Lecture I-Pharmaceutical Biotechnology
Introduction

for 5th grade pharmacy students

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References

1. **Pharmaceutical biotechnology(5th edition)**

J . A . Crommelin , Robert D. Syinder

2. **Biophrmaceuticals**

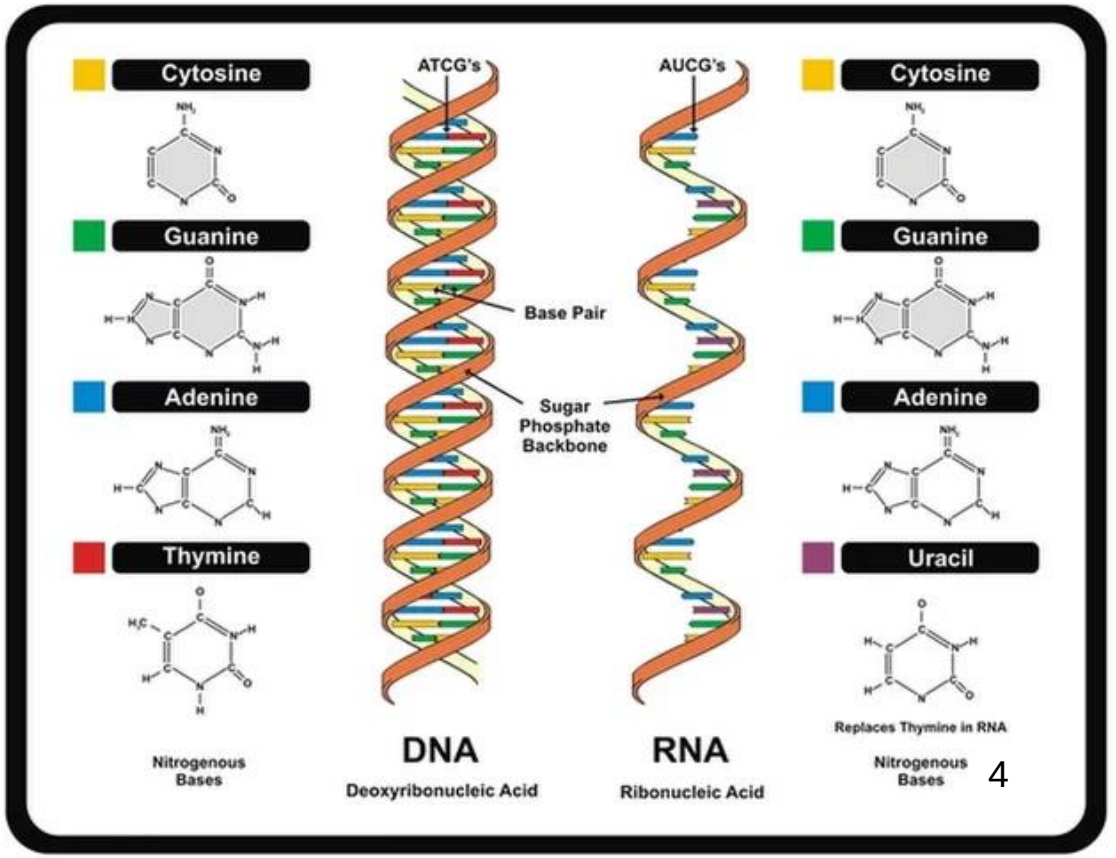
Walsh G.

Abbreviations

AA	amino acid(s)
Ab	Antibody
Ag	Antigen
BRCA1	breast cancer gene 1
cDNA	copy DNA/ complementary deoxyribonucleic acid
EDTA	Ethylene di amine tetra acetic acid
EMBL	European Molecular Biology Laboratory
FDA	Food and Drug Administration
DDBJ	DNA Data Bank of Japan
FSH	follicle-stimulating hormone
GF	Growth Factor
HPLC	high-performance liquid chromatography
IV	intravenous
Kb	thousand base pairs of DNA
HIV	Human immunodeficiency virus
EMEA	European Agency for the Evaluation of Medicinal Products
BSA	Bovine serum albumin
PCR	Polymerase chain reaction

General concepts

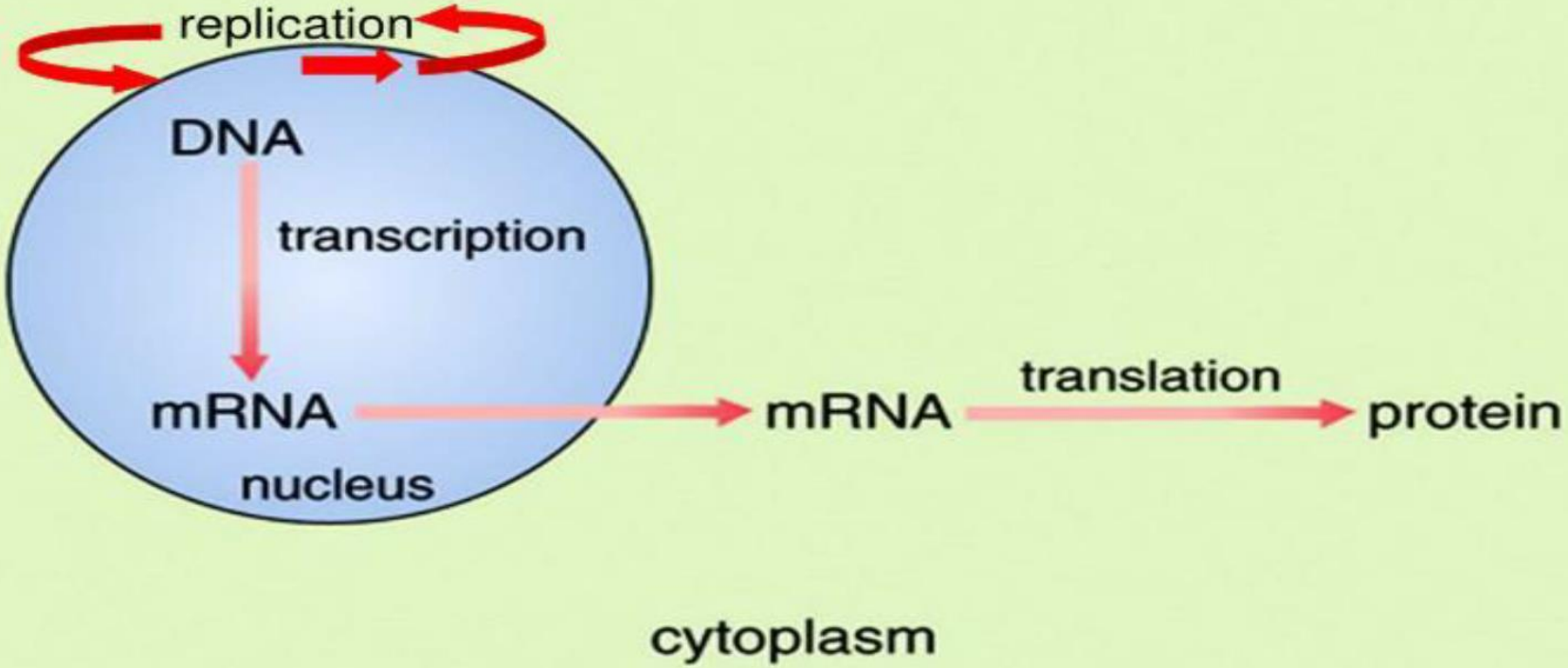
- **Genetic material!!!**
- **Building Blocks!!**
- **Gene expression**
- **Codons**



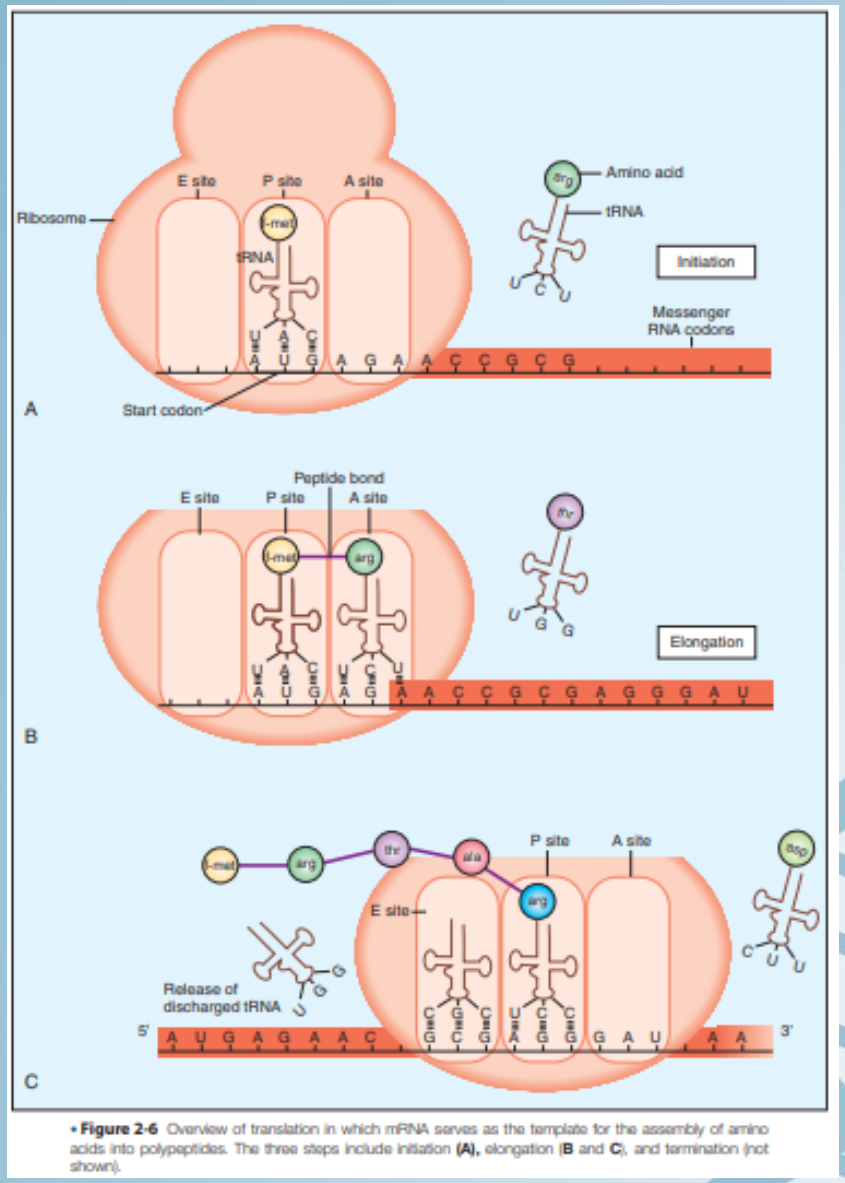
Central Dogma or Gene Expression

The central dogma of molecular biology was first stated by Francis Crick in 1958 and deals with the information flow in biological systems and can best be summarized as “DNA makes RNA makes protein” (this quote is from Marshall Nirenberg who received the Nobel Prize in 1968 for deciphering the genetic code). The basis of the information flow from DNA via RNA into a protein is pairing of complementary bases; thus, adenine (A) forms a base pair with thymidine (T) in DNA or uracil in RNA and guanine (G) forms a base pair with cytosine (C).(Reference-for details)

The Central Dogma of Life.



Translation & protein synthesis



• **Figure 2-6** Overview of translation in which mRNA serves as the template for the assembly of amino acids into polypeptides. The three steps include initiation **(A)**, elongation (**B** and **C**), and termination (not shown).

		2nd		Base			
		U	C	A	G		
	U	Phe	Ser	Tyr	Cys	U	
		Phe	Ser	Tyr	Cys	C	
		Leu	Ser	Stop	Stop	A	
1		Leu	Ser	Stop	Trp	G	3
s	C	Leu	Pro	His	Arg	U	R
t		Leu	Pro	His	Arg	C	D
		Leu	Pro	Gln	Arg	A	
b		Leu	Pro	Gln	Arg	G	B
a	A	Ile	Thr	Asn	Ser	U	A
s		Ile	Thr	Asn	Ser	C	S
e		Ile	Thr	Lys	Arg	A	E
		Met	Thr	Lys	Arg	G	
	G	Val	Ala	Asp	Gly	U	
		Val	Ala	Asp	Gly	C	
		Val	Ala	Glu	Gly	A	
		Val	Ala	Glu	Gly	G	

Table 1.2 ■ The genetic code

PHARMACEUTICAL BIOTECHNOLOGY

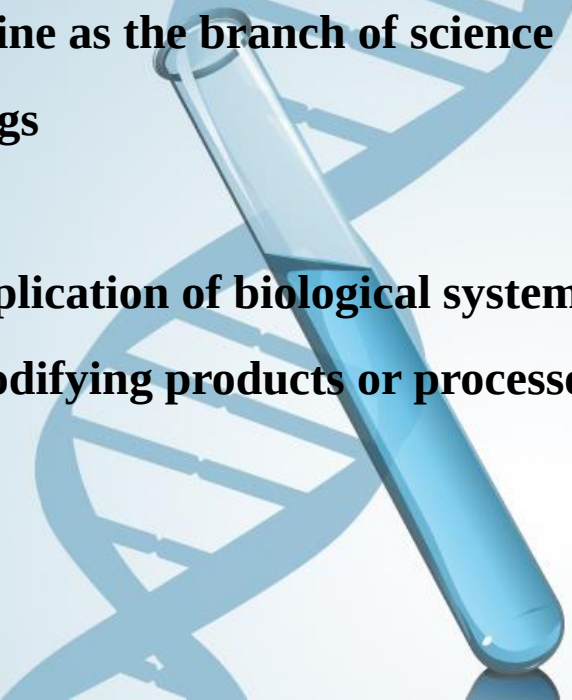
INTRODUTION:

Pharmaceutical biotechnology consist of the combination of two branches which Are "PHARMACEUTICAL SCIENCE" AND "BIOTECHNOLOGY".

DEFINATION:

PHARMACEUTICAL SCIENCE: Can simply be define as the branch of science that deals with the formulation and dispensing of drugs

BIOTECHNOLOGY: Can simply be define as the application of biological system, living organisms, or their derivatives in making or modifying products or processes for specific use.



PHARMACEUTICAL BIOTECHNOLOGY :Can simply be define as the science that covers all technologies required for the production, manufacturing and registration of biological drugs.

The aim of this pharmaceutical biotechnology is to design, produce drugs that are adapted to each persons genetic make up, which can give the maximum therapeutic effect.

Biotechnology plays an important role in pharmaceutical science most especially in the pharmaceutical industries by creation of genetically modified organisms that can be used in industrial production.

Biotechnology makes use of findings from various research areas, such as:

molecular biology

Separation technologies

Genetics

cell biology

Bioinformatics

Biochemistry

Microbiology

Development of Biotechnology

- 1953: Discovering the double helical structure of DNA
  by(----------&----------).
- 1971: Restriction enzymes (H.W.) discovered
- 1975: Production of monoclonal antibodies by hybridoma technology
- 1982: recombinant human insulin approved by the FDA .(sequence??).

- Until now biopharmaceuticals are primarily proteins.

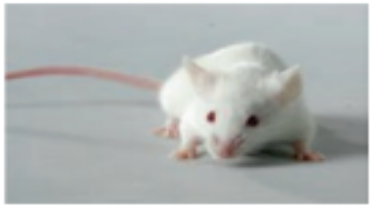
- but therapeutic DNA or RNA based molecules (think about gene therapy products, DNA vaccines, and RNA interference-based products--------

  (**COVID19 vaccines**)!!!!

In 1978, the first recombinant DNA human insulin was prepared by David Goeddel and his colleagues (of Genentech) by utilizing and combining the insulin A- and B- chains expressed in *Escherichia coli* .Thereafter, Genentech and Lilly signed an agreement to commercialize rDNA insulin. In 1982, the first insulin utilizing rDNA technology, Humulin® R (rapid) and N (NPH, intermediate-acting), were marketed

Hybridoma Technology

- First therapeutic mAb was produced by hybridoma technology! (H.W)
- Muromonab-CD3 (1985): immunosuppressant in organ transplants



Immunization with antigen

Isolation of B-lymphocytes from the spleen

PEG-mediated fusion of the lymphocytes with myeloma cells

Selection in HAT medium
Hybridomas survive

Picking and expanding of individual clones

Testing supernatants for antigen binding in an ELISA

Functional testing of obtained monoclonal antibodies

Selection and further expanding of those hybridomas that produce antibodies with the desired charcteristics

Figure 1.14 ■ The making of a mouse monoclonal antibody

Biopharmaceuticals vs. small molecule(traditional) drugs

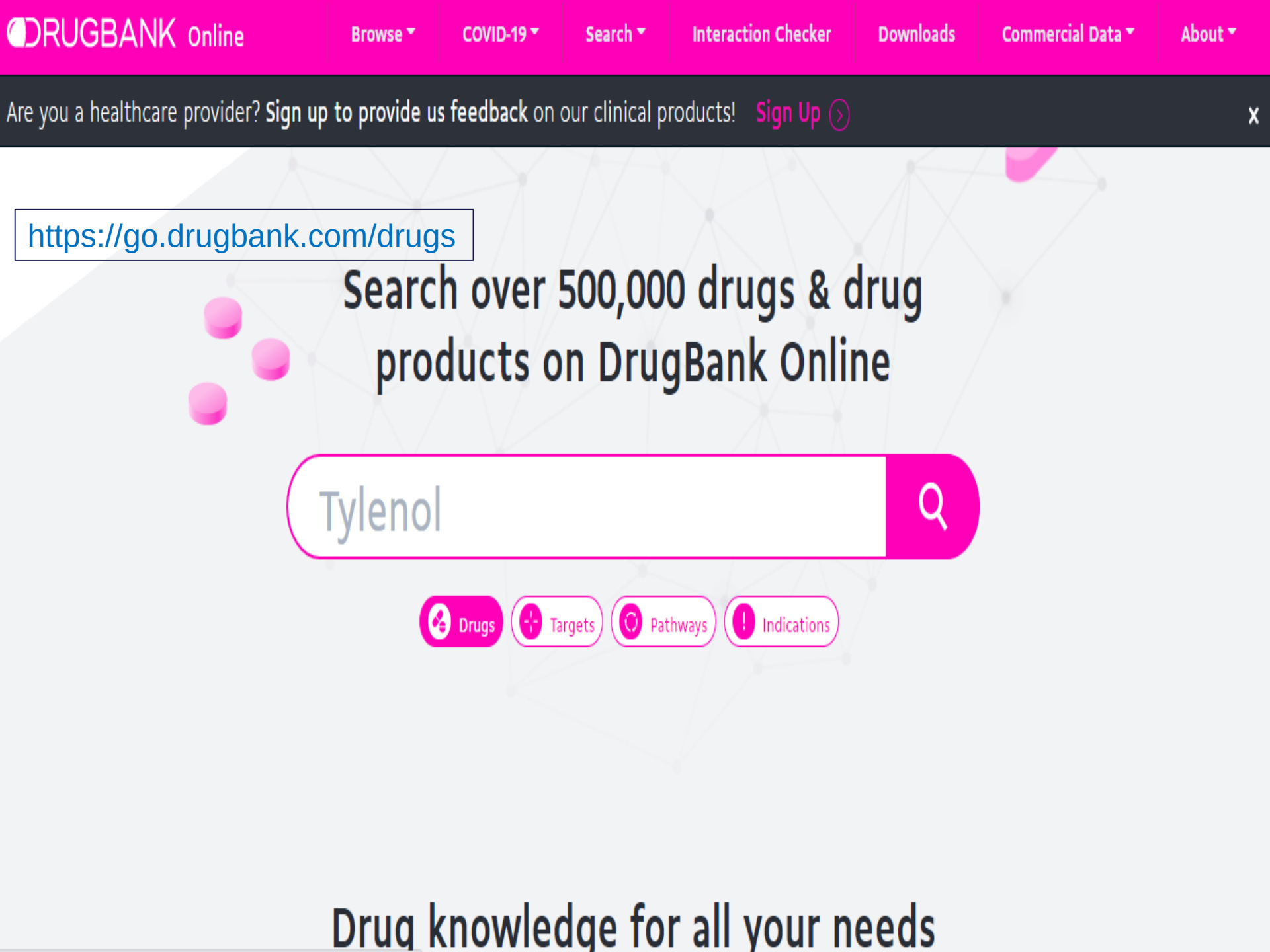
Since then (insulin production) a large number of biopharmaceuticals (biotechnology drugs) have been developed.

Until now biopharmaceuticals are primarily proteins

Therapeutic proteins differ in many aspects from classical, small molecule drugs. They differ in size, composition, production, purification, contaminations, side effects, stability, formulation, regulatory aspects, etc.

The main differences between Biopharmaceuticals and small drug molecules

Biopharmaceuticals	Small molecule drugs
Produced by living cell cultures	Produce by chemical synthesis
High molecular weight	Low molecular weight
Complex, heterogeneous structure	Well-defined structure
Strongly process-dependent	Mostly process-independent
Impossible to fully characterize the molecular composition and heterogeneity	Completely characterized
Unstable, sensitive to external conditions	Stable
Often injected or infused	Mostly oral route
Example: trastuzumab (m.wt = 145531 Da)	Example: atorvastatin (m.wt = 558 Da)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Drugs

Trastuzumab

Targets (13)

IDENTIFICATION

Name	Trastuzumab
Accession Number	DB00072 (BTD00098, BIOD00098)
Type	Biotech
Groups	Approved, Investigational
Biologic Classification	Protein Based Therapies Monoclonal antibody (mAb)
Description	Produced in CHO cell cultures, trastuzumab is a recombinant IgG1 kappa, humanized monoclonal antibody [6] that selectively binds with high affinity in a cell-based assay (Kd = 5 nM) to the extracellular domain of the human epidermal growth factor receptor protein (HER2) [Label]. It is used as

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Drugs

Atorvastatin

Targets (5) | Enzymes (10) | Carriers (1) | Transporters (10) | Biointeractions (28)

IDENTIFICATION

Name	Atorvastatin
Accession Number	DB01076 (APRD00055)
Type	Small Molecule
Groups	Approved
Description	Atorvastatin, also known as the brand name product Lipitor, is a lipid-lowering drug belonging to the statin class of medications. By inhibiting the endogenous production of cholesterol in the liver, statins lower abnormal cholesterol and lipid levels and ultimately reduce the risk of cardiovascular disease. More specifically, statin medications competitively inhibit the enzyme hydroxymethylglutaryl-coenzyme A (HMG-CoA) Reductase,[8] which catalyzes the conversion of HMG-CoA to mevalonic acid. This conversion is a critical metabolic reaction involved in the

Protein chemical formula	$C_{6460}H_{9946}N_{1720}O_{2043}S_{56}$
Protein average weight	146189.7 Da

Sequences

>Muromonab-CD3 light chain
QIVLTQSPAIMSASPGEKVTMTCSASSSVSYMNWYQQKSGTSPKRWIYDTSKLASGVPAH
FRGSGSGTSYSLTISGMEAEDAATYYCQQWSSNPFTFGSGTKLEINRADTAPTVSIFPPS
SEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTL
TKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC

