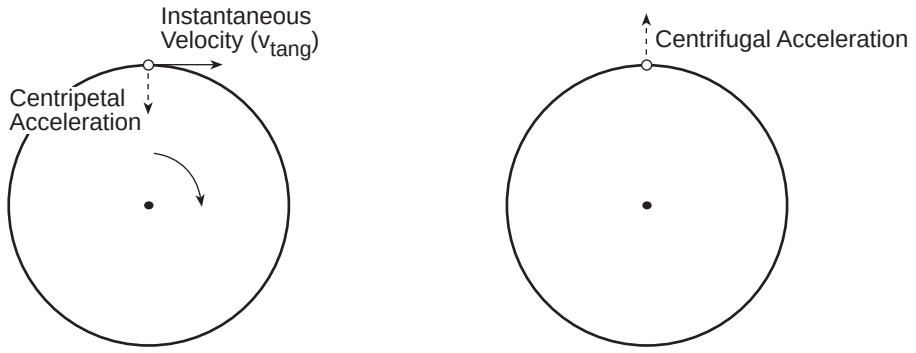
Sedimentation for Analysis and Separation of Macromolecules

A 'centrifugal force' as experienced by particles in a centrifuge actually reflects the natural tendency of the particle to move in a straight line in the absence of external forces. If we view the experiment in the (fixed) reference frame of the laboratory, we observe that the particle is deflected from a linear trajectory by a **centripetal** force (directed toward the central axis of the rotor). However, when we consider the motion of the particle with respect to the (rotating) reference frame of the rotor, it appears that at any instant the particle experiences an outward or **centrifugal** force:



As seen in laboratory reference frame

As seen in (rotating) reference frame of rotor

Ignoring buoyancy effects for the moment, the magnitude of the centrifugal force (which, in the rotating reference frame of the rotor, is as real as any other force) is

$$F_{centrif} = \frac{m_{object} \cdot v_{tang}^2}{r} = m_{object} \cdot r\omega^2 \qquad [1]$$

where r is the distance from the axis of rotation, v_{tang} is the linear velocity at any instant (in the direction perpendicular to the radial axis) and ω is the angular velocity (in radians sec^{-1} – **note that 1 rpm = 2π/60 rad sec^{-1}**). The magnitude of this force can be compared to that of the gravitational force at the earth's surface:

$$F_{grav} = \frac{G \cdot (m_{earth}) \cdot (m_{object})}{r_e^2} \qquad [2]$$

where G is the universal gravitational constant (= 6.67 x 10^{-8} g^{-1} cm^3 sec^{-2}) and r_e and m_e are the earth's radius (= 6.37 x 10^8 cm) and mass (5.976 x 10^{27} g), respectively.

Exercise 1

If a centrifuge rotor is spun at 50,000 rpm, calculate the centrifugal force experienced by a particle at radius 5 cm as a multiple of the gravitational force (= m·g, where g = $G \cdot m_e / r_e^2$ = 9.807 x 10^2 cm sec^{-2}). We will ignore buoyancy effects (discussed later).

Answer - From the above discussion we know that the centrifugal force $F_{centrif} = m_{part}r\omega^2$ where m_{part} is the mass of the particle. Thus

$$\frac{F_{centrif}}{F_{grav}} = \left(\frac{m_{part} \cdot r\omega^2}{m_{part} \cdot g} \right)$$

[3]

Canceling the mass term in the numerator and denominator, using the value of g indicated above and noting that ω (in rad sec^{-1})= (rpm x 2π)/60, we calculate that $\mathbf{F_{centrif} = 1.398 \times 10^5 \times F_{grav}}$. We would more commonly say that the centrifugal force (again, ignoring buoyancy effects) is equivalent to 139,800 x g.

In solution, the situation is more complex than one would predict from the physics of centrifugal acceleration of a single isolated particle in a vacuum. In this case at least three factors complicate the simple picture described above:

- A **buoyant force** will arise because the molecule displaces a volume of solvent equal its solvated volume (V_{solv}). The buoyant force has a sign opposite to that of the centrifugal force and a magnitude (ρ_{solv} = solvent density) given by

$$F_{buoyant} = -\rho_{solv} \cdot V_{solv} \cdot r\omega^2$$

[4]

- **Diffusion** will cause the molecule to move down its gradient of chemical potential when the chemical potential is not everywhere the same (i.e., when the system is not at equilibrium). In general the relative importance of diffusion decreases as the centrifugal force increases, but except for the largest particles it is seldom entirely negligible.
- The molecule will encounter **frictional resistance** to its motion through the solvent, the magnitude of which is given by

$$F_{fric} = -f \cdot \mathrm{v}$$

[5]

We will consider the effects of these factors in subsequent sections.

<u>Overview of the Varieties of Centrifugation Experiments</u>

Below are summarized some of the methods we will discuss in the following sections:

- **<u>Analytical boundary-sedimentation</u>** measurements and the closely related **<u>analytical zonal-sedimentation</u>** technique – These both use small quantities of material and are intended to produce accurate, reliable measurements of the **molecular sedimentation coefficient** (see below). The rotor is run at a relatively **high speed**, so that at 'infinite time' all of the molecules would accumulate at or very

near the bottom of the centrifuge cell. These methods require **analytical ultracentrifuges** with special sector-shaped cells and sophisticated optical systems that can monitor the profile of molecular concentration along the radial axis of the centrifuge cell **during the run**. Both monitor the evolution of the boundary or band as a function of time **before** the system reaches equilibrium.

- **Sedimentation equilibrium** – This method also uses a small amount of material and is intended to furnish accurate, fairly direct estimates of **molecular weight**. The rotor is run at a comparatively **low speed** to achieve an **equilibrium distribution** of the molecule along the length of the centrifuge cell, which is again measured in an analytical ultracentrifuge.
- **Zonal centrifugation in a preparative ultracentrifuge** – The sample is applied as a thin band over a **preformed gradient** of a substance such as sucrose, glycerol, etc.. This method does not provide a highly accurate estimate of the sedimentation coefficient, but with suitable calibration it can provide approximate s-values and can separate useful quantities of different molecules based on their relative sedimentation rates. A **relatively high rotor speed** is used, and the sample is not centrifuged to 'equilibrium' but simply long enough to allow the component(s) of interest to migrate partway down the length of the centrifuge tube. This technique (unlike the above methods) can be employed using an ordinary ultracentrifuge and cylinder-shaped centrifuge tubes. The distribution of material is analyzed **after the run has ended**.
- **Equilibrium density-gradient centrifugation** – The molecule(s) of interest is centrifuged into a (usually self-generating) **gradient** whose density increases along the length of the centrifuge tube. The sample is normally centrifuged **to equilibrium** but at a relatively high rotor speed (needed to generate a relatively steep gradient of density). At equilibrium the molecule will band where the local solvent density equals the **buoyant density** of the (solvated) macromolecule.

