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EXCLUSION CHROMATOGRAPHY OF CONCENTRATED HEMOGLOBIN SOLUTIONS

COMPARISON OF THE SELF-ASSOCIATION BEHAVIOR OF THE OXY AND DEOXY FORMS OF THE $\alpha_2\beta_2$ SPECIES

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Theory pertaining to the interpretation of partition chromatography results obtained with self-associating protein systems studied at high total concentrations is extended to permit consideration of situations in which both monomeric and dimeric states partition. This development, which includes considerations of thermodynamic nonideality effects, permits a quantitative correlation of human oxyhemoglobin results reported previously and obtained in this work employing a different stationary matrix of controlled-pore glass beads. The two sets of results, obtained at pH 7.3 and 20°C, indicate that the $\alpha_2\beta_2$ species of oxyhemoglobin self-associates. Two types of association pattern, discrete dimerization and an indefinite self-association, are examined. This is done for a realistic range of values for the radius, r, of the effective hard sphere appropriate to the calculation of the covolume of the $\alpha_2\beta_2$ species in the assessment of the thermodynamic nonideality contribution. Assessed values of the isodesmic association constant range from $66 \pm 23\ M^{-1}$ ($r = 2.84$ nm) to $154 \pm 26\ M^{-1}$ ($r = 3.13$ nm). This mode of indefinite association is marginally favored over a dimerization when the larger value of r is considered, the two patterns becoming virtually indistinguishable for the lower value of r. Partition chromatography results are also presented for human deoxyhemoglobin up to a total concentration of 225 g/l, and are analyzed in a similar fashion to show that the indefinite self-association pattern is favored, governed by an isodesmic constant in the range $91 \pm 9\ M^{-1}$ ($r = 2.84$ nm) to $223 \pm 84\ M^{-1}$ ($r = 3.13$ nm). Comparison of the constants assessed for the oxy and deoxy systems permits discussion of the concept that oxygen binds preferentially to the $\alpha_2\beta_2$ species of deoxyhemoglobin in comparison with its polymers.

1. Introduction

In a previous communication [1] frontal chromatographic experiments on human oxyhemoglobin up to a total concentration of 160 g/l were interpreted in terms of the joint operation of thermodynamic nonideality effects and an association of the $\alpha_2\beta_2$ form of the protein. Recently, this interpretation of the chromatographic results has been questioned [2] on the following grounds. First, the analysis was based on theory written for an experimental design in which all polymeric forms of the $\alpha_2\beta_2$ species were considered to be excluded from the stationary phase, with only the $\alpha_2\beta_2$ form capable of partitioning: as noted by the original workers [1], distribution of the dimer of this form could occur to a slight extent. Secondly, the use of an average concentration in the stationary phase, specified as a product of the experimental partition coefficient and the concentration in the mo-

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Abbreviation: Bistris, 2-[bis(2-hydroxyethyl)amino]-2-hydroxymethyl-1,3-propanediol.

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bile phase, may not be appropriate for the calculation of a species activity coefficient in the stationary phase if a nonuniform distribution of solute pertains throughout the phase. Thirdly, a question arose concerning the magnitude of the radius of the effective hard sphere appropriate to the calculation of the covolume of the $\alpha_2\beta_2$ species.

It is one purpose of this investigation to examine each of these points in relation to the previous results [1], and to reassert that a self-association reaction is indicated, in agreement with other indications from small-angle X-ray [3,4], spectral [5–8] and dielectric [9,10] studies. In addition, the nature of this self-association is explored further by analyzing results obtained with oxyhemoglobin on a column matrix with larger pore size in terms of theory specifically written to account for partitioning of the $(\alpha_2\beta_2)_2$ species. Finally, the extent of the corresponding self-association of deoxyhemoglobin is also examined in an attempt to comment on the influence of oxygen binding on this phenomenon.

2. Theory

2.1. Partitioning of a single nonassociating solute

The equation describing the concentration-dependence of the partition coefficient, σ, of a single nonassociating solute has been presented by Minton [2] as,

$$\sigma = \sigma^0 \exp\left\{ \sum_{k=2} \frac{B_k (c^\gamma)^{k-1} \left(1 - (\alpha\sigma)^{k-1}\right)}{(k-1)} \right\} \qquad (1)$$

where σ^0 is the partition coefficient at infinite dilution, c^γ the weight-concentration of solute in the mobile phase, and B_k denotes successive virial coefficients defined previously [1,11]. Eq. (1) is identical with that originally derived [1] with the exception that α was assumed to be unity, α being a parameter introduced by Minton [2] in an attempt to account for a possible nonuniform distribution of solute in the stationary phase. Minton [2] attempted to fit the experimental hemoglobin