>Muromonab-CD3 heavy chain
QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQGLEWIGYINPSRGYTNY
NQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAVYYCARYYDDHYCLDYWGQGTTLTVSSA
KTTAPSVYPLAPVCGGTTGSSVTLGCLVKGYFPEPVTLTWNSGSLSSGVHTFPAVLQSDL
YTLSSSVTVTSSTWPSQSITCNVAHPASSTKVDKKIEPRPKSCDKTHTCPPCPAPELLGG
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDE
LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Download FASTA Format

a

```
                    signal peptide                                  beta chain
human       MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAED
porcine     MALWTRLLPLLALLALWAPAPAQAFVNQHLCGSHLVEALYLVCGERGFFYTPKARREAEN
bovine      MALWTRLAPLLALLALWAPAPARAFVNQHLCGSHLVEALYLVCGERGFFYTPKARREVEG
            **** ** *********.* ** ********************************:***.*

                                                                alpha chain
human       LQVGQVELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN
porcine     PQAGAVELGGGLG--GLQALALEGPPQKRGIVEQCCTSICSLYQLENYCN
bovine      PQVGALELAGGPG-----AGGLEGPPQKRGIVEQCCASVCSLYQLENYCN
             *.* :**.** *       .***  **********:*:***********
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https://www.ebi.ac.uk/Tools/msa/clustalw2/

b

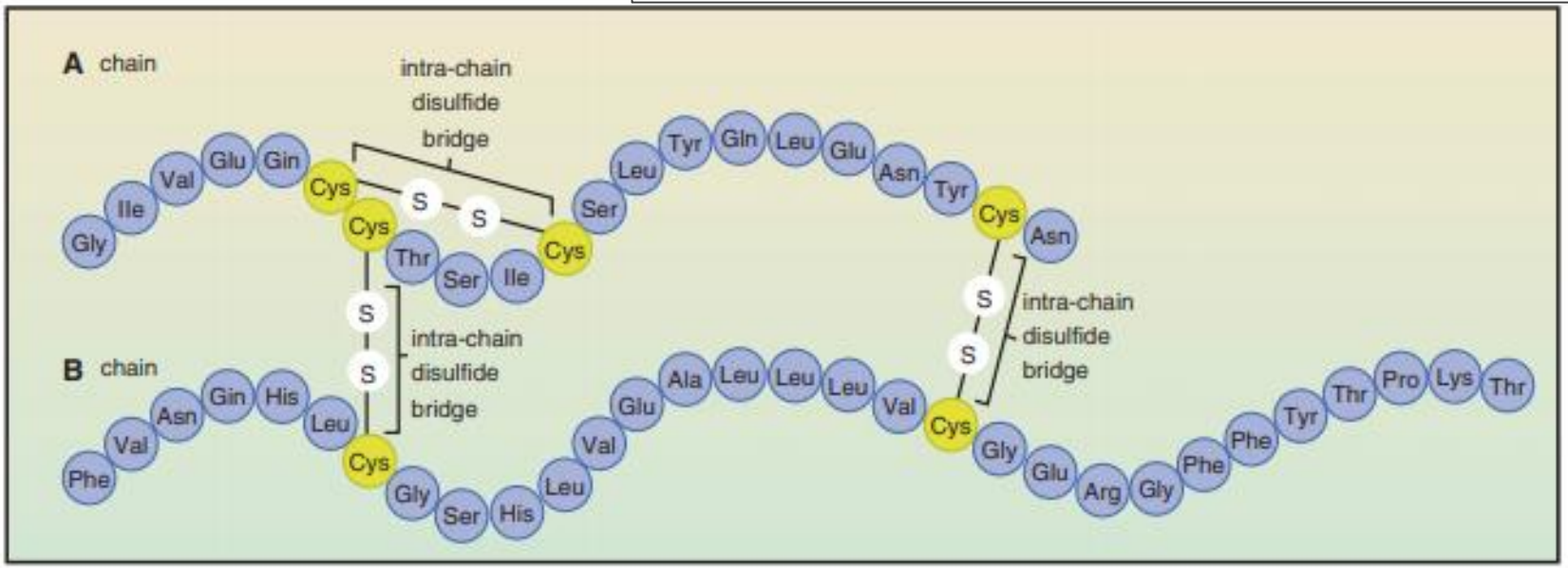

Figure 1.1 ■ (**a**) Multiple alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2) of the amino acid sequences of human, porcine, and bovine preproinsulin. (*): identical residue. (**b**) Schematic drawing of the structure of insulin. The alpha and beta chain are linked by two disulfide bridges. Both the one-letter and three-letter codes for the amino acids are used in this figure: alanine (Ala, A), arginine (Arg, R), asparagine (Asn, N), aspartic acid (Asp, D), cysteine (Cys, C), glutamic acid (Glu, E), glutamine (Gln, Q), glycine (Gly, H), histidine (His, H), isoleucine (Ile, I), leucine (Leu, L), lysine (Lys, K), methionine (Met, M), phenylalanine (Phe, F), proline (Pro, P), serine Sser, S), threonine (Thr, T), tryptophan (Trp,W), tyrosine (Tyr, Y), and valine (Val, V) (**b** is taken from Wikipedia)

Amino Acid	3-Letters	1-Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

❖ Most common biotech drugs are listed below:

- **Hormones (insulin, glucagon, growth hormone, gonadotrophins)**
- **Monoclonal antibodies (mAbs)**
- **Blood factors (Factor VIII and Factor IX)**
- **Thrombolytic agents (tissue plasminogen activator)**
- **Haematopoietic growth factors (Erythropoietin, colony stimulating factors)**
- **Interferons (Interferons-α, -β, -γ)**
- **Interleukin-based products (Interleukin-2)**
- **Vaccines (Hepatitis B surface antigen)**
- **Additional products (tumour necrosis factor, therapeutic enzymes)**

Lecture II–Pharmaceutical Biotechnology

Formulation of Biotechnology products (biopharmaceutical consideration)

for 5th grade pharmacy students

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References

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2. Biophrmaceuticals

Walsh G.

Topics

- **Genetic Engineering(Recombinant DNA Technology)**
- **Formulation of pharmaceutical proteins**
- **The technical aspects of production (upstream processing) and purification (downstream processing) of recombinant therapeutic proteins**

Abbreviations

LPS	**lipopolysaccharide**
rDNA	**Recombinant deoxyribonucleic acid/recombinant-DNA**
HSA	**Human serum albumin**
IP	**Intra peritoneal**
LDL	**low-density lipoprotein**
SOD	**Superoxide dismutase**
WHO	**World Health Organization**
IA	**Intra-arterial**
IC	**Intracoronary**
tRNA	**Transfer RNA**
cDNA	**Complementary DNA**

❑DNA Sequence

DNA, mRNA, and amino acid sequence of every protein in the human genome can be obtained from publicly available gene and protein databases, such as those present at the National Center for Biotechnology Information (NCBI) in the USA and the European Molecular Biology Laboratory (EMBL). Their websites are

http://www.ncbi.nlm.nih.gov/ and http://www. ebi.ac.uk, respectively.

DNA sequences in these databases are always given from the 5′ end to the 3′ end and protein sequences from the amino- to the carboxy-terminal end .These databases also contain information about the gene (e.g., exons, introns, and regulatory sequences).

a

>gi|109148525|ref|NM_000207.2| Homo sapiens insulin (INS), transcript variant 1, mRNA

5'AGCCCTCCAGGACAGGCTGCATCAGAAGAGGCCATCAAGCAGATCACTGTCCTTCTGCCATGGCCCTGT
GGATGCGCCTCCTGCCCCTGCTGGCGCTGCTGGCCCTCTGGGGACCTGACCCAGCCGCAGCCTTTGTGAAC
CAACACCTGTGCGGCTCACACCTGGTGGAAGCTCTCTACCTAGTGTGCGGGGAACGAGGCTTCTTCTACAC
ACCCAAGACCCGCCGGGAGGCAGAGGACCTGCAGGTGGGGCAGGTGGAGCTGGGCGGGGGCCCTGGTGCAG
GCAGCCTGCAGCCCTTGGCCCTGGAGGGGTCCCTGCAGAAGCGTGGCATTGTGGAACAATGCTGTACCAGC
ATCTGCTCCCTCTACCAGCTGGAGAACTACTGCAACTAGACGCAGCCCGCAGGCAGCCCCACACCCGCCGC
CTCCTGCACCGAGAGAGATGGAATAAAGCCCTTGAACCAGCAAAA 3'

b

>gi|4557671|ref|NP_000198.1| insulin preproprotein [Homo sapiens]

(NH_2)MALWMRLLPLLALLALWGPDPAAAFVNQHLCGSHLVEALYLVCGERGFFYTPKTRREAEDLQVGQV
ELGGGPGAGSLQPLALEGSLQKRGIVEQCCTSICSLYQLENYCN-(COOH)

Figure 1.3 ■ DNA sequences are always written from the 5′ → 3′ direction and proteins sequences from the amino-terminal to the carboxy-terminal. (**a**) nucleotide code, (**b**) amino acid code, see legend to Fig. 1.1

- When producing proteins for therapeutic use, a number of issues must be considered related to the manufacturing, purification, and characterization of the products.

- Biotechnological products for therapeutic use have to meet strict specifications especially when used via the parenteral route.

- The regulatory agencies both in Europe (EMA: European Medicines Agency) and in the United States of America (FDA: Food and Drug Administration) play a pivotal role in providing legal requirements and guidelines

Recombinant DNA technology

Other names:

(**Genetic modification**) OR (**molecular cloning**) OR (**genetic engineering**)

Molecular cloning is defined as the assembly of recombinant DNA molecules (most often from two different organisms) and their replication within host cells.

Adding of foreign DNA(desired gene) to microbial cells, plant cells or animal cells to form genetically Modified Organism(GMO).

A **GMO** is any organism, except for human beings, in which the genetic material has been altered in a way that does not occur naturally by mating or natural recombination.

Plasmids usually used as a vector for a foreign DNA after treating them with Restriction enzymes such as ECORI.

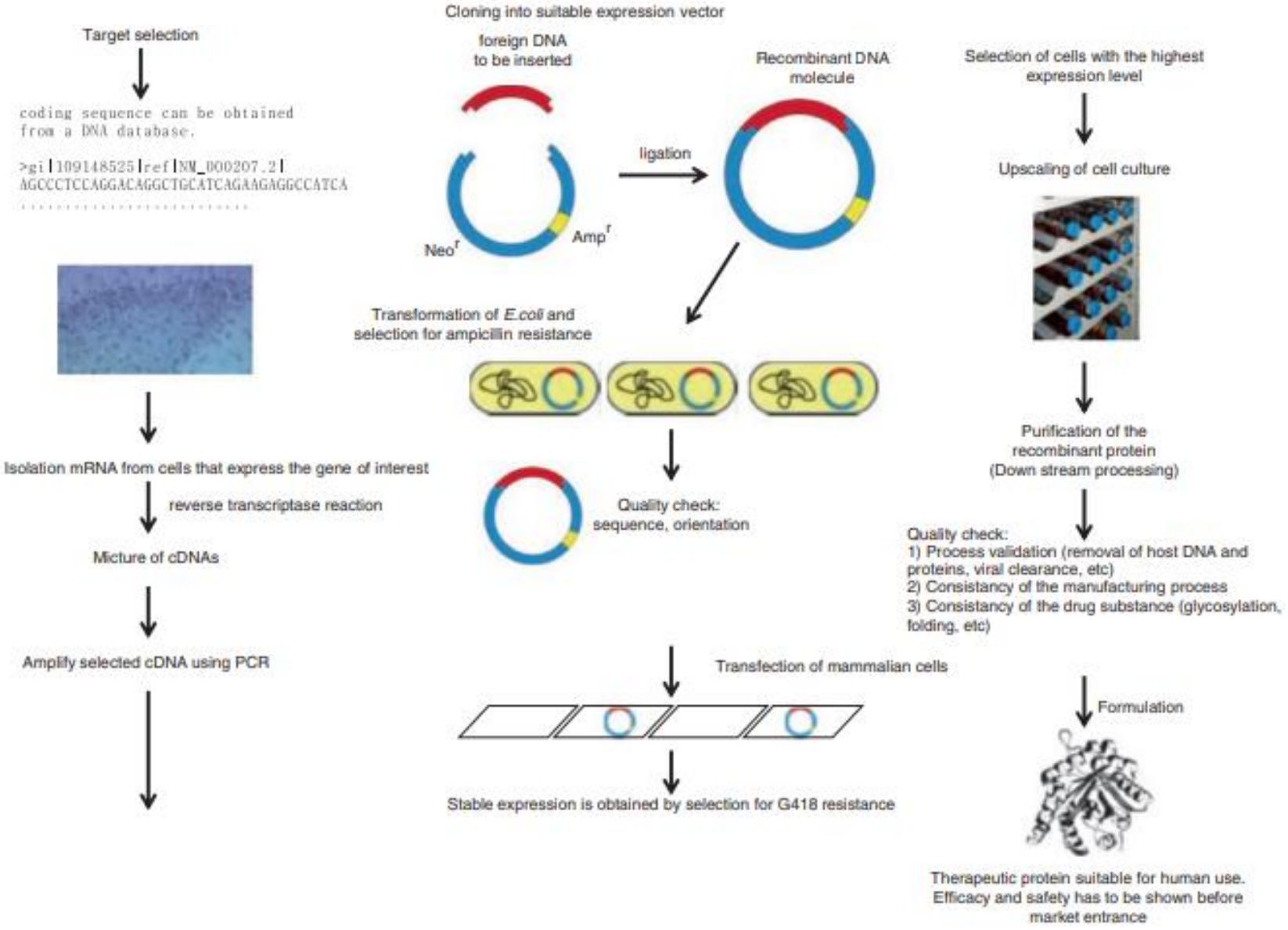


Figure 1.2 ■ Schematic representation of all the steps required to produce a therapeutic protein. *cDNA* copy DNA, *PCR* polymerase chain reaction

■ Selection of Expression Host

- Recombinant proteins can be produced in

✓ E. coli,

✓ yeast,

✓ plants (e.g., rice and tomato),

✓ mammalian cells,

✓ and even by transgenic animals.

All these expression hosts have different pros and cons

	Prokaryotes	Yeast	Mammalian cells
	E. coli	***Pichia pastoris Saccharomyces cerevisiae***	**(e.g., CHO or HEK293 cells)**
+	Easy manipulation Rapid growth Large-scale fermentation Simple media High yield	Grows relatively rapidly Large-scale fermentation Performs some posttranslational modifications	May grow in suspension, perform all required posttranslational modifications
–	Proteins may not fold correctly or may even aggregate (inclusion bodies) Almost no posttranslational modifications	Posttranslational modifications may differ from humans (especially glycosylation)	Slow growth Expensive media Difficult to scale up Dependence on serum (BSE)

Table 1.1 ■ Pros and cons of different expression hosts

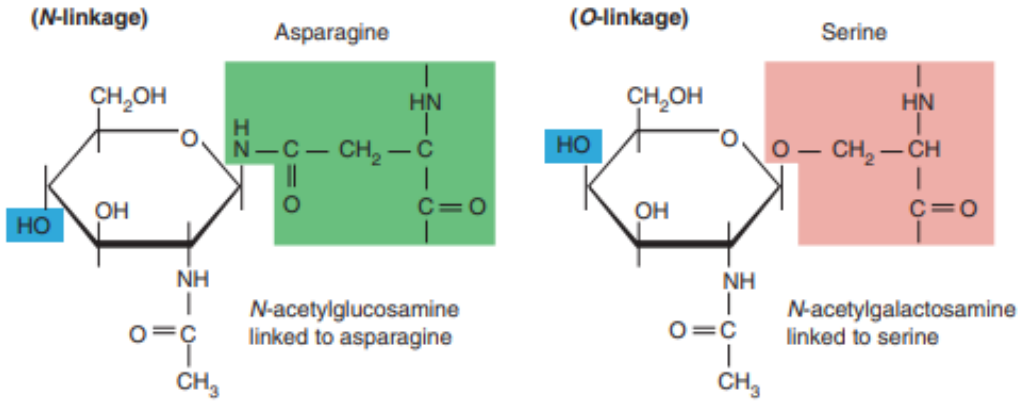
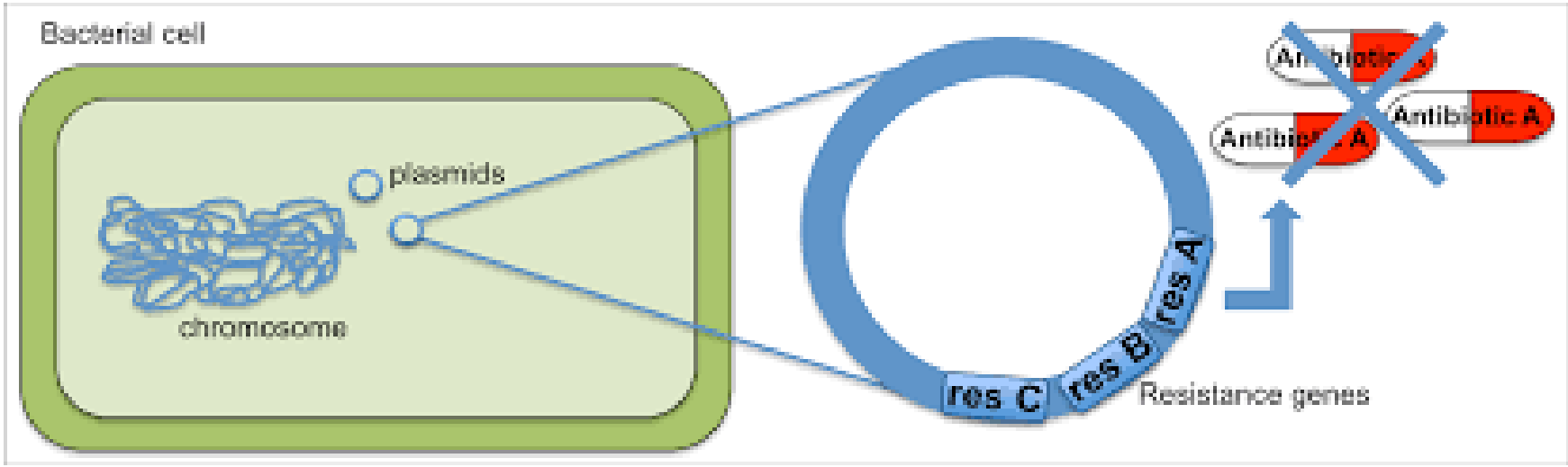
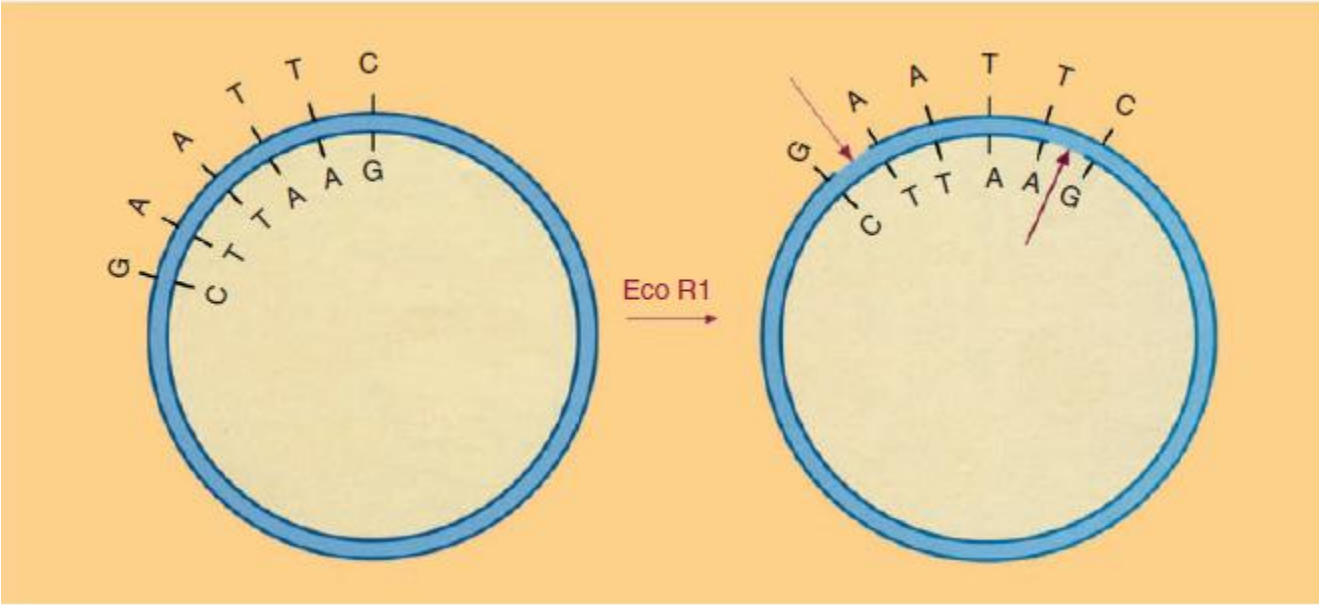


Figure 1.4 ■ Glycosylation takes place either at the nitrogen atom in the side chain of asparagine (N-linked) or at the oxygen atom in the side chain of serine or threonine. Glycosylation of asparagine takes place only when this residue is part of an Asn-X-Ser or Ans-X-Thr (X can be any residue except proline). Not all potential sites are glycosylated. Which sites become glycosylated depends also on the protein structure and on the cell type in which the protein is expressed

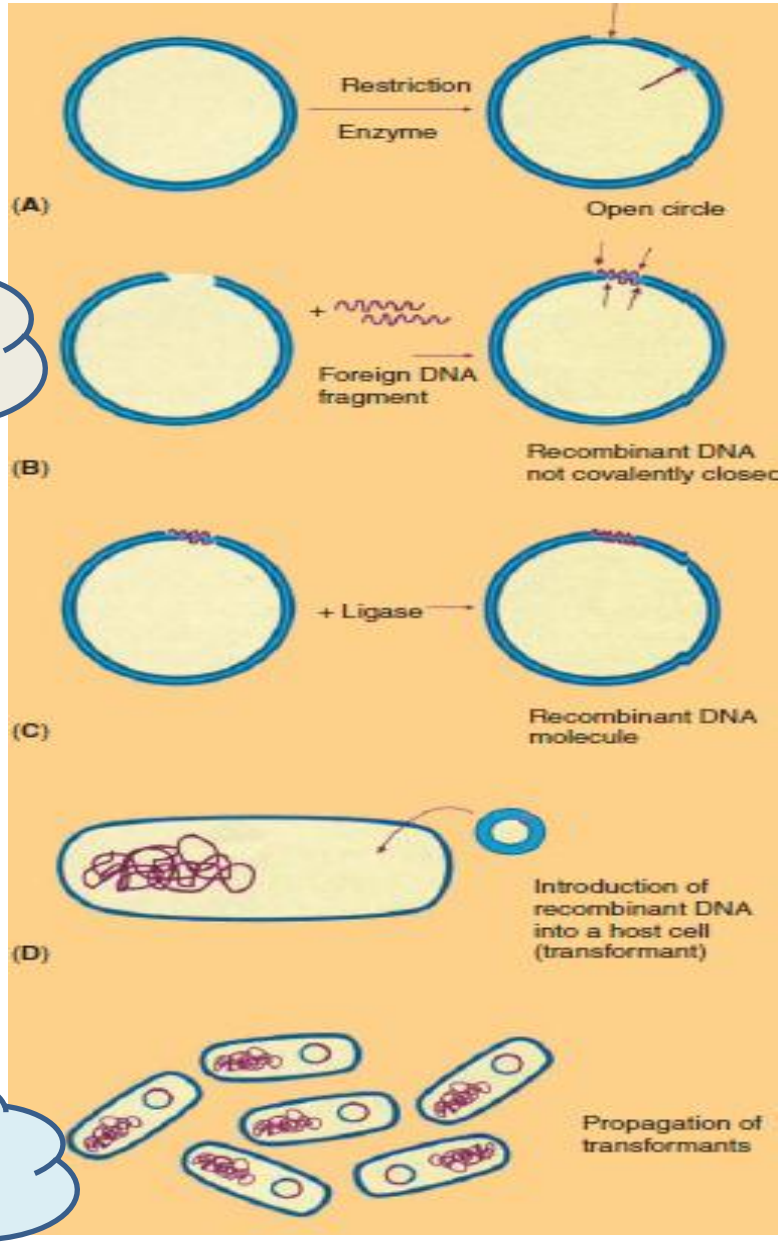
Plasmids


Bacterial cell
plasmids
chromosome
res C
res B
res A
Resistance genes
Antibiotic A



Treatment of a plasmid with an unique EcoR1 site. This restriction enzyme will open the plasmid and make it amenable for manipulation.

Bovine albumin



Bacillus subtilis

Principle of cloning a foreign DNA fragment

Recombinant DNA technology

Benefits:

The cloning technique is very suitable to obtain large amounts of a specific DNA fragment, by fusing such a fragment to an appropriate vector and transferring the construct to a host that can easily be cultivated to high cell densities. The recombinant DNA molecules, which can then be isolated from the cell mass, form an abundant source for the specific DNA fragment and it is important for pharmaceutical biotechnology.

Enzyme	Source	Cutting sequence*
EcoR1	*Escherichia coli*	G↓AATT C
		C TTAA↑G
Pst1	*Providencia stuartii*	C TGCA↓G
		G↑ACGT C
Taq1	*Thermus aquaticus*	T↓CG A
		A GC↑T
Hinf1	*Hemophilus influenzae*	G↓ANT C
		C TNA↑G
Msp1	*Moraxella species*	C↓CG G
		G GC↑C
HaeIII	*Hemophilus aegyptus*	GG↓CC
		CC↑GG

*N, no base preference.

Note: Open space in the recognition site indicates the endonucleolytic cut by the enzyme.

Some restriction enzymes, their origin, and their recognition site.

Foreign DNA fragment may be synthetic DNA(Oligonucleotides linked at laboratory and under certain conditions)

Recombinant DNA transfer

Transfer of a recombinant DNA molecule to a cell (host cell) is an essential step in DNA technology.

I. Natural transformation: DNA are taken up under physiological conditions.

