Reciprocal Interaction of Hemoglobin with Oxygen and Protons. The Influence of Allosteric Polyanions†

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ABSTRACT: The interaction of three inositol esters, inositol hexaphosphate (IHP), inositol pentaphosphate (IPP), and inositol hexasulfate (IHS), with hemoglobin has been investigated. The proton uptake method was used to obtain the six binding constants for deoxy- and oxyhemoglobin. These data combined with oxygen binding curves over a range of cofactor concentrations were used to test theoretical and empirical equations relating the affinity of hemoglobin for oxygen and allosteric effectors. The Bohr and Haldane coefficients in the presence of the inositol esters are unequal at low, but not at high, concentration of the cofactors. The maximum value reached by both parameters increases with the number of negative charges on the polyanion. 2,3-Diphosphoglycerate (DPG) differs sharply from the inositol esters since even at high concentrations of this cofactor, the Haldane coefficient remains elevated. This is a reflection of the negligible affinity of DPG for fully oxygenated hemoglobin.

The reversible oxygenation of the hemes of hemoglobin is accompanied by a conformational change in the globin (Muirhead and Perutz, 1963) which results in an increase in the acid strength of certain residues (Kilmartin and Rossi-Bernardi, 1969; Kilmartin and Wootton, 1970). Therefore, protons are liberated when oxygen is bound and absorbed when oxygen is released. This is known as the Haldane effect (Christiansen et al., 1914). The reciprocal phenomenon, i.e. the inverse relation between oxygen affinity and hydrogen ion concentration, is called the Bohr effect (Bohr et al., 1904). The linkage between the two effects was first derived by Wyman (1948), i.e.:

$$-\left(\frac{\partial \ln pO_2}{\partial \ln a_{H^+}}\right)_{\overline{V}_{O_2}} = \left(\frac{\partial \overline{V}_{H^+}}{\partial \overline{V}_{O_2}}\right)_{a_{H^+}} \quad (1)$$

where a_{H^+} is the proton activity and $\overline{V}_{H^+}$ and $\overline{V}_{O_2}$ are the number of molecules of H^+ and oxygen, respectively, bound per heme. Tyuma and Ueda (1975) have recently tested eq 1 experimentally and have shown that the commonly used expression, i.e. $\Delta \log p_{50}/\Delta pH = \Delta H^+$ (where p_{50} is the oxygen pressure at half-saturation and ΔH^+ is the difference per O_2 in the number of protons bound by oxy- and deoxyhemoglobin), is a good approximation in the absence of allosteric cofactors. Thus, under these conditions, the Bohr coefficient, $\Delta \log p_{50}/\Delta pH$ is equal to the Haldane coefficient, ΔH^+. However, in the presence of any anion which has a different affinity for oxy- and deoxyhemoglobin, eq 1 is no longer valid and therefore the magnitudes of the Bohr and Haldane coefficients need not be the same (Wyman, 1964; Benesch and Rubin, 1975). It was found previously (Benesch and Rubin, 1975) that in the presence of 2,3-diphosphoglycerate (DPG)[1] the two coefficients do indeed differ, especially at higher DPG concentrations.

We have now extended these investigations to the polyanions inositol hexaphosphate (IHP), inositol pentaphosphate (IPP), and inositol hexasulfate (IHS).

Experimental Section

Hemoglobin was prepared from adult human blood as described previously (Benesch et al., 1972). *myo*-Inositol hexaphosphate (IHP) was obtained from Sigma Chemical Company, St. Louis, Mo., as sodium phytate, type V, and the dihydrate of the hexapotassium salt of *myo*-inositol hexasulfate (IHS) was prepared by Terra Marine Bioresearch, La Jolla, Calif., by the method of Fatiadi (1970). *myo*-Inositol pentaphosphate (IPP) which was purchased from Calbiochem, San Diego, Calif., as the barium salt, was converted to the free acid by shaking a suspension with Dowex 50. The concentration of solutions of the phosphate esters was determined by phosphate analysis (Ames and Dubin, 1960), and that of the sulfate by direct weighing based on a mol wt of 924.

The Bohr coefficient was derived from oxygen equilibrium curves which were determined on 1×10^{-5} M hemoglobin solutions in 0.05 M Bistris buffers and 0.1 M Cl^- at 20 °C at pH 7.15 and 7.45 as described before (Benesch et al., 1965, 1973).

Haldane coefficients were obtained under conditions as nearly identical with the above as possible, i.e. at the same hemoglobin concentration, Cl^- concentration, and temperature, but, of course, in the absence of buffer and at pH 7.30. Aliquots (5–25 mL) of the hemoglobin solution were alternately purged with argon and oxygen in the presence of 15 μL of caprylic alcohol to prevent foaming. For each cycle the amount of 0.01 N HCl or 0.01 N NaOH required to maintain the pH at 7.30 was recorded using a Radiometer PM 64 pH meter with a coaxial electrode. At the end of each experiment the amount of ferrohemoglobin was determined spectrophotometrically (Benesch et al., 1973) and these values were used to calculate ΔH^+. The methemoglobin content at the end of the experiment varied from 5 to 8% of the total.

