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CHROMATOGRAPHIC EVIDENCE OF THE SELF-ASSOCIATION OF OXYHEMOGLOBIN IN CONCENTRATED SOLUTIONS: ITS BIOLOGICAL IMPLICATIONS

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Expressions that take into account the effects of thermodynamic non-ideality, described in terms of a high-order virial expansion, are derived for the concentration-dependence of the weight-average partition coefficient in exclusion chromatography of a single solute and of a solute undergoing reversible self-association. Comparison of the concentration-dependences predicted by those expressions with results obtained for bovine and human oxyhemoglobins on CPG-10-120 porous glass beads in 0.156 I phosphate-chloride buffer, pH 7.3, shows that neither oxyhemoglobin conforms with the concept of it being a single $\alpha_2\beta_2$ entity with Stokes radius of 3.13 nm, the experimental value. Previously published osmotic pressure and sedimentation equilibrium results are also shown to be inconsistent with this concept. On the other hand, both sets of exclusion chromatography results are consistent with the joint operation of thermodynamic non-ideality and reversible association of the $\alpha_2\beta_2$ species. From the magnitude of the equilibrium constant, derived for either of two possible modes of association, it is calculated that only half of the oxyhemoglibin would be in the $\alpha_2\beta_2$ states under conditions of oxygen saturation and a concentration of 320 g/liter, that pertaining in the red blood cell. The consequences of this association phenomenon are discussed in relation to the oxygen binding curves obtained by others in the presence and absence of 2,3-diphosphoglycerate (DPG). An explanation is provided of the observed dependence on hemoglobin concentration of oxygen-binding in the presence of DPG, and of the absence of such an effect in DPG-free solutions. It is concluded that the control of oxygen binding to hemoglobin in the physiological situation involves the joint operation of self-association and allosteric effects.

1. Introduction

The reversible dissociation of the $\alpha_2\beta_2$ form of hemoglobin at neutral pH has been the subject of many physicochemical investigations [1–9], primarily because of its occurrence in a very low concentration range that is eminently suited to spectrophotometric assay. Despite this well-established tendency of hemoglobin subunits to undergo association, far less attention has been given to possible association beyond the $\alpha_2\beta_2$ form at the high concentrations (approx. 320 g/liter) of this protein that occur in the red blood cell. On the one hand, it has been claimed on the basis of re-analysis of sedimentation equilibrium and osmotic pressure results obtained over a wide concentration range [10–12] that no long-range intermolecular interactions operate in hemoglobin solutions near the isoelectric point [13,14]. On the other hand, intermolecular interactions have been suggested on the basis of results obtained in small angle X-ray scattering [15,16], electron spin resonance [17], dielectric constant [18] and nuclear magnetic resonance [19,20] studies. In the present investigation the interpretation given to the non-spectral equilibrium methods is re-explored together with an examination of the concentration-dependence of weight-average partition coefficients obtained with human and bovine oxyhemoglobin. This dependence is established by ex-

clusion chromatography on porous glass beads, a medium dictated by the need to avoid complications arising from osmotic shrinkage of conventional gel media [21–23].

In a previous communication on exclusion chromatography [24], we considered concentration-dependence of the weight-average partition coefficients of both non-interacting and self-associating solutes. However, the theoretical formulation was in terms of a single virial coefficient and thus limited the range of solute concentration to which it could be validly applied. As a preliminary to the study of concentrated hemoglobin solutions, the problem of concentration-dependent partitioning has been examined using higher order virial coefficients.

2. Theory

2.1. Concentration-dependence of the partition coefficient for a single non-interacting solute

It has been shown previously [24] that the distribution of solute i between mobile phases (γ) and stationary phases (β) in the solute plateau regions of two frontal chromatography experiments (I and II) is governed by the expression

$$\frac{(c_i^\beta)_I(y_i^\beta)_I}{(c_i^\gamma)_I(y_i^\gamma)_I} = \frac{(c_i^\beta)_{II}(y_i^\beta)_{II}}{(c_i^\gamma)_{II}(y_i^\gamma)_{II}}, \tag{1}$$

where y_i is the activity coefficient of solute i of weight-concentration c_i (g/liter). The two experiments are visualized as being conducted at the same temperature with different weight concentrations of solute, c_i^γ. In the γ (mobile) phase only physical interactions of i with itself need to be considered, and thus the activity coefficient appropriate to this phase may be expressed as follows:

$$(y_i^\gamma)_I = \exp\left\{\sum_{k=2} [B_k(c_i^\gamma)_I^{k-1}/(k-1)]\right\} \tag{2a}$$

