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Electrostatic Interaction of Hemoglobin and Benzenepentacarboxylate

Enrico Bucci

ABSTRACT: The interaction of deoxyhemoglobin and benzenepentacarboxylate was followed by potentiometric measurements between pH 7.1 and 9.6. Up to pH 9 the difference in proteins bound by deoxyhemoglobin in presence and absence of benzenepentacarboxylate could be interpreted as the pK shift of three groups from 7.25, in the absence, to 8.55 in the presence of the effector. These were tentatively identified with the two 1β-valines and one histidine. Two more groups with p$K \geq 9$ appeared to interact with benzenepentacarboxylate. They may be either two lysines or a lysine and an arginine. The pH dependence between pH 7 and 9 of the affinity constant of deoxyhemoglobin for benzenepentacarboxylate appeared related only to the ionization and pK shift of the three groups detectable below pH 9. Cooperative protonation of these groups was not detectable. The protons absorbed by the interaction of deoxyhemoglobin with benzenepentacarboxylate were released upon oxygenation of the complex. These protons, liberated in addition to those liberated by the Bohr effect groups, constitute the additional Bohr effect (ABE).[1] The ABE decreased at concentrations of benzenepentacarboxylate higher than 10^{-4} M, indicating binding of benzenepentacarboxylate to oxyhemoglobin. The affinity of benzenepentacarboxylate for oxyhemoglobin was estimated to be about 1000 times less than the corresponding affinity for deoxyhemoglobin at the same pH. It is expected that at neutral pH and at high concentrations of benzenepentacarboxylate ($>10^{-3}$ M) both oxy- and deoxyhemoglobin would be saturated with the effector.

The interaction of hemoglobin and 2,3-diphosphoglycerate is relevant to respiratory physiology since it regulates the transport of both oxygen and carbon dioxide by hemoglobin (Benesch *et al.*, 1969; Tomita and Riggs, 1971; Pace *et al.*, 1970; Brenna *et al.*, 1972). A complete description of the system would be possible if the equilibrium constant for the binding of 2,3-diphosphoglycerate by hemoglobin could be measured under various conditions of pH, temperature, ionic strength, partial pressure of CO_2, fractional saturation with oxygen, etc. Unfortunately no simple way is available at present for measuring the binding of 2,3-diphosphoglycerate to deoxyhemoglobin. Measurements of modifications of the oxygen affinity of hemoglobin in the presence of 2,3-diphosphoglycerate are a very indirect procedure, based on assumptions, in regard to the mechanism of the interaction, which could be proved only by direct-binding experiments. However, these experiments are not easily performed in anaerobiosis. A third possible procedure is the measurement of the absorption and release of protons produced by the electrostatic interaction of hemoglobin and 2,3-diphosphoglycerate.

According to models of Arnone (1972) and Perutz (1970) the interaction between hemoglobin and 2,3-diphosphoglycerate is essentially an electrostatic phenomenon in which five salt bridges are established between the negative charges of 2,3-diphosphoglycerate and positive groups present in the B chains of hemoglobin. In the pH range where these groups ionize, hemoglobin is bound to absorb protons when it resets with 2,3-diphosphoglycerate. A direct measurement of these protons would give most of the information necessary to characterize the phenomenon. The ionization of some of the

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[1] Abbreviations used are: Bis-tris, bis(2-hydroxyethyl)iminotris-(hydroxymethyl) methane; ABE, additional Bohr effect.

negative charges of 2,3-diphosphoglycerate overlap with that of the positive groups in hemoglobin (Benesch *et al.*, 1969; DeBruin and Janssen, 1973) so that a simultaneous release and absorption of protons is produced in the reaction. For this reason 2,3-diphosphoglycerate substitutes were sought whose negative charges would be completely ionized at low pH, in order to leave an ample region of pH in which only the ionizable groups of hemoglobin would be responsible for the absorption of protons which occurs upon addition of the effector.

In a previous paper (Shimizu and Bucci, 1973) it was shown that benzenepentacarboxylic acid had an effect very similar to that of 2,3-diphosphoglycerate on the functional characteristics of human adult and fetal hemoglobin. As shown in this paper all of the carboxyl groups of benzenepentacarboxylate appeared to be completely ionized near pH 7, therefore, a direct measurement of the number of protons absorbed at various pH values by the interaction of human adult deoxyhemoglobin and benzenepentacarboxylate could be performed between pH 7 and 9. The picture which emerges is that of an interaction regulated only by electrostatic phenomena. It also appeared that benzenepentacarboxylate was bound by both oxy- and deoxyhemoglobin, and that in deoxyhemoglobin the affinity constant was about 1000 times higher than in oxyhemoglobin.

