*J. Biochem. (Tokyo) 13*, 117.
Brieskorn, C. H., and Kabelitz, L. (1971), *Phytochemistry 10*, 3195.
Bryce, T. A., Welti, D., Walsby, A. E., and Nichols, B. W. (1972), *Phytochemistry 11*, 295.
Capon, B., and Thacker, D. (1964), *Proc. Chem. Soc.*, 369.
Casu, B., Reggiani, M., Gallo, G. G., and Vigevani, A. (1966), *Tetrahedron 22*, 3061.
Fischer, E., and Bergmann, M. (1918), *Ber. Deutsch. Chem. Ges. 51*, 1760.
Hakomori, S. I. (1964), *J. Biochem. (Tokyo) 55*, 205.
Haworth, R. D. (1961), *Proc. Chem. Soc.*, 401.
Kochetkov, N. K., and Chizhov, O. S. (1966), *Advan. Carbohyd. Chem. 21*, 39.
Nichols, B. W., and Wood, B. J. B. (1968), *Nature (London) 217*, 767.
Park, J. T., and Johnson, M. J. (1949), *J. Biol. Chem. 181*, 149.
Roe, J. H. (1955), *J. Biol. Chem. 212*, 335.
Smith, P. R., and Mayberry, W. R. (1968), *Biochemistry 7*, 2706.
Sweeley, C. C., Ray, B. D., Wood, W. I., Holland, J. F., and Krichensky, M. I. (1970), *Anal. Chem. 42*, 1505.
Walsby, A. E., and Nichols, B. (1969), *Nature (London) 221*, 673.
Welsh, K., Shaw, N., and Baddiley, J. (1968), *Biochem. J. 107*, 313.
Winkenbach, F., Wolk, C. P., and Jost, M. (1972), *Planta 107*, 69.
Wolk, C. P. (1968), *J. Bacteriol. 96*, 2138.
Wolk, C. P., and Simon, R. D. (1969), *Planta 86*, 92.
Young, N. D. (1972), unpublished data.

# Analyses of Oxygen Equilibria of Native and Chemically Modified Human Adult Hemoglobins on the Basis of Adair's Stepwise Oxygenation Theory and the Allosteric Model of Monod, Wyman, and Changeux†

Kiyohiro Imai

ABSTRACT: Precise oxygen equilibrium curves of human adult hemoglobin and chemically modified derivatives prepared by the treatment of the hemoglobin with iodoacetamide, *N*-ethylmaleimide, or carboxypeptidase A were determined in the absence and presence of 2,3-diphosphoglycerate. These equilibrium data were analyzed according to Adair's stepwise oxygenation theory and the allosteric model of Monod, Wyman, and Changeux. The parameters involved in the oxygen saturation function of the theory and model were estimated with considerable accuracy by a least-squares method. It has been shown that 2,3-diphosphoglycerate increases the cooperativity of oxygenation of iodoacetamide-treated and *N*-ethylmaleimide-treated hemoglobins by reducing their affinity to the first, second, and third oxygen molecules without affecting the affinity to the fourth molecule as previously observed in native hemoglobin. Both the oxygen affinity and the cooperativity of carboxypeptidase-treated hemoglobin were scarcely affected by the phosphate. The oxygen association constant for R state, $K_R$, was insensitive to 2,3-diphosphoglycerate whereas the association constant for T state, $K_T$, was markedly reduced by the phosphate except for carboxypeptidase-treated hemoglobin. Both the chemical modifications of protein and the addition of 2,3-diphosphoglycerate influence not only the allosteric constant, $L$, but also the ratio $c$ ($=K_T/K_R$), contrary to early assumptions. From estimates of Monod–Wyman–Changeux's parameters, the principal courses of oxygenation in the T- and R-state system were deduced. The degree of ligation at which the allosteric transition takes place and the switching rate of transition at that degree of ligation were also obtained. 2,3-Diphosphoglycerate shifts the switching point toward later stages of oxygenation and increases the switching rate, making the oxygenation course and oxygen equilibrium curves asymmetrical. The phosphate increases cooperativity by reducing $c$; however, this effect is partially canceled out by the enhancement of the deviation of $Lc^2$ from unity. All these effects caused by the phosphate can be explained by assuming that the phosphate preferentially combines with the T-state molecules. The dependence of $c$ and $K_T$ on the allosteric effector, 2,3-diphosphoglycerate, was not anticipated in the original model and the present study suggests that the model needs modifications to explain the heterotropic effects in hemoglobin.

Hemoglobin has excited much investigation because of its physiologically and physically interesting action in oxygen binding which results from heme–heme interactions: indirect interactions mediated by conformational changes in the protein moiety between the distinct specific sites for oxygen binding.

In the discussion of oxygen equilibrium characteristics of hemoglobin, the oxygen affinity and the magnitude of cooperativity of oxygenation have usually been expressed by the oxygen pressure for 50% oxygen saturation, $P_{50}$, and Hill's coefficient, $n$, respectively (Rossi-Fanelli *et al.*, 1964; Antonini and Brunori, 1971). These measures, however, have no direct physical meaning and are too simple to describe quantitatively the functional behavior of hemoglobin. Roughton and his coworkers (Roughton *et al.*, 1955; Roughton, 1963; Roughton

† From the Department of Physicochemical Physiology, Medical School of Osaka University, Osaka, Japan.

and Lyster, 1965) determined very precise oxygen equilibrium curves of mammalian hemoglobins and estimated sets of the four association constants for oxygen according to Adair's stepwise oxygenation theory (Adair, 1925). Their data enabled a quantitative description of the oxygenation process and provided much information on the mechanism of action of hemoglobin.

Monod, Wyman, and Changeux (MWC)[1] (1965) proposed a model for the mechanism of action of allosteric proteins. This model is attractive from a point of view that it can explain not only homotropic effects, *i.e.*, interactions between identical ligands, but also heterotropic effects, *i.e.*, interactions between different ligands. This model has been applied to experimental data of the reaction of hemoglobin with oxygen and values of the parameters involved in the oxygen saturation function in the model have been estimated (Monod *et al.*, 1965; Edelstein, 1971; Hopfield *et al.*, 1971). In these attempts, however, the merit of the model does not seem to have been utilized sufficiently: major attention has been paid to the homotropic effect, *i.e.*, the heme–heme interaction, and not to the heterotropic effects. Therefore, a useful approach to examine the validity of the model would be a study of the heterotropic effect using allosteric effectors in addition to a study of the homotropic effect using hemoglobins with different cooperativities.

In this study the oxygen equilibrium curves of human adult hemoglobin and its chemically modified derivatives with different cooperativities have been determined in the absence and presence of the allosteric effector, 2,3-diphosphoglycerate ($P_2$-glycerate). The equilibrium data have been analyzed according to Adair's theory and the MWC model. The purpose of this study is to approach the problem of the mechanism of allosteric effects in hemoglobin through quantitative analyses of the equilibrium data. Through the analysis based on the MWC model various properties of the model will be clarified. These results will provide data to examine the validity of the model.

## Materials and Methods

*Materials.* Hemoglobins used are human adult hemoglobin (Hb A, native Hb) and three kinds of its derivatives prepared by the treatment with iodoacetamide [Hb(AcAm)] (Riggs, 1961; Taylor *et al.*, 1966), *N*-ethylmaleimide [Hb(MalN)] (Benesch and Benesch, 1961; Riggs, 1961), and carboxypeptidase A [Hb(CPase)] (Antonini *et al.*, 1961), which are classified into fully, slightly less, intermediately, and noncooperative hemoglobins, respectively. The tetrameric nature of all the modified hemoglobins has already been established to be equivalent to native Hb (Antonini *et al.*, 1961; Guidotti, 1967). Preparation of these hemoglobins and the stripping of phosphates were carried out as previously described (Imai, 1973). The reagents and solvents used were also the same as described in that paper.

*Determination of Oxygen Equilibrium Curves.* Oxygen equilibrium curves were determined by the automatic recording method of Imai *et al.* (1970) on $6.0 \times 10^{-5}$ M (on heme basis) hemoglobin in 0.05 M bis-tris-HCl buffer (pH 7.4) in the absence and presence of 2 mM $P_2$-glycerate.

