Proton-Dependent Dissociation Equilibrium of Hemoglobin. 1. A 700-Nanometer Light-Scattering Study on Horse Methemoglobin in the pH Range 4.8 to 7.2†

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ABSTRACT: The effect of proton concentration upon the subunit dissociation of horse methemoglobin has been investigated at two ionic strengths by light scattering photometry at 700 nm. Differential refractometry revealed a slight but systematic decrease of the specific refractive index increment with decreasing protein concentration for solutions in dialytic equilibrium with the solvent. In the pH range 4.8–7.2 the dissociation can be described by a simple equilibrium between tetramers and dimers. The dissociation constant K_d of the met derivative is found to be very similar to those of the O_2- and CO-ligated states. From the slope of a plot of log K_d vs. pH, the number of protons bound is $n = 1.3 \pm 0.1$ resulting from an increase in the pK values of two groups upon dissociation. These two groups must be identical because the dissociation is symmetrical.

Tetrameric hemoglobins reversibly dissociate into dimeric half-molecules under a variety of conditions. Most studies of this phenomenon are concerned with the dissociation effected by high concentrations of neutral salts. This publication deals with the proton-induced dissociation in slightly acidic solution. The enhancement of dissociation with increasing proton concentration indicates that the process is associated with proton binding. This implies differences in the proton affinity of certain groups in the dimer and in the tetramer.

With respect to the origin of this affinity change, it seems sensible to differentiate between conformation dependent alterations of the intrinsic pK values of distinct groups and alterations of the electrostatic interaction of the protein with small ions in the solvent affecting all charged groups of the polyelectrolyte. Extreme pK shifts arise if groups nontitratable in the tetramer become accessible upon dissociation. The change in electrostatic interaction can be assessed quantitatively on the basis of model approximations requiring measurements at different ionic strengths. To investigate such phenomena one can follow the concentration dependence of either the average molecular weight or the proton binding. In the work reported here, the average molecular weight was measured in the pH range 4.8 to 7.2 by means of light-scattering photometry.

Materials and Methods

Hemoglobin. Horse hemoglobin was prepared from freshly drawn blood following the method of Taylor and Hastings (1939) and oxidized by a twofold molar excess of $K_3Fe(CN)_6$ at 4 °C. All salts were removed by gel filtration (Sephadex G-25, pH 8.5) and mixed bed ion-exchange chromatography. Complete oxidation was checked spectrophotometrically (Cary 16). The eluate was lyophilized and stored at 4 °C.

For light-scattering experiments, freeze-dried samples were dissolved in aqueous solutions of NaCl, 0.1 and 0.01 M, respectively. pH was adjusted by careful titration with 0.1 M HCl avoiding local denaturation. All solutions were dialyzed to equilibrium (30 h minimum) at 4 °C vs. a 30-fold volume NaCl solution of equal pH and ionic strength.

Dust was removed from the Hb[1] solutions and solvents by filtration through membrane filters of 0.2-μm pore size (Sartorius-Membranfilter GmbH, Göttingen) under small excess pressure of nitrogen. In several cases, part of the solution was subjected to centrifugation (1 h at 90 000g, Beckman Spinco L2 65B). The procedures were of equal efficiency.

Hemoglobin concentrations were determined according to Bethke and Savelsberg (1950) from the absorbance of the CN derivative at 540 nm ($\epsilon_{540}^{Hb(III)CN} = 44.350$ l. mol^{-1} cm^{-1}) based on a molecular weight of Hb of 64 500.

Concentration determination was carried out on three samples of every solution in the light-scattering cell immediately after the measurement.

Light-Scattering Photometry and Differential Refractometry. A Brice-Phoenix light scattering photometer was modified to allow for measurements on hemoglobin in the wavelength range 600–700 nm (Schroeder, 1970).

The light source was a 24 V/250 W quartz halogen lamp supplied with a highly stabilized voltage utilizing a reference signal for voltage stabilization. Monochromatic light was obtained by means of interference filters (Oriel) of 100-Å half-band width. For all measurements the sample cell was kept at 25 ± 0.05 °C in a thermostated cell holder. A specially selected red-sensitive photomultiplier (HTV, type R 446) was used as a detector. The photocurrent was registered after amplification (1:10, signal-noise ratio 40 db) simultaneously with a digital voltmeter and a recorder. The recorder output served for controlling the stability of the scattered intensity with time. Contamination of the solutions with large particles are immediately detected by unstable recorder tracings.

