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Measurement of thermodynamic nonideality arising from volume-exclusion interactions between proteins and polymers

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Abstract

The effective thermodynamic radii of 23 ribosomal proteins from the 50 S subunit have been determined by gel chromatography on Sephadex G-50, thereby supporting the contention that most of the proteins of the 50 S ribosomal unit exhibit reasonably globular structures. To investigate further the usefulness of modelling proteins as spheres, the second virial coefficient describing excluded volume interactions of some ribosomal proteins with two inert polymers, polyethylene glycol (PEG) and dextran, has been determined by gel chromatography and/or sedimentation equilibrium techniques. Protein–polymer excluded volumes obtained with PEG 20000 and Dextran T70 as the space-filling solute are shown to conform reasonably well with a quantitative expression describing interaction between an impenetrable sphere and an ideal Brownian path (K.M. Jansons and C.G. Phillips, J. Colloid Interface Sci., 137 (1990) 75).

Keywords: Excluded volume; Molecular crowding; Protein–polymer interactions; Second virial coefficients; Thermodynamic nonideality

1. Introduction

Excluded-volume interactions between macromolecules give rise to thermodynamic nonideality that can often be taken into account, as a first approximation, by considering the deviation from ideal behaviour to be linear in solute concentration. Determination of the second virial coefficient is the paradigm, but the connection between the osmotic virial expansion and the thermodynamic activity coefficients of solute species means that results obtained by a wide variety of biophysical techniques can be analyzed in this way [1–3]. Measuring the effects of thermodynamic nonideality, together with a microscopic interpretation in terms of intermolecular forces, is fundamental to our understanding of the processes which occur in the crowded environment of living cells, where the 'infinite dilution' approximation fails [4]. Volume-exclusion interactions, which arise simply because matter occupies space, account for many effects which are observed upon inclusion of an inert solute in a dilute protein solution [5–15]. These effects are frequently explored with a polymer such as dextran or polyethylene glycol as the inert solute, on the grounds that the dimensions of such molecules are relatively large because of their extended configuration.

Quantitative analysis of the interactions between globular proteins and chain polymers has been hin-

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dered by the complexity of calculating the excluded volume between a solid geometrical object (the protein) and a configurationally averaged irregular chain (the polymer). Hermans [16,17] made some progress on the problem of excluded volume calculations for a protein and a polymer chain comprising flexible segments; and established rule-of-thumb consistency between experimental results and predicted behaviour. However, Jansons and Phillips [18] have now derived a general formula which can be adapted readily to many contexts. As a first approximation it is convenient to regard the protein as an effective sphere, and the configuration of the polymer chain as a Brownian path. Such action allows the mutually excluded volume to be expressed in terms of the radius of the protein and the radius of gyration of the polymer.

In this communication we report measurements of protein–polymer covolumes obtained from gel chromatographic and sedimentation equilibrium data on protein solutions supplemented with either dextran or polyethylene glycol. By this relatively simple methodology [19], we have been able to confirm the superiority of the Jansons and Phillips calculation [18] over previous expressions for the covolume.

2. Theory

2.1. Gel chromatography of protein alone

In gel chromatography the elution volume of a protein, V_A, is normalized to a column-independent partition coefficient, σ_A, by means of the relationship [20,21]

$$\sigma_A = (V_A - V_0)/(V_t - V_0) = C_A^\beta / C_A^\alpha \qquad (1)$$

which takes into account the total accessible volume of the column (V_t) and the volume of the mobile phase (V_0). The second equality in Eq. 1 follows from mass conservation considerations, which show that the partition coefficient also defines the ratio of solute concentrations in the stationary (β) and mobile (α) phases of the column. Although the concentration scale is immaterial in Eq. 1, we shall, for subsequent contexts, regard C_A^α and C_A^β as being defined on the molar scale.

Parameters emanating from gel chromatography are thermodynamic rather than hydrodynamic [22,23], in which case the chemical potential of the partitioning solute (μ_A) as well as that of solvent (μ_s) must be identical in the two phases because of the equilibrium nature of the partition between them. These two chemical potentials of solute are given by

$$(\mu_A)^\alpha_{T,\mu_s} = (\mu_A^0)_{T,\mu_s} + RT\ln z_A^\alpha = (\mu_A^0)_{T,\mu_s} + RT\ln(\gamma_A^\alpha C_A^\alpha) \qquad (2a)$$

