenation theory (Adair, 1925). Four successive association constants of hemoglobin for the binding of oxygen estimated by the least-squares method on accurately determined oxygen equilibrium curves in the absence and presence of the organic phosphates will be given together with standard errors involved in the estimation. The binding constant of P_2glycerate for hemoglobin in various oxygenation stages calculated from the four association constants will also be shown. Preliminary accounts of this study, where the association constants were estimated by a trial-and-error curve-fitting procedure on the Hill and Scatchard plots, have been published elsewhere (Tyuma *et al.*, 1971a,b).

Materials and Methods

Materials. Hb A prepared from the blood of normal human adults (nonsmokers) and human fetal hemoglobin (Hb F) isolated from cord blood by the method of Zade-Oppen (1963) were freed from phosphates according to Benesch

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[1] Abbreviations used are: P_2glycerate, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; Hb A, human adult hemoglobin; Hb F, human fetal hemoglobin; bisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane.

et al. (1968). The molar ratio of residual P_2glycerate to the hemoglobins was less than 0.01 as determined by the method of Lowry *et al.* (1964). The concentrations of hemoglobin solutions were estimated by spectral analysis after conversion into the cyanomet derivatives at 540 nm (ϵ 46.0 × 10^3 per tetramer) and were given on a tetramer basis.

The pentacyclohexylammonium salt of P_2glycerate obtained from Calbiochem was converted to the acid form by treatment with Dowex 50 resin. The sodium salt of IHP was purchased from Sigma. The source of bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (bisTris) was Aldrich. All other chemicals used were reagent grade and were purchased from standard sources.

Oxygen Equilibrium Measurements. The oxygen equilibrium curves were recorded on the apparatus of Imai *et al.* (1970). Special care was directed to the precise determination of the top and bottom of the curves as described elsewhere (Imai, 1973). The equilibrium measurements were performed at 25° with 1.5×10^{-5} M (tetramer) hemoglobins in the absence or presence of 0.1 M NaCl. The buffer employed was 0.05 M bisTris-HCl or 0.01 M Tris-HCl (pH 7.40), which gave exactly the same equilibrium curve.

The concentration of methemoglobin was checked by the method of Benesch *et al.* (1965) on the absorption spectrum of the hemoglobin solution taken before and after the equilibrium measurement, and the experiments with more than 3% methemoglobin formation during the measurement were discarded.

Data Analysis. The fractional saturation, Y, or percentage saturation, y ($= 100Y$), of hemoglobin with oxygen and the corresponding oxygen pressure, p, thus obtained were analyzed by the successive oxygenation theory of Adair (1925), and the four intrinsic association constants (corrected for statistical factors), k_i, for the reversible reaction (eq 1, where $i = 1$–4)

$$\mathrm{Hb(O_2)}_{i-1} + \mathrm{O_2} \rightleftarrows \mathrm{Hb(O_2)}_i \tag{1}$$

were estimated on a set of 20 experimental points by the least-squares method described previously (Imai, 1973).

If the four hemes in hemoglobin, as usually has been assumed, are equivalent in function, Y can be described in terms of four k values and p as follows

$$Y = \frac{k_1p + 3k_1k_2p^2 + 3k_1k_2k_3p^3 + k_1k_2k_3k_4p^4}{1 + 4k_1p + 6k_1k_2p^2 + 4k_1k_2k_3p^3 + k_1k_2k_3k_4p^4} \tag{2}$$

Thus, we can construct the simulated oxygen equilibrium curve from four k values by eq 2. The degree of deviation of the simulated curve from the experimental points, D, was expressed in terms of the root-mean-square of the residual of appropriately weighted Y (Imai, 1973). The overall free energy of interaction among oxygen binding sites, ΔF_I (cal/site), and the median ligand activity, P_m (mm), (Wyman, 1964) were evaluated by the relations

$$\Delta F_\mathrm{I} = RT \ln (k_4/k_1) \tag{3}$$

and

$$P_\mathrm{m} = 1/(k_1k_2k_3k_4)^{1/4} \tag{4}$$

respectively. Hill's parameter (Hill, 1910), n, defined by

$$n = \frac{\mathrm{d} \ln [Y/(1 - Y)]}{\mathrm{d} \ln p} \tag{5}$$

was calculated on the simulated Hill plots constructed by using eq 2 at $\Delta \log p = 0.1$ intervals, and the maximum value is expressed as n_max. P_{50} (mm), *i.e.*, the oxygen pressure at $Y = 0.5$, was also estimated on the simulated plots. The extent of asymmetry of the Hill plots is shown by the symmetry factor (Roughton *et al.*, 1955), $W = (k_1 \times k_4)/(k_2 \times k_3)$; if $W = 1$ the plot is symmetrical about $Y = 0.5$, *i.e.*, $\log [Y/(1 - Y)] = 0$.