Correction factors for the scattering volume C_v (Kerker et al., 1964) and the refractive index C_n (Hermans and Levinson, 1951) were applied to all measured scattering intensities. The

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[1] Abbreviations used: Hb, hemoglobin; Hb(III), methemoglobin; Hb(III)CN, cyanomethemoglobin; Hb(III)H_2O, aquomethemoglobin; Hb(II)O_2, oxyhemoglobin; Hb(II)CO, carbon monoxyhemoglobin.

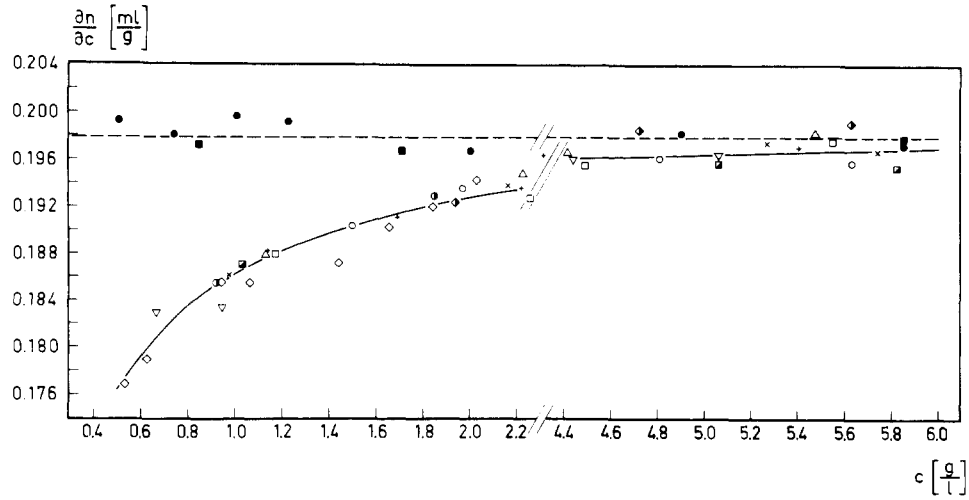

FIGURE 1: Effect of dialysis on the refractive index increment, $\partial n/\partial c$, of horse Hb(III). Upper curve: nondialyzed solutions in 0.1 M NaCl (●) pH 6.95 and (■) pH 5.0. Lower curve: dialyzed solutions in 0.01 M NaCl (○) pH 6.95, (□) pH 6.25, (◇) pH 6.05, (▽) pH 5.75, (△) pH 5.35, (+) pH 5.00; 0.01 M Bistris buffer (×) pH 7.35; 0.1 M NaCl (◑) pH 6.65, (◪) pH 5.35, (◆) pH 5.05. The dashed line represents the average value $(\partial n/\partial c)_m$ = 0.198 ± 0.001 (ml/g) for nondialyzed solutions.

instrument was calibrated at five wavelengths: 436, 546, 600, 669, and 700 nm with three different fractions of monodisperse polystyrene (Pressure Chemical Co., Pittsburgh, Pa.) in cyclohexane. The weight over number average molecular weight ratio M_w/M_n was specified to be 1.06.

The specific refractive increments $\Delta n/c$ were determined with an improved Brice-Phoenix differential refractometer. In order to increase precision and to avoid subjective readouts of scale deflections, the microscope eyepiece was replaced by a photometric device consisting of a photomultiplier with a slit mounted on a sliding carriage perpendicular to the optical axis. Light source and power supply were similar to the equipment used in the scattering photometer. More than 50 calibration measurements on aqueous solutions of highly purified red-heated KCl were performed using the Δn values determined interferometrically by Kruis (1936). The accuracy of the modified differential refractometer was estimated to be $\pm 10^{-6}$ for Δn.

