Eur Biophys J (2003) 32: 406–411
DOI 10.1007/s00249-003-0295-x

ARTICLE

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Adair was right in his time

Received: 11 June 2002 / Revised: 19 January 2003 / Accepted: 19 January 2003 / Published online: 24 April 2003

Abstract The subunit molar mass of hemoglobin was established in the 19th century by chemical analysis, the tetramer structure by osmotic pressure determination in 1924 and by the newly developed analytical ultracentrifuge in 1926, which became a powerful tool for biological macromolecule molar mass determinations. The Svedberg equation was derived by eliminating the translational friction coefficient relating to sedimentation and diffusion in the ultracentrifuge in a strictly solute/solvent vanishing concentration two-component system analysis. A differential equation describing the radial equilibrium concentration distribution in the ultracentrifuge was also derived, both yielding the buoyant molar mass $(1-\bar{v}_2\rho)M_2$ term. Many years later it was realized that solutions of biological macromolecules are multicomponent systems and the two-component analysis leads to minor or major erroneous results. Thermodynamic derivation of an equation for multicomponent systems redefines the buoyant molar mass terms by $(\partial\rho/\partial c_2)_\mu M_2$, leading to correct molar mass (g/mol) values following determination of the density increment at constant chemical potentials of diffusible solutes, and powerfully connects the analytical sedimentation equation to the osmotic pressure concentration derivative and, in a broad complementary sense, to light, X-ray and neutron scattering experiments. Macromolecular interactions can be studied with high precision and solute-solvent interactions yield powerful information relating to ''thermodynamic'' hydration, closely related to hydration derived from X-ray diffraction, as well as solute-cosolute interactions. A series of examples is given to demonstrate the correctness and usefulness of the thermodynamic multicomponent system approach. It is a strange fact that in current analytical ultracentrifugation analysis the elegant and powerful multicomponent solution technology is almost totally disregarded and the classical limited validity Svedberg approach is used uniquely.

Keywords Analytical ultracentrifugation · Light, X-ray and neutron scattering · Multicomponent systems · Osmotic pressure · Solute-solvent interactions

Presented at the Conference for Advances in Analytical Ultracentrifugation and Hydrodynamics, 8–12 June 2002, Grenoble, France

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Introduction

The determination of the correct molar masses of biological macromolecules was contested by leading chemists into the middle of the 20th century (Eisenberg 1996); however, protein scientists had developed the macromolecular, rather than the colloidal aggregation, structure of proteins already much earlier (Tanford and Reynolds 1999). The subunit molar mass of hemoglobin was established in the 19th century by chemical analysis (Tanford and Reynolds 2001), the tetramer structure by osmotic pressure determination (Adair 1924) and by the newly developed analytical ultracentrifuge (Svedberg and Fåhraeus 1926), which became a powerful tool for molar mass determinations of biological macromolecules. Svedberg and Fåhraeus (1926) were not aware of the osmotic pressure results of Adair (1924), who was highly active in hemoglobin research and other protein studies at that time. A relationship between osmotic pressure and analytical ultracentrifugation was unfortunately not established then. The Svedberg equation (Svedberg and Pedersen 1940):

$$s/D = (1 - \bar{v}_2\rho)M_2/RT \quad (1)$$

was derived by eliminating the translational friction coefficient relating to the sedimentation s and diffusion D coefficients in the ultracentrifuge in a strictly infinite

dilution solute/solvent two-component system analysis. A differential equation was also derived:

$$d \ln c_2/dr^2 = \left(\omega^2/2RT\right)(1 - \bar{v}_2\rho)M_2 \quad (2)$$

both yielding the buoyant molar mass $(1-\bar{v}_2\rho)M_2$ of the macromolecular component 2. Here the partial specific volume $\bar{v}_2$ is in units of mL/g and the molar mass in g/mol. The solvent, component 1, density ρ, is in g/mL. Many years later it was realized by leading investigators and by us (reviewed in Casassa and Eisenberg 1964; Eisenberg 1976) that solutions of biological macromolecules are multicomponent systems and the two-component analysis leads to minor or major erroneous results. Thermodynamic derivation of an equation for multicomponent systems redefines the buoyant molar mass infinite dilution term by $(\partial\rho/\partial c_2)_\mu M_2$, leading to correct molar mass values following determination of the density increment $(\partial\rho/\partial c_2)_\mu$ at constant chemical potentials μ of diffusible solutes (Kratky et al. 1973).