In order to obtain precise equilibrium curves which can be subjected to statistical analysis, the accuracy of measurements was improved as follows. Substitution of a very thin Teflon membrane (10 $\mu$ thick) for 50 $\mu$ thick Teflon membrane originally attached to the oxygen electrode (39065 Polarographic Oxygen Sensor, Beckman) made response of the electrode fast enough to eliminate error involved in oxygen pressure measurement. Consequently, deoxygenation and successive reoxygenation curves accorded well with each other without any limit to the flow rate of nitrogen gas (Imai *et al.*, 1970). This shortened the measurement time for a single curve, which was 40–60 min for all the hemoglobins, and consequently decreased extent of methemoglobin formation during the measurement below that described previously (Imai *et al.*, 1970). When assayed after the measurements by the method of Benesch *et al.* (1965), methemoglobin contents were less than 5% except for Hb(CPase) which contained up to 8%. In order to read the oxygen saturation accurately, curves which were appropriately enlarged along the ordinate, *i.e.*, the transmittance scale, were recorded on the chart. Especially, the top portion of the curves (about 90% to 100% saturation) was enlarged by more than ten times the usual recording of the entire curve. The spectrophotometer was stable enough to produce no significant noise on the recorded curve. This procedure was very useful for the determination of precise equilibrium curves over wide ranges of saturation.

The oxygen saturation of hemoglobin was calculated from the transmittance reading on 20 points in each recorded curve. The set of 20 experimental points was used for the analyses as described below.

*Analysis of the Oxygen Equilibrium Data According to Adair's Theory.* According to Adair's theory (Adair, 1925) the oxygen saturation function is expressed as

$$Y = \frac{a_1 p + 2a_2 p^2 + 3a_3 p^3 + 4a_4 p^4}{4(1 + a_1 p + a_2 p^2 + a_3 p^3 + a_4 p^4)} \quad (1)$$

where $Y$ and $p$ are fractional oxygen saturation of hemoglobin and partial oxygen pressure, respectively, and $a_i$ ($i$ = 1, 2, 3, 4) is a constant composed of several equilibrium constants of the intermediate reactions (Hb + $O_2 \rightleftarrows HbO_2$, etc.). Equation 1 is of general validity as far as the hemoglobin molecule reacts in the tetrameric form with oxygen whether the four heme groups are identical or differ in their activity toward oxygen and other ligands ($P_2$-glycerate, $CO_2$, etc.) are absent or present.

The constants, $a$'s, were estimated by the least-squares method for nonlinear functions described by Rubin (1963) with minor modifications. In order to obtain an initial set of the constants which is to be subjected to successive least-squares iteration, a certain set of four experimental points ($p_j$, $Y_j$,) was suitably chosen from the set of 20 points and was substituted into eq 1, then the resulting four simultaneous equations were solved for $a$'s. This set of $a$'s was subjected to the successive iteration until the third significant figure of each $a_i$ was not altered any longer by further iteration. The iteration gave the identical values of $a$'s irrespective of the way of choosing the first set of four experimental points. Standard error, $S_Y$, involved in the fractional saturation, $Y$, determined by the automatic recording method depends on $Y$ itself (see Figure 6(b) in the paper of Imai *et al.*, 1970). Therefore, each experimental point ($p_j$, $Y_j$) (the subscript, $j$, denotes the $j$th point among the 20 points) was weighted by $w_j$, where

$$w_j = S_{Y,j}^{-2} \Big/ \left( \sum_{j=1}^{20} S_{Y,j}^{-2} \right)$$

[1] Abbreviations used are: $P_2$-glycerate, 2,3-diphosphoglycerate; Hb, hemoglobin; native Hb, human adult hemoglobin untreated; Hb (AcAm), Hb (MalN), and Hb (CPase), human adult hemoglobin treated with iodoacetamide, *N*-ethylmaleimide, and carboxypeptidase A, respectively; bis-tris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; MWC, Monod, Wyman, and Changeux.

$$S_{Y,j} = 0.08Y_j(1 - Y_j)$$

which roughly simulates the dependence of experimental $S_Y$ on $Y$. Errors involved in the most probable values of $a$'s were simultaneously estimated during the least-square procedures. The degree of deviation of the fitted curve from the experimental points, $D$, was expressed in terms of the root mean square of the residual of $Y$ weighted by $w_j$, as follows

$$D = \sqrt{\frac{1}{20}\sum_{j=1}^{20} w_j(Y_j^{\exp} - Y_j^{\mathrm{cal}})^2}$$

The oxygen pressure at the half-saturation, $P_{50}$, and the maximum slope of the Hill plot, $n_{\max}$, were obtained from the fitted curves.

When the $a$'s are known, the median ligand activity, $P_m$, is given by the equation

$$P_m = 1/\sqrt[4]{a_4} \tag{2}$$

which can easily be derived from the definition of the median ligand activity (Wyman, 1964) and eq 1. $P_m$ is directly related to the total free-energy change of oxygenation and is a reasonable measure of oxygen affinity (Wyman, 1964). Overall free energy of interaction among the oxygen binding sites, $\Delta F_I$ (Wyman, 1964), was evaluated from $a$'s by

$$\Delta F_I = RT \ln (16a_4/a_1a_3) \tag{3}$$

since $\Delta F_I$ is given by the normal distance between the upper and lower asymptotes of the Hill plot multiplied by $(2)^{1/2}$ (Wyman, 1964) and these asymptotes are expressed as: $\log [Y/(1 - Y)] = \log p + \log (4a_1/a_3)$ and $\log [Y/(1 - Y)] = \log p + \log (a_1/4)$, respectively.

If the four heme groups are identical in their activity toward oxygen, the $a$'s are expressed by a simple set of association constants as follows

$$\begin{aligned} a_1 &= 4k_1 \\ a_2 &= 6k_1k_2 \\ a_3 &= 4k_1k_2k_3 \\ a_4 &= k_1k_2k_3k_4 \end{aligned} \tag{4}$$

where $k_i$ ($i = 1, 2, 3, 4$) is intrinsic association constant, *i.e.*, the constant corrected for statistical factor, for oxygen corresponding to the oxygenation stage, $Hb(O_2)_{i-1} + O_2 \rightleftarrows Hb(O_2)_i$. In this study, the oxygen equilibrium characteristics of hemoglobin were compared with each other and discussed in terms of $P_m$, $\Delta F_I$, and $k$'s which were evaluated from the $a$'s.

*Analysis of the Oxygen Equilibrium Data According to the MWC Model.* In the original model of Monod *et al.* (1965) it is assumed that the four heme groups are identical in function, that is, the oxygen affinity of the binding sites is independent of the degree of ligation. Then, the oxygen saturation function is expressed as

$$Y = \frac{Lc\alpha(1 + c\alpha)^3 + \alpha(1 + \alpha)^3}{L(1 + c\alpha)^4 + (1 + \alpha)^4} \tag{5}$$

where $L$ is an equilibrium constant (called "allosteric constant") for the equilibrium between unliganded T and R states, $c$ is the ratio of the intrinsic association constant[2] for oxygen for T state ($K_T$) to that for R state ($K_R$), and $\alpha$ is the oxygen activity including $K_R$ ($\alpha = K_R p$). Among these parameters $L$ and either two of the other parameters, $K_T$, $K_R$, and $c$, are independent. An initial set of the MWC parameters which is to be subjected to successive least-squares iteration was obtained by relating these parameters to Adair's. Comparing eq 5 with eq 1, into which the $a$'s in eq 4 were substituted, we have the following relationship among the individual parameters in the MWC model and Adair's theory

$$k_i = \frac{Lc^i + 1}{Lc^{i-1} + 1} K_R \tag{6}$$

where $i = 1, 2, 3, 4$. The enhancement factor (Roughton *et al.*, 1955) for the $i$th stage of oxygenation, $f_i$, is expressed in terms of the MWC parameters as

$$f_i = \frac{k_i}{k_1} = \frac{(Lc^i + 1)(L + 1)}{(Lc^{i-1} + 1)(Lc + 1)} \tag{7}$$

Here $f_4$ is known by substituting the estimates of $k_1$ and $k_4$ into eq 7. We substitute a certain positive value less than unity for $c$ into eq 7 for $i = 4$ and solve the equation for $L$. Thus we obtain many sets of $c$ and $L$ letting $c$ vary over a wide range. It is sufficient only to examine $c$ less than unity because when $c$ is larger than unity we have the same model by exchanging T and R states, *i.e.*, by replacing $c$, $L$, and $\alpha$ by $1/c$, $1/L$, and $c\alpha$, respectively. This is followed by the calculation of $f_2$ and $f_3$ after substituting each set of $c$ and $L$ into eq 7 and by comparing them with $f_2$ and $f_3$ which have been obtained directly from $k$'s estimated by the least-squares method. Then it is found that only narrow-ranged series of the sets of $c$ and $L$ can give a good fit of all $f$'s. Thus, approximate values of $c$ and $L$ can be obtained through the fitting procedure of the enhancement factors. $K_R$ is obtained from eq 6 for either of $i = 1, 2, 3$, or 4. These approximate values of $L$, $c$, and $K_R$ were subjected to the least-squares procedures as described in the preceding section.

