BIOC4004 - Industrial Biochemistry
Lecture 10 - Wed Feb 04, 04

Topics for the Day:

- Mammalian Cell Culturing (continued)
- Producing protein !!!
- Scaling-up production
- Protein Purification

Extension for Research proposal Topics :
due next Friday (Feb 13th, 2004)

Special Requirements for Mammalian Cell Cultivation

Because it is very difficult to obtain stable mammalian transformants, the transformed cell is a "valuable commodity"

- Cell Preservation
    - Bacteria:
        - freeze drying with storage at room temp
        - storage at -70C in 30% glycerol
    - Mammalian Cells:
        - Long term storage in liquid nitrogen (-196 C)
        - Use cryoprotectants:
            - 10% DMSO, 10-30% Glycerol, etc…
        - Need very rapid rate of freezing to avoid ice crystals

Slow growth and rich media, need really high aseptic technique to avoid contamination

- Can't use antibiotics to control bacterial contamination
- Very tightly controlled entry points into the culturing system
- All equipment used for mammalian work **only**
- Medium often has heat labile components
    - membrane filtration to sterilize

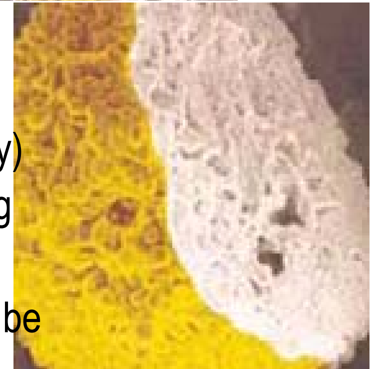
Mammalian cells are extremely finicky as far as water quality

- Extremely pure water (WFI grade - water for injection)
    - low metal toxicity
    - no endotoxin
    - low inorganics and organics
    - no traces of detergents or plant decay products

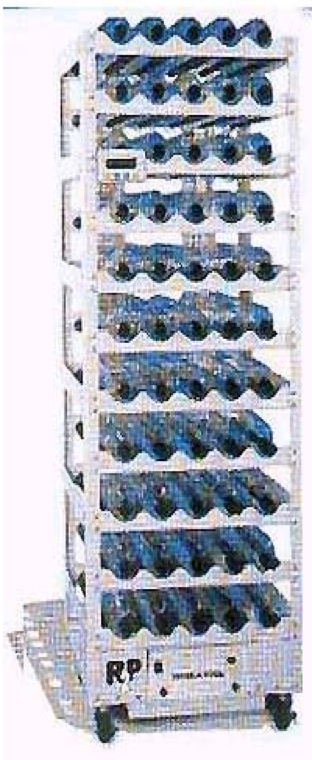
New Types of Mammalian Bioreactors

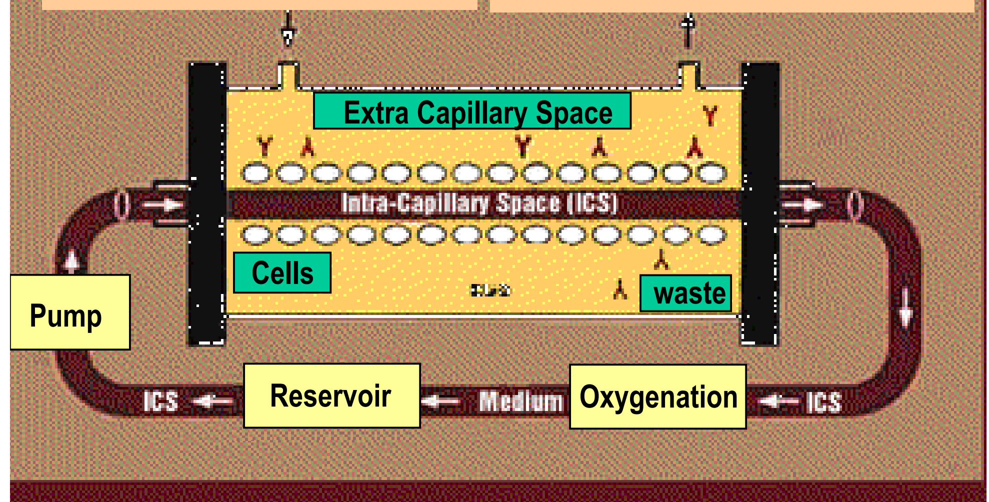
Conventional fermentors can be used as Bioreactors

- need to tailor for use with mammalian cells
  - lecture 7, slide 9
    - pH, temperature, mixing issues !
- Some cells require attachment to a matrix:
  - use carrier particles
    - cells grown on the particles
      - provides attachment point
      - increases surface area (higher cell density)
      - increases mechanical resistance to mixing
- Fermentor-type bioreactors are very predominant
- New types of specialized bioreactors have (and continue) to be developed



Carrier particles

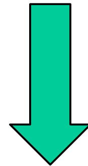




For production of secreted proteins (eg. Antibodies)

Steps in recombinant protein production (I)

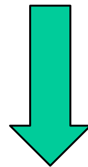
Starter culture of recombinant micro-organism or infected/transfected cell line



Bioreactor:

- establish exponential phase growth
- induce at optimal stage for protein expression

Harvest culture supernatant or cells(centrifugation/microfiltration)



- Kill cells remaining in supernatant
- Disrupt cells to release contents

Purification of product to necessary standards

Frozen cell stock → Starter culture → 1st stirred tank reactor (cell expansion) → 12,000 liter stirred tank reactor (cell expansion, product formation)