Materials and Methods

Human hemoglobin was prepared from freshly drawn blood by lysing the washed red cells in presence of toluene according to Drabkin (1946). It was freed from organic and inorganic ions by recycling for 1 hr in the cold through a mixed-bed resin column in a closed system saturated with O_2. Hemoglobin concentration was measured spectrophotometrically on the basis of $\epsilon = 56{,}000\ cm^{-1}\ M^{-1}$ per tetramer at 540 nm for the oxy and carboxy derivatives.

Benzenepentacarboxylic acid was purchased from Aldrich Chemical Co. and used as such. All reagents were analytical grade or better.

All pH measurements and acid–base titrations were performed with a Radiometer M26 instrument equipped with a GK2301B combination electrode, and a scale expander, so that the full-scale pen deflection of 10-in. Sargent recorder corresponded to 0.2 pH unit.

Anaerobic titrations of deoxyhemoglobin with benzenepentacarboxylate were performed by adding with a microsyringe-deoxygenated solutions of the pentacarboxylate to deoxyhemoglobin. The solutions were back-titrated to the original pH using deoxygenated 0.2 N HCl or NaOH delivered from a Methrom E457 microburet. These experiments were performed in 0.05 M NaCl at 20° under a stream of nitrogen. The gas was humidified in water after passage through hot copper and soda lime in order to remove the last traces of oxygen and CO_2.

Deoxygenation of the reagents was obtained flushing or bubbling nitrogen in the various solutions before filling the microsyringes and microburets used to deliver the reagents into the titration vessel.

Deoxygenation of hemoglobin was obtained by flushing nitrogen through the solutions, and was checked spectrophotometrically measuring the ratio between the optical density at 555 and 540 nm, which in deoxyhemoglobin is 1.24 (Benesch *et al.*, 1965). The presence of the ferric form was also checked spectrophotometrically measuring the ratio between the optical density at 576 and 541 nm, which in the oxy

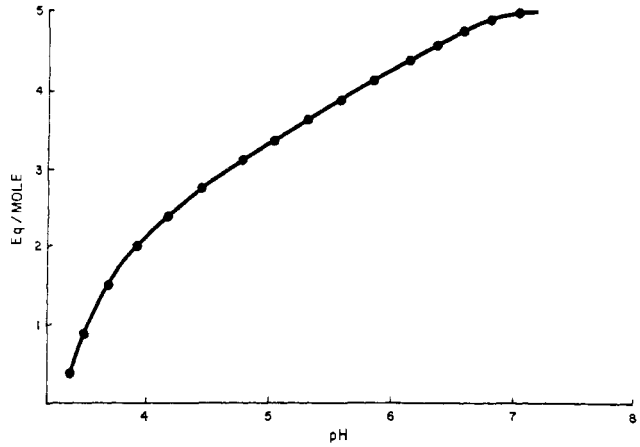


FIGURE 1: Acid–base titration of benzenepentacarboxylic acid (BPC) in 0.05 M NaCl at 20°; BPC concentration 3.2×10^{-4} M.

derivative was measured to be 1.062 ± 0.002. Measurements performed at the end of the experiments showed ratios not lower than 1.055, indicating that ferric hemoglobin was practically absent. In order to expedite the experiments a large stock solution of hemoglobin was deoxygenated, and aliquots were forced into the titration vessel by nitrogen pressure. At the end of the experiments the volumes of hemoglobin solutions were measured by transferring them into a 10-ml graduated cylinder or by careful aspiration into a 10-ml pipet. Remeasuring in this way known volumes of water an average loss of 0.1 ml was estimated. This quantity was added to the measured volumes.

The Bohr effect in the presence and absence of benzenepentacarboxylate was measured by flushing deoxyhemoglobin with oxygen until a constant pH was reached. The solution was then back-titrated to the original pH, under a stream of oxygen. The gas was humidified and purified from CO_2 as described for nitrogen.

Result

The Acid–Base Titration Curve of Benzenepentacarboxylate. This reagent showed a constant buffer capacity from pH 4.0 to 6.5 after which it decreased in order to end near pH 7, as shown in Figure 1. The interaction between benzenepentacarboxylate and deoxyhemoglobin was measured at pH 7.1 or above.

The Stoichiometry of the Reaction between Deoxyhemoglobin and Benzenepentacarboxylate. The stoichiometry was measured by following the pH changes produced by the titration of deoxyhemoglobin with benzenepentacarboxylate. The sharp break of the curves, as shown in Figure 2, was used to estimate the end point of the titrations. In Table I the values so obtained

TABLE I: Titrations of 10^{-4} M Deoxyhemoglobin with 10^{-2} M Benzenepentacarboxylate in 0.05 M NaCl at 20°.