$$(y_i^\gamma)_{II} = \exp\left\{\sum_{k=2} [B_k(c_i^\gamma)_{II}^{k-1}/(k-1)]\right\}, \tag{2b}$$

where B_k denote constant virial coefficients, and the summations may be extended to include sufficient terms to desrcibe with reasonable precision the magnitude of the activity coefficients at each c_i^γ considered. In the β (stationary) phase, interactions with the matrix j as well as the physical self-interactions must be considered, whereupon

$$(y_i^\beta)_I = \exp\left\{\alpha_{ij}(m_j^\beta)_I + \sum_{k=2} [B_k(c_i^\beta)_I^{k-1}/(k-1)]\right\}, \tag{2c}$$

$$(y_i^\beta)_{II} = \exp\left\{\alpha_{ij}(m_j^\beta)_{II} + \sum_{k=2} [B_k(c_i^\beta)_{II}^{k-1}/(k-1)]\right\}. \tag{2d}$$

With the substitutions $\sigma_i = (c_i^\beta)_I/(c_i^\gamma)_I$ and $\sigma_i^0 = (c_i^\beta)_{II}/(c_i^\gamma)_{II}$ in the limit that $[(c_i^\gamma)_{II} - (c_i^\beta)_{II}] \to 0$, plus the assumed identity of the molar matrix concentrations $(m_j^\beta)_I$ and $(m_j^\beta)_{II}$, combination of eq. (1) and (2) yields

$$\sigma_i = \sigma_i^0 \exp\left\{\sum_{k=2} [B_k(c_i^\gamma)^{k-1}(1-\sigma_i^{k-1})/(k-1)]\right\}. \tag{3}$$

It is noted that eq. (3) reduces to eq. (4) of the previous paper [24], where only the term $k = 2$ was considered. Eq. (3) shows that no concentration dependence of σ_i is predicted for the extreme values of zero (total exclusion of solute) and of unity (equal partition between phases).

In the experimental context, σ_i may be found as a function of c_i^γ, since

$$\sigma_i = (V - V_0)/(V_t - V_0) \tag{4}$$

where V denotes the elution volume of species i from a column with void volume V_0 and total accessible volume V_t. The partition coefficient at infinite dilution, σ_i^0, may be estimated by extrapolating these experimental results to zero concentration. At first sight it appears, therefore, that eq. (3) provides a means of evaluating the B_k values from the experimental results; but as Ross and Minton [13] have pointed out, such evaluations are not likely to be reliable when k assumes a relatively large integral value. These authors [13] prefer to compute estimates of the B_k from considerations of covolume and charge. In the present context this approach is also of value since it permits the construction from eq. (3) of a theoretical plot of σ_i versus c_i^γ for any given σ_i^0; this plot may then be compared directly with the ex-

perimental results. In the event that this comparison is unfavourable, consideration should be given to the possibility that solute i, rather than comprising a single species, self-associates so that monomer ($i = 1$) is in equilibrium with higher polymers.

2.2. Self-associating systems

As shown previously [24], the most readily interpreted situation is a chromatographic experiment in which the stationary phase has been selected to ensure that all species other than monomer are confined to the void volume of the column. In this situation, irrespective of the type of self-association (definite or indefinite), the measured weight-average partition coefficient, σ_w, is related to the total weight concentration of solute $\bar{c}^\gamma$ used in the frontal experiment by the expression

$$\sigma_w = \sigma_1 c_1^\gamma / \bar{c}^\gamma, \tag{5}$$

where σ_1 and c_1^γ denote, respectively, the partition coefficient and concentration of monomer in the solution. With the use of eq. (5) and eq. (2) written for monomer ($i = 1$) in terms of total concentrations $\bar{c}^\gamma$, it may readily be shown, by a derivation analogous to that of eq. (3), that [24]

$$\sigma_w = c_1^\gamma \sigma_1^0 \exp\left\{ \sum_{k=2} [B_k (\bar{c}^\gamma)^{k-1} \times (1 - \sigma_w^{k-1})/(k-1)] \right\} / \bar{c}^\gamma, \tag{6}$$

where the virial coefficients, B_k, refer to monomer self-interactions (physical). If the solute i is not associating ($\sigma_w = \sigma_i$, $\bar{c}^\gamma = c_i^\gamma$ and $c_1^\gamma = \bar{c}^\gamma$), eq. (6) is identical with eq. (3): but if the solute does self-associate, it is evident from eq. (6) that an experimental plot of σ_w versus $\bar{c}^\gamma$ cannot conform with a theoretical plot calculated from eq. (3). This type of examination is to be exemplified with studies on hemoglobin, for which estimates of B_k for the $\alpha_2\beta_2$ species are available [13].