Both instruments were calibrated for the five wavelengths mentioned above to rule out any systematic errors and to permit a check on the required wavelength dependency of the Rayleigh ratios and the specific refractive increments $\Delta n/c$ of the calibration substances used.

Measurements on Hb are handicapped by strong light absorption except at the long-wavelength end of the visible region. At 600 nm, for instance, concentration-dependent absorption corrections still proved incapable of yielding reasonable values of the 2d virial coefficient. At 700-nm absorption correction was negligible for Hb concentrations $C \leq 8$ g/l. Therefore, all measurements on Hb solutions were carried out at 700 nm despite the appreciable loss of scattering intensity.

As a result of equilibrium dialysis, the chemical potential of the diffusible components was the same in the solution and in the solvent. Thereby the system is reduced to a pseudo-two-component system to which the following equation can be applied (Ooi, 1958):

$$H\frac{c}{\tau} = \frac{1}{M_w(P(\theta)} + 2Bc \qquad (1)$$

where c = protein concentration in g/l., τ = absolute excess turbidity, M_w = weight average molecular weight, $P(\theta)$ = intensity distribution function, B = 2d virial coefficient. H is the usual light scattering constant, $H = 32\pi^3 n^2 (\partial n/\partial c)_\mu^2 / 3N\lambda^4$, where n is the refractive index of the solvent, N is Avogadro's number, λ is the wavelength of light, and $(\partial n/\partial c)_\mu$ is the refractive index increment at constant chemical potential obtained with dialyzed solutions.

Results and Discussion

Calibration Measurements with Polystyrene in Cyclohexane. The specific refractive increments of three polystyrene fractions with different molecular weight proved to be independent of molecular weight. All data obtained at five wavelengths fit very well to a simple Cauchy dispersion relation, i.e., $(\partial n/\partial c)$ is a linear function of λ^{-2}.

As required by theory, the linear relationship between the reduced relative scattering intensities $(c/R_{c=0}$ and $1/M$ was confirmed, and the corresponding absolute turbidities τ were proportional to λ^{-4}. At 25 °C the second virial coefficient B is negative as is typical for a poor solvent. Its absolute value decreases with increasing molecular weight. As far as the polystyrene molecular weights and temperatures are comparable, B is of the same order of magnitude as reported by other authors (Krigbaum and Flory, 1953; Krigbaum, 1954).

Horse Hb(III) in the pH Range between 4.8 and 7.2. The specific *refractive increment* $\partial n/\partial c$ of solutions of horse Hb(III) under different conditions is plotted as a function of concentration in Figure 1. With dialyzed solutions—indexed μ—a slight systematic decrease of $(\partial n/\partial c)_\mu$ occurs at low concentrations. This concentration dependence has also been reported by Stauff and Jaenicke (1961) for bovine serum albumin in guanidine hydrochloride solution and by Noelken and Timasheff (1967) for bovine plasma albumin at concentrations smaller than 0.3 g/l. The phenomenon, though not yet systematically analyzed, has been claimed to be an artefact. Kratohvil et al. (1964), for instance, has suggested that the Δn values are underestimated when measured by the Brice Phoenix refractometer in the low concentration range.

However, if any redistribution of solvent components was avoided simply by omitting dialyzation, the expected concentration independence could well be demonstrated with our improved instrument also in the low concentration range. Seventeen measurements on nondialyzed samples—indexed m—in 0.1 M NaCl at pH 7 and 5 are included in Figure 1, their average value being $(\partial n/\partial c)_m$ = 0.198 ml/g. If this constant value were used in eq 4, the extent of dissociation

would be calculated 8–10% higher than with the actual concentration-dependent $(\partial n/\partial c)_\mu$ values. It should further be noted that a deviation from proportionality between scale deflection Δd and refractive index difference Δn is much more likely to appear at larger Δd, i.e., high concentration, and that the variations due to the poorer accuracy of conventional refractometers compared with interferometric devices should be statistical rather than systematic. To exclude any systematic error, linearity between scale deflection and Δn was rechecked by additional calibration measurements with KCl. For polystyrene and myoglobin, Δn proved to be linear with c.

