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THE STUDY OF MULTIPLE POLYMERIZATION EQUILIBRIA BY GLASS BEAD EXCLUSION CHROMATOGRAPHY WITH ALLOWANCE FOR THERMODYNAMIC NON-IDEALITY EFFECTS

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Two related aspects are explored of the frontal exclusion chromatography of proteins employing controlled-pore glass beads as the stationary phase. First, it is shown theoretically that, despite the absence of osmotic shrinkage effects previously encountered with Sephadex matrices, the experimentally measurable partition coefficient of a single non-associating solute will be dependent on its concentration due to the differing ratios of activity coefficients in mobile and stationary phases at different total concentrations. The effect is demonstrated with results obtained using ovalbumin in phosphate buffer of pH 7.4, and is shown to be consistent (up to a solute concentration of 5 g/litre) with theoretical prediction formulated in terms of a single virial coefficient. Secondly, it is shown for self-associating systems that it is possible to determine the monomer concentration as a function of total concentration, provided the stationary phase is selected to ensure exclusion of all oligomeric species except monomer: the relation derived for this purpose accounts for the concentration-dependence of the partition coefficient of monomer, again as a first approximation involving one virial coefficient. Such information on the monomer concentration permits elucidation of the polymerization characteristics of the system in terms of the types of species present and the relevant equilibrium constants. The feasibility of the method, its likely sources of error and the relative contribution of the non-ideality effect are investigated using bovine glutamate dehydrogenase (up to a total concentration of 5.4 g/litre) in phosphate buffer of pH 6.9. This system was selected since comparison was possible with results obtained by other methods, which have established the enzyme polymerization pattern as an isodesmic indefinite self-association. The isodesmic equilibrium constant of 1.5 ± 0.3 litre/g found in this work is in reasonable agreement with previous findings.

1. Introduction

The use of gel permeation (exclusion) chromatography for the study of protein systems comprising monomer in equilibrium with a single higher polymer has been studied extensively [1–4], but its use for systems involving several polymeric forms has received far less attention. Two procedures [4,5] have been suggested for the analysis of results obtained with such systems, but each requires the assignment of the elution volumes (or partition coefficients) of all polymeric species present in the equilibrium mixture. The accuracy of any polymerization constants so determined thus relates directly to the reliability of the values assessed or assumed for these parameters, a point exemplified in a previous experimental study [4]. In this investigation, we consider the possibility of selecting a stationary phase such that only monomer distributes between mobile and stationary phases; the chromatographic characteristics of all polymeric forms are thus not only defined unequivocally but are also identical. It is shown that the con-

sequent simplification of the analysis leads to no loss in generality of its application to the elucidation of a diverse range of self-associating systems.

This elucidation requires, for a given system, distinction between several possible polymerization patterns and, in general, this is only possible if results have been obtained over a wide range of total solute concentration. At relatively high concentrations, osmotic shrinkage of conventional gel chromatographic media [6–8] complicates the quantitative analysis of association equilibria because neither the elution volumes of excluded polymers nor those of any partitioning species (monomer in the present context) can be regarded as invariant. This problem of osmotic shrinkage may be overcome by choosing controlled-pore glass beads as the stationary phase, thereby guaranteeing constancy of the volume of the mobile phase. The difficulty remains, however, that a penetrating solute might still exhibit concentration-dependent partition due to thermodynamic non-ideality. In this work, the latter point is explored theoretically and examined experimentally using ovalbumin as a model non-associating solute. The findings are extended to the consideration of a monomer (capable of partition) in equilibrium with excluded polymers and the feasibility of characterizing such multiple association equilibria is illustrated with bovine liver glutamate dehydrogenase, an enzyme that undergoes indefinite self-association [9–11].

2. Theory

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

Consider the distribution of a solute, i, between mobile phase (γ) and stationary phase (β) in the solute plateau region of a frontal exclusion chromatography experiment. The condition of partition equilibrium is given in terms of chemical potential by eq. (1a), rearrangement of which yields eq. (1b).