For finite macromolecular concentrations a powerful connection is established between the analytical equation and the osmotic pressure concentration derivative $(d\Pi/dc_2)$:

$$d \ln c_2/dr^2 = \frac{\left(\omega^2/2\right)(\partial\rho/\partial c_2)_\mu}{d\Pi/dc_2} \quad (3)$$

where r is the distance from the center of rotation in, and ω is the angular velocity of, the ultracentrifuge. The osmotic pressure concentration derivative can be expanded in a virial series in multicomponent biological macromolecule systems, yielding the molar mass at vanishing macromolecular concentrations:

$$(d\Pi/dc_2)/RT = M_2^{-1} + 2A_2c_2 + \ldots \quad (4)$$

Analytical ultracentrifugation powerfully connects in a broad complementary sense to light, X-ray and neutron scattering experiments (Eisenberg 1981, 1994, 1999). The heydays of analytical ultracentrifuge use lasted throughout the period in which the determination of molar masses of biological macromolecules and subunits was a major objective. However, its usefulness declined when replaced by alternate methods such as, for instance, sequencing and mass spectrometry. The development of the newly created Optima XL-A analytical ultracentrifuge and powerful computer technology led to renewed interest in terms of the study of macromolecular interactions and hydration analysis. However, multicomponent aspects were not referred to in this revival (Schachman 1992). Macromolecular interactions can now be studied with higher precision and solute-solvent interactions yield powerful information relating to ''thermodynamic'' hydration, closely related to hydration derived from X-ray diffraction, as well as solute-cosolute interactions (Eisenberg 1994, 1999, 2000). Thermodynamic hydration has deeper significance than hydration related to the modelling of a hydration layer supposedly covering a spherical, ellipsoidal or rodlike macromolecular assembly. Well-chosen examples will be used in this paper to demonstrate the correctness and usefulness of the thermodynamic multicomponent system approach. It is a strange fact that in current analytical ultracentrifugation analysis (Harding et al. 1992; Schuster and Laue 1994; Laue and Stafford 1999) the elegant and powerful multicomponent solution technology (Fujita 1975, 1994) is almost totally disregarded and the classical limited validity Svedberg approach is almost uniquely used. For instance, in a new, modern and updated biophysical text (Van Holde et al. 1998) and in recent contributions (Hansen et al. 1994; Hensley 1996; Schuster and Toedt 1996; Lebowitz et al. 2002), no mention is made of multicomponent analysis and only the Svedberg two-component approach is given. It is hoped that the presentation given here will lead to reevaluation in the primary approach and evaluation of analytical ultracentrifugation and hydration assessment.

In the presentation following, constructive arguments and equations will be discussed; however, figures will not be shown as they have often been given and will be referred to only, allowing the dedicated reader to examine the original presentations on which this work is based. It was a unique experience to attend the Advances in Analytical Ultracentrifugation and Hydrodynamics European Workshop and Conference in Autrans in June 2002 and it is my hope that this work presented at the Conference will lead to the expected satisfactory result.

