are binding simultaneously whereas in the presence of ATP, only one head binds at a time. In this regard it is of interest that subfragment 1, a further tryptic digestion product of HMM which has only one active site, has been reported to bind more weakly to actin than does HMM (Lowey *et al.*, 1971). To clarify this point we are presently carrying out further studies directly comparing the binding of actin to subfragment 1 in the presence and absence of ATP.

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Equilibria of Organic Phosphates with Horse Oxyhemoglobin†

Bo Hedlund, Constance Danielson, and Rex Lovrien*

ABSTRACT: Organic phosphates, ATP, AMP, and 2,3-diphosphoglycerate (DPG) were interacted with horse oxyhemoglobin. Binding parameters were obtained by means of dialysis equilibrium in buffers at 5°, and from calorimetry at 5 and 25°, all in the pH range 6.5–7.3. The calorimetric results were evaluated assuming a single strong site, and assuming that the pH shifts which occur in the absence of added salt are due to electrostatic effects and not to changes in side-chain titration, upon mixing samples previously adjusted to the same pH. There was obtained for ATP and 2,3-diphosphoglycerate, respectively, $\Delta G^\circ = -6.5$ and -4.9 kcal mol^{-1}, $\Delta H^\circ = -5.2$ and -9.1 kcal mol^{-1}, and $\Delta S^\circ = +5$ and -14 cal deg^{-1} mol^{-1}, at pH 6.9 and 25°. These binding parameters are for the case of no added salt. In dialysis equilibrium experiments, buffer and supporting electrolyte were used. In that case, the results indicate strong competition by inorganic phosphate, lesser competition by chloride, while cacodylate possibly is noncompetitive or nearly so. Competition by salt ions also is manifest in calorimetry of organic phosphate binding. Equilibria of AMP, in the presence of orthophosphate, may engage in a somewhat different mechanism involving the protein. There appears to be chemical linkage between binding sites, when AMP is the substrate. There are indications that for organic phosphates, there exists more than one site available on oxyhemoglobin. However there is only one strong site. Certain of the pH-dependent phenomena were roughly accounted for by electrostatic effects using the smeared charge model. Calorimetry at 5°, compared to 25°, indicates a ΔC_P of binding of $+260$ cal deg^{-1} mol^{-1} for ATP-deionized oxyhemoglobin interaction, and a ΔC_P of about $+100$ cal deg^{-1} mol^{-1} for DPG interaction.

This paper reports on organic phosphate binding to horse oxyhemoglobin, HbO_2.[1] The binding of a variety of organic phosphates to both oxy and deoxy forms of hemoglobin has been rather widely studied and reviewed. However, there is considerable controversy regarding the extent of binding to both forms of hemoglobin. The actual difference in free energy between organic phosphate binding to the two forms of hemoglobin, *i.e.*, the amount of free energy that is available for control of oxygenation by these compounds, has not been satisfactorily determined.

The data presented here for organic phosphate binding

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[1] Abbreviations used are: HbO_2, oxyhemoglobin; ATP, adenosine 5′-triphosphate; AMP, adenosine 5′-monophosphate; DPG, 2,3-diphosphoglycerate.

to horse HbO_2 are comparable to those reported by Chanutin and Hermann (1969) for human HbO_2. Comparison between data here and results obtained by others using human HbO_2 should be done with care. However, in certain respects, especially with regard to current understanding of DPG–hemoglobin interaction, horse and human hemoglobin are similar (Bunn, 1971).

It is impractical to measure dialysis equilibrium of organic ions without supporting electrolyte. The membranes bind the organic ions, equilibrium is difficult to achieve, and Donnan effects occur. Yet if electrolytes are used at reasonable concentrations, 0.01 M or greater, they often compete for binding sites, although their association constants are of the order of 10^2 or 10^3 M^{-1}. This pertains to numerous proteins and enzymes, and especially so to hemoglobin.

Because of the fragility of HbO_2, and the length of time needed to reach equilibrium, dialysis equilibrium experiments were carried out at 5° with cacodylate buffer in most experiments. This buffer was employed by Chanutin and Hermann (1969). From their results and ours, it appears that cacodylate is less competitive for HbO_2 ion binding sites than other commonly used systems. Support for this view is provided by calorimetry with deionized HbO_2. In the calorimeter, equilibrium can be reached with low or zero competing electrolyte concentration, depending on pH. The data from calorimetry can then be compared to dialysis equilibrium results with buffer. In both kinds of studies, the HbO_2 was equilibrated with air to keep the protein saturated with oxygen.

The notations used are: y = (mol of substrate added)/(mol of HbO_2 tetramer, mol wt 64,460); K_{app} = apparent molar association constant from a Scatchard plot; n = abscissa intercept on a Scatchard plot; K = overall association constant; n_i = number of sites of class i; k_{A_1}, k_{A_2} = association constants for substrate A in classes 1, 2 . . . . ; k_{B_1}, k_{B_2} . . . = association constants for competitor B in sites 1, 2 . . . . ; C_A = molar concentration of nonbound A; C_B = molar concentration of nonbound B; A_T = total concentration of A; B_T = total concentration of B; P_T = total concentration of protein; ν_{A_1}, ν_{B_1} . . . = mol of A bound into site class 1 per mol of protein, mol of B bound in site class 1, etc., ν_{AT} = $\nu_{A_1} + \nu_{A_2}$ = experimentally observed value of ν_A; Q = calorimetric heat; w = electrostatic interaction parameter (simplified model); Z_P = charge on the protein; z_A = charge on the substrate; r_{H^+} = number of H^+ bound relative to the isoionic pH.

Experimental Methods

Materials. The oxyhemoglobin was isolated from fresh horse erythrocytes, initially collected in citrate. The isolation was carried out at 5°. The steps were: centrifuge, discard plasma; wash erythrocytes three times with 1.0% NaCl, lyse with one volume of water–ethyl ether (50:50, vv), shake gently for 1 min; centrifuge at 10,000*g*; decant off ether, pump with an aspirator, and blow a gentle air stream over the hemolysate while gently stirring; add NaCl to 5%; centrifuge; remove floating debris by suction; dialyze three times against 0.05 M NaCl to remove ether, then against water to remove chloride; again centrifuge and decant. The HbO_2 solution is then ready for resin deionization, also in the cold, using the method of Dintzis (1952) over about 16 hr. The first quarter of the hemoglobin was discarded.