Using the estimates of the MWC parameters, we can represent some characteristics of the allosteric behavior of hemoglobin which are anticipated when the MWC model is applicable to the hemoglobin system. $\Delta F_I$ is expressed in terms of $L$ and $c$ as follows

$$\Delta F_I = RT \ln \frac{k_4}{k_1} = RT \ln \frac{(Lc^4 + 1)(L + 1)}{(Lc^3 + 1)(Lc + 1)} \tag{8}$$

which is derived from eq 3, 4, and 7. $\Delta F_I$ given by the above equation takes a maximum value for a particular value of $L$ when $c$ is fixed and $L$ is varied. The maximizing condition is expressed as

$$L = 1/c^2 \tag{9}$$

[2] In this paper the oxygen equilibrium constants for the T- and R-state hemoglobins are expressed in terms of intrinsic *association* constants, $K_T$ and $K_R$, respectively, instead of *dissociation* constants which were used by Monod *et al.* (1965). The parameter, $c$, defined by $K_T/K_R$, however, gives an identical value with that used by Monod *et al.*

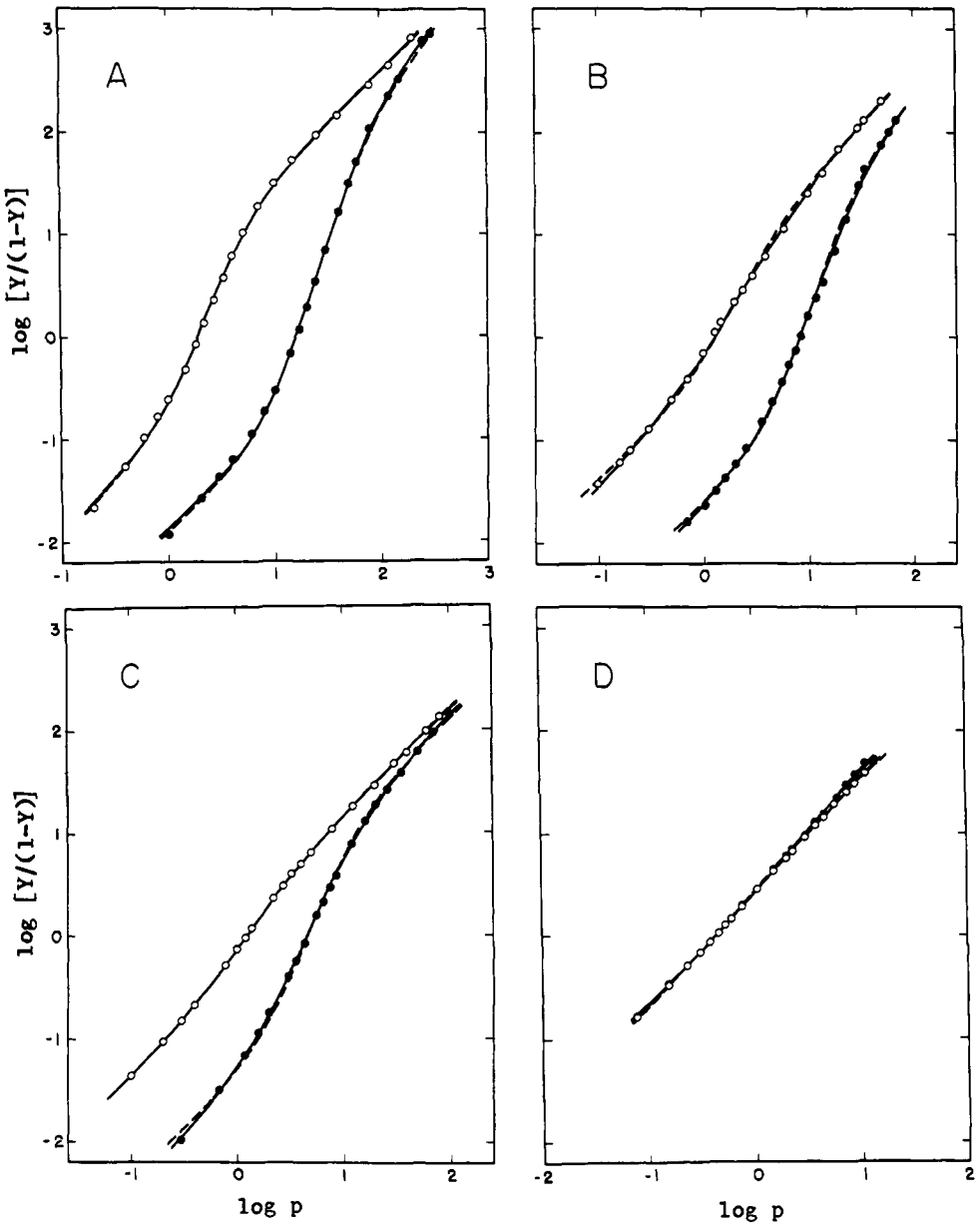


FIGURE 1: The Hill plots of oxygenation of A, native Hb; B, Hb(AcAm); C, Hb(MalN); D, Hb(CPase). (○) Stripped; (●) in 2 mM $P_2$-glycerate. Hemoglobin concentration, $6.0 \times 10^{-5}$ M on heme basis; temperature, 25°; in 0.05 M bis-tris-HCl buffer (pH 7.4). Solid and broken lines are simulated curves constructed from the estimates of the Adair parameters in Table I and the MWC parameters in Table III, respectively.

Under this condition $\Delta F_I$ is given by

$$\Delta F_I(\max) = RT \ln \frac{(c^2 + 1)^2}{c(c + 1)^2} = RT \ln \frac{(L + 1)^2}{\sqrt{L}(\sqrt{L} + 1)^2} \tag{10}$$

We denote the molecular species which have combined $i$ molecules of oxygen as $T_i$ and $R_i$ for T and R states, respectively. The ratio of the population of $T_i$ to that of $R_i$ is given by

$$T_i/R_i = Lc^i \tag{11}$$

where $i = 0, 1, \ldots, 4$. Now let us regard $i$ as a continuous number; then the switchover point in allosteric transition, $i_s$, *i.e.* $i$ at $T_i = R_i$, is obtained from eq 11 and is written as follows (Hopfield *et al.*, 1971)

$$i_s = -(\log L)/(\log c) \tag{12}$$

Differentiating the fraction of $R_i$ in $(T_i + R_i)$, $1/(Lc^i + 1)$, with respect to $i$ and substituting $i_s$ for $i$, we obtain a quantity expressed as

$$r_s = -(\ln c)/4 \tag{13}$$

This quantity, $r_s$, represents the switching rate of allosteric transition from T to R state and is called "switchover rate" in this paper.

The least-squares estimation of the Adair and MWC parameters and other computations were made using a NEAC 2200 digital computer (Nippon Electric Co., Tokyo) of the Computation Center, Osaka University, and a Hitac 10 digital computer (Hitachi Co., Tokyo).

## Results

*The Adair Parameters and Oxygen Equilibrium Characteristics of the Hemoglobins.* Figure 1 shows the Hill plots of

TABLE I: Summary of the Estimates of the Adair Parameters and $D$.

