Analytical ultracentrifugation;

The analytical ultracentrifuge is a high speed centrifuge with an optical system allowing observation of material during the sedimentation process. Several types of optical system are available for observation of the concentration of macromolecules as a function of radius and time.

There are two basic modes of operating the ultracentrifuge: velocity mode and equilibrium mode. In the equilibrium mode, relatively low speeds are used such that a an exponential gradient of macromolecule is allowed to build up with sedimentation proceeding until it is opposed by diffusion so that no further concentration changes occur. When the system is at equilibrium, the radial dependence of the concentration ditribution is determined be the molar mass distribution. Appropriate analysis of the concentration gradient allows determination of molar mass, stoichiometry and equilibrium constants for interacting sand non-interacting systems. The equilibrium mode has been discussed in this series by Tom Laue (Current Protocols, ...). This article concerns the velocity mode of operation.

Sedimentation Velocity Theory

In sedimentation velocity experiment one observes the evolution of the concentration distribution as a function of both radius and time. Normally, relatively high speeds are chosen so that a boundary is formed between the solution of sedimenting macromolecule and the buffer in which it is dissolved. Analysis of the rate boundary movement and evolution of its shape can yield information about the molar masses of species present as well as stoichiometries and equilibrium constants for their interactions. It is convenient to classify systems into two main categories:(1) non-interacting and (2) interacting systems. Each of these can be either ideal or non-ideal. In addition, interacting systems can be either rapidly reversible so that chemical equilibrium is maintained during sedimentation or kinetically limited so that chemical equilibrium is not maintained during sedimentation.

Non-ideality:

Before we proceed with the theory, a few words about non-ideality are in order. Non-ideality can be manifested either hydrodynamically through concentration dependence of the frictional coefficient or thermodynamically through concentration dependence of the activity coefficient. The former affects both sedimentation and diffusion while the latter affects diffusion through its effect on the chemical potential gradient which is the driving force for diffusion. Hydrodynamic concentration dependence comes in two main varieties, one due to backflow created as the macromolecule displaces buffer as it sediments, the other due to the so-called primary charge effect. The primary charge effect is a result of the requirement of electroneutrality. At low ionic strength sedimentation of the macromolecule is retarded by the drag of its more slowly sedimenting counter ions. At higher ionic strengths, where there is an excess of counter-ions the effect of counter ion drag becomes negligible. The effect of counter ions on the diffusion coefficient is to increase the rate of diffusion because the counter ions are more mobile than the macromolecule and tend to pull it along. The non-ideal

effects result in a decrease in sedimentation coefficient with an increase in protein concentration and are treated commonly by the following mathematical form:

$$s(c) = \frac{s(c = 0)}{(1 + k_s c)}$$

Where Ks is the concentration dependence of the sedimentation coefficient and depends on a number of factors including the ionic strength, the shape and charge of the molecule. Sometimes a linear form is used but the inverse form is applicable over a wider range of concentration. For the diffusion coefficient we have:

$$D(c) = D(c = 0)\frac{(1 + 2BM_1 c)}{(1 + k_s c)}$$

where M_1 is the molecular mass of the diffusing component, B is the colligative second virial coefficient and depends on both excluded volume and charge.

When determining either molar mass or shape from frictional information, it is best to work at the lowest concentrations possible to avoid non-ideality. For interacting systems, one must work at concentrations near the value of the dissociation constant, and for many systems of interest, these concentrations will usually be sufficiently low that non-ideality effects can also be ignored. At moderate concentrations, non ideality can be treated using the above equations. At high concentrations, other theoretical treatments are required (Minton et al)

Monodisperse Ideal System.

The simplest type of system that one is likely to encounter is the monodisperse, ideal system. For a monodisperse macromolecular solution, one can determine the molar mass and frictional coefficient in a sedimentation velocity experiment. In the classical sedimentation velocity experiment, one measures the rate of movement of the mid-point of the boundary to determine the sedimentation coefficient of the macromolecule. More sophisticated methods accessible with desktop and larger computers are now more commonly used.

