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BINDING OF ORGANIC PHOSPHATES BY HUMAN HEMOGLOBIN AT ALKALINE pH-VALUES

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Summary. The hemoglobin-oxygen equilibrium binding curve was found to be sensitive to the addition of inositol hexaphosphate at pH 9.1. A solution of hemoglobin A in 0.050 M sodium borate was half-saturated with oxygen at a partial pressure of 0.55mm Hg. Hemoglobin A in 0.050 M sodium borate, 0.001 M inositol hexaphosphate, pH 9.1 was half-saturated with oxygen at a partial pressure of 0.95mm Hg. The Hill plot was linear with a slope of 2.0 in the absence of phosphates. In the presence of inositol hexaphosphate the slope of the Hill plot increased from 1.0 to 2.56. The dependence of fractional saturation of hemoglobin with oxygen on concentration of inositol hexaphosphate was determined at partial pressures of oxygen of 0.46 and 1.07mm Hg.

The hemoglobin-oxygen binding curves obtained by Roughton using the combination of syringe-capillary and Van Slyke gasometric techniques are precise and accurate data. The majority of these studies, however, were completed before an appreciation of the effect of organic phosphate esters on the affinity of hemoglobin for oxygen was obtained(1,2). The concentration of phosphate esters, particularly DPG*, was not controlled in the studies of HbA carried out using these precise, but laborious, methods of gas analysis.

The binding of DPG to HbA at pH 9.1 was reported to be so weak that organic phosphate esters are not expected to influence ligand binding properties(3). Gray and Gibson(4) have observed characteristic similarities in the kinetics of formation of

*Abbreviations used: HbA, human hemoglobin; DPG, 2,3-diphosphoglyceric acid; IHP, inositol hexaphosphate; $\bar{F}$, fractional saturation of heme moieties with ligand; P_{ox}, partial pressure of O_2.

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carboxyHbA in solutions of neutral pH free of phosphates and alkaline solutions containing IHP. In both instances the progress of the reaction, determined as percentage of absorbance excursion, was wavelength independent and isoabsorption points could be observed. In the presence of IHP at neutral pH the progress curves for formation of carboxyHbA were biphasic, variously dependent on the analytical wavelength and, isoabsorption points could not be observed. More directly, the time-course of formation of carboxyHbA at pH 9.1 was insensitive to IHP.

Insofar as organic phosphates are thought to be without effect on hemoglobin-ligand interactions at pH 9.1, the dissociation curves obtained by Roughton and Lyster(5) using the combination of syringe-capillary and Van Slyke gasometric techniques are considered to be free of the perturbing effects of endogenous DPG. In the course of our efforts to establish binding data for the HbA-O_2 equilibrium(6) we have conducted experiments using HbA freed of endogenous DPG and rechromatographed on a column of Sephadex G-50 equilibrated with 0.050 M sodium borate, pH 9.1. The partial pressure of oxygen, P_{ox}, required for half-saturation of HbA at 20.0° was approximately half of the value reported by Roughton and Lyster(5). Addition of IHP restored the curve to the expected position. The purpose of this communication is to describe the results of experiments revealing the surprising influence of phosphate esters on the affinity of HbA for O_2 at alkaline pH-values.

Methods. Blood was obtained from a single donor. HbA was prepared as described by Gibson(7) and subjected to a photolysis procedure to remove endogenous carbon monoxide. Chromatography on CM-cellulose(Whatman, CM-52) removed membrane fragments as well as other components which introduced uncertainties into the spectrophotometric analysis. The sample of HbA eluted from the CM-cellulose column was between 0.006 and 0.008 M in heme. This sample was subjected to chromatography on a column of Sephadex G-50 equilibrated with 0.050 M sodium borate. pH 9.1. Samples containing IHP were prepared by mixing 0.010 M IHP, 0.050 M sodium borate, pH 9.1 with the HbA sample.

A gas-liquid equilibration apparatus was used in conjunction with a gas-mixture generator to prepare sample of HbA in equilibrium with a gas mixture containing oxygen at a known partial pressure. A digital spectrometer was used to obtain sample spectra at 160 analytical wavelengths between 460 and 620nm. Sample spectra were resolved into the best fitting components of oxyHbA and HbA by a least-squares curve fitting procedure. Statistical data on the curve fitting operations were obtained. The RMS of residuals(predicted minus observed absorbance values) was generally less than 0.0005 over the entire spectrum. With the exception of the gas mixture generator these methods have previously been described (6). Mass flowmeters produced by the Tylan Company(Torrance, Cal.) were used in place of the Matheson flowmeters.

