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Review

Analytical ultracentrifugation in a Gibbsian perspective ☆

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Abstract

The analytical ultracentrifuge has come into new intensive use following complete instrumental redesign and the use of advanced computer technologies for the analysis and interpretation of experimental results. Major attention is now devoted to the evaluation of interactions between similar and dissimilar biological macromolecules in dilute and concentrated systems. Electrostatically charged biological solute systems additionally comprise low molecular weight charged and non-charged cosolvents. Solvent/cosolvent interactions, insufficiently considered in most current analytical ultracentrifugation analyses, may quantitatively affect solute/solute interactions. For comprehensive analysis the Svedberg derivation considering a buoyant molar mass $(1-\rho^0\bar{v}_2)M_2$ and valid at vanishing solute concentration for strictly two component systems only, should be replaced, following classical thermodynamic analysis, by the ratio $(\partial\rho/\partial c_2)_\mu/d\Pi/dc_2$ of the density increment at constant chemical potential of diffusible cosolvents, to the derivative of the osmotic pressure with solute concentration. Disregard of the solvent/cosolvent and solute/cosolvent interactions should be avoided.

Keywords: Thermodynamics; Analytical ultracentrifugation; Multi-component systems; Density increments; Light; X-ray; Neutron scattering

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1. Introduction

The macromolecular nature of organic large molecules was contested by leading chemists into the 1930s, in an attempt to establish their preference for the variably sized colloidal aggregation mechanism in the creation of large molecular structures [1]. Hermann Staudinger was accorded the Nobel prize in 1953 for dismantling this concept and establishing the covalent macromolecular nature of organic substances composed of carbon, hydrogen and oxygen, such as rubber and cellulose, for instance [2]. It has, however, been emphasized [3] that the concept recognizing the macromolecular nature of proteins goes back to 1838, haemoglobin was first crystallized in 1840, and a reasonable value of its (subunit) molecular mass was obtained by the analytical determination of sulfur, available in all proteins containing methionine and cysteine amino acids, and iron, a basic haemoglobin component. The beliefs of the protein chemists were thus not affected by the colloidal structural interpretation, and they were able to obtain molar masses which were later identified with protein monomeric units. Thé Svedberg, in creating the analytical ultracentrifuge in 1923 [4], was able to show that haemoglobin has a molar mass of 67 000 g/mole, and is thus a tetramer of the originally found approximate 16 700 g/mole mass. It is interesting to note that Svedberg was accorded the Nobel prize for his earlier work on colloidal systems and not, as would generally be assumed, for his creation of the powerful analytical ultracentrifuge, whose main purpose, for many years, was the reliable determination of molecular masses.

With time the significance of the analytical ultracentrifuge decreased as methodologies were developed and improved for the precise determination of molecular masses by sequence determination [5,6] and mass spectrometry [7]. Gel filtration proved to be of unusual usefulness and versatility as well, requiring minute amounts of material [8], however, its usefulness is limited by the requirements of calibration, at times deviating from absolute correct correspondence.

State of the art advanced technology in the novel reconstruction of the analytical ultracentrifuge [9,10] and the development of sophisticated computer controlled analysis [11,12] has, in recent years, led to the evaluation of homologous and heterologous solute interactions from velocity and equilibrium ultracentrifugation. However, as described below, the analysis of solute/solvent and solute/cosolvent interactions are not receiving corresponding adequate support in the current context of analytical ultracentrifugation, although their significance in the analysis of biological structure and function is important and well established. This will be emphasized in the following. Emphasis will also be extended to show that analytical ultracentrifugation, primarily derived by thermodynamic analysis from osmotic pressure differentiation with solute concentration at constant chemical potentials of membrane diffusible solvents and cosolvents [13,14], connects in complementary fashion to methods of light, X-ray and neutron scattering [15–17], increasing the complementarity and the significance of the experimental analysis which should not be restricted to a single analytical approach [18]. An additional extension includes the use of density gradients and isotope substitutions the usefulness of which have been demonstrated in significant fashion [19–22].

