KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY, KUMASI
COLLEGE OF SCIENCE
DEPARTMENT OF BIOCHEMISTRY AND BIOTECHNOLOGY

Course: BCHEM 365
Biophysics
First Semester, January to May 2022
Credits: 3

Instructor: Prof. (Mrs.) Antonia Y. Tetteh
Department of Biochemistry and Biotechnology
Office: GF 9 Aboagye-Menyeh Building
Phone: (032)206-0298
Cell: (020)113-4416
(054)771-3575
Email: atetteh.sci@knust.edu.gh
aytetteh@gmail.com
Office hours: Friday 8:00 – 10:00 am or contact me to set up an appointment

Dr. S.P. Salifu
Office: Department of Biochemistry and Biotechnology
Phone: (020)958-3359

TA: Ben Diawuo
Office: GF9 Aboagye-Menyeh Building
Phone: (059)647-9012

Class Period and Location: Monday 13:00 -14:55 pm, SCB-FF1; Tuesday 13:00 -13:55, SCB- FF1

Course Objectives

During this course we will explore the physical properties of macromolecules of the cell and study the techniques used to investigate their properties, functions, and modifications. Knowledge of these macromolecules would determine their utilities in medical, industrial, pharmaceutical, and agricultural applications. The principles of electrophoresis, microscopy and radiobiology and their applications will be addressed.

Student Learning Outcomes

By the end of this course, the student will be able to:

1. Describe the physical structure of nucleic acids and proteins
2. Explain how the physical structure of these macromolecules determines the technique for their isolation, purification, characterization and modification
3. Explain how researchers use these techniques to elucidate function, mechanism and manipulation of macromolecules to produce products of importance to health, agriculture, and environment

4. Apply critical thinking to problem solving and decision-making in the use of these techniques
5. Examine scholarly articles and appreciate writing style of articles in the use of electrophoresis, microscopy and radiotracer techniques
6. Obtain understanding of macromolecule structure and physical properties
7. Obtain practical understanding of the use of the physical characteristics of macromolecules in their isolation, characterization and elucidating their functions
8. Learn the basic biophysical techniques used in molecular biology and biochemistry
9. Appreciate the writing style of research articles related to biophysics

Reading List
Pattabhi, V. and Gautham, N. (2002). Biophysics. Kluwer Academic Publishers, London.

Course Format
Class will be conducted in lecture format and reading/discussion of scientific research articles, and evidence of learning. All students are expected to be prepared for each class through careful reading of the assigned materials. There will be examination at the end of every four weeks.

Access to Class Notes
All class files will be posted at vclass.knust.edu.gh. Files include lecture notes, homeworks, and articles.

Prerequisites: none
Required text:

Attendance Policy
You are strongly urged to attend all lectures. Attendance will be taken daily.

Grading

2 exams at 10 % each	20%
Mid-Semester exam	10%
Final exam	70%
Total	100%

A	70- 100
B	60-69
C	50-59
D	40-49
F	<40

Exams
Each exam will cover approximately ⅓ of the material covered in the course. See the tentative schedule for dates and material covered on each exam. Exams will cover material from lecture including slides and handouts. Exams will be multiple choice questions, matching questions, and short essays. Missed exam will be recorded as “0”.

Academic Integrity
University regulations require that Instructors remind students that the code of student conduct defines a university policy on academic integrity already pledged by each student at the time of your acceptance

of offer of admission to the university. Guidelines set forth in the KNUST Policy on Academic Integrity will be strictly followed. These can be obtained in the student handbook. Plagiarism, cheating and helping others if independent work is requested constitute a violation of the code of conduct. Plagiarism is unethical and will affect your grade. When reading scientific material, take notes. Then take notes on your notes before composing your own sentences. This process will help you avoid copying someone's writing *verbatim*. Anytime you paraphrase a reference, you are required to cite that reference in your bibliography.

Course Outline

Mon Feb 28	Lecture 1- Review of syllabus; Introduction: Importance of biophysical techniques in the study of the biomolecules, nucleic acids and proteins as well as membranes
Tue Mar 01	Lecture 2- Physical and chemical structure of DNA; Alternative forms of DNA structures; Circular and Superhelical DNA.
Mon Mar 07	Lecture 3- Physical and chemical structure of RNA, proteins
Tue Mar 08	Lecture 4- Physical and chemical structure of proteins: primary, secondary and tertiary structures.
Mon Mar 14	Lecture 5- Theory and methods of electrophoresis. Native and denaturing Gel Electrophoresis; Electrophoresis of nucleic acids. Agarose gel electrophoresis.
Tue Mar 15	Lecture 6- Electrophoresis of Proteins on SDS polyacrylamide gel electrophoresis and their applications
Mon Mar 21	Lecture 7- Isoelectric Focusing (IEF); Two dimensional Gel Electrophoresis; Protein detection techniques
Tue Mar 22	Lecture 8- Electrophoresis of nucleic acids: RFLP, Southern blotting, Northern blotting and Western blotting. Electrophoresis of nucleic acids:
Mon Mar 28	Lecture 9- Genetic fingerprinting, Chain Termination sequencing, Maxam-Gilbert sequencing; Pulsed Field Gel Electrophoresis (PFGE).
Tue Mar 29	Lecture 10 - Characterization of nucleic acids by Affinity Electrophoresis. **Exam #1**
Mon Apr 04	Lecture 11 - Applications of protein electrophoresis: serum protein electrophoresis; cardiac markers in myocardial infarction, electrophoresis in detection of sickle-cell anemia.
Tue Apr 05	Lecture 12 - Characterization of proteins by Capillary Electrophoresis. Recap on Electrophoresis
Mon Apr 11	Lecture 13- Important parameters in Microscopy. Magnification; Resolving power; Contrast. Phase Contrast Microscopy; DIC Microscopy
Tue Apr 12	Lecture 14- Fluorescence Microscopy. Confocal Microscopy; Electron Microscopy: Transmission Electron Microscopy
Mon Apr 18	Lecture 15 - Scanning Electron Microscopy; Practical Microscopy-Stereomicroscopy
Tue Apr 19	Lecture 16 - Radioisotope techniques: Origin and properties of radioactivity; types of radiation; artificial radioactivity; Atomic Transmutation, Kinetics of radioactive decay; Detection and measurement of Radioactivity
Mon Apr 25	Lecture 17 - Labeled tracers: Autoradiography; Phosphorimaging; Liquid Scintillation Counting - technique and application; Non-radioactive tracers
Tue Apr 26	**Mid-Semester Exams**

Mon May 02	Lecture 18 - Imaging Techniques; Isotope Dilution; Radiotracer Techniques in Metabolic studies; Autoradiography; Advantages and Restrictions of Radiotracer Techniques
Tue May 03	Lecture 19- Applications of radiotracer techniques in Biological Sciences Biological effects of Nuclear Radiation; Radiation Dosimetry; Activation Analysis; Safe Handling of Radioisotopes; Disposal of Radioactive Wastes
Mon May 09	Lecture 20 - Introduction to nanotechnology

Last day of class

Final exam: May 2022

General Information

1. There are no extra expenses associated with this course
2. Students are asked to turn cell phones and pagers off during lectures
3. Grades are determined as outlined above. Grades will not be curved.
4. Exams are to be exclusively the student's own work. Students are encouraged to ask questions during class and participate in class discussions; however, students should refrain from extraneous conversations with each other when the instructor or TA is presenting information. Talking at inappropriate times violates academic integrity since you are interfering with another student's desire to hear and see what is going on in class.
5. An 'I' (Incomplete) grade will be given for work not completed because of serious interruption in a student's work, which is not caused by his/her own negligence. This must be supported by a documented serious event. An 'I' grade must be made up for during the Supplementary Examination.

BCHEM 365
Lecture 1
February 28, 2022

What is Biophysics?

- Biophysics: An interdisciplinary science- the physics of biology
- What is physics? - the study of matter and energy
- The universe exists in two forms: matter (the tangible) and energy (the intangible, yet exists, such as heat, light, radiation, force, etc.)
- Biophysics makes use of the principles, theories, and methods of physics to study and understand biological systems, specifically, (*i*) their composition at all levels, from atoms, molecules, cells, tissues, to organisms in their environment –hence covers molecular level to whole organisms and ecosystems; (*ii*) physical properties and behavior

What is Biophysics?

- In biophysics, four to five areas of study merge: physics, chemistry, biology, and mathematics

- Molecular biophysics addresses questions in biochemistry and molecular biology such as ‘’How do atoms join to make DNA and proteins?’. How do proteins work? How are variations in genes and proteins expressed in disease occurrence, disease control, and drug design?

What is biophysics

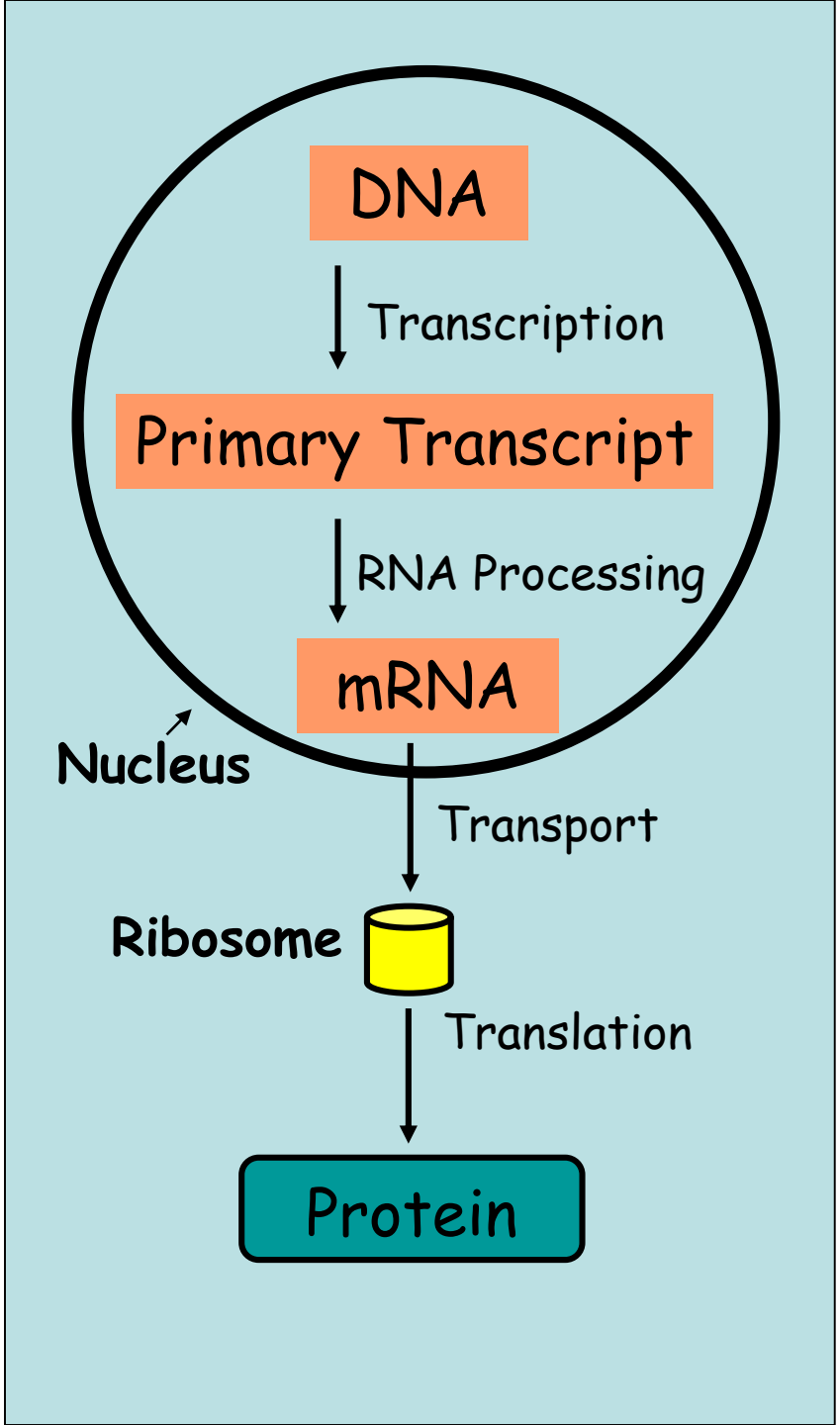
- Study of biophysics leads to understanding of the interactions between **macromolecules (**nucleic acids, proteins), regulation of protein biosynthesis, and membrane structure and function
- Biophysics explains how complex systems in our bodies work—the brain, circulation, immune system, and others
- Biophysics has contributed to many therapeutic techniques and devices such kidney dialysis, radiation therapy, cardiac defibrillators, pacemakers, and artificial heart valves.
-

What is biophysics?

- Through biophysics, the structure of DNA was elucidated
- Biophysical inventions were used to decode human genes in the Human Genome Project of the 2000s.
- Many genes have been sequenced through biophysics, as well as their relationships in organisms
- Other applications of biophysics include- preparation of vaccines, development of tools for understanding diseases such as cancer
- Biophysics creates tools for medical imaging technologies including PET scans, MRI, CT scans, and sonograms for disease diagnosis

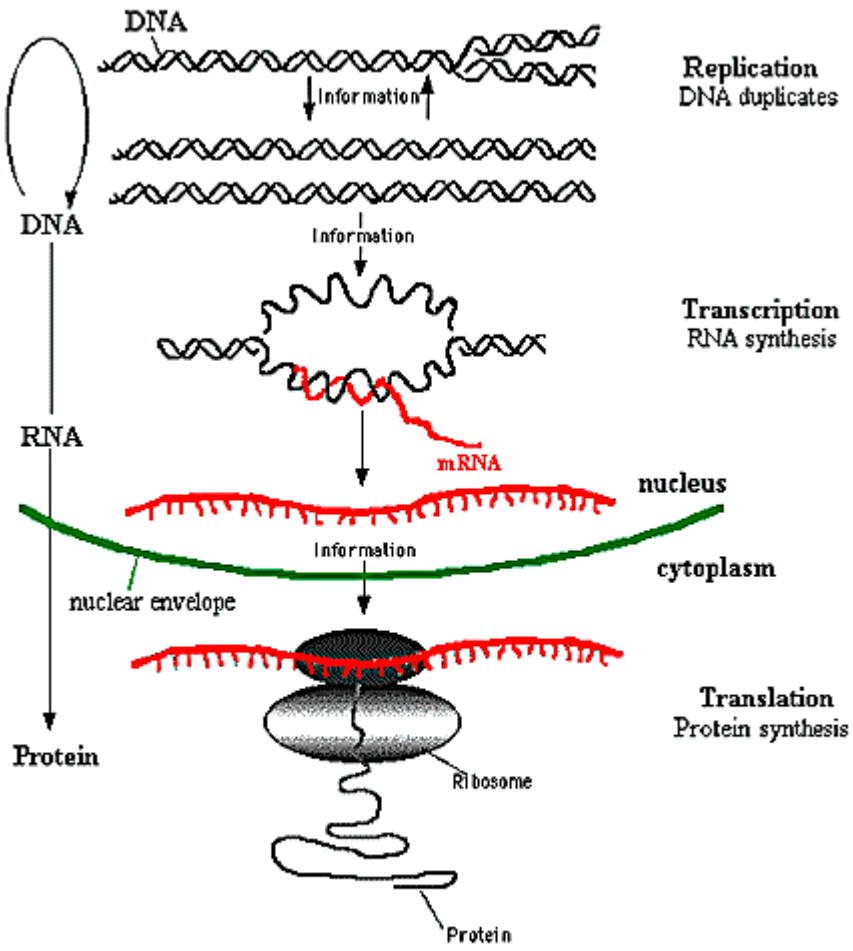
What is biophysics?

- The crises of the 21st century require innovations and better understanding of physics, chemistry and math to provide solutions
- Food security, climate change, water resources, new and emerging diseases can be surmounted through biophysics
- Biophysics researchers are exploring bioenergy and bioeletricity as alternatives over petroleum and coal



The fundamental dogma of molecular genetics

The Central Dogma of Molecular Biology



The Central Dogma of Molecular Biology

Macromolecules

- Are polymers present in a cell. MW ranges from 10^4 to 10^{12} Daltons
- Made up of proteins, glycoproteins, nucleic acids
- Determine a cell's shape, size and function
- Knowledge of the properties of macromolecules contributes to understanding of living processes, and their manipulation for the production of useful products that find use as diagnostics, therapeutic agents, degradation of wastes, etc.

Identification of macromolecules and Investigation into their structure and function

- Molecular biologists routinely carry out
  - Identification of genes and their location
  - Investigation of structure and function of genes and gene products
  - Perform molecular separations
  - Locate the position and movement of macromolecules in the cell
  - Modification of macromolecules for specific functions
  - Design macromolecules for many purposes

- The most popular techniques used:
  - Gel electrophoresis
  - Radiolabeled tracers
  - Nucleic acid hybridization

Common techniques

- Gel electrophoresis was used to provide evidence of DNA as the genetic material
- Gel electrophoresis is used to separate different nucleic acids or proteins
- Molecular biologists and biochemists use labeled (radioactive) tracers to detect tiny quantities of substances (e.g. to measure the appearance of a transcript during gene expression). The radiotracer techniques to cover in this module include:
  - Autoradiography
  - Phosphor imaging
  - Liquid scintillation counting

Common techniques

- Nucleic acid hybridization combines electrophoresis and labeled probes to identify specific DNA or map and quantify transcripts. Methods include
  - Southern blots
  - Northern blots
  - DNA fingerprinting/profiling/typing/testing
  - DNA sequencing
  - Restriction mapping
  - S1 mapping

Common techniques

- For detecting protein and DNA-protein interactions, a combination of electrophoresis and labeling with antibodies or radioactive material is used in
  - Immunoblotting (Western blotting)
  - Gel mobility shifts
  - DNase footprinting
  - Microscopy

What is the nature of genetic material?

Griffith's experiment- 1928

- Frederick Griffith laid the foundation for identification of DNA as genetic material
- In his experiment, he performed transformation in the bacterium *Pneumococcus* (*Streptococcus pneumoniae*). Two strains occur: wild and mutant
- The wild type is spherical, surrounded by a mucous coat- **capsule**
- Cells of the wild type form large glistening colonies characterized as Type III **smooth** (Type III-**S**)
- This wild type is **virulent**

Wild = spherical, capsule around it, large, smooth and glistening colonies, virulent

Griffith's experiment

- A **mutant** strain was identified
- Had no protective coat
- Cells formed small, rough colonies, Type II (**R**) -(Type II-**R**)
- Cells were **avirulent** because no protective coat. Why?
- Because host's white blood cells engulf them, and without the protective coat, are destroyed before they can multiply to cause disease
- He found that heat-killed virulent colonies alone could not cause disease
- However, heat-killed virulent **S** strain mixed together with live avirulent **R** strain caused disease

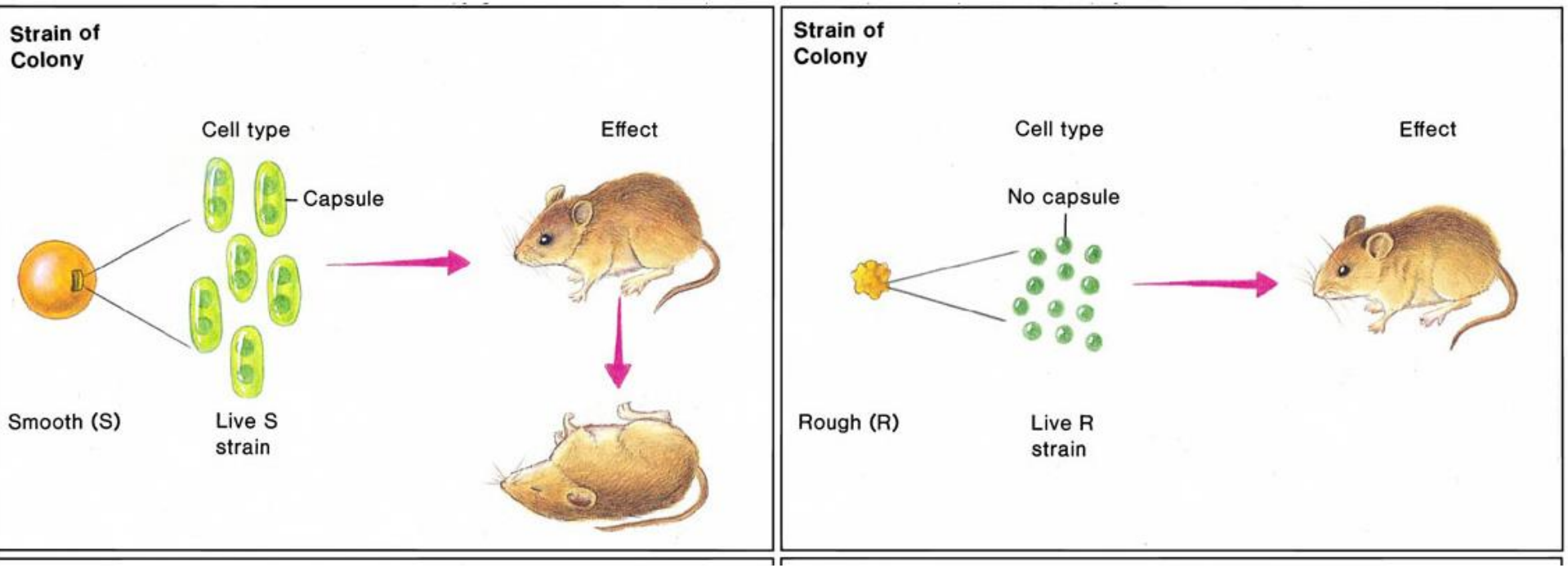
Mutant= no capsule, small and rough colonies, avirulent

Griffith's interpretation and conclusion

- Somehow, the virulent trait passed from dead, virulent cells to the live, avirulent cells, leading to transformation
- The transformation of avirulence to virulence in Type II-R was not short-lived but was passed on to their descendants as a heritable trait
- The mechanism of transfer, now termed horizontal gene transfer (HGT) is still not understood though similar mechanisms have also been discovered.
- In HGT, living bacterial cells which are competent (ability to pick up naked DNA) pick up foreign DNA across their cell membrane and incorporate it into their own genome by genetic recombination, hence, a transformation.
- Meaning the **transforming substance** in the heat-killed bacteria was probably the gene for virulence

1928

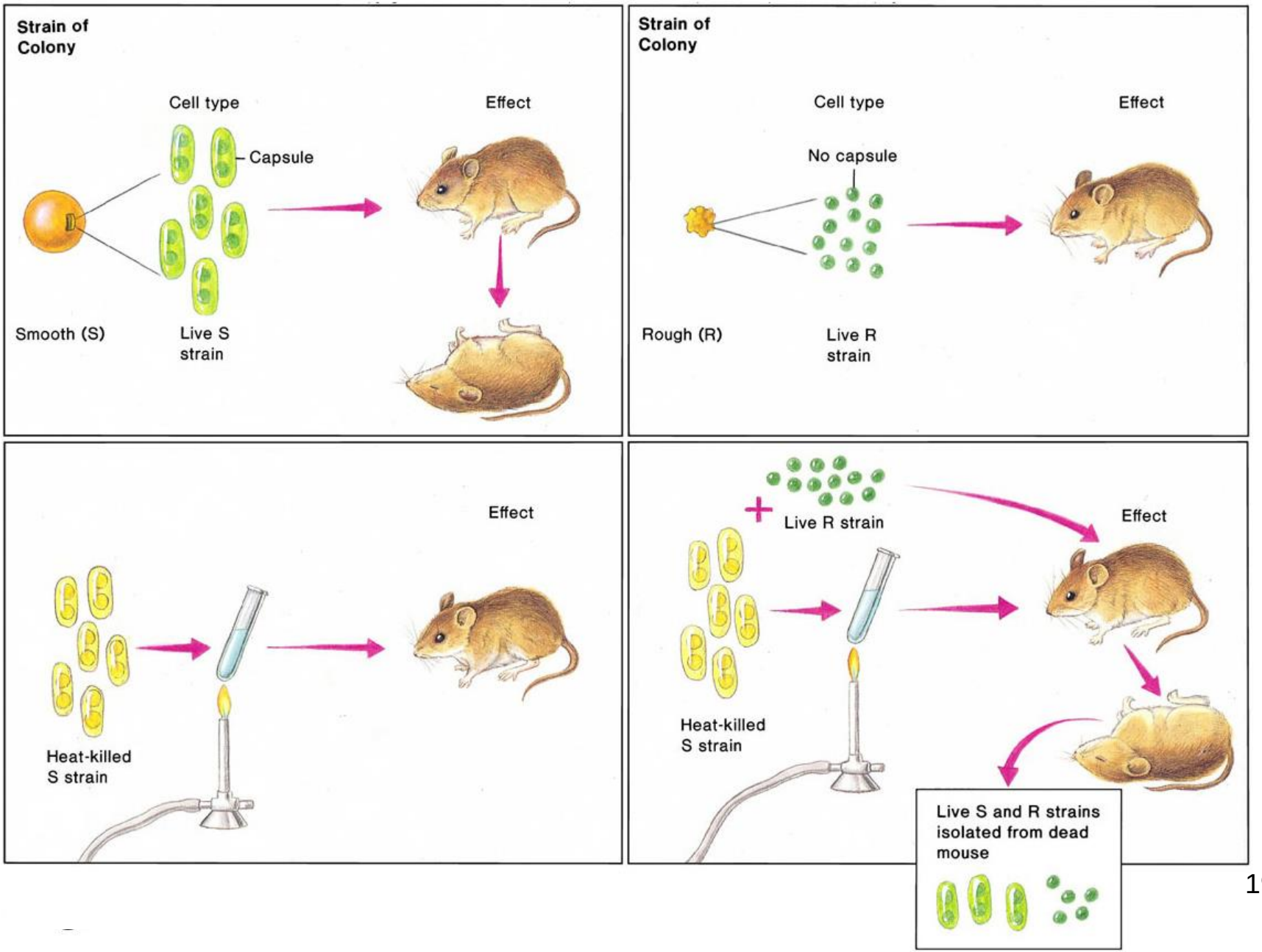
Griffith's transformation experiments



S = smooth colonies (virulent)
Contain protective coat

R = rough colonies (avirulent)
No protective coat
Engulfed by white blood cells
Cannot cause disease

Griffith's transformation experiments



1928 Frederick Griffith

Conclusion: Molecules that carry heritable information are present in the S strain cells, likewise the R strain carried material that could store the heritable material received from the S strain

Neither live Type II-R strain nor heat-killed Type III-S strain alone could cause the disease and kill the mice

The Type III-S strain contained some material that could transform the Type II-R strain bacteria

What is this transforming material?

Information missing here is the chemical nature of the transforming substance

Oswald Avery, Colin MacLeod, Maclyn McCarty Experiment - 1944

- The researchers demonstrate that the transformation of *Streptococcus pneumoniae* from avirulent Type II-R to a virulent Type II-S is the result of the transfer of DNA from dead Type II-S organisms to live Type II-R ones organisms.

- At that time it was believed that protein was the genetic material

- How did they do this?
- 1. They prepared serum from infected mice and heat-killed the bacteria in it
- 2. Removed protein from extract with the organic solvent chloroform, mixed it with live **R** strain and infected healthy mice with it. Mice got diseased. Extract from diseased mice shows **S** + **R** strain. Result: transformation occurred
- 3. They treated extract with trypsin and chymotrypsin to destroy proteins; repeated steps as before
- Result: transformation occurred
-

Oswald Avery, Colin MacLeod, Maclyn McCarty Experiment - 1944

- 4. They treated extract with RNase, repeated steps as before. Result: transformation occurred

  Interpretation

  These steps ruled out RNA and protein as transforming material
- 5. Treated extract with deoxyribonuclease (DNase); repeated steps as before. Extract from diseased mice shows only R avirulent strain.
- Result: No transformation occurred. Transformation ability destroyed by DNase. Transforming material may be DNA

Direct evidence that transforming substance is DNA

1. Ultracentrifugation –sedimented rapidly
   means transforming material has
   high molecular weight
2. Electrophoresis – showed high mobility (i.e., moved fast
   down the gel under electric current),
   meaning the transforming principle has
   high charge-to-mass ratio
3. UV light absorption spectrum– maximum at 260 nm

4. Elemental chemical analysis – nitrogen to phosphorus ratio of
   1.67 (expected for pure DNA)
   Protein: rich in nitrogen and low in phosphorus (would have a
   higher ratio)
   Even a slight contamination with protein would have raised the
   ratio

Conclusion: DNA is the transforming material

Today, we know that the "transforming principle" Griffith observed was
the DNA of the Type III-S strain

Hershey-Chase Experiment provided evidence that genes were made of DNA

1952 Hershey-Chase Experiment

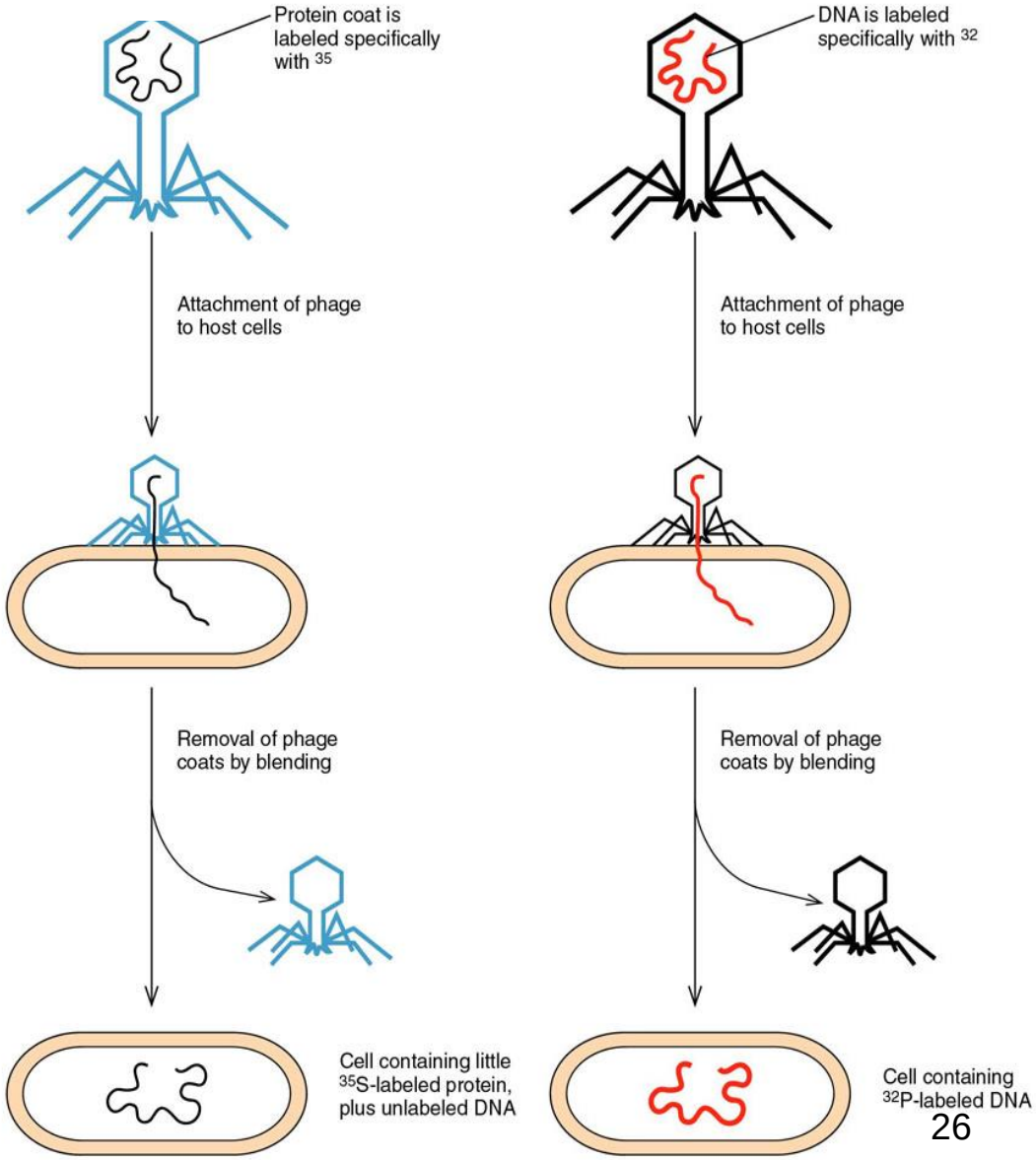
- A.D. Hershey and Martha Chase used the virus, bacteriophage T2 to infect *E. coli*
- During infection, phage genes enter host cells and direct the synthesis of new phage particles
- Phage made up of DNA surrounded by a protein coat
- First part: they labeled the phage protein with ^{35}S (blue) leaving the DNA unlabeled (black)
- Second part: labeled phage DNA with ^{32}P (red), leaving the protein unlabeled (black)

1952 Hershey-Chase Experiment

Known: that phage infects bacteria

Do the genes reside in protein or DNA?

E. coli



1952 Hershey-Chase Experiment

- Since phage genes must enter the cell, the type of label found within the infected cell would indicate the nature of the genes
- In (a) most of the labeled protein remained on the outside
- In (b) most of the labeled DNA entered the infected cells

- Conclusion: genes of this phage are made of DNA

Chemical nature of polynucleotides

DNA consists of

Nitrogenous bases
- adenine (A)
- cytosine (C)
- guanine (G)
- thymine (T)

Phosphoric acid

Sugar: deoxyribose

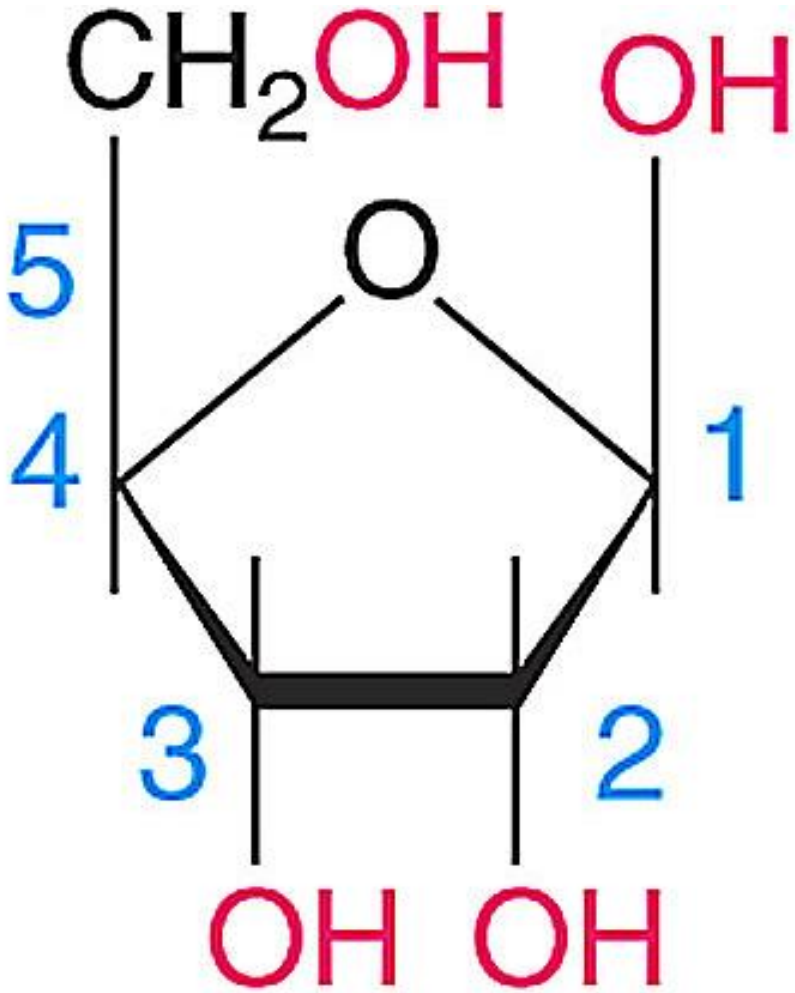
RNA consists of

Nitrogenous bases
- adenine (A)
- cytosine (C)
- guanine (G)
- uracil (U)

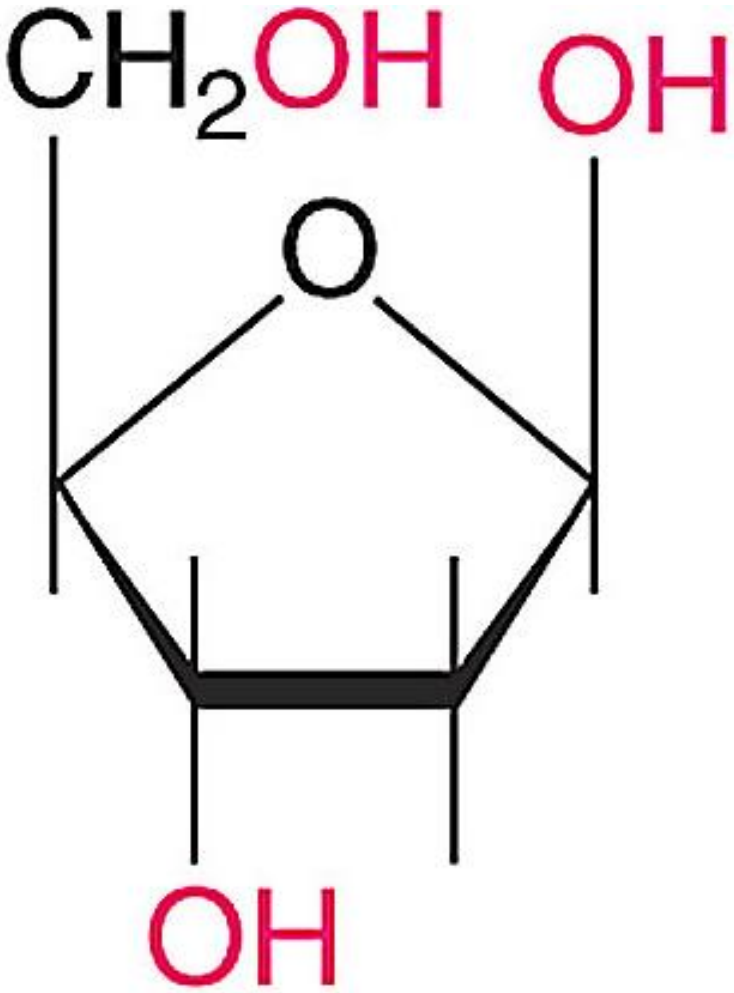
Phosphoric acid

Sugar: ribose

Sugars of nucleic acids

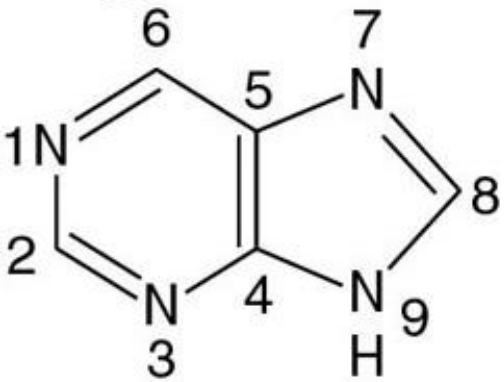


Ribose



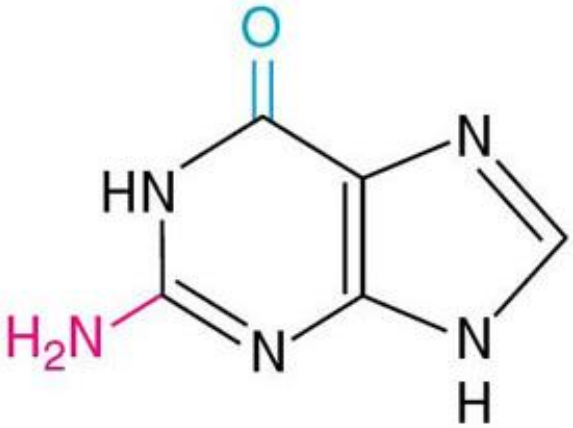
2-deoxyribose

Bases

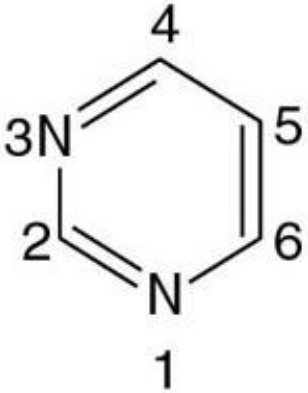


Purine

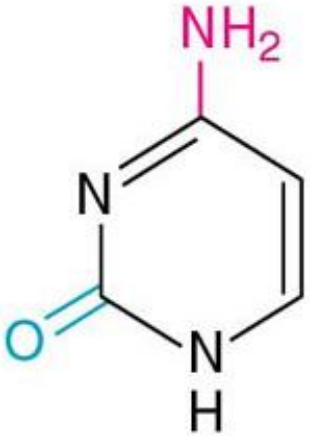
Adenine



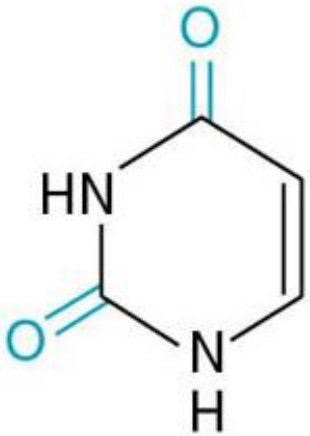
Guanine



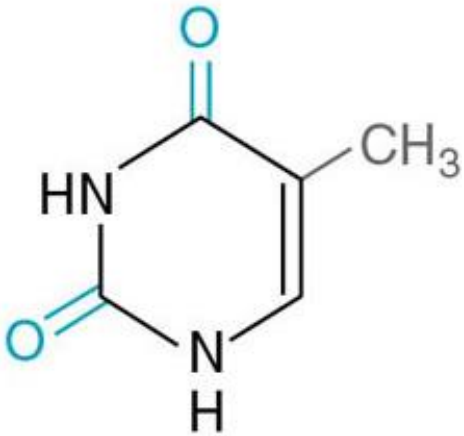
Pyrimidine



Cytosine



Uracil



Thymine

Nomenclature

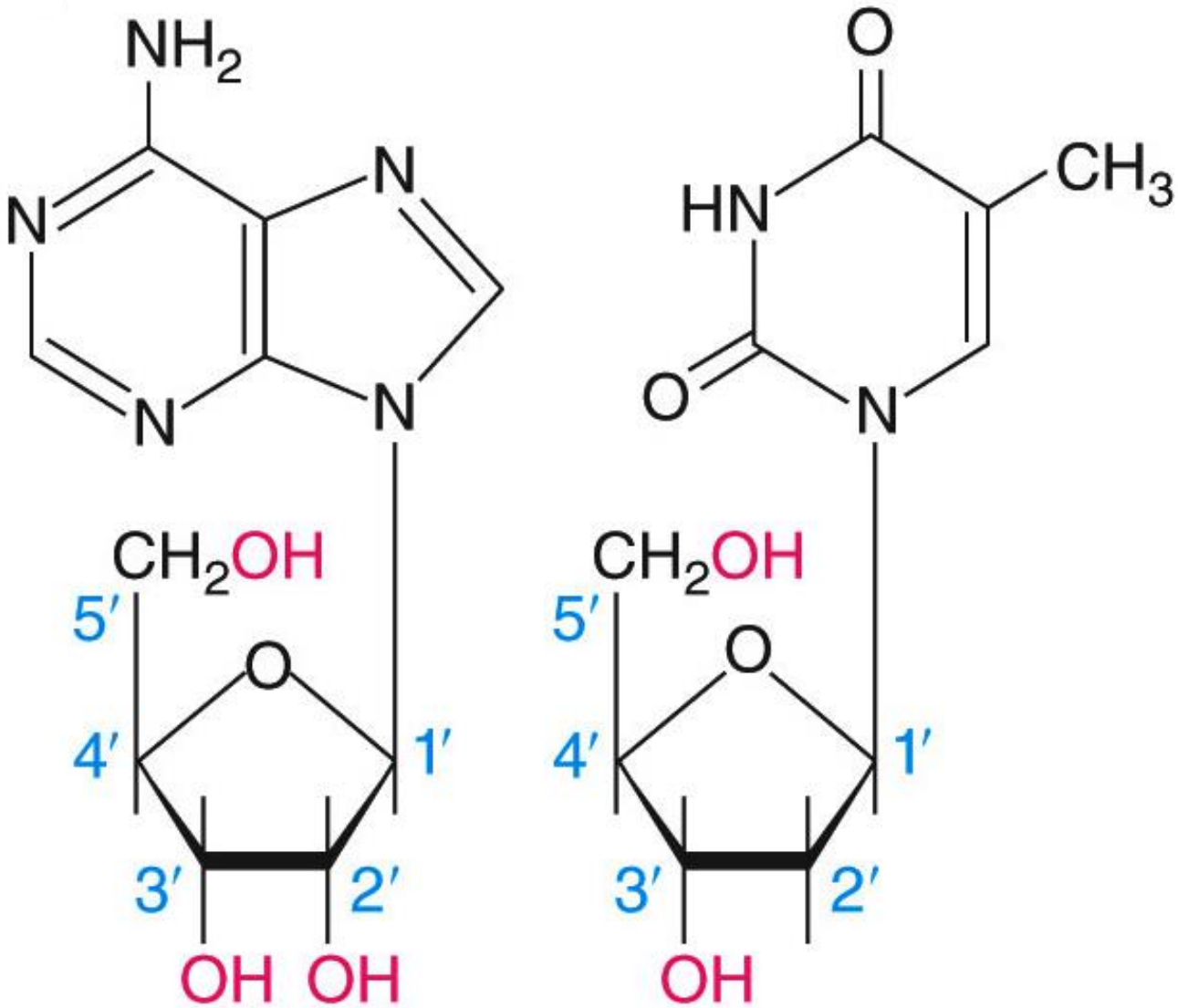
bases

(no sugar or phosphate)

nucleosides = sugar + base

nucleotides = sugar + phosphate group + base

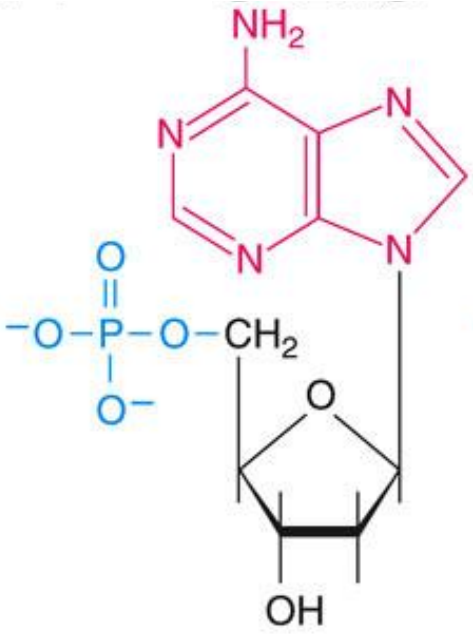
Nucleosides



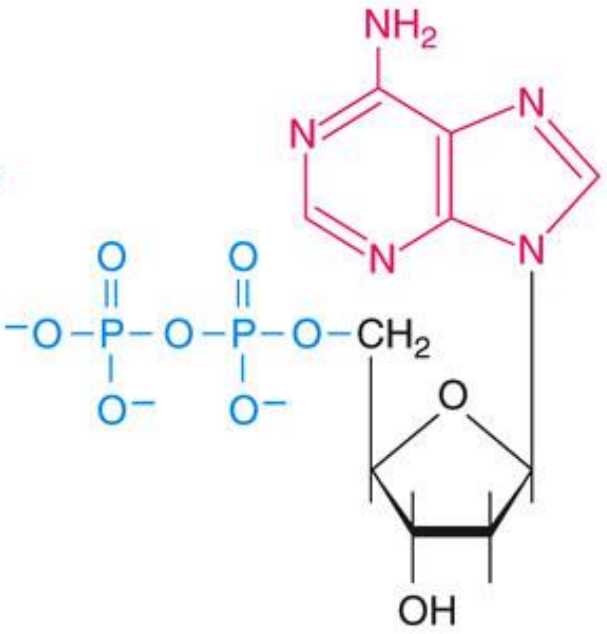
Adenosine

2′ -Deoxythymidine

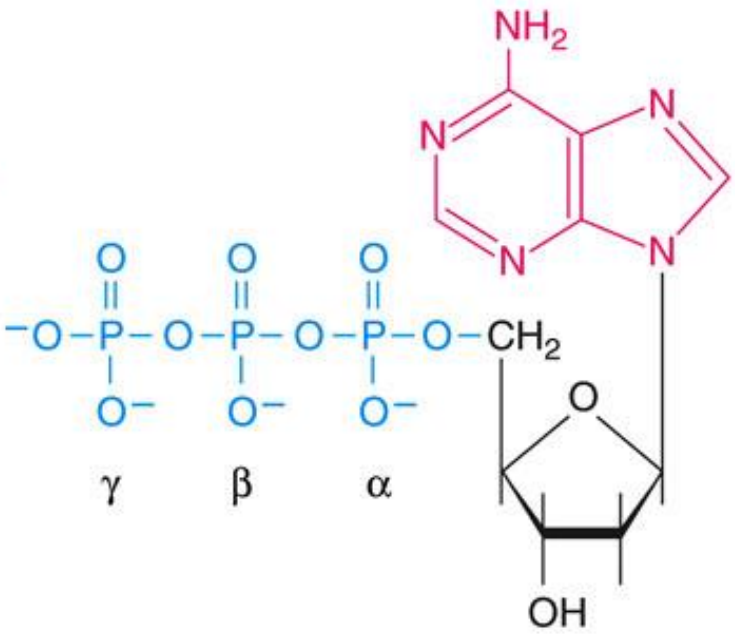
Nucleotides



Deoxyadenosine-5′-monophosphate (dAMP)

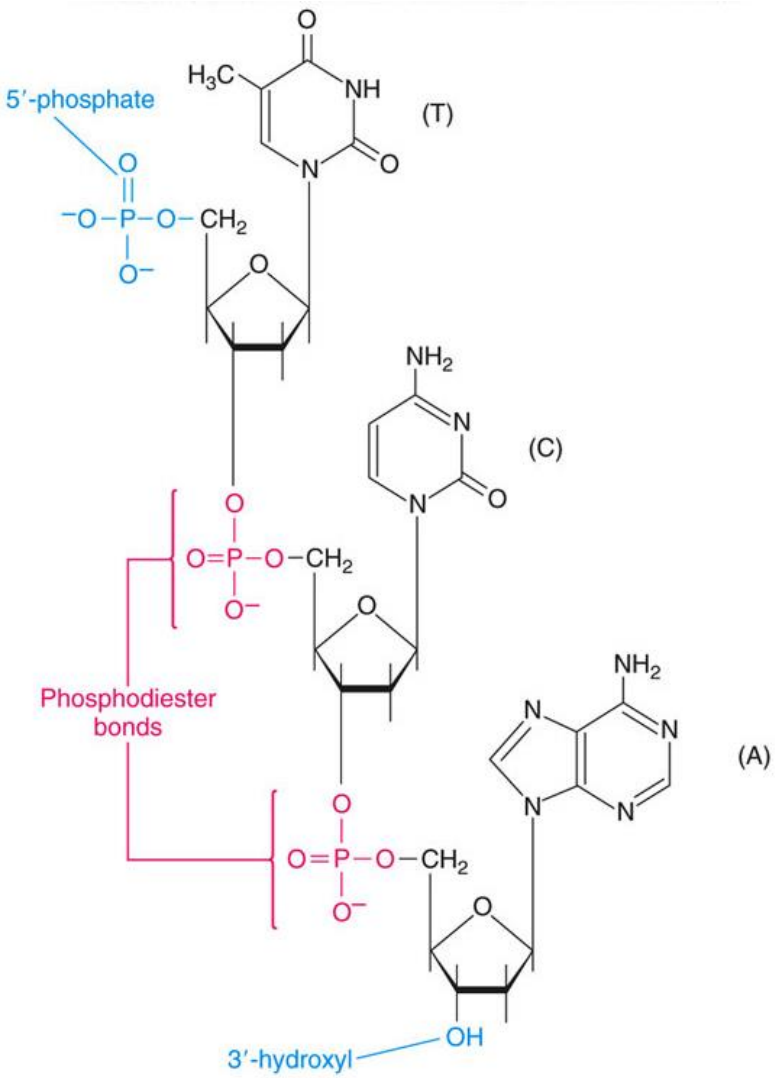


Deoxyadenosine-5′-diphosphate (dADP)



Deoxyadenosine-5′-triphosphate (dATP)

A trinucleotide

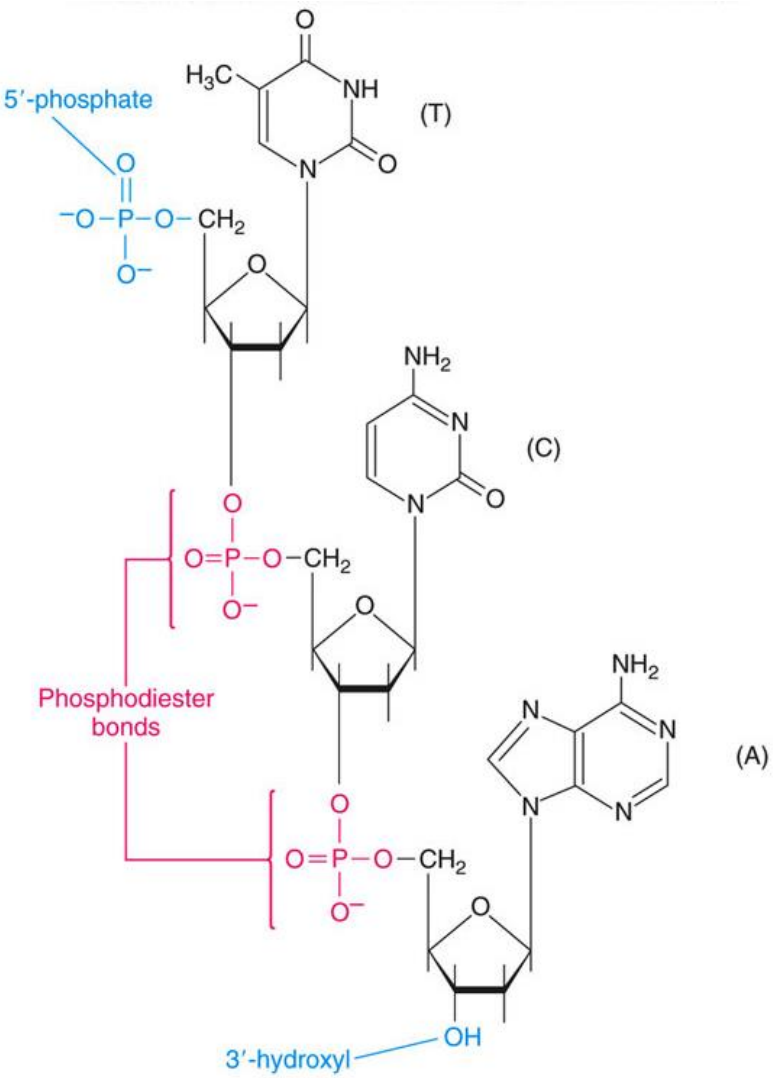


BCHEM 365
Lecture 2
Mar 01, 2022

Chemical composition of DNA and RNA

- DNA and RNA are chain-like molecules
- The basic subunit of DNA and RNA is a nucleotide
- A nucleotide contains a base linked to the 1’- position of a sugar and a phosphate
- The phosphate joins the sugars in a DNA or RNA chain through their 5’-OH group of one sugar and 3’-OH group of another sugar to create a phosphodiester bond
- Phosphodiester bond in a ribonucleic acid easily breaks down by alkaline hydrolysis owing to the presence of the -OH group on carbon 2 whereas that on deoxyribonucleic acid resists alkaline hydrolysis and is fairly stable

A trinucleotide



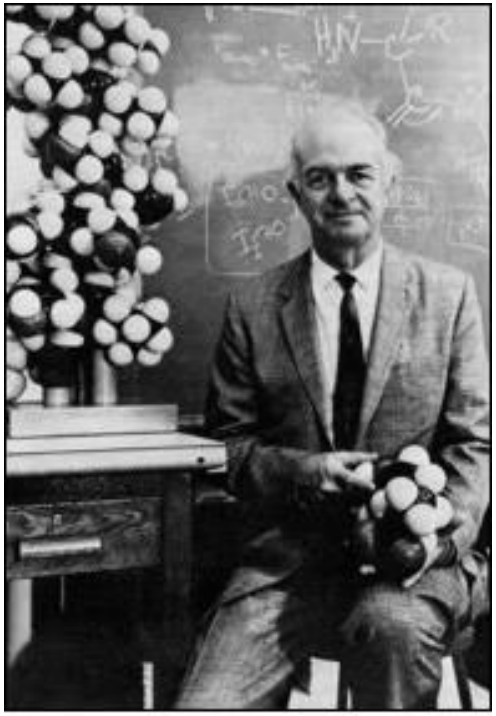
Three-dimensional structure of DNA

DNA structure

Determination of 3-D structure of DNA was preceded by elucidation of protein structure

Linus Pauling (1953): a theoretical chemist at California Institute of Technology elucidated the α-helix structure of proteins held together by hydrogen bonds

Linus proposed the triple helix model of DNA in which the phosphates (predominantly negative) form the helical core, and the bases point outwards



Linus Pauling

DNA structure

Linus Pauling's triple helix DNA

Linus Pauling's triple helix DNA would be impossible under normal cellular conditions as the many negative charges forced together within the core would repel each other and break down the structure

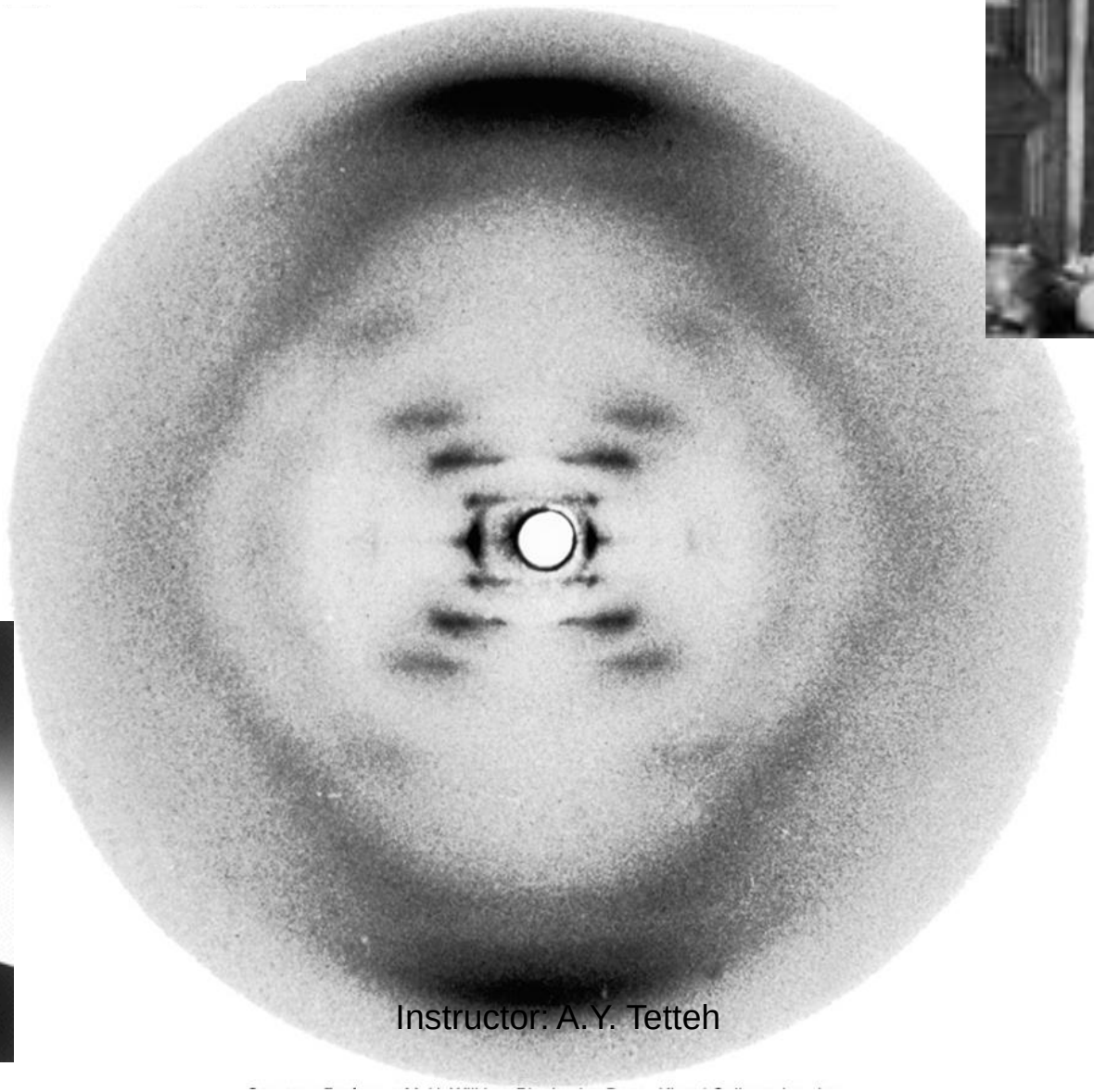
- The α-helix structure of proteins laid the groundwork for the double-helix model of DNA proposed by Watson and Crick
- Maurice Wilkins and Rosalind Franklin at Kings College in London used X-ray diffraction studies to analyze 3D structure of DNA

Maurice Wilkins and Rosalind Franklin experiment

- They made a highly concentrated, viscous solution of DNA
- Reached in with a needle and pulled out a fiber
- Fiber made of many molecules of DNA forced into side-by-side alignment by the pulling action
- The DNA fiber was exposed to X-ray. The fibers diffracted X-rays to produce a simple pattern
- Pattern was a series of spots arranged in an ‘X’ shape indicating that DNA structure must be very simple
- Regularity of pattern indicated DNA is a repeating molecule arranged in a cockscrew (helix) structure
- In contrast, protein gives a complex X-ray diffraction pattern

Wilkins-Franklin's X-ray diffraction picture of DNA

Rosalind



Wilkins

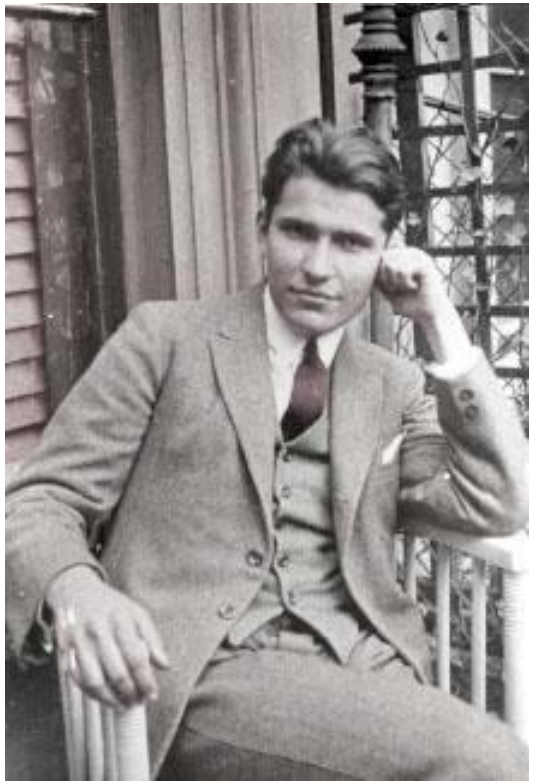
How the repeating unit of DNA was determined

DNA thought to be a repeat of four nucleotides: **ACGT**

1950
Erwin Chargaff

Examined the base composition of different organisms. Found that different species have
different base composition and content of purines was always roughly equal to content of pyrimidines

A=T and C=G known as **Chargaff's rules**



Chargaff's study: Base composition (expressed as G+C content) varies from one species to another

Table 2.3 Relative G + C Contents of Various DNAs

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Dictyostelium (slime mold)	22
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Bacillus cereus	37
B. megaterium	38
Hemophilus influenzae	39
Saccharomyces cerevisiae	39
Calf thymus	40
Rat liver	40
Bull sperm	41
Streptococcus pneumoniae	42
Wheat germ	43
Chicken liver	43
Mouse spleen	44
Salmon sperm	44
B. subtilis	44
T1 bacteriophage	46
Escherichia coli	51
T7 bacteriophage	51
T3 bacteriophage	53
Neurospora crassa	54
Pseudomonas aeruginosa	68
Sarcina lutea	72
Micrococcus lysodeikticus	72
Herpes simplex virus	72
Mycobacterium phlei	73

Source: From Davidson, *The Biochemistry of the Nucleic Acids,* 8th edition revised by Adams et al. Lippencott.

A=T and C=G

Table 2.1 Composition of DNA in moles of base per mole of phosphate

	Human						Avian	Bovine				
	Sperm		Thymus	Liver Carcinoma	Yeast		Tubercle Bacilli	Thymus			Spleen	
	#1	#2			#1	#2		#1	#2	#3	#1	#2
A:	0.29	0.27	0.28	0.27	0.24	0.30	0.12	0.26	0.28	0.30	0.25	0.26
T:	0.31	0.30	0.28	0.27	0.25	0.29	0.11	0.25	0.24	0.25	0.24	0.24
G:	0.18	0.17	0.19	0.18	0.14	0.18	0.28	0.21	0.24	0.22	0.20	0.21
C:	0.18	0.18	0.16	0.15	0.13	0.15	0.26	0.16	0.18	0.17	0.15	0.17
Recovery:	0.96	0.92	0.91	0.87	0.76	0.92	0.77	0.88	0.94	0.94	0.84	0.88

Source: E. Chargaff "Chemical Specificity of Nucleic Acids and Mechanism of Their Enzymatic Degradation," *Experientia* 6:206, 1950.

Watson and Crick - model builders

- performed no experiment but drew conclusions from:

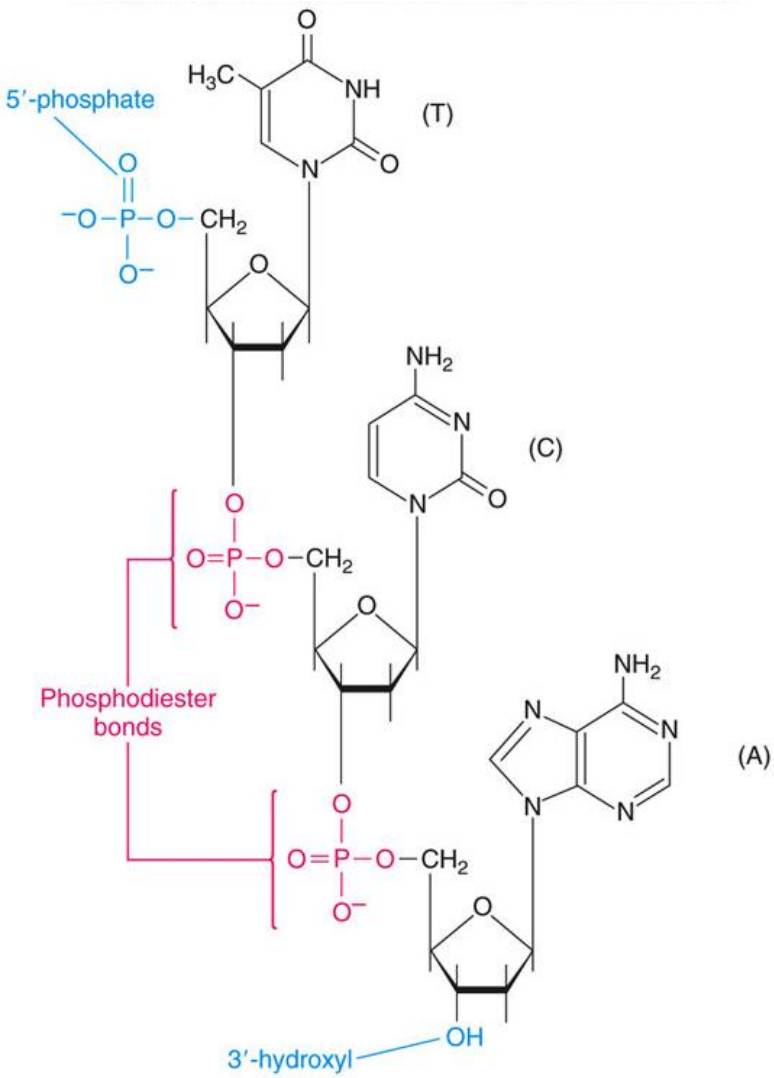
The very simple X-ray diffraction pattern

DNA structure must be simple, unlike proteins

Simplest repeating structure:

that a long, thin molecule
can take a corkscrew or helix
structure

A trinucleotide



The substituents, -OH, $-NH_2$, C=O, PO_4^{3-} participate in non-covalent interactions with each other to cause the formation of a helix

Paradox:

Franklin: DNA was a helix with a **regular**, repeating structure

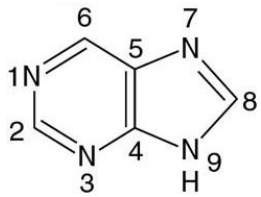
But, for DNA to serve its genetic function

it must have an **irregular sequence** of bases

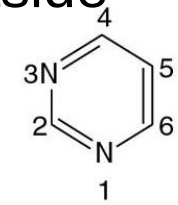
Watson and Crick solved this paradox and satisfied Chargaff's rules at the same time

A=T and C=G

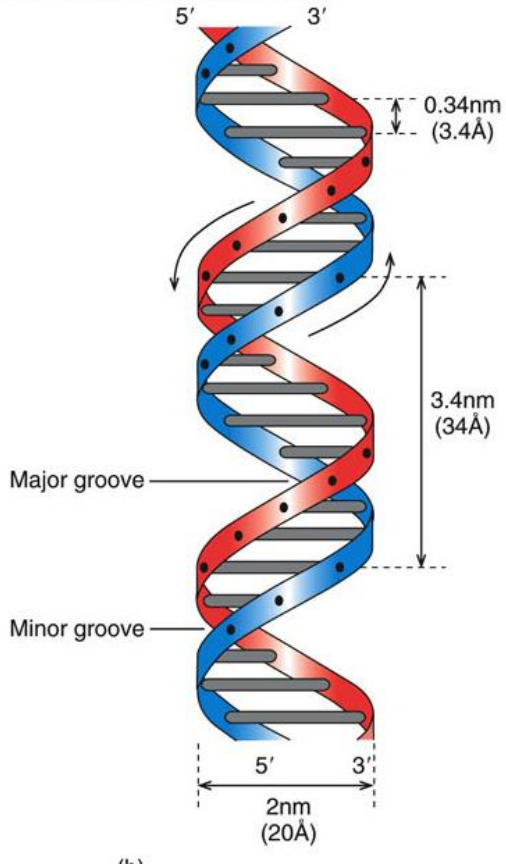
DNA is a double helix



Purine



Pyrimidine



DNA must be a **double helix**:

sugar and phosphate on the outside and bases on the inside

Bases must be paired :

purines in one strand pair with pyrimidines in the other strand

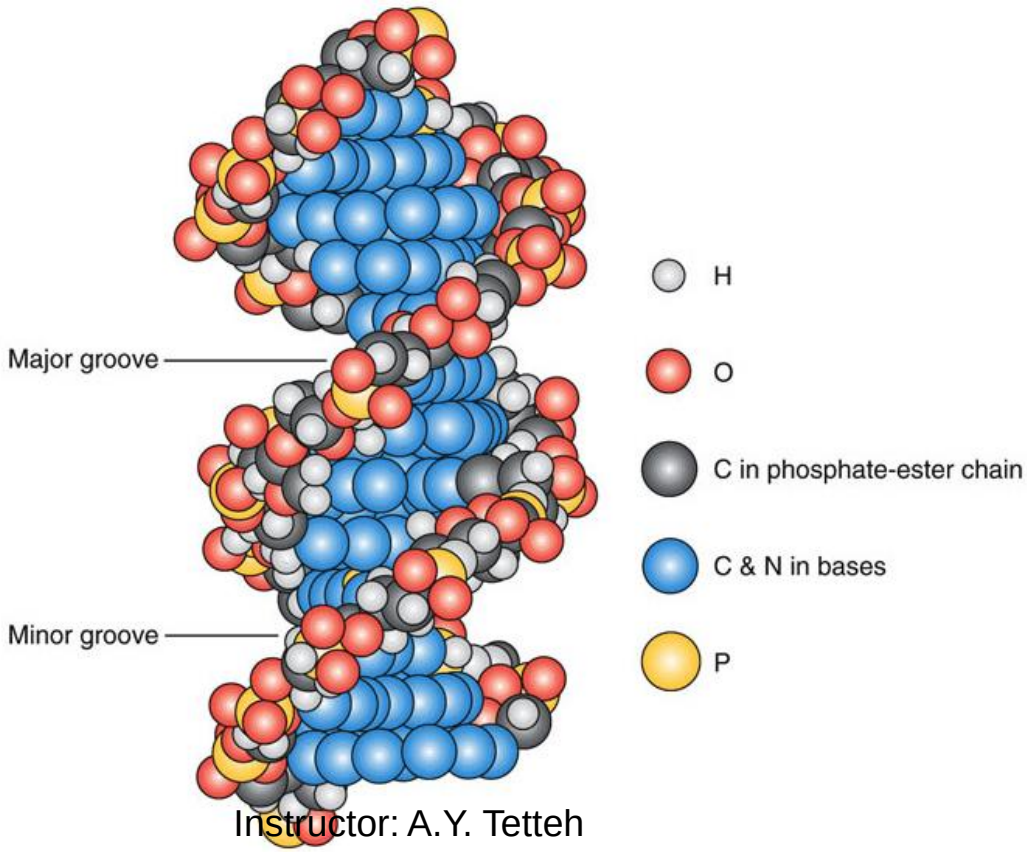
This would make the helix uniform

No bulges (purine-purine) and

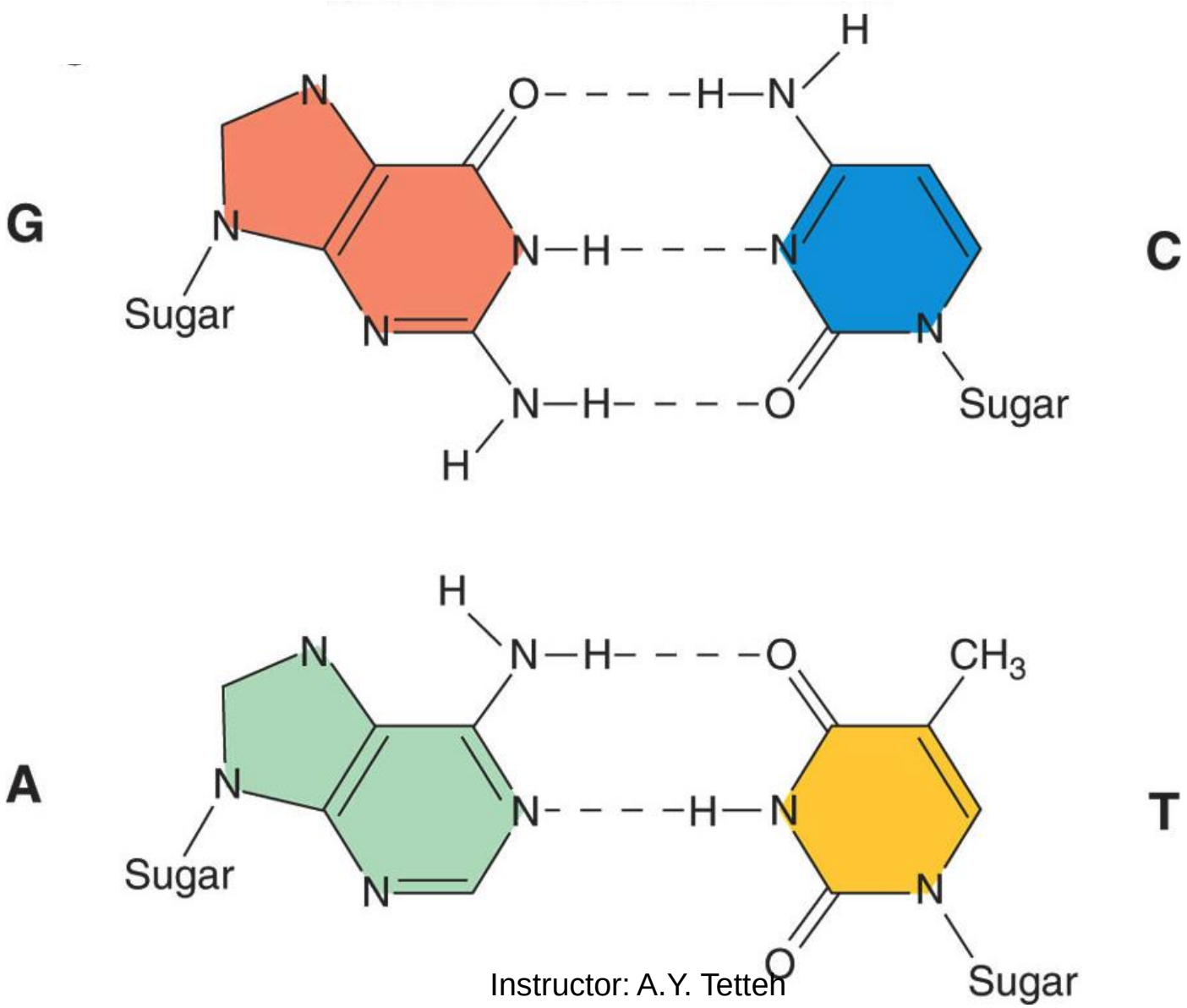
No constrictions (pyrimidine-pyrimidine)

DNA is a double helix

with sugar and phosphate on the outside
and bases on the inside



Purines pair with pyrimidines



Nature of purine-pyrimidine pair

- Guanine-cytosine pair held together by three hydrogen bonds
- This pair has the same shape as an adenine-thymine pair held together by two hydrogen bonds
- Similarity in shape makes DNA regular molecule
- Differences in bases make DNA irregular and functional

Watson and Crick (Nature 1953)

"It has not escaped our notice that the specific base pairing we have proposed, immediately suggests a possible copying mechanism for the genetic material."