$$(\mu_A)^\beta_{T,\mu} = (\mu_A^0)_{T,\mu_s} + (\mu_A^\beta)_{T,\mu_s} + RT\ln z_A^\beta = (\mu_A^0)_{T,\mu_s} + (\mu_A^\beta)_{T,\mu_s} + RT\ln(\gamma_A^\beta C_A^\beta) \qquad (2b)$$

where $(\mu_A^0)_{T,\mu_s}$ is the standard chemical potential of solute, defined under constraints of constant temperature and chemical potential of solvent, and $(\mu_A^\beta)_{T,\mu_s}$ is the contribution to the chemical potential of A due intrinsically to interaction between the solute molecules and the gel matrix: z_A^α and z_A^β are the respective thermodynamic activities of solute in the aqueous and gel phases. In the limit of zero solute concentration the two activity coefficients (γ_A^α, γ_A^β) become unity, and hence

$$\lim_{C_A \to 0}\left(C_A^\beta / C_A^\alpha\right) = \exp\left\{\left[(\mu_A^0)_{T,\mu_s} - (\mu_A^\beta)_{T,\mu_s}\right]/RT\right\} = \sigma_A^0 \qquad (3)$$

The above derivation of the expression for the limiting partition coefficient attributes the magnitude of σ_A^0 to the difference between the standard chemical potential of A (in bulk solvent) and its value in the stationary phase (gel matrix). In the present study C_A^α is sufficiently small for the partition coefficient measured in the absence of polymer in the mobile phase to correspond, within experimental error, to σ_A^0.

In general, the activity coefficient, γ_i, of solute i in a multicomponent solution can be written as a virial expansion in the concentration of all solute species. Specifically,

$$\ln\gamma_i = 2B_{ii}C_i + \sum_j B_{ij}C_j + \ldots \qquad (4)$$

where B_{ii} and B_{ij} are the osmotic second virial coefficients describing interactions between identical and dissimilar solute species, respectively.

2.2. Gel chromatography in the presence of inert polymer

We now focus attention on the situation in which a very dilute solution of protein is subjected to gel chromatography on the same column but in the presence of a relatively high molar concentration C_M^α of a polymer (macromolecule M) that is excluded from the stationary phase ($C_M^\beta = 0$). Under those circumstances the activity coefficient of A in the mobile phase becomes (from Eq. 4)

$$\ln \gamma_A^\alpha \simeq B_{AM} C_M^\alpha \tag{5}$$

The partition coefficient in the presence of polymer, $(\sigma_A)_M$, is then related to the polymer concentration by the expression

$$\ln \sigma_A \simeq \ln \sigma_A^0 + B_{AM} C_M^\alpha \tag{6}$$

The magnitude of the second virial coefficient B_{AM} for the excluded-volume interaction between protein and inert polymer may thus be obtained as the limiting slope of the dependence of ln σ_A upon polymer concentration C_M^α. If the weight-concentration of polymer, c_M^α, is used instead of the molar concentration (C_M^α), then B_{AM}/M_M is obtained, where M_M is the molecular weight of the polymer. In previous investigations [19,24] the limiting slope was erroneously [2] designated as $(B_{AM}/M_M - \bar{v}_M)$, where $\bar{v}_M$ is the partial specific volume of the polymer.

2.3. Measurement of B_{AM} by sedimentation equilibrium

Consider a situation in which parallel sedimentation equilibrium experiments are conducted on protein alone and on a protein–polymer mixture. Subject to the availability of a wavelength at which the protein but not the polymer exhibits significant absorbance, the absorption optical system provides a record of the sedimentation equilibrium distribution of only the protein throughout the cell. On the other hand, the Rayleigh interference pattern provides a measure of total solute distribution at sedimentation equilibrium. By employing a low concentration of protein and a relatively high concentration of polymer, it is therefore possible to obtain sedimentation equilibrium distributions for the two separate components.

Provided that a reference radial distance, r_F, can be selected that is common to both experiments, the dependencies of the thermodynamic activity of protein, z_A, upon radial distance r can be related to the activities at the reference radial position in the two centrifuge cells by the expressions [25]

$$z_A(r) = z_A(r_F)\exp\left[\phi M_A(r^2 - r_F^2)\right] \tag{7a}$$

$$\left[z_A(r)\right]_M = \left[z_A(r_F)\right]_M \exp\left[\phi M_A(r^2 - r_F^2)\right] \tag{7b}$$

