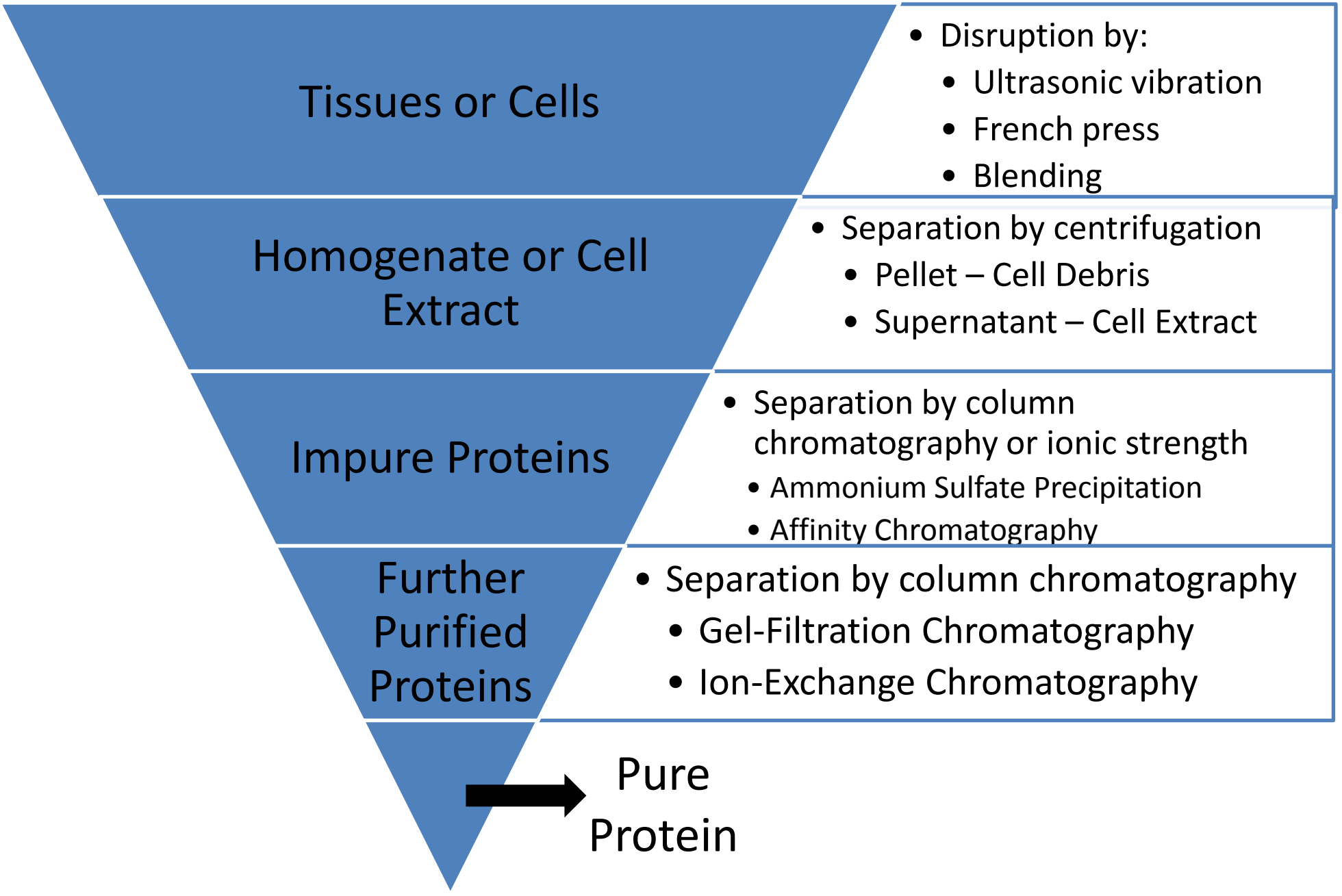
Chapter 3 – Week 1
Parts A,B,C

Purification of Lactate Dehydrogenase (LDH)

Purpose:

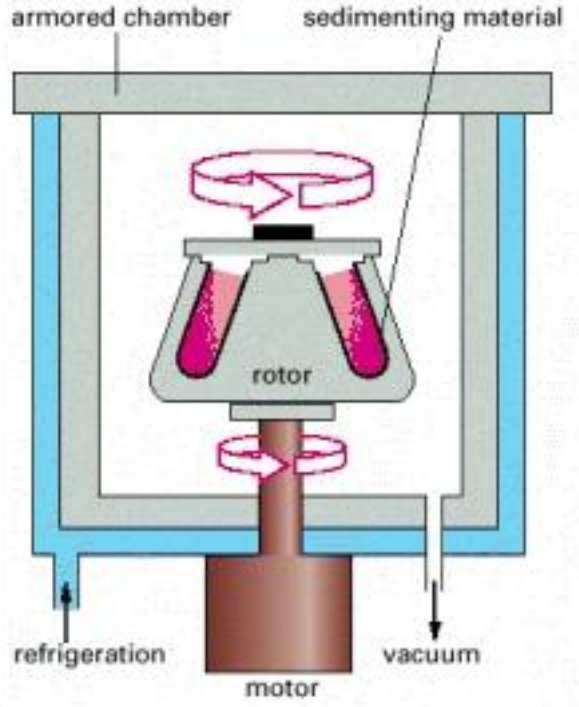
1. Learn basic techniques for protein purification
2. Prepare crude extract
3. Run enzyme assay

Protein Purification Process



Centrifugation

- Uses centrifugal force applied to sample to separate complex mixtures
  - Breaking down of cells into various components
- Largest units experience largest centrifugal force, moving rapidly to bottom of tube
- Speed is observed in RPM – revolutions per minute
- Can compare between different centrifuges using RCF – relative centrifugal force:



$$RCF = [r(2\pi N)^2]/g = (1.118 \times 10^{-5})(r_{cm})(N_{RPM})^2$$

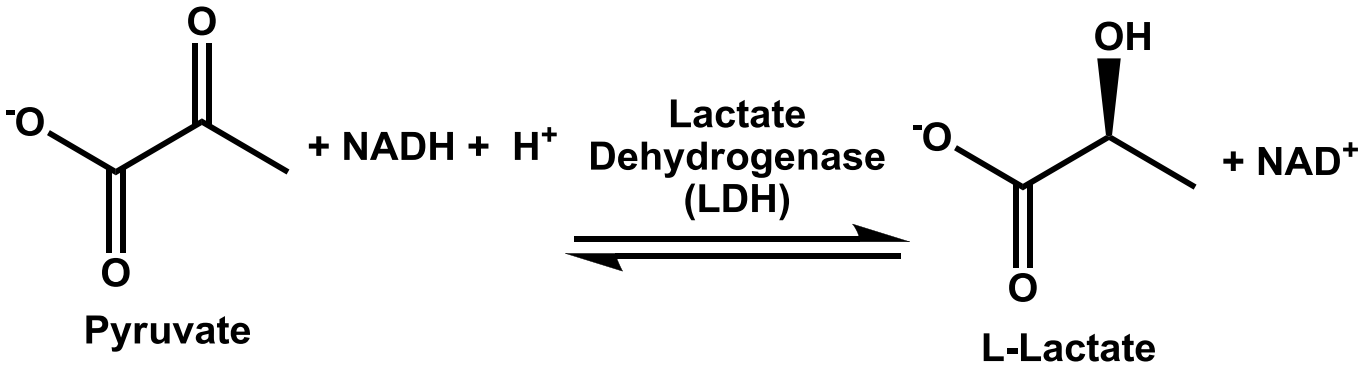
g = gravitational acceleration = 9.8 m/s^2

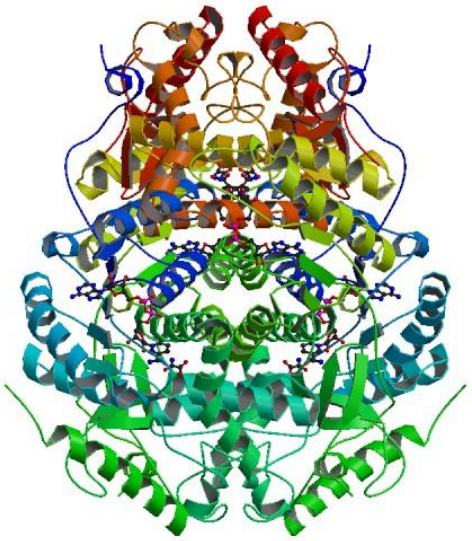
r = radius of rotor

N = rotational speed in RPM

Lactate Dehydrogenase (LDH)