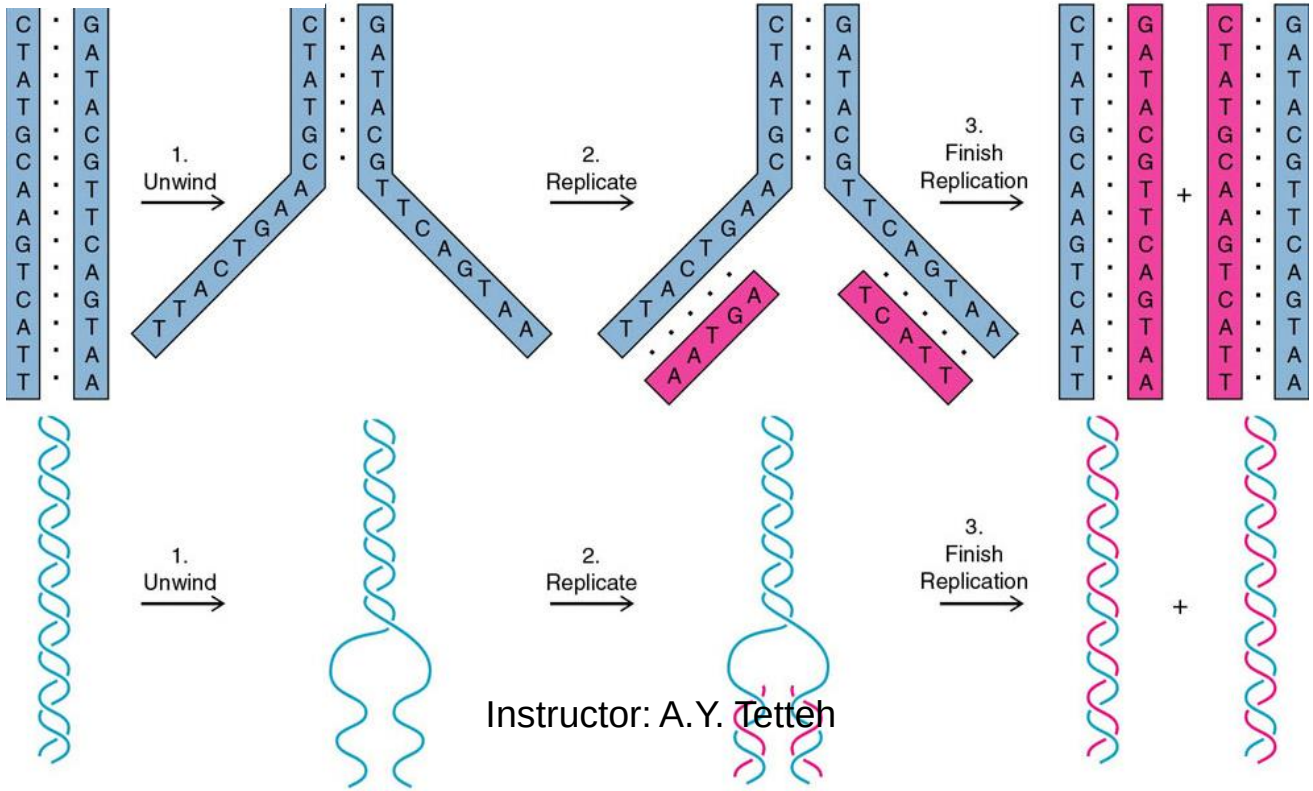
One strand is the complement of the other- complementary strands

The strands can be separated and each serve as the template for the other

Semiconservative replication ensures two daughter DNA duplexes are exactly the same as the parent

Watson and Crick 1953

Semiconservative replication



The double helix

- Spacing between base pairs is 3.4 Ångstrom units
- Overall helix repeat distance is about 34 Ångstrom units
- It means there are about 10 bp per turn of the helix
- The arrows indicate that the 2 strands are antiparallel: 5'→3' polarity from top to bottom
- The other must have 5'→3‘ polarity from bottom to top

‘Take home message’

- DNA is double helix; the two strands are complementary and anti-parallel

Physical structure of DNA

- DNA and RNA molecules can assume several different structures and behave differently under certain conditions
- Can assume certain conformations
- Can be denatured – i.e., the two strands can be separated
- Can anneal - the two separated strands can come together

DNA conformations

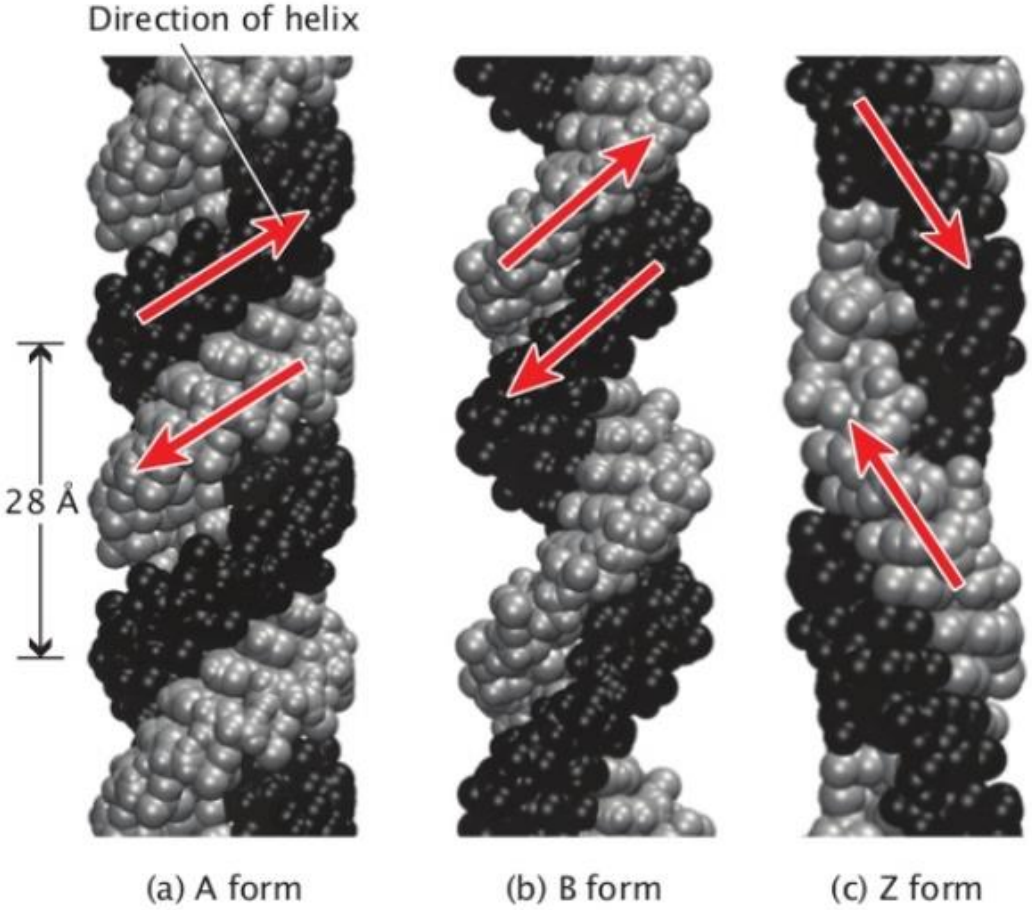
- Three DNA conformations have been found, *A*, *B*, and *Z* forms
- The sodium salt of DNA fiber at high relative humidity (92% RH) assumes the ***B*** form: the structure proposed by Watson and Crick, the most common conformation in most living cells. *B*-DNA is a right-handed helix This has 10 bp per turn as specified in Watson and Crick structure. A turn occurs after every 34 Ångstrom units

- The sodium salt of DNA fiber at relative humidity (75% RH) has ***A*** form with 11 bp per turn. *A*-DNA is short, wide and right-handed helix. Each turn occurs in 25 Ångstrom units. Found rarely under normal physiological conditions. Present in RNA-DNA hybrids
- Right-handed: helix turns clockwise away from you

DNA conformations

- ***Z***- form - first discovered in 1979
- a left-handed helix, i.e., the double helix winds to the left in a zig-zag pattern. It is much elongated
- A transient form of DNA which exists occasionally in response to certain types of biological activity
- Scientists have since discovered that certain proteins bind very strongly to *Z*-DNA, suggesting that *Z*-DNA plays an important biological role in protection against viral disease

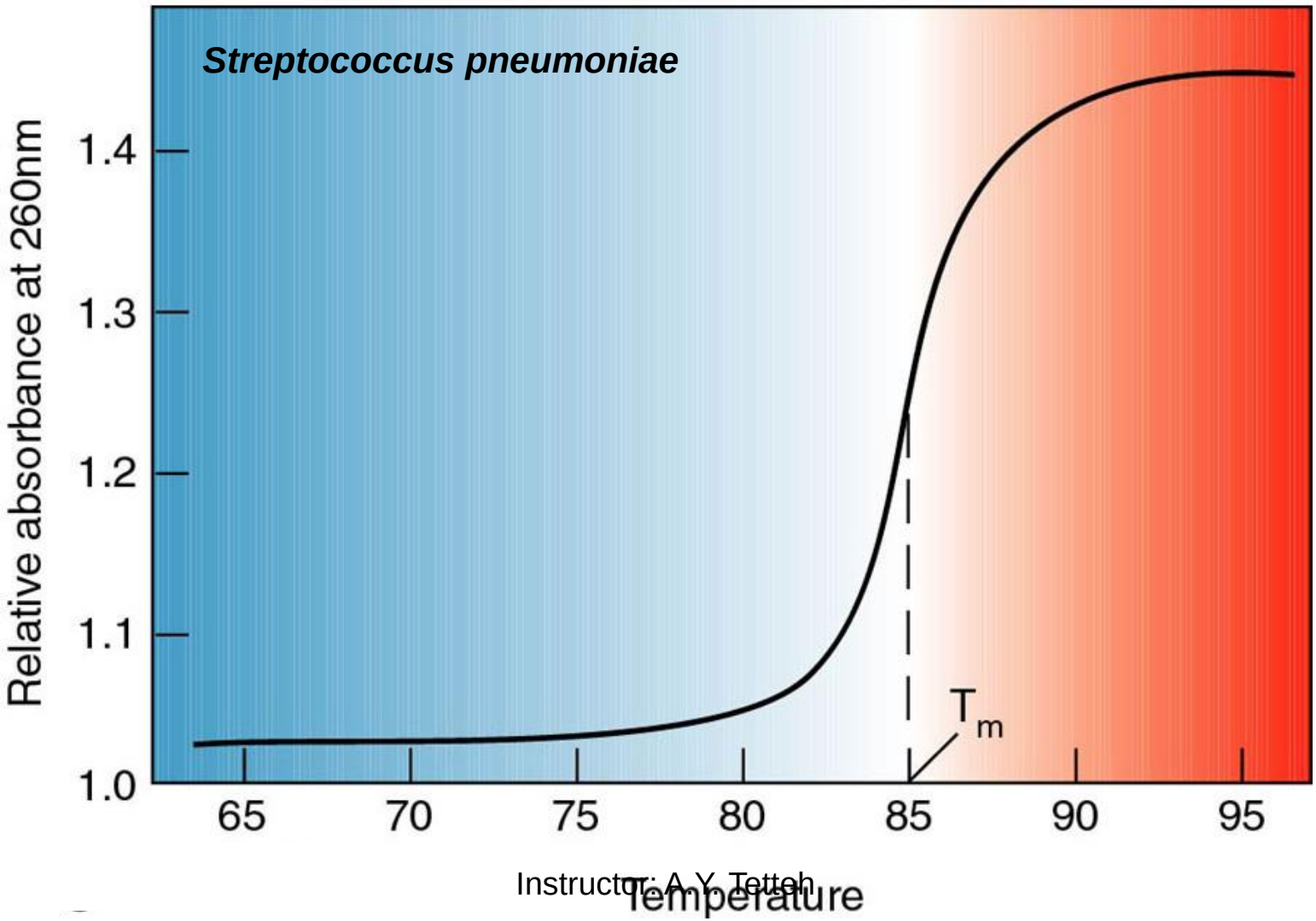
DNA conformations



Denaturation of DNA- by heating

- Heat DNA solution
- the noncovalent forces (H-bonds) holding the 2 strands together weaken and break
- The two strands come apart in a process known as **DNA denaturation/melting**
- The amount of strand separation or melting is measured by absorbance at λ_{260} against temperature
- Plot a graph of absorbance against temperature to follow DNA melting

Melting curve of DNA



T_m

T_m or melting temperature is the temperature at which ½ of the DNA strands are melted

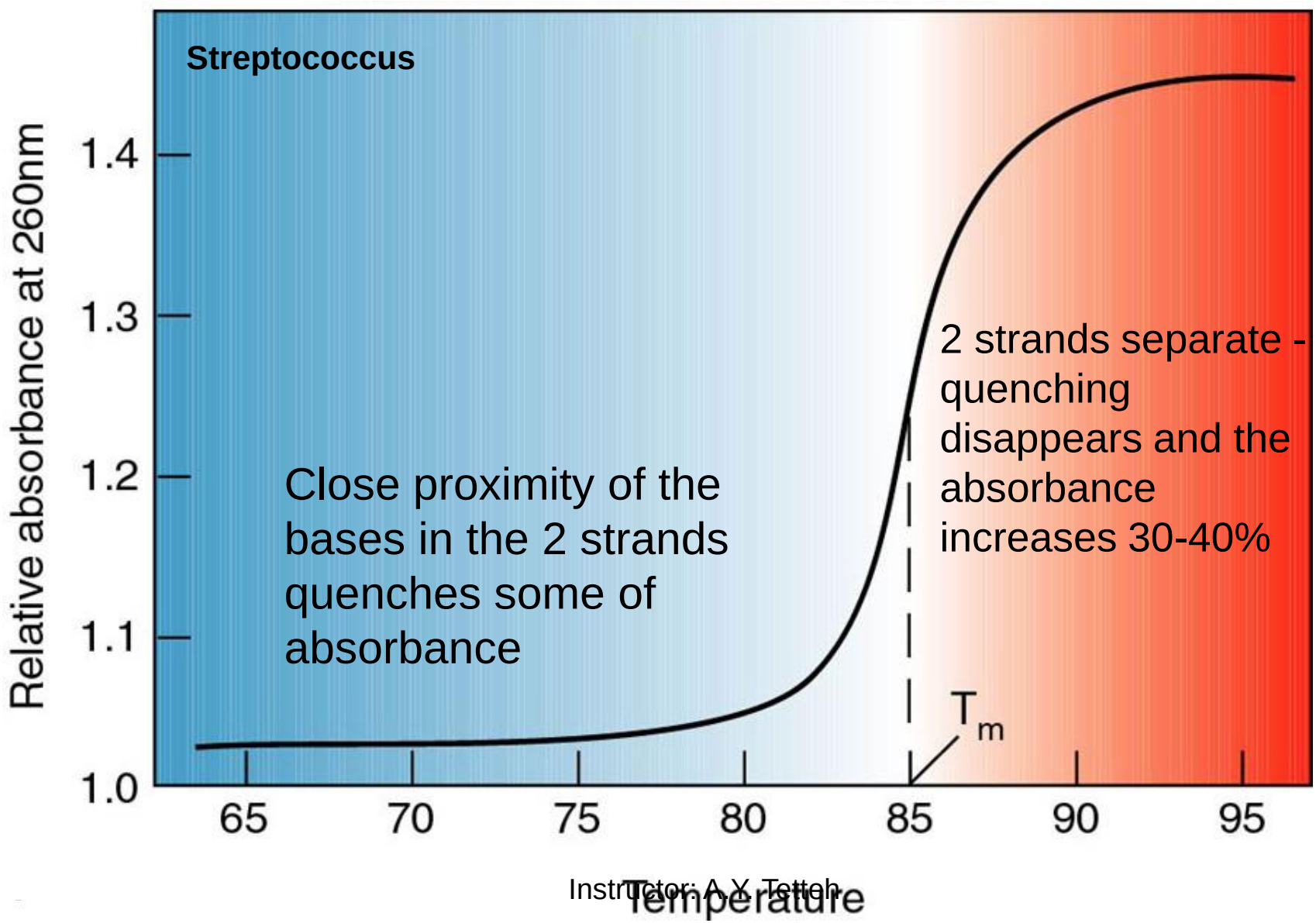
or

temperature at which melting is half complete

Hyperchromic shift

- The initial slow rise in the curve shows that the two strands hold fast at low temperature, hence low absorbance
- When temperature increases and approaches T_m, the strands separate and absorbance increases sharply until a plateau is reached when there is no more separation

Hyperchromic shift



Hyperchromic shift

Nucleic acids absorb light at wavelength of 260 nm because of the electrical nature of the bases

Close proximity of the bases in the 2 strands (double helix) quenches some of absorbance

When the 2 strands separate upon heating, quenching disappears and the absorbance increases by 30-40%. This is hyperchromic shift

Hyperchromic shift occurs when nucleic acids change from double stranded to single stranded structure

Denaturation of DNA – separating the two strands of the double helix

Differences in GC content produce differences in physical properties of DNA

GC (G + C) content can vary from one DNA to the other

Values range from 22-73%

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Bull sperm	41
Streptococcus pneumoniae	42
Wheat germ	43
Chicken liver	43
Mouse spleen	44
Salmon sperm	44
B. subtilis	44
T1 bacteriophage	46
Escherichia coli	51
T7 bacteriophage	51
T3 bacteriophage	53
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Sarcina lutea	72
Micrococcus lysodeikticus	72
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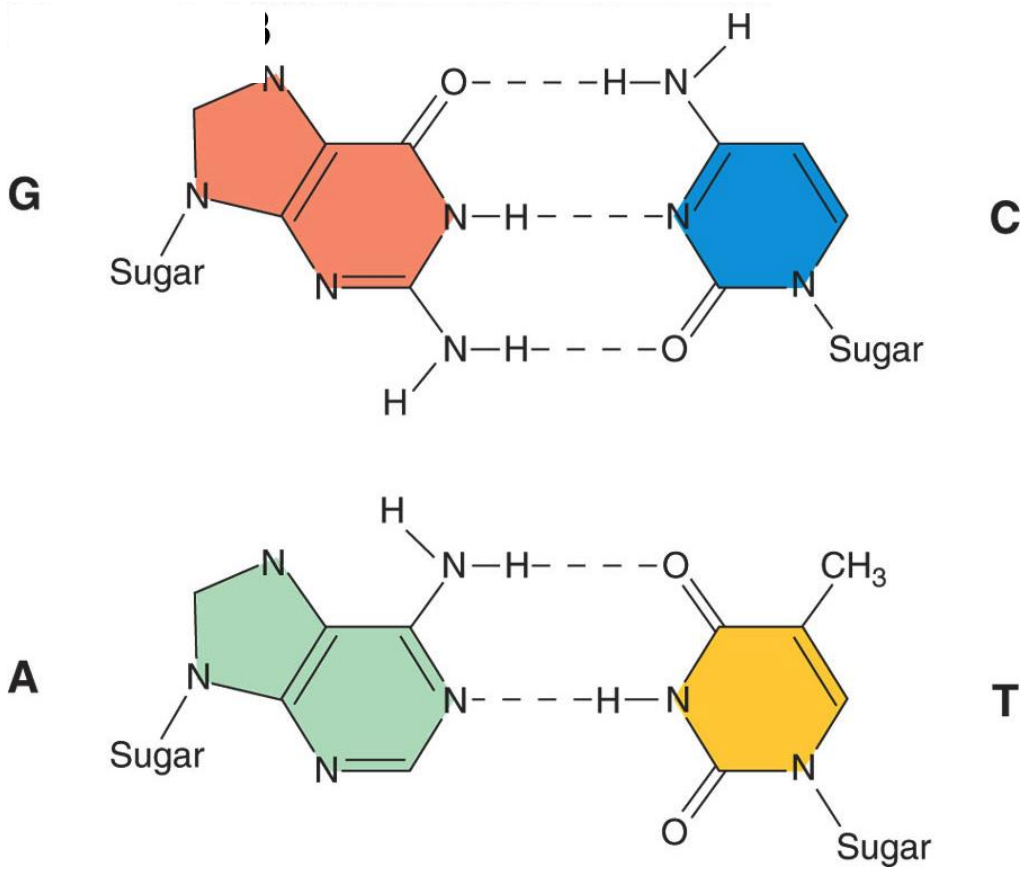
Mycobacterium phlei	73

Source: From Davidson, *The Biochemistry of the Nucleic Acids*, 8th ed. revised by Adams et al. Lippencott.

Higher the GC content - higher the T_m



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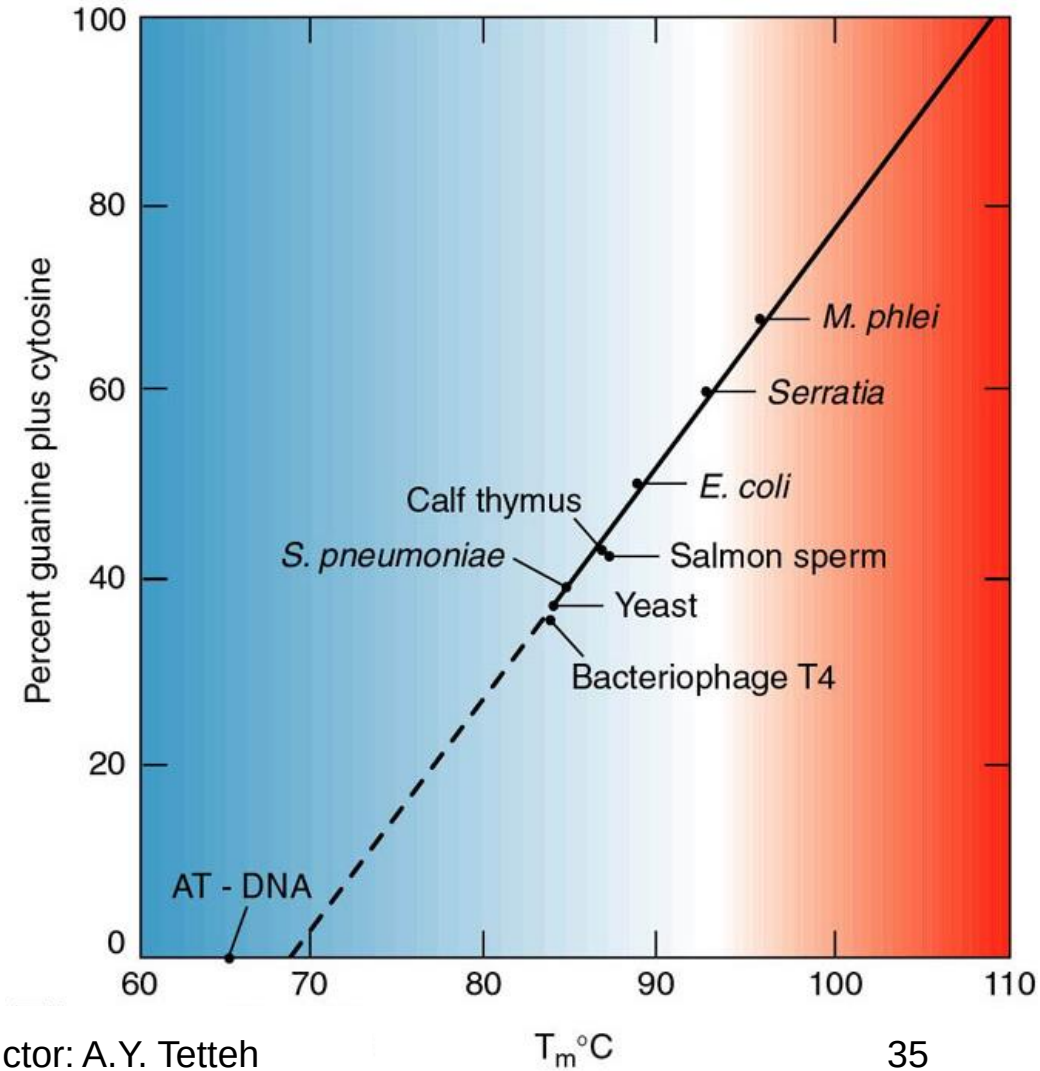


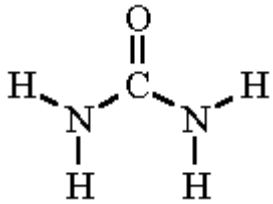
G-C content and Tm

- G-C pairs form three H-bonds , whereas A-T pairs form two H bonds
- So the two strands of DNA rich in G+C will hold to each other more tightly than those of A-T-rich DNA
- Harder to separate GC-rich than AT-rich DNA

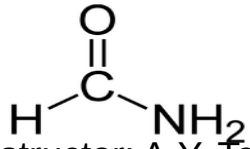
Other ways to **denature DNA**:

organic solvents such as dimethyl sulfoxide (DMSO), **urea** and **formamide** (denaturants)

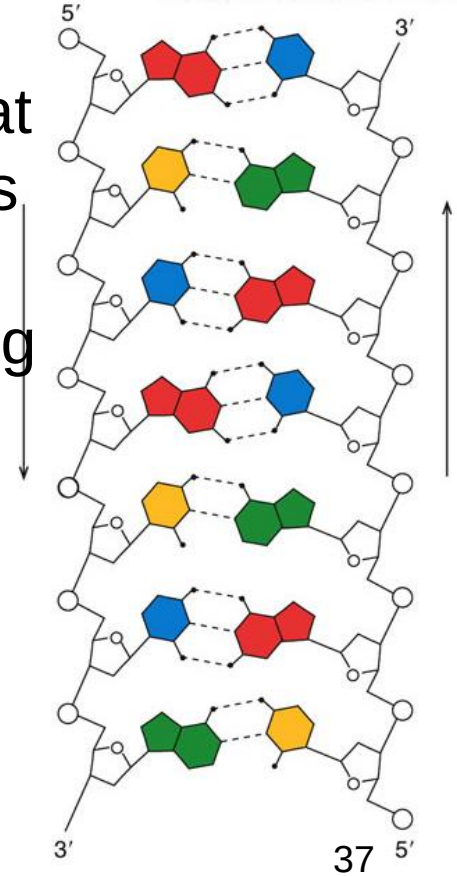
Urea and formamide contain functional groups that can form H-bonds with the electronegative centers of the N-bases. High concentrations of urea (8M) or formamide (70%) compete for H-bonds favoring interactions between the denaturant and the N-bases rather than between complementary bases. As a result, the two strands separate.



Urea



Formamide



Conclusions

DNA is the genetic material

DNA consists of nucleotides that contain

bases
sugars
phosphate groups

The structure of DNA is a double helix
The strands are complementary and antiparallel

The double helix is denatured at high temperatures

Other ways to **denature DNA**

High pH

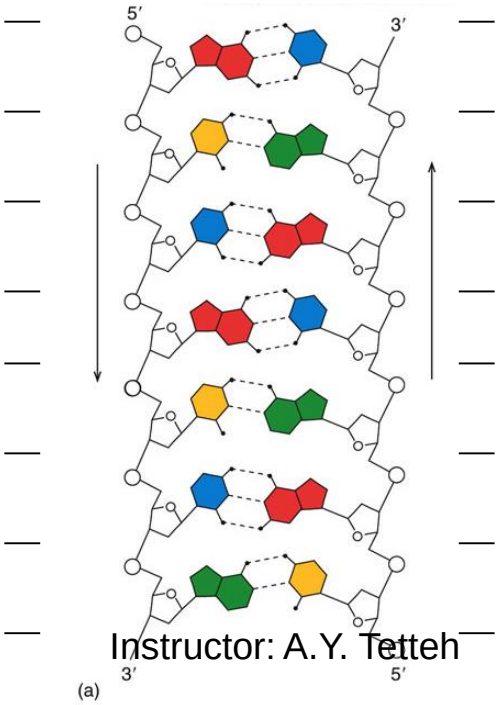
Disrupts hydrogen bonds between DNA strands, and promote denaturation. Bases such as NaOH raise the pH until the H^+ shared between the N-base electronegative centers (N-H and O=) is stripped from the H-bond.

Low salt - removes ions that shield the negative charges on the two strands from each other

Denaturation of DNA

At low ionic strength

mutually repulsive forces of the negative charges
are strong enough to denature the DNA
at relatively low temperatures



Summary

These conditions promote DNA denaturation

1. High temperature

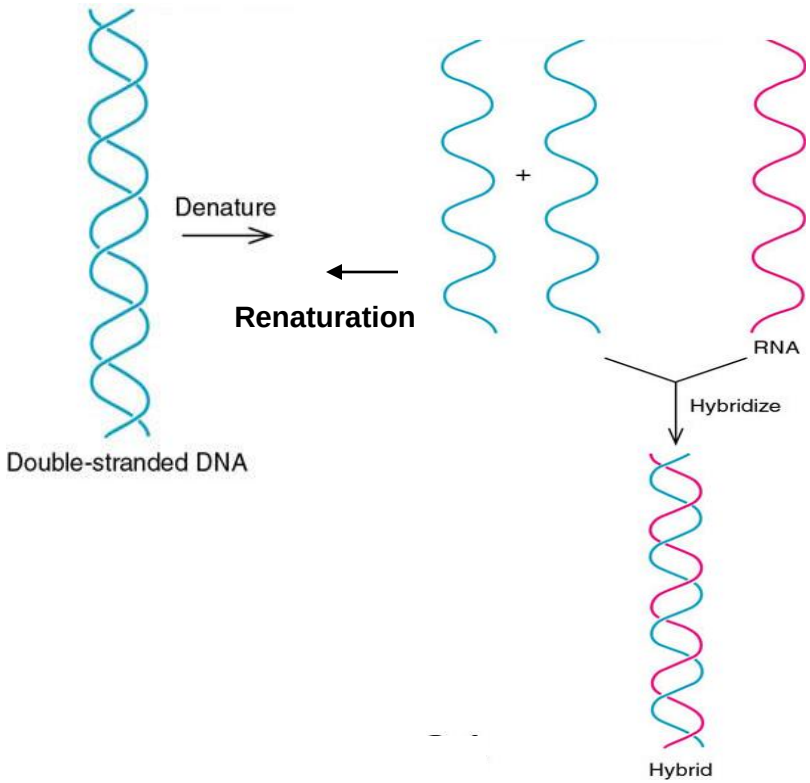
2. Organic solvents

3. High pH

4. Low salt

Reuniting the separated DNA strands

- Under proper conditions, the separated strands can come back together
- Known as annealing or renaturation



Renaturation

Depends on

1. Temperature

the best temperature for renaturation is about 25 ^{o}C below T_m

This temperature is low enough

-Does not promote further denaturation

This temperature high enough to

- allow rapid diffusion of DNA molecules
- weaken transient bonding between mismatched sequences and intrastrand base-pairing

Rapid cooling prevents renaturation by slowng down diffusion

2. DNA concentration

3. Time

4. Complexity

Renaturation

DNA concentration

the higher the concentration, the more likely two complementary strands will encounter each other within a given time and participate in H-bond formation

The higher the concentration, the faster the annealing

Renaturation time

- the longer the time allowed for annealing, the more annealing will occur

4. Complexity

-Cot curves

BCHEM 365
Lecture 3
March 14, 2022

DNA melting and renaturation

Complexity of DNA –C_ot curves

DNA reassociation kinetics was the forerunner of the concept of C_ot and complexity of DNA

C_ot = initial DNA concentration (C_o) in moles of nucleotides per liter × time (*t*) in seconds

If all other factors (concentration, pH, ionic concentration) are equal, extent of renaturation of complementary strands depends on the C_ot (i.e. complexity)

One particular value that is useful is $C_o t_{½}$, the $C_o t$ value where half of the DNA has annealed

There is a linear relationship between DNA structural complexity and $C_o t_{½}$

Complexity of DNA –$C_o t$ curves

There is a linear relationship between DNA complexity and $C_o t_{½}$

For example, compare calf genome size - 2×10^9 bp of DNA to T4 bacteriophage genome size - 2×10^5 bp of DNA, that is,

10,000 times as many base pairs in calf DNA

Implication: it takes 10,000 times as long to anneal denatured calf DNA making $C_o t$ value of calf DNA is 10,000 times larger

Complexity of DNA –C_ot curves

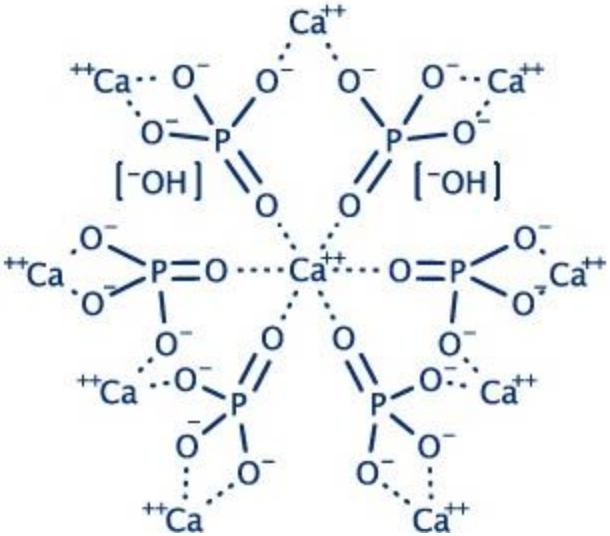
To construct a Cot curve for any DNA sequence, heat the DNA sample to denature it

Cool the sample slowly for reassociation to take place

Dilute the sample to further slow down reassociation

Determine DNA fraction reassociated and plot against log Cot

Pour sample onto a hydroxyapatite [a crystal of two units of $Ca_5(PO_4)_3(OH)$ making $Ca_{10}(PO_4)_6(OH)_2$] column



Hydroxyapatite

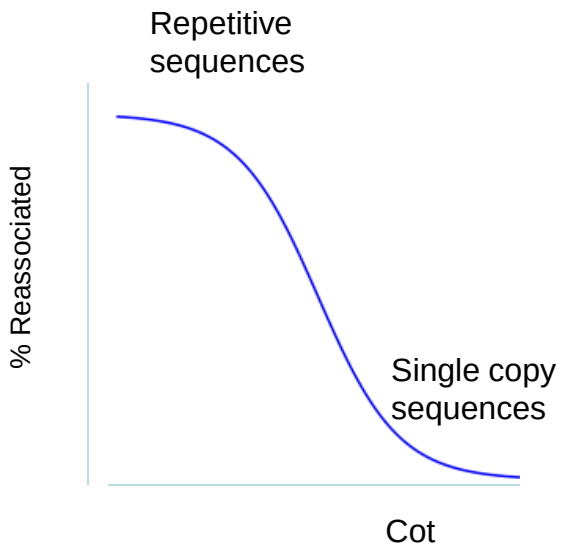
Complexity of DNA –C_ot curves

- The Ca^{2+} in the hydroxyapatite bind specifically to the negatively charged PO_4^{3-} in DNA; PO_4^{3-} in the hydroxyapatite bind to the partially positive charges and Na^+ in DNA
- When the column is treated with a low concentration phosphate buffer, the single-stranded DNA (fraction not annealed) is eluted. Similarly, elution with a high phosphate concentration buffer washes off the double-stranded DNA.
- Concentration of each eluted sample is measured with a spectrophotometer (Absorbance α concentration× path length)

Complexity of DNA –C_ot curves

- The percentage reassociated DNA is calculated and used to construct the curve. The y-axis is % anealled DNA (fraction reassociated). The x-axis having DNA concentration in moles/L multiplied by time (*Co*t) on a logarithmic scale.
- Percentage reassociated = concentration of double-stranded (fraction annealed) DNA (*C*) to the total concentration of the initial DNA (C_o), that is, C/C_o
- Alternatively, the Y-axis may also be expressed as percent of the DNA that remains single stranded or not annealed
-

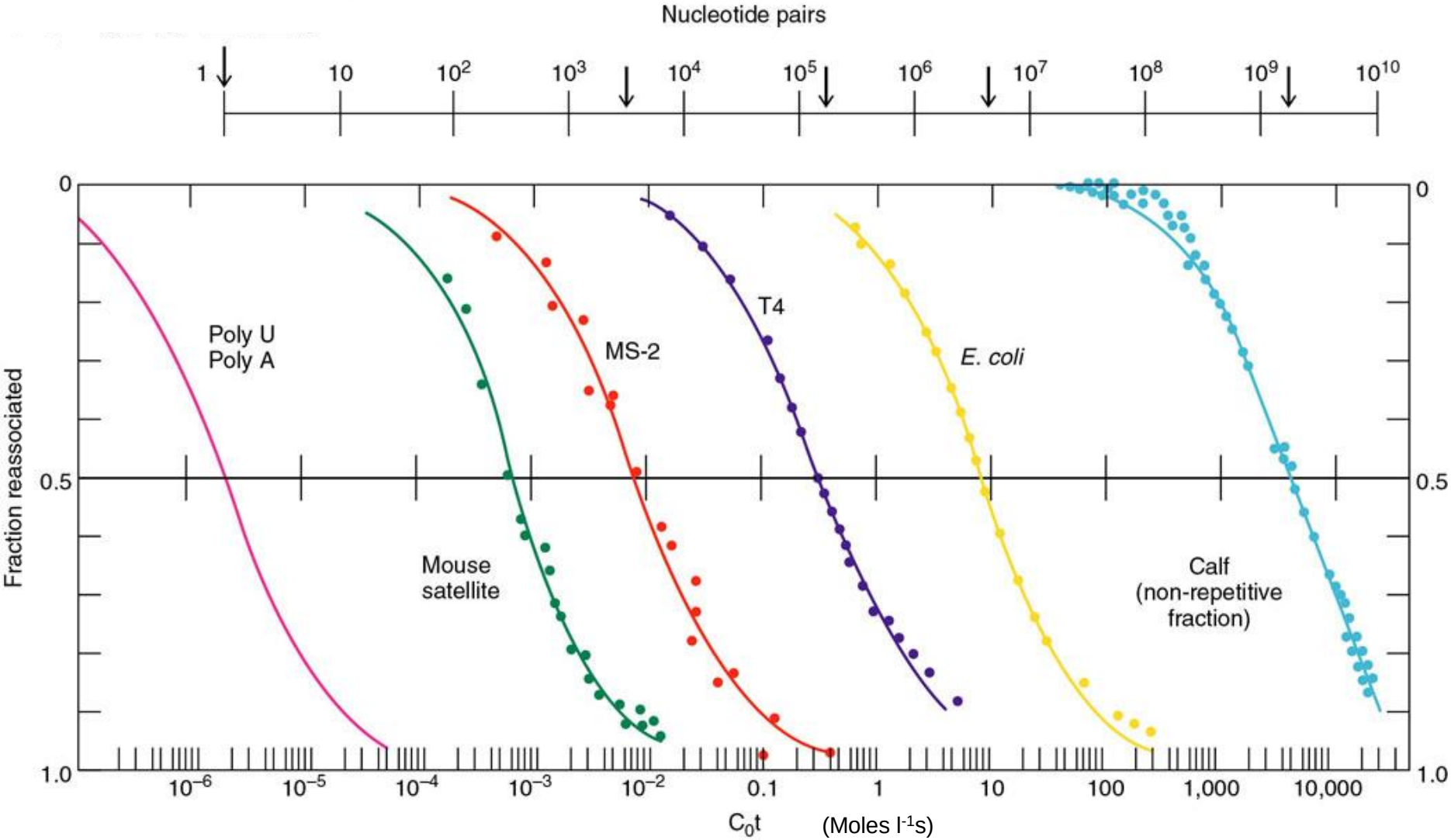
Complexity of DNA – C_ot curves



The kinetics of C_ot curve show the following characteristics:

- A sigmoidal curve
- Repetitive sequences reassociate faster and their concentration dominate the genome
- Nonrepetitive sequences are fewer and rarely reassociate, howbeit at a slow rate
- The curve is smooth which indicates that annealing occurs gradually over a period of time

Cot curves



It takes DNA toward right side of graphs much longer time to reanneal. The converse is true. Each dot represents a DNA sequence. It takes nonrepetitive DNA much longer time to reanneal. Mouse satellite DNA consists of highly repetitive tandem sequences located in the centromeric region and forms the main structure of heterochromatin. It is noncoding.

Applications of Cot curves

- The shape of a "Cot" curve for a given species is a function of two factors:
  - the size or complexity of the genome
  - the amount of repetitive DNA within the genome

  Cot curves reveal information about the following:

  - Repetitiveness of the DNA
  - Copy number of repetitive sequences
  - Information about the number of genes
  - Guide to primer sequences in noncoding regions (SSRs, VNTRs, etc.)
  - Facilitates sequencing of genomes

Conclusions from Cot curves

- As genome size increases, a higher concentration is required for the denatured DNA to anneal
- As genome size increases and nonrepetive sequences increase, a longer time is required for denatured DNA to anneal

DNA Complexity

$Cot_{1/2}$ indicates the total length of different sequences present = complexity (usually in bp)

$Cot_{1/2}$ of any genome is proportional to its complexity

Complexity of any DNA is determined by comparing its $Cot_{1/2}$ with a standard DNA of known complexity (usually *E.coli*).

$$\frac{Cot_{1/2}\text{ (DNA of any genome)}}{Cot_{1/2}\text{ (}E.\ coli\text{ DNA)}} = \frac{\text{Complexity of any genome}}{4.2 \times 10^6 \text{ bp}}$$

$$Cot_{1/2}\ E.\ coli\ DNA = 4.2 \times 10^6 bp$$

Therefore $Cot_{1/2}$ of DNA of any genome = what complexity of the genome?

$$\text{complexity of unknown genome} = \frac{Cot_{1/2}\text{ of DNA of any genome}}{Cot_{1/2}\ E.\ coli\ DNA} \times 4.2 \times 10^6\ bp$$

DNA Complexity: bacterial vs. eukaryotic

Eukaryotic genomes have Cot values
up to 8 orders of magnitude –
much broader than prokaryotes

Eukaryotic genome includes several components
each reassociating with characteristic kinetics

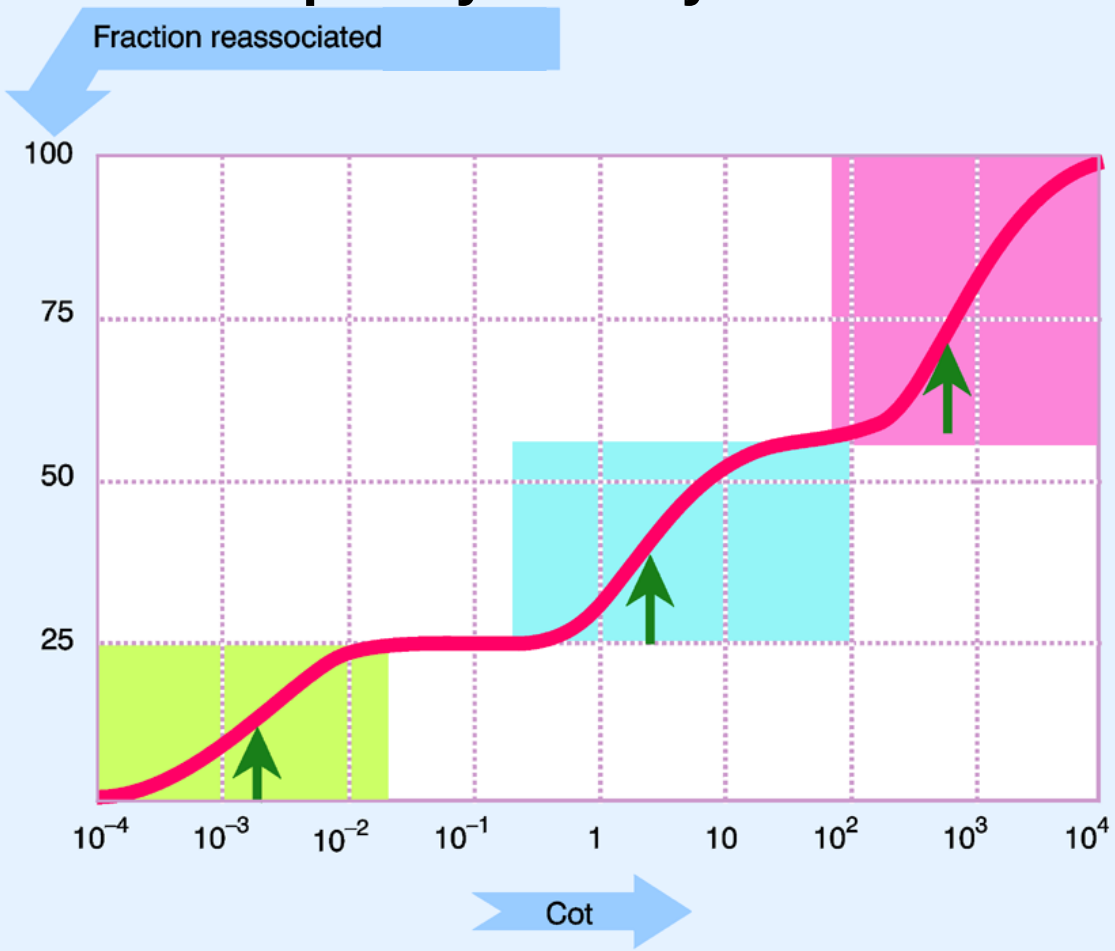
Cot curve reveals a crucial difference between bacteria and eukaryotic genomes:

bacteria genomes - a single kinetic component

eukaryotic genomes – varying kinetic components (much more complex)

Complexity: eukaryotic

The reassociation kinetics of eukaryotic DNA show three types of component (indicated by the shaded areas). The arrows identify the $Cot_{1/2}$ values for each component.



	Fast component	Intermediate component	Slow component
Percent of genome	25	30	45
$Cot_{1/2}$	0.0013	1.9	630
Complexity, bp	340	6.0×10^5	3.0×10^8
Repetition frequency	500,000	350	1

Intermediate and fast components

Intermediate and fast components are made up of moderately repetitive and highly repetitive DNA, respectively

	Fast component	Intermediate component	Slow component
Percent of genome	25	30	45
$Cot_{1/2}$	0.0013	1.9	630
Complexity, bp	340	6.0×10^5	3.0×10^8
Repetition frequency	500,000	350	1

Intermediate component

Not a single length of DNA

but a variety of individual sequences, whose total length together comes to 6×10^5 bp

Dispersed throughout the genome and their average repetition is 350

	Fast component	Intermediate component	Slow component
Percent of genome	25	30	45
$Cot_{1/2}$	0.0013	1.9	630
Complexity, bp	340	6.0×10^5	3.0×10^8
Repetition frequency	500,000	350	1

Repetitive DNA

If repetitive DNA is heated, the double strands melt gradually over a wide temperature range

If allowed to renature, the reassociated DNA do not consist of exactly paired molecules, some mispairing occur

The more mispairing, the lower the T_m because mismatches unwind easily

Moderately repetitive and highly repetitive DNA

Moderately repetitive DNA is dispersed throughout genome

- are relatively short individual sequences
- Many are ribosomal genes

Highly repetitive DNA often forms discrete clusters

- Found at centromeres

Usually not transcribed nor translated into protein

Mouse

40% of mouse DNA is repetitive

100,000 -1 million copies per genome

Highly repetitive is about 10% of the total DNA

Certain short sequences are repeated 1 million times in genome

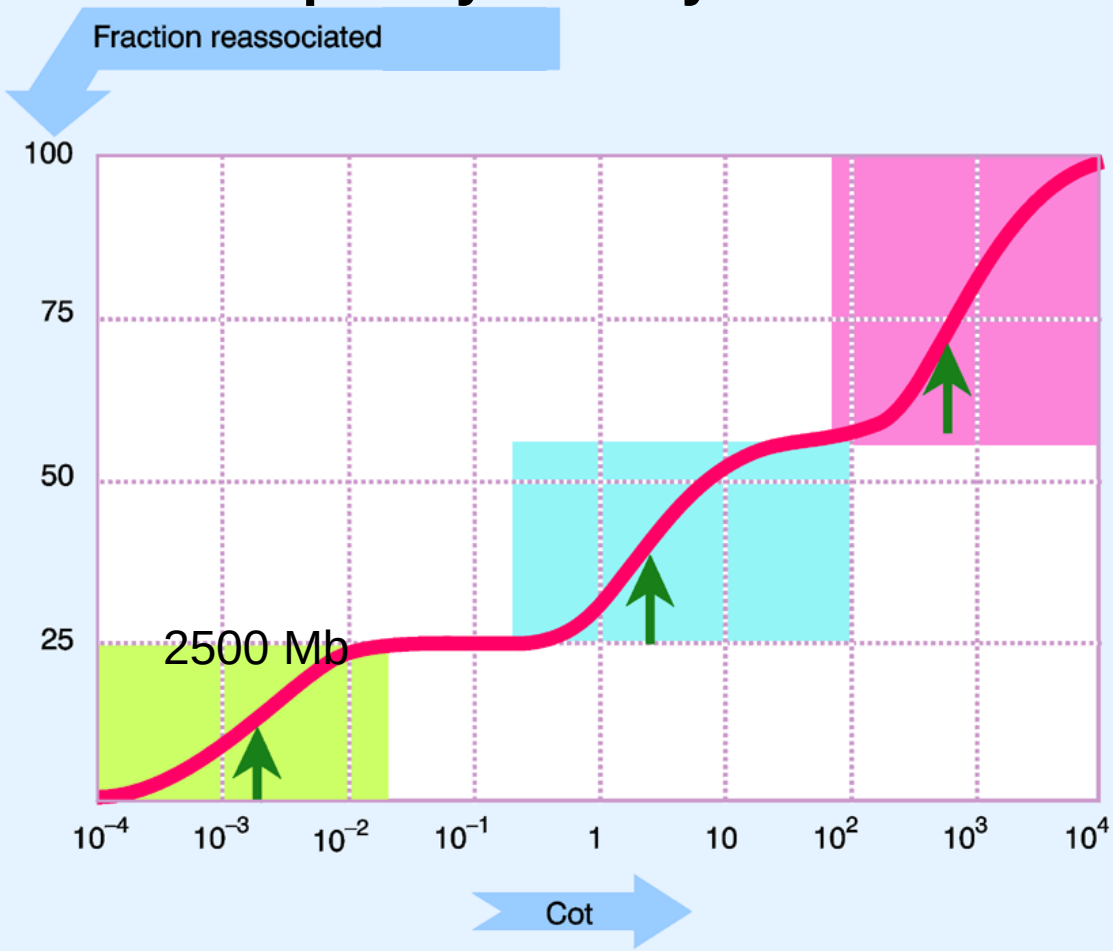
Moderately repetitive is about 30% of the total DNA

Single copy DNA

- Forms the slow component
- Are relatively longer sequences
- Are not repetitive
- Make up genes, therefore they are the coding DNA sequence (transcribed and translated)
- Takes higher concentration and longer time to reanneal

Figure 3.6 The reassociation kinetics of eukaryotic DNA show three types of component (indicated by the shaded areas). The arrows identify the $Cot_{1/2}$ values for each component.

Complexity: eukaryotic



	Fast component	Intermediate component	Slow component
Percent of genome	25	30	45
$Cot_{1/2}$	0.0013	1.9	630
Complexity, bp	340	6.0×10^5	3.0×10^8
Repetition frequency	500,000	350	1

Single copy DNA

- Maize genome size is 2.5 billion bp. Number of genes (protein-coding genes) is 32,000 spread across 10 chromosomes

- The human genome (23 chromosomes) is estimated to be about 3.2 billion bp long, contains 20,000–25,000 distinct protein-coding genes

Conclusions

The double helix is denatured at high temperatures

Cot is the product of
the initial DNA concentration (C_o)
in moles of nucleotides per liter

and time (t) in seconds

and is a measure of the **complexity** of DNA
Complexity = size of the DNA; type of sequence
(highly repetitive, mod. repetitive, or single copy)

DNA sizes and shapes

- DNA sizes expressed in three ways
  - Molecular weight
  - Base pairs
  - Length
    - All are related

Sizes of DNA from various sources

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Table 2.4 Sizes of various DNAs

Source	Molecular weight	Base pairs	Length
Subcellular Genetic Systems:			
SV40 (mammalian tumor virus)	3.5×10^6	5226	1.7 μm
Bacteriophage ϕX174 (double-stranded form)	3.2×10^6	5386	1.8 μm
Bacteriophage λ	3.3×10^7	5×10^4	13 μm
Bacteriophage T2 or T4	1.3×10^8	2×10^5	50 μm
Human mitochondria	9.5×10^6	16,596	5 μm
Prokaryotes:			
Haemophilus influenzae	1.2×10^9	1.83×10^6	620 μm
Escherichia coli	3.1×10^9	4.65×10^6	1.6 mm
Salmonella typhimurium	8×10^9	1.1×10^7	3.8 mm
Eukaryotes (content per haploid nucleus):			
Saccharomyces cerevisiae (yeast)	7.9×10^9	1.2×10^7	4.1 mm
Neurospora crassa (pink bread mold)	$\approx 1.9 \times 10^{10}$	$\approx 2.7 \times 10^7$	≈9.2 mm
Drosophila melanogaster (fruit fly)	$\approx 1.2 \times 10^{11}$	$\approx 1.8 \times 10^8$	≈6.0 cm
Rana pipiens (frog)	$\approx 1.4 \times 10^{13}$	$\approx 2.3 \times 10^{10}$	≈7.7 m
Mus musculus (mouse)	$\approx 1.5 \times 10^{12}$	$\approx 2.2 \times 10^9$	≈75 cm
Homo sapiens (human)	$\approx 2.3 \times 10^{12}$	$\approx 3.5 \times 10^9$	≈120 cm
Zea mays (corn, or maize)	$\approx 4.4 \times 10^{12}$	$\approx 6.6 \times 10^9$	≈2.2 m
Lilium longiflorum (lily)	$\approx 2 \times 10^{14}$	$\approx 3 \times 10^{11}$	≈100 m

DNA sizes and shapes

- To convert bp to length
  -About 10.5 bp equal one helical turn of 34 Å long

Question

Phage P1 has a double stranded DNA with 91,500 bp (91.5 kb). How many full double-helical turns are present in this DNA?

-10.5 bp make one turn, therefore 91,500 bp?

$$\frac{91{,}500 \text{ bp}}{10.5 \text{ bp}} \text{ x } 1 \text{ helical turn} = 8{,}710 \text{ helical turns}$$

DNA sizes and shapes

- How long is this DNA in microns?

  -The spacing between one base pair is about 3.4 Å or 3.4 × 10^{-4} μm along the helical axis.

Therefore 91,500 bp ?

$$\frac{91{,}500 \text{ bp}}{1 \text{ bp}} \text{ x } 3.4\text{x}10^{-4}\mu\text{m} = 31.11\ \mu\text{m}$$

DNA sizes and shapes

- To convert bp to molecular weight

  - Multiply bp by 660 D, the approximate molecular weight of one nucleotide pair

- What is the molecular mass of the phage P1 DNA?

  -One bp has a molecular weight of about 660 D, therefore 91, 500 bp?

$$\frac{91{,}500\ \text{bp}}{1\ \text{bp}} \times 660\ \text{D} = 6.039 \times 10^{7}\ \text{D}$$

DNA sizes and shapes

- Circular DNA
- Supercoiled DNA

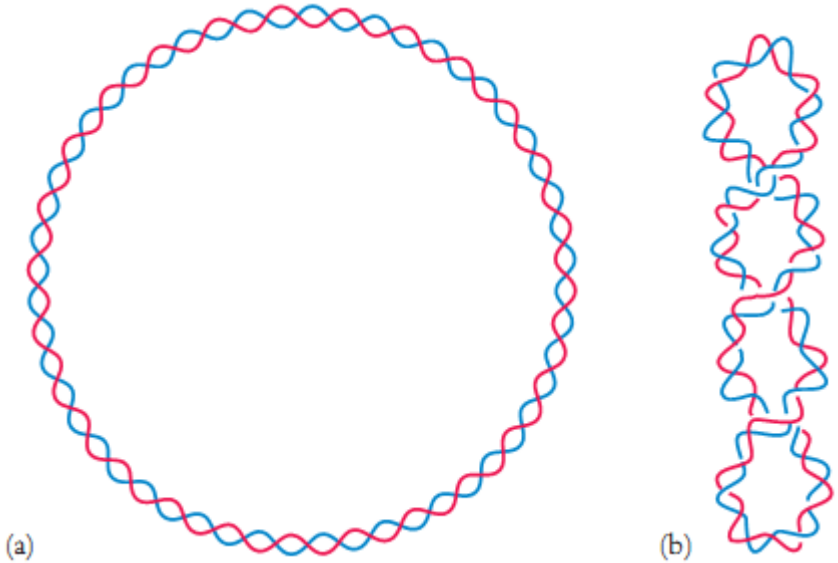
  DNA coils around itself like a twisted rubber band

DNA Forms

(a) The DNA double helix of almost all bacteria is in the shape of a closed circle.

(b) The circular DNA strands, already coiled in a double helix, are twisted a second time to produce supercoils. There are a few exceptions to the above picture.

The form of DNA influences their mobility on a gel



Supercoiling

- DNA can be twisted like a rope in a process called DNA supercoiling
- If the DNA is twisted in the direction of the helix, this is positive supercoiling, and the bases are held more tightly together
- If they are twisted in the opposite direction, this is negative supercoiling, and the bases come apart more easily.
- In nature, most DNA has slight negative supercoiling that is introduced by enzymes called **topoisomerases**.
- These enzymes are also needed to relieve the twisting stresses introduced into DNA strands during processes such as transcription and DNA replication

Open circular DNA (left bottom); Supercolied form (upper right) of phage PM2

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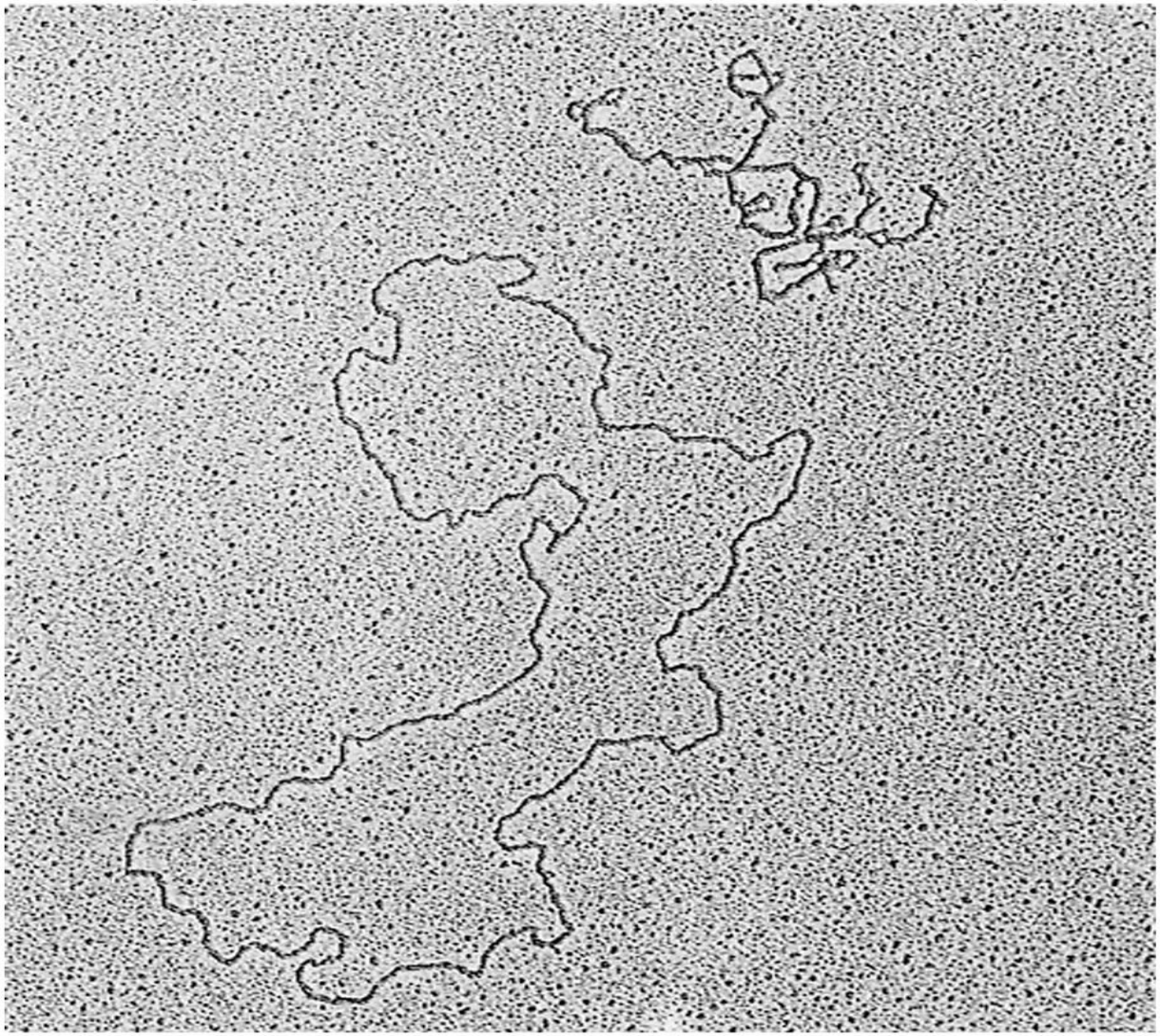

(Source: © Jack Griffith.)