Hemoglobin thus prepared is free from phosphate, as determined on digested hemoglobin by standard total phosphate assay (Bartlett, 1959). When the protein is converted to methemoglobin by twofold molar excess $K_3Fe(CN)_6$, it gives the optical density ratios (570 nm/630 nm) reported by Cameron and George (1969). Native HbO_2 concentration was determined using extinction coefficients from Anusiem's (1966) work with human HbO_2 listed in Table I. In this, we assume also with Anusiem that the absorption spectra of horse and human HbO_2 are very similar. The molecular weight used here is 64,460. The molar extinction coefficients from Table I were also used to determine methemoglobin content in HbO_2 preparations, which usually was 2% in new HbO_2 and increased to 10–15% upon storage at 5° for three weeks. Stock solutions were routinely centrifuged to remove precipitated material from deionized HbO_2.

TABLE I: Molar Absorption Coefficients for Met- and Oxyhemoglobin; Phosphate Buffer, pH 6.0, μ = 0.05; from Anusiem (1966).

λ (nm)	MHb, M^{-1} cm^{-1} × 10^{-4}	HbO_2, M^{-1} cm^{-1} × 10^{-4}
500	3.60	2.02
510	3.40	1.91
542	2.14	5.68
577	1.27	6.04

Dry weight, iron analysis by Stookey's (1970) method for Fe^{II} and Cameron's (1965) digestion method, and disc gel electrophoresis gave results which, in summary, indicate the presence of up to 3% of other proteins in our deionized HbO_2 preparations. Therefore, absorption spectra were routinely used to determine HbO_2 concentration itself.

ATP and AMP were used as received from Sigma Co.; ϵ_{259} = 1.54 × 10^4 at neutral pH (Dunn and Hall, 1970). 2,3-DPG, pentacyclohexylammonium salt, came from Boehringer-Mannheim.

Methods. Dialysis equilibrium methods used the apparatus from Lovrien and Anderson (1969). It was found that pretreatment of the Visking cellulose membranes according to Chanutin and Hermann (1969) is indeed necessary for the present kind of work. The membranes were treated with bicarbonate and EDTA over steam overnight, thoroughly rinsed, and then equilibrated with the buffers being used in the experiments for about a day, before installing them. Hemoglobin concentrations were usually in the 4–8 × 10^{-4} M range (constant in a series of experiments). It usually required about 48 hours at 5° to achieve equilibrium; air was present in each cell. DPG was determined colorimetrically by Bartlett's (1959) assay for total phosphate.

Microcalorimetry used a twin reaction cell batch instrument, an improved version of an earlier model (Lovrien and Anderson, 1969). The present instrument was used in the 0- to 10-mcal range, error ±0.2 mcal in a single run. Chemical calibration was used: Tris-HCl and KOH–HCl reactions. The heats for these calibrating systems are by Nelander (1964) and Leung and Grunwald (1970), respectively.

In a typical series of experiments a constant amount of protein (2.00 ml of 0.8–1.4 × 10^{-3} M) was mixed with 1.00 ml of organic phosphate solution containing up to 3.0 μmol of ligand. In the second reaction cell either protein or organic phosphate is mixed with solvent. This heat of dilution is automatically subtracted from the heat produced in the reaction cell. Under most conditions employed the heats of dilu-

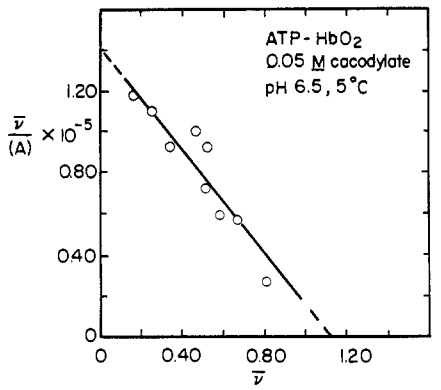

FIGURE 1: Scatchard plot of ATP binding to horse oxyhemoglobin. Hemoglobin concentration: 5.2×10^{-4} M.

tion are negligible and can be substituted with solvent–solvent mixing in the blank cell. The calorimeter is connected through an amplifier to an integrating recorder. The heat produced is proportional to the area under the curve.

Calorimetry of pentacyclohexylammonium chloride with HbO_2 showed that about 5% of the total DPG interaction heat with HbO_2 was probably due to the cation interaction, and corresponding corrections were made for cation binding heat production.

Calorimetric data were plotted as a function of ligand concentration added. Plots of this type were fitted with a least-squares method similar to the method used by Olofson (1969) to yield both association constant and the enthalpy change. It was assumed that, under the conditions employed, there is only one binding site for the ligand in question. The method was satisfactory only under conditions where moderately strong and well-defined binding seemed to occur. In the presence of competing ions or with very weakly binding compounds the method cannot be used. The pH was measured before and after mixing. A Beckman Research pH meter was used and temperature was controlled to within 0.5°.

Results

ATP–Horse HbO_2 Dialysis Equilibrium Data. The data were plotted according to Scatchard's method, ν_{AT}/C_A *vs.* ν_{AT}. The large majority of the data were concentrated in the region $\nu_{AT} < 1$, which gave apparently quite linear plots in some cases. These were calculator fitted by least squares. If there were a single site, an abscissa intercept of unity should occur. Despite the apparent linearity over the concentration ranges in which we worked, abscissa intercepts greater than one were consistently obtained, indicating at least one weaker site, in agreement with Chanutin and Hermann (1969) for human HbO_2. Figure 1 illustrates a typical plot. Scatchard's equation is $\nu_{A_i}/C_A = K_i n_i - K_i \nu_A$ for each class of sites i. We measure ν_{AT}. Hence in cases of the kind here, the intercepts on the axes are unresolved averages of n and Kn for whatever sites there are. Such values are listed in Table II, for a series in which pH was varied. As expected, the product, nK, and K itself, increases rapidly with decreasing pH in the neutral pH region.

TABLE II: Parameters from ATP–HbO_2 Scatchard Plots; 0.005 M P_i Buffer, 0.04 M Cl^-, 5°.

