Current Protocols in Immunology (in press)

Measuring Protein-Protein Interactions by Equilibrium Sedimentation

Andrea Balbo*, Patrick H. Brown*, Emory H. Braswell[&], Peter Schuck*[#]

*Protein Biophysics Resource, National Institute for Biomedical Imaging and Bioengineering, National Institutes of Health, Bethesda, MD, [&]National Analytical Ultracentrifugation Facility, Biotechnology Center, University of Connecticut, Storrs, CT

[#]to whom correspondence should be addressed

keywords: sedimentation equilibrium, sedimentation velocity, chemical equilibria, reversible interactions, multi-protein complex, analytical ultracentrifugation

Mini-abstract: This Unit describes basic principles and practice of sedimentation equilibrium analytical ultracentrifugation for the study of reversible protein interactions, such as the characterization of self-association, heterogeneous association, binding stoichiometry, and the determination of association constants. Advanced tools such as mass conservation, multi-wavelength, and global analysis are introduced and discussed in the context of the experimental design. A detailed protocol guiding through the experimental steps and the data analysis is available as an internet resource.

Address for Correspondence:

Dr. Peter Schuck
National Institutes of Health
13 South Drive, Bldg. 13, Rm. 3N17
Bethesda, MD 20892

Phone: (301) 435-1950
Fax: (301) 496-6608
Email: pschuck@helix.nih.gov

1. Introduction

Analytical ultracentrifugation (AU) is one of the oldest techniques for the study of biological macromolecules (Svedberg and Pedersen 1940; Schachman 1959). In his book, "The Molecular Biology of the Gene", J. D. Watson characterized AU "as perhaps the most striking contribution of physical chemistry to the study of biological macromolecules". While the use of the technique was in decline during the 1970s and the 1980s, it has experienced a renaissance in the 1990s with increasing interest in reversible protein-protein interactions. Equipped with modern instrumentation, sensitive detection systems, and new computational data analysis methods, the AU technique called sedimentation equilibrium (SE) is one of the most effective methods for the detection and characterization of protein interactions. Applications include antibody-antigen interactions, receptor-ligand interactions (e.g., with T-cell receptor, MHC molecules, superantigens, Fc- and IgE receptors, cell adhesion molecules, cell surface receptor interactions with chemokines and cytokines, recognition of viral or bacterial proteins, or interaction with small molecules), protein complexes in signal transduction, and other immunologically important types of transient self- or heterogeneous biomolecular interactions and their characterization with regard to stoichiometry and affinity (Burrows et al. 1994; Shire 1994; Advant et al. 1995; Ghirlando et al. 1995; Malchiodi et al. 1995; Dall'Acqua et al. 1996; Junghans et al. 1996; Kim et al. 1996; Philo et al. 1996; Horan et al. 1997; Leong et al. 1997; Paliwal et al. 1997; Rajarathnam et al. 1997; Shi et al. 1997; Silkowski et al. 1997; Leder et al. 1998; Fairman et al. 1999; Natarajan et al. 1999; Schuck et al. 1999; Center et al. 2000; Holtzer et al. 2000; Ikemizu et al. 2000; Jones et al. 2000; Arkin and Lear 2001; Center et al. 2001; Li et al. 2001; Dam et al. 2003; Rosovitz et al. 2003; Davis et al. 2004; Guan et al. 2004; Boulant et al. 2005; Burgess et al. 2005; Mancheno et al. 2005; Perugini et al. 2005; Weber et al. 2005; Yu et al. 2005; Dam et al. 2006; McLellan et al. 2006; Datta et al. 2007; Fernandez et al. 2007; Li et al. 2007a; Li et al. 2007b).

The measurements take place in dilute solution, and due to the effect of sedimentation, a concentration range of 10 to 1000-fold is typically established in a single experiment, while maintaining chemical equilibrium between the species at each position in the cell and therefore at all concentrations. This occurs without the interaction of a matrix or a surface, and usually without the requirements of labeling or other protein modifications. Generally, affinities in the range of 10^4 - 10^8 M^{-1} can be studied, and in some cases this range is much larger. The molar masses of the macromolecules under study and their complexes can range from less than 10^3 to more than 10^6. Suitable molecules include soluble protein and protein domains, as well as detergent-solubilized membrane proteins (Schubert and Schuck 1991; Howlett 1992; Fleming et al. 1997).

The underlying principle is the application of a gravitational (centrifugal) force to a solution of proteins while observing the resulting changes in the concentration distribution of the proteins by optical methods. At rotor speeds low enough to allow diffusion to balance the gravitational force, simple theoretical expressions describe the equilibrium distribution that will be attained after sufficient time. The interpretation of such SE experiments is directly based on first principles and equilibrium thermodynamics, and allows the measurement of the molar mass of the proteins, their state of association and the free energies of binding. Although the experiment spans usually one or more days, the extended experimental time can sometimes be an advantage when studying equilibria with slow interconversions of species (reactions that are associated with

large activation energies). In sedimentation velocity (SV) experiments, a higher rotor speed is chosen to apply a large centrifugal force that causes rapid sedimentation of the proteins. The SV experiment requires less time, and gives additional information on the purity and hydrodynamic shape of the proteins, but provides only more indirect information on the molar mass.

The present commentary is mainly concerned with the SE technique. However, references to SV experiments as described in Unit [SV Unit in this Series] will also be made, because they are highly complementary and can frequently reveal relevant additional information on the proteins under study. In fact, a strongly recommended practice adhered to in many AU laboratories is that all protein samples and their interactions be studied by SV first, in order to facilitate the reliable analysis of the following SE experiment. Due to the unique ability of AU to determine the number and size of protein complexes in self-association and hetero-association processes in free solution, SE and SV analytical ultracentrifugation is frequently used in conjunction with other biophysical techniques, including, for example, isothermal titration calorimetry, dynamic light scattering, surface plasmon resonance biosensing, nuclear magnetic resonance and x-ray crystallography.

SE is very versatile and has been refined during the last eight decades, making it in many aspects a highly technical discipline. Therefore, the scope of this Unit is only an introductory description of the principles involved, what can be learned about proteins, the considerations entering the experimental design and the data analysis, and the difficulties encountered in the process. Furthermore, only the so-called long-column (2-5mm solution columns) SE method for reversible interactions is described, as it is the most commonly used approach. More general reviews have recently been published (Arisaka 1999; Laue 1999; Rivas et al. 1999; Lebowitz et al. 2002; Cole 2004; Scott and Schuck 2005; Howlett et al. 2006), as well as recent reviews on the use of SE for protein-nucleic acid interactions (Ucci and Cole 2004), multi-protein complexes (Schuck 2007b), and the experimental protocols (Balbo and Schuck 2005; Schuck 2006).

As in many biophysical disciplines (and in contrast to common preparative centrifugation), the mathematical data analysis is an important part of SE. Where the present commentary relates to practical steps of data analysis, a review of different software is beyond the scope of the present work. For obvious reasons we focus on the application of the public domain software SEDPHAT written in our laboratory (Schuck 2006). It is a general tool for multi-method biophysical analysis including SE and SV analytical ultracentrifugation, isothermal titration calorimetry, dynamic light scattering, and general binding isotherms (Schuck 2003; Vistica et al. 2004; Houtman et al. 2007). It contains all the approaches for SE analysis described in the present Unit, and is being applied in many AU laboratories (Schuck 2007a). For the practical execution of the experiments, we refer to a detailed step-by-step protocol describing the design, execution, and analysis of SE experiments, which is provided as a separate internet resource and hosted at www.AnalyticalUltracentrifugation.com\SEprotocols.htm), and to a workshop being held semi-annually at the National Institutes of Health, Bethesda, MD.

2. Basic Principles

a) Sedimentation of Ideal Proteins in the Centrifugal Field

In the ultracentrifuge, the chemical potential of a macromolecule contains a mechanical component which is dependent on the radial position in the centrifugal field. As a result, at equilibrium, the concentration distribution of single, ideally sedimenting protein species in a solution column is described by the following Boltzmann exponential

$$c(r) = c(r_0)\exp\left(M(1-\bar{v}\rho)\omega^2(r^2-r_0^2)/2RT\right) \qquad (1)$$

where r denotes the distance from the center of rotation, ω the angular velocity of the rotor, M and $\bar{v}$ the protein molar mass and partial-specific volume, respectively, ρ the solvent density, T the absolute solution temperature, R the molar gas constant, and r_0 an arbitrary reference radius (*e.g.* the meniscus). Thus, from a single loading concentration a concentration gradient is obtained, with regions more dilute and more concentrated than the original sample, and from the shape of the gradient information about the protein molar mass can be derived.

Following the principle of Archimedes, the buoyancy of the protein has to be considered. As can be seen in Equation 1, the sedimentation of the protein is governed by its buoyant molar mass $M^* = M(1-\bar{v}\rho)$ (or in the thermodynamically more correct form, M* = Mdρ/dc, with dρ/dc denoting the density increment of the protein, see below). The implications of this expression with respect to the required determination of the buffer density and the protein partial specific volume will be discussed. For the study of protein interactions, it is generally assumed that no volume change occurs with binding (see below). As a consequence, the buoyant molar masses of the complexes can be calculated as the sum of the buoyant molar masses of the protein species that form the complex.

For a protein that is in a reversible monomer-dimer self-association equilibrium, the chemical equilibrium is described by the mass action law $c_2 = K_{12}c_1^2$, with K_{12} denoting the dimerization constant. This leads to the radial distribution of the dimer:

$$\begin{aligned} c_2(r) = K_{12}c_1^2(r) &= K_{12}\left\{c_1(r_0)\exp\left[M_1^*\frac{\omega^2(r^2-r_0^2)}{2RT}\right]\right\}^2 \\ &= K_{12}c_1^2(r_0)\exp\left[2M_1^*\frac{\omega^2(r^2-r_0^2)}{2RT}\right] \end{aligned} \qquad (2)$$

and the total protein distribution

$$c_{tot}(r) = c_1(r_0)\exp\left[M_1^*\frac{\omega^2(r^2-r_0^2)}{2RT}\right] + K_{12}c_1^2(r_0)\exp\left[2M_1^*\frac{\omega^2(r^2-r_0^2)}{2RT}\right] \qquad (3)$$

As can be seen, the mass action law is *fulfilled at all positions in the cell*, and the dimer sediments like an ideal species with the buoyant molar mass $2M_1^*$, twice the monomer buoyant molar mass M_1^*. More generally, the sedimentation profile of a protein in a multi-step self-association equilibrium is described by

$$c_{tot}(r) = \sum_{\{i\}} K_{1i} c_1^i(r_0) \exp\left[iM_i^* \frac{\omega^2 (r^2 - r_0^2)}{2RT} \right], \quad with\ K_{11} = 1 \tag{4}$$

and $\{i\}$ denoting the set of oligomers formed by the protein with association constants K_{1i}.

For the not uncommon case where all oligomers exist and their equilibrium relationship to oligomers longer by one unit is governed by the same equilibrium constant K (infinite isodesmic association) the sedimentation equilibrium distribution can be written in the closed form

$$c(r) = \frac{c_1(r_0) \exp\left(M^* \xi(r) \right)}{\left(1 - K c_1(r_0) \exp\left(M^* \xi(r) \right) \right)^2} \tag{5}$$

and variations for decaying indefinite self-associations and for isodesmic associations with a separate initial step have been derived and applied to some proteins (Adams and Lewis 1968; Howlett and Jeffrey 1973; Morris and Ralston 1989; Rivas et al. 2000).

The same principles apply for hetero-associations: For two ideal species A and B that interact reversibly to form a 1:1 complex, the buoyant molar mass M_{AB}^* of the complex can be calculated as

$$M_{AB}^* = M_{AB}(1 - \bar{v}_{AB}\rho) \cong M_A(1 - \bar{v}_A\rho) + M_B(1 - \bar{v}_B\rho) = M_A^* + M_B^* \tag{6}$$

(assuming the absence of volume changes), and the chemical equilibrium in dilute solution is described by the mass action law $c_{AB} = K_{AB} c_A c_B$. Analogously to Eqs. (2) and (3), this leads to

$$\begin{aligned} c_{tot}(r) = {} & c_A(r_0) \exp\left[M_A^* \frac{\omega^2 (r^2 - r_0^2)}{2RT} \right] + c_B(r_0) \exp\left[M_B^* \frac{\omega^2 (r^2 - r_0^2)}{2RT} \right] \\ & + K_{AB} c_A(r_0) c_B(r_0) \exp\left[(M_A^* + M_B^*) \frac{\omega^2 (r^2 - r_0^2)}{2RT} \right] \end{aligned} \tag{7}$$

as schematically illustrated in Figure 1. Again, the mass action law is fulfilled at all positions in the cell. The sedimentation profiles for general case of reversible interactions of A and B forming a set $\{ij\}$ of complexes with *i:j* stoichiometry can be described as

$$c_{tot}(r) = \sum_{\{ij\}} K_{ij} c_A^i(r_0) c_B^j(r_0) \exp\left[(iM_A^* + jM_B^*) \frac{\omega^2 (r^2 - r_0^2)}{2RT} \right] \qquad (8)$$

with K_{ij} denoting the macroscopic binding constants for complex formation.