$$\mu_i^\gamma = \mu_i^\beta = (\mu_i^\gamma)^0 + RT\ln a_i^\gamma = (\mu_i^\beta)^0 + RT\ln a_i^\beta, \quad (1a)$$

$$a_i^\beta/a_i^\gamma = \exp\{[(\mu_i^\gamma)^0 - (\mu_i^\beta)^0]/RT\}, \quad (1b)$$

where a_i is the thermodynamic activity of solute, i. Since the right hand side of eq. (1b) is a constant independent of total concentration at constant temperature, it follows that:

$$\frac{(c_i^\beta)_\mathrm{I}(y_i^\beta)_\mathrm{I}}{(c_i^\gamma)_\mathrm{I}(y_i^\gamma)_\mathrm{I}} = \frac{(c_i^\beta)_\mathrm{II}(y_i^\beta)_\mathrm{II}}{(c_i^\gamma)_\mathrm{II}(y_i^\gamma)_\mathrm{II}}, \quad (1c)$$

where subscripts I and II refer to two frontal experiments conducted with different total weight concentrations of solute, c_i^γ. In general, each activity coefficient y_i may be expressed [12] as:

$$\ln y_i = \alpha_{ii}m_i + \sum\alpha_{ij}m_j + \text{higher terms}, \quad (2a)$$

where m_i denotes molal (assumed molar) concentrations and α_{ii} and α_{ij} are constants. In the γ (mobile) phase only physical interactions of i with itself need be considered, whereupon eq. (2a) gives (neglecting the higher terms):

$$(y_i^\gamma)_\mathrm{I} = \exp\{(\alpha_{ii}/M_i)(c_i^\gamma)_\mathrm{I}\};$$
$$(y_i^\gamma)_\mathrm{II} = \exp\{(\alpha_{ii}/M_i)(c_i^\gamma)_\mathrm{II}\}, \quad (2b)$$

where M_i is the molecular weight of solute i. In the β (stationary) phase, interactions with the matrix (j) as well as self-interactions must be considered, whereupon the similarly truncated eq. (2a) gives:

$$(y_i^\beta)_\mathrm{I} = \exp\{(\alpha_{ii}/M_i)(c_i^\beta)_\mathrm{I} + \alpha_{ij}(m_j^\beta)_\mathrm{I}\},$$
$$(y_i^\beta)_\mathrm{II} = \exp\{(\alpha_{ii}/M_i)(c_i^\beta)_\mathrm{II} + \alpha_{ij}(m_j^\beta)_\mathrm{II}\}. \quad (2c)$$

With the substitution [5], $\sigma_i = c_i^\beta/c_i^\gamma$, combination of eq. (1c), (2b) and (2c) gives:

$$\sigma_i^\mathrm{I} = \sigma_i^\mathrm{II}\exp[(\alpha_{ii}/M_i) \times \{[(c_i^\gamma)_\mathrm{I} - (c_i^\beta)_\mathrm{I}] - [(c_i^\gamma)_\mathrm{II} - (c_i^\beta)_\mathrm{II}]\}]. \quad (3)$$

Derivation of eq. (3) assumes identity of the matrix concentrations $(m_j^\beta)_\mathrm{I}$ and $(m_j^\beta)_\mathrm{II}$, which is reasonable for a matrix that is subject to no (or extremely little) osmotic shrinkage.

If σ_i^II is now equated with σ_i^0, the value of the partition coefficient at infinite dilution, it follows that the difference $[(c_i^\gamma)_\mathrm{II} - (c_i^\beta)_\mathrm{II}]$ may be neglected, and eq. (3) then becomes:

$$\sigma_i = \sigma_i^0\exp\{(\alpha_{ii}/M_i)c_i^\gamma(1-\sigma_i)\}. \quad (4)$$

Eq. (4) shows that no concentration dependence of σ_i is predicted for the extreme σ_i values of zero (no

partition, i.e., total exclusion) and of unity (equal partition between phases). On the other hand, for all other values of σ_i concentration-dependent partition is predicted by eq. (4). This point is examined more fully later in relation to chromatographic results obtained with ovalbumin on a column of porous glass beads. From the experimental viewpoint it could be noted that σ_i is identical with the commonly used distribution coefficient parameter K_D [13], and hence,

$$\sigma_i \equiv (K_D)_i = (V - V_0)/(V_t - V_0), \tag{5}$$

where V denotes the elution volume of species i from a column with void volume V_0 and total accessible volume V_t.