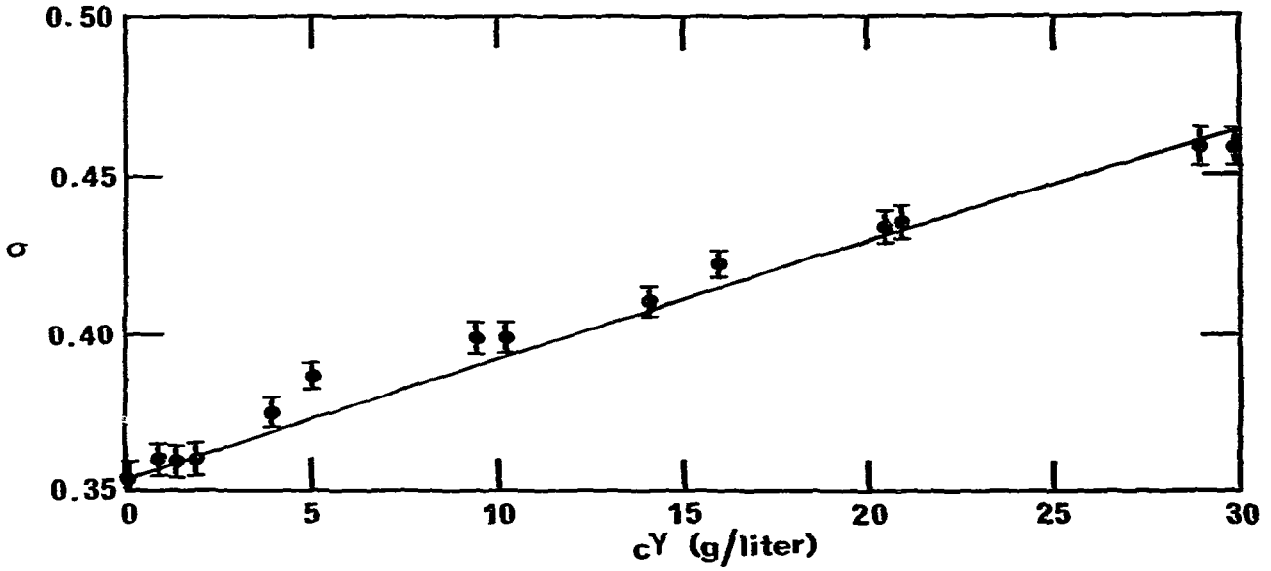

Fig. 1. Prediction of the effect of thermodynamic nonideality on the exclusion chromatography of ovalbumin on CPG-75 glass beads equilibrated with 0.156 I phosphate-chloride, pH 7.4. Experimental points are taken from fig. 1 of ref. [11] (where the matrix was incorrectly reported as CPG-120), and the solid line is the theoretical relationship based on eq. (1) with $\alpha = 1$ and on eq. (A.2) and (A.9) of ref. [13] plus the following magnitudes of parameters: $U_{\prime\prime} = 500$ l/mole, $M_\prime = 45000$, $\bar{v} = 0.000748$ l/g, $r_\prime = 2.92$ nm (all from ref. [14]), $\kappa = 1.29$ nm^{-1} and $z_\prime = -16$ [11], leading to a value of 770 l/mole for $B_2 M$.

results [1] on the basis of no self-association of the $\alpha_2\beta_2$ species by arbitrarily selecting values of α greater than unity.

Evidently, it would be desirable to have an experimental assessment of the value of α appropriate to porous glass beads, for otherwise it will remain merely as a curve-fitting parameter. For this purpose fig. 1 presents partition results for ovalbumin obtained on a column of controlled-pore glass beads in an environment where ovalbumin has been shown not to self-associate [11]. Inspection of Table 5 of Ross and Minton [12] indicates that such results obtained for a globular protein should reasonably be fitted by eq. (1) in terms of only a second virial coefficient, since the concentration is seen to extent to only 30 g/l. The previous attempt [11] to do this was only partially successful because an oversimplified assessment of the charge-charge contribution to the second virial coefficient was considered. It is of particular interest, therefore, to reinterpret these results utilizing a value of B_2M calculated from eq. (A.2) and (A.9) of ref. [13], which incorporate in addition to the covolume contribution an improved term for the charge-charge interaction ($B_2M = \alpha_{ii}$ in the terminology of ref. [13]). The values of the parameters relevant to the calculation of B_2M are reported in the caption to fig. 1. The solid line in fig. 1 presents the results of calculations based on eq. (1) with $\alpha = 1$, which describe the concentration dependence of the partition coefficient of ovalbumin. It is evident, for this system at least, that a choice of α equal to unity is appropriate to controlled-pore glass bead chromatography. It could also be noted that the arbitrary selection of α values of either 1.15 or 1.3 [2] failed to describe adequately the experimental oxyhemoglobin data [1], in that the form of the experimental results was sigmoidal while that of the theoretical plots was concave to the concentration axis. Moreover, it could be noted that these theoretical plots could be brought into the range of the experimental results only by invoking a value of σ^0 on the lower verge of the confidence limit assessed experimentally for this parameter. On the basis of these observations and the experimental results for ovalbumin, we conclude that the only reasonable course of action to be taken in the analysis of chromatographic results obtained with globular proteins on porous glass beads is to utilize eq. (1) with $\alpha = 1$.