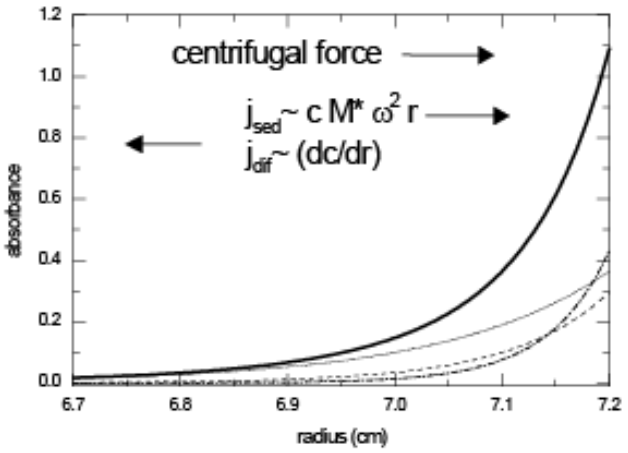

Figure 1: SE is attained when the sedimentation flux (j_{sed}, which is proportional to the local concentration c, the species buoyant molar mass M^*, and the local centrifugal field $\omega^2 r$) and the diffusional flux (j_{dif}, which is proportional to the local concentration gradient dc/dr) are balanced at each point in the solution column. This balance results in an exponential concentration distribution for each species, with the buoyant molar mass determining its steepness. In case of interacting systems, a superposition of several exponentials are observed (bold line). The example shown is the interaction of two protein species with molar masses of 30,000 and 50,000, which form a 1:1 complex and are in equilibrium at a rotor speed of 15,000 rpm and 4°C. The individual distributions of all species are also shown: the 30,000 Da species (dotted line), 50,000 Da species (dashed line) and the 80,000 Da complex (dash-dotted line). Assuming extinction coefficients of 1 OD/(mg/ml) for both proteins, the amount of complex simulated here would correspond to an equilibrium constant of K_D = 0.22 μM.

For example, if a protein A is in a monomer-dimer self-association equilibrium (with constant K_{12}) and also in reversible equilibrium with a protein B (with constant K_{AB}), a case which frequently occurs in membrane receptor – ligand interactions, there are several different possible modes of binding. If the dimer of A is the functional unit for binding of a single B, the SE profile can be described as

$$\begin{aligned} c_{tot}(r) = {} & c_A(r_0) \exp\left[M_A^* \Phi(r) \right] + K_{12} c_A(r_0)^2 \exp\left[2M_A^* \Phi(r) \right] + c_B(r_0) \exp\left[M_B^* \Phi(r) \right] \\ & + K_{AB} K_{12} c_A(r_0)^2 c_B(r_0) \exp\left[(2M_A^* + M_B^*) \Phi(r) \right] \end{aligned} \qquad (9)$$

now using the abbreviation $\Phi(r) = \omega^2 (r^2 - r_0^2) / 2RT$. If the protein B binds to all protomers of A independent of the oligomeric state, the radial distribution follows:

$$\begin{aligned} c_{tot}(r) = {} & c_A(r_0) \exp\left[M_A^* \Phi(r) \right] + K_{12} c_A(r_0)^2 \exp\left[2M_A^* \Phi(r) \right] + c_B(r_0) \exp\left[M_B^* \Phi(r) \right] \\ & + K_{AB} c_A(r_0) c_B(r_0) \exp\left[(M_A^* + M_B^*) \Phi(r) \right] \\ & + 2K_{AB} K_{12} c_A(r_0)^2 c_B(r_0) \exp\left[(2M_A^* + M_B^*) \Phi(r) \right] \\ & + 0.5 K_{AB}^2 K_{12} c_A(r_0)^2 c_B(r_0)^2 \exp\left[(2M_A^* + 2M_B^*) \Phi(r) \right] \end{aligned} \qquad (10)$$

It should be noted that this expression contains only 2 binding constants – the dimerization constant K_{12} , which can be determined in independent experiments with species A alone, and the microscopic constant for the hetero-association, K_{AB}. The apparent complexity of the sedimentation profiles arises from the multiple possible modes of interactions, with stoichiometries 1:1, 2:1, and 2:2 and their statistical factors. Analogous variants describe interactions with cooperativity, or binding at distinguishable independent binding sites. These and other models for binary and ternary interactions are implemented in the software SEDPHAT, for use in conjunction with the fitting strategies outlined below. In favorable cases, these different models can be discriminated, and thereby the mechanism of binding can be derived.

In principle, similar equations hold for multi-component systems, with Boltzmann exponentials describing all complexes and all free components, and with the molar mass of all species determining their respective exponents. Consequently, the complexity of systems that can be studied by SE is limited only by the ability to unravel the resulting exponential distributions in the data analysis. Several sophisticated data modeling strategies aiding in this process will be discussed below. Also, the combination of SE with other techniques, such as surface plasmon resonance biosensors, static and dynamic light scattering, fluorescence methods, or isothermal titration microcalorimetry, which may allow independent determination of binding constants or stoichiometry, can be highly useful.

Deviations from the exponential expressions above may arise in certain circumstances: If a significant change of the partial-specific volume of the protein does occur upon complex formation, a pressure dependence of the binding constant will result (invalidating Equation 6). Consequently, the observed binding constant will change with rotor speed and length of the solution column. However, at the relatively low speeds commonly used in SE experiments of proteins, pressure effects are usually not significant (since typically the maximum pressures are below 1 MPa), and therefore will not be treated in this Unit. (Pressure effects may be occasionally relevant, however, in relatively high-speed and very long-column (over 1 cm) sedimentation velocity experiments and may become apparent in comparison with SE results; for a more detailed reference on pressure-dependent interactions in the ultracentrifuge, see (Harrington and Kegeles 1973)). Pressure effects mediated through the compressibility of the solvent (even H_2O) can influence the SE profiles at the high rotor speeds and long solution columns required for the study of small peptides (~ kDa).

Another potential complication of the treatment outlined above is thermodynamic non-ideality of one or both binding partners. This can occur, e.g., if proteins are highly charged and studied in low ionic strength buffers, or at high concentrations, such that electrostatic or steric repulsion becomes a significant force opposing the sedimentation. It can be detected by a distinct deviation from the exponential sedimentation profile in Equation 1, exhibiting a smaller slope at higher concentrations. Methods for the analysis of nonideal behavior by introduction of virial coefficients into Eq. 1

$$c(r) = c(r_0)\exp\left(-2BM[c(r)-c(m)] + M^*\omega^2(r^2 - r_0^2)/2RT\right) \qquad (11)$$

(with B denoting the second virial coefficient, and m denoting the meniscus position of the solution column) (Haschemeyer and Bowers 1970; Holladay and Sohpianolpoulos 1972) and by

composition dependent activity coefficients have been described (Yphantis 1964; Wills et al. 1980; Chatelier and Minton 1987; Wills et al. 1993; Ralston 1994). The analysis of interactions under non-ideal conditions is significantly more difficult, because of partial masking of the repulsive non-ideality effect by attractive interactions. For the study of weak interactions, it is prudent to monitor the highest protein concentration generated (or considered in the analysis), and for heterogeneous interactions to examine the individual protein components in parallel at similarly high concentrations. However, for moderately charged proteins, if the protein concentration is below 1 mg/ml and the buffer ionic strength in the 10 mM range or higher, nonideal sedimentation is usually not observed and will be assumed absent for the remainder of this Unit.

It can be of interest to study protein interactions in the presence of high concentrations of unrelated macromolecules as a model for molecular crowding, where the repulsive non-ideality of the solution can significantly alter the thermodynamics of binding. This requires a post-centrifugal fractionation followed by SE analysis, and we refer to the method described by Rivas et al, 1999, and applied by Zorilla et al 2004.

The goal of an equilibrium ultracentrifugation experiment is the measurement of the sedimentation profiles of the different species present in solution after mechanical and chemical equilibrium is attained. As indicated above, preliminary experiments using the SV analytical ultracentrifugation technique can be crucial to aid in building the correct model for the interaction. From the SE data one can determine the association constants from the measured amplitudes (termed $c(r_0)$ in above equations) of the sedimentation profile of each species. After a brief outline of ultracentrifugal detection systems, which make possible the measurement of the concentration profiles of the proteins, the analysis of SE data of reversible interactions will be described.

b) Detection Systems

The optical system is the key experimental device that distinguishes analytical from preparative ultracentrifugation. It allows measuring the concentration gradients while the rotor is spinning, in order to subject the data to quantitative first-principle analysis. The measurement is based on the optical comparison of a protein sample with a buffer reference, which are both arranged in sectors side-by-side in a centerpiece of an ultracentrifugal cell, and enclosed with an assembly of optically transparent windows. The most commonly used detection systems are absorbance optics (Hanlon et al. 1962; Schachman et al. 1962) and interferometrical Rayleigh optics (Richards and Schachman 1959; Yphantis et al. 1994) which are present on the Beckman Optima XL-A (absorption only) and the XL-I (both optical systems) analytical ultracentrifuges. They have very different properties, and the optimal choice and combination of optical signals can be crucial for the analysis of complex interacting systems. The selection of the optical detection system has implications for the choice of buffer and experimental design. Detailed steps for planning can be found in the supporting online protocol (Balbo et al. 2007).

Conceptually, the absorbance optics is equivalent to a dual-beam spectrophotometer that is integrated into the ultracentrifuge. The light source is triggered by the rotor revolution and a slit assembly that can be moved to different radial positions, such that each ultracentrifuge cell containing a sample solution can be scanned in the radial direction at a wavelength between 190 nm and 800 nm. Detection of proteins is achieved on the basis of their extinction, e.g., that

caused by aromatic amino acids at 280 nm, the protein backbone extinction in the far UV (usually at 230 nm, where the xenon flashlamp has an emission peak and many buffers are still transparent), or by exploiting an intrinsic or extrinsic chromophore in the VIS. Importantly, the detection is selective; it allows different proteins to be discriminated that have different absorbance spectra, e.g., as a result of chromophoric labeling, while allowing some buffer components (such as salts and detergent micelles) to remain invisible.

The absorbance optical system has a linear range from 0 to ~ 1.5 OD (or less if a wavelength is chosen on the steep parts of the protein extinction outside the minima and maxima), which can limit the highest concentrations to be studied. In order to alleviate this, detection at the protein extinction minimum at ~250 nm can be very useful to extend the concentration range, and to permit some discrimination of protein components that differ in the composition of aromatic amino acids. Since data at multiple wavelengths can be acquired 'simultaneously', a combination of wavelengths of 230 nm, 250 nm and 280 nm can be conveniently integrated into a global multi-signal analysis for detection of the largest concentration range (see below, and the online protocol (Balbo et al. 2007)). A further strategy to increase the concentration range of the experiments is the use of centerpieces with 3 mm optical pathlengths in combination with the commonly used 12 mm pathlength centerpieces.

On the other hand, the interference optical system is a spatially resolved differential refractometer. The recorded quantity is the displacement of interference fringes generated at the superposition of two laser beams projected into a camera, arising from differences in the refractive index of the protein solution and the buffer reference due to the macromolecular concentration gradient. This optical system has the virtues of very high precision and sensitivity, by using a virtually unlimited dynamic range, and very high radial resolution. The latter enable the investigator to work with higher rotor speeds that produce steeper concentration gradients, and allows the use of substantially shorter solution columns (Correia and Yphantis 1992). While this does not have the discrimination potential to record different signals for species with different spectral properties, it has the advantage that the refractive index increment of proteins is largely independent of amino acid composition (although it will change with glycosylation or bound detergent). In this way, one can estimate absolute protein concentrations. Commercial instruments are configured so that a protein concentration of 1 mg/ml in 12 mm centerpieces will generate a signal of 3.3 fringes. This corresponds to a molar signal increment analogous to the extinction coefficients of $2.75 \times M_w$ (fringes/M×cm); in comparison, the typical instrumental noise is ~0.005 fringes.

A complication, however, is a radially-dependent signal offset, which can be of a magnitude similar to that of the signal from the protein concentration gradient. It is a consequence of the exquisite sensitivity of this detection system for changes in the optical pathlength in the nm range, and of unavoidable imperfections on this scale in the optical configurations. However, as long as the ultracentrifuge cell housings are not disassembled, and more mechanically stable components are used (such as sapphire windows), the signal offset pattern remains virtually unchanged (Ansevin et al. 1970). Therefore, after a mechanical stabilization procedure (see the protocol (Balbo et al. 2007)), the signal acquired from a water blank can be subtracted from the signal obtained in a SE experiment (both obtained under the same conditions), thereby canceling the systematic signal offsets. Special ultracentrifuge cell housings have been designed for this

purpose (Ansevin et al. 1970). Alternative to the water blank subtraction, computational methods have been established that take advantage of the data acquired at different rotor speeds and data synchronously acquired with the absorbance optical system in order to identify the signal contribution of the baseline imperfections (Vistica et al. 2004).