| | $k_1$[a] | $k_2$[a] | $k_3$[a] | $k_4$[a] | $D$[b] |
|---|---|---|---|---|---|
| Native Hb | | | | | |
| Stripped | 0.114 ± 0.009 (0.116)[c] | 0.165 ± 0.039 (0.155) | 1.17 ± 0.27 (1.23) | 4.04 ± 0.26 (4.02) | $6.14 \times 10^{-5}$ |
| In 2 mM $P_2$-glycerate | 0.0135 ± 0.0007 (0.0128) | 0.00894 ± 0.00262 (0.0129) | 0.0431 ± 0.0134 (0.0284) | 4.23 ± 0.50 (4.78) | $5.23 \times 10^{-5}$ |
| Hb(AcAm) | | | | | |
| Stripped | 0.297 ± 0.019 (0.407) | 0.954 ± 0.089 (0.424) | 0.391 ± 0.035 (0.733) | 4.46 ± 0.30 (3.87) | $1.66 \times 10^{-4}$ |
| In 2 mM $P_2$-glycerate | 0.0207 ± 0.0014 (0.0253) | 0.0748 ± 0.0127 (0.0263) | 0.0333 ± 0.0111 (0.150) | 5.19 ± 1.55 (2.66) | $2.98 \times 10^{-4}$ |
| Hb(MalN) | | | | | |
| Stripped | 0.405 ± 0.015 (0.411) | 0.624 ± 0.040 (0.601) | 1.07 ± 0.06 (1.10) | 1.59 ± 0.06 (1.59) | $1.38 \times 10^{-4}$ |
| In 2 mM $P_2$-glycerate | 0.0295 ± 0.0012 (0.0408) | 0.246 ± 0.016 (0.0713) | 0.200 ± 0.012 (0.615) | 1.58 ± 0.060 (1.26) | $1.38 \times 10^{-4}$ |
| Hb(CPase) | | | | | |
| Stripped | 2.09 ± 0.06 (1.95) | 2.20 ± 0.08 (2.11) | 2.11 ± 0.06 (2.53) | 3.49 ± 0.09 (3.33) | $1.55 \times 10^{-4}$ |
| In 2 mM $P_2$-glycerate | 2.01 ± 0.16 (1.86) | 2.71 ± 0.25 (2.02) | 1.54 ± 0.14 (2.58) | 4.43 ± 0.29 (3.93) | $3.33 \times 10^{-4}$ |

[a] Expressed in mm $Hg^{-1}$. [b] Degree of deviation of the simulated curve from the experimental points expressed in terms of the root-mean square of the residual of $Y$ weighted by $w_j$. [c] Figures in parentheses indicate the values of $k$'s recalculated from the MWC parameters in Table III.

oxygen equilibrium of the native and modified hemoglobins. The experimental points are compared with the curves constructed from the most probable values of $a$'s. The intrinsic association constants, $k$'s, and the degree of deviation of the simulated curve from the experimental points, $D$, are summarized in Table I. The fit of the simulated curves to the experimental points is satisfactory as judged from the values of $D$ and as seen in Figure 1. The errors involved in the Adair parameters, $k$'s, are fairly small. The errors for native Hb are comparable to those obtained by Roughton and his coworkers (Roughton *et al.*, 1955; Roughton, 1963; Roughton and Lyster, 1965) for sheep, horse, and human hemoglobins by a very accurate gasometric method. Generally, the errors involved in $k_2$ and $k_3$ are larger than those involved in $k_1$ and $k_4$ for all the hemoglobins as previously observed by these investigators. The estimates of $k_1$ and $k_4$ for all the hemoglobins agree well with those estimated by analysis of the Scatchard plot of oxygen equilibrium (Imai, 1973). The estimates of $k$'s for native Hb also agree well with those previously estimated by a trial-and-error curve-fitting procedure of the Hill and Scatchard plots (Tyuma *et al.*, 1971a). The effect of $P_2$-glycerate on the $k$'s of Hb(AcAm) and Hb(MalN) is quite similar to that of native Hb; the phosphate markedly reduces $k_1$, $k_2$, and $k_3$ whereas it scarcely affects $k_4$, which remains unchanged within experimental error for each hemoglobin. In Hb(CPase), on the other hand, $P_2$-glycerate has little effect on all $k$'s. It is noteworthy that the values of $k_4$ for Hb(AcAm) and Hb(CPase) are similar to those for native Hb whether $P_2$-glycerate is present or absent. The values of $k_4$ for Hb(MalN) are, however, somewhat smaller than those for the other hemoglobins.

Table II summarizes the oxygen equilibrium parameters obtained from the $a$'s and the simulated equilibrium curves. $P_{50}$ is close to $P_m$ irrespective of the kind of hemoglobin and whether $P_2$-glycerate is present or absent, indicating that $P_{50}$ can be regarded as a good measure of the overall oxygen affinity even in the presence of $P_2$-glycerate which exaggerates the asymmetry of oxygen equilibrium curve (Tyuma *et al.*, 1971b). The effect of $P_2$-glycerate on the oxygen affinity is expressed in terms of a ratio, $P_m^{phos}:P_m^{st}$, where $P_m^{phos}$ and $P_m^{st}$ are $P_m$ in the presence and absence, respectively, of 2 mM $P_2$-glycerate. The ratio for Hb(AcAm) is slightly smaller than that for native Hb and the ratio for Hb(MalN) is about a half of that for native Hb. $P_2$-glycerate remarkably increases the magnitude of cooperativity in oxygen binding, which is measured by $\Delta F_I$ or $n_{max}$, in Hb(AcAm) and Hb(MalN) as previously observed in native Hb (Tyuma *et al.*, 1971a) and in abnormal hemoglobin Hiroshima (HC3$\beta$, histidine → aspartate) (Imai *et al.*, 1972). The increment in $\Delta F_I$ due to the addition of $P_2$-glycerate for Hb(AcAm) and Hb(MalN) is similar to that for native Hb. The oxygen equilibrium functions of Hb(CPase) are scarcely affected by $P_2$-glycerate. This is consistent with the result of Tomita and Riggs (1971) obtained on carboxypeptidase A treated mouse hemoglobin, but does not agree with the result of Chanutin and Curnish (1968) obtained on carboxypeptidase A treated human hemoglobin.

*The MWC Parameters and the Allosteric Properties of the Hemoglobins.* The estimated values of MWC parameters and $D$ are summarized in Table III. The simulated curves constructed from these parameters are compared with the experimental points and the curves simulated by the Adair function in Figure 1. The fit of the simulated curves to the experimental points is satisfactory. $D$ for the simulation using the MWC function is, however, slightly larger than that for

TABLE II: Oxygen Equilibrium Parameters Obtained from the Adair Constants.

| | $P_{50}$[a] | $P_m$[b] | $P_m^{phos}/P_m^{st}$[c] | $n_{max}$[d] | $\Delta F_I$[e] | $\Delta(\Delta F_I)$[f] |
|---|---|---|---|---|---|---|
| Native Hb | | | | | | |
| Stripped | 1.9 | 1.83 | 7.98 | 2.51 | 2110 ± 60 | 1290 ± 100 |
| In 2 mM $P_2$-glycerate | 15.3 | 14.60 | | 3.09 | 3400 ± 80 | |
| Hb(AcAm) | | | | | | |
| Stripped | 1.2 | 1.19 | 6.57 | 1.63 | 1600 ± 50 | 1670 ± 190 |
| In 2 mM $P_2$-glycerate | 8.5 | 7.82 | | 2.71 | 3270 ± 180 | |
| Hb(MalN) | | | | | | |
| Stripped | 1.2 | 1.24 | 4.39 | 1.44 | 810 ± 30 | 1550 ± 50 |
| In 2 mM $P_2$-glycerate | 4.7 | 4.57 | | 2.27 | 2360 ± 30 | |
| Hb(CPase) | | | | | | |
| Stripped | 0.44 | 0.41 | 1.03 | 1.15 | 300 ± 20 | 160 ± 60 |
| In 2 mM $P_2$-glycerate | 0.44 | 0.40 | | 1.23 | 460 ± 60 | |

[a] Oxygen pressure (mm Hg) at the half oxygen saturation. [b] Median ligand activity (mm Hg). [c] Ratio of $P_m$ in the presence of 2 mM $P_2$-glycerate to $P_m$ in the absence of $P_2$-glycerate. [d] Maximum slope of the simulated Hill plot. [e] Overall free energy of interaction (cal/site). [f] Increment of $\Delta F_I$ due to addition of 2 mM $P_2$-glycerate (cal/site).