The proton absorption method described previously (Bucci, 1974; Benesch et al., 1976; Edalji et al., 1976) was chosen for the determination of the binding constants of these compounds

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[1] Abbreviations used are: DPG, 2,3-diphosphoglycerate; IHP, *myo*-inositol hexaphosphate; IPP, *myo*-inositol pentaphosphate; IHS, *myo*-inositol hexasulfate; Bistris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

TABLE I: Dissociation Constants of Hemoglobin-Polyanion Complexes.[a]

Compd	No. of − charges at pH 7.3	$K_D \times 10^6$	$K_{ox} \times 10^4$	K_{ox}/K_D
DPG	4	10		
IHS	6	0.8	4.0	500
IPP	6	1.0	4.0	400
IHP	8	0.06	0.9	1500

[a] The constants were measured at 20 °C and pH 7.30 in 0.1 M NaCl with hemoglobin concentrations of about 0.07 mM for K_D (DPG), 0.01 mM for K_D (IPP and IHS), 0.002 mM for K_D (IHP), and 0.4 mM for K_{ox} (IHP, IPP, and IHS).

since, with the exception of DPG, they are too highly charged for such techniques as equilibrium dialysis and ultrafiltration. In the case of DPG the constant obtained by the proton absorption method (Table I) agrees very well with that found by ultrafiltration under the same conditions (Renthal, 1972). The end point of the titrations with deoxyhemoglobin corresponded to 1 mol of cofactor per hemoglobin tetramer for all compounds and, at pH 7.3, involved the uptake of 2.6 H^+ for IHP, 1.7 H^+ for IPP, 2.0 H^+ for IHS, and 1.0 H^+ for DPG.

Binding of the inositol esters to oxyhemoglobin also showed a stoichiometry of 1 mol per tetramer with uptakes of 2.4 H^+, 1.6 H^+, and 2.2 H^+ for IHP, IPP, and IHS, respectively. The weaker binding made it necessary to employ much higher hemoglobin concentrations for the determination of K_{ox} (Table I).

Results and Discussion

Relation of Oxygen and Cofactor Affinities. The tightness of binding of the four polyanions to deoxyhemoglobin and therefore the magnitude of their allosteric effect on the oxygen affinity are directly related to the number of their negative charges (Tables I and II). The inositol esters show an affinity for deoxyhemoglobin which is at least 10 times greater than that of DPG at pH 7.3 in 0.1 M NaCl. Under these conditions, unlike DPG, they are also bound by oxyhemoglobin sufficiently strongly to permit measurement of their binding constants (Table I).

Complex formation between IHP and oxyhemoglobin was first detected by Gibson and Gray (1970) and the dissociation constant was estimated to be of the order of 1 μM at pH 7.0 in 0.11 M NaCl (Gray and Gibson, 1971). A detailed analysis of oxygen equilibrium curves in the presence of IHP also led to the conclusion that this compound is bound by fully oxygenated hemoglobin (Tyuma et al., 1971).

The mechanism by which the inositol esters are bound to fully liganded hemoglobin is not clear, but two possibilities must be considered. The binding site specific for the deoxy conformation, which is destroyed on ligand binding (Arnone, 1972; Arnone and Perutz, 1974; Benesch and Benesch, 1974), might be restored by these polyanions. This is in agreement with the demonstration that the T state is favored even in liganded hemoglobin in the presence of IHP (Olson and Gibson, 1970, 1971, 1972; Lindstrom et al., 1971; Cassoly et al., 1971; Lindstrom and Ho, 1972; Ogawa and Shulman, 1971). Alternately, these polyanions could be located elsewhere, for example, at the anion binding site at the N termini of the α chains (Arnone et al., 1976; Nigen et al., 1976; Chiancone et al., 1975).

The new experimental values of K_D and K_{ox} for the inositol esters shown in Table I make it possible for the first time to test the theoretical equation derived by Baldwin (1975) and by Szabo and Karplus (1976) which relates the numerical value of these constants to the change in oxygen affinity. A comparison of the measured and calculated values for $\Delta \log p_{50}$

TABLE II: Observed and Calculated Oxygen Affinities in the Presence of Three Inositol Esters.