A second important difference from the absorbance system is that refractometric optics gives a protein signal that is superimposed on gradients of all other solution components that have a refractive index different from water, including gradients from salt redistribution in the centrifugal field. This leads to more stringent requirements for the preparation of the protein sample and the reference solution, in that the buffer and sample must be in dialysis equilibrium. Further, it requires that both solution columns have exactly the same volume. (In contrast, when the absorbance optics is the solely used acquisition system, the volume of the reference solution is conventionally chosen slightly larger for the easier identification of the menisci – a convenience that must be abandoned when data are collected simultaneously with the IF system.).

Other detection systems for SE have been designed. A fluorescence optical system was developed by Riesner and colleagues (Schmidt and Riesner 1992), and more recently by Laue and colleagues (Laue et al. 1997; MacGregor et al. 2004). The latter is commercially available, and equipped for the detection of GFP and FITC. However, due to inherent limitations in the linearity of the signal, analysis approaches for protein interactions different from those presented here will have to be established (MacGregor et al. 2004). Such techniques, and experience from the practical application of the commercial fluorescence detector, have not yet been reported in the literature.

Another important alternative design is postcentrifugal detection with a microfractionator, which is commercially available and can be used in conjunction with a preparative ultracentrifuge (Darawshe et al. 1993; Darawshe and Minton 1994; Liu et al. 1997). Here, sample tubes are placed in a swinging-bucket rotor, and after attainment of SE their contents are carefully fractionated into small aliquots. These aliquots can be separately subjected to a variety of analytical methods, such as gel electrophoresis, scintillation counting, or enzymatic assays. If radio-labeled proteins are used, post-centrifugal microfractionation can allow the study of extremely tight protein interactions by SE. The drawback of this method compared to conventional analytical SE is that the radial resolution is much lower and only permits the calculation of weight-average molar masses, so that the interaction has to be studied by titration of the reactants in a series of experiments.

3. Strategies for the Analysis of Reversible Interactions

In order to assess the potential and the limitations of long-column SE for the study of any particular protein-protein interaction, consideration of three simple aspects is helpful. (1) As in many other techniques for the study of reversible interactions in which concentrations are measured, and the binding constant is derived from the mass action law, it is essential that the concentrations of free reactants and the complex can be measured independently. Otherwise, if the binding is too tight for free reactants to be detectable, only the stoichiometry of the complex may be determined. This requirement can be alleviated by the consideration of the total amount

of each protein component inserted into the experiment ('mass conservation constraints' (Roark 1976; Pörschke and Labuda 1982; Lewis and Youle 1986; Philo 2000; Arkin and Lear 2001; Vistica et al. 2004). (2) Since each species distributes throughout the entire solution column, exponentially increasing in concentration with increasing distance from the center of rotation, the species must exhibit significant differences in the steepness of their sedimentation profile in order to be distinguished. However, it is sometimes possible to study heterogeneous interactions by using absorbance optics and a multi-wavelength approach (Servillo et al. 1981; Lewis et al. 1993; Schuck 1994; Dölle and Schubert 1997), or a combination of absorbance and interference optics in a multi-signal approach (Vistica et al. 2004), for the detection of the various species on the basis of their extinction spectrum. Mass conservation approaches can address this problem, as well. (3) A consequence of the simple exponential contribution for each species in solution is that from a single experiment, stable (irreversible) complexes cannot be distinguished from complexes in a reversible association/dissociation equilibrium. This makes it essential to conduct several experiments side-by-side comprising different loading concentrations and/or to obtain sedimentation equilibrium data sequentially at different rotor speeds, such that the redistribution of species in chemical equilibria can be monitored.

From the first condition, it directly follows that the lowest measurable equilibrium dissociation constant can be estimated as the concentration at which any of the reactants has a signal in the same order of magnitude as the noise. For the absorbance optical system this is, $K_{D,min} \sim 1/(100\times\varepsilon_{M,\lambda})$, where $\varepsilon_{M,\lambda}$ is the molar extinction coefficient at the wavelength used for detection. This can be in the nM range for large proteins, when exploiting the protein backbone extinction in the far UV (e.g. 230 nm), or in the μM range for peptides with molar masses of only a few thousand and correspondingly lower molar extinction coefficient. On the other hand, the upper limit of $K_{D,max}$ for a detectable interaction, can be an order of magnitude greater than the maximal concentration where the protein exhibits ideal sedimentation behavior, or higher if special techniques are applied. In practice, depending on the molar mass, this can be in the mM range.

Unless advanced data analysis strategies are applied, the second condition results in constraints on the relative sizes of the proteins whose heterogeneous interactions can be studied by long-column SE. If the molar masses of the free protein reactants are very similar, e.g., if they differ by less than 20%, their resulting SE profiles can be too similar to allow reliable quantitation of their relative amounts from noisy experimental data. On the other hand, if the molar masses of the reactants are too far apart, e.g., if they differ by more than a factor of 5, the reactants can be well discriminated, but the larger species cannot be easily distinguished from the complex. There are several experimental strategies that can be applied to minimize these restrictions and optimize the information content of a SE experiment.

As indicated above, the global analysis of data acquired at different wavelengths or with different optical systems can overcome this problem, if the interacting components have differences in their extinction spectra. Instead of modeling the data with Eq. 7, a global data analysis of all different signals at once is achieved, taking into account each species contributions to the respective signal. For a simple reaction of two proteins *A* and *B* forming a reversible 1:1 complex, the signal a_λ observed by the absorbance optical system, at a wavelength λ is

$$a_\lambda(r) = c_A(r_0)\varepsilon_{A,\lambda} d \exp\left[M_A^*\Phi(r)\right] + c_B(r_0)\varepsilon_{B,\lambda} d \exp\left[M_B^*\Phi(r)\right] + K_{AB}c_A(r_0)c_B(r_0)(\varepsilon_{A,\lambda}+\varepsilon_{B,\lambda})d \exp\left[(M_A^*+M_B^*)\Phi(r)\right] + b_\lambda \quad (12)$$

where $\varepsilon_{A,\lambda}$ and $\varepsilon_{B,\lambda}$ denote the molar extinction coefficients of the proteins *A* and *B*, respectively, b_λ denotes a small unknown baseline offset that is usually superimposed on to the protein signal, and d denotes the optical pathlength (1.2 cm with the usual ultracentrifuge cell centerpieces). For interactions where complexes of higher-order stoichiometry occur analogous extensions of Eq. 7 and 8 can be made. The extinction coefficients should be determined from the analysis of the individual protein components in control experiments conducted in parallel in other rotor positions of the same ultracentrifugal run. (These experiments can also be used to determine M_A* and M_B*, see below.)

A very powerful analysis strategy is the principle of mass conservation. We have knowledge about the total amounts of each component inserted into the experiments. Although the absolute knowledge of concentration is typically not precise enough to be used directly as a constraint, the components' concentrations in the different rotor positions can be designed as a titration or dilution series such that precise relationships between the total amounts of material across the different experimental data sets are generated (Vistica et al. 2004). Further, when after a first sedimentation equilibrium a second and third sedimentation equilibrium at higher rotor speeds is attained, the redistribution of material that occurs in each sample will leave the total amount invariant (Roark 1976) (barring precipitation or other degradation processes, see below).

This idea can be conveniently expressed by calculating from the exponential concentration gradients, for each species, the equivalent 'average' (i.e. uniformly distributed) loading concentration that corresponds to the same total amount:

$$\overline{c} = c(r_0)\frac{\exp\left(M^*\Phi(b)\right) - \exp\left(M^*\Phi(m)\right)}{M^*\left(\Phi(b)-\Phi(m)\right)} \quad (13)$$

(This expression is based on a standard sector-shaped ultracentrifugal cell; other geometries are implemented in the SEDPHAT software.) m and b are the meniscus and bottom of the solution column. It can be observed that the calculation of the total mass depends critically on the assignment of the bottom of the solution column. Unfortunately, this parameter is not easily experimentally accessible, since obscured from optical detection, and common ad hoc assumptions will easily lead to apparent discrepancies in the mass balance (Vistica et al. 2004). However, if data at several rotor speeds are collected, this parameter can, for each centrifugal cell, be treated as a fitting parameter and derived from the experimental data (Vistica et al. 2004). Corrections accounting for the small displacement of the bottom of the cell from elastic stretching of the rotor at different angular velocities can be made (Vistica et al. 2004). In this way, the mass conservation constraint can be implemented, accounting for the contribution of each protein component to each free and complex species in each gradient.

Whether or not mass conservation holds depends on the absence of protein degradation and surface adsorption during the run. In the configuration with multiple rotor speeds, the absolute

amount does not have to be constrained and initial surface adsorption or errors in the estimated loading concentrations it allowed for. However, it is assumed that the mass of soluble material redistributing between different rotor speeds is constant ('soft mass conservation') (Vistica et al. 2004). With these assumptions, we found mass conservation to be fulfilled in the overwhelming majority of proteins studied in our laboratory, and many examples from different laboratories can be found in the published literature. Control experiments where each of the interacting components is studied separately can highlight degradation and loss of material during the run. For the mixtures, mass conservation constraints then allow the total protein molar ratio (in dilution series), or the total mass of one of the components (in titration series) to be held constant in the analysis. These are very powerful constraints generated through judicious experimental design, which depend on the ability to use mass conservation calculations (Vistica et al. 2004).

In summary, it is desirable to apply these strategies simultaneously in order to optimize the discrimination of different association models and to optimize the precision of the binding constants. For typical SE experiment, one runs side-by-side in different rotor positions the control samples for each component, as well as several mixtures, arranged either in dilution or titration series spanning a large range of concentrations. Sedimentation equilibria are then attained at several rotor speeds, and at each rotor speed, absorbance data are acquired at multiple wavelengths (such as 230 nm, 250 nm, and 280 nm or taking advantage of intrinsic or extrinsic chromophores), frequently in conjunction with interference optical detection.

In the following, we discuss in more detail the sample requirements, experimental parameters, as well as the data analysis.

4. Experimental Considerations

a) Size, Partial-Specific Volume, and Prior Characterization of the Proteins

Studies of protein-interactions can be performed for small peptides of molar mass in the range of one thousand, in a manner similar to that for very large proteins, or stable protein complexes, with molar masses of several hundred thousand or more, simply by adjustment of the rotor speed. It is a consequence of Equation 1, that proteins of molar masses 100,000 and 1,000 will produce the same equilibrium profiles if the smaller species are studied at a 10 fold higher rotor speed. Although SE was historically used to measure molar masses, it is very useful if prior knowledge of these values can be used in the characterization. The molar mass may be calculated from the usually known amino acid composition or may be found by mass spectrometry.

Knowledge of the amino acid composition is also useful because it allows the estimation of the partial specific volumes. Because SE is governed by the buoyant molar mass $M(1-\bar{v}\rho)$, and since typical values for $\bar{v}$ for proteins are in the range 0.70 – 0.75 ml/g, relative errors in $\bar{v}$ will be amplified by a factor of from 3 to 4. Therefore, the buoyancy factor $\bar{v}\rho$ has to be evaluated with some care. Compositional partial specific volumes are based on tabulated values of the partial specific volumes of amino acids (Durchschlag 1986), and can be calculated, e.g., using the free-ware software SEDNTERP (Laue et al. 1992). Because these values change slightly with solution conditions, the error (for most proteins without glycosylation and prosthetic groups) of these estimated $\bar{v}$ values can be as large as +/-0.01- 0.02 mg/ml. Therefore, experimental

buoyant molar mass values may be acceptable if they differ by less than *ca.* 5 – 10% from the predicted $M(1-\bar{v}\rho)$ from the amino acid composition.

Significantly larger deviations from the compositional $\bar{v}$ values can be expected under solvent conditions where preferential hydration occurs (and/or where the solvent density is significantly different from that of water), since then the solvation shell of the protein ceases to be neutrally buoyant and has to be considered. Preferential hydration is relevant, for example, in solvents typically used to stabilize proteins such as glycerol and sucrose solutions. However, in the absence of self-association, when the protein molar mass is known, the experimentally measured buoyant molar mass can be used to determine an experimental value for the partial-specific volume (for the particular buffer conditions), on which the further study of heterogeneous interactions may be based.

If the proteins contain prosthetic groups, or other components beyond the amino acids, the partial specific volume can be estimated using 'Traube's rule' described in (Hoiland 1986). Glycosylated proteins can pose an additional difficulty because most carbohydrates have a significantly lower partial specific volume than proteins, and the carbohydrate composition of glycoproteins is dependent on the expression system, and frequently both unknown and heterogeneous. Methods for the estimation of average partial specific volumes have been described by (Shire 1992; Junghans et al. 1996; Fairman et al. 1999; Lewis and Junghans 2000). For glycosylated proteins, the availability of molar mass data from mass spectrometry is extremely helpful, because together with the molar mass of the protein component as calculated from amino acid composition, the molar mass of the carbohydrate component can be estimated (Fairman et al. 1999; Center et al. 2000). A technique in which two SE experiments are performed *simultaneously*, one in D_2O and one in H_2O, (Edelstein and Schachman 1967) can be useful in the study of glycosylated proteins. H-D exchange can be estimated as about 5 replaceable hydrogens per glucose moiety (this number is not very critical for moderate degrees of glycosylation). Given the molar mass and $\bar{v}$ of the protein component, one can then determine the degree of glycosylation and $\bar{v}$ of the sugar moiety for moderately glycosylated proteins. This method only requires micrograms of material as compared to densitometric techniques. However, if the protein is only slightly glycosylated, by assuming a partial specific volume of the carbohydrate moiety to be between 0.62-0.64 ml/mg, one may obtain acceptable results with only one SE experiment in aqueous buffer (Shire 1994).

