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Mechanism of the Enhancement of the Bohr Effect in Mammalian Hemoglobins by Diphosphoglycerate

(computer/association constants/oxygen/carbon dioxide)

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ABSTRACT **The number of protons released from several mammalian hemoglobins as a consequence of oxygenation is greater in the presence of low concentrations of 2,3-diphosphoglycerate than in its absence. A mechanism for this enhancement of proton release is proposed. The basis of this mechanism is that 2,3-diphosphoglycerate binds primarily between the protonated α-NH_2 terminal groups of the two β chains in deoxyhemoglobin. This binding will shift the ionization equilibria in favor of the protonation of the deoxyhemoglobin. Partial release of 2,3-diphosphoglycerate upon oxygenation of the hemoglobin is then accompanied by a release of protons. The apparent enthalpy of diphosphoglycerate binding appears to be close to zero. The previously reported temperature dependence appears to be due entirely to the associated protonation reaction. If only a single diphosphoglycerate binding site is assumed, the intrinsic association constant is estimated to be 3.9×10^5 M^{-1} for deoxyhemoglobin and 1.05×10^4 M^{-1} for oxyhemoglobin at 20°C in 0.1 M NaCl.**

Schmidt-Nielsen and Larimer (1) showed that the oxygen transport properties of the bloods of different mammals are generally adapted to the metabolic need for oxygen: the smaller the mammal and the higher its metabolic rate, the higher is the oxygen pressure at which the blood tends to release a given fraction of combined oxygen. Later measurements (2) on hemoglobin solutions have shown that this relationship depends on differences in the Bohr effect among the hemoglobins from various mammals: the smaller mammal tends to possess a hemoglobin that releases more protons upon oxygenation than are released by the hemoglobin of larger mammals. This adaptation has recently been shown (3) to depend in part on the presence of 2,3-diphosphoglycerate (P_2G). The Bohr effect can be considered to be composed of two parts: the first part is believed to be independent of P_2G and the second part occurs only in the presence of P_2G. I wish here to propose a molecular mechanism for the enhancement of the Bohr effect that constitutes this second part, and to outline some of the consequences of the model.

The relevant observations are as follows: (*a*) P_2G lowers the affinity of hemoglobin for oxygen (4, 5); (*b*) P_2G is bound primarily to deoxyhemoglobin, and to a much lesser extent to oxyhemoglobin (6–9): (*c*) the stoichiometry at low concentrations of P_2G appears to be 1:1, although measurements at very high concentrations of P_2G and at very low salt concentrations indicate that additional sites are present (6, 8, 10); (*d*) the binding appears to be electrostatic: raising the ionic strength sufficiently displaces the P_2G (10); (*e*) the binding of P_2G is pH dependent and is greatest at low pH (refs. 3, 8, and 10); (*f*) CO_2 can abolish the effect of P_2G on the oxygen equilibrium of hemoglobin at high pH, but not at low pH (ref. 3); (*g*) CO_2 exerts its specific and direct effect on oxygen-binding by hemoglobin by combining with the uncharged α-NH_2 groups of all four subunits to form carbamino compounds: R–$NHCOO^-$ (11); (*h*) the α-NH_2 groups of the α-chain, but not of the β-chain, contribute to the Bohr effect in the absence of P_2G (11); (*i*) digestion of hemoglobin with carboxypeptidase A (EC 3.4. 2.1) removes the carboxyl terminal residues of the β-chain, and hemoglobin so digested does not show an effect with P_2G (3).