From the independence of $\partial n/\partial c$ in the dialyzed as well as the nondialyzed series (see Figure 1) of pH also in the range where dissociation into dimers occurs (see below), it can be concluded that the specific refractive increment is the same for Hb tetramers and dimers. In principle information on preferential binding of solvent-constituent ions to the protein can be deduced from the difference between $(\partial c/\partial n)_\mu$ and $(\partial n/\partial c)_m$ at zero protein concentration (Noelken and Timasheff, 1967). Quantitative statements are withheld here since for $(\partial n/\partial c)_\mu$ this extrapolation remains doubtful.

The angular *scattering intensity* distribution of horse Hb(III) proved symmetric, i.e., $P(\theta) = 1$ under all conditions applied. Consequently, all measurements were made at an angle of 90° relative to the incident beam. In total, the scattering intensities of 15 concentration series of Hb solutions in 0.1 and 0.01 M NaCl with concentrations between 0.9 and 7.2 g/l. were measured in the pH range from 4.8 to 7.2. The concentration dependence of the reduced absolute turbidities of Hb solutions at different values of pH and ionic strength is depicted in Figure 2.

In the pH range 4.8 to 7.2, no scattering intensities corresponding to apparent molecular weights $M_w < 32\,000$ were observed. This is in accordance with the fact commonly accepted that no detectable amounts of monomers are present under the conditions applied (Kellet, 1971; Kellet and Schachman, 1971; Bucci, 1971; Hanlon et al., 1971). Formation of monomers only appears below pH 4.8 (Bucci, 1971) or at high salt concentration (Elbaum and Herskovits, 1974). Some of our experiments at pH 4.5 yielded scattering intensities which could be attributed to $M_w < 32\,000$. However, it should be noted that light-scattering experiments on horse Hb(III) below pH 4.8 are poorly reproducible. In most cases extremely high and fluctuating scattering signals were obtained, indicating that formation of single chains was irreversible and accompanied by an unspecific aggregation. For the calculations of the dissociation constants of the tetramer–dimer equilibrium, no data below pH 4.8 were used.

Linear extrapolation of a series of measurements on horse Hb(III) in 0.1 M phosphate buffer at pH 7.2 to zero concentration yielded a molecular weight of $M_w = 63\,700 \pm 2000$. The second virial coefficient B is negative. In contrast to this result plots for lower pH values in Figure 2 show a shift to higher $H(c/\tau)$ values, an upward curvature at low protein concentration, and an increase of the slope of the linear part at higher protein concentration. The first two observations indicate increasing dissociation with decreasing Hb concentration and increasing proton concentration. The third phenomenon, increase of one order of magnitude in B, reflects the enforced protein–solvent interaction due to higher net charge.

With reversibly interacting proteins such as hemoglobin, the concentration dependence of the scattering intensity is governed not only by the second virial coefficient but also by the dissociation. Unambiguous analysis of the data in terms

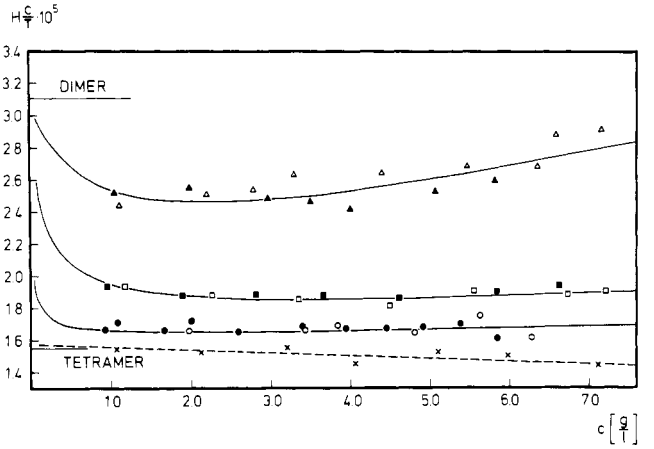

FIGURE 2: Light-scattering data for horse Hb(III). Solvent: 0.1 M phosphate buffer (X) pH 7.2; 0.01 M NaCl (open symbols) and 0.1 M NaCl (closed symbols), pH adjusted by titration with 0.1 M HCl, (○, ●) pH 6.95; (□, ■) pH 6.25; (△, ▲) pH 5.30. The dashed line represents linear extrapolation to $c = 0$ at pH 7.2 yielding $M_w = 63\,700$. The solid lines represent calculated curves with K_d and B obtained from the fitting program accumulating the data for 0.1 and 0.01 M NaCl.

of the dissociation constant K_d is impossible without assumptions or independent information about B. There is no principle justification for presuming the second virial coefficients to be identical for the different molecular species involved in the equilibrium. If they are not, the apparent mean B—which is a z average in light scattering—will change with the fractional composition of the molecular population, i.e., will be concentration dependent on its part.