The binding constant of P_2glycerate for hemoglobin in various oxygenation stages was calculated as follows. If we assume that 1 mol of the phosphate, A, combines with 1 mol of hemoglobin tetramer in any oxygenation stage, the equilibria can be described in terms of the five reversible reactions, where $j = 0$–4

$$\mathrm{Hb(O_2)}_j + \mathrm{A} \rightleftarrows \mathrm{Hb(O_2)}_j\mathrm{A} \tag{6}$$

Letting M_j be the binding constant for the jth equilibrium, the fractional oxygen saturation of hemoglobin in the presence of the phosphate is written as

$$Y = \frac{k_1R_1p + 3k_1k_2R_2p^2 + 3k_1k_2k_3R_3p^3 + k_1k_2k_3k_4R_4p^4}{1 + 4k_1R_1p + 6k_1k_2R_2p^2 + 4k_1k_2k_3R_3p^3 + k_1k_2k_3k_4R_4p^4} \tag{7}$$

where

$$R_i = \frac{1 + M_i[\mathrm{A}]}{1 + M_0[\mathrm{A}]} \tag{8}$$

Then, let k_i and m_i be the intrinsic oxygen association constants in the absence and presence of the phosphate, respectively. They are mutually related by the equation

$$m_i = \frac{1 + M_i[\mathrm{A}]}{1 + M_{i-1}[\mathrm{A}]}k_1 \tag{9}$$

Then, the binding constants are expressed as

$$\begin{aligned}
M_1 &= \frac{m_1}{k_1}M_0 - \frac{1}{[\mathrm{A}]}\left(1 - \frac{m_1}{k_1}\right)\\
M_2 &= \frac{m_1m_2}{k_1k_2}M_0 - \frac{1}{[\mathrm{A}]}\left(1 - \frac{m_1m_2}{k_1k_2}\right)\\
M_3 &= \frac{m_1m_2m_3}{k_1k_2k_3}M_0 - \frac{1}{[\mathrm{A}]}\left(1 - \frac{m_1m_2m_3}{k_1k_2k_3}\right)\\
M_4 &= \frac{m_1m_2m_3m_4}{k_1k_2k_3k_4}M_0 - \frac{1}{[\mathrm{A}]}\left(1 - \frac{m_1m_2m_3m_4}{k_1k_2k_3k_4}\right)
\end{aligned} \tag{10}$$

Therefore, if we know the values of four k's, four m's, and one M, we can calculate the remaining M values by eq 10. The calculation was performed on the k values and m values obtained in the presence of 0.1 M NaCl by assuming $M_4 = 0$ and [A] = added DPG concentration. The latter assumption is plausible as a first approximation since the lowest concentration of P_2glycerate used is more than ten times higher than that of Hb A.

All the computations were made with a Hitachi Hitac-10 digital computer, and errors involved in the estimation were expressed in terms of standard error and/or the coefficient of variation, *i.e.*, (standard error/estimated value) × 100%.

TABLE I: Estimates of k Values (mm^{-1}) and Deviation of the Simulated Curves, D, in the Absence of Added NaCl.

	k_1	k_2	k_3	k_4	D
Hb F					
stripped	0.0739 ± 0.0077	0.227 ± 0.045	0.297 ± 0.059	4.65 ± 0.53	3.38×10^{-4}
	(10.5%)	(19.6%)	(19.8%)	(11.5%)	
in 2 mM	0.0251 ± 0.0018	0.0198 ± 0.0096	0.0905 ± 0.0513	4.91 ± 1.47	3.52×10^{-4}
P_2glycerate	(7.0%)	(48.8%)	(56.7%)	(29.9%)	
Hb A					
stripped	0.114 ± 0.009	0.165 ± 0.039	1.17 ± 0.27	4.04 ± 0.26	6.14×10^{-5}
	(7.5%)	(23.7%)	(23.1%)	(6.5%)	
in 2 mM	0.0135 ± 0.0007	0.00894 ± 0.00262	0.0431 ± 0.0134	4.23 ± 0.50	5.23×10^{-5}
P_2glycerate	(4.9%)	(29.3%)	(31.0%)	(11.8%)	
in 1.7 mM IHP	0.00450 ± 0.00023	0.00801 ± 0.00133	0.00371 ± 0.00111	0.415 ± 0.108	3.16×10^{-4}
	(5.0%)	(16.6%)	(30.0%)	(25.9%)	

Results

Effect of P_2glycerate on Hb F and A in the Absence of Added NaCl. Figure 1 shows the effects of P_2glycerate on the Hill plots of the oxygen equilibrium of Hb F in the absence of added NaCl. The plots for Hb A under the same experimental conditions reported previously (Imai, 1973) and that in the presence of IHP are also shown for comparison. The intrinsic association constants, k values, and the degree of deviation of the simulated curve from the experimental points, D, are summarized in Table I. The fitness of the simulated curves to the experimental points is satisfactory as judged from the values of D and as seen in Figure 1. The estimates of k values agree fairly well with those previously determined by a trial-and-error curve fitting on the Hill and Scatchard plots (Tyuma *et al.*, 1971a,b).