Boundary Sedimentation Measurements

This is the 'thoroughbred' of sedimentation methods: capable of yielding the sedimentation coefficient with high accuracy, but also very exacting in its requirements and prone to difficulties if these are not met. Before we consider the method itself, let us consider the migration of an individual molecule in a centrifugal field and introduce the sedimentation coefficient (s).

Basic Molecular-Level Theory of the Method

Suppose that a molecule is sedimenting in a simple aqueous buffer (without sucrose, glycerol, etc.) in a centrifugal field and that diffusion could be ignored. The steady-state velocity of the molecule would then be determined by the balance of the centrifugal and the frictional forces:

$$F_{centrif} + F_{buoyant} + F_{fric} = 0$$

$$\Rightarrow m_{part} r\omega^2 - V_{solv}^{part} \rho_{solv} r\omega^2 - f\mathrm{v} = 0$$

[6]

which can be rearranged to give the expression

$$v = \frac{(m_{part} - V_{part}\rho_{solv}) \cdot r\omega^2}{f}$$

[7]

Note that this expression combines terms that depend on intrinsic properties of the molecule (and solvent) with a term ($r\omega^2$) that depends only on the position of the molecule and the rotor speed. Since it is the molecular properties that most interest us, we can factor out the latter terms by defining a **sedimentation coefficient** $s = v/r\omega^2$, which has cgs units of sec but is often reported in Svedbergs (S), where $1\ S = 10^{-13}$ sec.

It remains to better define the expression (m_{part}-$V_{part}^{solv}\rho_{solv}$) in equation (7) above to make it more useful for computation. In a simple dilute aqueous buffer (without glycerol, sucrose, etc.), as is usually used for boundary-sedimentation measurements, V_{part}^{solv} is simply the hydrated volume V_h and $\rho_{solv} \approx \rho_w$, the density of water. We can then dip into what we have already learned about hydrated particles, using the facts that

$$\begin{aligned} m_{part} &= m(unhydrated\ particle) + m(water\ of\ hydration) \\ &= (M/N_o) + (M/N_o) \cdot \delta_w \end{aligned}$$

[8]

and

$$V_h = (M/N_o) \cdot \left(\frac{\partial V_{system}}{\partial m_{macromol.}} \right) + (M/N_o) \cdot \delta_w \cdot \left(\frac{\partial V_{system}}{\partial m_{bnd\ H_2O}} \right)$$

$$\approx (M/N_o) \cdot \left(\bar{v}_p + \delta_w / \rho_w \right)$$

[9]

where we make the approximation that the second partial-derivative term above (the partial specific volume of bound water) is equal to ($1/\rho_w$), the reciprocal of the density of bulk water at the same temperature. We can then write that

$$\begin{aligned} &\left(m_{part} - V_{part}^{solv}\rho_{solv}\right) = \left(m_{part} - V_h\rho_w\right) \\ &= (M/N_o)\left(1 + \delta_w - \rho_{solv}\left(\bar{v}_p + (\delta_w/\rho_w)\right)\right) \end{aligned}$$

[10]

and since $\rho_{solv} \approx \rho_w$ for a dilute aqueous solution, the two terms in δ_w in the above equation will effectively cancel. Thus to good approximation

$$\left(m_{part} - V_h \rho_{solv}\right) = \left(M/N_o\right)\left(1 - \rho_w \bar{v}_p\right)$$

[11]

Plugging the above expressions into equation [7], we obtain the equation

$$v = \frac{r\omega^2 \cdot M \cdot \left(1 - \bar{v}_p \rho_{solv}\right)}{N_o f}$$

[12]

This equation can be expressed in a more generally useful form if we recall that $s = v/r\omega^2$ and if we **assume** that the relevant frictional coefficient f is the same as the one we can determine from diffusion measurements ($D = kT/f$). With these substitutions (plus the fact that the gas constant $R = N_o \cdot k$) and a bit of rearrangement, we obtain the equations

$$s = \frac{M \cdot D \cdot \left(1 - \rho_{solv} \bar{v}_p\right)}{RT} \quad \Rightarrow \quad M = \frac{sRT}{D\left(1 - \rho_{solv} \bar{v}_p\right)}$$

[13a,b]

where M is the unhydrated molecular weight of the macromolecule (including any truly bound ions or prosthetic groups). Equation [13b] is known as the **Svedberg equation**.

<u>Exercise 2</u>

A protein has a sedimentation coefficient of 7.16 S, a diffusion coefficient $D = 4.45 \times 10^{-7}$ cm^2 sec^{-1} and $v_p = 0.73$ cm^3 g^{-1} (all measured at 20°C). **(a)** How long will it require to migrate from r = 10 cm to r = 10.1 cm in a rotor spinning at 50,000 rpm (5×10^4 rpm)? (Assume constant velocity throughout this time). **(b)** What is the molecular weight of the protein?

Answers: **(a)** Time = (distance migrated)/v = (0.1 cm)/sw^2r =**509 sec**. **(b)** From equation [13b], 'plugging in' the above values for D, s and v_p and using ρ_w= .9982 g cm^{-1} at 20°C, we calculate that **$M = 1.45 \times 10^5$ Da**.

In this section, which has focussed on the behavior of individual molecules, we have derived the Svedberg equation as a powerful tool to determine the molecular weights of particles from measurements of the sedimentation and diffusion coefficients. In the following two sections we will see some additional real-world complications that arise when we analyze the behavior of **populations** of molecules, and specifically of a **sedimenting boundary**, to determine experimentally the value of s for a macromolecule.

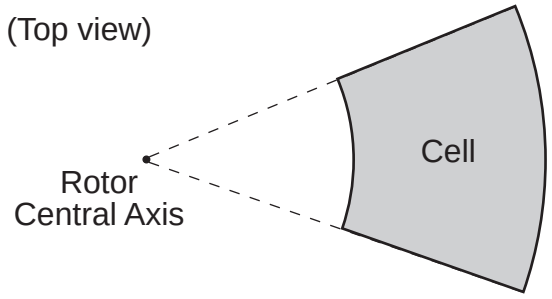
Analysis of the Migration of a Sedimenting Boundary

Suppose that we centrifuge an **initially homogeneous** solution of a macromolecule in an analytical centrifuge. We observe how the concentration profile of the macromolecule along the cell evolves during centrifugation. We can ask two questions:

- How can the experiment be set up so that the time-dependent changes in the concentration profile can yield useful, reliable information about the migration of individual molecules?
- How can the temporal evolution of the concentration profile then be analyzed to yield the value of the sedimentation coefficient s?