where $\phi = (1 - \bar{v}_A \rho_s)\omega^2/2RT$, ω being the angular velocity and ρ_s the solvent density [1,2]: the M subscript is used to denote thermodynamic activities in the centrifuge cell containing the polymer. After substituting the product $\gamma_A c_A / M_A$ for z_A, the ratio of protein concentrations at a given radial distance becomes

$$\frac{c_A(r)}{\left[c_A(r)\right]_M} = \frac{c_A(r_F)\left[\gamma_A(r)\right]_M}{\left[c_A(r_F)\right]_M\left[\gamma_A(r_F)\right]_M} \tag{8}$$

on noting that assumed ideal behaviour in the experiment on protein alone [$\gamma_A(r) \simeq \gamma_A(r_F) \simeq 1$] is a reasonable approximation in view of the low concentration being used. Introduction of Eq. 5 to relate the activity coefficients of A at radial positions r and r_F then leads to the relationship

$$\ln\left\{c_A(r)/\left[c_A(r)\right]_M\right\} \simeq \ln\left\{c_A(r_F)/\left[c_A(r_F)\right]_M\right\} + (B_{AM}/M_M)\left[c_M(r) - c_M(r_F)\right] \tag{9}$$

which signifies that the second virial coefficient may be obtained from the slope of the limiting dependence of ln $\{c_A(r)/[c_A(r)]_M\}$ as a function of the difference in polymer concentrations at r and r_F.

2.4. Statistical-mechanical representation of B_{AM}

For an uncharged, inert chain polymer the second virial coefficient, B_{AM}, is simply the covolume — the volume from which a polymer molecule and a protein mutually exclude one another. As a first approximation, a globular protein can be considered to be represented by an effective impenetrable sphere with radius R_A [16–19], whereas the extended form of chain polymers is most appropriately represented

as an effective Brownian walk of specified root-mean-square end-to-end length ℓ_M. This single parameter approach to the representation of the disparate molecular geometries of globular proteins and extended chain polymers should be regarded as a heuristic *ansatz* devised to allow the major effects of interactions between such molecules to be manifest. The covolume for this combination is given [18] by

$$B_{AM} = N\left[(2\pi/3)R_A\ell_M^2 + 4(2\pi/3)^{1/2}R_A^2\ell_M + (4\pi/3)R_A^3\right] \quad (10)$$

Inasmuch as ℓ_M is a constant for a given batch of inert polymer, the reasonableness of the Brownian path approximation for the description of polymers such as polyethylene glycol and dextran may be tested by comparing experimental values of B_{AM} with those predicted by Eq. 10, provided that suitable magnitudes may be assigned consistently to R_A for a range of proteins. The assignment of an 'effective radius' to a protein often enables hydrodynamic measurements (sedimentation velocity, diffusion or intrinsic viscosity) and thermodynamic measurements (sedimentation equilibrium and gel partition) to be compared with one another. Both size and shape of the protein influence the effective radius, in a manner dependent upon the technique through which it is defined. In some instances a combination of more sophisticated calculations and experimental measurements may be used to estimate the rough shape of a protein in terms of an ellipsoid of revolution. However, such extensions of Eq. 10 are not yet available.

Effective protein radii

Strictly speaking, the parameter R_A required for substitution into Eq. 10 is an effective radius of the solvated protein whose magnitude needs to be determined by quantifying thermodynamic nonideality due to molecular crowding under the specific conditions in question. In principle, measurements of σ_A^0 therefore have the potential to provide estimates of such a radius because the partitioning of a solute depends upon the spaces from which it is excluded in the stationary phase of the gel. However, the indeterminable character of the internal molecular geometry of the gel matrix necessitates recourse to an empirical procedure whereby R_A is determined from a calibration plot of σ_A^0 as a function of an effective radius determined by other means. Although an impediment to this approach is the sparsity of proteins for which an effective radius has been determined, it would appear that the Stokes radius, R_S, gives a reasonable first estimate of the magnitude of the parameter R_A defined for a range of thermodynamic interactions between globular proteins and other species [19]. We have therefore estimated R_A for a range of ribosomal proteins from a calibration plot [24] which uses the known Stokes radii of a number of common globular proteins.

Radii of gyration of polymers

Inasmuch as expressions for the molecular weight dependence of the radius of gyration (R_g) for polyethylene glycol [26] and dextran [27,28] are available, the required root-mean-square end-to-end distance (ℓ_M) may be estimated on the basis of the Brownian path assumption ($\ell_M^2 = 6R_g^2$). However, a major question at issue is whether polymers such as polyethylene glycol and dextran may be represented adequately as effective Brownian paths, an assumption needed to justify use of the Jansons–Phillips expression for covolume [18]. We shall therefore regard ℓ_M in Eq. 10 as a parameter to be evaluated by curve-fitting the dependence of B_{AM} upon R_A; and then rationalize the value in terms of the root-mean-square end-to-end distance that arises from the radius of gyration.