Initial pH	Final pH	Ratio Hb:BPC[a] at Breakpoint
7.101	7.272	1.07
7.465	7.598	0.93
7.675	7.842	0.95

[a] BPC = benzenepentacarboxylate.

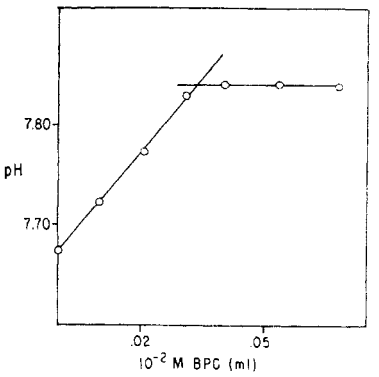


FIGURE 2: Titration of 10^{-4} M deoxyhemoglobin with 10^{-2} M benzenepentacarboxylate in 0.05 M NaCl at 20°.

at various pH's are reported. They indicated essentially a 1:1 reaction. The small deviations can be attributed to the approximation, implied in these experiments, of a constant buffer capacity of the solution through all the pH interval covered by the titration. These results were consistent with those obtained in a previous paper (Shimizu and Bucci, 1973) which showed that at pH 6.5 the oxygen affinity of hemoglobin did not decrease any further for ratios of benzenepentacarboxylate to hemoglobin higher than 1:1.

Determination of the Affinity Constant of Deoxyhemoglobin for Benzenepentacarboxylate at Various pH's. If in hemoglobin a certain number of groups shift their pK to a higher value in the presence of benzenepentacarboxylate, $\bar{h}$, the average number of protons per mole bound by those groups in the presence of the effector will be

$$\bar{h} = (\bar{m}[\mathrm{E}] + \bar{n}[\mathrm{C}])/([\mathrm{E}] + [\mathrm{C}]) \quad (1)$$

where $\bar{m}$ is the average number of protons per mole bound by those groups in free hemoglobin, [E] is the concentration of hemoglobin free of effector, $\bar{n}$ is the average number of protons per mole bound by those groups in the complex benzenepentacarboxylate–hemoglobin, and [C] is the concentration of the complex. The difference in protons bound per mole of protein in the presence and absence of the effector will be

$$\bar{h} - \bar{m} = (\bar{n} - \bar{m})\{[\mathrm{C}]/([\mathrm{E}] + [\mathrm{C}])\} \quad (2)$$

or

$$\Delta\bar{h} = YH_{\max} \quad (3)$$

where $Y = [\mathrm{C}]/([\mathrm{C}] + [\mathrm{E}])$ is the fractional saturation of hemoglobin with benzenepentacarboxylate and $H_{\max} = \bar{n} - \bar{m}$ is the maximum difference in protons bound by hemoglobin in the presence and absence of benzenepentacarboxylate at that pH. In practice at a chosen pH a deoxyhemoglobin solution was titrated by the addition of small amounts of effectors to produce a small pH change which was back-titrated in order to measure the correspondent $\Delta\bar{h}$ value. When further additions of effectors failed to produce pH changes, the value $H_{\max} = \Sigma\Delta\bar{h}$ gave the maximum number of protons per mole absorbed at that pH by the interaction of benzenepentacarboxylate with deoxyhemoglobin. Care was taken in order to keep the combined volumes of benzenepentacarboxylate and HCl (used for the back-titration) below 5% of the final volume. At each step Y was calculated from

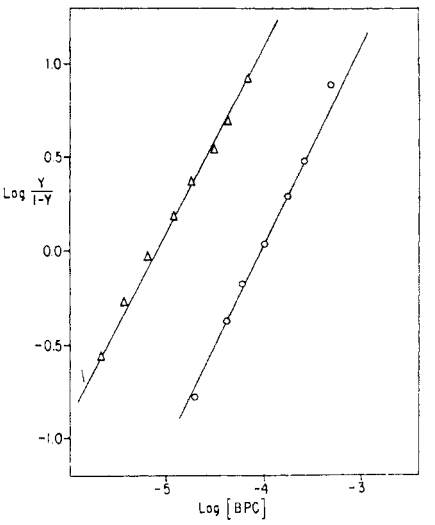


FIGURE 3: Hill plots relative to titrations of deoxyhemoglobin with benzenepentacarboxylate in 0.05 M NaCl at 20°: (△) at pH 8.01; (○) at pH 8.74; protein concentrations 0.96×10^{-4} M.

eq 3 which also gave the amount of benzenepentacarboxylate bound to the protein. By difference the amount of free benzenepentacarboxylate was calculated, and the equilibrium constant for the reaction computed by classical procedures. In the various calculations the volume of the reaction was assumed to be the average between initial and final volume, and the protein concentration was readjusted to that volume.