- LDH catalyzes the last step of anaerobic glycolysis

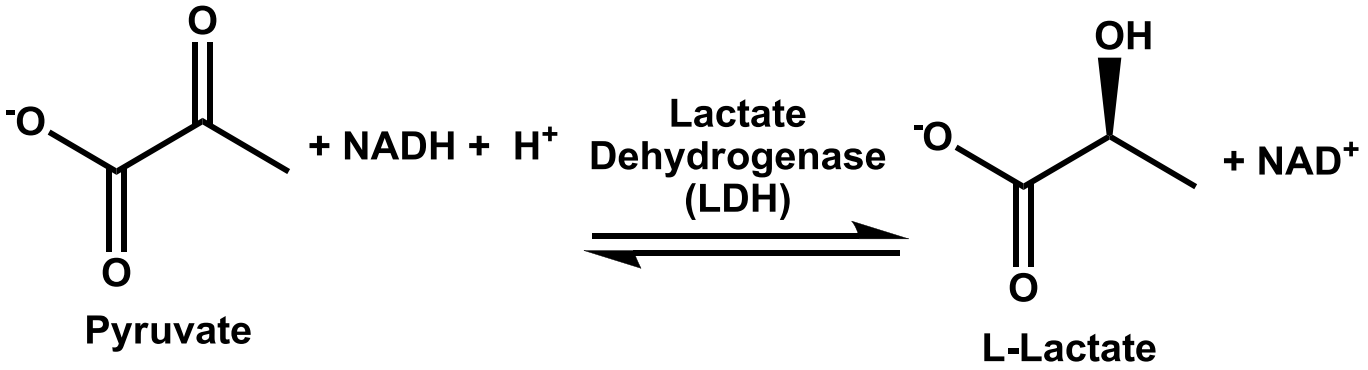




- Known kinetic parameters and crystal structure
- Multiple forms of LDH found in different tissues – Isozymes
  - Each isozyme has slightly different kinetic and structural properties, but same function and overall structure

Cofactors of LDH

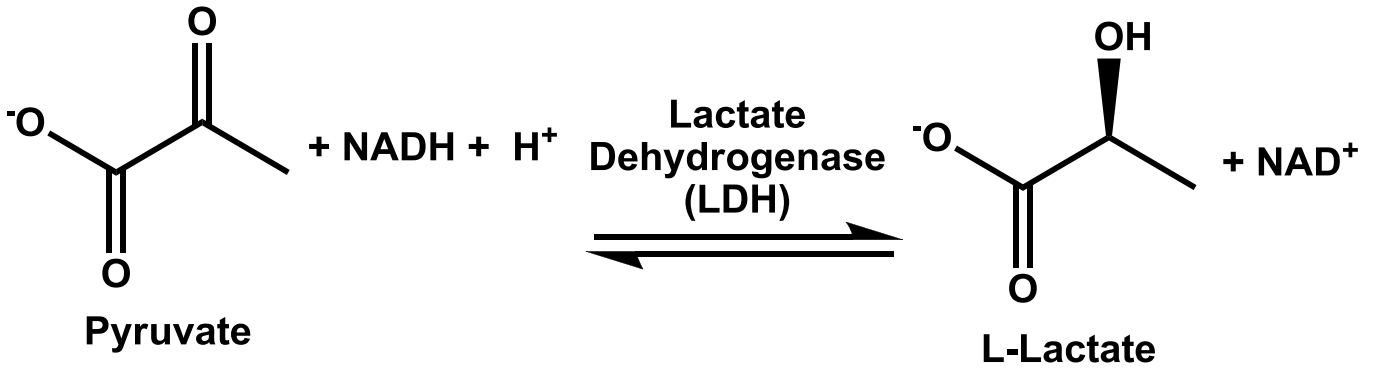
- LDH uses pyridine nucleotide coenzymes as cofactors to transfer reducing equivalents
  - H^+ and a pair of electrons
- NAD^+ – Nicotinamide adenine dinucleotide → Oxidized form
- NADH → Reduced form



- Pyridine nucleotides have characteristic absorbance spectra
  - NAD^+: No absorbance at 340 nm
  - NADH: λ_{max} = 340 nm, ε = 6210 $M^{-1}cm^{-1}$