TABLE III: Summary of the Estimates of the MWC Parameters and $D$.

| | $K_T$[a] | $K_R$[a] | $c$ | $L$ | $D$ |
|---|---|---|---|---|---|
| Native Hb | | | | | |
| Stripped | 0.115 ± 0.006 | 4.31 ± 0.15 | 0.0266 ± 0.0010 | $(3.89 \pm 0.56) \times 10^3$ | $6.15 \times 10^{-5}$ |
| In 2 mM $P_2$-glycerate | 0.0128 ± 0.0033 | 8.70 ± 1.68 | 0.00148 ± 0.00026 | $(2.56 \pm 1.98) \times 10^8$ | $5.48 \times 10^{-5}$ |
| Hb(AcAm) | | | | | |
| Stripped | 0.406 ± 0.106 | 7.78 ± 1.55 | 0.0522 ± 0.0087 | $(7.91 \pm 6.39) \times 10^3$ | $3.21 \times 10^{-4}$ |
| In 2 mM $P_2$-glycerate | 0.0253 ± 0.0041 | 3.17 ± 0.39 | 0.00797 ± 0.00084 | $(3.82 \pm 1.89) \times 10^5$ | $4.08 \times 10^{-4}$ |
| Hb(MalN) | | | | | |
| Stripped | 0.355 ± 0.011 | 1.82 ± 0.05 | 0.195 ± 0.003 | $(2.53 \pm 0.30) \times 10$ | $1.39 \times 10^{-4}$ |
| In 2 mM $P_2$-glycerate | 0.0398 ± 0.0030 | 1.30 ± 0.07 | 0.0306 ± 0.0017 | $(1.28 \pm 0.26) \times 10^3$ | $4.34 \times 10^{-4}$ |
| Hb(CPase) | | | | | |
| Stripped | 1.87 ± 0.44 | 5.54 ± 1.07 | 0.335 ± 0.046 | $(3.97 \pm 2.52) \times 10$ | $5.05 \times 10^{-4}$ |
| In 2 mM $P_2$-glycerate | 1.80 ± 0.80 | 7.05 ± 2.42 | 0.255 ± 0.073 | $(8.81 \pm 8.63) \times 10$ | $1.01 \times 10^{-3}$ |

[a] Expressed in mm $Hg^{-1}$.

the simulation using the Adair function (compare $D$'s in Table I and III). The Adair parameters were recalculated from the estimates of the MWC parameters by using eq 6 and are compared with those directly estimated by the least-squares method on the Adair function in Table I, generally giving a good agreement. $K_T$, $K_R$, and $c$ were estimated within a reasonable error but the estimates of $L$ were less accurate.

$L$ shows a great dependence on the cooperativity. $L$ is decreased markedly as the cooperativity is decreased by the chemical modifications of the $\beta$ chains or by the stripping of $P_2$-glycerate. The value of $c$ also markedly depends on the cooperativity. However, $c$ is increased as the cooperativity is decreased. $K_T$ and $K_R$ are influenced by the chemical modifications and by $P_2$-glycerate in a way similar to that for $k_1$ and $k_4$, respectively, since $K_T$ and $K_R$ are close to $k_1$ and $k_4$, respectively.

$\Delta F_I$'s calculated from the MWC parameters (Table IV) agree well with those calculated from the Adair parameters (Table II). It is an interesting question to what extent the values of $\Delta F_I$ actually observed are close to the maximum value of $\Delta F_I$ given by eq 10. Hence, the $\Delta F_I$ calculated from eq 8 using each observed value of $c$ was plotted against the logarithm of $L$ and the results are shown in Figure 2. These plots yielded symmetrical bell-shaped curves which have a maximum at $L = c^{-2}$ (refer to eq 9) and diminish both to the right and left of the maximum. The maximum values of $\Delta F_I$ for different $c$'s were also calculated from eq 10 and are plotted against $\log L$ ($= \log c^{-2}$) in Figure 2. This plot expresses a locus of the maximum points of the bell-shaped curve when $c$ is varied. The observed point for each hemoglobin is placed on the corresponding bell-shaped curves. The observed point of Hb(MalN) is very close to the maximum point both in the absence and presence of $P_2$-glycerate. The points for the other hemoglobins are far from the maximum points especially in

TABLE IV: Allosteric Parameters and Principal Oxygenation Courses Obtained from the MWC Parameters.

| | $\Delta F_I$[a] | $Lc^2$ | $i_s$ | $r_s$ | $T_0$—$T_1$—$T_2$—$T_3$—$T_4$ / $R_0$—$R_1$—$R_2$—$R_3$—$R_4$ |
|---|---|---|---|---|---|
| Native Hb | | | | | |
| Stripped | 2090 | 2.75 | 2.28 | 0.91 | |
| In 2 mM $P_2$-glycerate | 3490 | $5.57 \times 10^2$ | 2.97 | 1.63 | |
| Hb(AcAm) | | | | | |
| Stripped | 1330 | $2.16 \times 10$ | 3.04 | 0.74 | |
| In 2 mM $P_2$-glycerate | 2750 | $2.42 \times 10$ | 2.64 | 1.21 | |
| Hb(MalN) | | | | | |
| Stripped | 800 | 0.97 | 1.98 | 0.41 | |
| In 2 mM $P_2$-glycerate | 2020 | 1.19 | 2.05 | 0.87 | |
| Hb(CPase) | | | | | |
| Stripped | 320 | 4.46 | 3.37 | 0.27 | |
| In 2 mM $P_2$-glycerate | 440 | 5.73 | 3.28 | 0.34 | |

[a] Expressed in cal/site.

the presence of $P_2$-glycerate. All the observed points are located to the right of the locus of the maximum points.

Figure 3 shows the dependence of the fraction of $T_i$ in $(T_i + R_i)$, $Lc^i/(Lc^i + 1)$, upon the degree of ligation, $i$, which was obtained from the values of $L$ and $c$ in Table III. In each curve in the figure, the real state at which $i$ takes either one of the integers, 0, 1, ..., 4, is indicated by the open circles. If the fraction of $T_i$ is above 0.9 at a real state, we consider that $T_i$ is the major fraction and $R_i$ is negligible at that state and *vice versa* if the fraction of $T_i$ is below 0.1. If the fraction of $T_i$ is between 0.1 and 0.9 at a real state, we consider that $T_i$ and $R_i$ essentially coexist at that state. With these criteria, we can deduce a principal course of oxygenation in the MWC T- and R-state system from the curves in Figure 3. The principal courses of oxygenation obtained in this manner are presented in Table IV. Closed circles in Figure 3 indicate the switchover points in allosteric transition. The values of $i$ corresponding to these points, which can be also obtained from eq 12, are listed in Table IV. The curves in Figure 3 also illustrate the switchover rate: the negative slope of the curves at $i = i_s$ gives the rate defined by eq 13. The values of the rate are also listed in Table IV. A single oxygenation course can be specified when the cooperativity is comparably large, but the course diverges and the allosteric transition takes place at several stages of ligation as the cooperativity becomes smaller. In Hb(MalN) the transition occurs mainly at or around $i = 2$ corresponding to its $i_s$ close to 2. In this case, the oxygenation courses are symmetrical. In native Hb, the oxygenation course is also symmetrical in the absence of $P_2$-glycerate, but the addition of the phosphate shifts the transition stage to a later stage of ligation and makes the oxygenation course asymmetrical which corresponds to $i_s$ deviating from 2. The switchover rate, $r_s$, is increased on the addition of $P_2$-glycerate in all the hemoglobins, implying that the allosteric transition occurs more sharply in the presence of the phosphate. Table IV summarizes the values of $Lc^2$. The values of $Lc^2$ are close to unity for Hb(MalN), $\Delta F_I$'s of which are approximately maximized. For the hemoglobins other than Hb(MalN), however, the values of $Lc^2$ are greater than unity, especially in the presence of $P_2$-glycerate. The deviations of $Lc^2$ from unity shift the observed points to the right of the locus of maximum points as shown in Figure 2.

## Discussion

*Least-Squares Estimation of the Adair Parameters.* The present study clearly indicates that relatively accurate and unique values of $k$'s can be estimated by the least-squares method in which the experimental points in the lower and higher ranges of oxygen saturation are weighted more than those in the middle range. It has been shown previously that the $k$'s can be easily estimated from an oxygen equilibrium which has been determined precisely, particularly in the top and bottom portions, through a trial-and-error curve-fitting procedure using the Hill and Scatchard plots (Tyuma *et al.*, 1971a). The curve fitting of the Hill plot inevitably involves weighting of the experimental points in the lower and higher ranges of oxygen saturation since those ranges are expanded in the plot compared to the middle range.

*Structure–Function Relations in the Modified Hemoglobins.* According to the stereochemical model of Perutz (1970), the C-terminal histidine and penultimate tyrosine residues of the $\beta$ chains play a key role in keeping the tertiary and quaternary structure of hemoglobin molecule constrained. The similarity

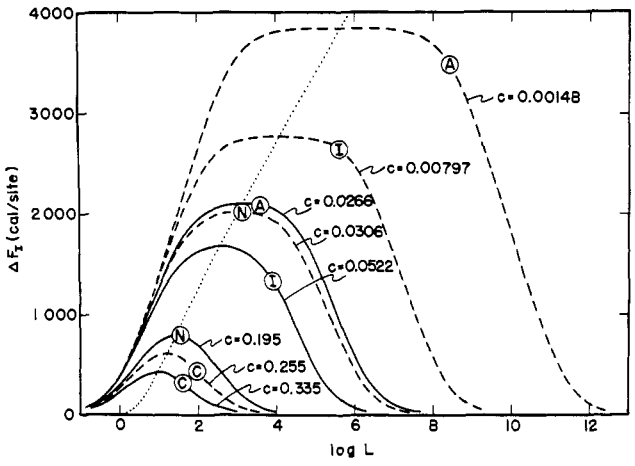


FIGURE 2: Dependence of the overall free energy of interaction upon the logarithm of the allosteric constant. (——) Stripped; (– – –) in 2 mM $P_2$-glycerate. Dotted line is the locus of the maximum points of the bell-shaped curves when $c$ is varied. Circles in the bell-shaped curves, Ⓐ, Ⓘ, Ⓝ, and Ⓒ, indicate the observed points for native Hb, Hb(AcAm), Hb(MalN), and Hb(CPase), respectively.