II. Creation of Non-physiological conditions:

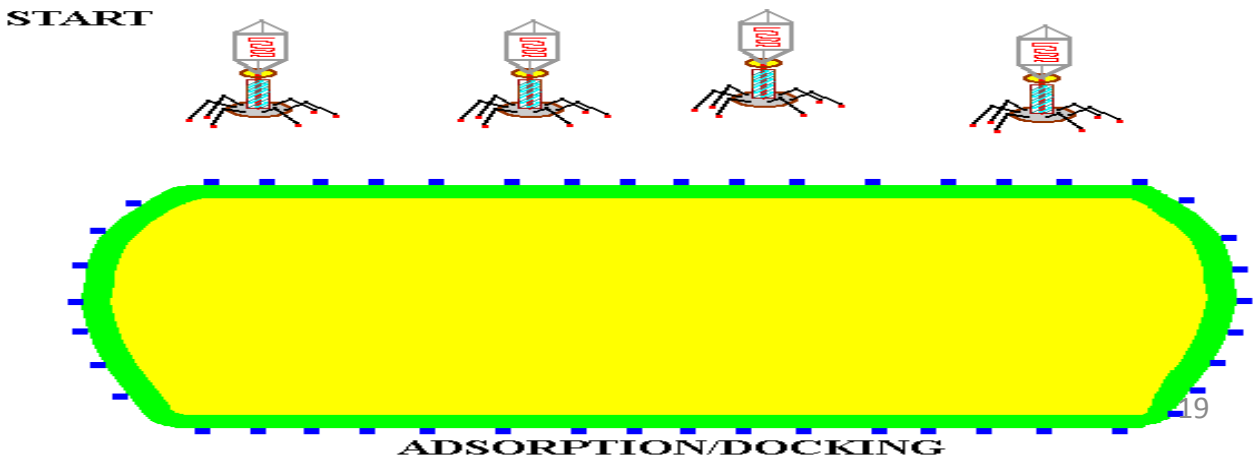
1. heat shock to the host cells in the presence of high amounts of Ca+2 ions.
2. **Electroporation**: DNA and cells are brought together in a cuvette which is then subjected to a vigorous electrical discharge. Under those artificial conditions the cell envelope is forced to open itself, after which DNA may enter through the“holes” that are created, The technique of electroporation is widely applicable and frequently used

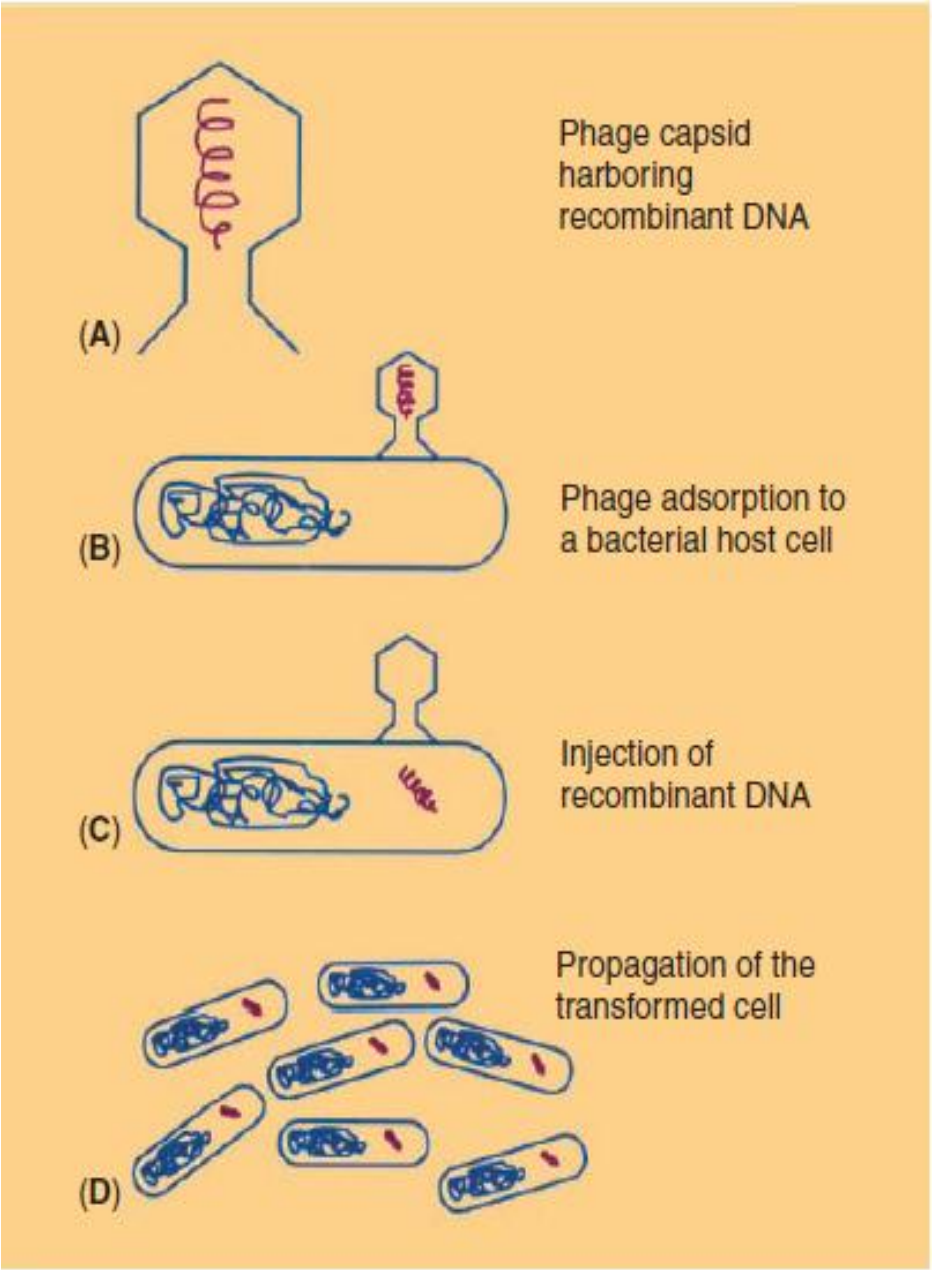
DNA transfer

III. Transduction:

package DNA in a bacteriophage capsid and then to mimic the normal bacteriophage infection procedure.

Lytic or Lysogenic?? H.W





Phage as a mediator for transfer of recombinant DNA

IV. **Conjugation:**

is a process where DNA transfer takes place by cell–cell mating conjugation a special class of plasmids is required, so called conjugative plasmids. If a cell with such a plasmid—the donor—meets a cell without such plasmid—the recipient—they may form together cell aggregates.

➢Transfection

- Introducing DNA into a mammalian cell is called transfection (and as already mentioned above, transformation in *E. coli*). There are several methods to introduce DNA into a mammalian cell line. Most often,

- the plasmid DNA is complexed to cationic lipids (such as Lipofectamine) or polymers (such as polyethyleneimines or PEI) and then pipetted to the cells. Next, the positively charged aggregates bind to the negatively charged cell membrane and are subsequently endocytosized .Then, the plasmid DNA has to escape from the endosome and has to find its way into the nucleus where mRNA synthesis can take place.

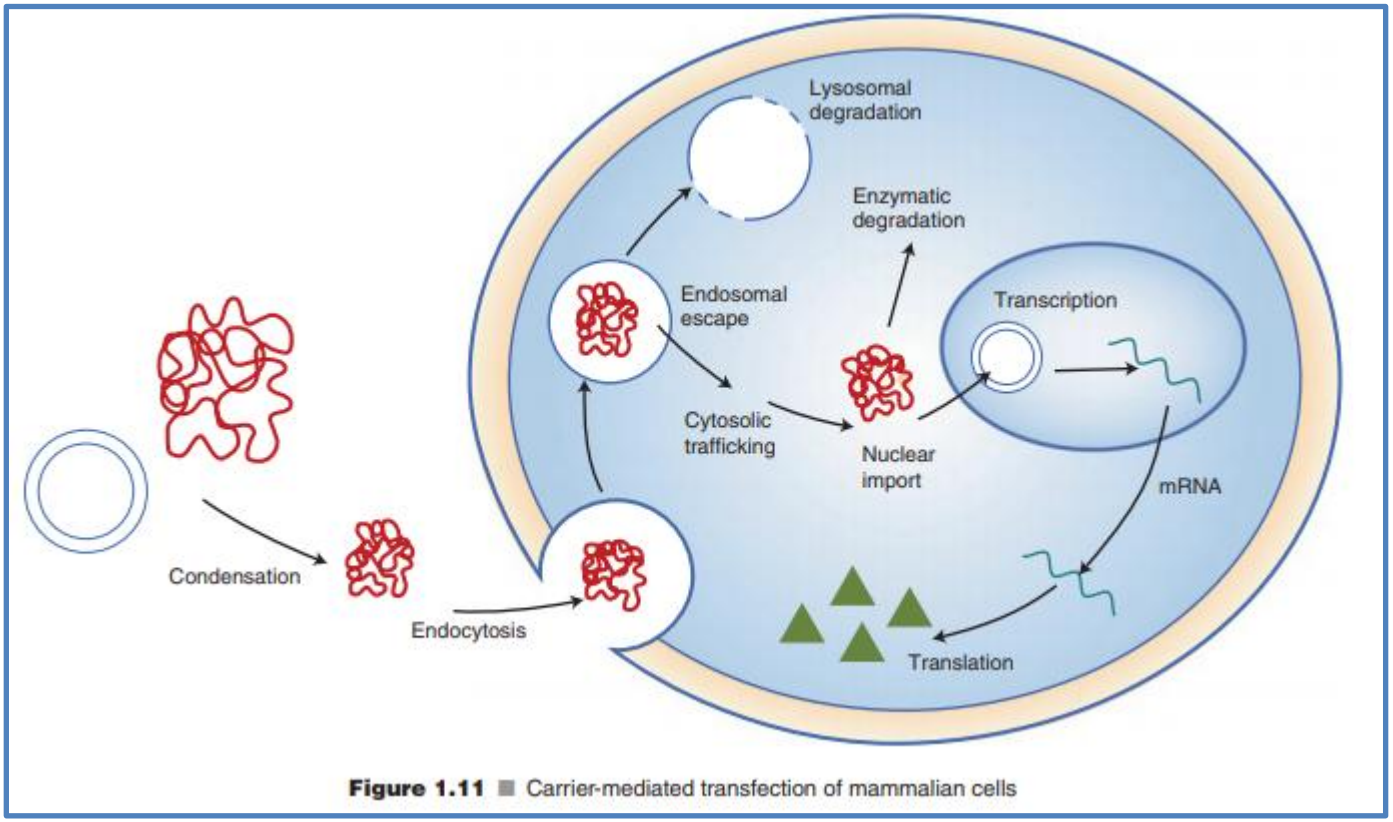


Figure 1.11 ■ Carrier-mediated transfection of mammalian cells

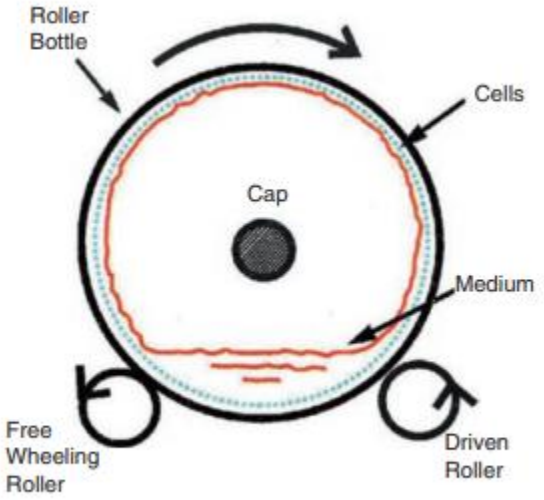


Figure 1.12 ■ Cell culturing in roller bottles

The term “biopharmaceuticals” is used to describe biotechnologically derived drug products. Biopharmaceuticals are protein-based macromolecules and include, insulin, human growth hormone, the families of the cytokines and of the monoclonal antibodies, antibody fragments, and nucleotide based systems such as antisense Oligonucleotides , siRNA and DNA preparations for gene delivery.

These are large complex molecules and are often heterogeneous mixtures compared to synthetically manufactured, pure small molecules.

Biopharmaceuticals

By mid-2002, some 120 biopharmaceutical products had gained marketing approval in the USA and/or EU.

Production by Recombinant DNA Technology

production of human insulin

The structural gene for human insulin is 1430 nucleotides long

The protein encoded by the gene is 110 amino acids in length and called Preproinsullin.

Processing steps, enzymes

mature protein encompasses a total of 51 amino acids. It consists of two separate chains: an A chain of 21 amino acids and a B chain of 30 amino acids. Chains A and B are held together by S bonds between the amino acids cysteine on the adjacent chains.

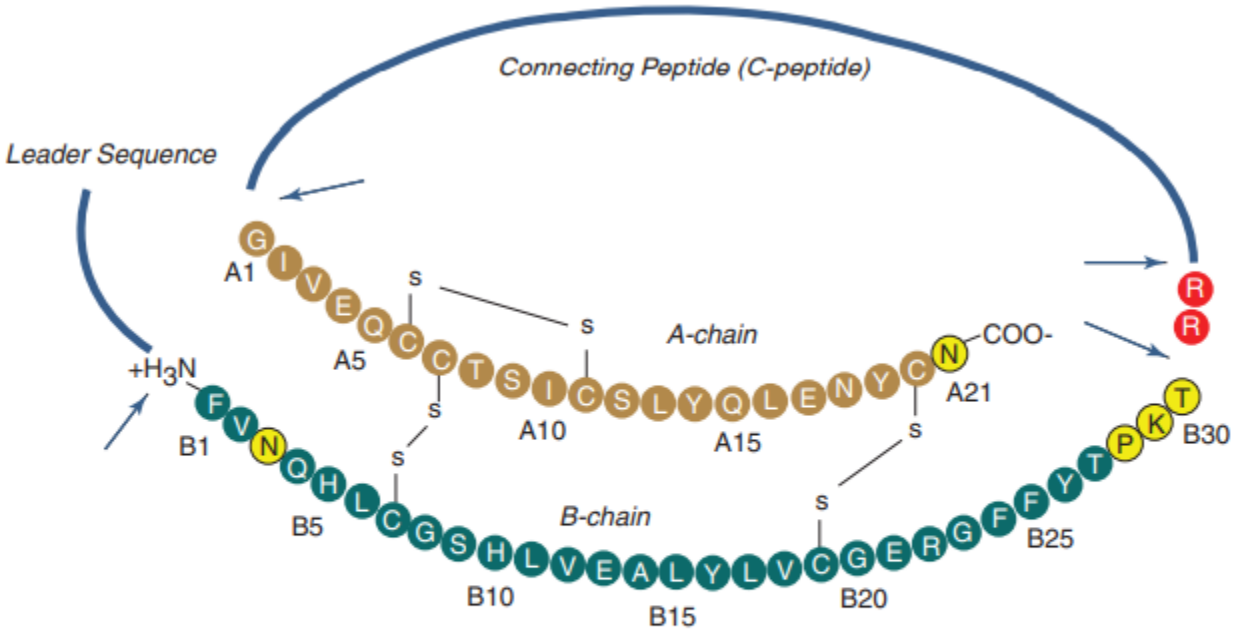


Figure. 18.1 ■ Primary sequence of insulin. The A-chain is displayed in tan. The B-chain is displayed in teal. The yellow-highlighted amino acids represent sites of sequence alterations denoted in Table 18.1. The arrows indicate sites of enzymatic processing that remove the leader sequence and connecting peptide from the expressed proinsulin. The sequences of the leader and connecting peptide are not shown, with the exception of the two arginine residues (red) that are retained in an analog to alter their time action (e.g., insulin glargine)

Species	A^{21}	B^{3}	B^{28}	B^{29}	B^{30}	B^{31}	B^{32}
human insulin (Humulin®, Novolin®, Afrezza®)	Asn	Asn	Pro	Lys	Thr	–	–
insulin lispro (Humalog®, Liprolog®, Admelog®)	Asn	Asn	Lys	Pro	Thr	–	–
insulin aspart (NovoRapid®, NovoLog®)	Asn	Asn	Asp	Lys	Thr	–	–
insulin glulisine (Apidra®)	Asn	Lys	Pro	Glu	Thr	–	–
insulin glargine (Lantus®, Basaglar®, Toujeo®)	Gly	Asn	Pro	Lys	Thr	Arg	Arg
insulin detemir (Levemir®)	Asn	Asn	Pro	Lys-(*N*-tetradecanoyl)	–	–	–
insulin degludec (Tresiba®)	Asn	Asn	Pro	Lys-(N^{ε}-hexadecandioyl-γ-Glu)	–	–	–

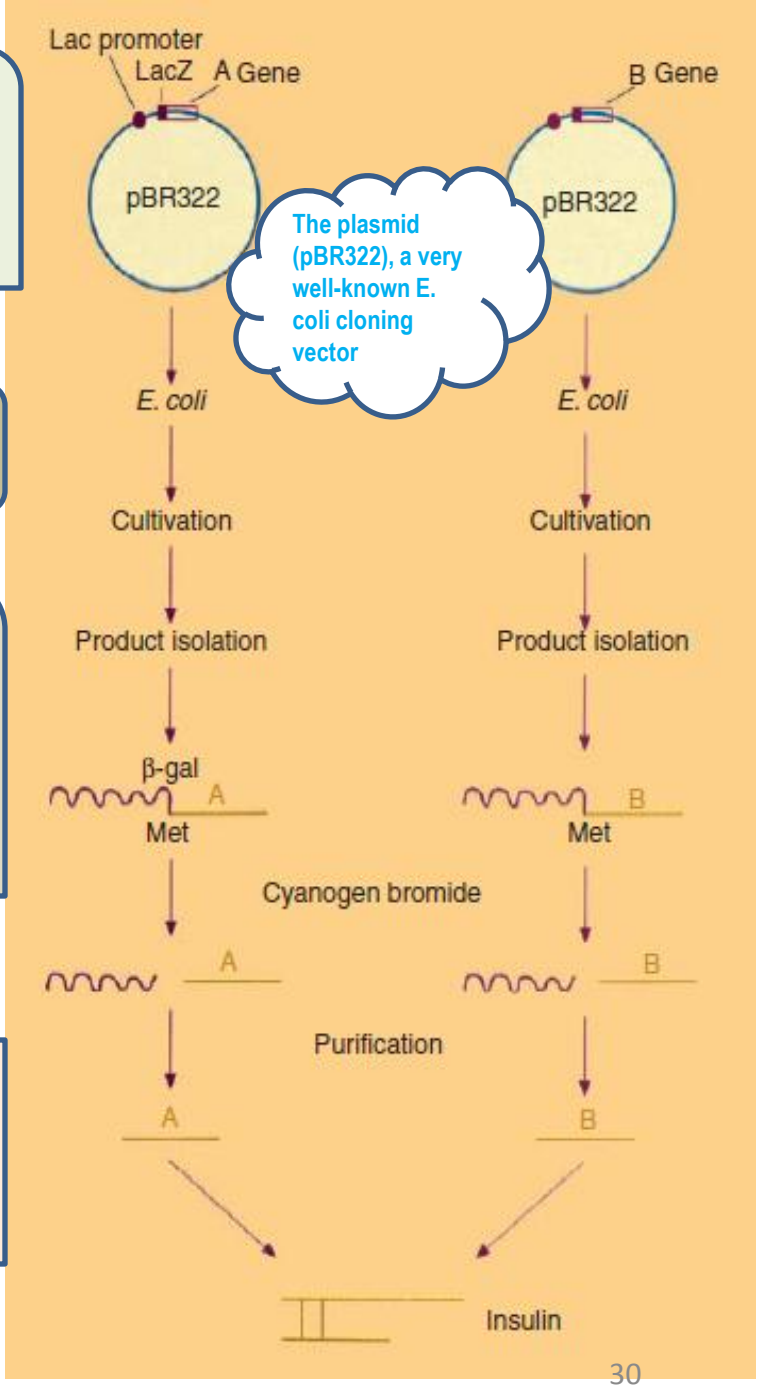
Table 18.1 ■ Amino acid substitutions in insulin analogs compared to human insulin

Synthesis of A-chain gene and B-chain gene from Oligonucleotides
Both parts were linked together and fused at the end of the lacZ gene in the plasmid pBR322. Again the codon for the amino acid methionine was built in at the fusion point

the peptides A and B are synthesized as products fused to ß-galactosidase

Cynogen Bromide
This agent has the ability to cleave peptides whenever the amino acid methionine is present and cleaves immediately after this amino acid. Since neither fragment A nor B of insulin contain methionine and the cloning strategy guaranteed the presence of methionine at the fusion point.

The final step consists of mixing A and B and allowing the S bonds to form spontaneously

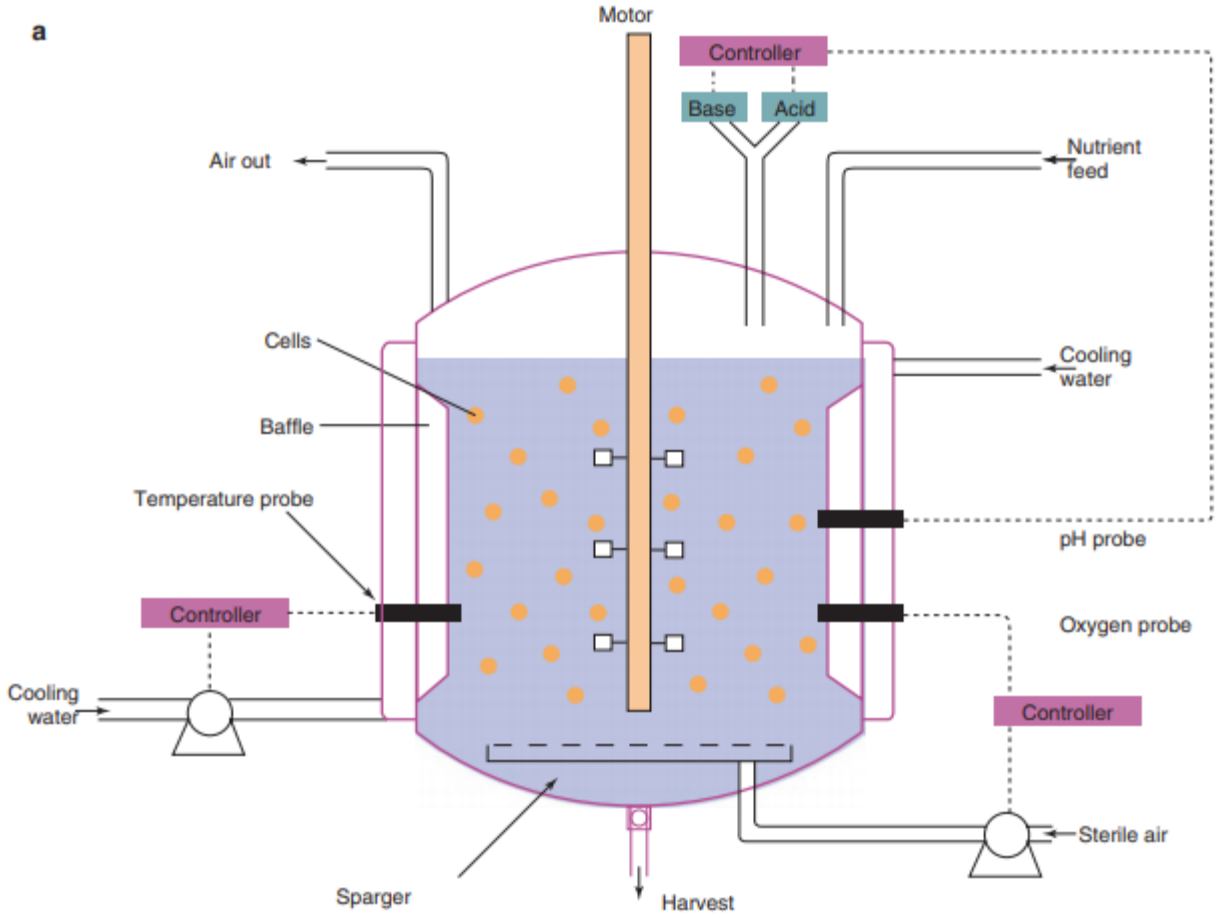


Synthesis of insulin by synthetic DNA.

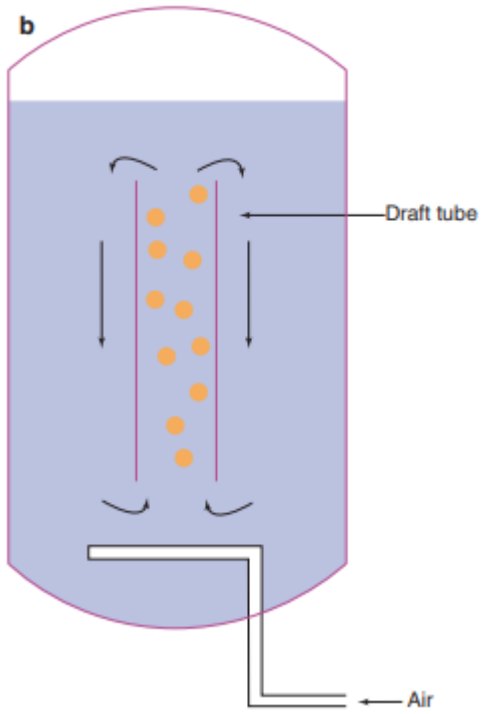
Cultivation Systems

Production-scale cultivation is commonly performed in fermentors, used for bacterial and fungal cells, or bioreactors, used for mammalian and insect cells. Bioreactor systems can be classified into four different types.

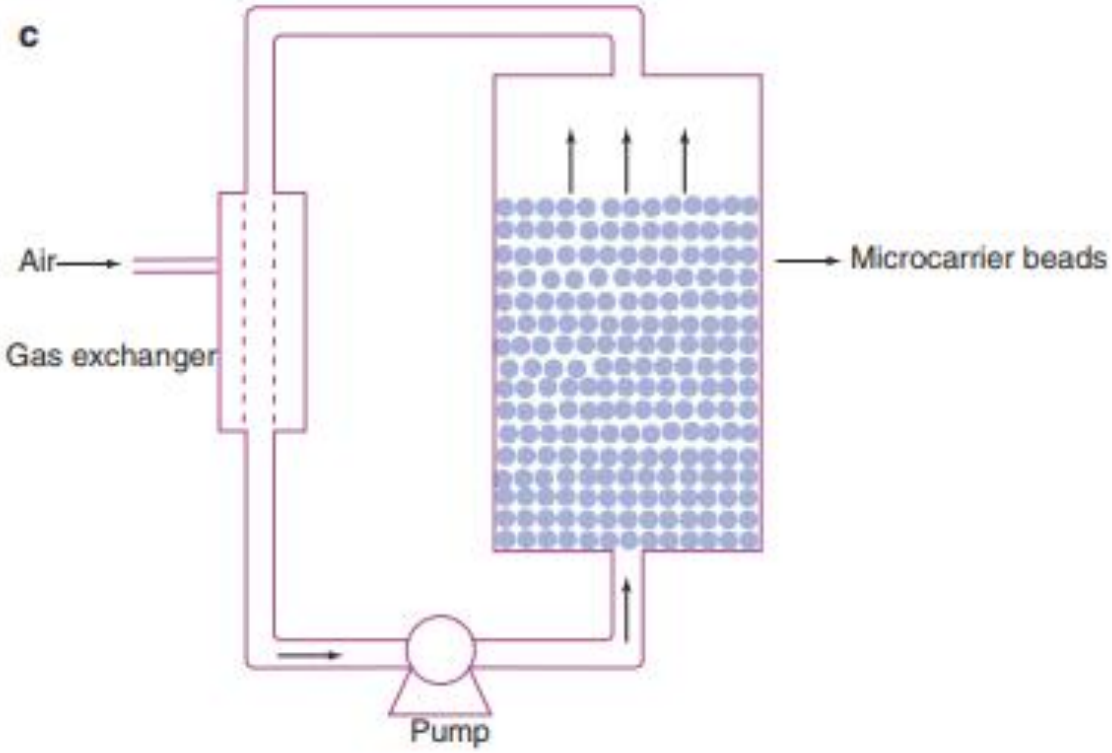
- Stirred tank
- Airlift
- Fixed bed
- Membrane bioreactors



Schematic representation of stirred –tank bioreactor



Schematic representation of airlift bioreactor



Schematic representation of fixed-bed stirred-tank bioreactor

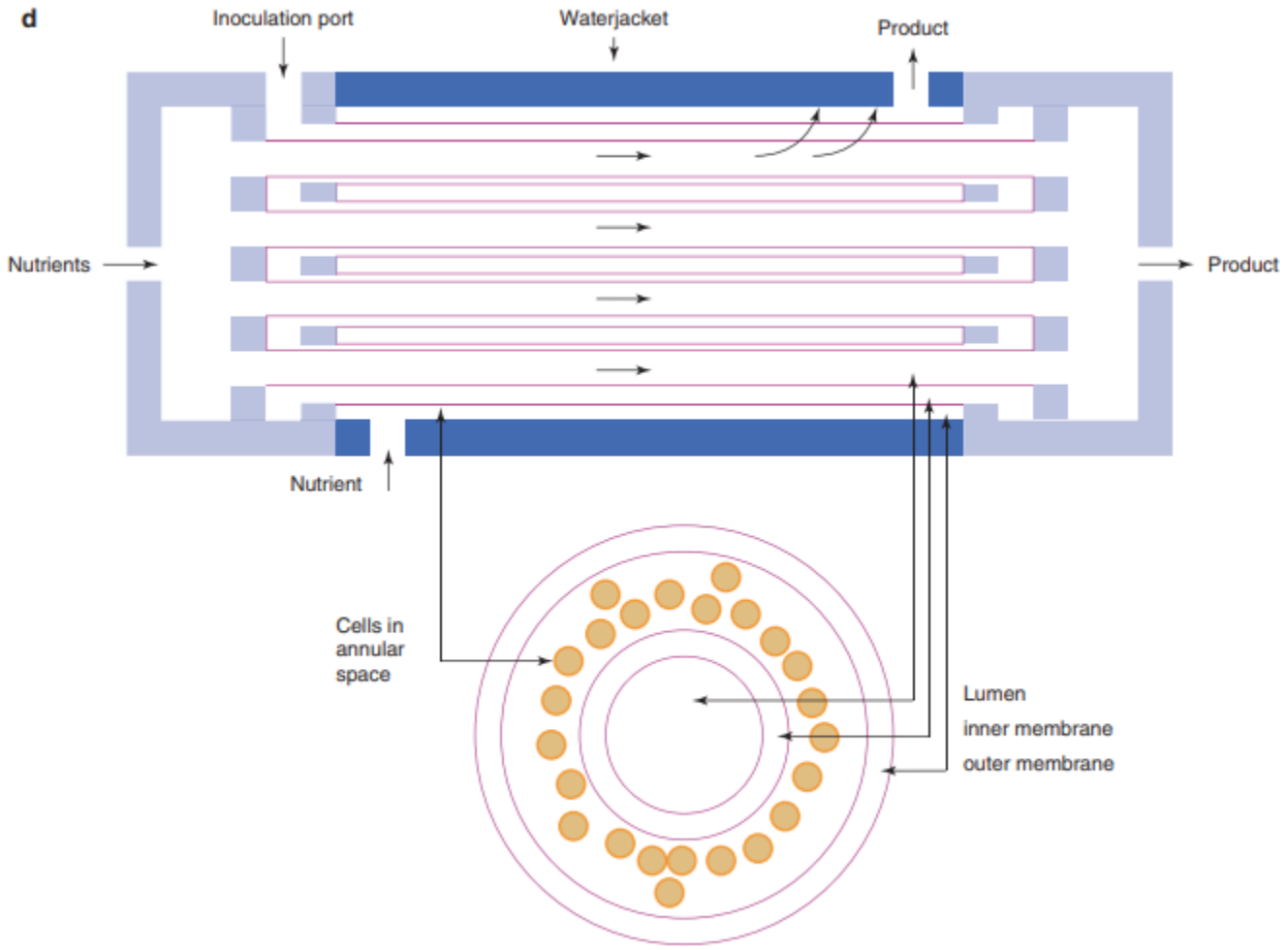


Figure 4.1 ■ (continued)

Schematic representation of hollow fiber perfusion bioreactor

➢ Purification; Downstream Processing

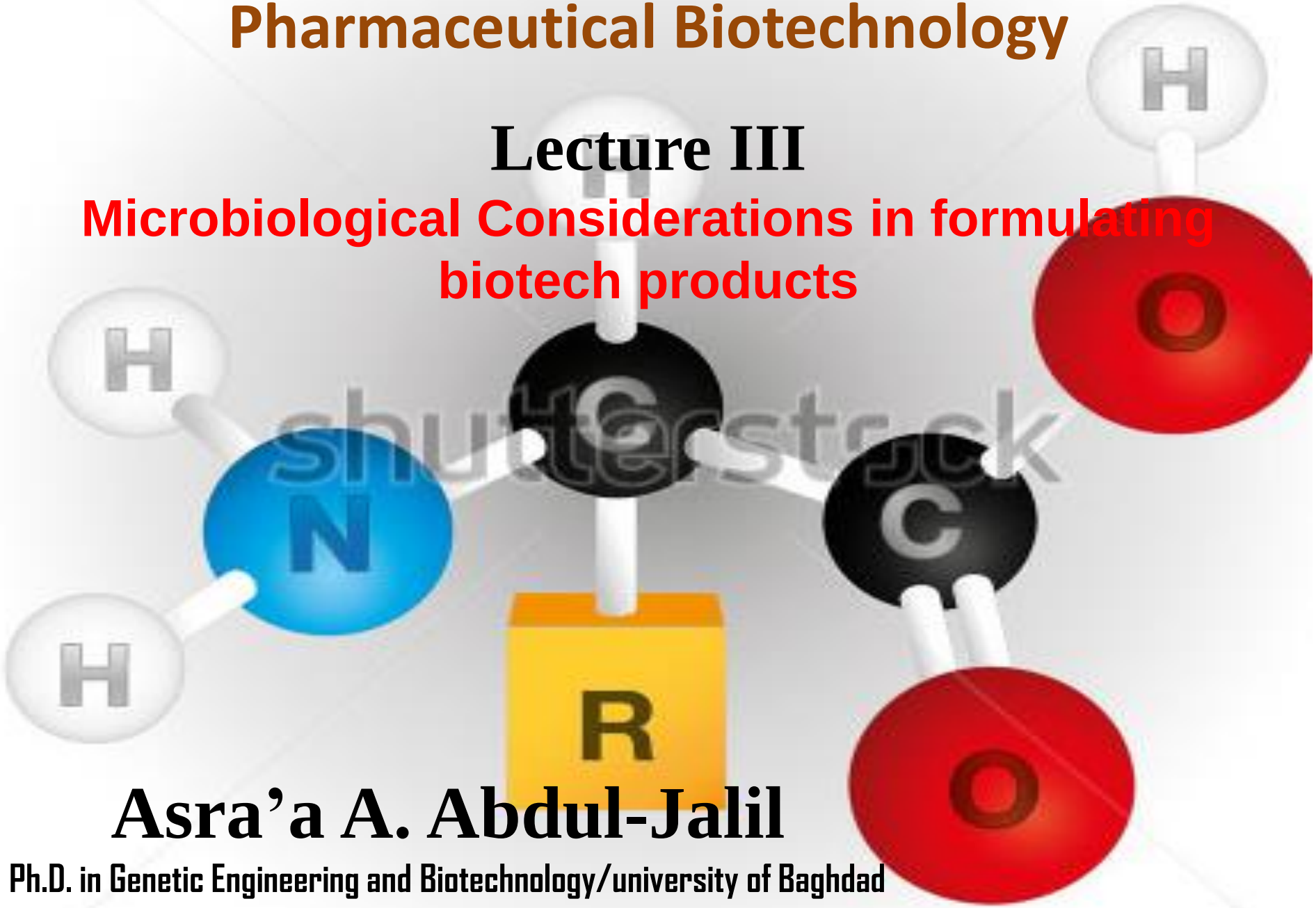
- Recombinant proteins are usually purified from cell culture supernatants or cell extracts by filtration and conventional column chromatography, including affinity chromatography .

- The aim of the downstream processing (DSP) is to purify the therapeutic protein from (potential) endogenous and extraneous contaminants, such as host cell proteins, DNA, and viruses.

Pharmaceutical Biotechnology

Lecture III

Microbiological Considerations in formulating biotech products



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MICROBIOLOGICAL CONSIDERATIONS

- **Sterility**
- Most proteins are administered **parenterally** and have to be sterile.
- In general, proteins are sensitive to heat(Why?) and other regularly used sterilization treatments; they cannot withstand autoclaving, gas sterilization, or sterilization by ionizing radiation.
- Consequently, sterilization of the protein end product is **not possible**

Sterility

- protein pharmaceuticals have to be assembled under aseptic conditions.

- Equipment and excipients are treated separately and autoclaved, or sterilized by dry heat (> 160C). chemical treatment (example:H.W) or gamma radiation to minimize the bioburden.

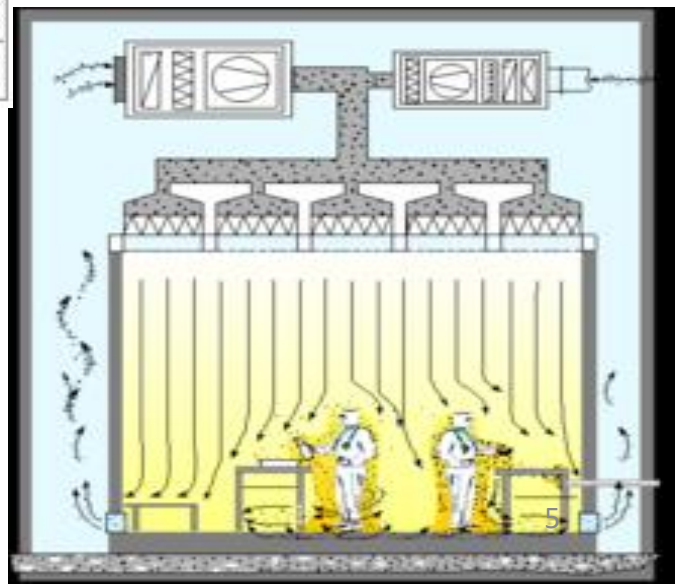
Sterility

- Filtration techniques are used for removal of microbial contaminants.

Pre-filters remove the bulk of the bioburden and other particulate materials. The final “sterilizing” step before filling the vials is filtration through 0.2 or 0.22 µm membrane filters.

Assembly of the product is done in class 100 rooms with laminar airflow that is filtered through high efficiency particulate air (HEPA) filters

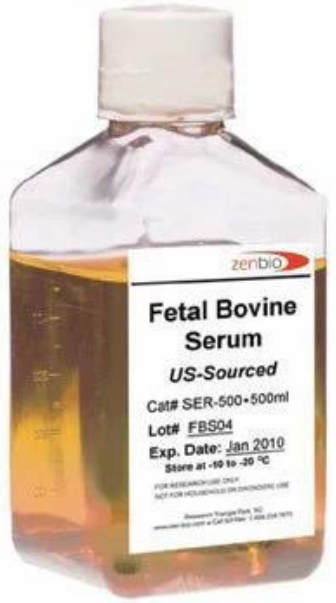
particles						
Class	0.1 µm	0.2 µm	0.3 µm	0.5 µm	1 µm	5 µm
1	35	7	3	1		
10	350	75	30	10	1	
100		750	300	100	10	1
1,000				1,000	100	10
10,000				10,000	1,000	100
100,000				100,000	10,000	1,000



"human factor" is a major source of contamination. Well-trained operators wearing protective cloths (face masks, hats, gowns, gloves, or head-to-toe overall garments) should operate the facility.

❖ Viral Decontamination

As recombinant DNA products are grown in microorganisms, these organisms should be tested for viral contaminants and appropriate measures should be taken if viral contamination occurs. In the rest of the manufacturing process, no (unwanted) viral material should be introduced. Excipients with a certain risk factor such as blood-derived human serum albumin should be carefully tested before use and their presence in the formulation process should be minimized.

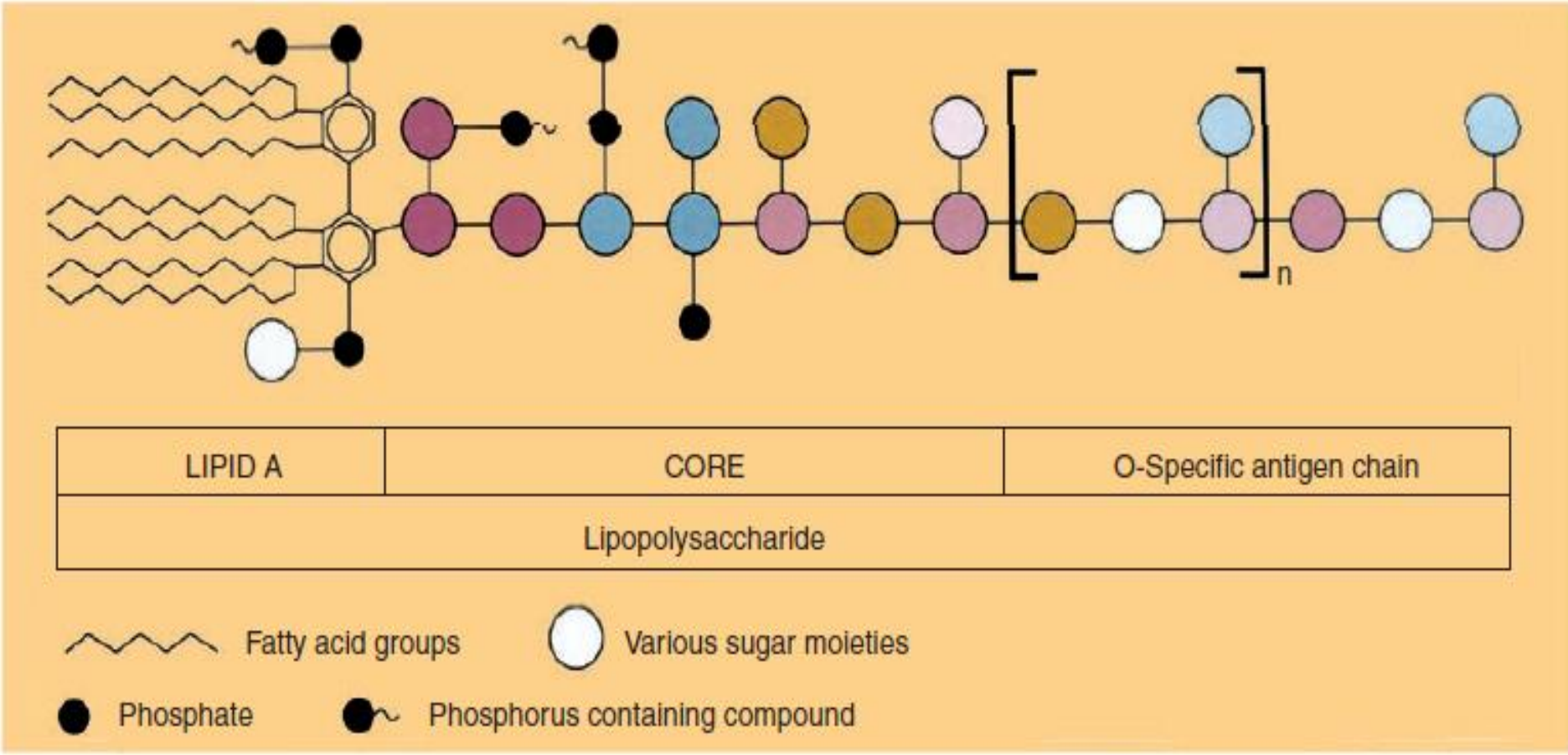


Category	Types	Example
Inactivation	Heat treatment	Pasteurization
	Radiation	UV-light
	Dehydration	Lyophilization
	Cross linking agents, denaturating or disrupting agents	β-propiolactone, formaldehyde, NaOH, organic solvents (e.g., chloroform), detergents (e.g., Na-cholate)
	Neutralization	Specific, neutralizing antibodies
Removal	Chromatography	Ion-exchange, immuno-affinity, chromatography
	Filtration	Nanofiltration
	Precipitation	Cyroprecipitation

Table 4 Methods for reducing or inactivating viral contaminants.

❖ Pyrogen Removal

- Pyrogens are compounds that induce fever.
- Exogenous pyrogens (pyrogens introduced into the body, not generated by the body itself) can be derived from bacterial, viral or fungal sources.
- Bacterial pyrogens are mainly endotoxins shed from gram negative bacteria. They are lipopolysaccharides.
- Humans are sensitive to pyrogen contamination at very low concentrations (**picograms/mL**).
- Another general property shared by endotoxins is their high, negative electrical charge. Their tendency to aggregate and to form large units with MW of over 10^6 in water and their tendency to adsorb to surfaces indicate that these compounds are amphipathic in nature.