Figure 3 shows the double-logarithmic plot (Hill plot) for two typical measurements performed at pH 8.01 and 8.74. The slopes of these lines were 1 ± 0.05. This is consistent with the assumption that 1 mol of benzenepentacarboxylate was absorbed per mol of hemoglobin.

Measurements below pH 7.5 were difficult because of the high affinity of deoxyhemoglobin for benzenepentacarboxylate, so that solutions of 10^{-6} M deoxyhemoglobin still showed a stoichiometric absorption of benzenepentacarboxylate. Above pH 9 the low affinity produced uncertain end points for the titrations. Also the relatively large amount of benzenepentacarboxylate necessary for saturating the protein produced a substantial increase of the ionic strength of the solution, and might have produced binding of benzenepentacarboxylate to additional sites on the protein.

Difference in Protons Bound by Deoxyhemoglobin in Presence and Absence of Benzenepentacarboxylate. Figure 4 shows the pH dependency of $H_{\max}$. In these experiments deoxyhemoglobin was titrated with benzenepentacarboxylate until no further pH changes were obtained, and the solution was back-titrated to the original pH and checked again to ascertain that no pH change was produced by the addition of benzenepentacarboxylate. Alternatively $H_{\max}$ values were calculated from the equation $H_{\max} = \Sigma\Delta\bar{h}$ as above described. The correlation between $H_{\max}$ and the hydrogen ion concentration $[\mathrm{H}^+]$ is described by

$$H_{\max} = \sum_i \left(\frac{K_i}{K_i + [\mathrm{H}^+]} - \frac{K_i'}{K_i' + [\mathrm{H}^+]} \right) \quad (4)$$

where K_i' and K_i are the ionization constant of the ith group in the presence and the absence of benzenepentacarboxylate, respectively. The line drawn in Figure 4 was obtained by assuming three groups with pK = 7.25 in the absence of

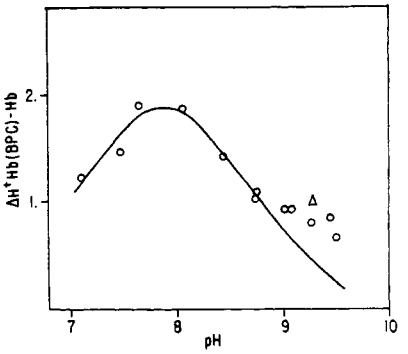


FIGURE 4: Difference in protons bound by deoxyhemoglobin in the presence and absence of benzenepentacarboxylate in 0.05 M NaCl at 20°. The interpolating line was calculated on the basis of three groups shifting their pK from 7.25 to 8.55 in the presence and absence of the effector, respectively.

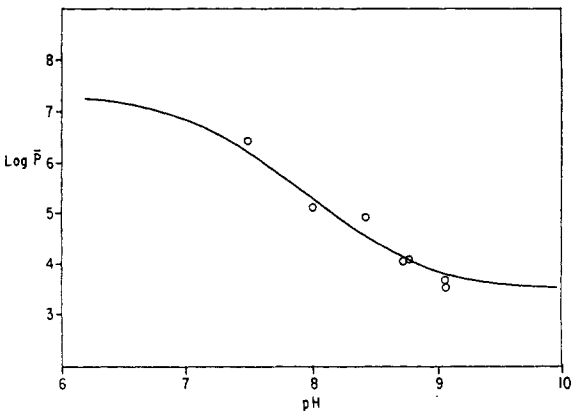


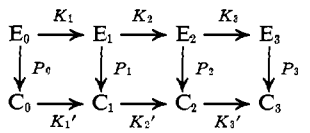
FIGURE 5: Variation with pH of the affinity constant of deoxyhemoglobin for benzenepentacarboxylate in 0.05 M NaCl at 20°. The interpolating line was calculated as described in the text.

benzenepentacarboxylate and pK = 8.55 in the presence of the effector. These parameters were found by trial and error and with the help of the Marquardt (1963) algorithm.

In this case either the three parameters were left floating or to some of them fixed values were assigned. In all instances the fitted parameters were very similar to those above reported.