Steps in recombinant protein production (II) : Downstream processing

Disruption of cells

Mechanical
- Freeze-thaw
- French Press
- Homogenisation
- Sonication

Chemical
- Alkali
- detergents
- organic solvents

Enzymatic (for cell wall degradation)
- Lysozyme (Gram-positive bacteria)
- Lysozyme + EDTA (Gram-negative bacteria)
- β-1,3-glucanase, β-1,6-glucanase, chitinase, mannase (Yeasts)

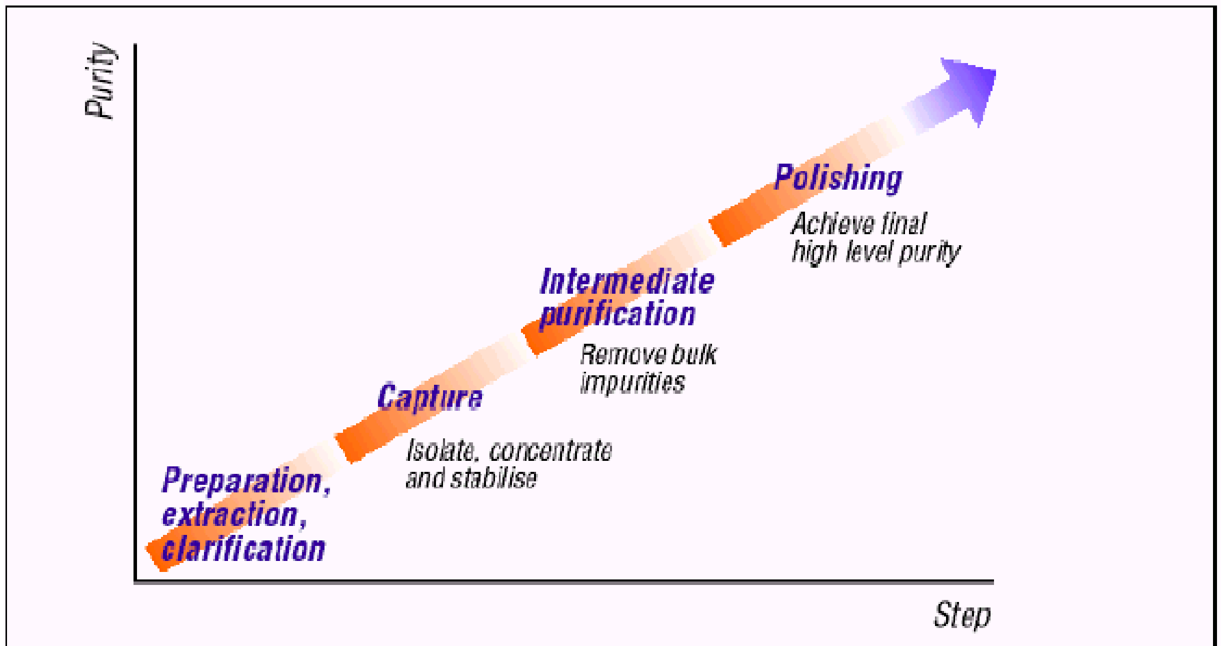
• Mechanical disruption of cells is cost-effective and highly efficient yet may be limited in through-put
• Chemical disruption of cells can be harsh and detergents are expensive. This method is efficient and useful for solubilising some products
• Enzymatic disruption of cells is highly specific. The enzymes themselves can be produced by recombinant micro-organisms to cut costs

Preliminary Purification

• Clarification by high speed centrifugation
• Precipitation using salts (eg.Ammonium Sulfate), organic solvents (eg.TCA)
• Concentration by ultrafiltration

• Many overexpressed proteins can be relatively pure following simple precipitation and concentration steps
• For other proteins, particularly those destined for pharmaceutical use, further purification is necessary

Four Phase Purification Strategy



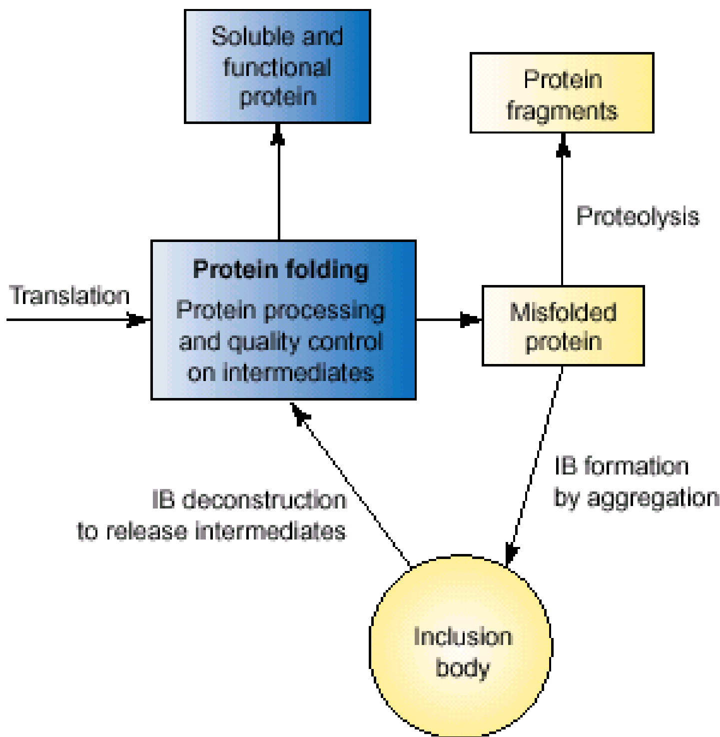
Concentration: centrifugation, salting out, $AmSO_4$