2.2. Self-associating systems

Regardless of the nature of the polymerization pattern, a series of frontal chromatography experiments with different plateau concentrations $\bar{c}^\gamma$ allows the determination of the concentration-dependence of the weight-average elution volume V_w and hence of the weight-average partition coefficient σ_w. The latter quantity, which may be obtained experimentally via eq. (5), is defined by:

$$\sigma_w = \sum_i (\sigma_i c_i^\gamma)/\bar{c}^\gamma; \quad \bar{c}^\gamma = \sum_i c_i^\gamma, \tag{6a}$$

where $i = 1$ denotes monomer, $i = 2$ dimer, etc. Provided that a stationary phase is chosen which excludes all polymers ($i \geqslant 2$), it follows that all σ_i ($i \neq 1$) are zero and hence that eq. (6a) simplifies to:

$$\sigma_w = \sigma_1 c_1^\gamma/\bar{c}^\gamma, \tag{6b}$$

where σ_1 is the partition coefficient of monomer in an experiment with total solute concentration $\bar{c}^\gamma$. The limiting value σ_1^0 may be obtained by extrapolating the plot of σ_w versus $\bar{c}^\gamma$ to infinite dilution; but an expression analogous to eq. (4) is required to estimate σ_1 at each $\bar{c}^\gamma$ before use may be made of eq. (6b).

Formulation of the required expression is based on eq. (1) and (2a), which remain valid when written with $i = 1$; the resulting expressions refer to monomer, the only species capable of partition. In the mobile (γ) phase only interactions between monomer and all other oligomer species need be considered, and hence from the truncated form of eq. (2a) we may write:

$$y_1^\gamma = \exp\left\{\frac{\alpha_{11} c_1^\gamma}{M_1} + \frac{\alpha_{12} c_2^\gamma}{M_2} + \frac{\alpha_{13} c_3^\gamma}{M_3} + \ldots\right\}. \tag{7a}$$

If the assumption is made that all $\alpha_{1j}/M_j = BM_1$, eq. (7a) becomes that suggested by Adams and Fujita [14] and used extensively in relation to equilibrium sedimentation analysis. With this simplification we may write:

$$(y_1^\gamma)_I = \exp\{BM_1 \bar{c}_I^\gamma\}; \quad (y_1^\gamma)_{II} = \exp\{BM_1 \bar{c}_{II}^\gamma\}. \tag{7b}$$

For the stationary (β) phase, eq. (2a) becomes:

$$\begin{aligned}(y_1^\beta)_I &= \exp\{BM_1 (c_1^\beta)_I + \alpha_{1j} (m_j^\beta)_I\},\\ (y_1^\beta)_{II} &= \exp\{BM_1 (c_1^\beta)_{II} + \alpha_{1j} (m_j^\beta)_{II}\},\end{aligned} \tag{7c}$$

which is entirely analogous with eq. (2c). Combination of eq. (1c), (7b) and (7c) yields, in conjunction with the identities $\sigma_1^{II} = \sigma_1^0$, $\{\bar{c}_{II}^\gamma - (c_1^\beta)_{II}\} = 0$ and $(m_j^\beta)_I = (m_j^\beta)_{II}$;

$$\sigma_1 = \sigma_1^0 \exp\{BM_1 (\bar{c}^\gamma - c_1^\beta)\}. \tag{8a}$$

On noting that $c_1^\beta = \sigma_1 c_1^\gamma$ by definition and that it therefore equals $\sigma_w \bar{c}^\gamma$ (from eq. (6b)), eq. (8a) may be rewritten as:

$$\sigma_1 = \sigma_1^0 \exp\{BM_1 \bar{c}^\gamma (1 - \sigma_w)\}, \tag{8b}$$

which is consistent with eq. (4) as required.

Four points may be made in relation to the assumption $\ln y_i = iBM_1 \bar{c}^\gamma$. First, it has been shown to be strictly valid for association of highly charged species [15] and for end-to-end association of long cylinders [16] and of long rods [12]. Secondly, its use permits identification of the thermodynamic equilibrium constants defined as ratios of oligomeric activities with equivalent ratios expressed on a weight-concentration scale [14]. Thirdly, calculations based on the association of uncharged spherical molecules, the most unfavourable case theoretically from the viewpoint of the approximation, have shown [12] that use of the Adams–Fujita approximation may still yield a reliable value of the thermodynamic equilibrium constant. Fourthly, in exclusion chromatography the importance of the $BM_1 \bar{c}^\gamma$ term as a correction for the operation of non-ideality effects is counteracted to some extent (eq. (8b)) by the factor $(1 - \sigma_w)$ which is necessarily less than unity.