Stability of DNA

- The structure of duplex DNA is governed by a balance of three forces:
- 1. **Noncovalent forces**
- 2. **Van der Waals dispersive forces**
- **3**. **Electrostatic effects**

Noncovalent forces

- Stabilizing and destabilizing
- Stabilizing (or favorable interactions): base stacking and hydrogen bonding

Stability of DNA- noncovalent forces

- Destabilizing: electrostatic repulsion between phosphate groups within same strand and repulsion of phosphates between strands.
- Similarly, different sizes of bases impose different steric effects. Purine bases impose more repulsion than pyrimidine bases. These are steric effects
- Variation in base pairing contributes to varying steric effects

Base stacking

- It is a noncovalent interaction controlled by several noncovalent forces.
- 1.“Aromatic stacking” refers to the geometry of face-to-face juxtaposition of two aromatic molecules such that the pi-electron cloud systems of the aromatic nucleus are in direct contact, though not directly aligned for purpose of maximizing surface area of contact
- In general, the distance between two aromatic planes is about. 3.4 Å in a stacked structure
- In the aqueous environment of the cell, the conjugated *pi* (π) bonds of nucleotide bases align perpendicular to the axis of the DNA molecule, minimizing their interaction with the aqueous environment. This character of DNA is spontaneous, hence the ΔG, Gibbs free energy, is small and negative

Stability of DNA

- 2.**Van der Waals dispersive forces**

  This is made up of dipole-induced dipole attractions which stabilize the stacking orientation

- 3. **Electrostatic effects** of interacting dipoles also influence stacking stability; this favorable or unfavorable contribution depends on the bond dipoles of the molecules

Stretching of DNA

- DNA is usually subjected to mechanical forces within the cell, namely, folding, unfolding, coiling and uncoiling, unzipping and zipping back
- During DNA isolation, it undergoes mechanical manipulations such as shear force
- Shear force damages the DNA in centrifugation-based isolation methods

Stretching of DNA

- When dsDNA is stretched at either ends, it undergoes a transition involving three stages (1) Initial resistance to stretching;
- (2) Stretching to a force of 65 picoNewtons (pN) causes ds DNA to unzip as the hydrogen bonds at the ends break apart and the middle part remains intact.
- This force causes the DNA to extend by 70% of its length; (3) At much larger force, the DNA becomes resistant to further stretching

Stretching of DNA

- The dynamics of DNA stretching is important for understanding the thermodynamics and energetics of DNA as applied to DNA-protein interactions, and for understanding Pulsed Field Gel Electrophoresis

Protein structure

- Proteins are polymers, long, chain-like molecules
- The monomers are amino acids
- Informational relationship between DNA and protein: three nucleotides in a DNA gene stands for one amino acid in a protein – the triplet codon
- Proteins contain 20 different amino acids

Outline of gene expression

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Gene: ATGAGTAACGCG Nontemplate strand
TACTCATTGCGC Template strand

↓ Transcription

mRNA: AUGAGUAACGCG

↓ Translation

Protein: MetSerAsnAla

Structures of amino acids

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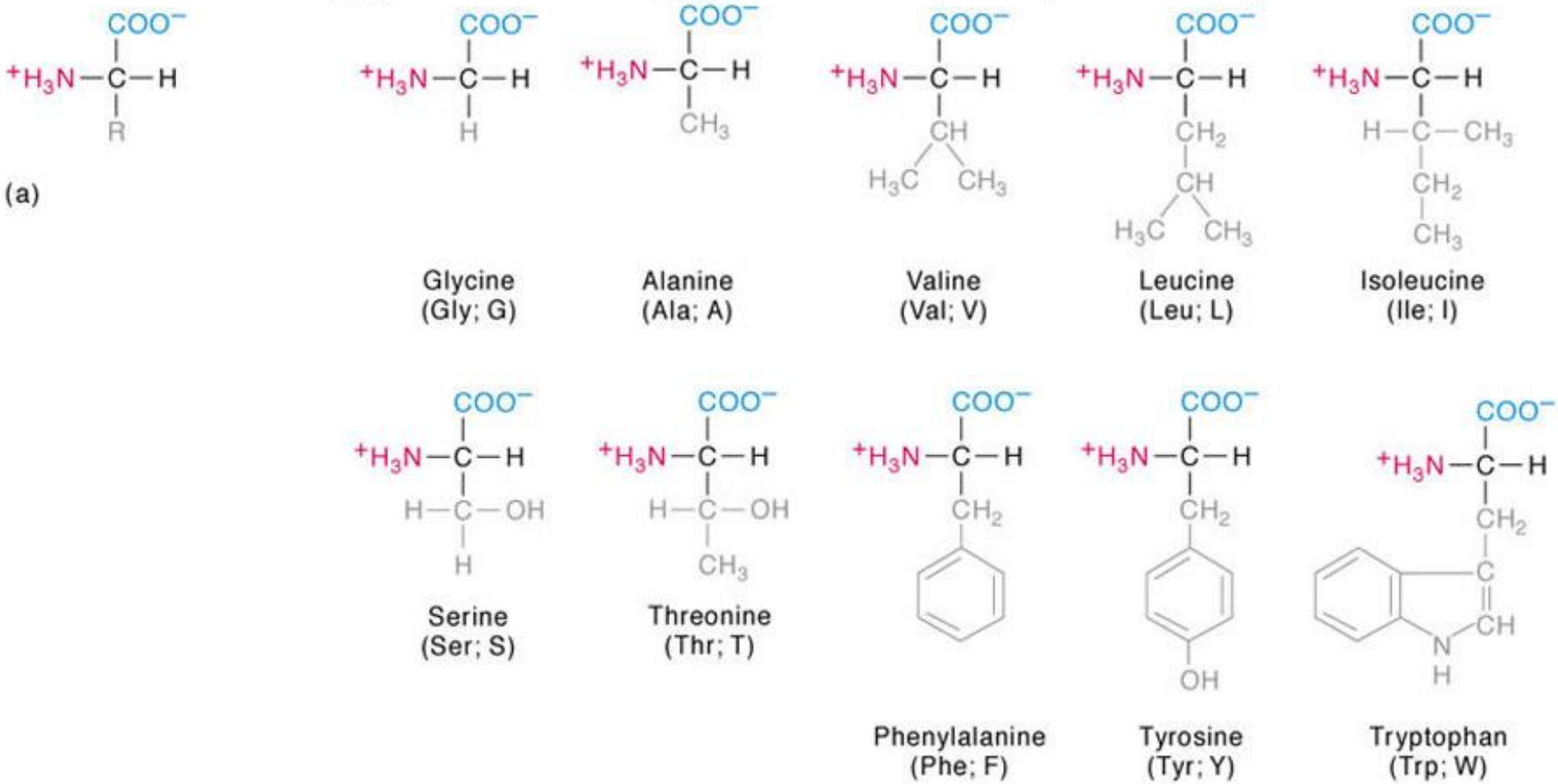

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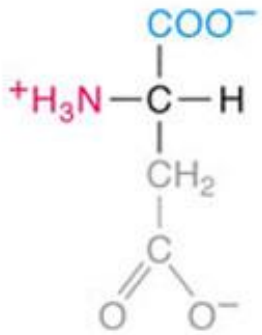
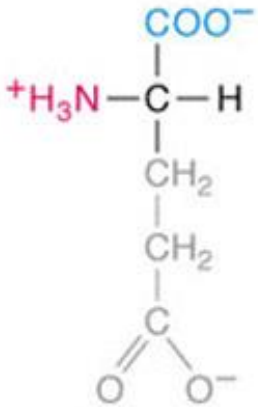
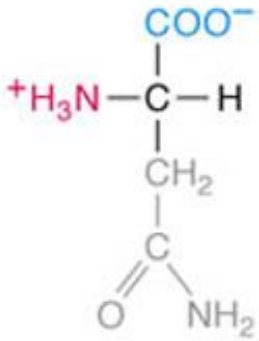


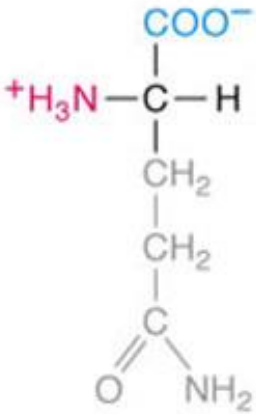
Aspartate
(Asp; D)



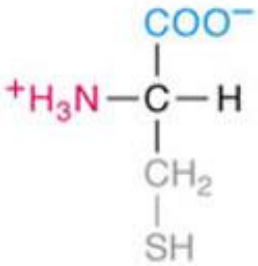
Glutamate
(Glu; E)



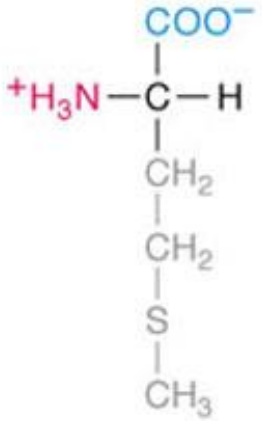
Asparagine
(Asn; N)



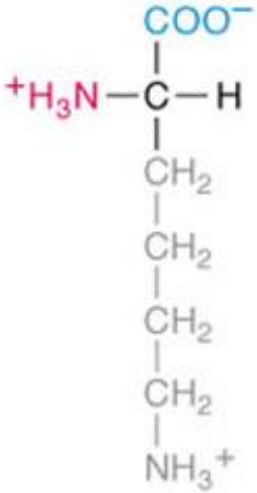
Glutamine
(Gln; Q)



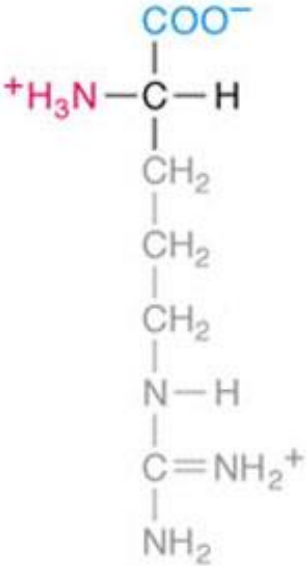
Cysteine
(Cys; C)



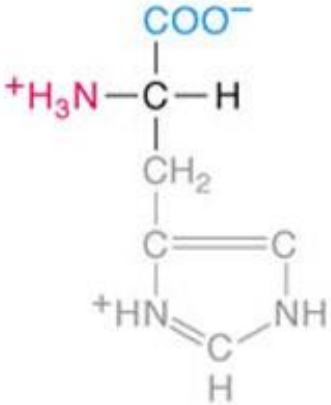
Methionine
(Met; M)



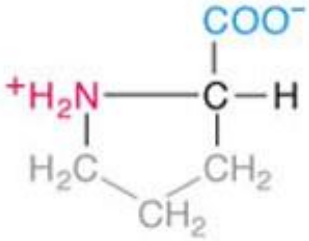
Lysine
(Lys: K)



Arginine
(Arg: R)



Histidine
(His; H)



Proline
(Pro; P)

(b)

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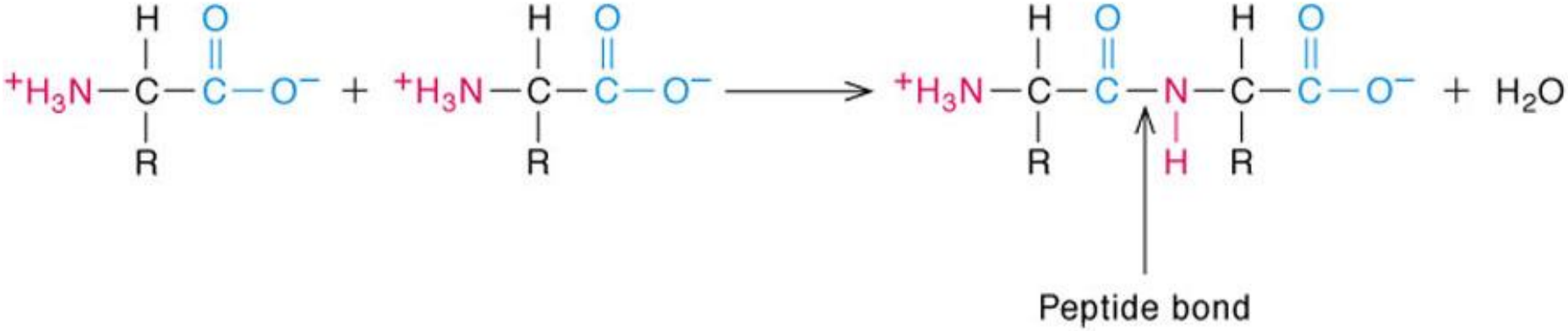
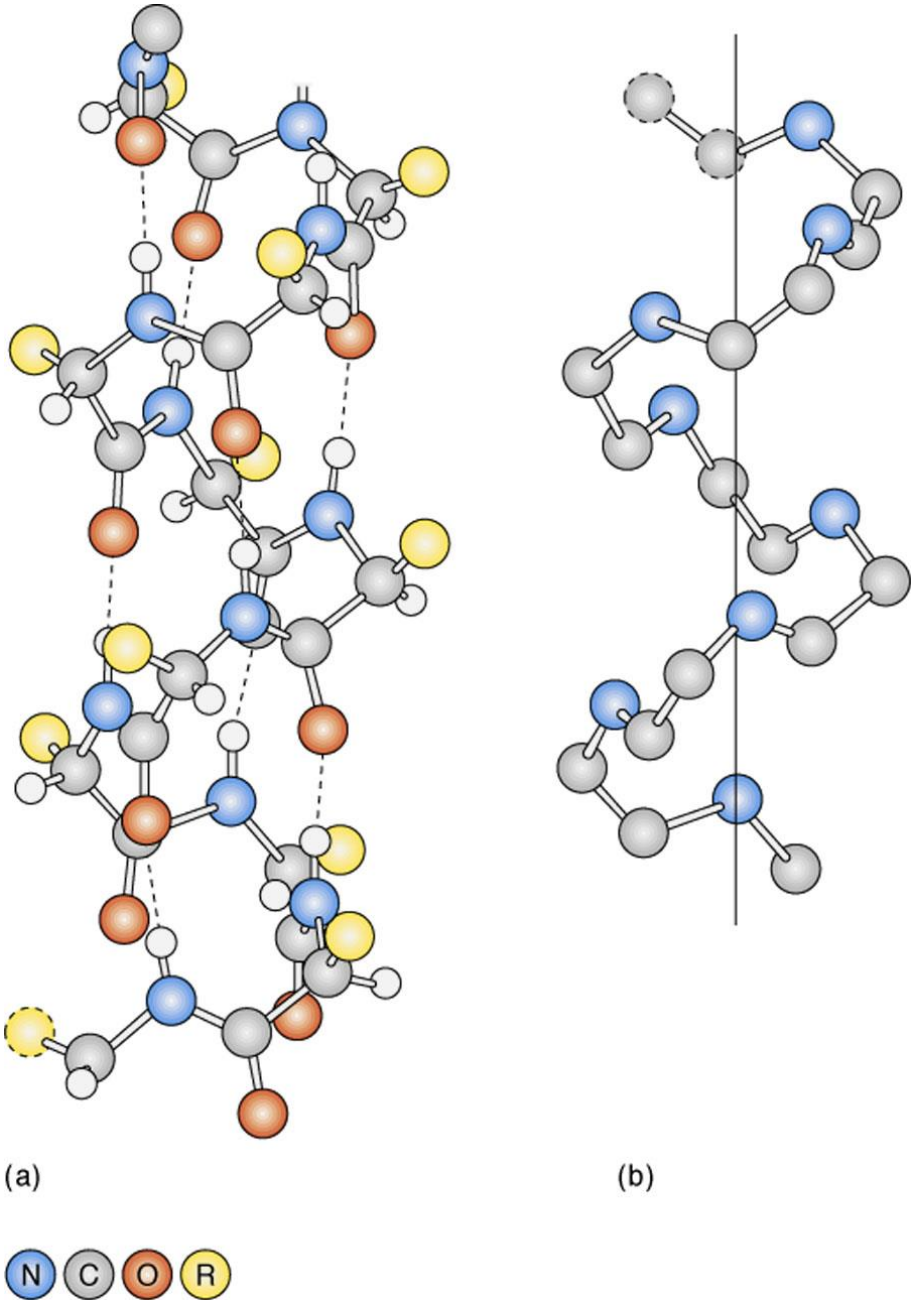


Protein structure

- The amino acid sequence of a protein is the primary structure
- Interaction of amino acids with their neighbours gives rise to secondary structure
  - α-helix: results from H- bonding among nearby amino acids
  - β-Pleated sheet: extended protein chains packed side by side and interact by H- bonding
- The three dimensional shape of a polypeptide is its tertiary structure: involves twisting and folding of the chains

α-helix



β-pleated sheet

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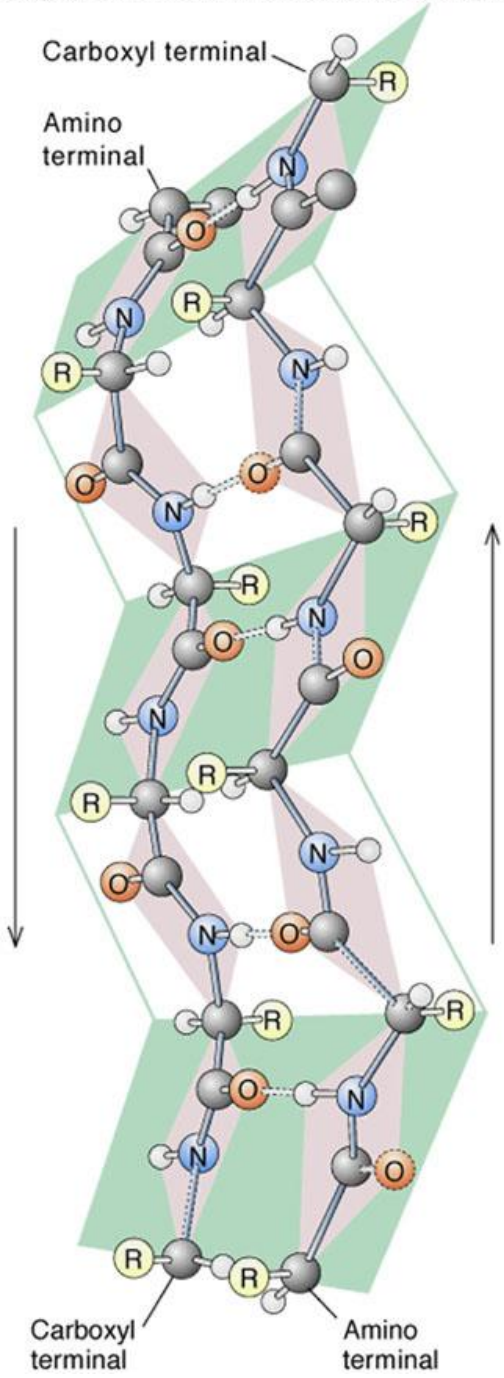
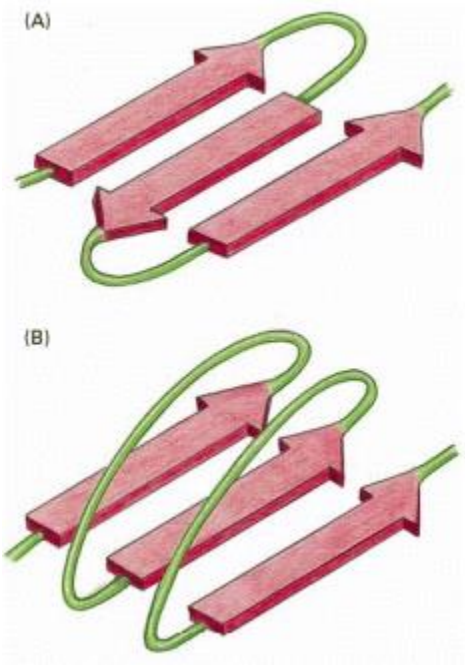


Protein structure

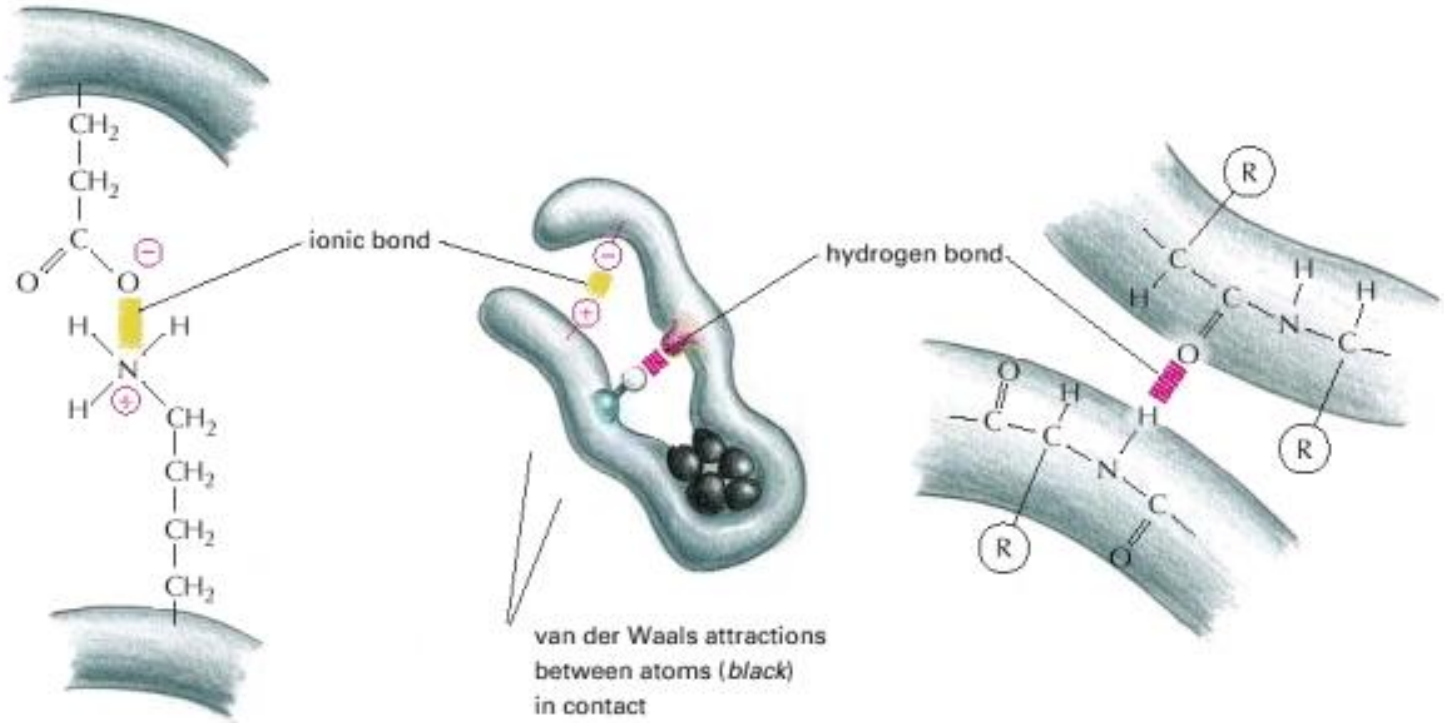
- The distribution of its polar and nonpolar amino acids govern the folding of any protein.
- Three noncovalent bonding systems drive folding in proteins – ionic interactions, hydrogen-bonding, van der Waals interaction. Though weak individually, their combined effect creates a strong bonding.
- An additional bonding system, the hydrophobic interaction also determines shape of the protein

Protein structure

- An alpha helix is generated when a single polypeptide chain twists around on itself to form a rigid cylinder.
- A hydrogen bond is made between every fourth peptide bond, linking the C=O of one peptide bond to the N–H of another to produce a regular helix with a complete turn every 3.6 amino acids.



Protein structure



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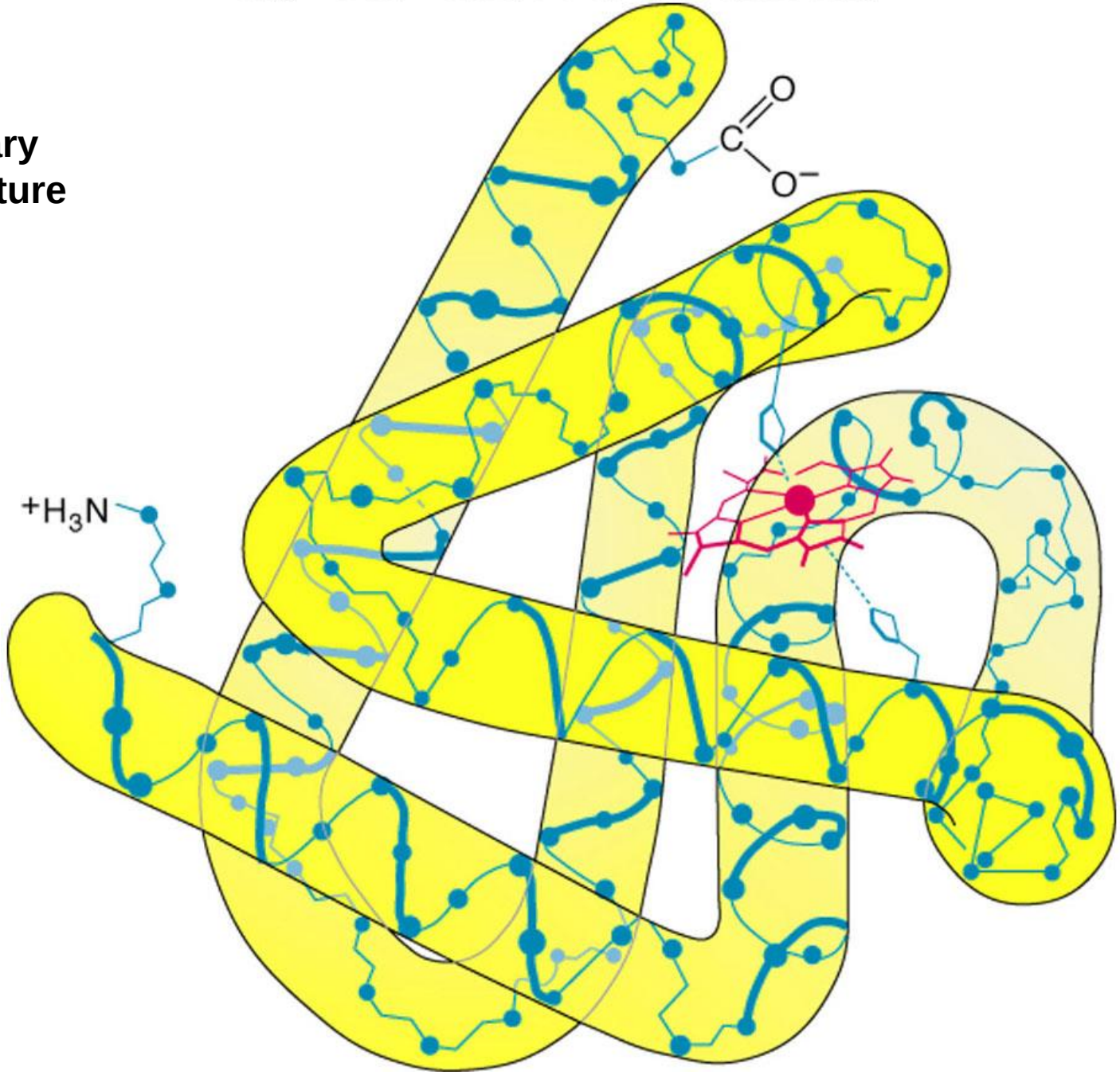
Tertiary structure



BCHEM 365
Lecture 4
March 15, 2022

Theory and methods of electrophoresis

- What is electrophoresis?
- It is the motion of charged particles in a colloid under the influence of an electric field
- Particles with a positive charge move toward the cathode and particles with a negative charge move toward the anode
- The phenomenon was observed for the first time by F. Reuss in 1807
- In his experiment, he found that application of constant electric field caused clay particles dispersed in water to migrate
- Electrophoresis is therefore a technique for separating different types of molecules based on their patterns (size, shape, charge) of movement in an electric field
- Many important biomolecules, such as amino acids, peptides, proteins, nucleic acids possess ionizable groups. At any given pH, these exist in solution as electrically charged particles. Under influence of electric field, the charged particles migrate either to the cathode or to the anode, depending on their net charge

Theory and methods of electrophoresis

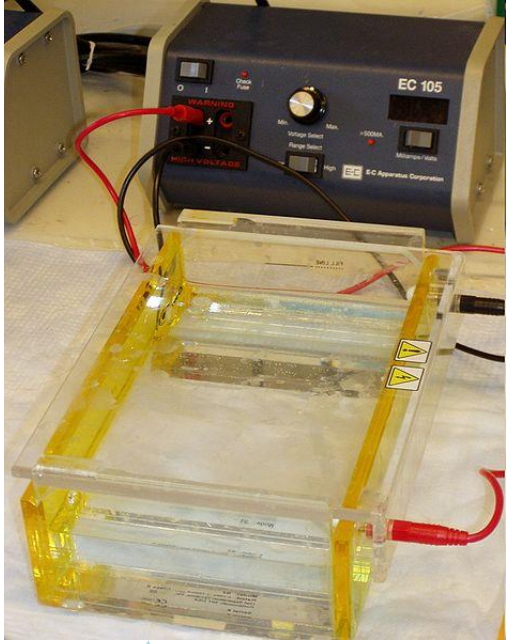
Equipment required:

1. A power pack
2. An electrophoresis unit

Electrophoresis units are available for running either **vertical** or **horizontal** gel system

Vertical gel systems are routinely used for protein separation on acrylamide gels

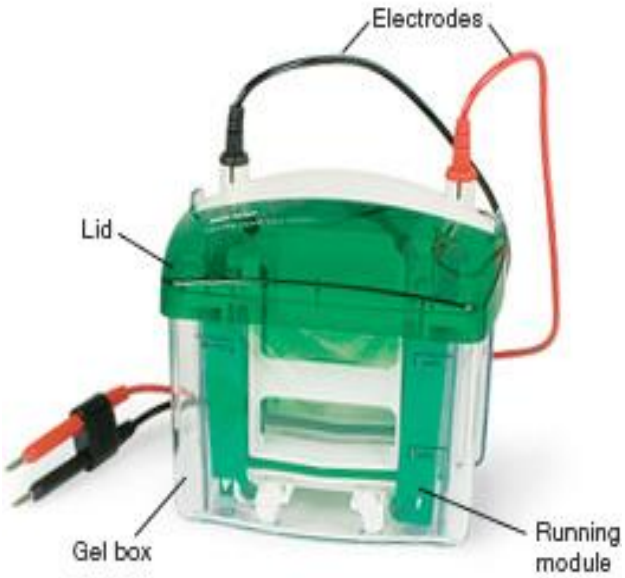
Horizontal gel systems are used for DNA separation on agarose gels



Power pack

Horizontal unit

Electrophoresis unit



Vertical unit

Equations relating to electrophoresis

- When a potential difference (voltage) is applied across the electrodes, it generates an electric gradient, *E*
- *E* is the applied voltage, V, divided by the distance, *d*, between the electrodes (E=V/d)
- In the presence of this electric gradient *E*, the force on a molecule bearing a charge of *q* coulombs is given by *Eq* newtons.
- *Eq* is the force that drives a charged molecule toward an electrode

Equations relating to electrophoresis

- As the charged particle moves toward the electrode, it encounters frictional resistance imposed by the medium that retards its movement

This frictional force is governed by the following four characteristics of the medium and particle:

  - Hydrodynamic size of particle
  - Shape of the particle
  - Pore size of the medium in which it is moving
  - Viscosity of the buffer in which it is immersed

- The velocity, *v*, of a charged particle in an electric field is therefore given by the force on the particle divided by the resistance to movement, i.e.,

$$v = Eq/f$$

where *f* is the frictional coefficient

Equations relating to electrophoresis

- A more common term used to describe the characteristic movement of a particle in electrophoresis is electrophoretic mobility, μ,
- μ is the ratio of the velocity of the charged particle to the field strength (the electric gradient)

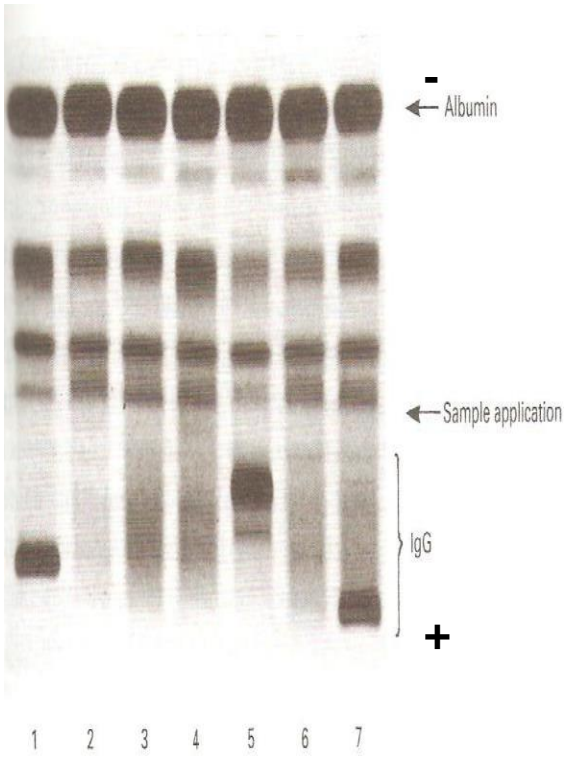
$$\mu = v/E$$

- When a potential difference is applied, molecules with different overall charges will begin to separate out owing to their different electrophoretic mobilities
- Molecules with similar charges will begin to separate if they have different sizes, since they will experience different frictional forces

Equations relating to electrophoresis

- Some forms of electrophoresis rely totally on the different charges on the molecules to achieve separation
- Other methods exploit differences in molecular size and therefore encourage frictional effect to bring about separation
- Movement must be monitored to prevent particles from moving beyond the electrodes
- By the time the electric field is removed, mixtures of molecules would have moved at different velocities toward the electrodes, thereby separation is achieved

Equations relating to electrophoresis



- The separated samples are then located by staining with an appropriate dye or by autoradiography if the sample is radiolabeled

Some technical problems with electrophoresis- Heating

- The current in the solution between the electrodes is conducted mainly by the buffer ions, with a small proportion conducted by the sample ions
- Ohm's law expresses the relationship between current, I, voltage V, and resistance R
- Ohm's law: $V=IR$
- Increase in applied voltage leads to increase in current, hence acceleration of electrophoretic separation

Some technical problems with electrophoresis- Heating

- The velocity of migration of particles, hence the distance moved will be proportional to both current and time
- Increase in current causes faster movement of particles and ions, which leads to generation of heat, a major problem in electrophoresis

Equations relating to electrophoresis

- During electrophoresis the power, W in watts, generated in the support medium is given by

$$W = I^2 R$$

- Most of this power is dissipated as heat
- Heating of the electrophoretic medium produces the following effects:
  1. Increased rate of diffusion of sample and buffer ions leading to broadening of separated samples
  2. Formation of convection currents, which leads to mixing of separated samples
  3. Denaturation of thermally unstable samples, such as proteins,
  4. Decrease in buffer viscosity, hence reduction in the resistance of the medium

Elecctrophoresis and heating problems

- Constant heat generation is a problem. One way of overcoming the heating problem is to run electrophoresis at very low power, i.e. low current
- Running electrophoresis at low current slows down movement of ions, long separation times are required, poor resolution

Electrophoresis and heating problems

- Compromise conditions have to be sought, with reasonable power settings in order to have acceptable separation times, and an appropriate cooling system to remove liberated heat
- With the use of such systems, heating is not always totally eliminated. With the use of cylindrical tubes or gel slabs, heat is removed only from the edges, resulting in a temperature gradient within the gel, with the center being hotter than the edges
- Since the warmer fluid at the center is less viscous, electrophoretic mobilities are greater in this region. Note that for every 1°C rise in temperature, electrophoretic mobility increases by 2%
- Electrophoretic zones develop a bowed shape, with the zone center migrating faster than the edges

Electrophoresis and electroendosmosis

- Presence of charged groups on the surface of the support medium establishes a phenomenon known as <u>electroendosmosis</u> (or electroosmotic flow). For example,
- Paper support medium contains carboxyl groups on its surface
- Agarose, depending on the purity grade contains sulfate groups
- The surface of glass walls used in capillary electrophoresis contains silanol (Si-OH) groups

Electroendosmosis

- **Electroendosmosis (EEO)** - movement of liquid through the gel. This liquid originates from (1) the water used to prepare the gel, which indeed has to be trapped by the gel material, (2) movement of counter cations from both the gel and buffer toward the cathode, while anionic groups in the gel are affixed to the matrix and cannot move, giving rise to EEO.
- Since electrophoretic movement of biopolymers is usually toward the anode, EEO can disrupt separations because of internal convection.

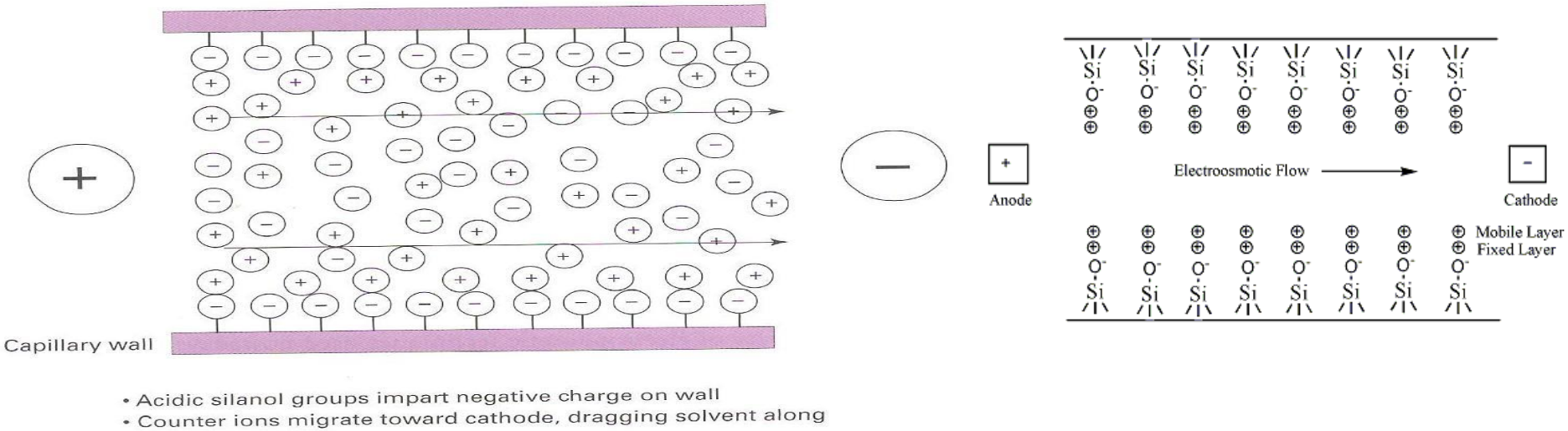


Fig. 1. Electroosmotic flow through a glass capillary. electrolyte cations are attracted to the capillary walls, forming an electrical double layer. When voltage is applied, the net movement of electrolyte solution toward the cathode is known as endosmotic flow

Electrophoresis and electroendosmosis

- Silanol groups on the surface of glass ionize above pH 3 generating negatively charged sites
- It is these charges that generate electroendosomosis. The ionized silanol groups create an electrical double layer, or a region of charge separation at the capillary wall/electrolyte interface. When voltage is applied, cations in the electrolyte near the capillary wall migrate towards the cathode, pulling electrolyte solution with them. This creates a net electroosmotic flow toward the cathode.
- It is a diffusion process which is undesirable as it affects the performance of electrophoresis

Support media

- The pioneering work on electrophoresis by Arne Tiselius et al. (1930) was performed in free solution
- It was soon realized that many of the problems associated with this approach, particularly the adverse effects of diffusion and convective currents could be minimized by stabilizing the medium
- Stabilization was provided by use of a porous mechanical support
- The support medium cuts down convective currents and diffusion so that the separated components remain as sharp zones

Support media

- The earliest support materials were filter paper or cellulose acetate strips, wetted in electrophoresis buffer. Not in common use in recent times, though cellulose acetate still has its uses
- For many years, small molecules like amino acids, peptides and carbohydrates were separated and analyzed on supports such as paper or thin-layer plates of cellulose, silica or alumina, but presently analyzed by more sensitive methods such as high performance liquid chromatography (HPLC)
- Separation of macromolecules such as proteins and nucleic acids on paper or thin-layer plates was found to be poor
- **Gels** were therefore developed as support media for macromolecules.

The gel

- The earliest gel system used was starch gel. Nowadays majority of electrophoretic techniques involve agarose or polyacrylamide gels as support media
- In most cases, the gel is a crosslinked polymer whose composition and porosity is chosen based on the specific weight and composition of the molecules being separated

- When separating **proteins**, small **nucleic acids** or oligonucleotides, different concentrations of **acrylamide** and a **cross-linker** molecule are used to produce varying- sized mesh of networks of polyacrylamide
- When separating larger nucleic acids, usually greater than a few 100 bases the preferred matrix is purified **agarose**

Agarose gels

- Agarose is a linear polysaccharide (average molecular mass = 12,000 Daltons), made up of the basic repeating unit agarobiose
- Agarobiose comprises alternating units of 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose; may have carboxylate, pyruvate, or sulfate substitutions.
- Agarose is one of the components of agar, a mixture of polysaccharides isolated from certain seaweeds

Seaweed

Gelling Temp: >35° C

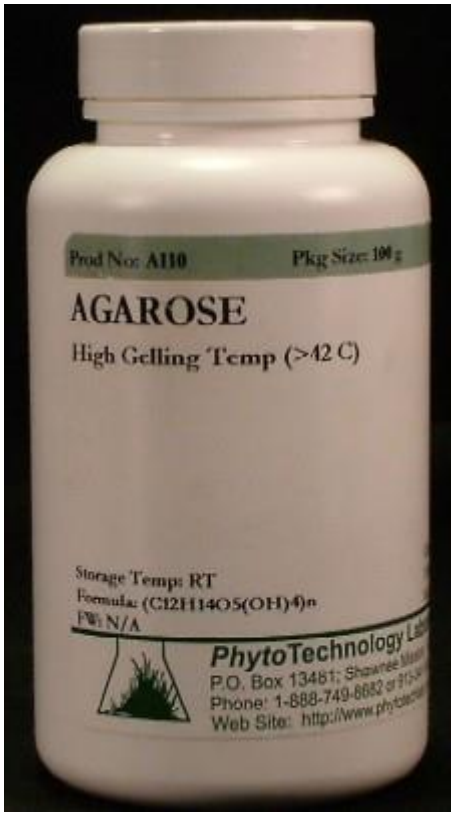
► Formula: $(C_{12}H_{14}O_5(OH)_4)_n$

► Soluble in Hot Water

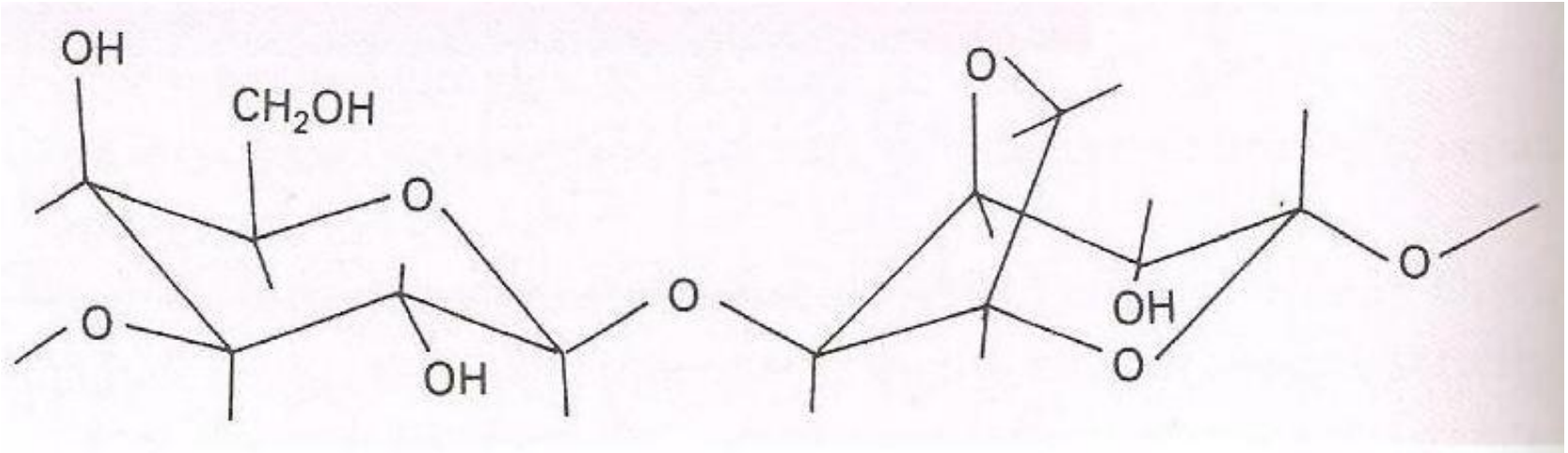
► 100 g costs $307.75

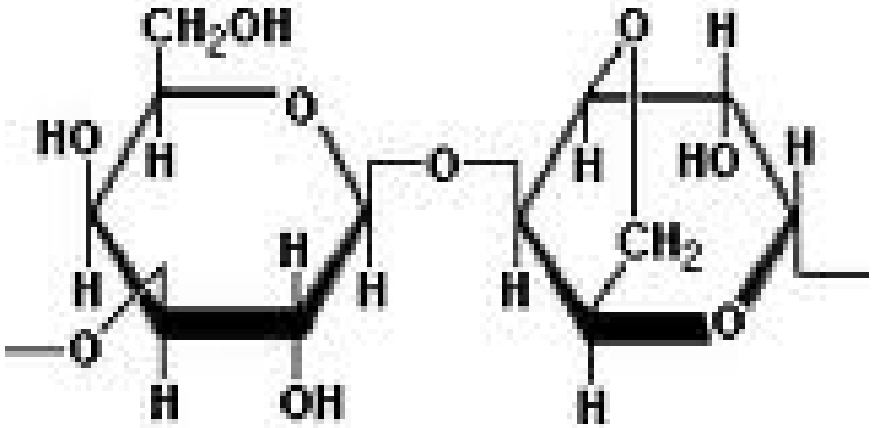
► Manufacturers prepare special grades of agarose for scientific experimentation

► Purified agarose is in powdered form, and is insoluble in water (or buffer) at room temperature



Agarobiose - the repeating unit of agarose





Preparation of Agarose gels

- Agarose is usually used at concentrations of between 1% and 3%. For large DNA fragments (5–10kb) 0.7% gels give good resolution. For small 0.2–1kb fragments 2% gels give good resolution. Beyond 3%, gel become brittle and breaks easily
- Agarose dissolves in boiling water. In solution it forms a random coiled structure.
- When it starts to cool, the agarose chains form helical fiber bundles held together by hydrogen bonding. It undergoes **polymerization,** where the long polysaccharides crosslink with each other in junction zones by formation of more hydrogen bonds, causing the solution to "gel" into a semi-solid matrix

Preparation of Agarose gels

- Increase in agarose concentration produces a firmer gel
- While the solution is still hot, we pour it into a mold called a "casting tray" so it will assume the shape of the tray
- To make 40 ml of 1% agarose: weigh 0.4 g of agarose, dissolve in 40 ml TAE buffer (Tris-Acetate-EDTA). Pour on gel plate and allow to cool to room temperature to form a rigid gel

Preparation of Agarose gels

- Gelling properties of agarose attributed to both inter- and intramolecular hydrogen bonding within and between the long agarose chains: cross-linkages
- This cross-linked structure gives the gel good anticonvectional properties

Casting tray

Agarose gels

- Pore size of the gel is controlled by the initial concentration of agarose
- Low concentrations form large pore sizes while high concentrations form small pore sizes
- Also present on the agarose are substitutions of alternating sugar residues with carboxyl, methoxyl, pyruvate and sulfate groups to varying degrees
- This substitution can result in electroendosmosis during electrophoresis: this is undesirable
- Also ionic interactions between gel and sample may occur: unwanted
- Agarose is therefore sold in different purity grades, based on the sulfate concentration
- The lower the sulfate content, the higher the purity

Agarose gels

- Agarose gels are used for electrophoresis of both proteins and nucleic acids
- For proteins, pore sizes of a 1% agarose gel are large relative to the sizes of proteins
- Agarose gels are therefore used in techniques such as immunoelectrophoresis or flat-bed isoelectric focusing, where the proteins are required to move unhindered in the gel matrix according to their native charge
- Such large pores of agarose gels are used to separate large molecules such as DNA and RNA because the pore sizes in the gel are large enough for these molecules to pass through the gel
- If molecular size increases, frictional effects begin to play a role in the separation of these molecules

Agarose gels

- Advantage of using agarose is the availability of low melting temperature agarose (62-65°C) gels which can be reliquefied by heating to 65°C. After separation of the samples, DNA bands can be cut out from the gel, returned to solution and recovered
- Horizontal slab gels which make use of agarose are used for immunoelectrophoresis and isoelectric focusing, as well as routine separation of DNA and RNA

Other support media for gel electrophoresis

- Polyacrylamide, starch
- Polyacrylamide gels: most commonly used gel for the following reasons:
- i) They are very stable
- ii) They can be prepared in a wide range of concentrations to give a variety of pore sizes
- iii) They can be made to have a gradient of concentrations in one gel slab
- iv) Polyacrylamide gels good for separating proteins of size 5 to 2,000 kDa because unlike agarose it gives uniform pore size even at high concentration

Other support media for gel electrophoresis

- Polyacrylamide gel was widely used in the Maxam-Gilbert or Sanger DNA sequencing for separation of small fragments up to 1 bp difference

- Electrophoresis in acrylamide gels is often referred to as PAGE (**p**oly**a**crylamide **g**el **e**lectrophoresis)

Formation of polyacrylamide gel

- Two methods are used to polymerize acrylamide: chemical polymerization and photopolymerization

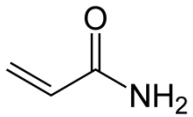
Polyacrylamide gels

Chemical polymerization

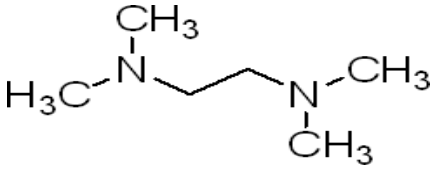
You need the following reagents:

- Acrylamide as the monomer
- A cross-linking agent: N,N’-methylene bis-acrylamide (normally referred to as bis-acrylamide)
- An initiator of the polymerization process: ammonium persulfate
- A catalyst: N,N,N’,N’-tetramethylenediamine (TEMED)

Reagents for chemical polymerization of acrylamide



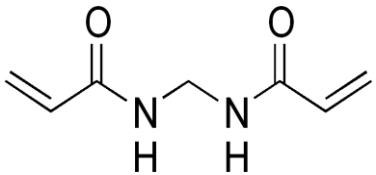
Acrylamide: molecular formula C_3H_5NO
Molar mass: 71.08 g/mol. A white odorless crystalline solid. A known neurotoxin and suspected carcinogen



N,N,N' N'-tetramethylenediamine (TEMED)- the catalyst

$(NH_4)_2S_2O_8$

Ammonium persulfate: molar mass 228.2 g/mol. Free radical is produced in this way:
$S_2O_8^{2-}$ + initiator catalyst → $SO_4^{\bullet-}$ + $SO_4^{\bullet-}$



N,N'-methylene-bis-acrylamide: molecular formula $C_7H_{10}N_2O_2$
Molar mass 154.17 g/mol
A crosslinking reagent

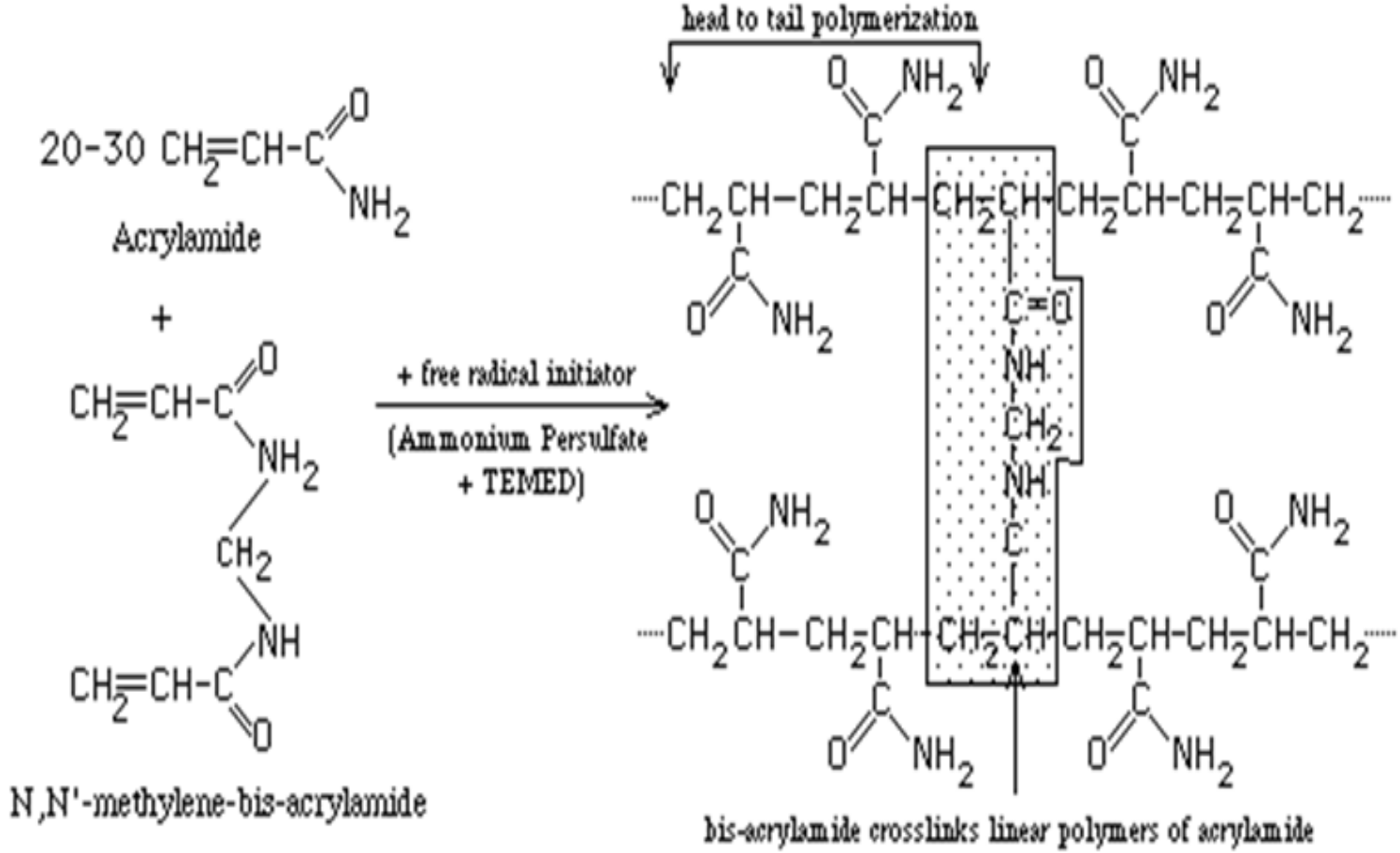
Chemical polymerization

- Proceeds by a free radical polymerization process. Upon addition of ammonium persulfate to acrylamide, the ammonium persulfate dissolves in the water (used to prepare the acrylamide reagent) to form free radicals This process is short-lived, slow, hence needs a catalyst
- An additional catalyst/initiator TEMED is added to enhance the free radical production from persulfate and continue the polymerization process
- TEMED catalyzes the decomposition of persulfate ion to give a free radical
- $S_2O_8^{2-}$ + initiator => $SO_4^{\bullet -}$ + $SO_4^{\bullet -}$
- The free radical initiates polymerization process through **vinyl addition** of the monomers

Chemical polymerization

- Vinyl addition of acrylamide monomers occurs in a **head-to-tail fashion** to build long and linear chains of polyacrylamide
- Addition of bis-acrylamide leads to **copolymerization** of the straight polymer chains to produce a cross-linked polyacrylamide
- Bis-acrylamide is essentially two acrylamide molecules linked by a methylene group and therefore is able to set up a copolymerization reaction in which long and linear polyacrlamide chains are occasionally interspersed with bis-acrylamide molecule, thereby introducing a second site for chain extension

Head-to-tail and copolymerization of acrylamide to form a polyacrylamide network (gel) held together by bis-acrylamide crosslinks



Formation of polyacrylamide gel from acrylamide and bis-acrylamide

- The result is a fairly well-defined three dimensional network of crosslinked polyacrylamide having a range of **pore sizes** and **rigidity**
- The pore size and rigidity can be controlled by the experimenter to enable separation of a wide range of sample sizes.
- Pore size is controlled by changing the **concentration** and **ratio** of acrylamide monomer and bis-acrylamide

Rate of polymerization of acrylamide gels

- The rate of polymerization depends on

(1) The net concentration of monomers and initiators

(2) Temperature

(3) Purity of the reagents

All three should be controlled for reproducibility. Reagents should be electrophoresis grade and deionized water should be used

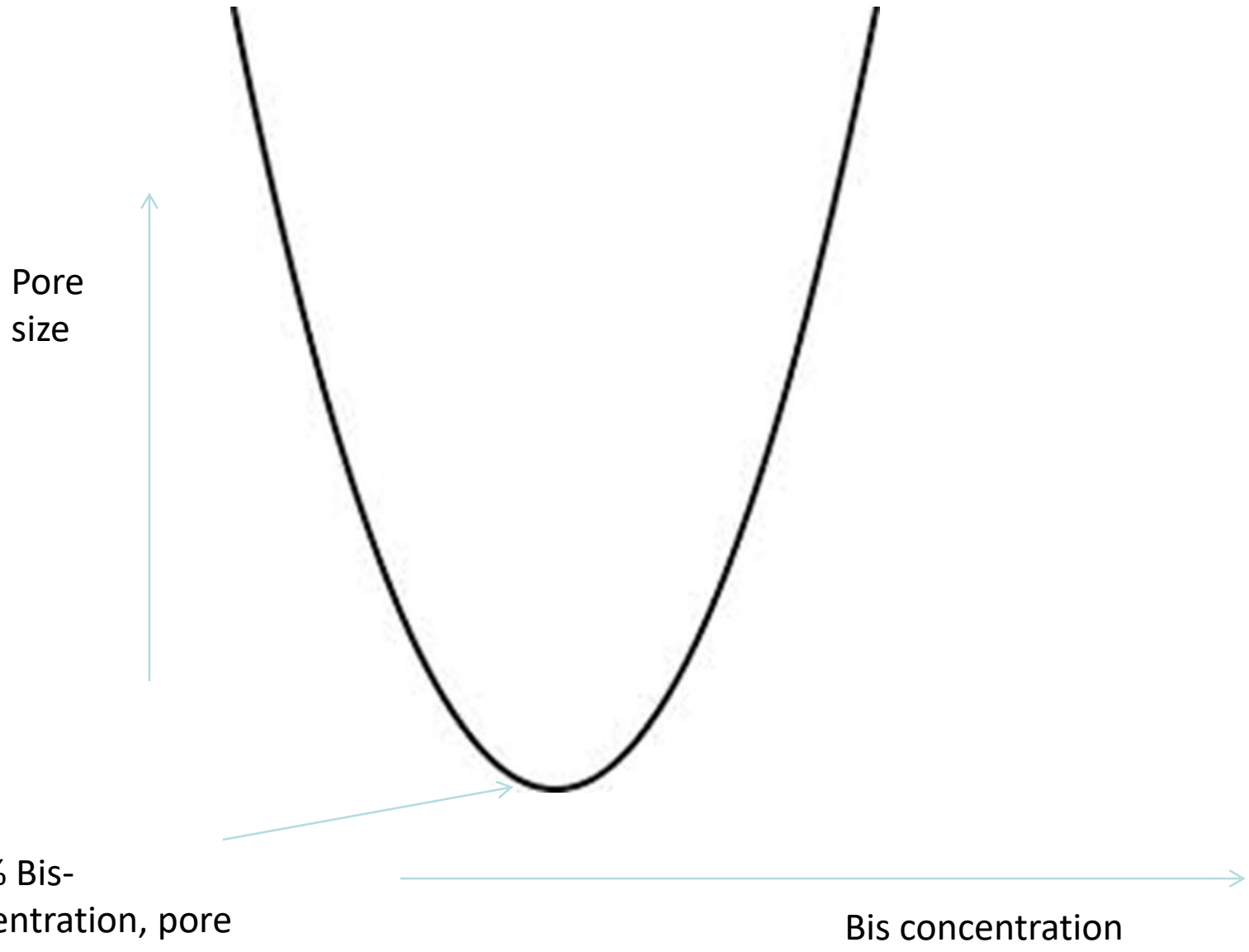
- For highest quality results, dissolved oxygen should be removed from the monomer mixtures by degassing them, since oxygen decreases the rate of polymerization. This is done by briefly placing it under vacuum to remove dissolved air prior to use . Degassing of the gel also serves to remove heat that is liberated during polymerization.
- Polymerization of acrylamide is an exothermic reaction. Warming up of the gel solution as it sets can liberate air bubbles that become trapped in the polymerized gel.
- Air bubbles in the gel affect resolution of the sample

Polyacrylamide gels: pore size and rigidity

- The ratio of bisacrylamide to acrylamide, as well as the total concentration of both components affect the pore size and rigidity of the final gel matrix. These, in turn, affect the range of protein sizes (molecular weights) that can be resolved

- By convention, polyacrylamide gels are characterized by a pair of values, %T and %C, where %T is the concentration of total monomer (acrylamide + bis) in g/100 ml and %C is the proportion of **bis** alone as a percentage of total monomer.

- The effective pore size of a polyacrylamide gel is an **inverse function** of the total monomer concentration (%T) and a **biphasic function** of %C. When %T is increased at a fixed %C, the number of chains increases and the pore size decreases continuously. On the other hand, when %T is held constant and %C is increased from low values, pore size decreases to a minimum at about 5%C.


Pore size
At 5% Bis-concentration, pore size is minimum
Bis concentration

Polyacrylamide gels: pore size and rigidity

- With further increase in %C from the minimum of 5%, pore size increases, probably due to the formation of shorter, thicker bundles of polymer chains.
- A plot of pore size against %C should give a parabola
- Typically 5-20% by weight (5%, 7.5%, 10%, 12.5%, 15%, 20% are commonly used values). To vary pore size, fix concentration of BIS at 5% and vary monomer concentration. Implies gel is mostly water.
- Gels with low %T (e.g., 7.5%T) are used to separate large proteins, while gels with high %T (e.g., 15%T) are used with small proteins.

Properties of polyacrylamide gels

- Polyacrylamide gels are hydrophilic and electrically neutral at the time they are cast. They are transparent to light at wavelengths above 250 nm and do not bind to protein stains.
- The gel has both solid (rigid fibers) and entrapped liquid components which create a three-dimensional shape for the gel. Without the liquid, the gel will dry to a thin film. At the same time, the polymer fibers hold the liquid firmly and prevent it from escape.
- Polyacrylamide gels are well suited for protein electrophoresis

Polyacrylamide gels

- TEMED is a base, hence polymerization is most efficient at alkaline pH. Polymerization efficiency falls rapidly at pH values below 6
- Photopolymerization with riboflavin is used for low-pH gels

Polyacrylamide gels

Photopolymerization

- In this method, riboflavin replaces ammonium persulfate and TEMED. When the gel is poured, it is placed in front of bright light for 2-3 hours
- Riboflavin is decomposed by light to generate free radicals that initiate polymerization of acrylamide

RE-cap on pore size of acrylamide gels

- Polyacrylamide gels can be made to have varying pore sizes
- The size of the pores within the gel can be varied/controlled by changing the concentrations of both the acrylamide and bis-acrylamide

BCHEM 365
Lecture 5
March 21, 2022

Gel Electrophoresis

- Gel electrophoresis is a broad subject encompassing many different techniques
- There are many variations of gel electrophoresis of proteins
- Gel electrophoresis reveals information about **molecular weight**, **subunit structure**, **purity**, and **charges on proteins**
- The most common use of gel electrophoresis is preparative and qualitative analysis of complex mixtures of proteins
- When combined with microanalytical methods and sensitive, linear image analysis systems, gel electrophoresis can be used for quantitative studies of proteins
- Gel electrophoresis provides the highest resolution of all methods available for separating proteins. Polypeptides differing in molecular weight by as little as a few hundreds of Daltons, and proteins which differ by less than 0.1 pH unit in their isoelectric points are routinely resolved on electrophoresis gels.

Gel Electrophoresis

- Gel electrophoresis of proteins produce pure proteins for further work such as:
- Determination of the protein's amino acid sequence
- Use of the protein in fingerprinting studies to reveal polymorphisms and evolutionary relationships, such as in genetic diversity studies
- A pure protein is required to elucidate a protein's tertiary structure, hence its biochemical function by means of X-ray crystallography

Websites for electrophoresis equipment and knowhow

- www.cleaverscientific.com
- www.biorad.com
- www.cbdscientific.com

Variations in gel electrophoresis of proteins

- Tube vs. slab gel electrophoresis
- One dimensional (1D) vs. two dimensional (2D) gel electrophoresis
- Native vs. denaturing electrophoresis
- Continuous vs. discontinuous gel systems

Tube vs. slab gel electrophoresis

- Acrylamide can be polymerized into any desired shape
- Two shapes in common use in electrophoresis
  - **Tube Gels** : polymerize in glass tubing to give cylindrical shaped gel
  - **Slab Gels:** polymerize between glass plates to give a rectangular gel

Tube gel electrophoresis

- Polymerization is carried out in glass tubes of dimensions 7 mm × 10 cm in length
- For isoelectric focusing (I.E.F.) gels and 1st dimension of 2-D electrophoresis
- **Advantages of tube gels:**
- Tube gels are easy to load
- Technique requires minimum use of apparatus
- **Disadvantages:**
- Only one sample can be run per tube, however, there are many places for 12, 24, etc. tubes per vacutainer
- Inaccurate comparison among different samples as different tubes would have different conditions

Apparatus includes solid vacutainer stoppers for variable size tubes. Has a cooling jacket for temperature control and interlocking safety cover

Slab Gel Electrophoresis

- Vertical gel slab sizes range from small (2 cm × 3 cm) to large (15 cm × 18 cm)
- **Advantages of small vertical gels**
  - Require less time and reagents than large gels
  - They are good for rapid screening of samples
- **Advantages** of large vertical gels
  - Provide better resolution
  - Good for separating proteins of similar sizes , as well as a large number of proteins
- Precast slab gels are commercially available for techniques such as SDS-PAGE, native gels, and iso-electric focusing

Precast protein gels for SDS-PAGE

A Thermo Scientific Pierce Precise Protein Gel Cassette. The plastic cassette contains a mini-gel that is 1 mm thick. Dividers along the top provide 10 wells for loading protein samples and molecular weight markers (ladder). Ordinarily, protein bands would not be visible until after electrophoresis they are disassembled from the cassette and and stained.

Slab gel electrophoresis

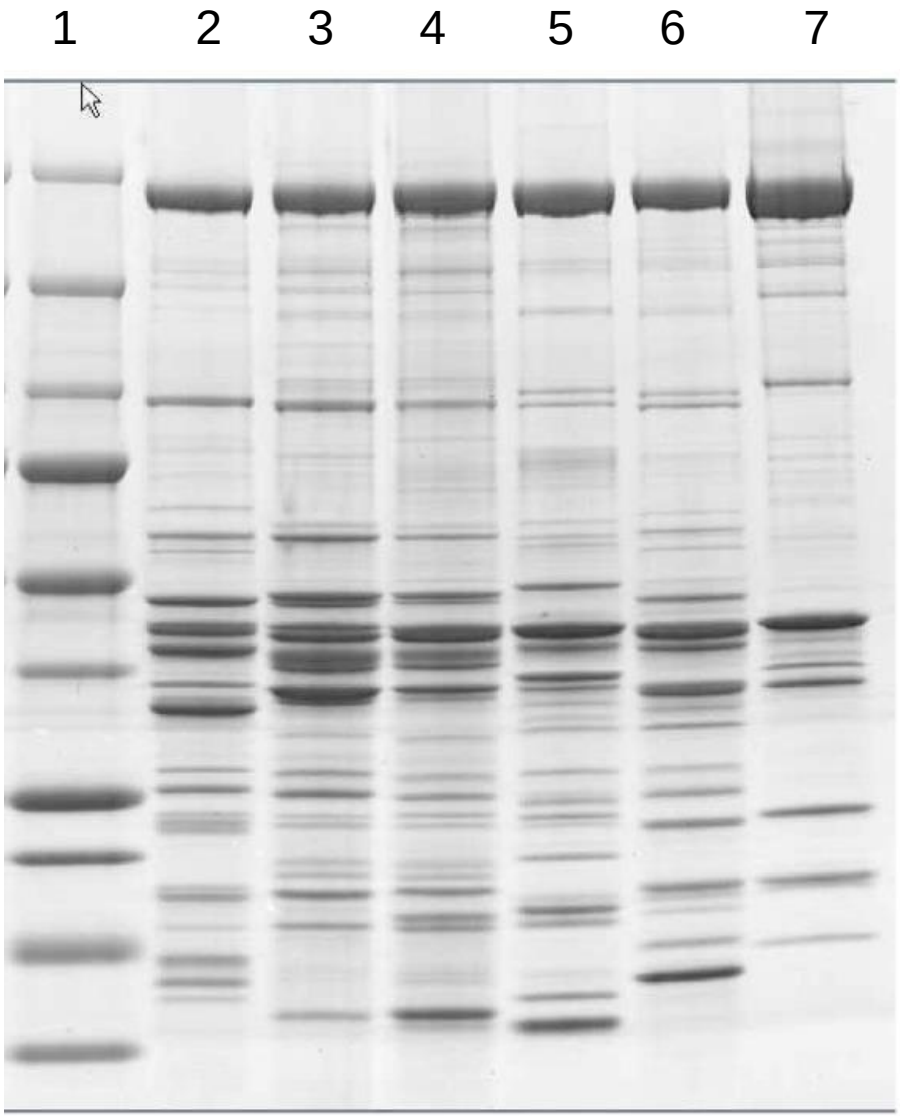
- **Slab gels offer the following advantages**
  - Permit analysis of up to 20 samples on a single gel slab having identical gel conditions
- Applications of vertical gel slabs
  - For routine analysis of proteins
  - For separation of DNA fragments during DNA sequence analysis

One dimensional vs. two dimensional electrophoresis

- Electrophoresis can be one dimensional (1D) (i.e. one plane of separation) or two dimensional (2D)
- One dimensional electrophoresis is used for most routine protein and nucleic acid separations
- It is also used for comparative analysis of multiple samples
- Gel sizes range from 2 cm × 3 cm (tiny) to 15 cm × 18 cm (large format). The most popular size (8 cm × 10 cm) is usually referred to as a "Mini-gel“
- An example of a one-dimensional separation of proteins is shown below. In this configuration, the protein pattern is one of multiple bands with each band containing one protein or a limited number of proteins with similar molecular weights

A typical analytical SDS-PAGE gel showing muscle proteins from five fish varieties and a control from rabbit muscle extract. Samples were separated by SDS-PAGE in a precast mid-size-gel and stained with colloidal CBB G-250.

Lanes are from left to right: marker proteins, shark, salmon, trout, catfish, sturgeon, and rabbit (actin and myosin). The salmon and trout patterns (lanes 4 and 5) are very similar, as expected given the close evolutionary relationship between the two species. All of the fish samples appear to contain muscle proteins similar to those of rabbit.



One Dimension vs. Two dimension Electrophoresis

- Samples that are loaded in adjacent wells and electrophoresed together are easily compared to each other after staining or some other detection system
- The intensity of staining and "thickness" of protein bands are indicative of their **relative abundance**
- The position (distance) of bands measured from the well in each lane indicates the **relative size in Daltons for protein or in base pairs for nucleic acids. Distance move or mobility is a function of size, shape, and charge.**
- Below, we find an electrophoregram of **protein lanes and bands in 1D SDS-PAGE.** Photograph of a mini-gel after removal from the cassette and staining with Coomassie dye (Thermo Scientific GelCode Blue Stain Reagent). The mini-gel has ten lanes, each containing many protein bands of varying abundance

Precast protein gels for SDS-PAGE

A Thermo Scientific Pierce Precise Protein Gel Cassette. The plastic cassette contains a mini-gel that is 1 mm thick. Dividers along the top provide 10 wells for loading protein samples and molecular weight markers (ladder). A common ladder for SDS-PAGE is PageRuler Prestained Ladder. This was loaded in lanes 5 and 8. Another Ladder was loaded in lanes 1 and 6

One dimensional vs. two dimensional electrophoresis

- Two dimensional separation of proteins is used for fingerprinting, and when properly constructed can be extremely accurate in resolving all of the proteins present within a cell (greater than 1,500).
- Two-dimensional separation of nucleic acids is used to resolve over 3,000 transcripts in gene expression studies

Native vs. denaturing gel electrophoresis

- Native: electrophoresis is carried out in the absence of denaturing agents such as heat, reducing agents
- For proteins: the reducing agents, β-mercaptoethanol and Dithiothreitol remove the disulfide linkages between polypeptides. Sodium dodecyl sulfate plus heat denature the tertiary structure and unwind the protein into a single fairly straight polypeptide
- For nucleic acids, urea and formamide denature by forming hydrogen bonds with the bases and occupying the H-bond sites. H-bonds can therefore not form between the bases of the two strands. Again, urea and formamide lower the melting temperature of double stranded DNA and allow denaturation to proceed at low temperature.

Native vs. denaturing gel electrophoresis

- The buffer system in native gel electrophoresis is nonreducing and nondenaturing and maintains the protein's secondary, tertiary, and quaternary structures, as well as its native charge density; and the native structure of dsDNA
- Separation of proteins and nucleic acids in nondenaturing gels is therefore based on their charge, size, and shape, particularly, their charge-to-mass ratio

<u>Separation of proteins by native gels electrophoresis</u>

- Migration of proteins in native gels is governed by the intrinsic charge (the combination of amino acids and post-translational modifications, e.g., addition of phosphates, sialic acid, etc.) on the protein at the pH of the running buffer.

Native vs. denaturing gel electrophoresis

- Migration of the protein also depends on the hydrodynamic size of the unfolded protein, hence, the conformation of the protein.
- A higher mobility is expected for more compact conformations, and lower mobility for larger structures like oligomers
- Native –PAGE for most proteins may be carried out at different pH for acidic proteins, basic proteins, and near-neutral proteins
- The idea is to prevent acid or alkaline denaturation of the protein so that the conformation, self aggregation, and binding of other proteins or compounds is preserved and may be studied

Native Gel Electrophoresis

- There are two methods:

(1) Nondenaturing polyacrylamide gel electrophoresis of proteins

(2) Nondenaturing polyacrylamide gel electrophoresis of nucleic acids

- For proteins, the nondenaturing gel retains the activity of native proteins, because the folded structure, charge, shape and size are preserved allowing the structure of the protein to drive its mobility in the gel. For DNA, the small pore sizes of the polyacrylamide gel is used for small amplified DNA sequences in fingerprinting (10 bp to 3,000 bp)
- Native PAGE does not provide direct measurement of molecular weight, but can be used to estimate protein charge or subunit composition

Native Gel Electrophoresis

- Electrophoretic migration is ensured by running the electrophoresis in alkaline running buffers (usually, pH 8-9 of the Laemmli system). At this pH most proteins carry a net negative charge and the magnitude of the charge is influenced by the intrinsic charges on the protein.
- The higher the negative charge density (more charges per molecular mass), the faster the protein migrates.
- During migration, the proteins encounter the frictional resistance of the gel matrix which creates a sieving effect
- The proteins therefore migrate according to their size and three-dimensional shape. Small proteins face only a small frictional force, while larger proteins face a larger frictional force. Thus native PAGE separates proteins based on both charge and mass.

Native Gel Electrophoresis

- Because no denaturants are used in native PAGE, subunit interactions within a multimeric protein are generally retained and information can be gained about the quaternary structure.
- In addition, some proteins retain their enzymatic activity (function) following separation by native PAGE. Thus, this technique may be used for preparation of purified, active proteins.

Native Gel Electrophoresis

- Native PAGE is good for separating proteins of identical molecular weight but different charges which cannot be resolved with SDS-PAGE.
- Because proteins on native PAGE usually retain their activity, the technique can be used to detect enzymes in their biologically active form

Native Gel Electrophoresis of proteins

- Separation of proteins on native gels depends on the mobility of the protein
- Mobility on native gels is a function of charge, mass, and isoelectric point of the protein and is governed by the equation

$$\log R_m = \log Y_0 - K_R T \ldots\ldots(1)$$

where R_m = relative mobility, normalized to the tracking dye front or some other standard; analogous to retention factor

Y_0 = relative mobility of the protein in free solution, i.e., in the absence of any sieving matrix or a gel. Y_o is related to the charge on a protein

K_R = retardation coefficient or mechanical resistance of the gel. It measures the extent to which a gel matrix impedes mobility. It is related to MW of the protein

T = % monomer (acrylamide)of the gel matrix

Native Gel Electrophoresis

- In the presence of SDS, an anionic detergent, all proteins have the same charge-to-mass ratio and identical relative mobilities in free solution, Y_0
- In such a case, a simple relationship exists between R_m and K_R when %T is known. In a free solution therefore, SDS-treated proteins will migrate at the same speed. Knowing K_R means that one can calculate mobility
- K_R is directly related to molecular weight and so molecular weight can be calculated from K_R

Native Gel Electrophoresis

- In native gels, the situation is more complicated. Both Y_0 and K_R can vary between proteins. Y_0 is related to the charge, while K_R varies with molecular mass or size of the protein.
- Y_0 and K_R are obtained from graphs known as **Ferguson Plots**
- To plot such graphs, protein mixtures and standards are separated on native polyacrylamide gels of varying percentages of acrylamide (%T)

Native Gel Electrophoresis

- The distance travelled by a protein, log R_m , is plotted against %T.

$$\log R_m = \log Y_0 - K_R T.....(1)$$

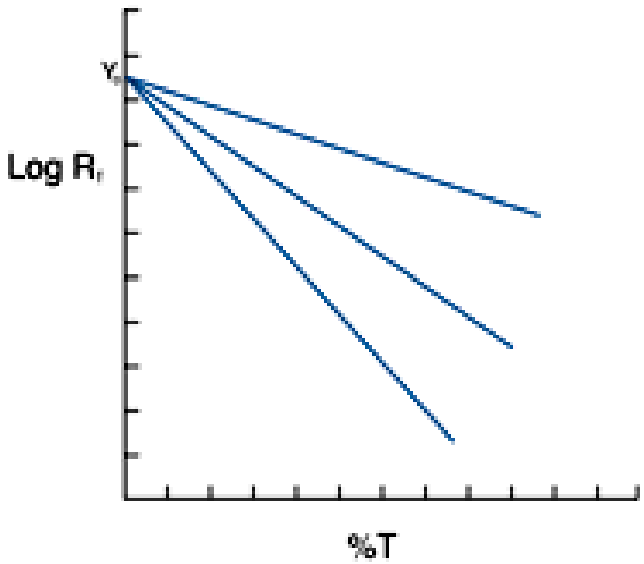
- From equation (1), the graph would have a slope of

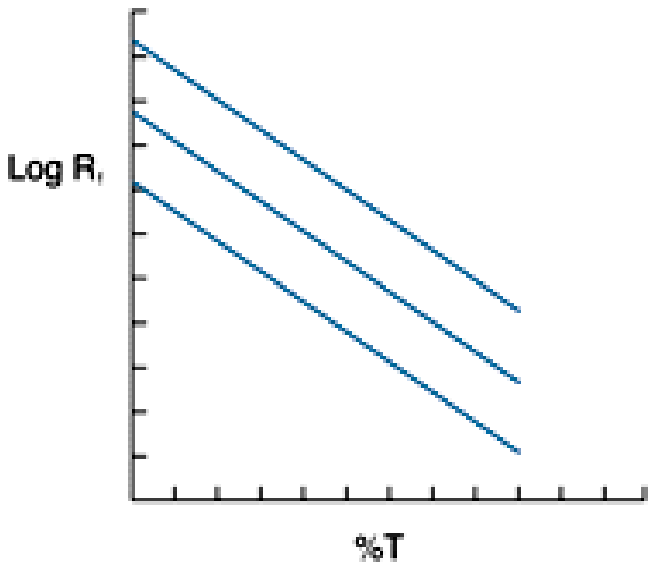
$-K_R$ and a Y intercept (when %T = 0) of Y_o

- Comparison with standards of known charge and size allows determination of the charge and molecular weight of the unknown samples.

Native Gel Electrophoresis

Ferguson Plots





Three proteins of the same charge (Y_0) but different masses

Three proteins of the same mass, i.e., slope (K_R) but different charges

Native Gradient PAGE

- Gradient gels offer a simpler method of interpretation of native gels.
- Gradient gel is made with increasing acrylamide concentration along the length of the gel, hence pore size gradually decreases
- As proteins migrate they enter smaller pore size range and their mobility decreases.
- Eventually, each protein reaches its "pore-limit", at which point it stops migrating.

Native Gradient PAGE

- At the pore limit, there is no movement and the relative positions are a direct reflection of their molecular weight.
- A plot of log MW against the distance moved by each protein (R_m) gives a sigmoid curve. Within the linear gradient range of this curve, log MW is proportional to log R_m over a wide range of molecular weights

Native Gradient PAGE

- Proteins separated by native gradient gels may be visualized by the general protein staining reagents as well as **activity stains** where the protein is likely to be an enzyme. These are stains which enable visualization of enzyme activity.
- Activity staining involves the use of **chromogenic** or **chemiluminescent** compound which reacts with the enzyme (the protein separated) to deposit colored, insoluble compound in the gel, or a luminous product, respectively.
- If the reaction is a direct involvement with the enzyme to produce color, we have a positive stain. If the colored compound is formed in a reaction inhibited by the enzyme, then we have a negative staining technique

Native gel electrophoresis for DNA

Nondenaturing polyacrylamide gel electrophoresis is used for DNA fingerprinting in marker systems such as RAPDs and SSRs to examine small fragment sizes (10-300 bp)

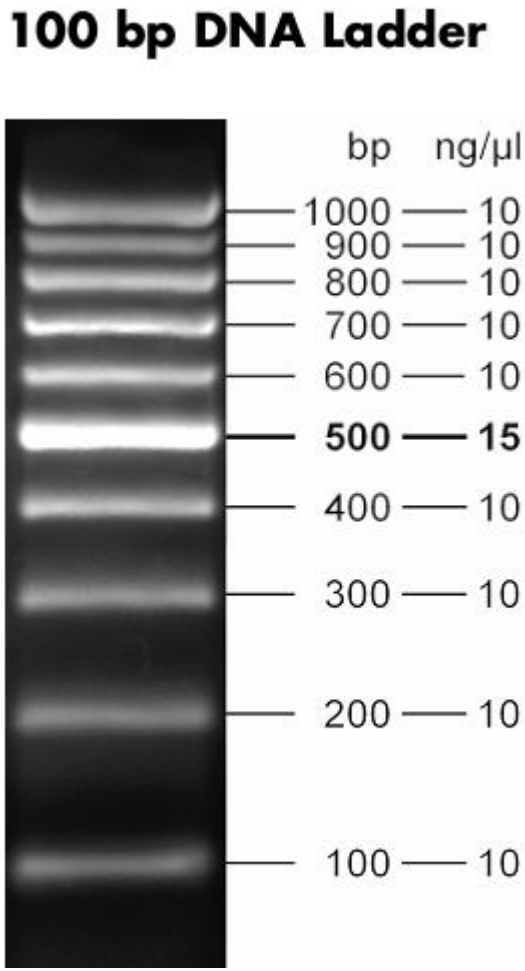
amplified by the primers

RAPDs = Random Amplified Polymorphic DNA sequences

SSRs = Simple Sequence Repeats

It is recommended that the researcher begins with 12% of 29:1 acrylamide/bis-acrylamide as the starting point

Concentration may be reduced (e.g., to 8%) or increased (e.g., to 12%) for larger or smaller fragments, respectively. For nondenaturing gels, Tris-glycine buffer (25 mM Trizma-base, 192 mM glycine) may be used.

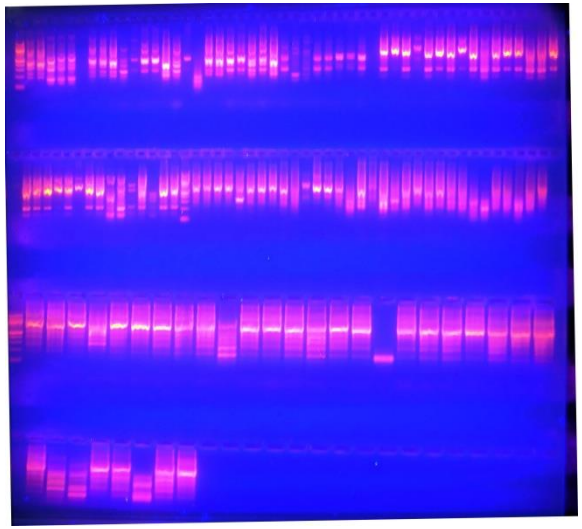


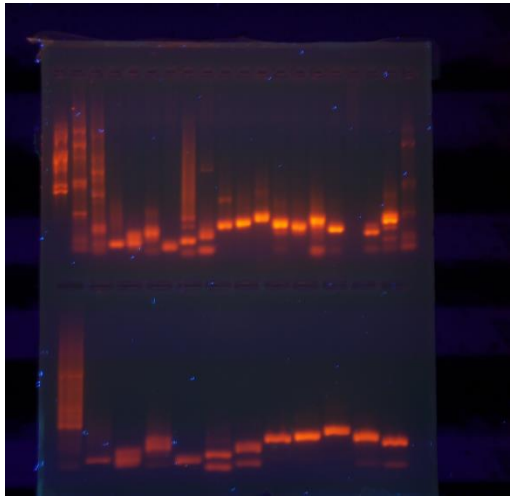
Nondenaturing gels for DNA

Run the gels at constant volatge of 250 V for 2-5 h, depending on the acrylamide concentration.