For dilute protein solutions, the second virial coefficient B is made up of two additive contributions (Tanford, 1961), a charge-independent excluded volume term B_e and a charge-dependent term B_c:

$$B = B_e + B_c \tag{2}$$

The main contribution within B_c is the Donnan term:

$$B_{\text{Donnan}} = \frac{1000 v_1 Z^2}{4 m_3 M^2} \tag{2a}$$

where v_1 = specific volume of the solvent (ml g^{-1}), m_3 = NaCl molality (mol kg^{-1}), and Z is the average net charge of the protein.

The excluded volume term can be calculated from the molecular weight M and the partial specific volume $\bar{v}$ of the protein:

$$B_e = 4\bar{v}/M \tag{2b}$$

Assuming a hard sphere model and $\bar{v} = 0.75$ ml g^{-1} to be the same for the tetrameric and dimeric Hb, one obtains $B_e = 4.7 \times 10^{-8}$ l. mol g^{-2} for the tetramers and 9.4×10^{-8} l. mol g^{-2} for the dimers. At the isoelectric point ($Z = 0$), B depends on the excluded volume term only. However, since in the neutral pH range dissociation is negligible, the apparent mean B_e (and in turn B) is practically constant within a concentration series. On the other hand, at acidic pH, i.e., high Z, where dissociation is substantial, the possible change in the mean excluded volume term is always one order of magnitude smaller than the corresponding value of the Donnan term. Therefore, it seems justified to assume a constant B within each concentration series of these experiments.

The second virial coefficient B determines the deviation of the system from ideality. A calculation of this deviation (Adams, 1965) on the basis of the B values encountered in

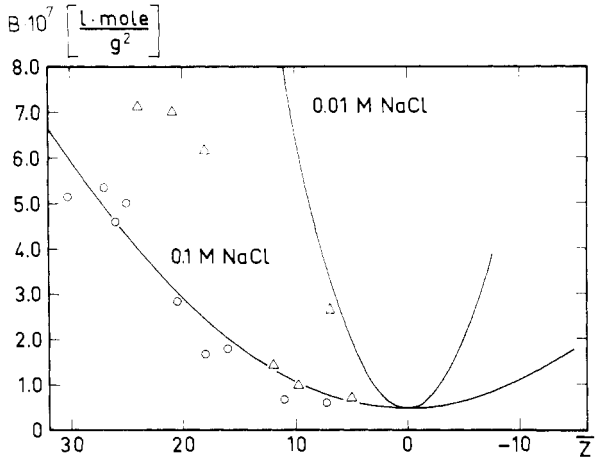

FIGURE 3: Comparison of second virial coefficients of horse Hb(III) obtained from light scattering with calculated values (solid lines). $\bar{Z}$ is the net average proton charge per tetramer obtained from titration experiments (Kubicki, 1970) on horse Hb(III) under identical conditions. Solvent: 0.1 M NaCl (○), 0.01 M NaCl (△).

these experiments demonstrates that the error introduced by setting concentrations for activities is substantially less than the experimental error. Therefore K_d in moles per liter can be expressed as:

$$K_d = \frac{c}{M_4/4}\left(\frac{\alpha^2}{1-\alpha}\right) \tag{3}$$

with

$$\alpha = c_{dim}/c \tag{3a}$$

designating the fraction of protein dissociated and M_4 the molecular weight of the tetramer (64 450). After introduction of α using the definition of M_w, eq 1 rearranges to:

$$H\frac{c}{\tau}\left(1-\frac{\alpha}{2}\right) - \frac{1}{M_4} = 2Bc\left(1-\frac{\alpha}{2}\right) \tag{4}$$

where

$$\alpha = \frac{M_4}{8}\frac{K_d}{c}\left(\sqrt{1+\frac{16c}{K_d M_4}} - 1\right) \tag{5}$$

A plot of the left-hand side of eq 4 against $c(1-(\alpha/2))$ gives a straight line with zero intercept and a slope $2B$.