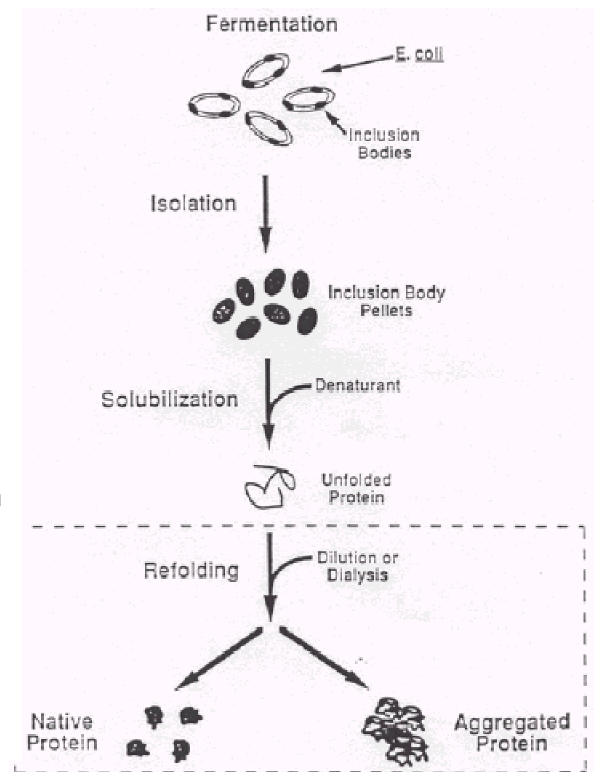
Capture: affinity chromatography, ion exchange, hydrophobic

Intermediate: affinity chromatography, ion exchange, hydrophobic

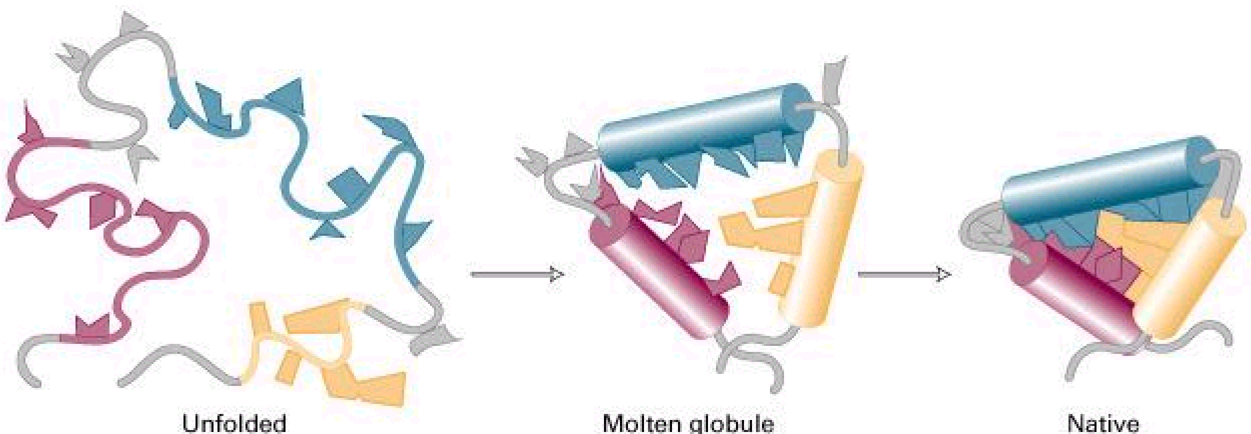
Polishing: affinity chromatography, ion exchange, gel exclusion

Protein Folding and Refolding (revisited)





Three stages in unassisted protein folding.



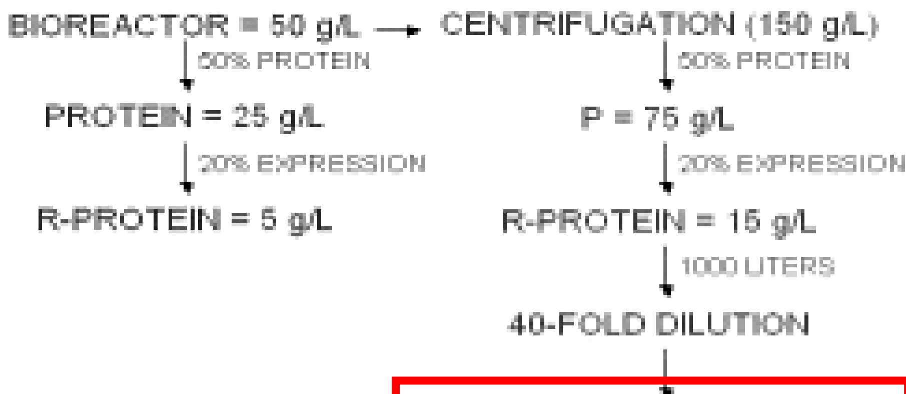
- Sometimes denaturing and refolding is acceptable
- You take a loss on yield (refolding is usually quite inefficient, if it works at all)
- Who cares (if you are first to market !!!)