Transport by sedimentation and diffusion in centrifugal field is described by the continuity equation, also know as the Lamm Equation (Lamm,1928),

$$\left(\frac{\partial c}{\partial t}\right)_r = -\frac{1}{r}\left(\frac{\partial}{\partial r}\right)\left[r\left(J_{sed} - J_{dif}\right)\right],$$

where c is the concentration, which is a function of radius and time, r is the radial distance from the center of rotation, t is the time of sedimentation from the beginning of the run. The Lamm equation relates the time dependence of the concentration distribution to the fluxes due to sedimentation, J_{sed}, and diffusion, D_{dif}, respectively. The Lamm

equation is essentially a statement of the conservation of mass. the flux due to sedimentation is just the concentration time the velocity and is given by

$$J_{sed} = c\frac{dr}{dt} = cs\omega^2 r$$

The sedimentation coefficient is a primary physical property of the molecule and is defined as the velocity of the particle in a gravitational field divided by the field strength

$$s \equiv \frac{dc/dr}{\omega^2 r}$$

where c is the concentrations, ω is the angular velocity of the rotor and r is the radius.

The sedimentation coefficient depends both on the molecular mass and shape according to the following relationship

$$s = \frac{M(1 - \bar{v}\rho)}{Nf}$$

where f is the frictional coefficient related to the shape by the Stokes equation:

$$f = 6\pi\eta_o R_s$$

where η_o is the viscosity of the solvent and R_S is the Stokes radius and R_o is the radius of the corresponding hydrodynamically equivalent sphere, which includes hydration. The Stokes radius is the radius of a hydrodynamically equivalent sphere. The frictional ratio, which is a measure of the deviation of the macromolecule from sphericity, is the ratio of the observed Stokes radius to the radius of a sphere with the same hydrated volume as the macromolecule and is defined by

$$\frac{f}{f_o} = \frac{R_s}{R_o}$$

where R_O is the radius of sphere with the same volume as the macromolecule (including hydration) and is given by

$$f_o = 6\pi\eta_o R_o$$

where R_O is given by

$$R_o = \left(\frac{3\left(\bar{v}_2 + \delta_1 v_1^o \right)M}{4\pi N} \right)^{1/3}$$

where v_2 is the partial specific volume of the macromolecule, δ_1 is the hydration coefficient, v_1^o is the specific volume of pure water, M is the molar mass of the macromolecule and N is Avogadro's number.

The frictional ratio forms the basis of shape analysis of macromolecules and can be compared to values of f/f_o for various models of macromolecular shape ranging from ellipsoids of revolution to detailed bead or shell models computed from crystallographic coordinate data.

The diffusion coefficient, D, is related to the frictional coefficient through the Stokes-Einstein equation:

$$D = \frac{RT}{Nf}$$

and is defined by Fick's First Law of diffusion:

$$J_{dif} = -D\left(\frac{\partial c}{\partial x} \right)_t$$

where J_{diff} is the flux per unit cross sectional area due to diffusion.

after substituting, the continuity equation becomes:

$$\left(\frac{\partial c}{\partial t} \right)_r = \frac{1}{r}\left(\frac{\partial}{\partial r} \right)\left[rD\left(\frac{\partial c}{\partial r} \right)_t - 2\omega^2 r^2 cs \right]$$

A non-interacting system can be composed of one or more independently sedimenting species which give rise to overlapping, additive essentially Gaussian boundaries whose spreading is determined solely by diffusion. Interacting systems, on the other hand, give rise to so called reaction boundaries. Reaction boundaries by their very nature are not able to be resolved into individual contributions from the several species present. For example, for a self-associating system, at each point in a reaction boundary, the Law of Mass Action must be obeyed such that the amount of each species is determined by the equilibrium constant and total concentration. The shape of the boundary is determined by coupling sedimentation transport with re-equilibration such that mass action is obeyed at each point in the boundary. Having made that distinction, we will proceed to describe each type of system and the ways in which they may be analyzed.