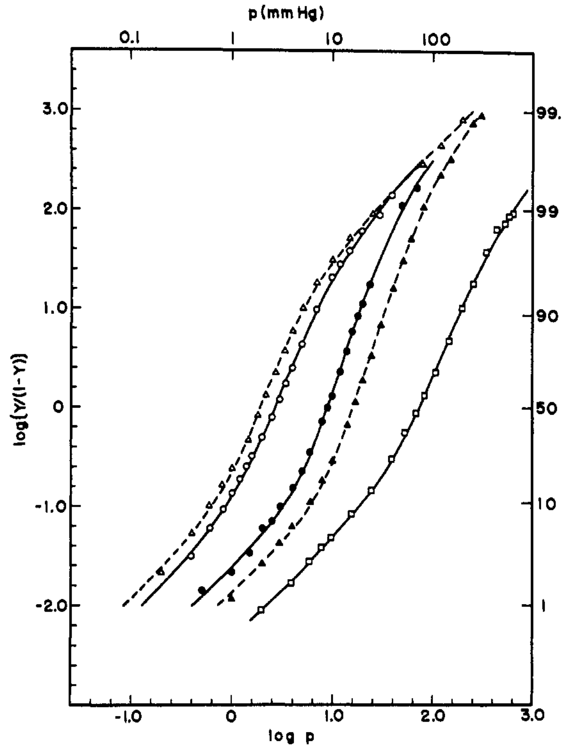

FIGURE 1: Hill plots of the oxygenation of Hb A and F in the absence of added NaCl; 0.05 M bisTris or 0.01 M Tris buffer, pH 7.4, 25°: (△) stripped Hb A; (▲) Hb A in 2 mM P_2glycerate; (□) Hb A in 1.7 mM IHP; (○) stripped Hb F; (●) Hb F in 2 mM P_2glycerate. Solid and broken lines are simulated curves constructed from the estimates of k values shown in Table I.

As seen in Table I, 2 mM P_2glycerate reduces k_1 and k_3 of stripped Hb F to about one-third and k_2 to about one-tenth. In accord with our previous observation (Tyuma and Shimzu, 1969, 1970) the magnitude of the reduction is smaller than in Hb A, where k_1, k_2, and k_3 are reduced to about $^1/_8$, $^1/_{20}$, and $^1/_{30}$, respectively, by the same concentration of the phosphate. On the other hand, it is very interesting to note that k_4 is not only very similar in value but also insensitive to P_2glycerate in both the hemoglobins. The similarity of k_4 for human, horse, and sheep hemoglobins has been demonstrated by Roughton and Lyster (1965).

Therefore, the magnitude of the shift of log p due to P_2glycerate depends upon the degree of hemoglobin oyxgenation as depicted in Figure 2. As can be easily shown, the limiting value of the magnitude of the shift (Δ log p) due to the phosphate at $y = 0\%$ is log k_1 (stripped) − log k_1 (P_2glycerate). In both Hb A and F, the magnitude slightly increases with increasing y reaching the maximum at about $y = 10\%$; then it decreases gradually up to 80% oxygenation, above which the rate of decrease becomes larger and larger with the increase of y. The limiting value of Δ log p at $y = 100\%$ is log k_4 (stripped) − log k_4 (P_2glycerate) and hence a negative value near 0.

Table II includes the oxygen equilibrium parameters obtained from k values and the simulated curves. P_{50} is close to P_m irrespective of the kind of hemoglobin and even in the presence of P_2glycerate, which makes the equilibrium curve markedly asymmetric as indicated by the large value of W. In the stripped condition, P_{50} and ΔF_I for Hb F are significantly larger than those for Hb A. The situation, however, is reversed in the presence of 2 mM P_2glycerate due to the decreased sensitivity of the fetal hemoglobin when compared with the adult one. Thus, the increase in ΔF_I due to 2 mM P_2glycerate amounts to about 1270 and 670 cal/site for Hb A and F, respectively. The maximum value of Hill's parameter, n_{max}, is essentially the same in both the stripped hemoglobins and increased by a similar extent by the addition of P_2glycerate.

Effect of IHP on Hb A in the Absence of Added NaCl. As shown in Figure 1 and Table I, the effect of IHP on the oxygen

TABLE II: Oxygen Equilibrium Parameters in the Absence of NaCl.

	P_{50} (mm)	P_{m} (mm)	n_{max}	ΔF_{I} (cal/site)	W
Hb F					
stripped	2.7	2.56	2.44	2450 ± 90	5.1
in 2 mM P_2glycerate	8.9	8.21	2.98	3120 ± 180	69
Hb A					
stripped	1.9	1.83	2.51	2080 ± 60	2.4
in 2 mM P_2glycerate	15.3	14.6	3.09	3350 ± 70	148
in 1.7 mM IHP	70.8	65.1	2.38	2680 ± 160	63

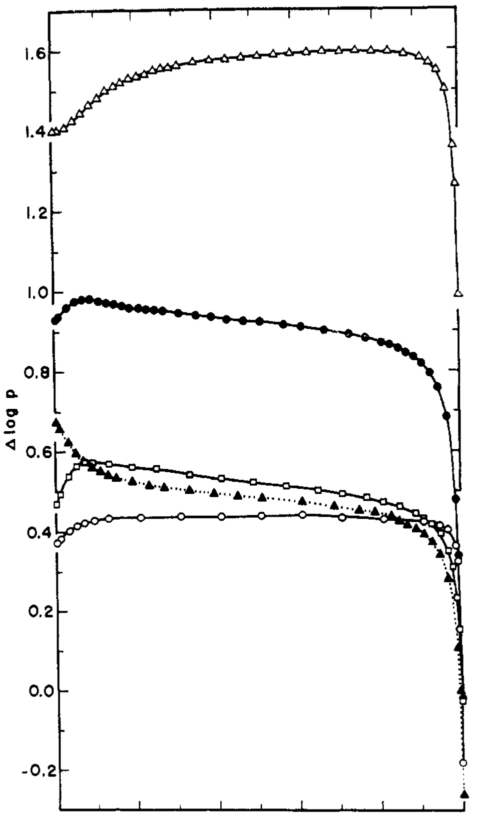

FIGURE 2: Dependence of the magnitude of the shift of log p, Δ log p, due to organic phosphates and salt upon the degree of hemoglobin oxygenation, y: (●,○) log p (2 mM P_2glycerate) − log p (stripped) for Hb A in the absence and presence of 0.1 M NaCl, respectively; (△) log p (1.7 mM IHP) − log p (stripped) for Hb A in the absence of NaCl; (□) log p (2 mM P_2glycerate) − log p (stripped) for Hb F in the absence of NaCl; (▲) log p (0.1 M NaCl) − log p (no salt) for stripped Hb A.