HbO_2 Concn ($M \times 10^4$)	pH	Abscissa Intercept, n	$(nK)_{app}$	(nK) pH 6.9 Calcd
5.4	6.50 ± 0.05	1.40	21×10^3	8×10^3
5.7	6.75 ± 0.05	1.50	13×10^3	10×10^3
5.8	7.10 ± 0.10	1.45	5×10^3	7×10^3

TABLE III: Parameters from ATP–HbO_2 Scatchard Plots, 5°.

P_i (M)	Cl^- (M)	μ	pH	Abscissa Intercept	$(nK)_{app}$	(nK) pH 6.9 Calcd
0.005	0.04	0.046	7.1	1.45	4.4×10^3	6.7×10^3
0.025	—	0.048	7.1	2.7	2.5×10^3	3.8×10^3
0.050	—	0.073	6.6	3.4	3.1×10^3	2.0×10^3

The buffer system listed in Table II is quite competitive with the ATP substrate for HbO_2 binding sites. Table III lists results from Scatchard plots in which P_i concentration varied considerably. The data for Table III were partly gathered in the presence of 0.04 M chloride which probably also competes. Some salt has to be present in dialysis equilibrium experiments. Both P_i and Cl^- decrease apparent association constants for the ATP–HbO_2 interaction. Benesch *et al.* (1969) found that a variety of buffers and salts similarly influence the 2,3-DPG–deoxyhemoglobin binding process. By starting with cacodylic acid and adding base, one can obtain a buffer without chloride. Cacodylate buffers alone seem nearly noncompetitive, which perhaps accounts for some of the alignment between our results and those of Chanutin and Hermann (1969), although such a comparison should be made cautiously, since they used human HbO_2 and ours was from horse. Our dialysis equilibrium K values in cacodylate, on the other hand, also are comparable to K values generated in fitting of calorimetric data described below.

All the data are pH dependent, and in order to make comparison of various data, it is of some interest to try to normalize results to the same pH. As shown in the discussion, if we assume that some of the pH dependency of binding is of the classical electrostatic variety, we obtain

$$\frac{\partial \log K}{\partial \mathrm{pH}} = -\frac{2}{2.303} w z_A \frac{\partial Z_p}{\partial \mathrm{pH}} \qquad (1)$$

and use this expression with experimentally determined values to correct $(nK)_{app}$ to pH 6.9. Table II shows that reasonably consistent corrected values for the value of nK at pH 6.9 are obtained in this way, with 0.005 M P_i. Table III shows that as the P_i concentration increases, such a correction is not even roughly adequate. The correction factor represented in eq 1 is only semiquantitative at best, but is perhaps better than no correction at all. It has been applied by us to some of the DPG data from Benesch *et al.* (1969), with results comparable to those obtained in Table III. We do not consider in this, that all pH dependence reflects only electrostatic effects. The latter is only one of the more obvious effects which are under pH control. In fact, Table III indicates that, even after normalization of all K values as best we can, to pH 6.9, there is still considerable variation in K_{app}.

TABLE IV: Parameters from ATP–HbO_2 Scatchard Plots, 5°, 0.05 M Cacodylate.

Cl^- (M)	μ	pH	Abscissa Intercept	$(nK)_{app}$
—	0.033	6.5	1.12	1.44×10^4
0.012	0.045	6.5	1.23	6.0×10^4
—	0.043	7.0	1.27	1.6×10^4
0.012	0.055	7.0	1.40	0.5×10^4
0.067	0.107	6.8	1.18	1.5×10^4

Table IV reflects three matters: (i) the expected pH dependency of the binding data; (ii) the decrease in binding caused by chloride, in a way rather unlikely due to minor ionic strength variations; (iii) the binding constants for ATP to horse HbO_2 in the absence of competitive ions such as P_i and Cl^-. The association constant at pH 6.5, 5° is probably best summarized by:

$$ATP + HbO_2 \rightleftarrows ATP \cdot HbO_2 \qquad K_1 = 1.3 \times 10^5\ M^{-1} \quad (2a)$$

$$ATP + B \cdot HbO_2 \rightleftarrows ATP \cdot HbO_2 + B \qquad K_{app} < K_1 \quad (2b)$$

in which B is a competing salt ion. The value of K_{app} depends on the nature and total concentration of B, which is B_T.

Dialysis Equilibria of 2,3-DPG and P_i With Horse HbO_2. The principal results from experiments with these ions, in cacodylate buffer, are summarized in Table V from data as in Figure 2 and 3. In the DPG systems there was also present a certain amount of chloride used in adjusting the buffers. Hence the apparent K values, K_{app}, are probably lower than the true values which probably exist for the noncompetitive binding case. As in the case of ATP, the abscissal intercepts of the Scatchard plots for DPG binding were between 1 and 2, indicating participation by a weaker second site at the higher DPG concentrations, $y > 1$.

Similar experiments with P_i itself, with 0.05 M cacodylate buffer and no chloride at pH 6.8 and 5°, gave significant association, 400 ± 200 M^{-1}. The association constant for P_i is estimated to be 800 ± 200 M^{-1}, at pH 6.5, in the same solvent.

Dialysis Equilibria of AMP with Horse HbO_2. Binding data for AMP, with P_i present are plotted in Figure 4. In this case, unusual Scatchard plots result, with no possibility of obtaining slopes and intercepts in the usual way. The experiments were repeated, examined with various HbO_2 preparations,

TABLE V: Parameters from DPG–HbO_2 Scatchard Plots, 5°, 0.05 M Cacodylate.