Generalized structure of endotoxins. Most properties of endotoxins are accounted for by the active, insoluble “lipid A” fraction being solubilized by the various sugar moieties (different colored circles). Although the general structure is similar, individual endotoxins vary according to their source and are characterized by the O-specific antigenic chain. Source: Adapted from Groves, 1988.

❖ Pyrogen Removal

- ✓ They are stable under standard autoclaving conditions, but break down when heated in the dry state. For this reason equipment and container are treated at temperatures above 160C for prolonged periods(e.g., 30 minutes dry heat at 250C).

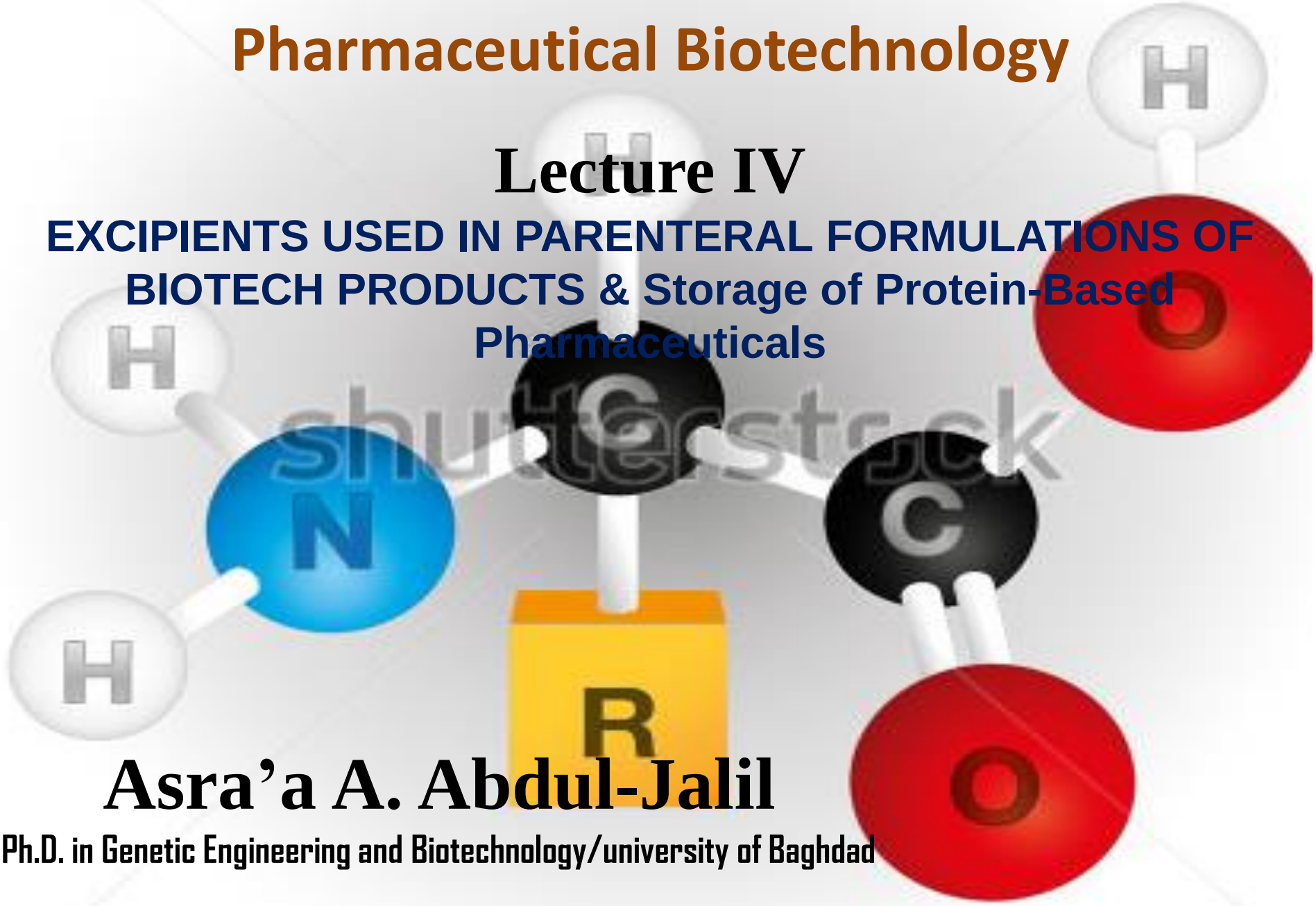
- ✓ Ion exchange chromatographic procedures (utilizing its negative charge) can effectively reduce endotoxin levels in solution.

- ✓ Endotoxins can also be inactivated on utensil surfaces by oxidation (e.g., peroxide) or dry heating (e.g., 30 minutes dry heat at 250C).

Pharmaceutical Biotechnology

Lecture IV

EXCIPIENTS USED IN PARENTERAL FORMULATIONS OF BIOTECH PRODUCTS & Storage of Protein-Based Pharmaceuticals



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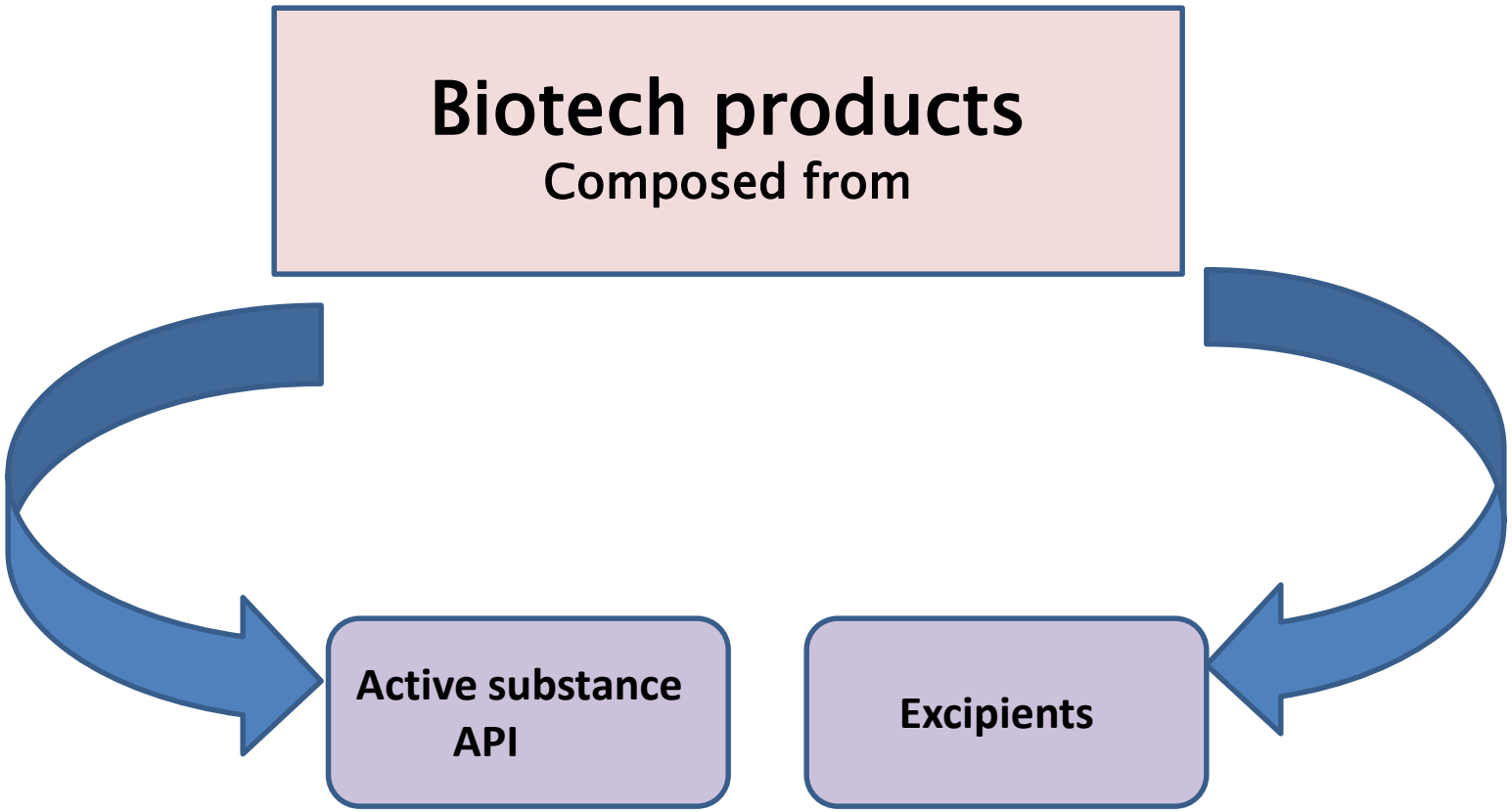

Biotech products
Composed from
Active substance
API
Excipients

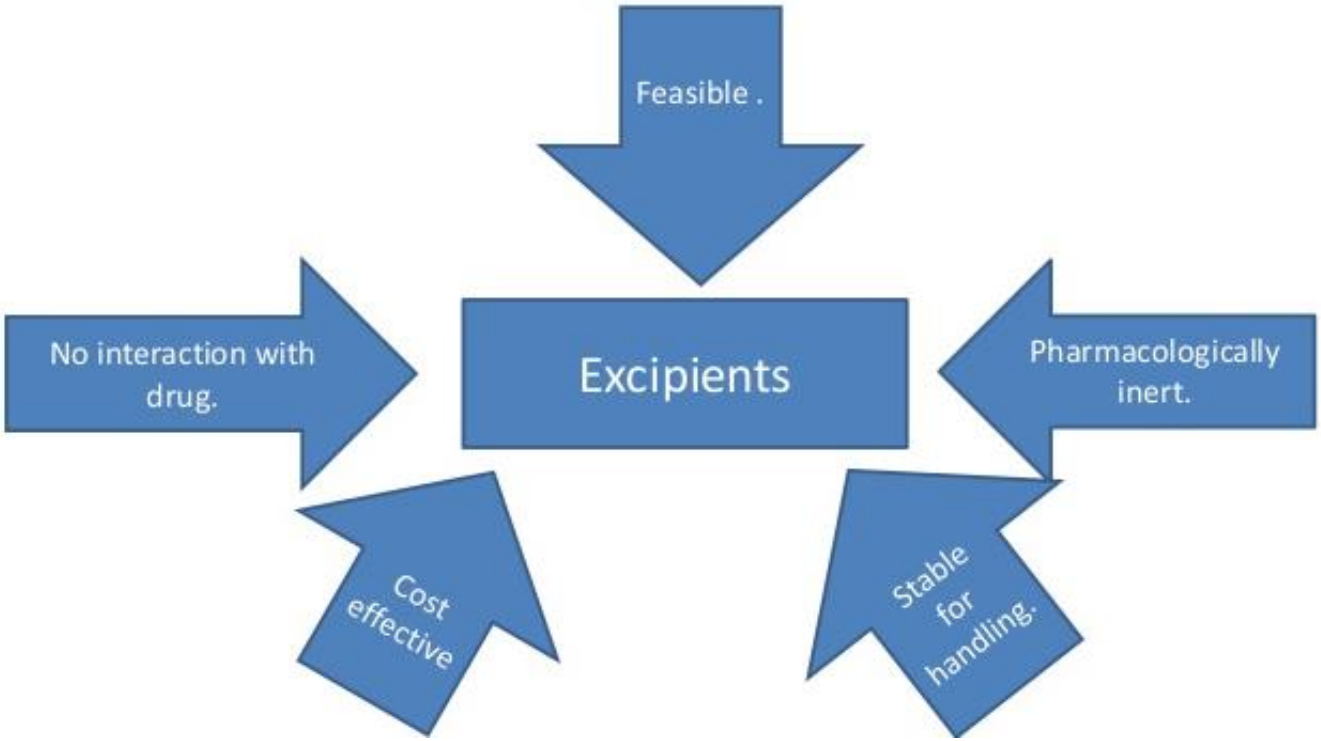
Goals of protein formulation

- The formulation should stabilize protein from chemical and physical degradations.

- Easily administered
- Efficacious
- Adequate stability for shelf life for marketing.
- Simple


excipient has potential interaction with protein drug which could block activity.

Ideal properties of Excipients:



Aggregation

- **Physical** based on hydrophobic and/or electrostatic interactions between molecules

- **Chemical** based on the formation of covalent bridges between molecules through disulfide bonds and ester or amide linkages. In those cases, proper conditions should be found to avoid these chemical reactions.

Excipients

- substances other than the active ingredient(s) which, for example, stabilize the final product or enhance the characteristics of the final product in some other way.

- Serum albumin.
- Addition of serum albumin has been shown to stabilize various different polypeptides. Human serum albumin (HSA) is often employed in the case of biopharmaceuticals destined for parenteral administration to humans. In many cases, it is used in combination with additional stabilizers, including amino acids (mainly glycine) and carbohydrates.

Table 3.23. Various biopharmaceutical preparations for which human serum albumin (HSA) has been described as a potential stabilizer

α- and β-Interferons	Tissue plasminogen activator
γ-Interferon	Tumour necrosis factor
Interleukin-2	Monoclonal antibody preparations
Urokinase	γ-Globulin preparations
Erythropoietin	Hepatitis B surface antigen

Excipient class	Function	Examples
Buffers	pH control, tonicity	Histidine, phosphate, acetate, citrate, succinate
Salts	Tonicity, stabilization, viscosity reduction	Sodium chloride
Sugars[a], polyols	Tonicity, stabilization, cryoprotection, lyoprotection[b], bulking agent[b], reconstitution improvement[b]	Sucrose, trehalose, mannitol, sorbitol
Surfactants	Adsorption prevention, solubilization, stabilization, reconstitution improvement[b]	Polysorbate 20, polysorbate 80, poloxamer 188
Amino acids	Stabilization, viscosity reduction, tonicity, pH control, bulking agent[b]	Arginine, glycine, histidine, lysine, proline
Anti-oxidants	Oxidation prevention	Methionine, sodium edetate
Preservatives[c]	Bacterial growth prevention	m-cresol, benzyl alcohol, phenol

Adapted from Weinbuch et al. (2018)
[a]Only non-reducing sugars
[b]For freeze-dried products
[c]Multi-dose containers

Table 5.6 ■ Common excipients in protein drug products

- **Components found in parenteral formulations of biotech products. All of the above are not necessarily present in one particular protein formulation.**

NOTES

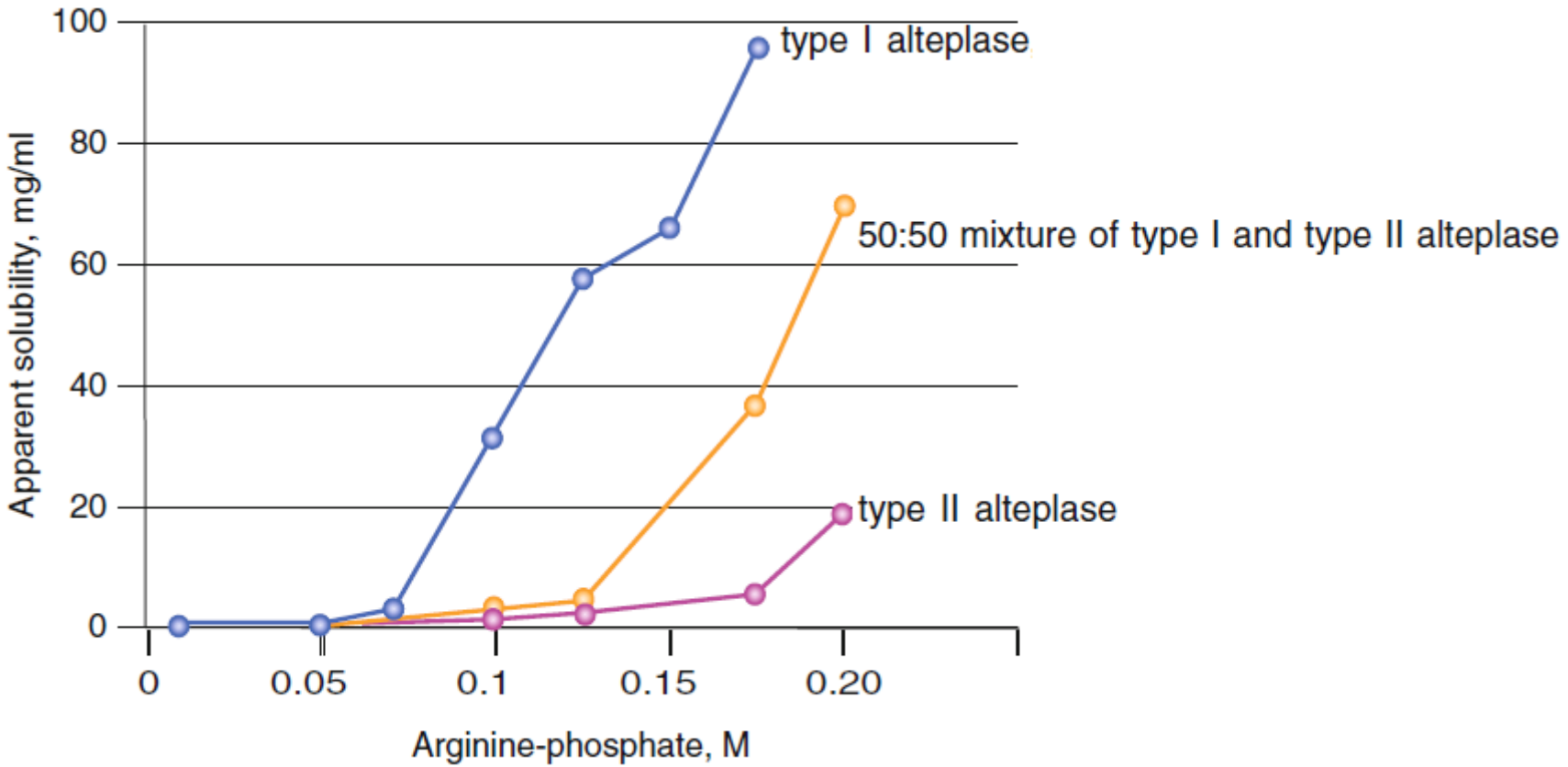
- Both the choice of the excipient and its concentration are important. For instance, low concentrations of polysorbates may stabilize the protein , while higher concentrations may cause denaturation.
- Excipients used in the protein formulation should be essentially endotoxin-free. For solutions “water for injection” (compendial standards) is (freshly) distilled or produced by reverse osmosis.
- The aggregated endotoxins cannot pass through the reverse osmosis membrane. Removal of endotoxins immediately before filling the final container can be accomplished by using activated charcoal or other materials with large surfaces offering hydrophobic interactions.
- The freeze-drying of a protein solution without the proper excipients causes, as a rule, irreversible damage to the protein .
- Bulking agent prevent Blowout is the loss of material taken away by the water vapor that leaves the vial. It occurs when little solid material is present in the vial.

❑Solubility Enhancers

Proteins, in particular those that are non-glycosylated, may have a tendency to aggregate and precipitate.Examples on solubility enhancers:

- ❖ proper pH and ionic strength conditions can enhance the solubility of proteins.
- ❖ Addition of amino acids such as lysine or arginine (used to solubilize tissue plasminogen activator, t-PA),
- ❖ or surfactants, such as sodium dodecylsulfate to stabilize non-glycosylated IL-2, can also help to increase the solubility

Solubility Enhancers



Effect of arginine on type I and type II alteplase at pH 7.2 and 25 C. A, type I alteplase; B, type II alteplase; C, 50:50 mixture of type I and type II alteplase.

❑ Anti-Adsorption and Anti-Aggregation Agents

- **Anti-adsorption agents are added to reduce adsorption of the active protein to interfaces.**
- **Some proteins tend to expose hydrophobic sites, normally present in the core of the native protein structure when an interface is present.**
- **These interfaces can be water/air, water/container wall or interfaces formed between the aqueous phase and utensils used to administer the drug (e.g., catheter, needle) .**
- **These adsorbed, partially unfold protein molecules form aggregates, leave the surface, return to the aqueous phase, form larger aggregates and precipitate.**
- **Example, the proposed mechanism for aggregation of protein such insulin in aqueous media through contact with a hydrophobic surface is presented in the following figure.**

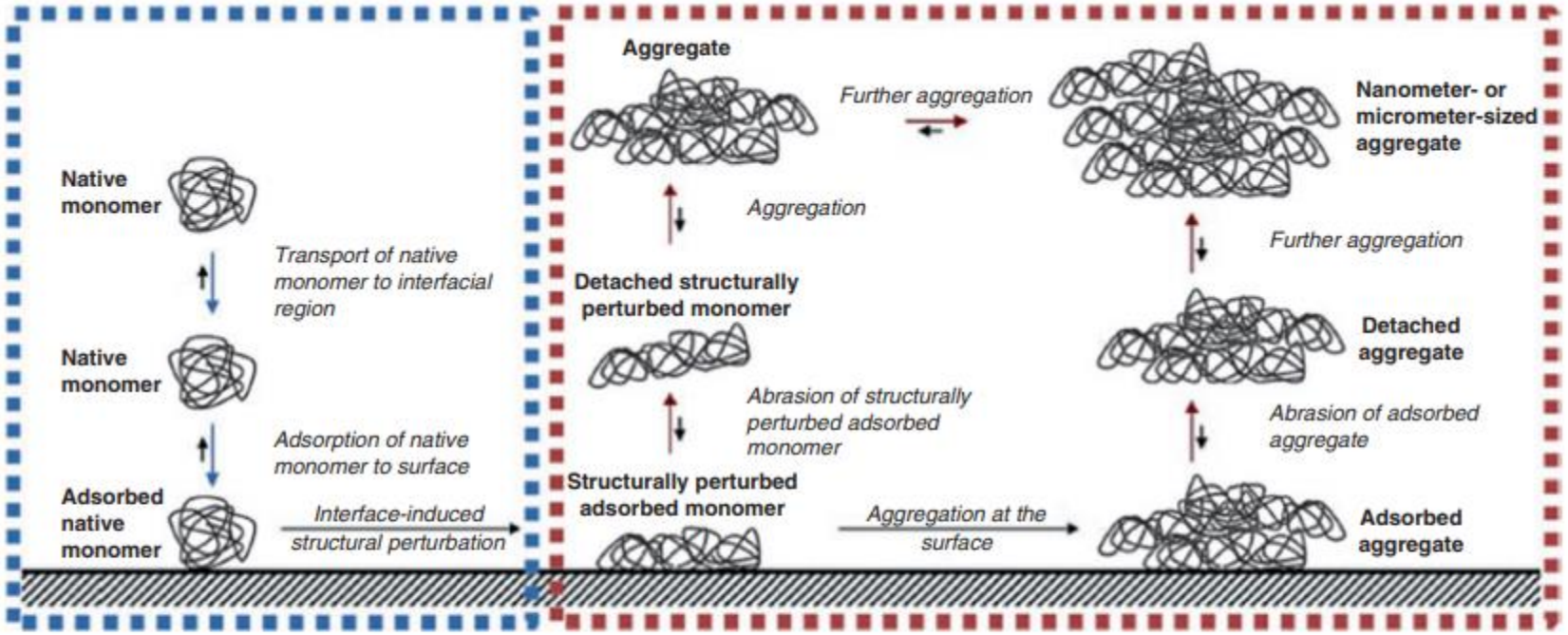


Figure 5.4 ■ Schematic representation of the suggested mechanism of stirring-induced protein aggregation. The left part (framed in blue) depicts the process of protein adsorption onto solid surfaces with potential perturbation of the native structure of the protein on adsorption. This process is followed by aggregation at the surface and in the bulk (framed in red). Contact sliding results in abrasion of the adsorbed protein layer, leading to renewal of the surface for adsorption of a fresh protein layer. Addition of surfactants, such as polysorbate 20, and avoidance of contact stirring will inhibit the steps shown as blue and red arrows, respectively. Adapted from Sediq et al. (2016)

Native insulin in solution is in an equilibrium state between monomeric, dimeric tetrameric, and hexameric forms . The relative abundance of the different aggregation states depend on the pH, insulin concentration, ionic strength, and specific excipients (e.g., Zn2þ and phenol). It has been suggested that the dimeric form of insulin adsorbs to hydrophobic interfaces and subsequently forms larger aggregates at the interface. This explains why anti adhesion agents can also act as anti-aggregation agents, Insulin is one of the many proteins that can form fibrillary precipitates (long rod-shaped structures with diameters in the 0.1 mm range).

- **Low concentrations of phospholipids** and **surfactants** have been shown to exert a fibrillation-inhibitory effect The **selection of the proper pH** can also help to prevent this unwanted phenomenon.

❑Buffer Components:

- Buffer selection is an important part of the formulation process, because of the pH dependence of protein solubility and physical and chemical stability.

- **Buffers used in biotech formulation are:**

✓ phosphate
✓ citrate
✓ acetate

- **temporary pH changes can cause aggregation. These conditions can occur, for example during the freezing step in a freeze-drying process, when one of the buffer components is crystallizing and the other is not. In a phosphate buffer, Na_2HPO_4 crystallizes faster than NaH_2PO_4. This causes a noticed drop in pH during the freezing step. Other buffer components do not crystallize, but form amorphous systems and then pH changes are minimized.**

❑Protection Against Oxidation

- **Methionine, cysteine, tryptophan, tyrosine, and histidine are amino acids that are readily oxidized.**
- **Proteins rich in these amino acids are susceptible to oxidative degradation.**
- **The sensitivity of an amino acid residue towards oxidation depends on its position within the protein, as this determines its accessibility for oxidative reagents.**
- **Replacement of oxygen by inert gases in the vials helps to reduce oxidative stress.**
- **Moreover, one may consider the addition of antioxidants, such as methionine, which competes with methionine residues for oxidation. Interestingly, some antioxidants can accelerate protein oxidation, Ascorbic acid, for example, can act as an oxidant in the presence of trace amounts of heavy metals which may be present as impurities. To reduce the catalytic activity of heavy metals, one may consider introducing chelators such as EDTA (ethylenediaminetetraacetic acid).**

❑ Protection Against Oxidation

- Certain proteins are formulated in containers designed for multiple injection schemes.
- After administering the first dose, contamination with microorganisms may occur and preservatives are needed to minimize growth.