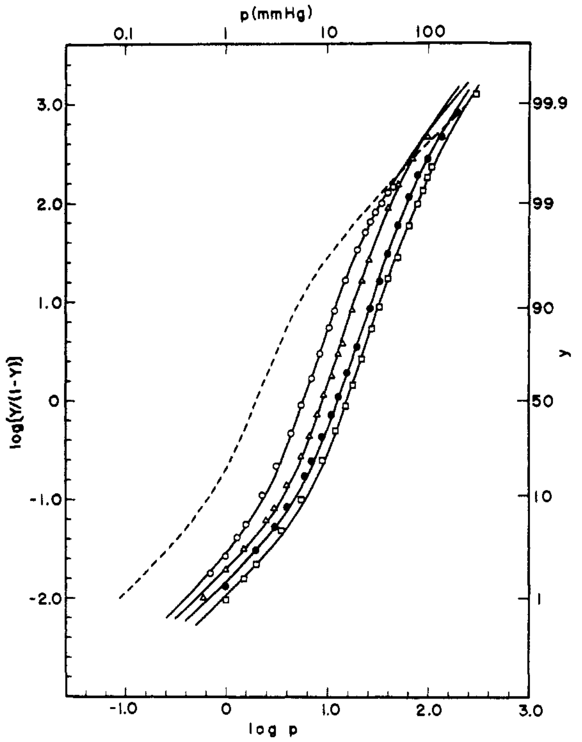

FIGURE 3: Hill plots of the oxygenation of Hb A in the presence of 0.1 M NaCl and 0.05 M bisTris buffer (pH 7.4) at 25°: (○) stripped; (△, ●, and □) in 0.2, 0.5, and 2.0 mM P_2glycerate, respectively; (—) simulated curves constructed from the estimates of k values shown in Table III; (- - - - -) the simulated curves for stripped Hb A in the absence of NaCl indicated in Figure 1.

equilibrium of Hb A is more marked than that of P_2glycerate; in 1.7 mM IHP k_1, k_2, and k_3 decrease to about $^1/_{25}$, $^1/_{20}$, and $^1/_{300}$, respectively. Moreover, in contrast to P_2glycerate, the hexaphosphate also reduces k_4 to approximately $^1/_{10}$, lowering n_{max} more than in stripped Hb A. ΔF_{I} in 1.7 mM IHP, however, is 2680 cal/site, which is about 700 cal lower than in 2 mM P_2glycerate but still approximately 600 cal higher than in the stripped condition. The dependence of the effect of IHP upon y also differs from that of P_2glycerate (Figure 2); Δ log p exhibits the maximum near $y = 75\%$ and a large positive value even at $y = 100\%$.

Effect of P_2glycerate on Hb A in the Presence of 0.1 M *NaCl.* Figure 3 illustrates the effect of various concentrations of P_2glycerate on the oxygen equilibrium of Hb A in the presence of 0.1 M NaCl. The curve for 1 mM P_2glycerate is not indicated to avoid the overcrowding of curves and the curve for stripped Hb A in the absence of NaCl is included for comparison. Tables III and IV summarize the estimated k values and some oxygen equilibrium parameters computed from the experimental points.

The profound influence of salt on the oxygen equilibrium function of hemoglobin has been recognized for a long time (Barcroft and Camis, 1909; Rossi-Fanelli *et al.*, 1961). This is clearly seen in the curves for stripped Hb A in Figure 2 for 0.1 M NaCl. The comparison of corresponding oxygen equilibrium parameters (Tables I–IV) reveals that the effect of NaCl is roughly similar to that of P_2glycerate except that

TABLE III: Estimates of k Values (mM^{-1}) and Deviation of the Simulated Curves, D, of Hb A in the Presence of 0.1 M NaCl and Various Concentrations of P$_2$glycerate.

[P$_2$glycerate] (mM)	k_1	k_2	k_3	k_4	D
0.0	0.0240 ± 0.0019 (7.8%)	0.0743 ± 0.0179 (24.1%)	0.0858 ± 0.0273 (31.8%)	7.37 ± 1.64 (22.3%)	2.22×10^{-4}
0.2	0.0199 ± 0.0012 (6.2%)	0.0207 ± 0.0075 (36.2%)	0.0499 ± 0.00234 (46.8%)	10.5 ± 3.2 (30.5%)	1.30×10^{-4}
0.5	0.0147 ± 0.0013 (9.1%)	0.0213 ± 0.0065 (30.4%)	0.0195 ± 0.0089 (45.8%)	9.08 ± 3.33 (35.8%)	1.03×10^{-4}
1.0	0.0139 ± 0.0010 (7.2%)	0.00719 ± 0.00405 (56.4%)	0.0318 ± 0.0213 (67.0%)	8.76 ± 3.27 (37.3%)	9.77×10^{-5}
2.0	0.0103 ± 0.0006 (6.0%)	0.0231 ± 0.0041 (17.9%)	0.00838 ± 0.00321 (38.3%)	11.2 ± 3.9 (34.7%)	6.15×10^{-5}

the effect is larger at low oxygen saturation (Figure 2) and the neutral salt slightly but significantly increases k_4: 0.1 M NaCl reduces k_1, k_2, and k_3 to about $^1/_5$, $^1/_2$, and $^1/_{15}$, respectively, and increases P_{50} to about threefold, $n_{\max}$ by 0.47, and ΔF_{I} by about 1300 cal/site.