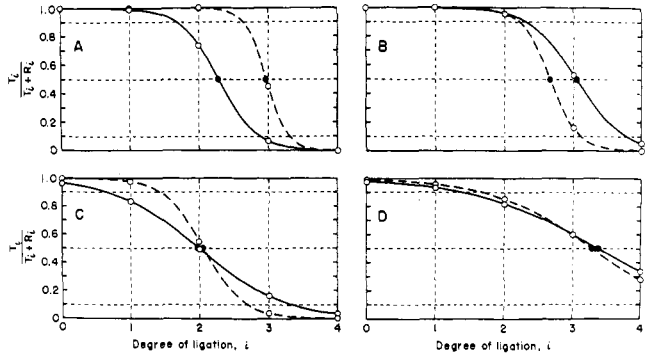


FIGURE 3: Dependence of the fraction of $T_i$ in $(T_i + R_i)$ upon the degree of ligation, $i$. (A) Native Hb; (B) Hb(AcAm); (C) Hb(MalN); (D) Hb(CPase). (——) Stripped; (– – –) in 2 mM $P_2$-glycerate. Open circles indicate the real states taken by hemoglobin during the ligation and closed circles the switchover points. Horizontal lines intersecting the ordinate at 0.1 or 0.9 indicate the boundaries used for deducing the principal courses of oxygenation.

of $k_4$'s for native Hb and Hb(CPase), which has lost the C-terminal histidine and penultimate tyrosine residues, implies that the constraint produced by these two residues is released before the fourth heme is liganded. A value of $k_4$ insensitive to $P_2$-glycerate and similar to that native Hb has also been obtained for a genetically modified hemoglobin, Hiroshima, which has lost the histidine residues due to the replacement by aspartate (Imai *et al.*, 1972).

On the other hand, Hb(MalN) has a somewhat smaller $k_4$ than that of native Hb. In the deoxy form, the *N*-ethylsuccinimide group attached to the F9$\beta$ cysteine has been shown to displace the HC3 histidine residue in the same $\beta$ chain from its normal position but not in the oxy form (Perutz *et al.*, 1969). If we assume that Hb(MalN) takes a conformation similar to that of native Hb in the fully oxygenated state, its smaller $k_4$ indicates that Hb $Hb(O_2)_3$ is still in a constrained state in the former hemoglobin.

The fact that the $k_4$'s of native Hb, Hb(AcAm), and Hb(MalN) are not significantly influenced by $P_2$-glycerate suggests that the phosphate has been expeled from these hemoglobins before the fourth oxygen molecule is combined as in native Hb (Perutz, 1970; Tyuma *et al.*, 1971a). The effect of $P_2$-glycerate on the cooperativity of Hb(AcAm) and Hb(MalN) can be considered as essentially the same as that of native Hb, since $\Delta(\Delta F_I)$ is not too different for these hemoglobins (Table II). Interestingly, even in hemoglobins such as Hb(AcAm) and Hb(MalN) which have a reduced "intrinsic cooperativity (*i.e.*, the $\Delta F_I$ in the absence of $P_2$-glycerate)," the $\Delta F_I$ is increased on the addition of $P_2$-glycerate by an extent similar to that in native Hb. The same phenomenon has also been observed in Hb Hiroshima which has an intermediate cooperativity comparable to that of Hb(MalN) (Imai *et al.*, 1972). The increase in the cooperativity due to $P_2$-glycerate is practically absent in essentially noncooperative hemoglobins as observed in Hb(PCase) in this study and in the hemoglobin treated with bis(*N*-maleimide methyl) ether (Salhany, 1971).

The insensitivity of Hb(CPase) to $P_2$-glycerate indicates that the phosphate has either the same or no affinity for both the oxy and deoxy forms of this hemoglobin. Recent X-ray data (Perutz and TenEyck, 1972) have shown that the enzymatic modification gives rise to a partial unwinding of the H helix as far back as H18$\beta$ alanine in the deoxy form. This structural disturbance will diminish, at least in part, the response of Hb(CPase) to the phosphate by making the imidazole groups of the H21$\beta$ histidines, which constitute one pair of the $P_2$-glycerate binding groups (Bunn and Briehl, 1970; Perutz, 1970), incapable of binding to the phosphate.

*Effects of $P_2$-Glycerate and Chemical Modifications of Hemoglobin Chains on the MWC Parameters.* The present study clearly shows that not only $L$ but also $c$ is strongly dependent on $P_2$-glycerate and chemical modifications. The dependence of $c$ results from the strong and weak dependence of $K_T$ and $K_R$, respectively, on the phosphate and the chemical modifications.

Recently, Edelstein (1971) estimated the values of $L$ for several types of hemoglobins from their oxygen affinities relative to those of the isolated chains. The assumption used by Edelstein was that $c$ is independent of pH, the stripping of $P_2$-glycerate, and amino acid substitutions and is invariably equal to 0.01. This assumption is not valid in the light of the present results. There is no physical reason that $c$ should be invariant to the change of hemoglobin species or solvent conditions as pointed out by Minton (1971). In the present study, all parameters have been estimated without any supposition on the parameters. The estimation of $L$ by the use of curve-fitting procedures of oxygen equilibrium data is usually difficult because the middle range of the equilibrium curve is not too sensitive to $L$. Therefore, it is not possible to estimate the parameters accurately by using the apparent Hill coefficient, $n$, which only represents the nature of the middle portion of the curve. In the present study, an accurate estimation of the MWC parameters was made possible by the precise determination of the equilibrium curves over a wide range of oxygen saturation and by appropriately weighting the experimental points in the least-squares procedure.

It is reasonable to ascribe the change of $K_T$ caused by $P_2$-glycerate to a preferential binding of the phosphate to the T-state molecules, noting that the major fraction of completely oxygenated and deoxygenated hemoglobins is in the R and T states, respectively, and that the phosphate combines with an overwhelming preference for deoxyhemoglobin (Benesch *et al.*, 1968). The present results suggest that the oxygen affinity of the T-state molecules is lowered by $P_2$-glycerate which is bound to those molecules. The following data also suggest

that the properties of the T-state molecules are modified by $P_2$-glycerate. Maeda *et al.* (1970) showed that reactivity of the sulfhydryl groups of F9(93)$\beta$ cysteine for deoxyhemoglobin toward 5,5′-dithiobis(3,3′-nitrobenzoic acid), which is lower than that for oxyhemoglobin, is significantly reduced on the addition of $P_2$-glycerate.

The observed change of the rate of reaction of the reagent with deoxyhemoglobin may be explained in part by a shift of the allosteric equilibrium from the $R_4$ state to the $T_4$ state. The shift of the allosteric equilibrium, however, cannot be responsible for the entire change of the reaction rate because the increase in the fraction of the $T_4$ state is, even if the equilibrium is extremely shifted to the $T_4$ state, very small (see Figure 3) compared with the change of the reaction rate. Thus, the result of Maeda *et al.* suggests that the properties of the T-state molecules are modified by $P_2$-glycerate which is bound to those molecules.

According to the MWC model, heterotropic effects would be due exclusively to displacement of the equilibrium between R and T states of protein. This predicts that the effector, $P_2$-glycerate, would alter only $L$ without affecting the other parameters. The present results are contrary to this prediction. It was impossible to simulate the experimental equilibrium curves both in the absence and presence of $P_2$-glycerate by using the same $c$ and different $L$'s. This contradiction originates from the fact that $P_2$-glycerate preferentially changes the oxygen affinity of T state. Thus, the present results suggest that an allosteric effector cannot alter the allosteric constant without modifying the properties of T- and/or R-state molecules preferentially.