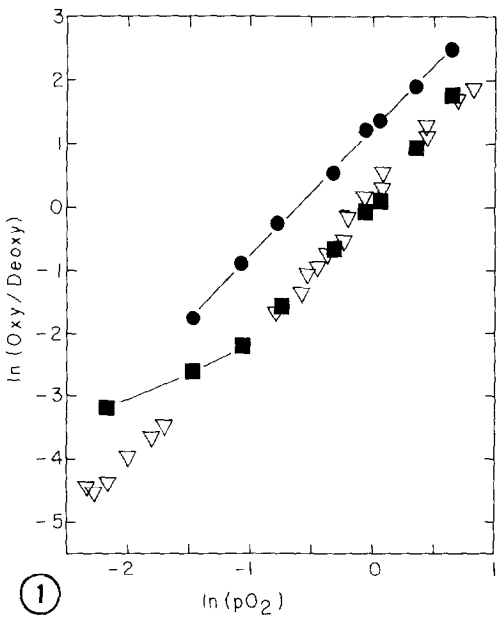
Results. The HbA-O_2 binding curve in 0.050 M sodium borate (pH 9.1, 20.0°), graphed according to Hill's equation[+](8), is presented in Figure 1. Without added IHP the plot was linear, the n-value being 2.0 and half-saturation being obtained at P_{ox} of 0.55mm Hg. Under the conditions of the experiment the interaction of HbA with O_2 obeys the mass action expression given in Equation I, the value of K being 3.31 mm^{-2}.

$$K = (Hb_2(O_2)_2)/((Hb_2)(O_2)^2) \qquad \text{Eq. I}$$

Addition of IHP to a final concentration of 0.001 M resulted in a lower affinity of HbA for O_2, half-saturation being obtained at P_{ox} of 0.95mm Hg. The Hill plot was not linear, the n-value being 1.0 at low values of fractional saturation, $\bar{F}$, and rising to 2.36. These data, along with those obtained by Roughton and Lyster(5), are included in Figure 1.

The binding of IHP by HbA at pH 9.1 was monitored by observing the effect of added IHP on $\bar{F}$ at a constant P_{ox}. Samples of HbA containing IHP at various concentrations were equilibrated with a gas mixture of invariant composition and $\bar{F}$ was determined. Gas mixtures with P_{ox} of 0.46 and 1.07mm Hg were used. At each value of P_{ox}, the addition of IHP resulted in a decrease in $\bar{F}$. The concentration of IHP required for half the observed excursion in $\bar{F}$

+ $Hb_n + nO_2 = Hb_n(O_2)_n$, where n is an interger.



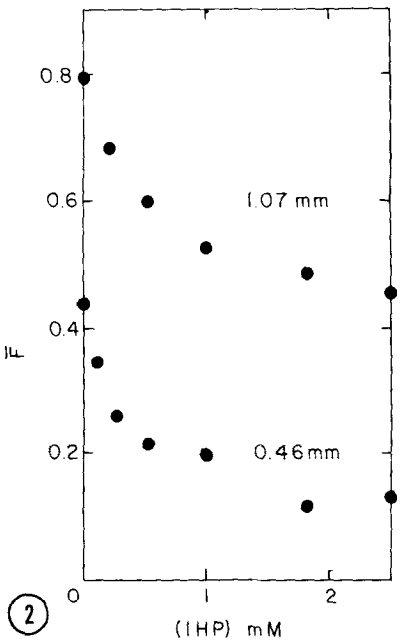


Figure 1. Hill plots of HbA-oxygen equilibrium binding curves in 0.050 M sodium borate, pH 9.1. Without added phosphate and free of endogenous phosphate(●) and complemented with 0.001 M IHP(■). The data obtained by Roughton and Lyster using a combination of capillary-syringe and Van Slyke gasometric techniques(▽) are included for comparison. The experiments were conducted at 20 degrees C.

Figure 2. Titration of hemoglobin with IHP at constant partial pressure of oxygen.

was 0.15 x 10^{-3} M at P_{ox} of 0.46mm Hg and 0.30 x 10^{-3} M at P_{ox} of 1.07mm Hg. The results are illustrated in Figure 2.

Discussion. Contrary to expectation the HbA-O_2 equilibrium has been found to be sensitive to the addition of IHP at alkaline pH. Hill plots of binding data obtained in phospnate-free borate buffer and IHP-containing borate buffer are markedly different in shape albeit not so greatly different in position. It may be pointed out that these curves lie in a region where tonometer experiments are not convieniently conducted and so the finding does not stand in opposition to a large body of accepted data. The results of Roughton and Lyster(5) are well regarded and the

discrepancy between their results and those described herein can be accounted for by the endogenous DPG found in human red blood cells. The procedure followed by Roughton and Lyster did not include dialysis or any other operation which would have diminished the concentration of intracellular salts and esters. The observations of Gray and Gibson(4) are reproducible and organic phosphates are without effect on the kinetics of binding of ligands to HbA at pH 9.1. Not surprisingly then, preliminary experiments have shown that the effect of IHP at pH 9.1 is reflected in the release of ligands from HbA(unpublished results, Knowles and Gibson).

The phosphate-dependent change in the shape of the Hill plot indicates a change in the mechanism of transmission of cooperative effects within the HbA tetramer. The n-value of unity obtained at low values of $\bar{F}$ in the presence of IHP clearly shows that binding of the initial ligand is without effect on the remaining unoccupied heme moieties. That the n-value was not observed to be less than 2.0 in phosphate-free buffer indicates, as clearly, that binding of the initial ligand results in transmission of the cooperative effect to a second ligand binding site. Remarkably, the cooperative effect reaches only one heme moiety since the n-value is not greater than 2.0. Knowles(9), using HbA derivatized at Cys-β 93 with the S-trifluoroethyl residue, has demonstrated a preferred order of binding of ligand to the α-chain in the presence of IHP. The results of the ^{19}F-nmr study taken together with those described herein suggest that the cooperative effect is manifested in the α-chain as a result of ligation of the β-chain. These fundamentally different roles for the α- and β-heme moieties do not appear to be interchangeable.

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