Let's begin with the first question. Once the centrifuge is turned on, we ideally want our molecules to be able to migrate in an unobstructed manner all the way to their final 'equilibrium' position (which typically means all piled up at the bottom of the tube). This requires that the molecules should **not** be pushed toward the **lateral** walls of the centrifuge cell by the centrifugal force. If molecules **were** pushed by this force toward the lateral walls of the cell, they could concentrate there to such a degree that they would form a solution whose density exceeded that of the surrounding medium. In this case the molecules that accumulated near the lateral walls could then move to the bottom of the cell (by convection) at rates **much faster** than would a single, isolated molecule in the bulk solution. We would then **not** be observing the sedimentation of individual molecules in the bulk solution, which is what we want to study, and the s-values determined would be useless for molecular-weight determination.

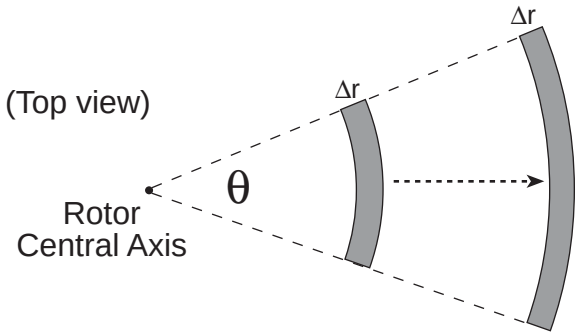
To avoid the potential problem just noted, for analytical sedimentation experiments we use a special sector-shaped cell:



Since the lateral walls of the cell run parallel to the (radially directed) centrifugal force, molecules are pushed into the walls of the cell only at the outer ('bottom') edge. For highly accurate measurements of the sedimentation coefficient, a sector-shaped cell must be used. Swinging-bucket rotors fitting 'normal' straight-walled tubes do not provide equally accurate estimates of sedimentation coefficients, in part for the reason noted above. (As an aside, fixed-angle rotors are **hopeless** for such analyses, since they are specifically designed to **promote** concentration of the particles at the tube walls in order to enhance the speed of sedimentation).

A full description of the sedimentation of molecules in a sector-shaped cell requires consideration of the following factors:

- The centrifugal acceleration as a function of the rotor speed ω and the (time-varying) distance r from the central axis of the rotor;
- The molecular sedimentation coefficient (which is constant **if** the solvent density and viscosity are constant and if there are no interactions between macromolecules);
- The tendency of the macromolecule to show net mass movement by **diffusion** in regions where there is a concentration gradient; and
- The sector-shaped geometry of the cell, which creates the phenomenon known as **radial dilution**. The effect of radial dilution can be most easily understood if we imagine that we gradually move a thin band of constant thickness Δr, containing a fixed total amount of the macromolecule, from the 'top' to the 'bottom' of the cell (inner edge to outer edge):



The volume of the region of the band is given (for small Δr) by r(θ/2π)(hΔr), where h is the height (thickness) of the cell. The volume of the region of the band would thus increase linearly with r, the distance of the band from the rotor axis, and the concentration of the macromolecule within the band would fall off as (1/r) as the band moves outward in the cell, **even if there was no broadening of the band through diffusion**.

The above factors are summarized in the **Lamm equation**, which in its general form is nasty in appearance and cannot be solved exactly. It is however instructive to study the solution of the Lamm equation in the idealized case where s is constant and diffusion is negligible. In this case this equation reduces to the form

$$\left(\partial c \big/ \partial t\right) = -s\omega^2\left(r\left(\partial c/\partial r\right) + 2c\right) \qquad [14]$$

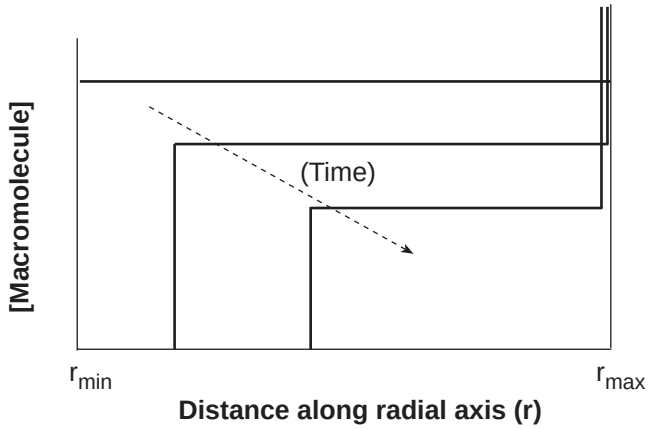
which has the solution

$$\begin{aligned} c(r,t) &= 0 && \left(r < r_{bndry}\right) \\ &= c_o \exp\left(-2s\omega^2 t\right) && \left(r > r_{bndry}\right) \end{aligned} \qquad [15]$$

where c_o is the (uniform) concentration of the macromolecule along the length of the cell at t = 0 and r_{bndry} is given by

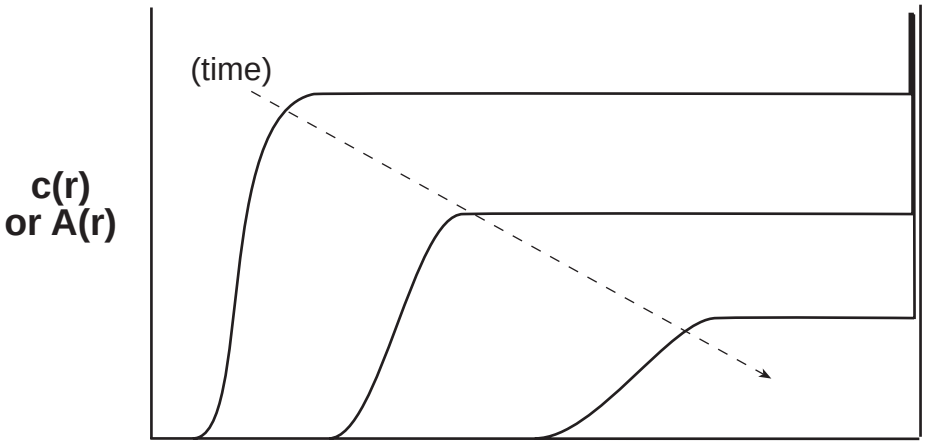
$$r_{bndry} = r_{\min} \exp\left(s\omega^2 t\right) \qquad [16]$$

The concentration profile of the macromolecule along the cell would thus vary with time during the run as shown below:



The above analysis gives us three important results, for this idealized case at least. First, as the boundary gradually migrates outward during the run, we expect to see a **plateau** in the concentration profile for $r > r_{bndry}$. Second, the height of this plateau will **decrease** with time as $c_o\exp(-2s\omega^2 t)$; analysis of the **decay of the plateau height with time** thus allows determination of the sedimentation coefficient s. (The origin of this decrease is a combination of radial-dilution effects and the gradual piling up of more and more of the total macromolecule at the outer edge of the cell.) Finally, analysis of the **position of the boundary** as a function of time provides a second potential means to determine s.

