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Allosteric Effectors of Hemoglobin. Interaction of Human Adult and Fetal Hemoglobins with Poly(carboxylic acids)†

Katsuiko Shimizu and Enrico Bucci*

ABSTRACT: Aliphatic and aromatic poly(carboxylic acids) interacted with hemoglobin lowering its oxygen affinity. The magnitude of the effect appeared unrelated to the structure of the polyacids and proportional to the number of carboxyl groups present in the compounds. The largest effect was produced by benzenepentacarboxylic acid which at 20° in 0.05 M bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane buffer at pH 7.3 increased log $P_{1/2}$ of human adult hemoglobin from about 0.3 to *ca.* 1.10 and that of human fetal hemoglobin from *ca.* 0.5 to *ca.* 0.95. Its affinity for fetal hemoglobin appeared less than that for adult hemoglobin. It increased the Bohr effect of both hemoglobins, indicating the release, in its presence, of extra protons in addition to those of the alkaline Bohr effect. The number of extra protons liberated in this way upon oxygenation was similar for adult and fetal hemoglobin. The value of the Hill parameter n for fetal hemoglobin appeared to be pH dependent both in the presence and absence of benzenepentacarboxylate, being near 2 below and near 3 above pH 7 with a sharp transition around this pH. The combined effect of benzenepentacarboxylate and CO_2 on adult hemoglobin was much less than the sum of the separate effects, suggesting a competition between the two reagents. The great similarity of the effects of benzenepentacarboxylate and 2,3-diphosphoglycerate on human adult and fetal hemoglobin would suggest that the two compounds bind to hemoglobin in a similar way.

The models proposed by Perutz (1970) and Arnone (1972) for the interaction of 2,3-diphosphoglycerate with human hemoglobin show that the 1β-valines, the 143β-histidines, and one of the 82β-lysines form salt bridges with the five negative charges of 2,3-diphosphoglycerate. The 82β-lysine would interact with the carboxyl group of 2,3-diphosphoglycerate and, according to Arnone (1972), the 2β-histidines can also participate in the binding, in this case substituting the 1β-valines. These groups are present in a crevice, between the two β chains, which only in deoxyhemoglobin is large enough to accommodate 2,3-diphosphoglycerate, thus explaining the preferential binding of 2,3-diphosphoglycerate to this form of hemoglobin (Perutz, 1970).

The electrostatic interaction is bound to modify the proton binding behavior of the groups involved, increasing the pK of the positive charges and decreasing that of the negative groups, so that within certain pH ranges hemoglobin is expected to absorb protons upon interaction with 2,3-diphosphoglycerate. This phenomenon contains much information. As it will be discussed in another manuscript, it might help to measure the affinity constant of the effector for hemoglobin, the number of groups which in hemoglobin participate in the interaction, their pK, and their shift to higher pK values in the presence of 2,3-diphosphoglycerate. This absorption of protons also affects the oxygen equilibrium; in fact the preferential binding of the effector to deoxyhemoglobin implies that they will be released all or in part upon oxygenation, in addition to those liberated by the alkaline Bohr effect groups. In this way the pH dependency of oxygen affinity in hemoglobin is increased. This phenomenon can be defined as the "additional Bohr effect" (ABE)[1] and is present in the complex 2,3-diphosphoglycerate–hemoglobin as demonstrated by Benesch *et al.* (1969), Bailey *et al.* (1970), Riggs and Imamura (1972), and DeBruin and Janssen (1973). A comparison between the number of ABE protons released and the number of protons absorbed by deoxyhemoglobin upon the interaction with 2,3-diphosphoglycerate would constitute a measure of the amount of effector displaced from hemoglobin upon oxygenation.

In the case of 2,3-diphosphoglycerate a direct measurement of these protons is made difficult by the high pK (Kumler and Eiler, 1943) of some of the negative charges of 2,3-diphosphoglycerate, which makes the ionization of these groups overlap with that of the positive groups of hemoglobin, so that simultaneous absorption and liberation of protons occur upon their interaction. Large corrections would have to be introduced in the experimental data in order to calculate the number of protons absorbed by hemoglobin. On the other hand it appears that the specificity of binding of effectors by hemoglobin is not limited to 2,3-diphosphoglycerate and that other chemicals can interact in a similar way (Chanutin and Curnish, 1967; Benesch and Benesch, 1967). Following this reasoning we began searching 2,3-diphosphoglycerate substitutes whose negative charges would be completely ionized below pH 7.

