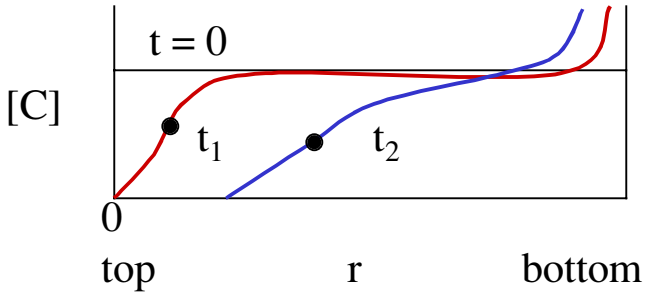
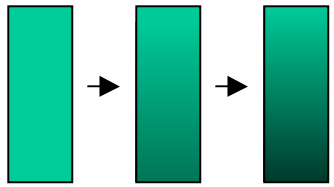
Measuring S using an analytical ultracentrifuge

Moving boundary





r_b = boundary position

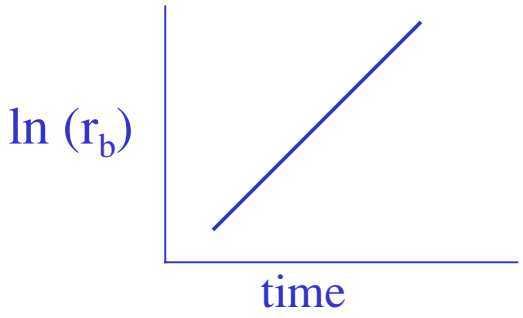
$$\text{velocity} = \frac{dr_b}{dt}$$

$$\frac{1}{r_b\omega^2} \cdot \frac{dr_b}{dt} = S \qquad \ln \frac{r_b(t)}{r_b(t_o)} = \omega^2 S\,(t-t_o)$$

$$\ln r_b(t) = \ln r_b(t_o) + \omega^2 S\,(t-t_o)$$

plot $\ln r_b(t)$ vs time

slope S ⇒ for known ω



Using the Preparative Ultracentrifuge

Finding the S-value

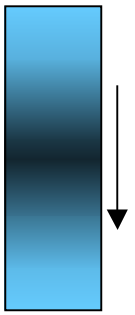
typically a sucrose gradient is used to prevent turbulence due to convection

$$S = \frac{M\,(1-\bar{V}_2\rho)}{Nf}$$

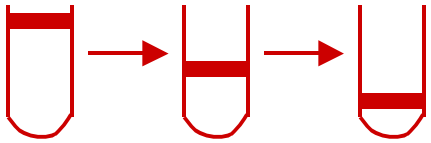
But the gradient makes computation of the S-value very difficult because in the sucrose density gradient,

ρ is not constant

and η is not constant ($f = 6\pi\eta R_s$)



A set of "standards" of known $\bar{S}$-value is used to get S for the unknown.



Using the Sedimentation Coefficient to get molecular parameters

Once you measure it
there are 3 unknowns : S =

$$\frac{M\,(1 - \bar{V}_2\rho)}{Nf}$$

$$f = 6\pi\eta R_s$$

Practical strategy to get M

1 measure, calculate or estimate V_2 (inverse density)

for proteins: $\bar{V}_2 \approx 0.75$ mL/g

-can compute from amino acid composition

2 **f** can be obtained from another experiment: gel filtration, diffusion (Stokes radius ⇒ f)

combination of sedimentation and gel filtration can yield **M**.

Combining Sedimentation and Diffusion

$$D = \frac{kT}{f} = \frac{kT}{6\pi\eta R_s}$$

Any method yielding R_s can be used. This is now most frequently obtained by gel filtration chromatography

$$S = \frac{M\,(1 - \bar{V}_2\rho)}{Nf}$$

$$\left(\frac{S}{D}\right) = \frac{M(1-\bar{V}_2\,\rho)}{RT}$$

The frictional coefficient drops out of the equation

This used to be one of the standard methods to obtain protein molecular weight

Sedimentation Coefficients and Molecular Weights of Some Proteins and Viruses

	$s^0_{20,w} \times 10^{13}$	$D^0_{20,w} \times 10^7$	$\bar{v}_2$	M
Ribonuclease[47]	1.64	11.9	0.728	12,400
Lysozyme[48]	1.87	10.4	0.688	14,100
Chymotrypsinogen[37]	2.54	9.5	0.721	23,200
β-Lactoglobulin[51]	2.83	7.82	0.751	35,000
Ovalbumin[49]	3.55	7.76	0.748	45,000
Serum albumin[67]	4.31	5.94	0.734	66,000
Hemoglobin[60]	4.31	6.9	0.749	60,000
Catalase[39]	11.3	4.1	0.73	250,000
Fibrinogen[69]	7.9	2.02	0.706	330,000
Urease[50]	18.6	3.46	0.73	480,000
Myosin[72]	6.4	1.0	0.728	570,000
Bushy stunt virus[45]	132	1.15	0.74	10,700,000
Tobacco mosaic virus[64]	170	0.3	0.73	50,000,000

infinite dilution

$S^o_{20,w}$

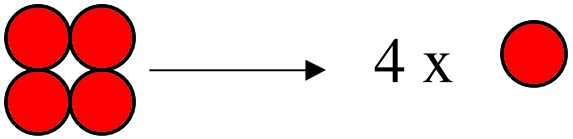
20^oC

in water (correct for viscosity and temperature from conditions of actual measurement)

To obtain the molecular weight of the native form of an enzyme (subunit association), the combination of sedimentation - gel filtration (R_S) is very useful

(from Tanford)