Polydisperse, non-interacting systems

For a polydisperse, non-interacting system, one must determine that the system is in fact non-interacting by demonstrating that sedimentation rate and boundary shape are independent of concentration. Once concentration independence has been established, the boundary may be analyzed by various methods capable of resolving independent boundaries. A non-interacting system will be composed of several superimposable independent boundaries that are essentially Gaussian in shape. The boundary for each species is transported according to its sedimentation coefficient and it spreads according to its diffusion coefficient. In the cylindrical coordinate system of the rotating reference frame of the centrifuge cell, the shape of the boundary is not exactly a Gaussian but its shape is sufficiently close that it can be treated as Gaussian. The variance of the Gaussian curve that represents the boundary is proportional to the product Dt.

Polydisperse, interacting systems

For interacting systems, a different approach has to be applied to determine the parameters describing the system. Interacting systems can be either single component or multi-component. An example of a single component interacting system would be a self-associating system like a monomer-dimer or monomer-dimer-tetramer system. It can be characterized by a single initial total concentration and the equilibrium constants for each reaction. For an incompressible system, the concentration of each macromolecular species is uniquely determined by the equilibrium constants and the total concentration according to the Law of Mass Action. Therefore, for a given monomer-dimer system, for example, the amount of monomer and dimer present at each point in the cell is uniquely determined by the concentration at that point. Because the system is in reversible chemical equilibrium at each point, no resolution into monomer and dimer boundaries can occur. This type boundary is called a reaction boundary and its shape and evolution with time is characteristic of the stoichiometry and equilibrium constants.

Weight average sedimentation coefficient.
　　　The weight average sedimentation coefficient is defined as the mass concentration weighted average of the sedimentation coefficients of the species comprising the system and is defined by

$$s_w = \frac{\sum_{i=1}^{N} c_i s_i}{\sum_{i=1}^{N} c_i}$$

Since the values of c_i are uniquely determined by the equilibrium constants and total concentration, s_w is a quantity completely determined by the thermodynamics of the system. The value of s_w is derived from the measurement of the equivalent boundary position obtained by integrating over the boundary (Schachman, 1959; Stafford and Schuster, 199x). The observed dependence of s_w on plateau concentration can be modeled to determine the stoichiometry and equilibrium constants. (Timasheff et al. and Correia et

al.). An advantage of using s_w is that its determination is completely independent of boundary shape since it is determined solely by the composition of the solution at the plateau concentration. The weight average value of s can be determined from g(s*) analysis(Stafford,1994).

Least squares fitting to solutions to the Lamm equation.

A disadvantage of using s_w is that it does not make use of the information contained in the evolution of the shape of the boundary. One way to make use of the boundary shape is to curve fit the reaction boundary with numerical solutions to the Lamm equation solved for various reaction schemes (Todd and Haschemeyer, 1981). Various programs are available for fitting using numerical solutions to the Lamm equation. For non-interacting ans self-associating systems one can use the software (Ultrascan running under LINUX) available from Demleler and Saber(199x) or (SEDFIT running under Windows)) from Schuck(19xx). For non-interacting, self-associating and hetero-associating systems the software (ABCD_Fitter running under LINUX, DOS and MacOS or SedAnal running under Window9x; Stafford 1998,2003) available from Stafford, 2003.

With the previous considerations in mind, we now proceed to the practical aspects of sedimentation velocity analysis. Many of the choices to made in the practical application of the techniques will be made with regard to the theory. Most of the results of sedimentation analysis are interpreted in terms of the theory and if the experiments are not designed to reflect the requirements of the theory, the data will usually be useless.

Experimental Design and Protocols.

Rotors and cells

See the accompanying article by Tom Laue for a discussion of the available rotors and cells.

Sample Preparation for Sedimentation Velocity Analytical Ultracentrifugation:

For initial runs, samples should be in the concentration range of 0.5 to 1.0 mg/ml for use with the Rayleigh optical system (refractive index) or in the range of 0.5 to 1.0 a.u. at the appropriate wavelength for the absorbance optics.