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

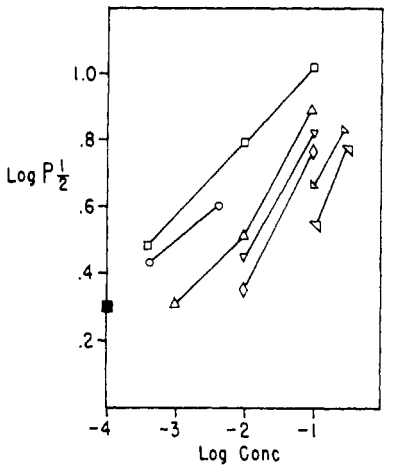

FIGURE 1: Effect on the oxygen affinity of human adult hemoglobin of various concentrations of: (□) citrate; (○) ethylenediaminotetraacetate; (△) oxalate; (▽) malonate; (◊) succinate; (◺) NaCl; (▽) formate; (■) hemoglobin control. Measurements performed at 20° in 0.05 M bis-tris buffer at pH 7.3; total Cl^- concentration, 0.05 M. The hemoglobin concentration was near 0.2×10^{-4} M in all cases.

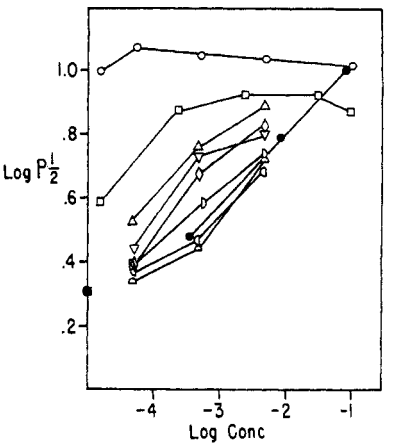

FIGURE 2: Effect on the oxygen affinity of human adult hemoglobin of various concentrations of: (○) benzenepentacarboxylate; (△) 1,2,3,4-benzenetetracarboxylate; (▽) 1,2,4,5-benzenetetracarboxylate; (◊) 1,3,4,5-benzenetetracarboxylate; (D) 1,2,3-benzenetricarboxylate; (◭) 1,2,4-benzenetricarboxylate; (◓) 1,3,5-benzenetricarboxylate; (●) citrate; (■) hemoglobin control; (□) effect of benzenepentacarboxylate on human fetal hemoglobin. Measurements were performed at 20° in 0.05 M bis-tris buffer at pH 7.3. The total Cl^- concentration was 0.05 M. The hemoglobin concentration was near 0.2×10^{-4} M in all cases.

This paper presents results relative to the interaction of hemoglobin with poly(carboxylic acids) and in particular with benzenepentacarboxylic acid which proved to be a very good 2,3-diphosphoglycerate substitute. The main purpose of the data presented is to stress the similarity between the action of 2,3-diphosphoglycerate and benzenepentacarboxylate on hemoglobin. It should open the way to the investigation of the electrostatic interaction of hemoglobin and its effectors, using model compounds which allow the direct measurement of the number of protons absorbed by hemoglobin upon the interaction with the effectors, and of the number of ABE protons released upon oxygenation of the complexes of hemoglobin and the effectors.

Materials and Methods

Human adult hemoglobin was prepared from washed red cells lysed in the presence of toluene according to Drabkin (1946). It was purified from organic and inorganic ions by recycling through a mixed bed resin column for 1 hr in the cold. Fetal hemoglobin was prepared from toluene hemolysates of umbilical cord blood by chromatography on carboxymethyl-Sephadex according to Zade-Oppen (1963). After preparation it was extensively dialyzed against distilled water and then recycled through a mixed bed resin column as described. On some occasions the recycling was omitted without any effect on the results obtained.