Materials and methods

Theoretical analysis

In a two-component osmotic experiment the solvent (component 1) equilibrates across the semipermeable membrane, whereas the macromolecule (component 2) is restricted to the interior osmometer compartment (Eisenberg 2000). The presence of component 2 lowers the chemical potential μ_1, leading to solvent influx into the interior compartment, balanced by an increase in pressure until equilibrium is achieved upon increase of μ_1 to its value μ_1' in the outside pure solvent compartment. The osmotic pressure concentration derivative $d\Pi/dc_2$ can be expanded into a virial series (Eq. 4), and at vanishing concentrations c_2 the reciprocal of M_2 is obtained. Π/RT extrapolated to $c_2 = 0$ equals c_2/M_2 (mol/mL) and the osmotic pressure thus determines the number of molecules, or moles, per mL. Depending on the units of concentration used, corresponding macromolecular units are obtained. Thus if, for instance, concentrations are expressed in moles of nitrogen per mL, the macromolecular component is determined in moles of nitrogen per mole of component 2.

When charged biological macromolecules are examined, osmotic pressure equilibrium can be achieved; however, a virial expansion as indicated in Eq. (4) cannot be performed because of long-range electrostatic interactions. Positively or negatively charged counterions cannot penetrate the semipermeable membrane and move into the outside compartment because of electroneutrality requirements. To restore the ability to perform a virial expansion, a low molecular weight electrolyte (component 3) is added, having an ion in common with the component 2 counterion. This brings us into the realm of multicomponent, three-component in this simplest case, systems, an essential requirement in the study of biological macromolecules. Long-range electrostatic interactions are now reduced and a virial expansion again becomes possible. A question still often raised is what molar or molecular mass of a

charged biological macromolecule is determined in an osmotic pressure, or related, experiment, whether equilibrium sedimentation or scattering of light, X-rays or neutrons. The answer again is that macromolecules are *counted* as moles (or molecules) per mL, not *weighed*, and the molecular mass is again given in terms of the concentration units used. If, for instance, NaDNA is dissolved in a high concentration of CsCl, the molar mass M_2, including the counterions, will be that of sodium and not of CsDNA, if the DNA concentration is expressed in terms of NaDNA (by weight added or by optical density). The experiment at this stage does not yield information regarding coion-counterion interactions, or counterion condensation as applied to highly charged DNA.

Having duly examined osmotic pressure results for charged macromolecules, we next move to an interpretation of analytical ultracentrifugation (Eq. 3). The density increment $(\partial\rho/\partial c_2)_\mu$ equals the difference in solution to solvent density at dialysis equilibrium, divided by component 2 concentration. The osmotic pressure derivative $d\Pi/dc_2$ is given by Eq. (4). Analytical ultracentrifugation, similarly to osmotic pressure, *counts* molecules per unit volume; therefore the units used for the concentration lead to the interpretation of the final result. Equation (3) applies to any number of components, yet the three-component discussion is maintained here for simplified presentation.

In the limit of vanishing concentration c_2, Eqs. (3) and (4) reduce to:

$$d\ln c_2/dr^2 = \left(\omega^2/2RT\right)(\partial\rho/\partial c_2)_\mu M_2 \tag{5}$$

which replaces the classical two-component Eq. (2). Equation (1) in the limit of vanishing component 2 concentration is replaced in similar fashion by:

$$s/D = (\partial\rho/\partial c_2)_\mu M_2/RT \tag{6}$$

The density increment $(\partial\rho/\partial c_2)_\mu$ for the three-component system is given by:

$$(\partial\rho/\partial c_2)_\mu = (1-\rho\bar{v}_2) + \xi_3(1-\rho\bar{v}_3) \tag{7}$$

$$= (1+\xi_3) - \rho(\bar{v}_2 + \xi_3\bar{v}_3) \tag{8}$$

where $\xi_3 = (w_3' - w_3)/w_2 = (\partial w_3/\partial w_2)_\mu$ is an interaction parameter indicating the change in gram molality w_3 with the change in gram molality w_2 at constant chemical potentials of components 1 and 3 diffusible through a semipermeable membrane, and ρ is the density of the solvent in the absence of component 2. For symmetry reasons, Eqs. (7) and (8) can also be written as:

$$(\partial\rho/\partial c_2)_\mu = (1-\rho\bar{v}_2) + \xi_1(1-\rho\bar{v}_1) \tag{9}$$

$$= (1+\xi_1) - \rho(\bar{v}_2 + \xi_1\bar{v}_1) \tag{10}$$

where $\xi_1 = (\partial w_1/\partial w_2)_\mu$ and ξ_1 and ξ_3 are related by:

$$\xi_1 = -\xi_3/w_3 \tag{11}$$

The interaction coefficients ξ_1 and ξ_3 cannot be associated with specific interaction with only either component 1 or 3, but should each be considered as relating to both components 1 and 3. A zero value of either ξ_1 or ξ_3 does not indicate a lack of interaction with component 1 or 3. Furthermore, a positive value of ξ_1 yields a negative value of ξ_3 or vice versa by Eq. (11). We also note from an examination of Eqs. (7) and (9) that, for a strictly two-component system, Eqs. (7) and (8) reduce to the Svedberg equation in terms of the *Archimedes* buoyancy term.

The density increment $(\partial\rho/\partial c_2)_\mu$ is sometimes substituted by $(1-\rho\Phi')$, mimicking the two-component buoyancy equation. This should be avoided as Φ' has no defined meaning and therefore should not be used.

For practical reasons, study of macromolecular solute-cosolute interactions by velocity or equilibrium analytical ultracentrifugation should be preceded by the determination of $(\partial\rho/\partial c_2)_\mu$ following dialysis and density determination. Objection to this, claiming the need for larger amounts of materials and experimental complications, and the use of $\bar{v}_2$ (usually obtained by calculation from amino acid composition), disregarding solvent-cosolvent and solute-cosolvent interactions, is not justified and may lead to erroneous results. Reasonable milligram amounts of biological macromolecules (reusable after the density experiment) are now available from cloning procedures and precise density measurements can be performed by the Kratky mechanical oscillator technique (Kratky et al. 1973). In cases in which M_2 is known from alternate methods (sequence or mass spectrometry), $(\partial\rho/\partial c_2)_\mu$ can be obtained from equilibrium sedimentation extrapolated to vanishing macromolecular concentration, and can then be analyzed for partial volumes and solvent-cosolvent and solute-cosolvent interactions. The density increment $(\partial\rho/\partial c_2)_\mu$ is the basic quantity in the analysis and should be given adequate presentation and attention. The mutually related thermodynamic interaction parameters ξ_1 and ξ_3 can be interpreted by the use of a variety of molecular models, leading to explicit physical interpretation. As an example, the *invariant particle* model (Tardieu et al. 1981), applicable in many circumstances, leads to simple mathematical expressions:

$$\xi_3 = B_3 - B_1 w_3 \tag{12}$$

or, equivalently:

$$\xi_1 = B_1 - B_3/w_3 \tag{13}$$

where B_1 and B_3 are defined as grams of solvent and grams of cosolvent *bound* per gram of component 2. *Bound* signifies in this instance the creation of volumes (to be added to the volume of component 2) *excluded* to the penetration of the *other* components. In the case of charged macromolecular component 2, B_3 is replaced by $B_3' = B_3 - E_3$, where E_3 is the result of Donnan exclusion. We now write:

$$(\partial\rho/\partial c_2)_\mu = (1-\rho\bar{v}_2) + B_1(1-\rho\bar{v}_1) + B_3'(1-\rho\bar{v}_3) \tag{14}$$

$$= \left(1 + B_1 + B_3'\right) - \rho^0\left(\bar{v}_2 + B_1\bar{v}_1 + B_3'\bar{v}_3\right) \tag{15}$$

which indicates that from the intercept and slope of the linear dependence of $(\partial\rho/\partial c_2)_\mu$ with ρ^0 it is possible to evaluate B_1 and B_3' if the partial specific volumes $\bar{v}_i$ are known and reasonably constant. The value of $V_{tot} = \left(\bar{v}_2 + B_1\bar{v}_1 + B_3'\bar{v}_3\right)$ can be cross-checked by the determination of the radius of gyration, R_g, from small-angle X-ray (Reich et al. 1982) or neutron scattering (Zaccai et al. 1986) or by total volume calculation of data derived from X-ray crystallography.