Example 1: Hemoglobin Dissociation at low pH



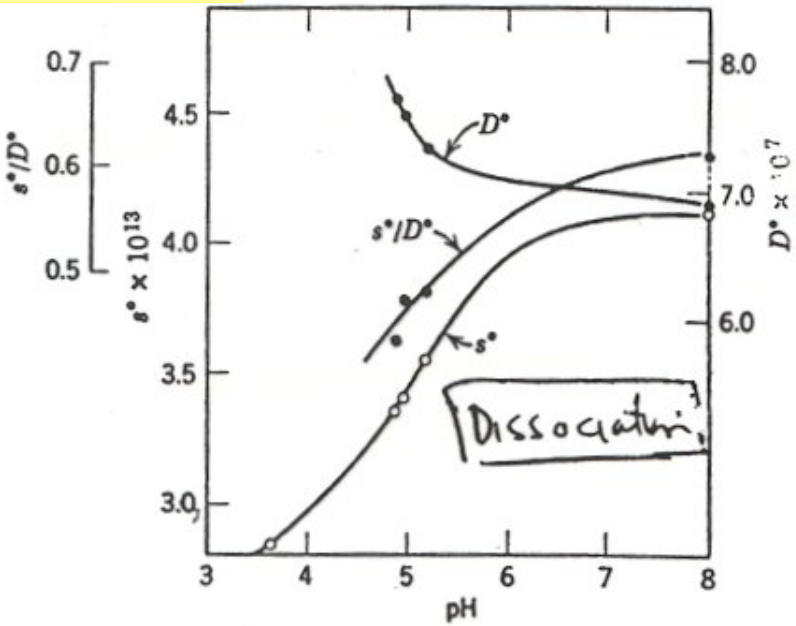
4 x

$D\uparrow$ $(R_s\downarrow)$

$S\downarrow$

$\left(\frac{M}{R_s}\right)\downarrow$

$\left(\frac{S}{D}\right)\downarrow$

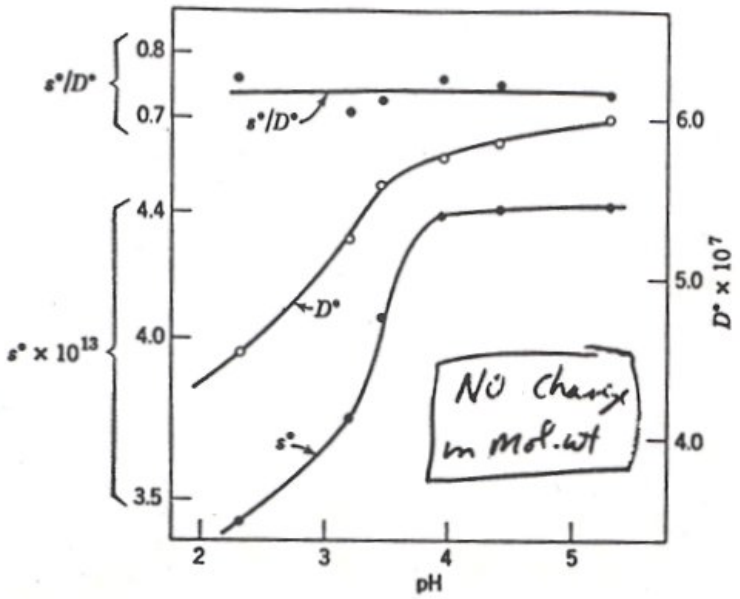


$$D = \frac{kT}{6\pi\eta R_s}$$

$$S = \frac{M\,(1 - \bar{V}_2\rho)}{N\,6\pi\eta R_s}$$

$$\frac{S}{D} = \frac{M(1-\bar{V}_2\,\rho)}{RT}$$

Example 2: Expansion of BSA at low pH



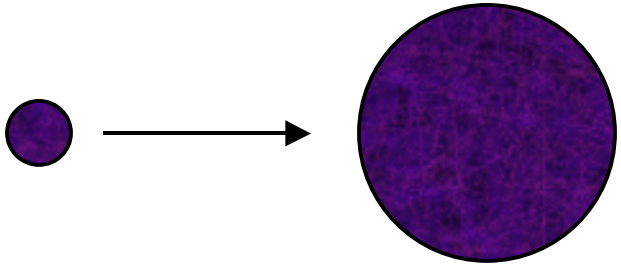
D ↓
(R_s ↑)

S ↓

$\frac{M}{R_s}$ ↓

$\frac{S}{D}$ constant

$$D = \frac{kT}{6\pi\eta R_s} \qquad S = \frac{M\,(1 - \bar{V}_2\rho)}{N\,6\pi\eta R_s}$$



$$\frac{S}{D} = \frac{M(1-\bar{V}_2\,\rho)}{RT}$$

Using Standards of known S-value to estimate molecular weight of an unknown

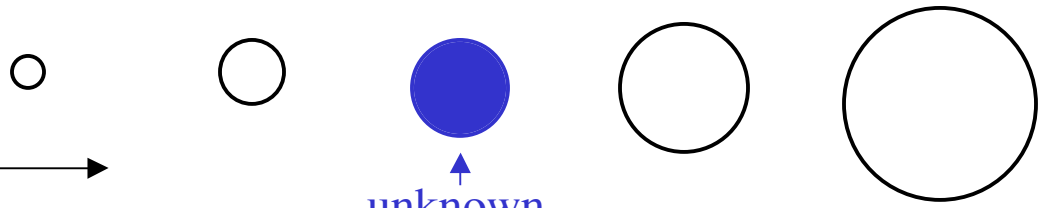
1 The best method to obtain M from a measured S-value is to independently get V_2 and f, and then obtain M in the equation below:

$$S = \frac{M(1 - \bar{V}_2\rho)}{Nf} \qquad f = 6\pi\eta R_s$$

2 Can convert S ⇒ M if you compare with known standards.

However: M must be the only independent parameter

Example stands set of "spherical" proteins



≈ same $\bar{V}_2$ ⟶

increasing M ⟶

$f \propto R_s \propto M^{1/3}$ ⟶ In general (for spherical compact proteins)

$S \propto M^{2/3}$ ⟶ increasing

But: if unknown is ellipsoid $f > f_{sphere}$ (then f and M are not correlated)

$S > S_{sphere}$, get wrong M

Using standards to get M from the S-value

The key to using a comparison to standards to obtain M is that molecular weight be the only independent parameter. (Also true for electrophoresis and for gel filtration chromatography)

$$S = \frac{M(1 - \bar{V}_2 \rho)}{Nf} \qquad f = 6\pi\eta R_s$$

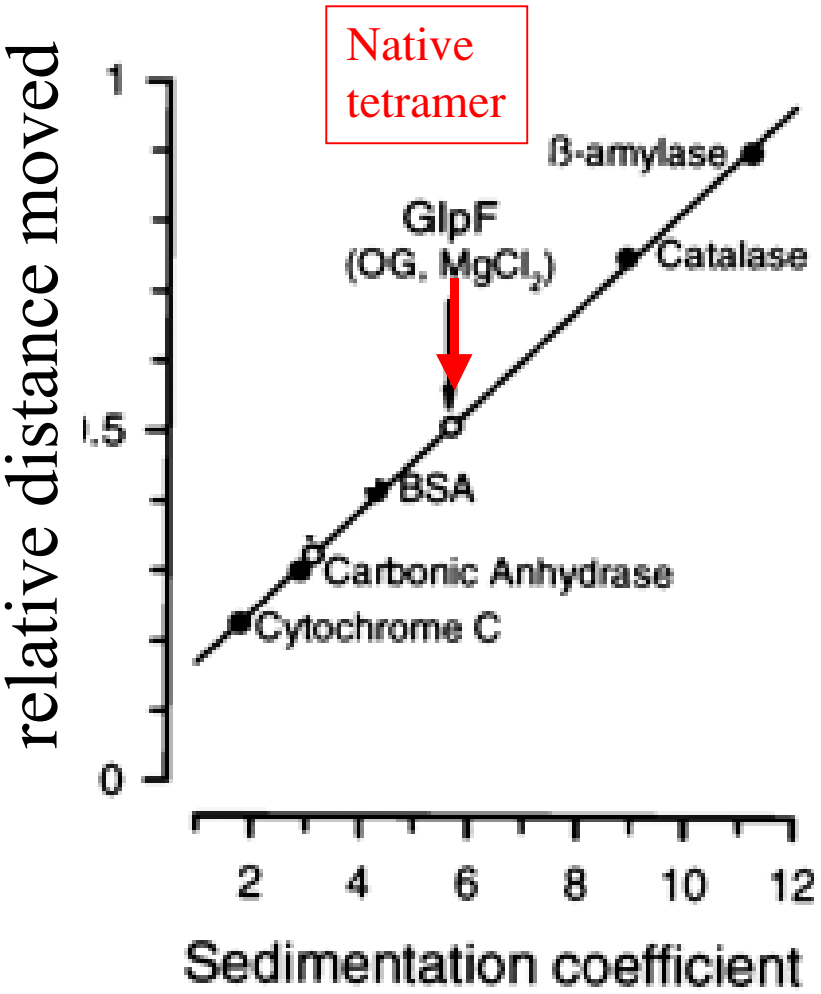
If all standards and the unknown are same shape and have the same $\bar{V}_2$, then S will be a smooth and predictable function of Molecular weight.

$$S \propto \frac{M}{f} \propto \frac{M}{f(M)}$$

You will get the wrong value of M if the shape of the unknown is not the same as the shape of the standards to which it is compared.