Generally it takes 2 h for 8%, and 3 h for 12%, 5 h for 16% gels when the tracking dyes, bromophenol blue and xylene cyanol reach the bottom of the gel

RAPDs on agarose gel





SSRs on PAGE

Buffers for electrophoresis

- The electric current in an electrophoresis cell is carried largely by ions in the buffer
- Note that proteins constitute only a small portion of this current
- The buffer system also:

  -maintains the desired pH for native systems and for SDS-PAGE

  -provides a medium for heat dissipation

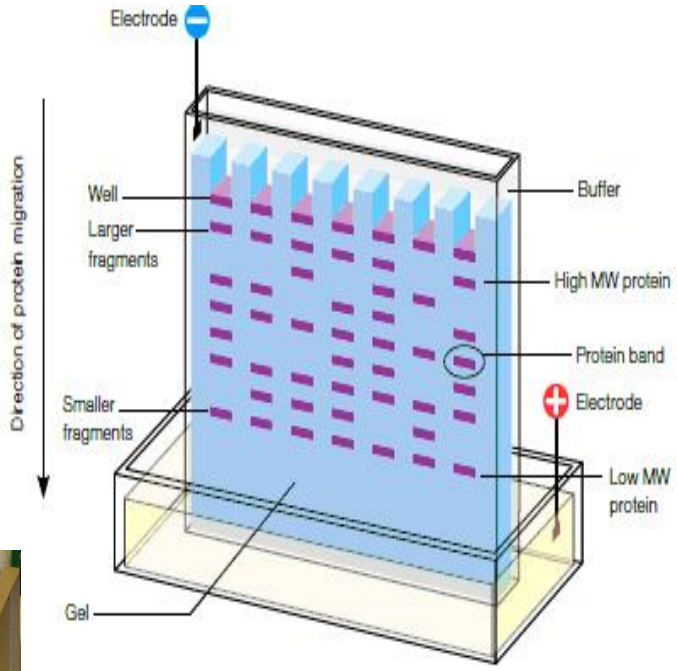
Buffers for electrophoresis

Two classes of buffer systems are commonly used in electrophoresis: continuous or discontinuous

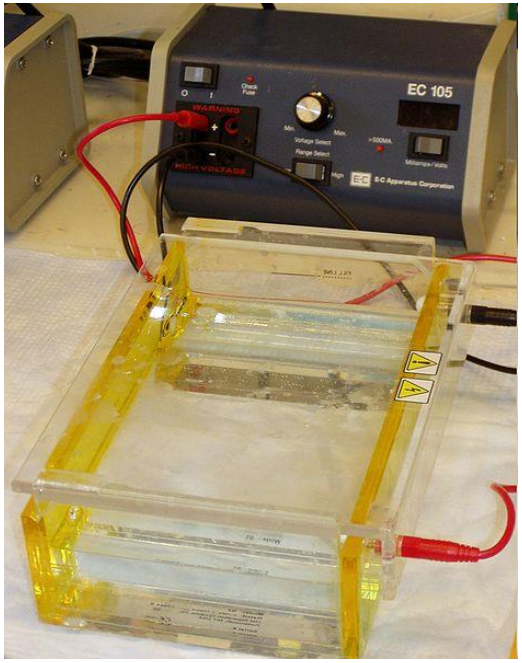
Continuous buffer systems

Continuous systems use one and the same buffer ions at constant pH in the gel, sample, and electrode reservoirs/tank. A **single** separating/resolving gel is used

- Schematic for continuous gel electrophoresis



PAGE



Agarose gel

Continuous gel buffers

- In continuous systems, the sample is loaded directly on the gel in which separation will take place, like a resolving gel.
- Continuous gels are easy to prepare and give adequate resolution for some applications, however, bands tend to be broader and resolution consequently poorer
- To improve on resolution, the strength of the sample buffer is diluted. Dilution leads to decreased buffer ions, a decrease in conductivity and hence localized voltage drop across the sample
- Voltage drop in the sample helps drive proteins into the gel

Continuous gel buffers

- As proteins migrate through the pores of the gel they are separated on the basis of electrophoretic mobility differences.
- The width of bands formed is highly dependent on the height of the applied sample volume
- To prevent smearing of bands, small sample volumes (5-15 microliter) must be applied. This can be achieved by using highly concentrated samples
- In continuous systems, molecular charge density and gel pore size are the only factors that control mobility and resolution
- Larger molecules are better resolved on continuous buffer systems than small molecules because of a small difference between their free solution mobility and their mobility in a gel. Continuous buffer systems work best with highly concentrated samples

Buffers for electrophoresis

- Almost any buffer can be used for continuous gel electrophoresis, but for best results, solutions of relatively low ionic strengths are used
- Buffers of low ionic strength keep heat generation at a minimum. On the other hand, protein aggregation may occur if the ionic strength is too low.
- Buffers for DNA/RNA include TAE (Tris-Acetate-EDTA) or TBE(Tris-Borate-EDTA) . TAE offers a lower buffering capacity, hence conducts lower current and requires more time, but offers better separation.
- Different buffers exist for different proteins : TRIS-glycine, TRIS-tricine, etc.
- Concentrations of electrophoresis buffers are in the range of from 0.01 to 0.1 M

Buffers for electrophoresis

Discontinuous buffer systems

- Discontinuous buffer systems employ different buffers and different pH for tank and gel, and often two different buffers within the two gel systems
- Discontinuous systems are designed to enhance the sharpness of the separated bands by sharpening the starting zones. This is achieved in a process called "**stacking**". It involves concentrating protein samples into a very narrow and tight zone prior to separation. It results in improved band sharpness and resolution.
- Stacking is an electrochemical phenomenon that establishes an ion gradient in the early stage of electrophoresis on the basis of mobility differences between (1) proteins, (2) leading buffer ions and (3)trailing buffer ions.
- Differences in mobilities cause all the proteins to focus into a single sharp band (isotachophoresis)

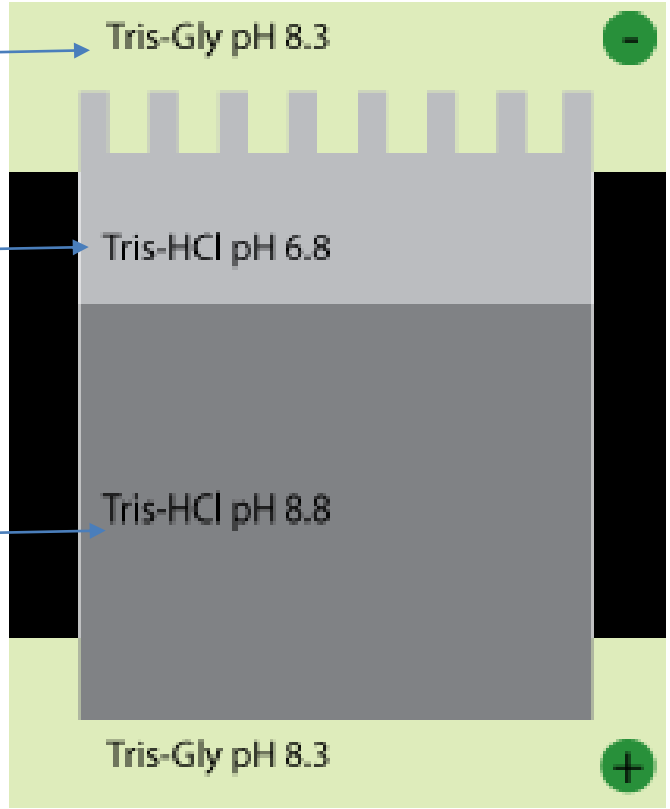
Discontinuous Gel Electrophoresis

<u>A discontinuous gel system</u>

Electrode/tank buffer

Stacking gel buffer, lower concentration gel with large pores

Resolving gel buffer, higher concentration gel with smaller pores



History of discontinuous gel electrophoresis

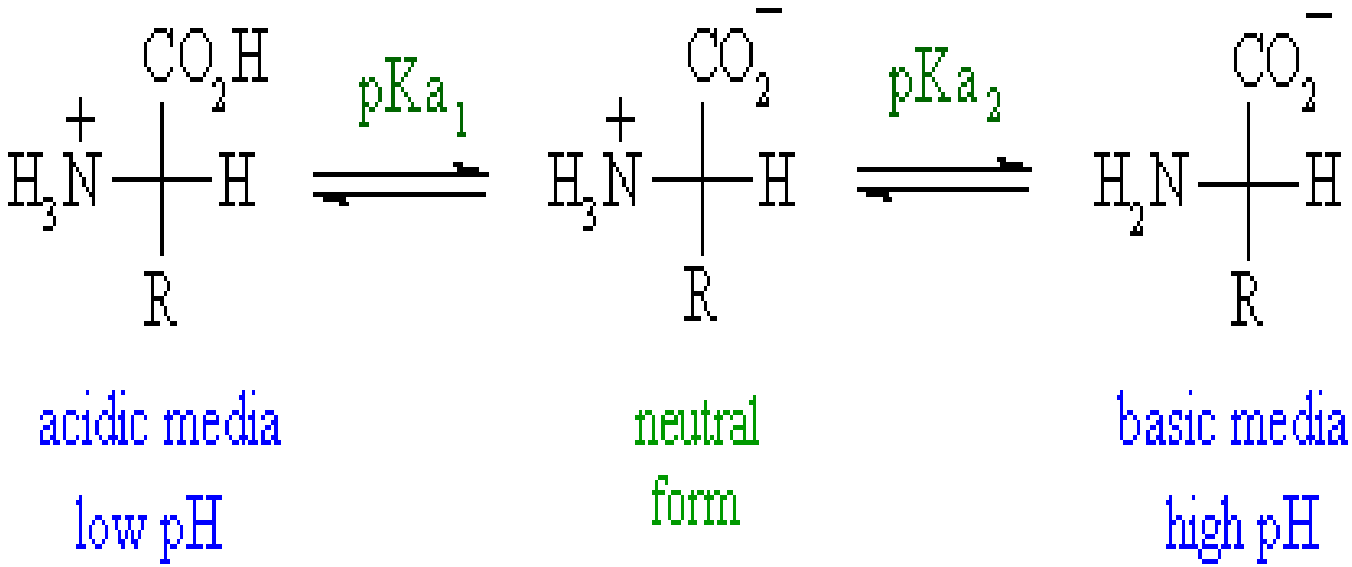
- Starch gels were first used in protein separation
- In 1964, starch gels were replaced by polyacrylamide gels (Ornstein, 1964; Davis, 1964)
- Shapiro et al.(1967) developed SDS-PAGE by including sodium dodecyl sulfate (SDS) to the polyacrylamide gel as well as the sample
- Laemmli (1970) developed protocols (the discontinuous system) to greatly improve the resolution and power of protein identification by SDS-PAGE

History of discontinuous gel electrophoresis

- Laemmli did two things:
- (a) He introduced the discontinuous gel system having different pore sizes:
- (i) Stacking gel: low acrylamide concentration (usually 3%), so no sieving effect; low pH 6.8
- (ii) Resolving (separating) gel: high acrylamide concentration (usually 10%), sieving effect, proteins move freely into the resolving gel; high pH 8.8
- (b) He added glycine and TRIS to improve the ionic strengths and pH of the buffers
- Three buffer systems were produced (1) Stacking gel, pH 6.8 TRIS-HCl buffer; (2) Resolving gel pH 8.8 TRIS-HCl buffer; (3) Tank or electrode buffer pH 8.3 TRIS-glycine
- Control of the charge state of glycine by the different buffers drives the stacking process

Discontinuous gel electrophoresis

- Glycine can exist in three charge states, namely, positive, neutral or negative, depending on pH of the medium shown below



Discontinuous gel electrophoresis

- At very acidic pH (below pKa_1) the amino acid will have an overall +ve charge and at very basic pH (above pKa_2) the amino acid will have an overall -ve charge
- The isoelectric point is average of the two pKas, *i.e.* **$pI = 1/2\ (pKa_1 + pKa_2)$**= ½(2.34 + 9.6), pI = 5.97

Discontinuous gel electrophoresis

- The stacking gel works like this: because pI of glycine is 5.97, in the tank buffer with pH 8.3, glycine will be negatively charged
- When power is turned on, the negatively-charged glycine ions in the tank buffer are forced to enter the stacking gel, where the pH is 6.8.
- At this relatively lower pH close to the pI, glycine switches predominantly to the zwitterion state causing them to move very slowly in the electric field

Discontinuous gel electrophoresis

- In contrast, the Cl^- ions (from Tris-HCl) in the stacking gel with their small size and negative charge move much more quickly in the electric field and form an ion front (leading ion) that migrates ahead of the almost neutral to positive glycine (trailing ion)
- The separation of Cl^- from the TRIS counter-ion (which is now moving toward the anode) creates a highly mobile Cl^- front and a slowly migrating glycine with a narrow zone of steep voltage gradient between them
- The gradient pulls along the glycine but remains behind the Cl^-

Discontinuous gel electrophoresis

- The abrupt voltage drop causes the proteins in the gel sample having an electrophoretic mobility that is intermediate between the extreme of the mobility of the glycine and Cl^-, to move rapidly (sweeping) to fill the gap and prevent a break in the circuit (Kolrausch discontinuity)
- By this, proteins are concentrated (stacked) into a tight band between the Cl^- and glycine fronts, thereby improving resolution

Discontinuous buffer systems

- The width of the stack therefore becomes very thin and compact, having about 100 µm thickness and with protein concentrations of about 100 mg/ml
- Electrophoretic stacking concentrates proteins into regions narrower than can be achieved by mechanical means. This has the effect of minimizing spreading of overall band widths and increasing resolution.
- In order to allow the stack to develop, gels used with discontinuous systems are usually divided into two distinct segments.

Discontinuous buffer systems

- The smaller, upper portion is called the stacking gel. It is cast with appreciably larger pores than the lower resolving gel (or separating gel) and serves mainly as an anticonvective medium during the stacking process. In the stacking gel, no sieving effect occurs.
- Separation takes place in the resolving gel, which has pores of roughly the same size as the proteins of interest. Once proteins enter the resolving gel their migration rates are slowed by the sieving effect of the small pores

Discontinuous buffer systems

- In the resolving gel, the trailing ions pass the proteins and electrophoresis continues in the environment supplied by the electrode buffer. The proteins are said to become “unstacked” in the resolving gel. They separate there on the basis of size and charge
- The electrophoresis monitored and timed by means of the dye front. Migration of the buffer front as it moves through the gel can be followed by the change in refractive index between the regions containing the leading and trailing ions. Addition of a tracking dye that moves with the buffer front aids in visualization of the protein front
- The major advantage of a discontinuous buffer system is increased resolution and sharpness of the sample band

Gel electrophoresis of proteins

Many techniques are available

- **S**odium-**d**odecyl **s**ulfate-**p**olyacrylamide **g**el **e**lectrophoresis (SDS-PAGE)
- ✓ Native (buffer) gels
- ✓ Gradient gels
- Iso-electric focusing
- Two-dimensional polyacrylamide gel electrophoresis
- Cellulose acetate electrophoresis
- Western blotting

SDS-PAGE

- SDS-PAGE is the most widely used method for analyzing **protein** mixtures qualitatively
- It is an example of **denaturing** gel electrophoresis
- A demerit of native PAGE is that the native structure of a protein affects mobility in a gel matrix such that the rate of migration is not a true estimate of the charge:mass ratio nor an accurate reflection of the molecular weight
- In their native structure, proteins fold into a variety of shapes to produce compact or elongated forms. Nature of folding depends on the amino acid sequence of the protein
- To overcome structural effects on mobility, denaturation of the protein is carried out

SDS-PAGE

- Denaturation allows separation of proteins on a true charge/mass ratio basis. It also separates proteins into their individual subunits and permits analysis of large, complex protein aggregates
- Sodium dodecyl sulfate (also called sodium lauryl sulfate) is the most common denaturant used. Other denaturants are Triton X-100 and β-octylglucoside. Both are nonionic.
- The molecular formula of SDS is given below:

SDS-PAGE

- MW: 288.38 $(CH_3-(CH_2)_{10}-CH_2OSO_3^-Na^+)$

- SDS denatures proteins by binding to the protein chain with its hydrocarbon tail, exposing normally buried regions and coating the protein chain with negatively charged surfactant molecules. SDS disrupts most noncovalent bonds, thereby decreasing protein folding.
- Proteins bind the SDS detergent uniformly along their length to a level of 1.4 g SDS/g protein or one SDS to 2 amino acid residues. This creates a charge/mass ratio which is uniform or consistent between proteins.
- Each protein in the mixture is therefore fully denatured by this treatment and opens up into a rod-shaped structure with a series of negatively charged SDS molecules along the polypeptide chain.

SDS-PAGE

- For this reason, separation on a polyacrylamide gel in the presence of SDS occurs by **mass alone,** hence the sieving effect of the gel governs the separation.

- SDS PAGE offers a rapid and relatively accurate way to determine protein molecular weights. Molecular mass determined by SDS-PAGE is usually accurate within 5 - 10% error rate

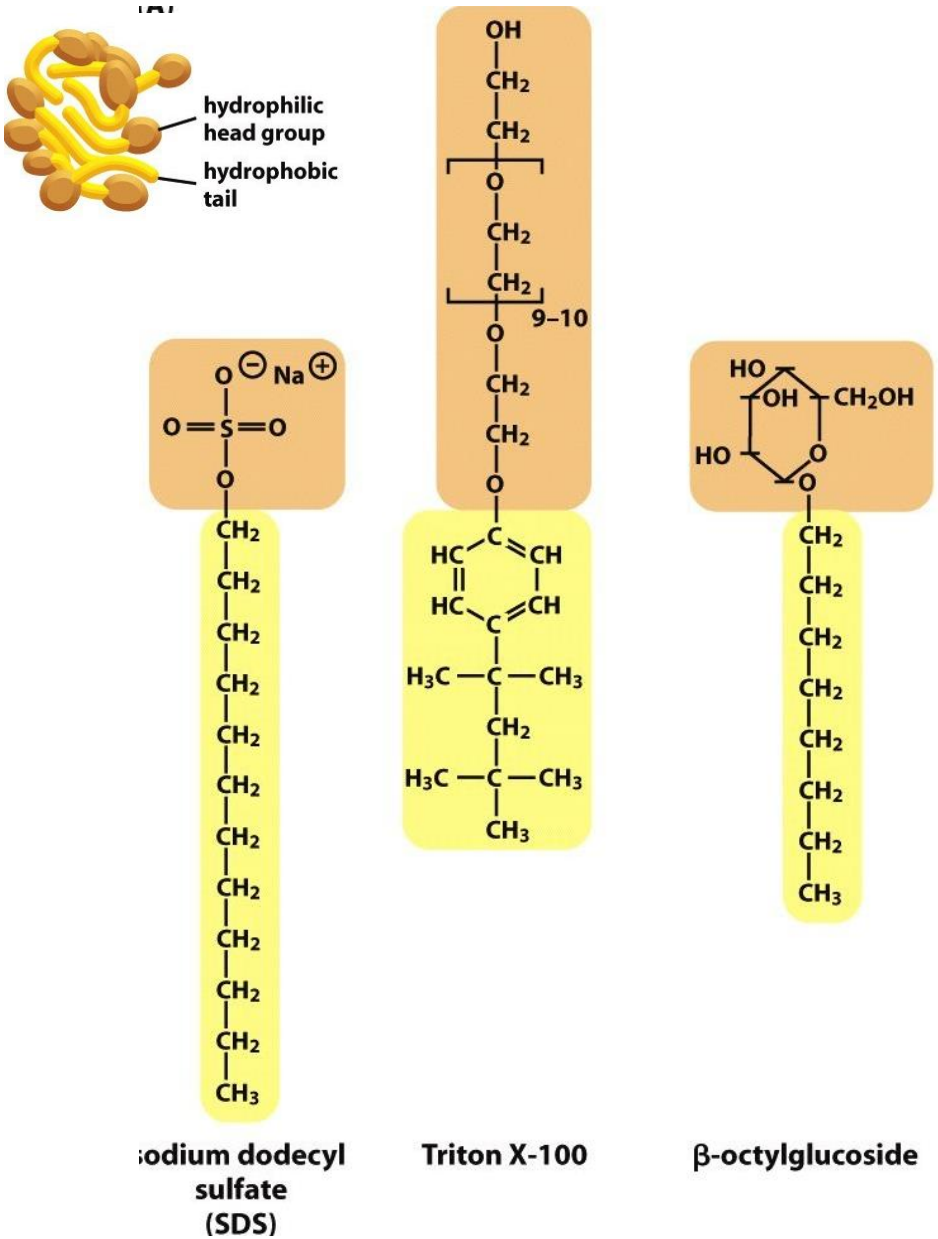
SDS-PAGE

- An exception: some proteins, such as, histones may not be fully denatured and may retain enough secondary structure or contain sufficient charged groups to migrate anomalously so that molecular weight will not be a direct reflection of their mobility. In such a case, Triton X-100 combined with urea and acetic acid are used to enhance denaturation

Denaturation of proteins for SDS-PAGE

- Complete dissolution of sample is required. Undissolved particles present in the sample will lead to clogging of the gel and **smearing** of the protein band from the well to the end of the gel.
- Complete denaturation of protein sample is required. Incomplete denaturation will not fully saturate the proteins with SDS and will lead to blurred bands or altered mobilities

Structure and function of detergent micelles. The three commonly used detergents are sodium dodecyl sulfate (SDS), an anionic detergent; Triton X-100 and β- octylglucoside are two nonionic detergents. Triton X-100 is a mixture of compounds in which the region in brackets is repeated 9 or 10 times. The hydrophobic portion of each detergent is shown in yellow, and the hydrophilic portion is shown in orange.



SDS-PAGE

- SDS is a strong detergent which completely dissolves many cells and tissues by heating to 95°C in loading buffer
- To solubilize more difficult samples such as plant tissue, a stronger loading buffer, containing more SDS and dithiothreitol (DTT) at high pH is used
- **Reagents: sample buffer for tissue culture cells and soft tissue**

  0.5M Tris-HCl, pH 6.8
  4.4% SDS
  300 mM Mercaptoethanol
  10mg/ml Bromophenol blue
- Mercaptoethanol reduces the disulfide bridges holding together the protein tertiary and quaternary structure
- Mix sample with an equal volume of 2× sample buffer and heat to 95° C for 10 min, cool to room temperature and centrifuge at 14,000 rpm for 5 min in a microcentrifuge to remove all particles. Load the gel

SDS-PAGE

- **Reagents: sample buffer for plant and hard tissue – CHES buffer**

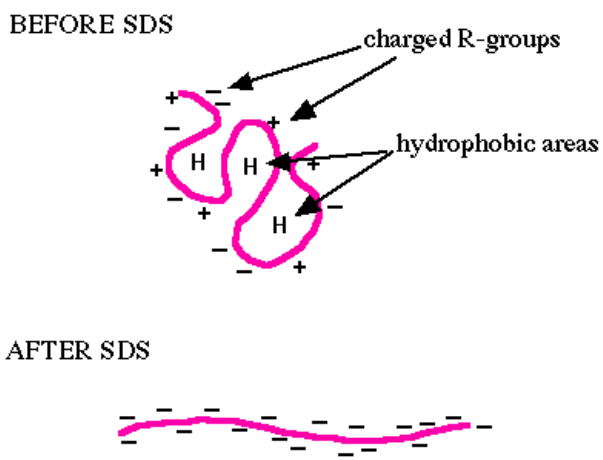
  1% CHES (N-cyclohexyl-2-aminoethanesulfonic acid), adjust pH to 9.5 with NaOH
  2% SDS
  1% DTT
  10% glycerol
  Can be stored at -20° C up to 6 months

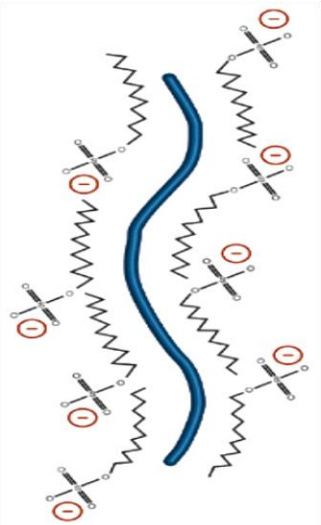
Homogenize sample in 5-15 volumes of CHES buffer in a homogenizer and heat to 95° C for 10 min, cool to room temperature and centrifuge at 14,000 rpm for 15 minutes. Load the gel

The lipopolysaccharide (LPS) layer of the capsule of yeasts, fungi, and Gram negative bacteria cell walls requires enzymatic digestion with lysozyme or zymolyase prior to homogenization

SDS-PAGE

- In SDS gel electrophoresis of samples SDS imparts identical charge:mass ratio to all proteins
- The rod-like structure remains stable as any attempt that tends to fold up the protein chain would result in repulsion between negative charges on different parts of the protein chain, returning the conformation back to the rod shape



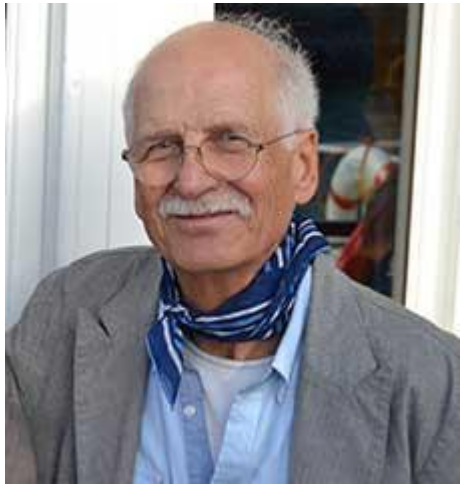


SDS attaches to protein by its hydrophobic chains. Negatively charged SO_4^{2-} groups are exposed to the medium

SDS-PAGE

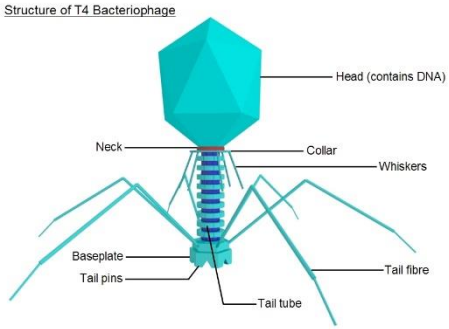
- The sample buffer also contains the following:
- An ionizable tracking dye, usually bromophenol blue These allow the electrophoretic run to be monitored so samples do not run beyond the plate
- Sucrose or glycerol, which gives the sample solution some density, allows the sample to settle easily through the electrophoresis buffer to the bottom when injected into the well

Preparation of Denaturing Protein Gels



U. K. Laemmli

Professeur Emeritus
University of Geneva, Switzerland.
Department of Molecular Biology



- SDS-PAGE system was developed by Laemmli (article in Nature, 1970).
- The classic Laemmli SDS-PAGE method uses a discontinuous gel system
- Discontinuous buffer systems use different buffers for tank and gel, and often two different buffers within the gel, with a third buffer in the tank.
- Because discontinuous systems concentrate, or stack the protein samples into a very narrow zone prior to separation, two gels are prepared: stacking gel and resolving gel

Denaturing gels

- The upper stacking gel is prepared with low acrylamide percentage (i.e. large pore size to allow proteins to move freely and concentrate into a tight band) and low pH of 6.8 [low %T, low pH]
- The lower resolving gel is prepared with high acrylamide percentage (means smaller pores) with a pH of 8.8 [high %T, high pH]
- Both gels contain only Cl^- as the mobile anion. The tank buffer has glycine as its anion, at a pH of 8.3.

SDS-PAGE

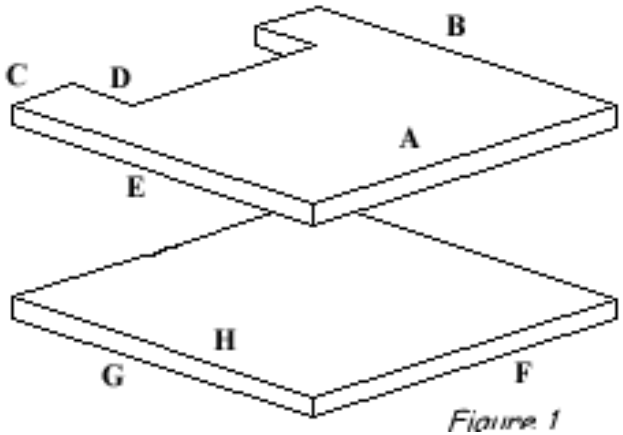
Photograph showing samples being loaded into the wells of an SDS-PAGE minigel. Six wells that have been loaded can be identified by the blue dye (bromophenol blue) added to the loading buffer

Gel electrophoresis apparatus and reagents

- Many variations in apparatus available from different manufacturers: Bio-Rad PROTEAN, Mini Plate, Thermoscientific, Cleaver Scientific, etc.

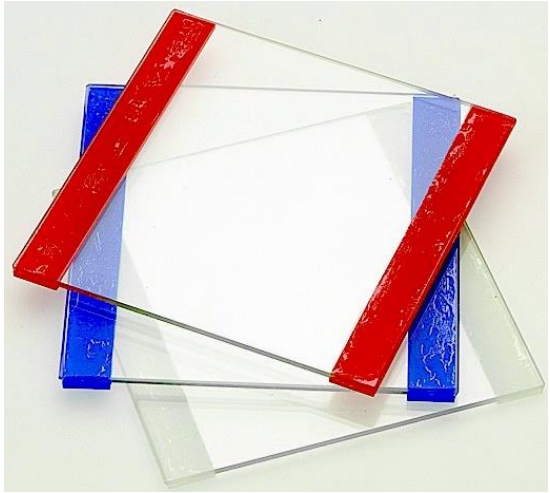
Apparatus

1) A set of 2 glass plates: a long plate (rectangular), a short plate (notched)
2) Spacer (1mm thick): 2 are needed, one for each opposite side. You can use a flexible tape for this
3) Gel casting system (casting frame to sandwich glass plate; casting stand for glass plates, or casting frame)
4) Clamping frame and electrode assembly for precast gel
5) Comb (1 mm thick) for glass plates; precast gels already have comb attached (gel cassette)
6) Electrophoresis tank or Gel box with lid; lid brings the power from the power supply
7) Micropipettes with slender tips



Rectangular and notched plate

Two spacers



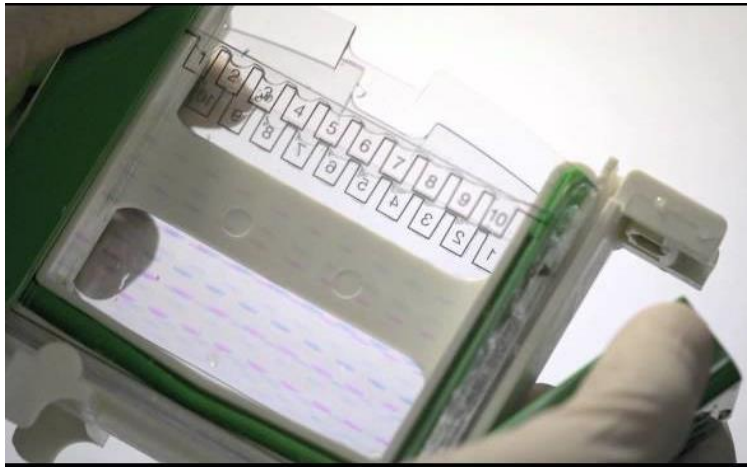
Glass plates with two spacers attached

Casting frame (bottom) and casting stand (up)

Combs

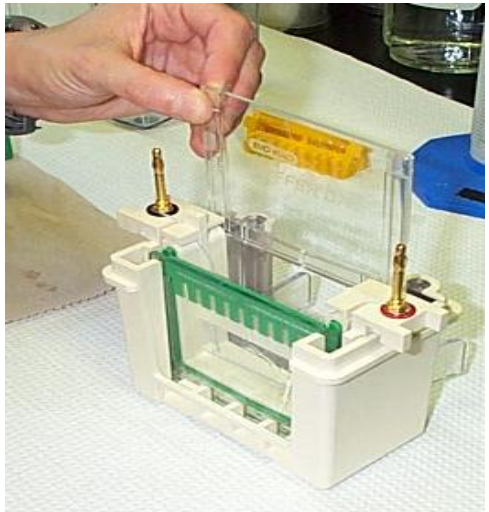
Casting frame with comb fitted to a casting stand

Gel cassette



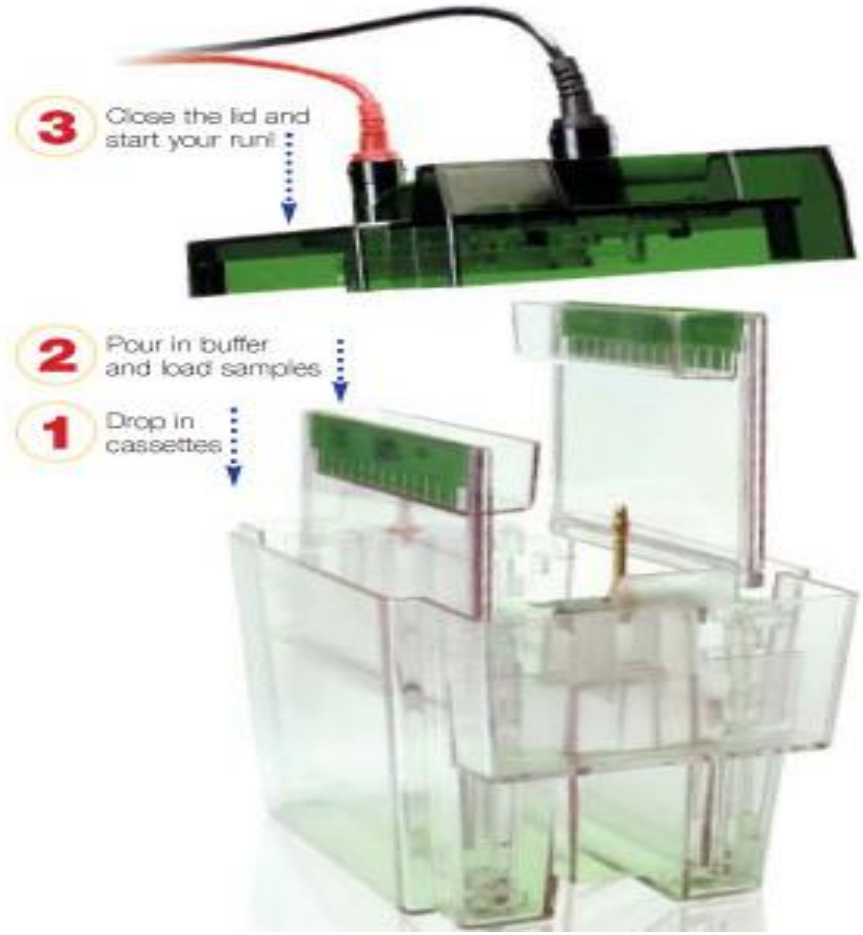
Clamping frame and electrode assembly with gel cassette in place (comb removed to show numbered wells

Buffer dam

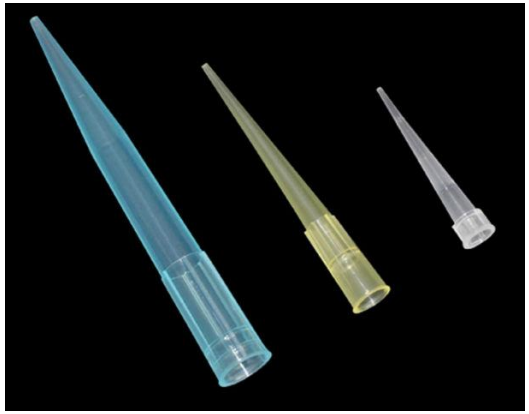


Clamping frame and electrode assembly with casting gel or gel cassette in front and buffer Dam behind; has two ears with red and black coding

Gel box or electrophoresis tank with lid

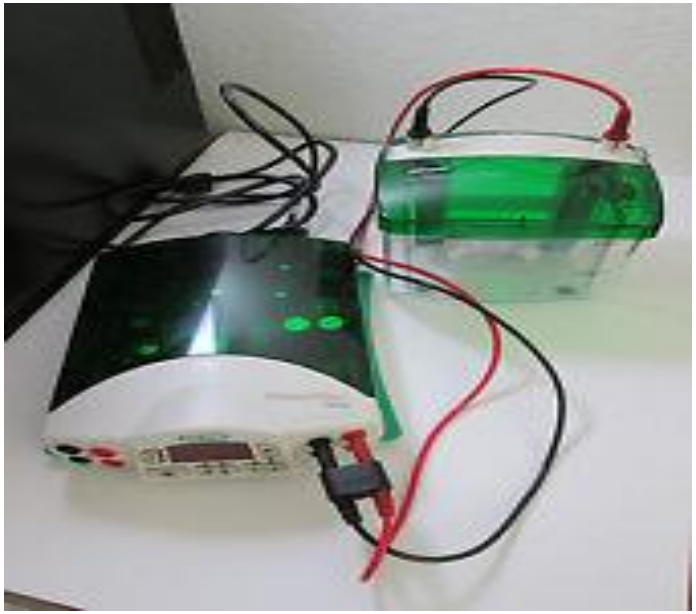


Pipette tips with slender ends for loading a gel



Normal pipette tips

Power pack



Gel box connected to power pack

Reagents for vertical gel electrophoresis

1) Acrylamide
2) Laemmli sample buffer – to prepare protein samples for SDS-PAGE
3) Running buffer

   Acts as an electrolyte for running the gel (counteracts changes in pH arising from electrolysis of water and has enough electrolytes to conduct electricity to run gel in a short time. It is sold as a 10x concentrate.
4) Stacking gel
5) Resolving gel
6) Ethanol
7) SDS (10%)
8) Staining solution
9) Destaining solution and reservoir

Wear gloves at all times during reagent preparation and casting gel. Acrylamide is a neurotoxin; precast gel in gel cassette is stored in sodium azide, a bacterial inhibitor and toxic to humans

Reagents for vertical gel electrophoresis

Stock solutions

Solution A: Acrylamide

- 30 g acrylamide/0.8 g Bis to 100 ml with Super Q water, sterilize by filtration through 0.2 µm membrane filter

Solution B:

1.5 M Tris, pH 8.8 = 36.3 g Tris in 100 ml water. Adjust pH to 8.8 and adjust volume to 200ml.

Solution C:

- 0.5 M Tris, pH 6.8 = 6 g Tris in 40 ml water. Adjust pH to 6.8 and adjust volume to 100 ml.

Solution D: ammonium persulfate (APS)

- 10% APS = 0.1 g in 1 ml water

Solution E: 10% SDS

3% Stacking gel

6.3 ml water/2.5 ml soln C/0.1 ml 10% SDS/1.2 ml soln A/10 µl TEMED/100 µl APS, (lower pH of 6.8)

5X Tray or Running Buffer/liter (Tris-Glycine-SDS)

- 15 g Tris/72 g glycine/5 g SDS. Dilute 1:5 for upper tray and 1:10 for lower tray. This is equivalent to 25 mM Tris /192 mM Glycine/0.1 % SDS (Higher pH of 8.8)

Reagents for vertical gel electrophoresis

- **2x Laemmli Bio-Rad sample buffer** – to prepare protein samples for SDS-PAGE. Based on Tris-glycine-SDS running buffer. Ensures optimal band resolution

Contains:

- 2.5 mM Tris-HCl, pH 6.8
- 25 % glycerol to make sample denser so it can sink to bottom of well
- 2 % SDS
- 0.01 % Bromophenol Blue

To use: (50:50 with sample)

- Dilute 1 part sample with 1 part Laemmli sample buffer.
- Add reducing agent to Laemmli : ß -mercaptoethanol (β-ME) or dithiothreitol (DTT)

The reducing agent breaks disulfide linkages in proteins

Add 50 µl of β-ME per 950 µl of sample

buffer to a final concentration of 5 % ß –ME equivalent to 710 mM. Alternatively, use DTT at a final concentration of 350 mM (54 mg/ml).

Add 1 mM EDTA to disperse cells

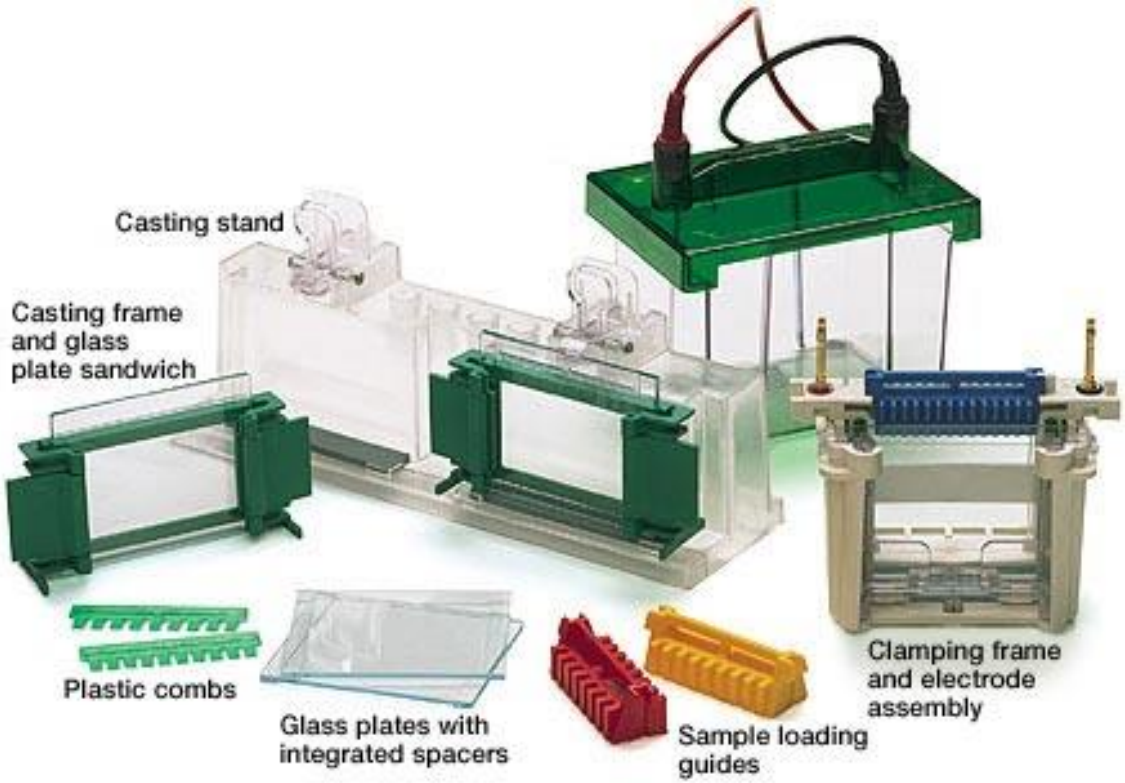
	10 % Resolving gel pH 8.8	**3 % Stacking gel pH 6.8**
	45 ml	12 ml
Solution A (ml)	15	3.6
Solution B (ml)	7.5	-
Solution C (ml)	-	2.5
10% SDS (ml) (Solution E)	0.45	0.1
TEMED (µl)	20	1
APS (µl)	200	19
Water (ml)	21.83	5.78

Resolving gel composition

29 ml				45 ml		
Component	5%	7.5%	10%	5%	7.5%	10%
Soln. A (ml)	5	7.5	10	7.5	11.25	15
Soln. B (ml)	7.5	7.5	7.5	7.5	7.5	7.5
SDS (ml)	0.3	0.3	0.3	0.45	0.45	0.45
TEMED (µl)	15	15	15	20	20	20
APS (µl)	150	150	150	200	200	200
Water	16.1	13.6	11.7	24.15	20.4	17.55

Preparation of PAGE gels

- To obtain optimal resolution of proteins, two gel systems are used: a "**stacking**" gel which is cast over the top of the "**resolving**" gel
- Stacking gel: has lower concentration of acrylamide (e.g., 3% for larger pore size), lower pH of 6.8 and different ionic content
- These properties of the stacking gel allow proteins in a loaded sample to be concentrated into a tight band during the first few minutes of electrophoresis before entering the resolving portion of a gel
- If using gradient gel, the use of a stacking gel is not necessary as the gradient, with its continually decreasing pore size performs this function
- To make stacking gel: Mix all solutions, add TEMED last
- To make resolving gel: Mix all solutions, add TEMED last



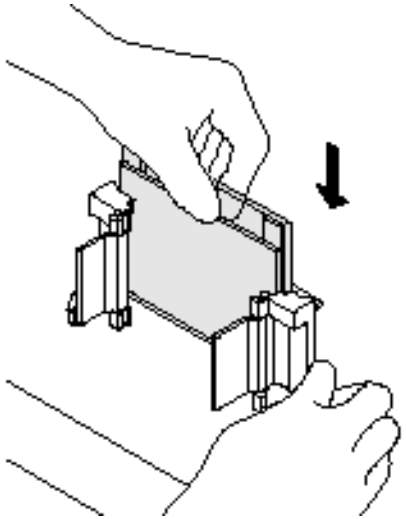
Vertical gel electrophoresis

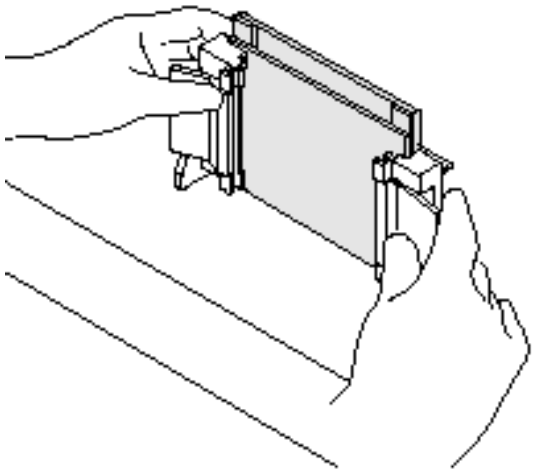
Assembly of apparatus and casting a gel

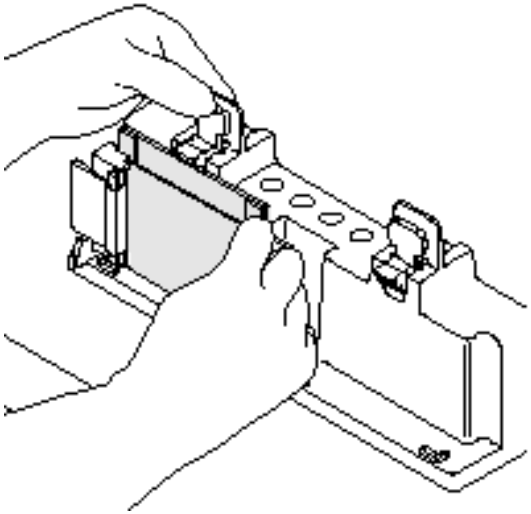
- The gel is cast between two glass plates, separated by spacers, typically less than 2mm thick

<u>To assemble</u>

- Place the spacers on the long plate (spacer plate). Place short notched plate on top of spacer plate so spacer is between the two glass plates. Keep the short plate in front. Top side of spacer plate is marked by manufacturer
- Slide both plates into the casting frame and secure it by locking the pressure clamps. To tighten and lock, move clamps through 90°
- Insure that both plates reach the bottom and align them perfectly. A gasket in the casting frame makes a seal. Check that the bottom is flat, i.e., glass plates do not protrude at the base of casting frame. Place casting frame on the casting stand
-

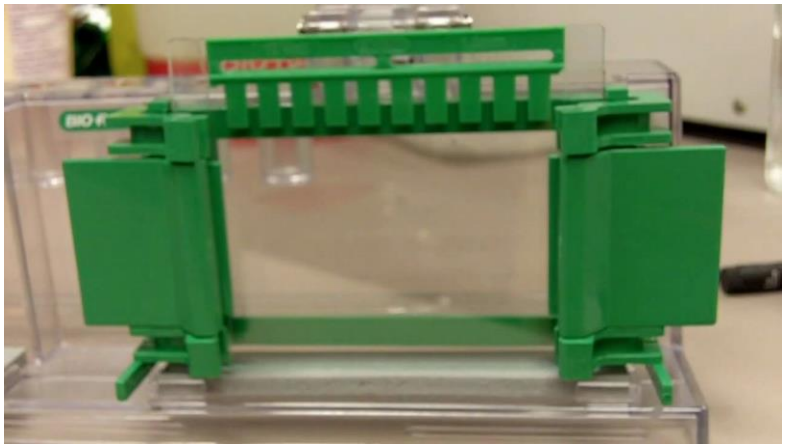


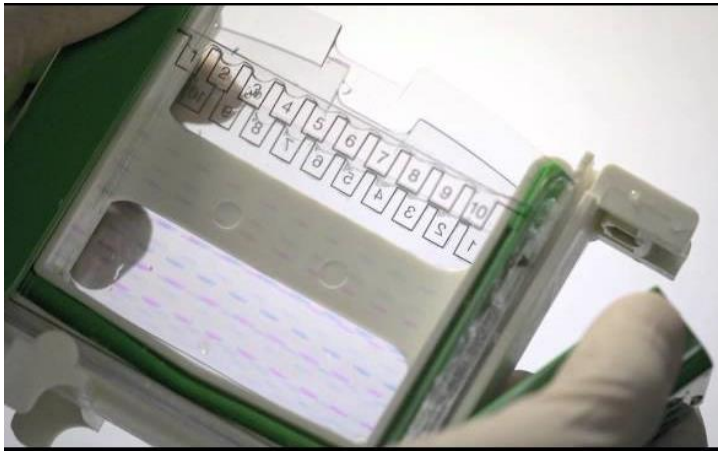




Assembly of apparatus and casting a gel

- Place comb in between glass plates. Make a mark on the right of glass plate, about 1 cm down the comb. Check for leakages by spraying water into the space between the glass plate. If no leakages, blot the water with filter paper. Remove the comb and cast the resolving gel





Precast gel in clamping frame and electrode assembly

Assembly of apparatus and casting a gel

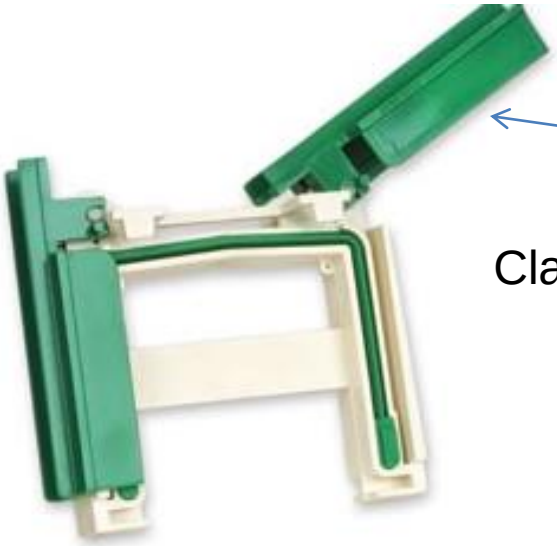
- To cast the resolving gel, pour gel mix between the two glass plates to the black mark you made outside the glass, avoiding bubbles. Gel must be poured right away after adding TEMED
- Remove any bubbles by adding a layer of isopropanol on top of the gel way up to the top.
- Wait for gel to polymerize. Blot out the isopropanol using a strip of filter paper or pour it off. After drying the isopropanol, cast the stacking gel immediately after adding TEMED
- Pour stacking gel on top of resolving gel to fill up the plates. Be careful not to introduce any air bubble. Place comb in the stacking gel which will create wells for samples. Wait for gel to polymerize. Remove the comb and place unit in a gel box.

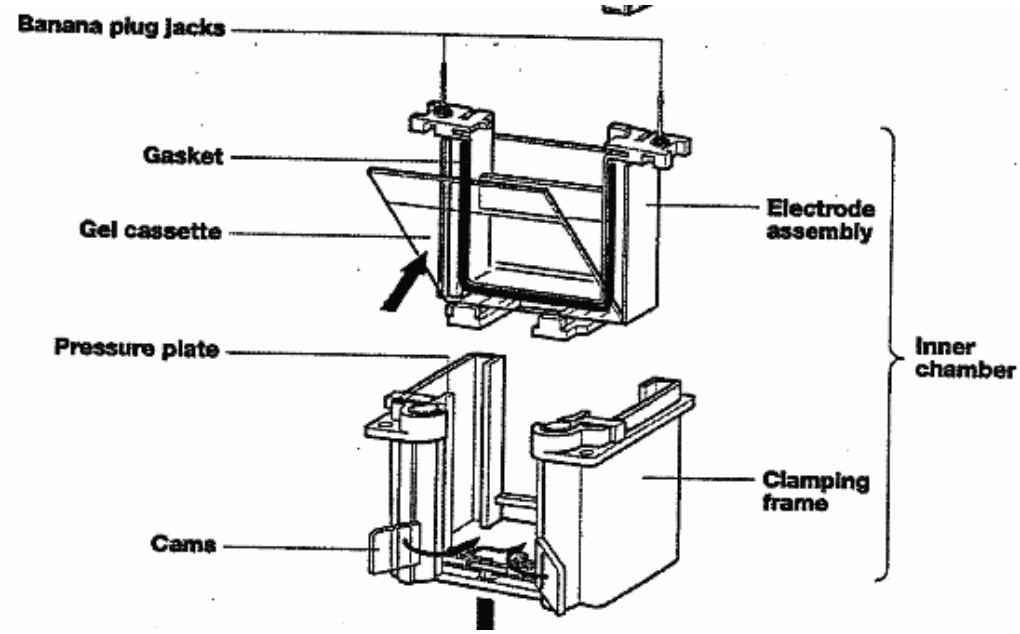
Using a precast gel

Use a gel casting clamping frame. Place buffer dam in the gel casting frame having two sides and gaskets to hold plates in place. This frame can run 2 gels, one on each side, or run one gel with the other side occupied by the buffer dam.

Place buffer dam in frame and clamp side arms to secure the plate. Remove gel cassette from storage pouch and remove the protective tape from the bottom to prevent insulation of the gel cassette.

This cassette has a comb already in place, and when placed in the frame must face the inside of the gel compartment.





Electrophoresis

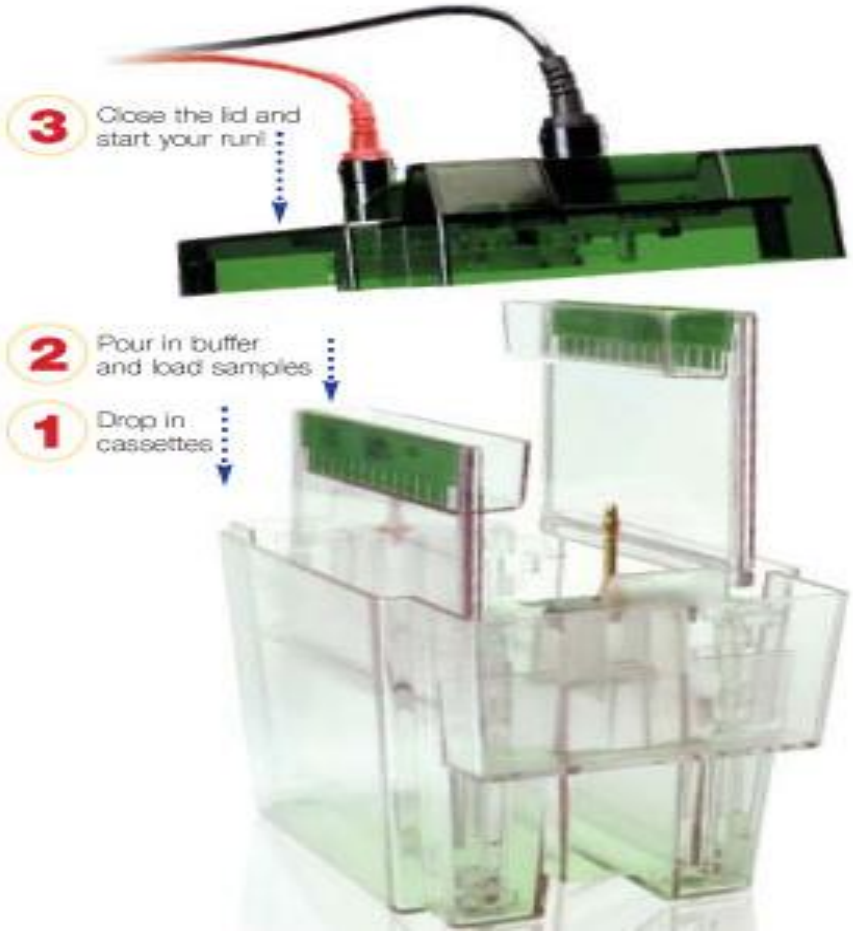
- Remove the glass plates from the stand and clamp and place in gel box/electrophoresis tank
- Add buffer to outer and inner reservoir. Fill the outer compartment with buffer to the mark indicated on gel box.
- Fill the inner compartment with buffer to the mark on gel box. The level of buffer on the inner compartment will be higher than that of the outer. This is used to ensure that there is no leakage, since as if there is, the level of buffer in the inner will drop down. Remove the comb gently and straight up
- Add more buffer to cover the inner glass plate to flood the wells with the buffer
- Load gel: prepare samples with the loading buffer (Laemmli loading buffer) and, if required, denature by heating a sample and adding denaturing agents.

Electrophoresis

- Use micropipette tips with slender ends to load sample wells. Load 1st and 2nd wells with molecular marker and positive control. Load sample wells
- After loading attach lid to gel box, red to red and black to black. Connect to power pack, red to red and black to black.
- Turn on power supply and choose either constant current or constant voltage. Press the run icon on power supply and observe the development of a line of bubbles in the tank- a sign that electrophoresis has began.
- The bubbles come from electrolysis of water and so the bubbles are hydrogen gas.

Electrophoresis

About 20 min into running of the gel, you want to see sample compressed into a narrow line caused by the sample moving through the fairly low percentage of the stacking gel into the high percentage and stiffer resolving gel causing the sample to compress into a tight line.



Electrophoresis

- Run electrophoresis till the dye front reaches appreciable length down the gel, before bromophenol blue reaches end
- When the electric field is turned off, the proteins stop moving. The gel matrix holds the proteins at their final positions long enough for them to be stained to make them visible.
- The gel material can also withstand high voltage gradients, feasible for various staining and destaining procedures, and can be cut to extract separated fractions or dried for autoradiography and permanent recording

Polyacrylamide gels

- Separation is achieved by (1) net charge carried by the protein and (2) sieving effect of the gel matrix.
- Proteins segregate into discrete zones, corresponding to their individual gel-mediated mobilities
- Lanes of protein bands are created

Polyacrylamide gels

- Separation by charge: in native PAGE, migration occurs because most proteins carry a net negative charge at slightly basic pH. The higher the negative charge, the faster the protein will migrate
- Separation by sieving effect of the gel: the frictional force created by the pore sizes of the gel matrix creates a sieving effect, retarding the movement of proteins according to their size.
- Small proteins encounter small frictional force while large proteins face larger frictional force and are retarded the most

Gradient gels

- Gradient gels can be applied to protein and nucleic acid separation
- Main features of a gradient gel
- Low percentage of acrylamide at the top (beginning of sample path) and high percent-acrylamide at the bottom (end) unlike homogeneous gels
- Pore size increases linearly as proteins move down the gel, hence migration rates decreases down the gel
- Are required for separation of both large and small molecular weight proteins on a single gel slab
- Used for a quick estimate of the range of protein sizes in a mixture permitting proper selection of a single concentration gel
- Resolution is much better in homogeneous gel

Gradient gels

- Another form of gradient gel

- Increasing concentration of denaturing reagent (e.g SDS for protein; urea and formamide for DNA).
- Denaturing gradient gel electrophoresis is used to resolve PCR amplicons up to a single base changes, and separate out DNA on the basis of variations in GC content.
- Find applications in elucidating molecular diversity among microbial organism

Properties of proteins

- Since proteins are charged molecules, they migrate under the influence of electric fields. In electrophoresis, the two most important physical properties of proteins are their

  (1) Electrophoretic mobilities : this depends on the charge, size, and shape of the protein

  (2) Isoelectric points: depends only on the net overall charge of the protein

- Various electrophoresis systems have been developed to exploit differences between proteins in these two fundamental properties.
- Electrophoretic mobility, μ, is the rate of migration of a protein per unit of field strength , $\mu = v/E$

- Units of electrophoretic mobility are those of velocity (cm/sec) divided by the units of electric field (V/cm), which gives $cm^2/V\text{-}sec$.

Properties of proteins

Electrophoretic mobility of a protein is dependent on

- Its charge
- Frictional coefficient (shape) $f=(6\pi r\eta)$ where r=radius of the particle, η= viscosity of the medium
- The ratio of charge: mobility
- Both charge and frictional coefficients are established by the composition of the protein and by the makeup of the surrounding medium.
- Electrophoretic mobilities are influenced by factors such as pH and the amounts and types of counter ions and denaturants that are present in the medium.
- Proteins are amphoteric molecules, that is, they can carry positive, negative, or zero net charge depending on the pH of their environment.
- For every protein there is a specific pH at which its net charge is zero. This pH is called the “isoelectric point”, or pI, of the protein. A protein is positively charged in solution at pH values below its pI and negatively charged when the pH is above its pI.

Properties of proteins

- This pH dependence on charge affects the mobilities of proteins in terms of both magnitude and the direction of migration and is exploited in gel electrophoresis, especially in the technique of isoelectric focusing
- The electrophoretic mobilities of proteins are very different in gels than in free solution.
- Gels can act as molecular sieves for molecules such as proteins. They consist of three-dimensional networks of solid material and pores. During electrophoresis in gels the polymeric material acts as a barrier to the motion of proteins forcing them to move between the buffer-filled pores of the gels.

BCHEM 365
Lecture 6
April 19, 2022

Applications of SDS-gel electrophoresis

- 1. Molecular weight determination
- 2. Determination of number of subunits in a protein
- 3. Purity of a protein

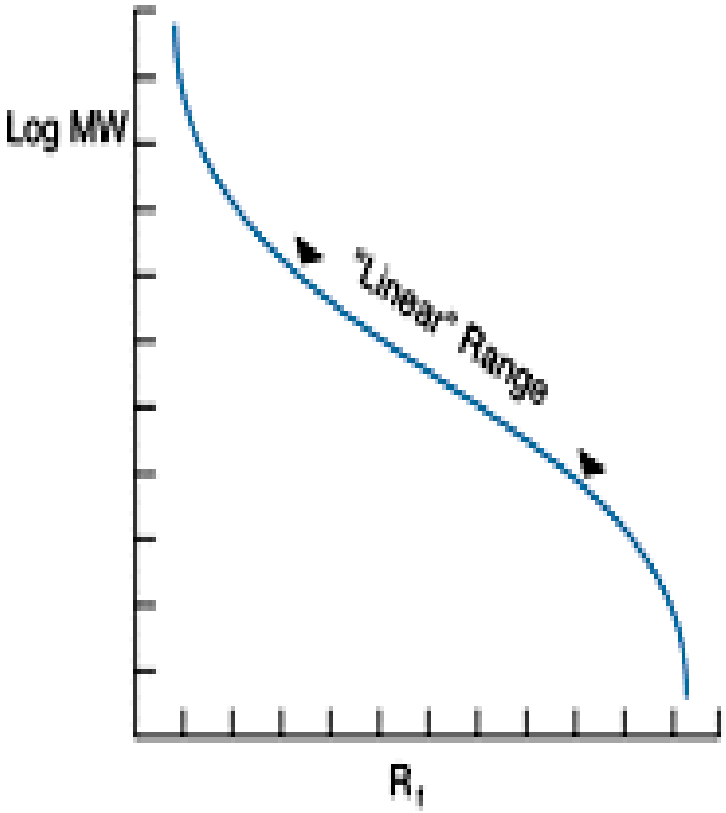
SDS-PAGE

- Typically, the resolving gel used is a 15 % polyacrylamide gel. This gives a gel of pore size in which proteins of relative molecular mass (M_r) 10,000 move through the gel relatively unhindered, whereas proteins of M_r 100,000 can only just enter the pores of this gel
- Gels of 15 % polyacrylamide are therefore useful for separating proteins in the range M_r 10,000 to 100,000
- However, proteins having M_r >150,000 would require a larger pore gel (e.g., a 10% or even 7.5% gel)

Protein molecular weight determination

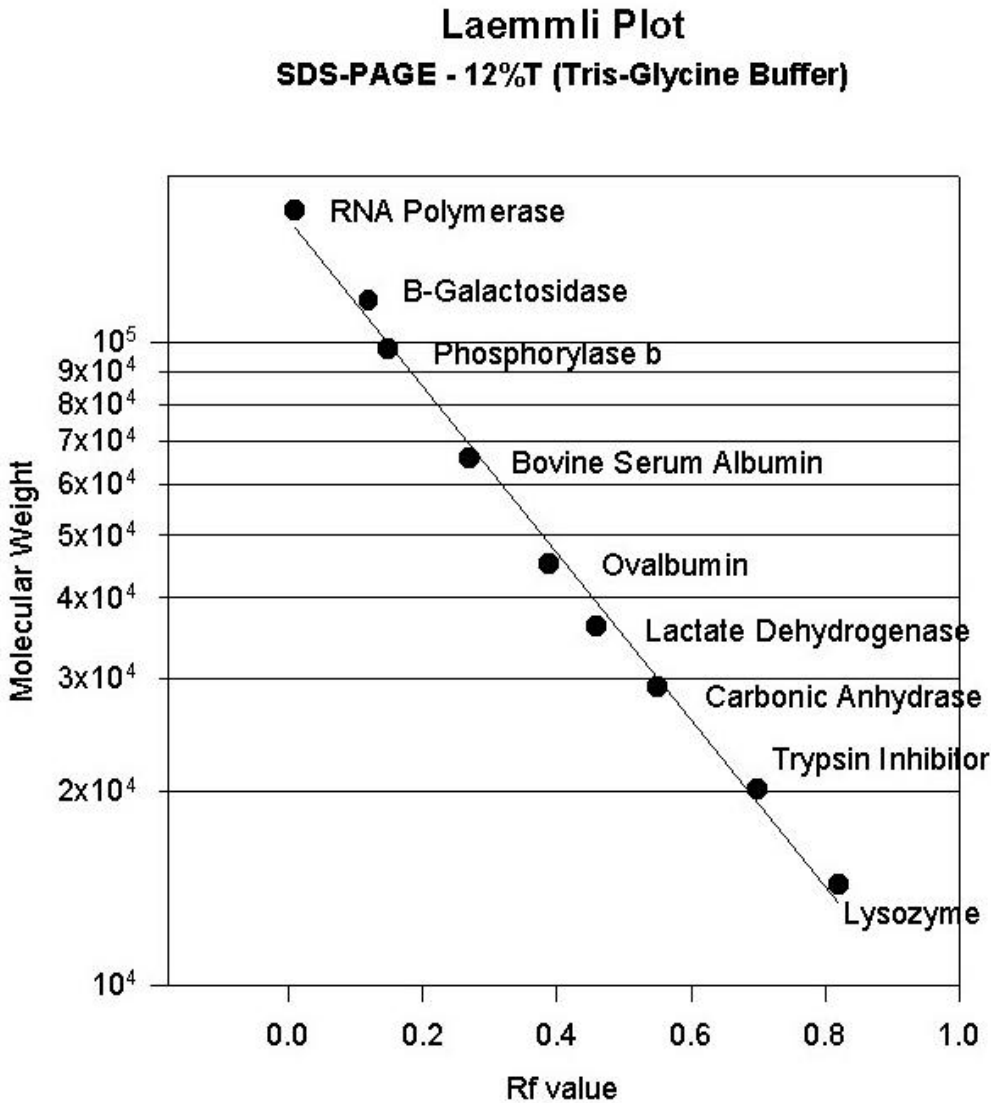
- The relative molecular weight (M_r) of a protein can be determined by comparing its mobility with those of standard proteins run on the same gel
- The M_r of the unknown protein is obtained from a graph of calibration curve of distance moved (R_f) against log M_r for each of the standard proteins (MW ladder)
- The actual plot of log(MW) vs. R_f is sigmoidal because at high MW the sieving effect of the gel hinders penetration of most proteins, while at low MW the sieving effect is negligible and proteins migrate almost at their free mobility, which in SDS is independent of MW

A graph of log(MW) of proteins vs. R_f is sigmoidal but is nearly linear for a range of molecular weights depending on concentration of acrylamide. The linear or proportionality range , in which log of MW is directly proportional to th distance travelled on gel requires that proteins are fully denatured and appropriate gel concentration is selected



Protein molecular weight determination

An empirical plot of log(MW) against R_f for several protein standards used to determine the MWs of proteins. It is required that the protein be fully denatured.



Worked example

- Question: Table 1 shows the distance moved in SDS-PAGE by a series of marker proteins of known relative molecular mass (M_r)
- A purified bacterial protein (X) run on the same gel showed a two bands, A and B that had moved a distance of 45 mm and 64 mm, respectively. What is the nature of this protein? Find the M_r of protein X? Assign reasons for separation into two bands.
- Note: Beta-Lactoglobulin is a dimer with a relative molecular mass of 36,800. Under the reducing conditions, the disulfide bridges linking the subunits are reduced so that the two monomer polypeptide chains are seen on the gel

Table 1. Data for worked example

Protein	M_r	Distance (mm)
Transferrin	78,000	6.0
Bovine serum albumin	66,000	12.5
Ovalbumin (egg albumin)	45,000	32.0
Glyceraldehyde-3-phosphate dehydrogenase	36,000	38.0
Carbonic anhydrase	29,000	50.0
Trypsinogen	24,000	54.0
Soybean trypsin inhibitor	20,100	61.0
Beta-Lactoglobulin	18,400	69.0
Myoglobin	17,800	69.0
Lysozyme	14,300	79.0
Cytochrome c	12,400	86.5

Solution

- Construct a calibration graph by plotting log M_r versus distance moved for each of the marker proteins. Determine the relative molecular mass of protein X from the graph. M_r is approximately 31,000.
- Note that this method is accurate with an error rate of 5 to 10 %, so the answer is 31,000 ±3100 for band A.
- You are to calculate the Mr for band B and discuss the result.

Monitoring purity of proteins

- SDS-gel electrophoresis is often used to assess the purity of a sample
- A pure protein should give a single band on SDS-PAGE, unless the molecule is made up of two unequal subunits, where two bands, corresponding to the two subunits, will appear
- Observation of two bands or more shows an impure protein

Isoelectric focusing- Properties of proteins

- Since proteins are charged molecules, they migrate under the influence of electric fields. In electrophoresis, the two most important physical properties of proteins are their:

  (1) Electrophoretic mobilities: this depends on the charge, size, and shape of the protein

  (2) Isoelectric points: depends only on the net charge of the protein

- Various electrophoresis systems have been developed to exploit differences between proteins in these two fundamental properties.
- Electrophoretic mobility, μ, is the rate of migration of a protein per unit of field strength , $\mu = v/E$
- Units of electrophoretic mobility are those of velocity (cm/sec) divided by the units of electric field (V/cm), which gives $cm^2/V\text{-}sec$.

Isoelectric focusing

- Proteins are amphoteric molecules, that is, they can carry positive, negative, or zero net charge depending on the pH of their environment
- The charges on proteins arise from functional groups present on the amino acids which make up the protein. Twenty amino acids varying in shape, size, charge, and chemical reactivity are found in proteins
- Each of the functional groups has a pK_a value
- $HA \rightarrow A^- + H^+$ with an acid dissociation constant, K_a
- pK_a is the pH at which half of the members of that functional group are protonated
- At pH values above the pK_a that group can be considered fully protonated, and below the pK_a, is fully deprotonated
- Thus, as the pH of the environment changes, the net charge carried by a protein will change
- At high pH, most proteins will have many deprotonated surface groups and will carry net negative charge

Properties of proteins

Electrophoretic mobility of a protein is dependent on

- Its charge
- Frictional coefficient (shape) $f=(6\pi r\eta)$ where r=radius of the particle, η= viscosity of the medium
- The ratio of charge: mobility
- Both charge and frictional coefficients are established by the composition of the protein and by the makeup of the surrounding medium.
- Electrophoretic mobilities are influenced by factors such as pH and the amounts and types of counter ions and denaturants that are present in the medium.
- Proteins are amphoteric molecules, that is, they can carry positive, negative, or zero net charge depending on the pH of their environment.
- For every protein there is a specific pH at which its net charge is zero. This pH is called the “isoelectric point”, or pI, of the protein. A protein is positively charged in solution at pH values below its pI and negatively charged when the pH is above its pI.