Therefore, although Figure 4 indicates that k_1, k_2, and k_3 evidently decrease with increasing P$_2$glycerate concentration, the effect of the phosphate on the oxygen equilibrium parameters is less pronounced in the presence of 0.1 M NaCl than in its absence. As seen in Table IV, the increase in P_{50} and ΔF_{I} due to 2 mM P$_2$glycerate is only about threefold and 750 cal/site, respectively. The extra ΔF_{I} is near half of the observed in the absence of NaCl. The insensitivity of $n_{\max}$ to P$_2$glycerate concentration in 0.1 M NaCl confirms the earlier reports of Benesch *et al.* (1968). The dependence of the P$_2$glycerate effect upon oxygen saturation is also less remarkable than in the absence of NaCl, Δ log p being essentially independent of y between 10 and 90% oxygenation (Figure 2). On the other hand, the insensitivity of k_4 to P$_2$glycerate is substantiated at four different concentrations of the phosphate in the presence of 0.1 M NaCl (Figure 4) as in its absence.

Benesch *et al.* (1969) have shown that P$_2$glycerate competes with NaCl for Hb A, and the efficacy of the phosphate is about three orders of magnitude greater than that of the neutral salt. Thus, in the presence of 2 mM P$_2$glycerate the effect of 0.1 M NaCl is almost undiscernible, except for an increased ΔF_{I} and W primarily caused by the increase of k_4; the middle portion of the corresponding Hill plots is superimposable.

Binding Constant of P$_2$glycerate for Hb A. Table V includes the binding constant of P$_2$glycerate for Hb A in various oxygenation stages calculated from k values listed in Table III by assuming $M_4 = 0$, *i.e.*, the phosphate has no affinity to the fully oxygenated hemoglobin. The validity of the assumption and one P$_2$glycerate binding site per hemoglobin tetramer have been established by Benesch *et al.* (1971) in this experimental condition, *i.e.*, in the presence of 0.1 M NaCl.

TABLE IV: Oxygen Equilibrium Parameters of Hb A in the Presence of 0.1 M NaCl and Various Concentrations of P$_2$glycerate.

[P$_2$glycerate] (mM)	P_{50} (mm)	P_{m} (mm)	$n_{\max}$	ΔF_{I} (cal/site)	W
0.0	5.8	5.46	2.98	3390 ± 140	28
0.2	8.9	8.25	3.14	3710 ± 180	200
0.5	12.6	11.57	3.05	3800 ± 220	320
1.0	15.1	13.77	3.18	3820 ± 220	532
2.0	15.5	14.56	3.05	4140 ± 200	595

Since k_4 is insensitive to P$_2$glycerate, it is clear from eq 9 that $M_3 = M_4 = 0$, *i.e.*, the phosphate has no affinity to Hb(O$_2$)$_3$. Expectedly, P$_2$glycerate shows the highest affinity to the fully deoxygenated hemoglobin, and the affinity decreases stepwise on each oxygenation, M_2 being approximately one-tenth of M_0. Recently, Benesch *et al.* (1971) have redetermined the binding constant of P$_2$glycerate for fully deoxygenated Hb A in 0.1 M NaCl by the direct binding experiment. Their

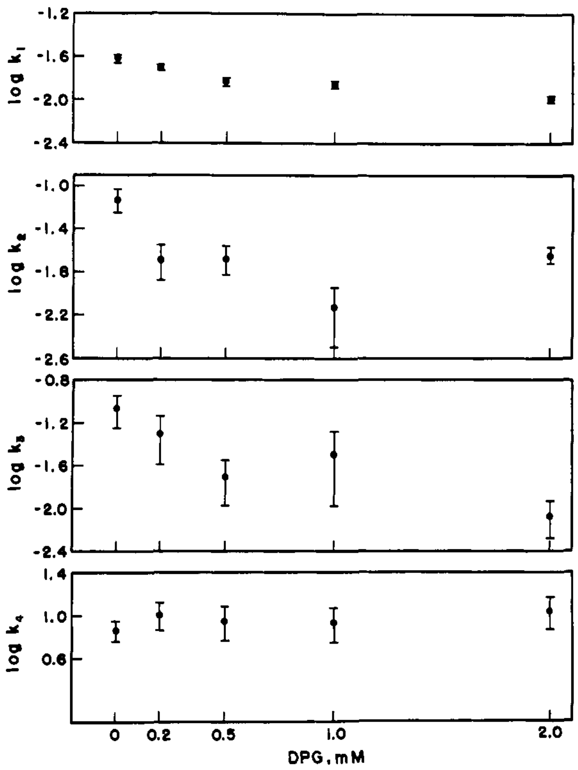

FIGURE 4: Dependence of the oxygen association constants, k's, upon P$_2$glycerate concentration in the presence of 0.1 M NaCl.