3. Experimental

3.1. Materials

Bovine erythrocyte carbonic anhydrase was a commercial preparation supplied by Boehringer Mannheim GmbH, and bovine α-lactalbumin was obtained from Sigma Chemical Co. Bovine chymotrypsinogen A, soybean trypsin inhibitor, horse heart cytochrome *c*, horse-spleen ferritin, whale muscle myoglobin, lysozyme and ovalbumin (both chicken egg white) were all products of Serva Feinbiochemica GmbH.

Individual ribosomal proteins from the 50 S subunit were isolated by a mild purification procedure

involving salt extraction of the subunit followed by ion-exchange chromatography and gel filtration [29]. This procedure does not lead to the isolation of all 32 ribosomal proteins, some of which do not survive the purification procedure because of extensive aggregation under the conditions used.

Studies of protein–polymer excluded-volume interactions entailed the use of polyethylene glycol (PEG 20000 for gas chromatography) obtained from E. Merck, Darmstadt, Germany; and of dextran (Dextran T 70) from Pharmacia Fine Chemicals, Uppsala, Sweden. Values of 21000 (±2000) and 70000 (±2000) were obtained for the molecular weights (M_{SD}) of the respective polymer preparations by use of the Svedberg equation.

3.2. Gel chromatography

To a column (1.0 × 145 cm) of Sephadex G-50 (Superfine), equilibrated at 4°C with phosphate–chloride buffer, pH 7.0, I 0.39 (0.008 M NaH_2PO_4–0.012 M Na_2HPO_4–0.35 M NaCl), were added 1-ml zones of protein solution (0.2 or 1.0 mg/ml) in the same buffer. The column effluent, maintained at a flow-rate of 4 ml/h by means of a peristaltic pump, was monitored continuously at 230 nm. Elution volumes (V_A), taken as the effluent volume corresponding to the maximum absorbance of the symmetrical elution peak, were converted to partition coefficients ($\sigma_A \simeq \sigma_A^o$) on the basis of respective values of 41.6 ml and 109.2 ml for V_0 and V_t (Eq. 1). The void volume was measured as the elution volume of ferritin, whereas the total accessible volume (V_t) was determined by monitoring conductimetrically the elution profile for a 1-ml zone of salt solution.

Hydrated radii (R_A) of the isolated ribosomal proteins were obtained on the basis of the linear dependence of the inverse error function of $(1 - \sigma_A)$ upon R_A [24] observed for several well-characterized globular proteins (Fig. 1). As noted in Section 2.3.1, published Stokes radii have been substituted for R_A in this empirical calibration plot. To gain some insight into the extent to which the solvated protein differs from its most compact anhydrous spherical configuration, the effective hydrated radius, R_A, has been compared with the minimum value,

$$R_{min} = \left(3M_A\bar{v}_A/4\pi N\right)^{1/3} \quad (11)$$

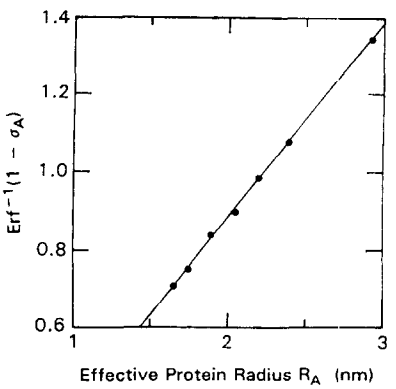

Fig. 1. Empirical calibration plot [26] for the evaluation of solvated protein radii (R_A) from the partition coefficient obtained in gel chromatography on Sephadex G-50. Standard proteins, in order of increasing radius, were cytochrome c, α-lactalbumin, myoglobin, soybean trypsin inhibitor, chymotrypsinogen A, carbonic anhydrase and ovalbumin. The solid line corresponds to the relationship $\text{erf}^{-1}(1 - \sigma_A) = -0.128 + 0.503R_A$.

by calculating the frictional ratio (f/f_{min}) as R_A/R_{min}. The required values of molecular weight and partial specific volume for substitution in Eq. 14 were obtained from the amino acid sequence (and composition) of each individual ribosomal protein [30].