[Hb] (μM)	μM	n	$\Delta \log p_{50}$ Calcd[a]	$\Delta \log p_{50}$ Calcd[b]	$\Delta \log p_{50}$ Exptl[c]
	$[IHP]_{total}$				
5	25	2.3	0.90	0.62	0.89
5	50	2.2	0.97	0.68	0.96
10	200	2.2	1.02	0.76	1.03
10	500	2.0	1.09	0.79	1.09
50	1000	2.0	1.11	0.80	1.11
	$[IHS]_{total}$				
5	25	2.5	0.55	0.36	0.58
5	50	2.5	0.65	0.43	0.67
10	100	2.5	0.71	0.50	0.68
50	250	2.6	0.74	0.56	0.71
50	600	2.6	0.80	0.62	0.82
50	1000	2.5	0.82	0.64	0.81
	$[IPP]_{total}$				
5	25	2.5	0.59	0.34	0.53
10	100	2.5	0.69	0.48	0.71
50	1000	2.5	0.86	0.62	0.84

[a] These values were calculated with the empirical equation (Benesch et al., 1971): $\Delta \log p_{50} = (1/n) \log \{[1 + (C/K_D)]/[1 + (C/K')]\}$. $\Delta \log p_{50}$ is the difference in the oxygen pressure at 50% oxygenation in the presence and absence of the cofactor, n is the experimentally found Hill's coefficient, C is the molar concentration of cofactor, K_D is the dissociation constant of the deoxyhemoglobin-cofactor complex, and K' is that for intermediate oxygenation states. The value of K' was estimated from the oxygenation data at high cofactor concentration as explained previously (Edalji et al., 1976; Benesch et al., 1976). [b] These values were calculated from the equation (Baldwin, 1975; Szabo and Karplus, 1976): $\Delta \log p_{50} = (1/4) \log \{[1 + (C/K_D)]/[1 + (C/K_{ox})]\}$. K_{ox} is the dissociation constant of the oxyhemoglobin-cofactor complex. [c] These values were derived from oxygen equilibrium curves measured at 20 °C in 0.05 M Bistris buffer, pH 7.30, 0.1 M total chloride.