Properties of proteins

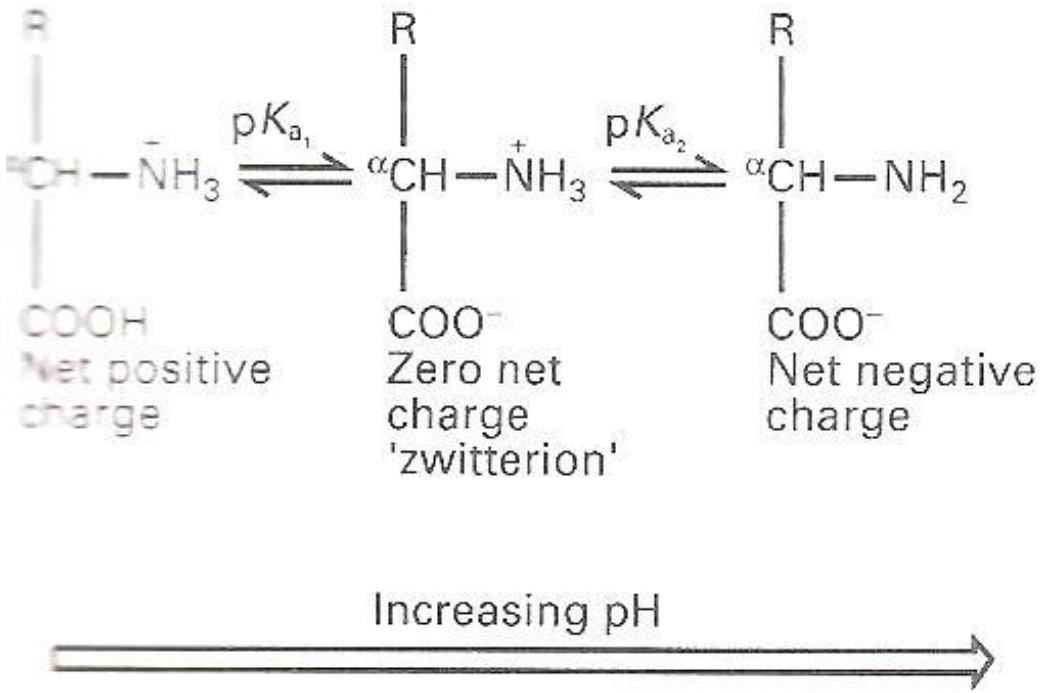
- This pH dependence on charge affects the mobilities of proteins in terms of both distance moved and the direction of migration and these are exploited in gel electrophoresis, especially in the technique of isoelectric focusing
- The electrophoretic mobilities of proteins are very different in gels than in free solution

Isoelectric focusing

- At low pH, H^+ are in excess and most proteins have a net positive charge
- At some intermediate pH, different for every protein, the net charge on the protein will be zero, that is, the protein carries equal numbers of positive and negative charges.
- The pH at which a protein has a net charge of zero is its **isoelectric point (pI**). At this pH, the protein has no net electrophoretic mobility.
- In solution, a protein is positively charged at pH values below its pI and negatively charged when the pH is above its pI

Isoelectric focusing

- Separation of proteins on the basis of differences in isoelectric points is called **Isoelectric focusing**
- The method has high resolution, and can separate proteins that differ in their isoelectric points by as little as 0.01 pH unit
- The numerical value of isoelectric point is related to the pKa values, and is given by the equation:



$$pI = \frac{pK_{a1} + pK_{a2}}{2}$$

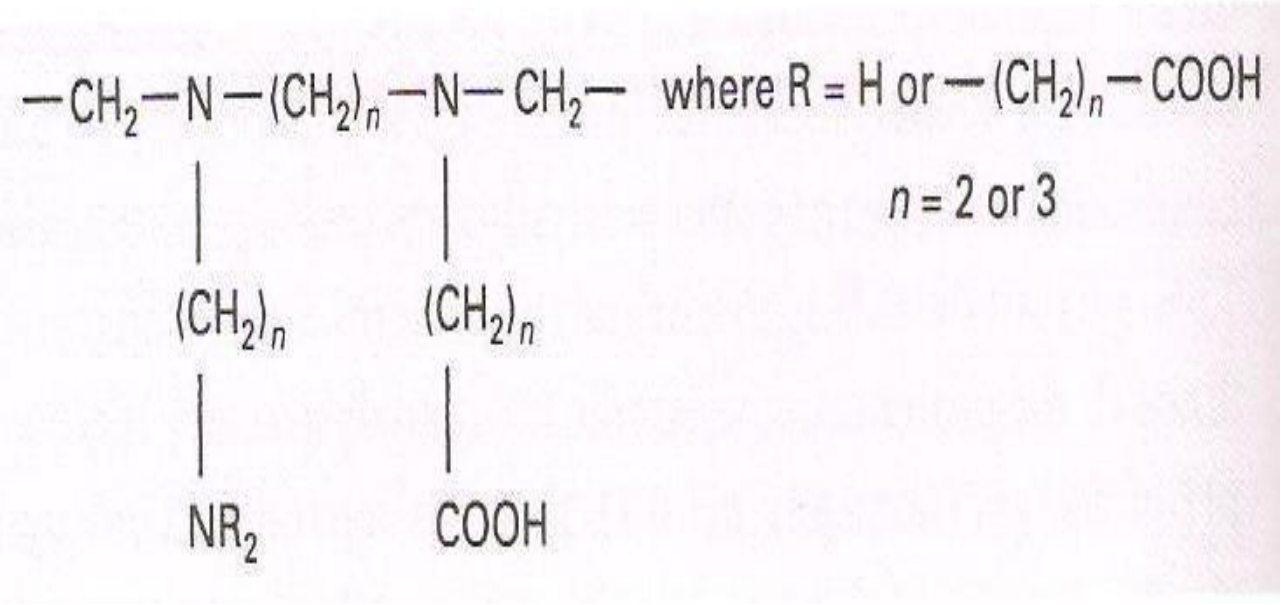
Isoelectric focusing (IEF)

- In isoelectric focusing (IEF) a **pH gradient** is established along the length of a gel. Proteins migrate through this gradient until they reach their pI where they stop moving

Making of pH gradient for IEF gels

- pH gradient for IEF is prepared using a mixture of **ampholytes**, each having a different pI
- The most common ampholytes are complex mixtures of synthetic polyamino-polycarboxylic acids (Fig. 6.1).
- Concentration of ampholytes used must be high enough to change their surrounding pH
- Like protein molecules, the ampholytes migrate through the gel until they reach a region where the pH is equal to their pI

Fig. 6.1. General formula of ampholytes



Isoelectric focusing

- The gel is set up with a uniform mixture of ampholytes throughout, and its anode and cathode ends are immersed in dilute acid and base, respectively.
- A well designed ampholyte system will produce a smooth and gradual gradient of pH with no abrupt charges or 'steps'
- Ampholytes near the positive electrode will be positively charged, and negatively charged if near the negative electrode.
- Ampholytes are sold in **broad range** covering pH 2 - 12 or **narrow range** of 2 pH units across the length of the gel, say pH 5 – 7
- Commercial ampholytes include Bio-Lyte and Pharmalyte

Materials used as ampholytes for IEF gels

- Materials which behave as ampholytes: amino acids, proteins, and polyacidic and polybasic synthetic molecules
- Advantages and disadvantages
- Amino acids: have poor conductivity and poor buffering capacity in their zwitterionic state, making them poor ampholytes
- Proteins are good ampholytes, but they interfere with analysis of the sample, by introducing new proteins on the gel
- Polyamino-polycarboxylic acids have good buffering capacity and conductivity across a broad pH range, and usually manufactured in a relative molecular weight range of 300 - 500, which is small enough to avoid interference with most subsequent processing
- Polyamino-polycarboxylic acids have a disadvantage of binding tightly to the proteins being separated, due to ionic interactions, and can be very difficult to remove

Ampholytes

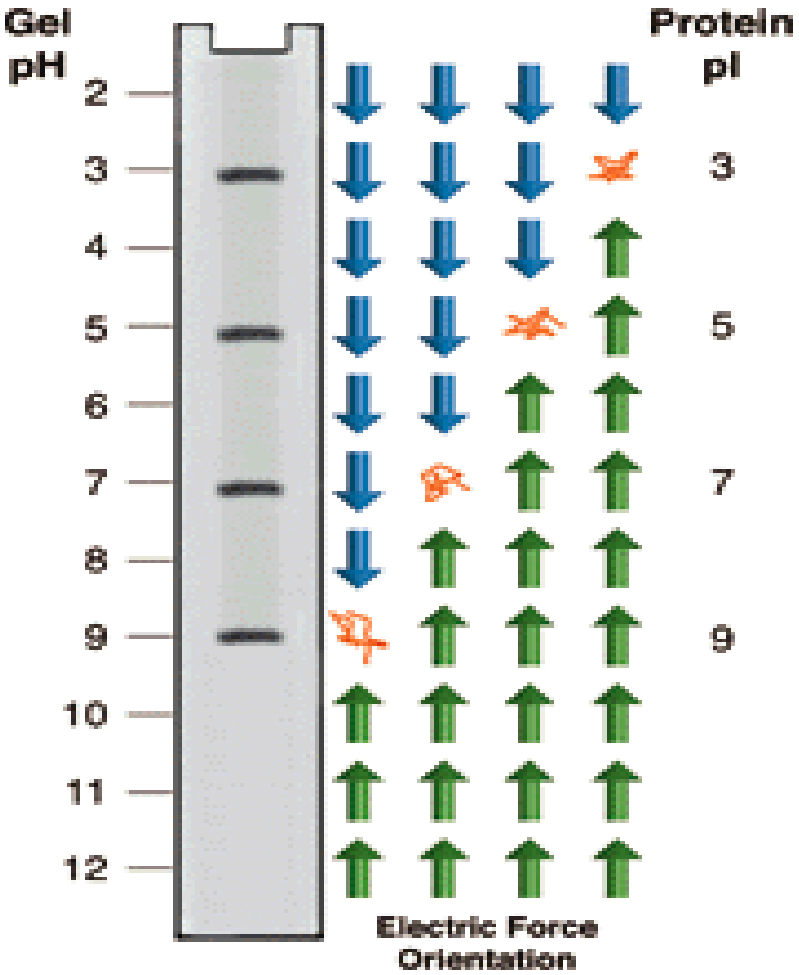
- Optimum concentration of ampholytes for IEF gels is 2 % (w/v)
- Ampholyte concentrations below 1% (w/v) often result in unstable pH gradients
- Concentrations above 3% (w/v) are difficult to remove from gels and can interfere with protein staining

Isoelectric focusing

- On the pH gradient gel, if a protein is in a pH region above its pI, it has a negative charge and will move into lower pH regions in attempt to look for its pI
- If it is in a pH region below its pI, it has a positive charge and will move into higher pH regions to look for its pI
- Hence, proteins are continually swept back or forward until they settle on the appropriate pI (Fig. 6.2)
- This sweeping back process is called "focusing“. IEF is thus an equilibrium electrophoresis system, run until protein movement ceases

Isoelectric focusing (IEF)

Fig. 6.2. A gel is prepared having pH gradient 2 to 12 extending the length of the gel. Protein samples soaked on paper discs are layered on the gel. Protein diffuses from paper into gel and is swept back or forward by the electric field depending on its pI and wherever it was placed on the gel. A protein stops migrating when it enters a zone in which the surrounding pH equals its isoelectric point. At any other position on the gel, the protein acquires a charge which causes it to migrate toward its pI (green and blue arrows). On the gel are 4 protein molecules having pI 3,5,7 and 9 (red)



Isoelectric focusing

- When the current is turned on, proteins begin to migrate into the gel, with the most charged, i.e., those furthest from their pI moving fastest
- Gradually, proteins separate into zones of defined pH
- IEF is most frequently carried out as the **first step in 2-dimensional gel electrophoresis.** The apparatus best suited for this use is a **tube gel** system. The gels are cast and run in glass tubes with an internal diameter matched to the thickness of the second dimension gel. 1.5 mm gels are commonly used

Isoelectric focusing

- After the IEF gel is run, it is extruded from the tube and laid across the top of the second dimension gel. Only one sample can be resolved per tube
- IEF gels **slabs** allow separation of many samples in one gel and after electrophoresis cut into strips for loading onto second dimension gels
- Since the method requires that proteins move freely according to their charge under the electric field, IEF is carried out at **low percentage polyacylamide gels** (4%) or agarose gels to avoid any sieving effect within the gel

Isoelectric focusing (IEF)

- To prepare a thin-layer IEF gel slab, carrier ampholytes, covering a suitable pH range, and riboflavin are mixed with the 4 % acrylamide solution
- The mixture is poured between the glass plates (typically 25 cm × 10 cm), which contains the spacer. The second glass plate is then placed on top of the first to form the gel cassette, and the gel polymerized by photoplymerization
- Photodecomposition of riboflavin generates a free radical, which initiates polymerization. This takes 2-3 hours.
- Once gel has set, the glass plates are pushed apart gently to reveal the gel stuck to one of the glass plates

Isoelectric focusing (IEF)

- Two electrode wicks, made of thick (3 mm) strips of filter paper, one soaked in H_3PO_4 (as anode) and the other soaked in NaOH (as cathode) are laid along the long length of each side of the gel and power is turned on
- Ampholytes form a pH gradient between the anode and cathode. The power is then turned off and protein samples soaked on small discs of filter paper are layered on the gel
- Voltage is again applied for about 30 min to allow protein samples to electrophorese off the filter paper and into the gel, at which time the paper squares can be removed from the gel
- Depending on which point on the pH gradient the sample has been loaded, proteins that are initially at a pH region below their pI will be positively charged and will initially migrate toward the cathode

Isoelectric focusing (IEF)

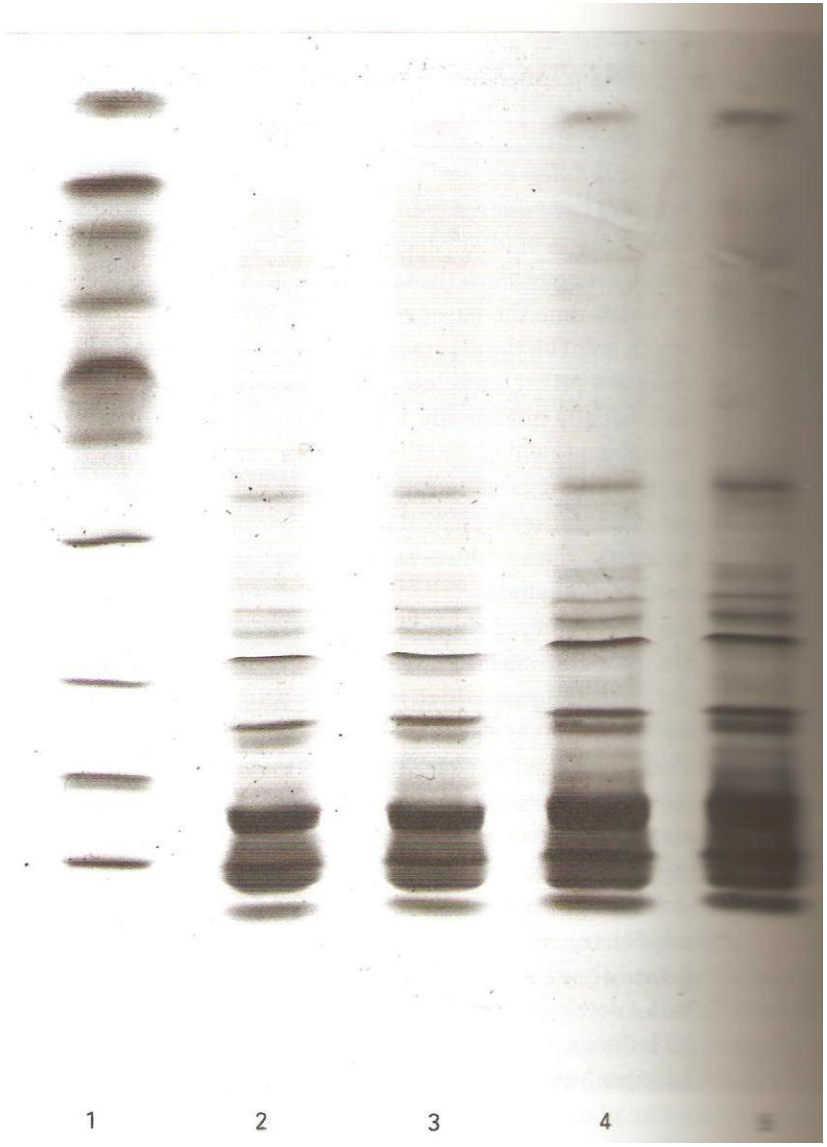
- Proteins that are initially at pH regions below their pI will be positively charged. As the proteins move, the surrounding pH will be steadily increasing, and therefore, the positive charge on the protein will decrease correspondingly until eventually the protein arrives at a point where the pH is equal to its isoelectric point
- The protein will now be in the zwitterion form with no net charge, so further movement will cease. Likewise, proteins that are initially at pH regions above their pI will be negatively charged and will migrate toward the anode until they reach their isoelectric point and become stationary.
- Because samples will always move toward their pI, it is not critical where on the gel they are placed
- To achieve rapid separation (i.e., 2-3 hours of running the gel), relatively high voltage (up to 2,500 V) are used. As considerable heat is produced, gels are run on cooling plates (10°C) and power packs are used to stabilize the power output and minimize thermal fluctuations

Isoelectric focusing (IEF)

- Following electrophoresis, the gel must be stained to detect the protein samples. However, this cannot be done directly, because the ampholytes will stain too, giving a totally blue gel
- The gel is therefore first washed with fixing solution, such as 10 % trichloroacetic acid (TCA)
- TCA precipitates proteins in the gel and allows the much smaller ampholytes to be washed out
- Gel is then stained with Coomassie Brilliant Blue and then destained. A typical IEF gel is shown in Fig. 6.3

Isoelectric focusing (IEF)

Fig. 6.3. A typical isoelectric focusing gel. Lane 1 contains a mixture of standard proteins of known isoelectric points. Lanes 2-5 show increasing loadings of venom from the Japanese water moccasin snake. Source: Bio-Rad Labs.



Applications of Isoelectric focusing

1. For preparative purposes
2. Determination of pI of a protein
3. Determination of heterogeneity of a protein
4. Separating isoenzymes

Preparative IEF

- For separation of mixtures of proteins, that is protein purification

Determination of pI of a protein

- The pI of a given protein may be determined by running a mixture of proteins of known isoelectric points on the same gel
- A number of mixtures of proteins with differing pI values are commercially available covering the pH range 3.5 -10. After staining, the distance of each band from one electrode is measured and a graph of distance for each protein against its pI plotted
- By means of this calibration line, the pI of an unknown protein can be determined from its position on the gel

Heterogeneity in a protein

- IEF is a highly sensitive analytical technique and is particularly useful for studying microheterogeneity in a protein
- For example, a protein may show a single band on an SDS gel, but may show more than one band on an IEF gel
- This may occur, for example, when a protein exists in mono-, di-, and tri-phophosphorylated forms.
- The difference of a couple of phosphate groups has no significant effect on the overall relative molecular mass of the protein, hence a single band on SDS gels. However, the small charge difference introduced on each molecule by PO_4^{3-} groups can be detected by IEF

Isoelectric focusing

- IEF is useful for separating isoenzymes, which are different forms of the same enzyme often differing by only one or two amino acid residues. Different amino acids introduce different charges, hence, different pIs
- The approach has found particular use in forensic science, where traces of blood or other biological fluids from suspect, victim, or perpetrator can be analyzed and compared according to the composition of certain isoenzymes
- In this case, detection may be carried out by overlaying the corresponding substrate on the IEF gel

BCHEM 365
Lecture 7
April 26, 2022

Two-Dimensional Gel Electrophoresis (2DGE)

- 2DGE is one of the two major technologies for protein separation in proteomics, the other method being Multidimensional Liquid Chromatography (a technique that improves resolution of chromatographic separation of complex substances by passing sample through two different separation stages having different stationary phase. Each stage consists of many columns arranged at 90° to each other
- SDS-PAGE gives very good resolution of polypeptides, however, sometimes, a mixture of polypeptides is so complex that we need a better method to resolve them

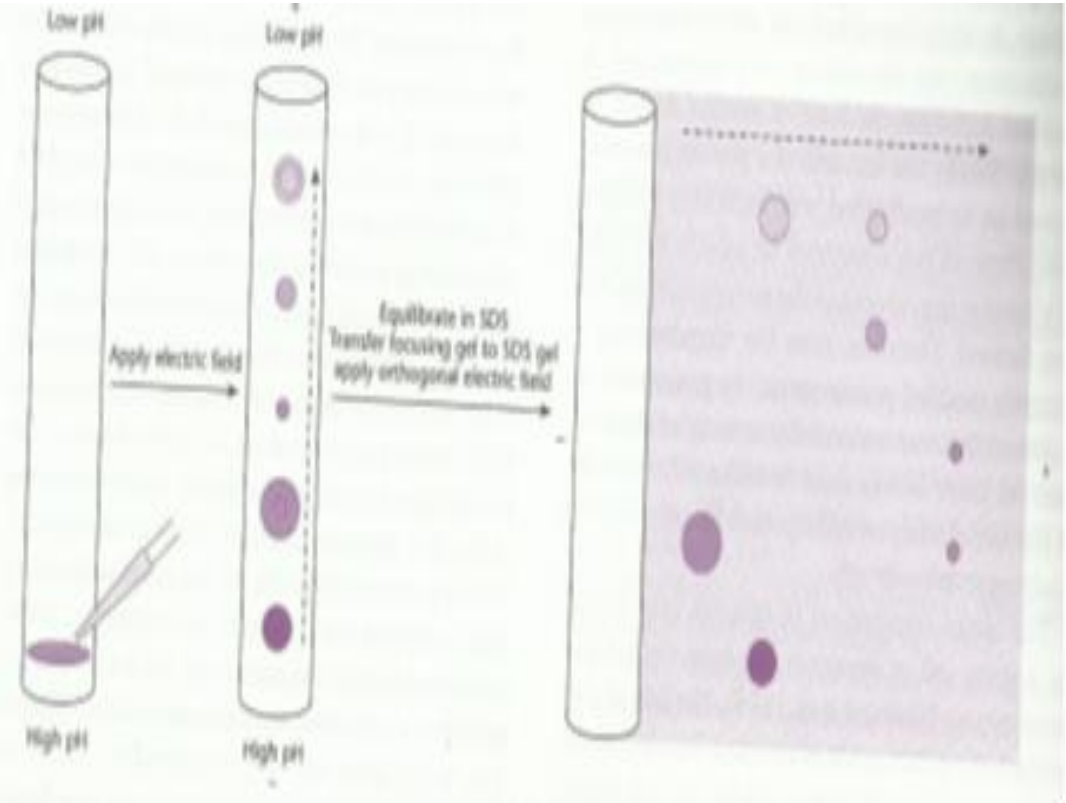
Two-dimensional gel electrophoresis (2DGE)

- In proteomics, one may want to separate all of the numerous polypeptides present at a given time in a cell. This is a common practice in proteomics during gene expression studies
- 2DGE produces a visual display of the complex protein mixtures such as those extracted from whole cells or tissues
- 2D gel electrophoresis offers the best method for high-resolution profiling of minute concentrations of proteins and RNA (Northern blotting) in complex biological samples

Two-dimensional polyacylamide gel electrophoresis

- 2-D PAGE separates proteins in two steps according to two independent properties:
- First dimension is isoelectric focusing (IEF) in a tube gel, which separates proteins on the basis of their pI
- Second-dimension is carried out in a slab gel for SDS-PAGE which separates proteins on the basis of their size or MW
- In this way, complex mixtures of thousands of different proteins can be resolved and the relative amount of each protein can be determined

- 2DGE



2-D PAGE

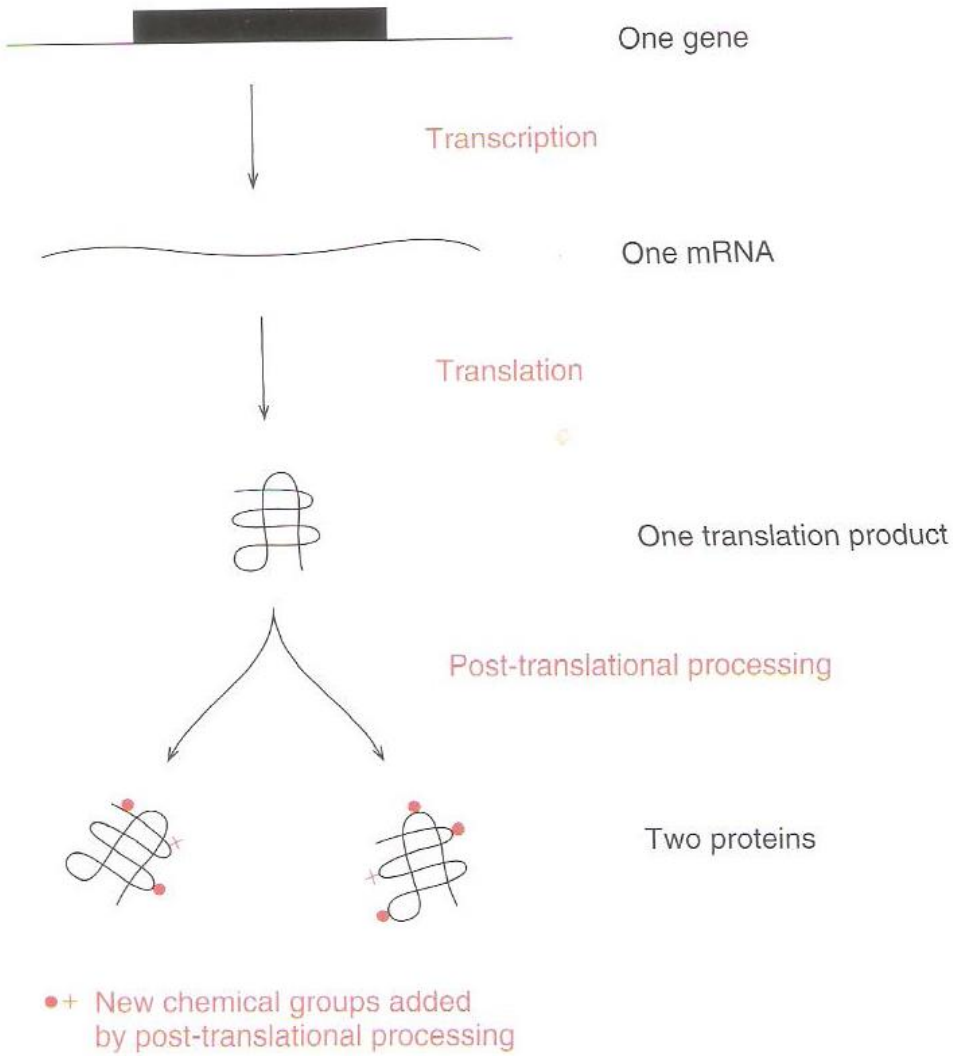
- The IEF gel contains synthetic ampholytes for forming the pH gradient to allow each protein to migrate to its pI
- The IEF gel is then incubated in a sample buffer containing SDS which binds nonspecifically to all proteins and confers a uniform negative charge.
- The focused proteins are then separated in a perpendicular plane to the first separation
- In the original study of 2DGE, the researchers separated *E. coli* proteins by IEF in a tube gel after which the tube was cracked open and the proteins exposed to SDS by immersion of the gel in SDS solution
- The tube gel was then attached to SDS-PAGE slab gel cast between two glass plates for separation by size to proceed
- When electrophoresis begins, the SDS-bound proteins run into the gel and separated according to size, as described previously

2-dimensional gel electrophoresis

- Currently, immobilized pH gradient gels (IPG), where the acrylamide is copolymerized with the pH gradient to give a more stable gradient, are used to improve resolution and reproducibility of separation
- After fractionation the gradient gel is treated with sensitive fluorescent stain, SYPRO dyes, which have a broad linear range and do not introduce covalent modifications into proteins as does silver staining
- This produces a unique pattern of dots , each dot representing a protein, hence a fingerprinting of the proteins in a cell

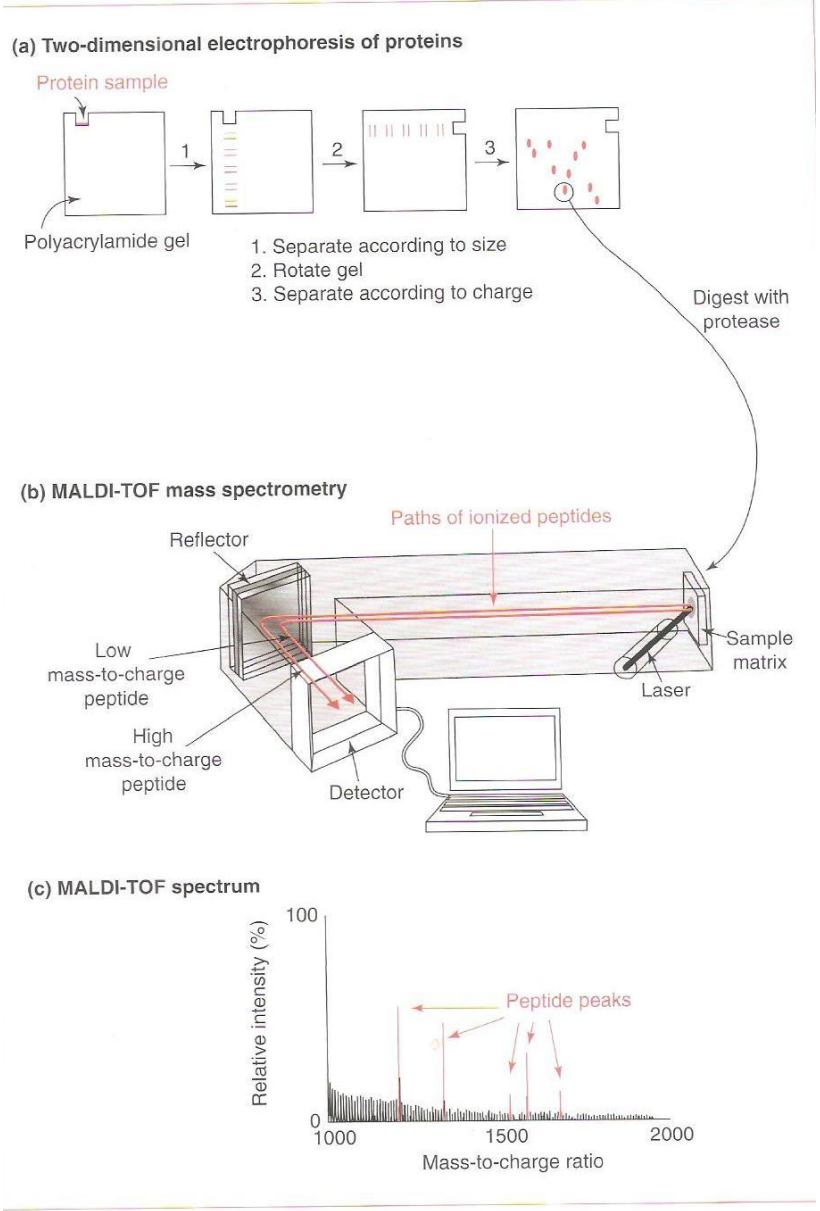
2D gel electrophoresis

The proteome is the entire collection of proteins in a cell. Proteome studies (proteomics) provide additional information that cannot be obtained by examining the transcriptome because a single mRNA (hence a gene) can give rise to more than one protein because of post-translational processing. To the protein is added an additional chemical compound such as a glycan, phosphoryl group, etc., in order to activate it



2D gel electrophoresis

To study the proteome, the entire protein content of a cell or tissue is first separated by 2D electrophoresis. In this technique, the proteins are loaded into a well on one side of a square of polyacrylamide gel and separated according to their molecular masses. The square is then rotated by 90° and a second electrophoresis is performed, this time separating the proteins on the basis of their charges, (IEF). The result is a 2-dimensional pattern of spots, of different sizes, shapes and intensities, each representing a different protein or related group of proteins. Differences between two proteomes are apparent from differences in the pattern of spots when the two gels are compared



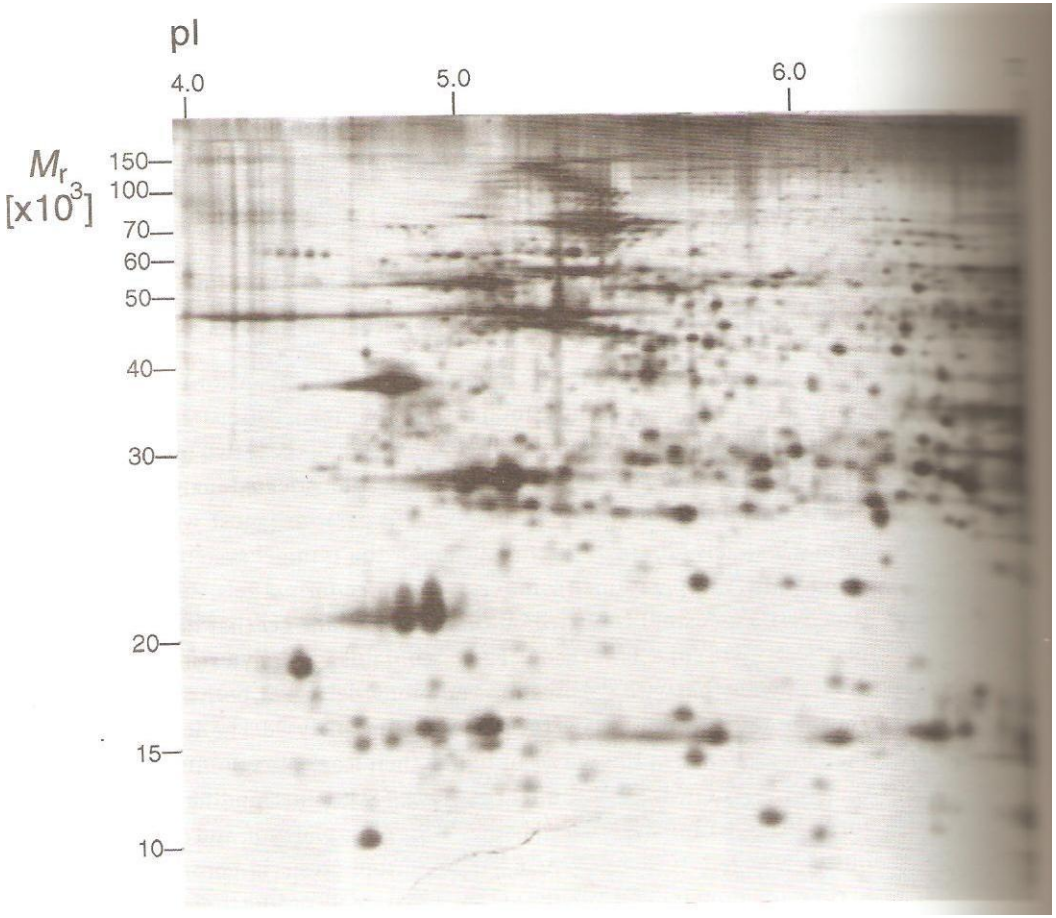
Identification of a protein by 2D electrophoresis followed by MALDI-TOF (Matrix-assisted laser desorption/ionization) spectrometry

2-D PAGE

- The IEF gels are provided as dried strips and need rehydrating overnight. The first dimension IEF run takes 6-8 hours , the equilibration step with SDS sample buffer takes about 15 min, and then SDS-PAGE step takes about 5 hours. A typical 2-D gel is shown in Fig. 7.1.
- Using this method, one can resolve up to about 10,000 proteins in a sample

Fig. 7.1. A typical two-dimensional gel

The sample applied was 100 µg of total protein extracted from a normal dog heart ventricle. The first dimension was carried out using a pH 4-7 isoelectric-focusing gel. The second dimension was a 12 % SDS-PAGE vertical slab gel. The pattern was visualized by silver staining. Source: Heinke and Dunn, Heart Science Center, UK)



Cellulose Acetate Electrophoresis

- Cellulose acetate (or paper and starch) electrophoresis is an old method which currently finds uses in clinical analysis of serum proteins, urine proteins, hemoglobin variants, isozymes, and glycoproteins
- It is a type of horizontal electrophoresis in which separation is based on charge differences
- Cellulose acetate, unlike paper, is a more homogeneous medium, with uniform pore size, and does not adsorb proteins the way paper does. There is therefore much less smearing of protein bands and resolution is better

Cellulose Acetate Electrophoresis

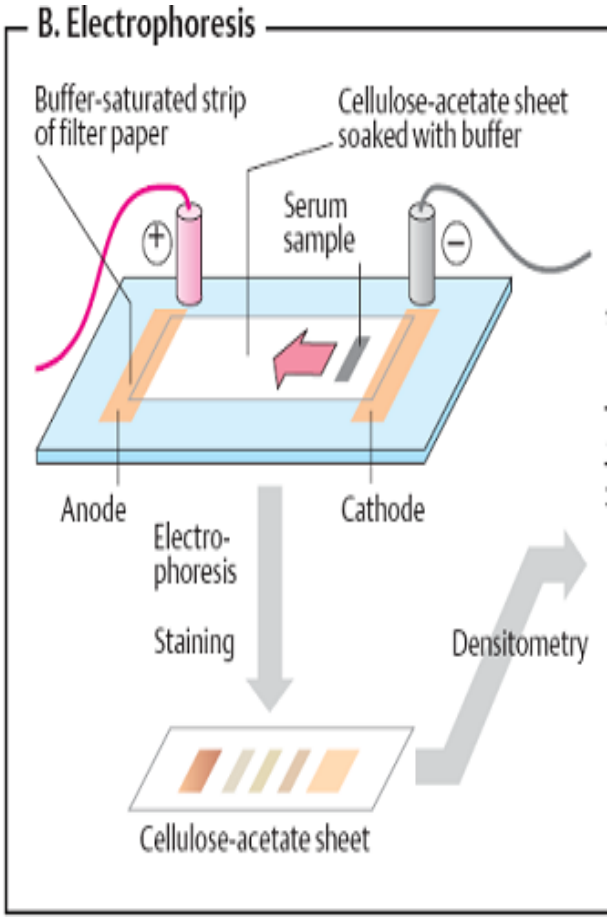
Preparation of cellulose acetate from cellulose, example, cotton

$$\text{Cellulose} \xrightarrow[CH_3COOH,(CH_3CO)_2O,\ H_2SO_4,\ 50\text{-}85^oC]{} \text{Crude Cellulose acetate}$$

$$\text{Crude Cellulose acetate} \xrightarrow{H^+/H_2O,110\text{-}120^oC} \text{Cellulose acetate}$$

Cellulose acetate electrophoresis

The method is simple to set up and run. Single samples are normally run on cellulose acetate strips (2.5 cm × 12 cm) while multiple samples are run on wider sheets. Cellulose acetate sheet is first wetted in electrophoresis buffer (pH 8.6 for serum samples) and the sample (1-2 µl) is loaded on the sheet as a 1-cm wide strip in the third bottom of the strip. The ends of the sheet make contact with the electrophoresis buffer tanks via filter paper strips that overlap the ends of the cellulose acetate sheet. Electrophoresis is conducted at 6-8 V /cm for about 3 h



Cellulose acetate electrophoresis

- The pH, ionic strength, and nature of the buffer may be varied according to the proteins being separated, e.g. serum proteins are separated at a pH of 8.6 using barbitone buffer
- At this alkaline pH all serum proteins and hemoglobin will have a net negative charge and will migrate toward the anode
- The magnitude of the charges will differ among the protein

Cellulose acetate electrophoresis

- After the electrophoresis, the proteins are fixed to the solid cellulose acetate support by means of a fixative such as acetone or methanol. Then it is stained with a dye such as Amido Schwartz, naphthalene black, Ponceau S or Coomassie Blue)
- The sheet is then destained by using dilute acetic acid to remove background color for visualization of the proteins
- For quantitative estimation of protein, the sheet may be scanned using a densitometer and each band quantified. In the densitometer, light is passed through the cellulose acetate; the absorption of light will be proportional to the quantity of protein present on a band
- Another method is to elute the stained protein from the support and to quantify each fraction colorimetrically

Cellulose acetate electrophoresis

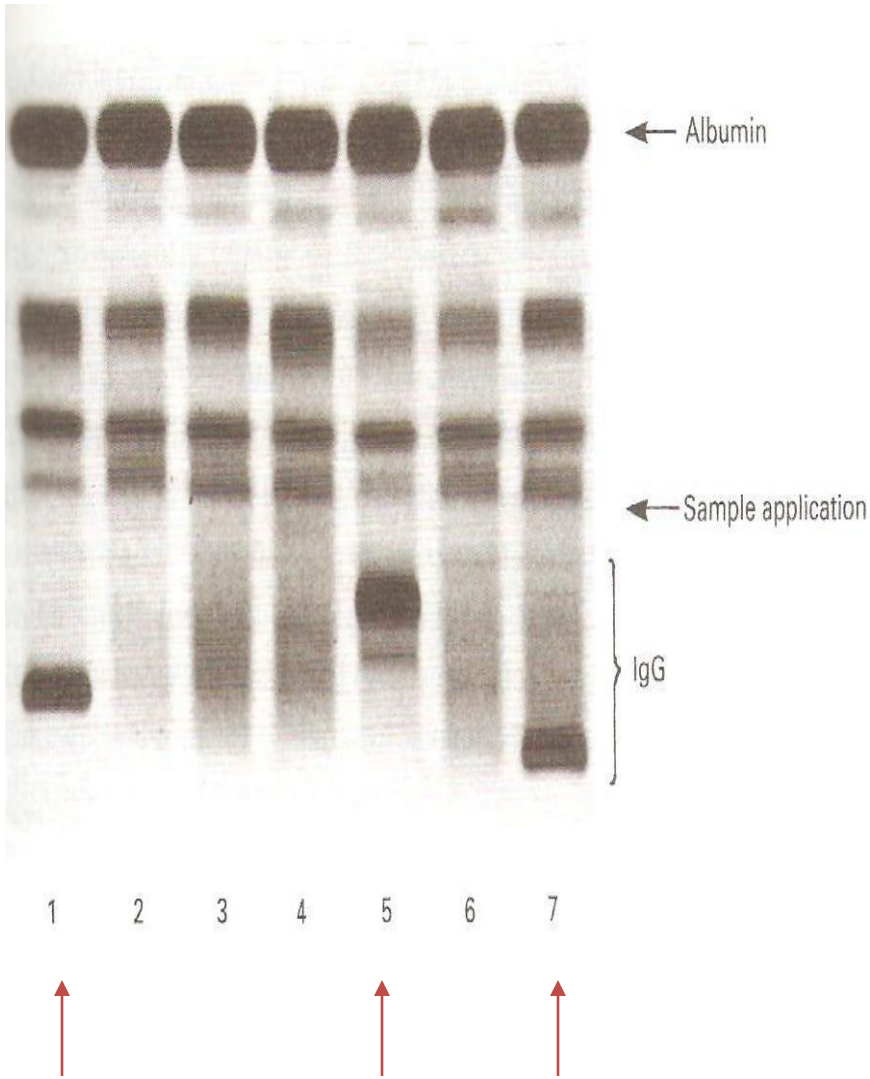
- Another method of detection and semiquantitative estimation of protein on the paper strip is to treat the strip as a blot and probe for the test protein using a primary antibody, followed by an enzyme-linked secondary antibody
- An enzyme-substrate complex formed will produce color whose intensity is equivalent to the protein detected on the strip.
- Although still frequently used for serum analysis, electrophoresis on cellulose acetate is frequently replaced with agarose gel, which gives similar but somewhat better resolution

Cellulose acetate electrophoresis

- Typically, a normal serum protein separation pattern shows about six major bands. This arrangement of the bands is altered in many diseased conditions Clinicians use the serum protein pattern for diagnosis
- A typical pattern of serum proteins on cellulose acetate or agarose gel is shown below

Electrophoresis of human serum samples on an agarose gel

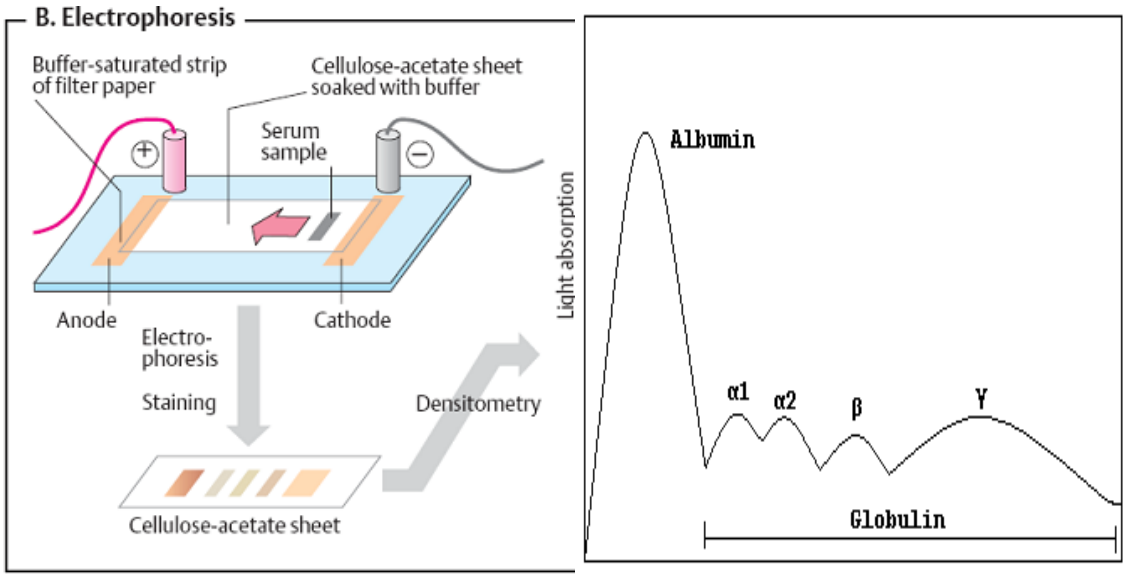
Lanes 2, 3, 4, and 6 show normal serum protein profiles. Lanes 1, 5, and 7 show myeloma patients, who are identified by the excessive production of a particular monoclonal antibody seen in the IgG fraction. (Source: Andrews and Cundy, Edgware General Hospital, London)



Serum protein separation pattern

This pattern shows a typical serum separated on a high resolution electrophoresis gel. The vertical marks define the five standard fractions from left to right, albumin, alpha-1 globulin, alpha-2 globulin, beta globulin and gamma globulin.

Two peaks may be visible in the alpha-2 region and two to four in the beta region. A faint prealbumin peak, which moves faster than albumin, is sometimes visible in serum



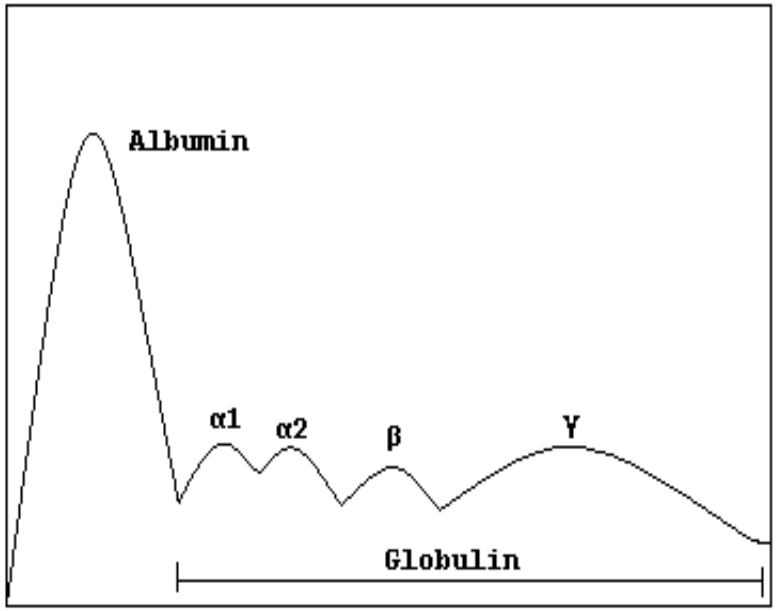
Normal ranges of serum proteins

	%	g/dl	mean
Albumin	62.8	3.60 - 5.20	4.46
Alpha-1	3.6	0.15 -0.40	0.26
Alpha-2	9.4	0.50 - 1.00	0.67
Beta	14.7	0.60 - 1.20	1.04
Gamma	9.5	0.50 - 1.60	0.67

Population Total Protein range 6.2 -8.3 g/dl
Population mean Total Protein 7.1 g/dl

Serum proteins

- Each of these five protein groups moves at a different rate in an electric field and together form a specific pattern. This pattern is used for identification of some diseases.
- A separate protein peak, the prealbumin is usually not visible on routine serum protein electrophoresis
- Prealbumin: also known as transthyretin; a major protein in the blood produced in the liver and released into the blood.
- Function: a binding protein for thyroxine and retinol-binding protein, so a transport protein for vitamin A and thyroxine; serum concentration of prealbumin reflects the synthesis capacity of the liver, hence a marker for nutritional status.
- Normal level 19-38 mg/dL; low values of 0 to 5 mg/dL= indicate severe malnutrition; 5 to 10 mg/dL moderate, and 10 to 15 mg/dL= mild protein depletion



Classification of serum proteins:

(1) Albumin

(2) (2) Alpha-1 globulin= 90% alpha-1-anti-trypsin

(3) Alpha-2 globulin = haptoglobin and alpha-2-macroglobulin.

(4) Beta globulin = transferrin, beta-2-lipoprotein, hemopexin (a heme-binding protein expressed in liver; high levels observed in inflammation) and immunoglobulins.

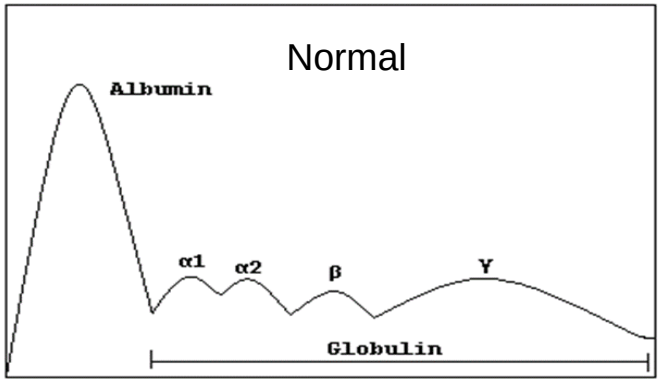
(5) Gamma globulin immunoglobulins and also contains lysozyme and C reactive protein

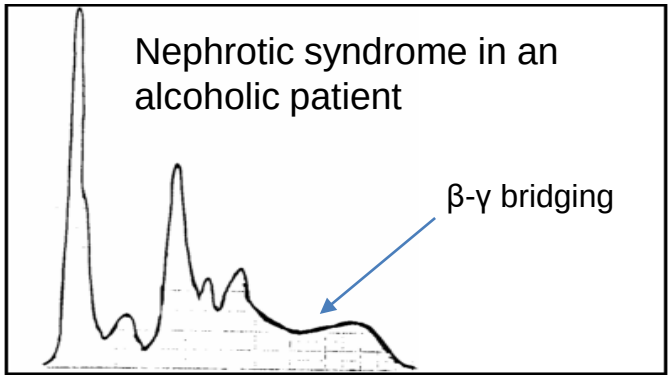
Electrophoresis of human serum samples on an agarose gel

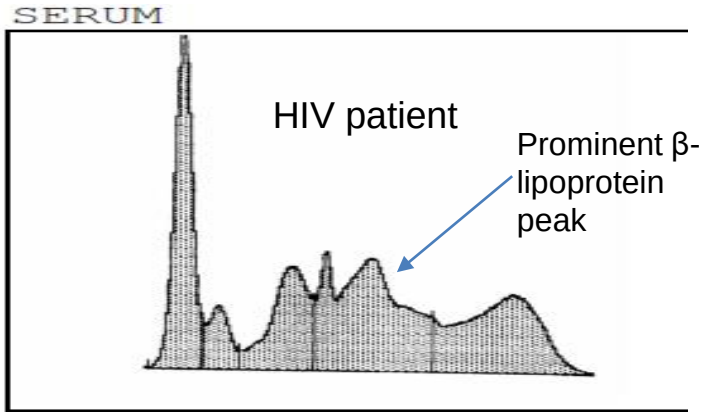
- Albumin, the first visible peak, is synthesized in the liver, for keeping fluid from leaking out from the blood vessels into the tissues.

Serum proteins in disease

	Normal	Alcoholic	HIV	Normal	Alcoholic	HIV
	%			g/dl		
Albumin	62.8	31.4 ↓	35.3 ↓	4.46	2.4	0.417
Alpha-1	3.6	5.9 ↑	10.2 ↑	0.26	0.5	0.120
Alpha-2	9.4	28.9 ↑	10.9 ↑	0.67	2.2	0.129
Beta	14.7	19.6 ↑	16.6 ↑	1.04	1.5	0.196
Gamma	9.5	14.2 ↑	27.0 ↑	0.67	1.1	0.319
Total				**7.10**	**7.70**	**1.181**







<u>Alcoholic serum protein profile</u>: low albumin; elevation of alpha-2 peak, specifically alpha-2-macroglobulin; a prominent beta-2-lipoprotein peak; beta-gamma bridging; a very high serum IgA, typical of post-necrotic cirrhosis

<u>HIV patient</u>: extremely low albumin; the patient has severe uremia and nephrosis, losing 28 grams of protein per day; beta-gamma bridging; prominent betalipoprotein peak; alpha fractions normal

Other serum proteins examined by electrophoresis

Protein	Function	Diseased condition
α_1-antitrypsin	Protease inhibitor	Reduced in α_1 –antitrypsin deficiency
β_2-micrglobulin	A subunit of the HLA (Human Leukocyte Antigen) on all cell membranes.	Raised in renal tubular dysfunction
Caeruloplasmin	Oxidizing enzyme	Reduced in Wilson's disease
C-reactive protein (CRP)	Involved in immune response	Increased in acute illness, especially infection
Ferritin	Binds iron in tissues	Gives an indication of body iron stores
Haptoglobin	Binds hemoglobin	Reduced in hemolytic conditions
Thyroid-binding globulin (TBG)	Thyroid hormone binding	Investigation of thyroid disease
Sex hormone binding globulin (SHBG)	Binds testosterone and oestradiol	Investigation of raised testosterone
Transferrin	Iron transport	Assessing response to nutritional support

Estimation and recovery of protein in gels

- Three methods are used to measure relative abundance of proteins in a gel

(1) Scanning densitometry

(2) Cutting out stained bands, elution, and measurement of absorbance of the color

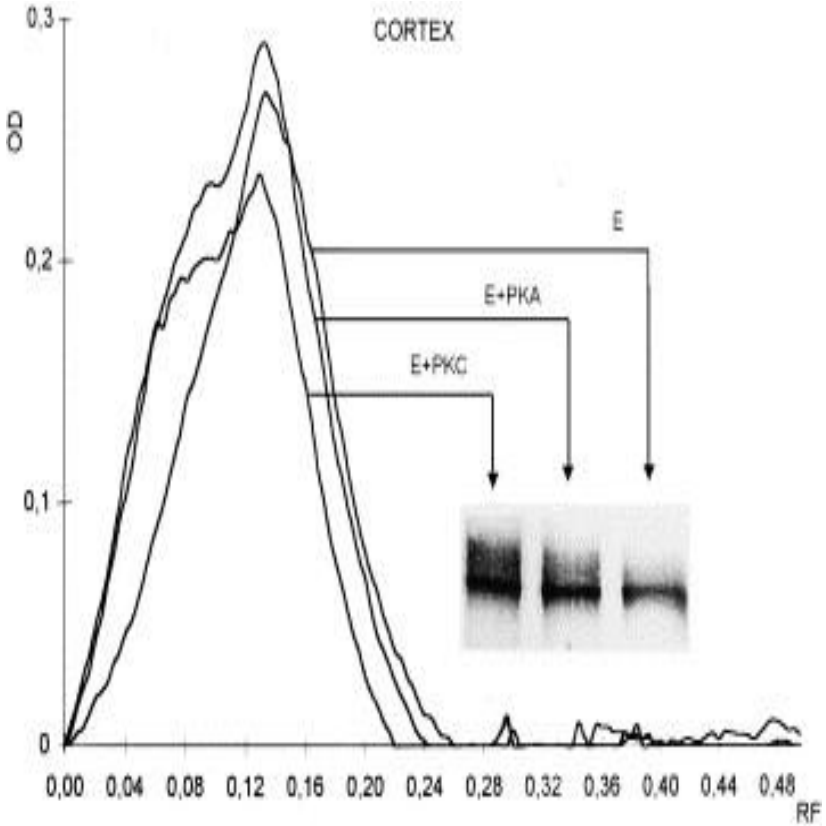
(3) Gel documentation systems

Estimation and recovery of protein in gels

- **Scanning densitometry** works by passing the stained gel lane over a beam of light (laser) and measuring the transmitted light
- A graphic presentation of protein zones (peaks of absorbance) against migration distance is produced, and peak areas can be calculated to obtain quantitative data
- Disadvantages:

1. Linear relationship between absorbance and concentration (A α conc. × path length) exists only within limited range
2. Equal amounts of different proteins do not always stain equally so comparison can only be semiquantitative

- Interpret densitometric data with caution



Densitometric scan of protein electrophoresis with three protein fractions

Cutting protein bands, elution and absorbance reading

- Protein bands can be cut out of protein blots or gel and the protein recovered by electrophoresis of the protein out of the gel piece by electroelution
- Further studies such as sequencing, determination of 3-dimensional conformation, molecular weight, etc., may be performed on the eluant
- Different designs of electroelution cells are commercially available, but the easiest method is to seal the cut gel piece in a buffer in a dialysis sac and place the sac in buffer between electrodes.
- Proteins will electrophorese out of the gel piece toward the appropriate electrode but will be retained by the dialysis sac.
- After electroelution, the current is reversed for a few seconds to drive off any protein that has adsorbed to the wall of the dialysis sac and then the protein solution within the sac is recovered

Cutting protein bands, elution and absorbance reading

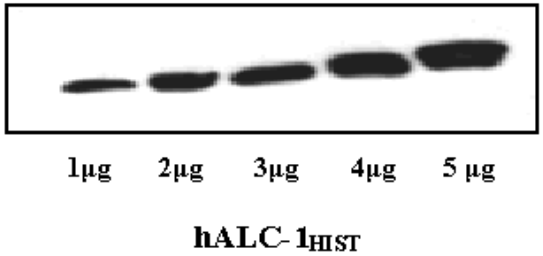
This method is much cheaper than scanning densitometry

Elute cut bands in 50% pyridine

hALC= Human atrial myosin light chain, a protein expressed in the ventricles of patients with hypertrophic cardiomyopathy and congenital heart diseases

Analysis of bands on gels is now enhanced with Image Quant TL software developed by GE Healthcare for detection of disease

A



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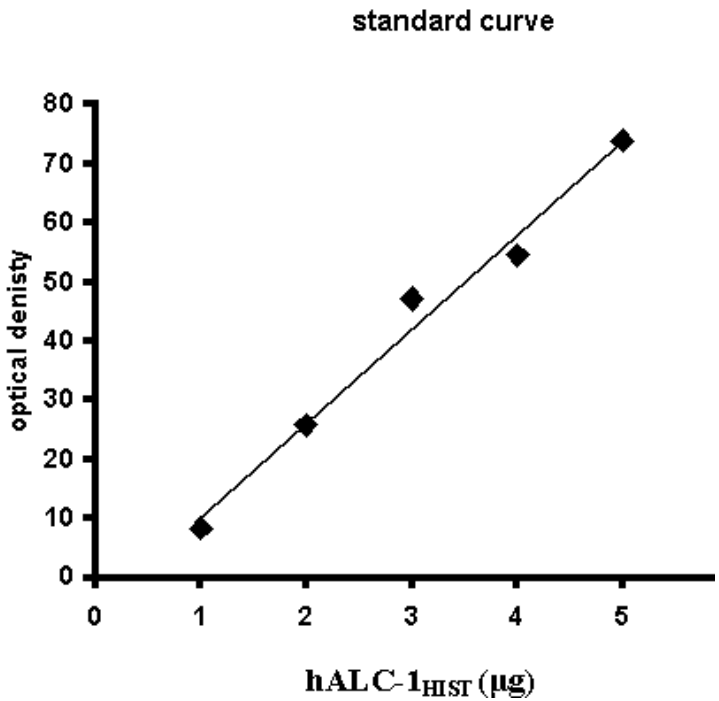


Gel documentation systems

- Gel docs comprise a video imaging unit, linked to a computer and attached to a small darkroom unit that is fitted with a choice of white or UV transilluminator.
- Gel images are produced which can be stored on a computer, enhanced accordingly and printed as required on a thermal printer

Gel documentation (gel doc) systems

For capturing of color images of nucleic acid and proteins suspended within agarose and polyacrylamide gels. Gels are typically stained with (ethidium bromide) EtBr or other fluorophores such as SYBR green. Generally, a gel doc includes an UV light transilluminator, a hood to shield external light sources and protect the user from UV exposure, and a camera for image capturing

Variations in native gel electrophoresis

- There are three methods of native PAGE: blue native (BN-PAGE), clear native (CN-PAGE), and quantitative preparative native continuous (QPNC-PAGE).

Blue Native PAGE

- BN-PAGE is the oldest native PAGE technique, where the Coomassie blue dye provides the necessary charges to the protein complexes for electrophoretic separation

- The disadvantage of Coomassie is that in binding to proteins it can act like a detergent causing complexes to dissociate

- Another drawback is the potential quenching of chemiluminescence (e.g. in subsequent Western blot detection or activity assays) or fluorescence of proteins with prosthetic groups (e.g. heme or chlorophyll) or labeled with fluorescent dyes.

Clear Native PAGE

- CN-PAGE separates acidic water-soluble and membrane proteins in a polyacrylamide gradient gel
- It uses no charged dye so the electrophoretic mobility of proteins in CN-PAGE depends only on the intrinsic charge of the proteins and on the pore size of the gel
- In many cases this method has lower resolution than BN-PAGE, but CN-PAGE offers advantages whenever Coomassie dye would interfere with further analytical techniques.

- Also CN-PAGE is milder than BN-PAGE so it can retain assemblies of membrane protein complexes that dissociate under the conditions of BN-PAGE

Quantitative Preparative Native Continuous PAGE

- QPNC-PAGEis a high-resolution technique applied in biochemistry and bioinorganic chemistry to separate proteins by isoelectric point.

- This method is used by biologists to isolate active or native metalloproteins in biological samples and to resolve properly and improperly folded metal cofactor-containing proteins in complex protein mixtures

Gel properties

- In order to obtain a fully polymerized gel for a PAGE run, the acrylamide mixture, at a low concentration, is polymerized for 69 hr at room temperature. As a result, the prepared gel is homogeneous, mechanically stable and free of monomers or radicals.
- The pore sizes of the prepared gel are very large and therefore, sieving effects become minimized during the electrophoretic separations. The proteins are separated based on their pI.
- The separated metalloproteins are not dissociated into apoproteins and metal cofactors.

QPNC-PAGE buffer

- The QPNC-PAGE procedure is accomplished in a special electrophoresis chamber for separating bioactive molecules

- Due to the specific properties of the prepared gel and electrophoresis buffer solution (which is basic and contains Tris-HCl and NaN_3), most proteins of a biological system are charged negatively in the solution, and will migrate from the cathode to the anode when placed in an electric field

- Although the pH value (10.00) of the electrophoresis buffer does not correspond to a physiological pH value within a cell or tissue type, the protein isomers are eluted continuously by a physiological buffer solution and isolated in different fractions. The separation system including the electrophoresis chamber and a fraction collector is cooled in a refrigerator

Identification

- After separation of the metalloproteins, the metal cofactors can be identified and quantified by mass spectrometry techniques
- Some of the metal cofactors which have been identified by this method are Fe, Co, Mn, Cu, and Zn

Protein detection techniques

- Fixing proteins on the gel
- Staining/dyes
- Enzyme-linked staining
- Chemiluminiscence
- Autoradiography
- Silver staining
- The location of protein on a gel can be determined by Coomassie blue staining, silver staining, or fluorescent staining. Coomassie staining is easier and more rapid than silver staining and fluorescent staining, but both are more sensitive. Prior to staining, the gel must be fixed.

Fixation

- Fixation is a procedure which employs chemical fixatives to denature and precipitate proteins into **large insoluble aggregates** within a gel matrix
- **Purpose of fixation**
  - Prevents diffusion of proteins, thus keeping protein bands sharp and resolved during the staining process
  - Removes gel buffer components, most importantly SDS, which may interfere in the staining process
  - Prevents protein from being washed out during staining
  - Modifies the proteins to enhance the staining reaction

  Disadvantage:

  Fixation reduces the amount of protein that can be recovered from the gel as gel matrix traps the protein samples

Fixation

- Requirements of a fixative: fast-acting; convenient to handle and nonhazardous; must preserve fine detail of the gel
- Commonly used fixatives: solutions of short chain alcohols and acetic acid in water, such as methanol:water:glacial acetic acid
- The combination of low pH (from the acetic acid) and highly organic solvent (the methanol) disrupts the hydrogen bonds which hold protein structures together, and exposes hydrophobic portions of the protein core
- This acid:methanol mixture acts as a denaturant to precipitate or fix the protein in the gel matrix, thus, preventing the protein from being washed out while it is stained
- As a result, the polypeptide chains uncoil
- The chains then participate in irreversible associations to produce complexes which are trapped inside the gel matrix

Fixation

- A disadvantage of the short chain alcohol:acetic acid:water fixatives is that the solutions are moderately denaturing, and may not fully fix small or unusually soluble proteins
- Stronger fixatives may be required
  - Trichloroacetic acid (TCA) (12% in water)
  - Sulfosalicylic acid
  - Aldehydes

Fixation

- TCA and sulfosalicylic acid are relatively stronger acids which disrupt the secondary structure of proteins by protonating weak acids in the protein structure, weakening salt bridges and charge interactions
- Aldehydes, such as formaldehyde and glutaraldehyde, react with amines on the surface of proteins, creating covalent cross links between protein molecules, resulting in truly irreversible denaturation

Examples of fixative formulations

- Methanol:Water:Glacial acetic acid (45:45:10) and Isopropanol:Water:Acetic acid (25:65:10) by volume), which can be stored at 4°C for 4 months
- To fix proteins with this class of fixatives: soak gel for 1 h by agitation, rinse the gel and proceed to staining

Protein detection techniques - staining

- The most commonly used general protein stain for detecting protein on gels is the sulfated trimethylamine dye, Coomassie Brilliant Blue R-250 (CBB).
- Staining is usually carried out using 0.1% (w/v) CBB in methanol:water:glacial acetic acid (45:45:10, by volume)
- Staining of most gels is accomplished in 2 hours and destaining , usually overnight, is achieved by gentle agitation in the same acid-methanol solution but in the absence of the dye.

Protein detection techniques

- Some proteins are difficult to fix

(A) Small proteins and unusually soluble proteins

- These proteins move through and out of the gel when fixed with methanol/acid class of fixatives.
- Require prefixing of the gel in 12 % trichloroacetic acid for 1-3 hours at room temperature prior to fixing by the MeOH/acid fixatives.

(B) Small peptides, heavily glycosylated and strongly basic proteins are resistant and cannot be precipitated by acid-based fixatives

- These need covalent cross-linking of the proteins with formaldehyde or glutaraldehyde

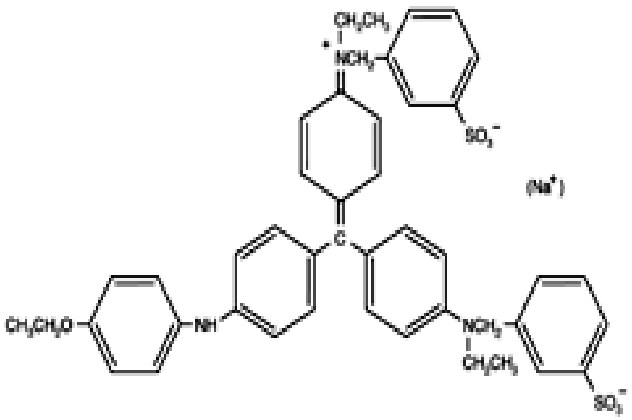
Protein detection techniques

- Formaldehyde fixative reagent: EtOH:Formalin:$H_2$0 (25:15:60 by volume). (Formalin is 35 % formaldehyde)
- Soak gel in fixative solution for 1 hour prior to staining
- Glutaraldehyde fixative reagent: soak gel in 10 % aqueous glutaraldehyde for 30 minutes, then wash with water for 2x lasting 20 min each. This treatment denatures proteins, fixes them in the gel, and also inserts reactive aldehyde groups on the surface of the proteins. This step is beneficial for silver staining

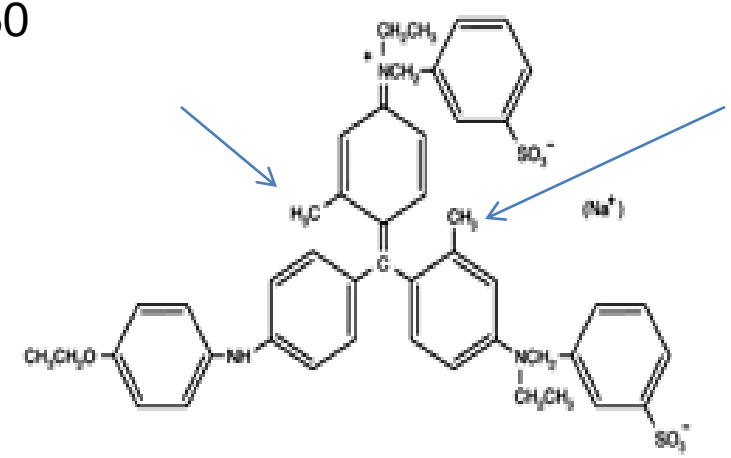
Coomassie brilliant blue dye

R-250 and G-250 differ by only two methyl groups

R-250

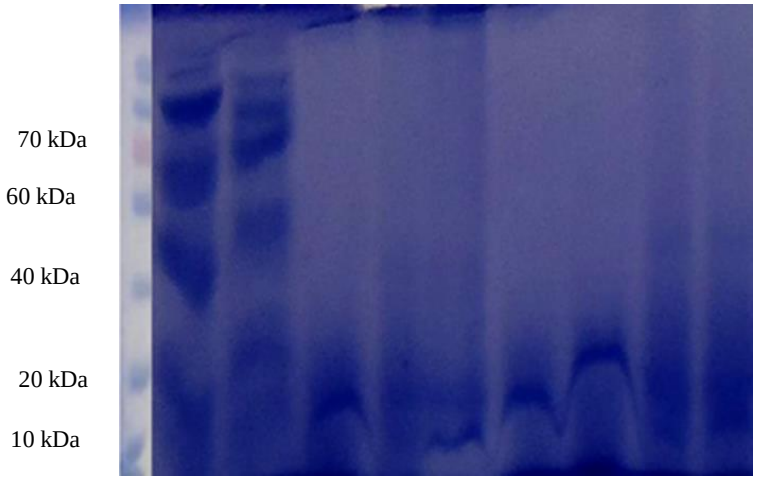


G-250



Protein detection techniques

SDS-PAGE of cocoa proteins stained with CBB dye. Note the intense blue bands and the Page Ruler protein ladder



- CBB binds to proteins through ionic interactions between dye sulfonic acid moieties and amino groups of protein and also through Van der Waals attraction.
- R-250 is more sensitive requiring less dye (0.1 µg of protein) than G-250 (0.5 µg). The G-250 dye shows a property of shifting from white or colorless form at pH 2 to dark blue black at pH 7. Upon binding to protein, the dye recovers its blue color, apparently due to the more neutral pH of the environment around the protein molecule.
- A gel placed in an acidified solution of G-250 will manifest blue protein bands on a light amber background

Protein detection techniques

Staining with Coomassie R-250

- Fix gel in isopropanol:water:acetic acid for 30-60 min
- Stain gel in 10% acetic acid in water, containing 60 mg/L of R-250. Bands will appear in 30 min. Allow staining to proceed until desired band intensity is reached.
- Because a low dye concentration is used, background staining is low. Destain gel in 10% acetic acid for 2 hours. Store gel in 7% acetic acid

Protein detection techniques

Staining with Coomassie G-250

- Make G-250 staining reagent : dissolve 0.2 g dye in 100 ml H_2O by warming to ~50°C. Cool and add 100 ml 2N H_2SO_4. Incubate at room temperature overnight, then filter. Carefully add 22.2 ml 10 N KOH, 28.7 g TCA. Incubate for at least 3 hr, then filter to obtain amber-brown solution without blue precipitate
- Immerse gel in the staining solution. Bands begin to appear in 15 min. Allow staining to proceed for several hours to increase intensity of band and sensitivity

Silver staining

- Silver staining is a highly sensitive method for visualization of protein bands on electrophoresis with detection limit of 2-5 ng/protein band
- Silver stains are based on photographic process
- In silver staining, silver binds to sulfhydryl and carboxyl moieties in proteins. Ag^+ cations then undergo reduction by the glutaraldehyde fixative to produce insoluble metallic silver (Ag^0) deposits which appear as dark brown or black bands on the gel

Silver staining

- A Sterling silver staining kit is commonly used in the lab. Minor bands, not detected with CBB are resolved using silver stain. It is at least 100 times more sensitive than CBB, detecting proteins down to 1 ng amounts.
- To visualize, place gel on a light box with fluorescent lamps and photograph the gel

Protein detection techniques- glycoproteins

- Detection of glycoproteins by lectins
- Lectin is a protein derived from plants which bind specifically to carbohydrates and produce agglutination of some animal cells and precipitates glycoconjugates
- Glycoproteins: are proteins glycosylated by covalent addition of oligosaccharide moiety predominantly to the nitrogen of the amide group of asparagine (N-glycosylation) or the oxygen of serine or threonine (O-glycosylation) polypeptide in post-translational modification
- The glycan added on to the protein may be made of the monosaccharides- glucose, galactose, fucose, mannose, N-acetylglucosamine, N-acetyl galactosamine, or N-acetylneuraminic acid
- Role of the sugars
  - enhance the water-holding capacity of proteins
  - Influence folding of the protein
  - Stabilize proteins
  - Make proteins resistant to proteolysis by digestive enzymes

Miracle berry (*Synsepalum dulcificum*) of West Africa

- Glycoproteins are present in cytosol, as extracellular secretory proteins; as integral membrane proteins, the immune system, for the ABO blood group recognition, as hormones (FSH, LH, TSH, etc.), in miraculin, the miracle berry

Protein detection techniques

- A far more sensitive method used currently to detect glycoproteins is to **blot** the gel and use **lectins** for detection
- Lectins are protein molecules that bind carbohydrates. Lectins have different specificities for different carbohydrates
- For example, certain lectins recognize mannose, fucose or terminal glucosamine of the carbohydrate side chains of glycoproteins
- The glycoprotein samples to be analyzed are run on a number of lanes of an SDS-polyacrylamide gel
- The gel is blotted onto nitrocellulose or Polyvinylidene difluoride (PVDF) membrane
- The membrane is blocked for 1 hour with RIPA (radioimmuno precipitation assay) buffer at room temperature
- Each lane, having the glycoproteins, is incubated with a different lectin to form a glycoprotein-lectin complex

Detection of glycoproteins by lectins

- For color to develop, the glycoprotein on the blot is incubated with horseradish peroxidase (HRP)-lectin conjugate (commercially available) with RIPA buffer for 2 hours at room temperature
- RIPA - Radioimmunoprecipitation assay buffer (composition- TRIS, NaCl, Na deoxycholate, EDTA, Na pyrophosphate, and others: check Sigma website)
  To this conjugate is linked an antibody specific to the lectin on the conjugate, and then a substrate of the peroxidase is added

Glycoprotein detection

Glycoprotein-Lectin complex on blot + Antibody (HRP-linked)+ substrate + oxidizing agent (H_2O_2)

↓ HRP

Oxidized substrate

(produces detectable color or luminescence)

Horseradish peroxidase (HRP), is a metalloenzyme found in the roots of the plant, horseradish. It is used extensively in biochemistry, proteomics and cancer studies for the detection of proteins, glycans, etc., where it amplifies a weak signal and increases detectability of a target molecule

HRP

- Horseradish peroxidase is found in the roots of horeradish plant. It is a glycoprotein metalloenzyme with many isoforms, with form C most studied. It is an alpha helix protein which binds heme as a cofactor.
- HRP contains 21.8% carbohydrate made from N-acetylglucosamine, mannose, fucose, xylose
- Its presence must be made visible using a substrate, which when oxidized by HRP using H_2O_2 as the oxidizing agent produces color that can be measured by a spectrophotometer
- HRP may also be utilized to produce chemiluminescence or fluorescence when the appropriate substrate is used.
- MW= 44,173.9 Dalton glycoprotein with 6 lysine residues which can be conjugated to a labeled molecule

Glycoprotein detection

- Chromogenic substrates include **TMB** (3,3’,5,5’-Tetramethylbenzidine) and **ABTS** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) both of which act as hydrogen donors for the reduction of hydrogen peroxide to water by the peroxidase enzyme such as HRP
- Chemiluminescent substrate: Luminol + H_2O_2 → 3-aminophthalate + light

Glycoprotein detection

<u>Examples of glycoproteins supplied by Sigma Aldrich</u>

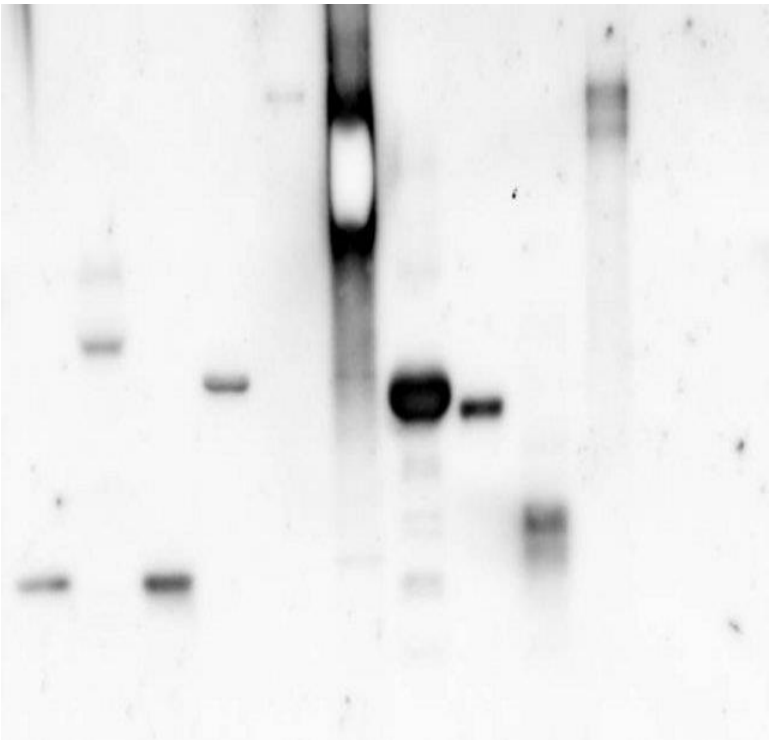
- 1. Urokinase from human urine
- 2. Factor IX Human
- 3. Complement component C1q from human serum
- 4. α1-Antitrypsin from human plasma
  5. Fetuin
- 6. Invertase glycoprotein Standard
- 7. IgG1 Monoclonal Anti-Flag M2 Ab produced in mouse
- 8. IgG2a Monoclonal Anti-AMY1A Ab produced in mouse
- 9. Recombinant Human t-Plasminogen Activator/tPA
  10. Epidermal growth factor receptor human
- 11. Apolipoprotein C-III from human plasma
- 12. Thrombospondin from human platelets

Glycoprotein detection

- Examples of lectin-HRP conjugates
- *Arachis hypogea*- peanut
- *Canavalia ensiformis* - Jackbean
- *Glycine max*- Soybean
- *Triticum vulgaris*- wheat
- Price per 1 mg lectin conjugate: EUR 178.00

Glycoprotein detection

By testing a protein sample against a series of lectins, it is possible to determine both the presence of the glycoprotein and the type of glycosylation



Western blot of twelve glycoproteins using HRP conjugated *Canavalia ensiformis* lectins

Glycoprotein staining

- Glycoproteins occur at many locations in eukaryotic cells
  - Glycoproteins of the plasma membrane
  - Some enzymes are glycoproteins, e.g., glycosylated invertase (a polymannan), pancreatic ribonuclease B (RNase B, having 5 to 9 mannose units attached to a chitobiose core), and peroxidase, e.g. horseradish peroxidase (HRP)
  - Immunoglobulins
- Besides detection with lectins, glycoproteins can be detected on SDS-PAGE gels and on Western blot membranes by the periodic acid-Schiff (PAS) stain
- However, the PAS stain is not very sensitive and often gives very weak magenta bands with a light pink or colorless background

Glycoprotein staining with PAS

- Detection limit is 25-100 ng of carbohydrates depending on the nature and degree of glycosylation of the protein
- Peroxidase from horseradish, having a carbohydrate content of approximately 16% is used as a positive control in the kit

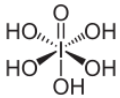
Periodic acid Schiff stain – a general or selective stain for glycoproteins

Following fixation, the gel is treated with an oxidizing reagent, periodic acid, the orthoperiodate form (MW= 227.94 g/mol).