2.3. The virial coefficients for hemoglobin

First it is noted that Ross and Minton [13] expressed the activity coefficient of hemoglobin, viewed as a non-associating $\alpha_2\beta_2$ species, as

$$\ln y_i = \sum_{k=2} [B_k c_i^{k-1}/(k-1)], \tag{7}$$

which is entirely consistent with eq. (2). Furthermore, they showed that the various virial coefficients B_k are inter-related by the expression

$$B_k = k\Gamma_k - (k-1)\Gamma_{k-1}\bar{v}_i, \tag{8}$$

where $\bar{v}_i$ denotes the partial specific volume of hemoglobin, and Γ_k denote the osmotic virial coefficients with concentrations expressed on a g/liter scale. Because of the essentially isoelectric nature of hemoglobin at neutral pH it seems reasonable to neglect any charge contribution to non-ideality [13], whereupon the various Γ_k may be calculated from considerations of exclusion volume. For a hard sphere model

$$\Gamma_2 = 16\pi N r_i^3 / 3M_i \tag{8b}$$

where N is Avogadro's number, r_i is the Stokes radius of hydrated hemoglobin and M_i its unhydrated molecular weight. The magnitudes of the next five Γ_k ($3 \leqslant k \leqslant 7$) are related to this value of Γ_2 by the expressions given in the top line of table 1 of Ross and Minton [13]. It follows that eq. (8a) may be used to calculate the corresponding B_k for use in either eq. (3) or eq. (6).

In the use of eq. (8b) to calculate Γ_2, consideration must be given to the degree of hydration, w, the number of grams of solvent bound per gram of dry solute. In these terms, $r_i = (3M_i^H \bar{v}_i^H / 4\pi N)^{1/3}$ where $M_i^H = M_i(1 + w)$ and $\bar{v}_i^H = (\bar{v}_i + w v_1)/(1 + w)$ with v_1 being the partial specific volume of solvent. A value of 0.746 ml/g was taken for $\bar{v}_i$ [13], the unhydrated partial specific volume, with a corresponding value of 64,500 for the unhydrated molecular weight, M_i. With $w = 0$ (a hypothetical situation assuming no hydration), $r_i = 2.67$ nm and $\Gamma_2 = 2.98 \times 10^{-3}$ liter/g. However, the mean value of r_i obtained from measurements of the translational diffusion coefficient [25–29], the sedimentation coefficient [2,30, 31] and the second virial coefficient [23] is 3.13 nm, which corresponds to $w = 0.45$ g/g and $\Gamma_2 = 4.80 \times 10^{-3}$ liter/g. While both sets of values of these parameters are explored in what follows, that corresponding to the hydrated particle of Stokes radius 3.13 nm must be judged the more realistic.

3. Experimental

3.1. Preparation of hemoglobin solutions

Oxyhemoglobin was prepared from freshly obtained samples of bovine and normal human blood essentially as described by Garby et al. [32], dialysed exhaustively (4—5 days) against 0.156 I phosphate-chloride buffer (0.002 M NaH_2PO_4—0.008 M Na_2HPO_4—0.110 M KCl—0.020 M NaCl), pH 7.3, and stored at 4°C for up to three weeks. Within this time the extent of methemoglobin formation was less than 5% as judged from spectrophotometric measurements at 500 nm and 630 nm. Before each chromatography experiment the stock oxyhemoglobin solution (approx. 160 g/liter) was diluted with buffer to the required concentration and re-dialysed for 16 h at 4°C against more phosphate-chloride buffer. The solution was then subjected to membrane filtration (5 μm Millipore) and equilibrated at 20°C for 2 h immediately prior to exclusion chromatography. Concentrations of suitably diluted samples were determined at 576 nm using molar heme extinction coefficients of 1.58×10^4 and 1.59×10^4 for the human [33] and bovine [34] oxyhemoglobin, respectively.

For experiments conducted in the presence of 2,3-diphosphoglycerate (DPG) the oxyhemoglobin was dialysed for 16 h at 4°C against phosphate-chloride buffer to which sufficient DPG (pentacyclohexylammonium salt) had been added to give a four-fold molar excess of DPG over hemoglobin: the pH of the DPG-containing buffer was readjusted to 7.3 (by addition of HCl) prior to the dialysis step.