Protein Folding and Refolding (revisited)

- DENATURATION (e.g., UREA = 8 M)
  - PROTEIN CONC. HIGH: 40-80 g/L
- DILUTION REQUIRED
  - PROTEIN AGGREGATION AT HIGH CONCENTRATIONS
  - METHODS FOR DILUTION AND REMOVAL OF DENATURANT:

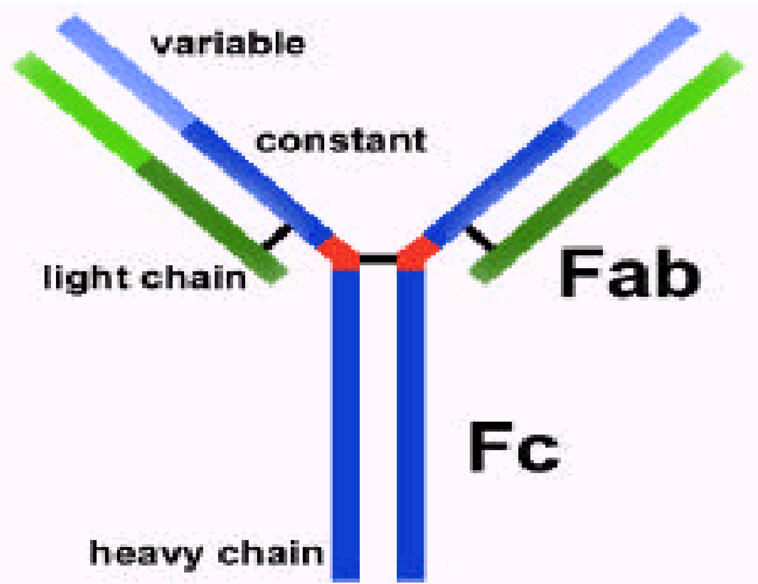
    - DIALYSIS (DIAFILTRATION)
      MEMBRANE WITH BUFFER EXCHANGE

    - DILUTION WITH BUFFER TO
      REDUCE DENATURANT CONC. AND PROTEIN CONC.
  - TYPICAL PROTEIN CONCENTRATION AFTER DILUTION
    e.g., 8 M UREA → 0.2 M UREA (40 FOLD)
    ∴ 40 g/L → 1 g/L PROTEIN CONC.
  - AT LABORATORY SCALE: DILUTION ACCEPTABLE
  - INDUSTRIAL SCALE: 1,000 LITERS

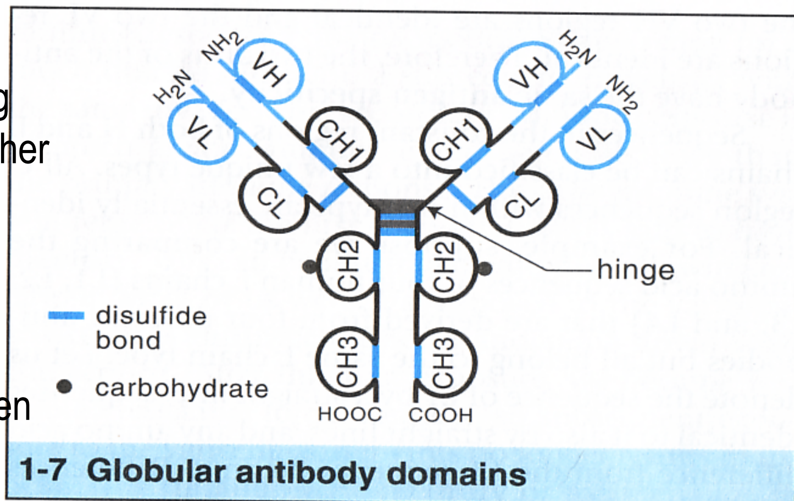


STOP THE INSANITY →

An informal intro to Antibodies (Ab)

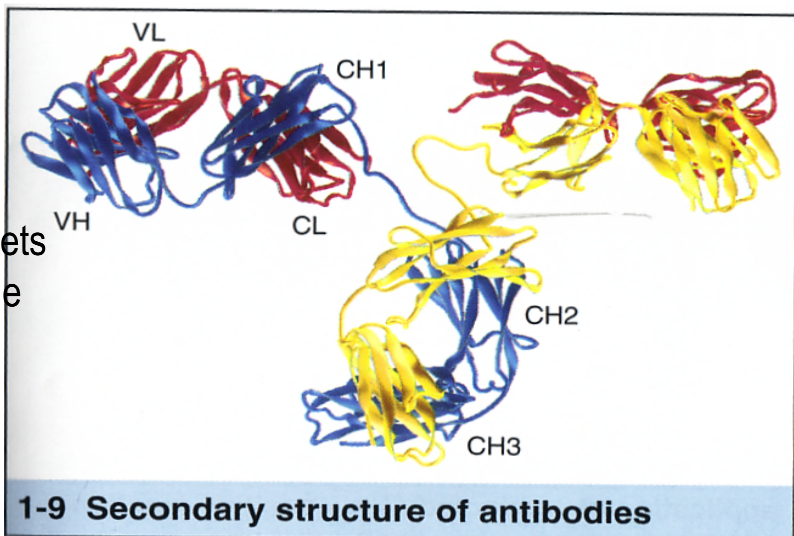


- Produced in B-cells
  - secreted
  - cell surface
- Two heavy chains
  - conserved domain
  - variable domain
- Two light chains
  - conserved domains
  - variable domains



1-7 Globular antibody domains

- Fab responsible for antigen binding
- Fc responsible for mediating "all other interactions"
  - transport
  - cell recruitment
- Heavy chain is glycosylated
- Disulfide bridges within and between the chains