2.2. Concentration dependence of the weight-average partition coefficient of a self-associating system

Eq. (6) of our previous communication [1] provides the appropriate description for a situation in which only the monomer of a self-associating system is capable of partitioning. As mentioned in section 1, it is desired to expand this formulation to include cases where both monomeric and dimeric forms of the protein partition by virtue of the available average pore diameter of the matrix. The weight-average partition coefficient, σ_w, for such a system is given by,

$$\sigma_w = (\sigma_1 c_1^\gamma + \sigma_2 c_2^\gamma)/\bar{c}^\gamma = \bar{c}^\beta/\bar{c}^\gamma \qquad (2)$$

where σ_1 and σ_2 are the partition coefficients of monomer and dimer, respectively, appropriate to an experiment with total concentration $\bar{c}^\gamma$ in the mobile phase and total concentration $\bar{c}^\beta$ in the stationary phase. Eq. (2) is evidently appropriate to a monomer-dimer system, and is also valid for an isodesmic indefinite association (since $\sigma_{i>3} = 0$), the two types of self-association pattern particularly relevant to the hemoglobin association [1]. The partition coefficients of monomer and dimer may be related to their values at infinite dilution by [13],

$$\sigma_i = \sigma_i^0 y_i^\gamma \exp(G_i)/y_i^\beta;\ i = 1, 2 \qquad (3)$$

where y_i denotes the activity coefficient of species i in the phase denoted by the superscript, and $\exp(G_i)$ is the contribution to the ith species activity coefficient in the stationary phase due to ith species-matrix interaction.

Combination of eqs. (2a) and (2c) of ref. [1], written in terms of total concentrations [11], yields, for the monomer ($i = 1$),

$$(y_1^\gamma/y_1^\beta) = \exp\left\{\sum_{k=2} \frac{B_k(\bar{c}^\gamma)^{k-1}\left[1 - (\bar{c}^\beta/\bar{c}^\gamma)^{k-1}\right]}{(k-1)}\right\} \times \exp(-G_1) \qquad (4)$$

It then follows from combination of eqs. (2)–(4)

that,

$$\sigma_1 = \sigma_1^0 \exp\left\{ \sum_{k=2} \frac{B_k(\bar{c}^\gamma)^{k-1}(1-\sigma_w^{k-1})}{(k-1)} \right\} \quad (5)$$

It has been necessary to assume that in both phases $y_2 = y_1^2$, an approximation introduced originally by Adams and Fujita [15] in treating homogeneous solution equilibria and one with some justification over a total concentration range where the weight fraction of monomer remains reasonably large. With this assumption it follows that $(y_1^\gamma/y_1^\beta)^2 = (y_2^\gamma/y_2^\beta)$, that $\exp(G_2) = \exp(2G_1)$, and thus from eqs. (2)–(4) that,

$$\sigma_2 = \sigma_2^0 \exp\left\{ 2 \sum_{k=2} \frac{B_k(\bar{c}^\gamma)^{k-1}(1-\sigma_w^{k-1})}{(k-1)} \right\} \quad (6)$$

Adoption of the Adams and Fujita [15] approximation also makes it possible to interrelate the species concentrations in the mobile phase by the expression,

$$\bar{c}^\gamma = c_1^\gamma + \left[2K_2(c_2^\gamma)^2/M_1\right] \quad (7a)$$

for a monomer-dimer system (K_2 in l/mole) and by,

$$\bar{c}^\gamma = c_1^\gamma/\left[1 - K_1(c_1^\gamma/M_1)\right]^2 \quad (7b)$$

for an isodesmic, indefinite self-association (K_1 in l/mole): M_1 is the monomeric molecular weight.

Eqs. (5) and (6) make it possible to calculated σ_1 and σ_2 from experimental (σ_w, $\bar{c}^\gamma$) points. For a monomer-dimer system c_1^γ is then readily calculated from eq. (2) on noting that $c_2^\gamma = \bar{c}^\gamma - c_1^\gamma$ for that case. The consequent knowledge of c_1^γ appropriate to each $\bar{c}^\gamma$ allows the evaluation of K_2 from eq. (7a). Determination of K_1 for an isodesmic indefinite self-association is also feasible, but in this case the analysis is less straigthforward. Substitution of the expression $2K_1(c_1^\gamma)^2/M_1$ for the concentration of dimer (c_2^γ) in eq. (2), and elimination of the isodesmic constant K_1 by use of eq. (7b) lead to the polynomial,

$$\bar{c}^\gamma\left[(\sigma_1 + 2\sigma_2)c_1^\gamma - \sigma_w\bar{c}^\gamma\right]^2 - 4\sigma_2^2(c_1^\gamma)^3 = 0 \quad (8)$$

from which c_1^γ may be found by numerical solution for corresponding values of σ_1, σ_2, σ_w and $\bar{c}^\gamma$. The magnitude of K_1 then follows directly from eq. (7b). It follows that alternative self-association patterns may be examined on the basis of constancy of apparent equilibrium constants over a range of $\bar{c}^\gamma$ for cases where both monomeric and dimeric states partition. A similar potential is offered by the experimental design in which only monomer partitions ($\sigma_{i\geq 2} = 0$) with the advantage that c_1^γ values may be calculated from (σ_w, $\bar{c}^\gamma$) points without assuming any particular pattern of self-association [11]. However, in counterbalance, partitioning of the monomer and dimer may be advantageous in certain circumstances for the distinction between possible association patterns: for example, the observed σ_w values, for a monomer-dimer system, must always lie between the values of σ_1 and σ_2 calculated from eqs. (5) and (6).