3.2. Exclusion chromatography

Controlled pore glass beads (CPG-10-120, 80/120 mesh) with a mean pore diameter of 12.6 ± 1.0 nm were obtained from Electro-Nucleonics Inc., Fairfield, N.J., and treated with polyethylene glycol to minimize adsorption effects [35]. According to technical literature supplied by the manufacturer, this treatment decreases the effective pore diameter by about 1 nm.

Solutions of oxyhemoglobin were subjected to frontal exclusion chromatography on a column (0.9 × 69 cm) of CPG-10-120 glass beads pre-equilibrated with degassed 0.156 I phosphate-chloride buffer, pH 7.3, and thermostatically maintained at 20°C. Approximately 30 ml of protein solution was applied to the column by upward flow, the flow-rate being maintained at 16 ml/h by means of a peristaltic pump. Fractions (1.0—1.25 ml) were collected in pre-weighed tubes so that the precise weight of each fraction could be determined. The weight of each fraction was then converted to a volume on the basis of the protein concentration $\bar{c}$ of the fraction and the expression $\rho = \rho_b + 0.25\,\bar{c}$, in which the buffer density ρ_b was 1.0055 g/ml. This relationship, derived from density measurements at 20°C on an Anton Paar DMA 02C precision density meter, is also predicted from the value of 0.746 ml/g used for the partial specific volume of oxyhemoglobin.

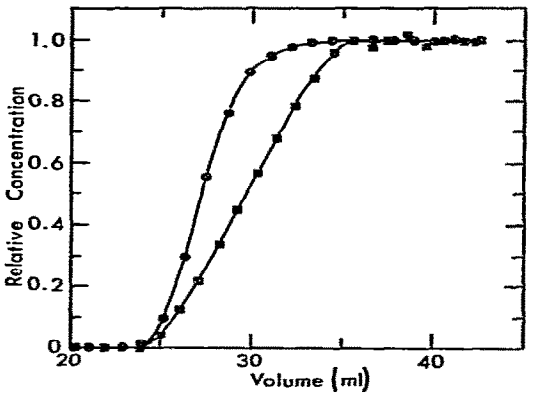

Fig. 1. Elution profiles (advancing side) obtained in frontal chromatography of human oxyhemoglobin in 0.156 I phosphate-chloride buffer, pH 7.2, at 20°C on a column (0.9 × 69 cm) of CPG-10-120 porous glass beads. The ordinate refers to the ratio of the total concentration at any point to that in the plateau in experiments with applied concentrations of 19.4 g/liter (•) and 164.9 g/liter (▪).

4. Results and discussion

4.1. Exclusion chromatography of oxyhemoglobin

Fig. 1 shows normalized elution profiles for the advancing side in frontal chromatography of 19.4 g/liter (•) and 164.9 g/liter (▪) solutions of human oxyhemoglobin. In order to take into account any variation in the shapes of such profiles the median bisector has been used to define V_w, the weight-average elution volume, which has then been converted to the

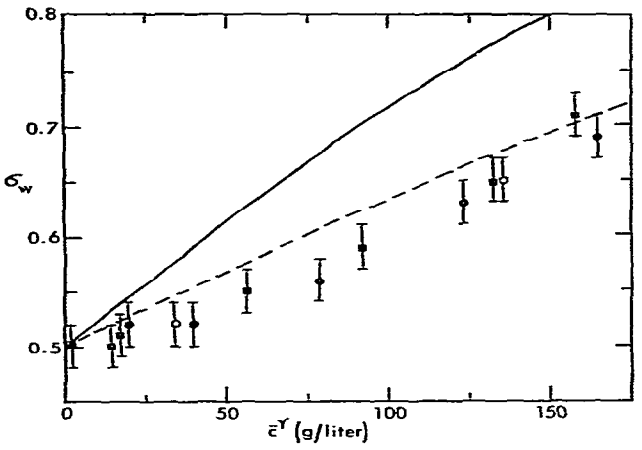

Fig. 2. Dependence of the weight-average partition coefficient (σ_w) of human (•) and bovine (▪) oxyhemoglobin on total concentration ($\bar{c}^Y$). Open circles refer to human oxyhemoglobin in the presence of a four-fold molar excess of DPG. The curvilinear plots denote theoretical calculations of the concentration-dependence based on eqs. (3) and (8) with the Stokes radius (r_i) taken as 3.13 nm (——) and 2.67 nm (— —).

corresponding partition coefficient σ_w via eq. (4) with values of 21.3 ml for V_0 and 34.3 ml for V_t: these were obtained from chromatography of bovine liver glutamate dehydrogenase (Sigma type III) and K_2CrO_4 respectively. The experimental concentration dependence of σ_w is shown in fig. 2, from which it is evident that oxyhemoglobin of human (circles) and bovine (squares) origin exhibit indistinguishable exclusion chromatographic behavior. Secondly it is noted that the partition coefficient of human oxyhemoglobin is unaffected by the presence of a four-fold molar excess of DPG (open symbols in fig. 2).