Combination of eqs. (6b) and (8b) yields an expression for the concentration of monomer as a function of total concentration which permits direct evaluation of c_1^γ from experimental $(\sigma_w, \bar{c}^\gamma)$ points, provided that BM_1 may be estimated. Thus,

$$c_1^\gamma = \sigma_w \bar{c}^\gamma / [\sigma_1^0 \exp\{BM_1 \bar{c}^\gamma (1 - \sigma_w)\}]. \quad (9)$$

The estimation of BM_1 may proceed either empirically [14] or by recalling that it may be equated (approximately) with α_{11}/M_1, where

$$(\alpha_{11}/M_1) = [U_{11} + (Z^2/2I)]/M_1 \quad (10)$$

and U_{11} denotes the covolume of monomer, Z its valence and I the ionic strength of the medium [12].

The determination of $(c_1^\gamma, \bar{c}^\gamma)$ from measurements of $(\sigma_w, \bar{c}^\gamma)$ is of value in establishing the polymerization pattern of the self-associating system. This may be exemplified by considering an isodesmically self-associating system, pertinent to results to be presented later. For such an indefinitely associating system governed by a single equilibrium constant X (on a molar scale), it has been established that the total weight-concentration is related to monomer weight-concentration by the expression [17]

$$\bar{c}^\gamma = c_1^\gamma / \{1 - (Xc_1^\gamma/M_1)\}^2; \quad (Xc_1^\gamma/M_1) < 1. \quad (11a)$$

This quadratic equation may be solved uniquely for X, since only the smaller root is acceptable because of the requirement that $(Xc_1^\gamma/M_1) < 1$. Hence,

$$X = (M_1 \bar{c}^\gamma - M_1 \sqrt{c_1^\gamma \bar{c}^\gamma}) / \bar{c}^\gamma c_1^\gamma. \quad (11b)$$

Clearly, eq. (11b) may be used to evaluate X from each of a series of $(c_1^\gamma, \bar{c}^\gamma)$ points: constancy of X over a range of $\bar{c}^\gamma$ is the criterion for the validity of the isodesmic model. Similar closed solutions relating c_1^γ to $\bar{c}^\gamma$ are available for other types of indefinite self-association [18,19] which may therefore also be examined in the same terms. Furthermore, the analysis may be applied to definite self-associations, where $\bar{c}^\gamma$ is also expressable as a polynomial in c_1^γ with a finite number of terms. In the present context, an interesting example of such a system is a simple monomer-dimer equilibrium, because it is also governed by a single equilibrium constant and (in exclusion chromatography) by two species elution volumes. For such a system it is readily shown that:

$$\bar{c}^\gamma = c_1^\gamma + \{2X(c_1^\gamma)^2/M_1\}, \quad (12a)$$

$$X = M_1(\bar{c}^\gamma - c_1^\gamma)/2(c_1^\gamma)^2, \quad (12b)$$

where, as before, X is the association constant expressed on a molar scale. Despite the qualitative similarities between the isodesmic and the two-state system, results to be presented for glutamate dehydrogenase serve to show that eqs. (11) and (12) predict pronounced quantitative differences. Thus conversion of the isodesmic case to a two-elution-volume system by excluding all polymers from the stationary phase does *not* revert the system to a comparable monomer-single higher polymer case.

3. Experimental

3.1. Studies of ovalbumin

Ovalbumin (Grade V, crystallized, freeze-dried and salt-free) and bovine plasma fraction I were obtained from Sigma Chemical Co., St. Louis, Mo., the latter protein fraction being used as a source of fibrinogen, which was prepared by the method of Sturtevant et al. [20]. Ovalbumin and fibrinogen samples were dissolved in 0.156 I phosphate-chloride buffer (0.008 M Na_2HPO_4-0.002 M NaH_2PO_4-0.110 M KCl-0.020 M NaCl), pH 7.4, and dialysed against the same medium prior to use. Concentrations of the dialysed solutions were determined spectrophotometrically at 280 nm on the basis of specific absorptivity $(A_{1\,cm}^{1\%})$ of 6.6 and 16.0 for ovalbumin [21] and fibrinogen [22] respectively.

