Analytical Ultracentrifugation (AUC)

Theodor “The” Svedberg, University of Uppsala, Sweden. 1884-1971
Inventor of the Analytical Ultracentrifuge
Nobel Prize in Chemistry for “work on disperse systems” in 1926

Swedish physical chemist Theodor "The" Svedberg studied the chemistry, distribution, light absorption and sedimentation of colloids and molecular compounds. In 1923 he invented the analytical ultracentrifuge, a high-speed centrifuge used to measure molecular weight of biopolymers.

AUC Background

Analytical Ultracentrifugation:

- **A solution-based separation technique used to characterize molecules on the nanoscale**
- **Informs about hydrodynamic radius, mass, globularity, and density**
- **Provides information about interactions between molecules**
- **AUC is a first-principle measurement technique that does not need references (provided the instrument is working properly)**
- **Widely used in biomedical and material science applications**
- **Different optical systems allow detection and characterization of virtually any colloidal molecule**
- **Essential for Biopharma to characterize injectables/liquid pharmaceuticals**

AUC Background

What can be learned from AUC?

- **Molecules can be studied in <u>a physiological environment</u> – solution conditions can be adjusted (concentration dependency, effect of pH, ionic strength, buffer type, ligands, oxidation state, temperature, etc.)**
- **<u>Very large</u> size range (10^2 – 10^8 Dalton), rotor speed is adjustable**
- **<u>Dynamics and Interaction Analysis</u> - measure polymerization and molecular growth of self- or hetero-associations, ligand binding, slow kinetics and Kd**
- **<u>Composition analysis</u> – purity, number of components, their partial concentration, molecular weight, density, and globularity**
- **<u>Conformational analysis</u> - folding/melting studies of biopolymers, conformational changes based on changes in solution properties**

AUC Background

9 things to remember about AUC:

What does it measure?

AUC measures 3 things:

1) **Sedimentation Coefficients (s)**
2) **Diffusion Coefficients (D)**
3) **Concentration**

AUC Background

9 things to remember about AUC:

Using centrifugal force, AUC will separate molecules in solution based on:

4) **Size**
5) **Anisotropy**
6) **Density**

AUC Background

<u>9 things to remember about AUC:</u>

How does it detect molecules?

By using 3 different optical systems:

7) UV-visible absorbance (200-800 nm)
(can also be used in MW-AUC mode)

8) Refractive Index (Rayleigh Interference)

9) Fluorescence Emission

AUC Applications

What types of systems can be characterized?

Short answer: Anything colloidal!

- **Biopolymers: Proteins, nucleic acids, lipids, carbohydrates**
- **Nanoparticles: LNPs, silica, synthetic polymers, metal NPs, carbon nanotubes, quantum dots, etc…**

What types of questions can be answered?

- **Composition: Purity, aggregation/degradation, particle sizing**
- **Thermodynamics: mass action, oligomerization, k_d, k_{off}, temperature**
- **Interactions: protein-protein/nucleic acids/nanoparticles, complex formation, stoichiometries, ligand binding, binding strength**
- **Vector cargo loading: viral vectors, LNP vaccines**
- **Solvent effects: pH, ionic strength, redox state, small molecules**
- **Conformation: IDPs, unfolding, anisotropy, allosteric changes**
- **Density: partial specific volume distributions (cargo loading)**

AUC Background

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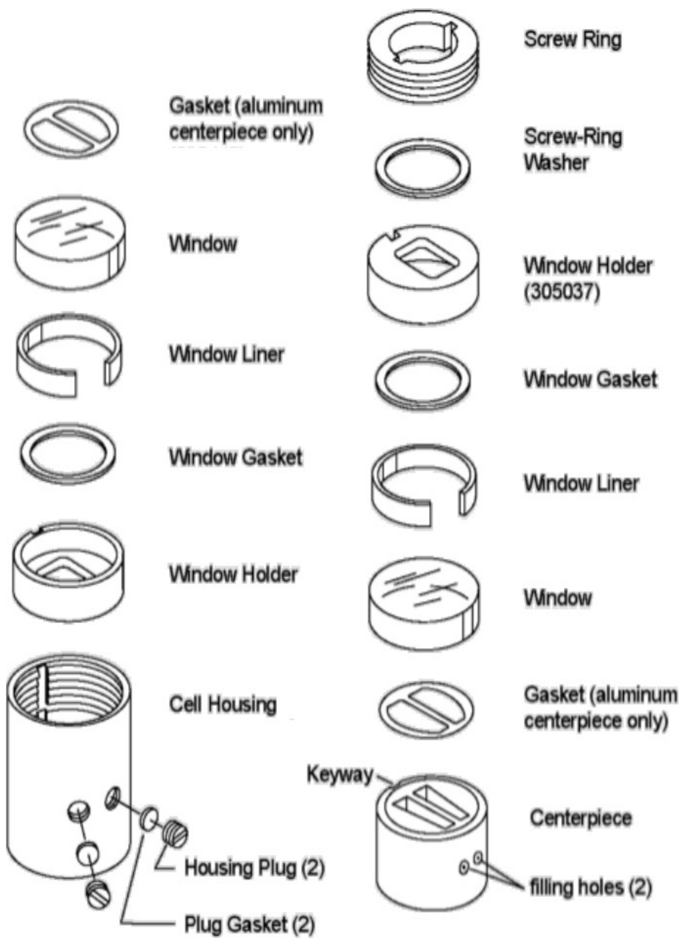
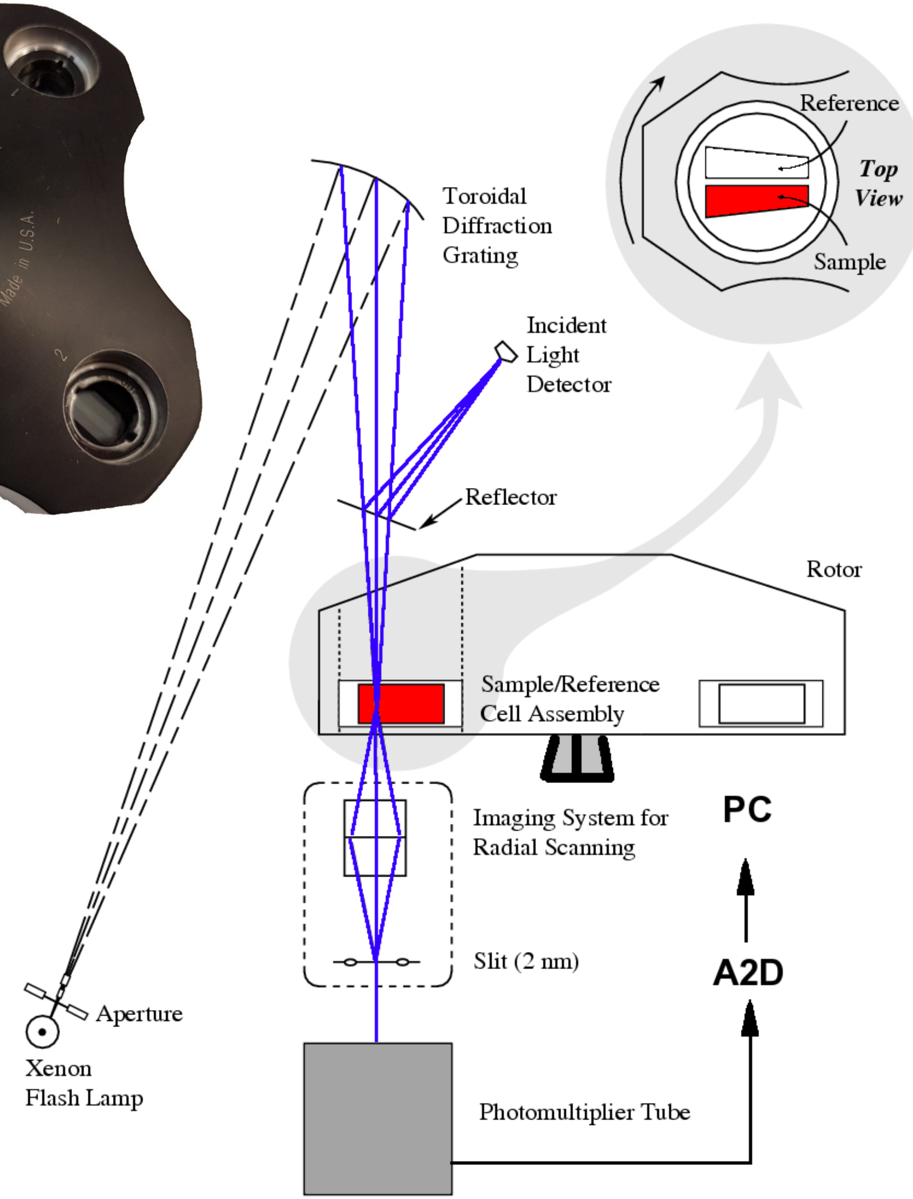
AUC Instrumentation