The solid line in fig. 2 represents the theoretical concentration-dependence of the partition coefficient predicted from eq. (3) and (8) for a solute with a Stokes radius of 3.13 nm and a value of 0.50 for σ_i^0: clearly the agreement between theory and experiment is extremely poor. Although a better (though still not good) theoretical description of the experimental results may be obtained by selecting the minimal Stokes radius of 2.67 nm (broken line in fig. 2), this value of r_i is unrealistic since it corresponds to hemo-

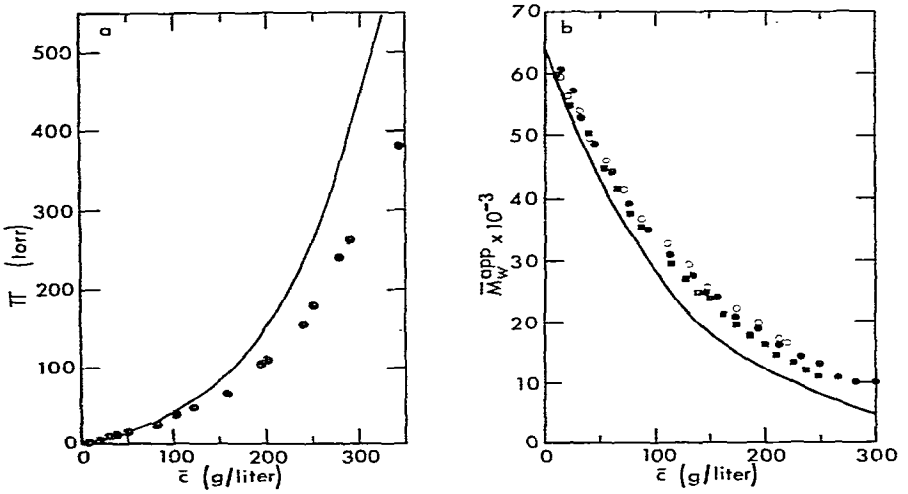

Fig. 3. Comparison of the experimental concentration-dependence observed in (a) osmometry and (b) equilibrium sedimentation with that calculated by combining eq. (8) with eqs. (9) and (10), respectively, using a value of 3.13 nm for the Stokes radius r_i. In (a) the experimental data refer to ovine hemoglobin and are taken from Adair [12]. In (b) adapted from fig. 3 of ref. [13] the symbolism is as follows: •, human deoxyhemoglobin [10]; ○, human deoxyhemoglobin S [10]; ▪, human carbonmonoxyhemoglobin [11]. Although the observed concentration-dependence for the latter systems differ, none are in accord with the behavior predicted for a non-interacting $\alpha_2\beta_2$ species (solid line).

globin devoid of any hydration ($w = 0$). It could also be noted that the solid line in fig. 2, calculated using the Stokes radius of the hydrated particle, is likely to underestimate the effect of thermodynamic non-ideality because of the assumption inherent in eq. (8) that the hemoglobin molecule is a hard, impenetrable, uncharged sphere. We therefore conclude that the exclusion chromatographic behavior of human and bovine oxyhemoglobin is *not* consistent with the concept that the $\alpha_2\beta_2$ species exists alone in the range of concentration examined. This point is now explored in relation to results obtained by others who also utilized equilibrium methods.

4.2. Correlation with osmotic pressure and sedimentation equilibrium studies

Fig. 3a presents results obtained by Adair [12] for the concentration-dependence of osmotic pressure (π) for ovine hemoglobin, together with the theoretical dependence calculated from the expression

$$\pi = (RT/M_i)\left\{c_i + \sum_{k=2} [\Gamma_k c_i^k]\right\} \qquad (9)$$

In obtaining the theoretical curve (solid line) values of Γ_k were those used to calculate the solid line in fig. 2 and thus refer to a non-interacting hydrated particle of Stokes radius 3.13 nm. Experimental measurements of the concentration-dependence of apparent weight-average molecular weight (M_i^{app}) of deoxyhemoglobin [10], its carbonmonoxy derivative [11] and also deoxyhemoglobin S [10] are shown in fig. 3b. The theoretical curve in this instance was calculated, with B_k values corresponding via eq. (8a) to the same Γ_k, from the relationship [13]

$$M_i^{app} = M_i \Big/ \left\{1 + \sum_{k=2} [B_k c_i^{k-1}]\right\} . \qquad (10)$$