Solutions of ovalbumin (0.2–30 g/litre) were subjected to frontal gel chromatography [1] on a column (0.9 × 51 cm) of CPG-10 120 A glass beads (Electro-Nucleonics, Inc., Fairfield, N.J.) which had been pretreated with polyethylene glycol to minimize adsorption effects [23]. The column was equilibrated with the phosphate-chloride buffer, pH 7.4, at a flow rate of 14.7 ml/h: the column eluate was divided into 1.2 ml fractions which were then diluted appropriately for spectrophotometric assay at 280 nm. Bovine fibrinogen was used to determine the void volume V_0 of the column (18.4 ml), the total acessible volume V_t of which was taken as the elution volume of K_2CrO_4 (26.6 ml). Elution volumes were converted to partition coefficients via eq. (5).

3.2. Studies of glutamate dehydrogenase

L-glutamate dehydrogenase from bovine liver was obtained as a freeze-dried powder (type III) from Sigma Chemical Co., and dissolved directly in 0.46 I phosphate buffer (0.13 M Na_2HPO_4-0.07 M NaH_2PO_4), pH 6.9, containing 10 mg/litre of sodium azide to prevent bacterial contamination. Before each experiment solutions were dialysed at 4°C for 16 h and subjected to membrane filtration (0.8 μm pore size) immediately prior to exclusion chromatography on columns (0.9 X 62 cm) of CPG-10 170 B glass beads (batch mean pore diameter of 15.9 nm) which had also been pre-treated with polyethylene glycol. Two such columns, thermostatically maintained at 25°C, were pre-equilibrated with degassed phosphate buffer, pH 6.9: the void volumes (determined with blue dextran) were 21.8 and 22.0 ml, and the corresponding total volumes (determined with K_2CrO_4) were 35.7 and 35.9 ml. Protein solution (~ 30 ml) was applied to the column at 20 ml/h and approximately 1 ml fractions were collected in tared tubes so that the precise volume of each fraction could be determined by weight: the buffer density was 1.025 g/ml. Concentrations of glutamate dehydrogenase were determined spectrophotometrically on suitably diluted (by weight) samples at 279 nm using a specific absorptivity ($A^{1\%}_{1\,cm}$) of 9.7 [24]. Elution volumes were again converted to partition coefficients via eq. (5).

4. Results

4.1. Exclusion chromatography of ovalbumin

Results obtained from frontal gel chromatography of ovalbumin on CPG-10 120A equilibrated with 0.156 I phosphate-chloride buffer, pH 7.4, are shown as the experimental points in fig. 1, which clearly establish the existence of concentration-dependent partition. In order to compare these results with the behaviour predicted by eq. (4) it is first necessary to evaluate α_{ii}/M_i from eq. (10). The covolume U_{ii} may be assigned a value of 500 litre/mole [25] and M_i = 45 000. Comparison of the electrophoretic mobility under the present conditions with that in phosphate-free medium with identical pH and ionic strength

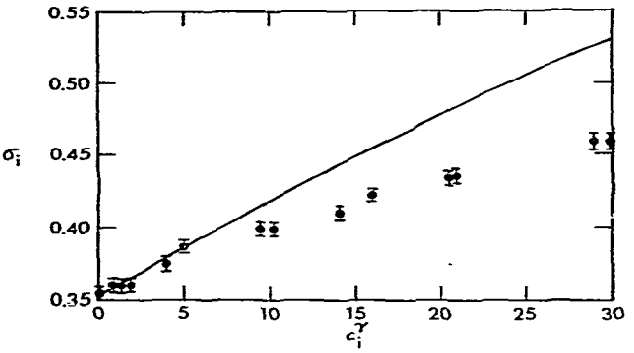

Fig. 1. The concentration-dependence of the partition coefficient (σ_i) of ovalbumin found in a series of chromatography experiments performed with different plateau concentrations (c_i^γ, g/litre) in phosphate buffer, 0.156 I, pH 7.4, 25°C on a column (0.9 X 51 cm) of CPG-10 120 A glass beads. Experimental points and assessed error bars are shown, together with the theoretical curve calculated using eq. (4).

yields a value of −16 for Z, the protein valence: this estimate is based on the valence of −14 for ovalbumin at this pH in phosphate-free buffer [25]. The solid line drawn in fig. 1 has been constructed on the basis of eq. (4), a value of 1300 litre/mole for α_{ii} (= U_{ii} + $Z^2/2I$) and a σ_i^0 of 0.353. In the concentration region $0 < c_i^\gamma < 5$ g/litre, this theoretical curve provides an entirely satisfactory description of the experimental results: thereafter, the theoretical curve lies above the experimental σ_i because of the neglect in eq. (4) of higher order virial terms [7]. From the experimental viewpoint the agreement between experimental results and the theoretical predictions on the basis of the truncated eq. (4) in the range $0 < c_i^\gamma < 5$ g/litre lends justification to use of the similarly truncated eq. (7) in analysis of results obtained with glutamate dehydrogenase over a similar concentration range ($0 < \bar{c}^\gamma < 5.4$ g/litre).