The light, $I(0)$, X-ray, $I_{el}(0)$, and neutron, $I_N(0)$, forward-scattering intensities are related to the refractive index, electron and neutron scattering density increments in the limiting case of low macromolecular concentrations (cf. Eq. 5) (Eisenberg 1981):

$$N_A I(0)/c_2 = (\partial n/\partial c_2)_\mu^2 M_2 \tag{16}$$

$$N_A I_{el}(0)/c_2 = (\partial\rho_{el}/\partial c_2)_\mu^2 M_2 \tag{17}$$

$$N_A I_N(0)/c_2 = (\partial\rho_N/\partial c_2)_\mu^2 M_2 \tag{18}$$

where N_A is Avogadro's number. At finite component 2 concentrations, M_2 is replaced in Eqs. (16)–(18) by $RT/(d\Pi/dc_2)$. Similarly to the mass density increments (Eq. 9), the radiation scattering length density increments can be expressed as:

$$(\partial\rho_{el}/\partial c_2)_\mu = l_2 + \xi_i l_i - \rho_{el}^0(\bar{v}_2 + \xi_i\bar{v}_i) \tag{19}$$

$$(\partial\rho_N/\partial c_2)_\mu = b_2 + \xi_i b_i - \rho_N^0(\bar{v}_2 + \xi_i\bar{v}_i) \tag{20}$$

where l_i and l_2 are electrons per g and b_i and b_2 (cm g^{-1}) the scattering lengths per g of water ($i=1$) or cosolvent ($i=3$) and protein (index = 2) calculated from the chemical composition, respectively; ρ_{el}^0 (e cm^{-3}) is the electron density and ρ_N^0 (cm^{-2}) its neutron scattering density. The values of the l_i and b_i are listed in Table 1,

Table 1 Neutron scattering lengths, b_i, per gram (Zaccai et al. 1986)and l_i, electrons per gram (Reich et al. 1982), in water buffers in hMDHsolutions

Neutrons (H_2O) (cm/g)	X-rays (e/g)[a]
$b_1 = -5.62\times10^9$	$l_1 = 3.343\times10^{23}$
$b_2 = 14.8\times10^9$	$l_2 = 3.23\times10^{23}$
$b_3 = 13.59\times10^9$	$l_3 = 2.885\times10^{23}$

[a] 1 electron/gram is equivalent to 2.81×10^{-13} cm g^{-1}

from a study of halophilic malate dehydrogenase (Zaccai et al. 1986). The important feature characterizing neutron scattering, or diffraction, is that, in distinction to the other parameters, b_1 is negative for hydrogen (Jacrot and Zaccai 1981; Zaccai and Jacrot 1983). Light scattering and refractive index increments have been analyzed (Bonnété et al. 1993), but will not be discussed in this work.

We can now express the equations for X-ray and neutron scattering in the formulation corresponding to the mass density expression Eq. (15):

$$(\partial\rho_{el}/\partial c_2)_\mu = \left(l_2 + B_1 l_1 + B_3' l_3\right) - \rho_{el}^0 V_{tot} \tag{21}$$

$$(\partial\rho_N/\partial c_2)_\mu = \left(b_2 + B_1 b_1 + B_3' b_3\right) - \rho_N^0 V_{tot} \tag{22}$$

If, as previously observed, the "particle" volume is constant, as well as B_1 and B_3', then $(\partial\rho/\partial c_2)_\mu$, $(\partial\rho_{el}/\partial c_2)_\mu$ and $(\partial\rho_N/\partial c_2)_\mu$ versus ρ^0, ${\rho_{el}}^0$ and ${\rho_N}^0$, respectively, are straight lines with the same slope, which is equal to the total volume of the particle, V_{tot}. It is thus possible to determine three parameters (B_1, B_3', $\bar{v}_2$) by solving Eqs. (15) and (22) or Eqs. (21) and (22), but a combination of Eqs. (15) and (21) is not useful in our work because of the similarity of the mass/electron ratios (cf. Table 1).