The question of buoyant molar mass is slightly more complex for detergent solubilized membrane proteins, because of the additional mass and volume of the detergent micelle, and because integral membrane proteins frequently exhibit self-association. Experimental techniques for this case have been developed, and include experiments at different solvent densities (e.g. mixtures of H_2O and D_2O) and with detergents that have different densities, or detergent/solvent combinations for which the detergent micelle is neutrally buoyant (Tanford and Reynolds 1976; Reynolds and McCaslin 1985; Schubert and Schuck 1991; Howlett 1992; Schubert et al. 1994). Density matching with H_2O and D_2O can also applied to proteins reconstituted in lipid vesicles to make the lipid neutrally buoyant, however, this requires small lipid vesicles of relatively uniform size, and high precision in the adjustment of the buffer density (Lindenthal and Schubert 1991). For some detergents (or for other practical reasons) density matching may have to be achieved with other densifying co-solvents, but care should be taken to consider the degree of

preferential hydration on the protein $\bar{v}$ and to determine whether the uncertainty introduced is acceptable in view of the conclusions drawn from the experiment (Mayer et al. 1999; Lustig et al. 2000; Center et al. 2001; Lebowitz et al. 2003) (e.g., a 10 % error will be tolerable for the discrimination of protein monomers and dimers).

As indicated above, if the molar masses of two interacting protein species are similar, it may be helpful to chromophorically label one of the proteins. This can also be very useful or even essential for the study of small peptides binding to much larger proteins. In this case, labeling of the smaller component usually gives better ultracentrifugal data. Labeling can be done using standard techniques, e.g., attaching any of the well-known fluorescent dyes (such as FITC), and requires the usual controls for the functional activity of the labeled protein. An alternative procedure is the expression of proteins in the presence of 5-hydroxytryptophan, which extends the Trp extinction distinctly into the longer wavelength range with a shoulder extending to about 300 nm (Laue et al. 1993).

Besides the obligate knowledge of molar mass (from mass spectrometry if contributions other than amino acids are present, or from amino acid composition), the partial-specific volume, and one extinction coefficient at one of the wavelengths used (both can be calculated from the amino acid composition), it is prudent to be familiar with the expression system and purification protocol, and the details of the method for the final buffer exchange (see below). As a common step preceding any biophysical analysis, it is recommended to examine the absorption spectrum of each protein in the final buffer, in order to catch unexpected solution components (e.g. from the ratio of OD_{260}/OD_{280}) or the presence of aggregates (e.g. from the baseline at OD_{320}). Inspection of elution profiles from size-exclusion chromatography or the spreading of the sedimentation boundaries in sedimentation velocity is very helpful in assessing the homogeneity of the individual proteins. In particular, SV analytical ultracentrifugation additionally provides an orthogonal approach to verify the association model. In some cases, this may reveal previously undetected self-association of the proteins.

b) Purity

The study of protein interactions by SE requires proteins at a purity of greater than 95%. It is important to note that the assessment of purity by gel electrophoresis is generally insufficient, because this does not allow detection of proteins that may be poorly stained and of small peptides or large aggregates that are outside the size-range of the gel. In particular, the presence of contaminating small peptides, which may not be relevant for the study of proteins by other techniques and may therefore go undetected, can be highly detrimental for the analysis by SE. A strongly recommended precaution is the application of size-exclusion chromatography as the last preparative step or as an additional step prior to the centrifuge experiment, which usually results in samples of sufficient quality. In many laboratories, SV analytical ultracentrifugation is routinely applied prior to the SE experiment, in order to assess the purity of the material and to obtain additional information on the protein interactions, such as the number and approximate size of species involved (for details, see the Unit [SV Unit in this Series]).

The contamination of the sample by a population of misfolded proteins, which may be incompetent to participate in the reversible interaction of interest, can lead to problems in the SE data analysis and errors in the estimated binding constants. If recognized, the SE analyses can be

performed while taking into account the existence of populations of incompetent protein fractions (either incompetent free monomers, or incompetent (covalent) complexes). In some cases, these incompetent conformations may be detected by chromatography, or sedimentation velocity.

The protein samples also should not contain covalent or non-covalent (artificial) aggregates of the protein of interest. Usually, these can be detected very efficiently by light scattering and sedimentation velocity (Schönfeld et al. 1998; Berkowitz 2006). A small population of very large aggregates may be tolerable, if they are stable, and if the size of the aggregates is distinctly above the largest species of interest, such that they sediment to the bottom of the ultracentrifuge cell and pellet. If the absorbance optical system is used in conjunction with a specifically labeled protein (e.g. detection in the VIS using FITC), inert contaminating species that do not absorb are tolerable, as long as they do not produce a high refractive index gradient resulting in optical artifacts such as Wiener skewing (Yphantis 1964).

c) Concentrations, Buffers, and Volumes

The choice of loading concentrations, buffer conditions, and optical detection systems are intimately related. Since the analysis is based on the mass action law, ideally, a range of concentrations covering the entire binding isotherm should be studied, i.e. for a simple bimolecular reaction this should be at least from 1/10 to 10 fold that of the expected K_D. If this cannot be accomplished because of limiting sample concentrations or amounts, equimolar loading concentrations at approximately K_D are the best compromise.

The sensitivity of the detection system may also be a limiting factor, since at signal/noise ratios below 10:1 (maximum signal in the gradient) not much information can be gained from the equilibrium profiles. With typical proteins and when using the interference optics or the absorbance data at a wavelength of 230 nm, this corresponds to a minimal loading concentration of 20-30μg/ml while with absorbance at 280 nm, this minimal concentration would be approximately 100 μg/ml. The possibility of using the protein backbone extinction at 230 nm for the low concentration experiments, however, depends strongly on the buffer used (see below).

Regarding the maximal concentration, it should be noted that the centrifugal force will generate regions of the solution in the lower (higher radii) part of the centrifugal cell where the concentration is higher than the loading concentration. The extent of this effect depends on the rotor speed, and can be simulated prior to the experiment. If protein samples are available at only very low stock concentrations, this effect can be exploited in order to achieve better signal/noise ratios by increasing the solution column length, even though at the cost of a longer experimental time, see below. Therefore, it is not always necessary to span cover the entire range of the binding isotherm with the set of loading concentrations used in different ultracentrifugal cells. It should be noted that no data at optical densities greater than 1.5 will be useable (which imposes concentration limits dependent on the detection wavelength), and that non-ideality will likely be encountered at concentrations much greater than mg/ml. In the absence of other considerations, with typical choices of sample volumes and rotor speeds, and within the usual constraints of the detection system, frequently the sample loading concentrations

are between 0.05 – 0.75 OD_{280}.

The sample volumes needed are typically between 100 and 180 µl per equilibrium experiment (180 µl samples are standard in our laboratory). The larger sample volume is desirable due to the ability to record more curvature from the protein gradients which will significantly increase the information content of the data, resulting in improved discrimination of different models and higher precision of the binding constants. However, this comes at the expense of longer experimental time (see below). For a typical set of experiments for the characterization of a heterogeneous protein interaction of the form A + B ⇔ AB, which includes characterization of the individual proteins, approximately 300 µl of each protein at a concentration of 10fold of the expected K_D could be required. It should be noted that usually 80 to 90% of the sample can be recovered from the ultracentrifuge cells after the run.

As indicated above, it can be advantageous to study interacting systems with titration or dilution series such that additional mass conservation constraints are generated. In preparing these samples, it is critical that they be pipetted such as to ensure the highest possible consistency (for example, for the dilution series to make a stock mixture and use different dilutions), keeping in mind that the absolute volumes and absolute concentrations are typically not sufficiently well-known quantities to be used as constraints in the data analysis.

The sample should be brought into a buffer (by dialysis or gel filtration) that provides sufficient ionic strength for the protein to be thermodynamically ideal over the concentration range studied (the ionic strength of buffers used are typically above 10 mM). Both the interference and the absorbance optical systems require the reference sector be filled with solvent. Because interference optics is sensitive to the redistribution of salt during centrifugation, dialysis of the protein sample (applied sufficiently long to attain dialysis equilibrium), and the use of the dialysis buffer in the reference sector is mandatory. Also, in order to ensure the correct redistribution of salt in both the sample and the reference sector, it is imperative that both sectors be filled to the same volume (superseding the custom to use slightly higher volumes in the reference buffer permitting convenient identification of the menisci). Because the buoyancy factor of the protein requires the knowledge of the solvent density, measurement by densitometry may be required; however, for most standard buffer substances and salts, tabulated density increments are available for calculating the solution density.

If possible, no substance should be in the buffer that increases the viscosity of the solution, such as glycerol or sucrose, because even at low concentrations this can lead to an excessive time required to attain equilibrium. As mentioned above, high concentrations of co-solvents also can be expected to lead to preferential hydration (usually this is the basis for the stabilizing effect of co-solvents on the protein) changing the estimates of the partial-specific volume.

It is obvious that no buffer component should be used that interferes with data acquisition. With the absorbance optical system, substances such as β-mercaptoethanol, DTT, nucleotides, or protease inhibitors which absorb at 280 nm cannot be used in high concentrations. If they cannot be avoided, then their optical density should be below 0.1 – 0.2 OD_{280}. It should be noted that some substances, e.g. DTT and mercaptoethanol change their absorbance with oxidation state during the ultracentrifuge run. (For technical reasons, it is advisable to have little or no absorbance in the reference sector, in particular, none of the reducing agents because their

oxidation in the sample and reference sector can be different.) Many nonionic detergents are transparent at 280 nm, which allows the study of detergent solubilized membrane proteins with the absorbance optical system. Detection at 230 nm places more stringent requirements on the buffer. EDTA, EGTA, TRIS and HEPES all absorb in the far UV, while phosphate or borate buffer with sodium chloride is transparent at 230 nm. Absorbance spectra of the sample and the solvent should be taken with a dual-beam spectrophotometer before the sedimentation experiment to verify sufficient transparency of the buffer and absorbance of the sample.

Since the interference optical system is based on the detection of the refractive index of the components in the solution, small molecules that do not sediment but have strong absorbance, such as GTP, do not hinder the measurement of concentration profiles. However, as all other species in solution they do contribute to the refractive index signal. (This includes those species that are transparent in absorbance optics, such as salts).

a) Rotor Speed, Rotor Temperature, and Experimental Time

As described above, in order to choose the equilibrium rotor speed, one must consider the molar masses of all species as well as the length of the solution column. There is no sharp optimum of rotor speed (in the sense that a 10% different rotor speed will usually not dramatically increase or diminish the information content of the run), but remember that sedimentation depends on the square of the rotor speed. If only a single rotor speed is to be used, an approximate rotor speed can be estimated with the formula $1.4\times10^5/M^{1/2}$ for solution column heights of ca. 2.5 – 3 mm (Laue 1999), or $1.2\times10^5/M^{1/2}$ for solution columns of 4.5 – 5 mm (Balbo and Schuck 2005) (with the rotor speed in units of rpm, and an average molar mass (M) in kDa). It should be noted that the use of refractometric optics permits the use of higher rotor speeds because of higher radial resolution, less Wiener skewing, and greater linearity of the signal.

As mentioned above, it can frequently be highly advantageous to acquire data at multiple rotor speeds in order to enable mass conservation constraints to be established. In this case, it is most effective to combine information from those in 'low-speed' conditions (Richards et al. 1968) ($c(b) \sim 3 \times c(m)$) with those obtained from intermediate rotor speeds ($c(b) \sim 10 \times c(m)$) and those in traditionally termed 'high-speed' or 'meniscus depletion' conditions (Yphantis 1964) ($c(b) >> 10 \times$ loading concentration, and $c(m) \sim 0$). For 4.5 – 5 mm columns, this can be achieved with the formula $\omega_1 = 0.75\times10^5/M^{1/2}$, $\omega_2 = 1.2\times10^5/M^{1/2}$, $\omega_3 = 1.5\times10^5/M^{1/2}$. The lower speeds will produce data defining the larger oligomers while the highest speed should help determine the buoyant mass of the monomer and the baseline offsets. As a rule of thumb, the lowest rotor speed in a set of experiments should allow detection of the species with the highest molar mass at least over the bottom third of the solution column. In this sequence of equilibrium experiments at several rotor speeds, it is best to start the first experiment at the lowest speed. This will minimize the back-diffusion of aggregates that may form at the relatively high concentration in the vicinity of the bottom of the solution column.

When we are concerned with an interacting system, multiple species exist and multiple possible M values could be inserted in above formulas. As a rule of thumb, one can estimate the lowest rotor speed with the highest expected M-value, and the highest rotor speed with the lowest M-value. If the molar mass or the oligomeric state of the protein is unknown, it can be estimated from the time-course of depletion at the meniscus during the first hours of the experiment using

the modified Archibald method with the software SEDFIT (Schuck and Millar 1998) (provided the experiment was started with the rotor and samples in thermal equilibrium).