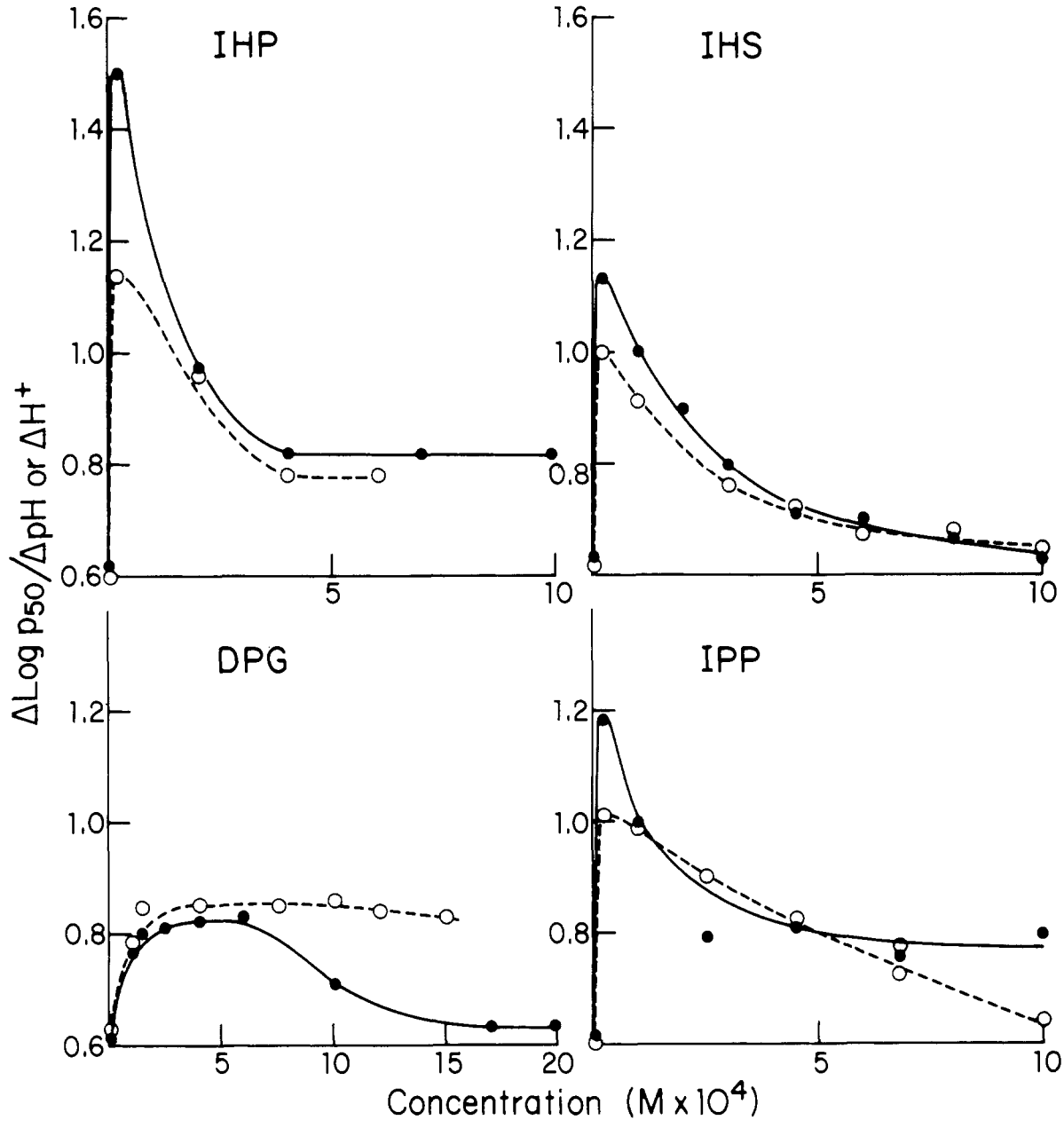

FIGURE 1: The effect of polyanions on the Bohr and Haldane coefficients: (○) ΔH^+ (Haldane coefficient); (●) $\Delta \log p_{50}/\Delta$pH (Bohr coefficient). The data for DPG are taken from the paper by Benesch and Rubin (1975).

(Table II) shows that the shift in oxygen affinity is much larger than that predicted by this equation. A simple resolution of this discrepancy is unlikely, since an adequate model for the effect of these cofactors on oxygen binding will probably require a consideration of binding constants to the various intermediates associated with the assymetric relation of one allosteric effector for four ligand molecules as well as the effect of the cofactors on the tetramer-dimer equilibrium of the protein (White, 1976; Wiedermann and Olson, 1975). The results in Table II also show that the empirical equation of Renthal (1972) and Benesch et al. (1971) gives values which are in very good agreement with the experimental ones.

Bohr and Haldane Effects. In the presence of allosteric effectors both the Bohr and Haldane coefficients are made up of two quite distinct components (De Bruin et al., 1971; Shimizu and Bucci, 1974): one of these, 0.6 H^+/ heme, is the value in the absence of cofactors and is due to the change in the acid strength of several groups on the globin when the conformation of the protein changes with ligand binding. The added component is due to the protons which are bound along with the allosteric effectors at the specific binding site formed by the termini of the β chains. The Haldane effect [(ΔH^+)/(deoxy − oxy)] is increased by an amount which depends on the ratio of K_{ox}/K_D, i.e. the relative affinity for the oxy and deoxy conformations (Table I). The additional Bohr effect (Δ log p_{50}/ΔpH), on the other hand, is due to the pH dependence of cofactor binding, i.e. its tighter binding with decreasing pH. The variation of the additional increment of both coefficients with cofactor concentration is shown in Figure 1. A number of conclusions can be drawn from these results.

(1) At the lower concentrations, the inositol esters increase the Bohr effect much more than the Haldane effect. It is clear that the maximum value reached by the Bohr coefficient is proportional to the number of negative charges carried by the cofactor. This is not surprising since the number of positive residues on the protein involved in holding the polyanion will

determine the pH dependence of the binding constant.

(2) The additional Bohr coefficient approaches zero for all four compounds when the concentration of the cofactor becomes sufficiently large to eliminate the pH dependence of the binding by saturating the protein at both pH values used for the measurement.

(3) For the three inositol esters the Haldane effect also reaches a maximum at low concentrations and then declines toward the original value of 0.6 H^+/heme. The affinity of these compounds for the oxy conformation is evidently sufficiently strong (Table I) to approach saturation of the binding site comparable to that in the deoxy conformation, as the concentration of the cofactor is increased.

(4) By contrast, the Haldane effect in the presence of DPG does not decrease from its maximum value, up to a concentration of 1.5 mM. As found previously, DPG, unlike the other polyanions, is bound so weakly to oxyhemoglobin that the proton uptake associated with binding to the deoxy conformation is not noticeably decreased by a similar proton uptake due to binding to the oxy conformation.

The increased Haldane effect in the presence of DPG, therefore, differs sharply from the inositol esters and it remains constant over a wide concentration range (Figure 1). This illustrates that the inositol compounds cannot simply be regarded as more powerful DPG analogues and that this compound, because its affinity for hemoglobin is so sensitive to oxygenation, is rather unique among the allosteric effectors of oxygen binding.

As has been pointed out elsewhere (Benesch et al., 1975), this maintenance of an increased Haldane effect over the physiological concentration range has important implications for carbon dioxide transport. Carbon dioxide is transported both as carbamate on the N-terminal amino groups and as carbonic acid, neutralized by the Haldane effect. Since DPG decreases carbamate formation by competing for the same site, the increased Haldane effect will compensate for the displacement of carbon dioxide by DPG from the β-chain N-terminal amino groups.

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