4.2. Association of bovine liver glutamate dehydrogenase

Glutamate dehydrogenase has been selected as a well-characterized model system with multiple association equilibria because of the accumulated evidence that the monomer of this enzyme undergoes indefinite association characterized by an isodesmic associa-

tion constant (X/M_1) of 1.4–2.3 litre/g [9–11, and references cited therein]. The value of BM_1 for use in eq. (9) may be calculated as 0.023 litre/mole from eq. (10) with M_1 = 309 000 [11], U_{11} = 5 400 litre/mole and Z = –40. The covolume U_{11} refers to a right cylindrical monomer with a length of 13.3 nm and a radius of 4.63 nm [26]; the charge Z has been calculated from the reported electrophoretic mobility [24] by the method of Abramson et al. [27]. Calculation of the Stokes diameter of monomer either from the dimensions of the cylindrical model [26] or from M_1 and the monomeric sedimentation coefficient ($s^0_{20,w}$) of 11.4 S [28] yields a value of 12.0 nm. On the basis of the relationship between translational diffusion coefficients of polymers that is used to assess quasielastic light scattering measurements on glutamate dehydrogenase (fig. 1 of [10]) the Stokes diameter of dimer is then 17.2 nm. A batch of porous glass beads with a specified mean pore diameter of 15.9 nm has therefore been used in order to meet the requirement of the theory that all polymeric species be excluded from the stationary phase.

Fig. 2 shows normalized elution profiles obtained by applying glutamate dehydrogenase (30 ml) at concentrations of 0.01 g/litre (■) and 1.62 g/litre (●) to a column of CPG-10 170B (0.9 × 62 cm) equilibrated with 0.46 I phosphate buffer, pH 6.9. First it is noted from the profile obtained at the higher plateau concentration where comparable proportions of monomer coexist with higher polymers that the advancing front is sharper than its trailing counterpart, a characteristic feature of profiles for systems in rapid association equilibrium [1,2]. Secondly, because of the essentially symmetrical nature of the advancing boundary the volume at which $c = \bar{c}^\gamma/2$ has been used routinely as a sufficiently accurate estimate of the weight-average elution volume V_w: check calculations using the latter quantity have verified that no experimentally significant error has been introduced by this approximation. Thirdly, the two profiles clearly demonstrate pronounced concentration-dependence of the elution volume, the variation being qualitatively in accordance with the existence of glutamate dehydrogenase as a series of polymers in rapid association equilibrium. This qualitative inference is given quantitative significance in table 1, which presents the weight-average partition coefficients (column 2) obtained from a series of frontal experiments with different plateau concentrations $\bar{c}^\gamma$ (column 1). Extrapolation of these results to infinite dilution yields a value of 0.56 ± 0.04 for σ^0_1. Column 3 gives the con-

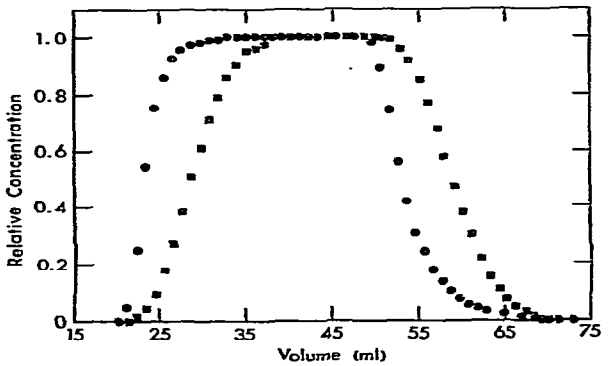

Fig. 2. Elution profiles obtained in the chromatography of bovine liver glutamate dehydrogenase in phosphate buffer, 0.46 I, pH 6.9 at 25°C on a column (0.9 × 62 cm) of CPG-10 170 B glass beads. The plateau concentrations, $\bar{c}^\gamma$, were 0.01 g/litre (■) and 1.62 g/litre (●). The ordinate refers to the ratio of the total concentration at any point to that in the plateau ($\bar{c}^\gamma$).