A probable primary binding site in deoxyhemoglobin consistent with these observations is on the diad axis of the tetramer between the two α-NH_2 groups of the β-chain (12, 15, 16). The effects of pH and ionic strength on P_2G binding suggest that binding only occurs when the α-NH_2 groups of each β-chain are protonated. The scheme shown in Fig. 1 is completely consistent with these facts. This model explains the competitive interaction between the effects of P_2G and CO_2 (3) first reported by Bauer (13). Kilmartin and Rossi-Bernardi (11) showed that, under certain conditions, CO_2 abolishes the total Bohr effect because the release of protons associated with carbamino formation exactly cancels the number of protons normally taken up by hemoglobin as a consequence of deoxygenation. Formation of the P_2G hemoglobin complex will tend to shift reaction 1 (Fig. 1) to the left in favor of protonation. Thus, deoxyhemoglobin will tend to become more protonated in the presence of P_2G. This means that protonation of the α-NH_2 groups of the β-chains becomes linked to oxygenation in the presence of P_2G *even though* the protonation of these groups does not appear to be so linked in its absence. This scheme suggests (3) a common mechanism to explain why both CO_2 and P_2G lower the affinity of hemoglobin for oxygen. The x-ray data, obtained in the absence of P_2G, indicate that the terminal –COOH histidine of one β-chain is about 12 Å from the α-NH_3^+ group of the neighboring β-chain in deoxyhemoglobin (14). This distance shrinks to about 6 Å in oxyhemoglobin (14). The presence of either the negatively charged carbamino group, –$NHCOO^-$, or of the P_2G anion would tend, by electrostatic repulsion, to prevent the approach of the –COO^- terminus toward the α-NH_3^+ group that otherwise occurs during oxygenation: the deoxy conformation would therefore be favored. In addition, the total space available for accomodation of the P_2G anion is less in oxy- than deoxyhemoglobin (12).

Abbreviation: P_2G, 2,3-diphosphoglycerate.

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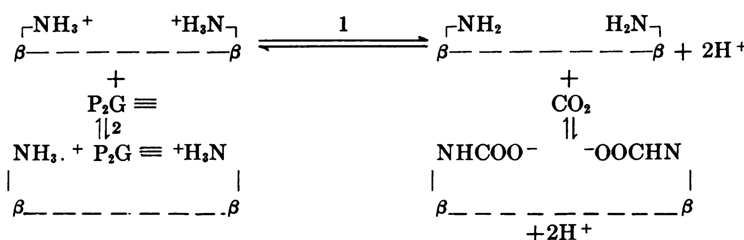

FIG. 1. Scheme for the relationship between P_2G and CO_2 binding by hemoglobin. Reaction 1 involves one microscopic dissociation constant, k; the two groups are assumed to ionize independently. Reaction 2 is assumed to occur only when both groups are protonated.

The scheme shown in Fig. 1 can be made quantitative as follows. Benesch, Benesch, and Yu (10) give the association constant for P_2G binding at three different values of the pH. Evidently, their association constant (K_B) includes a protonation reaction and, therefore, all of their thermodynamic calculations (10) for P_2G binding must also include this pH dependence. The two α-NH_2 groups of the β-chain appear to be too far apart to influence each other electrostatically: they are about 16 Å apart in deoxyhemoglobin and 20 Å apart in oxyhemoglobin (12). Therefore, we can describe their ionization by a two-step reaction, $Hb^{2+} \xrightarrow{K_1} Hb^{+} \xrightarrow{K_2} Hb$, where K_1 and K_2 are the macroscopic dissociation constants: $K_1/K_2 = 4$ if the groups are independent. In terms of a microscopic constant, k, $K_1 = 2k$, and $K_2 = k/2$. Hb^{2+} symbolizes the hemoglobin tetramer with both α-NH_2 groups of the β-chain protonated, and Hb^+ symbolizes the tetramer with only one of these groups protonated. We assume that $Hb^{2+}\cdot P_2G$ is the *only* form of P_2G complex, and that Benesch *et al.* (10) measured the ratio of this form to all other forms, Hb, Hb^+, Hb^{2+}. If so, the intrinsic P_2G association constant, K_D, can be expressed in terms of K_B, the pH-dependent constant:

$$K_D = K_B\left(1 + \frac{k}{(H^+)}\right)^2 \qquad [1]$$

A plot of $1/\sqrt{K_B}$ against $1/(H^+)$ yields a straight line, of slope $k/\sqrt{K_D}$ and intercept $1/\sqrt{K_D}$. The Benesch data fit such a plot within about 0.8%, and yield a value of $K_D = 4.07 \times 10^5\ M^{-1}$ and $k = 7.7 \times 10^{-8}$ M, corresponding to an intrinsic pK of about 7.11 at 25°C. This pK value is slightly higher than the value (7.14) found by Forster *et al.* (17) for the ionization of the α-NH_2 groups associated with CO_2 binding by human deoxyhemoglobin at 37°C, when the latter value is corrected to 25°C. We can make this correction approximately if we assume that the enthalpy, $\Delta H°$, for the ionization of an α-NH_2 group is about 11 kcal/mol, which is typical of such groups (18). The results of Forster *et al.* (17) can be considered as only a rough approximation, because their preparation was dialyzed against distilled water, which only partially removes diphosphoglycerate. Therefore, their pH dependence for the ionization of the α-NH_2 group must have been shifted. The similarity of pK values, and the fact that CO_2 combines only with the uncharged α-NH_2 groups and competes with P_2G for binding, strengthens the identification of the groups responsible for the pH dependence of P_2G-binding. Although the β-143 histidyl residues appear to be within hydrogen-bonding distance of bound P_2G (12), and therefore might contribute to the ionization reaction, the presently available data can be completely described in terms of only two groups that ionize independently of one another, without invoking any additional groups. The Benesch data cannot be fitted at all to a model that assumes only a single ionizing group, and the fit is relatively poor, as expected, if a concerted model is assumed in which the two α-NH_2 groups are both either protonated or unprotonated.

We seek to obtain the temperature dependence of P_2G binding exclusive of any effect of pH. Benesch, Benesch, and Yu (10) provide data on the temperature dependence of K_B, but K_B depends on pH. With the aid of these data, Eq. [1] permits the calculation of the temperature dependence of K_D, provided we know that for k. This can be estimated if we assume that the enthalpy, $\Delta H°$, for ionization of the α-NH_2 group is about 11 kcal (18), and use the thermodynamic relationship, $\partial pK/\partial(1/T) = \Delta H°/2.3\,R$, where R is the gas constant. If we then make the appropriate substitutions in Eq. [1], the temperature dependence of K_D is found to be virtually zero. The $\Delta H°$ value reported (10) appears to be due entirely, or almost entirely, to the protonation reaction and not to diphosphoglycerate binding; $\Delta S°$ becomes + 24 e.u., not −22 e.u. as reported (10). These considerations suggest that the true $\Delta H°$ for P_2G binding is compensated for completely by the concomitant displacement of other ions. The site of P_2G binding has six positively charged groups; in the absence of P_2G, these charges are presumably balanced by Cl^- ions when the solvent is 0.1 M NaCl. At low ionic strength, $\Delta H°$ (observed) would not be expected to be close to zero. This picture helps explain why dialysis against distilled water is ineffective in removing diphosphoglycerate (10, 3).

The average number of protons, $\bar{h}_D$, bound per tetramer by the P_2G-linked acid groups on the β-chains can be expressed as follows.

$$\bar{h}_D = \frac{1}{C_H}\left(2\,[Hb^{2+}\cdot P_2G] + 2[Hb^{2+}] + [Hb^+]\right) = \frac{2\,\frac{(H^+)}{k}\left(1 + \frac{(H^+)}{k}\right) + 2K_D\,(P_2G)_D\,\frac{(H^+)^2}{k^2}}{\left(1 + \frac{(H^+)}{k}\right)^2 + K_D\,(P_2G)_D\,\frac{(H^+)^2}{k^2}} \qquad [2]$$

Here C_H is the total molar concentration of hemoglobin; (H^+) is the hydrogen ion activity; K_D is the association constant for P_2G, and k is the microscopic constant for the dissociation of either α-NH_3^+ group. The free P_2G concentration, $(P_2G)_D$, when the hemoglobin is in the deoxy state, can be expressed in terms of the total P_2G concentration, C_D, by the expression:

$$(P_2G)^2 + \left(\frac{1}{K_D}\left(\frac{k}{(H^+)} + 1\right)^2 + C_H - C_D\right) - \frac{C_D}{K_D}\left(\frac{k}{(H^+)} + 1\right)^2 = 0 \qquad [3]$$