Ogata and McConnell (1971, 1972) have presented a generalized MWC model where the $\alpha$ and $\beta$ subunits are treated as nonequivalent and they have shown that the oxygen equilibrium curves of native, cyanmet hybrid, and abnormal hemoglobins both in the absence and presence of spin-labeled organic phosphates can be fitted to the generalized oxygen saturation function where only $L$ is influenced by the phosphates. Their oxygen equilibrium data used for the curve fitting were, however, less accurate than those used in this study and lacked the experimental points at the top and bottom of equilibrium curves to which the parameters are sensitive. Therefore, their result that $c$ ($c_\alpha$ and $c_\beta$ in their papers) is not affected by the phosphates does not appear conclusive.

The present result that $K_T$ and $K_R$ are affected by the chemical modifications of the $\beta$ chains suggests that the modified hemoglobins assume T and R conformations different from those of native Hb. Figure 3 shows that an allosteric transition does occur even in Hb(CPase). Since $c$'s of Hb(CPase) are close to unity, indicating that the T and R conformations are similar to each other, the present result is not necessarily incompatible with the result of Zito *et al.* (1964) who showed that Hb(CPase) does not appear to undergo conformational changes on oxygenation.

*Nature of the Allosteric Transitions.* $L$ has a particular value which maximizes the cooperativity measured by $\Delta F_I$. If $L$ becomes smaller or larger than this value, the cooperativity is diminished. It has been shown that the Hill coefficient, $n$, an empirical measure of the cooperativity, also exhibits a similar dependence on $L$ (Rubin and Changeux, 1966). The condition for maximum $\Delta F_I$, $Lc^2 = 1$ (eq 9), is also the condition for $i_s = 2$ and for the symmetry of both the oxygen equilibrium curve and the oxygenation course in the MWC T- and R-state system.

What is the physical meaning of this condition? An explanation for the question may be given as follows. In the MWC system, there are two pathways at each stage of ligation, *i.e.*, the ligation of either $T_i$ or $R_i$. $\Delta F_I$ will be increased as more of the unliganded molecules are liganded through the pathway, $T_0 \rightarrow T_1$, and more of the triply liganded molecules through the pathway, $R_3 \rightarrow R_4$, since the overall free energy of interaction, $\Delta F_I$, is determined only by the ratio of $k_4$ to $k_1$ (eq 7). Such a situation is realized when the ligation course is symmetrical. This is the case of stripped Hb(MalN) (Figure 3). The addition of 2 mM $P_2$-glycerate to this hemoglobin makes the situation more conspicuous by increasing the switchover rate, and in addition, it reduces $K_T$ to about one-tenth without a significant change of $K_R$. Thus, $\Delta F_I$ is markedly increased (Table IV). In the case of native Hb, the cooperativity increasing effect of $P_2$-glycerate is less efficient; the phosphate increases $\Delta F_I$ by reducing $K_T$ to about one-tenth with smaller change of $K_R$, but this increase is canceled out in part because a large deviation of $Lc^2$ from unity (Table IV) allows the existence of $T_3$ in a considerable amount (Figure 3).

Noting that $P_2$-glycerate combines preferentially with the T-state molecules, we can consider that $P_2$-glycerate molecule is expelled simultaneously when the allosteric transition takes place during oxygenation. The oxygenation course in the MWC system (Table IV) suggests that in native Hb $P_2$-glycerate is expelled mainly after the binding of the third oxygen molecule. This result is not compatible with the Perutz model which suggests that the expulsion of $P_2$-glycerate precedes the third oxygen binding.

The switchover point and the expelling stage of $P_2$-glycerate for native Hb are shifted toward later stages of the ligation beyond the half-ligation ($i = 2$) (Table IV). This indicates that $P_2$-glycerate bound to the T-state molecules stabilizes that state and restrains the allosteric transition into the R state. According to the model of Perutz (1970), hemoglobin molecule is free of constraints in the fully oxygenated state since all of the several salt bridges which constrain the tertiary and quaternary conformations of the molecule in the deoxygenated state are broken successively during oxygenation. The oxygenation courses shown in Table IV suggest that these constraints are removed around the half-ligation in stripped hemoglobins except for Hb(CPase). $P_2$-glycerate will stabilize these intersubunit salt bridges by forming additional salt bridges with a pair of the $\beta$ chains, in consequence stabilizing the T conformation. Thus, the rupture of the intersubunit salt bridges will be postponed and concerted on the stage of $P_2$-glycerate expulsion. This is a plausible explanation of the deviation of $Lc^2$ from unity and the increase in the switchover rate caused by $P_2$-glycerate.

It is well known that $P_2$-glycerate acts as a regulator for oxygen transport by regulating the overall oxygen affinity of hemoglobin in the red cell (Benesch and Benesch, 1969). The shift of the switchover point to later stages beyond the half-ligation yields an asymmetrical oxygen equilibrium curve which retains high cooperativity up to a high range of oxygen saturation. The slope of the Hill plot at $Y = 0.99$ ($\log [Y/(1 - Y)] = 2.0$) is still above 2 in 2 mM $P_2$-glycerate though it is very close to unity for stripped native Hb (Tyuma *et al.*, 1971b). This effect may raise the efficiency of oxygen transport by hemoglobin since usually only the upper portion ($Y$ = about 0.7–1.0) of the curve has physiological significance.

$L$ expresses the difference of stabilities of the T and R states. When one hemoglobin molecule undergoes the transition from the T to R state in the absence of oxygen, then the free energy change for the transition, $\Delta F_t$, is given by $\Delta F_t = RT$

ln $L$. Using the estimates of $L$ in Table III we have $\Delta F_t$ = 4890 ± 90 cal/mol for stripped native Hb and $\Delta F_t$ = 11,470 ± 460 cal/mol for native Hb in 2 mM $P_2$-glycerate. If we assume that the stability of the T state relative to that of the R state is attributable to the six salt bridges among the subunits (Perutz, 1970) and the bond energy per salt bridge is the order of 1000–2000 cal/mol (Perutz, 1970), the estimates of $\Delta F_t$ for stripped native Hb is somewhat smaller than the expected value of 6000–12,000 cal/mol. The increment in $\Delta F_t$ on the addition of 2 mM $P_2$-glycerate, $\Delta(\Delta F_t)$ = 6570 ± 290 cal/mol, may be reasonably ascribed to the additional salt bridges formed between the $P_2$-glycerate molecule and the pair of the $\beta$ chains in deoxyhemoglobin since $P_2$-glycerate carries four negative charges per mole at pH 7.4 (Benesch *et al.*, 1969).

*Validity of the MWC Model.* The curves simulated using the MWC oxygen saturation function deviate from the experimental points slightly more than those simulated using the Adair function. This does not necessarily indicate that the MWC model is less suitable for the hemoglobin–oxygen equilibrium system than the Adair theory, because the former involves only three independent parameters as compared to four in the latter. Reasonable estimates of the Adair parameter, $k_i$, sometimes do not show a monotonic increase with $i$ (Table I). On the other hand, the original MWC model always yields a cooperative effect for each ligation stage, consequently a monotonic increase in $k_i$ with $i$ (refer to $k_i$ recalculated from the MWC parameters in Table I). This nature of the model is liable to facilitate the deviation of the simulated curves from the experimental points. The generalized model of Ogata and McConnell (1971) involves one more parameter to introduce the nonequivalence of the $\alpha$ and $\beta$ subunits and may fit to the experimental data better than the original model. It seems difficult, however, to draw conclusions on the validity of the MWC model only from the results of curve fitting of oxygen equilibrium data. To test the model further, accumulation of data from various kinds of independent experiments is necessary.

In the MWC model each protomer has the same number of homologous sites for the binding of allosteric effectors. $P_2$-Glycerate may not be an allosteric effector in that sense because it is considered that it combines with a pair of protomers, *i.e.*, the sister $\beta$ chains, forming interprotomer bridges, and has no binding site on the other protomers (Perutz, 1970). The phosphate can be, however, regarded as an allosteric effector in a wide sense, because its affinity to the oligomer is closely related to the quaternary structure of the oligomer and its binding to the stereospecific sites, which are arranged symmetrically along the twofold axis of the oligomer, conserves the molecular symmetry. Therefore, in this study, the heterotropic effect was examined by regarding $P_2$-glycerate as an allosteric effector. The present result on the heterotropic effect does not support the assumption in the MWC model that allosteric effectors affect the allosteric constant, $L$, only. In this sense, the original model is oversimplified and the present study suggests that the model needs modifications to explain the heterotropic effect in hemoglobin. The modification may be attained by taking into account more than two accessible states as suggested by Monod *et al.* (1965).