Above pH 9 a discrepancy was present between the calculated line and the experimental points. As already discussed the measurements were more difficult in this pH region and this is probably the reason for the scattering of the data. The point, indicated in Figure 4 with a triangle, was obtained by measuring the number of protons liberated by oxygenation of the benzenepentacarboxylate–hemoglobin complex. Apparently all of the protons absorbed by the interaction of hemoglobin and the effector in that pH range were released upon addition of oxygen.

pH Dependence of the Affinity Constant of Hemoglobin for Benzenepentacarboxylate between pH 7 and 9. In this pH interval, the interaction of hemoglobin and benzenepentacarboxylate produced in the protein a pK shift of three ionizable groups with very similar pK. Assuming that the three groups were independent and equivalent in their interaction with benzenepentacarboxylate, the following model can be derived



(5)

in which E_i's (i = 0, 1, 2, 3) are the species of hemoglobin free of benzenepentacarboxylate and protonated in 0, 1, 2, 3 of these groups, respectively, C_i's (i = 0, 1, 2, 3) are the corresponding species for the benzenepentacarboxylate–deoxyhemoglobin complex, K_i's (i = 1, 2, 3) are the affinity constants of species E_{i-1} for the additional proton, k_i' values (i = 1, 2, 3) are the correspondent constants for species C_i, and P_i's (i = 0, 1, 2, 3) are the affinity constants of species E_i for benzenepentacarboxylate. Neglecting the small negative interaction due to the electrostatic interaction predicted by the Linderstrøm–Lang model all K_i's can be assumed to be identical. Assuming that there is no cooperative protonation of the same groups in C_i's, all K_i values will also be identical. Therefore K_i'/K_i = const = R. From eq 5 it can be seen that

$$P_i = P_0 R^i \tag{6}$$

The overall apparent affinity constant of hemoglobin for benzenepentacarboxylate is

$$\bar{P} = \sum_i \frac{[C_i]}{[B]\sum_i [E_i]} \tag{7}$$

where [B] represents the concentration of free effector. Defining the quantity $w_i = [E_i]/\Sigma_i[E_i]$ and substituting in eq 7

$$\bar{P} = \sum_i w_i \frac{[C_i]}{[B][E_i]} = w_i P_i \tag{8}$$

is obtained.

In free hemoglobin the three groups have identical pK values, therefore, the various w_0, w_1, w_2, and w_3 will be equal to α^3, $3\alpha^2\beta$, $3\alpha\beta^2$, and β^3, respectively, where α is the degree of ionization and $\beta = 1 - \alpha$. Consequently

$$\bar{P} = \alpha^3 P_0 + 3\alpha^2\beta P_0 R + 3\alpha\beta^2 P_0 R^2 + \beta^3 P_0 R^3 \tag{9}$$

In this equation all parameters have been experimentally measured except P_0. In fact R = 20, as it corresponds to a pK shift of 1.3, and α corresponds to a pK = 7.25. P_0 represents the affinity constant for benzenepentacarboxylate of deoxyhemoglobin deprotonated in all of the three interacting groups, which having a pK = 7.25, are only 2% protonated at pH 9. Therefore the affinity constants measured near that pH gave an indication of the P_0 value. Figure 5 shows the fitting to the experimental data of a line calculated with eq 9 when P_0 = 3000 M^{-1}.

The fit is good and it appears that the system was described only by the ionization of the three groups considered, and by their pK shift upon interaction with benzenepentacarboxylate. It is worth noting that in the model chosen no cooperative protonation was assumed for the groups involved in the E_i and C_i species.

Additional Bohr Effect. It is expected that when, in the proper pH range, benzenepentacarboxylate is dissociated from hemoglobin all of the protons absorbed upon the formation of the complex should be released. Therefore, if oxygenation is lowering the affinity of hemoglobin for benzenepentacarboxylate in such a way that all or part of it is dissociated from hemoglobin, protons will be released in addition to those liberated by the alkaline Bohr effect of

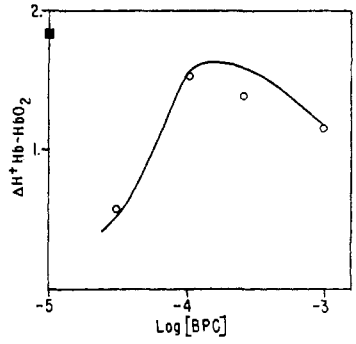


FIGURE 6: Additional Bohr effect of hemoglobin in the presence of different concentrations of benzenepentacarboxylate at pH 7.7 in 0.05 M NaCl at 20°. The line was calculated as described in the text taking $\bar{P} = 600{,}000\ M^{-1}$ and $\bar{P}' = 600\ M^{-1}$; protein concentration 0.94×10^{-4} M; (■) H_{max}.

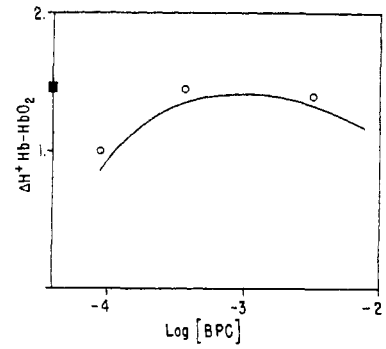


FIGURE 8: Additional Bohr effect of deoxyhemoglobin in presence of different amounts of benzenepentacarboxylate at pH 8.40 in 0.05 M NaCl at 20°. The continuous line was calculated taking $\bar{P} = 30{,}000\ M^{-1}$ and $\bar{P}' = 30\ M^{-1}$; protein concentration 0.98×10^{-4} M; (■) H_{max}.

hemoglobin. This will constitute the additional Bohr effect (ABE) produced by the oxygenation of hemoglobin in presence of benzenepentacarboxylate.