Beckman Optima AUC

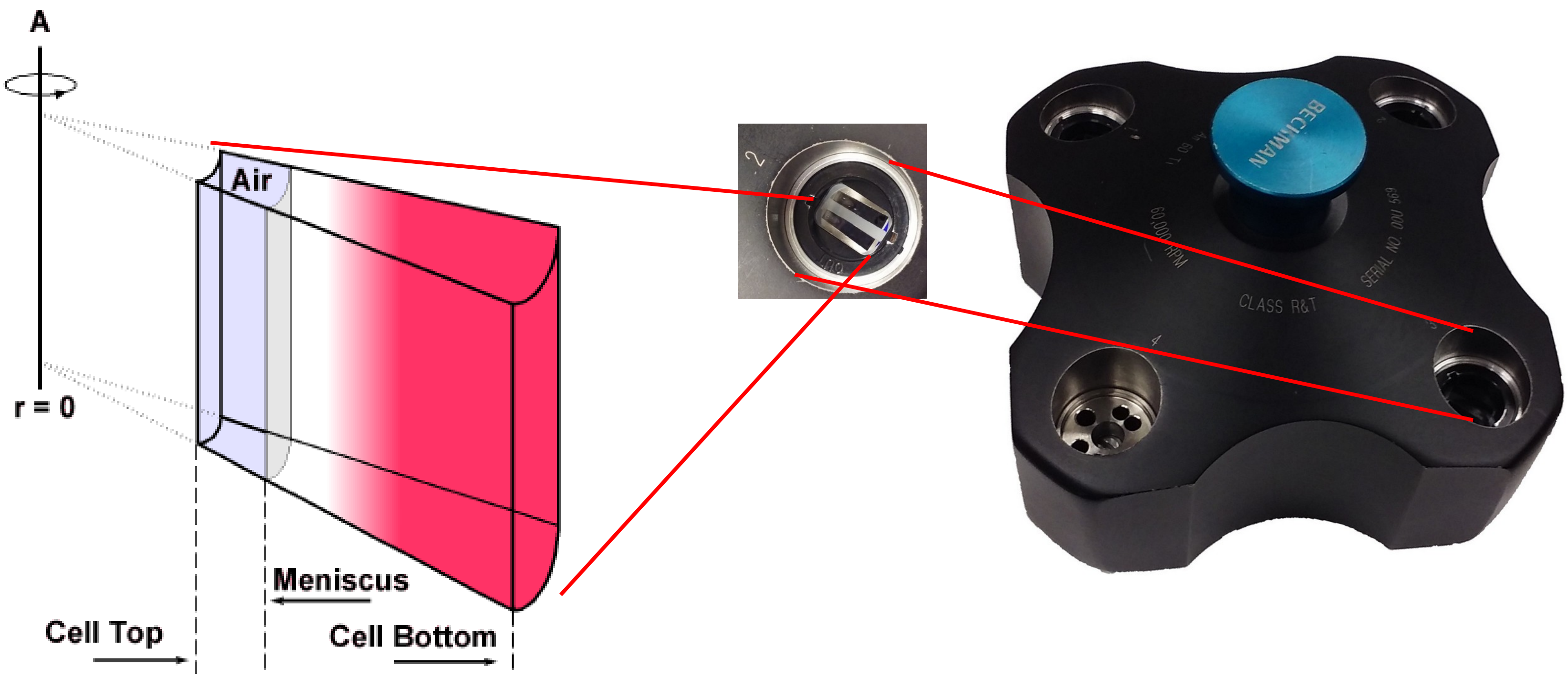
- 10 micron radial resolution
- 0.5 nm wavelength resolution
- 8 seconds per scan
- Network interface
- Multi-wavelength capable
- Built-in database for data acquisition

Macromolecular Transport - Diffusion

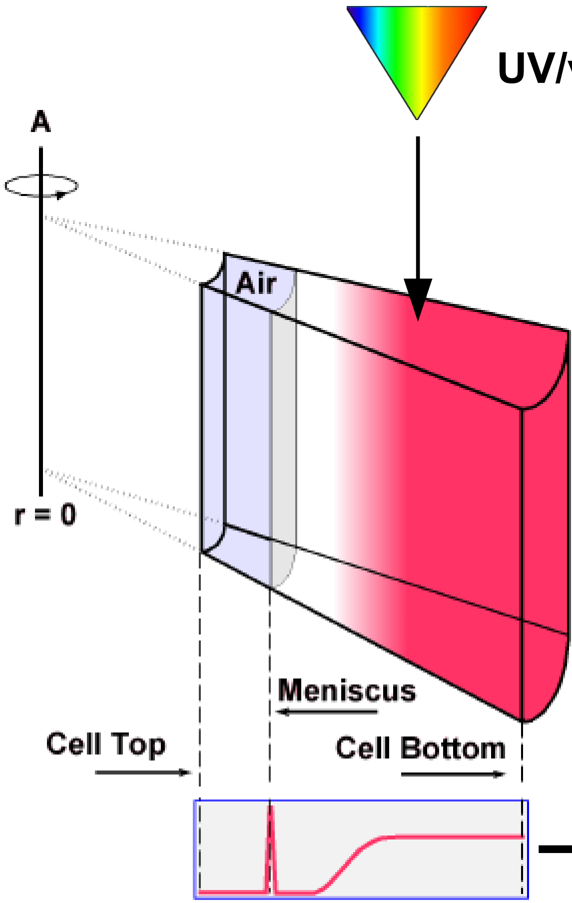




Sedimentation Instrumentation

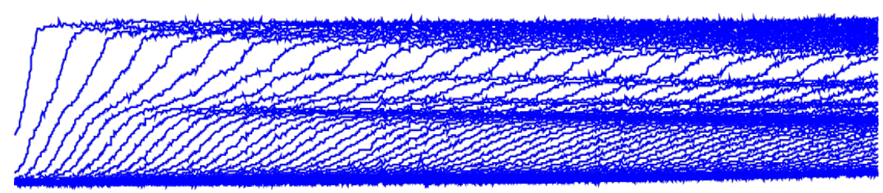


Sedimentation Instrumentation



UV/vis absorbance, Rayleigh interference, fluorescence

Multiple recordings over time...

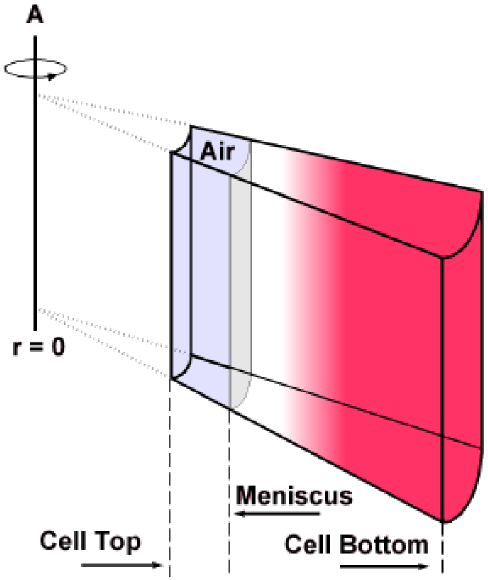


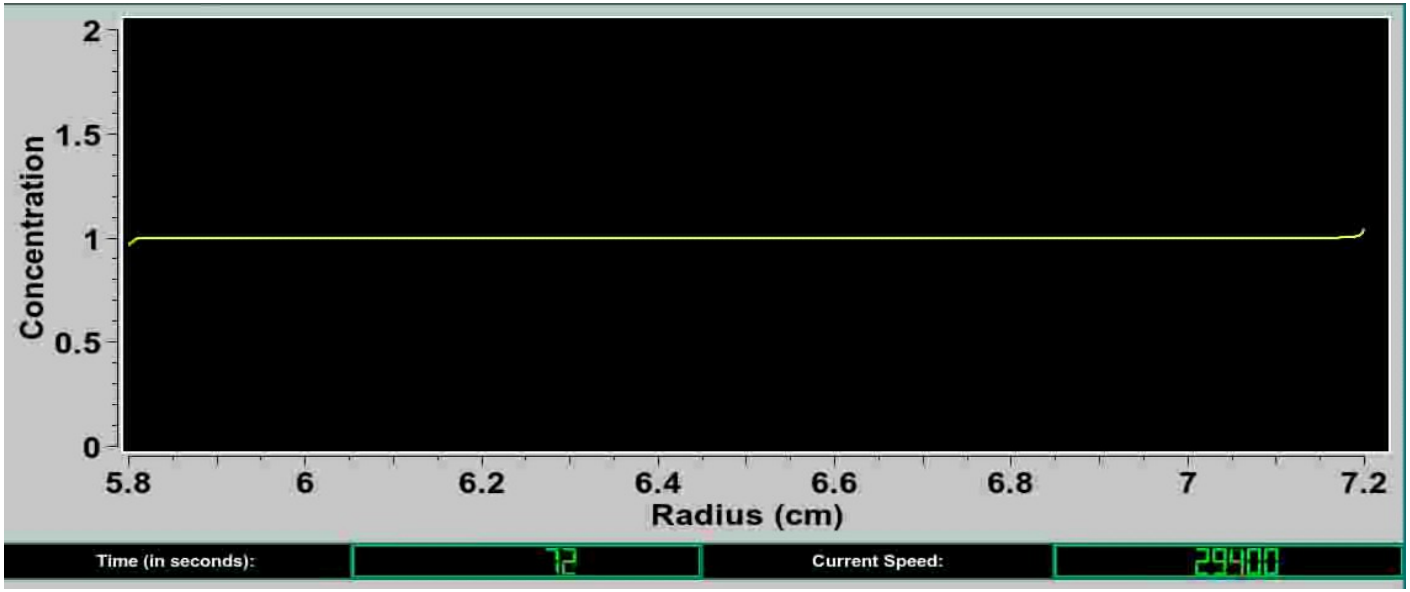
Transport Processes – Sedimentation and Diffusion

$$\left(\frac{\partial C}{\partial t}\right)_r = \frac{-1}{r}\frac{\partial}{\partial r}\left[s\,\omega^2 r^2 C - D r \frac{\partial C}{\partial r}\right]_t$$