- Usually, these preservatives are present in concentrations that are bacteriostatic rather than bactericidal in nature.
- Antimicrobial agents are the phenol, meta-cresol, benzyl alcohol, and chlorobutanol.
- These preservative molecules can interact with the protein, which may compromise both the activity of the protein and the effectivity of the preservative.

❑Protection Against Freezing and Drying

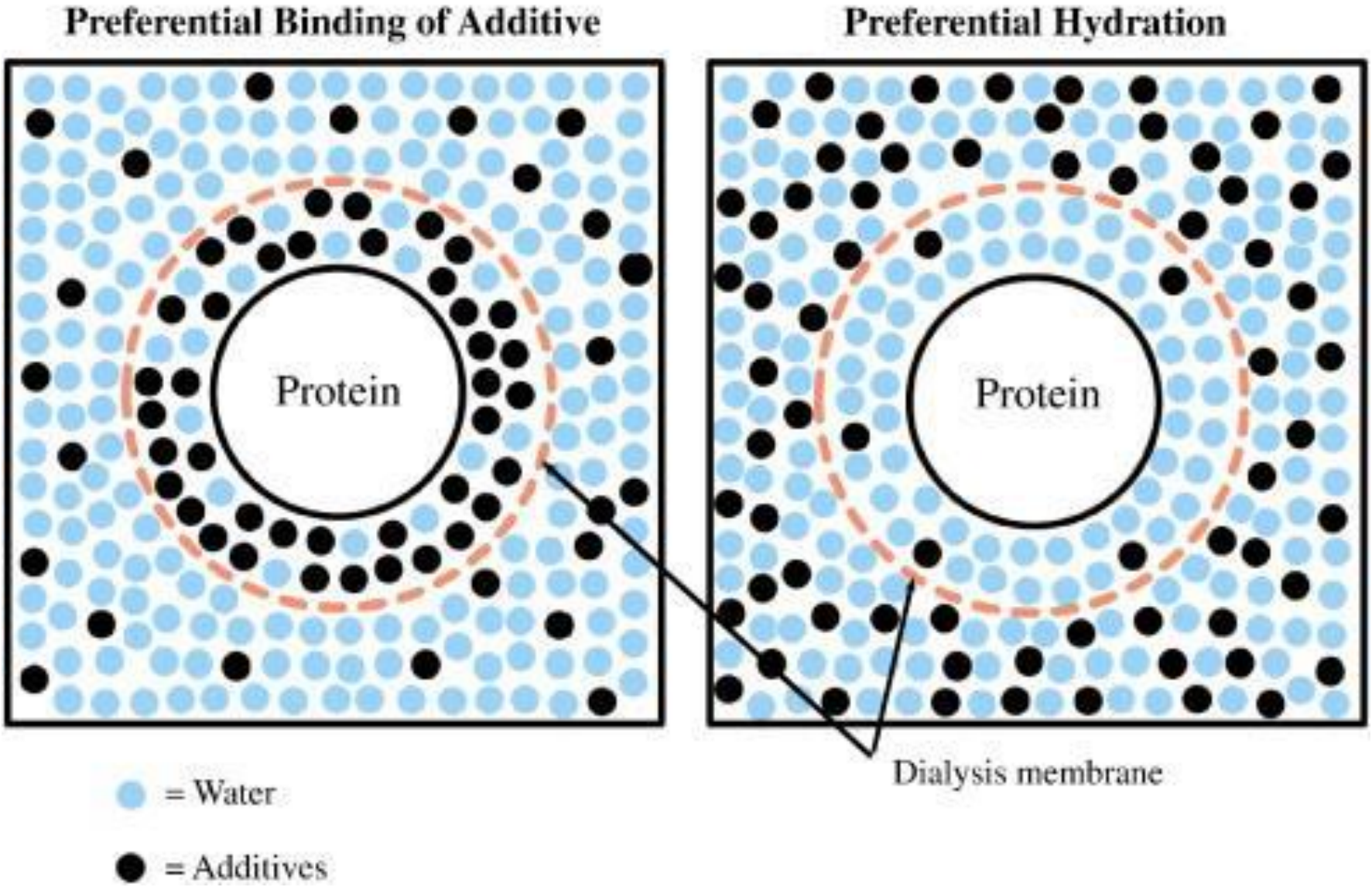
- Cryoprotectants are excipients that protect a protein during freezing or in the frozen state (mainly sugars: sucrose, trehalose and sugar alcohols: mannitol, sorbitol).

- These excipients may not be inert; they may influence protein structural stability.

- The key stabilizing mechanisms is ‘preferential exclusion’ stabilize the protein structure through the principle of “**preferential exclusion**”.

- These additives enhance the interaction of the solvent with the protein and are themselves excluded from the protein surface layer; the protein is preferentially hydrated.

Dialysis Equilibrium



❑ Protection Against Freezing and Drying

- Lyoprotectants protect the protein in the lyophilized state (e.g., sugars). The key mechanisms of protection described for lyoprotectants are :
- ✓ (1) the ‘water replacement theory’: replacement of water as stabilizing agent by forming hydrogen bonds with the protein and
- ✓ (2) the ‘vitrification theory’: formation of a glassy amorphous matrix keeping protein molecules separated from each other

SHELF LIFE & STORAGE OF PROTEIN-BASED PHARMACEUTICALS

Shelf life??

Proteins can be stored as

(i) an aqueous solution.

(ii) freeze-dried form.

(iii) in dried form in a compacted state (tablet).

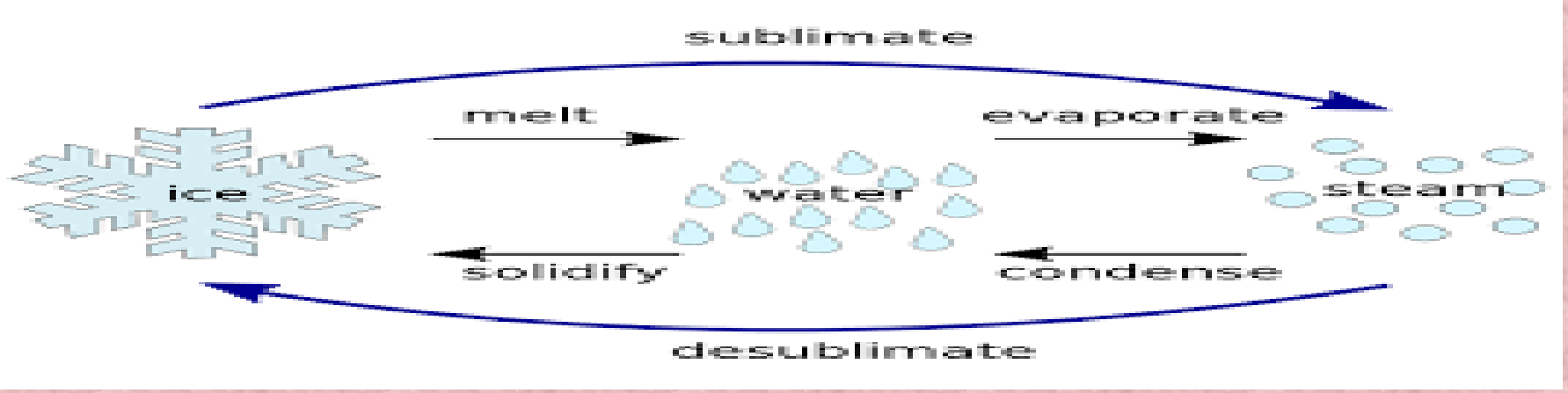
- **Freeze-drying:**

also known as lyophilization, is a dehydration process typically used to preserve a perishable material or make the material more convenient for transport. Freeze-drying works by freezing the material and then reducing the surrounding pressure to allow the frozen water in the material to sublime directly from the solid phase to the gas phase.

Freeze Drying of Proteins

- Freeze drying may provide the requested stability.

- During freeze-drying water is removed through sublimation and not by evaporation.



Freeze Drying of Proteins

Proteins in solution often do not meet the preferred stability requirements for industrially produced pharmaceutical products (> 2 years), even when kept permanently under refrigerator conditions (cold chain). The abundant presence of water promotes chemical and physical degradation processes.

Lab-scale freeze-drier

Freeze Drying of Proteins

- During freeze-drying water is removed through sublimation and not by evaporation.
- **Three stages can be discerned in the freeze-drying process**:

(i) freezing step: The temperature of the aqueous system in the vials is lowered to –15C° or lower have been reached. During the cooling stage, concentration of the protein and excipients occurs because of the growing ice crystal mass at the expense of the aqueous water phase.

(ii) the primary drying step: Sublimation of the water mass in the vial is initiated by lowering the pressure. The water vapor is collected on a condenser, the supply of heat from the shelf to the vial, so the shelf is heated during this stage. The end of the primary drying stage is reached when product temperature and shelf temperature become equal.

(iii)the secondary drying step: When all frozen or amorphous water that is non-protein and non-excipient bound is removed, the secondary drying step starts, In the secondary drying stage the temperature is slowly increased to remove “bound” water; the chamber pressure is still reduced, the secondary drying step ends when the product has been kept at 20C for some time.

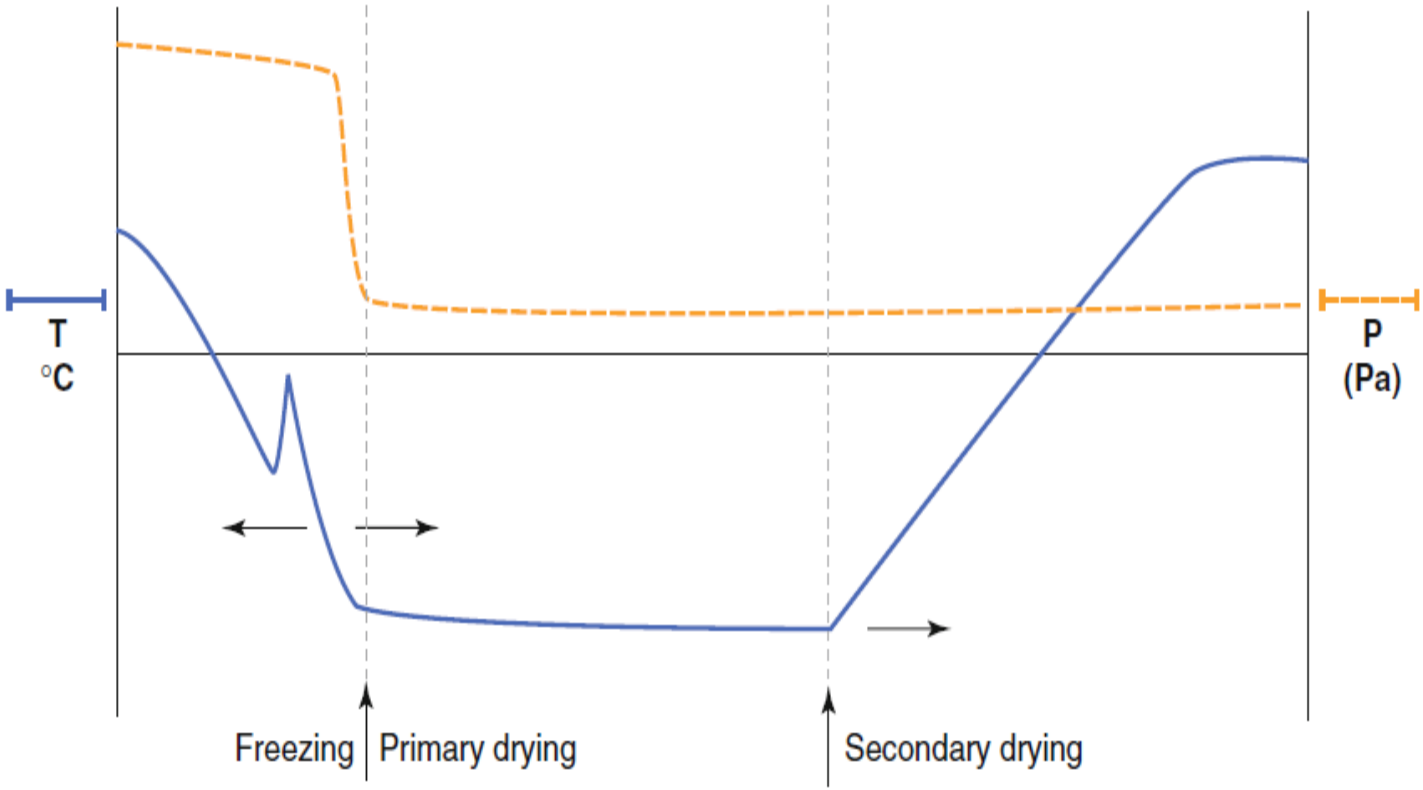


Figure 4.6 Example of freeze-drying protocol for systems with crystallizing water. *T* temperature, *P* pressure.

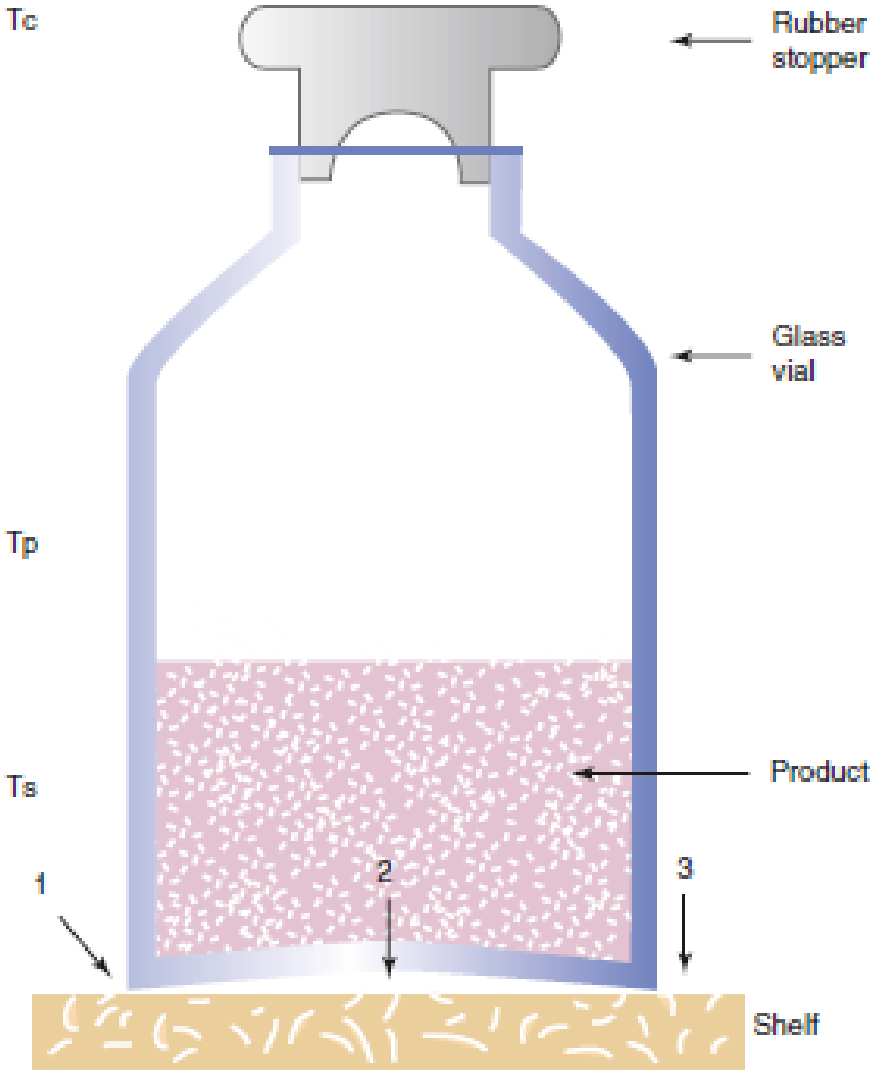


Figure 5.10 ■ Heat transfer mechanisms during the freeze-drying process: (1) Direct conduction via shelf and glass at points of actual contact. (2) Gas conduction: contribution heat transfer via conduction through gas between shelf and vial bottom. (3) Radiation heat transfer. Ts shelf temperature, Tp temperature sublimating product, Tc temperature condensor. Ts > Tp > Tc

Pharmaceutical Biotechnology

Pharmacokinetic of peptides and proteins

-Lecture VI-

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For a recombinant protein to become a human therapeutic, its biophysical and biochemical characteristics
must be well understood.

PROTEIN STRUCTURE

- **PRIMARY STRUCTURE:**

The functional properties of proteins are derived from their folding into distinct three dimensional structures

This alignment of the 20 amino acids, called a primary sequence, has in general all the information necessary for folding into a distinct tertiary structure comprising different secondary structures such as a-helices and b-sheets.

- All of the 20 amino acids consist of a Cα carbon to which an amino group, a carboxyl group, a hydrogen, and a side chain bind in L configuration. These amino acids are joined by condensation to yield a peptide bond consisting of a carboxyl group of an amino acid joined with the amino group of the next amino acid

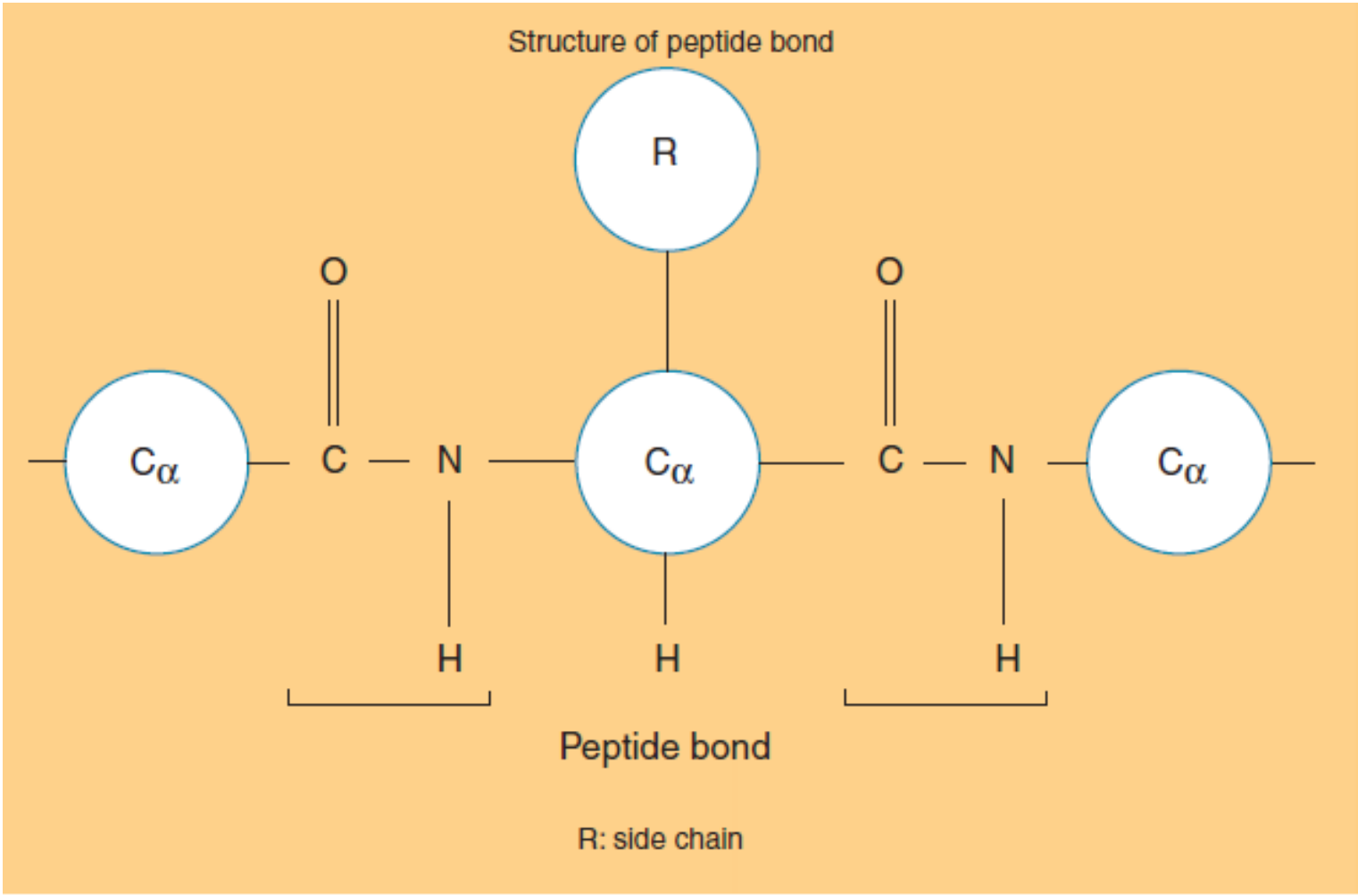
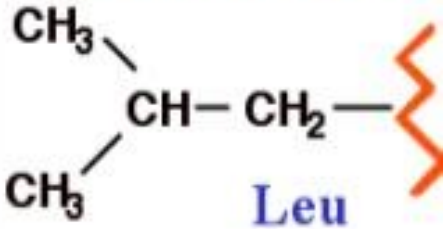
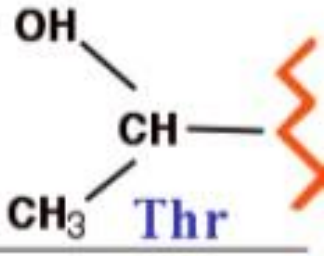
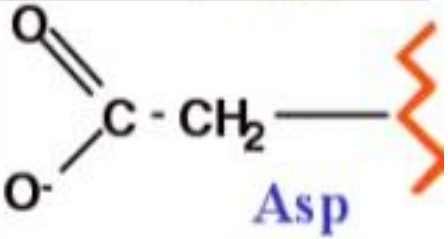
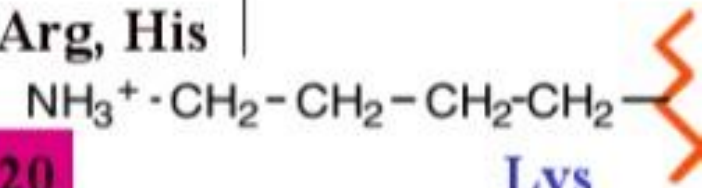


Figure 2 Structure of peptide bond.

Nomenclature

Amino Acid	3 letter code	1 letter code	Amino Acid	3 letter code	1 letter code
Glycine	Gly	G	Threonine	Thr	T
Alanine	Ala	A	Cysteine	Cys	C
Valine	Val	V	Tyrosine	Tyr	Y
Leucine	Leu	L	Asparagine	Asn	N
Isoleucine	Ile	I	Glutamine	Gln	Q
Methionine	Met	M	Aspartic Acid	Asp	D
Proline	Pro	P	Glutamic Acid	Glu	E
Phenyl alanine	Phe	F	Lysine	Lys	K
Tryptophan	Trp	W	Arginine	Arg	R
Serine	Ser	S	Histidine	His	H

Amino acids groups

Group	Characteristics	Names	Example (-Rx)
non-polar	hydrophobic	Ala, Val, Leu, Ile, Pro, Phe Trp, Met	 Leu
polar	hydrophilic (non-charged)	Gly , Ser, Thr, Cys, Tyr, Asn Gln	 Thr
acidic	negatively charged	Asp, Glu	 Asp
basic	positively charged	Lys, Arg, His	 Lys

Total = 20

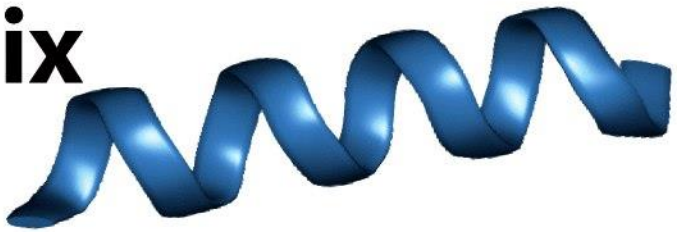
❑SECONDARY STRUCTURE

α-Helix

Immediately evident in the primary structure of a protein is that each amino acid is linked by a peptide bond. The amide, NH, is a hydrogen donor and the carbonyl, C=O, is a hydrogen acceptor, and they can form a stable hydrogen bond when they are positioned in an appropriate configuration of the polypeptide chain. Such structures of the polypeptide chain are called secondary structure. Two main structures, α-helix and β-sheet.

Secondary structure

α helix



β sheet

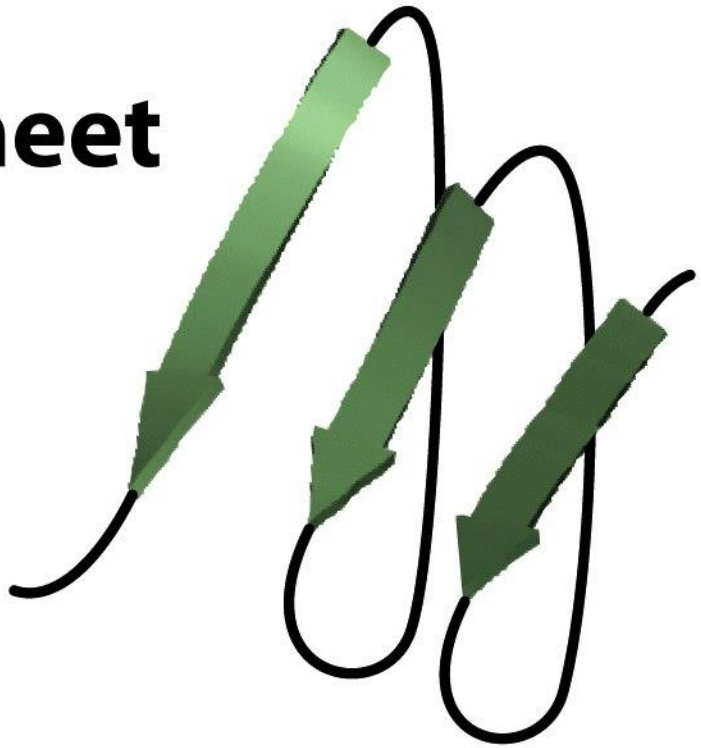


Figure 3-2b
Molecular Cell Biology, Sixth Edition

Tertiary structure

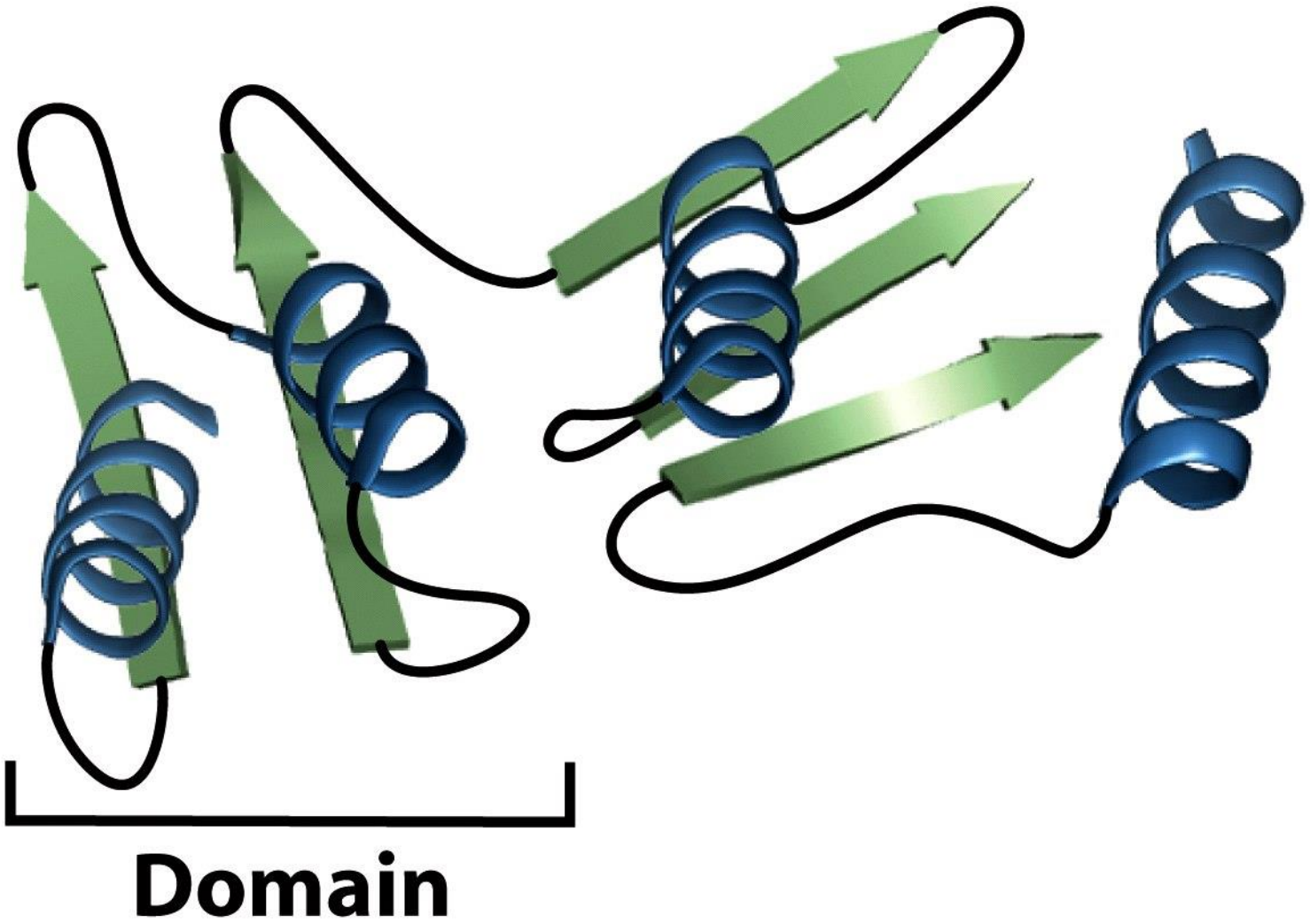


Figure 3-2c
Molecular Cell Biology, Sixth Edition

Pharmacokinetics and Pharmacodynamics of Peptide and Protein Drugs

The dose–exposure–response relationship and thus the dose of a drug required to achieve a certain effect are determined by the drug's pharmacokinetic and pharmacodynamic properties

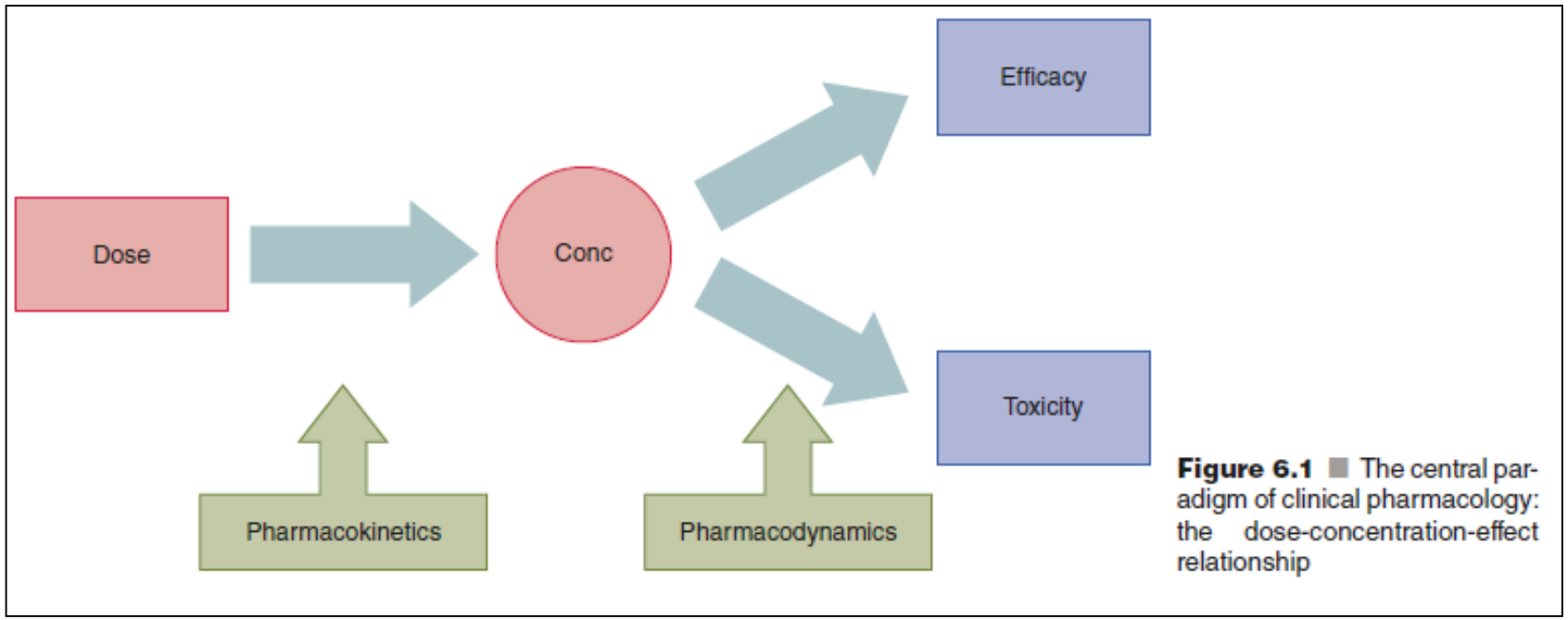


Figure 6.1 The central paradigm of clinical pharmacology: the dose-concentration-effect relationship

Pharmacokinetics and Pharmacodynamics

Pharmacokinetics describes the time course of the concentration of a drug in a body fluid, preferably plasma or blood, that results from the administration of a certain dosage regimen. It comprises all processes affecting drug absorption, distribution, metabolism, and excretion. Simplified, pharmacokinetics characterizes

what the body does to the drug

In contrast,

pharmacodynamics characterizes the intensity of a drug effect or toxicity resulting from certain drug concentrations in a body fluid, usually at the assumed site of drug action. It can be simplified to

what the drug does to the body

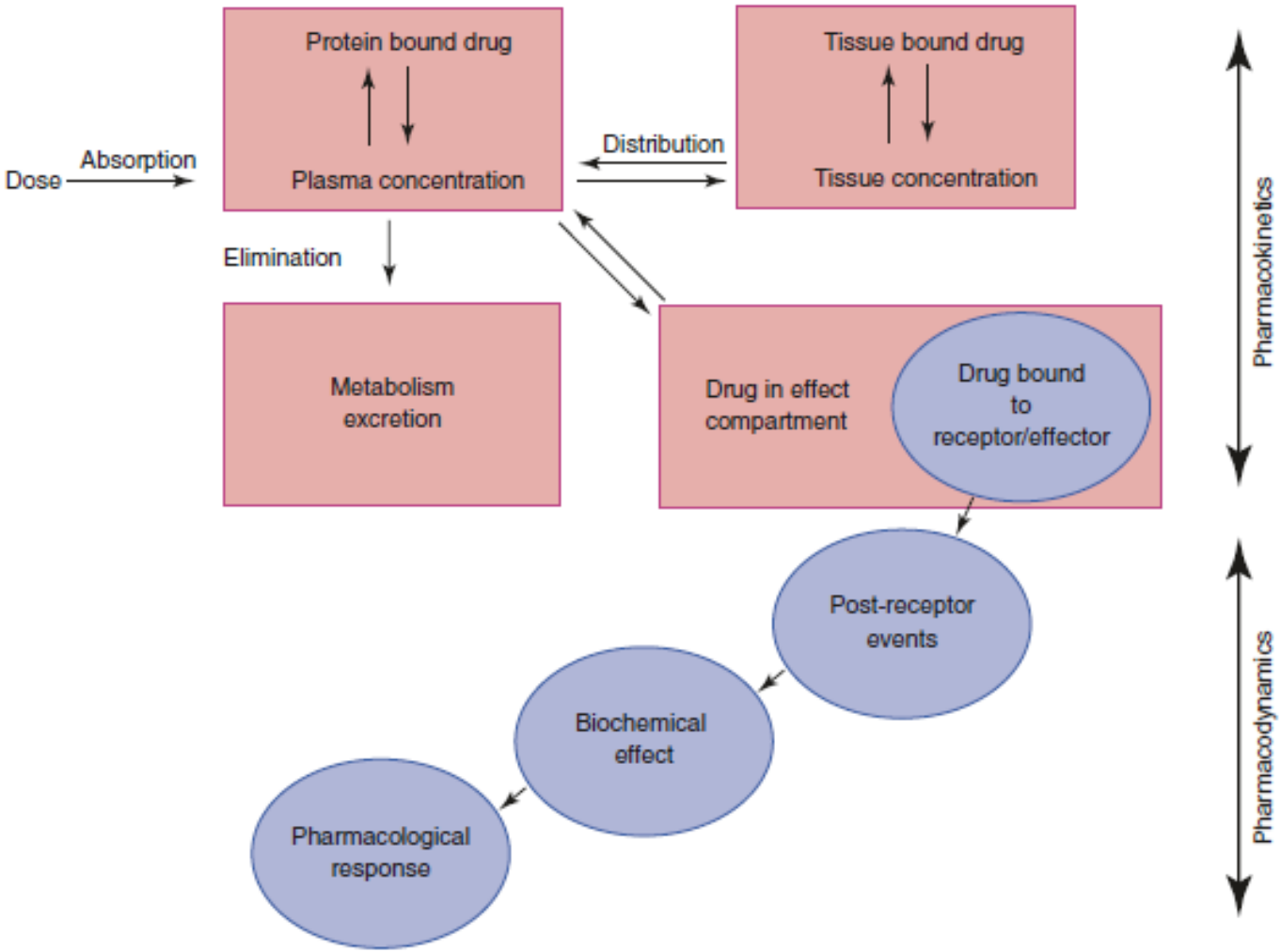


Figure 6.2 ■ Physiological scheme of pharmacokinetic and pharmacodynamic processes

Pharmacokinetics

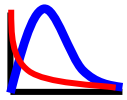
Absorption

Distribution

Metabolism

Excretion

Christopher Town, Ph.D.



The characterization of the pharmacokinetics and pharmacodynamics of peptide and protein therapeutics, however, arise from some of their specific properties:

a. Their structural similarity to endogenous structural or functional proteins and nutrients.

b. Their intimate involvement in physiologic processes on the molecular level, often including regulatory feedback mechanisms.

c. The analytical challenges to identify and quantify them in the presence of a myriad of similar molecules.

d. Their large molecular weight and macromolecule character (for proteins)

PHARMACOKINETICS OF PROTEIN THERAPEUTICS

Peptides which frequently have hormone activity, usually have short elimination half-lives, which is desirable for a close regulation of their endogenous levels and thus function. **Insulin**, for example shows dose-dependent elimination with a relatively short half-life of **26 and 52 minutes** at 0.1 and0.2 U/kg, respectively. Contrary to that, proteins that have transport tasks such as albumin or long-term immunity functions such as **immunoglobulins** have elimination half-lives of several days, which enables and ensures the continuous maintenance of physiologically necessary concentrations in the bloodstream. This is for example reflected by the elimination half-life of antibody drugs such as the anti-epidermal growth factor receptor antibody cetuximab, an IgG1 chimeric antibody for which a half-life of approximately 7 days has been reported.