2. Theoretical analysis

The classical equation of Svedberg [23] for the sedimentation coefficient $s = u/\omega^2 r$ (seconds), (u is the velocity of the particle in the ultracentrifuge per unit gravitational acceleration; ω (radians per second), is the angular velocity and r (cm), is the distance of the particle from the axis of rotation), is in the limit of vanishing particle concentration, and for strictly two component systems only,

$$s = \left(1 - \rho^0 \bar{v}_2\right) M_2 / N_{\mathrm{Av}} f \qquad (1)$$

where M_2 (g/mole) is the molar mass of the macromolecular component 2, $\bar{v}_2$ (ml/g) is the partial specific volume, ρ^0(g/ml) is the density of the solvent, N_{Av} is Avogadro's number and f

(g/s) is the frictional coefficient which depends on the shape and size of the particle. $(1-\rho^0\bar{v}_2)M_2$ is the Archimedes buoyant weight of component 2, capable of assuming positive, zero or negative values depending on solvent density, leading to sedimentation, layering at matching macromolecule solvent density, or flotation. The diffusion coefficient $D = RT/N_{Av}f$ depends on the size and shape of the particle and can be determined by the rate of the spreading of the boundary between solution and solvent in an ultracentrifuge experiment [24]. By combination of the sedimentation and diffusion experiments the frictional coefficient can be eliminated and the Svedberg equation is obtained

$$s/D = \left(1-\rho^0\bar{v}_2\right)M_2/RT \tag{2}$$

with all experimental quantities extrapolated to vanishing concentration of the macromolecular component 2. In an experiment balancing sedimentation and diffusion to reach an equilibrium distribution of component 2, a differential relation between the logarithm of the concentration c_2 (g/ml) and r^2 is obtained

$$\mathrm{d}\ln c/\mathrm{d}r^2 = (\omega^2/2RT)\left(1-\rho^0\bar{v}_2\right)M_2 \tag{3}$$

Eq. (3) is integrated in the analysis of finite macromolecular concentrations and non-interacting or interacting macromolecular species, assuming variable apparent molar masses M_2^*.

In charged synthetic polyelectrolyte or biological systems additional components such as low molecular weight salts, or neutral molecules, such as sugars for instance, have to be considered. In these situations the Archimedes buoyancy term $(1-\rho^0\bar{v}_2)$ in Eqs. (1)–(3) does not apply in this classical form and has to be extended to include solvent and cosolvent interactions [13–17]. To keep matters simple in this presentation a three component system containing a cosolvent component 3 only in addition to the solvent component 1 and the macromolecular component 2 is considered. This does not limit extension to any number of low molecular or macromolecular components. The presentation is based on a strict Gibbsian thermodynamic approach, though at certain advanced stages of discussion molecular models are introduced for practical evaluations.

In a two component osmotic experiment the solvent component 1 can equilibrate across the semipermeable membrane whereas the macromolecular component 2 is restricted to the interior osmometer compartment. Presence of component 2 lowers the chemical potential μ_1 leading to solvent influx into the interior compartment, balanced by 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 $\Pi = P - P'$ (primes indicate the outside 'open' solution) can be expanded into a virial series, or related to the activity coefficient γ on the c-concentration scale,

$$\Pi/c_2RT = M_2^{-1} + A_2c_2 + ... \tag{4a}$$

$$(\mathrm{d}\Pi/\mathrm{d}c_2)/RT = M_2^{-1} + 2A_2c_2 + ... = M_2^{-1}\{1 + (\mathrm{d}\ln\gamma/\mathrm{d}\ln c_2)\} \tag{4b}$$

At vanishing concentrations c_2 the reciprocal of M_2 will be obtained.