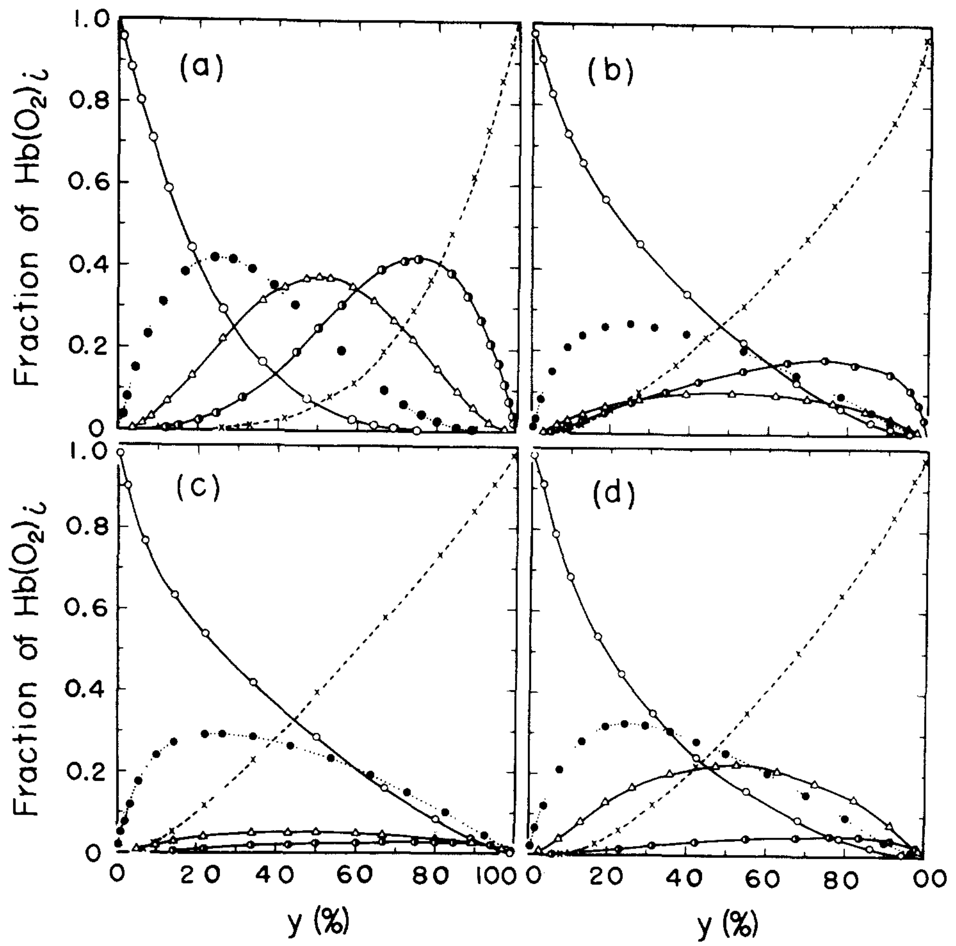

FIGURE 5: Fractions of Hb A in various oxygenation stages, $Hb(O_2)_i$, as a function of oxygen saturation, y, calculated from the values of k shown in Table I: (a) no homotropic interaction, *i.e.*, $k_1 = k_2 = k_3 = k_4$; (b) stripped; (c) in 2 mM P_2glycerate; (d) in 1.7 mM IHP; (○) fully deoxygenated hemoglobin; (●) $Hb(O_2)_1$; (△) $Hb(O_2)_2$; (◑) $Hb(O_2)_3$; (×) $Hb(O_2)_4$.

data, after correction for slight differences in pH and temperature by the published relations (Benesch *et al.*, 1969), gives 3.6×10^4 M^{-1} at pH 7.4 and 25°, which agrees quite well with the average value of M_0, 3.10×10^4 M^{-1}.

If we assume $M_4 > 10^3$ M^{-1}, the resulting M_0 shows a marked dependence upon P_2glycerate concentration; the value of M_0 increases with increasing the phosphate concentration.

TABLE V: Binding Constant (M_j, 10^4 M^{-1}) of P_2glycerate for Hb A in Various Intermediate Oxygenation Stages in the Presence of 0.1 M NaCl.

[P_2glycerate] (mM)	M_0	M_1	M_2
0.2	2.11 ± 0.13 (6.1%)	1.67 ± 0.24 (14.5%)	0.104 ± 0.257 (247%)
0.5	3.85 ± 0.26 (6.9%)	2.28 ± 0.34 (14.8%)	0.513 ± 0.268 (52.2%)
1.0	3.96 ± 0.26 (6.6%)	2.25 ± 0.29 (13.0%)	0.127 ± 0.138 (108%)
2.0	2.49 ± 0.14 (5.8%)	1.03 ± 0.12 (11.9%)	0.287 ± 0.098 (34.0%)
Av	3.10	1.81	0.258

Fractions of Hemoglobins in Various Oxygenation Stages. The values of four k's being defined, it is possible to calculate the single fraction of $Hb(O_2)_i$ at given oxygen saturation. It is also easily shown that the maximum fraction of $Hb(O_2)_1$, $Hb(O_2)_2$, and $Hb(O_2)_3$ is always obtained at $y = 25$, 50, and 75% and if there is no homotropic interaction ($k_1 = k_2 = k_3 = k_4$) the values are $^{27}/_{64}$, $^{3}/_{8}$, and $^{27}/_{64}$, respectively, irrespective of the absolute value of k's, as indicated in Figure 5a.

Figures 5b, c, and d illustrate the fractions of $Hb(O_2)_i$ as a function of oxygenation in the absence of NaCl. Evidently, homotropic interaction delays the disappearance of the fully deoxygenated species and accelerates the appearance of $Hb(O_2)_4$, reducing the maximum value of the fraction of other hemoglobin species in various intermediate oxygenation stages. In the presence of the organic phosphates, the maximum fraction of $Hb(O_2)_3$ is below 5% and its presence can be neglected except in the region near $y = 100\%$. In 0.1 M NaCl the maximum is 4.2% for stripped Hb A and further decreased to only 1.1% by the addition of 2 mM P_2glycerate. Thus, it can be said, as a first approximation, that the third and fourth oxygen molecules combine with hemoglobin simultaneously.