What happens when we introduce diffusion into the picture? The answer is that the moving boundary will gradually **broaden** over the course of the run (recall what happens in a boundary-diffusion measurement to determine D), but that there will still be a plateau, whose amplitude still in fact decreases as $c_o\exp(-2s\omega^2 t)$:



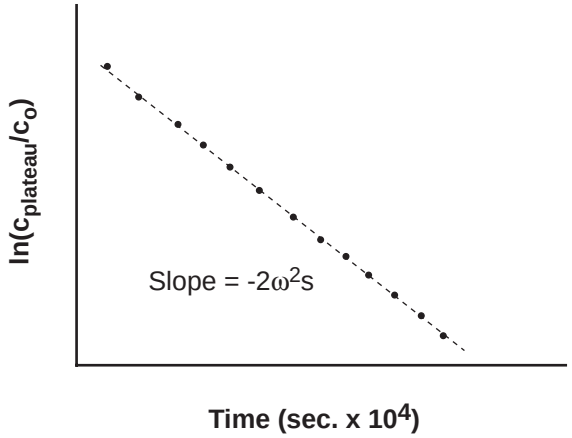
We can thus analyze the **rate of decay of the plateau concentration** to determine s:

$$c_{plateau}(t) = c_o \exp\left(-2s\omega^2 t\right)$$

$$\Rightarrow \quad \ln\left(c_{plateau}(t)/c_o\right) = -2\omega^2 st$$

[17]

Thus a plot of $\ln(c_{plateau}/c_o)$ vs. time will have slope $-2s\omega^2$ and likewise, if we are measuring absorbance, a plot of $\ln(A_{plateau}/A_o)$ will be linear with the same slope.



Exercise 3

An initially homogeneous solution of a protein is centrifuged at an angular velocity of 70,000 rpm in an analytical ultracentrifuge. At different times the absorbance profile at 230 nm is read along the length of the cell, giving the following measurements of the plateau absorbance vs. time:

Time (hr.)	0	1	2	3	5	8	12
$A_{plateau}$	2.04	1.84	1.66	1.50	1.22	0.90	0.60

From these data determine the sedimentation coefficient for the protein.

Answer: From a linear least-squares fit (or a graphical fit by eye) to a plot of $\ln(A_{plateau}(t))$ vs. time, we calculate a slope of -1.021×10^{-1} hr^{-1} = -2.835×10^{-5} sec^{-1}. Since the slope is equal to $-2s\omega^2$, we can readily calculate that **s = 2.64 x 10^{-13} sec, or 2.64 S** (note again that ω (in rad sec^{-1}) = $(2\pi/60)\cdot$(rpm) = 7.330×10^3 rad sec^{-1}. Cantor and Schimmel have a nasty habit of giving ω in rpm, which is useless for calculations if not outright wrong).

Can we also analyze the **position of the boundary** to determine the sedimentation coefficient when diffusion is occurring? The answer is a qualified yes. The qualification lies in how we define 'the' boundary when the boundary is being broadened by diffusion. We might initially guess that 'the' position of the boundary could be defined by the inflection point, i.e., the point where the concentration changes most rapidly as a function of distance along the radial axis. It turns out, however, that this is only approximately correct, and estimates of s based on measuring the position of the inflection point with time will be slightly but significantly in error. The correct approach is to plot the parameter r as a function of time, where

$$\bar{r} = \left(\frac{1}{c_{plateau}} \int_{r_{\min}}^{r_{\max}} (\partial c / \partial r) r^2 dr \right)^{1/2} \qquad [18]$$

This is a somewhat formidable expression but one that can be readily evaluated from the measured concentration profile along the centrifuge cell; it typically lies at a slightly higher r-value than the inflection point.

Nonideal Behavior in Boundary-Sedimentation: Speed-dependent Effects

The Svedberg equation assumes that s is speed-dependent, and that the value of the frictional coefficient f for the molecule is identical in a centrifugation experiment and in the parallel diffusion experiment used to determine f experimentally (as kT/D). This is a correct assumption **only** if these conditions are fulfilled:

(1) During its sedimentation in the centrifuge the molecule must be (on average) **randomly oriented** in space, just as in a diffusion measurement. If a nonspherical molecule is **not** randomly oriented during a centrifugation experiment (i.e., if it spends more time in some orientations than in others), it may exhibit an average frictional resistance during sedimentation that is **different** from that which it exhibits during a simple diffusion measurement. An extreme example would be the case of an elongated particle. During sedimentation at high rotor speeds, such a molecule may show significant **preferential alignment** along the **r**-axis and move to the bottom of the cell like a 'javelin.'

(2) The molecule must have the **same (average) conformation** during the sedimentation and the diffusion measurements. Application of excessively large centrifugal forces to long, flexible molecules (e.g., long ds-DNAs) can cause them to adopt a more extended conformation than they would in the absence of the centrifugal force. This can provide a second source of error in our above assumption that the frictional coefficient in a sedimentation experiment has the same magnitude as that in a diffusion experiment.

(3) A corollary (and fairly obvious) assumption related to (2) above is that the molecule must exist in the **same state of oligomerization** in the sedimentation and diffusion experiments. Thus for example we must be sure that the molecule does not exist as a monomer under the conditions of the diffusion experiments and an oligomer under the conditions of the sedimentation experiments. For this reason it is usually desirable to use the same aqueous buffer and a similar protein concentration for both types of measurements.

The effects described in (1) and (2) above can be negligible for relatively small symmetrical macromolecules, such as globular proteins, but are often very significant for large nucleic acids, long protein fibrils, elongated viruses, etc.. In these latter cases it is important to measure the sedimentation coefficient s at several different rotor speeds and to **extrapolate s to zero speed**. There is no universal formula for such an extrapolation; instead one typically determines s at lower and lower rotor speeds until the measured s-value levels off at a constant value. When this is not possible, one can usually find an empirical formula for the form of the speed dependence which is appropriate for the specific type of molecule/particle of interest and which can be used as the basis for an extrapolation to zero speed.

Nonideal Behavior in Boundary-Sedimentation: Concentration-dependent Effects

There are two major ways in which the rate of sedimentation of an individual macromolecule (we'll call it **M**) can be **perturbed** by the presence of other macromolecules:

(1) The presence of other macromolecules in the immediate vicinity can increase the **local viscosity** and thereby reduce the rate at which the molecule **M** sediments.