3. Experimental

3.1. Preparation of hemoglobin solutions

Oxyhemoglobin was prepared from samples of human blood (type O) essentially by the method of Garby et al. [16] and dialysed exhaustively against 0.15 I Bistris-chloride buffer (0.020 M Bistris, 0.004 M HCl, 0.130 M KCl, 0.018 M NaCl), pH 7.3. After concentration of the sample to 225 g/l by ultrafiltration, this stock solution was stored at 4°C for up to 6 weeks, there being very little methemoglobin (< 5%) detectable spectrophotometrically even on storage for this length of time. In preparation for each experiment, this stock solution was diluted appropriately with Bistris-chloride buffer and then subjected to membrane filtration (5 μm Millipore) and equilibrated for 1 h at 20°C immediately prior to exclusion chromatography. For experiments on deoxyhemoglobin the same procedure was adopted except that a deoxygenation step was inserted after the membrane filtration stage. Sufficient dry sodium dithionite to provide a 5 mM excess of the reducing agent over the heme concentration was added to the oxyhemoglobin solution. Oxygen-free nitrogen was then flushed over the deoxyhemoglobin solution throughout the final equilibration step (1 h at

20°C) and also during the application of the sample to the chromatography column. Concentrations of hemoglobin were measured at 570 nm; a wavelength at which oxy and deoxy forms exhibit molar heme extinction coefficients of 1.18×10^4 and 1.11×10^4, respectively [17].

3.2. Exclusion chromatography

Glyceryl-coated controlled-pore glass beads (Glyceryl-CPG-120B; 120/200 mesh) with a mean pore diameter of 11.6 ± 1.0 nm were used as supplied by Electro-Nucleonics Inc., Fairfield, NJ. Prior to frontal chromatography of hemoglobin solutions the column (0.9×69 cm) of porous glass beads, thermostatically maintained at 20°C, was preequilibrated for 2 h either with degassed 0.15 I Bistris-chloride buffer, pH 7.3, or with the same buffer to which dithionite (5 mM) had been added. Protocol for these experiments was essentially that used in the previous hemoglobin study [1], upward flow through the column at a rate of 16 ml/h being maintained by means of a peristaltic pump. The size of each fraction (1.0–1.2 ml) was determined by weight, and the conversion to a volume was made on the basis of the determined protein concentration $\bar{c}$ of the fraction and the expression $\rho = \rho_b + 0.25\ \bar{c}$. A values of 1.0062 g/ml for the buffer density, ρ_b, was obtained from measurements on an Anton Paar precision density meter. Weight-average elution volumes were converted to the corresponding partition coefficients, σ_w, by means of eq. (4) of ref. [1] with values of 20.9 and 33.4 ml for the void (V_0) and total (V_t) volumes, respectively, of the column: Blue dextran 2000 and potassium chromate were used to obtain these column parameters. Experimental errors in the estimates of σ_w have been assessed on the basis of an uncertainty of 0.25 ml in the measurement of elution volumes. Values of σ_1^0, the limiting partition coefficient of the $\alpha_2\beta_2$ hemoglobin species, were obtained by extrapolating plots of σ_w versus $\bar{c}^\gamma$ to infinite dilution, whereas those of σ_2^0, the corresponding parameter for the $(\alpha_2\beta_2)_2$ entity, were again considered to be given the partition coefficient obtained for lactate dehydrogenase, a protein with a similar Stokes' radius [1].

4. Results and discussion

One of the major objections [2] to our previous interpretation of exclusion chromatography results for hemoglobin [1] concerned the size of the effective radius of the $\alpha_2\beta_2$ species that should be used in predictions of thermodynamic nonideality based on the covolume concept. The uncertainty surrounding the magnitude of this parameter was in fact recognized in the previous study, a point evident from fig. 2 of ref. [1], where theoretical relationships based on two estimates (3.13 and 2.67 nm) of this effective radius were presented for the concentration dependence of the partition coefficient of $\alpha_2\beta_2$ hemoglobin. In retrospect, it was an unfortunate decision to restrict consideration of the data in terms of an envelope of possible theoretical descriptions to this particular case of the nonassociating model. We therefore revert to this practice in the present investigation, using values of 3.13 and 2.84 nm as the two extremes for the radius. The larger value is based on estimates of the Stokes' radius from measurements of the translational diffusion coefficient [18–22] and the sedimentation coefficient [23–25], and also of the effective covolume radius deduced [26] from the osmotic pressure data of Adair [27]. The smaller value, which is the radius favored by Minton and co-works [2,12,28,29], seems more realistic than the previously used [1] lower limit, which corresponded to the Stokes' radius of a completely unhydrated hemoglobin moiety. Values of B_k ($2 \leqslant k \leqslant 7$) appropriate to eqs. (5) and (6) were calculated for these radii on the basis of zero charge and spherical geometry, as described previously [1,12].