Fraction of Hemoglobin Combined with P_2glycerate and Oxygen Saturation. Figure 6 shows the plots of the fraction of hemoglobin species combined with P_2glycerate, z, against

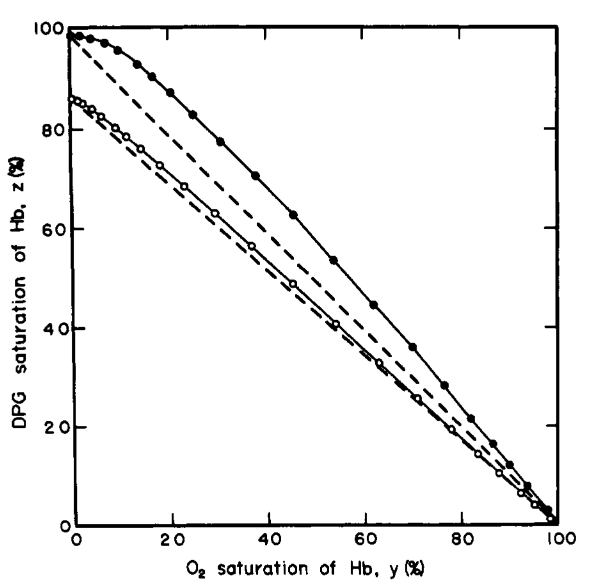

FIGURE 6: Dependence of the fraction of Hb A combined with P$_2$glycerate, z, upon the degree of hemoglobin oxygenation, y: (○) in 0.2 mM P$_2$glycerate; (●) in 2 mM P$_2$glycerate; (- - - - -) straight lines connecting the limiting values of z at y = 0 and 100%.

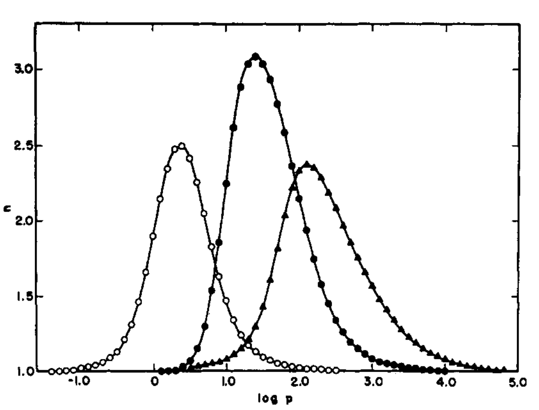

FIGURE 7: Plots of the slope, n, of the simulated Hill plots of Hb A shown in Figure 1 against log p: (○) stripped; (●) in 2 mM P$_2$glycerate (▲) in 1.7 mM IHP.

oxygen saturation, y, in the presence of 0.1 M NaCl. The calculation has been carried out by using the average values of M and k for stripped Hb A. Clearly the plot deviates from linearity and the deviation increases with increasing concentration of the phosphate. Figure 6 also predicts the result of a direct binding experiment of P$_2$glycerate performed on Hb A at various oxygen saturations.

Implication of Hill's Parameter. Hill's parameter, n, defined by eq 5 has usually been determined from the slope of the Hill plot in the middle range and taken as a good index of the heme–heme interactions. As shown in Table II, however, $n_{\max}$ is not always proportional to ΔF_{I}. The slope, n, calculated on the simulated Hill plots in Figure 1 at different log p values is illustrated in Figure 7. Apparently, the value of n strongly depends upon log p except at the very top position of the curves. Further, although the curve for stripped Hb A is essentially symmetric, the curve in the presence of the organic phosphates, especially IHP, shows a remarkable asymmetry.

This explains an inconsistency between the above-mentioned ΔF_{I} and $n_{\max}$, since the magnitude of ΔF_{I} is proportional to the area under the plot shown in Figure 7 (Wyman, 1964) and not to n itself. Nonproportionality of n to ΔF_{I} has already been indicated by Wyman (1964) between *Spirographis* hemoglobin containing about 80 hemes/molecule and mammalian hemoglobin. The above results, in addition, clearly demonstrate that even in hemoglobin molecules having the same number of hemes, Hill's parameter is not invariably a pertinent measure of the interactions among oxygen combining sites.

Discussion

Since the pioneer work of Roughton and his school (Roughton *et al.*, 1955; Roughton and Lyster, 1965), very little data have been accumulated on the absolute values of four association constants (k's) in Adair's successive oxygenation theory, owing to the experimental difficulties involved in the determinations. The present results clearly show, however, that the combined use of the automatic recording apparatus for the oxygen equilibrium measurement and the method of least squares (Imai, 1973) enables one to estimate four k values with considerable accuracy and without skill. The errors involved in k values are comparable to those obtained by the above investigators (Tables I and III). The good fit of the simulated oxygen equilibrium curves constructed from the estimates of k values to the experimental points (Figures 1 and 3) indicates the validity of the Adair equation (2) in describing the oxygen equilibrium of hemoglobin at least phenomenologically. If the four hemes in Hb A ($\alpha_2\beta_2$) are functionally nonequivalent as recently described by Olson and Gibson (1972) and Lindstrom and Ho (1972), each of four k values will be a composite of several microscopic constants for equilibria of the type $\alpha_2\beta_2 + O_2 \rightleftharpoons \alpha\alpha(O_2)\beta_2$, $\alpha_2\beta_2 + O_2 \rightleftharpoons \alpha_2\beta\beta(O_2)$, etc. Thus, the incorporation of the chain heterogeneity into the Adair scheme enormously increases the number of undetermined constants, which are hardly estimated uniquely. In this paper, therefore, the functional equivalence of the four hemes is assumed as a first-order approximation.