Cl^- (M)	μ	pH	Abscissa Intercept	$(nK)_{app}$
0.012	0.045	6.5	1.33	3.7×10^4
0.012	0.055	7.0	1.28	0.9×10^4
0.10	0.131	6.4	1.08	1.3×10^4

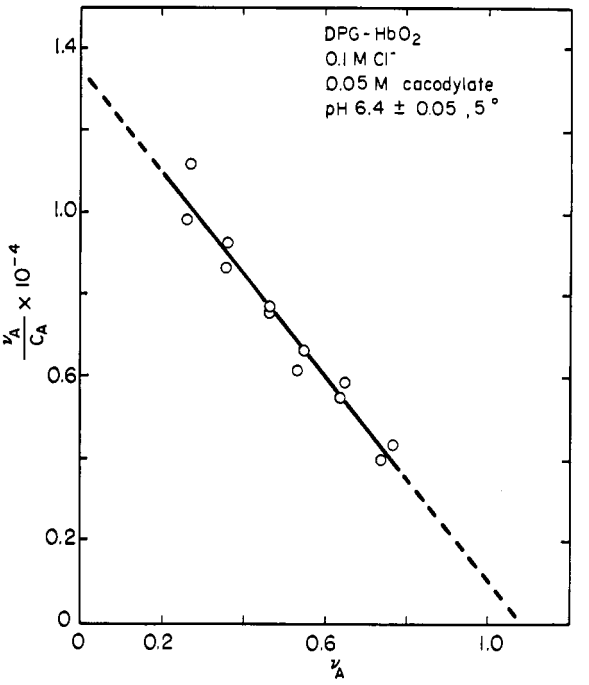

FIGURE 2: Scatchard plot for DPG binding to horse oxyhemoglobin.

and so on. We cannot assign this behavior to obvious experimental errors, at least in the sense of doing anything different from the ATP experiments which led to normally behaving plots. As it turns out, other protein–substrate interactions probably give qualitatively the same behavior upon using Scatchard's method of plotting; this is amplified below.

Calorimetry of Organic Phosphate–Horse HbO_2 Interaction. Data points from calorimetry of mixing ATP with deionized

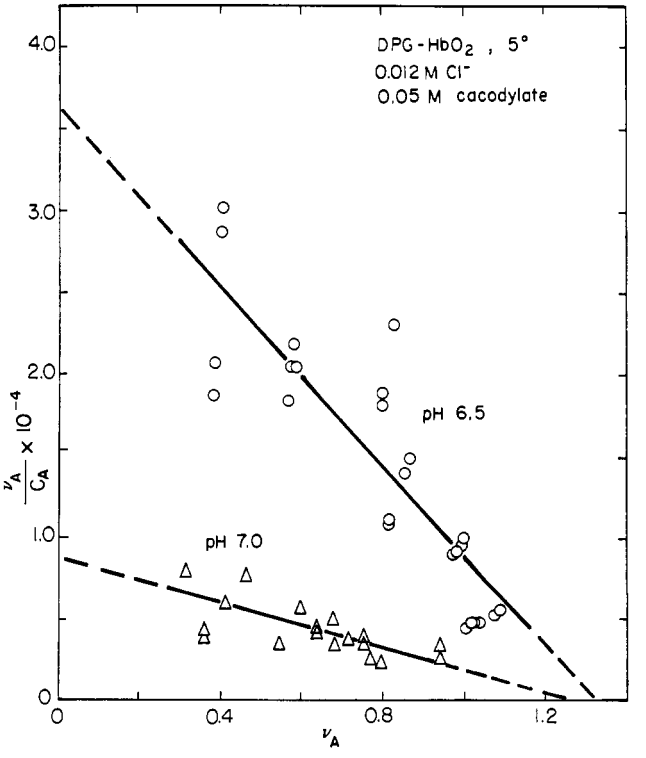

FIGURE 3: Scatchard plots for DPG binding to horse oxyhemoglobin at two different pH values.

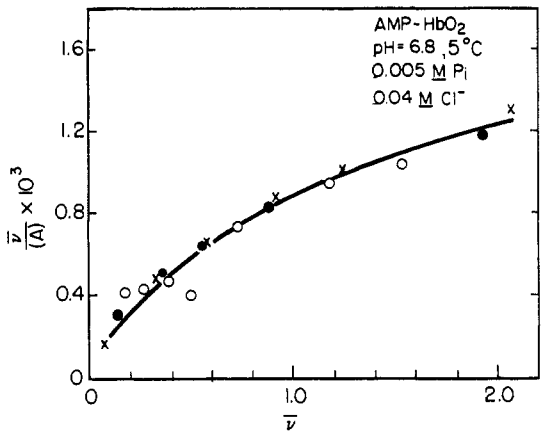

FIGURE 4: Scatchard plot for AMP binding to horse oxyhemoglobin in a competing buffer system.

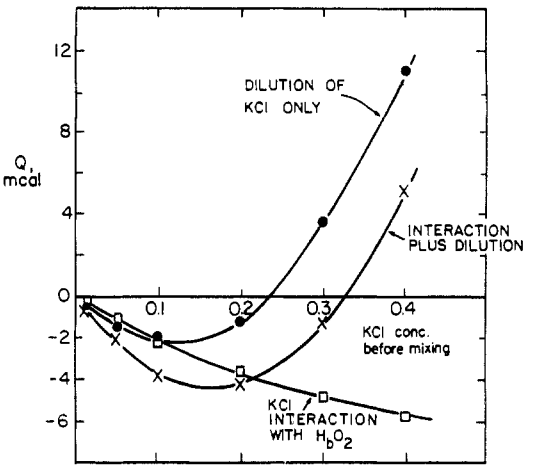

FIGURE 6: Calorimetry of chloride interaction with 1.35 μmol of deionized horse oxyhemoglobin and calorimetry of KCl dilution at 25°. See Resnlts section for additional details.

HbO_2 at 25°, with no added salt in either component, are plotted in Figure 5. The ATP solutions were adjusted to the same pH as that of the HbO_2 before mixing (pH 6.93 ± 0.02) by use of carbon dioxide free KOH. On the same plot, calorimetric data are illustrated from the case in which both solutions were 0.10 M in KCl before mixing. Figure 5 shows that chloride considerably decreases the heat evolved, relative to the case for no added KCl. Equivalent effects are wrought by P_i, in concentrations *ca.* 0.01 M. There are two general ways in which this probably occurs: (i) prevention of ATP binding by P_i, or by Cl^-; (ii) endothermic displacement of P_i or Cl^-, upon exothermic ATP binding. The instrument, of course, measures the sum of the heats.