The mass of the monomer may be so low that one cannot attain speeds high enough to produce the necessary gradient. In this case, one may trade off column height for speed: By doubling the column height one can decrease the needed speed by a factor of the square root of two. However, this requires a fourfold increase in the time to reach equilibrium. Therefore, in general, despite the somewhat higher diffusion rate of small molecules, the time to reach equilibrium for these materials is long because of rotor speed limitations, which result in longer solution columns. An example for the study of a very low molar mass species (M_r = 300) can be found in (Braswell and Lary 1981).

Generally, it is a good practice to simulate the sedimentation equilibrium profiles for the species expected to be present in the solution. This can be done with the software SEDFIT and/or SEDPHAT. Simulation of the profiles is useful, in particular, for column lengths different from those on which above formulas are based. In addition to the shape of the equilibrium profiles, the simulation permits one to estimate the minimal time required to attain equilibrium (see below), and to perform a mock data analysis in order to test whether the planned experiment can be expected to reveal the desired information.

As mentioned above, in order to maximize the information content of the sedimentation data, it is advantageous to work with long solution columns of 100 – 180 µl. The drawback of this method is the relative long time required for attaining equilibrium, which for a 3 – 5 mm column height can be one or two days for the first equilibrium, and slightly less for the following equilibria at higher rotor speeds. To improve stability of the protein over this time period, the experiment can be conducted at a temperature of 4 °C. (It should be noted that the choice of the temperature will influence the observed binding constants, since $RT \ln K = \Delta G = \Delta H - T\Delta S$.) In principle, the Beckman Optima XLA/I allows the experiments to be conducted at any temperature between 0 and 40 °C. Because reaching the temperature set-point in the vacuum chamber is very slow, the rotor and the cell assembly may need to be pre-chilled when working with proteins that need to be kept continuously at low temperatures. Generally, we have observed that proteins in the centrifugal field frequently appear to exhibit higher stability than when kept at 1 g in the refrigerator. The origin of this is unknown, but one could speculate that it may be due to the rapid depletion of larger artifical oligomers of misfolded material, which may otherwise constitute nuclei for irreversible aggregation.

Generally, the time to equilibrium increases approximately with the square of the height of the solution column, and it increases with the molar mass of the species present (because of the smaller diffusion coefficient of larger species). Although formulas are available for predicting the experimental time (van Holde and Baldwin 1958; Yphantis 1964), these are not applicable in the presence of a chemical reaction (such as protein interaction), which in practice may be the rate-limiting step. It should be noted that many models for the equilibrium distribution of interacting systems can be modeled surprisingly well to sedimentation profiles that are close to, but not yet in equilibrium, and that these can give quite different results from those in true equilibrium.

A good experimental method for long-column experiments, therefore, is the acquisition of scans in time-intervals of 3 h or 6 h, for short and long columns, respectively. If the difference between the scans shows no systematic residuals, equilibrium has been attained. This method may not work well, however, if the absorbance data is taken somewhat off the peak of an extinction curve (e.g. at 230 nm for proteins) i.e. where the absorbance is changing rapidly. This may produce false gradients as a result of inaccuracies in the optical alignment, which can cause small wavelength changes across the cell. If it is necessary to use wavelengths that are on the slope of an extinction curve, a scan taken at low speed before significant sedimentation has occurred will reveal such false gradients.

By far the safest assessment for attainment of equilibrium is the inspection of the complete time-course of the profiles measured in 3 – 6 h intervals, e.g. using the software programs SEDFIT or MATCH. When a sequence of N scans is available $\vec{s}_1(t_1)$, $\vec{s}_2(t_2)$, … $\vec{s}_N(t_N)$ and the root-mean-square difference between the last scan and all previous scans is plotted as $\Delta(t_i) = |\vec{s}_i(t_i) - \vec{s}_N(t_N)|$, this function $\Delta(t_i)$ should asymptotically reach a constant value, which is the noise in the data acquisition. In SEDFIT, such a graph can be displayed in real-time for all cells and signals, automatically updating when new scans have been acquired by the XLA/I system software.

One way to shorten the experimental time for the first equilibrium is the application of a higher initial centrifugal field, which leads to an increased initial transport in the solution column (e.g., a rotor speed 3fold above the desired equilibrium rotor speed during the first few hours of the experiment). However, this procedure is not recommended for two reasons. First, a prolonged application of a high rotor speed may lead to the excessive accumulation of material at the bottom of the cell, which may take long to redistribute and can actually sharply increase the time required to reach equilibrium (Chatelier 1988), and in this way may make the over speeding strategy counterproductive. Second, the high concentration of the proteins at the base of the solution column may lead to irreversible or slowly dissociating large aggregates, which would lead to artifacts in the measured equilibrium profiles. If the initial overspeeding technique cannot be avoided, e.g. when studying proteins that lack sufficient stability for attaining SE conventionally, we recommend monitoring the overspeeding phase to prevent the accumulation of protein to concentrations at the base of the solution column to concentrations that are higher than the expected final equilibrium concentration at this position.

For SE absorption experiments, where only one rotor speed is used under conditions far from 'meniscus depletion', after equilibrium has been attained and SE data have been acquired, it can be useful to add a final overspeeding phase that completely depletes the meniscus region of the solution column, thus allowing the experimental determination of the baseline offset parameter (*b* in Equation 12). For scanning the depleted meniscus, the rotor speed should be dropped again to the rotor speed of the equilibrium experiment. Although the baseline parameter *b* should in theory vanish in an ideal experiment with dialyzed buffer in the optical reference sector, in practice, technical limitations of the absorbance measurement in the ultracentrifuge can lead to a signal offset typically in the range of ± 0.02 OD. While the baseline can be treated as a floating parameter in the data analysis, its experimental determination generally improves the analysis and helps the discrimination of different models. In the case of interference optics, one has no option, but that of allowing the offset to be calculated.

5. Data Analysis

The overriding difficulty in SE data analysis is the exponential nature of the species distribution, which requires solving the problem of exponential decomposition of noisy data. This problem is well-known from many other biophysical disciplines, and notoriously difficult because the error surface (the sum of squares residuals as a function of parameter values) to be minimized is usually highly complex and exhibits many local minima. The equations for SE are ill-conditioned and cannot provide reliable results for interacting systems if too many unknown parameters have to be determined from only one or few experimental data sets. However, even relatively complex systems can become tractable with proper experimental design when using the strategies outlined above. The following are useful principles to be applied to the data analysis:

(1) From the control experiments with each protein component separately in SE, determine the protein molar mass, or partial-specific volume, respectively. This fixes the exponents in the analysis of the interactions, and greatly simplifies the fitting problem. It is much more difficult (and requires superior data quality and concentration range) to fit for both M and the association constants, although this may not be avoidable in some cases of self-associations with insufficiently known mass or partial-specific volume of the monomer.

When studying heterogeneous interactions it is possible, to transform the molar mass and partial-specific volume values for both interaction partners to a uniform scale of apparent partial-specific volume $\bar{v}^{(app)}$, based on the formula $M^* = M^{(true)}(1-\bar{v}^{(true)}\rho) = M^{(app)}(1-\bar{v}^{(app)}\rho)$. Since the modeling of SE only depends on the buoyant molar mass M^*, this transformation leaves the model invariant. However, the choice of $\bar{v}^{(app)}$ to be the true $\bar{v}$ of one of the interacting proteins is frequently very convenient in the data analysis, since on the same $\bar{v}$ scale, the apparent molar mass values are simply additive in forming the complex molar mass values.

When multi-signal data acquisition is applied, one should also determine from these control experiments the relative extinction coefficients at the different wavelengths, or for interference detection, respectively (using one extinction coefficient as prior knowledge).

(2) Except for the most simple cases (e.g., non-interacting species), it is essential to assemble multiple scans into a global analysis project. Typically, this will comprise between 10 – 50 or more scans. Fitting parameters can be distinguished between: (a) the global parameters – those describing molecular parameters that will govern the theoretical distributions for all data sets (e.g., binding constants, extinction coefficients, etc.); and (b) the local parameters – those which are only applicable to a particular data set (e.g., baseline offsets, bottom positions of the solution column). (c) Generally, it is important to reduce the number of fitting parameters as much as possible, and therefore to take advantage of the possibility of introducing constraints (termed 'links' in SEDPHAT) between the local parameters: For example, scans taken from the same centrifugal cell will have the same meniscus and bottom (accounting for the usual magnitude of rotor stretching as a function of rotor speed), as well as the same concentrations, because they originate from physically the same solution column. Further, scans from the same cell taken at different rotor speeds usually share the same baseline offset (although this does not always apply

due to technical reasons or due to the properties of the buffer, see above). Similarly, scans from the same cell but acquired with different detection system/wavelength can be fitted with the same values for the solute concentrations.

(3) As indicated above, it is always useful to include scans from different detection systems and/or absorbance wavelengths. Even for the study of a single protein component, this allows the improvement of the signal/noise ratio at lower concentrations, to alleviate limitations in the linear range of the optical system and to permit data from a larger concentration range. For heterogeneous associations, this can significantly improve the discrimination between protein species that have different ratios of aromatic amino acids.

(4) Mass conservation greatly reduces the number of free parameters to be fitted, by eliminating the reference concentrations $c(r_0)$ from the model and substituting them with equivalent loading concentrations, which can be extensively shared among different data sets from the same cell, and – with the proper experimental design – also shared among data from different cells. By reducing the number of free concentration parameters, the error surface will considerably simplify and the precision of the derived parameters increases. Further, the effective loading concentrations are parameters that can be easily initialized for the fit, as opposed to the reference concentrations $c(r_0)$. Even if mass conservation is not strictly fulfilled (as assessed from the control experiments), it is advantageous to achieve a preliminary fit with mass conservation imposed as a strategy to obtain excellent starting values of all other parameters for the final fit without mass conservation. When using mass conservation, it is important to float the value of the bottom position of the solution column, otherwise the bottom position will be irrelevant for the model.

(5) It may be necessary to introduce additional parameters into the model in order to account for experimental imperfections. These may reside in the protein preparation itself, when, for example, a fraction of protein is misfolded and unable to participate in the reaction. In this case, a constant proportion of the loading concentration would sediment as a free monomer, and a global parameter termed in SEDPHAT 'the incompetent fraction' for each species may be floated. Imperfections may also arise in the protein purification, which may necessitate inclusion of a term for a protein that is unrelated to the interaction to be studied ('non-participating species' in SEDPHAT). This should be applied very carefully, and only if the molar mass of the contaminating macromolecule is well outside the range of masses of all species of interest. This may be established by SV ultracentrifugation. Finally, technical imperfections may arise from the ultracentrifugal detection causing baseline shifts, either shifting as a function of rotor speed or as a function of radius ('RI noise' and 'TI noise' in SEDPHAT, respectively). Although these terms introduce additional parameters, if they have little cross-correlation with the parameters of interest (a condition that may tested by inspection of the covariance matrix) and if a significantly better fit is achieved in their presence, these additional terms may well lead to an increase in the reliability and precision of the parameters of interest.

The analysis of SE data in SEDPHAT consists of distinct steps.

(1) After the analysis of the control experiment and the determination of the ancillary parameters,
(2) all experimental scans are loaded into the analysis software and the relationships

between the local parameters are established by setting up all appropriate links.

(3) Next, a model for the interaction is chosen. It is preferable that it be based on prior knowledge of the biological system under study and supported by data from SV ultracentrifugation.

(4) Next, the parameters of the model have to be initialized. For complex models, this may not be trivial, and may involve some trial-and-error. In SEDPHAT, by exploiting the optimization technique of separating linear and non-linear variables, only non-linear fitting parameter values need to be initialized. They can be assessed using the 'Run' function, which should result in theoretical distributions in the overall vicinity of the experimental scans. Sometimes, a good strategy to arrive at starting guesses for the global fit is to fix some parameters that seem obvious to initialize, while optimizing others in a preliminary global or local fit.

(5) After parameters have been initialized for the global fit, the configuration of loaded data, the links established between the local parameters, and the model with the initialized global parameters should be saved for later retrieval.

(6) The fit of the data floating all unknown parameters follows. This is a key step in the data analysis, and one where the complicated nature of the error surface to be minimized can become most obvious. Frequently, the fit can be 'caught' in local minima, and therefore alternating optimization algorithms should be used, including the simplex approach, Marquardt-Levenberg approach, and simulated annealing.

(7) After convergence of the fit, the inspection of the quality of fit should show stringently that both the rms error of the fit is similar to the instrument noise and that the residuals of the fits are evenly distributed, i.e. that the model describes the data well. If this is not the case, a refinement of the model is necessary.