Oxygen equilibria were measured spectrophotometrically in a way similar to that described by Allen *et al.* (1950). All measurements were performed at 20° in 0.05 M bis-tris buffer between pH 6 and 8, and in 0.05 M Tris buffer above pH 8. To keep the chloride ion concentration constant at all pH values a large amount of Tris or bis-tris solution was titrated to a pH slightly below 6 with HCl and then readjusted to the desired pH with NaOH. In this way we assumed the Cl^- concentration to be near 0.05 M in all cases. The protein concentration was near 0.2×10^{-4} M in all experiments. When oxygen absorption was measured in the presence of CO_2, that amount of gas was introduced in the tonometers which would give a partial pressure of 60 mm. This lowered the pH of the solutions; therefore, in order to have a final pH near 7 a buffer at pH near 8 was used. For a final pH near 7.3 the initial pH of the buffer was near 8.5.

All reagents were analytical grade or better. The various poly(carboxylic acids) were purchased from Aldrich Chemical Co. or K&K Laboratories and used without further purification.

Results

Effect of Aliphatic Poly(carboxylic acids) on Human Adult Hemoglobin. Figure 1 shows the effect of varying concentrations of poly(carboxylic acids) on the oxygen affinity of hemoglobin. Citrate and ethylenediaminotetraacetate showed similar effects. The effect of α,ω-dicarboxylic acids is also shown. The length of the carbon chains of these acids was not relevant to their interaction with hemoglobin, and all of those tested, from oxalate to suberate, showed an effect similar to that of malonate and succinate. The effect of formate was similar to that of sodium chloride.

Effect of Aromatic Poly(carboxylic acids) on Human Adult Hemoglobin. Figure 2 shows the effect on the oxygen affinity of hemoglobin of various concentrations of benzene tri-, tetra-, and pentacarboxylic acids. Their effect was proportional to the number of negative charges and not to the position of the carboxyl groups on the benzene ring. The similarity of the effects of citrate and benzenetricarboxylates confirmed that the chemical structure had very little importance in these interactions. By far the strongest effect was produced by benzenepentacarboxylate which at a concentration of 10^{-4} M produced a value of log $P_{1/2}$ similar to that obtained with 2,3-diphosphoglycerate under analogous circumstances (Benesch *et al.*, 1969).

The overall shape and symmetry of the oxygen saturation curve of hemoglobin was not changed by benzenepentacarboxylate or any other poly(carboxylic acid) tested. Low concentrations of effectors in some case produced low values of the Hill parameter n. This was never below 2.5 except in the case, to be described below, of fetal hemoglobin.

Interaction of Benzenepentacarboxylate with Human Adult Hemoglobin. The pH dependence of the oxygen affinity of

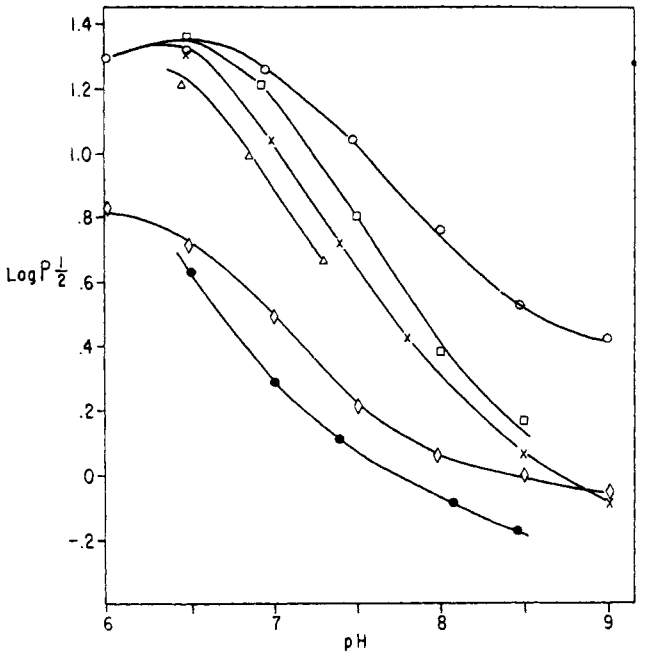

FIGURE 3: Dependency on pH of the oxygen affinity of human adult hemoglobin in the presence of various concentrations of benzene pentacarboxylate: (○) 5×10^{-3} M; (□) 10^{-4} M; (×) 2×10^{-5} M; (△) 10^{-5} M (◊) hemoglobin control; (●) hemoglobin at low chloride concentration. Measurements were performed at 20° in 0.05 M bis-tris buffer. The total Cl^- concentration was 0.05 M. The hemoglobin concentration was near 0.2×10^{-4} M in all cases.