The calorimetric mixing process was performed in a twin instrument. The "blank heat" is automatically subtracted out. When a protein in zero salt is mixed with an inorganic salt, besides the binding heat there will be produced some heat of tumbling and mixing the two solutions, in addition to the heat of dilution of the salt. Deionized HbO_2 was mixed with aqueous KCl (Figure 6) with the blank for the experiments that of mixing corresponding volumes of neat water in the second mixing vessel. This protocol would not subtract the heat of KCl dilution. A second set of experiments was performed with no protein, *i.e.*, dilution of KCl, in the same way, *vs.* a blank of water–water mixing. The results from those experiments are also plotted on Figure 6. The difference between the two plots is presumably caused by chloride–HbO_2 interaction. The difference quantity can be gotten by a single set of experiments, mixing deionized HbO_2 with KCl, and using as a blank the dilution of a corresponding amount of KCl. Figure 6 shows that, with the weak but finite interaction in which chloride probably engages, the heats of dilution are comparable in magnitude to whatever intrinsic heats of binding actually occur in the association of Cl^- with HbO_2. Also it is found that the heat production from KCl dilution is quite similar to that which would be expected from the thermal properties of aqueous electrolytes (Parker, 1965). The total heat of dilution of 1 ml of 0.15 M KCl, under the conditions used here (dilution to 0.05 M), is calculated to be −2.4 ± 0.2 mcal. The data gave −2.1 ± 0.2 mcal, for a single run. The Cl^- interaction heats with HbO_2 are ascribed to binding at Cl^- at an unknown level of $\bar{\nu}$. At 0.10 M KCl, about 74 mol of Cl^- are mixed with each mol of HbO_2 (ν = 74). Further work is necessary to determine binding constants of Cl^- with HbO_2.

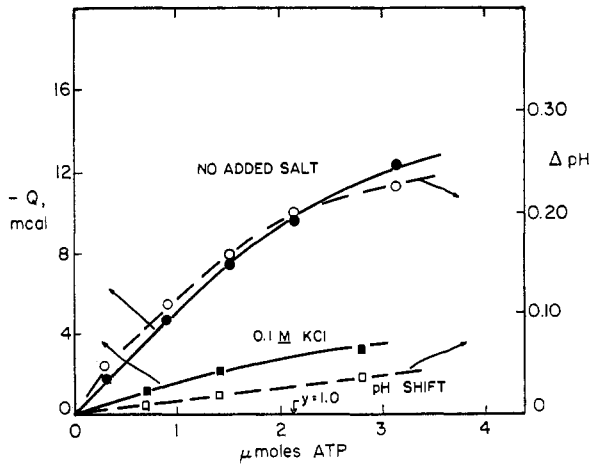

FIGURE 5: Calorimetry of mixing ATP with 2.1 μmol of deionized horse oxyhemoglobin at 25°. pH of the solutions were within 0.01 pH unit of one another before mixing. Solid circles and squares: right ordinate. Lines only connect data points.

The continuous lines on Figure 5 simply connect data points. The data were fitted later with parameters using Olofsson's method (1969). In Olofsson's method, the calorimetric heats yield the intensive quantity h. a and b are concentrations of the reactants after mixing; K and ΔH are the molar association constant and enthalpy change for formation of a simple bimolecular complex. The extensive quantity Q, the calorimetric heat developed in a volume V, is related to h by $h = Q/aV$. By keeping the amount of ATP relative to protein sufficiently low, *i.e.*, by keeping ν low, there can be held conformity to the restrictions involved in Olofsson's method, namely, that only one association complex forms. The equation used is

$$(\Delta H)^2 + (\Delta H)h\left(1 + \frac{a}{b} + \frac{1}{bK}\right) + h^2\frac{a}{b} = 0 \qquad (3)$$

Upon gathering enough data points, reasonably consistent values for ΔH and K finally were obtained. There is, of course, some latitude in the fitting parameters for Olofsson's equation, eq 3, which is manifest as error in the values for ΔH and

TABLE VI: Thermodynamic Parameters[a] from Calorimetric Studies of ATP–HbO_2 Interactions.

Initial pH	Cl^- (M)	Temp (°C)	K_1 (M^{-1})	ΔH (kcal mol^{-1})	ΔG (kcal mol^{-1})	ΔS (eu)
6.93 ± 0.03	—	25	$6.3 \pm 2.0 \times 10^4$	−5.2 ± 0.2	−6.5 ± 0.2	4.5 ± 1.5
7.05 ± 0.03	0.10	25	$8.0 \pm 0.2 \times 10^2$	−4.5 ± 0.5	−4.0 ± 0.2	−1.5 ± 2.5
7.32 ± 0.04	—	5	$6.0 \pm 2.0 \times 10^3$	−10.4 ± 1.4	−4.8 ± 0.2	−20 ± 6
6.50 ± 0.05	0.005	5	$1.3 \pm 0.5 \times 10^5$	−10.3 ± 0.2	−6.4 ± 0.3	−14 ± 2

[a] These are standard overall quantities. The free energies and entropies may be converted to unitary values according to Gurney (1953).

TABLE VII: Thermodynamic Parameters from Calorimetric Studies of DPG–HbO_2 Interactions.

Initial pH	Cl^- (M)	Temp (°C)	K (M^{-1})	ΔH (kcal mol^{-1})	ΔG (kcal mol^{-1})	ΔS (eu)
6.93 ± 0.03	—	25	$4.0 \pm 1.0 \times 10^3$	−9.1 ± 0.6	−4.9 ± 0.2	−14 ± 3
7.05 ± 0.03	0.10	25	$4.0 \pm 1.0 \times 10^2$	−8.5 ± 0.8	−3.5 ± 0.2	−16 ± 6
7.32 ± 0.04	—	5	$2.0 \pm 1.0 \times 10^4$	−10.8 ± 0.8	−5.4 ± 0.3	−19 ± 4
6.50 ± 0.04	0.005	5	$2.2 \pm 1.4 \times 10^5$	−11.2 ± 1.0	−6.7 ± 0.4	−16 ± 5

K. Table VI summarizes these for the ATP system, and Table VII summarizes them for DPG.

Two other potential contributions to the interaction heats need consideration. Even if the solutions before mixing are fairly precisely adjusted to the same pH (±0.01 pH unit, usually) and the work was carried out to insure that that was the case, there still occurred pH shifts upon mixing horse HbO_2 and some of the substrates. The magnitude of such shifts, ΔpH, for the system ATP–HbO_2, in which the initial pH for both components was 6.93, is shown in Figure 5, with the right-hand ordinate. The mixtures were drawn from the instrument after the calorimetric run, and were measured for pH value in the same way that the separate components were measured beforehand.