3.3. Determination of protein–polymer covolumes

Two procedures have been used to evaluate the second virial coefficient (B_{AM}) describing the excluded-volume interactions of a range of proteins with PEG 20000 and Dextran T 70.

Effect of polymer concentration on σ_A

After the re-equilibration of the above column of Sephadex G-50 with phosphate–chloride buffer containing either PEG 20000 (2.5–20 mg/ml) or Dextran T 70 (5–30 mg/ml), solutions (1 ml) of selected proteins in the same medium (0.2 or 1 mg/ml) were subjected to gel chromatography at 4 ml/h, as in Section 3.2. Because gel chromatographic media are subject to osmotic shrinkage [31–33], the polymer concentration was restricted to a range within which the decrease in column volume was below 1%. Such variations in column volume necessitated the redetermination of V_t and V_0 after each change to buffer supplemented with a new concentration of

polymer [19]. After conversion of the elution volumes, V_A, to the corresponding partition coefficients, σ_A, the second virial coefficient, B_{AM}, was determined from the dependence of ln σ_A upon polymer concentration (Eq. 6). In that regard, control experiments with refractometric recording of elution profiles of the two polymers were conducted to verify their elution at the void volume, and hence their exclusion from the stationary phase — an assumption inherent in the application of Eq. 6.

Evaluation of B_{AM} by sedimentation equilibrium

The second virial coefficient for the excluded-volume interaction of carbonic anhydrase with PEG 20000 was also determined by performing simultaneous sedimentation equilibrium experiments on enzyme alone (0.28 mg/ml) in phosphate–chloride buffer (pH 7.0, I 0.39) and on enzyme (also 0.28 mg/ml) in phosphate–chloride buffer supplemented with PEG 20000 (5.2 mg/ml). In these experiments, conducted at 14000 rpm in a Beckman model E analytical ultracentrifuge, the sedimentation equilibrium distributions of enzyme could be recorded spectrophotometrically at 342 nm, whereas that of polymer could be obtained from the Rayleigh record of total solute distribution at equilibrium. On the grounds that the carbonic anhydrase contributed negligibly to the interferometric pattern, the Rayleigh distribution was converted to one in terms of polymer concentration on the basis of a specific refractive increment of 0.136 ml/g for polyethylene glycol [34]. Corresponding experiments with lysozyme and cytochrome c as the protein were also performed.

Table 1
Gel chromatographic evaluation of hydrated radii (R_A) for isolated ribosomal proteins

Protein	σ_A [a]	R_A [b] (nm)	M_A [c]	$\bar{v}_A$ [d] (ml/g)	R_{min} [e] (nm)	R_A/R_{min}
L1	0.117	2.49	24 599	0.743	1.94	1.28
L2	0.036	3.27	29 416	0.731	2.05	1.60
L3	0.085	2.72	22 258	0.737	1.86	1.46
L5	0.152	2.28	20 171	0.742	1.81	1.26
L6	0.144	2.33	18 832	0.741	1.77	1.32
L9	0.152	2.28	15 531	0.745	1.66	1.37
L11	0.135	2.38	14 874	0.743	1.64	1.45
L13	0.195	2.08	16 019	0.741	1.68	1.24
L15	0.128	2.42	14 981	0.739	1.64	1.48
L16	0.186	2.14	15 296	0.750	1.66	1.29
L17	0.253	1.85	14 364	0.733	1.61	1.15
L19	0.237	1.91	13 002	0.742	1.57	1.22
L22	0.163	2.18	12 111	0.748	1.53	1.42
L23	0.119	2.41	11 013	0.753	1.49	1.62
L24	0.227	1.95	11 185	0.747	1.49	1.31
L25	0.395	1.54	10 694	0.746	1.47	1.05
L26	0.163	2.18	9 553	0.741	1.41	1.55
L27	0.189	2.11	8 993	0.727	1.38	1.53
L28	0.390	1.54	8 875	0.735	1.37	1.12
L29	0.374	1.56	7 273	0.745	1.29	1.21
L30	0.483	1.47	6 411	0.752	1.24	1.19
L32	0.288	1.74	6 315	0.726	1.22	1.43
L33	0.234	1.92	6 255	0.757	1.24	1.55

[a] Partition coefficient calculated from Eq. 1.
[b] Uncertainty of ±0.02 nm in these estimates.
[c] Deduced from amino acid sequence reported in Ref. [31].
[d] Deduced from amino acid composition data in the SwissProt library.
[e] Calculated from Eq. 11.