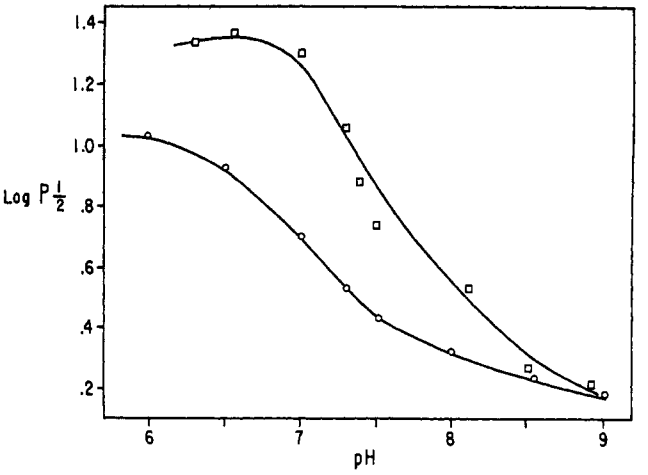

FIGURE 4: Dependency on pH of the oxygen affinity of human fetal hemoglobin in the presence of different amounts of benzenepentacarboxylate: (□) 5×10^{-3} M; (○) hemoglobin control. Measurements were performed at 20° in 0.05 M bis-tris buffer. The total Cl^- concentration was 0.05 M. The hemoglobin concentration was 0.2×10^{-4} M in all cases.

hemoglobin in the presence of various concentrations of benzenepentacarboxylate is shown in Figure 3. At pH 6.5 no additional increase in the value of log $P_{1/2}$ was obtained for molar ratios of benzenepentacarboxylate to hemoglobin higher than 1:1. In presence of 10^{-4} M benzenepentacarboxylate the steepness of the Bohr effect curve was greater than that of stripped hemoglobin, indicating that ABE protons were released upon oxygenation of the complex benzenepentacarboxylate–hemoglobin. When the concentration of benzenepentacarboxylate was increased to 5×10^{-3} M, the steepness of the Bohr effect decreased approaching again that of stripped hemoglobin. A similar effect produced by increasing concentrations of 2,3-diphosphoglycerate on the Bohr effect of human hemoglobin has been reported by Benesch *et al.* (1969). In all of our measurements the value of the parameter n in the Hill equation was between 2.7 and 3.1.

In Figure 3 two curves are shown for hemoglobin in the absence of effector. For the lower curve the various pH values were obtained titrating a 0.05 M bis-tris solution with HCl. In this way the chloride concentration was inversely proportional to pH and in any case less than 0.05 M. It appears that oxygen affinity was very sensitive to Cl^- concentration in hemoglobin. When the experiment was repeated in the presence of 5×10^{-3} M benzenepentacarboxylate no such effect was noticed and the curve obtained was indistinguishable from that shown.

Interaction of Benzenepentacarboxylate with Human Fetal Hemoglobin. The effect of increasing amounts of benzenepentacarboxylate on the oxygen affinity of fetal hemoglobin is shown in Figure 2. It had an effect very similar to that produced on adult hemoglobin, only larger concentrations were necessary for reaching the minimum oxygen affinity. The increase in the value of log $P_{1/2}$ was less for fetal than for adult hemoglobin. Both of these phenomena are similar to those reported for 2,3-diphosphoglycerate on adult and fetal human hemoglobin (Tyuma and Shimizu, 1969) and indicate that the affinity of benzenepentacarboxylate for fetal hemoglobin was less than that for adult hemoglobin.