However, one should not be confused by the units used. 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. Matters get more complicated when polyelectrolytes or charged biological macromolecules are examined (Fig. 1a). Osmotic pressure equilibrium can be achieved, however, a virial expansion as indicated in Eqs. (4a and b) cannot be performed because of long-range electrostatic interactions. Positively 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 compo-

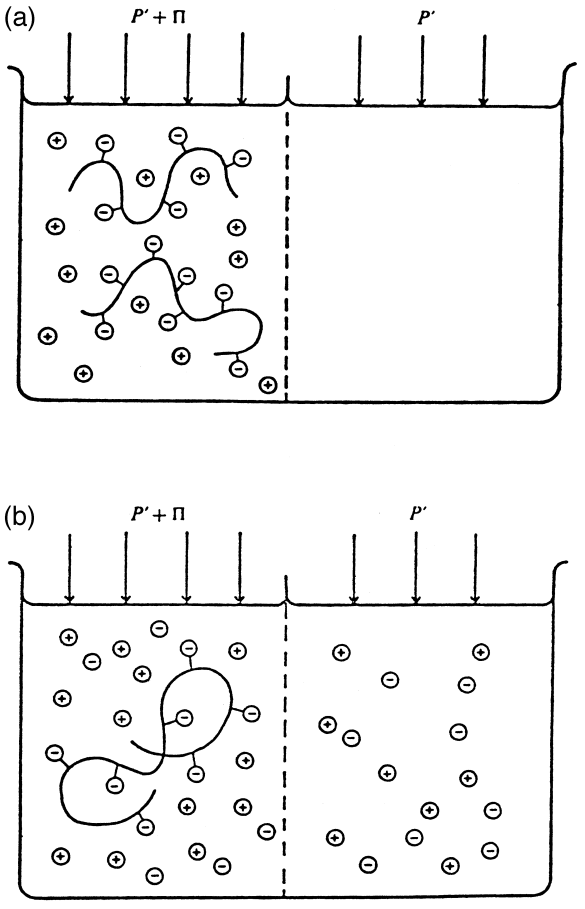

Fig. 1. Schematic representation of osmotic pressure experiments of charged macromolecules in the absence of (a) and the presence of (b) low molecular weight uni-univalent salts. With permission, from H. Eisenberg, in Photon Correlation and Light Beating Spectroscopy, H.Z. Cummins and E.R. Pike, editors, Plenum Press, New York, pp. 551–567, 1974.

nent 3 is added (Fig. 1b), having, to maintain the three component characterization, an ion in common with the component 2 counterion. Long range electrostatic interactions are now reduced and a virial expansion again becomes possible. Schematically osmotic pressure equilibrium, including chemical potential equilibrium of diffusible components, is presented in Fig. 2a. A question still often raised in contemporary advanced research circles is what molar or molecular mass of a polyelectrolyte or charged biological macromolecule is determined in an osmotic pressure, or related experiment, equilibrium sedimentation or scattering of light, X-rays or neutrons? Are the counterions included or not included in the experimental result? Here, as before, the answer is clear. Macromolecules are *counted* as moles (or molecules) per ml, not *weighed*, and the molecular mass is again given in terms of the