The number of ABE protons observed at various pH's was measured by back-titrating the pH changes obtained upon oxygenation of deoxyhemoglobin in the presence of various amounts of benzenepentacarboxylate, and subtracting the number of protons liberated by oxygenation of deoxyhemoglobin alone.

As can be seen from Figures 6, 7, and 8 the ABE first increased then decreased at concentrations of benzenepentacarboxylate higher than 10^{-4} M. Also, the maximum number of released ABE protons was less than H_{max} at the same pH. The various curves drawn in Figures 6, 7, and 8 were calculated in the following way. At constant pH the released ABE protons are

$$\bar{h} - \bar{h}' = [\bar{m}([E] - [E']) + \bar{n}([C] - [C'])]/[M] = [(\bar{n} - \bar{m})/[M]]([C] - [C']) \quad (10)$$

where $\bar{h}$ and $\bar{h}'$ are the averge number of protons per mole bound in deoxy- and oxyhemoglobin by those groups which participate to the interaction, [C] and [C'] are the concentrations of the deoxygenated and oxygenated complexes of hemoglobin with benzenepentacarboxylate, $\bar{m}$ and $\bar{n}$ retain their significance as in eq 1 and [M] is the total concentration of hemoglobin.

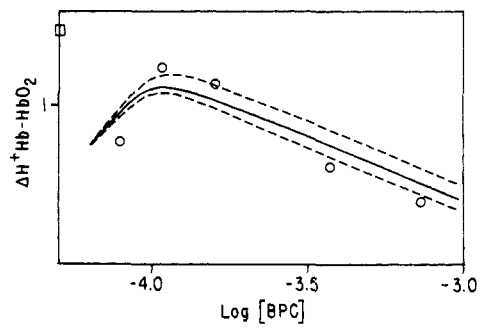


FIGURE 7: Additional Bohr effect of deoxyhemoglobin in presence of different amounts of benzenepentacarboxylate at pH 7.31 in 0.05 M NaCl at 20°; (□) H_{max}. All the three curves were calculated assuming $\bar{P} = 3{,}600{,}000\ M^{-1}$. The following values were used for $\bar{P}'$: upper dashed line 2000 M^{-1}, middle continuous line 2800 M^{-1}, lower dashed line 3600 M^{-1}; protein concentration 0.95×10^{-4} M.

The quantity [C] can be calculated by solving the second-degree equation

$$\bar{P} = [C]/([M] - [C])([T] - [C]) \quad (11)$$

where $\bar{P}$ is the apparent affinity constant of deoxyhemoglobin for benzenepentacarboxylate and [T] is the total concentration of effector. C' is computed in a similar way substituting $\bar{P}'$, the affinity constant for oxyhemoglobin, for $\bar{P}$. If the values of $\bar{P}$ and $\bar{P}'$ were the only difference in the binding of benzenepentacarboxylate to oxy- and deoxyhemoglobin, $\bar{P}'$ was the only unknown parameter in these equations. The numerical value for $\bar{P}$ was taken from the simulation in Figure 5, and $\bar{P}' = \bar{P}/1000$ was assumed in all cases. The obtained fit was good specially in consideration of the many numerical manipulations necessary for calculating the curves. Three lines have been computed in Figure 7 to show the sensitivity of the simulations to the parameter $\bar{P}'$.

It is worth noting that the number of ABE protons released upon oxygenation by the complex benzenepentacarboxylate–hemoglobin corresponded very well with the number of ABE protons calculated from the pH dependence of the oxygen affinity of the complex, as it is shown in Table II.

TABLE II: The Number of ABE Protons Measured Potentiometrically upon Oxygenation of the Benzenepentacarboxylate–Hemoglobin Complex, is Compared to That Calculated from the pH Dependence of the Oxygen Affinity of the Same Complex.[a]

pH	Measured ABE Protons/Mol of Hb	Interval of pH	Calcd ABE Protons/Mol of Hb
7.3	1.13	7.2–7.4	1.10
7.7	1.56	7.6–7.8	1.60

[a] The potentiometric measurements refer to the experiments shown in Figures 6 and 7 when the concentration of effector was near 10^{-4} M. The oxygen affinity data are from Shimizu and Bucci (1974). The oxygen absorption was measured at various pH in a 0.05 M Cl^-, 0.05 M Bis-tris buffer, and 10^{-4} M benzenepentacarboxylate. Hemoglobin concentration was 0.25×10^{-4} M. The equation was used $\Delta[H^+] = (\Delta \log P^{1}/_{2}/\Delta pH) \times 4$.