We now assume that for oxyhemoglobin, the protons bound, $\bar{h}_O$, can be expressed by an equation identical with [*2*] above, except that each K_D is replaced by K_O and each $(P_2G)_D$ is replaced by $(P_2G)_O$, the latter two terms being given by Eq. [*3*] with either K_D or K_O, respectively. The average number of protons released from the P_2G-linked groups as a result of oxygenation will be $\Delta\bar{h} = \bar{h}_D - \bar{h}_O$. As the P_2G concentration rises to sufficiently high levels, $\Delta\bar{h}$ approaches zero. Benesch, Benesch, and Yu (10) and Tomita and Riggs (3) provide data on the relationship between $\Delta \log P_{50}/\Delta pH$ and the total P_2G concentration, where P_{50} is the oxygen pressure required for 50% oxygenation, $\Delta \log P_{50}/\Delta pH$ gives the protons released per molecule of oxygen bound at 50% oxygenation. These data show that $\Delta\bar{h}$, the *additional* Bohr effect due to P_2G, does indeed approach zero at high P_2G concentrations. Furthermore, the return to zero is inconsistent with the conclusion maintained by Benesch *et al.* (10) that *no* P_2G is bound by oxyhemoglobin. If one assumes that $K_O = 0$, (i.e., no P_2G bound by oxyhemoglobin) and that the P_2G concentration is very large, $\Delta\bar{h}$ rises to a limiting plateau value of $2k/[k + (H^+)]$, and does *not* return to zero. This result shows that the lower the pH, the *lower* is the plateau level; $\Delta\bar{h}$ approaches 2 in alkaline solution, 1.0 where $k = (H^+)$, and $\Delta\bar{h} < 1$ where $(H^+) > k$. This helps explain qualitatively why Benesch, Benesch, and Yu obtained a smaller effect at a lower pH.

Eqs. [*2*] and [*3*] involve only a single unknown constant, K_O. The other constants, k and K_D, are independently determined by Eq. [*1*] from the pH-dependence data at 25°C provided by Benesch, Benesch, and Yu (10). The experimental data on $\Delta \log P_{50}/\Delta pH$ were obtained, however, at 20°C. The value of k at 20°C is estimated to be 5.62×10^{-8} on the assumption that $\Delta H°$ for the ionization of the α-NH_2 groups is 11 kcal. Since K_D appears to be essentially independent of temperature, we take the average value, $K_D = 3.92 \times 10^5$. With these values of k and K_D, a least-squares search program was used to determine the best-fit value of K_O, 1.05×10^4 M^{-1}, which is about 2.7% of the value of K_D at 20°C. Had we ignored this temperature correction and used the values of K_D and k at 25°C, 4.07×10^5 M^{-1} and 7.7×10^{-8} M, respectively, the calculated K_O would be 1.8×10^4 M^{-1}. The fit to the Benesch data (10) is shown in Fig. 2. Although some deviation from the theoretical relationship is apparent, the model accounts for the overall changes of $\Delta \log P_{50}/\Delta pH$ with P_2G concentration rather satisfactorily. Whether the deviations are real or due to experimental error cannot as yet be determined.

It should be recognized that several simplifying assumptions have been made. The model ignores changes in the ionization of P_2G with pH and assumes that only two ionizable groups are associated with P_2G binding. Ionization of the β-143 histidyl residues could also be involved, but does not appear necessary to describe the data. The possibility of additional P_2G binding sites has also been ignored in this approximation. Some error may result from the fact that the calculated $\Delta\bar{h}$ is that resulting from complete oxygenation at constant pH, whereas the values of $\Delta \log P_{50}/\Delta pH$ were derived from measurements near 50% oxygenation over a finite pH interval. These will only be exactly equal to $\Delta\bar{h}$ if the corrected value of $\Delta \log P_{50}/\Delta pH$ is independent of oxygenation, and if the pH interval is small. Although this is largely true in the absence of P_2G, it may not be completely valid in the presence of P_2G