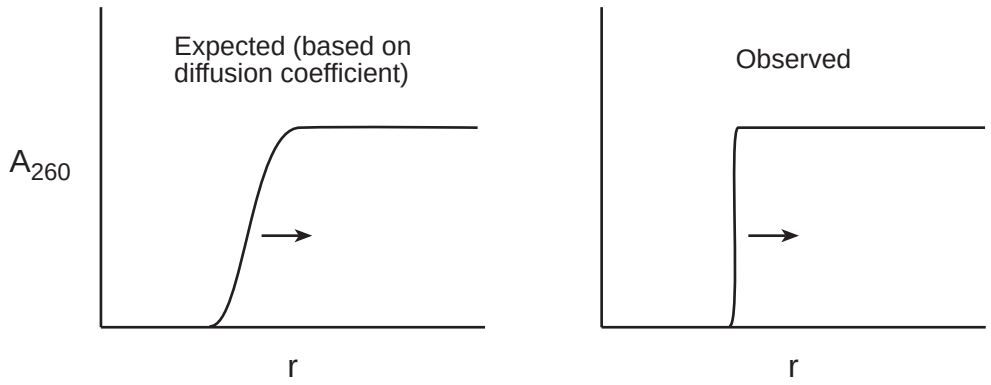
(2) The sedimentation of other macromolecules immediately 'ahead of' molecule **M** leads to a **displacement of solvent**, which must flow back along the cell (i.e., in the **–r** direction). This backflow acts like a local 'current' flowing against the direction of motion of **M**, and it thereby slows the rate at which **M** sediments.

These effects have two important consequences. The first is that the sedimentation coefficient we measure for any given species (even if no other macromolecular species are present) can **vary with the concentration** of that species. It is in fact common to **correct** measured s-values to **infinite dilution** to factor out such effects. Experimentally, this is done by making boundary-sedimentation measurements for several samples containing different initial concentrations c_o of the macromolecule. The resulting data are fitted to an equation that is usually of the form

$$s = s^o/(1+kc) \qquad \textbf{[19]}$$

where k is an empirical fitting constant and s^o is the 'true' sedimentation coefficient of the macromolecule at infinite dilution. A plot of (1/s) vs. concentration c_o will thus be linear with slope = kc, and extrapolation back to c = 0 yields the y-intercept ($1/s^o$).

Interactions between macromolecules can also affect the shape of a sedimenting boundary. This is not necessarily a problem for analyses like those described above, but it has one consequence that is worth noting when boundary-sedimentation experiments are undertaken for very long ds-DNA's. Since these species are typically very open/extended structures, intermolecular interactions can become significant even at relatively low concentrations. This leads to pronounced **sharpening** of the sedimenting boundaries for these species:



Why does this happen? The answer is that because of the intermolecular interactions just noted, the DNA molecules in the region of **higher concentration** (i.e. those in the plateau and the 'leading' edge of the boundary) will migrate significantly **slower** than those found where the local DNA concentration is lower (i.e., in the 'trailing' portion of the boundary). Thus the closer to the trailing edge of the boundary a DNA molecule is found (and hence the lower is the concentration of DNA in its immediate environment), the **faster** on average it will be sedimenting. The DNA molecules in the **trailing** (low-DNA-concentration) region of the boundary therefore sediment **faster** than those in the leading (high-DNA-concentration) region, and the boundary will become sharpened by this constant tendency of the DNA molecules in the rear to 'catch up and close the ranks.'

When interactions like those described above occur between macromolecules in a **mixture**, the sedimentation of any given species can affect that of other species in regions of the cell where they are present together. These effects can be both significant and complex (see for example the description in Cantor and Schimmel of the **Johnston-Ogston effect**, in which interactions between two or more species perturb the relative heights of the boundaries the different components create). The simple take-home message is that boundary-sedimentation analyses for multicomponent systems can become quite messy.

Correction of the Sedimentation Coefficient to Standard Conditions

We have seen that the sedimentation coefficient can be affected by speed- and/or concentration-dependent nonidealities in behavior of the macromolecule. These factors need to be corrected for by (a) determining s at decreasing rotor speeds until the value of s becomes speed-independent, and (b) determining the value of s at multiple initial concentrations of the macromolecule and extrapolating to zero concentration using equation [19] to give the 'infinite-dilution' value s^o.

Measured s-values are also functions of the solvent viscosity and density, which may in turn depend on the experimental temperature. When reporting s-values, it is desirable to express the value of s that would be obtained under **standard conditions**, which by convention are taken to mean 20°C and a solvent with the density and viscosity of water at this temperature. The appropriate formula to correct s-values to $\mathbf{s_{20,w}}$ values is as follows:

$$s_{20,w} = s_{\exp} \cdot \frac{1-\bar{v}_p \rho_{w,20^oC}}{1-\bar{v}_p \rho_{solv,T_{\exp}}} \cdot \frac{\eta_{w,T_{\exp}}}{\eta_{w,20^oC}} \cdot \frac{\eta_{solv}}{\eta_w}$$

[20]

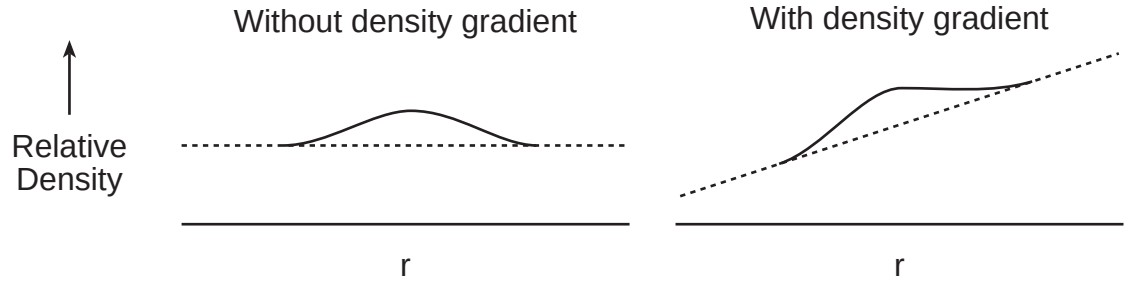
where s_{exp} is the value of s measured under the actual experimental conditions (temperature T_{exp}, solvent whose density at T_{exp} is $\rho_{solv,Texp}$ and whose viscosity relative to that of water at the same temperature is η_{solv}/η_w). In this equation $\eta_{w,Texp}$ and $\eta_{w,20oC}$ represent the viscosity of water at T_{exp} and at 20°C, respectively, and $\rho_{w,20oC}$ represents the density of water at 20°C (= 0.9982 g cm^{-3}).

Reporting s-values under standard conditions is always desirable, but it is perhaps most important when we wish to use the Svedberg equation (combining data from sedimentation and diffusion measurements) in cases where the diffusion and sedimentation coefficients were determined under different conditions. So long as we can assume that the molecular weight and the shape of the macromolecule are the same under the two conditions, we can then convert the experimental s- and D-values to $s_{20,w}$ and $D_{20,w}$ and use the latter values in the Svedberg equation.

Analytical Zonal-Sedimentation Measurements

The interference by different species in a boundary-sedimentation experiment arises because with the exception of the slowest-moving component, each component is moving through regions where other macromolecular components are also present. This difficulty could be overcome if the experiment could be set up so that at any given time different macromolecules were sedimenting in different regions. It might immediately occur to you that this would be the situation if the different species were migrating in different **bands**. Why not therefore simply carry out a sedimentation-velocity measurement in which the sample is initially layered as a thin band at the 'top' (i.e., next to the inner edge) of the cell and the rest of the cell is filled with a simple aqueous buffer?