(8) The statistical precision of the parameters of interest should be established. The Monte-Carlo method is recommended only in relatively simple models, because it relies on the ability to automatically optimize the error surface at each iteration – a requirement that is typically not fulfilled for global SE models of protein interactions. A rigorous approach is the projection method in conjunction with F-statistics (Bevington and Robinson 1992; Press et al. 1992; Johnson and Straume 1994; Schuck 1994). In this method, one parameter of interest is kept fixed at non-optimal values in the vicinity of the best-fit value, while all other parameters are allowed to adjust to compensate for the constraint. If the resulting decrease of the quality of the fit forced by the constraint, measured as the sum of the square deviations of the fit (or as χ^2, respectively), matches a critical value predicted by F-statistics, the constrained parameter value represents one limit of the confidence interval for this parameter. If the decrease in the quality of fit does not yet reach the preset critical value, the parameter of interest is fixed a value slightly further away from the best-fit value. While mapping the projections of the error surface in this way, different optimization strategies can be chosen and combined at each step, which makes it most reliable and can reveal sometimes that what was considered the best-fit was indeed sub-optimal. Analogous tests using the F-statistics can be used to discriminate between different models. It is important to note, that the confidence intervals from SE analyses are frequently asymmetric (Johnson 1983), or even one-sided. A more detailed analysis of the information content of the sedimentation data can be performed by plotting the sum of squares of the fit as a function of the constrained parameter value (Servillo et al. 1981; Schuck 1994; Schuck et al. 1999).

As mentioned in the introduction, all analysis methods described above are available in the public domain software SEDPHAT (Schuck 2003; Vistica et al. 2004; Houtman et al. 2007).

6. Summary

In this Unit, we have outlined the theory, practical requirements and the basic procedures of SE for the long-column technique. It should therefore be clear from the many considerations that may be necessary when SE experiments are conducted and analyzed, that no simple recipe can be given that adequately describes even a majority of all variations encountered in practice. However, systematic weighing and elimination of factors described above can provide a straightforward approach for the study of most systems, and lead to the recognition of complicating factors, if present. A typical approach may proceed, for example, along the following lines:

1) What is molar mass, and amino acid sequence? Recapitulate the purification protocol and establish a range of buffer conditions that the protein will tolerate, as well as essential buffer requirements. If possible, add a size-exclusion step for buffer exchange and purification as the last preparative step.
2) Is the protein sufficiently pure and available in sufficient quantities?
3) Determine the protein partial-specific volume and extinction properties.
4) Perform a SV experiment to examine purity and to obtain information on the oligomeric state and the number and size of reversible complexes (see Unit [SV Unit in this Series]).
5) Based on the buffer requirements and the protein extinction properties (for heterogeneous interactions exploiting specific differences in extinction) select the optical system. In the absence of other considerations, use the absorbance optics for simultaneous detection at 230 nm, 250 nm, and 280 nm. With strongly absorbing macromolecular or buffer components, one will typically choose the interference optics (after mechanical stabilization of the cell components). If in doubt, there is no harm in using both optical detection systems (using the mechanical setup for the interference detection).
6) Considering the factors listed above, plan the sample volumes. Typically, we usually use 180 μl, unless the molecules are very large (> 200 kDa) or very small (< 10 kDa), or have limited stability.
7) Based on the available material, make mixtures of the protein samples aiming to cover a large concentration range, organizing a set of experiments as dilution or titration series, and including separate controls for each protein.
8) Based on the expected molar masses and sample volumes, select a set of rotor speeds.
9) Execute the ultracentrifugation experiment, giving sufficient time at each rotor speed to attain equilibrium, as judged from a set of scans taken every 6 hours.
10) Assemble the data into an analysis project, establish as many constraints from prior knowledge as possible, fit the data (frequently sequentially using alternate models for comparison), scrutinize the quality of fit, and determine the statistical error limits for the parameters of interest.

On the homepage of our laboratory

(http://www.nibib.nih.gov/Research/Intramural/dbeps/pbr/auc) and on the website of the SEDPHAT software (Balbo et al. 2007) are available additional instructions for SE analytical ultracentrifugation. This includes step-by-step protocols for the planning, execution, and analysis of SE experiments, which also contains more details on the assembly of the ultracentrifugal cells, the use settings for the XLA/I operating software, a protocol for the mechanical stabilization of the cells for interference optical detection, etc. On-line tutorials for different typical problems are available as introduction to the practical data analysis. As mentioned in the introduction, all analysis methods described above are available in the public domain software SEDPHAT (Schuck 2003; Vistica et al. 2004; Houtman et al. 2007; Schuck 2007c). On the SEDPHAT homepage, an extensive searchable reference for all software functions, as well as a list of references to its application in the literature. Finally, our laboratory at the National Institutes of Health, Bethesda, Maryland, U.S.A., offers hands-on workshops twice a year that cover the material presented in this Unit.

Selected Key References:

General Reviews

Schachman, H. K. 1959. Ultracentrifugation in Biochemistry. Academic Press, New York.

Svedberg, T., and K. O. Pedersen. 1940. Die Ultrazentrifuge. Theodor Steinkopff, Dresden.

Lebowitz, J., Lewis M.S., and P. Schuck. 2002. Modern analytical ultracentrifugation in protein science: a tutorial review. Protein Sci. 11:2067-2079

Balbo, A., and P. Schuck. 2005. Analytical ultracentrifugation in the study of protein self-association and heterogeneous protein-protein interactions. In *Protein-Protein Interactions* (eds E. Golemis, and P.D. Adams) pp 253-277. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Scott, D.J., and P. Schuck. 2005. A brief introduction to the analytical ultracentrifugation of proteins for beginners. In *Modern Analytical Ultracentrifugation: Techniques and Methods* (eds D.J. Scott, S.E. Harding, and A.J. Rowe) pp. 1-25. The Royal Society of Chemistry, Cambridge

Howlett, G.J., Minton, A.P., and G. Rivas. 2006. Analytical ultracentrifugation for the study of protein association and assembly. Curr. Opin. Chem Biol. 10:430-436

Schuck, P. 2007. Sedimentation equilibrium analytical ultracentrifugation for multi-componenet protein interactions. In *Protein Interactions: Biophysical Methods for Complex Systems* (ed. P. Schuck) pp 289-316. Springer, New York.

References on Special Topics of Long-Column SE

Ansevin, A. T., D. E. Roark, and D. A. Yphantis. 1970. Improved ultracentrifuge cells for high-speed sedimentation equilibrium studies with interference optics. *Anal. Biochem.* 34:237-261.

Durchschlag, H. 1986. Specific volumes of biological macromolecules and some other molecules of biological interest. *In* Thermodynamic data for biochemistry and biotechnology. H.-J. Hinz, Springer, Berlin. 45-128.

Howlett, G. J. 1992. Sedimentation analysis of membrane proteins. *In* Analytical Ultracentrifugation in Biochemistry and Polymer Science. S. E. Harding, A. J. Rowe, and J. C. Horton, The Royal Society of Chemistry, Cambridge, U.K. 470-483.

Schubert, D., and P. Schuck. 1991. Analytical ultracentrifugation as a tool for studying membrane proteins. *Progr. Colloid Polym. Sci.* 86:12-22.

Tanford, C., and J. A. Reynolds. 1976. Characterization of membrane proteins in detergent solutions. *Biochim. Biophys. Acta.* 457:133-170.

Eisenberg, H. 2003. Modern analytical ultracentrifugation in protein science: look forward, not back. Protein Sci. 12:2657-2650

Lebowitz, J., Lewis M.S., and P. Schuck. 2003. Back to the future: A rebuttal to Henryk Eisenberg. Protein Sci 12:2649-2650

Vistica, J., Dam, J., Balbo, A., Yikilmaz, E., Mariuzza, R.A., Rouault, T.A., and P. Schuck. 2004. Sedimentation equilibrium analysis of protein interactions with global implicit mass conservation constraints and systematic noise decomposition. Anal. Biochem. 326:234-256

Data Analysis

Johnson, M. L., and M. Straume. 1994. Comments on the analysis of sedimentation equilibrium experiments. *In* Modern analytical ultracentrifugation. T. M. Schuster, and T. M. Laue, Birkhäuser, Boston. 37-65.

Lewis, M. S., R. I. Shrager, and S.-J. Kim. 1993. Analysis of protein-nucleic acid and protein-protein interactions using multi-wavelength scans from the XL-A analytical ultracentrifuge. *In* Modern Analytical Ultracentrifugation. T. M. Schuster, and T. M. Laue, Birkhäuser, Boston. 94-115.

Minton, A. P. 1997. Alternative strategies for the characterization of associations in multicomponent solutions via measurement of sedimentation equilibrium. *Progr. Colloid Polym. Sci.* 107:11-19.

Schuck, P. 1994. Simultaneous radial and wavelength analysis with the Optima XL-A analytical ultracentrifuge. *Progr. Colloid. Polym. Sci.* 94:1-13.

Servillo, L., H. B. Brewer, and J. C. Osborne. 1981. Evaluation of the mixed interaction between apolipoproteins A-II and C-I by equilibrium sedimentation. *Biophys. Chem.* 13:29-38.

Philo, J.S. 2000. Sedimentation equilibrium analysis of mixed associations using numerical constraints to impose mass or signal conservation. Methods in Enzymology 321:100-120

Vistica, J., Dam, J., Balbo, A., Yikilmaz, E., Mariuzza, R.A., Rouault, T.A., and P. Schuck. 2004. Sedimentation equilibrium analysis of protein interactions with global implicit mass conservation constraints and systematic noise decomposition. Anal. Biochem. 326:234-256

Some Experimental Alternatives

Correia, J. J., and D. A. Yphantis. 1992. Equilibrium sedimentation in short solution columns. *In* Analytical ultracentrifugation in biochemistry and polymer science. S. E. Harding, A. J. Rowe, and J. C. Horton, editors. The Royal Society of Chemistry, Cambridge, U.K. 231-252.

Darawshe, S., and A. P. Minton. 1994. Quantitative characterization of macromolecular associations in solution via real-time and postcentrifugation measurements of sedimentation equilibrium: A comparison. *Anal. Biochem.* 220:1-4.

Lebowitz, J., M. Teale, and P. W. Schuck. 1998. Analytical band centrifugation of proteins and protein complexes. *Biochem. Soc. Transact.* 26:745-749.

Literature Cited

Adams, E.T., Jr., and Lewis, M.S. 1968. Sedimentation equilibrium in reacting systems. VI. Some applications to indefinite self-associations. Studies with beta-lactoglobulin A. *Biochemistry* **7:** 1044-1053.

Advant, S.J., Braswell, E.H., Kumar, C.V., and Kalonia, D.S. 1995. The effect of pH and temperature on the self-association of recombinant human interleukin-2 as studied by equilibrium sedimentation. *Pharm. Res.* **12:** 637-641.

Ansevin, A.T., Roark, D.E., and Yphantis, D.A. 1970. Improved ultracentrifuge cells for high-speed sedimentation equilibrium studies with interference optics. *Anal. Biochem.* **34:** 237-261.

Arisaka, F. 1999. Applications and future perspectives of analytical ultracentrifugation. *Tanpakushitsu Kakusan Koso* **44:** 82-91.

Arkin, M., and Lear, J.D. 2001. A New Data Analysis Method to Determine Binding Constants of Small Molecules Using Equilibrium Analytical Ultracentrifugation with Absorption Optics. *Anal. Biochem.* **299:** 98-107.

Balbo, A., Brown, P.H., and Schuck, P. 2007. http://www.analyticalultracentrifugation.com/SEprotocols.htm.

Balbo, A., and Schuck, P. 2005. Analytical ultracentrifugation in the study of protein self-association and heterogeneous protein-protein interactions. In *Protein-Protein Interactions*. (eds. E. Golemis, and P.D. Adams), pp. 253-277. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Berkowitz, S.A. 2006. Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals. *AAPS J.* **8:** E590-605.

Bevington, P.R., and Robinson, D.K. 1992. *Data Reduction and Error Analysis for the Physical Sciences*. Mc-Graw-Hill, New York.

Boulant, S., Vanbelle, C., Ebel, C., Penin, F., and Lavergne, J.P. 2005. Hepatitis C virus core protein is a dimeric alpha-helical protein exhibiting membrane protein features. *J Virol* **79:** 11353-11365.

Braswell, E., and Lary, J. 1981. Equilibrium-sedimentation studies of some self-associating cationic dyes. *J. Phys. Chem.* **85:** 1573.

Burgess, B.R., Schuck, P., and Garboczi, D.N. 2005. Dissection of merozoite surface protein 3, a representative of a family of Plasmodium falciparum surface proteins, reveals an oligomeric and highly elongated molecule. *J Biol Chem* **280:** 37236-37245.

Burrows, S.D., Doyle, M.L., Murphy, K.P., Franklin, S.G., White, J.R., Brooks, I., McNulty, D.E., Scott, M.O., Knutson, J.R., Porter, D., et al. 1994. Determination of the monomer-dimer equilibrium of interleukin-8 reveals it is a monomer at physiological concentrations. *Biochemistry* **33:** 12741-12745.

Center, R.J., Earl, P.L., Lebowitz, J., Schuck, P., and Moss, B. 2000. The human immunodeficiency virus type 1 gp120 V2 domain mediates gp41-independent intersubunit contacts. *J Virol* **74:** 4448-4455.

Center, R.J., Schuck, P., Leapman, R.D., Arthur, L.O., Earl, P.L., Moss, B., and Lebowitz, J. 2001. Oligomeric Structure of Virion-Associated and Soluble Forms of the Simian Immunodeficiency Virus Envelope Protein in the Pre-Fusion Activated Conformation. *Proc. Natl. Acad. Sci. U S A* **98:** 14877-14882.