We have seen that mass density increments are obtained by direct density measurements, or from analytical ultracentrifugation (equilibrium or velocity sedimentation) in which the experimental parameter determined is $M_2(\partial\rho/\partial c_2)_\mu$. On the other hand, radiation-scattering increments are determined from the forward-scattering intensity as $M_2(\partial\rho_{el}/\partial c_2)_\mu^2$ and $M_2(\partial\rho_N/\partial c_2)_\mu^2$. Additional information from light, X-ray and neutron scattering is of course obtained from the study of the angular dependence of the scattering (Eisenberg 1971, 1976, 1981; Jacrot 1976; Wyatt 1993).

We have proposed a new plot (Bonnété et al. 1993), which allows a joint analysis of all the scattering and mass density increments. Equations (15), (21) and (22) are each divided through by their respective intercept at zero solvent density (ρ^0, ${\rho_{el}}^0$ and ${\rho_N}^0$ equal to zero). The resulting "reduced" density increments are dimensionless and can be plotted together as a function of solvent density also divided by the appropriate intercept. All points should now fall on the same straight line with an intercept of 1 and a slope of V_{tot}:

$$\begin{aligned}(\partial\rho/\partial c_2)_\mu^* &= \left(\partial\rho_y/\partial c_2\right)_\mu / \left(x_2 + B_1 x_1 + B_3' x_3\right) \\ &= 1 - [\rho_y^0/(x_2 + B_1 x_1 + B_3' x_3)] V_{tot} \\ &= 1 - \left(\rho_y^0\right)^* V_{tot}\end{aligned} \tag{23}$$

where subscript y is blank for mass, el and N for X-rays and neutrons, x_2 and x_i are unity for mass, l^2 and l_i for X-rays and b_2 and b_i for neutrons.

An interesting application in which the multicomponent system approach turned out to be of great significance is the analysis of equilibrium sedimentation in a density gradient. Whereas, in applications discussed so far, component 3 maintains a constant concentration distribution in equilibrium sedimentation in the ultracentrifuge, a mixed solvent, containing a light component 1 and a heavy component 3, generates a density gradient when sedimented at high speeds in the ultracentrifuge. Meselson et al. (1957) have shown that when DNA is sedimented at high speeds in a mixed solvent containing a high proportion of CsCl, a well-defined density gradient, $d\rho/dr$, forms (even in the absence of component 2) because of the redistribution of component 3 in the centrifugal field, and component 2 concentrates in a narrow band, centered at a point r_b in the ultracentrifuge cell. If equilibrium conditions with respect to all components are achieved, Eq. (3), or the more restricted form at low macromolecular concentrations, Eq. (5), applies for the analysis of the experimental results. The differential equation can be integrated if the dependence of $(\partial\rho/\partial c_2)_\mu$ on r is known. If a quantity χ:

$$\chi = \left[\mathrm{d}(\partial\rho/\partial c_2)_\mu/\mathrm{d}r\right]_{r_b} = \left[\mathrm{d}(\partial\rho/\partial c_2)_\mu/\mathrm{d}\rho\right]_{r_b} (\mathrm{d}\rho/\mathrm{d}r)_{r_b} \tag{24}$$

is defined, Eq. (5) can (for constant χ) be integrated to yield a Gaussian distribution about r_b with the standard deviation:

$$\sigma^2 = -RT/M_2\omega^2 r_b \chi \tag{25}$$

We have described a method (Eisenberg, 1967, 1976; Cohen and Eisenberg 1968) for evaluating χ which circumvents the necessity (Baldwin 1959; Hearst and Vinograd 1961; Fujita 1962) of obtaining a detailed description of the partial specific volumes (which may be affected at the high component 3 concentrations under study) and interaction parameters which enter into $(\partial\rho/\partial c_2)_\mu$, as well as their dependence on pressure and position in the cell. The method is based upon the important observation (Meselson and Stahl 1958) in the establishment of the semi-conservative form of DNA replication, an achievement now called "the most beautiful experiment in biology" (Holmes 2001), that upon isotopic substitution (^{15}N for ^{14}N) in *Escherichia coli* DNA, the center of the DNA band in the CsCl gradient shifts by $\Delta\rho$ density units to the denser side of the CsCl gradient. Elementary analysis (Eisenberg 1967) yields:

$$\chi = -(\alpha - 1)/\Delta r = -[\langle\alpha - 1\rangle/\Delta\rho](\mathrm{d}\rho/\mathrm{d}r)_{r_b} \tag{26}$$

where α [equal to about $1 + (15/4\times441) = 1.0085$ for a DNA with 50% GC content, i.e. 15 nitrogens per 4 nucleotides] is the ratio of the weights (per nucleotide) of the two DNA species. The true value of M_2 may thus be derived from Eq. (25), without any knowledge of partial volumes, interaction parameters and their dependence on pressure and r.

Additionally, the fact that $(\partial\rho/\partial c_2)$ vanishes at r_b permits the estimation of ξ_1 or ξ_3 (if the partial volumes are known) under specified conditions in the ultracentrifuge and the pertinent CsCl concentrations. For further details the reader is referred to Eisenberg (1976).

The presentation can now consider examples of solvent-cosolute and solute-cosolute, or to solute-solvent, interactions, with maintenance of a strictly correct analytical approach.

Results

A basket of results

Density increment and partial specific volume measurements enable the calculation of ξ_1 as a function of ρ or w_3 (Eqs. 9 and 10). Plots of ξ_1 versus ${w_3}^{-1}$ (Eq. 13) are usually linear (Cohen and Eisenberg 1968; Reisler et al. 1977, fig. 1; Eisenberg 1994) and allow the calculation of B_1 and $B_3' = B_3 - E_3$ (Table 2). In the DNA analysis, the Donnan exclusion E_3 outweighs "binding" of salt B_3. "Hydration" B_1 (~0.2 g H_2O/g DNA) leads to 5 molecules of water per nucleotide, with an uncertainty of one, in good agreement with subsequent X-ray crystallography studies (Kopka et al. 1983). B_1 of bovine serum albumin (BSA) changes little (0.23 to 0.18 g/g) in going from the native form in NaCl to the denatured form in GdmCl, indicating that hydration is related to a significant extent to the amino acid composition and is only

Table 2 Interaction parameters B_1 and $B'_3 = B_3 - E_3$ of proteins and DNA in salt cosolvents, in units of g water/g protein (DNA) and g cosolvent/gprotein (DNA) (Eisenberg 1994)

Substance	B_1 (g/g)	$B'_3 = B_3 - E_3$
hMDH, NaCl	0.35–0.45	0.08–0.14
BSA, NaCl	0.23	0.012
BSA,GdmCl	0.18	0.27
DNA, NaCl	0.20	−0.054
DNA, CsCl	0.24	−0.070