There may be, therefore, proton ionization shifts by both the substrate and protein, attending their interaction. The view we take of this, as a contributor to the overall heat, is in the Discussion section. In the meantime, there also was carried out (B. Boman and R. Lovrien, unpublished data), point by point potentiometric H^+ titration and calorimetry of H^+ addition to horse HbO_2 in the same pH region. The change in protonation on the protein as a function of pH was $\partial r_{H^+}/\partial pH = -9.5$, centered on pH 7 at 25°, in 0.02 M KCl, in fair agreement with Antonini *et al.* (1965). The enthalpy of adding H^+ to HbO_2 under the same conditions was −7.3 ± 0.3 kcal/mol of H^+, from 15 measurements. This is in agreement with the values expected for human hemoglobin; Tanford and Nozaki (1966) consider it to be −7 to −8 kcal per mol, from van't Hoff methods.

The resin deionized HbO_2 had a pH close to 6.9 at 25°, with no added salt, acid, or base. At 5°, the corresponding pH was 7.3. It was assumed that such HbO_2 samples have all their sites open to anion binding, in the calorimetric measurements in which no supporting electrolyte was used.

It is of interest to see how consistent some of our DPG binding data is, from two different methods, upon correcting in a simple way to conditions corresponding to more physiological conditions. These are at about 25°, 0.1 M chloride (pH 7.0). Using our dialysis equilibrium results, at pH 6.4 and 5°, in 0.1 M chloride, from Table V, we start with $K_{app} = 1.3 \pm 0.1 \times 10^4$. By correcting this to pH 7 *via* the exponential electrostatic factor (Discussion section), and then using the van't Hoff equation with the enthalpy from Table VII, we arrive at $K = 6 \pm 2 \times 10^2\ M^{-1}$ for DPG binding. The *K* value from calorimetry fitting under quite similar conditions (Table VII) is $4 \pm 1 \times 10^2\ M^{-1}$. This leads to −3.5 ± 0.3 kcal of free energy for DPG binding to horse HbO_2 under these conditions.

Discussion

It is impractical to compare our results to more than a fraction of the literature of organic phosphate binding to hemoglobin. For human HbO_2, with various temperatures and salt concentrations, Luque *et al.* (1969), Garby *et al.* (1969), and Chanutin and Hermann (1969) have reported that such phosphates do in fact bind. Keeping in mind the mammalian species differences, and that solvent parameters are often quite different, we generally agree most closely with the results of Chanutin and Hermann. Benesch *et al.* (1969) have considered that organic phosphates engage in negligible or zero binding with human HbO_2, in 0.1 M chloride at 20° and physiological pH.

In Chanutin and Hermann's results, it is evident that if they had extrapolated the steeply rising branch of their Scatchard plots back to the abscissa, they also would have intercepted on the abscissa between 1 and 2. They fitted their results with the standard polyequilibrium expression for more than one site. We agree that there probably exists more than one anion binding site. Their cacodylate buffering system is especially useful in that it is either noncompetitive, or less so than most other common electrolytes.

Competitive equilibria between inorganic electrolyte anions and organic phosphate anions is a topic previously discussed

by other authors. Benesch *et al.* (1969) found that at sufficiently high NaCl concentrations, the influence of DPG on hemoglobin's oxygen affinity disappears. Our results, at least for P_i, indicate that supporting electrolyte anions bind with association constants of the order of 10^2 to 10^3; $K_1 = 400 \pm 200$ M^{-1} for P_i at pH 6.8, 5°. Such values are lower than those for the organic phosphates by two to three orders of magnitude. However, in many *in vitro* experiments, and perhaps *in vivo*, chloride concentrations are in fact two to three orders of magnitude greater than those of endogenous organic phosphates. It is the product terms of K and C for the ions which are of central importance in governance of binding processes. Hence salt ions may exert considerable influence beyond their usual Debye–Hückel ionic strength effects, it they bind at all.

Let us consider a protein with two independent kinds of binding sites, n_1 and n_2 of them, and that both kinds engage in independent equilibria with substrate A and competitor B. The notation used is in the introduction above. There are n_1P_T total sites of class n_1 in solution, leaving n_1P free sites available to bind A and B. Likewise n_2P free sites are available to bind A and B. The equilibria are:

$$\begin{aligned} n_1P + A &\overset{k_{A1}}{\rightleftharpoons} P_1A \\ n_1P + B &\overset{k_{B1}}{\rightleftharpoons} P_1B \\ n_2P + A &\overset{k_{A2}}{\rightleftharpoons} P_2A \\ n_2P + B &\overset{k_{B2}}{\rightleftharpoons} P_2B \end{aligned} \tag{4}$$

The total amount of B, B_T is known if one used deionized protein and adds all salt. Mass conservation and site conservation lead to

$$\begin{aligned} B_T &= C_B + P_1B + P_2B \\ n_1P &= n_1P_T - \nu_{A_1}P_T - \nu_{B_1}P_T \\ n_2P &= n_2P_T - \nu_{A_2}P_T - \nu_{B_2}P_T \end{aligned} \tag{5}$$

Standard competitive binding equations may be utilized (Steinhardt and Reynolds, 1969), which in our notation are

$$\begin{aligned} \nu_{A_1} &= \frac{k_{A_1}C_A}{1 + k_{A_1}C_A}(n_1 - \nu_{B_1}) \\ \nu_{B_1} &= \frac{k_{B_1}C_B}{1 + k_{B_1}C_B}(n_1 - \nu_{A_1}) \end{aligned} \tag{6}$$

with similar expressions for ν_{A_2} and ν_{B_2}.