Absorption of Protein Therapeutics

❑ Enteral Administration

- Peptides and proteins, unlike conventional small molecule drugs, are generally not therapeutically active upon oral administration

- The lack of systemic bioavailability is mainly caused by two factors:

(1) high gastrointestinal enzyme activity and

(2) Low permeability through the gastrointestinal mucosa

- Since **oral administration is still a highly desirable route of delivery for protein drugs due to** its:

❑ **Convenience.**

❑ **Cost-effectiveness.**

❑ **Painlessness.**

❖ Suggested approaches to increase the oral bioavailability of protein drugs include :

➢ encapsulation into micro- or nanoparticles thereby protecting proteins from intestinal degradation

➢ **Other strategies are chemical modifications such as amino acid backbone modifications**

➢ **chemical conjugations to improve the resistance to degradation and the permeability of the protein drug.**

➢ **Coadministration of protease inhibitors has also been suggested for the inhibition of enzymatic degradation**

❑ Parenteral Administration

- Most peptide and protein drugs are currently formulated as parenteral formulations because of their poor oral bioavailability. Major routes of administration include intravenous (**IV**), subcutaneous (**SC**), and intramuscular (**IM**) administration.

In addition, other non-oral administration pathways are utilized, including **nasal, buccal, rectal, vaginal, transdermal, ocular and pulmonary drug delivery.**

❖ IV administration of peptides and proteins offers the **advantage** of **circumventing presystemic degradation**, thereby achieving the highest concentration in the biological system.

❖ Protein therapeutics given by the **IV route** include, among many others:**The tissue plasminogen activator (t-PA) analogs alteplase and tenecteplase. The recombinant human erythropoietin α.**

❖ One of the potential limitations of SC and IM administration, however, are the **presystemic degradation processes** frequently associated with these administration routes, resulting in a **reduced bioavailability compared to IV administration.**

Several peptide and protein therapeutics including insulin are administered as SC injections. Following an SC injection, peptide and protein **therapeutics may enter the systemic circulation either via blood capillaries or through lymphatic vessels.(diagram in last lecture)**

In general, macromolecules larger than 16 kDa are predominantly absorbed into the lymphatics whereas those under 1 kDa are mostly absorbed into the blood circulation. There appears to be a linear relationship between the molecular weight of the protein and the proportion of the dose absorbed by the lymphatics. This is of particular importance for those agents whose therapeutic targets are lymphoid cells (i.e., interferons and interleukins).

Distribution of Protein Therapeutics

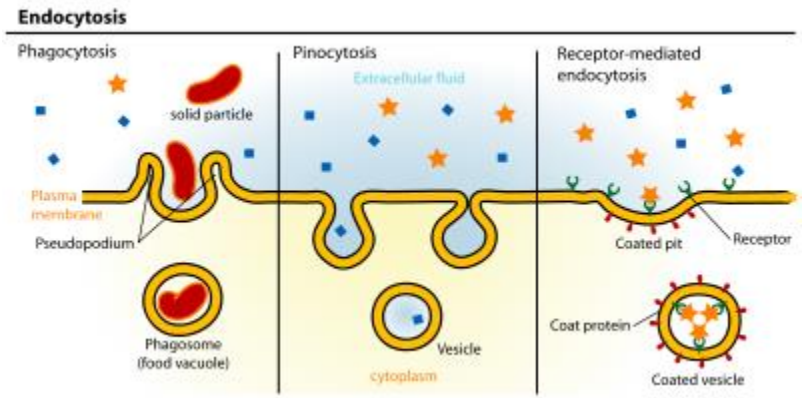
The rate and extent of protein distribution is determined largely by their:

- **Size and molecular weight.**
- **Physiochemical properties (e.g., charge, lipophilicity).**
- **Protein binding.**
- **Their dependency on active transport processes**

Since **most therapeutic proteins have high molecular weights** and are thus **large in size, their apparent volume of distribution is usually small** and limited **to the volume of the extracellular space** due to their limited mobility secondary to impaired passage through biomembranes.

In contrast to small molecule drugs, protein transport from the vascular space into the interstitial space of tissues is largely mediated by convection rather than diffusion, following the unidirectional fluid flux from the **vascular space through paracellular pores into the interstitial tissue space.** The subsequent removal from the tissues is accomplished by lymph drainage back into the systemic circulation.

Another, but much less prominent pathway for the movement of protein molecules from the vascular to the interstitial space is transcellular migration via endocytosis.



Besides the size-dependent sieving of macromolecules through the capillary walls, **charge** may also play an important role in the biodistribution of proteins. **It has been suggested that the electrostatic attraction between positively charged proteins and negatively charged cell membranes might increase the rate and extent of tissue distribution.**

- ✓ Most cell surfaces are negatively charged because of their abundance of glycosaminoglycans in the extracellular matrix.

Protein Binding of Protein Therapeutics

- Another factor that can influence the distribution of therapeutic peptides and proteins is **binding to endogenous protein structures. Physiologically active endogenous peptides and proteins** frequently interact **with specific binding proteins** involved in their transport and regulation.

Furthermore, interaction with binding proteins may enable or facilitate cellular uptake processes and thus affect the drug's pharmacodynamics.

Similarly, therapeutically administered proteins may interact with endogenous binding proteins

Specific binding proteins have been identified for numerous protein drugs, including **recombinant human DNase** for use as mucolytic in cystic fibrosis, **growth hormone**, and **recombinant human vascular endothelial growth factor (rhVEGF).**

Protein Binding of Protein Therapeutics

Recombinant cytokines, for example, may after IV administration **encounter various cytokine binding proteins including soluble cytokine receptors and anti-cytokine antibodies. (In either case, the binding protein may either prolong the cytokine circulation time** by acting **as a storage depot or it may enhance the cytokine clearance.**

Elimination of Protein Therapeutics

- Protein-based therapeutics is generally subject to the same **catabolic pathways as endogenous proteins.** The **end products** of protein metabolism are thus **amino acids.**

- **Non-metabolic elimination pathways such as renal or biliary excretion** are negligible for most proteins.

Proteolysis

The **metabolic rate for protein degradation generally increases with decreasing molecular weight** from large to small proteins to peptides (**Table below**), but is also dependent on other factors such as:

- Size.
- Charge.
- Lipophilicity.
- Functional groups.
- Glycosylation.
- Pattern.
- Secondary and tertiary structure.

Proteolysis

Molecular weight (kDa)	Elimination site	Predominant elimination mechanisms
<0.5	Blood, liver	Extracellular hydrolysis Passive lipoid diffusion
0.5–1	Liver	Carrier-mediated uptake Passive lipoid diffusion
1–60	Kidney	Glomerular filtration and subsequent degradation processes (see Fig. 6.4)
50–200	RES, endothelial cells (skin, muscle, gut), liver	Receptor-mediated endocytosis Pinocytosis
200–400	Immune system	Opsonization
>400	Phagocytic cells	Phagocytosis

Based on Meijer and Ziegler (1993) and Eigenmann et al. (2017)
Other determining factors are size, charge, lipophilicity, functional groups, sugar recognition, vulnerability for proteases, aggregation to particles, formation of complexes with opsonization factors, etc. As indicated, mechanisms may overlap. Endocytosis may occur at any molecular weight range; *RES* reticuloendothelial system

Table 6.1 ■ Molecular weight as major determinant of the elimination mechanisms of peptides and proteins

Gastrointestinal Protein Metabolism

The gastrointestinal tract is a **major site of protein metabolism with high proteolytic enzyme activity due to its primary function to digest dietary proteins.** Thus, gastrointestinal **metabolism of protein drugs is one of the major factors limiting systemic bioavailability of orally** administered protein drugs. The metabolic activity of the gastrointestinal tract, however, is not limited to orally administered proteins.

Parenterally administered peptides and proteins may also be metabolized in the intestinal mucosa following intestinal secretion. At least 20% of the degradation of endogenous albumin, for example, has been reported to take place in the GIT.

Proteolysis

- **Sites capable of extensive peptide and protein metabolism are not only limited to the liver, kidneys, and gastrointestinal tissue, but also include blood and vascular endothelium as well as other organs and tissues.** As proteases and peptidases are also located within cells.

- **The proteolytic activity of SC tissue, for example, results in a partial loss of activity of SC compared to IV administrated interferon-g.**

Renal Protein Metabolism and Excretion

- The **kidneys are a major site of protein metabolism for smaller sized proteins that undergo glomerular filtration.**

- **The size-selective cut-off for glomerular filtration is approximately 60 kD, Glomerular filtration is most efficient, however, for proteins smaller than 30 kDa**.

Renal metabolism of peptides and small proteins is mediated through three highly effective processes (Fig. 8). As a result, only minuscule amounts of intact protein are detectable in urine.

1. **The first mechanism** involves **glomerular filtration** of larger, complex peptides and proteins followed by: **Reabsorption into endocytic vesicles** in the proximal tubule. Subsequent **hydrolysis into small peptide fragments** and amino acids.

2. **The second mechanism** entails glomerular filtration followed by intraluminal metabolism, predominantly by exopeptidases in the luminal brush border membrane of the proximal tubule. The resulting peptide fragments and amino acids are reabsorbed into the systemic circulation.

3. The third mechanism of renal metabolism is peritubular extraction of peptides and proteins from post-glomerular capillaries with subsequent intracellular metabolism.