At a concentration of 5×10^{-3} M, benzenepentacarboxylate still produced a substantial increase of the alkaline Bohr effect in fetal hemoglobin. It is interesting to note that the Bohr effect curves for adult hemoglobin in the presence of 10^{-4} M benzenepentacarboxylate and fetal hemoglobin in the presence of 5×10^{-3} M benzenepentacarboxylate were very similar, suggesting that the same number of protons were involved in the two processes. In Table I the number of protons are shown liberated by adult and fetal hemoglobin upon oxygenation in the presence and absence of the effector. They are calculated from the Bohr effect in the region of pH between 7.0 and 7.8.

TABLE I: Additional Bohr Effect for Human Adult and Fetal Hemoglobin in the Presence of Benzenepentacarboxylate.[a]

Interval of pH	Protein	[Benzenepentacarboxylate] (M)	Total Bohr Effect (Protons per Tetramer)	Additional Bohr Effect (Protons per Tetramer)
7.0–7.3	Adult Hb		2.13	
		10^{-4}	3.20	1.07
	Fetal Hb		2.00	
		5×10^{-3}	3.20	1.20
7.5–7.8	Adult Hb		1.36	
		10^{-4}	3.12	1.76
	Fetal Hb		1.12	
		5×10^{-3}	2.72	1.60

[a] The number of protons per mole of hemoglobin released upon oxygenation was calculated from the relationship $\Delta H^+ = -(\Delta \log P_{1/2}/\Delta pH) \times 4$. Values of log $P_{1/2}$ at these pH values were either obtained experimentally or interpolated from the curves shown in Figures 3 and 4.

TABLE II: Effect of pH on the Hill Parameter n for the Oxygen Absorption by Fetal Hemoglobin.

[Benzenepentacarboxylate] (M)	pH	n
	5.92	2.1
	6.42	2.4
	7.0	3.0
	7.3	3.3
	7.4	2.75
	8.0	2.85
	8.54	2.80
	9.0	3.0
5×10^{-3}	6.3	2.5
5×10^{-3}	6.55	2.0
5×10^{-3}	7.0	2.2
5×10^{-3}	7.3	2.8
2×10^{-5}	7.3	3.0
2.5×10^{-4}	7.3	2.8
5×10^{-3}	8.1	3.1
5×10^{-3}	8.5	3.1
5×10^{-3}	8.9	3.16

The number of ABE protons released upon oxygenation appeared to be very near the same for adult and fetal hemoglobin.

Table II gives the values of the Hill parameter n obtained for fetal hemoglobin at various pH values in the presence and absence of benzenepentacarboxylate. They appeared to be near 2 below and near 3 above pH 7, with a sharp transition near this pH.

Competition between CO_2 and Benzenepentacarboxylate. Table III shows the results obtained when oxygen absorption by hemoglobin was measured in the presence of carbon dioxide with and without benzenepentacarboxylate in solution. It appeared that the combined action of benzenepentacarboxylate and CO_2 was far from being the sum of the separate effects, in a way similar to that reported for 2,3-diphosphoglycerate and CO_2 (Bauer, 1969; Pace *et al.*, 1970; Tomita and Riggs, 1971; Brenna *et al.*, 1972). This suggests a competition between CO_2 and benzenepentacarboxylate similar to that shown between CO_2 and 2,3-diphosphoglycerate.

Discussion

None of the chemical characteristics of the compounds used as effectors can justify the hypothesis of a direct binding to the ferrous heme, and it seems logical at present to assume that only a limited number of receptor sites are available on the surface of hemoglobin for the kind of interactions here examined. That only one site might be present would be inconsistent with the observation that the efficiency of the effectors, used in lowering the oxygen affinity of hemoglobin, was related only to the number of negative charges available on the reagents, and very little, if at all, to their chemical structure.

Although the mechanism by which these effectors decrease the oxygen affinity of hemoglobin is not completely elucidated, the preferential binding of the effectors to deoxyhemoglobin seems to play an important role (Ogata and McConnell, 1972; Imai, 1973). So, within the same type of isomer, if the higher affinity of one of them for hemoglobin does not produce a stronger preferential binding to deoxyhemoglobin, it would not necessarily produce a larger decrease of the oxygen affinity. The sensitivity of the oxygen affinity to the number of negative charges present in the effectors can be explained, considering that a higher number of interacting groups would sensitize the binding of the effector to conformational changes produced in hemoglobin by oxygen binding, so increasing the preferential binding to deoxyhemoglobin.