4. Results and discussion

This investigation was instigated many years ago to obtain further information on the general size/shape characteristics of the proteins that comprise the 50 S subunit of ribosomes [35], the interest in this problem at the time being prompted by the possibility that the extended configurations ascribed initially to these proteins may merely have reflected the study of proteins that had undergone structural changes during exposure to harsh isolation conditions [30]. We therefore continue a previous line of investigation [36,37] which had indicated essentially globular protein characteristics for four of six ribosomal proteins that had been prepared by a milder purification procedure: the other two exhibited slightly higher frictional ratios that possibly signified their existence in marginally extended or asymmetric configuration.

4.1. Solvated radii and frictional ratios of ribosomal proteins

Results of gel chromatographic evaluation of the effective hydrated radius for 23 of the 32 ribosomal proteins of the 50 S subunit are presented in Table 1, the second column of which lists the partition coefficients (σ_A) deduced from the elution volume via Eq. 1. The third column gives the effective radius inferred from the calibration plot (Fig. 1), while the fourth and fifth columns provide the information required for substitution into Eq. 11 to evaluate R_{min}. From the final column it would appear that only four of the proteins (L2, L23, L26 and L33) are characterized by frictional ratios that are possibly indicative of significantly extended or asymmetric configurations: the remainder exhibit R_A/R_{min} values consistent with their existence in a globular or marginally globular state.

Further comment on the compactness of the protein structures has been sought from measurement of protein–polymer covolumes (B_{AM}), because the functional form of the dependence of B_{AM} upon size of A (Eq. 10) is now known for proteins that can be approximated, for the purpose of analyzing some measurements of their thermodynamic properties, as impenetrable spheres with effective radius R_A. Noncompliance with this requirement may thus be identifiable by significant deviation of B_{AM} for an extended/asymmetric protein from the covolume-size relationship expected for globular proteins.

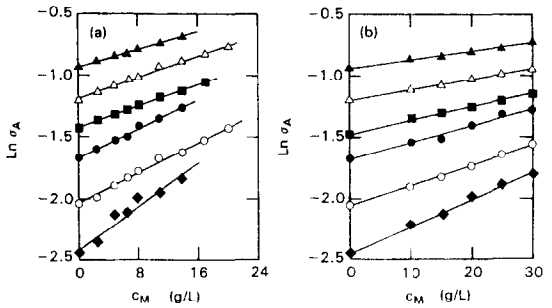

Fig. 2. Effect of the concentration of (a) PEG 20000 and (b) Dextran T 70 on the partition coefficient in gel chromatography of carbonic anhydrase (○), cytochrome *c* (△), and ribosomal proteins L3 (◆), L24 (□), L25 (▲) and L27 (●), on Sephadex G-50. Results are plotted in accordance with Eq. 6.

4.2. Protein–polymer covolume measurements

The determination of protein–polymer covolumes by gel chromatography is illustrated in Fig. 2, where results for several proteins in the presence of PEG 20000 (Fig. 2a) and Dextran T 70 (Fig. 2b) are plotted in accordance with Eq. 6. As predicted, these dependencies of ln σ_A upon polymer concentration are essentially linear; and hence second virial coefficients (covolumes) have been calculated from slopes obtained by linear regression analysis.

The application of Eq. 9 to the evaluation of protein–polymer covolumes by sedimentation equi-

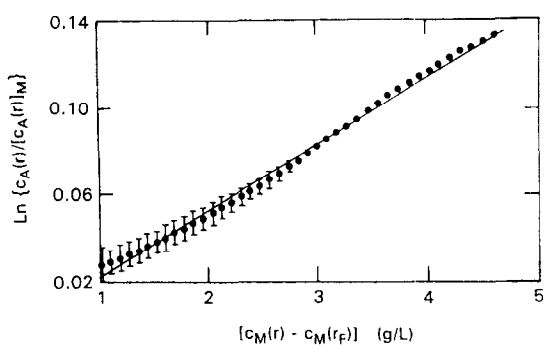

Fig. 3. Evaluation of B_{AM}/M_M for the excluded volume interaction between carbonic anhydrase and PEG 20000 by sedimentation equilibrium, the results being plotted in accordance with Eq. 9.

librium is illustrated in Fig. 3 for the carbonic anhydrase-PEG 20000 system. The apparent sigmoidality of the data set is probably attributable to polydispersity of the PEG 20000, which gives rise to systematic variation in its weight-average molecular weight across the equilibrium distribution. Because the polymer concentration range employed is similar to that used in the gel chromatography experiment, it is unlikely that this minor deviation from the linear relationship predicted by Eq. 9 reflects the influence of higher-order terms. A value of 31 (±3) ml/g for B_{AM}/M_M emanates from linear regression analysis of the data set (Fig. 3) in terms of Eq. 9.