Peritubular transport of proteins and peptides from the basolateral membrane has also been shown for insulin.

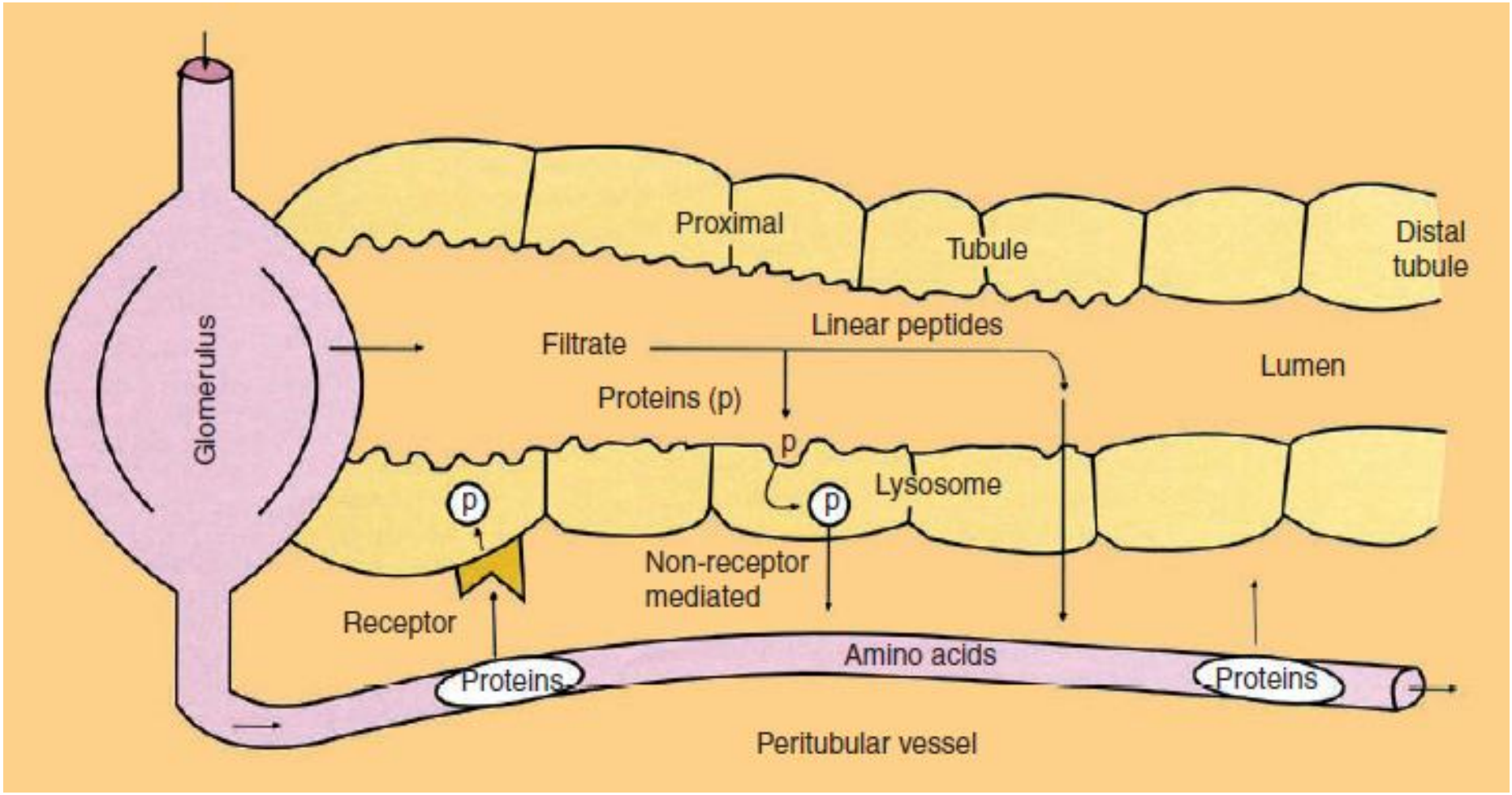


Figure : Pathways of renal metabolism of peptides and proteins: Glomerular filtration followed by either

(a) intraluminal metabolism or

(b) tubular reabsorption with intracellular lysosomal metabolism, and

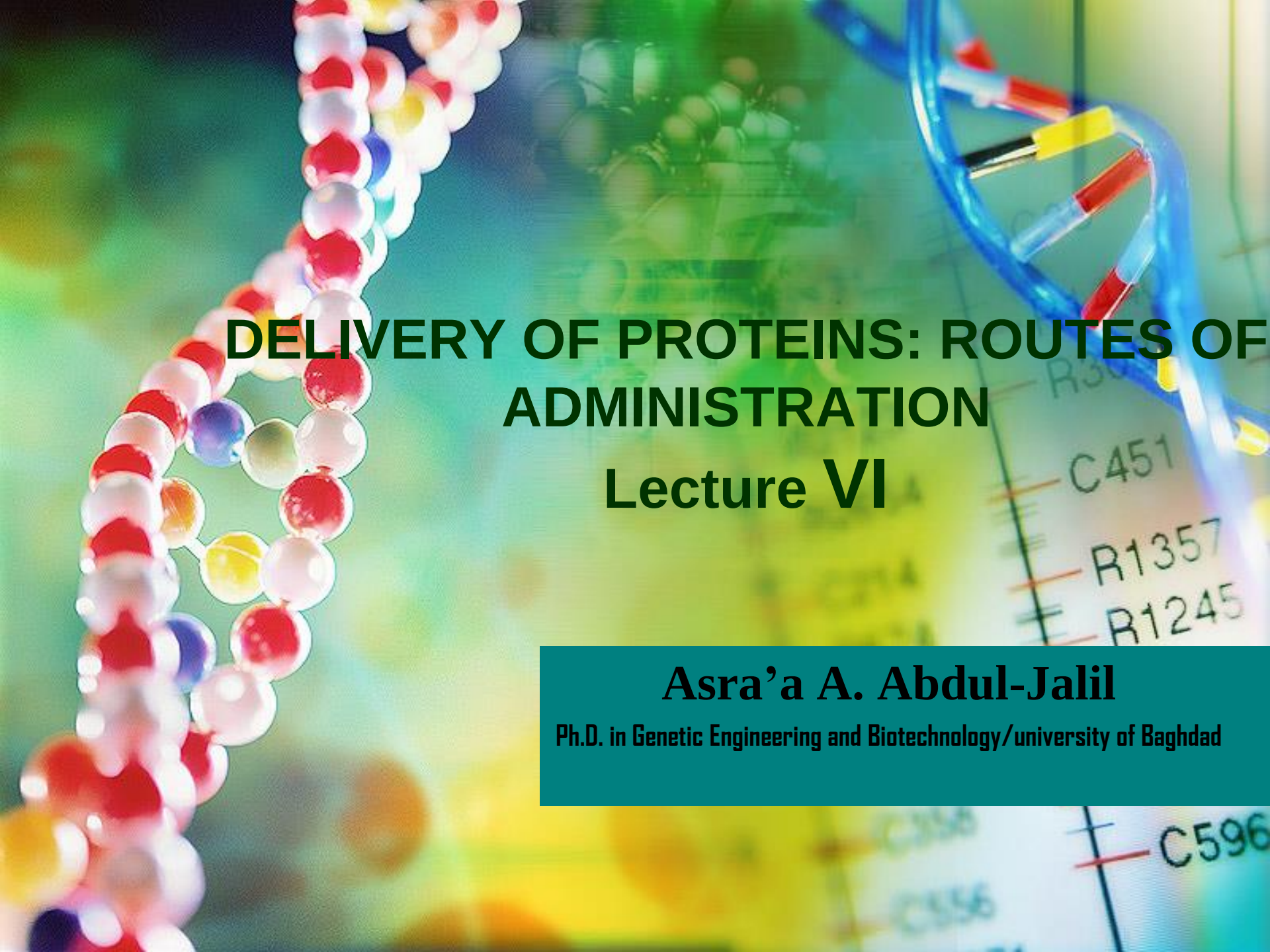
(c) peritubular extraction with intracellular lysosomal metabolism

Hepatic Protein Metabolism

Aside from renal and gastrointestinal metabolism, the liver may also play a major role in the metabolism of protein therapeutics.

Exogenous as well as endogenous proteins undergo proteolytic degradation to dipeptides and amino acids that are reused for endogenous protein synthesis.

Proteolysis usually **starts with endopeptidases** that attack in the middle part of the protein, and **the resulting oligopeptides are then further degraded by exopeptidases**



DELIVERY OF PROTEINS: ROUTES OF ADMINISTRATION

Lecture VI

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Routes of administration

- Is the path by which a drug, fluid, poison or other substance is brought into contact with the body.

- **Factors affecting choice of route**

✓ Drug-related factors

✓ Patient related factors

✓ Therapeutic action

A. The Parenteral Route of Administration

defined as administration via those routes where a needle is used, including:

- Intravenous (IV),
- Intramuscular (IM),
- Subcutaneous (SC)
- Intraperitoneal (IP) injections.

Table 1-1 Parenteral Routes

Route	Injection Site
Intravenous (IV)	Vein
Intramuscular (IM)	Muscle tissue
Intradermal (ID)	Dermis of the skin
Subcutaneous (subcut; SQ)	Subcutaneous tissue of the skin
Intrathecal (IT)	Subarachnoid space of the spinal cord
Epidural	Epidural space of the spinal cord
Intra-arterial	Artery
Intra-articular	Joint space
Intracardiac	Heart
Intraocular	Eye
Intraperitoneal	Peritoneal cavity

The Parenteral Route of Administration

- The blood half-life of biotech products can vary over a wide range. For example, the circulation half-life of Tissue Plasminogen Activator tPA is a few minutes, while **monoclonal antibodies (MAb)** reportedly have half-lives of a few days.

- Obviously, one reason to develop modified proteins through site directed mutagenesis(*In vitro* mutagenesis) is to enhance circulation half-life.

The method provides a means of introducing specific nucleotide changes into a gene and Leading to change in the type of amino acid.

- ✓ A simple way to expand the mean residence time for short half-life proteins is to switch from IV to IM or SC administration.
- ✓ absorption of SC administered protein injections (compared to IV administration) may be as low as 30%
- ✓ One should realize that by doing that, changes in disposition may occur, with a significant impact on the therapeutic performance of the drug & bioavailability. These changes are related to:

(i) the prolonged residence time at the IM or SC site of injection compared to IV administration, differences in protein environment upon injection and enhanced exposure to degradation reactions (peptidases), **(ii)** differences in disposition

(i) the prolonged residence

- For instance, diabetics can become “insulin resistant” through **high tissue peptidase activity**, Other factors that can contribute to absorption variation are related to differences in exercise level of the muscle at the injection site and also massage and heat at the injection site. The state of the tissue, for instance the occurrence of pathological conditions, may be important as well

- (ii) differences in disposition:
- Upon administration, the protein may be transported to the blood through the lymphatics or may enter the blood circulation through the capillary wall at the site of injection.

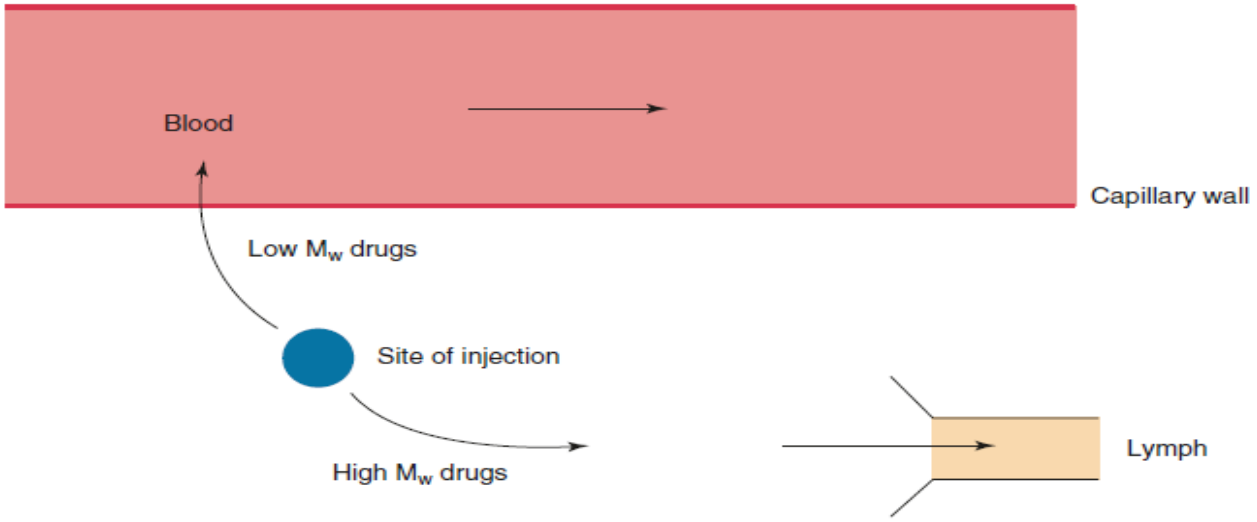

Figure 5.12 ■ Routes of uptake of SC- or IM-injected drugs

- The fraction of the administered dose taking this lymphatic route is molecular weight dependent.

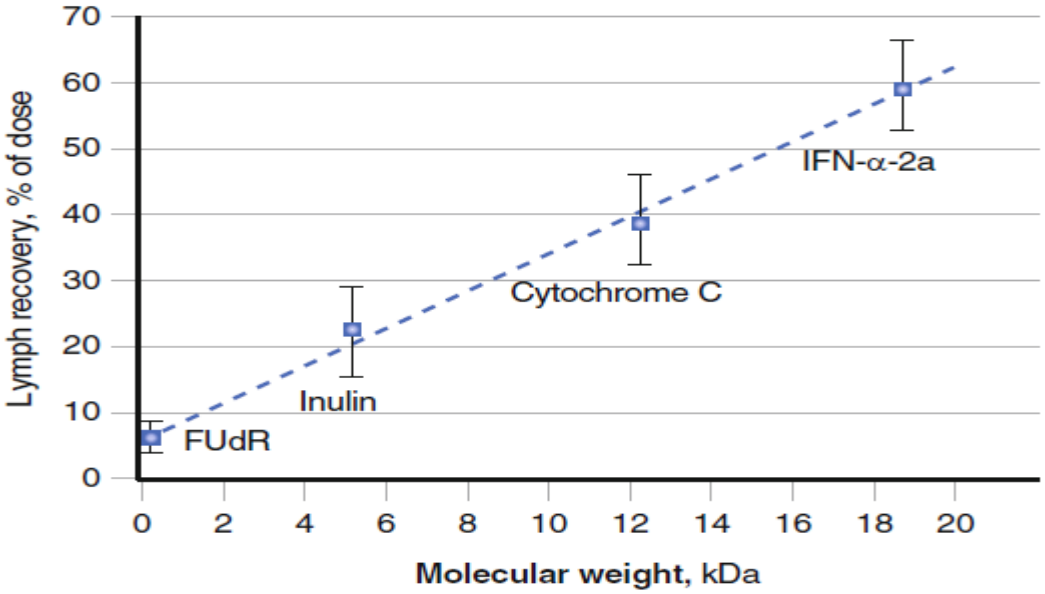

Figure 4.12 ■ Correlation between the molecular weight and the cumulative recovery of rIFN alpha-2a (M_w 19 kDa), cytochrome c (M_w 12.3 kDa), insulin (M_w 5.2 kDa), and FUDR (M_w 256.2 Da) in the efferent lymph from the right popliteal lymph node following SC administration into the lower part of the right hind leg of sheep. Each point and bar shows the mean and stan-

- Lymphatic transport takes time (hours) and uptake in the blood circulation is **highly dependent on the injection site.**

- On its way to the blood, the lymph passes through draining lymph nodes and **contact is possible between lymph contents and cells of the immune system such as macrophages, B- and T-lymphocytes residing in the lymph nodes.**

B. The Oral Route of Administration

- **Oral delivery of protein drugs would be preferable, because** :

  - it is patient friendly.
  - and no intervention by a healthcare professional is necessary to administer the drug

- **Protein oral bioavailability is usually very low, The two main reasons for this failure of uptake are**:

  - ✓ protein degradation in the gastrointestinal (GI) tract and
  - ✓ poor permeability of the wall of the GI tract in case of a passive transport process

The Oral Route of Administration

i. Protein degradation in the GI tract:

The human body has developed a very efficient system to break down proteins in our food to amino acids, or di- or tri-peptides. These building stones for body proteins are actively absorbed for use wherever necessary in the body. In the **stomach pepsins**, a family of aspartic proteases, are secreted. They are particularly active between pH 3 and 5 and lose activity at higher pH values. Pepsins are **endopeptidases** capable of cleaving peptide bonds distant from the ends of the peptide chain. They preferentially cleave peptide bonds between two hydrophobic amino acids. Other endopeptidases are active in the GI tract at **neutral pH values**, e.g., trypsin, chymotrypsin, and elastase. They have different peptide bond cleavage characteristics that more or less complement each other.

Continued..

Exopeptidases, proteases degrading peptide chains from their ends, are present as well. Examples are carboxypeptidase A and B. In the GI lumen the proteins are cut into fragments that effectively further break down to amino acids, di- and tri-peptides by brush border and cytoplasmic proteases of the enterocytes.

(ii) poor permeability:

High molecular weight molecules do not readily penetrate the intact and mature epithelial barrier if diffusion is the sole driving force for mass transfer. Their diffusion coefficient decreases with increasing molecule size. Proteins are no exception to this rule. Active transport of intact therapeutic recombinant proteins over the GI-epithelium has not been described yet.

Conclusions:

- The above analysis leads to the conclusion that nature, unfortunately, does not allow us to use the oral route of administration for therapeutic proteins if high (or at least constant) bioavailability is required.

Oral vaccines

- ✓ However, for the category of oral vaccines the above-mentioned hurdles of degradation and permeation are not necessarily prohibitive. For oral immunization, only a (small) fraction of the antigen (protein) has to reach its target site to elicit an immune response.

- ✓ The target cells are lymphocytes and antigen presenting accessory cells located in Peyer's patches. The B-lymphocyte population includes cells that produce secretory IgA antibodies.

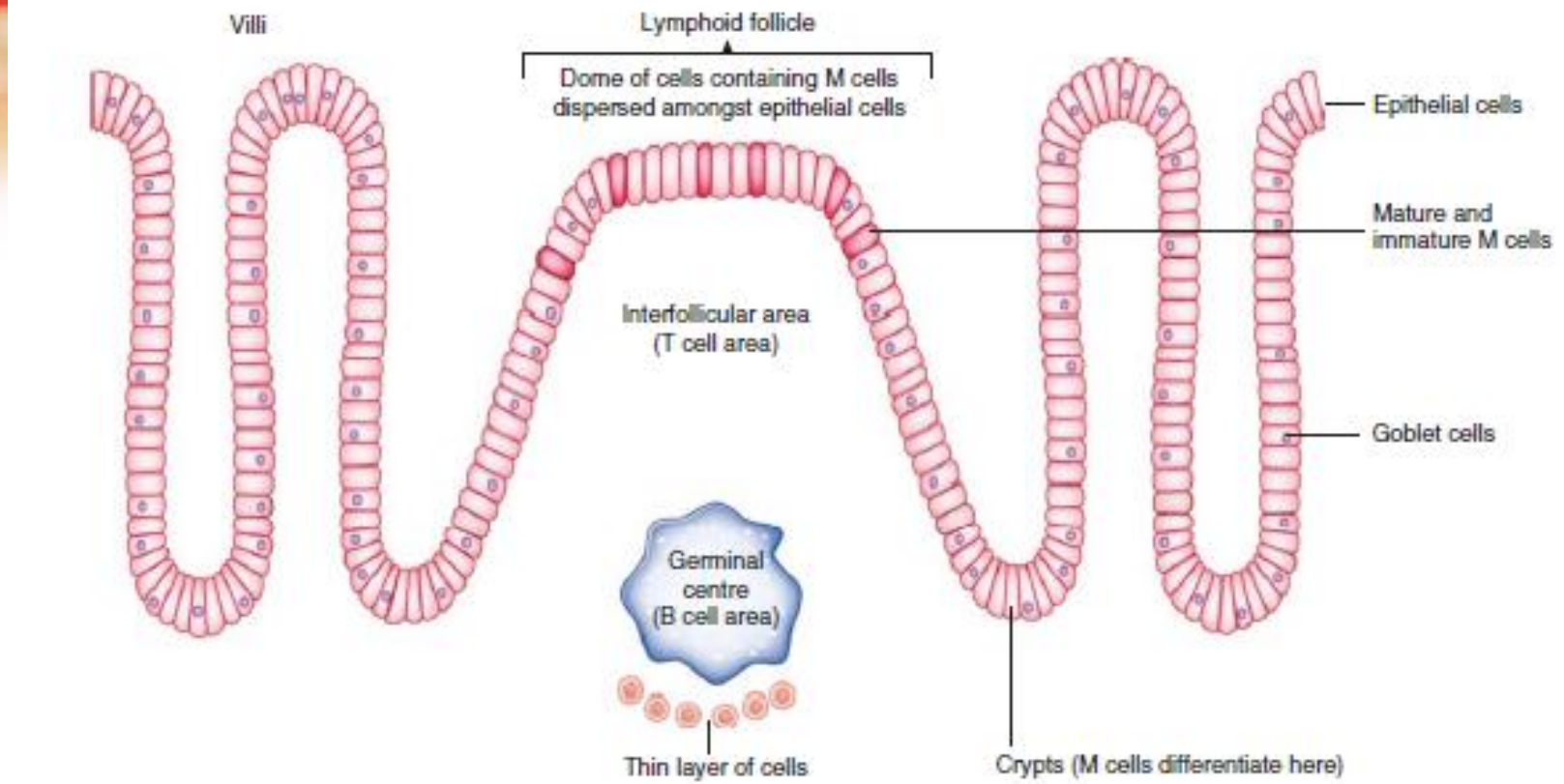

Figure 14.8 ■ Schematic diagram of the structure of intestinal Peyer's patches. M cells within the follicle-associated epithelium are enlarged for emphasis (from O'Hagan 1990)

- ✓ **These Peyer's patches are macroscopically identifiable follicular structures located in the wall of the GI tract.**

- ✓ Peyer's patches are overlaid with microfold (M) cells that separate the luminal contents from the lymphocytes. These M cells have little lysosomal degradation capacity and allow for antigen sampling by the underlying lymphocytes.

- ✓ Moreover, mucus producing goblet cell density is reduced over Peyer's patches. This reduces mucus production and facilitates access to the M cell surface for luminal contents.

- ✓ Attempts to improve antigen delivery via the Peyer's patches and to enhance the immune response are made by using **microspheres**, **liposomes** or modified live vectors, such as attenuated bacteria and viruses

C. Alternative Routes of Administration

Parenteral administration has disadvantages (needles, sterility, injection skills) compared to other possible routes. Therefore, systemic delivery of recombinant proteins by alternative routes of administration has been studied extensively. The nose, lungs, rectum, oral cavity, and skin have been selected as potential sites of application.

Route of administration
Oral
+ Easy to access, proven track record with "conventional" medicines, sustained/controlled release possible
– Negligible bioavailability for proteins
Nasal
+ Easily accessible, fast uptake, proven track record with a number of "conventional" medicines, probably lower proteolytic activity than in the GI tract, avoidance of first pass effect, spatial containment of absorption enhancers is possible
– Reproducibility (in particular under pathological conditions), safety (e.g., ciliary movement), negligible bioavailability for proteins
Pulmonary
+ Relatively easy to access, fast uptake, proven track record with "conventional" medicines, substantial –in the 10% range- fractions of insulin are absorbed, lower proteolytic activity than in the GI tract, avoidance of hepatic first pass effect