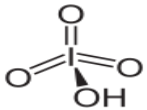
Periodic acid oxidizes the vicinal diols (or glycols) of the sugars in glycoproteins to produce aldehydes at the two free ends

Addition of Schiff's reagent (fuchsin sulfurous acid) forms a bright red color with aldehydes, and magenta color with glycoproteins

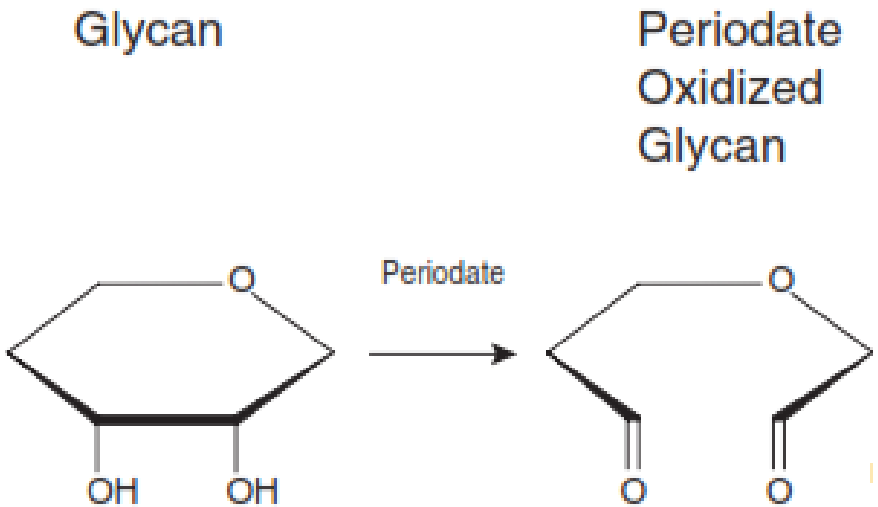
- Periodic acid exists in two forms- H_5IO_6



the orthoperiodate and HIO_4,



the metaperiodate



Glycoprotein detection

- A Glycoprotein Detection Kit provides a system for easy assay.

Composition of kit

- Oxidation Component (Periodic Acid)
- Reduction Component (Sodium Metabisulfite)
- Schiff’s Reagent, Fuchsin-Sulfite Reagent
- Peroxidase

Glycoprotein detection

Steps

- 1. Fixing 30 min
- 2. Washing 2 x 10 min
- 3. Oxidation 30 min
- 4. Washing 2 x 10 min
- 5. Staining 1-2 hours with Schiff's reagent until bands turn magenta
- 6. Reduction 60 min.
- 7. Wash with 2-3 changes of dH_2O, and band color will intensify
- 8. Storage overnight
- **Reading: Egito et al. (2001). Highly sensitive periodic acid/Schiff detection of bovine milk glycoproteins electrotransferred after nondenaturing electrophoresis, urea electrophoresis, and isoelectric focusing. Le Lait 81:775-785.**

Fluorescent staining

- Fluorescent stains are also used for protein detection. Examples are Sypro Orange and Sypro Red protein gel stain. Sypro Orange is slightly brighter, whereas Sypro Red has lower background fluorescence.
- Stained proteins can be visualized using a standard 300 nm UV Transilluminator or a laser scanner. They have detection limits of 1-2 ng/protein band, similar sensitivities to silver staining

Electrophoresis of nucleic acids

- Long chain DNA and RNA molecules may be resolved with agarose gel electrophoresis
- Short chain or fragments of DNA are separated by polyacrylamide gel electrophoresis

Agarose gel electrophoresis

- Agarose gel electrophoresis separates large DNA molecules and quickly estimates DNA concentration and size
- The following materials are required:
- A horizontal gel electrophoresis tank
- A power supply unit
- Analytical-grade agarose
- An appropriate running buffer, usually 1×TAE
- An intercalating DNA dye, eg., Ethidium bromide (EtBr)
- An appropriately sized DNA standard or DNA ladder

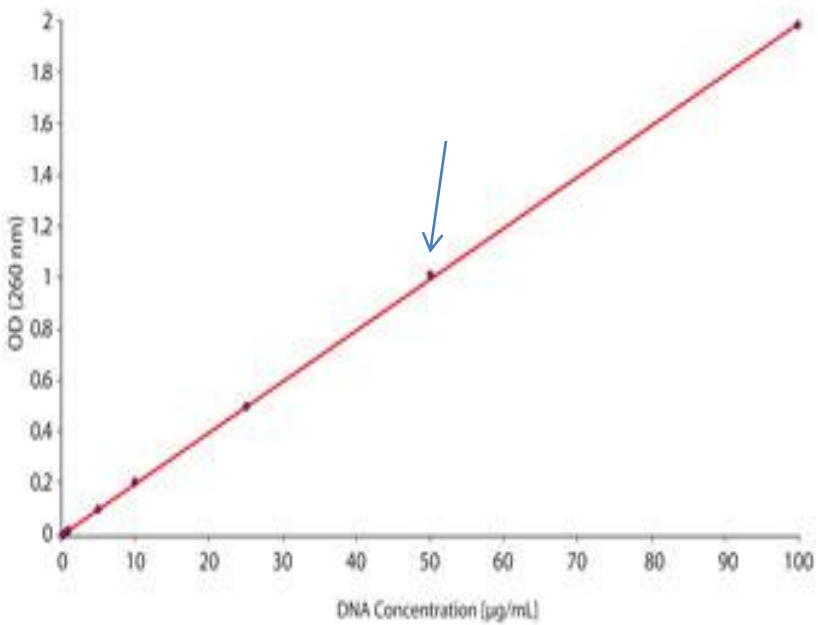
Agarose gel electrophoresis

- Load a sample of the isolated DNA into a well in the agarose gel slab (usually 1%)
- Connect to power supply and then expose to an electric field. The negatively charged DNA backbone migrates toward the anode. Small DNA fragments migrate faster than larger ones to achieve separation by size
- RNA and protein contaminants migrate at different rates compared to DNA. DNA bands will therefore be distinct

Agarose gel electrophoresis for DNA quantitation

- Concentration of DNA can be determined after gel electrophoresis by comparing the test DNA intensity to that of a DNA quantitation standard
- For example, if a 2 µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100 ng standard, then the sample concentration is 50 ng/µl (100 ng divided by 2µl). Standards used for quantitation are designed to have the relationship that an A_{260} of 1.0 = 50 µg/ml pure dsDNA

DNA Quantitation standard curve
Source: BMG Labtech

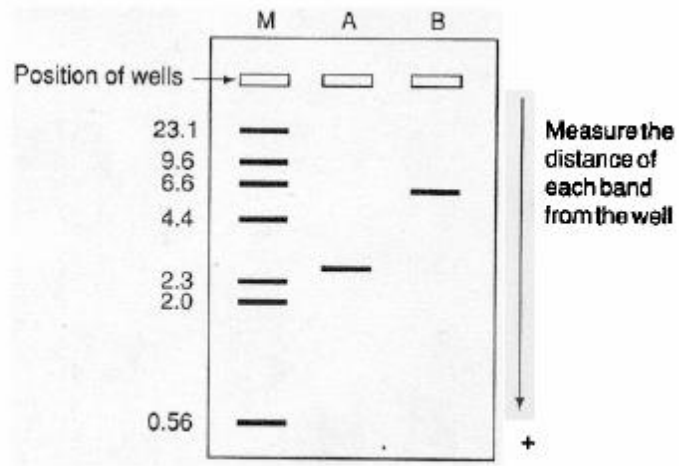


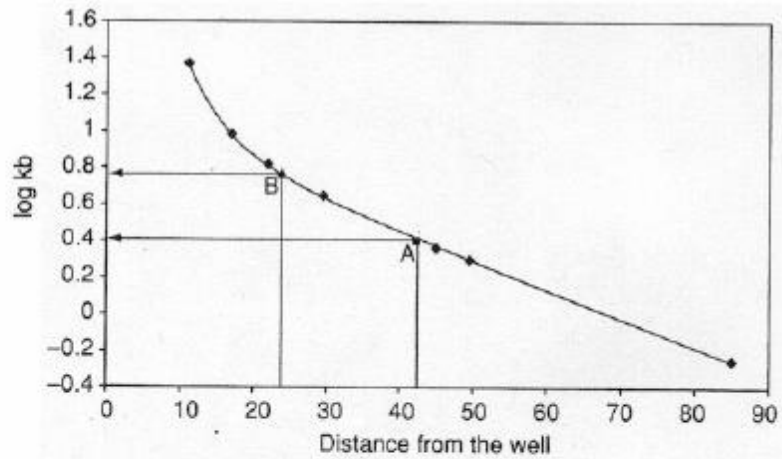
Conventional DNA quantitation

- Absorbance in a spectrophotometer is used
- dsDNA has max. absorbance at 260 nm
- A_{320} corrects for turbidity
- RNA and guanidine functional group of arginine also absorb at 260 nm, proteins at 280 nm (from aromatic functional group of amino acids), causing overestimation of DNA concentration
- DNA concentration (µg/ml) = (A_{260} reading – A_{320} reading) × dilution factor × 50 µg/ml
- Purity of dsDNA = A_{260}/A_{280} =1.7 to 1.9
- RNA A_{260}/A_{280} ratio is 2.0; Low ratio below 1.7 means protein contamination

DNA size by agarose gel electrophoresis

1. Eyeball it
2. Use calibration curve
   - Most gel imaging systems have this feature





For fragment A: Log kb- = 0.4. So kb= antilog 0.4 = 2.5 kb.

Similarly, antilog 0.8 = 6.3 kb from fragment B

Figure 4.4 Analytical gel electrophoresis; using the standard marker to provide a calibration curve, the size of fragment A is estimated as 2.5 kb and fragment B as 6.0 kb

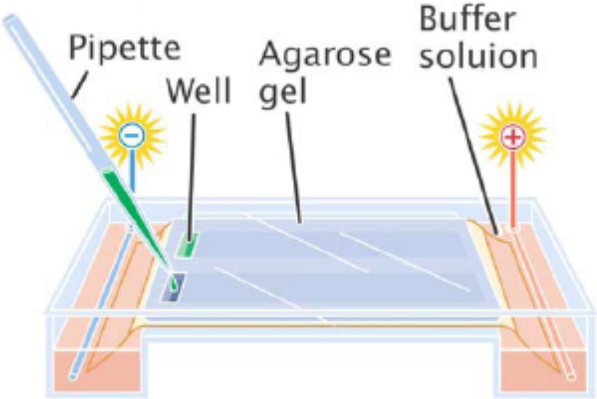
Agarose gel electrophoresis

- To visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR® Green is required. Caution: handle ethidium bromide with care, a known mutagen

Agarose gel electrophoresis for long DNA and RNA

Gel electrophoresis—separate molecules based on size and charge
DNA is – charged—place in electrical field, move towards opposite charge moves to + pole (anode)

Run DNA on agarose gel—visualize sample using ethidium bromide—intercalates into DNA between bases—fluoresces under UV light



Preparation of agarose gel

- Most agarose gels are made with between 0.7% (good separation or resolution of large 5–10 kb DNA fragments) and 2% (good resolution for small 0.2–1kb fragments) agarose dissolved in electrophoresis buffer.
- Up to 3% can be used for separating very tiny fragments. Low percentage gels are very weak and may break when you try to lift them. High percentage gels are often brittle and do not set evenly. 1% gels are common for many applications.
-

Buffers

- There are a number of buffers used for agarose electrophoresis. The most common being: Tris acetate EDTA (TAE), Tris/Borate/EDTA (TBE) and Tris-phosphate/EDTA.

- TAE is the most used. It has the lowest buffering capacity but provides the best resolution for larger DNA. This means a lower voltage and more time, but better result. Not suitable for extended periods of electrophoresis at high current.
- However, migration of double-stranded, linear and supercoiled DNA is much faster in this buffer than in TBE or TPE. Replacement of the buffer recommended under such conditions

- TBE and TPE are more expensive but have higher buffering capacity

Buffers

- **TAE buffer**
- Comes as a stock solution. 50× TAE buffer: 242g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5MEDTA (pH 8.0)
- **TBE buffer**
- Comes as a stock solution. 5× TBE buffer: 54g Tris base; 27.5 g boric acid; 20 ml 0.5M EDTA (pH 8.0)
- **TPE buffer**
- Stock solution. 10X TPE buffer: 108g Tris base; 15.5 ml 85% phosphoric acid; 40 ml 0.5M EDTA (pH 8.0)
- Alkaline buffer
  - Stock solution is 5 ml 10 N NaOH; 2ml 0.5 M EDTA
  - The buffer system used for denatured single-stranded DNA is alkaline buffer. Because agarose cannot melt in NaOH, the agarose must first be melted in water before adding the 50 mM NaOH/EDTA

The loading dye

- A color marker containing a low molecular weight dye such as bromophenol blue (to enable tracking the progress of the electrophoresis; bromophenol blue runs as 300 bp size) and glycerol (to make the DNA solution denser so it will sink into the wells of the gel) plus a negatively charged loading buffer is mixed with the DNA before loading
- The loading dye co-sediments with DNA (meaning they move at the same speed as DNA of a certain length).

- **Recipes for loading buffers**
- The precise amount of dye is not important. However, it is crucial that you prevent overlay of dye and expected DNA size. For example, if you are expecting a genotyping band of 200-400 bp, you shouldn't use bromophenol blue since it will obscure your product. In this case, you should use a smaller dye orange G which migrates at 50 bp
- **Ficoll & Orange G (6x)**
- 1.5g Ficoll 400, Orange G dye, dH_2O to 10mL
- Add very small amounts of Orange G dye such that the loading dye is dark orange. Store in small aliquots at 4°C (room temperature is okay too). To use, add and mix 1/5th volume of loading dye to DNA solutions prior to loading into the wells of gels.

Loading dye

- **Sucrose & xylene cyanol / bromophenol blue (6x)**
- 4 g sucrose
- 25 mg bromophenol blue or xylene cyanol (0.25%) Xylene cyanol migrates around 3,000 bp
- dH_2O to 10mL
- Add appropriate amount to DNA sample, e.g. 5µl to 25µl.
- Store at 4°C to avoid mould growing in the sucrose. 10mL of loading buffer will last for years.
- **Glycerol & bromophenol blue (6x)** (most often used)
- 3 ml glycerol (30%)
- 25 mg bromophenol blue (0.25%)
- dH_2O to 10mL

Visualization

- The most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis is ethidium bromide, (EtBr: 5.25 mg/ml in H_2O). It fluoresces under UV light when intercalated into DNA or RNA.
- By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than 20 ng DNA becomes distinctly visible. EtBr is a known mutagen, care must be taken during handling. The following are however, safer to use.
- *SYBR Green* is another dsDNA stain, produced by Invitrogen. It is more expensive, but 25 times more sensitive, and possibly safer than EtBr, though there is no data addressing its mutagenicity or toxicity in humans. A safer variant is *SYBR Safe*

Visualization of electrophoresis gels

- Since EtBr stained DNA is not visible in natural light – visualize under UV light with protection of the eye with UV protective goggles or shield

- Loading buffers contain bromophenol blue and xylene cyanol which are visible in natural light. These run about the same speed as DNA fragments that are 300 bp and 3,000 bp in length, respectively. Cresol red and Orange G which run at about 125 bp and 50 bp, respectively, are less frequently used

Agarose gel electrophoresis process

- Isolate genomic DNA or prepare cDNA from sample
- Measure concentration using the Nanodrop spectrophotometer
- Perform agarose gel electrophoresis on the DNA samples by:
- Prepare 1% agarose gel, heat in a microwave and allow to cool to about 60°C. Add 2.5 μl EtBr. Pour gel and insert comb. Mix 5 ul loading dye with 8 to 17 μl DNA sample and load onto gel. Also load 5 μl DNA ladder plus 5 μl loading dye. Connect to power source and run electrophoresis for about 20 to 25 min

Agarose gel electrophoresis process

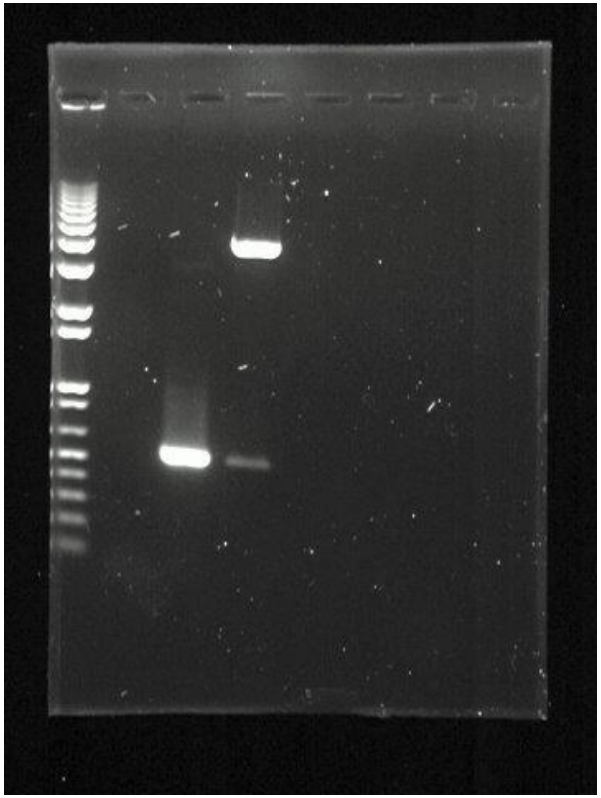
- The DNA molecule may range from oligonucleotides to chromosomes.
- During electrophoretic separation, the conformation of dn is not static, but changes between collapsed (coiled) to highly extended states as in its movement it encounters both electrical field and nonelectric forces of colliding with the gel matrix

Visualization

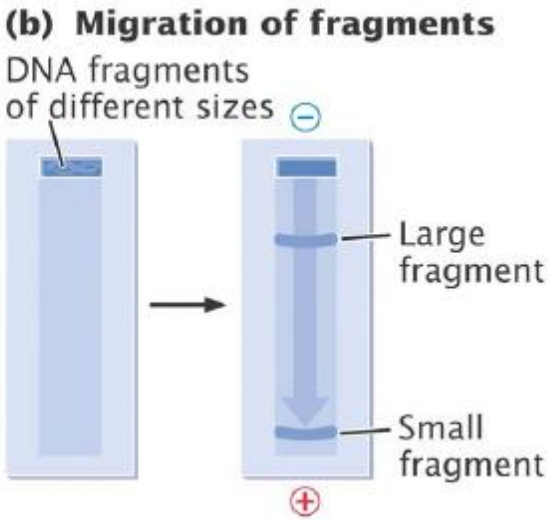
- After electrophoresis the gel is illuminated with UV light from a UV Transilluminator (protect yourself from exposure to UV radiation to view the DNA bands.
- The EtBr fluoresces reddish-orange in the presence of DNA.

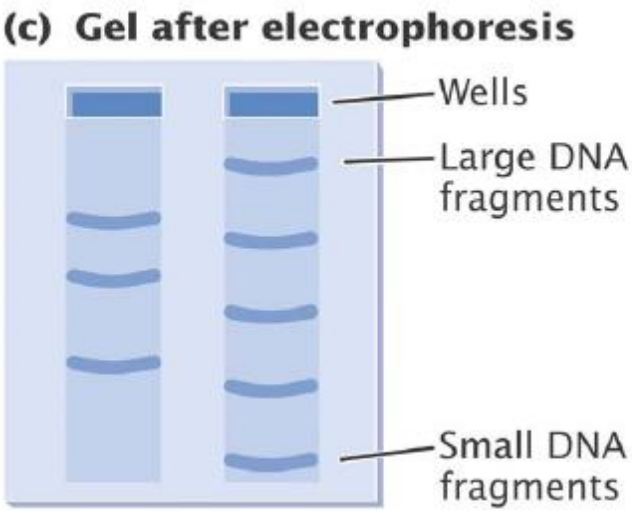
The gel can then be photographed usually with a digital or polaroid camera.

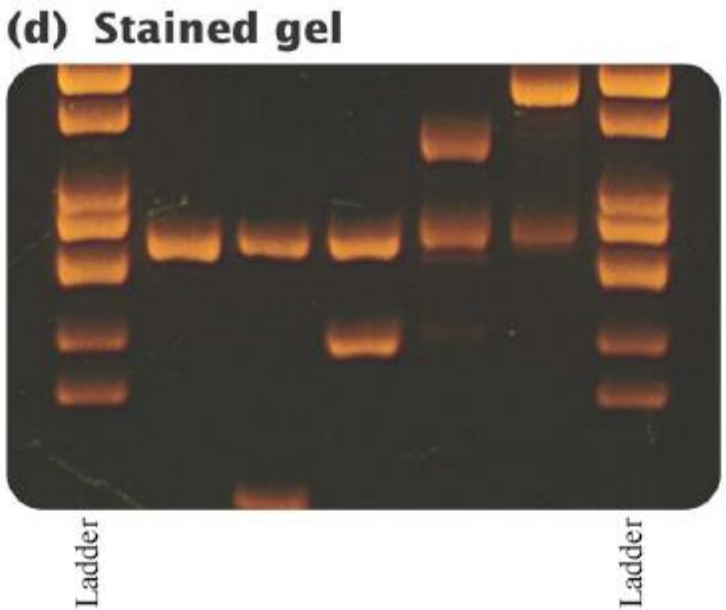
- Although the stained nucleic acid fluoresces reddish-orange, images are usually shown in black and white
- The DNA band can also be cut out of the gel, and can then be dissolved to retrieve the purified DNA.



An agarose gel picture. DNA bands appear white on a black background. A 1 kb DNA ladder on the left. The first intense band (from the bottom up) is 1 kb







Use agarose gel to determine quality of DNA

Can quantitate DNA if have known quantities for comparison

Use ladder to estimate size of unknown

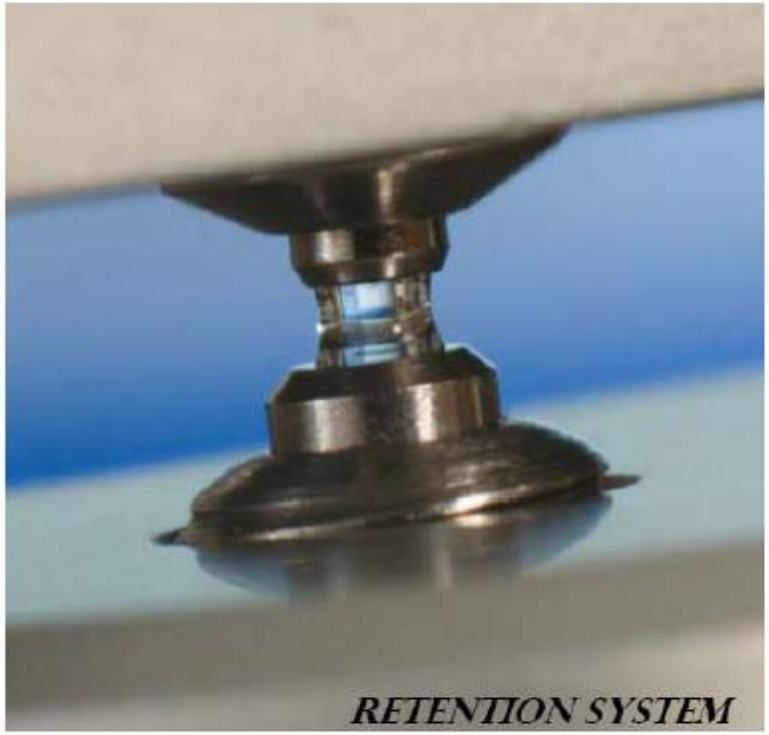
True size if linear molecule

Agarose gel electrophoresis

- A more sensitive detection method incorporates a radioisotope into the DNA molecules before electrophoresis
- ^{32}P is often used as it can be incorporated into DNA phosphates. It emits an energetic β particle that is easily detected by autoradiography

•Nanodrop—no cuvette, accurately detects from 2-3700 ng/µl without dilution

•No measure of sample integrity



Polyacrylamide gel electrophoresis of low MW nucleic acids

- The types of gel most commonly used for DNA electrophoresis are agarose (for relatively long DNA molecules) and polyacrylamide (for high resolution of short DNA molecules, for example in DNA sequencing
- Watch videos of Maxam-Gilbert DNA sequencing Method and Sanger DNA sequencing
- DNA preparation → Chemical modification → Electrophoresis → Detection by autoradiography

DNA sequencing

Two methods were developed:

1. **Maxam-Gilbert method**—specific chemical cleavage—use dimethylsulfate, hydrazine, piperidine, dilute acid, alkali solutions

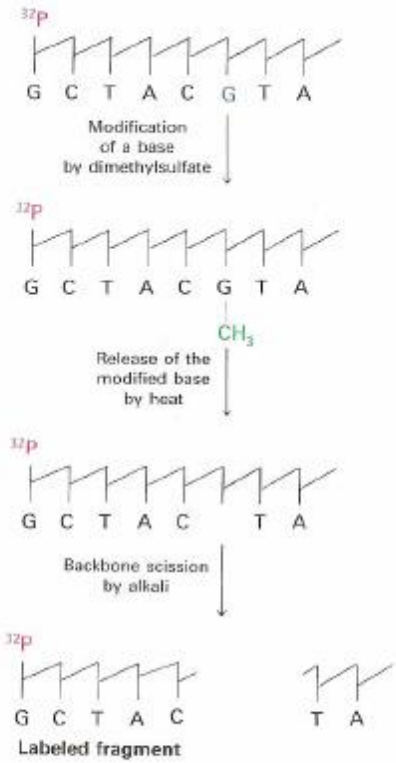
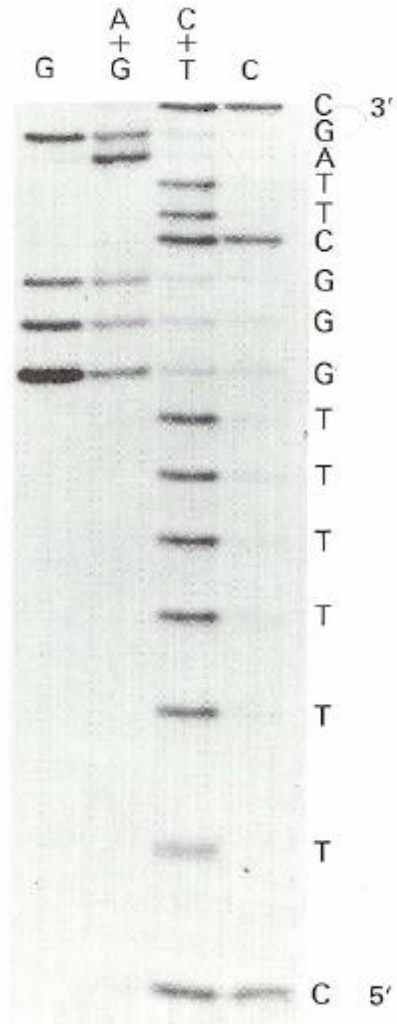
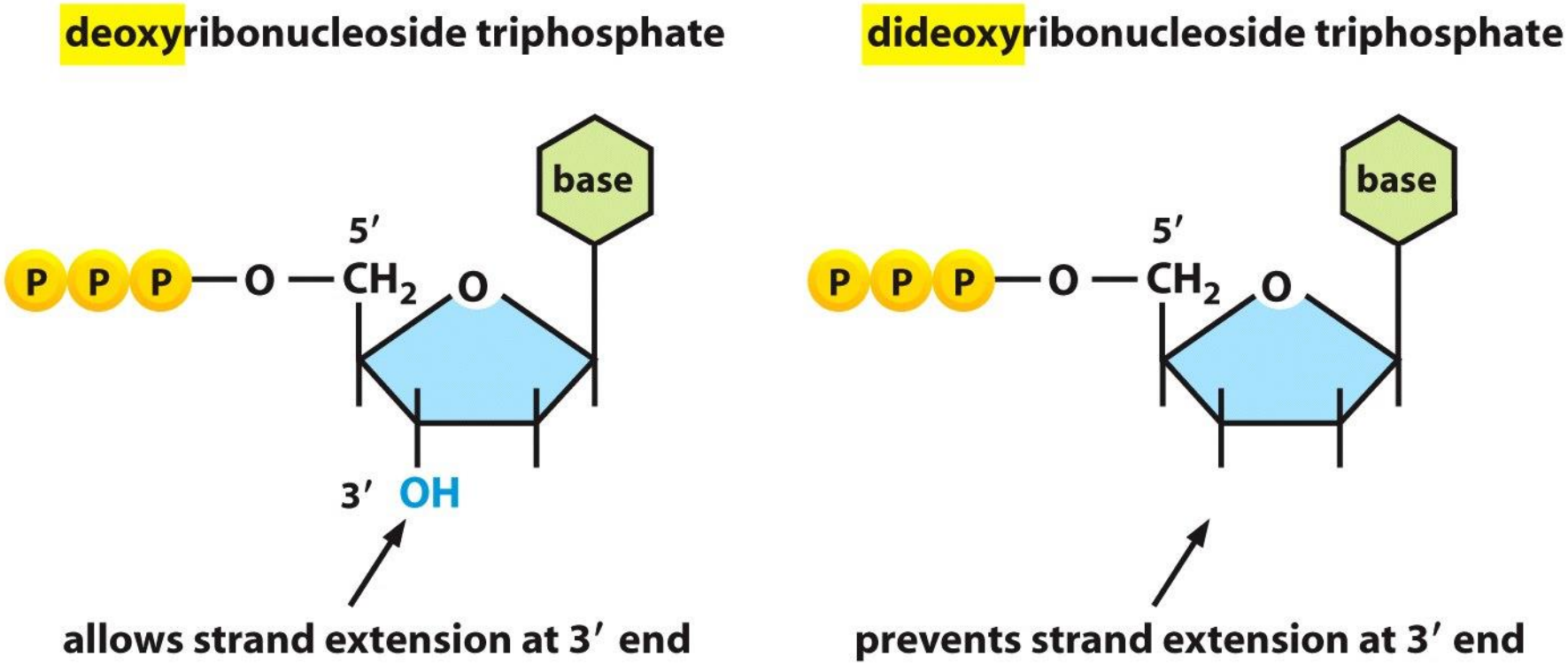


Figure 6-6
Strategy of the chemical-cleavage method for the sequencing of DNA. This particular procedure would produce the fragments visualized in the G lane of a set of gels.



Polyacrylamide gel electrophoresis of low MW nucleic acids

- 2. Frederick Sanger developed the chain termination method for DNA sequencing
- This method employs polyacrylamide gels with high resolution to separate fragments which differ by only one base at a time. This is also called the dideoxy method of sequencing
- Sanger's method is more efficient and uses fewer toxic chemicals and lower amounts of radioactivity than the method of Maxam and Gilbert
- Sanger's method relies on the use of dideoxyribonucleoside triphosphates. These are derivatives of the normal deoxyribonucleoside triphosphates that lack the 3'-hydroxyl group


deoxyribonucleoside triphosphate
base
5′
P P P — O — CH2 O
3′ OH
allows strand extension at 3′ end
dideoxyribonucleoside triphosphate
base
5′
P P P — O — CH2 O
prevents strand extension at 3′ end

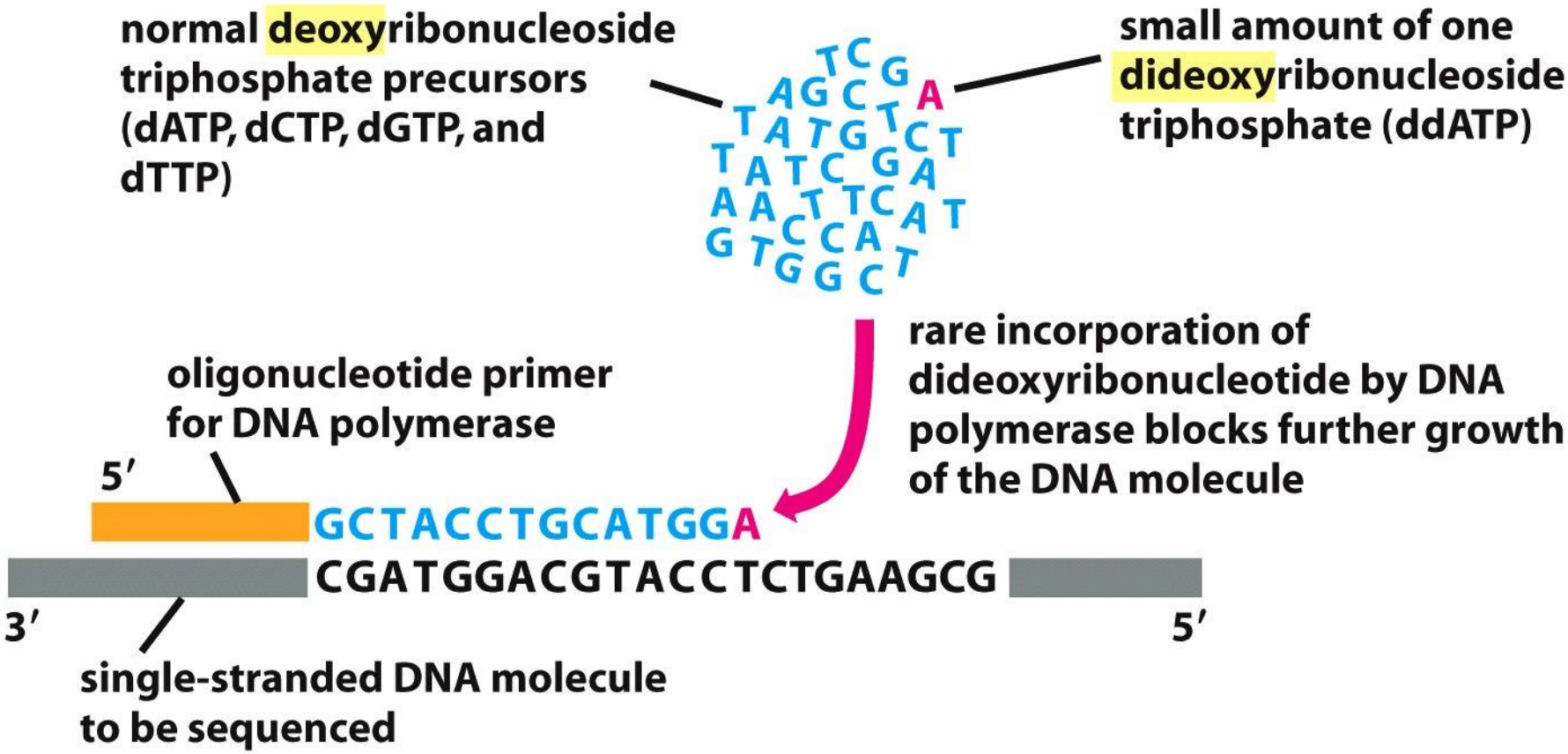
PAGE of low MW nucleic acids

- Purified DNA is synthesized in vitro in a mixture that contains single-stranded molecules of DNA to be sequenced, the enzyme DNA polymerase, a short primer DNA to enable the polymerase to start DNA synthesis, and the four deoxyribonucleoside triphosphates (dATP, dCTP, dGTP, dTTP)

- If a dideoxyribonucleotide analog of one of these nucleotides is added to the nucleotide mixture, where it can be incorporated into a growing DNA chain, because this chain now lacks a 3'-OH group, the addition of the next nucleotide is blocked, and the DNA chain terminates at that point. The reaction mixture will eventually produce a set of DNAs of different lengths complementary to the template DNA that is being sequenced and terminating at each of the different As, Cs, Gs or Ts

PAGE of low MW nucleic acids

- The exact lengths of the DNA synthesis products can then be used to determine the position of each of the nucleotides in the growing chain
- To determine the complete sequence of a DNA fragment, the Double-stranded DNA is first separated into single strands and one of the strands is used as a template for sequencing
- Four different chain-terminating nucleoside triphosphates – ddATP, ddCTP, ddGTP, ddTTP) are used in four separate DNA synthesis reactions using copies of the same single-stranded DNA template
- Each reaction produces a set of DNA copies that terminate at different points in the sequence
- The products of these four reactions are separated by polyacrylamide gel electrophoresis in four parallel lanes labeled A,T, C, and G


normal deoxyribonucleoside triphosphate precursors (dATP, dCTP, dGTP, and dTTP)
small amount of one dideoxyribonucleoside triphosphate (ddATP)
rare incorporation of dideoxyribonucleotide by DNA polymerase blocks further growth of the DNA molecule
oligonucleotide primer for DNA polymerase
5′
GCTACCTGCATGGA
CGATGGACGTACCTCTGAAGCG
3′
5′
single-stranded DNA molecule to be sequenced

PAGE of low MW nucleic acids

- A high resolution polyacrylamide gel under denaturing conditions is used so all DNAs are single stranded
- The electrophoretic analysis of single stranded nucleic acids is complicated by the secondary structures assumed by these molecules.
- The inclusion of denaturing agents unfolds the DNA or RNA strands and remove the influence of shape on their mobility.
- Nucleic acids form structures stabilized by hydrogen bonds between bases. Denaturing requires disrupting these hydrogen bonds.
- The most commonly used DNA denaturants are urea and formamide. Each of these forms hydrogen bonds with the DNA bases, "saturating" H-bond sites and preventing the formation of inter-base bonds.

PAGE of low MW nucleic acids

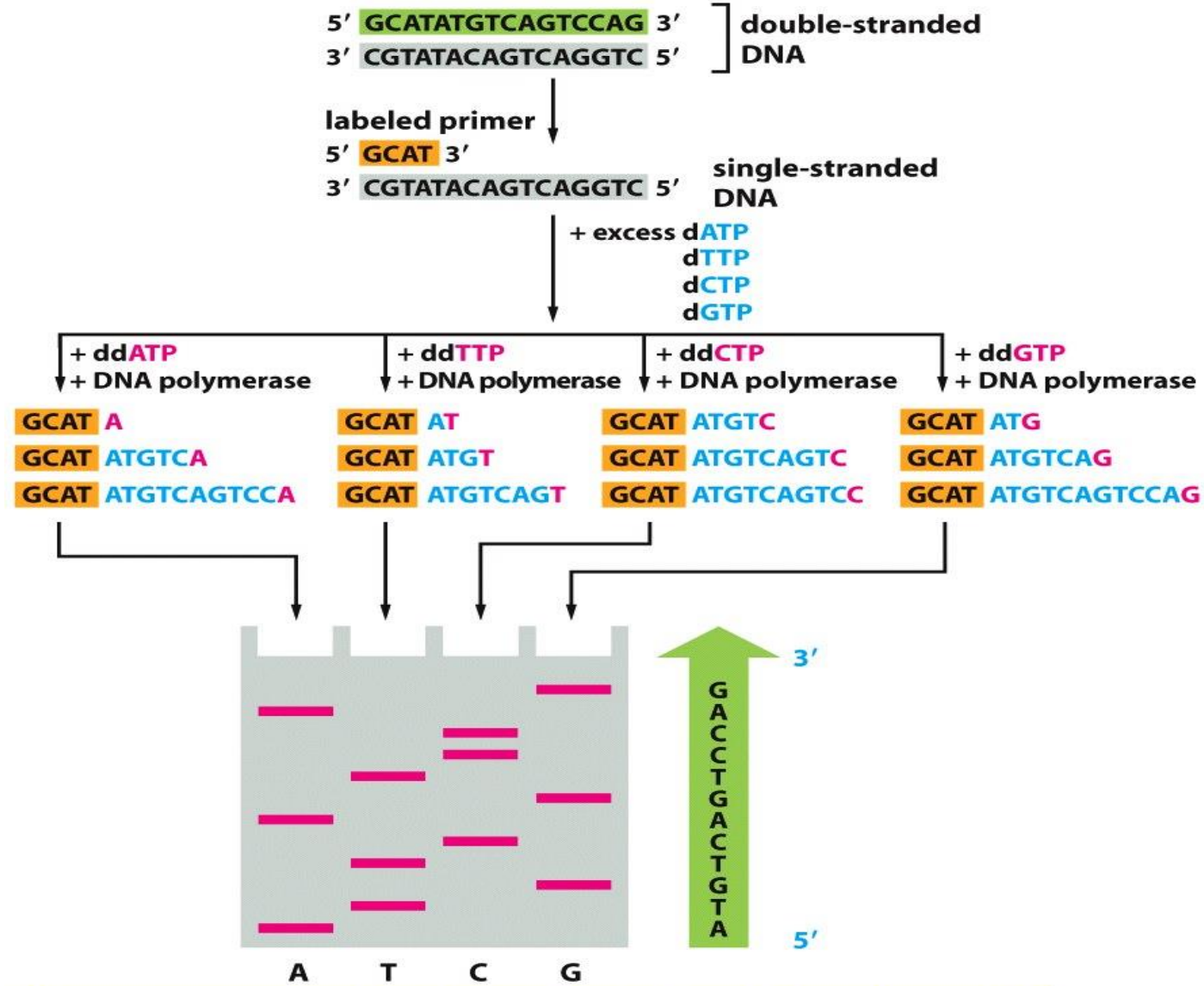
- Both formamide and urea effectively lower the melting point of the DNA molecules, allowing the structures to fall apart at lower temperatures.
- RNA is often denatured with harsher agents, because RNA tends to form stronger structures.
- This denaturing gel electrophoresis has such a good resolution that it can separate fragments, differing in length by only one base
- The newly synthesized fragments are detected by a label (radioactive or fluorescence) incorporated into the primer or into one of the dideoxynucleotides

Template

3′ ATCGGTGCATAGCTTGT 5′

Sequence reaction products

5′ TAGCCACGTATCGAACA* 3′
5′ TAGCCACGTATCGAA* 3′
5′ TAGCCACGTATCGA* 3′
5′ TAGCCACGTA* 3′
5′ TAGCCA* 3′
5′ TA* 3′



DNA sequence reading directly from the bottom of the gel upward, is

ATGTCAGTCCAG
1 12

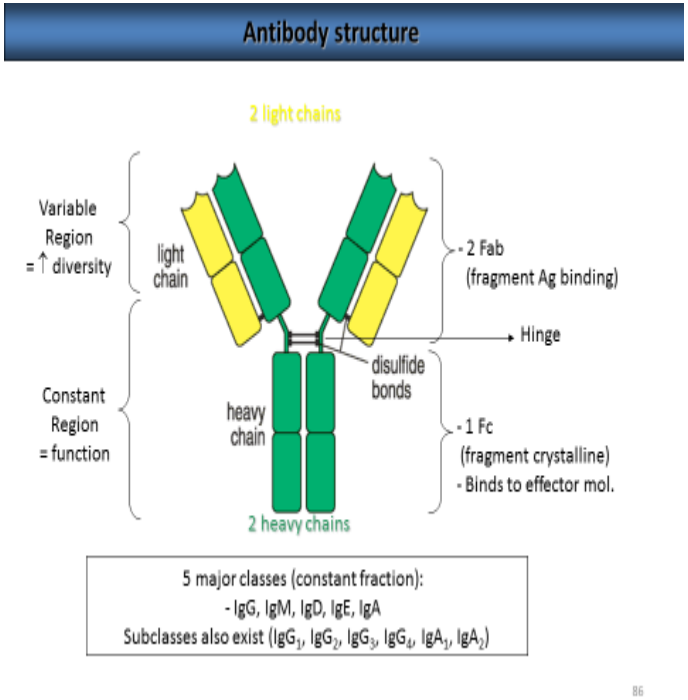
Western Blotting

- After electrophoresis, some proteins may be stained when they are present on a membrane
- This procedure requires that proteins be transferred and immobilized onto a membrane support as done in **Western Blotting**
- Staining of proteins immobilized on membranes may be done using Ponceau S or indelible ink

Western blotting

- Western blotting (immunoblotting) is an immunostaining technique introduced by Towbin et al. (1979) for identifying specific antibodies or proteins in which proteins are separated by electrophoresis, transferred to nitrocellulose membrane, and reacted with antibody.
- The term "blotting" refers to the transfer of biological samples from a gel to a membrane followed by detection on the surface of the membrane.
- The prefix –immuno, indicates that an antibody is used to specifically detect its antigen (the protein)
- Western blotting- a routine technique for qualitative detection and semiquantitative determination of a protein

Western blotting



- An antibody can be raised and/or purified against a single “epitope” (area on an antigen to which an antibody attaches) on a larger “antigen”
- So, the antibody possesses a specific binding site, the paratope ,whose structure fits a specific binding location (epitope) on an antigen
- When antibody and antigen are mixed, the antibody will bind tightly to the epitope it recognizes
- In this way, immunological detection answers questions about the structure and identity of a protein which cannot be detected through conventional chemical stains.
- The specificity of the antibody-antigen interaction enables a target protein to be identified in the midst of a complex protein mixture

Western blotting

- Immunological detection can be as much as 100 fold more sensitive than chemical stains

Steps in Western blotting procedure

- Separate the protein mixture using gel electrophoresis
- After electrophoresis, transfer or blot the separated protein bands onto a second matrix, generally a nitrocellulose or polyvinylidene difluoride (PVDF) membrane.
- Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface of the membrane
- Create a complex of the transferred protein with an enzyme-labeled antibody as a probe. Add an appropriate substrate (chromogenic or luminescence) to the enzyme and together they produce a detectable product such as a chromogenic precipitate or a luminescent product on the membrane for colorimetric or chemiluminescent detection, respectively

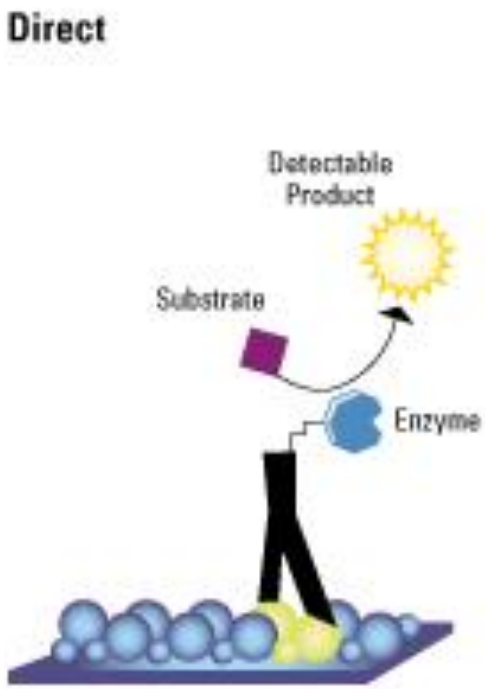
Western blotting

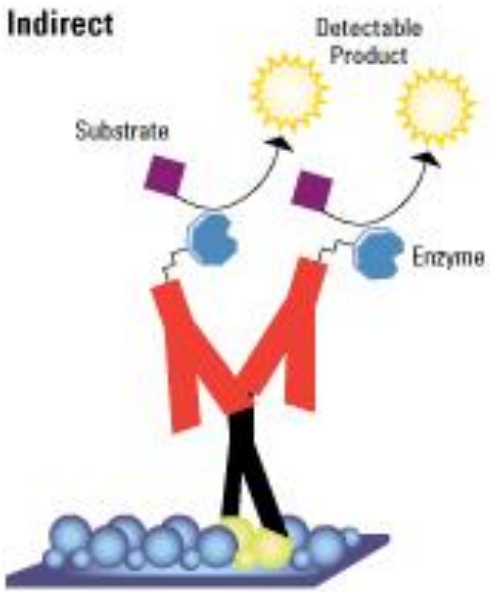
- Chemiluminescent detection is more sensitive than colorimetric detection
- The light output, which is a byproduct of the enzyme-substrate reaction can be captured using a photographic film, a CCD camera or a phosphorimager that is designed for detection.
- Alternatively, fluorescently tagged antibodies can be used, which are directly detected with the aid of a fluorescence imaging system. Whatever system is used, **the intensity of the signal should correlate with the abundance of the antigen on the membrane.**
- There are two major methods of detection of a Western blot: **direct** (not widely used) and **indirect** detection
- In the direct detection method, **a primary antibody** is used to detect an antigen on the blot. The primary antibody is labeled with an enzyme or fluorescent dye
- In the indirect detection method, **two antibody systems** are used: a primary antibody which is added first to bind to the antigen, followed by addition of a labeled secondary antibody that is directed against the primary antibody

Western blotting

- Compounds used as labels include biotin, fluorescent probes such as fluorescein or rhodamine, and enzyme conjugates such as horseradish peroxidase (HRP) or alkaline phosphatase.
- The indirect method offers many advantages over the direct method

Western blotting detection methods



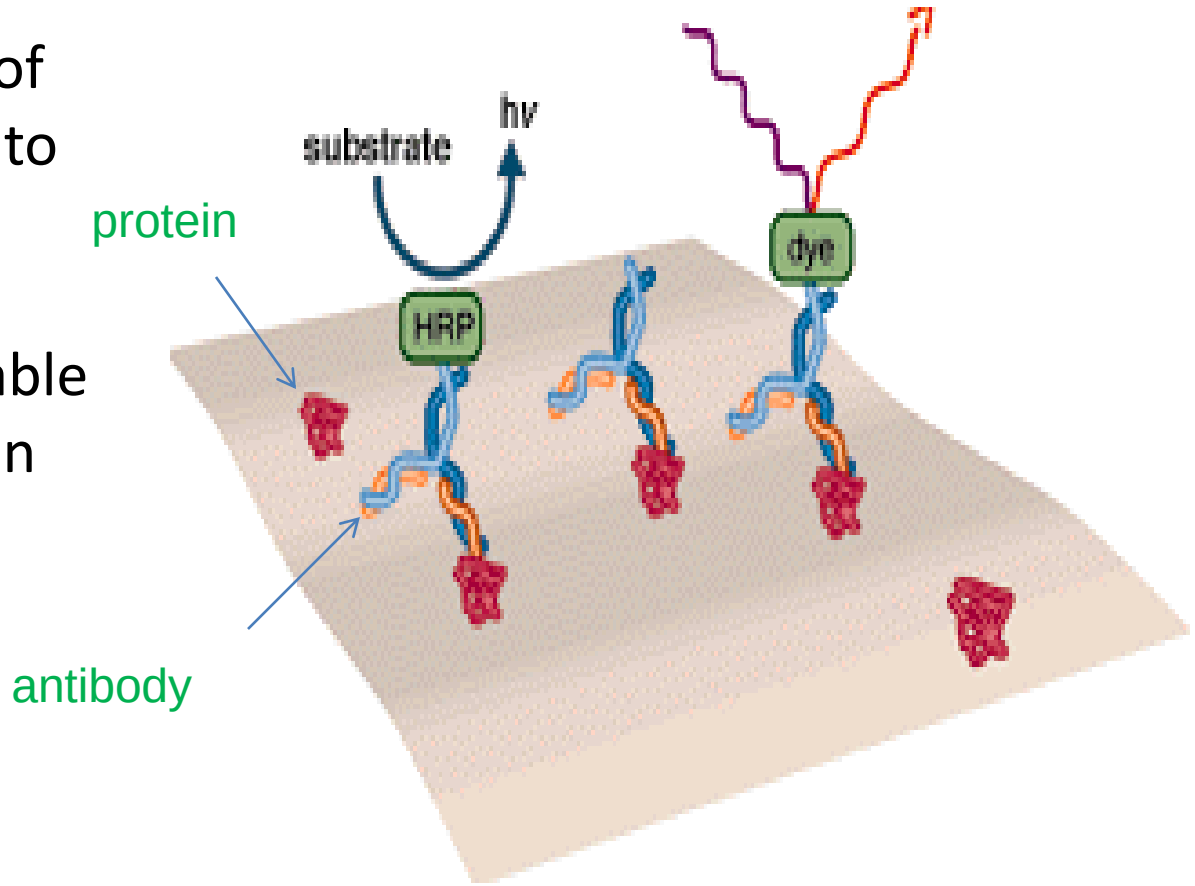


Mechanism of Immunostaining

- Enzymes are particularly suited to this role because they are proteins, and thus stable wherever antibodies are stable.
- Two enzymes are most commonly used for labeling antibodies: (1) Horseradish Peroxidase (HRP) and(2) Alkaline Phosphatase (AP).
- Chromogenic and luminogenic substrates are available for both of these enzymes.

Mechanism of Immunostaining

The basic method of immunostaining is to probe with an antibody which is bound to a detectable molecule such as an enzyme or a fluorescent dye.



Immunostaining with alkaline phosphatase

- Alkaline phosphatase catalyzes the removal of a phosphate group from its substrate.
- Artificially made phosphorylated substrates, such as BromoChloroIndoyl Phosphate (BCIP) coupled with Nitro Blue Tetrazolium (NBT) which produces color upon dephosphorylation are available
- BCIP is dephosphorylated by alkaline phosphatase to generate one half of an indigo dye molecule
- Dimerization of the molecule generates the full indigo dye, and liberates two reducing equivalents
- Reduction of NBT by the reducing compounds produces insoluble formazan dye which is intense blue

Immunostaining with alkaline phosphatase

- Another artificially made phosphorylated compound is para-nitrophenyl phosphate (PNPP)
- PNPP is hydrolyzed by alkaline phosphatase to liberate a soluble yellow dye. This is commonly used in Enzyme-Linked Immunosorbent Assay (ELISA) reactions.

Immunostaining with alkaline phosphatase

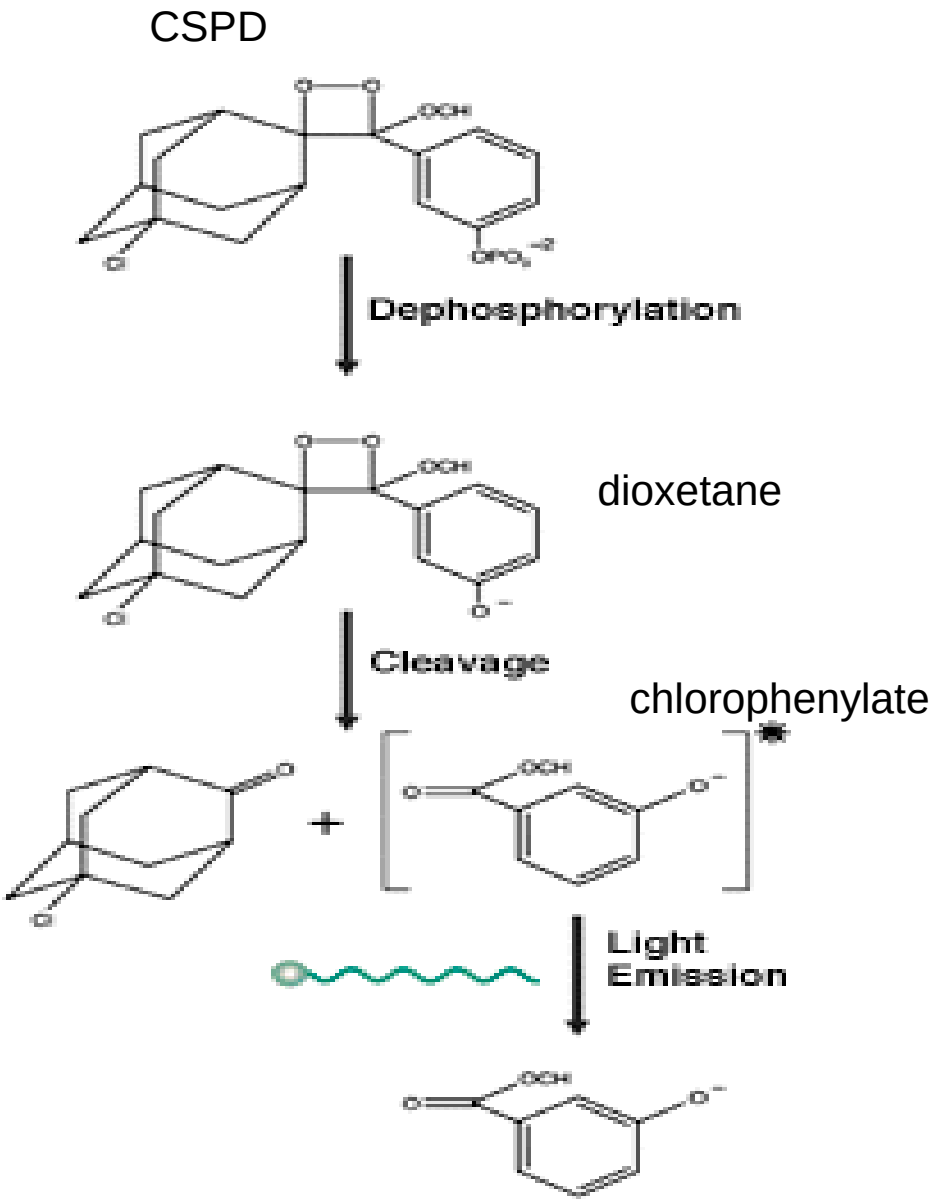
- A chemiluminescent substrate for alkaline phosphatase that enables extremely fast and sensitive detection of biomolecules is CSPD

Structure and formula of CSPD

- Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate or $C_{18}H_{20}ClO_7PNa_2$
- When CSPD is dephosphorylated, it produces an electrically excited product, dioxetane
- Dioxetane then cleaves, releasing a highly energetic chlorophenylate. This molecule decays within 1 minute to a ground state, releasing visible light of wavelength 466 nm. The light emission is recorded on X-ray film or on luminescence imager systems

Immunostaining with alkaline phosphatase

Mechanism of light emission by CSPD



Western blotting

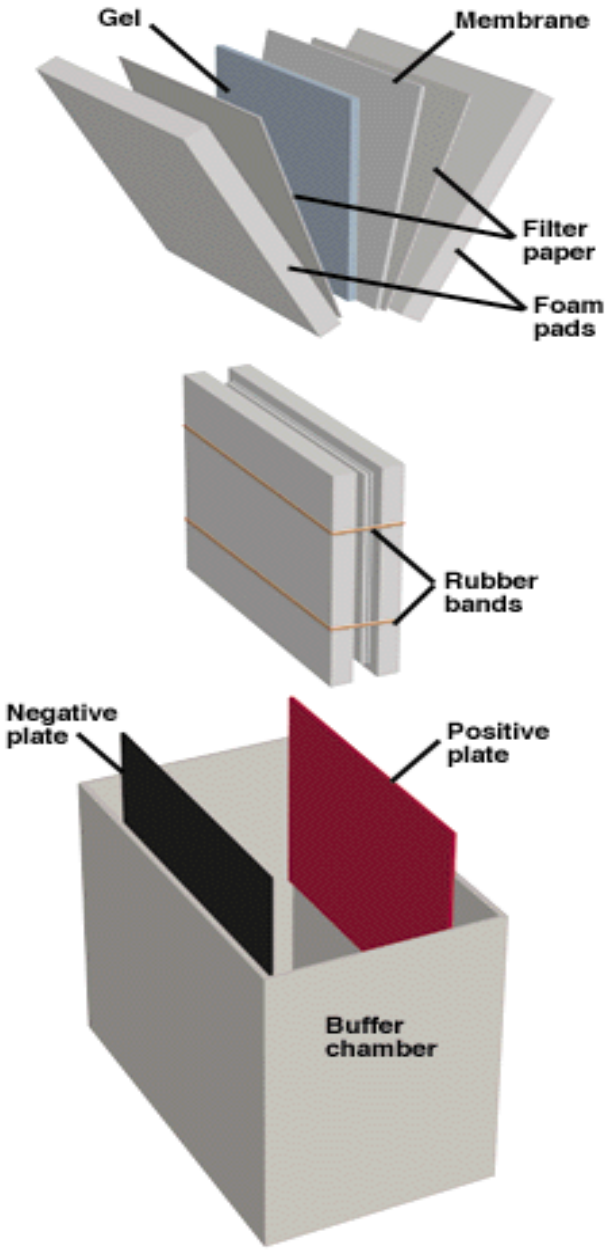
- The two primary advantages of Western blotting are sensitivity and specificity
- Compared to silver staining whose detection limit is ~2-5 ng of protein, and detects all proteins in a given sample, Western blotting can detect as little as 0.1 ng of protein, and is capable of selectively detecting only the protein of interest
- Thus a complex mixture containing only traces of the desired protein may be analyzed accurately with this technique.
- Disadvantage: Because antibodies are large molecules, they penetrate gels slowly.

Western blotting

- The first step in Western blotting is to transfer or blot out and immobilize the pattern of separated proteins from the gel onto a sheet of nitrocellulose paper.
- This method is known as protein blotting, or ‘western blotting’ by analogy with ‘southern blotting’, the equivalent method used to recover DNA samples from an agarose gel.
- " Electroblotting is the method used for blotting and immobilization of the proteins from the gel

Electroblotting

The gel is sandwiched within a membrane and compressed in a cassette and immersed, in buffer, between two parallel electrodes. Voltage is applied perpendicular to the plane of the gel. Proteins are electrophoresed from the gel to the membrane



Western blotting

- The two commonly used membranes are nitrocellulose and PolyVinylidine Difluoride (PVDF)
- Nitrocellulose binds proteins through hydrophobic interactions. In addition, the binding of proteins to the membrane is reversible under some circumstances, leading to sample loss and lowering of detection limits.
- PVDF is mechanically stronger than nitrocellulose. It binds proteins more tightly

Western blotting

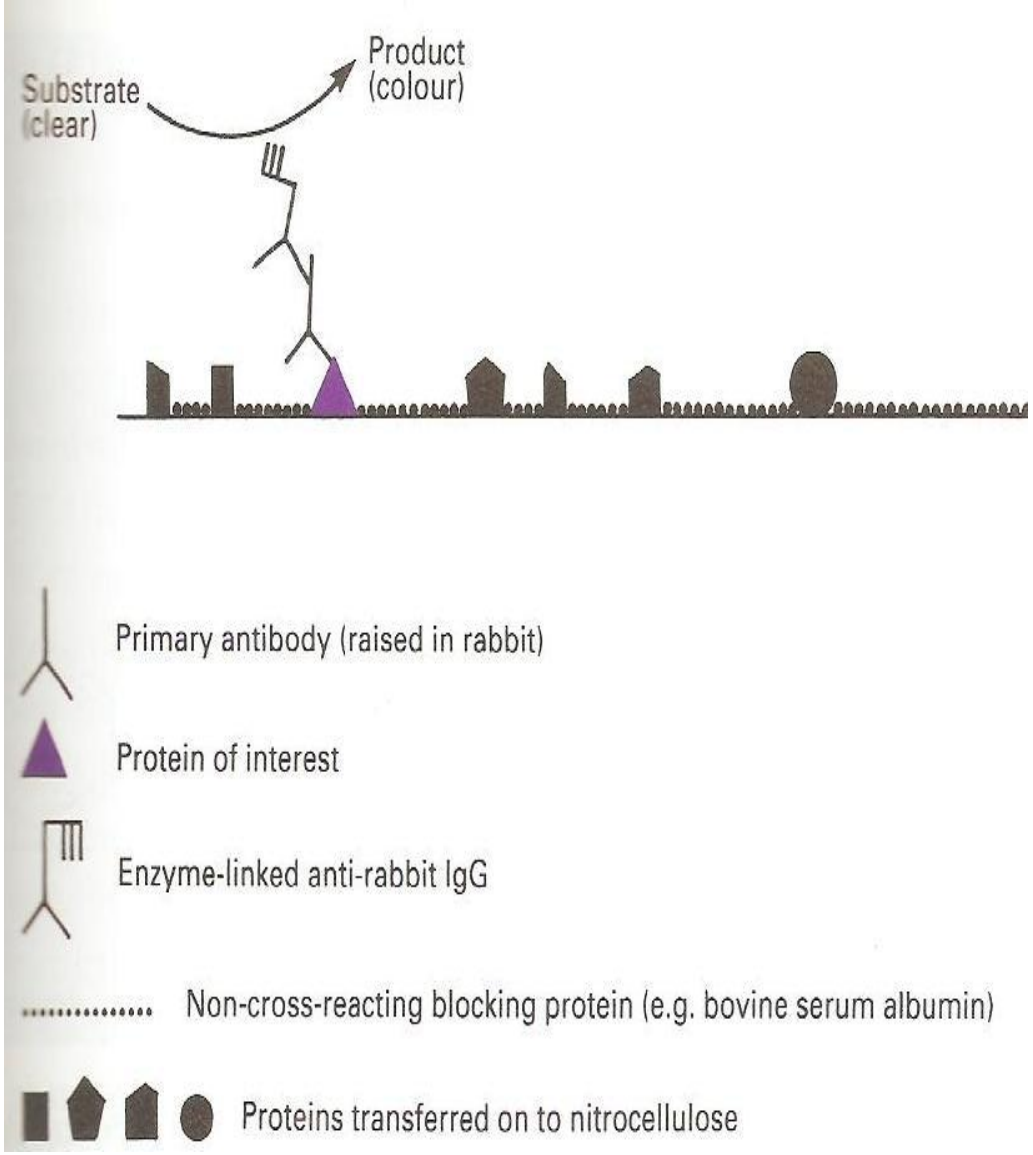
- The nitrocellulose with its transferred protein is referred to as a **blot**.
- Once transferred onto nitrocellulose, the separated proteins can be examined further. Examination of the protein involves probing the blot, usually by using an antibody to detect specific proteins.
- The blot is first incubated in a protein solution, for example 10 % (w/v) bovine serum albumin (BSA), or 5 % (w/v) non-fat dried milk (the blotto technique), which will **block** all remaining hydrophobic binding sites on the nitrocellulose sheet
- The membrane has high affinity for protein – besides the bound protein band, the remaining membrane surface will bind to the antibody in a nonspecific fashion
- This nonspecific binding is prevented by blocking

Western blotting

- The blot is then incubated in a dilution of an antiserum (primary antibody, usually an Immunoglobulin G, IgG) directed against the protein of interest
- This IgG molecule will bind to the blot if it detects its antigen (epitope), thus identifying the protein of interest
- In order to visualize this interaction, the blot is incubated further in a solution of secondary antibody, which is directed against the IgG primary antibody of the species that provided the primary antibody. For example, if the primary antibody was raised in a rabbit then the secondary antibody would be anti-rabbit IgG

Western blotting

The use of enzyme-linked secondary antibodies in immunodetection of protein blots. First, the primary antibody (e.g., raised in a rabbit) detects the protein of interest on the blot. Secondly, enzyme-linked anti-rabbit IgG detects the primary antibody. Thirdly, the addition of enzyme and substrate results in colored product deposited on the site of protein of interest on the blot.



Western blotting

- Following treatment with enzyme labeled secondary antibody, the blot is incubated in enzyme-substrate solution, where the enzyme converts the substrate into an insoluble colored product that is precipitated onto the nitrocellulose
- The presence of a colored band indicates the position of the protein of interest
- By careful comparisons of the blot with a stained polyacrylamide gel of the same sample, the protein of interest can be identified
- Antibodies which do not have their corresponding Antigen would be washed away

Western blotting

- The enzyme used in enzyme-linked antibodies is usually either (1) Alkaline phosphatase, which converts colorless 5-bromo-4-chloro-indoylphosphate (BCIP) substrate into a blue product, or
- (2) Horseradish peroxidase, which with H_2O_2 as a substrate, oxidizes either
  - 3-amino-9-ethylcarbazole into an insoluble brown product, or
  - (3) 4-chloro-1-naphthol into an insoluble blue product
- An alternative approach to the detection of horseradish peroxidase is to use the method of Enhanced Chemiluminescence (ECL).

Western blotting

- In the presence of H_2O_2 and the chemiluminescent substrate luminol (Fig. 4), horseradish peroxidase oxidizes the luminol with concomitant production of light, the intensity of which is increased 1,000-fold by the presence of a chemical enhancer.
- The light emission can be detected in a dark room and captured by exposing the blot to a photographic film. Corresponding Enhanced Chemiluminescence substrates are available for use with alkaline phosphatase-labeled antibodies.
- The luminol reaction is an oxidation-reduction reaction. Luminol is oxidized with H_2O_2, luminol is oxidized, H_2O_2 is reduced. Electrons of oxidized luminol are elevated to an excited state. When the electrons return to the ground state, visible light (a blue glow) is emitted.
- The principle behind the use of enzyme linked antibodies to detect antigens in blots is highly analogous to that used in enzyme-linked immunosorbent assays (ELISA)

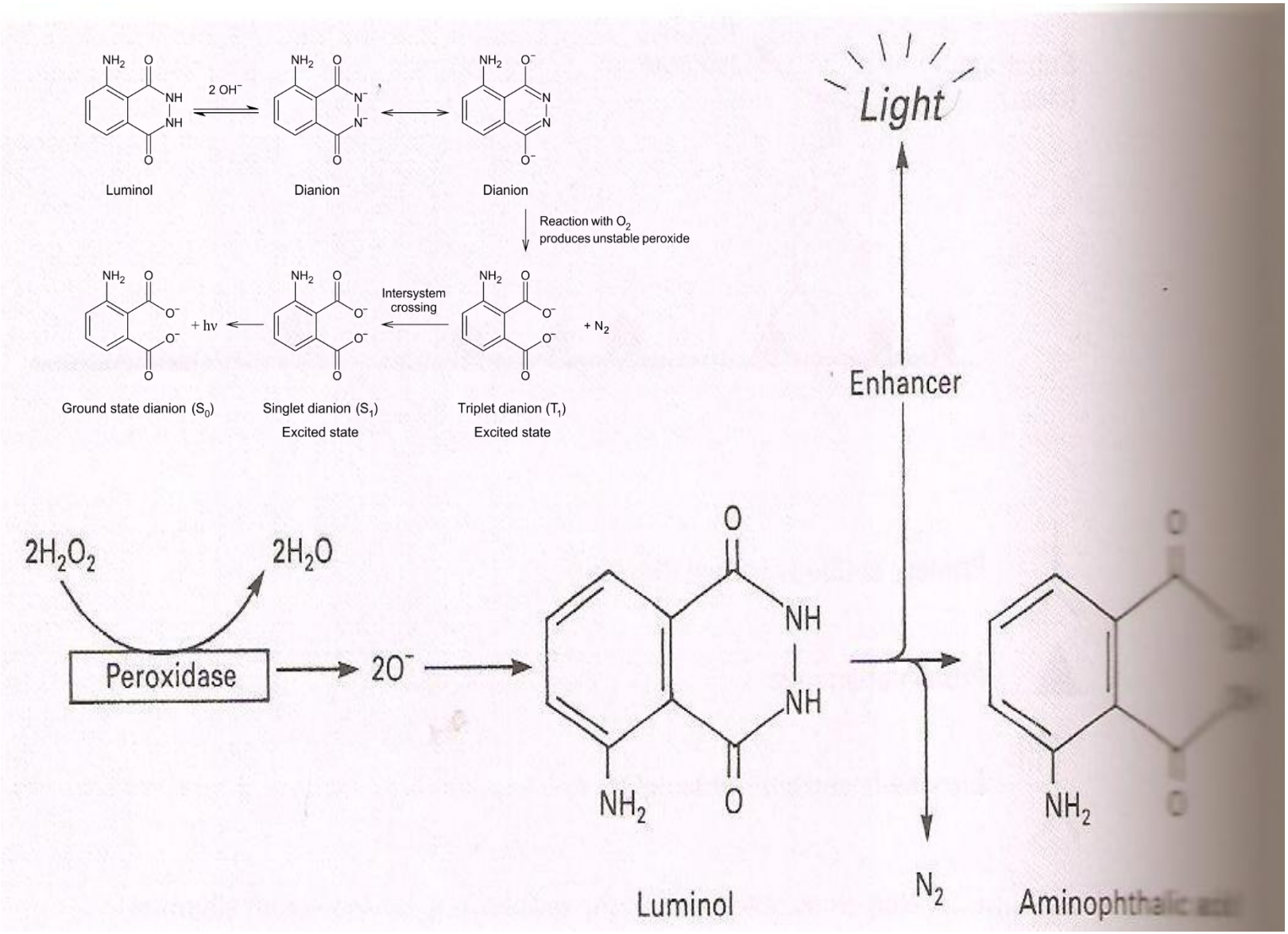


Fig. 4. The use of enhanced chemiluminescence to detect horseradish peroxidase

Western blotting

- Although enzymes are commonly used as markers for secondary antibodies, other markers can also be used. These include:
- (1) ^{125}I-labeled secondary antibody: binding to the blot is detected by autoradiography. Detection with ^{125}I requires placing blot on an X-ray film and exposing to X-ray. Upon development, the film will show bands corresponding to the position and intensity of detected antibody band
- (2) Fluorescein isothiocyanate (FITC)-labeled secondary antibody: this fluorescent label is detected by exposing the blot to UV light

Method of Western blotting

- Other labels for antibodies
- The most popular antibody labels are ^{125}I, HRP and alkaline phosphatase. ^{125}I is simple, straightforward to use, and gives reproducible results. However, the hazard of working with radioactivity must be considered.
- Detection with ^{125}I requires placing blot on an X-ray film and exposing to X-ray
- Upon development, the film will show bands corresponding to the position and intensity of detected antibody band

BCHEM 365
Lecture 8
May 11, 2022

Pulsed Field Gel Electrophoresis (PFGE)

- PGFE is a special technique used to separate out large (>15 kb) fragments of DNA on agarose gel that makes use of two alternating electric fields that periodically change direction.
- PFGE was developed by David Schwartz and Charles Cantor, 1984.