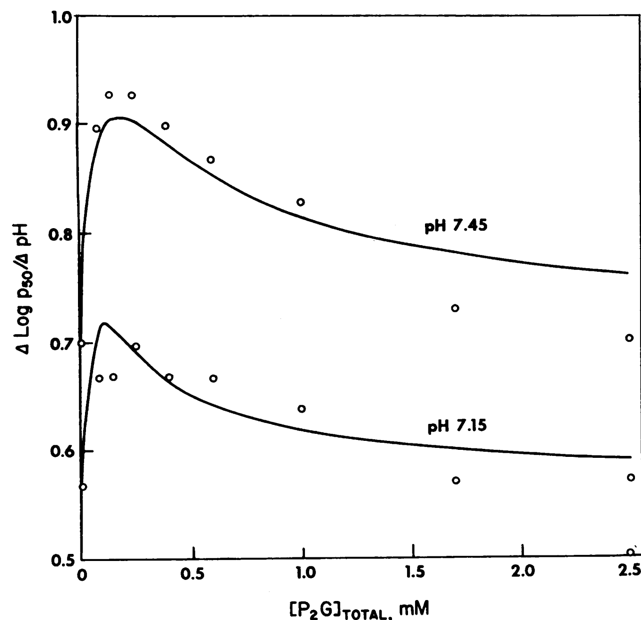

FIG. 2. The dependence of the Bohr effect (total protons released per O_2 bound) of human hemoglobin on the total diphosphoglycerate concentration. Experimental points are those of Benesch, Benesch, and Yu (10). The solid lines are computed on the basis described in the text, with $K_D = 3.92 \times 10^5$ M^{-1}, $K_O = 1.05 \times 10^4$ M^{-1}, and $k = 5.62 \times 10^{-8}$ M; hemoglobin concentration is 60 μM (tetramer basis). The value of $\Delta\bar{h}$ from Eq. [*1*] (text) has been added to the value of the Bohr effect in the absence of diphosphoglycerate to obtain the computed curve.

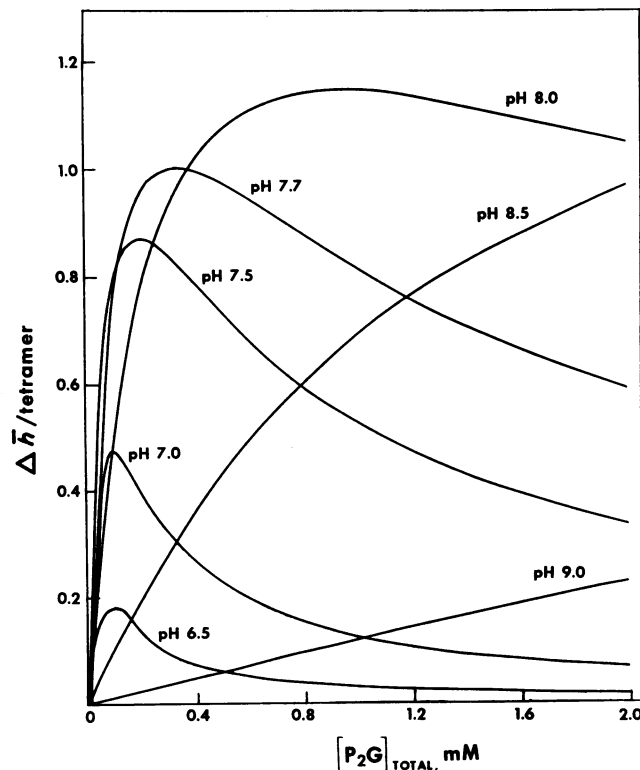

FIG. 3. Computed curves for the additional Bohr effect due to the presence of diphosphoglycerate. Here $\Delta\bar{h}$ is the number of protons released from the α-NH_2 groups of the β-chains upon formation of oxyhemoglobin, as a function of diphosphoglycerate concentration and pH. The constants are the same as in Fig. 1.