The experimental setup just proposed has an inherent problem, although as we will see shortly, there is also a potential remedy. A sedimenting band of a macromolecule is of course a region of (aqueous medium + macromolecule) surrounded by medium alone and hence, if the medium is everywhere the same, has a slightly elevated density:



As a result, if the aqueous medium is the same throughout the centrifuge cell, a migrating band will have a **slightly higher density** than does the immediately adjacent medium on either side, as illustrated in the left-hand panel above.

The **relative** density differences shown above can be only a very small fraction of the **absolute** density. Nonetheless, under the enormous g-forces generated in an ultracentrifuge, even a tiny local density inversion (i.e., a region where $(d\rho/d\mathbf{r} < 0)$ will lead to convective movement of material. (As a crude analogy, imagine what would happen if you attempted to layer a 5% sucrose solution over water). This can compromise the integrity of the band and/or artifactually enhance the rate at which the band moves down the cell.

The solution to the above problem is to cause the band to sediment through a **density gradient** which is sufficiently steep that, as shown in the right-hand panel above, the presence of a band of sedimenting macromolecules does not produce a local density inversion. Sucrose and glycerol are commonly used to create such gradients in **preparative** applications or for approximate determinations of the sedimentation coefficient, as we will discuss later. However, for **accurate** determination of s, it is most useful to sediment the macromolecules through a medium where the buoyancy factor $(1-\rho_{solv}v_p)$ and the medium viscosity are essentially constant throughout the entire cell. How do we resolve this apparent dilemma?

To solve this problem, we note that at the concentrations of macromolecules used in analytical sedimentation experiments, a migrating band of a macromolecule will create only a **very small** increase in the local density, which even a **very shallow** gradient of density of the medium can suppress. The usual way to generate a very shallow gradient of density along the cell is to apply the sample as a thin band in low-ionic-strength buffer over an aqueous medium containing 2 M NaCl (or CsCl). At the beginning of the run we will thus have a narrow zone of low salt concentration at the 'top' (inner edge) of the cell and a steep boundary between this region and the rest of the cell, which is filled with 2 M salt. With time during the run, diffusion of the salt will gradually cause this boundary to flatten out but also to **extend** toward the bottom of the cell. It turns out that after a brief period at the start of the run, the macromolecules will be migrating in a region where there exists a **very shallow gradient** of salt concentration (near, but a bit less than, 2 M in salt). In these conditions the local gradient of salt concentration, and hence of medium density, is large enough to stabilize bands of macromolecules but **not** large enough to perturb their s-values significantly. Moreover, the rate of spreading of the salt gradient is fast enough that the macromolecules can sediment for almost the entire run under these favorable conditions. This is known as **analytical zonal centrifugation** – 'analytical' because the method gives highly accurate s-values. This technique has been most commonly used for very large nucleic acids, which can give the most trouble in boundary-sedimentation experiments due to intermolecular interactions. (You should note that sucrose, glycerol and other agents that are typically used to generate **preparative** density gradients are not generally useful for **analytical** zonal centrifugation).

The Sedimentation-Equilibrium Method

A quite different approach to estimation of the molecular weight of a macromolecule is based on the fact that at relatively low rotor speeds, at equilibrium the molecule may not pile up completely at the bottom of the centrifuge cell but instead may be distributed with a gradually increasing concentration from the 'top' to the 'bottom' of the cell. The latter situation arises when both sedimenting forces and diffusion exert a significant influence over the distribution of the macromolecule within the centrifuge cell.

To predict the **equilibrium** distribution of a macromolecule in a spinning rotor, we begin by noting that the condition of equilibrium implies that the chemical potential of the macromolecule is the same at all points along the cell. We will assume ideal behavior of the macromolecule (i.e., that there are no interactions between macromolecules). Then to move a single molecule from a position r_1 to another position r_2 along the radial (**r**-) axis requires that we do work equivalent to the integral of the centrifugal force along the radial axis from r_1 to r_2;

$$\begin{aligned} Work(r_1 \rightarrow r_2) &= -\int_{r_1}^{r_2} (M/N_o)(1-\bar{v}_p\rho_{solv})r\omega^2 dr \\ &= (M/N_o)(1-\bar{v}_p\rho_{solv})(\omega^2/2)(r_1^2 - r_2^2) \end{aligned}$$

[21]

The total change in free energy accompanying the transfer of a single molecule from position r_1 to position r_2 along the radial axis is given by

$$\Delta\mu(r_1 \rightarrow r_2)/N_o = (1/N_o)\left(RT\ln c_2 - RT\ln c_1 + M(1-\rho_{solv}\bar{v}_p)(\omega^2/2)(r_1^2 - r_2^2)\right)$$

[22]

where $\mu^o(r)$ and c(r) are respectively the chemical potential and the concentration of the macromolecule at position r. However, at equilibrium the chemical potential for the macromolecule must be the same everywhere along the centrifuge cell. We can then set the left-hand side of equation [22] to zero and rearrange to give the distribution of the macromolecule along the length of the cell at equilibrium in the spinning rotor:

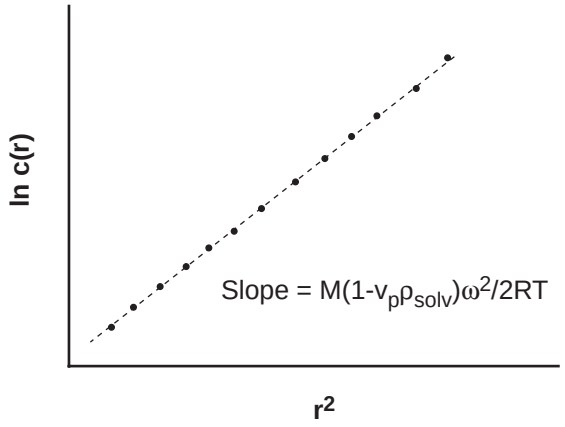
$$\ln\left(c(r_2)/c(r_1)\right) = \left(\frac{M(1-\rho_{solv}\bar{v}_p)\omega^2}{2RT}\right)\cdot(r_2^2 - r_1^2)$$

[23]

Again, note that we are assuming here that the molecule is behaving 'ideally' (i.e., that the thermodynamic activity of the molecule is equal to its concentration), a condition which is most easily fulfilled when the concentration of the macromolecule throughout the gradient is relatively low. (If you do not like chemical potentials, an alternative derivation of equation [23] based on the Boltzmann equation can be found in Freifelder.)