Preferably, samples should be of the highest purity possible with gel filtration being performed as the last step before sedimentation to remove aggregates. Remember: "Garbage in - garbage out".

Samples also should be at "osmotic" equilibrium with their respective buffers This can be achieved either by 24 hour dialysis or by gel filtration. Either ordinary column gel filtration by Sephadex, FPLC or HPLC can be used, or "spin" columns can be used (e.g.

cf. Christopherson and Jones, Anal. Biochem 100, 184-187 (1979)). Spin columns are convenient because there is effectively no dilution during buffer exchange.

For samples prepared by dialysis and transported to another location, it is best to deliver the samples still in their dialysis bags in about 25-50ml of dialysis buffer in a conical screw-top centrifuge tube, for example. Air should be removed from the dialysis bags as much as possible to minimize the potential for surface denaturation during transport.

Samples that are prepared by ordinary gel filtration or spin columns, must accompanied by about 25-50 ml of the column buffer which will be used to make dilutions and as an optical reference. This aliquot of buffer must be the identical buffer that was used to equilibrate the column. Since the refractive index match between the sample buffer and the reference buffer must be exact, an aliquot of buffer of nominally the same composition will not match sufficiently well to act as reference. Exhaustive dialysis (or its equivalent, gel filtration) has thermodynamic consequences related to the definition of macromolecular components: in order to maintain the composition of the macromolecular component (i.e. ratio of protein to counter-ions) upon dilution, the dilutions must be carried out with dialysate (c.f. Cassasa and Eisenberg, 1964). Therefore, dialysis is a good idea even if absorbance optics are being used.

Resolution in a sedimentation velocity experiment increases as the boundary is transported toward the cell bottom; therefore, one should fill the cell as full as possible to create the longest sedimentation path possible. In a polydisperse non-interacting sample, the separation between components is roughly proportional to the first power of time while the contribution to boundary spreading due to diffusion is roughly proportional to the square root of time. Therefore, even though the boundary is spreading during the time of sedimentation, the resolution will still increase as the square root of time and, therefore, distance.

Filling the Cells.

Looking at the assembled cell with the screw ring facing you, the reference solution should be loaded on the left and the sample on the right side. Load 430 uL on each side to match the meniscuses as closely as possible. This is very important with the interference optical system since it measures the refractive index contribution from the protein by subtracting two relatively large numbers, the refractive index of the buffer from the refractive index of the buffer plus protein. The protein makes a relatively small contribution to the total. If the menisci are not matched then the contributions from the buffer, which also redistributes to a significant extent during the experiment, will not cancel at corresponding radial positions on the sample and references sides; the result will a changing background refractive index gradient that will make a contribution to the overall sedimentation pattern. Needless to say, this variable background contribution will complicate the analysis. Similar but much smaller contributions can be expected from the absorbance optical system if the buffer absorbs appreciably at the wavelengths of observation. It should also be noted that with interference optics, proper cancellation of

the buffer contribution at corresponding radial positions requires that the axis of the cylinder lens be properly aligned perpendicularly to the radius bisecting the centerpiece. This is alignment can be checked and verified by the user by a simple procedure (vide infra); however, the actual adjustment must be made by a your BeckmanCoulter serviceman.

In my laboratory, we generally use capillary type synthetic boundary centerpieces to match the meniscuses exactly. Usually, the reference side is initially filled to 440uL and the sample side to 420uL. The rotor is accelerated to 3000-10000 RPM to allow the small amount of buffer to flow onto the sample side; when the menisci are matched, the rotor is stopped, removed and gently shaken to re-mix the solution so that the run starts with a uniform initial concentration distribution. The extra step to match the menisci is especially important when working at protein concentrations below about 0.1 mg/ml.