Inserting α calculated from assumed K_d values (eq 5) and the experimental data for H, c, and τ in eq 4, a linear regression line can be calculated for every K_d. A trial and error method is applied with asserted sets of K_d values with decreasing stepwidth. The optimum K_d is obtained from the best least-squares fit and B from the slope of the corresponding regression line.

The second virial coefficients for two different ionic strengths are given in Table I. At neutral pH, B is on the order of magnitude of B_e for tetramers. No further statements are possible concerning the difference in B at 0.1 and 0.01 M NaCl except a general tendency for it to be slightly smaller at 0.1 M NaCl concentration, reflecting the well-known fact that addition of neutral salt suppresses the deviation from nonideality. The influence of pH, i.e., the net charge of protein on B, is much more pronounced (see Figure 3). From titration experiments (Kubicki, 1970) under identical conditions the net charge $\bar{Z}$ of horse Hb(III) was determined as a function of pH at Hb concentration c = 3.5 g/l. which is an average value for the concentrations applied in this study. If we assume that $\bar{Z}$ equals the net proton charge $\bar{Z}_H$ and other charge effects be-

TABLE I: Virial Coefficients of Horse Hb(III) at Various pH for Two Salt Concentrations of Solvent.

0.1 M NaCl		0.01 M NaCl	
pH	$B \times 10^7$ (1 mol/g²)	pH	$B \times 10^7$ (1 mol/g²)
6.95	0.6	6.97	0.7
6.65	0.7	6.80	2.7
6.20	1.8	6.50	1.0
6.00	1.7	6.30	1.4
5.75	2.9	5.76	6.2
5.24	5.0	5.40	7.0
5.14	4.6	4.98	7.1
5.03	5.4		
4.80	5.2		

side the Donnan term are neglected, B_e can be calculated as a function of $\bar{Z}$. The net charge obtained from titration of dissociable proteins is an average of Z_4 (tetramer) and Z_2 (dimer) and includes the contribution of n additional groups (see below) that become accessible upon dissociations, thus

$$\bar{Z} = Z_4(1-\alpha) + 2\left(\frac{Z_4+n}{2}\right)\alpha \tag{6}$$

with $(Z_4 + n)/2 = Z_2$. To evaluate the contribution of dimers to the Donnan term B_{Do} is calculated as Z average from $\bar{B}_{Do} = \Sigma_{i,j}B_{ij}M_iM_j(w_iw_j/M_w^2)$ $(i, j = 2, 4)$, with $w_2 = \alpha$, $w_4 = 1 - \alpha$, and $B_{ij} = (1000v_1/4m_3)Z_iZ_j/M_iM_j$. Thereby $\bar{B}_{Do}$ becomes concentration dependent as in the case of $\bar{B}_e$. Using eq 6 we obtain:

$$\bar{B}_{Do} = \frac{1000v_1}{4m_3}\frac{\left[Z_4\left(1-\frac{\alpha}{2}\right)+n\frac{\alpha}{2}\right]^2}{M_4^2\left(1-\frac{\alpha}{2}\right)^2} \tag{7}$$

As an approximation m_3 was replaced by the molarity of NaCl and v_1 was taken to be 1 ml/g. For $n = 0$, it is obvious that $\bar{B}_{Do} = B_{44} = B_{22} = B_{24}$ for all α. Trial calculations for $n = 2$ (see below) exhibited only small changes of $\bar{B}_{Do}$ with α and that for all $\bar{Z}$ values $\bar{B}_{Do}$ is very close (within 2%) to a value obtained from the simple relation $B_{Do} = (1000v_1/4m_3)\bar{Z}^2/M_4^2$. As the change of the excluded volume term B_e with decreasing $\bar{Z}$ never exceeds 5% of the total value of B, the solid lines in Figure 3 were calculated with $B = 4\bar{v}/M_4 + (1000v_1/4m_3) \cdot \bar{Z}^2/M_4^2$. The experimental data for the solutions of higher ionic strength (0.1 M NaCl) are in good agreement with the calculated curves even for high $\bar{Z}$ values, although no electrostatic interactions besides those imposed by the Donnan effect were taken into account. The poor fit of data for 0.01 M NaCl indicates that the neglect of other charge effects, e.g., charge fluctuations which make a negative contribution to B, are of greater influence at lower ionic strength.