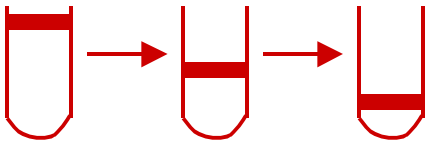
You will get the wrong value of M if the value of $\bar{V}_2$ is incorrect. Glycoproteins, proteins with lipids and/or detergents bound to them are examples where the anhydrous volume per gram of the particle is not a trivial matter to obtain.

Example: determination of the S-value of GlpF the glycerol transport facilitator protein from *E. coli*



$$S = \frac{M\,(1-\bar{V}_2\rho)}{Nf}$$

Empirical correlation of the S-value of soluble protein standards using sedimentation velocity in a 5-20% sucrose density (neither ρ nor f are constant!)



PNAS (2001) 98, 2888-2893

Using the Preparative Ultracentrifuge

Preparative Applications: **Differential Sedimentation**

1) Larger **size** particles (larger M/f): sediment faster (pellet vs supernatent.

2) Higher **density** particles (smaller $\bar{V}_2$): sediment faster

3) More **compact** particles (smaller f): sediment faster

$$S = \frac{M\,(1-\bar{V}_2\rho)}{N\mathbf{f}}$$

Using the Preparative Ultracentrifuge

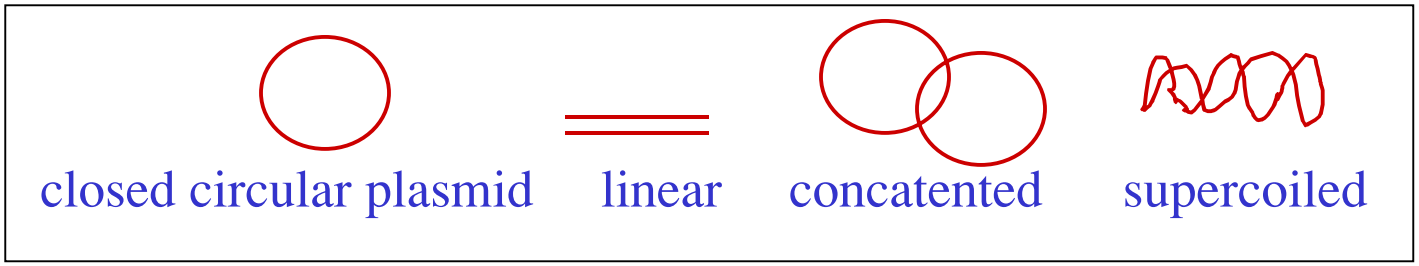
Examples:

1. **lipoproteins** (separate on the basis of density)

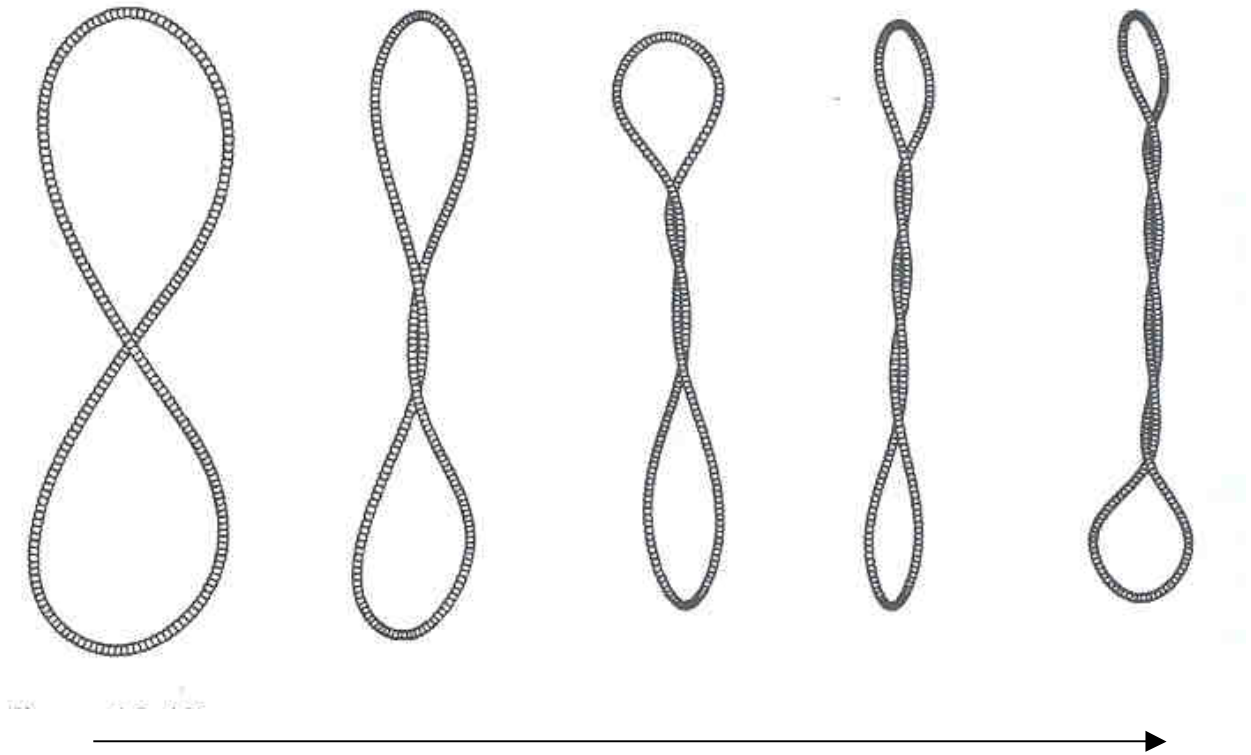
2. **protein - nucleic acid complexes** (separated from protein and DNA by density differences)

3. **RNA**: usually must denature to eliminate 3° structure to separate on the basis of size

4 .**DNA forms** - differ in shape/size from each other



DNA Supercoiling:
induced by binding intercalating molecules (such as ethidium bromide) that insert between basepairs and unwind the double helix