In both figs. 3a and b the theoretical curves, calculated using values of k in the range $2 \leqslant k \leqslant 7$, fail to describe adequately the experimental results. In this connection, we do note that Ross and Minton [13] reported agreement between theory and experiment for the same data by invoking a Stokes radius of 2.9 nm for hemoglobin: as mentioned above, a larger value of r_i is indicated by the experimental evidence. Consequently, we consider that there is agreement between studies of various forms of hemoglobin by exclusion chromatography, by osmometry and by equilibrium sedimentation that the concentration-dependence of the apparent activity coefficient indicated by the experimental results is less than that predicted from eq. (2) or eq. (7) written for the non-interacting hydrated $\alpha_2\beta_2$ species with Stokes radius 3.13 nm. This type of discrepancy is indicative of a self-associating system.

4.3. Consideration of oxyhemoglobin as a self-associating system

The interpretation of osmotic pressure or sedimentation equilibrium results obtained at high concentration in terms of an equilibrium mixture of oligomeric species is rendered exceedingly difficult by the need to consider non-ideality effects defined on the basis of the composition-dependence of the activity coefficients of each oligomeric species present [36,37]. However, it may be recalled from sect. 2.2 that the same difficulties do not arise in the interpretation of results obtained from chromatographic studies conducted with a stationary phase that excludes all species except monomer. Consideration of hemoglobin polymers as a rigid, linear string of spherical beads [38,39] leads to the conclusion that the dimer, that is $(\alpha_2\beta_2)_2$, would have a Stokes diameter of approximately 8.4 nm and, thus, it must be conceded that any dimer would have penetrated the stationary phase to some extent. In order to comment further on this point, experiments were conducted to determine the value of σ_1^0 for muscle lactate dehydrogenase, which has an identical Stokes diameter of 8.4 nm. The determined partition coefficient was only 0.05 and thus we have decided to make the approximation that the hemoglobin dimer, as well as any higher polymers, are effectively excluded. This permits the direct application of eq. (6) to the experimental results shown in fig. 2, but necessitates as we will see some caution in drawing quantitative conclusions.

Column 3 of table 1 presents values of c_1^γ obtained using eq. (6) with B_k values appropriate to the hydrated monomer and a value of 0.50 for σ_1^0. The uncertainty shown for these values is based on an experimental error of ±0.02 in the measurement of σ_w. Columns 4 and 5 of table 1 present the analysis of each (c_1^γ, $\bar{c}^\gamma$) pair in terms of a monomer–dimer equilibrium and an isodesmic indefinite self-association, respective-

Table 1
Analysis of weight-average partition coefficients of oxyhemoglobin

$\bar{c}^{\gamma}$ (g/liter)	σ_w	c_I^{γ} (g/liter) [a]	Assoc. const. X (M^{-1})	
			monomer–dimer	isodesmic
1.7 [b]	0.50	1.7 ± 0.1	–	–
13.9	0.50	13.0 ± 0.6	172	163
16.9	0.51	16.0 ± 0.7	113	109
19.4	0.52	18.5 ± 0.8	85	82
33.3	0.52	29.6 ± 1.3	136	125
39.1	0.52	33.7 ± 1.6	153	137
56.2	0.55	47.3 ± 2.3	128	113
78.5	0.56	59.2 ± 3.1	178	143
91.7	0.59	68.7 ± 3.8	157	126
122.9	0.63	83.2 ± 5.3	185	137
132.6	0.65	88.9 ± 5.9	178	131
135.6	0.65	88.9 ± 6.0	191	138
158.0	0.71	107.3 ± 8.2	142	106
164.9	0.69	98.2 ± 7.8	223	150
Average (and standard deviation)			157 ± 37	128 ± 21

[a] Values were calculated using eq. (6) with values of B_k appropriate to the hydrated monomer ($\alpha_2\beta_2$) species of Stokes radius 3.13 nm.
[b] The proportion of monomeric ($\alpha_2\beta_2$) species in this experiment is too high to permit reliable estimation of X.