Fig. 2 presents the partition results obtained previously for oxyhemoglobin on CPG-120 glass beads treated with poly(ethylene glycol) [1]. The broken curves are included to emphasize, for the indicated range of radii of the effective hard sphere used in covolume calculations, that with $\alpha = 1$ (eq. (1)) the results are not fitted by a model which assumes the absence of self-association of $\alpha_2\beta_2$ oxyhemoglobin. The solid curves in fig. 2 are computed on the basis of the joint operation of nonideality effects and an isodesmic indefinite self-association of $\alpha_2\beta_2$ oxyhemoglobin. These calcula-

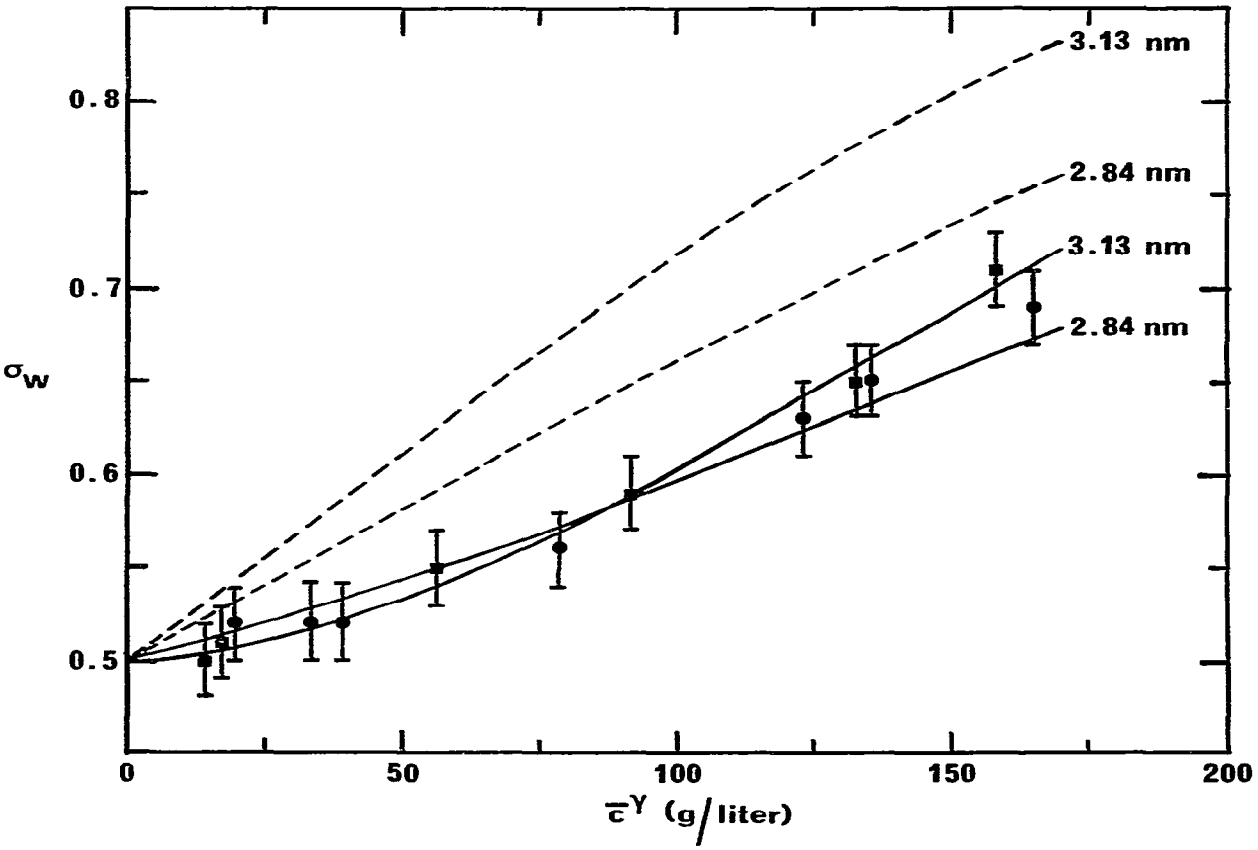

Fig. 2. Comparison of the exclusion chromatographic behavior of bovine (■) and human (●) oxyhemoglobin on CPG-120 porous glass beads with theoretical relationships predicted for various models of the system: (- - - - - -) concentration dependence predicted for a nonassociating $\alpha_2\beta_2$ species. (————) concentration dependent predicted for an $\alpha_2\beta_2$ species undergoing isodesmic indefinite self-association. Numbers adjacent to curves denote the effective covolume radius of the $\alpha_2\beta_2$ entity used in calculating these theoretical relationships. The experimental points, taken from fig. 2 of ref. [1], refer to experiments conducted in 0.156 I phosphate, pH 7.3, 20°C.

tions differ from those presented previously [1] both in examining a range of effective radii and in allowing for partitioning of the $(\alpha_2\beta_2)_2$ species according to eqs. (5)–(8) with $\sigma_2^0 = 0.05$ (see ref. [1]). Whereas a value for the mean isodesmic association constant, K_1, of 128 ± 21 M^{-1} ($r = 3.13$ nm) was reported in our earlier work, the values appropriate to the envelope described by the solid curves in fig. 2 are $K_1 = 154 \pm 26$ M^{-1} ($r = 3.13$ nm) and $K_1 = 66 \pm 23$ M^{-1} ($r = 2.84$ nm). Clearly, while consideration of the partitioning of the $(\alpha_2\beta_2)_2$ species leads to a slightly larger value of the mean association constant, the estimated value of K_1 does decrease with decreasing r but retains a nonzero value even in the lower limit that $r = 2.84$ nm. Similar calculations were performed on the basis of a definite self-association of the $\alpha_2\beta_2$ species to form solely a dimer of this species. The

relevant values of the mean dimerization constant, K_2, were found to encompass the range 227 ± 95 M^{-1} ($r = 3.13$ nm) to 74 ± 26 M^{-1} ($r = 2.84$ nm). Within the error bars on experimental points shown in fig. 2 these sets of values also sufficed to describe the results. Indeed, the curves calculated for the lower limit of r(2.84 nm) are virtually identical for both association models. This is expected since, for this radius, $K_1 \approx K_2$ and K_1 is sufficiently small to ensure that $\alpha_2\beta_2$ and $(\alpha_2\beta_2)_2$ species would predominate in the range of $\bar{c}^{\gamma}$ examined.