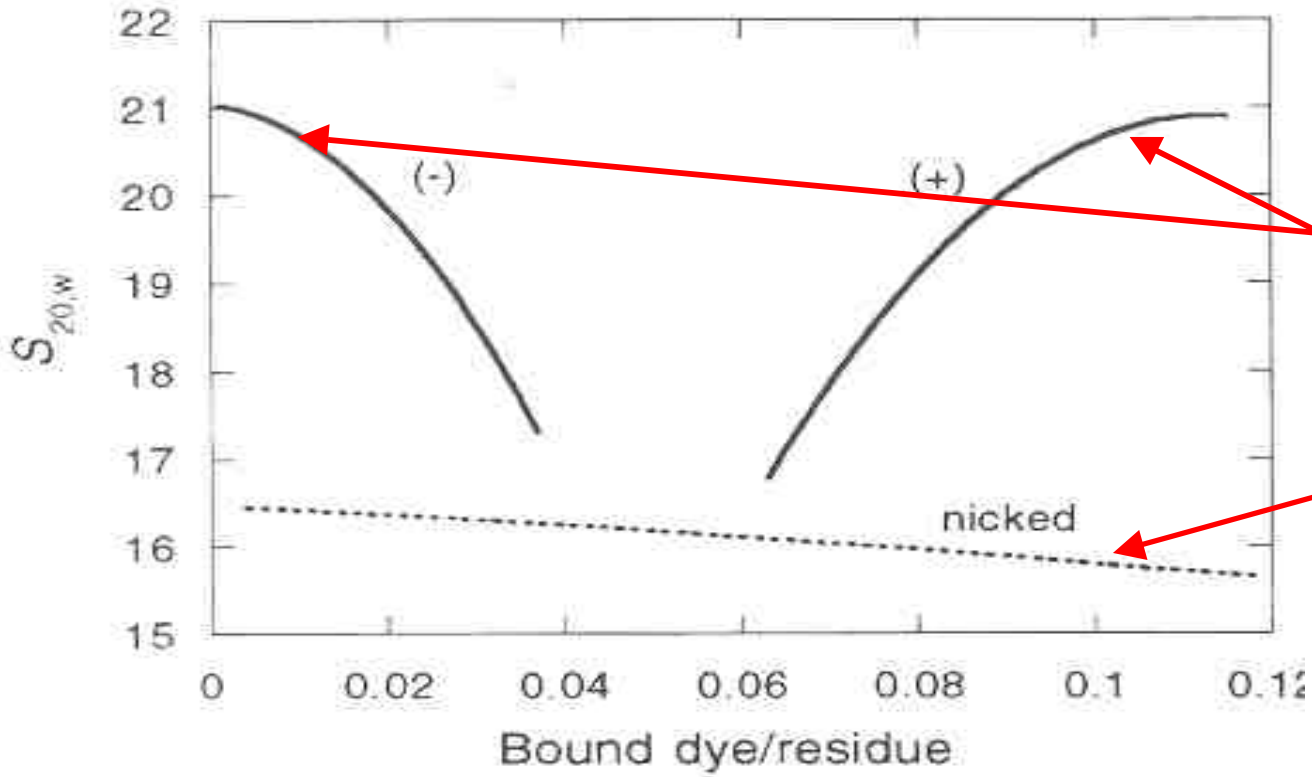


more supercoils makes the DNA more compact

change in shape results in a change in S-value

Discovery of DNA Supercoiling by Analytical Centrifugation (1968)

Titrate DNA with ethidium bromide



Closed circular DNA

Nicked DNA

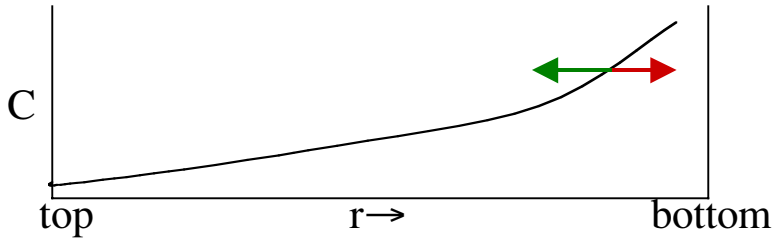
$$S = \frac{M\,(1-\bar{V}_2\rho)}{Nf}$$

Negative supercoils: Compact/high S value → No supercoils: Low S value → Positive supercoils: Compact/high S value

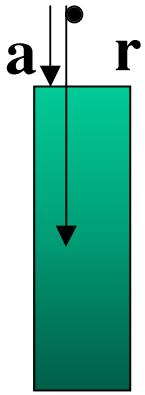
Sedimentation Equilibrium

$$\mu = \mu^0 + RT \ln c - \frac{\omega^2 r^2}{2} M (1-\bar{V}_2\rho)$$

ω = radial velocity
μ = partial molar free energy



$$\frac{d\mu}{dr} = 0 \quad \text{at equilibrium} \quad \boxed{0 = RT \frac{1}{c}\left(\frac{dc}{dr}\right) - \omega^2 r M (1-\bar{V}_p)}$$

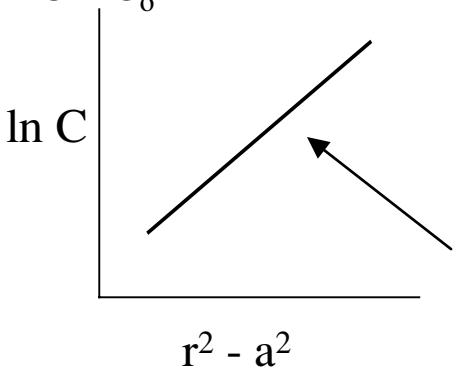


at the top of the sample (meniscus, r = a), $C = C_o$

$$\Rightarrow \frac{1}{c}\frac{dC}{dr} = \frac{\omega^2 r M (1-\bar{V}_2\rho)}{RT}$$

integrate: $$\boxed{\ln C(r) = \frac{\omega^2 M (1-\bar{V}_2\rho)}{2RT}(r^2 - a^2)}$$

meniscus



get M from the slope
-must know ω and $\bar{V}_2$ and ρ

Note: f is not in the equation

Sedimentation equilibrium

Best method is to use an analytical centrifuge

measure C(r) vs r optically

Need pure material to do this

Sedimentation equilibrium using an analytical ultracentrifuge

Alkyl hydroxyperoxide reductase from *S. typhimurium*

Recombinant heterodimer generated to study electron transfer properties

Two subunits are co-expressed in *E. coli*:
subunit size: 34,000 and 55,000 (AhpF and AhpC)

Question: what is the molecular weight of the isolated complex with enzymatic activity?

Biochemistry (2001) 40, 3912-3919

Sedimention equilibrium of AhpF/AhpC complex

Beckman Optima analytical ultracentrifuge: monitor A_{280} in the centrifuge cell

Conditions:

several protein concentrations from 3 to 34 µM

115 µl per sample

calculated $\bar{V} = 0.743$ cm^3/g

measured solvent density, $\rho = 1.0058$ g/cm^3

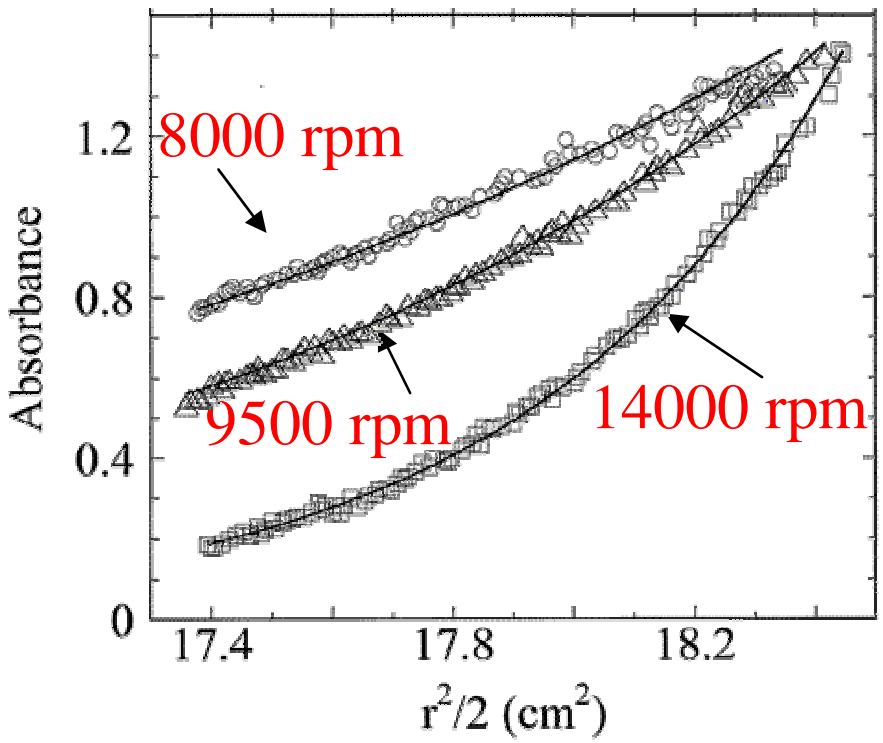
Temperature = 20^o C

Rotor speed varied from 8000, 9500 and 14000 rpm

collect data at 8, 10 and 12 h for each speed

Sedimentation equilibrium data of Alkyl hydroxyperoxide reductase

$$M = d \ln C / dr^2 (2 R T / (\omega^2 (1 - \bar{V}\rho)))$$



Result: M = 86,200 ± 200

Conclusion: Enzyme contains one copy of each subunit

Sedimentation Equilibrium without an analytical ultracentrifuge

Alternatives to the analytical ultracentrifuge

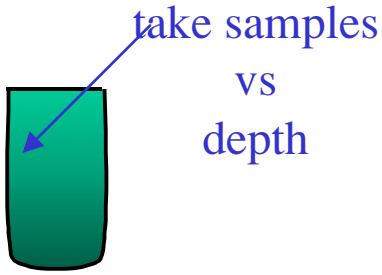
1) the airfuge

2) the preparative ultracentrifuge

Airfuge enables you to measure the distribution of material in a centrifuge tube qusing biochemical techniques

- small, high speed (desktop)
- sample solution for biochemical assays
- get ln c(r) vs r^2 from impure material

- enzyme assays
- binding assays
- antibody detection



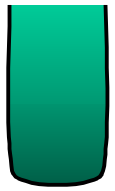
Preparative Ultracentrifuge

1. Can use like the airfuge and remove sample to measure material distribution

2. Can use a **density gradient centrifugation** to separate particles on the basis of $\bar{V}_2$

Sedimentation equilibrium in a density gradient

- use sucrose, CsCl, KBr, etc to establish a density gradient in the centrifuge tube
-either make the gradient prior to centrifugation or
establish the gradient during centrifugation



This creates a 3-component system

1) solute - 2) water - 3) density forming co-solvent (eg, sucrose)

must use the partial specific volume of hydrodynamic particle

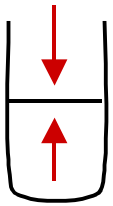
Vol/gram (particle)

Solution density (varies with position in the tube, r)

$$S = \frac{M\,[1 - \bar{V}_h \rho\,(r)]}{N\,f}$$

when $\rho(r) = \frac{1}{\bar{V}_h}$

$S = 0$



Sedimentation $S > 0$ ← $\rho < 1 / \bar{V}_h$ particle density greater than density of solution

Equilibrium $S = 0$ ← $\rho = 1 / \bar{V}_h$ particle density equal to density of solution

Flotation $S < 0$ ← $\rho < 1 / \bar{V}_h$ particle density less than density of solution

For a linear density gradient:

$$\rho(r) = \rho_o + (r - r_o)\left(\frac{d\rho}{dr}\right)$$

where r_o is where $\rho = \frac{1}{V_h}$

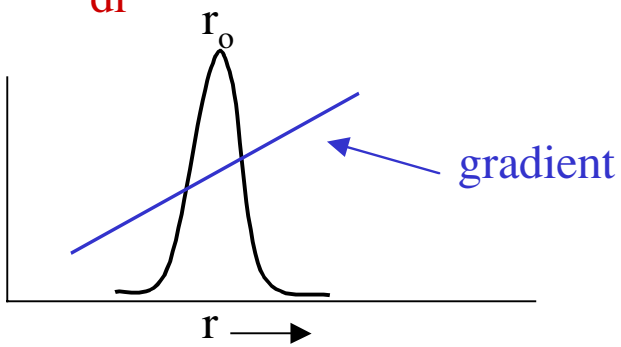
substitute

at equilibrium $\frac{d\mu}{dr} = 0$

$$\frac{1}{c}\frac{dc}{dr} = \frac{\omega^2 M [1 - V_h \rho(r)]}{RT}$$

$$c(r) = c(r_o)\, e^{-\omega^2 r_o M V_h \left(\frac{d\rho}{dr}\right)(r - r_o)^2 / 2RT}$$

Gaussian Distribution about $r = r_o$



Note: Width of the distribution depends on Molecular weight

At r_o: $\frac{1}{\rho_o} = \overline{V}_h = \frac{\overline{V}_2 + \delta_{H2O}}{1 + \delta_{H2O}}$ at the peak (this does not take into account any salt or ions)

Applications of sedimentation equilibrium in a density gradient for analysis and for preparative purposes