Combination of eq 4, 5, and 6 yields

$$\begin{aligned} \nu_{A_1} &= \frac{n_1k_{A_1}C_A}{1 + k_{A_1}C_A + k_{B_1}C_B} \\ \nu_{A_2} &= \frac{n_2k_{A_2}C_A}{1 + k_{A_2}C_A + k_{B_2}C_B} \\ \nu_{B_1} &= \frac{n_1k_{B_1}C_B}{1 + k_{A_1}C_A + k_{B_1}C_B} \\ \nu_{B_2} &= \frac{n_2k_{B_2}C_B}{1 + k_{A_2}C_A + k_{B_2}C_B} \end{aligned} \tag{7}$$

$$B_T = C_B\{1 + k_{B_1}P_T[n_1 - \nu_{A_1} - \nu_{B_1}] + k_{B_2}P_T[n_2 - \nu_{A_2} - \nu_{B_2}]\}$$

Scatchard plots may be calculated on the basis of equations (7), after choosing $k_{A_1}, k_{A_2}, \ldots, B_T, P_T$, and n_1 and n_2. The solutions depend on starting with a value for C_A and iterating until the expression for B_T is satisfied. At that point, all ν values are determined. Hence, since $\nu_{A_1} + \nu_{A_2} = \nu_{A_T}$, ν_{A_T}/C_A *vs.* ν_{A_T} may be plotted. Upon varying C_A and repeating the process, B_T is held constant, as is usually done in an experiment. A_T, the total amount of substrate, is readily calculated if desired.

In the present case, we let $n_1 = n_2 = 1$, and utilize values listed in Figure 7 to construct Scatchard plots. Two aspects are immediately apparent. (i) Even if the binding constants of competitor B are well below those of substrate A ($k_{B_1} = 8 \times 10^2$ and $k_{A_1} = 1.3 \times 10^5$) at B_T values of only a factor of about ten higher than P_T (5×10^{-3} and 3×10^{-4} M, respectively), the effect of B is to drastically drop the intercept on the ordinate. (ii) Most data are of poorer quality in the extreme ranges for ν_{A_T}, especially with low salt. In the ranges in which the best analytical data occurs, the points lead to indications of one strong site, but interception occurs on the abscissa at $\nu_T > 1$. With the equations above, utilizing only two classes of sites, a rather large amount of data can be fitted within experimental error. Hence we have no strong opinions concerning the existence of more than two sites, at least for very specific sites. Chanutin and Hermann (1969) invoke up to four total sites for some organic phosphates. Their total substrate concentrations ranged over values markedly higher than ours, *i.e.*, over higher ν values.

The AMP equilibria with HbO_2 (Figure 4) probably represents a different kind of problem than that of multiple but completely independent sites. The strongly positive sloping Scatchard plot as in Figure 4 actually occurs, however, with other proteins. We have recalculated binding data for other proteins reported in the literature, and find that if Scatchard's method is used, plots similar to Figure 4 occasionally ensue. Some Scatchard plots already in the literature obviously partake of the same behavior, *e.g.*, in the cytochrome *c*–dodecyl sulfate system studied by Burkhard and Stolzenburg (1972).

It is likely that, no matter what the total number of binding sites, nor what the levels and ratios of substrates, competitors, and proteins, only negative sloping Scatchard plots will occur for those cases in which all binding sites are truly independent throughout the accessible range of ν_{A_T}. The production of positively sloping Scatchard plots probably indicates intramolecular linkage between available and potentially available sites, governed by the extent of binding and kind of ions which bind. The term linkage is used in the sense of Edsall and Wyman (1958). We shall not analyze Figure 4 further here, except to remark that it is especially striking compared to other such cases, and probably is rather dependent on total competitor level, B_T.

The pH shifts with deionized protein, ΔpH in Figure 5, for example, should be considered with respect to possible contributions to calorimetric enthalpies upon changes in state of protonation, by both substrate and protein. The pK and standard enthalpy of ionizations for $H_2PO_4^-$ and $HATP^{3-}$ are at 25°, 6.78 and −0.80 kcal per mol, and 6.95 and −1.68 kcal per mol, respectively (Alberty, 1969). Thus, in the pH 7 region, an increase in pH might produce a few hundred small calories of exothermic heat from the reaction $HATP_3^-$

$\rightleftarrows H^+ + ATP^{4-}$ but not nearly enough heat to account for our observations. In the case of the protein, one might initially assume that upon binding of an anion, with $\Delta pH \sim +0.1$, about one imidazole side chain becomes protonated, according to the titration curve. This could, at first sight, produce -7 kcal of heat/mol of substrate, leaving a OH^- behind which increases the pH. However, in the first place, about $+13$ kcal of heat is involved in the reaction $H_2O \rightleftarrows H^+ + OH^-$, hence the net heat from such a course should be endothermic by about $+8$ kcal/mol. Secondly, the origins of the pH shift are rather likely those produced by changes in protein electrostatic interaction with its surroundings, which in turn depends on the binding process.

The ΔpH effect may have the same origins that Scatchard and Black studied (1949) in the serum albumin system. Binding of charged substrates may lead to appreciable pH shifts, according to Scatchard and Black's expression $\Delta pH = -0.868w\Sigma\nu_A z_A$. This is derived on the basis of no appreciable shift in side-chain titration upon binding, and has been reinvestigated by Cassel and Steinhardt (1969). The matter can be summarized in the following way. With 10^{-4} M protein, even if only one imidazole side chain possibly were involved, there would be 10^{-4} M imidazoles to be titrated. But at pH 7, even if $\Delta pH \sim +1$, only 10^{-7} M H^+ exists in free solution. Therefore, without an external source of H^+ or OH^-, major shifts in the state of side-chain ionization cannot occur from binding *per se*, unless a source were a proton transferring from $H_2PO_4^-$ or $HATP^{3-}$ and which binds to the protein side chain. We have no way of deciding about that, and leave it open as a possibility. Therefore in sum, there is a fair chance that the observed enthalpies are due to binding itself, and not to imidazole protonation, pH shifts during binding notwithstanding.