An interesting aspect of the evaluation of B_{AM}/M_M by the sedimentation equilibrium procedure is the absence of any terms (Eq. 9) in angular velocity or molecular weight of either solute. Nevertheless, those three factors play an important role in determining (i) the accuracy with which ln $\{c_A(r)/[c_A(r)]_M\}$ may be estimated, and (ii) the range of polymer concentration available within the ultracentrifuge cell for evaluation of the slope (Fig. 3). Inasmuch as the method relies upon an ability to record separate sedimentation equilibrium distributions for the two solute components (protein and polymer), the conduct of such experiments in the Beckman XL-A analytical ultracentrifuge will have to await the installation of interferometric optics on this Model E replacement. Alternatively, separate analytical procedures may always be used to determine the two concentration distributions after sedimentation equilibrium experiments in a preparative ultracentrifuge [38].

4.3. Comparison of covolume measurements with theoretical predictions

The dependence of measured protein–polymer covolumes upon effective hydrated protein radius R_A is presented in Fig. 4. From Fig. 4a it is evident that there is very good agreement between the value of B_{AM}/M_M obtained for carbonic anhydrase-PEG 20000 by sedimentation equilibrium (Fig. 3) and that of 32 (±2) ml/g obtained by gel chromatography. Likewise, for cytochrome *c* the value of B_{AM}/M_M from the ultracentrifuge experiment, 22 (±2) ml/g, duplicates the magnitude of the gel chromatographic estimate, 22.0 (±0.8) ml/g. A similar comparison

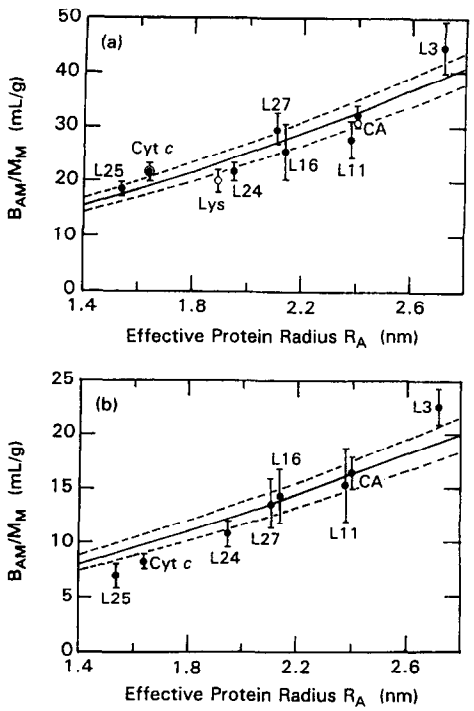

Fig. 4. Dependence of the second virial coefficient for protein–polymer interaction upon effective hydrated radius of the protein in experiments with (a) PEG 20000 and (b) Dextran T 70 as the inert polymer: ●, values of B_{AM}/M_M determined gel chromatographically; ○, values obtained by sedimentation equilibrium. Solid lines and encompassing uncertainty envelopes correspond to the best-fit descriptions of the results in terms of Eq. 10.

could not be made for lysozyme because of its affinity for the Sephadex matrix [39]; but the magnitude of B_{AM}/M_M, 20 (±2) ml/g, obtained at pH 4.3 to suppress enzyme self-association [40], conforms with the covolume-R_A relationship for PEG 20000 (Fig. 4a). Clearly there is good agreement between estimates of the enzyme–polymer covolume obtained by the two methods.

Also shown in Fig. 4 are the best-fit descriptions of the dependencies of B_{AM}/M_M upon R_A obtained by nonlinear regression analysis in terms of Eq. 10, an exercise that requires specification of the polymer molecular weight. Use of the M_{SD} value of 21000 g/mol obtained for PEG 20000 (see Section 3.1) leads to the best-fit relationship (and its uncertainty envelope) shown in Fig. 4a and a value of 11.7 ± 0.6 nm for ℓ_M, the root-mean-square end-to-end distance (and its uncertainty, 2SD) in PEG 20000. This