Discussion

Stoichiometry of the Reaction. It cannot be excluded that other sites were present in deoxyhemoglobin capable of binding benzenepentacarboxylate either without producing absorption of protons, or with such a low affinity that they were not detectable at the used concentrations of benzenepentacarboxylate. On the other hand, all of the data presented showed a very good internal consistency, and were all based on the assumption that the stoichiometry was 1:1. In particular, if this were not true notable distortions of the Hill plots shown in Figure 3 would have occurred. The conclusion is that if other sites were present they were binding negligible amounts of benzenepentacarboxylate under the conditions used.

Difference in Protons Bound by Deoxyhemoglobin in the Presence and Absence of Benzenepentacarboxylate. Between pH 7 and 9, this difference was explained by the pK shift of three residues in hemoglobin from 7.25 in the abence to 8.55 in the presence of benzenepentacarboxylate. Probably some averaging is present in these figures; however, a significant difference of the pK of these groups does not seem probable. The groups may be histidines or α-amino groups. The strong competition observed between CO_2 and benzenepentacarboxylate (Shimizu and Bucci, 1973) suggests, in analogy to what was shown for 2,3-diphosphoglycerate (Pace *et al.*, 1970; Brenna *et al.*, 1972; Tomita and Riggs, 1971), that the two 1β-valines are among them. The third group would be a histidine. In this way a pK = 7.25 is assigned to the NH_2-terminal groups of the β chains in deoxyhemoglobin.

Above pH 9 additional protons were absorbed by the interaction of benzenepentacarboxylate and deoxyhemoglobin, in addition to those absorbed by the pK shift of the three groups considered. Their absorption was due to the allosteric interaction of benzenepentacarboxylate and deoxyhemoglobin as proven by their release upon oxygenation of the benzenepentacarboxylate–deoxyhemoglobin complex. Therefore they probably represented the pK shift of other groups with a more alkaline pK.

From the pK shift of the three groups above mentioned free energy of formation of the salt bridges between hemoglobin and benzenepentacarboxylate can be evaluated to be 1.7 kcal/bond, and near pH 9 the affinity constant of deoxyhemoglobin for benzenepentacarboxylate was approximately 3000 M^{-1}, which corresponds to a free energy near 4.7 kcal/mol.

This might suggest that two additional salt bridges are formed between benzenepentacarboxylate and deoxyhemoglobin besides those investigated below pH 9.0. Of the additional two groups which would interact with benzenepentacarboxylate in hemoglobin, one would be a lysine as its pK shift began to show near pH 9.0, the other would be either a second lysine (symmetrically placed on the partner β chain) or an arginine.

The pH dependence of the affinity constant of benzenepentacarboxylate for deoxyhemoglobin was also consistent with the pK shift above discussed.

Cooperative protonation of these groups was not evident either in the curve-fitting procedure for evaluating the number and pK shift of the groups involved in the binding, or in the pH dependence of the affinity constant of benzenepentacarboxylate for deoxyhemoglobin. The assumption used by Riggs (1971) and DeBruin and Janssen (1973), that only the hemoglobin species protonated in the reactive groups are capable of binding the effector 2,3-diphosphoglycerate, is ruled out in this case.

Binding of Benzenepentacarboxylate to Oxyhemoglobin. Our experiments gave only semiquantitative data in this respect, and attempts to measure the binding directly by following the pH changes produced by additions of benzenepentacarboxylate to oxyhemoglobin was not possible. In fact, the low-affinity constants, which were regulating the process, produced uncertain end points for the titration of oxyhemoglobin with the effector. Nevertheless both those titrations and the behavior of the ABE indicated that, below pH 8, the reaction between benzenepentacarboxylate and oxyhemoglobin was absorbing a measurable number of protons at relatively low concentrations of benzenepentacarboxylate. For this reason its presence in deoxyhemoglobin should have been detected in all of the experiments performed below that pH. However, only one binding site was detectable in deoxyhemoglobin implying either that (i) the site present in oxyhemoglobin was sensitive to the conformation of the protein, so to disappear in deoxyhemoglobin, or that (ii) the same site bound benzenepentacarboxylate in both derivatives of hemoglobin. At present the simplest explanation of the phenomenon is the last. Additional binding of benzenepentacarboxylate might occur in both derivatives at high concentrations of the reagent, as seen by Hedlund *et al.* (1973), Garby *et al.* (1969), and Luque *et al.* (1969) for 2,3-diphosphoglycerate and deoxy and oxyhemoglobin.