Table 1
The analysis of weight-average partition coefficients of glutamate dehydrogenase

$\bar{c}^\gamma$ (g/litre)	σ_w	c_1^γ (g/litre)	(X/M_1), litre/g	
			iso-desmic	monomer-dimer
5.36	0.042	0.357	2.1	19.7
4.07	0.067	0.446	1.5	9.1
2.99	0.076	0.380	1.7	9.0
1.62	0.141	0.395	1.3	3.9
1.05	0.174	0.320	1.4	3.6
0.845	0.199	0.296	1.4	3.1
0.495	0.281	0.246	1.2	2.0
0.414	0.282	0.207	1.4	2.4
0.234	0.328	0.137	1.7	2.6
0.177	0.421	0.133	1.0	1.3
0.098	0.453	0.079	1.3	1.5
0.049	0.474	0.041	1.9	2.2
0.010 a)	0.530	0.009_5	–	–
Average (and standard deviation)			1.5 ± 0.3	5.0 ± 5.3

a) These data refer to the elution profile (■) shown in fig. 2, where approximately 95% of the plateau concentration is monomer, a proportion prohibiting reliable estimate of (X/M_1).

centration of monomer c_1^γ calculated from each (σ_w, $\bar{c}^\gamma$) pair by means of eq. (9) with BM_1 taken as 0.023 litre/mole. Estimation of c_1^γ has been completely independent of the nature of the association equilibria, which may therefore be assessed from the dependence of c_1^γ upon $\bar{c}^\gamma$. Columns 4 and 5 present the analysis of each (c_1^γ, $\bar{c}^\gamma$) pair in terms of an isodesmic indefinite self-association (eq. (11b)) and a monomer-dimer system (eq. (12b)), respectively.

Several points merit comment. (i) Description of the system as a monomer-dimer equilibrium is unacceptable because of the large and systematic variation in the calculated apparent association constant; but the results are consistent with the isodesmic model, the standard deviation amounting to an uncertainty of about ± 7 kcal/mole in ΔG^0. The ability of the results to distinguish between these two models is emphasized in fig. 3, where the solid line presents the dependence of σ_w upon $\bar{c}^\gamma$ that is predicted for the isodesmic model from eq. (11a), eq. (9) and the average value of 1.5 litre/g for (X/M_1): the broken line, calculated from eq. (12a) and eq. (9), is the corresponding curve for the monomer-dimer case. Similar curves for the monomer-dimer case based on different values of σ_1^0 in the range reported also failed to describe satisfactorily the experimental results. In contrast, any deviations of the results from the theoretical curve describing isodesmic association of glutamate dehydrogenase are clearly within experimental error. (ii) Repetition of the calculations for the isodesmic case with BM_1 in eq. (9) set equal to zero leads to an essentially identical estimate of 1.4 ± 0.3 litre/g for (X/M_1), revealing that the result is relatively insensitive to non-ideality effects in the concentration range 0–5 g/litre. In this connection it is noted that the value of 0.023 litre/mole used for BM_1 overestimates the effect of thermodynamic non-ideality [29], since U_{11} represents an upper limit of the covolume term (see fig. 1 of [26]). (iii) A potentially greater source of error in the present chromatographic procedure is the extrapolation required in the estimation of σ_1^0 (see fig. 3). However, use of the extreme values of 0.52 and 0.60 for σ_1^0 yielded isodesmic association constants of 1.2 ± 0.3 litre/g and 1.8 ± 0.4 litre/g, respectively: clearly, the value of 1.5 ± 0.3 litre/g reported in table 1 encompasses these values. (iv) The magnitude of (X/M_1) is in very good agreement with the literature reports of 1.4 litre/g [10], 1.7 litre/g [29], 2.0–2.1 litre/g [26,28] and 2.25 litre/g [11].