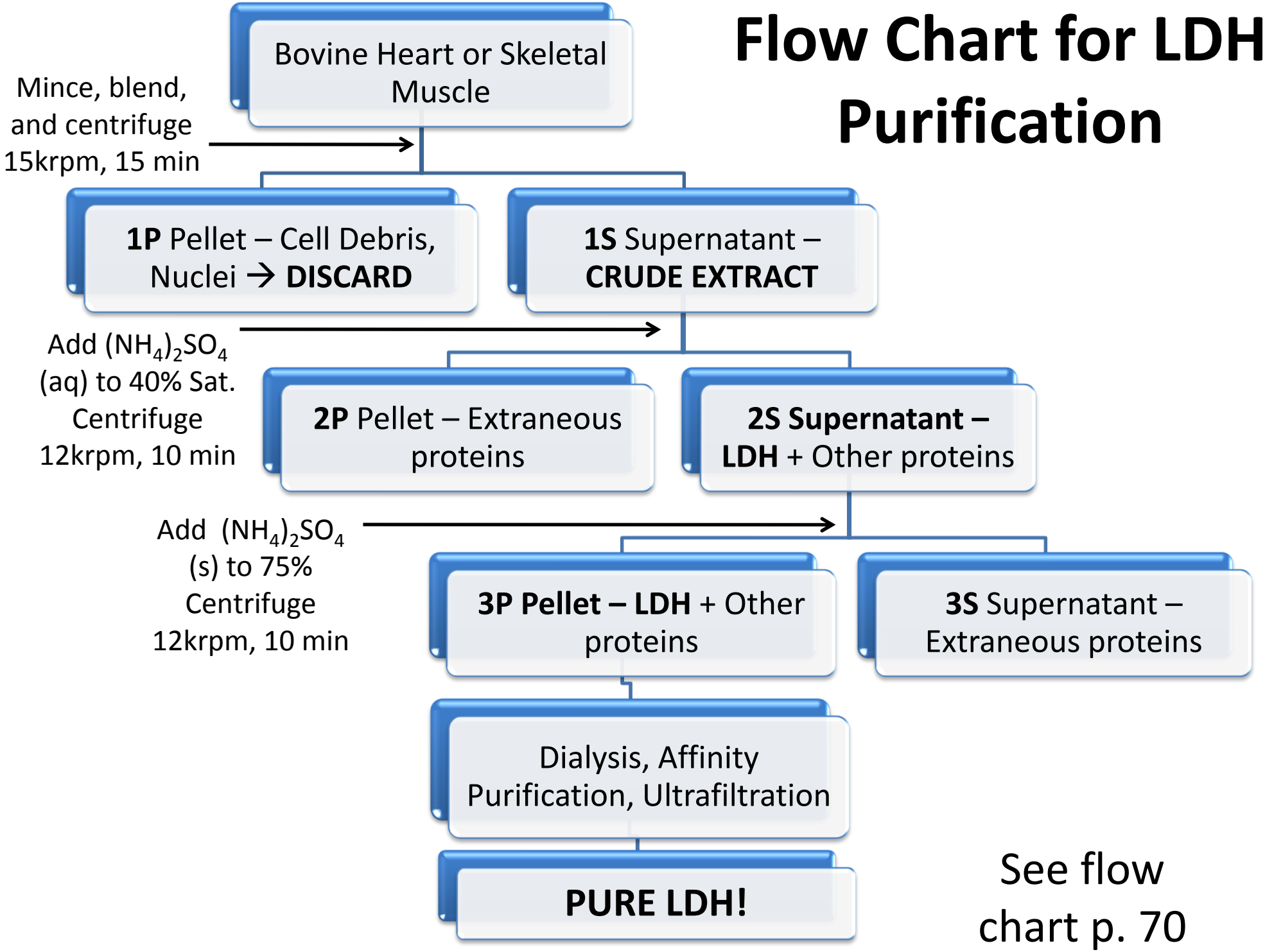
How to Measure if LDH is Present?

- Enzymes are catalysts – speed up the rate of the reactions without being consumed



- We can measure:
  - Rate of consumption of reactants (Pyruvate or NADH)
  - Rate of formation of products (L-Lactate or NAD^+)
- NADH has a visible absorbance at 340 nm – can follow its consumption during reaction
- Always want to monitor the initial rate because concentrations change during the reaction

Flow Chart for LDH Purification



See flow chart p. 70

Week 1: Procedure

- Purify Beef Heart or Skeletal Muscle
- Absorption Spectra & Calculate Extinction Coefficient
- Activity Assay
- Protein Concentration – Dye Binding Assay

Keep everything on ice!
Especially LDH extract!

Week 1: Procedure

- Purify Beef Heart or Skeletal Muscle
  - Blend with buffer, measure volume of homogenate
  - Centrifuge 15,000 rpm for 15 minutes
  - Discard 1P fraction, measure total volume of 1S fraction
  - Save ~1 mL of 1S fraction in separate aliquot for activity assays and dye-binding
  - Save rest of 1S fraction for Week 2

Record everything!
Masses, volumes, concentrations etc!
When in doubt, don't throw it out!