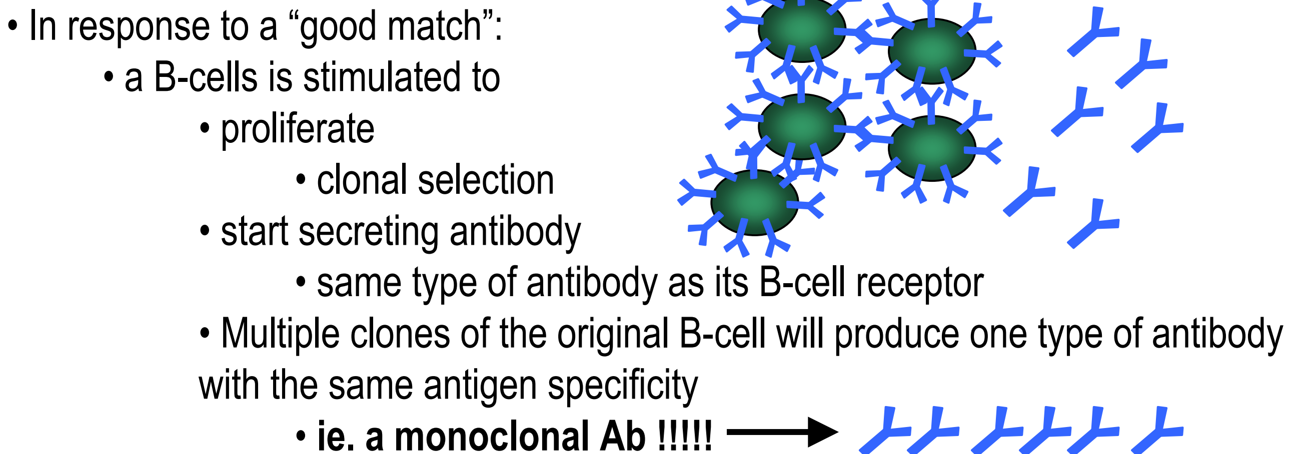


1-9 Secondary structure of antibodies

Each domain is globular in structure
- Ig folddomain
- 2 Beta sheets
  - Disulfide bridge between sheets
- In the variable domains, three of the loops connecting beta-strands
  - variable
  - antigen binding

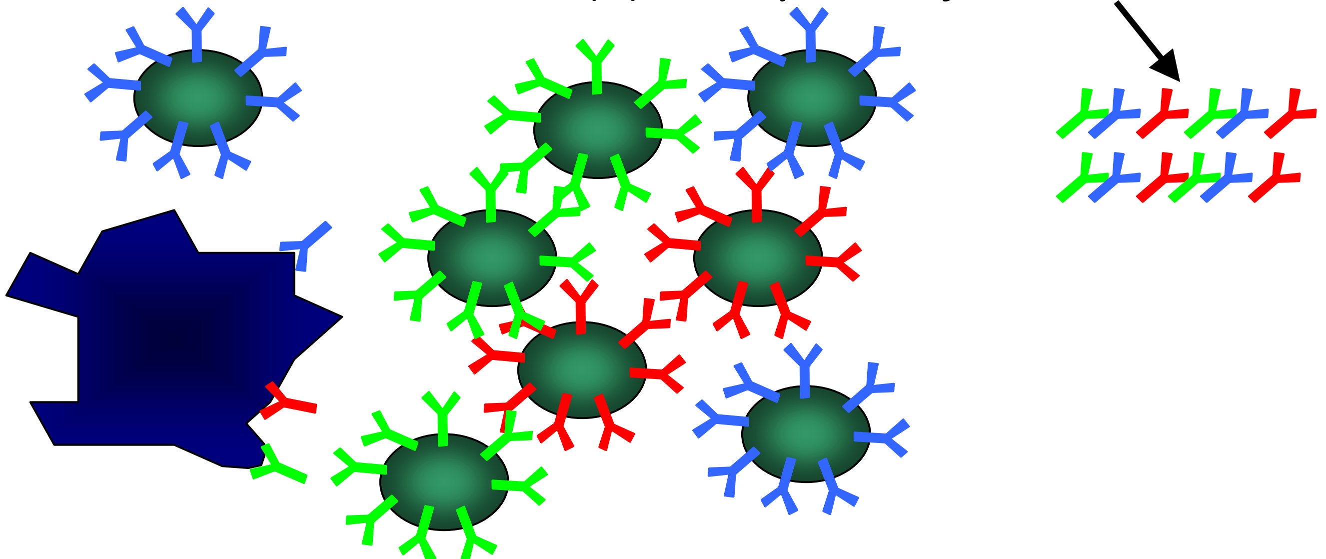
Monoclonal Ab vs Polyclonal Ab

- B-cells before an antigen encounter are called **naïve**
  - express B-cell receptors (essentially membrane-bound antibodies)
  - all of the receptors have the same antigen specificity
    - dictated by variable regions of the antibody
  - naïve cells go around "sampling" antigens until they find one that binds well



- In response to a "good match":
  - a B-cells is stimulated to
    - proliferate
      - clonal selection
    - start secreting antibody
      - same type of antibody as its B-cell receptor
    - Multiple clones of the original B-cell will produce one type of antibody with the same antigen specificity
      - **ie. a monoclonal Ab !!!!!**

- An antigen will have several different types of antibodies that can bind to it
  - each antibody will bind to a different **epitope**
    - ie. a different specificity determinant
  - each antibody type is generated by a clonal population of B-cells
    - The collection of clonal populations yields **Polyclonal Ab**



Monoclonal Antibodies (mAb)