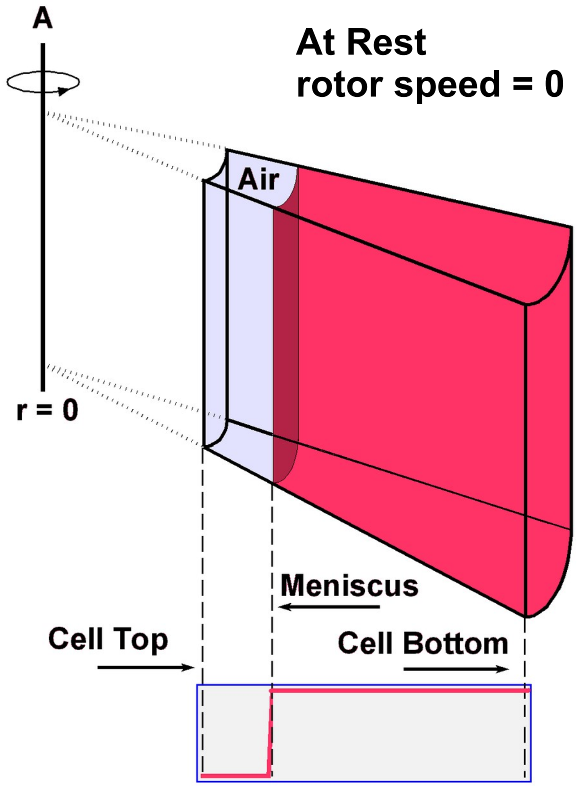
Lamm Equation, solved with finite element method

Cao, W and B. Demeler. Modeling AUC Experiments with an Adaptive Space-Time Finite Element Solution for Multi-Component Reacting Systems. Biophys. J. (2008) 95(1):54-65

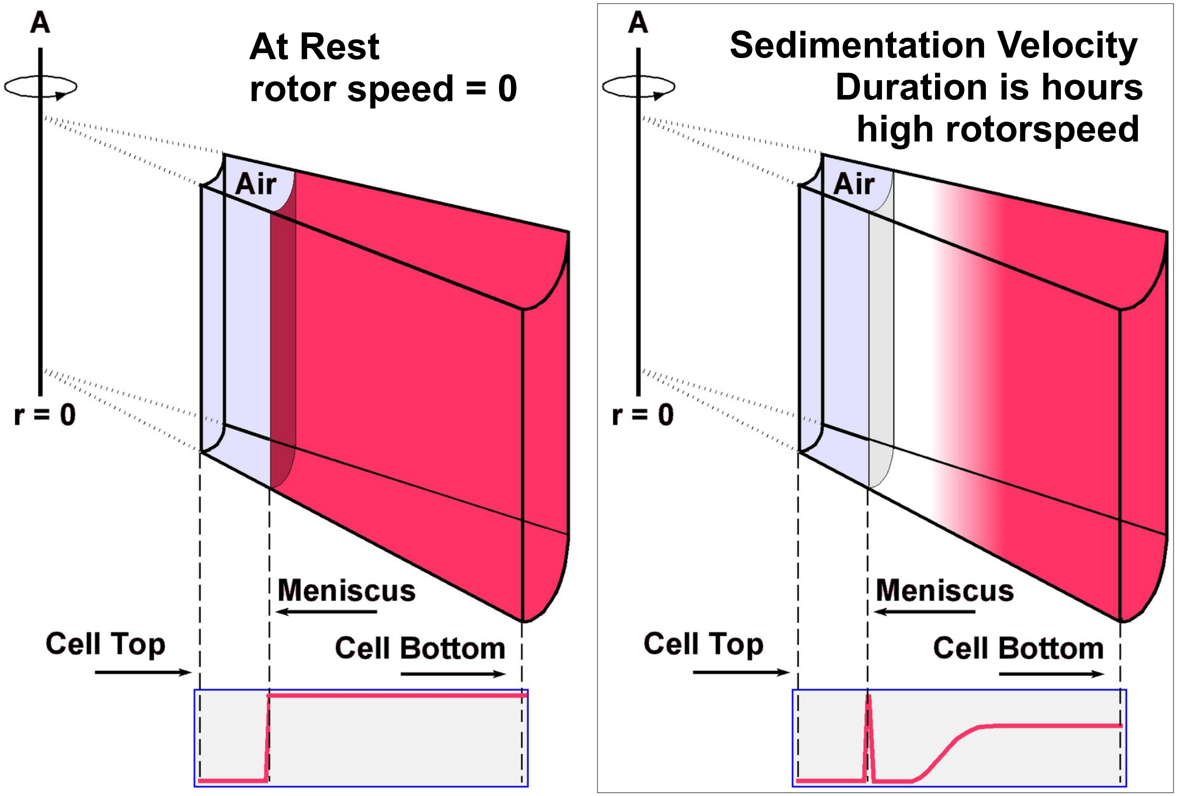




Transport Processes – Sedimentation and Diffusion

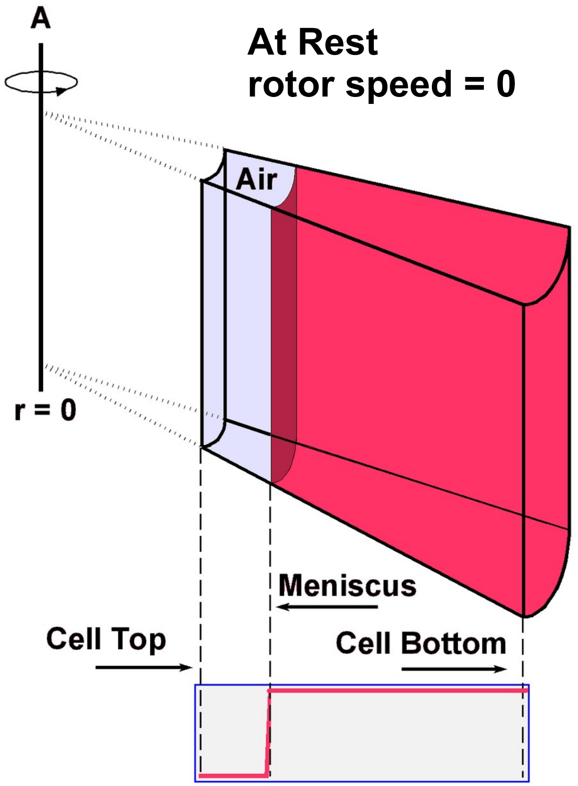


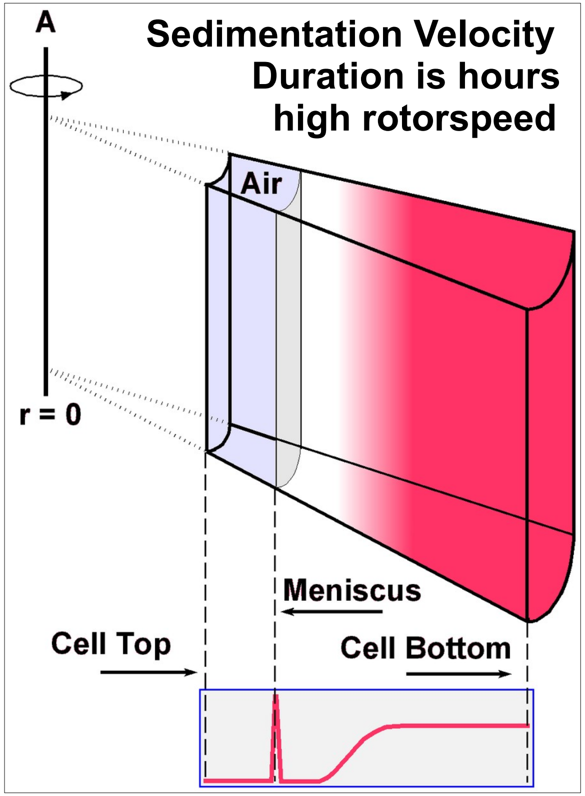
Transport Processes – Sedimentation and Diffusion



Transport Processes – Sedimentation and Diffusion

$$\left(\frac{\partial C}{\partial t}\right)_r = \frac{-1}{r}\frac{\partial}{\partial r}\left[s\,\omega^2 r^2 C - D\,r\,\frac{\partial C}{\partial r}\right]_t$$