Ionic strength affects the oxygen affinity of hemoglobin mainly through modifications of the alkaline Bohr effect (Antonini *et al.*, 1962). In our experiments the ionic strength of the solvents varied with pH and with the addition of polyvalent acids used as effectors. At neutral and alkaline pH benzenepentacarboxylate gives an ionic strength equal to 15 times its concentration; therefore at a concentration of 10^{-3} M it produced an ionic strength of 0.015. The oxygen affinity of free hemoglobin was measured at pH 7.4 in 0.05 M Tris or bis-tris buffers. At that pH the different pK values of the two buffers produced a difference in ionic strength of nearly 0.02 in favor of the bis-tris solution. The oxygen absorption by hemoglobin was very similar in the two circumstances.

Bull *et al.* (1973) have demonstrated that anions like chlorides bind to hemoglobin with a much higher affinity than cations. Also, Chiancone *et al.* (1972) have shown results which suggest a competition between chlorides and organic phosphates for the binding to hemoglobin. Our results are consistent with these reports, as they show that the effect of chlorides on the oxygen affinity of hemoglobin disappeared in the presence of benzenepentacarboxylate.

As discussed in the introductory statement, any effector which would preferentially bind to deoxyhemoglobin, through an interaction of its negative charges with alkaline positive groups on the protein molecule, would produce the appearance of an additional Bohr effect upon oxygenation of the complex. This was the case for 2,3-diphosphoglycerate (Benesch *et al.*, 1969; Riggs and Imamura, 1972; DeBruin and Janssen, 1973; Bailey *et al.*, 1970) and for benzenepentacarboxylate as shown here. It is interesting to note that in both cases the additional Bohr effect was dependent on the concentration of the effector. Upon increasing the concentration of 2,3-diphosphoglycerate or benzenepentacarboxylate the additional Bohr effect was first increased and then decreased, so at high concentrations of the effector the total Bohr effect was similar to that of "stripped" hemoglobin (Benesch *et al.*, 1969). The simplest explanation of this phenomenon is the one proposed by Riggs (1971), that the protons absorbed by the interaction of hemoglobin with the effector are not totally released, because not all of the effector is dissociated from hemoglobin. The binding of 2,3-diphosphoglycerate to oxyhemoglobin is a demonstrated phenomenon (Luque *et al.*, 1969; Garby *et al.*, 1969; Hedlund *et al.*, 1972) and anticipates also that benzene-

TABLE III: Effect of CO_2 on the Interaction between Human Adult Hemoglobin and Benzenepentacarboxylate.[a]

[Benzenepentacarboxylate] (M)	CO_2 (mm)	pH	Log $P_{1/2}$
		7.01	0.49
	60	7.03	0.86
2×10^{-5}		6.99	1.04
2×10^{-5}	60	7.02	1.18
10×10^{-4}		7.00	1.16
10×10^{-4}	60	7.10	1.32

[a] Hemoglobin concentration near 0.2×10^{-4} M in all cases.

pentacarboxylate binds to oxyhemoglobin, although with a much lower affinity than for deoxyhemoglobin.