Cell Alignment in the rotor:

It is extremely important that the cells be aligned properly in the centrifugal field. Because sedimentation proceeds in a radial direction, outward from the center of the rotor, the sample and reference compartments of the cells are sector shaped to prevent material from colliding with the cell walls. If a cell is slightly miss-aligned, material can collect against a cell wall and build up to concentration that will lead to unstable, convective sedimentation. Each hole in the rotor has a small line inscribed at the center of both the centripetal and centrifugal sides of the cell holes. The cell casing also has corresponding lines inscribed in the bottom. These lines must be aligned as carefully as possible to avoid convection. Use of a magnifying glass is recommended. One can check the alignment with the interference optics while the rotor is spinning by stepping through the delay until the image just starts to darken. The fringes should darken uniformly at across the cell as it is adjusted. If the fringes at the top or the bottom start to darken before the rest of the image, then the cell is miss-aligned. It is highly recommended that the run be stopped and the miss-aligned cell readjusted before proceeding. Convection, needless-to-say, will confound the analysis and is to be avoided. Convection can be caused by

Rotor temperature equilibration:

It is important that the rotor be at uniform temperature before starting the run. The BeckmanCoutler XL-A/I centrifuges have two temperature sensing mechanisms. At 100 microns of pressure the instrument switches from one to the other and there is a jump of as much as 4 degrees in the reported temperature. Therefore, it is important to make sure the pressure gets below 100 microns while you equilibrate the rotor so that the rotor equilibrates at the expected temperature. Since the rough vacuum pump may not be able to pull the vacuum below 100 microns, you must turn on the diffusion pump to get the full vacuum. The diffusion pump comes on only while the machine is running; therefore, one must run the machine at 0 RPM (not 3000 RPM) during the equilibration process by entering 0 RPM for the speed and pressing "START". I t may take up to an hour to equilibrate depending on the initial temperature difference.

It is important to note that the temperature of the rotor will decrease by about 0.8° upon acceleration from 0 to 60,000 RPM as the rotor stretches (Waugh, D.F. and Yphantis, D.A. 1952); there will also be an increase in the sample temperature due to compression of the solution that will result in a net temperature difference of about 1° between the sample and the rotor. It takes some time for this difference to dissipate, and it can lead to sample convection. The default acceleration rate of the XL is 400 RPM/sec. One might consider decreasing this value somewhat if convective disturbances of the boundaries are suspected to allow more time fore re-equilibration during acceleration.

Optical Systems

The XL series of analytical ultracentrifuges come equipped with either an absorbance optical system (XL-A) or both absorbance and interference optical systems (XL-I). The absorbance optical system gives a profile of optical density as a function radius and time; the interference optical system gives a profile of refractive index as a function of radius and time. The absorbance optical system allows analysis at up to three specific wavelengths but is hampered by buffer components that may absorb at the wavelengths of interest. The interference optical system has an intrinsically higher signal-to-noise ratio (about 5 times) than the absorbance optics and is not affected by absorbing buffer components unless they absorb at the laser wavelength.

Machine Set up

With either optical system, it is desirable to take data as frequently as possible. For most methods of analysis the greatest signal-to-noise ratio is achieved with the largest number of scans.

Absorbance Optics:

The absorbance optical system is composed of a dual-beam, multiwavelength spectrophotometer than scans an image of the cell to produce a plot of absorbance as a function of radius. Up to three wavelengths may scanned in a single experiment.

It is advantageous to collect each scan in the shortest possible time consistent with the desired point density and signal-to-noise ratio. The scan time on the XL-A is dependent on rotor speed and the radial increment: shorter scan rates can be achieved at higher speeds. The scan cycle time is typically 1-2 minutes. For typical velocity runs, one should set the radial increment to 0.03 to 0.05 and average 4 flashes of the lamp.

Interference Optics:

The scan cycle time for the interference system is on the order of 5-10 seconds. It is necessary when using time derivative analysis (DCDT), for example, to gather as much data as possible to take advantage of its signal averaging features to the increase signal-to-noise ratio of the g(s*) plots. curves. Most other methods of analysis will perform better if the data are sampled as frequently as possible.