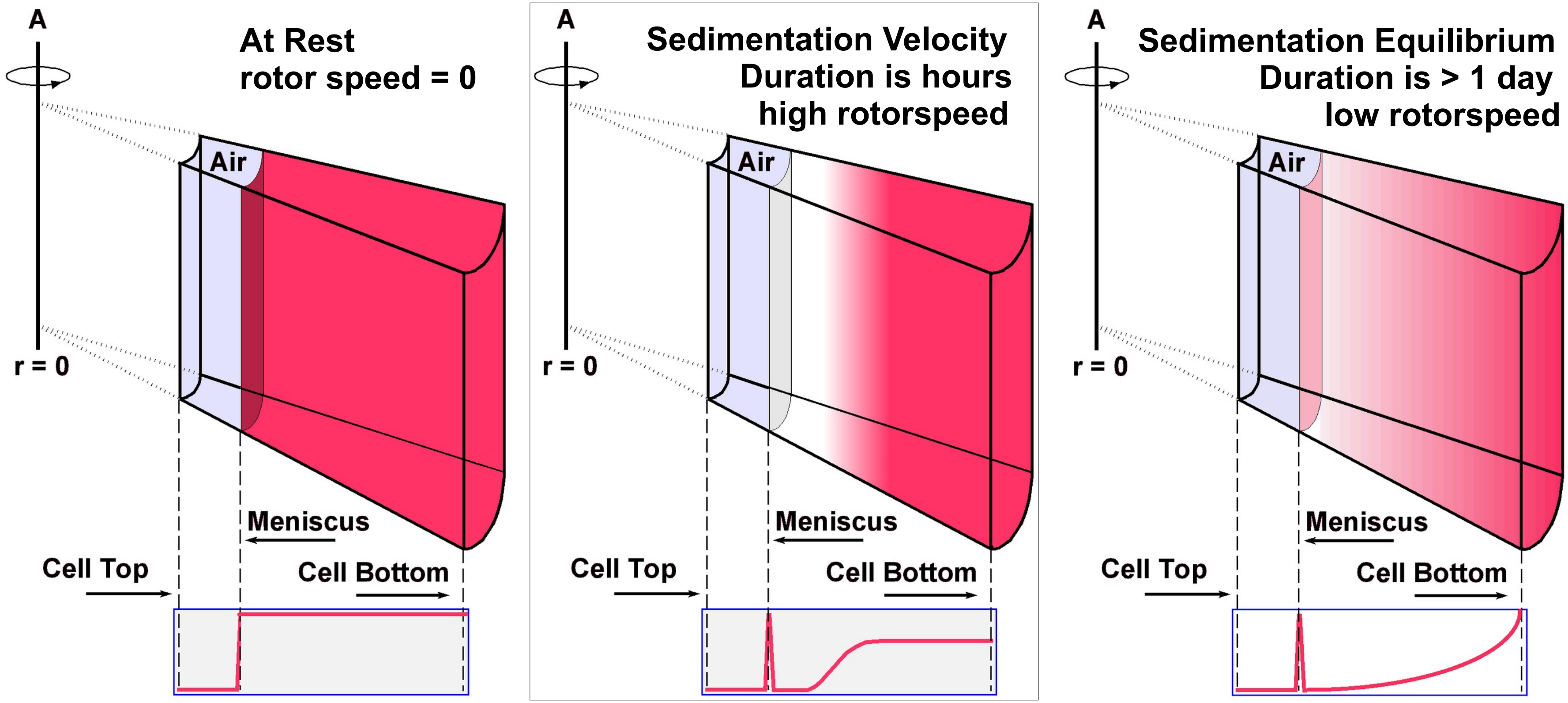


Question: ***What happens at the end of the sedimentation experiment?***

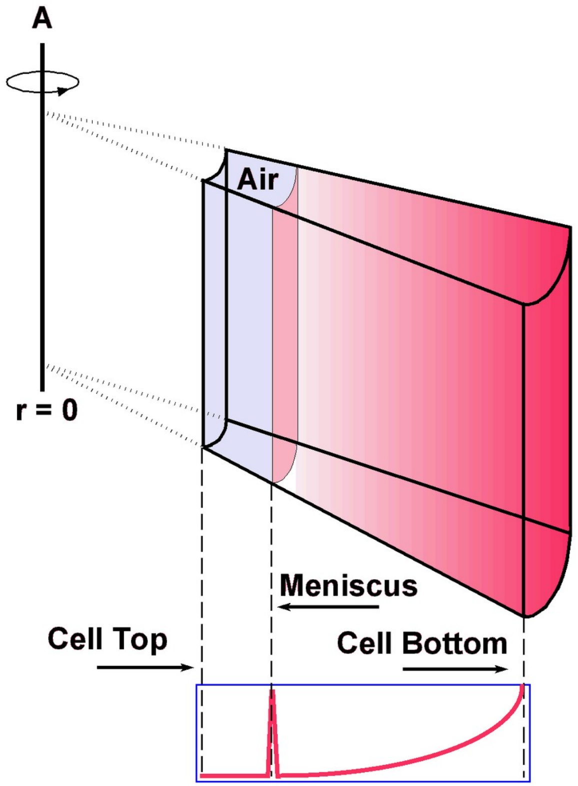
Transport Processes – Sedimentation and Diffusion

$$\left(\frac{\partial C}{\partial t}\right)_r = \frac{-1}{r}\frac{\partial}{\partial r}\left[s\omega^2 r^2 C - Dr\frac{\partial C}{\partial r}\right]_t$$

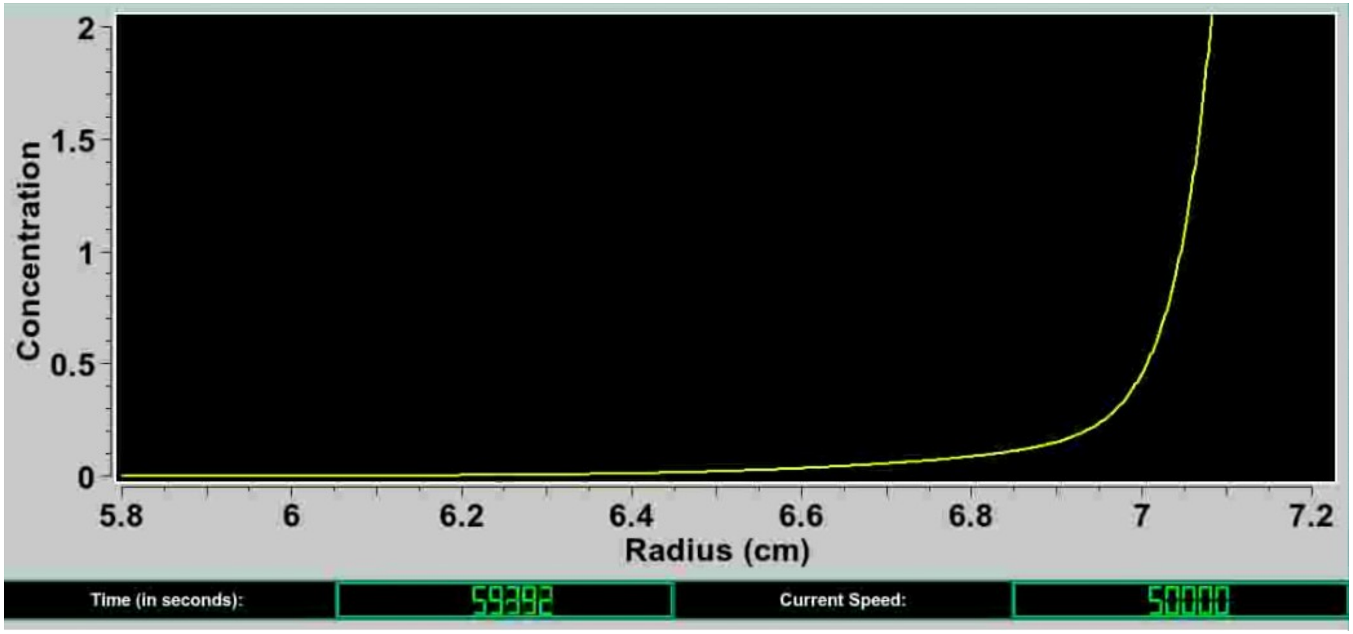
$$J = s\omega^2 r C - D\frac{\partial C}{\partial r} = 0$$



Transport Processes – Sedimentation and Diffusion



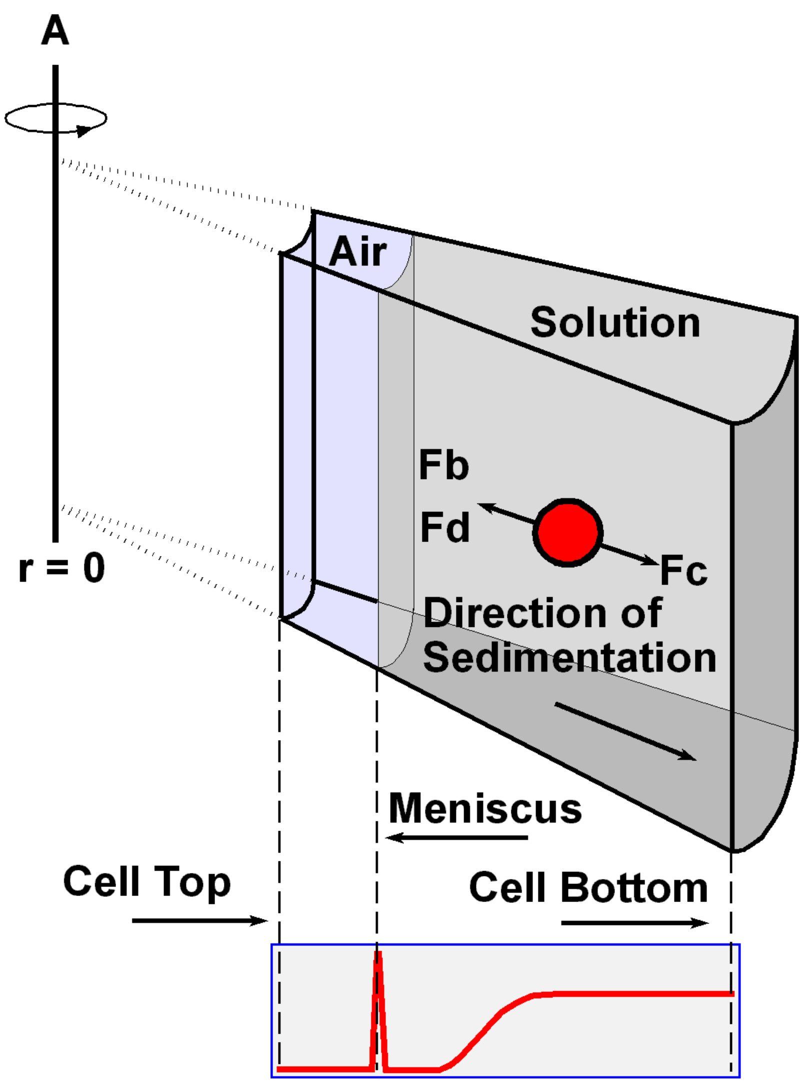
Sedimentation Equilibrium
Duration is > 1 day
low rotorspeed

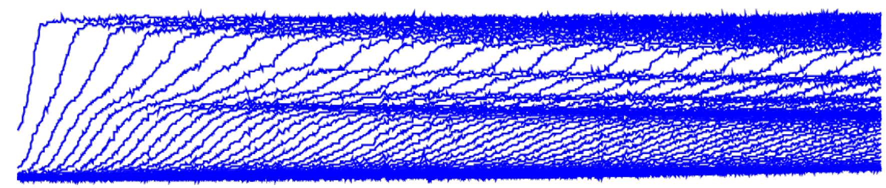


$$J = s\omega^2 r C - D\frac{\partial C}{\partial r} = 0$$

$$C = C_0 \, e^{\left[\sigma\left(r^2 - r_m^2\right)\right]}$$

Transport Processes – Sedimentation





Sedimentation:

Forces at Equilibrium:

Fc - Fb - Fd = 0

Fc (centrifugal force)	$= \omega^2 r m$
***Fb* (buoyancy)**	$= \omega^2 r m_s$
***Fd* (viscous drag)**	$= f v$

$$\omega = rpm * (\pi/30)$$

Explanation:

Fb is the buoyancy force - the force required to displace the buffer surrounding the solute, and $\boldsymbol{m_s}$ is the mass of the displaced solvent.