1 Different membranes or organelles can be separated on the basis of density

E.coli: inner + outer membranes
eukaryotic: rough + smooth ER

2 Serum lipoproteins: LDL, HDL etc

3 DNA with different GC content

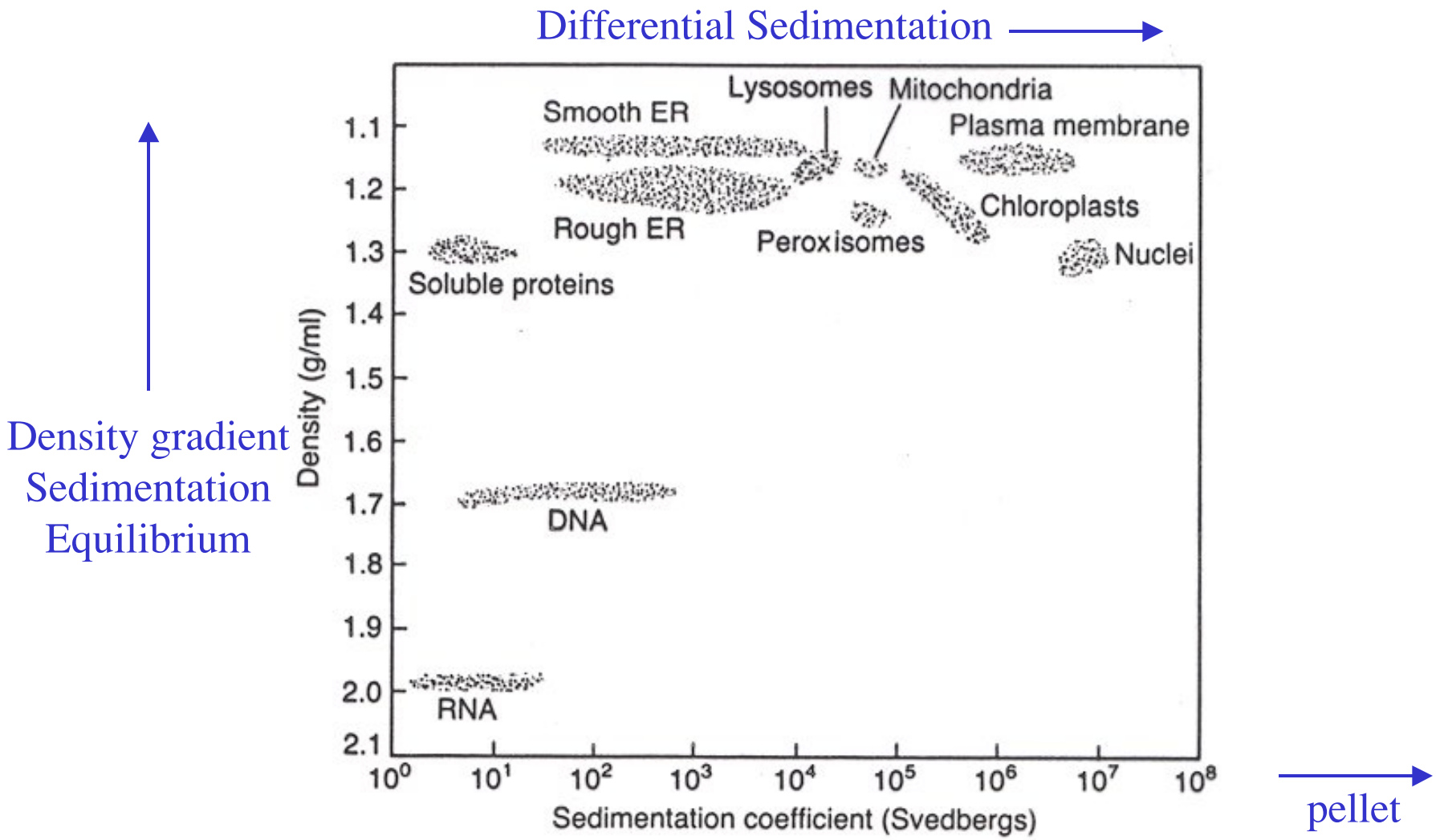
$$\rho = \frac{1}{\bar{V}_h} = 1.66 + 0.09 \bullet f_{GC}$$

4 Single vs double-strand DNA

5 ^{15}N vs ^{14}N containing DNA (or DNA with Br-U instead of T)

6 Supercoils with different amounts of bound ethidium bromide - have different $\bar{V}_h$ values

Some particles cannot be separated based on differences in S value



Case Study: Actin-binding Nebulin Fragments

Nebulin binds to the actin/tropomyosin/troponin thin filament of skeletal muscle

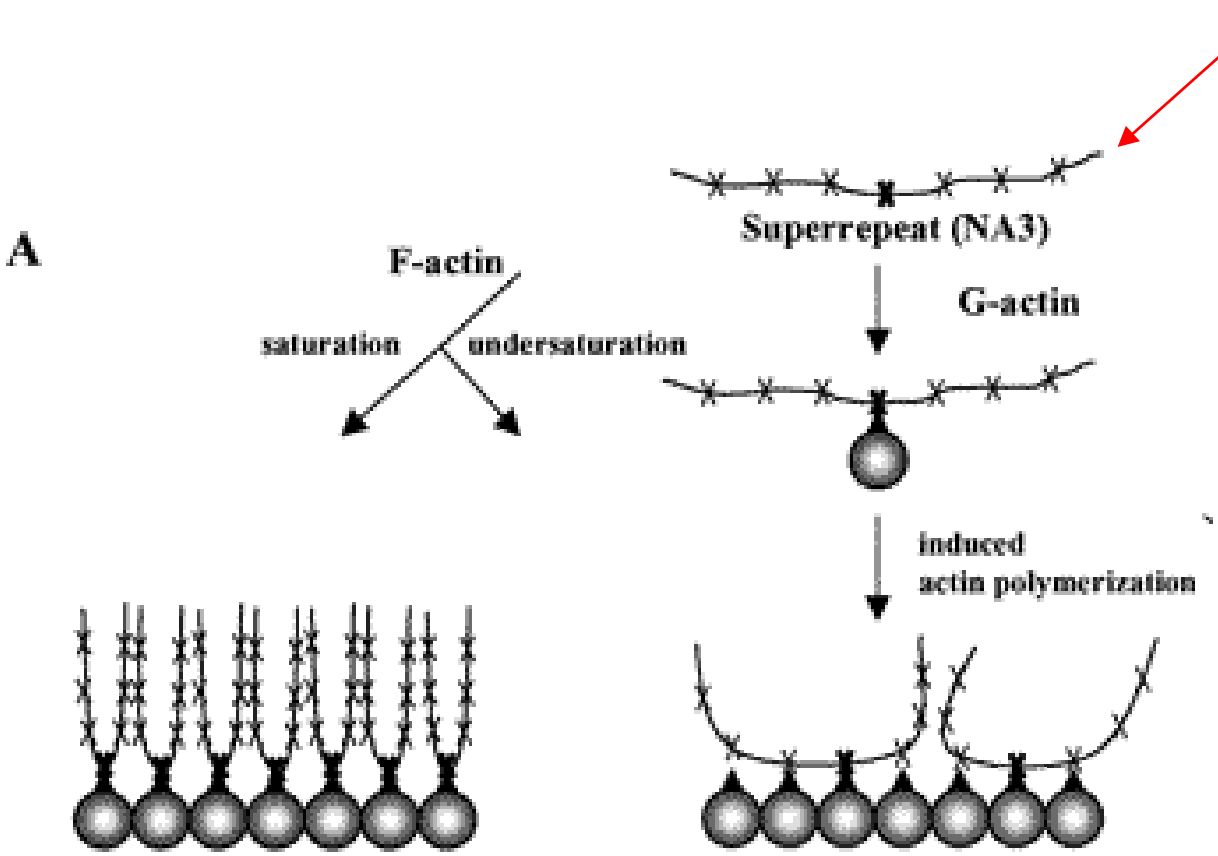
Multiples of repeated sequences arranged in modules

Fragments containing 2 - 15 modules retain actin binding function

Two fragments: NA3 and NA4

Question: Are NA3 and NA4 aggregated in solution?