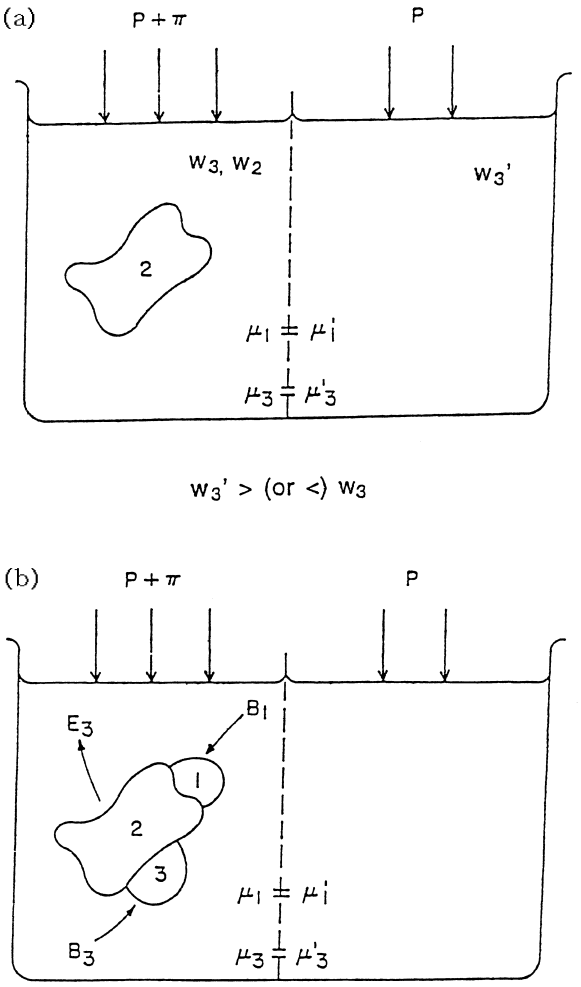

Fig. 2. Schematic representation of (a) osmotic equilibrium of three-component system, solvent component 1, macromolecular solute component 2 and low-molecular weight cosolvent component 3, and (b) schematic representation of binding B_1 g/g of component 1 and B_3 g/g of component 3 including Donnan exclusion E_3, to the macromolecular component 2. With permission, from E. Reisler, Y. Haik and H. Eisenberg, Biochemistry 16 (1977) 197–203.

concentration units used. Thus, if, for instance NaDNA is dissolved in a high concentration of CsCl, is M_2 of NaDNA or CsDNA obtained? Obviously, in an excess of caesium over sodium the majority of positive counterions surrounding the negatively charged DNA backbone will be caesium rather than sodium counterions. However, if we express the DNA concentration in terms of NaDNA (by weight added or by optical density), the molar mass M_2, including the counterions, is that of sodium and not of CsDNA. However, the experiment at this stage does not yield information regarding coion/counterion interactions, or counterion condensation as applies to highly charged DNA [25]. Having correctly interpreted the osmotic pressure results for charged macromolecules, we next proceed to an examination of analytical ultracentrifugation.

In equilibrium sedimentation, an equilibrium distribution of the macromolecular component 2 and the low molecular weight component 3 is achieved, however the latter usually maintains a practically uniform distribution. The basic equation for equilibrium sedimentation is derived [13,14,26,27] on the basis of the theory of heterogeneous equilibrium requiring uniform chemical and gravitational potentials of all components throughout the system in the spinning sample in the analytical ultracentrifuge. This leads to the basic equation

$$(\mathrm{d}\ln c_2/\mathrm{d}r^2) = (\omega^2/2)(\partial\rho/\partial c_2)_\mu/(\mathrm{d}\Pi/\mathrm{d}c_2) \quad (5)$$

The density increment $(\partial\rho/\partial c_2)_\mu$ at constant chemical potentials of components 1 and 3 will be discussed in the following; $(\partial\rho/\partial c_2)_\mu$ equals the difference in solution to solvent density at dialysis equilibrium, divided by component 2 concentration c_2. The osmotic pressure derivative $\mathrm{d}\Pi/\mathrm{d}c_2$ can be derived from Eq. (4b). Eq. (5) again clearly indicates that the units used for the concentration are inconsequential for the interpretation of the final result. Eq. (5) has been recalled and rederived by Fujita [28,29]. It applies to any number of components [13] yet we maintain here the three component discussion for practical reasons and simplified presentation. In the limit of vanishing concentration c_2 Eq. (5) and (4) reduce to

$$\mathrm{d}\ln c_2/\mathrm{d}r^2 = (\omega^2/2RT)(\partial\rho/\partial c_2)_\mu M_2 \quad (6)$$

which replaces the classical two component Eq. (3). The Svedberg Eq. (2) is replaced in similar fashion by

$$s/D = (\partial\rho/\partial m_2)_{P,\mu}/(\mathrm{d}\Pi/\mathrm{d}m_2) \quad (7)$$