Transport Processes – Sedimentation

Fb (buoyancy)	= $\omega^2 r\, m_s$
Fd (viscous drag)	= $f\, v$
Fc (centrifugal force)	= $\omega^2 r\, m$

Substitute the mass of the solvent, m_s, with the mass of the solute, m

$$m_s = m\bar{v}\rho, \quad Fb = \omega^2 r\, m\bar{v}\rho$$

Rearrange the force equation: $Fc - Fb - Fd = 0$ and substitute

$$\omega^2 r\, m - \omega^2 r\, m\bar{v}\rho = fv$$

Place molecular parameter on one side and experimental parameters on the other

$$\frac{m\left(1 - \bar{v}\rho\right)}{f} = \frac{v}{\omega^2 r}$$

Put into molar units by multiplying with Avogadro's number, N

$$\frac{M\left(1 - \bar{v}\rho\right)}{Nf} = \frac{v}{\omega^2 r} = s$$

Transport Processes – Sedimentation:

$$\frac{M\left(1-\bar{v}\rho\right)}{Nf} = \frac{v}{\omega^2 r} = s$$

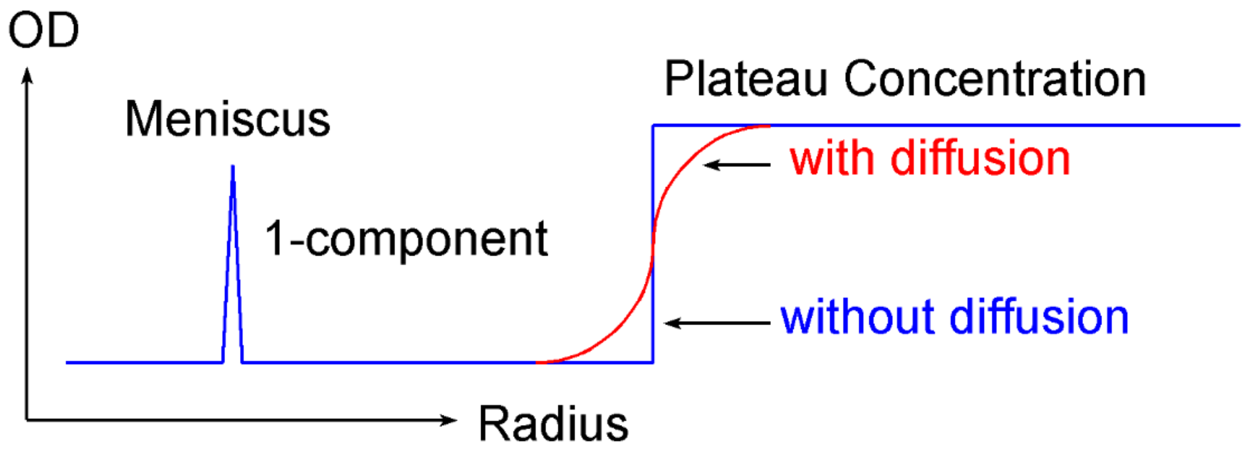
Definition:

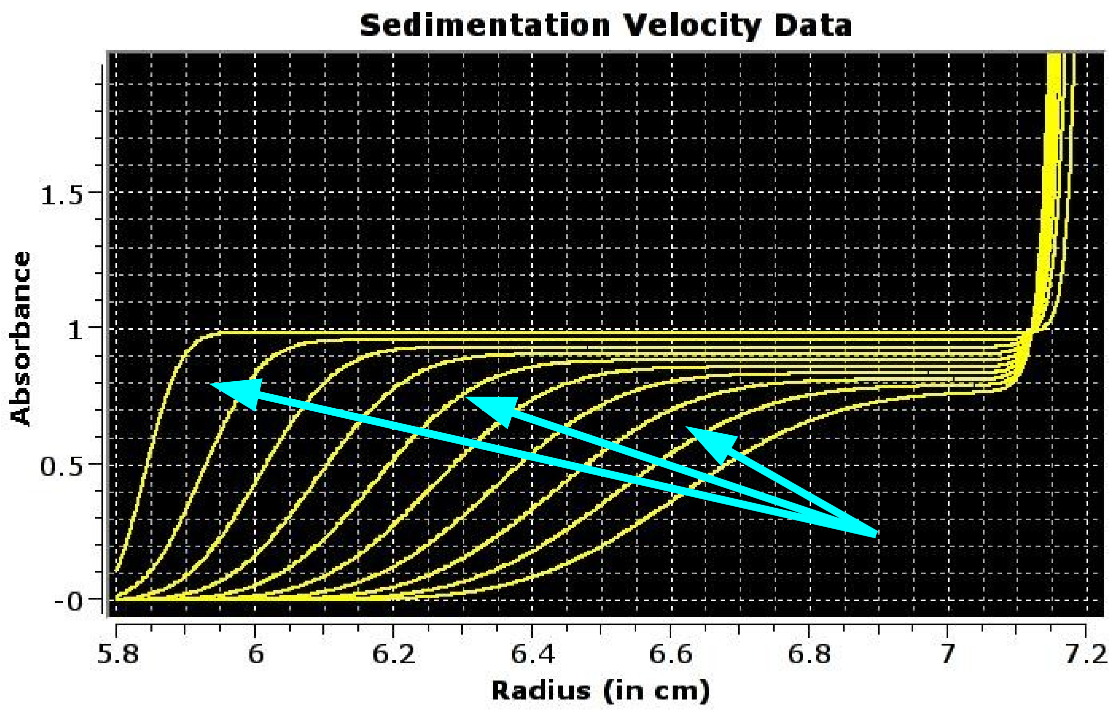
The sedimentation velocity, *v*, divided by the centrifugal field strength, $\omega^2 r$, is equal to the sedimentation coefficient, *s*

Take-home message:

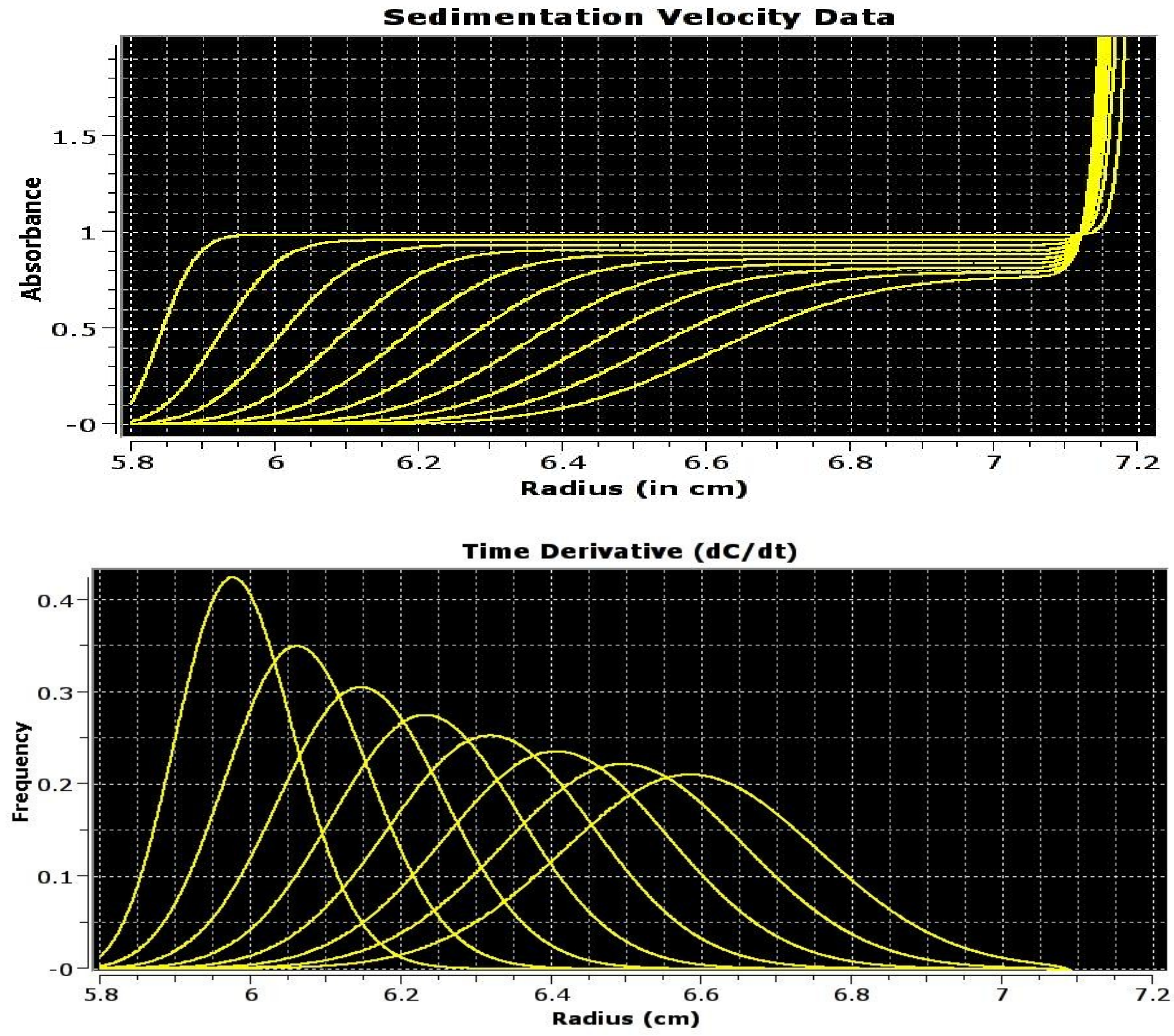
The sedimentation coefficient is proportional to *M* and inversely proportional to *f*