– Reproducibility (in particular under pathological conditions, smokers/nonsmokers), safety (e.g., immunogenicity), presence of macrophages in the lung with high affinity for particulates
Rectal
+ Easily accessible, partial avoidance of hepatic first pass, probably lower proteolytic activity than in the upper parts of the GI tract, spatial containment of absorption enhancers is possible, proven track record with a number of "conventional" drugs
– Negligible bioavailability for proteins
Buccal
+ Easily accessible, avoidance of hepatic first pass, probably lower proteolytic activity than in the lower parts of the GI tract, spatial containment of absorption enhancers is possible, option to remove formulation if necessary
– Negligible bioavailability of proteins, no proven track record yet
Transdermal
+ Easily accessible, avoidance of hepatic first pass effect, removal of formulation if necessary is possible, spatial containment of absorption enhancers, proven track record with "conventional" medicines, sustained/controlled release possible
– Negligible bioavailability of proteins
Intravitreal
+ Direct access to vitreous, delivery close to the target site
– Not suitable for systemic effects

+ Relative advantage, – Relative disadvantage

Table 5.7 ■ Alternative routes of administration to the IV, IM and SC route for biopharmaceuticals

Summary

- The nasal, buccal, rectal, and transdermal routes all have been shown to be of little clinical relevance if systemic action is required and if simple protein formulations without an absorption-enhancing technology are used.

- In general, bioavailability is too low and varies too much! The pulmonary route may be the exception to this rule.

- Absorption was strongly protein dependent, with no clear relationship with its molecular weight.

Molecule	*Mw* kDa	#AA	Absolute Bioavailability (%)
α-Interferon	20	165	>56
PTH-84	9	84	>20
PTH-34	4.2	34	40
Calcitonin (human)	3.4	32	17
Calcitonin (salmon)	3.4	32	17
Glucagons	3.4	29	<1
Somatostatin	3.1	28	<1

Adapted from Patton et al. (1994)

PTH recombinant human parathyroid hormone, *#AA* number of amino acids

Table 4.5 ■ Absolute bioavailability of a number of proteins (intratracheal vs. intravenous) in rats.

The first pulmonary insulin formulation was approved by FDA in January 2006 Uptake of insulin is faster than after a regular SC insulin injection (peak 5–60 minutes versus 60–180 minutes).

The fraction of insulin that is ultimately absorbed depends on:

(i) the fraction of the inhaled/nebulized dose that is actually leaving the device, (ii) the fraction that is actually deposited in the lung, and (iii) the fraction that is being absorbed

- i.e., total relative uptake (TO%) = % uptake from device × % deposited in the lungs × % actually absorbed from the lungs. For insulin, TO% is estimated to be about 10%.
- The reproducibility of the blood glucose response to inhaled insulin was equivalent to SC-injected insulin.

- Insulin absorption via the lung may be a promising route, but the fraction absorbed is small.

- Therefore, different approaches have been evaluated to increase bioavailability of the pulmonary and other non-parenteral routes of administration.

- The goal is to develop a system that temporarily decreases the absorption barrier resistance with minimum and acceptable safety concerns.

Approaches to enhance bioavailability of proteins

Classified according to proposed mechanism of action

1. Increase the permeability of the absorption barrier:

- Addition of fatty acids/phospholipids, bile salts, enamine derivatives of phyenylglycine, ester and ether type (non)-ionic detergents, saponins, salicylate derivatives of fusidic acid or glycyrrhizinic acid, or methylated β cyclodextrins
- Through iontophoresis
- By using liposomes: Soluble in both organic & aqueous media.

2. Decrease peptidase activity at the site of absorption and along the "absorption route": aportinin, bacitracin, soybean tyrosine inhibitor, boroleucin, borovaline.

3. Enhance resistance against degradation by modification of the molecular structure.

4. Prolongation of exposure time (e.g., bio-adhesion technologies).

Table 4.7 presents an example of the (apparently complex) relationship between nasal bioavailability of some peptide and protein drugs, their molecular weight, and the presence of the absorption enhancer glycocholate.

Molecule	#AA	Bioavailability (%)	
		Without	With glycocholate
Glucagon	29	<1	70–90
Calcitonin	32	<1	15–20
Insulin	51	<1	10–30
Met-hGH[a]	191	<1	7–8

Adapted from Zhou and Li Wan Po (1991b)

[a]See also Chap. 14

Table 4.7 ■ Effect of glycocholate (absorption enhancer) and molecular weight of some proteins and peptides on nasal bioavailability.

Pharmaceutical Biotechnology

Gene therapy- General Concepts

-Lecture VII-

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Gene therapy---importance

- ✓The genomic DNA of any organisms is constantly under the influence of various intrinsic and extrinsic agents.
- ✓These agents sometimes lead to the generation of thousands of genetic mutations.
- ✓Repairing, turning-off or replacing dysfunctional genes with exogenous DNA act as a novel approach to treat, cure, or ultimately prevent disease by changing (the expression) a person's genes.
- ✓The insertion, alteration, or removal of genetic material to treat a disease or to improve and manage the clinical status of a patient is commonly known as gene therapy.
- ✓gene therapy is the use of a DNA vector as a pharmaceutical agent to treat various diseases

- The first approved gene therapy case in the United States took place on September 14, 1990 at the National Institute of Health (NIH). The patient was a 4-yearold girl with severe combined immunodeficiency (SCID) disease caused by a defect adenosine deaminase (ADA) gene. In the therapy procedure, the medical group isolated patient's T lymphocytes through aphaeresis, exposed these cells ex vivo to a genetically engineered live nonvirulent retrovirus carrying the normal ADA gene, and transfused these genetically modified T-cells back into the patient's bloodstream. The treatment was successful.

Approaches in Gene therapy

- **In vivo gene therapy:** direct delivery of genes into the cells of a particular tissue in the body, usually with blood cells (lymphocytes or blood stem cells) for diseases affecting the hematopoietic system

- ***Ex vivo* gene therapy:** transfer of genes to cultured cells & reinsertion.
  Ex: Oncolytic adenoviruses for the treatment of cancer

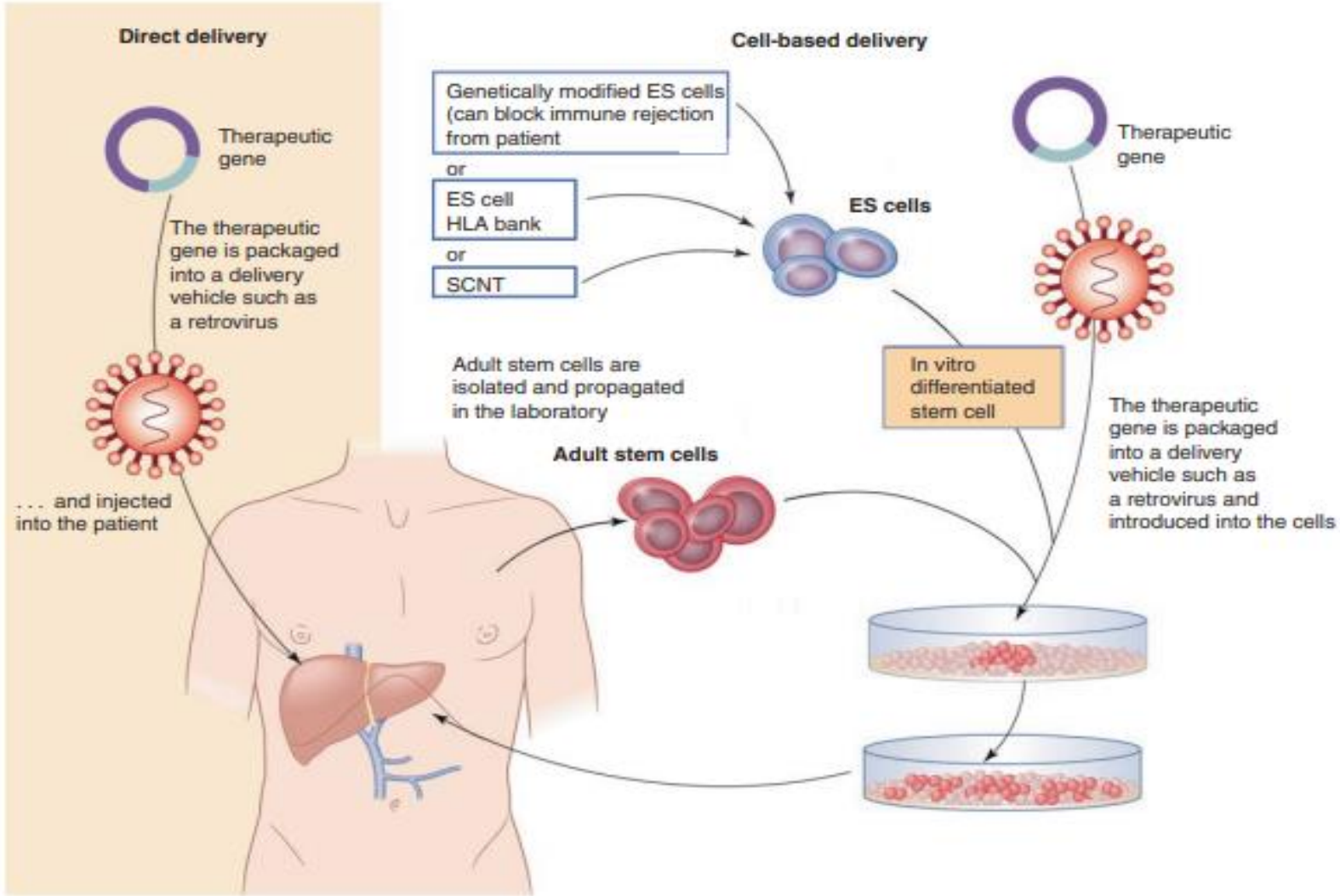


Figure 16.1 ■ Methods of administration of gene therapy vectors. In vivo gene transfer involves direct administration of the vector in the tissue of interest. Ex vivo gene transfer requires the collection of cellular targets from the patient. The cells are treated in culture with the vector. Cells expressing the therapeutic transgene are harvested and given back to the patient. *ES* Embryonic Stem Cell, *SCNT* somatic cell nuclear transfer (strategy). From Zwaka 2006; with permission to reprint (also cf. Chap. 17)

Vectors in gene therapy

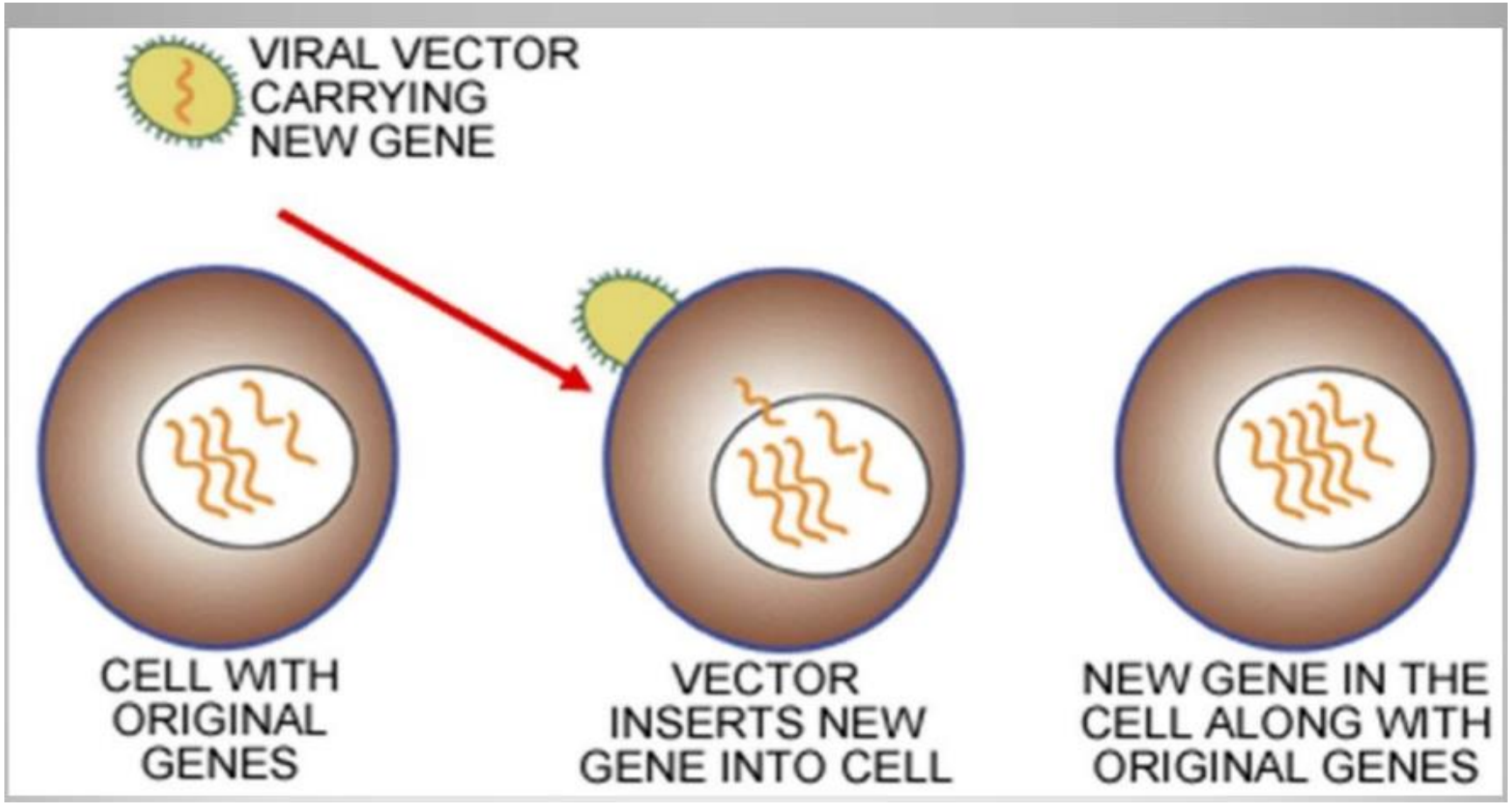
✓To transfer the desired gene into a target cells, a carrier is required. Such vehicle of gene deliver are known as vectors.

✓Two main classes:

- Viral vectors
- Non-viral vectors(nacked DNA plasmid, physical & chemical methods).

Viral vectors

- Viruses bind to their host and introduce their genetic material.
- Plausible strategy for gene therapy by removing the viral DNA and using the virus as a vehicle to deliver the therapeutic DNA.
- The viruses used are altered to make them safe, although some risks still exist with gene therapy.
- GT viruses: Retroviruses OR Adenoviruses
- Adenoviruses with DNA genome------good vector----e.g. Common Cold adenovirus.
- Deliver of genes by a virus is termed transduction and the infected cells are described as transduced.


VIRAL VECTOR
CARRYING
NEW GENE
CELL WITH
ORIGINAL
GENES
VECTOR
INSERTS NEW
GENE INTO CELL
NEW GENE IN THE
CELL ALONG WITH
ORIGINAL GENES

BASIC COMPONENTS OF PLASMIDS(Nacked DNA)

- A plasmid is a circular, double-stranded DNA molecule, which contains a complementary DNA (cDNA) sequence coding for the therapeutic gene. In addition, it also contains several other genetic elements including bacterial elements, transcription regulatory elements, multiple cloning sites (MCS), untranslated regions (UTR), introns, polyadenylation (polyA) sequences, and fusion tags, all of which have great impact on the functioning of the final genetic products. After constructing a plasmid, certain screening methods are needed to validate the construct. For example, DNA sequencing, polymerase chain reactions (PCR), restriction digestions, agarose gel electrophoresis, SouthernWestern blots and enzyme-linked immunosorbent assays (ELISA) are a few useful tools to validate the structure and function of the construct

➢ Other non biological methods

- physical (carrier free gene delivery) or Chemical approaches (Synthetic vector based gene delivery).
- Physical approach include

➢Needle injection

➢Electroporation

➢Gene gun

➢Ultrasound

➢Hydrodynamic delivery

Employ a physical force that permeates the cell membrane and facilitates intracellular gene transfer.

➢Chemical approaches **(Lipoplexes & Polyplexes)**

✓**DNA entry must be protected from damage.**

✓**Plasmid DNA can be covered with lipids.**

✓**Three types of lipids :**

✓**Anionic (negatively charged)**

✓**Cationic (positively charged)**

✓**Neutral**

❑ **Complexes of polymers with DNA are called polyplexes(especially cationic polymers)**

Common used of lipoplexes

- ✓In gene transfer into cancer cells, where the supplied genes have activated tumor suppressor control genes in the cell.
- ✓Decrease the activity of oncogenes.
- ✓Treatment of genetic respiratory disease such as cystic fibrosis.