Equation [23] is extremely useful because it can be used to determine the molecular weight of a molecule (or particle) without requiring additional information from techniques such as diffusion measurements. This equation tells us that a plot of ln(c) vs. r^2 will be linear and have a slope equal to

$$\frac{M\left(1-\bar{v}_p\rho_{solv}\right)\omega^2}{2RT} \qquad \textbf{[24]}$$



The sedimentation-equilibrium method has the important advantage that it can yield molecular weights without the need for independent data to determine the frictional coefficient (since this is an **equilibrium** method, the frictional coefficient does not influence the results – so long, of course, as we wait long enough to be sure that true equilibrium is reached). A limitation of the technique is that it can be difficult to apply to mixtures, even to mixtures of different oligomeric forms of the same species. The reason is that in this technique the distribution of a given macromolecule along the centrifuge cell exhibits no sharp, well-localized feature like a band or boundary – there is only a smooth, relatively gentle variation of concentration with position. When two or more species are present their distributions thus tend to blur together, and resolving the different components can be very difficult unless their molecular weights are very different and only a few species are present.

Exercise 4

A protein is centrifuged at a rotor speed of 12,000 rpm to equilibrium at 4°C in a dilute aqueous buffer (assume the density is the same as that of water). The measured absorbance profile over part of the cell (assume that the absorbance is suitably blanked, so that A ∝ [protein]) over part of the cell is tabulated on the next page. If the partial specific volume of the protein is 0.740 $cm^3\ g^{-1}$, what is the molecular weight of the protein?

r (cm)	5.00	5.05	5.10	5.15	5.20	5.25	5.30	5.35
A_{230}	0.263	0.341	0.442	0.576	0.752	0.985	1.292	1.700

Answer: First we plot $\ln(A_{260})$ vs. r^2 and determine the best-fit line either by eye or by a linear least-squares analysis. This yields a slope of 0.**51517 cm^{-2}** (we are carrying extra significant digits for intermediate calculations). Using R = 8.314 x 10^7 erg mol^{-1} (^{o}K)$^{-1}$, where 1 erg = 1 g cm^2 sec^{-2}, a density for water of **1.0000 g cm^{-3}** at 4^{o}C (from the data table) and calculating that ω = (1.2 x 10^4 rpm)($2\pi/60$) = **1.25664 x 10^3 rad sec^{-1}**, from equation [24] above we calculate that **M = 57,800 Da** (57,825 Da before rounding to an appropriate number of significant digits).

Density-gradient Centrifugation in the Preparative Ultracentrifuge

We have already touched on the use of density gradients to stabilize bands against density inversions and consequent convective mixing of materials during analytical rate-zonal centrifugation. However, this is not the most common application of density gradients in ultracentrifugation. Most biochemical researchers have routine access only to **preparative** ultracentrifuges (the type found in our department, for example), which use straight-walled rather than sector–shaped cells and which do not permit the concentration profile of the macromolecule(s) to be monitored during the course of the run. Using preparative ultracentrifuges, it is not possible to apply either the boundary-sedimentation method (which requires that we monitor the migration of the boundary **during the run**) nor the sedimentation-equilibrium method (in which we must determine the gradient of the macromolecule **while the rotor is spinning**) in order to determine the s-values or molecular weights.

Does this mean that preparative ultracentrifuges are useless for analyzing the sizes or sedimentation coefficients of macromolecules? The answer is no, **if** we are willing to use a modified approach that yields only **approximate** sedimentation coefficients. In this approach we apply the sample as a **thin band** at the top of a preformed **density gradient**, and we centrifuge for a predetermined time which is sufficient to cause the different particles to migrate partway down the gradient. We then stop the run and carefully remove and analyze the contents of the tube to reconstruct the distributions of the different species along the gradient. From the positions of the bands of the different species we can attempt to estimate their s-values.

Way must we use a gradient for such analyses? There are several answers to this question:

- The most basic answer, to stabilize bands against density inversions and convective mixing, was already described in the section on analytical zonal-sedimentation measurements. Under the conditions used in this method, a **very shallow** gradient can be sufficient to stabilize bands during the run itself.

- By contrast, for preparative work and to stabilize bands against not only density inversions but also vibrations, etc. that may occur during handling **after the run has ended**, a steeper gradient of density is required.
- Additionally, since the tubes used in a preparative swinging-bucket rotor are straight-walled along most of their length, the lines of centrifugal force during the run are **not** strictly parallel to the lateral walls of the tube. As a result, macromolecules can tend to be 'pushed' toward the walls where they will accumulate to higher concentrations than in the bulk solution. As we have already noted, the resulting higher concentration of macromolecules at the walls of the tube can lead to **density inversion** and **artifactually rapid sedimentation** of the macromolecules. The use of a sufficiently steep density gradient can overcome this problem by avoiding density inversions even if the macromolecule becomes more concentrated near the walls of the tube.

Gradients of species such as sucrose, glycerol, polymers such as Ficoll and relatively dense molecules like Metrizamide are commonly used in preparative ultracentrifuges. These agents typically change the **viscosity** as well as the **density** of the medium.

I have stated already that highly accurate sedimentation coefficients are difficult to obtain for species separated on a density gradient in a preparative ultracentrifuge. There are several reasons for this:

- In a preparative run we only have two 'time points,' the time before the run begins and the time at which we stop the run and analyze the distribution of materials along the length of the tube. (To complicate matters still more, note that bands can tend to "smear out' to some degree when we recover fractions from the gradient, no matter how careful we may be).
- The exact time of centrifugation is somewhat difficult to define precisely, since a nontrivial fraction of the total length of the run is required for acceleration and deceleration of the rotor.
- The sedimentation coefficient can be a function of position in the gradient, since the local density and viscosity typically vary along the length of the gradient. This problem can be overcome by constructing an **isokinetic gradient** such that the velocity (though not the s-value) of the molecule will be the same all along the length of the tube.

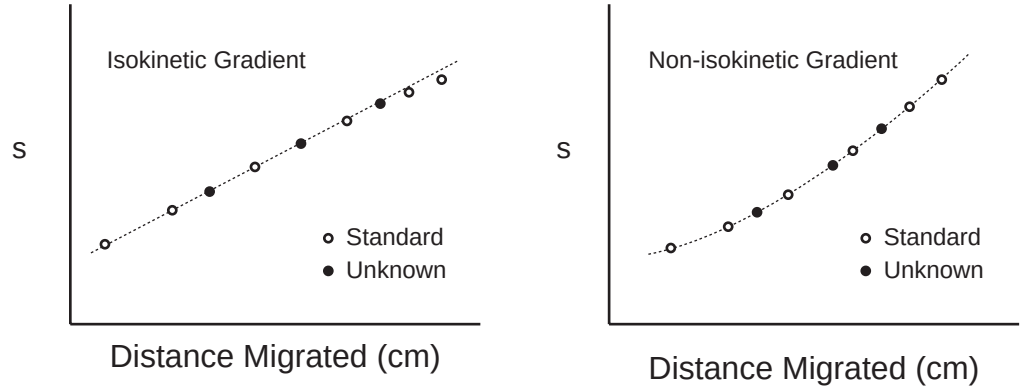
  Using equation [7] for the velocity of a sedimenting macromolecule and substituting $(6\pi\eta_{solv}r_hF)$ for f in this equation, we can readily obtain the condition for an isokinetic gradient:

$$v = \frac{r\omega^2 M\left(1-\rho_w \bar{v}_p\right)}{N_o\left(6\pi\eta_{solv} r_h F\right)} = (\text{constant})$$

$$\Rightarrow \frac{r\left(1-\rho_w \bar{v}_p\right)}{\eta_{solv}} = (\text{constant}) \qquad [25a,b]$$

where to focus on the essentials we have we have derived equation [25b] from [25a] by factoring out all the quantities that are not dependent on the position of the macromolecule on the gradient. The above expression is only approximate, since it is based on a hidden (and usually incorrect) assumption that water and the density-perturbing agent (sucrose, etc.). interact identically with the macromolecule. Note again that on an isokinetic gradient a given macromolecule does **not** exhibit the same value of s at all positions along the gradient, but rather simply the same **velocity** at all positions.

To circumvent the above complications, estimates of s using a preparative ultracentrifuge are usually obtained by running one or more macromolecules of interest on an appropriate density gradient along with **standards** with known s-values and having a similar composition. On an isokinetic gradient, the relationship between s and the distance migrated during the run will be fairly close to linear, while a more nonlinear relationship will be observed if the gradient is not isokinetic:.

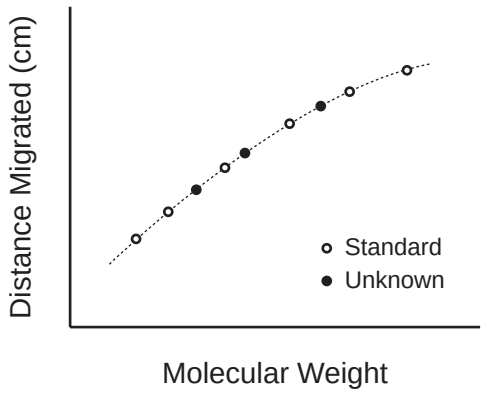


At the end of the run, the positions of the different bands are determined and the s-values of the unknowns determined by interpolation. Note that 'the' s-values of the reference standards are of course defined **for a particular set of solvent and temperature conditions** (e.g., $s_{20,w}$), and that these conditions are typically not the same as those actually used when we run our (standards + unknowns) on a density gradient in the preparative ultracentrifuge. If we want to report our results for a particular species, we therefore have two choices:

(1) We can report 'the' s-value itself, with the understanding that it is only a **relative** quantity. To illustrate this, suppose that the standards on the gradient shown above are RNA's whose sedimentation coefficients **under some standard condition** (temperature/solvent) are 11.3S, 14.5S, 17.1S, etc.. We can then report that by interpolation as shown above, our unknown RNA's migrate as '15.7S,' '18.6S' etc., **where it is understood that these s-values are defined with respect to those of the standard species**. The absolute values of s for the standard species are in turn defined for some specific set of reference conditions, **not** those of the gradient run we carried out. (To help to remember this, you can just remember for example that s is **not constant** on the density gradients we use in preparative ultracentrifuges).

(2) If we are comparing the sedimentation rates of standard and unknown molecules of the same type and composition, it may be safe to assume that both s and D vary in some

consistent manner with the molecular weight (this is reasonable for example for a set of long ds-DNA's, a set of denatured RNA's, a set of denatured DNA's, etc.). In this case we may plot the distances migrated by the standard species as a function of molecular weight and estimate the molecular weights of the unknowns by interpolation:



In this case it is important to remember that we must compare 'apples to apples,' i.e. to compare standards and unknowns of the same basic type and for which the shape and the sedimentation and diffusion coefficients vary in a very similar, **systematic** manner with molecular weight. This approach would not be very successful if applied for example to a series of globular proteins that all had very different shapes.

Equilibrium Density-gradient Centrifuugation

The last application of density gradients that we will consider is relatively rigorous and can be used for both analytical and preparative purposes; it consists of centrifuging macromolecules **to equilibrium** as bands on a suitably constructed gradient. To determine the condition for this to occur, we recall that the average velocity of a macromolecule in a centrifugal field is given by

$$v = \frac{r\omega^2\left(m_{part} - V_{part}^{solv}\rho_{solv}\right)}{f} \qquad [7]$$

In the bracketed term 'the' mass of the particle includes that of the associated water, ions, etc., and likewise for 'the' solvated volume of the particle. Under the conditions of a typical equilibrium density-gradient centrifugation (where the medium is **not** a dilute aqueous solution), we **cannot** rewrite this term as a simple function of the anhydrous molecular weight, the hydration factor and the partial specific volume. Instead we simply define the **buoyant density** of the particle as (m_{part}/V_{part}^{solv}). The particle will then band in the density gradient where its average velocity of sedimentation becomes zero, i.e., at the point where the buoyant density is equal to the local density of the medium, ρ_{solv}.

You will recall that equilibrium density-gradient centrifugation was used in the **Meselson/Stahl experiment** to demonstrate the semiconservative nature of DNA replication (remember ^{15}N- vs. ^{14}N-labeled strands and all that? The equilibrium density-gradient method is still used mainly for nucleic acids, typically with gradients of **cesium chloride**. The gradient is self-generating when a concentrated solution of CsCl is spun at high rotor speeds, making the method relatively convenient as well.

Historically, equilibrium density-gradient centrifugation was frequently used to separate species on the basis of their relative contents of (G+C) vs. (A+T) bases – the higher the relative (G+C) content, the higher the buoyant density. Cesium gradients have also been commonly used in conjunction with **ethidium bromide**, which intercalates into double-stranded nucleic acids (thereby perturbing their buoyant density) to different extents depending on their secondary structure (base-pairing and supercoiling). It is thereby possible to separate not only RNA from DNA (based on their intrinsically different buoyant densities) but also for example negatively-supercoiled from relaxed forms of ds-DNA (based on their differential binding of ethidium and consequent differences in their buoyant densities in an ethidium-containing medium). Until recently CsCl/ethidium gradients were the method of choice for purification of supercoiled (and hence un-nicked) plasmid DNA, although column-adsorption methods are now more widely used for preparative purposes.

A Final Note

In the above discussion we have consistently spoken of particles **sedimenting**, which will occur when the buoyancy factor $(1-\rho v) > 0$. However, under conditions where the buoyancy factor is **negative**, we will observe **flotation** of particles (migration in the **-r** direction). This effect is in fact exploited for centrifugal analyses of lipid-rich or other low-density particles, which migrate 'upward' (toward negative **r**-values) when centrifuged after initially layering **under** gradients of sucrose or even concentrated salt solutions. The principles discussed above for sedimentation are equally valid for particle flotation (just note the appropriate sign changes!).