Effect of Diffusion on the sedimenting boundary shape:





Transport Processes – Diffusion:



Question: ***When is the concentration gradient the steepest in the sedimentation experiment?***

Transport Processes – Diffusion:

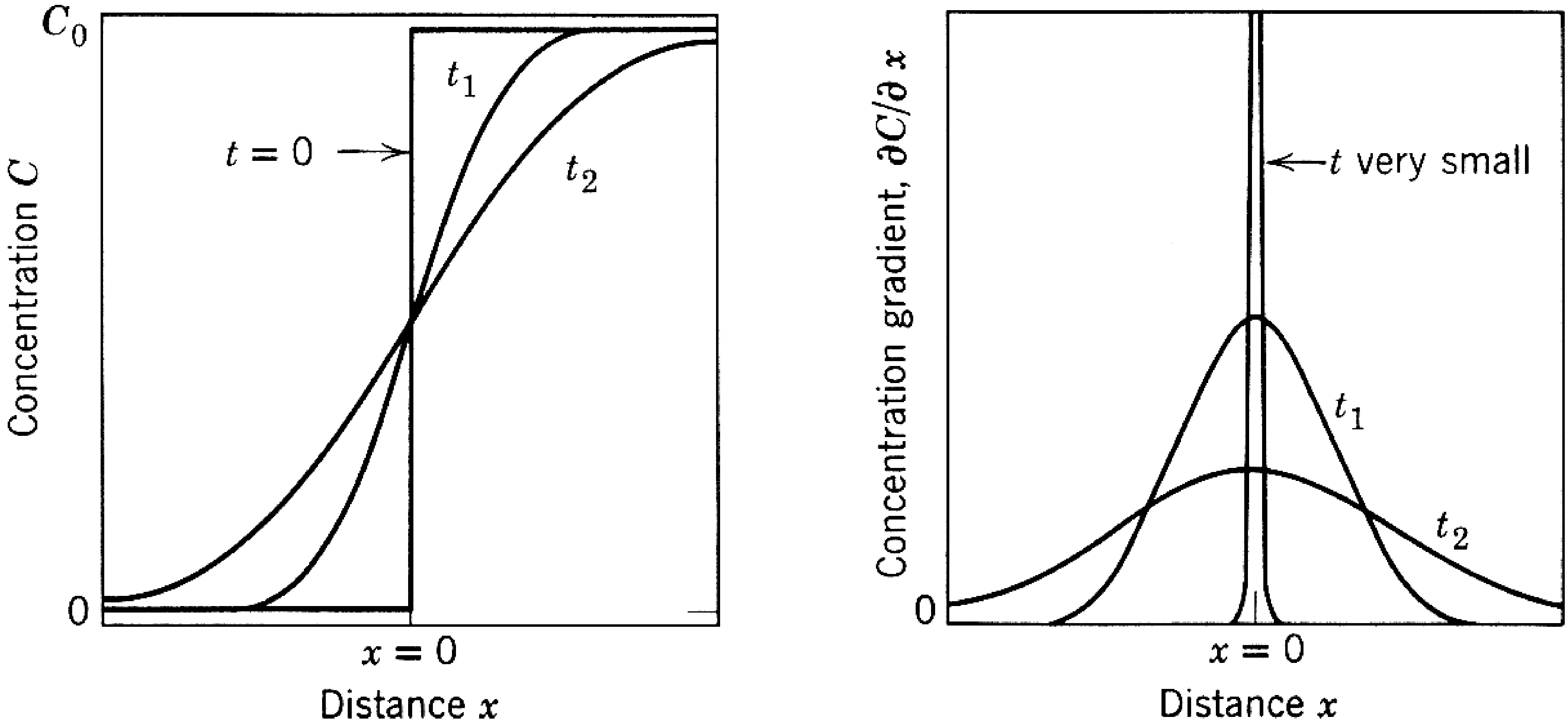
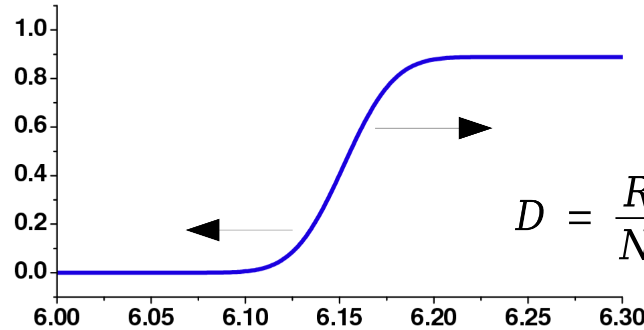


Fig. 21–1. Progress of a diffusion experiment with initially sharp boundary at $x = 0$.

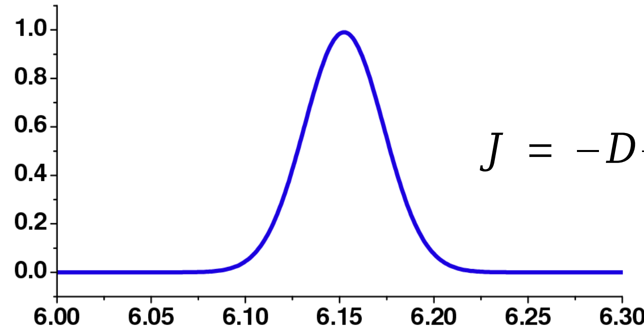
Transport Processes – Diffusion:

Random translation of a particle due to Brownian motion



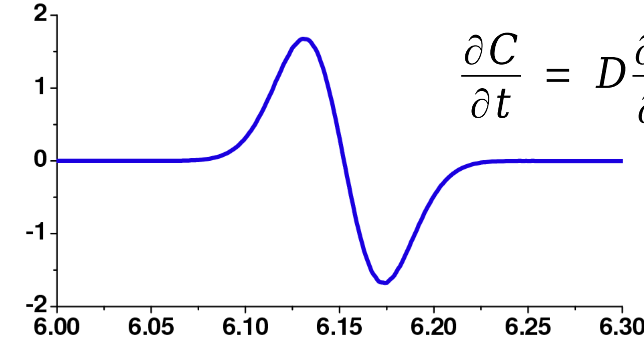
$$D = \frac{RT}{N f}$$

The flow due to diffusion is proportional to the concentration gradient and the diffusion coefficient (Fick's first law):



$$J = -D \frac{\partial C}{\partial x}$$

The rate of change of concentration is proportional to the change in steepness of the concentration gradient (Fick's second law):



$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2}$$

Transport Processes – Diffusion:

How do we measure Diffusion?

1. **Boundary method**
2. **Dynamic light scattering**
3. **Fluorescence Correlation Spectroscopy**
4. **Sedimentation Velocity**

Transport Processes – Diffusion:

Diffusion Equation: $$D = \frac{RT}{Nf}$$

Stokes-Einstein Equation: $$f = 6\pi\eta r \quad \text{(spherical shape only!)}$$

Stokes Radius: $$r = \frac{RT}{N 6\pi\eta D}$$

For a spherical particle, we can predict the frictional coefficient with the Stokes - Einstein relationship.

For any molecule, the measured frictional coefficient can then be used to calculate the corresponding radius. This is called the Stokes radius. This is the radius of a hypothetical sphere that has the same frictional coefficient as the molecule. The Stokes radius has a volume that is larger or equal to the volume of the actual molecule. Most macromolecules are NOT spherical.

If the volume is known, the radius r_0 of a hypothetical minimal sphere can be calculated, as well as its frictional coefficient, f_0.

The ratio of $\varphi = f/f_0$ is called the frictional ratio, and defines the anisotropy of the molecule.

Relationship between M, f, φ, s, and D

$$\left(\frac{\partial C}{\partial t}\right)_r = \frac{-1}{r}\frac{\partial}{\partial r}\left[s\omega^2 r^2 C - Dr\frac{\partial C}{\partial r}\right]_t$$

Concentration **Sedimentation** **Diffusion**

$$f = \frac{RT}{N\,D}$$

$$M = \frac{s\,N\,f}{1-\bar{v}\rho}$$

$$V = \frac{M\,\bar{v}}{N}$$

$$r_0 = \left(\frac{3V}{4\pi}\right)^{1/3}$$

$$f_0 = 6\pi\eta r_0$$

$$\phi = \frac{f}{f_0}$$

Relationship between M, f, φ, s, and D

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Concentration **Sedimentation** **Diffusion**

Shape Dependence (f, f_0)

$$f = \frac{RT}{N\,D}$$

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Relationship between M, f, φ, s, and D

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$$f_0 = 6\pi\eta r_0$$

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Size (→ M, r_0)

Relationship between M, f, φ, s, and D

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Concentration **Sedimentation** **Diffusion**

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$$M = \frac{s N f}{1 - \bar{v}\rho}$$

$$V = \frac{M \bar{v}}{N}$$

$$r_0 = \left(\frac{3V}{4\pi}\right)^{1/3}$$

$$f_0 = 6\pi\eta r_0$$

Anisotropy

$$\phi = \frac{f}{f_0}$$

Relationship between M, f, φ, s, and D

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Concentration **Sedimentation** **Diffusion**

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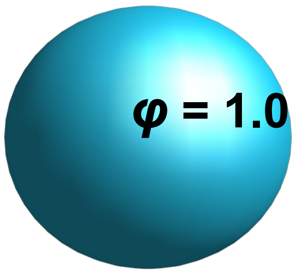
$$\phi = \frac{f}{f_0}$$

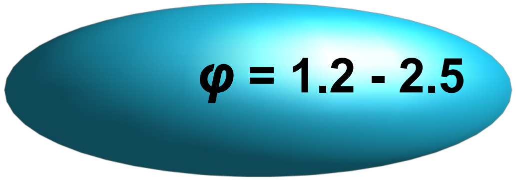
Partial specific volume and density

Frictional Ratio

The frictional ratio $f/f_0 = \varphi$ is a convenient way to parameterize the diffusion coefficient and the shape of a molecule .