ly: values of the appropriate equilibrium constant X were calculated from eq. (12b) and (11b), respectively, of ref. [24]. Several points merit comment in relation to table 1. (i) Although it is not made in the evaluation of c_I^{γ}, the Adams-Fujita [40] approximation, that $\ln y_i = \Sigma_{k=1}\beta_k M_i(\bar{c}^{\gamma})^k$ (where the β_k are constants independent of the value of i), is implicit in the calculation of X on the basis that ratios of oligomeric activities are given by the corresponding ratios expressed on a molar concentration scale. (ii) Description of the hemoglobin system as either a monomer–dimer system or as one involving isodesmic self-association of the $\alpha_2\beta_2$ species is adequate from the viewpoint of the requirement that the magnitude of X be independent of $\bar{c}^{\gamma}$. For either system the association constant corresponds to a ΔG^0 of –3 kcal/mole. It would be unwise to attempt a further distinction between these polymerization patterns because of the assumed exclusion of the $(\alpha_2\beta_2)_2$ species from the stationary phase. (iii) Consideration of hemoglobin in terms of either associating system provides a much better description of the observed concentration-dependence of the weight-average partition coefficient than that predicted on the basis of a non-associating $\alpha_2\beta_2$ entity.

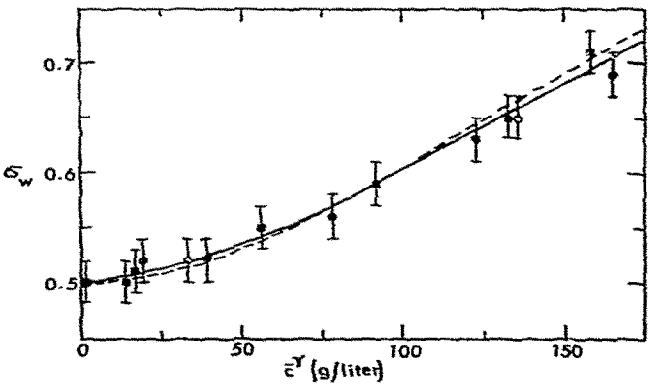

Fig. 4. Description of the concentration-dependence of σ_w for oxyhemoglobin in terms of the joint operation of non-ideality and self-association effects. Experimental points are taken from fig. 2, and the theoretical lines are based on eq. (6) and (8) for two different models of self-association: ——, isodesmic indefinite, $X = 128\ M^{-1}$; – – –, monomer–dimer, $X = 157\ M^{-1}$. Values of B_k employed to assess the non-ideality contribution were the same as those used to construct the solid line in fig. 2.

This point is emphasized by comparing fig. 2 with fig. 4, which presents the experimental results and the theoretical concentration-dependence of σ_w for monomer–dimer (– – –) and isodesmic (——) systems with the mean value of X reported in table 1. Of particular note is the distinct change in the overall shape of the theoretical curves that results from the superimposed effects of self-association equilibrium and thermodynamic non-ideality (fig. 4) compared with with that predicted on the basis of the latter effect alone (solid curve in fig. 2). Clearly, the postulate that both types of effect are operative is much more in accord with the experimental results. (iv) It appears that the addition of a four-fold molar excess of DPG, an organic phosphate present in the human erythrocyte in equimolar proportion, is without significant effect on the extent of association of oxyhemoglobin (open circles in fig. 4). This implies that, provided no other effector of the polymerization equilibrium exists in the erythrocyte, only about half of the hemoglobin in the red blood cell would be in the $\alpha_2\beta_2$ state, under conditions of near oxygen-saturation. The latter estimate was made employing the values of X in table 1 and a total concentration of 320 g/liter.

5. General discussion

The major point which emerges from this work is that the $\alpha_2\beta_2$ form of human oxyhemoglobin self-associates in accordance with the law of mass action under conditions physiologically relevant with regard to pH and solute concentration. This conclusion confirms and extends previous postulates [15–20] of macromolecular interaction in hemoglobin solutions: likewise, it is now evident that sedimentation equilibrium [10,11] and osmotic pressure [12] results are not consistent with the sole existence of the hydrated $\alpha_2\beta_2$ species in concentrated solutions, as claimed previously [13,14]. The coexistence of oligomeric species of hemoglobin in equilibrium at the saturating oxygen tension used in this work indicates that there is little if any preferential binding of oxygen to a particular oligomeric state. In cases where a ligand binds to equivalent and independent sites on each oligomeric state and to equal extents to the oligomeric states of an acceptor coexisting in equilibrium, it has been shown [41] that the binding curve is identical to that pertaining to the monomer and is independent of acceptor concentration. The same basic conclusions may be shown to apply when each acceptor state binds ligand equally but with cooperativity of binding to each state, a situation pertinent to hemoglobin where the $\alpha_2\beta_2$ form is known to exhibit positive cooperativity in its oxygen-binding behavior [42]. For such an allosteric system comprising monomeric and dimeric acceptor states ($2A \rightleftharpoons A_2$, governed by an association constant X), the appropriate expression for the binding function r, the moles of ligand bound per base mole of total acceptor, is given by