Fig. 3 summarizes results obtained in the frontal chromatography of oxyhemoglobin on glyceryl-CPG-120B glass beads. It is noted that the extrapolated value ($\sigma_1^0 = 0.62$) is greater than that of 0.50 pertinent to fig. 2. This implies that the mean pore diameter of the glyceryl-coated beads is greater than that of the poly(ethylene glycol)-treated beads, an observation supported by the finding that in control experiments the partition coefficient of lactate dehydrogenase was 0.20 on the former medium and 0.05 on the latter. These values provide suitable estimates for σ_2^0 [1] and highlight the necessity of employing eqs. (2), (5) and (6) to account for the increased partitioning of $(\alpha_2\beta_2)_2$ hemoglobin on the column of glyceryl-coated beads (fig. 3). Attention should be drawn to the possibility that, on this column with the larger mean pore diameter of 11.6 ± 1.0 nm, the $(\alpha_2\beta_2)_3$ species of estimated Stokes' diameter of 9.3–10.3 nm may also partition slightly. This effect is only relevant to results obtained with the glyceryl-coated beads in the event that $(\alpha_2\beta_2)_3$ species exist as postulated in the indefinite self-association model; but even in this instance estimations of the relative concentrations of this trimeric species in the range of $\bar{c}^{\gamma}$ examined and of its partition coefficient, $\sigma_3^0 < 0.05$, show that the contribution to σ_w of the partitioning of trimer would be insignificant. Indeed, the neglect of trimer partitioning on the

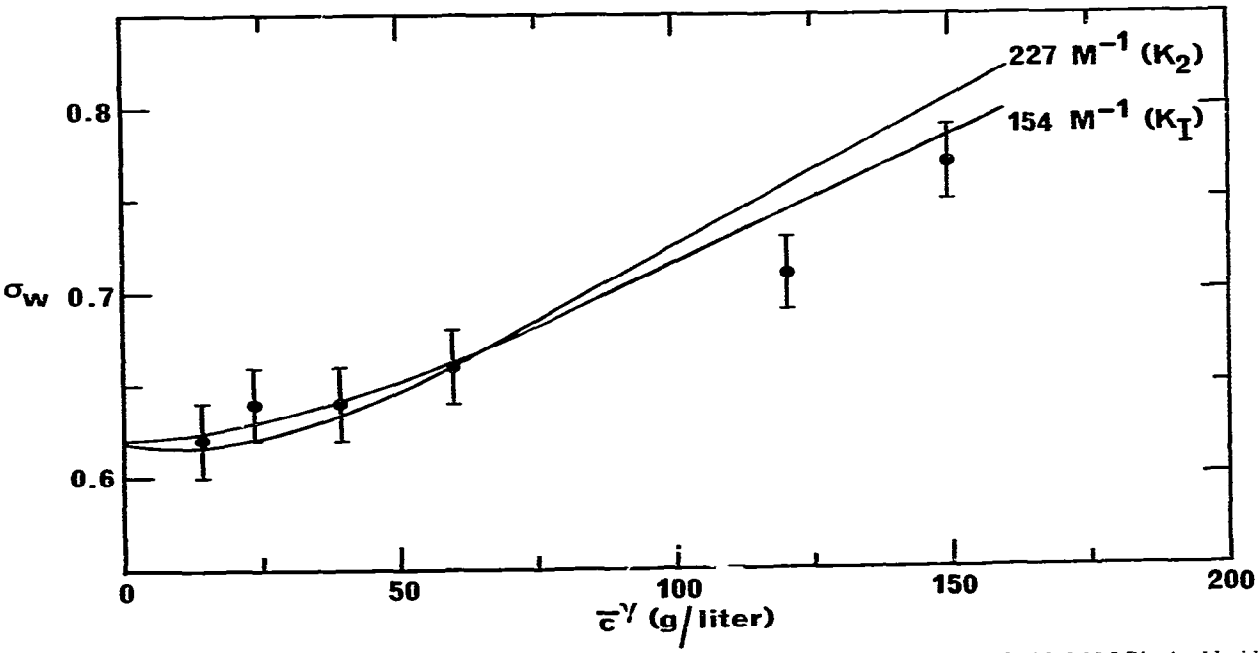

Fig. 3. Exclusion chromatography of human oxyhemoglobin on glyceryl-CPG-120 glass beads equilibrated with 0.15 I Bistris-chloride buffer, pH 7.3, 20°C. The solid curves are those predicted for two modes of $\alpha_2\beta_2$ self-association, discrete dimerization and isodesmic indefinite self-association, using the relevant mean association equilibrium constant deduced from the data in fig. 2 on the basis of a covolume radius of 3.13 nm for the $\alpha_2\beta_2$ entity.

column of glyceryl-coated beads parallels the neglect of dimer partitioning on the poly(ethylene glycol)-coated column [1], which as we have seen in relation to fig. 2 had the effect of underestimating (and then only marginally) estimated values of the association constants.