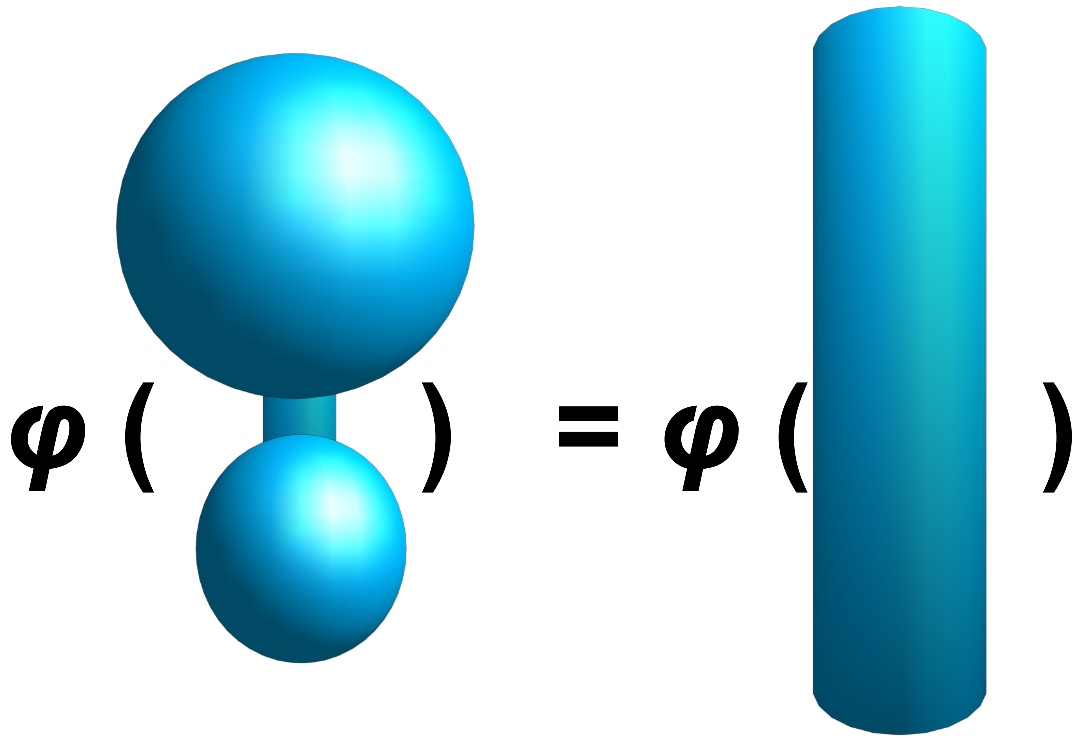
The frictional ratio φ is 1.0 for a sphere since $f = f_0$ and hence φ has a convenient lower limit



$1.0 \leq \varphi \leq 4.0$ for most proteins, higher for rod-shaped, disordered and unfolded proteins, DNA, fibrils and aggregates or linear molecules

$\varphi > 3$

Frictional Ratio



φ may be the same for different shapes, we cannot distinguish them by AUC, we can only measure the anisotropy!

Transport Processes – Fundamental Equations:

We have:

$$s = \frac{M\left(1 - \bar{v}\,\rho\right)}{N\,f} \quad and \quad D = \frac{RT}{N\,f}$$

Svedberg equation:

$$\frac{s}{D} = \frac{M\left(1 - \bar{v}\rho\right)}{RT}$$

Stokes-Einstein:

$$f = 6\pi\eta r$$

s depends on $\bar{v}$ and ρ

s and D are inversely proportional to f

f depends on the viscosity

Transport Processes – Viscosity and Density Corrections

The density and also the viscosity of the solvent affect the sedimentation and diffusion of the particle in solution, so the measured values need to be corrected to standard conditions. Moreover, temperature and buffer composition affect the solvent density and viscosity, so they need to be considered. To correct for density and viscosity, use these formulas:

$$s_{20,W} = s_{T,B} \frac{\left(1-\bar{v}\rho\right)_{20,W}\,\eta_{T,B}}{\left(1-\bar{v}\rho\right)_{T,B}\,\eta_{20,W}}$$

$$D_{20,W} = D_{T,B} \frac{293.15\,\eta_{T,B}}{T\,\eta_{20,W}}$$

Transport Processes – Partial Specific Volume and Buoyancy

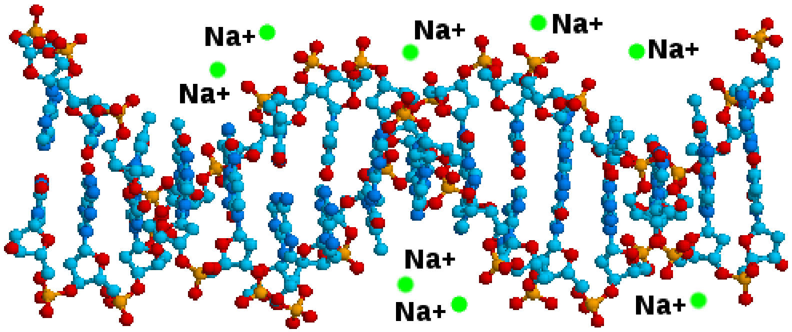
$$s = \frac{M\left(1 - \bar{v}\rho\right)}{N f}$$

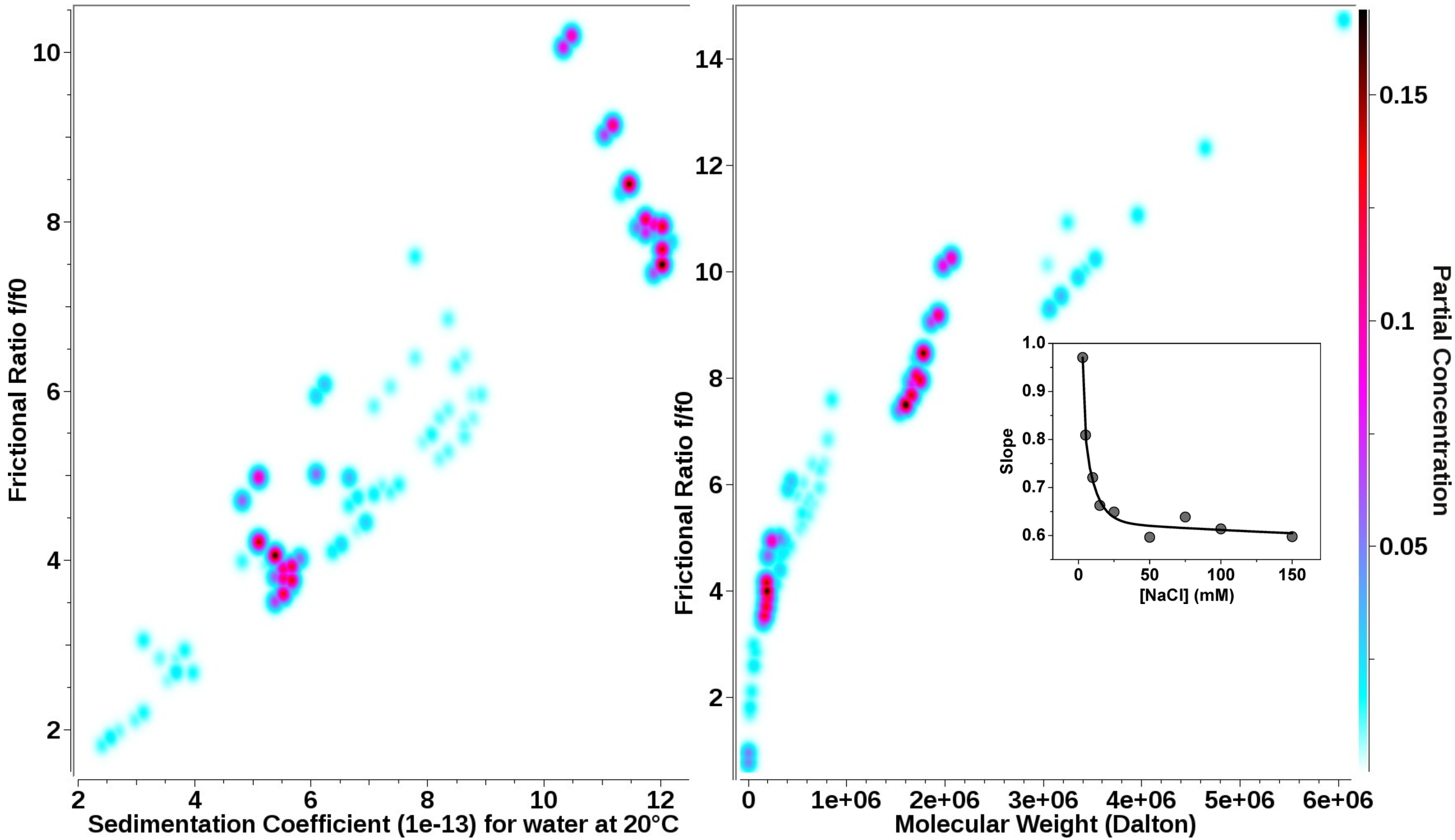
What effect does the *partial specific volume have on sedimentation*?