In analogy to the action of 2,3-diphosphoglycerate, the effect of benzenepentacarboxylate on the oxygen affinity of fetal hemoglobin was much less pronounced than in the case of adult hemoglobin. As proposed by Tyuma and Shimizu (1969) and Bunn *et al.* (1970) the phenomenon can be explained assuming that fetal hemoglobin has a lower affinity for 2,3-diphosphoglycerate (and benzenepentacarboxylate) than adult hemoglobin. The affinity would be lower because in fetal hemoglobin the 143β-histidine is substituted in the γ chains by a serine. Since that histidine contributes one of the positive groups which in hemoglobin interacts with 2,3-diphosphoglycerate (Perutz, 1970; Arnone, 1972), its absence would decrease the affinity for the effector of fetal hemoglobin. However, the situation, at least for benzenepentacarboxylate, might be more complicated. In fact a very similar number of ABE protons was calculated for fetal and adult hemoglobin in the presence of benzenepentacarboxylate. If the 143β-histidine interacts with benzenepentacarboxylate in adult hemoglobin, its replacement with a serine in fetal hemoglobin should produce a substantial decrease in the number of ABE protons. However, it is possible that this histidine has a very high or very low pK so that its participation in the ABE could not be detected in the pH range investigated. Also, a higher affinity of benzenepentacarboxylate for the oxy form of adult hemoglobin, rather than for fetal oxyhemoglobin, might produce a more incomplete dissociation of benzenepentacarboxylate from adult hemoglobin upon oxygenation, thus lowering the number of ABE protons liberated in this case. Another factor which might play a role is that fetal hemoglobin, as obtained from the hemolysate of cordonal blood by the procedure of Zade-Oppen (1963), contains 15–20% of hemoglobin F_1 (Allen *et al.*, 1958), which, as demonstrated by Schroeder *et al.* (1962), has the α-amino groups of the β chains acetylated, and, as demonstrated by Bunn *et al.* (1970), hardly binds 2,3-diphosphoglycerate. Extrapolating this behavior to benzenepentacarboxylate, it might explain at least in part the lower affinity of hemoglobin "F" for this reagent. Also it is possible that the 143β-histidine does not participate in the binding of benzenepentacarboxylate. Finally it might be asked if the use of the differential Wyman equation is valid in this case, for the effector is a third component, whose activity in solution is strongly dependent on oxygenation, especially at the low concentrations used in these kinds of experiments (Wyman, 1964). Riggs (1971) and Riggs and Imamura (1972) have largely used the Wyman equation for estimating the number of protons involved in these processes. A direct measurement of the ABE protons appears indispensable at this point. The use of benzenepentacarboxylate will facilitate this kind of experiment. In fact, this reagent is completely ionized at a pH near 7, so that *all* of the protons absorbed or liberated upon the interaction with hemoglobin can be referred to pK shifts of ionizable groups of the protein. Preliminary experiments show that there is a good correspondence between the number of ABE protons estimated with the Wyman equation and measured directly at the potentiometer.

A phenomenon not previously reported is the pH dependence of the Hill parameter n of fetal hemoglobin. It varied from a value near 2 below pH 7 to a value near 3 above this pH, in a way consistent with results obtained on a crude hemolysate of cord blood (Antonini *et al.*, 1964). At present we do not have any firm explanation for that. The heterogeneity of fetal hemoglobin, as discussed above, might contribute to the phenomenon. In fact if the two components have different functional properties in regard to oxygen affinity and Bohr effect, the result might be that in certain pH ranges, in this case below 7, the oxygen affinities of the two hemoglobins will be substantially different. This can lower the apparent value of the parameter n. At present there is little in the literature regarding the functional properties of hemoglobin F and F_1. Most of the available information was obtained on mixtures of the two proteins (Antonini *et al.*, 1964; Nechtman and Huisman, 1964). Probably any further investigation of the functional properties of hemoglobin F and of its interaction with benzenepentacarboxylate and 2,3-diphosphoglycerate should be conducted on the purified components F and F_1.

The competition between 2,3-diphosphoglycerate and CO_2 (Bauer, 1969; Brenna *et al.*, 1972; Pace *et al.*, 1970; Tomita and Riggs, 1971) is generally taken as a strong support for the hypothesis that the 1β-valines participate in the binding of 2,3-diphosphoglycerate. The similar competition between benzenepentacarboxylate and CO_2 would suggest that these groups interact also with benzenepentacarboxylate.

On the basis of this observation and of all the similarities shown in the behavior of the interaction of benzenepentacarboxylate and 2,3-diphosphoglycerate with human adult and fetal hemoglobin, we propose that benzenepentacarboxylate and 2,3-diphosphoglycerate bind to the same receptor site in hemoglobin.

It appears that in hemoglobin there is a positively charged crevice, as indicated by Perutz (1970) and Arnone (1972), ready to interact with any chemical which contains a sufficient number of negative groups, and whose overall dimensions are compatible with those of the crevice.

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Electrostatic Interaction of Hemoglobin and Benzenepentacarboxylate

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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.