(m_2 is in molality units, moles of component 2 per kg of component 1) and, in the limit of vanishing component 2 concentration

$$s/D = (\partial\rho/\partial c_2)_\mu M_2/RT, \quad (8)$$

The density increment $(\partial\rho/\partial c_2)_\mu$ for the three component system is given by [13–17]

$$(\partial\rho/\partial c_2)_\mu = \left(1 - \rho^0\bar{v}_2\right) + \xi_3\left(1 - \rho^0\bar{v}_3\right) \quad (9a)$$

$$= (1 + \xi_3) - \rho^0(\bar{v}_2 + \xi_3\bar{v}_3). \quad (9b)$$

where ρ^0 is the solvent density as defined by dialysis equilibrium, and $\xi_3 = (w'_3 - w_3)/w_2 = (\partial w_3/\partial w_2)_\mu$ 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 (Fig. 2a). For symmetry reasons Eqs. (9a and b) can also be written as

$$(\partial\rho/\partial c_2)_\mu = \left(1 - \rho^0\bar{v}_2\right) + \xi_1\left(1 - \rho^0\bar{v}_1\right) \quad (10a)$$

$$= (1 + \xi_1) - \rho^0(\bar{v}_2 + \xi_1\bar{v}_1), \quad (10b)$$

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

$$\xi_1 = -\xi_3/w_3. \quad (11)$$

The interaction coefficients ξ_1 and ξ_3 thus depend on cosolvent concentration and cannot be associated with specific interaction with only component 1 or 3, but should each be considered as relating to both. A zero value of either ξ_1 or ξ_3 does not indicate 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 that for a strictly two compo-

nent system Eq. (6) reduces to Eq. (3), as the first term only on the right hand side of Eq. (9a) and Eq. (10a) is conserved in this case.

For intuitive and practical reasons study of macromolecular solute/solute interactions by velocity or equilibrium analytical ultracentrifugation should therefore be preceded by the determination of $(\partial\rho/\partial c_2)_\mu$ by dialysis and density determination [30]. Objection to this based on 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 [30] 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 [31]. 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 [30]. The density increment $(\partial\rho/\partial c_2)_\mu$ is the basic quantity in the ultracentrifugation analysis and should be given adequate presentation and attention, beyond its application to synthetic polyelectrolytes [32].

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 [30]. As an example I mention the invariant particle model [33], applicable in some circumstances, leading to simple mathematical expressions:

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

or, equivalently

$$\xi_1 = B_1 - B_3/w_3 \tag{12b}$$

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 the other components (Fig. 2b). Note that whereas ξ_3 and ξ_1 depend on w_3, B_3 and B_1 are constants independent of w_3 in the invariant particle model [16]. Eqs. (9,10) can be rewritten in terms of this model as

$$(\partial\rho/\partial c_2)_\mu = \left(1 - \rho^0\bar{v}_2\right) + B_1\left(1 - \rho^0\bar{v}_1\right) + B_3\left(1 - \rho^0\bar{v}_3\right) \tag{13a}$$

$$= (1 + B_1 + B_3) - \rho^0(\bar{v}_2 + B_1\bar{v}_1 + B_3\bar{v}_3) \tag{13b}$$

the slope in the plot of $(\partial\rho/\partial c_2)_\mu$ against ρ^0 being the total volume of the component 2 particle. For alternative models cf. Ebel et al. [30].

Research can now veer to solvent/cosolute and solute/cosolute, or to solute/solvent interaction studies, with maintenance of a correct analytical approach. An interesting example confirming the invariant particle model concerns fractal probing of nucleosome core particles by analytical equilibrium ultracentrifugation in solutions containing variable amounts of sugars increasing in size [34,35]. Sucrose, raffinose and glycerol sugars penetrate inside the nucleosome core particle, probing excluded volume due to hydration. The larger sugar γ-cyclodextrin cannot penetrate inside the pores of the nucleosome core particle therefore it probes its outer surface, leading to volume calculation corresponding well to the volume determination by X-ray crystallography. Protein hydration does not lead to changes of $\bar{v}_2$ unless changes occur in the volumes of the water of hydration.