The partial specific volume is the volume that includes the hydration of the sedimenting particle, plus any ions bound. If the hydration shell is large (e.g., a charged nucleic acid or protein in low salt), the vbar will increase compared to the anhydrous molecule, while its density decreases. However, water molecules are only bound transiently, so they are not considered in the molecular weight of the macromolecule. Hence, given a measurement for *s*, *f* and *ρ*, the vbar value is the value that makes the molecular weight come out “correctly”, i.e., for the value expected from sequence or mass spectrometry.

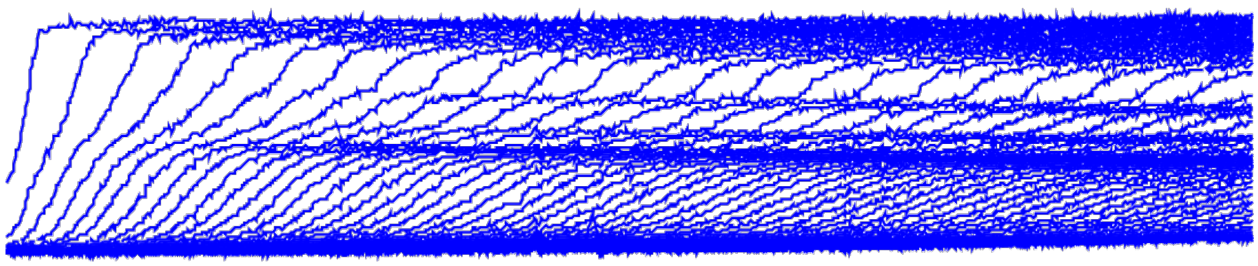
The value of vbar can be very sensitive to solution conditions.

Transport Processes – Partial Specific Volume and Buoyancy



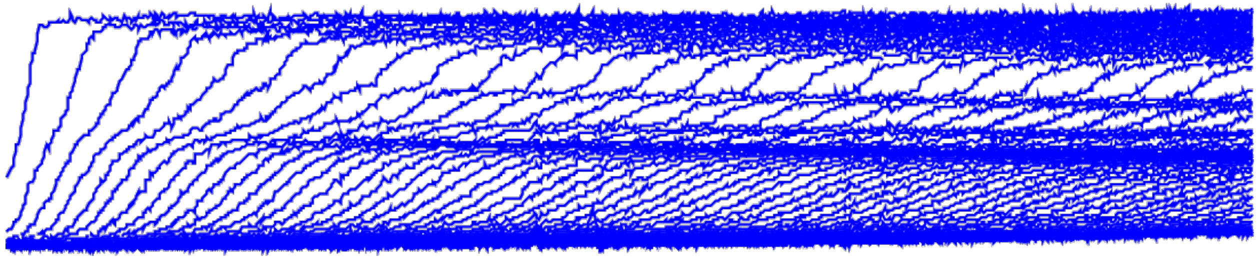


Transport Processes – Sedimentation Summary



Sedimentation velocity profile of a mixture of macromolecules over time

Transport Processes – Sedimentation Summary

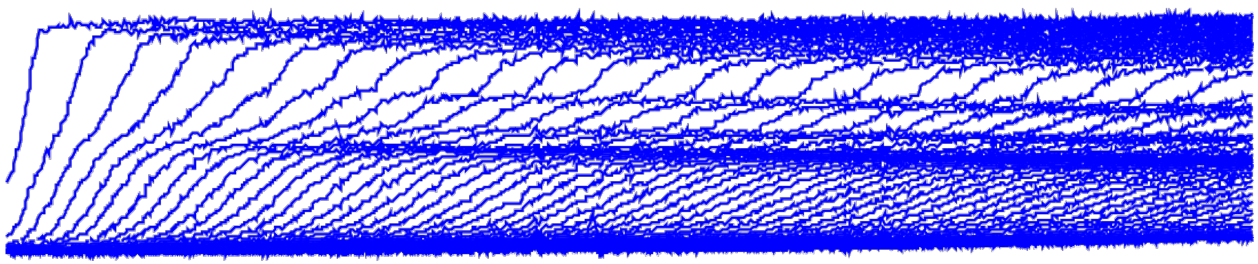


Composition Analysis

We can answer these questions:

How many components?

Transport Processes – Sedimentation Summary



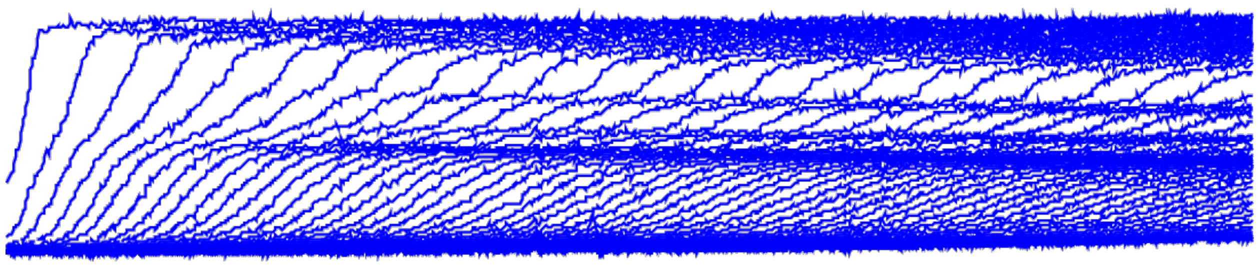
Composition Analysis

We can answer these questions:

How many components?

What are their sizes and molecular weights?

Transport Processes – Sedimentation Summary



Composition Analysis

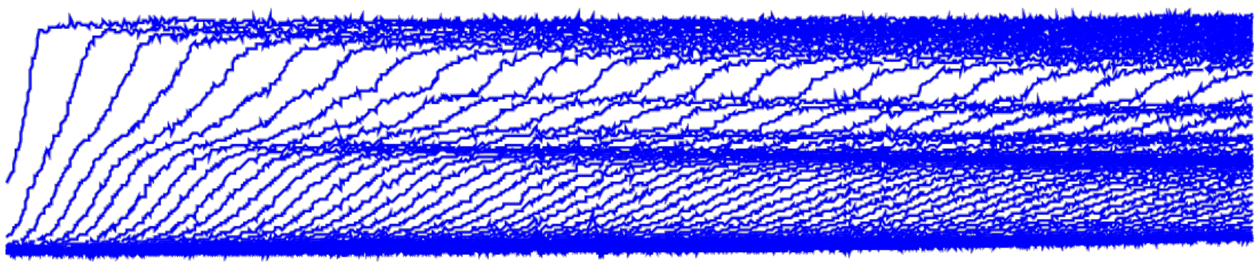
We can answer these questions:

How many components?

What are their sizes and molecular weights?

What are their anisotropies?

Transport Processes – Sedimentation Summary



Composition Analysis

We can answer these questions:

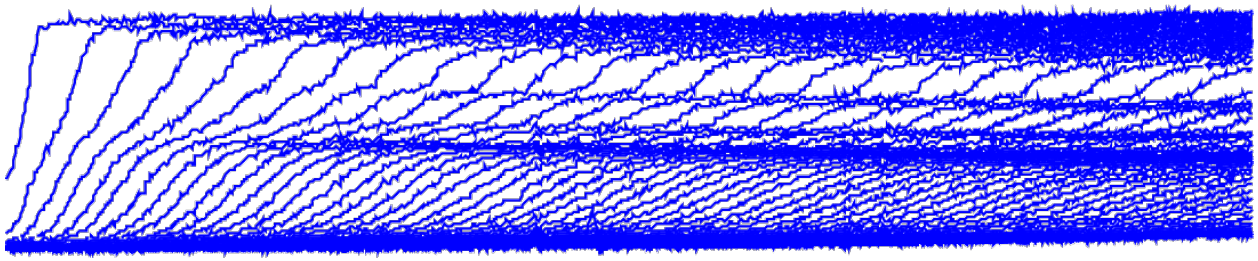
How many components?

What are their sizes and molecular weights?

What are their anisotropies?

What is the partial concentration of each component?

Transport Processes – Sedimentation Summary



Composition Analysis

We can answer these questions:

How many components?

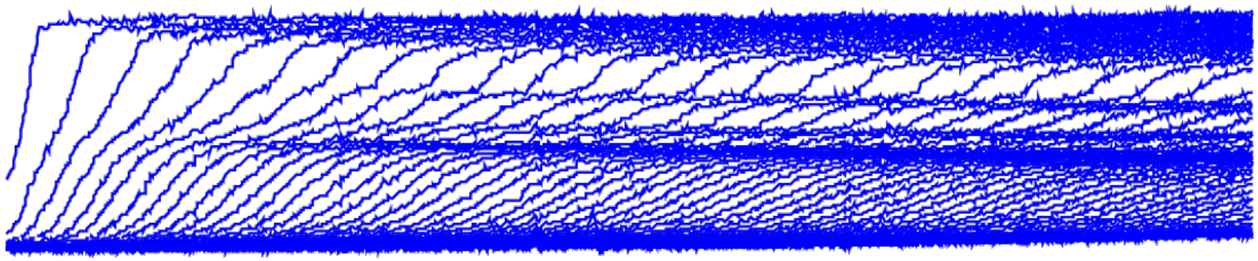
What are their sizes and molecular weights?

What are their anisotropies?

What is the partial concentration of each component?

Do the components interact (how fast, strong)?

Transport Processes – Sedimentation Summary



Composition Analysis

We can answer these questions:

How many components?

What are their sizes and molecular weights?

What are their anisotropies?

What is the partial concentration of each component?

Do the components interact (how fast, strong)?

What is the reliability of our measurement?