Model of Nebulin binding to actin



Sedimentation equilibrium of nebulin fragments Using the Beckman Airfuge

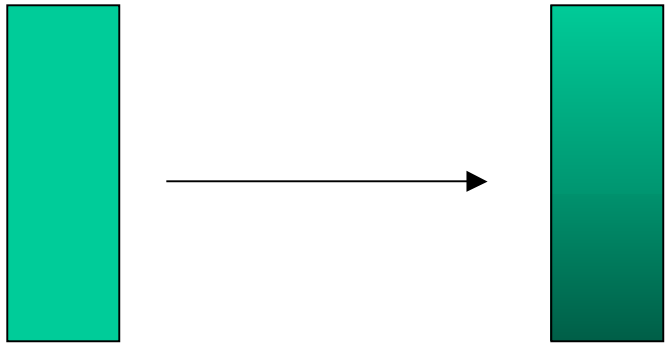
Conditions:

0.1 mg/ml protein

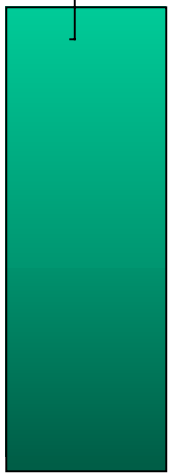
1 mM Ca^{++}, pH 7 buffer

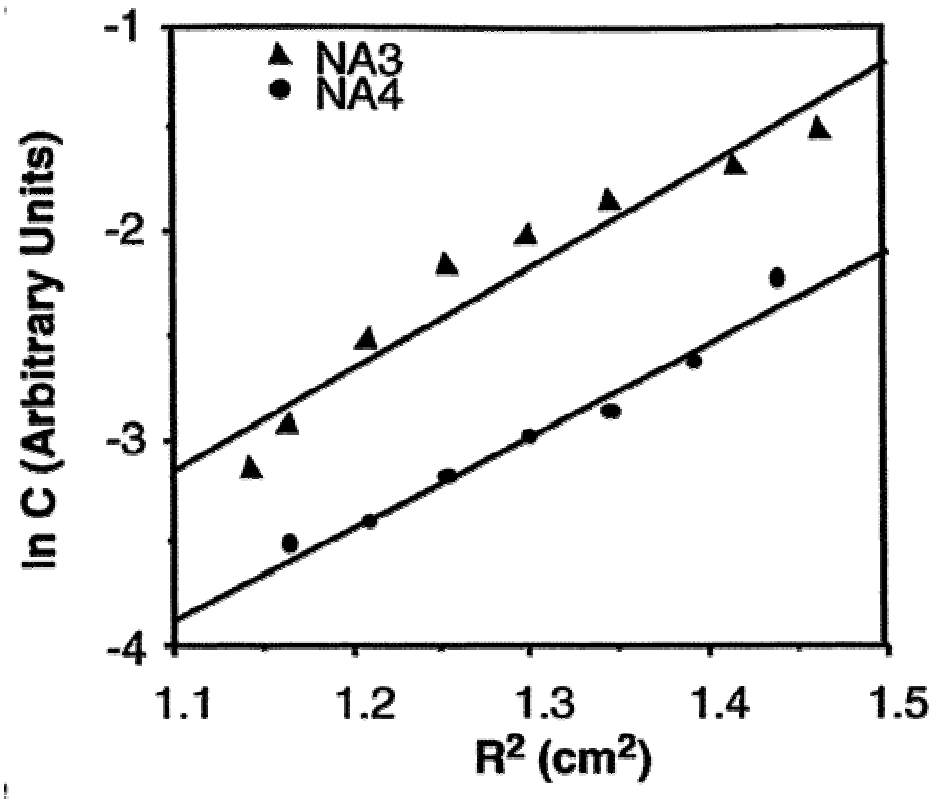
20% sucrose to stabilize the protein distribution during rotor deceleration

Spin solution for 80 h at 54,000 rpm ($\omega = 5700\ s^{-1}$)

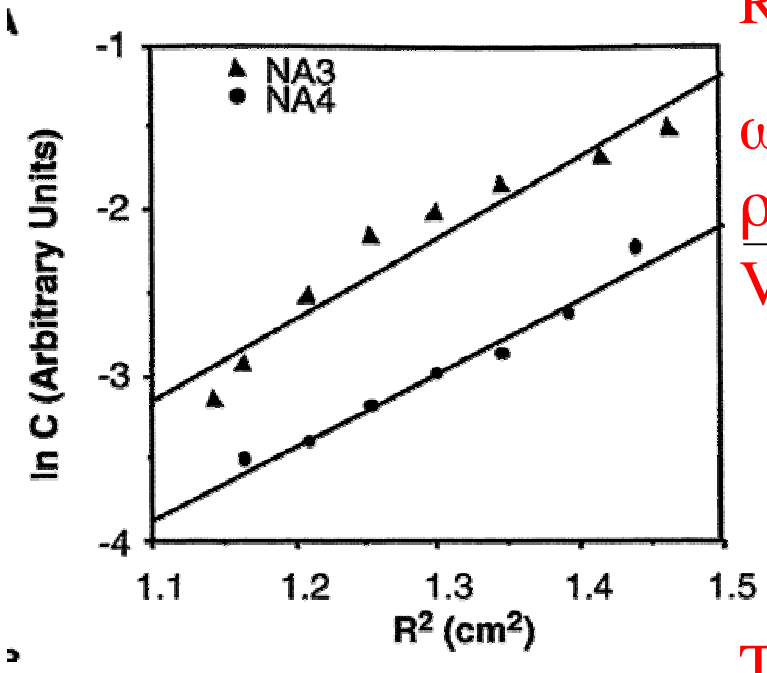


Using a syringe, remove liquid from the top of the tube and measure protein concentration for each fraction





$$M = d \ln C / dr^2 (2 R T / (\omega^2 (1 - \bar{V}\rho)))$$



R (gas constant) = 8.3×10^7 g cm^2 $s^{-2} mol^{-1} K^{-1}$

$\omega = 5700\ s^{-1}$

$\rho = 1.08$ g cm^{-3} density of 20% sucrose

$\bar{V}$ = partial specific volume of nebulin fragments in 20% sucrose.
Calculated from amino acid sequence
0.739 for NA3
0.745 for NA4

T = 300 °K

NA3: M = 37 kDa (monomer = 31kDa)
NA4: M = 35 kDa (monomer = 25 kDa)

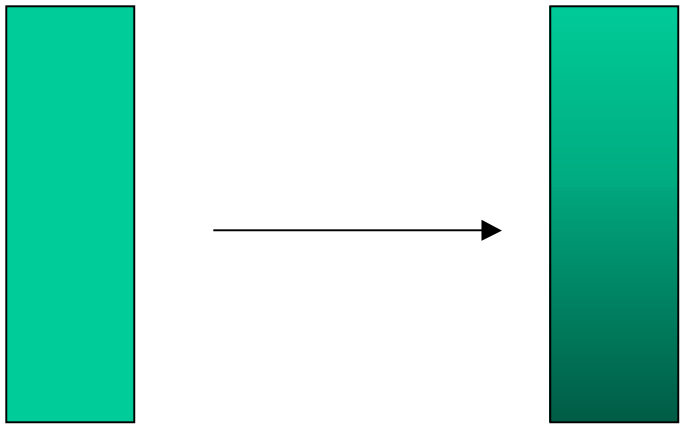
Conclusion:
Nebulin fragments
Are not aggregated

What is the shape of the nebulin fragment?