- In PFGE, mobility of DNA on a gel depends on the **direction** of applied electric field. The electric field is not static nor in one plane as in conventional electrophoresis, but changes **direction** and **holding time**. PGFE can also determine the size of really large DNA molecules.
- Reasons for development of PFGE:

Pulsed Field Gel Electrophoresis (PFGE)

- **1**. When large DNA, >15kb, moves down a gel, its movement does not depend on size anymore, unlike smaller fragments. Relationship between log of large DNA size and mobility deviates strongly from linearity
- 2. The double-stranded DNA is a rigid rod, long and thin, making it brittle
- 3. The standard gel electrophoresis technique runs on a unidirectional constant electric field and works best up to 15 kb DNA, beyond which DNA fragments migrate independent of size and produce a single large MW band at the top of the gel
- 4. The low concentration of agarose required to separate large MW DNA fragments does not make strong gels and the DNA breaks easily

Pulsed Field Gel Electrophoresis (PFGE**)**

- 5. Beyond 15 kb, large DNA fragments easily undergo mechanical breakage during preparation where swirling and pipetting create shearing forces sufficient to fracture DNA

- PGFE separates out DNA molecules >15 kb to several million base pairs (Megabases, Mb) and maintain a relatively linear relationship between the log of their sizes and their mobilities

- PFGE differs from normal gel electrophoresis. Normal gel electrophoresis uses constant voltage which runs down the gel in one direction. In PFGE two things keep changing at regular intervals: 1) The electric field periodically changes in direction, 2) Holding time for each direction, which may be same or different

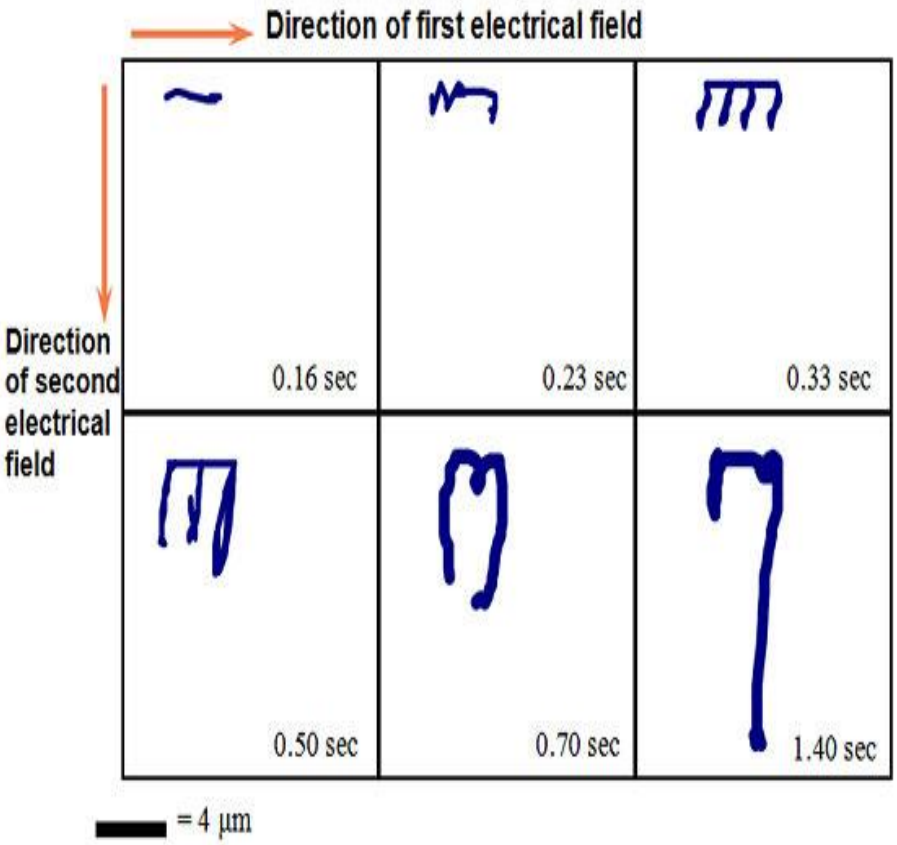
Pulsed Field Gel Electrophoresis (PFGE**)**

- Direction of current: the current is periodically switched among three directions - one current runs through the central axis of the gel; the second current runs at 60° from one side of gel; the third current runs at 60° from other side of gel. Current may also be changed at 90° to each other
- The pulse times are equal for each direction leading to a net forward migration of the DNA down the gel

Pulsed Field Gel Electrophoresis

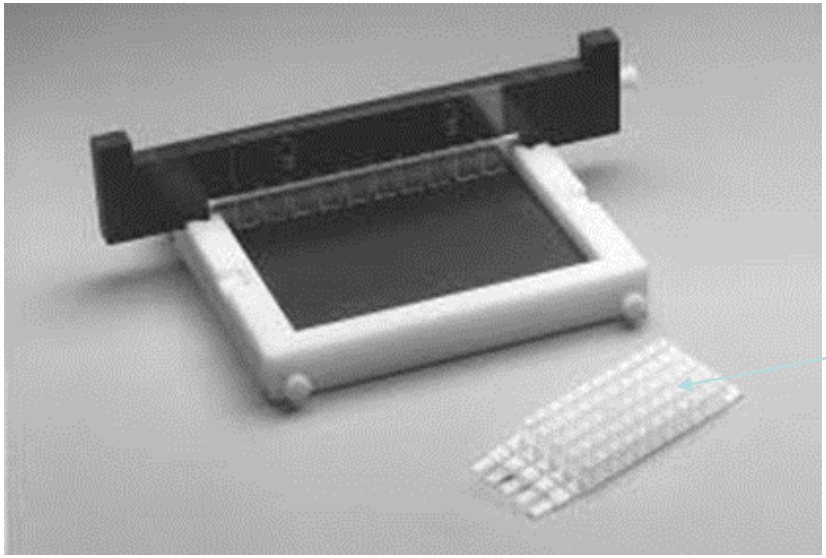
The result is that:

- 1. Different DNA sizes respond differently to the changed current direction at differing rates
- 2. DNA molecules elongate upon application of electric field and return to an unelongated (supercoiled) state upon removal of the electric field
- The size-dependent relaxation rate introduces <u>different rates of migration</u> through the gel to effect separation
- Technique can be used to separate chromosomes



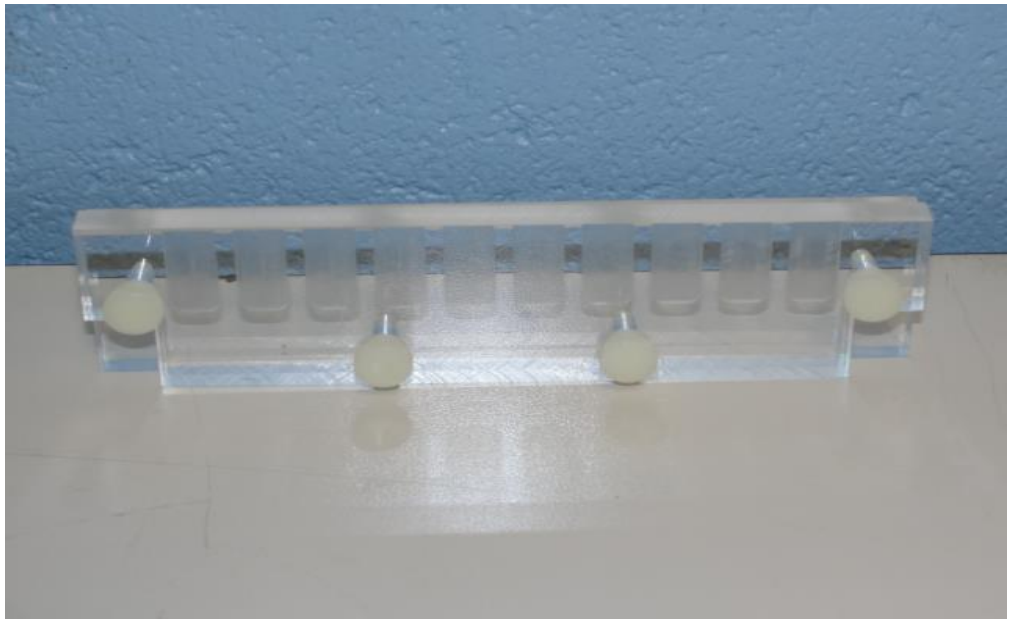
Protocol for PGFE

- Special apparatus is needed to carry out PGFE
- Because DNA molecules larger than 500 kb are fragile, they need to be protected from both mechanical breakage and nucleolytic degradation during isolation.
- To protect them, cells for DNA extraction are embedded in a protective agarose medium held on a mold, referred to as **agarose plug mold** or simply, **sample mold**.



Plug mold

Bio-Rad DRII Sample Mold, 10 plug mold for PFGE, for use with any CHEF system



https://www.youtube.com/watch?v=k_QAXLGuQ5w

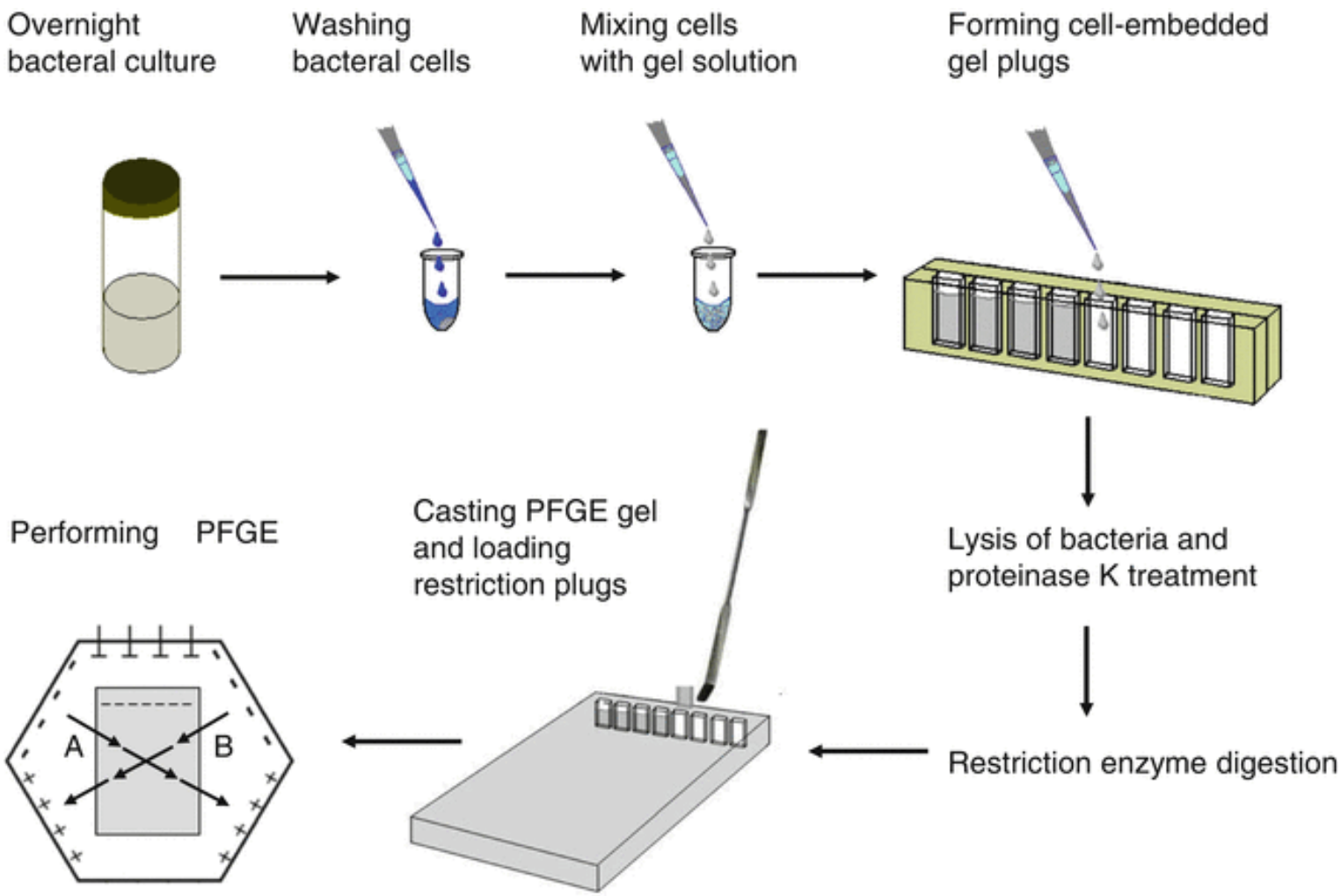
Protocol for PGFE

- The DNA is prepared as follows:
- The organism under study may be microorganisms (bacteria, fungi) or virus; animal or human cells, blood, or plant cells, etc.
- Pulsed field gel electrophoresis of *Pseudomonas aeruginosa*
- Prepare single cell suspension culture
- Wash the suspension culture by repeated pelleting and resuspending in buffer

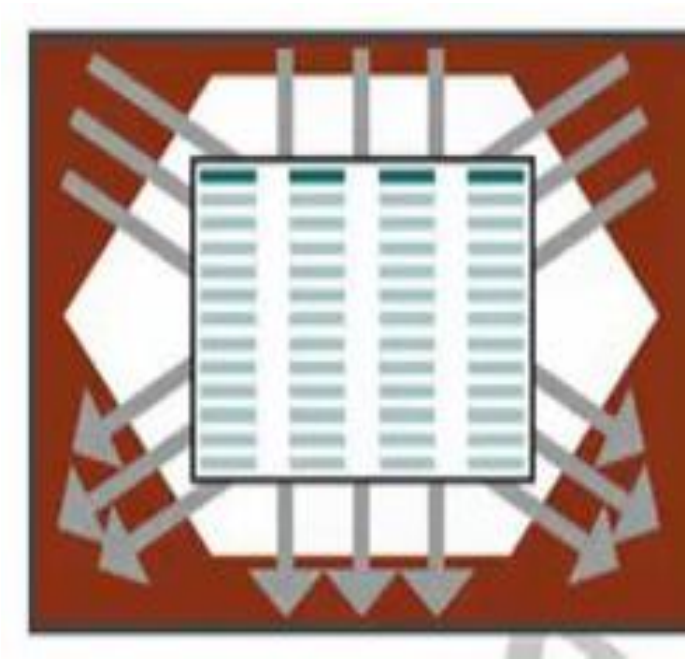
Protocol for PGFE

- The cell suspension is mixed with melted agarose at 65°C and poured into a plug mold
- Add a cell lysis buffer to the plug mold to break up the cells and free the DNA in the plug mold. The plugs are washed
- The plugs are treated with restriction enzymes
- PFGE gel is prepared and loaded with the digested DNA plugs, connected to power source and allowed to separate

Protocol for PGFE



Protocol for PGFE



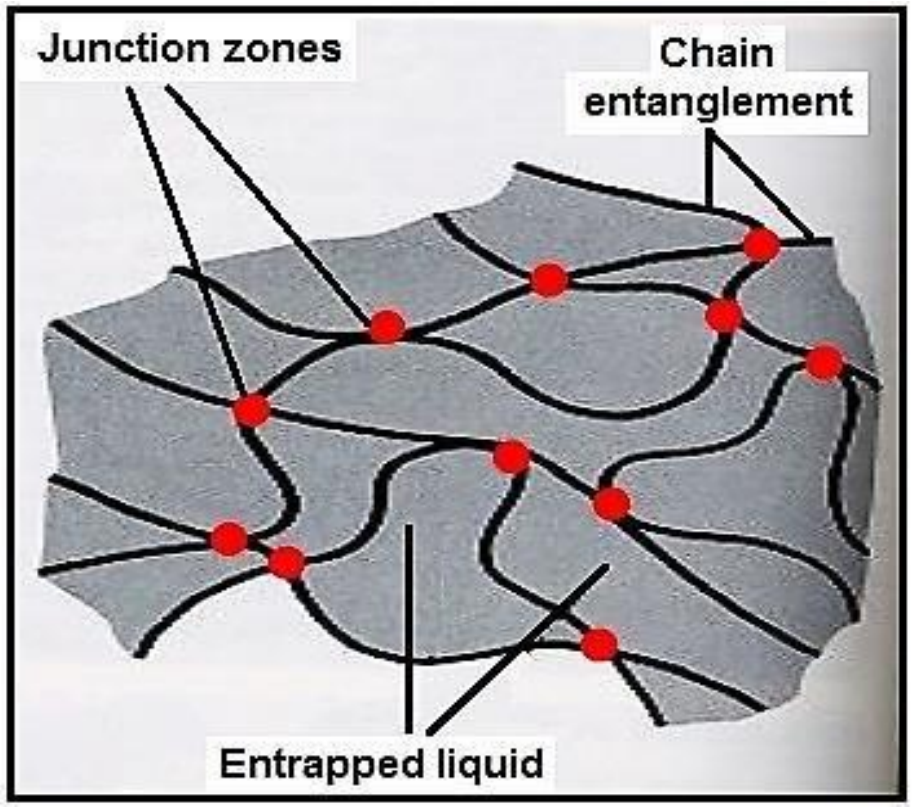
Alternating currents applied intermittently (pulsed) through gel in three directions: 60° obtuse left and right, 90°

Gel is stained for visualization with UV light

Source: Chang-Hui Shen (2019). Pulsed-Field Gel Electrophoresis for Large DNA Molecules. In Diagnostic Molecular Biology

Mechanism of Separation of DNA in PFGE

- Main driver of the separation of DNA is the electric field. Two events occur when pulsed electric field is applied to the agarose gel containing the DNA:
- (1) DNA stretches and becomes **oriented** in the direction of the electric field
- (2) Electric field disrupts the hydrogen bonds in the junction zones constantly, allowing the gel fibers and fiber bundles to orient in the direction of electric field.



Junction zone within a gel

- H-bonds in the junction zones break upon applying electric field
- Agarose chains align in the direction of electric field
- Large DNA fragments orient and move at different speeds within the gel

Mechanism of Separation of DNA in PFGE

- When the electric field is removed or changed in amplitude and/or direction, the DNA relaxes into its random coil conformation, for it to stretch again in the new direction of electric field
- Similarly, the agarose fiber and fiber bundles which were oriented in the first direction of current, now orient in the perpendicular direction when the electric field is reversed in polarity, thereby creating transient and larger pore sizes between the agarose fibers and fiber bundles in the gel matrix.

Mechanism of Separation of DNA in PFGE

- The “flip-flop” orientation and reorientation of agarose fibers and fiber bundles, allow large DNA molecules to migrate through the transient large pore gel during electrophoresis
- The rate of relaxation depends on DNA size, where large DNA fragments relax faster and smaller DNA fragments relax slower
- Consequently, large DNA molecules are trapped in the gel matrix and scarcely move. In the end, separation can occur by molecular size between 50 kb to 10 Mb.
- In the end, the process produces a DNA fingerprint with a characteristic pattern.

- PFGE of yeast chromosomes
- Identical samples of yeast chromosomes were electrophoresed in 10 parallel lanes and stained with ethidium bromide. The bands represent chromosomes having sizes ranging from 0.2 Mb (bottom) to 2.2 Mb (top). There are 16 chromosomes in yeast; no. of genes 6,275; and genome size 12,156,677 bp

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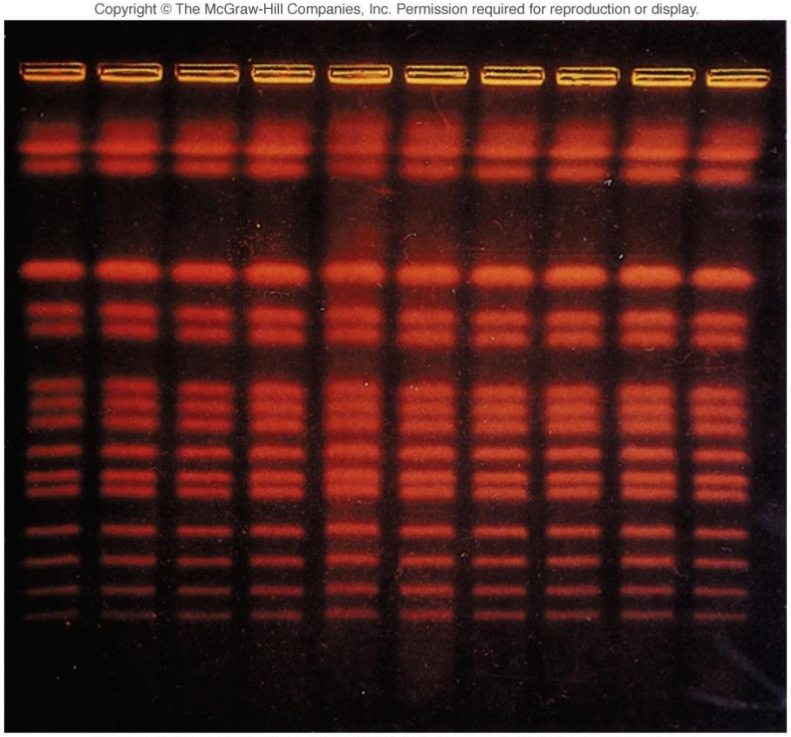


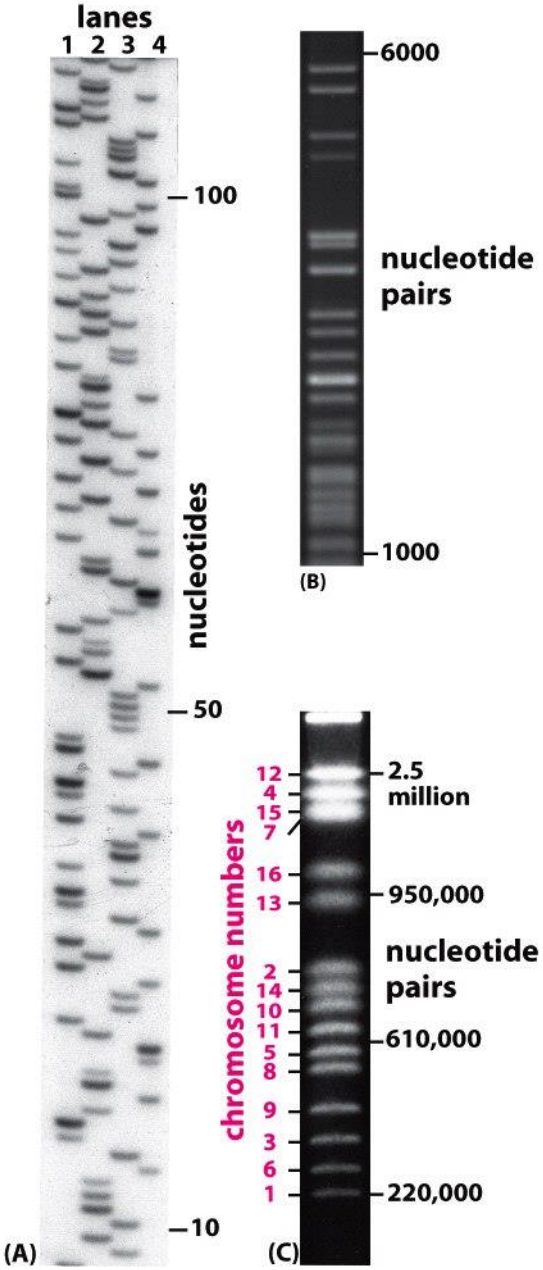
(Source: Courtesy Bio-Rad Laboratories/CHEF-DR(R)II pulsed-field electrophoresis systems.)

Applications of PFGE

- Estimates genome size of a microorganism
- Determines if a genome is circular or linear
- Reveals the presence of megaplasmids
- Determines number, size, and changes in bacterial and viral genomes (chromosomes), hence the strain. This method has been used for identification of pathogens in foodborne and zoonotic diseases; antibiotic resistant genes in methicillin resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa*
- Identification of bacteria in clinical samples and in epidemiological studies
- For detection of DNA viruses, such as Herpes simplex

Summary

Three gel electrophoresis techniques for separating DNA molecules by size. Electrophoresis is from top to bottom. Largest and therefore slowest-moving DNA are near the top of the gel. Smallest and supercoiled DNA molecules are near the bottom



Applications of electrophoresis

- The phenomenon of nucleic acid hybridization is very important in molecular biology

- Nucleic acid hybridization reactions provide a sensitive way to detect specific nucleotide sequences

- Northern and southern blotting facilitate hybridization with electrophoretically separated nucleic acid molecules
- Southern blot is used to identify specific DNA fragments
- Northern blot is used to measure gene activiy as RNA transcripts
- Northern blot is similar to Southern blot but it contains electrophoretically separated RNAs instead of DNAs

Nucleic acid hybridization

- Heat an aqueous solution of DNA at 100°C or expose to high pH (pH≥13) and complementary base pairs that hold the two strands of the double helix together are disrupted, double helix rapidly dissociates into single strands
- This is DNA denaturation
- When complementary strands reform at a lower temperature, renaturation or annealing occurs
- Similar hybridization can occur between any two complementary single stranded nucleic acid chains: DNA/DNA, RNA/RNA, RNA/DNA
- These specific hybridization reactions are widely used to detect and characterize specific nucleotide sequences in both RNA and DNA molecules
- The complementary strands which are used for detection of DNA or RNA on a blot are called **probes**

Nucleic acid hybridization

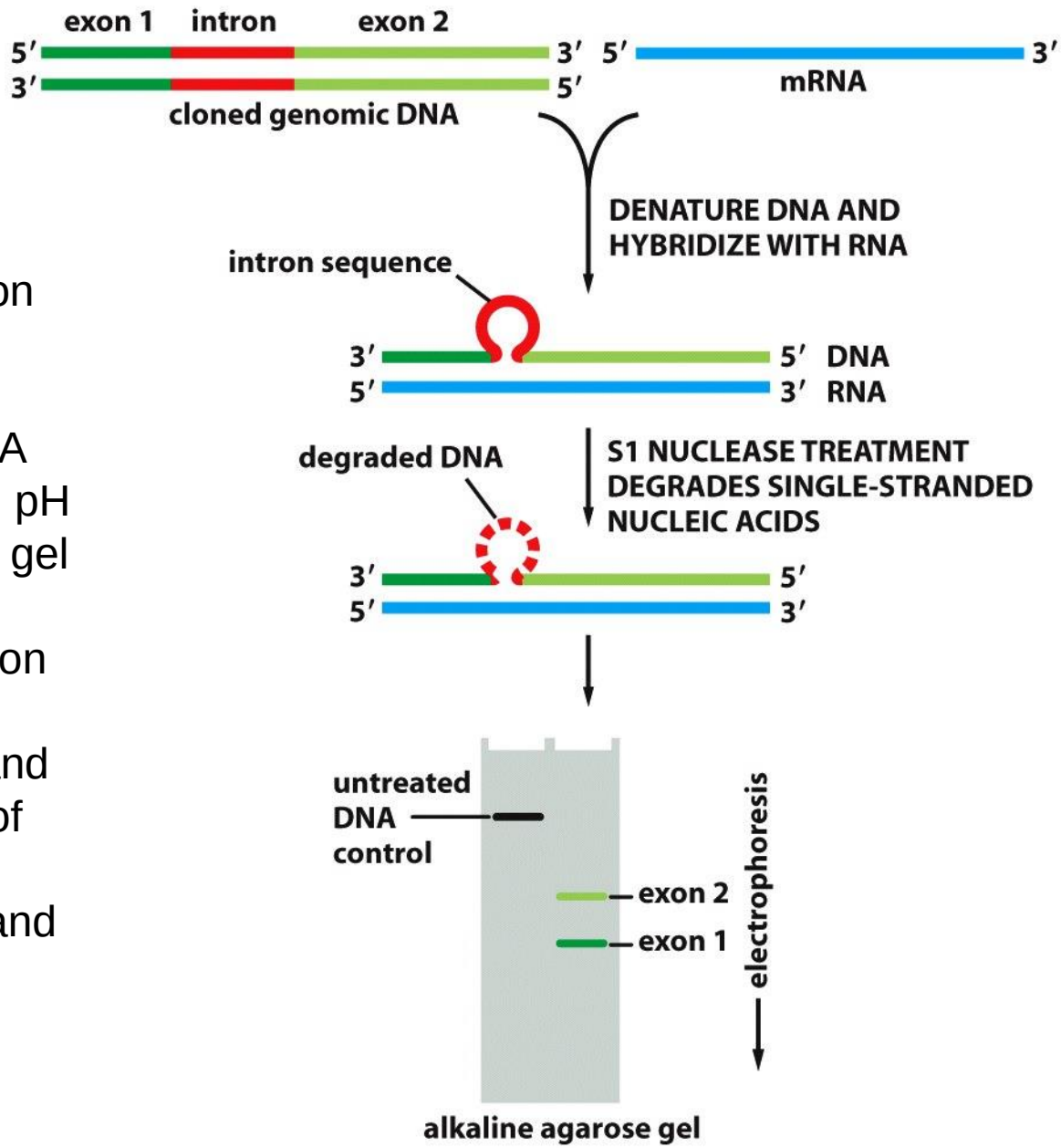
DNA probes

- These are single-stranded DNA molecules used to detect complementary sequences. They are usually labeled with a radioactive or chemical marker for detection. Can range from 15 to 1,000 nucleotides long
- DNA probes are very sensitive. Can detect complementary sequences present at concentrations as low as one molecule per cell therefore it can determine the number of copies of a DNA sequence present in a cell
- Can be used to find related but nonidentical genes
- Can be used to find a gene of interest in an organism whose genome has not yet been sequenced
- Can be used in hybridization reactions with RNA and allows one to determine if a particular gene is being transcribed

Nucleic acid hybridization

- In this case, a DNA probe that contains part of a gene's sequence is hybridized with RNA purified from the cell to see whether the RNA includes nucleotide sequences matching the probe DNA, and in what quantities
- After hybridization is complete, the DNA is treated with specific nucleases to determine the exact regions of the DNA that have paired with the RNA molecules.
- Through this, one can determine the start and stop sites for RNA transcription, as well as the precise boundaries of intron and exon sequences in a gene

Use of nucleic acid hybridization to determine the region of a cloned DNA fragment that is present in an mRNA molecule. The high pH in alkaline agarose gel electrophoresis causes loss of proton from guanine and thymine residues and prevent formation of hydrogen bonds between cytosine and adenine bases.



RFLP

- Restriction-fragment length polymorphism
  - Cut genomic DNA from two individuals with restriction enzyme
  - Run Southern blot
  - Probe with different pieces of DNA
  - Sequence difference creates different band pattern

Recognition site for enzyme: GTATCC

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Southern blot

- Named after its inventor, Edward Southern
- In this method, isolated DNA is first cut into readily separable fragments with restriction endonucleases
- Double-stranded fragments are then separated on the basis of size by agarose gel electrophoresis
- Denature the DNA with a base to obtain single-stranded DNA fragments.
- Transfer the DNA single strands from the gel to a nitrocellulose material
  - By diffusion, in which the buffer passes through the gel, carrying the DNA with it
  - By electrophoresis
- Hybridize the blot to a labeled probe and detect labeled bands by autoradiography or phophorimaging
- This probe will form base pairs with its complementary DNA sequence and bind to form a double-stranded DNA molecule.
- The degree of hybridization of a complementary probe is a measure of the amount of the specific target sequence in the sample.
- The probe is labeled before hybridization either radioactively or enzymatically (e.g. alkaline phosphatase or horseradish peroxidase).
- Those fragments complementary to a DNA probe are identified by blotting and hybridization

Southern blot

- Wherever the probe encounters a complementary DNA sequence, it hybridizes, forming a labeled band corresponding to the fragment of DNA containing the gene of interest

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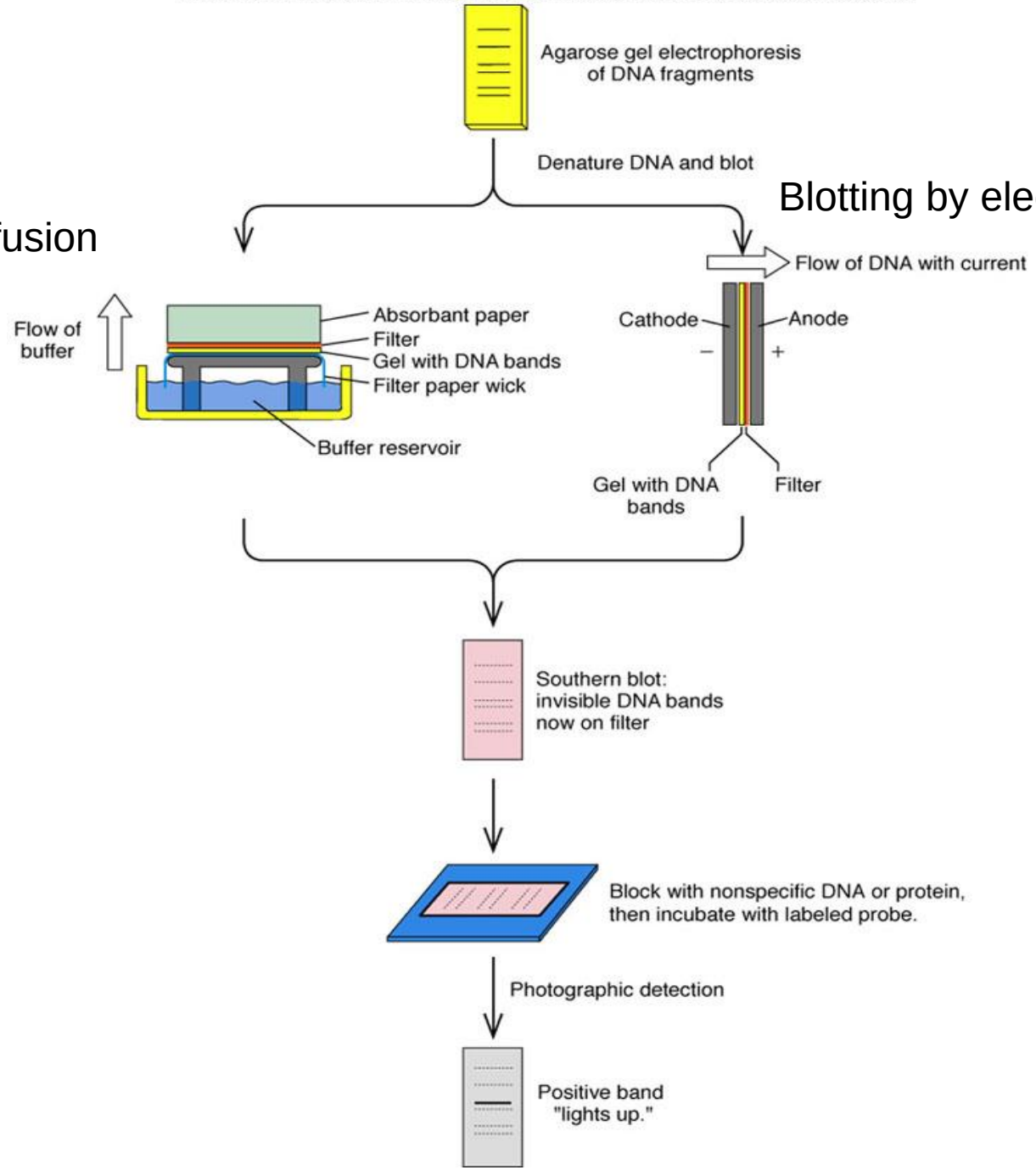


DNA fingerprinting

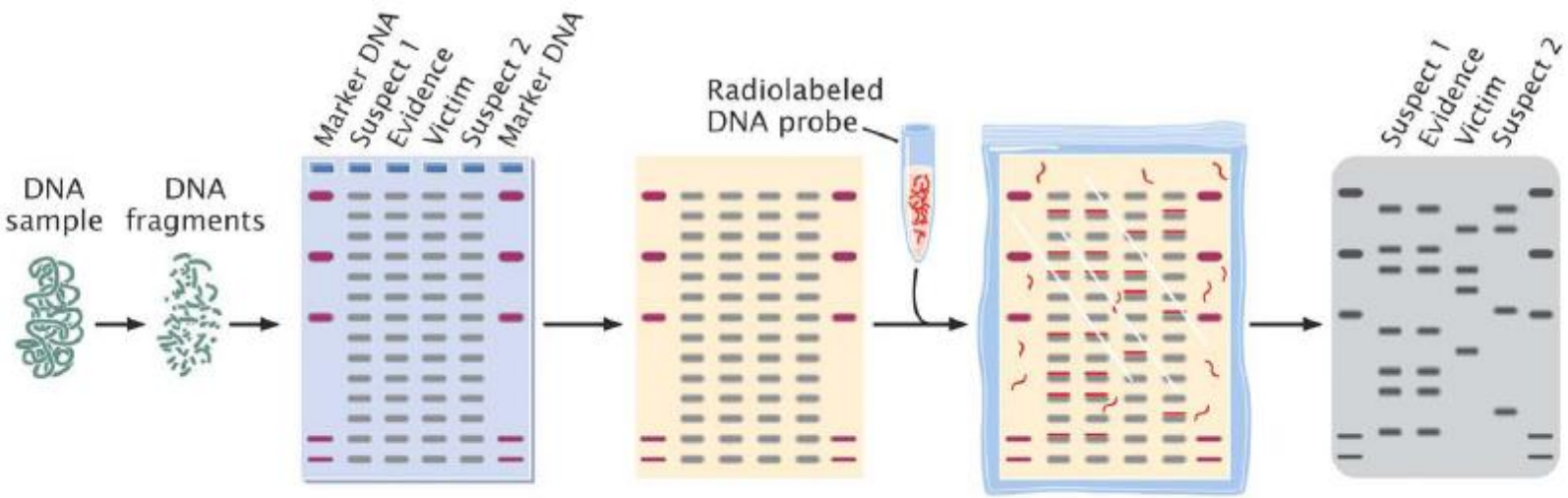
- It is a Southern blot technique
- Used in forensic labs to identify perpetrators, that is, suspects who have left blood or any DNA-containing tissue on a crime scene
- Makes use of sequences of bases repeated several times: minisatellites
- Individuals differ in the pattern of repeats of the basic sequence
- Two individuals have an extremely rare chance of having exactly the same pattern. Such patterns are like fingerprints hence DNA fingerprinting
- To do it, first cut the DNA with restriction enzyme such as *HaeIII* (GGCC nucleotide sequence)which cuts on either side of the repeat regions. This produces fragments bearing the repeated regions
- Perform electrophoresis of the fragments, denature and blot
- Probe with a labeled minisatellite DNA and detect bands with X-ray film

DNA fingerprinting—use of DNA sequences to identify a person

Used in paternity/forensics

Chance to 2 people having same set of bands is low unless monozygotic twins

If examine many markers—probability that 2 people have same sets of patterns—extremely small



DNA fingerprinting used in other criminal investigations:

- Identify human remains
- Identify source of other organisms—anthrax in mail

Northern blot

- A technique used in molecular biology research to study gene expression by detection of RNA (or isolated mRNA) in a sample.
- Northern blotting involves the use of electrophoresis to separate RNA samples by size, and detection with a hybridization probe complementary to part of or the entire target sequence
- Say you have cloned a cDNA and you want to know how the corresponding gene is expressed in a number of tissues
- Begin by collecting RNA from the tissues, then perform agarose gel electrophoresis of the RNA and blot them to a nitrocellulose or nylon support
- Hybridize the northern blot to a labeled cDNA probe.
- Wherever an mRNA complementary to the probe exists on the blot, hybridization will occur, resulting in a labeled band that can be detected by X-ray film. If you run the electrophoresis along with an RNA marker, you can tell the size of the RNA bands

Electrophoresis in the detection of sickle-cell anaemia

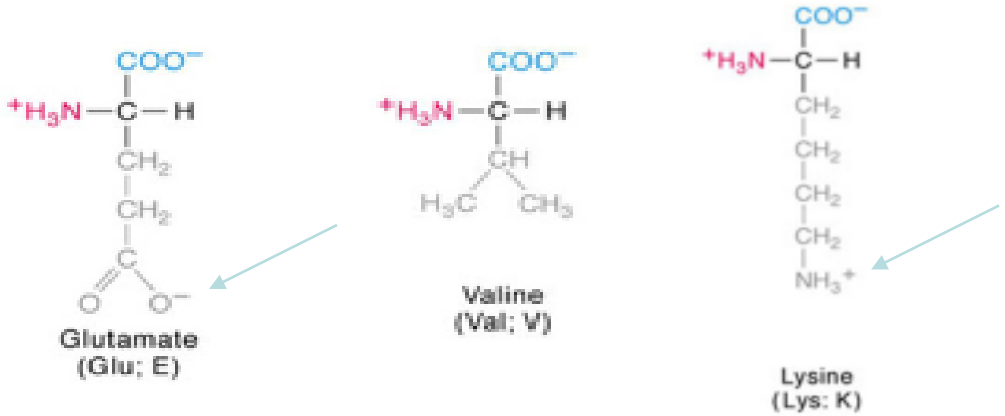
- Hemoglobin (Hb) is the iron-containing oxygen-transport metalloprotein in red blood cells
- In most humans, the hemoglobin molecule is an assembly of four globular protein subunits (tetramer), each composed of a polypeptide chain tightly associated with a non-protein heme group.

- There are variants of hemoglobin
- Normal hemoglobin, called hemoglobin A (HbA) consists of two alpha chains, and two beta chains as subunits non-covalently bound, each made of 141 and 146 amino acid residues, respectively. This is denoted as $\alpha_2\beta_2$.

- Hemoglobin S ($\alpha_2\beta^S{}_2$) –is found in sickle cell anemia. a variation in the β-chain gene, causing a change in the properties of hemoglobin which results in sickling of the red blood cells.

Electrophoresis in the detection of sickle-cell anaemia

- HbS arises from a point mutation of the HBB gene on the short arm of chromosome 11. This gene encodes the beta globin chain. Mutation in the β-globin which causes codon = GAA and GAG of the gene (the amino acid glutamic acid at the sixth position) to be replaced with codon GUA and GUG (the hydrophobic amino acid valine)



- HbS therefore assumes a less negative charge. In HbC, glutamic acid is replaced with lysine making it slightly more positive (or better still, much less negative than HbA and even HbS

Electrophoresis in the detection of sickle-cell anaemia

- The hydrophobic valine causes Hb to be less soluble at low oxygen concentration (i.e., deoxygenated Hb), enhances hydrophobic interaction among the β- chains, and leads to aggregation and distortion in the shape of the red blood cells

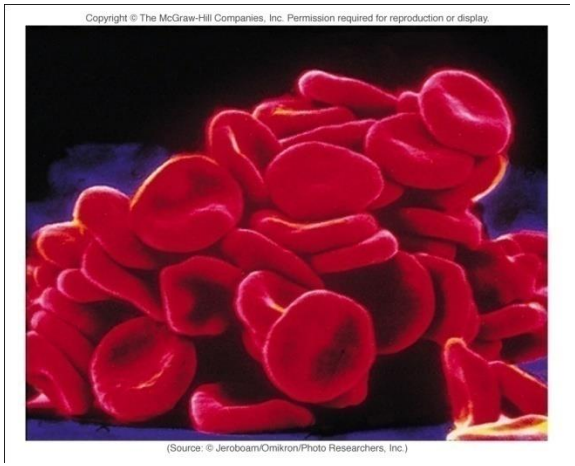
Electrophoresis in the detection of sickle-cell anemia

- Hemoglobin C ($\alpha_2\beta^C{}_2$) –variation in the β-chain gene. This variant causes a mild chronic hemolytic anemia.
- Heterozygotes have one normal and one sickle-cell gene and their red blood cells contain one HbS and one HbA and are said to have the sickle-cell trait. Others have one HbS and one HbC; Homozygotes have two HbS genes and have sickle cell anemia

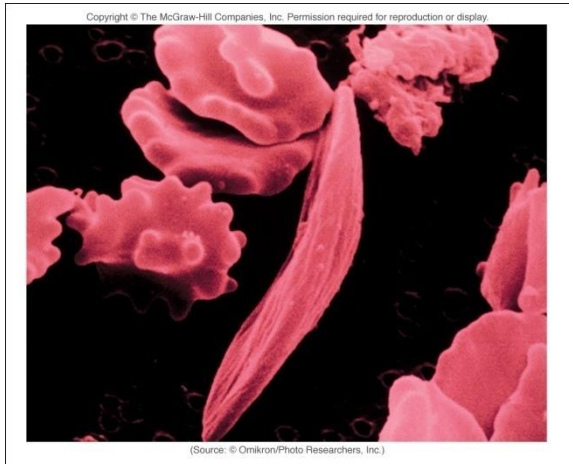
Electrophoresis in the detection of sickle-cell anemia

- Sickle-cell anemia, is a disease caused by a defective gene which produces a life-long blood disorder characterized by red blood cells that assume an abnormal, rigid, sickle shape (biconcave in normal cells).
- Sickle cell anemia affects about 1 in 500 infants and is common among blacks

A



B

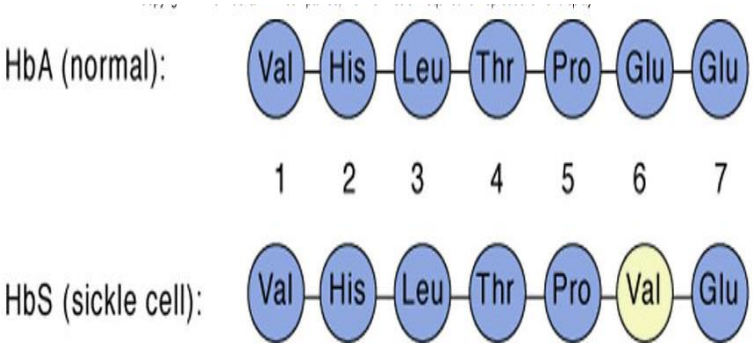


A) Normal red blood cells are disc shaped, biconcave, flexible, move freely through blood vessels. B) Sickle cells (like crescent), stiff, elongated, do not move freely, clog and stick to blood vessels, causing pain in limbs

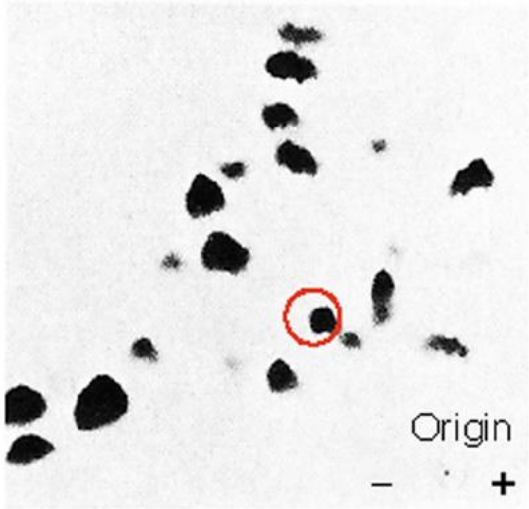
Electrophoresis in the detection of sickle-cell anemia

- During electrophoresis at alkaline pH, HbS with its less negative charge will migrate more slowly toward the anode than HbA does. HbC will migrate even more slower than HbS because it is much less negative
- Vernon Ingram in 1957 used a 2-dimensional fingerprinting method to find the difference between HbA and HbS. He digested the two globin subunits to obtain peptides and separated the peptides first by paper electrophoresis. Then the paper was turned 90° and the peptides separated further by paper chromatography in the second dimension
- The peptides appeared as spots on the paper; different peptides with different amino acid composition gave different pattern of spots. The patterns were named fingerprints.
- Comparing the fingerprints of HbA peptides and HbS peptides, he observed that all spots matched except for one, which had **a much slower mobility** in the HbS fingerprint than in the normal HbA fingerprint indicating that it had an altered charge, arising from a different amino acid composition
- He found that they differed in only one amino acid: the glutamic acid in the sixth position in HbA becomes valine in HbS

Fingerprint of hemoglobin A and hemoglobin S. All spots are identical except for one peptide which moves up and to the left in HbS



Hemoglobin A



Hemoglobin S

(Source: Dr. Corrado Baglioni.)

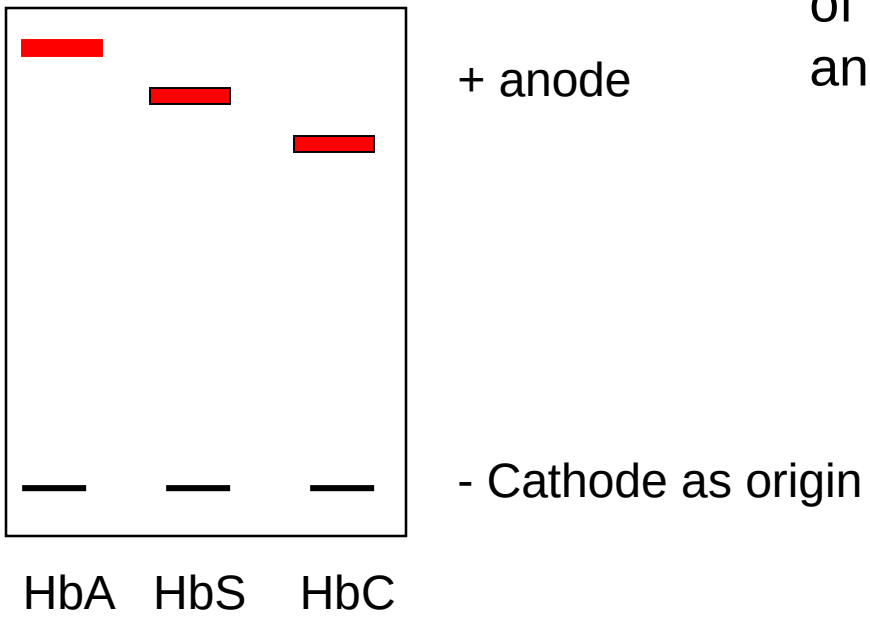
Electrophoresis in the detection of sickle-cell anemia

- Normal hemoglobin remains soluble under physiological conditions
- Sickle cell hemoglobin precipitates when blood oxygen level falls (e.g., during exercise), forming long, fibrous aggregates. Such cells are impeded in their path through the tiny capillaries, so they clog and rupture them, starving parts of the body of blood and causing internal bleeding and pain
- The hemoglobin electrophoresis test precisely identifies the hemoglobins in the blood by separating them. The separation of the different hemoglobins is possible because of the unique electrical charges they each have on their protein surfaces, causing them each to move characteristically in an electrical field.

Hemoglobin electrophoresis test

- Electrophoresis is carried out on cellulose acetate paper using an alkaline buffer at pH 8.6 to 9.2 and run for 30 min at 250 to 300 Volts. At the end of the process, the paper is stained and dried at 60 to 100°C. The pattern of the bands is a function of the differences in the mobilities of the variants.

Paper electrophoresis of haemoglobin A, S, and C



Serum protein electrophoresis

- This is a technique used to investigate the levels of specific proteins in the blood called albumin and globulins. It is used clinically to diagnose and monitor a variety of diseases, especially disorders of the immune system, liver dysfunction, kidney diseases, impaired nutrition, some genetic disorders, and cancer
- Blood serum contains two major protein groups: albumin and globulin. Both albumin and globulin carry substances through the bloodstream
- In this method, serum samples are placed on electrophoresis gels and exposed to an electric current to separate the two major serum protein components into five smaller group which differ in shape, size, and electrical charge. The intensity of the fractions are measured with a densitometer

Serum protein electrophoresis (SPEP)

- Albumin
  - More than half of the protein in serum is albumin. Albumin proteins keep the blood from leaking out of blood vessels. Albumin also helps carry some medicines and other substances through the blood and is important for tissue growth and healing

- Globulins
  - Alpha-1 globulin. Made up of α1-antitrypsin and α1-acid glycoprotein. High-density lipoprotein (HDL), the "good" type of cholesterol, is included in this fraction. α1-antitrypsin protects tissues from enzymes of inflammatory cells, especially elastase. In its absence, elastase is free to break down elastin, which contributes to the elasticity of the lungs, resulting in respiratory complications such as emphysema, or COPD (chronic obstructive pulmonary disease) in adults and cirrhosis in adults or children.

Serum protein electrophoresis

- Alpha-2 globulin. A protein called haptoglobin, that binds with haemoglobin, is included in this fraction.
- Also present are α2-macroglobin, a large plasma protein produced by the liver. It functions as an inhibitor of coagulation by inhibiting thrombin and an inhibitor of fibrinolysis.
- Also in this fraction is α2-antiplasmin which is responsible for inactivating plasmin, an important enzyme that participates in fibrinolysis and degradation of various other proteins.
- Ceruloplasmin, officially known as ferroxidase or iron(II):oxygen oxidoreductase, is the major copper-carrying protein in the blood, and in addition plays a role in iron metabolsm is an alpha-2 globulin

Serum protein electrophoresis (SPEP)

Beta globulin. This fraction consists of transferrin, LDL and the complement system. The **complement system** is a biochemical cascade (a group of plasma proteins) that helps clear pathogens from an organism.

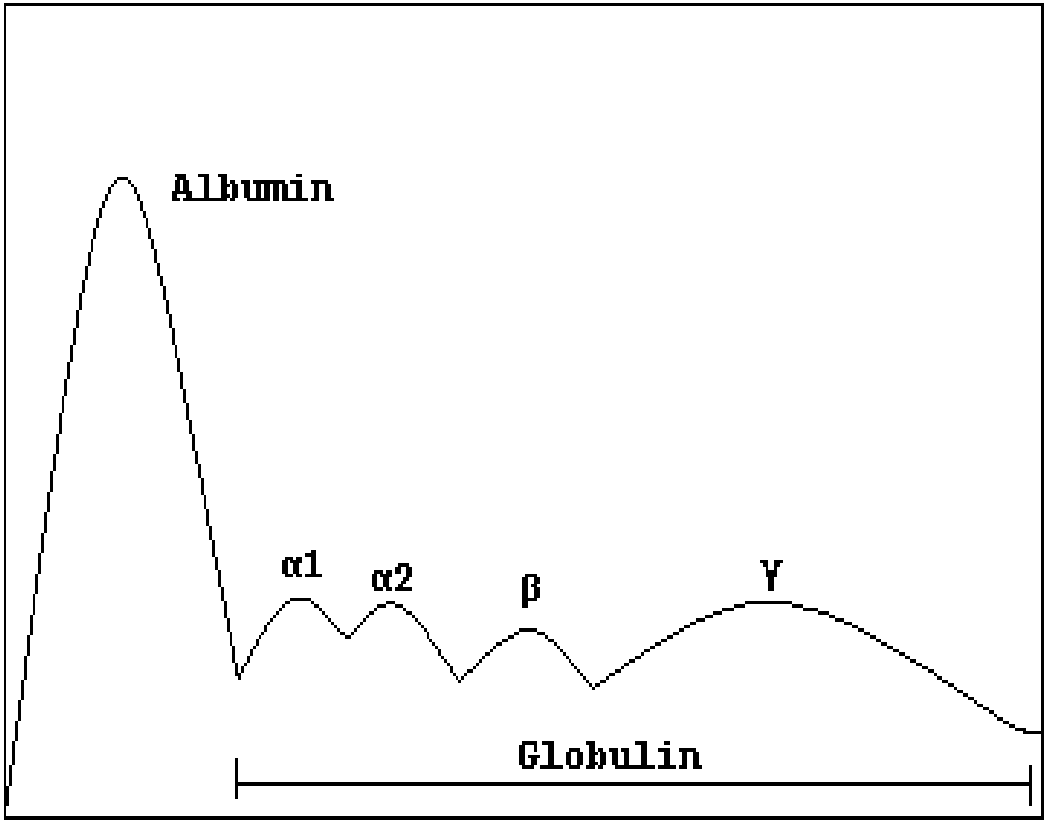
Transferrin is for iron transport

LDL is a type of lipoprotein that transports cholesterol and triglycerides from the liver to peripheral tissues. Also known as bad cholesterol.

Serum protein electrophoresis (SPEP)

- Gamma globulins
  - This fraction is made up of immunoglobulin (IgA, IgD, IgE, IgG and IgM) and paraproteins
  - The immunoglobulins are also called antibodies. They help prevent and fight infection. Gamma globulins bind to foreign substances, such as bacteria or viruses, causing them to be destroyed by the immune system
  - Paraproteins are a group of proteins most often associated with benign MGUS (monoclonal gammopathy of undetermined significance), and multiple myeloma

Each of these five protein groups moves at a different rate in an electrical field and together form a specific pattern. The topography of the pattern helps identify some diseases.



Classification of serum proteins: (1) Albumin (2) Alpha-1 globulin (3) Alpha-2 globulin (4) Beta globulin (5) Gamma globulin

Serum protein levels

- Protein tests may be measured in grams per deciliter (g/dL) or milligrams per deciliter (mg/dL) depending on the laboratory performing the test.

Total serum protein: Normal levels are 5.5 to 9.0 g/dL

- Albumin: Normal levels are 3.5 to 5.5 g/dL
- Globulins: Normal levels are 2.0 to 3.5 g/dL
- A/G ratio (albumin to globulin ratio): Normal levels are greater than one

Normal levels of a serum protein electrophoresis

- Alpha-1 globulin: Normal levels are 0.1 to .4 g/dL
- Alpha-2 globulin: Normal levels are 0.5 to 1 g/dL
- Beta globulin: Normal levels are 0.7 to 1.2 g/dL
- Gamma globulin: Normal levels are 0.5 to 1.6 g/dL

Abnormal results

- **Albumin levels**

  Abnormally high may indicate severe dehydration.

  A decreased level of albumin is common in many diseases and is especially important in liver disease Abnormally low may signal malnutrition, liver or kidney disease, lupus, rheumatoid arthritis, Hodgkin disease, uncontrolled diabetes, hyperthyroidism or heart failure.

  Globulin levels

  Alpha-1 globulin elevated in inflammatory diseases. Absence indicates a genetic disorder called juvenile pulmonary emphysema

  A decrease in alpha-1-globulin is seen in nephrotic syndrome and absence could indicate possible alpha-1-antitrypsin deficiency which eventually leads to emphysema

Abnormal results

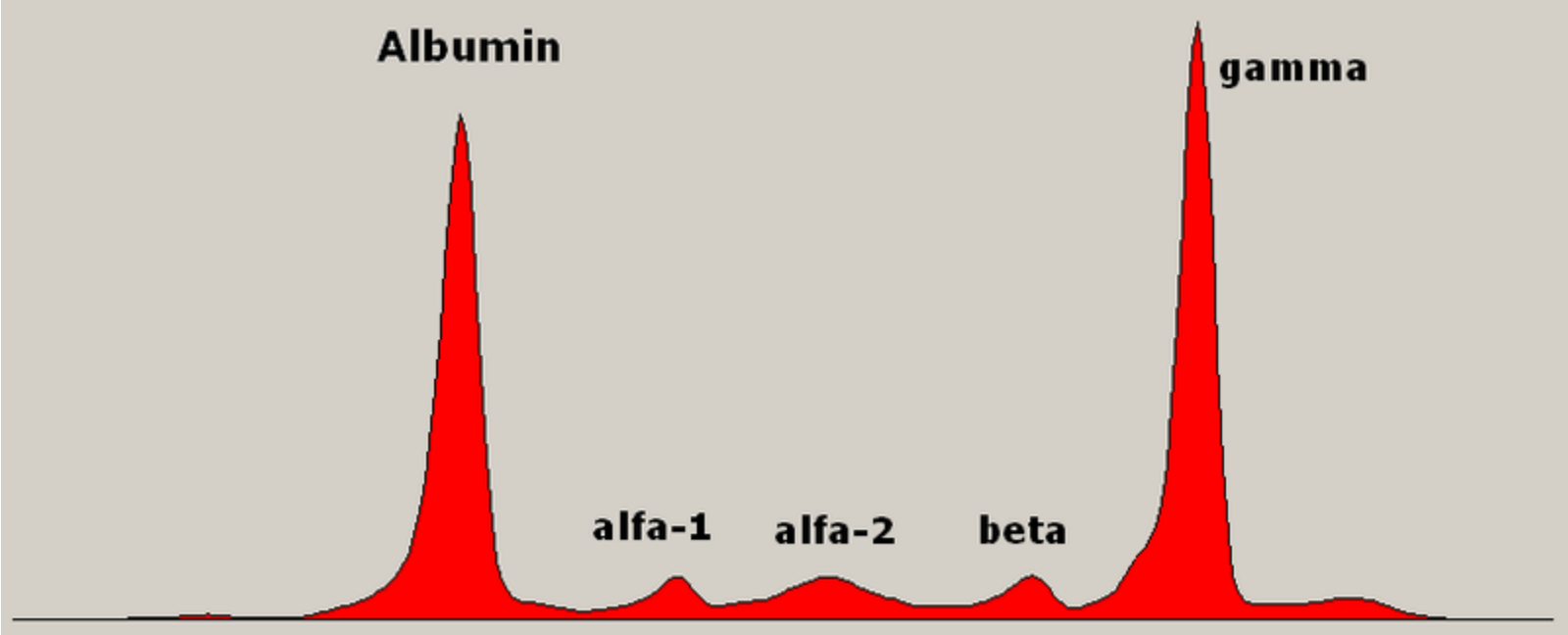
Alpha-2 globulin elevated in nephrotic syndrome of the kidney (from the macroglobin fraction). Decreased in hyperthyroidism or severe liver dysfunction and in blood clotting problems

A normal alpha-2 and a raised alpha-1 is typical in hepatic metastases and cirrhosis.

Beta globulins elevated in hypercholesterolemia and iron deficiency anemia. Abnormally low levels can indicate malnutrition

Gamma globulins elevated in chronic inflammatory diseases, in autoimmune diseases such as lupus or rheumatoid arthritis, cancer, kidney or liver disease, infection, multiple myeloma (a bone cancer), or Hodgkin disease (cancer of the lymphatic system).

Abnormally low levels can indicate gastrointestinal diseases such as Crohn’s disease, liver or kidney disease, blood clotting problems, emphysema, leukemia, hemolytic anemia, or problems with the immune system.



Serum protein electrophoresis showing a paraprotein (peak in the gamma zone) in a patient with multiple myeloma

Cardiac markers in myocardial infarction

- Myocardial infarction is the destruction of heart tissue resulting from obstruction of blood supply to the heart muscle
- It is the medical term for heart attack. This is most commonly caused by occlusion (blockage) of a coronary artery following rupture of a vulnerable atherosclerotic plaque, which is an unstable collection of lipids (like cholesterol) and white blood cells (especially macrophages) in the wall of an artery.
- The resulting ischemia (restriction in blood supply) and oxygen shortage, if left untreated for a sufficient period of time, can cause damage or death (infarction) of heart muscle tissue (myocardium)

Cardiac markers in myocardial infarction (MI)

- Heart attack is a leading cause of death for both men and women all over the world.
- Important risk factors :

  -previous cardiovascular disease (such as angina, a previous heart attack or stroke)

  -old age (especially men over 40 and women over 50)

  - tobacco smoking

  - high blood levels of certain lipids (triglycerides, LDL or "bad cholesterol")

  - low levels of high density lipoprotein (HDL, "good cholesterol")

  -diabetes, high blood pressure, obesity, chronic kidney disease

  -excessive alcohol consumption, the abuse of certain drugs such as cocaine

  - chronic high stress levels

Cardiac markers in myocardial infarction (MI)

- Patient will receive a number of diagnostic tests, such as electrocardiogram (ECG), a chest X-ray and blood tests to detect elevations in cardiac markers (abnormal protein levels signifying heart muscle damage).
- Commonly used markers are the creatine kinase-Myocardial B fraction (CK-MB) fraction and the troponin I (TnI) or troponin T (TnT) levels.
- These proteins normally reside in the heart cells but are released into the blood stream after a heart attack, although elevated levels may also signify problems with other muscles in the body so these tests are not specific for myocardial infarction

Cardiac markers in myocardial infarction (MI)

- Until the 1980s, the enzymes, serum glutamic oxaloacetic transaminase (SGOT) and lactate dehydrogenase (LDH) were used to assess cardiac injury

  -Cardiac troponins T and I which are released within 4–6 hours of an attack of MI and remain elevated for up to 2 weeks, have nearly complete tissue specificity and are now the preferred markers for assessing myocardial damage.

  -Elevated troponins in the setting of chest pain may accurately predict a high likelihood of a myocardial infarction in the near future. New markers such as glycogen phophorylase isozyme BB are under investigation

Cardiac markers in myocardial infarction (MI)

- Cytosolic creatine kinase (CK) enzymes consist of two subunits: *B* (brain type) or *M* (muscle) type. Three different isozymes therefore exist: CK-MM, CK-BB and CK-MB. The genes for these subunits are located on different chromosomes, *B* on 14*q*32 and *M* on 19*q*13. Isozyme determination has been used extensively as an indication for myocardial damage in heart attacks.
- Isozymes are enzymes that catalyze the same reaction but differ in their physical properties as a result of differences in their amino acid sequence which is genetically determined.
- Isozyme patterns differ in tissues. Skeletal muscle expresses CK-MM (98%) and low levels of CK-MB (1%). The heart muscle, in contrast, expresses CK-MM at 70% and CK-MB at 25–30%. CK-BB is expressed in all tissues at low levels and has little clinical relevance.
- Increase in myocardial-specific enzyme CK-MB is considered an evidence of acute myocardial infarction

Cardiac markers in myocardial infarction (MI)

- Troponins are measured in the blood to differentiate between unstable angina and MI. Cardiac troponin T and I are measured by immunoassay methods

Cardiac markers in myocardial infarction (MI)

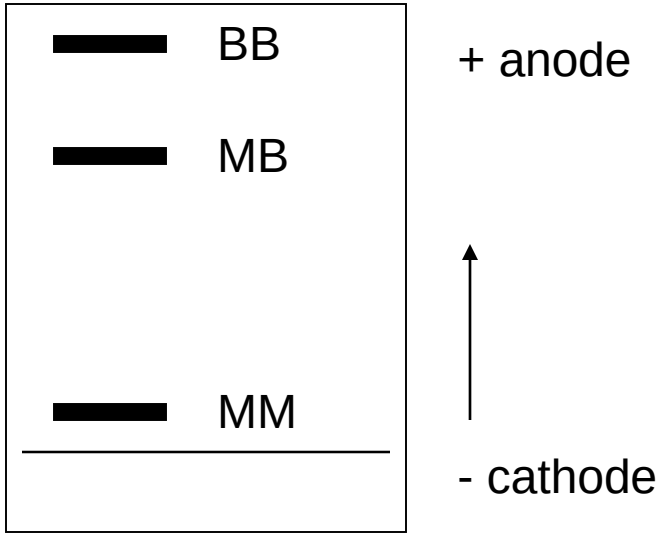
- CK-MB is known to exist in two forms: CK-MB_2, the gene product, and CK-MB_1, which is modified upon release into the bloodstream. Carboxypeptidase cleavage of the C-terminal lysine residue of the M subunit transforms CK-MB_2 into CK-MB_1.
- In healthy individuals, CK-MB_2 is in equilibrium with the modified CK-MB_1 subform at a ratio of approximately 1:1. In the early hours of myocardial infarction, the abrupt release of CK-MB_2 from myocardium produces an upward shift in the serum CK-MB_2/CK-MB_1 ratio, usually before total CK-MB ($CKMB_2$ + $CKMB_1$) exceeds normal levels.

- Determinations of the serum CK-MB_2/CK-MB_1 ratio are also proving useful in diagnosis of myocardial infarction

Cardiac markers in myocardial infarction (MI)

- Electrophoretic separation of the isozymes using agarose, cellulose acetate or polyacrylamide as support media achieves distinct bands that can be quantified by fluorescence densitometry.
- Separation is possible because the M and B subunits have different charges on them. The mobility of these isozymes at pH 8.6 toward the anode is BB> MB> MM with the MM remaining close to the point of application.
  The method is sensitive from 2 - 5 U/L

Electrophoretic separation of CK-MM, CK-MB, and CK-BB



BCHEM 365
Lecture 9
May 16, 2022

Radioisotope Techniques

The Nature of Radioactivity

Atomic structure

An atom is composed of a positively charged nucleus that is surrounded by a cloud of negatively charged electrons. The mass of an atom is concentrated in the nucleus, even though the nucleus makes only a small fraction of the total size of the atom.

Atomic nuclei are made up of two major particles: protons and neutrons.
Protons: are positively-charged particles with a mass of $1.67262158 \times 10^{-27}$ kilograms, approximately 1,850 times greater than that of an orbital electron. The number of protons found in the nucleus of an atom, that is, the atomic number (Z), defines the type of atom.

Neutrons are the uncharged particles in the nucleus with mass approximately equal to the mass of a proton. Because protons are unable to bind with each other due to their mutual electromagnetic repulsion being stronger than the attraction of the nuclear force, the presence of a neutron is required within an atomic nucleus as they bind with protons via the nuclear force.
Electrons: negatively-charged with a mass of $9.10938188 \times 10^{-31}$ kilograms, which is about 1/1850 times the mass of a proton.

The number of orbital electrons in an atom must equal the number of protons present in the nucleus, since the atom as a whole is electrically neutral.
The number of neutrons in the nucleus is given a notation N. The number of neutrons determines the isotope of an element. For example, the abundant ^{12}C isotope has 6 protons and 6 neutrons, while the very rare radioactive ^{14}C isotope has 6 protons and 8 neutrons.
The sum of protons and neutrons in a nucleus is the mass number (A).

Thus, $A = Z + N$

Since the number of neutrons in a nucleus is not related to the atomic number, it does not affect the chemical properties of the atom. Atoms of a given element may not necessarily contain the same number of neutrons. Such atoms, having same number of protons but different mass numbers (i.e. different numbers of neutrons) are called isotopes.

By convention, an atom/element and its isotope are represented by a subscript for atomic number, and a superscript for mass number, followed by the symbol of the element. For example, the isotopes of carbon are represented below:

$^{12}_{6}C$ $^{14}_{6}C$ $^{16}_{8}O$ $^{18}_{8}O$

However, in practice it is more conventional just to cite the mass number (e.g., ^{14}C). The number of isotopes of a given element varies: there are 3 isotopes of hydrogen, ^{1}H, ^{2}H, and ^{3}H, and 7 isotopes of carbon, ^{10}C to ^{16}C, and 20 or more isotopes of some of the elements of high atomic number.

Atomic stability and radiation

In general, the **ratio of neutrons to protons** in the nucleus will determine whether an isotope of a given element is **stable** enough to exist in nature. Stable isotopes for elements with low atomic numbers have neutron:proton ratio of 1, whereas stability for elements of higher atomic numbers is associated with a neutron:proton ratio in excess of 1. Unstable isotopes, also known as radioisotopes are often produced artificially, but many occur in nature.

Radioisotopes emit particles and/or electromagnetic radiation which arise from changes in the composition of the atomic nucleus. The process is known as radioactive decay and may occur either directly or as a result of a decay series in the production a stable isotope. Radioactive decay is the process by which an unstable isotope emits energy to reach a more stable state. Radioactive material decays until only stable substance is left and the decay of a substance is fixed and measurable.

Types of radioactive decay

There are several types of radioactive decay; only those relevant to biological scientists are considered below. A summary of these decay events and their properties is given in Table 9.1.

Table 9.1. Properties of different types of radiation

Alpha	**Beta**	**Gamma, X-rays**
Heavy charged particle	Light charged particle	EM radiation
More toxic than other forms of radiation	Toxicity same as electromagnetic radiation per unit of energy	Toxicity same as beta radiation per unit of energy
Not penetrating	Penetration varies with source	Highly penetrating

<u>Decay by negatron emission (also called beta emission)</u>

In this case a neutron is converted to a proton by the ejection of a negatively-charged beta (β) particle called a negatron (β^-):

Neutron → Proton + Negatron

A negatron is an electron, but the term negatron is preferred, although not always used, since this particle originates from the nucleus and not from the orbit. Negatron emission causes the nucleus to **lose a neutron but gain a proton**. The *N*/*Z* (number of neutrons/no of protons) ratio therefore decreases because *Z* increases by 1 and *A* (mass no.) remains constant. An isotope frequently used in biological work that decays by negatron emission is ^{14}C.

$$^{14}_{6}C \rightarrow ^{14}_{7}N + \beta^-$$

Beta (or negatron) emission is very important to biologist/biochemists because many of the commonly used radionuclides decay by this mechanism. Examples are: ^{3}H and ^{14}C, which can be used to label any organic compound; ^{35}S is used to label methionine, for example in the study of protein synthesis; and ^{32}P, is a powerful tool in molecular biology when used to label a nucleic acid.

<u>Decay by positron emission</u>

Some isotopes decay by emitting positively-charged β-particles referred to as positrons (β^+). This occurs when a proton is converted to a neutron:

Proton → Neutron + Positron

As a result of positron emission the nucleus loses a proton but gains a neutron, the *N*/*Z* ratio increases, *Z* decreases by 1 and *A* remains constant. An example of an isotope which decays by positron emission is ^{22}Na :

$$^{22}_{11}\text{Na} \rightarrow ^{22}_{10}\text{Ne} + \beta^{+}$$

Positrons are extremely unstable and have only a transient existence. Once they have dissipated their energy they interact with electrons and are completely destroyed. The mass and energy of the two particles are converted to two γ-rays emitted at 180° to each other. This phenomenon is frequently described as **back-to-back** emission.

Positron emitters are detected by the same instruments used to detect γ-radiation. They are used in a clinical technique known as Positron Emission Tomography (PET scanning) to scan the brain to identify active and inactive areas.

<u>Decay by alpha particle emission</u>

Isotopes of elements with high atomic numbers frequently decay by emitting alpha (α) particles. An α-particle is a helium nucleus without electrons; it consists of two protons and two neutrons ($^{4}\text{He}^{2+}$). Emission of α-particles results in the following:

- A considerable lightening of the nucleus
- A decrease in atomic number of 2
- A decrease in the mass number of 4.