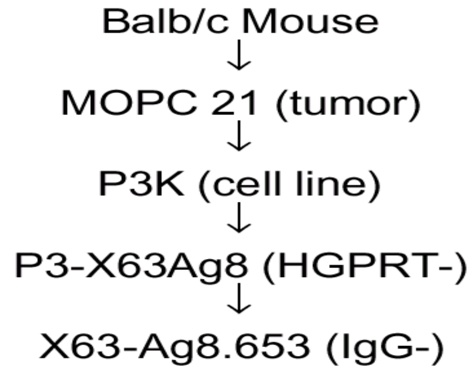
- Described in 1975 by Kohler and Milstien (1984 Nobel prize in medicine)
- Growth of clonal populations of cells secreting antibody of defined specificity
- The method consists of fusing B-cells with **myeloma cells** (a type of B-cell tumor).
- The fused cell is known as a **hybridoma**.
  - ability to grow in culture (phenotype acquired from myeloma)
  - secretes antibody of defined specificity (phenotype acquired from the B-cell)
- The antibody produced by hybridoma is a **monoclonal antibody** (mAb).

Uses of mAbs

• **Research**: study of specific biological or medical systems
ELISA, Western blotting, immunoprecipitation, Immunofluorescence, immunohistochemistry, flow cytometry, FACS...
• **Diagnostics (detection)** : determine presence or concentration of specific subatances in a given sample
• **Therapeutics**: as a treatment approach
• **Prophylaxis**: protective or preventatuve measure

- Advantages of mAbs
  - ♦ unlimited supply
  - ♦ defined reagent
- Disadvantages of mAbs
  - ♦ single epitope
  - ♦ time consuming

Making Hybridomas (Part I)



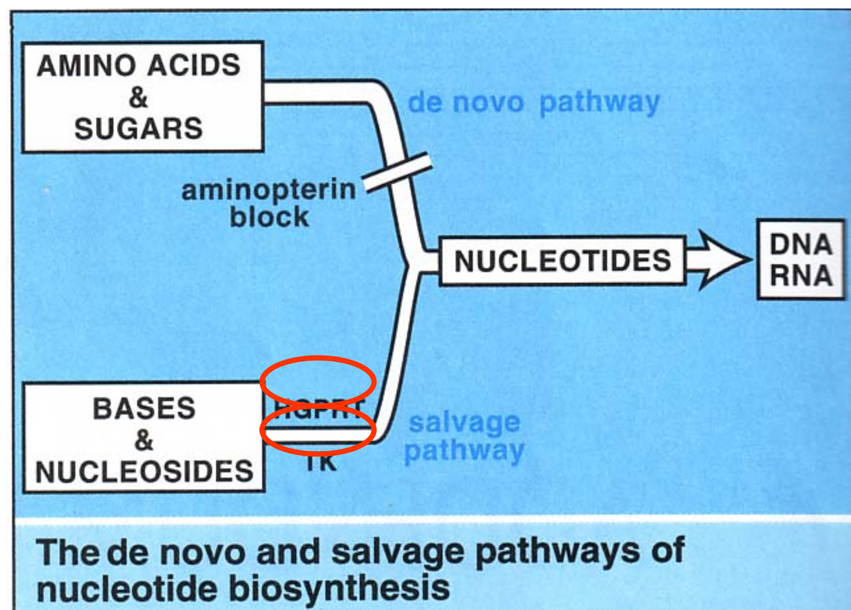
Hybridoma = cell fusion
- B-cell
- myeloma cell

Normal B-cell
- secretes a given Ab
- mortal

Myeloma cell (tumorigenic B-cell)
- mutated for **salvage pathway**
  Hypoxanthine **G**uanine **P**hospho**R**ibosyl **T**ranferase
- mutated for no Ab secretion
- immortal