The pH dependency of organic phosphate binding to hemoglobin perhaps should be viewed using the simplest model available. We assume that there exists general Coulombic interaction between charged protein and charged substrate, with pH control of both these charges, Z_P and z_A, respectively. The interaction treated here does not involve details of binding sites, as in the case of Rigg's (1971) treatment, but considers only the cruder model of a charged spherical macroion with interaction parameter w. If the intrinsic binding constant with $\bar{Z}_P = 0$ is K_0, and the pH-dependent binding constant is K_Z, in general (Tanford, 1961), $k_Z = k_0 e^{-2wZ_P z_A}$. The charge Z_P is a function of pH, and also changes sign over the pH range of interest. For DPG and ATP, we may use $z_A = -4$ and -3, respectively, from Benesch *et al.*'s (1969) analytical data. Under our conditions, where $\mu = 0.033$, we obtain $w = 0.046$ for the hemoglobin tetramer, with radius 30 Å. If k_0 is pH independent, then upon differentiation, $\delta \log K_Z/\delta pH = -(2/2.303)wz_A\delta Z_P/\delta pH$, which is eq 1, above. It is assumed $Z_P \cong Z_{H^+}$. From the foregoing values for w and z_A, and the slope of the titration curve, we obtain $\delta \log K_Z/\delta pH = -1.5$ as a calculated value under these conditions. We observed a value of about -1.2 in the pH 6.5–7 region, from the data of Table V. Benesch *et al.* (1969) obtain in 0.1 M NaCl, for deoxyhemoglobin, about -1.3 in the pH 7.0–7.8 region using their Table I data. Because of differing salt concentrations these can be only rough comparisons, but they serve to indicate the effects of invoking the simplest available Coulombic interaction model. Since the charge on the organic phosphates is rather large, the sign and magnitude of the $Z_P z_A$ term changes rapidly over relatively small pH ranges in the isoionic pH region. Roughly at least, some of the pH dependency of ion binding processes to hemoglobin probably can be accounted for from simple Coulombic effects, expressed *via* the $e^{-2wZ_P z_A}$ term. Consideration of Coulombic interactions probably can help in understanding some of the pH, ionic strength, and buffer-dependent binding of organic phosphates to HbO_2. However, even if some sorting out of pH dependency of binding can be made using the exponential factor as it was employed above, what one inserts into w for the radius remains a question. We assumed that the whole protein should be encompassed, but perhaps a more restricted constellation of charges as invoked by Riggs (1971) is more appropriate, with a smaller effective Z_P but also a smaller radius which would increase w. At the same time, the precise placement of charges in various discrete charge models with the same net charge, may represent large and variable electrostatic energy contents, as shown by Tanford (1961a).

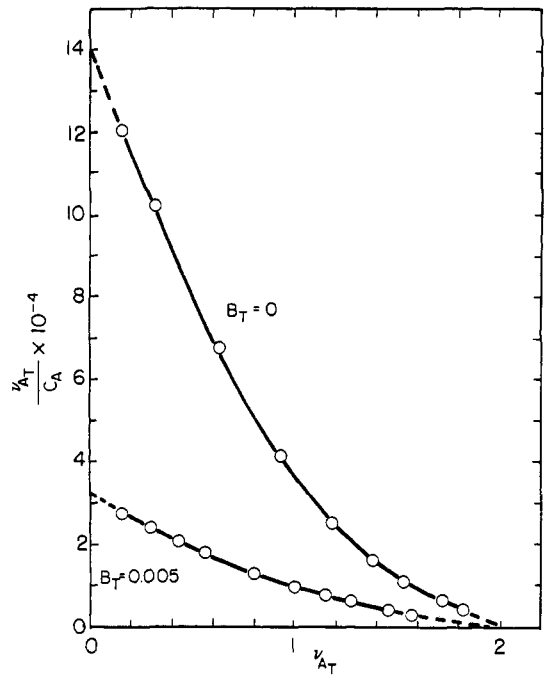

FIGURE 7: Calculated plots using eq 7. The following parameters were chosen: $n_1 = n_2 = 1$, $k_{A_1} = 1.3 \times 10^5$, $k_{A_2} = 1.0 \times 10^4$, $k_{B_1} = 8.0 \times 10^2$, $k_B = 4.0 \times 10^2$, $P_T = 3.0 \times 10^{-4}$ M.

From the temperature dependency of the calorimetric binding enthalpies for ATP and DPG to HbO_2 (Tables VI and VII), heat capacity changes accompanying binding may be estimated. That is, we assume $\partial\Delta H/\partial T \simeq \Delta\Delta H/\Delta T$ over the 20° temperature range, 5–25°. The value of ΔC_P for the DPG reaction is $+90 \pm 70$ cal deg^{-1} mol^{-1}, using deionized protein with no salt added. In ATP association, $\Delta C_P = +260 \pm 80$ cal deg^{-1} mol^{-1}.

This cannot readily be assigned to some mechanism simply involving transfer of a substrate moiety from a polar to a nonpolar solvent. The sign of ΔC_P is positive, and the adenosine group hardly resembles the model compounds for which numbers have been tabulated (Tanford, 1970; Wishnia, 1969). The following are possibilities. (i) ATP binding involves a macromolecular change, perhaps a loosening of structure. (ii) ATP binding induces a conformation change which is accompanied by exposure of nonpolar groups to water. (iii) The heat capacity effects stem from polar parts of ATP, transferred from water to a new environment. (iv) These contrast with the low ΔC_P of DPG binding. It perhaps fits into a site

without many requirements for reshuffling, whereas ATP requires some protein rearrangement. All such possibilities are open to question; we are dealing with HbO_2 here and not deoxyhemoglobin. Possibly the discussion of Perutz (1970), regarding the fitting of organic phosphates to deoxyhemoglobin, partly extends to HbO_2, but again this is speculation.

The ΔH and ΔS parameters for binding are fairly typical for anion binding to proteins in general, especially in the exothermic enthalpies. They quite likely are sums of values for perhaps numerous individual processes, needing dissection.

Likewise, the data of Figure 4, with AMP, probably represents the sum of several events. Whatever they are, some of them may well be different in kind from those of ATP and DPG. Such a plot probably is not the result of static binding to multiple sites, competition or not. It indicates some kind of chemical linkage, between sites set in motion upon binding the first AMP, making binding of more than one AMP progressively easier. If there is more than one site available, the species $HbO_2 \cdot (AMP)_n$ tends toward a population in which n is either zero, or $n > 1$, depending on the total amount of AMP which is present. The plot in Figure 4 does not necessarily extrapolate to zero, but probably has some intercept on the ordinate.

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