In a plot of $-\log K_d = pk_d$ as a function of pH, the points arrange along a straight line (see Figure 4) with a slope of 1.3 ± 0.1. The slope of a pK_d–pH curve is identical with the net number n of protons bound or released upon dissociation (Wyman, 1964). As mentioned in the introduction, two different, though interdependent, phenomena will determine the net number of protons involved in this process: shifts in the intrinsic pK_s values of distinct ionogenic groups and changes in the electrostatic interaction of the protein and small solvent ions. For the interpretation of the numerical value of the slope obtained, it must be kept in mind that hemoglobin dissociation is symmetrical. Therefore, the number of protons involved must

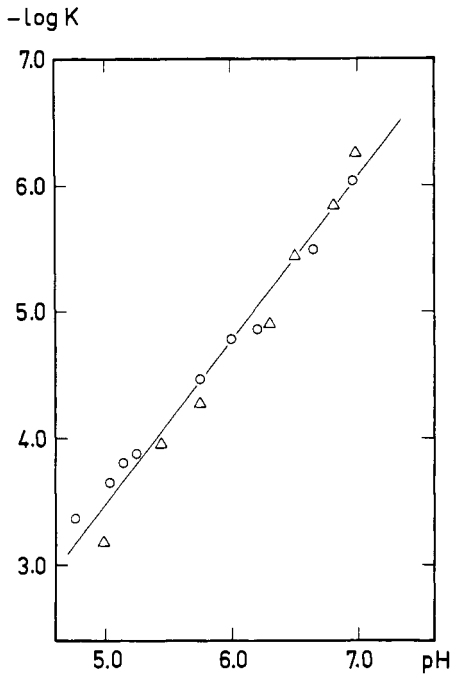

FIGURE 4: Variation of log K_d with pH for horse Hb(III) in 0.1 M (○) and 0.01 M (△) NaCl solutions. From a least-squares fit of data, the relation $-\log K_d = -2.9 + 1.3$ pH is obtained (solid line).

be even. With respect to a slope of 1.3 the involvement of 0 or 2 protons is the most probable alternative. If there were no distinct groups to be protonated, dissociation would be due exclusively to a change in electrostatic interactions. A diminution would be predicted by the theory of Linderstrøm-Lang for the splitting of a hard sphere into two hard spheres of half the volume each. This would result in a positive contribution to the pK_d-pH slope. Calculations based on this model of Linderstrøm-Lang yield values for the slope similar to those determined here. However, titration experiments to be reported later (Kubicki and Ohlenbusch, in preparation) indicate that in the acidic range there are at least two groups binding protons upon dissociation which must be identical.

There is no significant deviation of the pK_d-pH curve from linearity in the pH range covered by the measurements. Therefore, the position of the pK_s attributed to the groups in the dimers must be above and the position of the pK_s attributed to the groups in the tetramer below this range. Binding of two protons would be compatible only with relatively small simultaneous changes in electrostatic interactions which can also be concluded from the apparent insensitivity of the slope of the pK_d-pH curves to changes in ionic strength (see Figure 4).

In order to attempt identification of the proton-binding group, it seems reasonable to presume that this group would be liberated on dissociation. Regarding its pK_s value this means that it should be "normal" in the dimeric and "abnormally low" in the tetrameric state. In view of these requirements, of the proton-binding residues located in contact areas separated on dissociation, His FG4(97)β is the only candidate. In horse methemoglobin, this histidine may be hydrogen bonded to Thr C6(41)α (Bolton and Perutz, 1970). This hydrogen bond would account for the required lowering of the pK_s value in tetrameric hemoglobin.

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