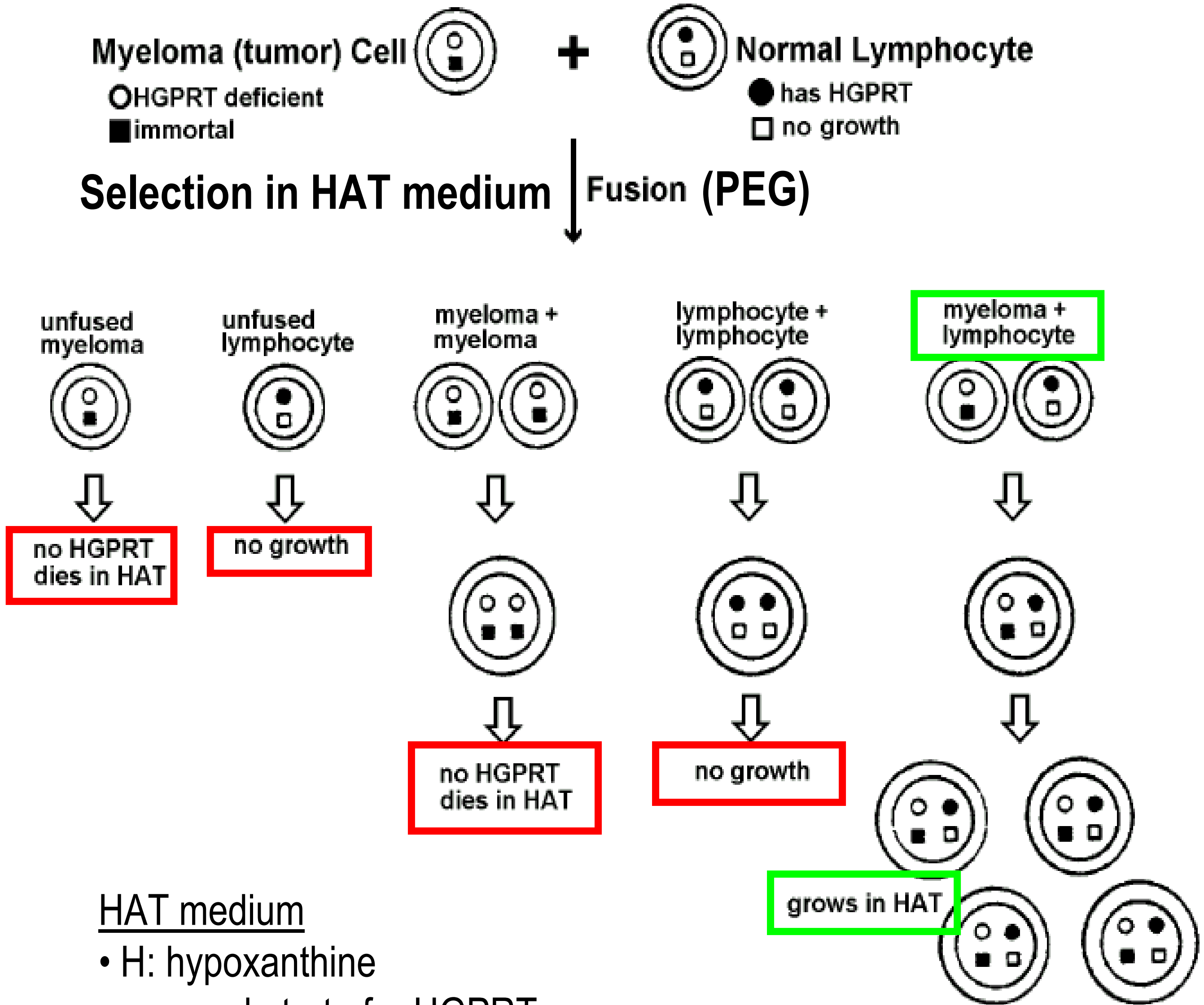
The Salvage Pathway

- Nucleotides are necessary for life!!!!
- Two pathways:
  - *de novo* synthesis
  - salvage pathway
    reuse and recycle
- if *de novo* synthesis is not available, can use salvage pathway



The de novo and salvage pathways of nucleotide biosynthesis

Making Hybridomas (Part II)



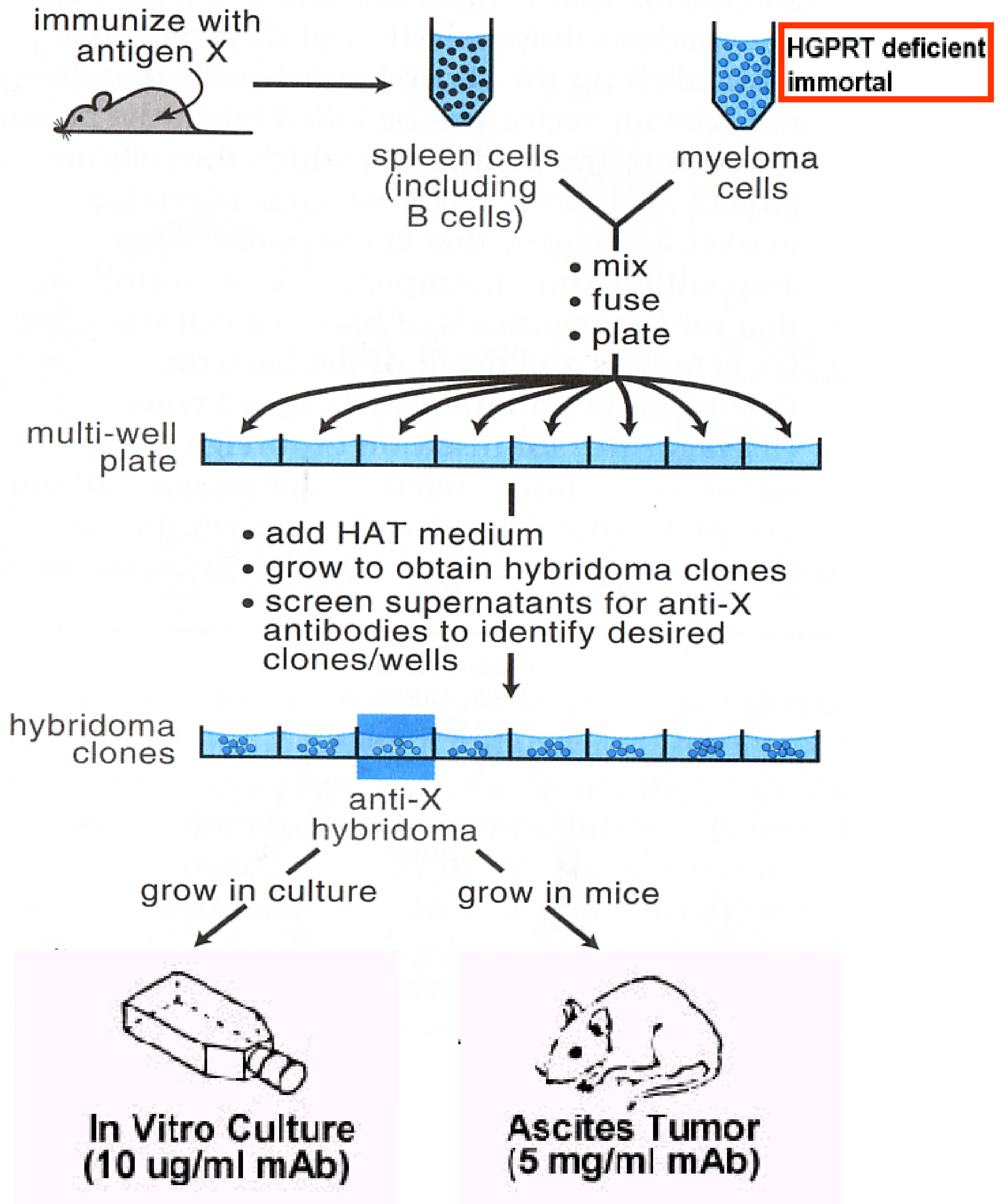
Frequency:
~ 1 in 10000 myeloma cells)