Changing of temperature in aldolase/sugar studies [30] led to correct electrostriction water volume changes determination related to changes in hydration. In studies of biological macromolecules in concentrated simple salt solutions the Donnan effect and hydration were correctly determined for DNA and changes in hydration and salt binding were correctly established for bovine serum albumin, in native and denatured form, and malate dehydrogenase from extreme halophilic bacteria [16]. The correct number of

Table 1
Density increments $(\partial\rho/\partial c_2)_\mu$ compared to the Svedberg Archimedes buoyancy term $(1-\rho^0\bar{v}_2)$ for a number of systems

Comp. 2	Comp. 3	C_3 mole/l	w_3 g/g	$\bar{v}_2$ ml/g	ρ^0 g/ml	ξ_1 g/g	B_1 g/g	B_3 g/g	$(\partial\rho/\partial c_2)_\mu$	$(1-\rho^0\bar{v}_2)$	$\xi_1(1-\rho^0\bar{v}_1)$
DNA[a]	NaCl	1.0	0.0598	0.528	1.037	1.118	0.2	−0.054	0.396	0.452	−0.042
BSA[b]	NaCl	1.0	0.0598	0.735	1.037	0.036	0.23	0.012	0.233	0.238	−0.001
BSA[b]	GdnCl	4.0	0.537	0.728	1.095	−0.358	0.18	0.27	0.238	0.203	0.034
Aldolase[c]	Sucrose	0.399	0.1488	0.737	1.0507	0.215	0.215	0	0.217	0.226	−0.011

[a] Eisenberg [16,17].
[b] Eisenberg [16].
[c] Ebel et al. [30].

rabbit muscle aldolase subunits could be established at a time when this was still under dispute [36].

In Table 1 experimental density increments $(\partial\rho/\partial c_2)_\mu$ are compared for a number of systems to the Svedberg Archimedes buoyancy term $(1-\rho^0\bar{v}_2)$ and the interaction term $\xi_1(1-\rho^0\bar{v}_2)$ from Eq. (10a) at selected cosolvent concentrations and densities. Notice that DNA in NaCl solutions is subject to significant corrections as a result of hydration and Donnan exclusion, BSA requires only minor corrections in NaCl solutions, however major corrections following GdmCl denaturation, and aldolase in concentrated sucrose solutions is also subject to corrections. Study of Table 1 should be helpful in promoting the use of density increments in preference to reliance on calculated partial specific volume values only.

Analysis of the scattering of light, X-rays and neutrons, calculated from thermodynamic fluctuations of component 2 concentrations at constant chemical potentials of diffusible solutes [13–17] leads to equations relating to osmotic pressure and analytical ultracentrifugation in complementary fashions, the density increments being replaced by related refractive index, electron density and neutron scattering length increments. Neutron scattering is of special added interest in view of the negative water neutron scattering length, returning to a positive value with exchange of hydrogen by deuterium. Complementary approaches leading to combined plots have been worked out [37]. Additional advantages from scattering experiments reside in the ability to determine molecular size and shape parameters from angular dependence of scattering. Dynamic light scattering determines translational diffusion D and thus provides size and shape in a hydrodynamic sense. Additional methodologies have been reviewed recently [18].

3. Conclusions

We have undertaken an excursion in the interpretation of thermodynamic aspects of osmotic pressure, density increments and analytical ultracentrifugation and have mentioned the complementarity with scattering of light, X-rays and neutron methodologies. The main take home message in this excursion is that the Svedberg Archimedes term $(1-\rho^0\bar{v}_2)M_2$ in analytical ultracentrifugation analysis should be replaced by the much more general and correct $(\partial\rho/\partial c_2)_{P,\mu}/(\mathrm{d}\Pi/\mathrm{d}c_2)$ ratio when proceeding to the evaluation of interactions in analytical ultracentrifugation experiments. This is aesthetically preferable, leads to deeper understanding, and avoids misinterpretation likelihoods. Additional quotations to the technologies discussed are to be found in the references given.

Note added in proof

The article by P.J.G. Butler, Use of density increment in assessing protein aggregation under unusual conditions, Advances in Ultracentrifugation Analysis, Biochemical Society Transactions 26 (1998) 749–753, pertinent to the present discussion, has recently come to my attention.

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