The solid curves in fig. 3 were computed with $r = 3.13$ nm and the corresponding values of K_1 (154 M^{-1}) and K_2 (227 M^{-1}) obtained from fig. 2 for the examined association models. For this radius it is evident from fig. 3 that of the two postulated modes of association that of isodesmic indefinite self-association is marginally favored, and that in these terms the different sets of results shown in figs. 2 and 3 are not only similar in form but also quantitatively consistent. However, it is not possible to exclude the monomer-dimer association pattern, since consideration of the lower radius (2.84 nm) with $K_1 = 66$ M^{-1} and $K_2 = 74$ M^{-1} (from fig. 2) resulted in curves (virtually identical) which also fitted the results shown in fig. 3.

Fig. 4a and b presents experimental results obtained with deoxyhemoglobin under identical conditions to those pertaining in fig. 3, but over a larger range of $\bar{c}^{\gamma}$. A value of 0.20 for σ_2^0 was again used in the analysis together with a value of $\sigma_1^0 = 0.67$, an extrapolated value from fig. 4 that is larger than the corresponding value of 0.62 found from fig. 3 for the oxygenated form of the protein. Fig. 4a examines the results in terms of the discrete dimerization pattern, $2\alpha_2\beta_2 \rightleftharpoons (\alpha_2\beta_2)_2$, and shows for the deoxy system that this mode of association could only pertain if the radius of the

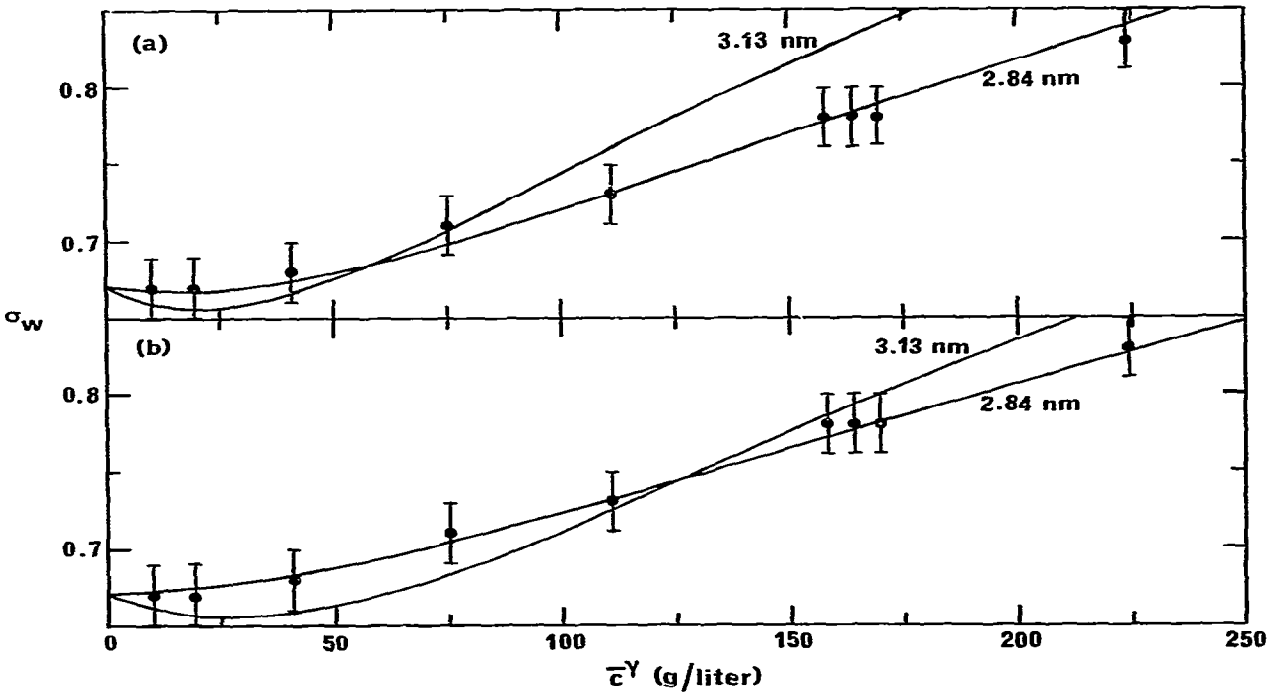

Fig. 4. Exclusion chromatography of human deoxyhemoglobin on glyceryl-CPG-120 glass beads equilibrated with 0.15 I Bistris-chloride buffer, pH 7.3, 20°C. Descriptions of the system on the bases that the $\alpha_2\beta_2$ species undergoes reversible (a) dimerization, and (b) isodesmic indefinite self-association. Numbers adjacent to curves denote the effective covolume radius of the $\alpha_2\beta_2$ entity used in calculating the theoretical relationships.

effective hard sphere for the deoxy $\alpha_2\beta_2$ state was in the vicinity of 2.84 nm, whereupon the calculated mean value of K_2 is 135 ± 33 M^{-1}. On the other hand, fig. 4b shows that the postulate of indefinite self-association is reasonable for deoxyhemoglobin within the envelope of parameters $K_1 = 223 \pm 84$ M^{-1} ($r = 3.13$ nm) and $K_1 = 91 \pm 9$ M^{-1} ($r = 2.84$ nm). Entirely similar sets of values for these parameters emerged from analyses of results obtained with deoxyhemoglobin at a higher temperature, 37°C, indicating that any overall enthalpy change involved in association of the $\alpha_2\beta_2$ form is small.