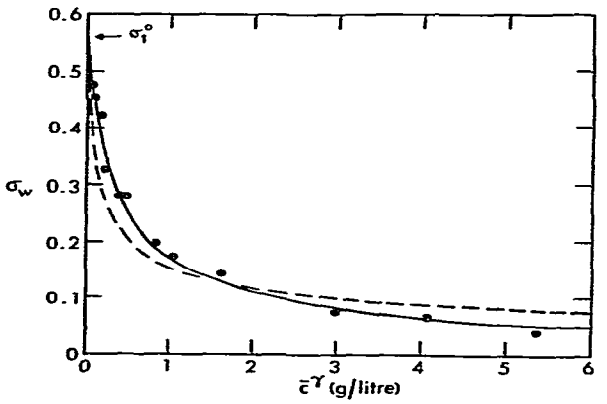

Fig. 3. The dependence of the weight-average partition coefficient (σ_w) of bovine liver glutamate dehydrogenase on total protein concentration ($\bar{c}^\gamma$). (•) experimental points; solid curve was calculated using eqs. (11a) and (9) referring to an isodesmic indefinite self-association with values $\sigma_1^0 = 0.56$, $(X/M_1) = 1.5$ litre/g and $BM_1 = 0.023$ litre/mole; broken curve was calculated using eqs. (9) and (12) for a monomer dimer model using the same values of parameters, but with $(X/M_1) = 5$ litre/g (table 1).

5. Discussion

The basic point which emerges from this work is that the concentration of monomer in self-associating protein systems of any type may readily be evaluated as a function of total protein concentration from weight-average partition coefficients determined in frontal chromatography in which all polymers are excluded from the stationary phase. This conclusion follows directly from eq. (6b) which predicts a simple linear relationship between weight-average partition coefficient and weight-fraction of monomer ($c_1^\gamma/\bar{c}^\gamma$) in the event that non-ideality effects are negligible and hence, that the partition coefficient of monomer may be taken as a constant (σ_1^0). Even when first-order non-ideality effects require consideration, eq. (9) together with an assessment of the second virial coefficient (eq. (10)) provides a pathway (illustrated in table 1) for the interpretation of chromatographic results in these terms. This type of information on the weight-fraction of monomer is basic to the thermo-

dynamic characterization of self-association patterns [18,19,30] and is available from other types of study; for example, from sedimentation equilibrium where it emerges from analysis of derived weight-average molecular weights [18] or more directly from the recently developed $\Omega(r)$ method [31]. In this sense, the present chromatographic procedure is complementary to other established methods, and, in the elucidation of certain systems, may offer little advantage except in simplicity of experimental design. On the other hand, situations have been encountered [32,33] where the ability to employ low plateau concentrations ($\bar{c}^{\gamma}$) and to monitor the elution profile by enzymic assay has proven essential to the detection of interactions governed by large association constants. For such systems, the present chromatographic approach does offer advantage in quantitative characterization by a means which does not require assessment of the partition coefficients of individual polymers. Moreover, with the elimination of osmotic shrinkage effects [7,8] by the use of glass beads as the stationary phase, the potential is offered of studying self-associating systems at relatively high total concentrations. In the latter instance, as exemplified by the results on ovalbumin (fig. 1) it would be necessary to utilize an expression accounting for non-ideality effects: in the absence of osmotic shrinkage effects, eq. (9) is certainly more appropriate for this purpose than the linear relation previously suggested [30], but even it may need to be expanded to include higher order virial coefficients [7] in the event that very high plateau concentrations are employed. In this connection, the finding is encouraging that the value of the isodesmic association constant for glutamate dehydrogenase found by the chromatographic method was relatively insensitive to the non-ideality correction, which includes (eq. (9)) the factor $(1 - \sigma_w) < 1$.

Finally, particular attention is drawn to the work of Chun et al. [30], which also discussed the interpretation of weight-average partition coefficients of glutamate dehydrogenase. These workers chose to employ a stationary phase (Sepharose 4B) which ensured that the partition coefficients of oligomeric species differed and, in contrast to the present study, did not in their initial analysis evaluate the weight-fraction of monomer from σ_w values. Instead, they utilized information on the self-association pattern derived from previous weight-average molecular weight studies, to examine possible models which empirically might govern the relationship between the partition coefficient of monomer and other σ_i ($i \geqslant 2$). Their chromatographic studies were therefore explicitly directed toward elucidation of polymer geometry (linear indefinite association in this case) rather than toward elucidation of the type of self-association itself. It follows that, in the investigation of systems of unknown polymerization pattern, two types of exclusion chromatography are useful. First, choice of a stationary phase which allows partition of only monomer would permit thermodynamic characterization of the polymerization pattern, as established in the present study. Secondly, complementary investigation employing a stationary phase which ensures differences in the partition coefficients of most oligomeric species provides a basis for comment on polymer geometry in terms of assumed models [30].

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