value compares very favourably with that of 11.2 nm that is deduced from the general relationship $\ell_M = (0.006\ M_M)^{1/2}$ nm obtained by Mark and Flory [26] for polyethylene glycol. This finding signifies the usefulness of the equivalent random path model for PEG 20000 from the viewpoint of interpreting measurements of its covolume with globular proteins. In contrast, modelling the polymer as an effective hard sphere requires use of an equivalent radius which varies with the size and shape of the protein [16,19,34]. The Jansons and Phillips result [18], which stems from a more comprehensive consideration of the random-path model than that presented by Hermans [16,17], allows a reasonable estimate to be made of protein–polyethylene glycol covolumes in terms of a single parameter characteristic of the polymer chain. This improvement has been effected by taking into account the variable probability of exclusion as a function of distance between the centres of mass of the extended polymer and proteins of different sizes. The unavailability of an expression taking into account this major effect has previously been a severe impediment to the interpretation of measured protein–polymer covolumes.

The corresponding analysis of the covolume measurements with Dextran T 70 as the inert polymer (Fig. 4b) is also based on the value of the molecular weight (M_{SD}) reported in Section 3.1. On that basis the best-fit relationship (solid line in Fig. 4b) indicates a root-mean-square end-to-end distance (ℓ_M) of 16.0 ± 0.8 nm, which is somewhat smaller than that of 19.2 nm inferred from the reported dependence of the radius of gyration upon molecular weight of dextran [27,28]. Part of this discrepancy may well reside in the value of M assigned to the polydisperse Dextran T 70 ($M_w/M_n \simeq 2$), but another contributing factor is almost certainly the poorer description provided by the random path model for a polymer comprising glucose (a cyclic monomer) that is linked α-1,6 with occasional α-2,6 branchpoints. Nevertheless, provided that M is merely regarded as an empirical curve-fitting parameter whose magnitude does not necessarily correspond exactly to the radius of gyration expected on the basis of the Brownian path assumption, results obtained using polymers such as Dextran T 70 may still be interpreted more confidently in such terms than on the basis of a hard-sphere model of the polymer molecule: but clearly, experiments with a range of proteins with known R_A are first required to calibrate the effective Brownian path representing the polymer. Thus, even though the absolute fit to the Jansons–Phillips [18] expression for covolume is less satisfactory in the experiments with Dextran T 70 (Fig. 4b) than with PEG 20000 (Fig. 4a) as inert polymer, the same general conclusions about the compactness of the ribosomal proteins is reached: of the six proteins examined, only L3 seems to deviate very markedly from the behaviour predicted on the basis of an equivalent spherical model of the protein. In that regard we note that L3 exhibited a relatively high frictional ratio (1.45).

5. Concluding remarks

This investigation has achieved its first goal of demonstrating the likelihood that most of the ribosomal proteins of the 50 S subunit behave as reasonably globular proteins in solution. However, our major finding concerns the thermodynamic nonideality of proteins caused by the molecular crowding effect of polymers. To that end gel chromatographic and sedimentation equilibrium procedures have both been illustrated as a means of evaluating the excluded volume per gram of polymer, and hence the second virial coefficient, B_{AM}, if a magnitude can be assigned to M_M. In addition, this study has broken new ground in the molecular interpretation of B_{AM} by demonstrating that the protein–polymer covolumes obtained with PEG 20000 and Dextran T70 as crowding agents are consistent with the functional form of the quantitative expression (Eq. 10) based on a random path model of the polymer. In principle, therefore, a useful effective thermodynamic radius of a hydrated protein may be defined consistently on the basis of the second virial coefficient describing its interaction with a polymer of root-mean-square end-to-end distance, ℓ_M: this may require prior determination of an empirical value of ℓ_M by constructing a calibration plot for proteins with specified effective radius, R_A.

The stage has thus been reached whereby better predictions can be made of excluded volume contributions to the thermodynamic nonideality of proteins imposed by the presence of high concentrations of

inert flexible chain polymers. Initially [34], the polymer was modelled as a long rod, whereas the solution for the protein–polymer covolume obtained by Hermans [16,17] is now seen explicitly to apply in the limit of infinite polymer chainlength (the first term of Eq. 10). The Jansons–Phillips [18] formulation, Eq. 10, clearly accommodates that limiting situation as well as the other extreme case of a small space-filling solute ($\ell_M \to 0$), whereupon B_{AM} becomes the hydrated volume of the protein (final term in Eq. 10). The obvious advantage of Eq. 10 is its ability to describe the intermediate (and more usual) situations. At last a more satisfactory solution seems to have been found to a problem raised by Edmond and Ogston [34] more than a quarter of a century ago.

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