Correlations between the Interaction of Benzenepentacarboxylate and 2,3-Diphosphoglycerate with Human Hemoglobin. According to the model of Arnone (1972) and Perutz (1970) five groups in hemoglobin interact with 2,3-diphosphoglycerate: the two 1β-valines, which according to Arnone (1972) can be substituted by the 2β-histidines, the two 143β-histidines, and one of the 82β-lysines. These would make four groups with a pK near the neutrality and one group with an alkaline pK. In the case of benzenepentacarboxylate three of the interacting groups had a pK near the neutrality and the remaining two were alkaline. Probably the two 1β-valines were involved, and the identity of the other groups is not certain at this point. In any case, even if we identify the two alkaline groups with the two 82β-lysines, the interacting groups are not identidal for 2,3-diphosphoglycerate and benzenepentacarboxylate.

Almost identical was the behavior of the ABE resulting from the interaction of hemoglobin with benzenepentacarboxylate and 2,3-diphosphoglycerate. It is known that when the affinity of hemoglobin for oxygen is measured as a function of pH, the slope of the curve is given by

$$-(\mathrm{d} \log P^{1}/_{2})/\mathrm{dpH} = \Delta[H^+] \qquad (12)$$

where $\Delta[H^+]$ is the amount of protons liberated by hemoglobin upon oxygenation (Wyman, 1964). When additional protons are liberated, because of the interaction of hemoglobin with these effectors, the slope of the curve increases. This increase was observed for 2,3-diphosphoglycerate and benzenepentacarboxylate (Benesch *et al.*, 1971; Shimizu and Bucci, 1973; Riggs and Imamura, 1972). Also, it appeared that at concentrations higher than 10^{-4} M the slope of the Bohr effect curve decreased again so that the ABE was nearly zero for benzenepentacarboxylate or 2,3-diphosphoglycerate concentration above 10^{-8} M (Shimizu and Bucci, 1973; Benesch *et al.*, 1969). The explanation proposed by Riggs (1971) and DeBruin and Janssen (1973) was that this decrease is due to a binding of 2,3-diphosphoglycerate to oxyhemoglobin was confirmed for benzenepentacarboxylate, providing additional evidences that both 2,3-diphosphoglyc-

erate and benzenepentacarboxylate bind to oxyhemoglobin. From these data and from the results here presented, it might be expected that at pH near 7 and at concentrations of the effectors above 10^{-3} M, both oxy- and deoxyhemoglobin will be nearly saturated with benzenepentacarboxylate or 2,3-diphosphoglycerate.

Validity of Eq 12. It might be asked if eq 12 is still valid in systems where hemoglobin is in the presence of effectors like benzenepentacarboxylate or 2,3-diphosphoglycerate. In fact, besides the protons, anions are present in these solutions whose activity varies with the saturation of hemoglobin with oxygen (Wyman, 1964). It is to be noted that in deriving his equations Wyman (1964) takes in consideration the *total* anionic activity of the solution. When the stoichiometry of the interaction between hemoglobin and the effector is 1:1, the maximum possible variation of the total anionic activity of the solution, upon oxygenation, is represented by the molarity of hemoglobin, corrected for the number of charges per molecule of effector. Consequently if the molarity of hemoglobin is much lower than that of the electrolytes present in solution, this variation becomes negligible. The results shown in Table II are consistent with this prediction.

Glossary of Symbols Used in Eq 1–11

[B], free benzenepentacarboxylate concentration

C and C′, complex of hemoglobin and benzenepentacarboxylate in the deoxygenated and oxygenated from, respectively

E and E′, hemoglobin free from effectors in the deoxygenated and oxygenated form, respectively

$\bar{h}$, average number of protons bond per mole of protein by deoxyhemoglobin in a mixture of E and C species

$\bar{h}'$, same as above for oxyhemoglobin in a mixture of E′ and C′ species

H_{max}, maximum number of protons absorbed by deoxyhemoglobin upon interaction with benzenepentacarboxylate

[H^+], hydrogen ions activity

K_i and K_i', affinity constant for protons of the groups which interact with benzenepentacarboxylate in E_i and C_i, respectively

[M], total hemoglobin concentration

$\bar{m}$ and $\bar{n}$, average number of protons bound per mole of hemoglobin by the groups which interact with benzenepentacarboxylate in E (or E′) and C (or C′), respectively

P_i, affinity constant of species E_i for benzenepentacarboxylate

$\bar{P}$ and $\bar{P}'$, overall affinity constant constant of hemoglobin for benzenepentacarboxylate in deoxygenated or oxygenated conditions, respectively

[T], total benzenepentacarboxylate concentration

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