HAT medium

- H: hypoxanthine
  - substrate for HGPRT
  - used in salvage pathway (A, G)
- A: aminopterin
  - *de novo* synthesis pathway inhibitor
- T: thymidine
  - substrate for Thymidine Kinase
  - used in salvage pathway (C, T)

- HAT medium forces cells into salvage pathway (which the myeloma is deficient for !!!)

Making Hybridomas (Part III): a protocol for mAb production



- FDA has approved 13 mAb for clinical use(two must be produced by ascites method)
- Most new-drug applications to FDA are for mAb that are produced in vitro.

Large-Scale Production of Monoclonal Antibodies

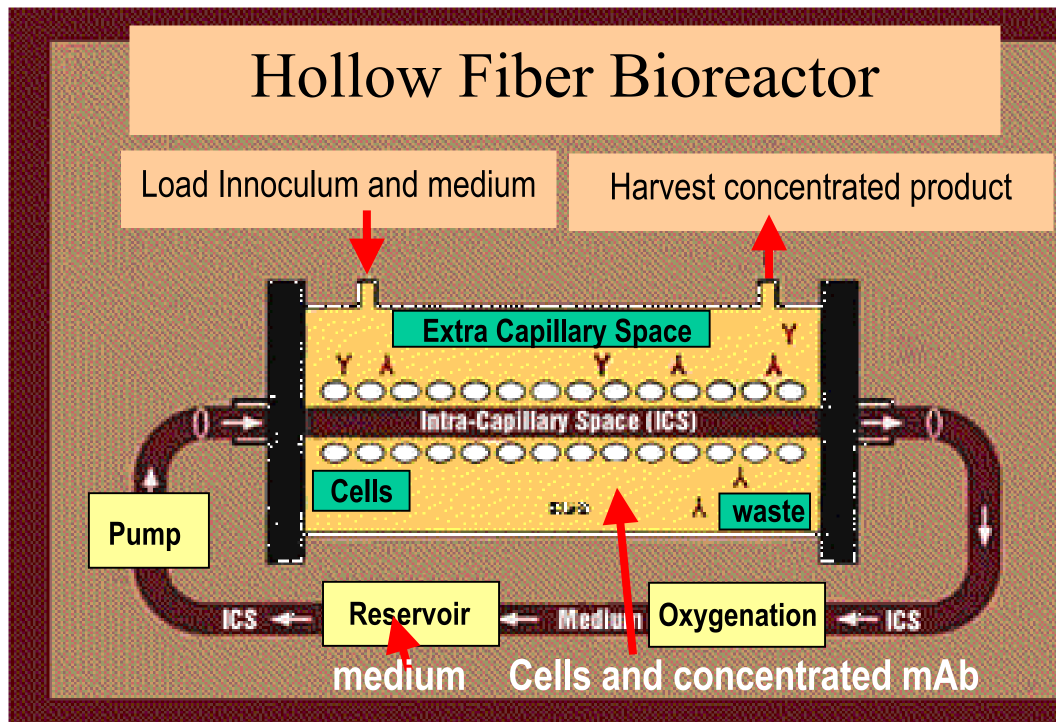
About 25,000 mAb are listed in *Linscott 's Directory* (1998-9)

- Most are produced in small quantities for bench-related research purposes (diagnostic and analytical)
- Some have become commercially successful
  - require a much higher scale of production
    - 0.1-10 g (small):
      research reagents (diagnostic and analytical)
    - 10-100 g (medium):
      diagnostics kits and small scale *in vivo* testing of therapeutics
    - over 100 g (large):
      routine diagnostics procedures and therapeutics
- Commercial-scale production produces mAb for three main purposes:
  - diagnosis
  - therapy
  - research and development of new therapeutic agents

Things to consider for commercial mAb production

- Culturing of large batches of cells (or injection into large numbers of mice)
- Considerable preproduction effort ensuring that the cell line
  - is stable
  - can produce commercially appropriate quantities of stable antibody
  - can produce an uncontaminated product.

In Vitro production of Monoclonal Antibodies



- Cells and mAb are produced in small chamber separated from medium
  - volume of medium can be large
  - cell growth chamber is kept small (1-15 ml)
- Barrier is a semipermeable fiber
  - cells and mAbs can't diffuse out
  - nutrients and growth factors can diffuse in
  - cell waste products can diffuse out (dilution !!!)
- Medium can be replaced without losing cells or mAbs
- Cells and mAbs can be harvested independently of growth medium
- Compartmentalization makes it possible to achieve mAb concentrations comparable with those in mouse ascites

The hollow-fiber bioreactor is designed to provide total yields of 500 mg + of mAb