Week 1: Procedure

- Absorption Spectra & Calculate Extinction Coefficient
  - Obtain spectra of NAD^+ and NADH with UV-Vis Spectrometer
  - Calculate the apparent extinction coefficient for your bench top spectrophotometers
    - Given extinction coefficients are for square cuvettes with 1 cm path length
    - Measure A_{340} of NADH with your spectrophotometer and UV-Vis spectrophotometer and calculate:

$$\varepsilon_\lambda(\text{apparent}) = [\varepsilon_\lambda(\text{UV})][A_\lambda(\text{test tube})/A_\lambda(\text{UV cuvette})]$$

Week 1: Procedure

- Activity Assay
  - Monitors $A_{340\ nm}$ to observe of the conversion of NADH to NAD^+ as the reaction proceeds
  - Requires cocktail with all other reagents except enzyme and water
    - Make enough for 10 assays (See Recipe, p. 73)

"The Cocktail"

Reagent	Volume
1 M KPO_4, pH 7.4	200 µl
6 mM Sodium pyruvate	300 µl
1 mM NADH	300 µl
DI Water	2.15 ml
Diluted Enzyme Solution	50 µl
Total Volume	**3.00 ml**

  - Will need to dilute enzyme substantially before starting assays
    - Typically 1:200 or 1:400 dilutions of enzyme are required

Week 1: Procedure

- Activity Assay
  - To run assays:
    - Use LDH Kinetics program
    - Blank spectrometer
    - Add cocktail and water to cuvette
    - Add enzyme, mix, and start run
  - Want to look at linear portion of graph
  - Readout is a **RATE**: $-\Delta A_{340}$/min
    - Why Negative?

- Need at least 4 dilutions in range ΔA_{340}/min -0.05-0.25
- Need table of time vs. A_{340} for 1 run

Week 1: Procedure

- Protein Concentration – Dye Binding Assay
  - Do only if time allows, otherwise save for week 2
  - You can use your standard curve from Lab 1 if it goes through the origin and is a good fit
    - When in doubt, make a new standard curve
  - Record protein concentration for 1S fraction
    - Dilute your protein with water!
    - Blank?
  - Use protein concentration with activity for calculations

Activity Calculation

Activity = Units = µmol of Substrate Consumed or Product Formed / min

Activity concentration (ΔC) = [Activity] = µmol/min*ml = Units/ml

From Beer's Law: $A = (\varepsilon)(l)(C)$

We are looking at Kinetic Rates: $\Delta A = (\varepsilon)(l)(\Delta C)$

Apparent extinction coefficient, ε_{app}, in mM^{-1} combines ε and l

$$\Delta A_{340/min} = (\varepsilon_{app} \text{ in } mM^{-1})\ (\Delta C)$$

$$[\text{Activity}] = \Delta C = \Delta A_{340/min} / \varepsilon_{app} \text{ in } mM^{-1}$$

$$[\text{Activity}] = (0.05/min)/(6.21\ mM^{-1}) = 0.0081\ mM/min =$$

0.0081 µmol/min*ml = **0.0081 units/ml**

mM = µmol/ml
mM^{-1} = ml/µmol

Enzyme Activity <u>in the Assay</u>

Activity Calculation

$$[\text{Activity}] = \text{Units/ml} = \frac{\mu\text{mol of Substrate Consumed or Product Formed}}{\text{min} * \text{ml}}$$

$$[\text{Activity}] = \Delta C = \Delta A_{340/\text{min}} / \varepsilon_{app} \text{ in } mM^{-1} = (0.05/\text{min})/(6.21\ mM^{-1}) =$$

0.0081 units/ml <u>in the assay</u>

You must account for the dilutions of your protein!

$$[\text{Activity}_{\text{Undiluted}}] = (\Delta C)(\text{Total Volume of Assay})(\text{Dilution Factor})$$

$$\frac{}{(\text{Volume of enzyme used in assay})}$$

$$[\text{Activity}_{\text{Undiluted}}] = \frac{(0.0081 \text{ units/ml})(3.0 \text{ ml})(400)}{(0.05 \text{ ml})} = 193 \text{ units/mL}$$

More Enzyme Calculations

- Total Activity = (Activity)(Total Volume) = Units/ml* ml = Units
- Protein = Mass Protein/Volume Extract = mg/ml
- Total Protein = (Protein)(Total Volume) = mg/ml* ml = mg
- Specific Activity = Total Activity/Total Protein = Units/mg
- % Yield = $\left[\frac{\text{Total Activity in Given Step}}{\text{Total Activity in Crude Extract}} \right] \times 100$

Remember to account for the dilutions of your protein!

For needed calculations, see purification table, p. 86