The results shown in Tables I and III indicate that the values of k's relative to k_1 (enhancement factor) of all the samples, except in the stripped condition in the absence of NaCl, are roughly in the same order of magnitude for the first three oxygenation reactions and increase by a factor of more than 100 at the last oxygenation step. This implies an almost exclusive contribution of the last step to ΔF_{I} and therefore suggests that the conformation of hemoglobin involved in the oxygen affinity changes abruptly after the third heme has been oxygenated.

As seen in Tables II and IV, the symmetry factor, W, for the hemoglobins in the presence of the organic phosphates markedly deviates from unity. This evidently shows an inapplicability of Pauling's square theory (Pauling, 1935) and Wyman's rectangular theory (Wyman, 1948), which are modifications of the Adair theory, to these materials, since both the modified theories predict $W = 1$. The same argument can be applied to the tetrahedral and square models of Koshland *et al.* (1966) as well.

Recently Perutz (1970) has proposed a model for the sequence of cooperative oxygen binding of Hb A in which six salt bridges constraining the fully deoxygenated hemoglobin are broken stepwise during the oxygenation. He estimated the total interaction energy as between 6 and 12 kcal/mol of hemoglobin, since the bond energy per salt bridge may reasonably be assumed as 1–2 kcal/mol. ΔF_{I} obtained for stripped

Hb A and F in the absence of NaCl is 2080 and 2450 cal/site (Table I), *i.e.* 8.3 and 9.8 kcal/mol, respectively, and within the estimated range.

The results with P$_2$glycerate are also consistent with the mechanism of action of the phosphate proposed in the Perutz model: P$_2$glycerate combined with four salt bridges involving valine NA1(1)β, histidine H21(143)β, and lysine EF6(82)β in the central cavity of fully deoxygenated Hb A is expelled after the second heme has been oxygenated. The increase in ΔF_{I} by 1270 cal/site, *i.e.*, 5080 cal/mol of Hb A, in the presence of 2 mM P$_2$glycerate and in the absence of NaCl (Table II) is comparable to the bond energy for the four additional salt bridges (4–8 kcal/mol) and the insensitivity of k_4 to P$_2$glycerate (*i.e.*, $M_3 = M_4 \pm 0$) substantiates the proposed mechanism. Further, the decreased effect of the phosphate on Hb F can reasonably be ascribed to the loss of two of the four salt bridges with P$_2$glycerate, since histidine H21(143)β is replaced by uncharged serine in the γ chains and the extra ΔF_{I} due to 2 mM P$_2$glycerate is 2680 cal/mol in Hb F, which corresponds to the formation of only two salt bridges.

The effects of NaCl on oxygen equilibrium function of Hb A are qualitatively similar to those of P$_2$glycerate, suggesting preferential binding of the salt by the deoxy as against the oxy form of hemoglobin. On one hand, the salt may weaken the salt bridges constraining the hemoglobin due to its shielding effect. On the other hand, as described by Benesch *et al.* (1969), anions such as chloride can fill the central cavity with specific effects similar to P$_2$glycerate on the oxygen equilibrium. The resulting net effect of NaCl will be the preponderance of the latter specific effect. The slight increase of k_4 by 0.1 M NaCl may be ascribed to the shielding effect on the remaining intrasubunit salt bridges in Hb(O$_2$)$_3$. Thus, the competition between P$_2$glycerate and NaCl makes the effect of the phosphate less pronounced in the presence of the salt. The insensitivity of k_4 to the phosphate, however, is still observed in this experimental condition (Table III and Figure 4).

From a physiological point of view, the invariance of k_4, *i.e.*, the upper part of the oxygen equilibrium curve, on the addition of P$_2$glycerate is an important advantage for the phosphate as a regulator for oxygen transport. Clearly this allows the red cells to increase the oxygen release in the tissue capillaries without an impaired oxygen uptake in the lung. The insensitivity of k_4 to inorganic phosphate has also been shown in the recent kinetic study of Gibson (1970).

The binding constants, M_j, of P$_2$glycerate for Hb A decrease monotonically with j, indicating a progressive weakening of the P$_2$glycerate interaction with its binding site brought about by the successive rupture of the salt bridges which link the polyanion to fully deoxygenated hemoglobin. M_j becomes zero at $j = 3$, but the phosphates still have considerable affinity to Hb(O$_2$)$_2$ (Table V). This sudden change of the binding constant suggests that the conformation of hemoglobin near the binding sites of the phosphate also changes abruptly after the third heme has been oxygenated. The conclusion that P$_2$glycerate is released with the binding of the third ligand has recently been reached by MacQuarrie and Gibson (1972) and Herzfeld and Stanley (1972) as well.

In contrast to P$_2$glycerate, IHP considerably reduced k_4, indicating that the hexaphosphate has an affinity to Hb(O$_2$)$_3$. Thus, extra ΔF_{I} due to the phosphate is appreciably lower than that for P$_2$glycerate. In view of the results obtained by Gibson and Gray (1970) that IHP significantly affects the rate of oxygen dissociation from the fully oxygenated Hb A, the hexaphosphate may also combine to Hb(O$_2$)$_4$.

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