Adjust the laser timing so that the laser dwell is centered over the cell sectors or slits if you are using interference window holders. In the case of wide window holders, set the laser pulse width to 0.4° and then set the laser delay so that there is an equal angle to the edge of the sectors. For example, for a given cell, find the smallest delay angle at which the fringes just appear; then find the largest angle at which they disappear. Set the delay half way between these two values. During this process note whether the fringes disappear and reappear uniformly across the cell as these limiting delay angles are approached. If not, then the cell is not oriented correctly in the rotor hole. It is likely, if the cell is miss-aligned, that convection of the sample will occur. The run should be stopped and the cell reoriented in the rotor with careful attention to the exact orientation of the scribe lines on the cell casing with respect to those on the rotor hole. For interference slits, the laser pulse width can be set to 1.4°. It is good idea to use interference window holders fi you can find them. They act to mask off a constant piece of the cell window and make the interference fringe patterns insensitive to any jitter in the laser delay timing pulses. This especially important for proper cell blank corrections when performing equilibrium runs, but that is another topic (cf T.M Laue this series).

Cylinder Lens Alignment Check

To check the cylinder lens alignment, exactly the same solution must be present in each sector. To accomplish this, a specially modified centerpiece is required. A double sector centerpiece must be modified (i.e. sacrificed) by removing the rib between the two sectors (a coping saw carefully applied, will do the trick). Assemble the cell and fill it with 60 mg/ml bovine serum albumin (or similarly expendable protein). Accelerate the rotor to 50,000 rpm for about an hour until the boundary is about 1/3 the way to the bottom and then decelerate the rotor to about 14,000 and allow the protein to diffuse so that gradients are present throughout most of the cell. Initially, the gradients will be so steep that the light is bent completely out of the optical system; black bands will appear on either side of the boundary position and at the base of the cell. The edges of these regions represent the regions of steepest gradient possible to observe with this optical configuration. Now, if the cylinder lens is properly aligned, the fringes will be straight and level up to the point where they disappear at the point of maximum gradient. If there is any significant curvature of the fringes as they enter the region of steepest gradient, the optics are not properly aligned: call your serviceman.

Centrifuge cells:

The centerpieces normally used for sedimentation velocity analysis are the ordinary double sector type. They are usually fabricated out of charcoal filled epon or aluminum filled epon. These centerpieces are nominally rated for 40,000 RPM; however if both sectors are filled to the same level there will negligible distortion even up to 60,000 RPM. However, it is important to make sure the cells will not leak because a leak from one sector will result in collapse of the center rib and destruction of the centerpiece.

Choice of cell window material depends on the optical system in use. When using the absorbance optics, quartz windows are sufficient. However, when using the interference optics, sapphire windows are required. Sapphire windows are much less subject to compression and distortion than quartz at high centrifugal fields. Sapphire windows may be used also with the absorbance optical system as long as they are optically clear at the wavelengths used.

The run:

When the rotor temperature has equilibrated, start the run by accelerating to the desired speed; start taking data immediately so that any larger material or aggregates can be seen. If the presence of a wide range of sizes is suspected in your sample, run for a while at a relatively low speed to determine if very large particles are present. then accelerate to the selected full speed for the rest of the run.

Set the time between scans to "0" so that the machine will take scans as rapidly as possible. the actual time between scans will be determined by the CPU speed of the computer used to operate the XLA/I. Hard disk storage is very cheap today especially with the available data compression algorithms. So there is no reason not to acquire the maximum amount of data. Failure to do so may preclude certain types of analysis.