The final point may be made in relation to the analysis of results presented in figs. 2–4 that the dissociation reaction $\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$ has been neglected. This is entirely reasonable, since the proportion of the $\alpha\beta$ form of both oxy and deoxy hemoglobin is negligible at high total concentrations, being at the most 5% at 10 g/l [30,31], the lowest concentration examined in the present work.

5. General discussion

A major point which emerges from this study is that the forms of curves described by the experimental results in figs. 2–4 are all basically sigmoidal. This type of behavior, which is consistently observed for both oxy- and deoxyhemoglobin, seemingly cannot be described on the basis of nonideality effects in the absence of self-association. For the latter type of system, comparable curves are concave to the abscissa as illustrated in fig. 1 and by the theoretical curves shown in fig. 2 (dashed line). As shown by Minton [2], this basic concave form arising solely from nonideality effects is not changed even by evoking values of α greater than unity, our experimental value (fig. 1). The sigmoidal form of the different sets of experimental results provides then one item of evidence in favor of the postulate of self-association of hemoglobin, and indeed, it is now evident that such results may be fitted quantitatively in this framework. Other items of evidence also favoring this postulate include the spectral and dielectric studies already cited [3–10], and the fact that deoxyhemoglobin A undergoes gelation in concentrated phosphate buffer [32] and copolymerizes with hemoglobin S [33], the genetic variant of hemoglobin that is known to self-associate to form fibers and gels [34].

The precise nature of the self-association pattern of normal adult hemoglobin is less certain. This point may be exemplified by reference to figs. 2–4, and to table 1 which summarizes the ranges of association constants reported in relation to these figures, and reflects the "goodness-of-fit" of the theoretical curves to the experimental results in terms of standard deviations of the constants. It is evident from table 1 that for both oxy- and deoxyhemoglobin, and for either value of r examined, the indefinite self-association pattern is favored over the dimerization model. This type of indefinite association is also consistent with observed fiber and gel formation [32–34], although in this connection it is stressed that the indicated extent of association of normal hemoglobin is certainly less than that of hemoglobin S at comparable total concentrations. The reservation is that if the lower value of r (2.84 nm) is indeed appropriate this distinction between definite and

Table 1

Summary of association equilibrium constants relevant to figs. 2–4

Protein	Dimerization constant, K_2 (M^{-1})		Isodesmic constant, K_1 (M^{-1})	
	$r = 2.84$ nm	$r = 3.13$ nm	$r = 2.84$ nm	$r = 3.13$ nm
Oxyhemoglobin	74 (±26)	227 (± 95)	66 (±23)	154 (±26)
Deoxyhemoglobin	135 (±33)	260 (±160) [a]	91 (± 9)	223 (±84)

[a] Only the points for the five lowest concentrations shown in fig. 4a were used to calculate this mean and standard deviation. Consideration of the next three points led to K_2 values orders of magnitude larger and for the highest concentration point the calculated value of σ_2 became larger than σ_w. These results indicate the inappropriateness of the dimerization model for deoxyhemoglobin with r taken as 3.13 nm.

indefinite association remains tenuous, but would become somewhat esoteric since in this event K_2 approaches K_1 and both are of small magnitude.

The final point requiring discussion pertains to the perturbation (or otherwise) of the association of deoxyhemoglobin by the addition of an effectively saturating concentration of oxygen. If in table 1 the mean values of the association constants for oxy- and deoxyhemoglobin are compared at any fixed values of r, the observation emerges that addition of oxygen has caused a decreased overall extent of association, suggesting that oxygen may bind preferentially to the $\alpha_2\beta_2$ form in comparison with its polymers. Such an effect would result in oxygen binding curves becoming more sigmoidal as the hemoglobin concentration is increased [35], and this type of behavior has been reported [36] although its magnitude remains somewhat controversial [37]. In a critical vein, it could be argued that in table 1 comparisons should not be made at fixed r values, since the appropriate magnitudes of this quantity may differ for oxy- and deoxyhemoglobin. It was also for this reason that a range of values for likely radii was explored. In this event, two possibilities arise. First, if r_{oxy} were significantly greater than r_{deoxy}, the ranges of association constants would completely overlap and no preferential binding of oxygen could be claimed. On the other hand, if $r_{oxy} < r_{deoxy}$, the effect of preferential oxygen binding would in fact be magnified. At present the weight of evidence favors the postulate that oxygen binds preferentially to the $\alpha_2\beta_2$ form of deoxyhemoglobin in comparison with its polymers, a concept which would also be of relevance to the extent of oxygen binding and release in vivo; since such an effect would act, together with cooperative effects, in determining the form of the binding response and may well be magnified in the presence of other erythrocyte components.

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