Chatelier, R.C. 1988. A parameterized overspeeding method for the rapid attainment of low-speed sedimentation equilibrium. *Anal. Biochem.* **175:** 114-119.

Chatelier, R.C., and Minton, A.P. 1987. Sedimentation equilibrium in macromolecular solutions of arbitrary concentration. II. Two protein components. *Biopolymers* **26:** 1097-1113.

Cole, J.L. 2004. Analysis of heterogeneous interactions. *Methods Enzymol* **384:** 212-232.

Correia, J.J., and Yphantis, D.A. 1992. Equilibrium sedimentation in short solution columns. In *Analytical ultracentrifugation in biochemistry and polymer science*. (eds. S.E. Harding, A.J. Rowe, and J.C. Horton), pp. 231-252. The Royal Society of Chemistry, Cambridge, U.K.

Dall'Acqua, W., Goldman, E.R., Eisenstein, E., and Mariuzza, R.A. 1996. A mutational analysis of the binding of two different proteins to the same antibody. *Biochemistry* **35:** 9667-9676.

Dam, J., Baber, J., Grishaev, A., Malchiodi, E.L., Schuck, P., Bax, A., and Mariuzza, R.A. 2006. Variable dimerization of the Ly49A natural killer cell receptor results in differential engagement of its MHC class I ligand. *J Mol Biol* **362:** 102-113.

Dam, J., Guan, R., Natarajan, K., Dimasi, N., Chlewicki, L.K., Kranz, D.M., Schuck, P., Margulies, D.H., and Mariuzza, R.A. 2003. Variable MHC class I engagement by Ly49 NK cell receptors revealed by the crystal structure of Ly49C bound to H-2Kb. *Nature Immunology* **4:** 1213-1222.

Darawshe, S., and Minton, A.P. 1994. Quantitative characterization of macromolecular associations in solution via real-time and postcentrifugation measurements of sedimentation equilibrium: A comparison. *Anal. Biochem.* **220:** 1-4.

Darawshe, S., Rivas, G., and Minton, A.P. 1993. Rapid and accurate microfractionation of the contents of small centrifuge tubes: application in the measurement

of molecular weights of proteins via sedimentation equilibrium. *Anal. Biochem.* **209:** 130-135.

Datta, S.A., Zhao, Z., Clark, P.K., Tarasov, S., Alexandratos, J.N., Campbell, S.J., Kvaratskhelia, M., Lebowitz, J., and Rein, A. 2007. Interactions between HIV-1 Gag molecules in solution: an inositol phosphate-mediated switch. *J Mol Biol* **365:** 799-811.

Davis, A.J., Perugini, M.A., Smith, B.J., Stewart, J.D., Ilg, T., Hodder, A.N., and Handman, E. 2004. Properties of GDP-mannose pyrophosphorylase, a critical enzyme and drug target in Leishmania mexicana. *J Biol Chem* **279:** 12462-12468.

Dölle, F., and Schubert, D. 1997. Dye-labelling as a means to study ternary protein complexes by analytical ultracentrifugation: the band 3/ankyrin/aldolase complex from erythrocyte membranes. *Progr. Colloid Polym. Sci.* **107:** 77-81.

Durchschlag, H. 1986. Specific volumes of biological macromolecules and some other molecules of biological interest. In *Thermodynamic data for biochemistry and biotechnology*. (ed. H.-J. Hinz), pp. 45-128. Springer, Berlin.

Edelstein, J., and Schachman, H. 1967. Simultaneous determination of partial specific volumes and molecular weights with microgram quantities. *J. Biol. Chem.* **242:** 306-311.

Fairman, R., Fenderson, W., Hail, M.E., Wu, Y., and Shaw, S.-Y. 1999. Molecular weights of CTLA-4 and CD80 by sedimentation equilibrium ultracentrifugation. *Anal. Biochem.* **270:** 286-295.

Fernandez, M.M., Bhattacharya, S., De Marzi, M.C., Brown, P.H., Kerzic, M., Schuck, P., Mariuzza, R., and Malchiodi, E.L. 2007. Superantigen natural affinity maturation revealed by the crystal structure of staphylococcal enterotoxin G and its binding to T-cell receptor Vbeta8.2. *Proteins: Structure, Function, Bioinformatics* **68:** in press.

Fleming, K.G., Ackerman, A.L., and Engelman, D.M. 1997. The effect of point mutations on the free energy of transmembrane alpha-helix dimerization. *J. Mol. Biol.* **272:** 266-275.

Ghirlando, R., Keown, M.B., Mackay, G.A., Lewis, M.S., Unkeless, J.C., and Gould, H.J. 1995. Stoichiometry and thermodynamics of the interaction between the Fc fragment of human IgG1 and its low-affinity receptor FcγRIII. *Biochemistry* **34:** 13320-13327.

Guan, R., Malchiodi, E.L., Wang, Q., Schuck, P., and Mariuzza, R. 2004. Crystal structure of the C-terminal peptidoglycan-binding domain of human peptidoglycan recognition protein Ialpha reveals sodium-mediated receptor dimerization. *Journal of Biological Chemistry* **279:** 31873-31882.

Hanlon, S., Lamers, K., Lauterbach, G., Johnson, R., and Schachman, H.K. 1962. Ultracentrifuge studies with absorption optics. I. An automatic photoelectric scanning absorption system. *Arch. Biochem. Biophys.* **99:** 157-174.

Harrington, W.F., and Kegeles, G. 1973. Pressure effects in ultracentrifugation of interacting systems. *Methods Enzymology* **27:** 106-345.

Haschemeyer, R.H., and Bowers, W.F. 1970. Exponential analysis of concentration or concentration difference data for discrete molecular weight distributions in sedimentation equilibrium. *Biochemistry* **9:** 435-445.

Hoiland, H. 1986. Partial molar volumes of biochemical model compounds in aqueous solution. In *Thermodynamic data for biochemistry and biotechnology*. (ed. H.-J. Hinz), pp. 17-44. Springer, Berlin.

Holladay, L.A., and Sohpianolpoulos, A.J. 1972. Nonideal associating systems. I. Documentation of a new method for determining the parameters from sedimentation equilibrium data. *J Biol Chem* **247:** 427-439.

Holtzer, M.E., Braswell, E.H., Angelleti, R.H., Mints, L., Zhu, D., and Holtzer, A. 2000. Ultracentrifuge and CD studies of folding equilibria in a retro-GCN4-like leucine zipper. *Biophys. J.* **78:** 2037-2048.

Horan, T.P., Martin, F., Simonet, L., Arakawa, T., and Philo, J.S. 1997. Dimerization of granulocyte-colony stimulating factor receptor: The Ig plus CRH construct of granulocyte-colony stimulating factor receptor forms a 2:2 complex with a ligand. *J. Biochem.* **121:** 370-375.

Houtman, J.C., Brown, P.H., Bowden, B., Yamaguchi, H., Appella, E., Samelson, L.E., and Schuck, P. 2007. Studying multisite binary and ternary protein interactions by global analysis of isothermal titration calorimetry data in SEDPHAT: application to adaptor protein complexes in cell signaling. *Protein Sci* **16:** 30-42.

Howlett, G.J. 1992. Sedimentation analysis of membrane proteins. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science*. (eds. S.E. Harding, A.J. Rowe, and J.C. Horton), pp. 470-483. The Royal Society of Chemistry, Cambridge, U.K.

Howlett, G.J., and Jeffrey, P.D. 1973. Theoretical behavior of interacting protein systems in density gradients at sedimentation equilibrium. *J Phys Chem* **77:** 1250-1258.

Howlett, G.J., Minton, A.P., and Rivas, G. 2006. Analytical ultracentrifugation for the study of protein association and assembly. *Curr. Opin. Chem. Biol.* **10:** 430-436.

Ikemizu, S., Gilbert, R.J., Fennelly, J.A., Collings, A.V., Harlos, K., Jones, E.Y., Stuart, D.I., and Davis, S.J. 2000. Structure and dimerization of a soluble form of B7-1. *Immunity* **12:** 51-60.

Johnson, M.L. 1983. Evaluation and propagation of confidence intervals in nonlinear, asymmetrical variance spaces. *Biophys. J.* **44:** 101-106.

Johnson, M.L., and Straume, M. 1994. Comments on the analysis of sedimentation equilibrium experiments. In

Modern Analytical Ultracentrifugation. (eds. T.M. Schuster, and T.M. Laue), pp. 37-65. Birkhäuser, Boston.

Jones, I.I., Vullev, V., Braswell, E.H., and Zhu, D. 2000. Multi-step photoinduced electron transfer in a de novo helix bundle: Multimer self-assembly of peptide chains including a chromophore special pair. *J.A.C.S.* **122:** 388-389.

Junghans, R.P., Stone, A.L., and Lewis, M.S. 1996. Biophysical characterization of a recombinant soluble interleukin 2 receptor (Tac). *J. Biol. Chem.* **271:** 10453-10460.

Kim, K.-S., Rajarathnam, K., Clark-Lewis, I., and Sykes, B.D. 1996. Structural characterization of a monomeric chemokine: monocyte chemoattractant protein-3. *FEBS Letters* **395:** 277-282.

Laue, T.M. 1999. Analytical centrifugation: equilibrium approach. *Current Protocols in Protein Science***:** 20.23.21-20.23.13.

Laue, T.M., Anderson, A.L., and Weber, B.W. 1997. Prototype fluorimeter for the XLA/XLI analytical ultracentrifuge. In *Ultrasensitive Biochemical Diagnostics II. SPIE Proceedings.* (eds. G.E. Cohn, and S.A. Soper), pp. 196-204. SPIE, Bellingham, WA.

Laue, T.M., Senear, D.F., Eaton, S., and Ross, J.B. 1993. 5-hydroxytryptophan as a new intrinsic probe for investigating protein- DNA interactions by analytical ultracentrifugation. Study of the effect of DNA on self-assembly of the bacteriophage lambda cI repressor. *Biochemistry* **32:** 2469-2472.

Laue, T.M., Shah, B.D., Ridgeway, T.M., and Pelletier, S.L. 1992. Computer-aided interpretation of analytical sedimentation data for proteins. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science*. (eds. S.E. Harding, A.J. Rowe, and J.C. Horton), pp. 90-125. The Royal Society of Chemistry, Cambridge.

Lebowitz, J., Lewis, M.S., and Schuck, P. 2002. Modern analytical ultracentrifugation in protein science: a tutorial review. *Protein Sci* **11:** 2067-2079.

Lebowitz, J., Lewis, M.S., and Schuck, P. 2003. Back to the future: A rebuttal to Henryk Eisenberg. *Protein Sci* **12:** 2649-2650.

Leder, L., Llera, A., Lavoie, P.M., Lebedeva, M.I., Li, H., Sékaly, R.-P., Bohach, G.A., Gahr, P.J., Schlievert, P.M., Karjalainen, K., et al. 1998. A mutational analysis of the binding of staphylococcal enterotoxins B and C3 to the T cell receptor β chain and major histocompatibility complex class II. *J. Exp. Med* **187:** 823-833.

Leong, S.R., Lowman, H.B., Liu, J., Shire, S., Deforge, L.E., Gillece-Castro, B.L., McDowell, R., and Hébert, C.A. 1997. IL-8 single-chain homodimers and heterodimers: Interactions with the chemokine receptors CXCR1, CXCR2, and DARC. *Protein Science* **6:** 609-617.

Lewis, M.S., and Junghans, R.P. 2000. Ultracentrifugal analysis of the molecular mass of glycoproteins of unknown or ill-defined carbohydrate composition. *Methods in Enzymol.* **321:** 136-149.

Lewis, M.S., Shrager, R.I., and Kim, S.-J. 1993. Analysis of protein-nucleic acid and protein-protein interactions using multi-wavelength scans from the XL-A analytical ultracentrifuge. In *Modern Analytical Ultracentrifugation.* (eds. T.M. Schuster, and T.M. Laue), pp. 94-115. Birkhäuser, Boston.

Lewis, M.S., and Youle, R.J. 1986. Ricin subunit association. *J Biol Chem* **261:** 11572-11577.

Li, H., Zhao, Y., Guo, Y., Li, Z., Eisele, L., and Mourad, W. 2007a. Zinc induces dimerization of the class II major histocompatibility complex molecule that leads to cooperative binding to a superantigen. *J Biol Chem* **282:** 5991-6000.

Li, H., Zhao, Y., Guo, Y., Vanvranken, S.J., Li, Z., Eisele, L., and Mourad, W. 2007b. Mutagenesis, biochemical, and biophysical characterization of Mycoplasma arthritidis-derived mitogen. *Mol Immunol* **44:** 763-773.

Li, Y., Li, H., Dimasi, N., McCormick, J.K., Martin, R., Schuck, P., Schlievert, P.M., and Mariuzza, R.A. 2001. Crystal structure of a superantigen bound to the high-affinity, zinc-dependent site on MHC class II. *Immunity* **14:** 93-104.