partially represented by a layer of water covering the native globular protein molecule. B_3 in the case of NaCl is minimal; however, it increases significantly in the case of GdmCl. The extreme halophilic malate dehydrogenase (hMDH) at high NaCl concentrations "binds" both water and salt (Bonnété et al. 1993). In our early studies (Reisler and Eisenberg 1969; Reisler et al. 1977), experiments in GdmCl established that rabbit muscle aldolase is a tetramer and not a trimer, as believed by some at that time. For $\bar{v}_2$ at 25 °C we obtained 0.734 mL/g for BSA (65,800 g/mol) in water, decreasing to 0.728 in 3–6 M GuaHCl in the denatured state. For the native aldolase tetramer (156,840 g/mol), we found $\bar{v}_2 = 0.739$ mL/g, decreasing to 0.733 in 3–6 M GuaHCl, in the denatured monomer state. For this range of molar masses the elaborate scaled particle theory (SPT) calculations of Chalikian and Breslauer (1996, fig. 1) predict increasing $\bar{v}_2$ following denaturation.

In a recent analysis (Eisenberg 2000, table 1) it was established that the error in using $(1-\rho^0\bar{v}_2)$ rather than $(\partial\rho/\partial c_2)_\mu$ in multicomponent systems, in multimolar cosolvent concentrations, is about 9.3% in case of DNA in NaCl, about 0.4% (negligible) in the case of BSA in NaCl (luckily also true for the Svedberg hemoglobin study); however, it is 16.7% for BSA in GdmCl and 4.9% for aldolase in sucrose (Ebel et al. 2000). Hydration of aldolase in raffinose increases with decreasing temperature (Ebel et al. 2000), leading to decreasing partial volume $\bar{v}_2$ values, a result of increasing water electrostriction with increasing hydration (cf. Svergun et al. 1998). The hydration parameter B_1 mildly increases with increasing sugar size (Ebel et al. 2000), but considerably less than by a calculation of rolling balls, the size of the cosolvent molecules, over the enzyme surface, determined by X-ray diffraction.

Varying the size of sugars as cosolvents in the study of biological macromolecules leads to additional benefits if the size of the sugars can be chosen for fractal analysis, distinguishing between sugar molecules which are able to penetrate the biological macromolecule, leading to information about hydration, and larger sized sugars which cannot penetrate the biological macromolecule, only probing its outer surface. We have in this way studied the nucleosome core particle (Eisenberg and Felsenfeld 1981; Greulich et al. 1985; Eisenberg 1994, fig. 4). A comparison between sucrose, capable of penetrating the nucleosome core particle, and γ-cyclodextrin, excluded from it, led to reasonable hydration, $B_1 = 0.318$ g/g, $\bar{v}_2 = 0.662$ mL/g and a total volume $V_t = 561$ nm^3 of the particle, compared to 542 nm^3 from X-ray scattering and hydrodynamics. In these experiments, $B_3' \approx 0$ and therefore $\xi_1 = B_1$.

We will only briefly refer here to the complementarity between neutron data, on the one hand, and X-ray and mass density data, on the other, by the plot allowing a joint analysis of all the scattering and mass density increment data (Bonnété et al. 1993; Eisenberg 1994, fig. 3). Neutron data, being close to null solvent scattering, do not define a precise slope; they are therefore not sensitive to the volume of the particle, but very sensitive to its composition through the value of the intercept at zero solvent density, which is well defined. On the other hand, the mass and X-ray values are quite far from the intercept at zero solvent density, but they define the slope of the line, and are therefore sensitive to the total volume of the particle.

In closing this discussion, and for those analytical ultracentrifugation experimentalists still unwilling to perform density increment measurements, there is a recent alternative, attractive approach (Anderson et al. 2002a, 2002b), still to be tested, based on water vapor pressure osmometry, including corrections to be applied in comparing equilibrium dialysis, electromotive force determination and isopiestic distillation (Eisenberg 1976). Vapor pressure osmometry and isopiestic distillation yield identical results; however, the advantage of vapor pressure osmometry lies in the availability of a highly attractive experimental procedure (Courtenay et al. 2000).

Acknowledgements The author acknowledges the encouraging understanding and support of Hiroshi Fujita, Jo (P.J.G.) Butler and Tom Laue.

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