## Acknowledgment

The author thanks Professor I. Tyuma for reading this manuscript and for discussion. The author also thanks Professor W. B. Elliott, Department of Biochemistry, State University of New York at Buffalo, for generous supply of thin Teflon membrane.

## References

Adair, G. S. (1925), *J. Biol. Chem. 63*, 529.
Antonini, E., and Brunori, M. (1971), *Hemoglobin Myoglobin, Reactions Ligands, 1971*, 1.
Antonini, E., Wyman, J., Zito, R., Rossi-Fanelli, A., and Caputo, A. (1961), *J. Biol. Chem. 236*, PC60.
Benesch, R., and Benesch, R. E. (1961), *J. Biol. Chem. 236*, 405.
Benesch, R., and Benesch, R. E. (1969), *Nature (London) 221*, 618.
Benesch, R., Benesch, R. E., and Yu, C. I. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 526.
Benesch, R., Benesch, R. E., and Yu, C. I. (1969), *Biochemistry 8*, 2567.
Benesch, R., Macduff, G., and Benesch, R. E. (1965), *Anal. Biochem. 11*, 81.
Bunn, H. F., and Briehl, R. W. (1970), *J. Clin. Invest. 49*, 1088.
Chanutin, A., and Curnish, R. R. (1968), *Arch. Biochem. Biophys. 123*, 163.
Edelstein, S. J. (1971), *Nature (London) 230*, 224.
Guidotti, G. (1967), *J. Biol. Chem. 242*, 3685.
Hopfield, J. J., Shulman, R. G., and Ogawa, S. (1971), *J. Mol. Biol. 61*, 425.
Imai, K. (1973), *Biochemistry 12*, 128.
Imai, K., Hamilton, H. B., Miyaji, T., and Shibata, S. (1972), *Biochemistry 11*, 114.
Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., and Kuroda, M. (1970), *Biochim. Biophys. Acta 200*, 189.
Maeda, T., Hiromi, K., and Ohnishi, S. (1970), *Biochem. Biophys. Res. Commun. 38*, 934.
Minton, A. P. (1971), *Nature (London), New Biol. 232*, 145.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Ogata, R. T., and McConnell, H. M. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36*, 325.
Ogata, R. T., and McConnell, H. M. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 335.
Perutz, M. F. (1970), *Nature (London) 228*, 726.
Perutz, M. F., Muirhead, H., Mazzarella, L., Crowther, R. A., Greer, J., and Kilmartin, J. V. (1969), *Nature (London) 222*, 1240.
Perutz, M. F., and TenEyck, L. F. (1972), *Cold Spring Harbor Symp. Quant. Biol. 36*, 295.
Riggs, A. (1961), *J. Biol. Chem. 236*, 1948.
Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1964), *Advan. Protein Chem. 19*, 73.
Roughton, F. J. W. (1963), *Clin. Chem. 9*, 682.
Roughton, F. J. W., and Lyster, R. L. J. (1965), *Hvalradets Skr. 48*, 185.
Roughton, F. J. W., Otis, A. B., and Lyster, R. L. J. (1955), *Proc. Roy. Soc., Ser. B 144*, 29.
Rubin, D. I. (1963), *Chem. Eng. Progr., Symp. Ser. 59*, 90.
Rubin, M. M., and Changeux, J.-P. (1966), *J. Mol. Biol. 21*, 265.
Salhany, J. M. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 14*, 11.
Taylor, J. F., Antonini, E., Brunori, M., and Wyman, J. (1966), *J. Biol. Chem. 241*, 241.
Tomita, S., and Riggs, A. (1971), *J. Biol. Chem. 246*, 547.
Tyuma, I., Imai, K., and Shimizu, K. (1971b), *Biochem.*

*Biophys. Res. Commun. 44*, 682.
Tyuma, I., Shimizu, K., and Imai, K. (1971a), *Biochem. Biophys. Res. Commun. 43*, 423.
Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.
Zito, R., Antonini, E., and Wyman, J. (1964), *J. Biol. Chem. 239*, 1804.

# Band Pattern of the Segment-Long-Spacing Form of Collagen. Its Use in the Analysis of Primary Structure†

Romaine R. Bruns* and Jerome Gross

ABSTRACT: The highly reproducible banded pattern of segment-long-spacing crystallites observed in the electron microscope has been characterized in terms of the number and location of bands in order to facilitate its use in the primary structure analysis of collagen. Positively stained crystallites of calf skin collagen show 117 characteristic dark and light bands distributed in an aperiodic pattern. The location in the pattern of individual bands, designated by simple numbers, has been determined by an optical averaging procedure. The data enable us to define and report the precise location of individual bands and segment-long-spacing fragments along the length of the molecule. Since the segment-long-spacing band pattern of calf skin collagen represents the pattern of crystallites prepared from a wide variety of collagens, it also has been possible to collocate, on a standard basis, a large number of published observations on segment-long-spacing fragments produced by enzymatic or specific chemical cleavage, and the most recent data correlating particular bands with specific amino acid sequences.

Electron microscopy of segment-long-spacing crystallites has played a significant role in both the amino acid sequence analysis of collagen and in studies on the specific sites of cleavage by various collagenases (Piez *et al.*, 1968; Rauterberg and Kuhn, 1968; Stark and Kuhn, 1968a; Fietzek *et al.*, 1970; Igarashi *et al.*, 1970; Gross and Nagai, 1965; Rauterberg *et al.*, 1970; Harper *et al.*, 1971). These applications of electron microscopy to the study of molecular structure are possible because of the unusual properties of the collagen molecule. The long (3000 × 14 Å), rigid, triple-helical molecules, in the presence of adenosinetriphosphoric acid (ATP) at low pH (Schmitt *et al.*, 1953; Gross *et al.*, 1954), align in perfect transverse register to form crystallites, called *segment-long-spacings*, that are the same length as the collagen molecule. When stained with solutions of phosphotungstic acid or uranyl acetate, such crystallites reveal a characteristic band pattern which reflects the distribution of clusters of charged and uncharged amino acids along the molecule (Hodge and Schmitt, 1960; Mark *et al.*, 1970b; Balian *et al.*, 1971); thus individual bands of a segment-long-spacing crystallite serve to identify loci (*ca.* 20 Å) on the collagen molecule.

In the amino acid sequence analysis of collagen, the linear order of large peptide fragments has been established by relating the fragments to particular groups of bands in the segment-long-spacing band pattern, *e.g.*, cyanogen bromide (CNBr) peptides, prepared from isolated $\alpha$ chains, re-form triple-helical structures upon temperature annealing (Rauterberg and Kuhn, 1968), and when allowed to react with ATP such structures form fragments of segment-long-spacing crystallites that have all the detailed banding characteristic of their region of origin in the native structure. To date it has been possible to renature and order by electron microscopy four of the nine CNBr peptides of the $\alpha$1 chain and three of the five peptides of the $\alpha$2 chain, which range in molecular weight from 13,500 to 30,000 (Rauterberg and Kuhn, 1968; Fietzek *et al.*, 1970; Igarashi *et al.*, 1970). By combining electron microscopic data on the larger fragments with biochemical data on the smaller ones, it has been possible to establish the proper order of all the cyanogen bromide peptides in the $\alpha$1 and $\alpha$2 chains of several mammalian collagens (Piez *et al.*, 1968, 1969; Vuust *et al.*, 1970).

The segment-long-spacing band pattern also has been used to locate sites of cleavage by specific enzymatic or chemical methods (Gross and Nagai, 1965; Stark and Kuhn, 1968b; Heidemann and Heinrich, 1970; Hörmann and Volpin, 1970; Nordwig and Bretschneider, 1971) and to visualize directly, by specific staining, bands containing particular reactive residues such as arginine or methionine (Weiss and Bowden, 1969a,b).

A serious limitation in all of these studies has been the absence of a quantitative characterization of the segment-long-spacing band pattern in terms of the number and location of bands. In this paper we establish the number and position of reproducible bands in segment-long-spacing crystallites of calf skin collagen and demonstrate the application of this information to the structural analysis of collagen molecules.

## Materials and Methods

*Collagen.* Purified acid-soluble calf skin collagen was prepared according to the method of Gross and Kirk (1958), lyophylized, and stored frozen until used. The collagen was solubilized in 0.5 M acetic acid (4°) at a concentration of 0.1%;

† From the Developmental Biology Laboratory, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114. *Received September 18, 1972.* This is publication No. 591 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities. This investigation was supported by Public Health Service Grants AM-12785 and AM-3564 and Training Grant AM-5067.