Sedimentation velocity in a Beckman L5-50 preparative ultracentrifuge: SW41 swinging bucket rotor

Conditions:

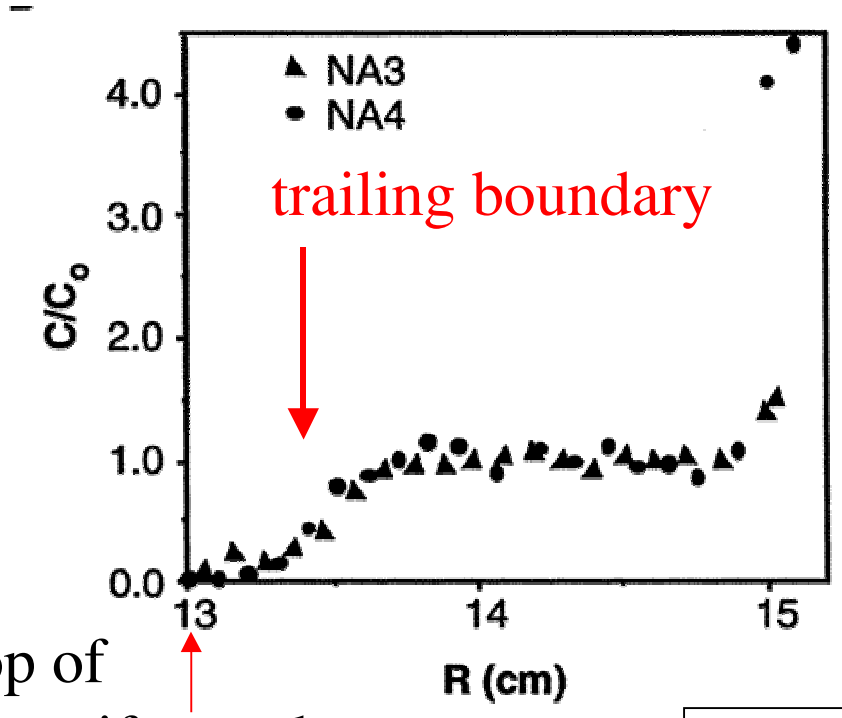
0.05 mg/ml protein concentration
1 mM Ca^{++}, pH 7 buffer
20% sucrose



Remove sample from the top and measure protein to determine the profile of the trailing boundary.

Spin for 18 h at 35,000 rpm at 20° C

Sedimentation Velocity of Nebulin Fragments



$$S_o = (\ln r_b - \ln r_m)/\omega^2 t$$

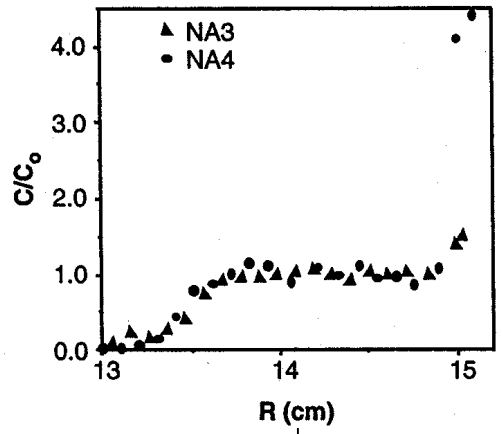
t = 64,800 sec

$\omega = 3665\ s^{-1}$

r_b = boundary position

r_m = meniscus position

Sedimentation velocity of nebulin fra



Must correct for the fact that the measurement is done in 20% sucrose:

1. Density is 1.08 (+sucrose) instead of 1.00 g/cm^3

2. Viscosity is 0.0195 P (+ sucrose) instead of 0.01 P

3. Partial specific volumes are different

   V = 0.739 (+ sucrose) instead of 0.728 cm^3/g for NA3

   V = 0.745 (+ sucrose) instead of 0.733 cm^3/g for NA4

$$S_{w,20} = S_o \frac{\eta(1 - \bar{V}_w\rho_w)}{\eta_w(1 - \bar{V}\rho)}$$

Measured in 20% sucrose

Results:

$S_{w,20}$ = 1.1 S for both NA3 and NA4

Interpretation of the Sedimentation of nebulin fragments

1. We can now calculate the Diffusion coefficients:

From sedimentation equilibrium

$$D_{w,20} = S_{w,20} RT/(M(1 - \bar{V}\rho)$$

2. From this, calculate f/f_{min}:

$$f/f_{min} = (kT/D_{w,20})(6\pi\eta(3M\bar{V}/4\pi N_o)^{1/3})$$

(Note: use values of η, ρ and $\bar{V}$ that apply to the protein in water)

Results: NA3	**$D_{w,20} = 3.2 \times 10^{-7}$ cm^{2}s^{-1}**	**$f/f_{min} = 3.27$**
NA4	**$D_{w,20} = 3.6 \times 10^{-7}$ cm^{2}s^{-1}**	**$f/f_{min} = 2.96$**

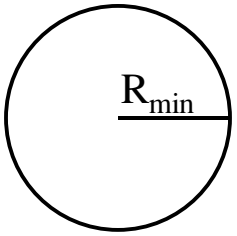
f/f_{min}

experimental $\quad D = \frac{kT}{f} = \frac{kT}{6\pi\eta R_S}$ $\quad$ since $f = 6\pi\eta R_s$

$$\mathbf{R_S = (kT/D_{w,20})/6\pi\eta}$$

Anhydrous sphere

$$\text{vol} = [\bar{V}_2 \bullet \frac{M}{N}] = \frac{4}{3}\pi R^3_{min}$$



$$\mathbf{R_{min} = (3V_2M/4\pi N)^{1/3}}$$

:

Define: $f_{min} = 6\pi\eta R_{min}$

measured

$$\left(\frac{R_s}{R_{min}}\right) = \left(\frac{f}{f_{min}}\right) = \frac{(kT/D_{w,20})/6\pi\eta}{(3V_2M/4\pi N)^{1/3}}$$

anhydrous sphere

Nebulin fragments are highly asymmetric

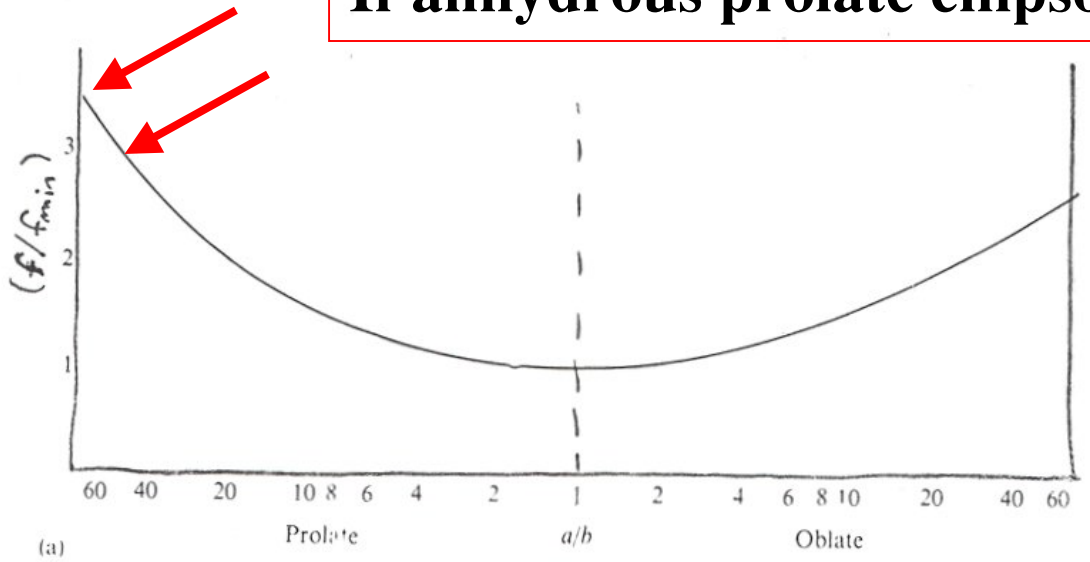
NA3

$f/f_{min} = 3.27$

NA4

$f/f_{min} = 2.96$

If anhydrous prolate ellipsoids: a/b = 50/1 to 60/1



about 600 Å long