Correction of sedimentation and diffusion coefficients to standard conditions

It is common practice to correct raw sedimentation and diffusion coefficients to standard conditions of water at 20°C for the purposes of comparison with other experiments. Both density and viscosity corrections must be made to the sedimentation coefficient while only viscosity corrections made to the diffusion coefficient.

$$S_{20,w} = S_{t,b} \left(\frac{\eta_t^o}{\eta_{20}^o} \right) \left(\frac{\eta_t^b}{\eta_t^o} \right) \left(\frac{\left(1 - \overline{v}_{2,o}\rho_o\right)}{\left(1 - \overline{v}_{2,b}\rho_b\right)} \right)$$

$$D_{20,w} = D_{t,b} \left(\frac{\eta_t^o}{\eta_{20}^o} \right) \left(\frac{\eta_t^b}{\eta_t^o} \right)$$

where η_t^o is the viscosity of water at the temperature, t, of the run; η_{20}^o is the viscosity of water at 20°C; η_t^b is the viscosity of the buffer at the temperature of the run; ρ_o is he density of water at 20°C and ρ_b is the density of the buffer at the temperature of the run; $\bar{v}_{2,o}$, and $\bar{v}_{2,b}$ are the partial specific volume of the macromolecular in water at 20° and in buffer at the temperature of the run, respectively. The buoyancy correction term, $\left(1 - \bar{v}_2 \rho\right)$, is used here by tradition; it is used to represent the density increment $(\partial \rho / \partial c_2)_{T,\mu}$.

Data analysis and interpretation:

The primary data are supplied as text files containing a two line header with information about the conditions of the run followed by columns of data that are radius and concentration and sometimes a third column of standard errors of the concentration data. These concentration profile data may be analyzed by various methods to extract the hydrodynamic and thermodynamic information the investigator desires.

There are several software packages available that either transform the data into suitable visual form or that fit the profiles directly by least squares techniques.

Before we apply any of these techniques, we must make some distinction between the different types of system we are likely to encounter and then choose the appropriate type of analysis. In general, we can divide systems into either ideal or non-ideal and either interacting or non-interacting and either monodisperse or polydisperse and single component or multi-component. An example of a single component, monodisperse, ideal system would be a globular protein near it's isoelectric point at an ionic strength of 0.1 at about 1 mg/ml and not undergoing self-association. An example of a single component, polydisperse ideal system would be a monomer-dimer-tetramer self-associating system in rapidly reversible equilibrium near its isoelectric point at an ionic strength of 0.1 at about 1 mg/ml. An example of a two component, polydisperse, ideal system would be a weakly binding antigen-antibody system composed of 4 species, free antibody, free antigen, singly ligated antigen and doubly ligated antigen at an ionic strength of 0.1 at about 1 mg/ml.

Experimental Design.
Starting with a sample of unknown properties, one should perform a run with three or four cells covering a wide range of loading concentrations. In this laboratory, the usual protocol with interference optics is to start with either1 mg/ml or 0.3 mg/ml as the highest concentration and make 3-fold serial dilutions spanning a 27 fold range. With absorption optics use a starting absorbance of 1.0 A.U. This initial run will provide information about both the polydispersity and possible concentration dependence. If concentration dependence can be ruled out at this stage, the system can be analyzed as

either a mono-disperse system or a polydisperse system composed of independent species. If concentration dependence is observed, it will be a combination of either association or non-ideality or both. Association is manifest as a weight average sedimentation coefficient that increases with concentration while non-ideality is manifest as a weight average sedimentation coefficient that decreases with concentration. A non-ideal, mono- or poly-disperse system can be analyzed as the sum of non-ideal independent components. An interacting systems, which is composed of a reaction boundary, must be analyzed by taking mass action into account during sedimentation. The distinction we made above between ideal, non-ideal, interacting and independent species will determine which method of analysis must be employed. Use of DCDT-g(s*) or least-squares-g(s*) analysis would be appropriate for the initial characterization and as a way of visualizing the general behavior of the system.