$$r = \left(m_A \sum_{i=1}^{i=4} i \left\{\prod_{l=1}^{l=i} K_l\right\} m_S^i + 2Xm_A^2 \sum_{i=1}^{i=4} i \left\{\prod_{l=1}^{l=i} K_l\right\} m_S^i\right) \times \left(m_A\left(1 + \sum_{i=1}^{i=4} \left\{\prod_{l=1}^{l=i} K_l\right\} m_S^i\right) + 2Xm_A^2\left(1 + \sum_{i=1}^{i=4} \left\{\prod_{l=1}^{l=4} K_l\right\} m_S^i\right)\right)^{-1}, \quad (11)$$

where m_A is the molar concentration of free $\alpha_2\beta_2$ possessing four oxygen-binding sites, S refers to oxygen and the K_l denote the equilibrium constants describing successive binding of ligand. Since no preferential oxygen binding to an oligomeric state is evident, the K_l in each term of eq. (11) are directly comparable which permits rearrangement of eq. (11) by factorization and cancellation to yield,

$$r = \sum_{i=1}^{i=4} i \left\{\prod_{l=1}^{l=i} K_l\right\} m_S^i \left(1 + \sum_{i=1}^{i=4} \left\{\prod_{l=1}^{l=i} K_l\right\} m_S^i\right)^{-1}. \quad (12)$$

The same simplification occurs when $\alpha_2\beta_2$ is considered to undergo indefinite self-association with each oligomeric state binding oxygen equally. Eq. (12), a ratio of polynomials, is of the same form used to describe the sigmoidal oxygen-binding curve relevant to the $\alpha_2\beta_2$ form [42]. The finding of Torelli and coworkers [43] in their fig. 1 that the oxygen-binding curve of human hemoglobin is sigmoidal and independent of hemoglobin concentration over the range 44–313 g/liter is consistent with eq. (11) and (12) and thus is in accordance with our interpretation of oxyhemoglobin as an equilibrium mixture of oligomeric states possessing identical affinities and site-cooperativity properties with respect to oxygen binding.

At the same time, the latter workers [43] in their fig. 2 have shown a distinct acceptor-concentration dependence of oxygen-binding curves in the presence of DPG. If it is assumed for simplicity that any DPG-hemoglobin complex formed with DPG in the β-cleft cannot bind oxygen [44], the numerator of eq. (11) remains unaltered but the denominator must contain additional terms to account for the contribution of these complexes to the total concentration of the acceptor. The result is that eq. (11) can no longer be simplified to eq. (12) and, indeed, can no longer be formulated as an expression independent of m_A. Thus, acceptor-concentration dependence of oxygen-binding curves is a necessary consequence of the self-association of oxyhemoglobin even though oxygen affinity is unaltered by this polymerization. The delineation of terms to be included in the denominator of eq. (11) to account for DPG-complexes requires specification of these complexes, for, indeed, interactions of the type $2\alpha_2\beta_2$ DPG $\rightleftharpoons (\alpha_2\beta_2$ DPG$)_2$, linked with the oxyhemoglobin self-association, may well operate. Two items of evidence presented in this work point to this possibility, namely, that DPG does not appear to affect the extent of association of oxyhemoglobin (fig. 4) and that the deoxy-state, of similar conformation to the DPG-complex [45], seemingly also self-associates (fig. 3b). The former observation offers no comment on the effect on the association of hemoglobin of specific DPG binding in the β-cleft (which does not occur at saturating oxygen tension); but it does show that non-specific DPG binding [32] does not affect the self-association.

It is not timely yet to formulate a comprehensive binding equation aimed at describing oxygen binding results found under physiological conditions since more detail is required on the possible self-association of the DPG-complex and more information is needed on the effects of other biologically relevant ligands such as ATP and magnesium ions [46]. However, it is becoming increasingly clear that the fundamental question of the control of oxygen binding to hemoglobin requires joint consideration of allosteric and self-association effects. The present study has stressed the relevance of the self-association of oxyhemoglobin and has described a method of exclusion chromatography which may be helpful in elucidating other association equilibria which may be operative in the physiological environment of the red blood cell.

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