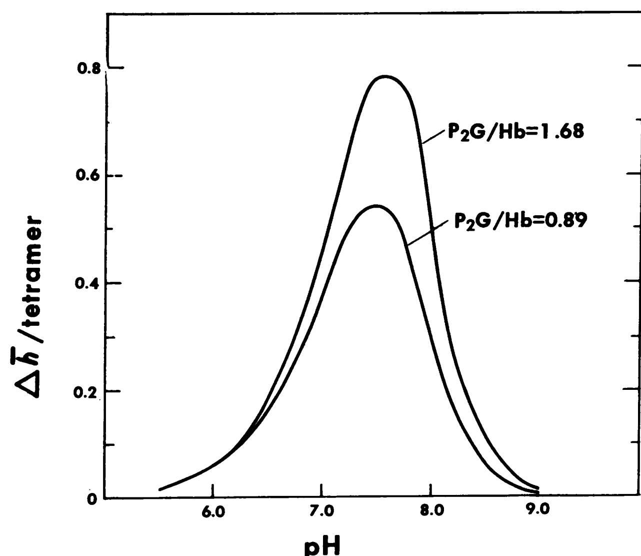

FIG. 4. The pH dependence of the additional Bohr effect computed on the same basis as in Fig. 3 for two ratios of diphosphoglycerate to hemoglobin. This pH dependence includes only the contribution from the α-NH_2 groups of the β-chains and does *not* represent the *total* Bohr effect.

because the shapes of the oxygen saturation curves become pH dependent in the presence of low concentrations of P_2G (3). Finally, k is considered to be independent of oxygenation because of the observations of Kilmartin and Rossi-Bernardi (11), but this may not be completely correct.

In the absence of P_2G, it appears that the α- and β-chains make about equal contributions to the Bohr effect [the α-NH_2 group and histidyl residue-122 of the α-chain and histidyl residue-146 of the β-chain are the groups believed to be involved (12)]. If P_2G binds primarily to the β-chains, the contributions of the two kinds of chain to the Bohr effect would no longer be equal. Under these conditions a change in the shape of the oxygen binding curve would be anticipated. This inequality would be diminished if P_2G binds also to the α-NH_2 groups of the α-chain as previously suggested (3).

The model predicts the way in which $\Delta\bar{h}$ depends on pH and the P_2G concentration. The data in Fig. 2 show that the model works reasonably well for two pH values. Calculated curves are given in Figs. 3 and 4 for the pH range 6.0–9.0. These curves show a well-defined pH optimum at about pH 7.5. Thus, in the physiological range, at or below pH 7.4, an *increase* in pH would lead to an *increase* in the number of protons released from the α-NH_2 groups of the β-chains during oxygenation, but above pH 7.5, an increase in pH would be associated with a decrease in $\Delta\bar{h}$. Although the reactions with CO_2 have not yet been incorporated into the model, it is clear from Fig. 1 that an increase in CO_2 at a constant pH value should have much the same effect as an increase in pH in the absence of CO_2; in either event reaction 1 is driven further to the right.

These considerations bear on comparative studies (3) that show that mouse, human, and elephant hemoglobins are functionally different from one another in their response to P_2G. Thus, in the presence of P_2G (about 1 mol/mol of hemoglobin) the Bohr effect at 20°C (protons released per oxygen bound) is 0.9, 0.65, and 0.45 for mouse, human, and elephant hemoglobins under the same set of conditions. These variations presumably result from differences in K_D, K_O, and k. Although an insufficient amount of data (3) exists for detailed analysis, the forms of the curves relating Bohr effect to P_2G concentration suggest that K_D is much larger in mouse than in human hemoglobin, perhaps in the range of about 10^7 M^{-1}. The value of K_O in mouse hemoglobin appears to be very much lower than in human hemoglobin; $\Delta\bar{h}$ never returns to zero, even at P_2G- to-hemoglobin ratios of 100. Further studies will be required to determine the quantitative and structural basis for these variations.

NOTE ADDED IN PROOF

An important paper by C. Bauer [*Respiration Physiology*, **10,** 10 (1970)] came to my attention after the completion of this manuscript. Bauer came to some of the same qualitative conclusions as those reported here.

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