Lindenthal, S., and Schubert, D. 1991. Monomeric erythrocyte band 3 protein transports anions. *Proc. Natl. Acad. Sci. USA* **88:** 6540-6544.

Liu, J., Reitz, B., Fox, J., and Shire, S.J. 1997. Determination of the average molecular weights of antibody and its complexes in serum using a preparative ultracentrifuge. *Pharm. Res.* **14:** S-348.

Lustig, A., Engel, A., Tsiotis, G., Landau, E.M., and Baschong, W. 2000. Molecular weight determination of membrane proteins by sedimentation equilibrium at the sucrose or Nycodenz-adjusted density of the hydrated detergent micelle. *Biochim.Biophys.Acta* **1464:** 199-206.

MacGregor, I.K., Anderson, A.L., and Laue, T.M. 2004. Fluorescence detection for the XLI analytical ultracentrifuge. *Biophys Chem* **108:** 165-185.

Malchiodi, E.L., Eisenstein, E., Fields, B.A., Ohlendorf, D.H., Schlievert, P.M., Karjalainen, K., and Mariuzza, R.A. 1995. Superantigen binding to a T cell receptor β chain of known three-dimensional structure. *J. Exp. Med.* **182:** 1833-1845.

Mancheno, J.M., Tateno, H., Goldstein, I.J., Martinez-Ripoll, M., and Hermoso, J.A. 2005. Structural analysis of the Laetiporus sulphureus hemolytic pore-forming lectin in complex with sugars. *J Biol Chem* **280:** 17251-17259.

Mayer, G., Ludwig, B., Muller, H.W., van den Broek, J.A., Friesen, R.H.E., and Schubert, D. 1999. Studying membrane proteins in detergent solution by analytical

ultracentrifugation:different methods for density matching. *Prog.Colloid Polymer Sci* **113:** 176-181.

McLellan, J.S., Yao, S., Zheng, X., Geisbrecht, B.V., Ghirlando, R., Beachy, P.A., and Leahy, D.J. 2006. Structure of a heparin-dependent complex of Hedgehog and Ihog. *Proc Natl Acad Sci U S A* **103:** 17208-17213.

Morris, M., and Ralston, G.B. 1989. A thermodynamic model for the self-association of human spectrin. *Biochemistry* **28:** 8561-8567.

Natarajan, K., Boyd, L.F., Schuck, P., Yokoyama, W.M., Eilat, D., and Margulies, D.H. 1999. Interaction of the NK cell inhibitory receptor Ly49A with H-2Dd: Identification of a site distinct from the TCR site. *Immunity* **11:** 591-601.

Paliwal, V., Ptak, W., Sperl, J., Braswell, E., and Askenase, P.W. 1997. Recombinant soluble alpha beta T cell receptors protect T cells from immuno suppresion - Requirement for aggregated multimeric, disulfide-linked alpha beta heterodimers. *J. Immunololgy* **159:** 1718-1727.

Perugini, M.A., Griffin, M.D., Smith, B.J., Webb, L.E., Davis, A.J., Handman, E., and Gerrard, J.A. 2005. Insight into the self-association of key enzymes from pathogenic species. *Eur Biophys J* **34:** 469-476.

Philo, J.S. 2000. Sedimentation equilibrium analysis of mixed associations using numerical constraints to impose mass or signal conservation. *Methods in Enzymology* **321:** 100-120.

Philo, J.S., Aoki, K.H., Arakawa, T., Narhi, L.O., and Wen, J. 1996. Dimerization of the extracellular domain of the erythropoietin (EPO) receptor by EPO: One high-affinity and one low-affinity interaction. *Biochemistry* **35:** 1681-1691.

Pörschke, D., and Labuda, D. 1982. Codon-induced transfer ribonucleic acid association: Quantitative analysis by sedimentation equilibrium. *Biochemistry* **21:** 53-56.

Press, W.H., Teukolsky, S.A., Vetterling, W.T., and Flannery, B.P. 1992. *Numerical Recipes in C*, 2nd (corrected) edition ed. University Press, Cambridge.

Rajarathnam, K., Kay, C.M., Dewald, B., Wolf, M., Baggiolini, M., Clark-Lewis, I., and Sykes, B.D. 1997. Neutrophil-activating peptide-2 and melanoma growth-stimulatory activity are functional as monomers for neutrophil activation. *J. Biol. Chem.* **272:** 1725-1729.

Ralston, G.B. 1994. The concentration dependence of the activity coefficient of the human spectrin heterodimer. A quantitative test of the Adams-Fujita approximation. *Biophys. Chem.* **52:** 51-61.

Reynolds, J.A., and McCaslin, D.R. 1985. Determination of protein molecular weight in complexes with detergent without knowledge of binding. *Methods Enzymology* **117:** 41-53.

Richards, E.G., and Schachman, H.K. 1959. Ultracentrifuge studies with Rayleigh interference optics. I. General applications. *J. Phys. Chem.* **63:** 1578-1591.

Richards, E.G., Teller, D.C., and Schachman, H.K. 1968. Ultracentrifuge studies with Rayleigh interference optics. II. Low-speed sedimentation equilibrium of homogeneous systems. *Biochemistry* **7:** 1054-1076.

Rivas, G., Lopez, A., Mingorance, J., Ferrandiz, M.J., Zorrilla, S., Minton, A.P., Vicente, M., and Andreu, J.M. 2000. Magnesium-induced linear self-association of the FtsZ bacterial cell division protein monomer. The primary steps for FtsZ assembly. *Journal of Biological Chemistry* **275:** 11740-11749.

Rivas, G., Stafford, W., and Minton, A.P. 1999. Characterization of heterologous protein-protein interactions via analytical ultracentrifugation. *Methods: A Companion to Methods in Enzymology* **19:** 194-212.

Roark, D.E. 1976. Sedimentation equilibrium techniques: multiple speed analyses and an overspeed procedure. *Biophys Chem* **5:** 185-196.

Rosovitz, M.J., Schuck, P., Varughese, M., Chopra, A.P., Mehra, V., Singh, Y., McGinnis, L.M., and Leppla, S.H. 2003. Alanine-scanning mutations in domain 4 of anthrax toxin protective antigen reveal residues important for binding to the cellular receptor and to a neutralizing monoclonal antibody. *J Biol Chem* **278:** 30936-30944.

Schachman, H.K. 1959. *Ultracentrifugation in Biochemistry*. Academic Press, New York.

Schachman, H.K., Gropper, L., Hanlon, S., and Putney, F. 1962. Ultracentrifuge studies with absorption optics. II. Incorporation of a monochromator and its application to the study of proteins and interacting systems. *Arch. Biochem. Biophys.* **99:** 175-190.

Schmidt, B., and Riesner, D. 1992. A fluorescence detection system for the analytical ultracentrifuge and its application to proteins, nucleic acids, viroids and viruses. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science*. (eds. S.E. Harding, A.J. Rowe, and J.C. Horton), pp. 176-207. The Royal Society of Chemistry, Cambridge.

Schönfeld, H.-J., Pöschl, B., and Müller, F. 1998. Quasi-elastic light scattering and analytical ultracentrifugation are indispensable tools for the purification and characterization of recombinant proteins. *Biochem. Soc. Transactions* **26:** 753-758.

Schubert, D., and Schuck, P. 1991. Analytical ultracentrifugation as a tool for studying membrane proteins. *Progr. Colloid Polym. Sci.* **86:** 12-22.

Schubert, D., Tziatzios, C., Broeck, J.A.v.d., Schuck, P., Germeroth, L., and Michel, H. 1994. Determination of the molar mass of pigment-containing complexes of intrinsic membrane proteins: Problems, solutions and application to the light-harvesting complex B800/820 of Rhodospirillum molischianum. *Progr. Colloid. Polym. Sci.* **94:** 14-19.

Schuck, P. 1994. Simultaneous radial and wavelength analysis with the Optima XL-A analytical ultracentrifuge. *Progr. Colloid. Polym. Sci.* **94:** 1-13.

Schuck, P. 2003. On the analysis of protein self-association by sedimentation velocity analytical ultracentrifugation. *Anal. Biochem.* **320:** 104-124.

Schuck, P. 2006. www.nih.gov/od/ors/dbeps/PBR/AUC.htm.

Schuck, P. 2007a. http://www.analyticalultracentrifugation.com/sedphat/references.htm.

Schuck, P. 2007b. Sedimentation equilibrium analytical ultracentrifugation for multi-component protein interactions. In *Protein Interactions: Biophysical Methods for Complex Systems*. (ed. P. Schuck), pp. 289-316. Springer, New York.

Schuck, P. 2007c. www.analyticalultracentrifugation.com/sedphat/sedphat.htm.

Schuck, P., and Millar, D.B. 1998. Rapid determination of molar mass in modified Archibald experiments using direct fitting of the Lamm equation. *Anal. Biochem.* **259:** 48-53.

Schuck, P., Radu, C.G., and Ward, E.S. 1999. Sedimentation equilibrium analysis of recombinant mouse FcRn with murine IgG1 and Fc fragment. *Mol. Immunol.* **36:** 1117-1125.

Scott, D.J., and Schuck, P. 2005. A brief introduction to the analytical ultracentrifugation of proteins for beginners. In *Modern Analytical Ultracentrifugation: Techniques and Methods*. (eds. D.J. Scott, S.E. Harding, and A.J. Rowe), pp. 1-25. The Royal Society of Chemistry, Cambridge.

Servillo, L., Brewer, H.B., and Osborne, J.C. 1981. Evaluation of the mixed interaction between apolipoproteins A-II and C-I by equilibrium sedimentation. *Biophys. Chem.* **13:** 29-38.

Shi, J., Ghirlando, R., Beavil, R.L., Beavil, A.J., Keown, M.B., Young, R.J., Owens, R.J., Sutton, B.J., and Gould, H.J. 1997. Interaction of the low-affinity receptor CD23/FcεRII lectin domain with the Fcε3-4 fragment of human immunoglobulin E. *Biochemistry* **36:** 2112-2122.

Shire, S. 1992. *Determination of molecular weight of glycoproteins by analytical ultracentrifugation*. Beckman Instruments, Palo Alto, CA.

Shire, S. 1994. Analytical ultracentrifugation and its use in biotechnology. In *Modern Analytical Ultracentrifugation*. (eds. T.M. Schuster, and T.M. Laue), pp. 261-297. Birkhäuser, Boston, MA.

Silkowski, H., Davis, S.J., Barclay, A.N., Rowe, A.J., Harding, S.E., and Byron, O. 1997. Characterization of the low affinity interaction between rat cell adhesion molecules CD2 and CD48 by analytical ultracentrifugation. *Eur. Biophys. J.* **25:** 455-462.

Svedberg, T., and Pedersen, K.O. 1940. *The ultracentrifuge*. Oxford University Press, London.

Tanford, C., and Reynolds, J.A. 1976. Characterization of membrane proteins in detergent solutions. *Biochim. Biophys. Acta* **457:** 133-170.

Ucci, J.W., and Cole, J.L. 2004. Global analysis of non-specific protein-nucleic interactions by sedimentation equilibrium. *Biophys Chem* **108:** 127-140.

van Holde, K.E., and Baldwin, R.L. 1958. Rapid attainment of sedimentation equilibrium. *J. Phys. Chem.* **62:** 734-749.

Vistica, J., Dam, J., Balbo, A., Yikilmaz, E., Mariuzza, R.A., Rouault, T.A., and Schuck, P. 2004. Sedimentation equilibrium analysis of protein interactions with global implicit mass conservation constraints and systematic noise decomposition. *Anal. Biochem.* **326:** 234-256.

Weber, A.N., Moncrieffe, M.C., Gangloff, M., Imler, J.L., and Gay, N.J. 2005. Ligand-receptor and receptor-receptor interactions act in concert to activate signaling in the Drosophila toll pathway. *J Biol Chem* **280:** 22793-22799.

Wills, P.R., Comper, W.D., and Winzor, D.J. 1993. Thermodynamic nonideality in macromolecular solutions: interpretation of virial coefficients. *Arch. Biochem. Biphys.* **300:** 206-212.

Wills, P.R., Nichol, L.W., and Siezen, R.J. 1980. The indefinite self-association of lysozyme: consideration of composition-dependent activity coefficients. *Biophys. Chem.* **11:** 71-82.

Yphantis, D.A. 1964. Equilibrium ultracentrifugation of dilute solutions. *Biochemistry* **3:** 297-317.

Yphantis, D.A., Lary, J.W., Stafford, W.F., Liu, S., Olsen, P.H., Hayes, D.B., Moody, T.P., Ridgeway, T.M., Lyons, D.A., and Laue, T.M. 1994. On-line data acquisition for the Rayleigh interference optical system of the analytical ultracentrifuge. In *Modern analytical ultracentrifugation*. (eds. T.M. Schuster, and T.M. Laue), pp. 209-226. Birkhäuser, Boston.

Yu, I.M., Gustafson, C.L., Diao, J., Burgner, J.W., 2nd, Li, Z., Zhang, J., and Chen, J. 2005. Recombinant severe acute respiratory syndrome (SARS) coronavirus nucleocapsid protein forms a dimer through its C-terminal domain. *J Biol Chem* **280:** 23280-23286.