Data Analysis:
There is a wide range of software available for analysis of sedimentation velocity data. The reader is referred to Table I for a list of the most commonly used software packages. The g(s*) methods are model independent and give one an idea of the range of sedimentation coefficient, number of species, their relative amounts and degree of concentration dependence. In the absence of concentration dependence, one could use any one of the curve fitting methods that use either approximate or numerical solutions to the Lamm equation to estimate number of components, sedimentation and diffusion coefficients, their relative amounts, and molecular weights. In the case of negative concentration dependent, single species, non-ideal systems, one can estimate hydrodynamic non-ideality and second virial coefficients (Solovyova, Schuck, Costenaro and Ebel 2001). For the analysis of interacting systems, Ultrascan, SEDFIT, Sedanal and ISODES_Fitter can be used for simple self-associations. For more complex interactions, ABCD_Fitter and SedAnal can be used for analysis of multi-component systems forming heterologous complexes. Sedanal can also deal with self-association of either reactants or products. SEDFIT is also useful for global fitting to data from dynamic light scattering used in conjunction with sedimentation data.

Current protocols: Sedimentation velocity

TABLE I		
Software available for analysis of sedimentation velocity data		
Package	**Author**	**Abilities**
BeckmanCoulter	various	General package for velocity and equilibrium sedimentation analysis
DCDT	Stafford(a)	Time derivative g(s*) analysis for Mac OS including multi-speed wide distribution analysis.
DCDT+	Philo(b)	Time derivative g(s*) analysis for Windows includes fitting routines for molecular weight determination.
DCDT-wd	Lary(c)	Time derivative g(s*) analysis for Windows including multi-speed wide distribution analysis.
LAMM	Behlke(d)	Direct least squares fitting to approximate solutions to the Lamm equation. Useful for low molecular weight proteins (M <20kDa) and peptides.
SEDFIT	Holladay(e)	Direct least squares fitting to concentration data to approximate solutions to the Lamm equation.
SVEDBERG	Philo(f)	Direct least squares fitting to concentration and concentration time difference data to approximate solutions to the Lamm equation for molecular weight of non-interacting systems. Useful for low molecular weight proteins (M <20kDa) and peptides.
SEDFIT	Schuck(g)	A powerful suite of procedures for the analysis for sedimentation velocity data. Lamm equation fitting, least-squares-g(s*), partial diffusion deconvolution with c(s) analysis.
SEDANAL	Stafford(h)	Least squares fitting of concentration time difference data to numerical solutions to the Lamm equation for single or multi-component, polydisperse interacting and non-interacting systems including self- and hetero- associating systems.
ABCD_Fitter	Stafford(i)	Least squares fitting to concentration and concentration time difference data to numerical solutions to the Lamm equation for two component hetero-associating systems. A+B=C; C+B=D
ISODES_Fitter	Stafford(i)	Least squares fitting to concentration and concentration time difference data to numerical solutions to the Lamm equation for a single component indefinite self-associating system.
ULTRASCAN	Demeler et al.(j)	A powerful general purpose package for the analysis of sedimentation velocity and equilibrium data.
VanHolde-Weischet	van Holde(k)	Extrapolation method for the reduction of the effects of diffusion. Useful for revealing heterogeneity in paucidiserse non-interacting systems.
SEDNTERP	Philo(l)	A general purpose tool for the interpretation of sedimentation velocity and sedimentation equilibrium experiments. Calculates partial specific volume, hydration and other parameter from the atomic composition.

References (a) (Stafford 1992) (b) (Philo 2000) (c) http://vm.uconn.edu/~wwwbiotc/uaf.html (d) (Behlke and Ristau 2002) (e) (Holladay 1979; Holladay 1980) (f) (Philo 1997; Philo 1997) (g) (Schuck 2000) (h) (Stafford 2003) (i) (Stafford 1998; Rivas, Stafford and Minton 1999) (j) http://www.ultrascan.uthscsa.edu/ (k) (Van Holde and Weischet 1978) (l) http:www.jphilo.mailway.com/download

Further reading:

The older literature contains a tremendous amount of information that is very much germane to modern day sedimentation analysis. The reader is encouraged to seek out some of the earlier classical treatises like "The theory of Sedimentation Analysis by Williams et al. (Williams, Van Holde, Baldwin and Fujita 1958),"Ultracentrifugation in Biochemistry" by Howard Schachman (Schachman 1959), "Ultracentrifugation" by S. Claesson and I. Moring-Claesson. (Claesson and Moring-Claesson 1961).

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