Isotopes that decay by α-emission are not frequently encountered in biological work. Radium-226 (^{226}Ra) decays by α-emission to radon-222 (^{222}Rn), which is itself radioactive, thus, beginning a complex decay series which culminates in the formation of ^{206}Pb :

$$^{226}_{88}\text{Ra} \rightarrow ^{4}_{2}\text{He}^{2+} + ^{222}_{86}\text{Rn} \rightarrow\rightarrow\rightarrow ^{206}_{82}\text{Pb}$$

Alpha emitters are extremely toxic if ingested, due to their large mass and ionizing power.

<u>Electron capture</u>

In this decay process, a proton captures an orbital electron in the innermost K shell and is converted to a neutron and the electron emits electromagnetic radiation (X-ray)

$$\text{Proton} + \text{Electron} \rightarrow \text{Neutron} + \textit{X-ray}$$

$$^{125}_{53}\mathrm{I} \rightarrow {}^{125}_{52}\mathrm{Te} + \mathrm{X-ray}$$

The loss of a proton reduces the atomic number by one and gain of a neutron keeps mass number constant.

<u>Decay by emission of Gamma (γ) -rays</u>

γ-radiation arises from a transformation in the nucleus when, for some atoms, an α- and/or β- decay event occurs. γ-emission is an electromagnetic radiation which similar to X-ray but has a shorter wavelength. γ-emission is not a particle, and does not lead to change in atomic number or mass number of the atom.

Gamma radiation has low ionizing power but high penetration and has toxicity similar to that of X-rays. For example, the γ-radiation from $^{60}\mathrm{Co}$ will penetrate 15 cm of steel.

Example:

$$^{131}_{53}\mathrm{I} \rightarrow {}^{131}_{54}\mathrm{Xe} + \beta^{-} + \gamma$$

γ-radiation from different sources of atoms vary in energy levels.

Radioactive decay energy

The usual unit used to express energy levels associated with radioactive decay is the **electron volt**. One electron volt (eV) is the energy required by one electron to accelerate through a potential difference of 1.0 V and is equivalent to 1.6×10^{-19}J. For majority of isotopes, the radiation energy is so high, typically in the millions, that is, mega electron volts (MeV). Alpha decay events have the highest energies, in the range of 4.0 – 8.0 MeV, whereas β- and γ- emissions have energies than 3.0 MeV. The energy of emissions depends on the source. There are weak and strong beta emitters and also weak and strong gamma emitters.

Rate of radioactive decay

Radioactive decay is a spontaneous process and it occurs at a definite/fixed rate characteristic of the source. This rate always follows an exponential law, such that, the decay events occurring at any time *t*, is proportional to the number of atoms of the isotope (*N*) present at that time. Because each decay event leads to loss of the isotope, except for some α-emitters which decay in series, a plot of number of isotopes remaining with time of decay gives an exponential curve (Fig. 9.1), the linear presentation of which, has a negative slope.

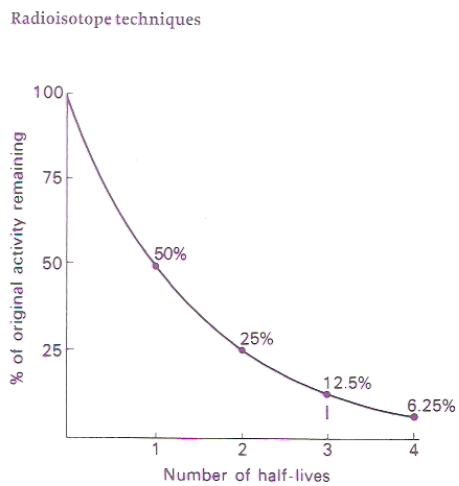


Fig. 9.1. The exponential nature of radioactive decay

Mathematically, the exponential equation is:

$$-\frac{dN}{dt}\alpha N \quad \text{or} \quad -\frac{\mathrm{dN}}{\mathrm{dt}} = \lambda \mathrm{N} \ldots (1)$$

where λ is the decay constant, a unique property of a given isotope. Decay constant is defined as the fraction of an isotope decaying per unit time (t^{-1}). To linearize this expression, we integrate and take the logarithm to give:

$$\ln \frac{\mathrm{N_t}}{\mathrm{N_0}} = -\lambda \mathrm{t} \ldots (2)$$

where N_t is the number of radioactive atoms present at time *t*, and N_0 is the number of radioactive atoms originally present. In practice, it is more convenient to express the decay constant in terms of half-life ($t_{½}$).

Half life - the time it takes for a radioactive isotope to decay to half of its original value and have it in a stable form. The half life of an isotope is a fixed parameter.

If N_t in equation (2) is equal to half of N_0, then t will equal the half-life of the isotope. Thus

$\ln ½ = -\lambda t_{½}$ ….(3)

or

$2.303 \log (½) = - \lambda t_{½}$ …(4)

So that

$t_{½} = 0.693/ \lambda$ …(5)

Values of $t_{½}$ vary widely from 3×10^{-7} s for polonium-212 (^{212}Po) to over 10^{19} years for lead-204 (^{204}Pb). The half-lives of some isotopes frequently used in biological work are shown in Table 9.2.

Table 9.2. Half-lives of some isotopes used in biological studies

Isotope	**Half-life**
^{3}H	12.26 years
^{14}C	5760 years
^{22}Na	2.58 years
^{32}P	14.20 days
^{33}P	25.4 days
^{35}S	87.20 days
^{42}K	12.40 hours
^{45}Ca	165 days
^{59}Fe	45 days
^{125}I	60 days
^{131}I	8.05 days
^{135}I	9.7 hours

In Table 9.2, we do not have the two important radioisotopes ^{15}O ($t_{½}$ = 2.03 min) and ^{13}N ($t_{½}$ = 10.00 min) because their half-lives are too short for most biological studies.

The advantages and disadvantages of working with isotopes of short half-lives are given in the table below.

Advantages and disadvantages of working with isotopes of short half-lives

Advantages	**Disadvantages**
High specific activity	Experimental design must be appropriate as isotope decays during time of experiment
Lower doses likely (e.g., in diagnostic testing of human subjects)	Cost of replacement for further experiments

Worked example

Given ln (N_t/N_0) = -λt and that the half-life of ^{32}P is 14.2 days, how long would it take a solution containing 42,000 d.p.m. of ^{32}P to decay to 500 d.p.m.?

Solution

Use equation (5) to calculate the value of λ.

$t_{½} = 0.693/\lambda$ or $\lambda = 0.693/t_{½}$

$\lambda = 0.693/14.2$ days

$\lambda = 0.0488$ days^{-1}

$$\ln \frac{N_t}{N_0} = -\lambda t....(2)$$

$$\ln \frac{500}{42,000} = -0.0488 \text{ days}^{-1}(t)$$

$$t = \frac{-4.43}{-0.0488 \text{ days}^{-1}} = 90.78 \text{ days}$$

<u>Units of radioactivity</u>

The Système International d'Unités (SI system) unit for radioactivity is Becquerel (Bq), defined as **one disintegration per second (1 d.p.s.)**. The older or imperial unit is curie (Ci). It is defined in terms of the disintegrations in radioactive radium, such that, one curie is the number of disintegrations per second in any isotope that is same as the disintegrations in 1 g of radium, namely 3.7 10^{10} d.p.s. (or 37 GBq).

For biological purposes, this unit is too large and the microcurie (μCi) and millicurie (mCi) are more relevant. It is important to realize that the curie refers to the number of disintegrations actually occurring in a sample and not to the disintegrations detected by the radiation counter (in counts per second, c.p.s.)., which will generally a fraction of the actual disintegrations.

Normally, in experiments with radioisotopes, a carrier of the stable isotope of the element is added. For example, in experiments with ^{14}C, ^{12}C is added as a carrier. It therefore becomes necessary to express the amount of radioisotope present per unit mass (sum of ^{14}C and ^{12}C). This is the specific activity. Specific activity may be expressed in a number of ways including disintegration rate (d.p.s./g or

d.p.m./g), count rate (c.p.s. /g or c.p.m. /g) or curies (mCi/g or μCi/g) or (d.p.s./mole or d.p.m./mole), count rate (c.p.s. /mole or c.p.m. /mole) or curies (mCi/mole or μCi/mole). An alternative method of expressing specific activity, which is not frequently used is atom percentage excess. This is defined as the number of radioactive atoms per 100 atoms of the compound.

Interaction of radioactivity with matter

α-particles

Alpha-particles have quite an amount of energy (4 -8 MeV) and all the particles from a given isotope have the same amount of energy. Alpha particles react with matter in two ways:

1. They may cause excitation: in this case, the energy associated with the decay event is transferred from the α-particle to the orbital electrons of neighboring atoms and the electrons are elevated to higher orbitals. The α-particle continues on its path with its energy reduced by a little more than the amount transferred to the orbital electron. The excited electron eventually falls back to its original orbital, emitting the energy it picked up as photons of light in the visible or near visible range.

2. α-particles may cause ionization when the energy received by the orbital electron causes it to be completely removed, that is, an electron is ejected. Thus, the atom becomes ionized and forms an ion-pair, consisting of a positively charged ion and an electron. Because of their size, slow movement, and double positive charge ($^{4}He^{2+}$), α-particles frequently collide with atoms in their path. Therefore, they cause intense ionization and excitation and their energy is rapidly dissipated. Because of this, though they have initial high energy, α-particles are not very penetrating.

Negatrons

Negatrons are very small, rapidly moving particles, which carry a single negative charge, compared to α-particles. They interact with matter to cause ionization and excitation in the same way as α-particles do. However, due to their speed and size, they are less likely to interact with matter and therefore are less ionizing but more penetrating than α- radiation.

Another difference between β-particles and α-particles is that, whereas for a given α-emitter all the particles have the same energy, β-particles are emitted over a range of energies so that negatron emitters

have a characteristic energy spectrum. The maximum energy level (E_{max}) varies from one isotope to the other, and ranges from 0.018 MeV for ^{3}H to 4.81 MeV for ^{38}Cl. The difference in E_{max} affects the penetration of the radiation.

For example:
β-particles from ^{3}H can travel only a few millimeters in air
β -particles from ^{32}P can penetrate over 1 m of air.

W. Pauli's (1931) explained that **neutrinos**, which are produced in many beta decay events, pick up some of the energy (E_{max}) of the decay events and the remaining energy is carried by β-particle. A neutrino is an electrically neutral particle with negligible mass. The proportion of the total energy taken by the negatron and the neutrino varies for each disintegration.

γ-Rays and X-rays

For simplicity, we will refer to these rays collectively as γ-rays. These rays are electromagnetic radiation and therefore have no charge or mass. They have the following properties:

- They rarely collide with neighboring atoms and travel great distances before dissipating all their energy, that is, they are highly penetrating.
- If they collide with neighboring atoms, they react with matter in many ways to eject orbital electrons, whose energies they carry, cause further excitations and ionizations. Three most important interactions of γ-rays with matter include:

a. Photoelectric absorption: here, low energy γ-rays interact with orbital electrons, transferring all their energy to the electron which is then ejected as a photoelectron. The photoelectron subsequently behaves as a negatron.
b. Compton scattering: medium energy γ-rays interact with orbital electrons, which gain energy and are ejected. The γ-ray is deflected and moves on with reduced energy and the ejected electron behaves as a negatron or a beta particle.
c. Pair production: very high energy γ-rays (highly penetrative) react with the nucleus of an atom and all the energy they carry is used to convert nuclear particles into positron and a negatron.

When high atomic number materials absorb high energy β-particles, the absorber gives out a secondary radiation, an X-ray called Bremsstrahlung. They are X-rays produced when energetic

electrons are deflected in the field of an atomic nucleus. Thus, Bremsstrahlung radiation is present during all beta decay processes as the emitted beta particles (both negatrons and positrons) slow down in matter. For this reason, shields of ^{32}P use low atomic number materials such as Perspex (polymethyl methacralate).

Detection and measurement of radioactivity

There are three commonly used methods for detecting and quantifying radioactivity. The methods are based on:

1. Ionization of gases
2. Excitation of solids or solutions
3. Autoradiography

Methods based on gas ionization

A radioactive substance produces charged particle ($^{4}_{2}He^{2+}$, β^{+}, β^{-}). As a charged particle passes through gas, its electrostatic field dislodges orbital electrons from atoms of the gas which are sufficiently close to its path and causes ionization (Fig. 9.2).

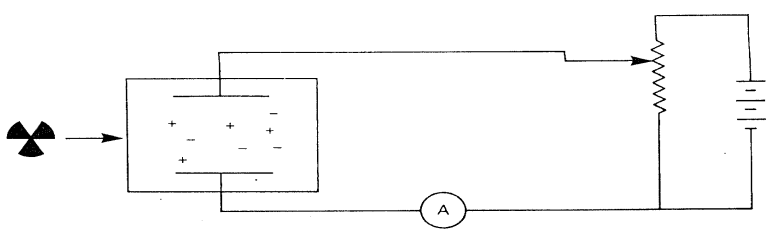


Fig. 9.2. Detection based on ionization

Ability to induce ionization decreases in the order $\alpha > \beta > \gamma$ (10,000 : 100 : 1). Because of this, α- and β-particles may be detected by gas ionization methods, but these methods are poor for detecting γ –radiation. If ionization occurs between a pair of electrodes enclosed in a suitable chamber filled with gas, ionization of the gas creates surge in the current (called a pulse), whose magnitude depends on:

1. The applied potential, that is, the voltage across the electrodes
2. The number of radiation particles entering the chamber.

Fig. 9.3 shows the effect of voltage on pulse flow.

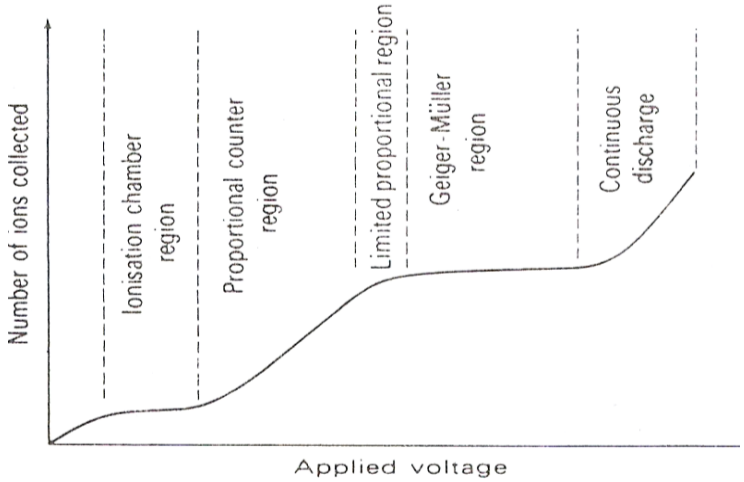


Fig. 9.3. Effect of voltage on pulse flow

Comments on the curve:

1. An overall rise in the curve demonstrates that as voltage across the electrodes increases, ionization increases, to create five characteristic regions.
   a) Ionization chamber region: at low voltage each radioactive particle produces only <u>one ion-pair per collision</u>. The corresponding current is low and one needs very sensitive measuring device to detect them. This range of voltage is little used in quantitative work, but various types of electroscopes, which operate on this principle, are useful in demonstrating the properties of radioactivity.
   b) Proportional counter region: At a higher voltage, electrons resulting from ionization move toward the anode much more rapidly. In their movement, they <u>cause secondary ionization of gas</u> in the chamber, resulting in the production of secondary ionization electrons, whose fast movement leads to further ionization, and so on. Hence, from the original event, a stream of electrons reaches the anode. This is the principle of gas amplification and is known as the Townsend Avalanche Effect, named after its discoverer. As a consequence of this gas amplification, current flow is much greater and more pulses are created. In the proportional counter region, we can see that the number of ion-pairs collected is directly proportional to the applied voltage.

c) Limited proportional region: as voltage is increased further, and more ion-pairs are produced, a point is reached where increase in voltage does not result in further increase in ionization - a plateau is reached (the Geiger-Müller region). Before the plateau is reached, there is a region known as the limited proportion region, which is not often used for detection and quantification of radioactivity. The main drawback of counters that are manufactured to operate in the proportional region is that they require very stable voltage supply because small fluctuations in voltage result in significant changes in amplification. Proportional counters are useful for detecting and quantifying α-emitting isotopes, but relatively few such isotopes are used in biological work.

d) In the Geiger-Müller region: voltage is high and all radiation particles, including weak β-particles, induce complete ionization of the gas in the chamber and no further increase in ionization occurs. Thus, the size of the voltage is no longer dependent on the primary ions produced. Since maximal gas amplification is produced in this region, the size of the output pulse from the detector will remain same over a considerable voltage range (the Geiger-Müller plateau). Therefore, it is not possible to distinguish between different isotopes using this type of counter.

Since it takes a finite time for the ion-pair to travel to the electrodes, other ionizing particles entering the tube during this time fail to produce ionization and hence are not detected, thereby reducing the counting efficiency. This is referred to as the dead time of the tube and is normally 100 to 200 μs.

e) Continuous discharge region: when the ions reach the electrode, they are neutralized. Inevitably, some escape and produce their own ionization avalanche. Because of this, if unchecked, a Geiger-Müller tube would tend to give continuous discharge. To overcome this, the tube is quenched by the addition of a suitable gas, which reduces the energy of the ions. Common quenching agents are ethanol, ethyl formate and the halogens.

Instrumentation

Counters based on gas ionization were previously the main methods employed in quantification of radioisotopes in biological samples. Currently, scintillation counting is commonly used. However, all

labs use small hand-held radioactivity monitors based on gas ionization, the end-window design being the most popular.

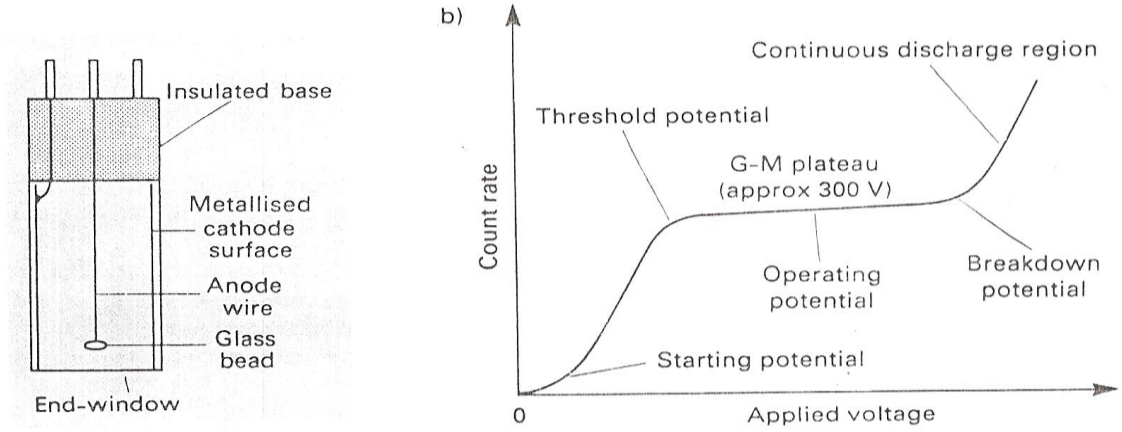


Fig. 9.4. (a) The Geiger-Müller (G-M) tube. (b) Effect of applied voltage on count rate.

The Geiger Counter

A Geiger counter is the most common radiation detector based on gas ionization. This device uses a Geiger-Müller tube as the sensor. This tube is filled with an inert gas, usually argon, helium or neon with halogens added as quencher, and voltage in the chamber is maintained over 100 V. When a radioactive particle passes the tube, the gas ionizes, becomes conductive and produces pulse which can be amplified by a cascade effect and outputs a magnified current or pulse, which is displayed visibly as glows of light or as audible clicks.

Fig. 9.5. A hand-held Geiger counter.

A large amount of radiation passing through the tube produces a higher reading and more clicks because of the greater amount of electric current generated inside the tube.

Geiger counters usually contain a sealed, gas-filled cylinder and a fine axial wire. The cylinder is operated at ground potential and the wire at a positive potential that is higher than that in the proportional counter. In this region, the discharge due to ionization spreads along the whole length of the

wire. Total number of electrons produced does not depend on the amount of primary ionization, and all of the discharge pulses become equal in amplitude. In other words, each particle crossing the cylinder produces a gas ionization that is roughly independent of the particle's nature and energy.
The number of pulses (clicks) over a certain time (count rate) is a quantitative measure of radiation. The count rate is referred to as counts per minute (c.p.m.).

Geiger counters are useful for detecting the ionizing radiations: alpha, beta and gamma rays. With gamma radiation, sensitivity can be lower, so rarely used. Most hand-held Geiger counters are at their best with alpha and beta rays. The density of the gas within the tube is usually sufficient for these two rays but not for high-energy gamma rays.

These counters have a thin end-window made of aluminum and can detect β-radiation from high energy (^{32}P) and weak emitters (^{14}C) but are incapable of detecting weaker ^{3}H because the radiation cannot penetrate the end-window. For the same reason, they are not very efficient detectors of gamma-radiation.
End-window ionization counters are used for routine monitoring of the radioactive laboratory to check for contamination. They are also useful in experimental situations where the presence or absence of radioactivity needs to be known rather than the absolute quantity, for example, quick screening of radioactive gels prior to autoradiography, or checking of chromatographic fractions for labeled components.

The inability of end-window counters to detect β-emitters presents a problem in biosciences because ^{3}H is a commonly used radioisotope. This problem can be overcome by using a windowless counter where a gas flow is used. These instruments are cumbersome and need to be conveyed by trolleys. They are useful for mass screening of premises for ^{3}H contamination but are rarely used routinely. Most labs monitor for ^{3}H by doing a **wipe test** regularly. This test uses wet paper towels or cotton wool to take swabs for scintillation counting.

Methods based on excitation

Radioactive isotopes interact with matter in two ways - they cause (1) ionization, which forms the basis of Geiger-Müller counting, and (2) excitation, which governs scintillation counting.

Excitation of a fluorescent compound (fluor or phosphor) leads to emission of photons of light in the visible region of the electromagnetic spectrum which can be detected and counted. This process is known as scintillation counting.

Scintillators (fluor or phosphor) are materials which fluoresce (or phosphoresce) in the presence of ionizing radiation. Scintillation counters combine this fluorescence with a well-known phenomenon — the photoelectric effect (emission of electrons when light shines on the surface of a material), to convert incident radiation from a radioisotope into an electrical signal and amplifies it by means of a photomultiplier tube. Albert Einstein (1879-1955) was known for the theory of relativity and the law of photoelectric effect. A photomultiplier tube is a photoemissive device in which the absorption of a photon results in the emission of an electron. It is useful for light detection of very weak signals.

TYPES OF SCINTILLATION COUNTING

There are two types of scintillation counting illustrated in Fig. 9.6.

1. Solid scintillation counting
2. Liquid scintillation counting

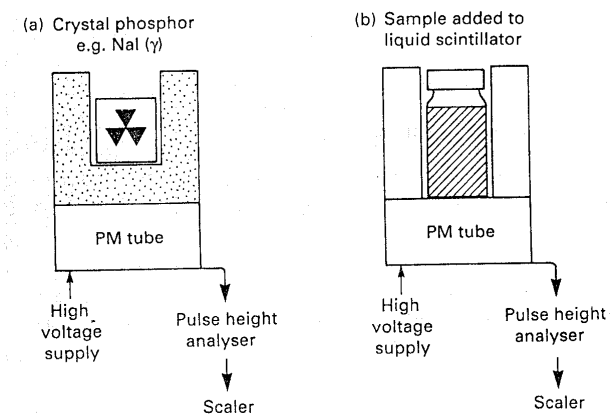
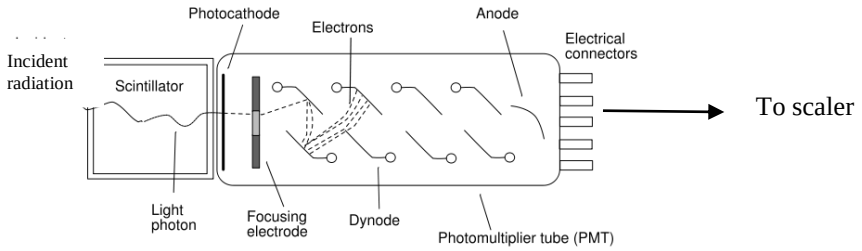


Fig. 9.6. Diagrammatic illustration of A) Solid scintillation counter. B) Liquid scintillation counter

When a radioactive particle hits the scintillator, its energy is absorbed and immediately reemitted as a photon of light of a given wavelength. The emitted photons are gathered and focused toward the photocathode, where they are absorbed and their energy is used to emit electrons via the photoelectric effect. The photoelectrons are in turn focused toward a series of metal electrodes known as dynodes (A dynode is an electrode in an electron tube that produces electrons through secondary emission). Photocathodes are made of materials such as silver oxide/cesium (Ag-O-Cs), and temperature-resilient sodium-potassium-antimony (Na-K-Sb), cesium iodide, and cesium terrulide.

When a photoelectron hits the first dynode, its kinetic energy is sufficient to knock several more electrons off the dynode surface via the Auger effect. These Auger electrons proceed through the rest of the dynode series, producing more and more electrons with each impact. Finally, the amplified electron signal is absorbed at the anode, measured, and quantified. The electrical pulse that results from the conversion of light energy to electrical energy in the photomultiplier is directly proportional to the energy of the original radioactive event. The advantage of this method is that two or more isotopes can be separately detected and measured in the same sample, provided they have sufficiently different emission energy spectra.

The mode of action of a photomultiplier is shown in Fig. 9.6. The source of light for the Photomultiplier is the light emitted by the scintillator. This light is directed through a glass or quartz window toward a photosensitive surface, known as a photocathode. The photocathode then releases electrons that are multiplied by electrodes known as metal channel dynodes. At the end of the dynode chain is an anode or collection electrode. The current flowing from the anode to the scaler is directly proportional to the photoelectron flux generated by the photocathode and proportional to the energy of the original radioactive event.



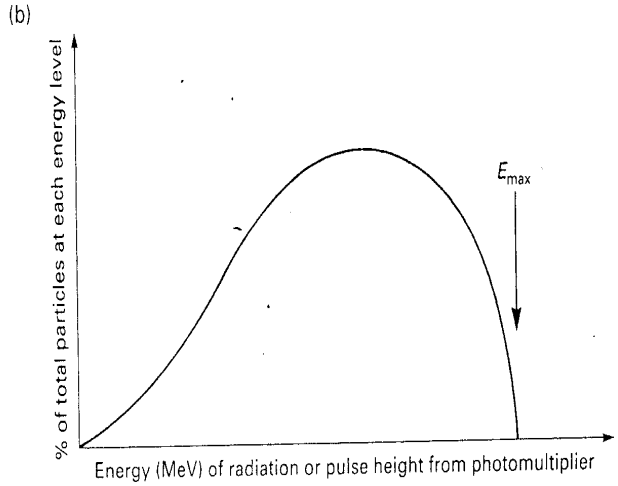


Fig. 9.6. (a) The mode of action of a photomultiplier and (b) the energy spectrum of a typical β-emitter.

Scintillation counting provides two kinds of information:

1. Quantitative: the number of scintillations is proportional to the rate of decay of the sample, i.e. the amount of radioactivity
2. Qualitative: the intensity of light given out and therefore signal from the photomultiplier is proportional to the energy of radiation

Solid Scintillation Counting

In solid scintillation counting, the radioactive sample is placed adjacent to a crystal of fluorescent material called a scintillation phosphor which is coupled to a suitable light-sensitive photomultiplier tube which in turn is connected to a high voltage supply and a scaler (Fig. 9.6). As radiation interacts with the scintillation crystal, energy is transferred to bound electrons of the crystal's atoms. These emit light, which can be amplified in a photomultiplier. The following crystals are used:

For γ–emitters - sodium iodide
For α-emitters – zinc sulphide
For β-emitters – organic scintillators such as anthracene

Two conditions are required of a scintillator: 1) must be able to absorb radiation, 2) must interact with the radiation. Medium- and low-energy beta particles cannot penetrate the crystal and, therefore, cannot interact. A thin scintillator is an excellent choice for low-energy gamma rays (in the range of 100 keV) and high-energy beta particles. The ray or particle will be absorbed within the thin scintillator, interact with it to produce light, then travel through the remaining crystal thickness for onward detection. A high-energy gamma ray is likely to pass right through the thin scintillator without interacting.

A thick scintillator is the choice for radionuclides emitting high-energy gamma rays. This scintillator is thick enough to absorb the gamma ray but not too thick to prevent the light that is produced from being detected. A thick scintillator is not very good for low-energy gamma rays; they will interact but the scintillator is too thick and will absorb the light that is produced before it can be detected.

Solid scintillation counting is particularly useful for γ-emitters because they produce electromagnetic radiation and collide only rarely with neighboring atoms to cause ionization or excitation. In a crystal,

the atoms are densely packed, making collisions (i.e., interactions) more likely. Conversely, solid scintillation counting is generally unsuitable for weak β-emitters such as ^{3}H and ^{14}C, because even the highest energy negatrons emitted by these isotopes would have hardly sufficient energy to penetrate the walls of the counting vials in which the samples are placed for counting.

Because many of the isotopes used in radioimmunoassay are γ-emitters, solid scintillation counting is frequently used in biological work.

Liquid Scintillation Counting

Liquid scintillation counting (Fig. 9.5) detects radioactivity via the same type of light emission events which are used in solid scintillation. The key difference is that in liquid scintillation counting the scintillation takes place in a solution of scintillator, rather than in a solid crystal. This allows close contact between the isotope atoms and the scintillator, which is not possible with solid scintillation. With liquid scintillation counting, the short path length of soft β- emissions is not an obstacle to detection.

In this method, the sample is mixed with a scintillation cocktail containing a solvent and one or more fluors. Liquid scintillation cocktails absorb the energy emitted by radioisotopes and re-emit it as flashes of light. To accomplish these two actions of absorption and re-emission, cocktails contain two basic components, the solvent and the fluor(s). The solvent carries out the bulk of the energy absorption. The fluor, which is dissolved in the solvent, converts the absorbed energy into light. Many cocktails contain additional materials to extend their range of use to other sample compositions but the solvent and the fluor provide the scintillation of the mixture. Liquid scintillation is particularly useful in quantifying weak β-emitters such as ^{3}H, ^{14}C and ^{35}S, which are frequently used in biological work.

BCHEM 365
Lecture 10
November 26, 2018

Energy transfer in liquid scintillation counting

Role of the solvent and fluors

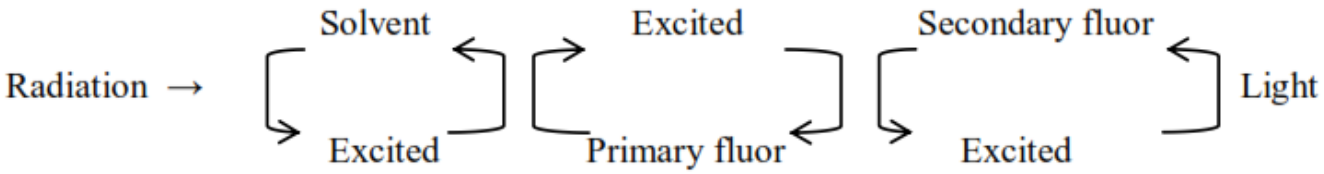
The solvent portion of a scintillation cocktail makes up about 60-99 % of the total solution. Properties required of a solvent are as follows:

1. The solvent must act as an efficient collector/absorber of energy
2. It must conduct that energy efficiently to the fluor molecules rather than dissipating it
3. The solvent must not quench the scintillation of the fluor
4. The solvent must dissolve the fluor to produce a stable and homogeneous counting solution

A small number of organic solvents fluoresce when bombarded with radioactivity, i.e., they also act as scintillators. However, the light emitted has a very short wavelength (Fig. 10.1) and cannot be efficiently detected by the photomultipliers, though they are gathered by the photocathode. If another compound is added to this cocktail that can accept the energy picked up by the solvent, and itself fluoresce at a longer wavelength, then the light emitted can be more efficiently detected. Such a compound is known as a primary fluor. The most frequently used primary fluor is 2,5-diphenyloxazole (PPO). It is included at a concentration of 0.3 % to 1 % of the total solution volume. Linked benzene rings rather than large aromatic systems make the best scintillators.

Unfortunately, the light emitted by PPO is not always detected with very high efficiency, so one needs to further aid the energy transfer by including a secondary fluor or wavelength shifter such as 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP). Combination of two fluors with the solvent makes the energy transfer process efficient. Because the solvent cannot transfer its energy directly to the secondary fluor, it becomes necessary to have both primary and secondary fluor in the scintillation system. Find below a path of energy transfer from

Sample ⟶ Solvent ⟶ Primary fluor ⟶ Secondary fluor ⟶ Photocathode



PPO and POPOP were among the first fluors used in liquid scintillation counting and are still commonly used. An alternative primary fluor 2-(4'-t-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole (Butyl-PBD) performs better as a primary fluor than PPO, but more expensive and is affected by extremes of pH. Bis-MSB is the most common secondary fluor. Napthalene can serve as both solvent and fluor. Fluors are organic and inflammable.

Most labs now purchase their scintillation cocktails already prepared and there are many different recipes on the market. Competition and increasing awareness of health and safety means that manufacturers are making less toxic scintillation cocktails with lower fire hazard.

Some cocktails are designed for organic samples and others for aqueous samples. For aqueous samples, emulsifiers such as Triton X-100 are added to the scintillation cocktail.

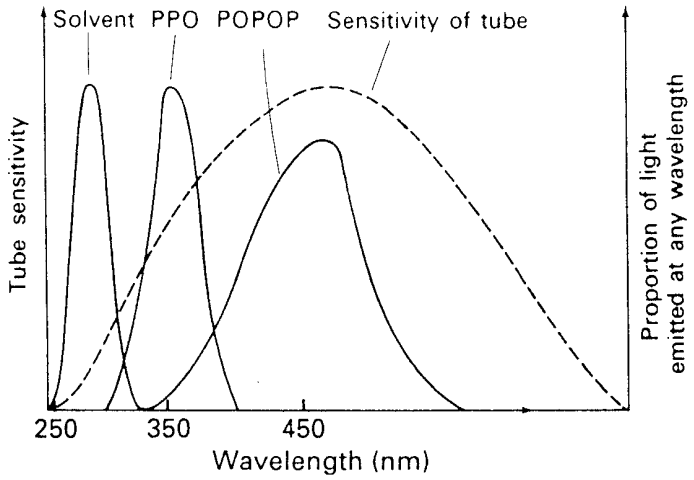
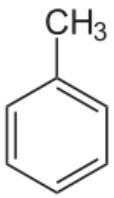
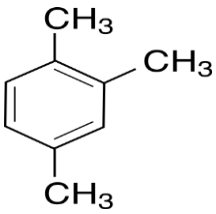


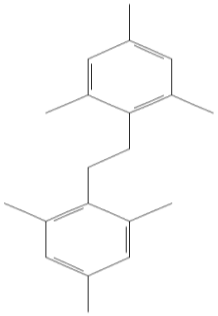
Fig. 10.1. Emission spectra of various fluors in relation to sensitivity of phototubes

The aromatic organics as solvents

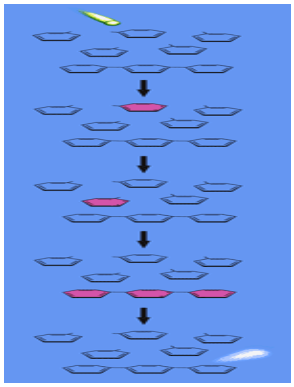
Aromatic organics are the best solvents for liquid scintillation counting. The π electron cloud of the toluene ring or any aromatic ring is a target for β-particle interaction, which captures the energy of the incident particle. The β-particle passing through toluene solution causes the production of energized toluene molecules. Energy from these molecules passes back and forth among the solvent ring system, allowing efficient energy capture by the dissolved fluors. The following solvents possess aromatic rings for efficient absorption of incident radiation.



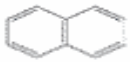
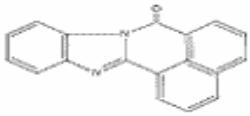




Toluene 1,2,4-trimethylbenzene (pseudocumene) Phenylxylyl ethane (PXE)



Solvent molecules in a scintillation cocktail absorb a portion of the alpha or beta particle's energy. The energy passes between solvent molecules until it reaches a fluor which absorbs the energy and re-emits it as light at a longer wavelength.

Primary Scintillators		
Scintillator	**Structure**	**Emission Wavelength**
Butyl PBD 2-[4-biphenylyl]-5-[4-*tert*-butyl-phenyl]-1,3,4-oxadiazole) **Order No. SFC-20**		363nm
Naphthalene **Order No. SFC-40**		322nm
PPO 2,5-diphenyloxazole **Order No. SFC-10**		357nm
p-Terphenyl **Order No. SFC-50**		340nm
Secondary Scintillators		
BBQ (7H-benzimidazo[2,1-a]benz[de]isoquinoline-7-one) **Order No. SFC-13**		477nm
Bis-MSB (1,4-bis[2-methylstyryl]-benzene) **Order No. SFC-90**		420nm
POPOP (1,4-bis[5-phenyloxazol-2-yl]benzene) **Order No. SFC-60**		410nm
TPB (1,1,4,4-tetraphenyl-1,3-butadiene) **Order No. SFC-15**		455nm

Advantages of scintillation counting

1. **No dead time** - Much higher count rates are possible with scintillation counting than gas ionization counting because of the speed of fluorescence decay (10^{-9} s). There is also no dead time as in a Geiger-Müller tube (10^{-4} s).
2. **No delay by end window** - Much higher counting efficiencies particularly for low energy β-emitters are obtained; over 50% counting efficiency for weak β-emitters, and over 90% counting efficiency for high energy emitters. This is because the negatrons do not have to travel through air or pass through an end-window of a Geiger-Müller tube dissipates much of the energy before causing ionization), but interact directly with the fluor so that energy loss before counting is minimal.
3. Ability to accommodate samples of any type, including polar and organic liquids, solids, suspensions and gels.

4. Ease of sample preparation.
5. Ability to count different isotopes separately in the same sample which means dual labeling experiments can be carried out.
6. Scintillation counters are highly automated and in-built computer facilities carry out many forms of data analysis, such as efficiency correction, graph plotting, radioimmunoassay calculations, etc.

Disadvantages of scintillation counting

Five disadvantages are associated with scintillation counting systems

1. **High cost**: High cost of counting per sample, however, sensitivity, versatility, ease and accuracy outweigh the cost for most applications
2. **Photomultiplier Noise**: At the high voltages applied to the photomultiplier, heat is generated and extraneous electronic events occur in the tube, which do not arise from the radioactivity, thus contributing to background count. This is called photomultiplier noise which must be reduced. There are four ways of reducing photomultiplier noise.

(a) **By Cooling** - Can be reduced by cooling the photomultiplier tube. Cooling results in improved counting efficiency.

(b) **Special design of the photomultiplier** - Use of low noise photomultipliers with greater temperature stability in ambient temperature systems.

(c) **Use of pulse height analyzer** – The data analysis tool can be regulated to electronically reject most of the photomultiplier noise which would usually have low energy. Regrettably, this approach also rejects the low energy pulses arising from low energy radioisotopes such as β-particles from ^{3}H (about 0.019MeV).

(d) **Use of coincidence counting systems** – this system employs two photomultiplier tubes (PMT) in one scintillation counter. When both PMTs produce pulse simultaneously, we describe it as coincidence pulse. An actual radioactive event, with its relatively higher energy than an electronic arising from heat would more often lead to simultaneously pulses (coincidence) in the PMT. The system is designed to allow only coincidence pulses to proceed to the scaler and reject single pulses. The photomultipliers are set in coincidence by mounting them opposite to each other to facilitate detection of only simultaneous pulses. In general, coincidence counting systems reduce photomultiplier noise to a very low level.

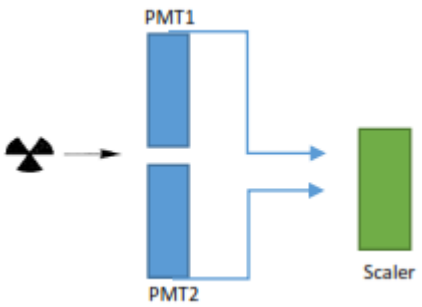


Fig. 10.2. Arrangement of photomultipliers for coincidence counting

3. **Quenching**: The greatest disadvantage of scintillation counting is quenching. Quenching occurs when the energy transfer process from solvent to fluor to photomultiplier tube is interfered with. Correcting quenching adds cost to scintillation counting.

Quenching

Quenching is the loss of counts due to some inherent property of sample, such as its components, and the properties of the cocktail reagents. There are three kinds of quenching: optical, chemical, and color quenching. Note that quenching is not a great problem in solid scintillation counting, but a major problem in liquid scintillation counting.

OPTICAL QUENCHING

This occurs if inappropriate or dirty scintillation vials are used. The dirt will absorb some of the light emitted by the fluors before it reaches the photomultiplier.

COLOR QUENCHING

This occurs if the sample is colored and absorbs light in the range of the wavelength emitted by the fluor. The light emitted is therefore absorbed within the scintillation cocktail before it leaves the sample vial. In this case the number of photons reaching the photomultiplier is reduced.

CHEMICAL QUENCHING

This form of quenching results when materials in the sample interfere with transfer of energy from solvent to primary fluor, or from primary fluor to secondary fluor. Quenchers absorb radioactive

energy before it is converted to light, hence reduction in the number of photons generated by each emission.

4. **Chemiluminescence and static electricity**

Some light emissions unrelated to excitation of the solvent and fluor system by radioactivity may arise from chemical reactions between sample components and reagents of the scintillation cocktail. These light emissions are generally low energy events and are rejected by the threshold setting of the photomultiplier in the same way as photomultiplier noise. Chemiluminescence can be overcome by storing samples for some time before counting, to permit the chemiluminescence to decay. In many modern instruments, chemiluminescence can be detected, subtracted or flagged on the print output.

Chemiluminescence can generate as high as 10^5-10^6 c.p.m., skewing both total c.p.m. data and counts ratio information. It is diagnosed by counting a sample twice within a period of about an hour between counts. A sharp decrease in counts over this period indicates presence of chemiluminescence. As the chemiluminescent reaction consumes its substrate, the rate of photon production decreases noticeably over an hour, and will usually decrease to zero over the course of 2-24 hours. By contrast, even a short-lived isotope like ^{32}P will decrease its emissions by only 5% over 24 hours (Fig. 10.3).

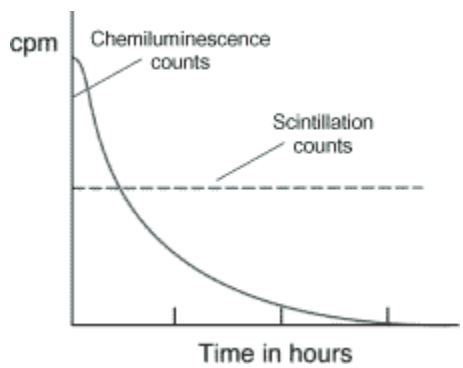


Fig. 10.3. Spurious counts due to chemiluminescence dissipates with time, while the true count stays nearly constant. With the common isotopes used in life science research, the rate of radioactive decay is much slower than the decay of chemiluminescent reactions.

Many scintillation counters use **coincidence counting** to eliminate counts due to chemiluminescence. Because chemiluminescence only generates one photon at a time, only one photomultiplier tube will be activated, hence, this count is rejected. In contrast, the burst of photons from a genuine decay event will activate both photomultiplier tubes simultaneously. Coincidence counting eliminates those emission events which do not appear at both photomultiplier tubes, thus

eliminating chemiluminescence counts. However, coincidence counting will also cause some low energy emission events to be missed.

A further source of false counts is static electricity. The energy from static electric buildup can be released as a burst of light from the cocktail. In dry environments, use of plastic vials and latex gloves cause build-up of high levels of static electricity, sufficient to give at least 10^4 c.p.m. Static is the likely cause if counts from an individual sample vary unpredictably from one measurement to the next. Static can be minimized by wiping the vials with wet paper towel (water dissipates static) or by wiping with an antistatic laundry dryer sheet or fabric softener.

5. **Phospholuminescence**: It results from components of the sample, including the vial itself, absorbing light and re-emitting it. Unlike chemiluminescence, which is a once-only effect, phospholuminescence will occur on each exposure of a sample to light. Samples that are pigmented are most likely to phosphoresce. To prevent this problem, samples should be adapted to darkness prior to counting and the sample holder should be kept closed throughout the counting process.

Despite the five disadvantages described above, scintillation counters are universal in Bioscience labs. This is because the instruments have automated systems for calculating counting efficiency.

Using scintillation counting for dual-labeled samples

A desirable feature of scintillation counting is that the size of electric pulse produced by the conversion of emissions to light energy and then to electrons in the photomultiplier is related directly to the energy of the original radioactive event. Because different β-emitters have different energy spectra, it is possible to quantify two isotopes separately in a single sample, provided their energy is sufficiently different. Examples of pairs of isotopes that have different energy spectra are ^{3}H and ^{14}C, ^{3}H and ^{35}S, ^{3}H and ^{32}P, ^{14}C and ^{32}P, ^{35}S and ^{32}P. The principle of counting dual-labeled samples is illustrated in Fig. 10.4.

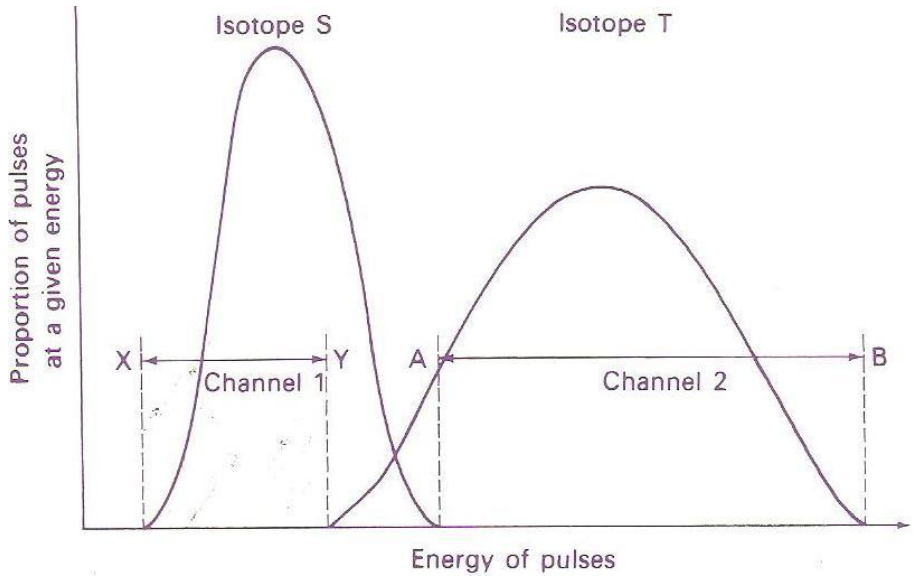


Fig. 10.4. Illustration of the principle of counting dual-labeled samples

In this diagram, the spectra of two isotopes, S and T overlap slightly. By setting up a pulse height analyzer to reject all pulses of energy below X (threshold X) and to reject all pulses of energy above Y (window Y) for isotope S,and also to reject below a threshold of A and accept pulses up to window B for isotope T, it is possible to separate counts from the two isotopes completely. A pulse height analyzer set with a threshold and window for a particular isotope is known as a channel.

Most modern counters operate with a so-called multichannel analyzer. They are based on an analog-to-digital converter; electronic signals from the photomultiplier are converted to digital signals and stored in a computer. Thus the entire energy spectrum is analyzed simultaneously. This greatly facilitates multi-isotope counting and in particular, allows the effect of quenching on dual-labeled counting to be assessed adequately.

Dual-label counting is useful in many aspect of molecular biology, such as nucleic acid hybridization and transcription; in elucidating metabolic pathways such as steroid synthesis, and in drug development.

Determination of counting efficiency

Quenching is a major problem encountered in scintillation counting and this problem makes it necessary to determine the counting efficiency of some samples in an experiment.

Counting efficiency can be determined by the following standardization methods:

1. Internal standardization
2. Channels ratio
3. External standardization

Internal standardization method

An internal standard is a known quantity of a substance that is added to every sample that is analyzed. The substance must behave in a similar way as the analyte and must provide a signal that can be distinguished from that of the analyte. Two approaches are employed in determination of quantitative measures by the internal standardization method: 1) A calibration curve, or 2) standard addition method.

<u>The standard addition method</u>

A sample is counted and gives a reading of, say, ***A*** in c.p.m. It is then removed from the counter, and a small amount of **standard** material of known d.p.m., say, ***B***, is added. The mixture is then recounted to get *C* c.p.m. and the counting efficiency of the sample is calculated.

$$\text{Counting efficiency} = \frac{C - A}{B} \times 100\% \quad .... \qquad (1)$$

Worked example: The internal standard method for calculating counting efficiency

QUESTION

An experimental sample of ^{3}H on a filter paper in scintillation fluid gave a count rate of 1,450 c.p.m. in a liquid scintillation counter. The filter was removed and 5,064 d.p.m. added to it. On recounting, the filter gave a reading of 2,878 c.p.m. What is the d.p.m. of the experimental sample?

SOLUTION

A = 1,450 c.p.m.

B = 5,064 d.p.m

C = 2,878 c.p.m.

First use equation (1) to calculate counting efficiency.

$$\text{Counting efficiency} = \frac{C - A}{B} \times 100\%$$

$$\text{Counting efficiency} = \frac{2,878 - 1,450}{5,064} \times 100\% = 28.2\%$$

Now, use this value to calculate actual radioactivity present in disintegrations per minute.

If 28.2% efficiency gives 1,450 c.p.m.

therefore 100% counting efficiency will give what?

$$\frac{100\%}{28.8\%} \times 1,450 \text{ c.p.m} = 5,142 \text{ d.p.m.}$$

It is necessary to use an internal standard that contains the same isotope as the one being counted, and to ensure that the standard itself does not act as a quenching agent. Suitable ^{14}C-labeled internal standards include [^{14}C]toluene and [^{14}C]hexadecane. Suitable ^{3}H -labeled internal standards are [^{3}H] benzoic acid and $^{3}H_2O$. Note that, benzoic acid and water are themselves quenching agents and must be used in only very small amounts.

Perkin Elmer C-14 Internal Standard

Internal liquid scintillation counting (LSC) standard. ^{14}C Toluene, ~5 x 10^5 DPM/g, 10 mL, List price USD108.00.

Advantages of internal standardization

1. Simple and reliable and corrects adequately for all types of quenching.
2. When carefully carried out it is the most accurate way of correcting for quenching.

Disadvantages of internal standardization

1. It requires very accurate measurement of volumes of the internal standard
2. The method is time-consuming because each sample must be counted twice
3. Sample cannot be recounted in the event of error because it will be contaminated with the standard.
4. Changes in quenching characteristics may occur in sample during the interval between the first and second counts which can lead to considerable inaccuracies, nevertheless, internal standardization is the method by which a scintillation counter is calibrated.

Demerits of scintillation counting

1. No radiation detection system is perfect because of background count.
2. No instrument is capable of recording all of the atomic disintegrations within a scintillation vial because of the counting systems available are not 100 % efficient.

Spurious counts

Actual radioactive events are usually accompanied by spontaneous flashes of light that are also recorded as counts because they cannot be differentiated from genuine decay process. Such false counts in equipment arise from the following:

1. Cosmic rays
2. Beta particles arising from decaying potassium (Half life: 1.251×10^{9} years) in the glass vial
3. Spontaneous discharges from the sensitive photodetectors
4. Chemicals dissolved in the scintillation fluid: chemiluminescence and phospholuminescence

The c.p.m. attributable to such sources are called **background count**. Background counts are often so low relative to the actual decay events being measured and so they are ignored. However, if the number of "real" counts is low, background counts can contribute to experimental error. Therefore, a **control** vial must be prepared which contains everything except added radioactivity in order to determine the background level. Background c.p.m. is then subtracted directly from the c.p.m. from the experimental samples

Efficiency of counting: Owing to the geometry of the vial and photoelectric detectors some events go undetected. The maximum efficiency with which a low energy emitter such as ^{3}H can be detected is

about 70 %. Worse yet, the energy of some photons is absorbed by chemicals in the solvent before the photon can reach the detector. The latter phenomenon is an example of quenching. With the chemical quenching that is typical of most experiments, the usual counting efficiency for ^{3}H is 30 to 40%, and sometimes much less. The amount of quenching can vary from sample to sample therefore it is often necessary to estimate the efficiency of counting for each individual sample.

Remember that the amount of light detected is proportional to the energy of the beta particle that was released by the disintegrating nucleus. When you prepare to count samples, you select appropriate "**windows**", that is, ranges of light intensity that the instrument will record as counts, rejecting all others. The instrument records the amount of light detected following an event, and if that amount is within the energy range for a particular window, the event is recorded as one count. It is ignored if it falls outside the selected range. Each window is given a **channel number**, and the count for each window is given as c.p.m. for the corresponding channel.

Quenching causes scintillation process to be less efficient, that is, less light is produced for a given quantum energy of radiation. Thus, the energy spectrum for a sample appears to be lower than for an unquenched sample (Fig. 10.5)

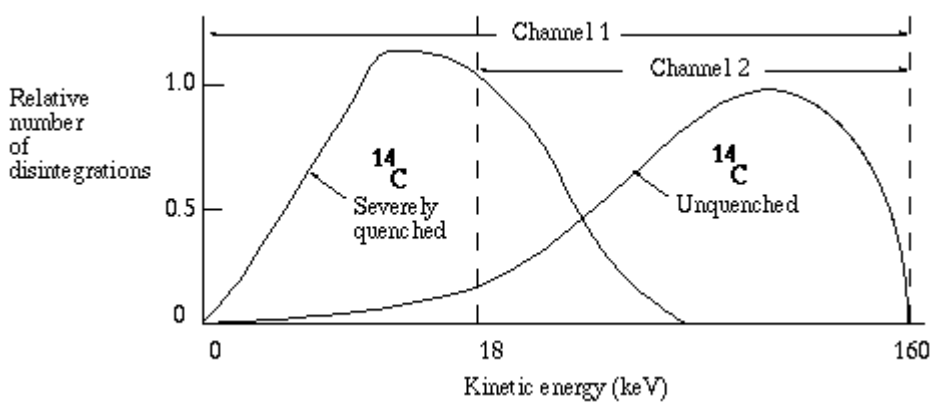


Fig. 10.5. Effect of quenching on a ^{14}C energy spectrum

The higher the degree of quenching, the more pronounced is the resulting decrease in the energy spectrum. As quenching takes place, the energy recorded for each event is less than it would be for an unquenched sample, since for each event the energy of some photons are absorbed before detection is possible. Counts in channel 2 therefore decreases with increasing concentration of

quenching material leading to the ratio of counts in channel 2 to counts in channel 1 becoming smaller after every addition. Similarly, counts in channel 1 decreases with increase in quenching material. The ratio of counts in the channels is the sample channels ratio, or SCR. This forms the basis of the **channels ratio** method for determining counting efficiency.

The channels ratio method

This involves preparation of a calibration curve based on counting in two channels that cover different but overlapping parts of the spectrum. As a sample is quenched, and the spectrum shifts gradually to lower apparent energies, the ratio of counts in each channel will vary.

To prepare the calibration curve

1. A standard (i.e., having known d.p.m.) is counted = unquenched standard. Because radioactivity in the standard is known, the efficiency of counting in each channel can be determined
2. Add **increasing concentrations** of a **quenching substance** to the standard and count after every addition. Calculate efficiency of counting.
3. As the energy spectrum of the quenched standard gradually moves to the left with increasing concentration of quenching substance, the appropriate windows are selected for channel 1 and channel 2 to pick energy ranges that correspond to the radiation being emitted.
4. Calculate standard sample channels ratio (SCR) for each concentration of quenched standard added and plot efficiency (*y*-axis) against SCR (*x*-axis) to produce the standard curve (Fig. 10.6).

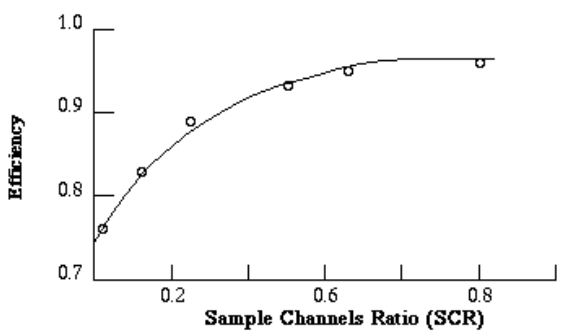


Fig. 10.6. Channels ratio quench correction curve for ^{14}C

Typical data for a set of ^{14}C quenched standards are shown in Table 10.1. It is important to note that one standard curve applies to only one circumstance, i.e., one radioisotope, counter and scintillation cocktail.

Table 10.1. Radioactivity recorded with gradually increasing concentration of quench substance (say, [^{3}H] benzoic acid) in two channels of a scintillation counter.

	c.p.m.		**Ratio**	**Counting efficiency**
Sample	Channel 1	Channel 2	Ch1 : Ch2	Counting efficiency in Channel 2 (%)
^{14}C standard (203,600 d.p.m.) unquenched	171,930	184,250	0.93	90.5= (184250/203600)
^{14}C standard (203,600 d.p.m.) With increasing quench	146,610	168,840	0.87	82.9 = (168,840/203,600)
	94,240	135,090	0.70	66.3 = (135,090/203,600)
	52,260	102,030	0.51	50.1 = (102,030/203,600)
	16,030	58,320	0.27	28.6 = (58,320/203,600)
	5,920	34,740	0.17	17.1 = (34,740/203,600)
	2,060	20,270	0.10	9.9 = (20,270/203,600)
	1,130	13,260	0.08	6.5 = (13,260/203,600)

Once the standard curve is prepared, the efficiency of counting experimental samples can be determined. Samples are counted in the same two channels, the ratio is calculated and efficiency is read from the graph. In practice, all the data can be stored in the counter's computer and corrected values printed automatically.

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Lecture 11
November 27, 2018

The channels ratio method cont'd

Multichannel scintillation counters operate on the same principle as the two-channel approach, however, instead of manual calculation of SCR, a graph is plotted (relative number of disintegrations against energy of the emission) from the electronic signals within the equipment and the **whole shape and position of the spectrum** is analyzed. Parameters obtained from the graph may be:

1. Area under the curves for unquenched and quenched sample
2. Height of the curves

The graph parameter is given a **digital value** that relates to counting efficiency. Manufacturers have developed their own titles for such parameters. For example, LKB Instruments use Automatic Quench Compensation and Hewlett Packard uses Packard's Automatic Efficiency Control as the digital value terminology. These systems have greater precision than the two-channel approach, as the whole spectrum of the graph is used for analysis.

Advantages of channels ratio method over internal and external standardization methods are:

1. The channels ratio method is suitable for all types of quenching.
2. Counting in more than one channel is simultaneous, less time consuming than either internal or external standardization.
3. Accurate method for determining counting efficiency, provided care is taken in preparation of the calibration curve.

Demerits of the channels ratio method

1. It is inaccurate at low count rates, because error on the counts per minute is high, (refer to statistic of counting decay events)
2. Method is inaccurate for very highly quenched samples

Channels ratio efficiency calculation

Counting efficiency is positively related to the sample channel ratio (SCR). To get a conversion factor between SCR and efficiency, equal amounts of the isotope (sample) are placed in a series of vials. Increasing amounts of a quenching agent, such as carbon tetrachloride, are added to each vial. The vials are counted and the c.p.m. of each is divided by known d.p.m. to get the counting efficiency. For example, if a vial with 20,000 d.p.m. of ^{3}H yields 5,000 c.p.m., the

counting efficiency would be 5,000/20,000 = 0.25, that is, only 25% of the atomic disintegrations were detected. The counting efficiency is plotted versus channels ratio to yield a quench curve.

The instrument prints out c.p.m. and sample channels ratio (SCR) for each sample, therefore to get actual d.p.m. in a sample the investigator must (1) subtract background or blank (i.e., without sample) c.p.m. from the c.p.m. for the sample, (2) determine counting efficiency from the SCR for the sample, and (3) divide net c.p.m. by the counting efficiency.

Remember, $\text{counting efficiency} = \frac{\text{c.p.m}}{\text{d.p.m}}$

Worked example

The efficiency of counting 100,000 d.p.m. of a [^{35}S]methionine was estimated in a scintillation counter using two channels, A and B, in a scintillation fluid containing increasing amounts of chloroform as the quencher. The following data were obtained:

Chloroform (ml)	c.p.m. A	c.p.m. B
0	48,100	54,050
1	31,612	42,150
2	17,608	28,400
3	7,400	15,000

An unknown sample of [^{35}S]methionine gave the following data:
Channel A 1,890 c.p.m.
Channel B 2,700 c.p.m.
How much radioactivity is present in the unknown sample?

Solution

Calculate efficiency for channels A and B. Plot counting efficiency in channel A or in channel B against channels ratio. Calculate the sample channel ratio $\frac{\text{c.p.m. A}}{\text{c.p.m.B}}$

<u>Steps</u>

Calculate counting efficiency for channel A
Calculate counting efficiency for channel B
Compute the channels ratio for the standard at each level of quenching
Plot a graph of efficiency of counting on the Y axis against channels ratio of standard on X axis
Compute the channels ratio for the sample
Read off efficiency of counting sample from the graph

Calculation of efficiency for channel A

100,000 d.p.m. = 100%

So 48,100 c.p.m. in channel A = $\frac{48{,}100 \text{ c.p.m.}}{100{,}000 \text{ d.p.m}} x\ 100 = 48.1\%$

Now calculate efficiency for the rest of the data. This is shown in Table 11.1.

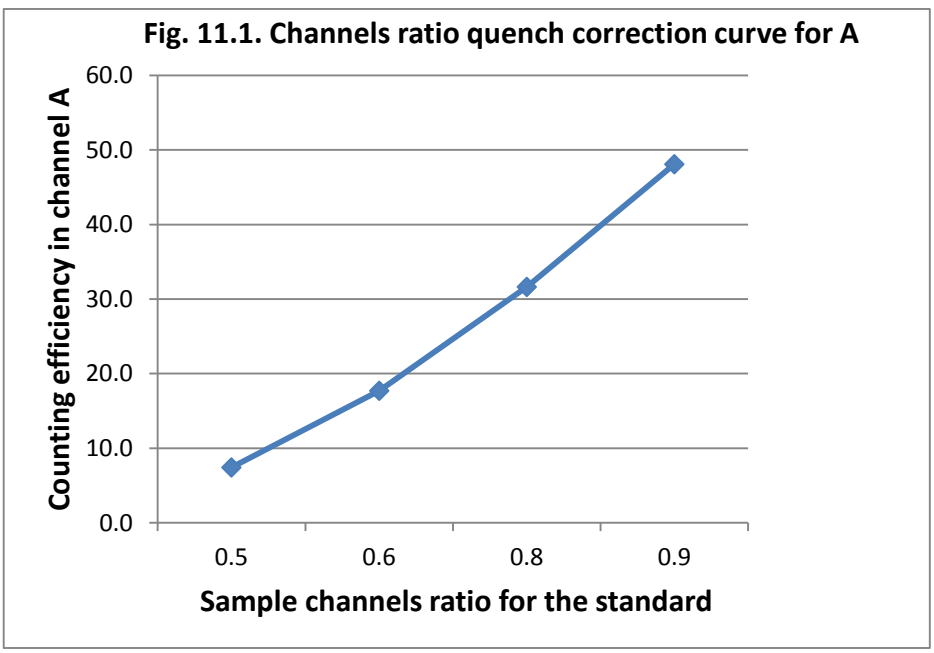
Table 11.1. Calculation of counting efficiency

Channel A	Counting eff. in A	Channel B	Counting eff. in B	Sample channel ratio for the standard $\text{c.p.m. A}/\text{c.p.m. B}$
$\frac{48{,}100 \text{ c.p.m.}}{100{,}000 \text{ d.p.m,}} x\ 100$	48.1	$\frac{54{,}050 \text{ c.p.m.}}{100{,}000 \text{ d.p.m,}} x\ 100$	54.05	$48{,}100/54{,}050 = 0.89$
$\frac{31{,}612 \text{ c.p.m.}}{100{,}000 \text{ d.p.m,}} x\ 100$	31.612	$\frac{42{,}150 \text{ c.p.m.}}{100{,}000 \text{ d.p.m,}} x\ 100$	42.15	$31{,}612/42{,}150 = 0.75$
$\frac{17{,}608 \text{ c.p.m.}}{100{,}000 \text{ d.p.m,}} x\ 100$	17.68	$\frac{28{,}400 \text{ c.p.m.}}{100{,}000 \text{ d.p.m,}} x\ 100$	28.4	$17{,}608/28{,}400 = 0.62$
$\frac{7{,}400 \text{ c.p.m.}}{100{,}000 \text{ d.p.m,}} x\ 100$	7.4	$\frac{15{,}000 \text{ c.p.m.}}{100{,}000 \text{ d.p.m,}} x\ 100$	15.0	$7{,}400/15{,}000 = 0.49$

Calculate c.p.m. A/c.p.m. B for the experimental sample.

$$\text{S.C.R}_{\text{experimental}} = 1890/2700 = 0.7$$

Read off the efficiency of counting the unknown sample from the graph.



Now, convert the c.p.m. for the experimental sample in channel A or B (depending on which one you choose to calculate the efficiency). If you use channel A, the corresponding efficiency from the graph is 26.5 %. So the corrected c.p.m. is

26.5% gives 1,890 c.p.m in channel A
Therefore 100% = ?

1890 × 100/26.5 = 7,130 d.p.m.

Note: try plotting the two graphs (efficiency in A or B versus c.p.m A/c.p.m B) and work out the answer using both in turn; you should get the same answer of 7,132 d. p.m. if you use channel B.

External standardization

This method is based on the principle that, the spectrum for an external standard varies according to the degree of quench in the sample vial and therefore, the efficiency of counting of the internal experimental sample also varies.

It is used to estimate counting efficiency. In this method, a **γ-emitting** external standard is built into the scintillation counter. Under the control of the counter, each radioactive sample to be counted is exposed to this external source. In addition, a quenching substance is added at increasing concentrations as explained before. The γ-radiation penetrates the sample vial and excites the scintillation fluid, and causes the ejection of an electron, called Compton electron) from the solvent molecules. This electron behaves like a beta particle beta particles, and will induce the same scintillation effects. The resulting spectrum is unique to the sample and is significantly different from that produced by the sample's radioactivity alone in the vial. the difference in spectrum is quantified and is assigned a value, such as transformed spectral index of External Standard, tSIE, based on the pulse height, or area under the curve. The γ-source commonly used are ^{137}Cs, ^{133}Ba, or ^{226}Ra and these may be varied with the make of the equipment. The spectrum obtained by ^{226}Ra is shown in Fig. 11.2.

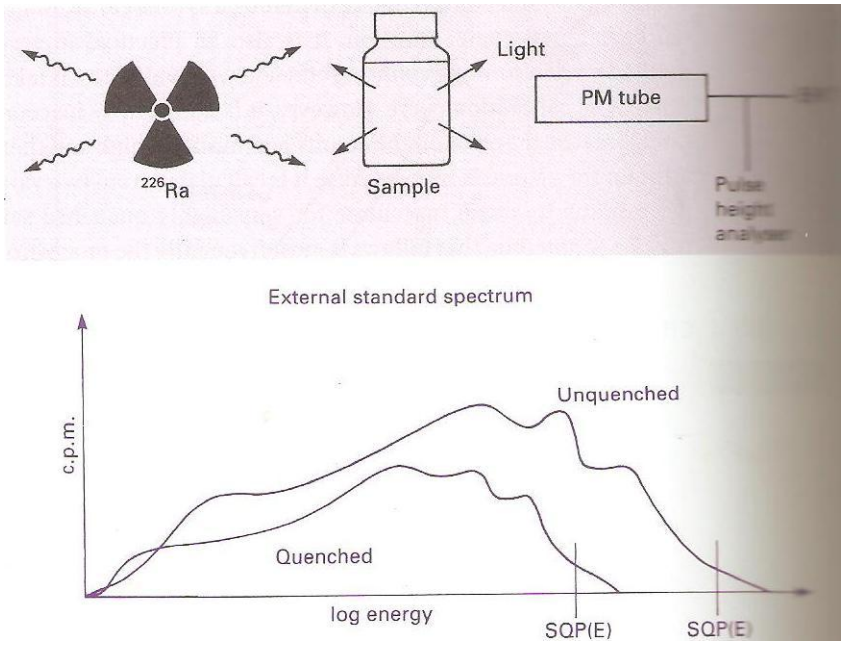


Fig. 11.2. The external standard for estimating counting efficiency. The external source of γ-emitter, ^{226}Ra, irradiates the sample. The counter analyzes the sample spectrum, which shifts to lower energies if the sample is quenched. The SQP(E), i.e., standard quench parameter (external), expressed without units, is derived from the log energy axis and relates to the extent of quench. The greater the quench in the sample, the lower the counting efficiency (Table 11.2)

Quenching agents present in the scintillation fluid will significantly affect the spectrum obtained. The instrument analyses this quenched spectrum and assigns a **quench parameter** to it. A standard quench parameter used by Perkin Elmer is the **transformed Spectral Index of the Sample (tSIS)**. Quench parameter is a numerical value obtained by **quantifying the change in spectral shape** caused by quenching and can be estimated by **calculating the mean pulse height**. The quench parameter then **correlates** changes in counting efficiency with changes in the shape (i.e., pulse height) of the sample spectrum; LKB Instruments refer to a standard quench parameter for the external source as (SQP(E)). Other manufacturers use slightly different approaches but the principle is the same, i.e.,

"the spectrum for an external standard varies by the degree of quench in the sample vial and therefore, the efficiency of counting of the internal experimental sample also varies."

Similar to the channels ratio method, a standard curve is required i.e., a range of quenched standards is counted and the external standard spectrum analyzed in each case. As an example, the resulting data (Table 11.2) are used to prepare a standard curve that is saved in the counter's computer. Unknown samples are then counted in the same way, the efficiency read from the standard curve and the sample counts corrected from c.p.m. to d.p.m.

Table 11.2. Recorded radioactivity from a ^{14}C standard sample with increasing quench detected by an external standard

Sample	c.p.m.	External quench parameter	Counting efficiency (%)
^{14}C standard (203,600 d.p.m.) unquenched	194,930	810	95.7
^{14}C standard (203,600 d.p.m.) with increasing quench	146,141	422	93.5
	181,171	207	89.0
	167,731	126	82.4
	145,879	76	71.6
	126,913	55	62.3
	108,641	42	53.3
	96,103	37	47.2

The external standard approach is now a routine in most labs. The main advantage it has over the channels ratio method is that it is **suited to samples with low count rates.**

Disadvantage: it requires a standard curve for each set of circumstances, as is seen with the channels ratio.

In all cases where an automated procedure for calculating counting efficiencies is used, it is important to count a few prepared samples in which the true amount of radioactivity is known.

Worked example: External standard efficiency calculation

Question

The efficiency of detecting ^{14}C in a scintillation counter was determined by counting a standard sample containing 105,071 d.p.m. at different degrees of quench analyzed by the external standard approach.

c.p.m	SQP
87,451	0.90
62,361	0.64
45,220	0.46
21,014	0.21

SQP = standard quench parameter

An experimental sample gave 2,026 c.p.m. at an SQP of 0.52. What is the true count rate?

Solution

Calculate efficiency for the counts at the various quench parameters. Note, standard sample contains 105,071 d.p.m

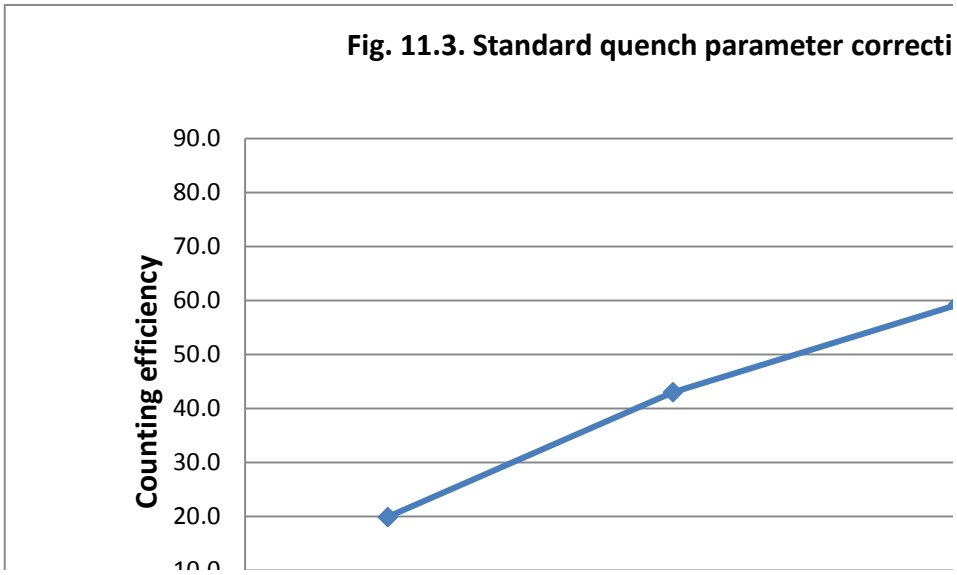
c.p.m	Counting efficiency calculation	Counting eff. (%)
87,451	$\frac{87{,}451 \text{ c.p.m.}}{105{,}071 \text{ d.p.m}} x\ 100$	83.2
62,361	$\frac{62{,}361 \text{ c.p.m.}}{105{,}071 \text{ d.p.m}} x\ 100$	59.3
45,220	$\frac{45{,}220 \text{ c.p.m.}}{105{,}071 \text{ d.p.m}} x\ 100$	43.0
21,014	$\frac{21{,}014 \text{ c.p.m.}}{105{,}071 \text{ d.p.m}} x\ 100$	19.9

Plot the SQP values against counting efficiency. Read off the graph the corresponding counting efficiency for the experimental sample at SQP of 0.52. This gives ~48% efficiency. Now, correct the 2,026 c.p.m to d.p.m. by:

48% = 2,026 c.p.m

Therefore 100% = ?

2,026 × 100/48 = 4,221 d.p.m.



Sample preparation for scintillation counting

Major considerations should be taken when carrying out scintillation counting. These are:

1. Sample vials
2. The scintillation cocktail

3. Volume of cocktail
4. Method of overcoming quenching
5. Tissue solubilizers used
6. Combustion methods.

SAMPLE VIALS

In solid scintillation counting, sample preparation is easy and only involves transfer of sample to a glass or plastic vial compatible with the counter. In liquid scintillation counting, sample preparation is more complex and starts with a decision on the type of sample vial to use. Sample vials may be glass, specifically low potassium glass, with low levels of ^{40}K that reduce background count. Vials may also be polyethylene.

Polyethylene is cheap, but not suitable for cleaning and reuse. Glass vials can be reused many times provided they can be thoroughly cleaned. Polyethylene vials give better light transfer and result in slightly higher counting efficiencies but are inclined to exhibit more phosphorescence than do glass vials. Presently, mini-vials, which use far smaller volumes of expensive scintillation cocktails, are in frequent use. Modern counters are able to accept many kinds of vials. Smallest sizes are chosen to save cost and give consideration to environmental issues, as scintillation fluids are toxic. Some counters are designed to accept very small samples in special polyethylene bags split into an array of many compartments; these are particularly useful to pharmaceutical industries which work with large numbers of receptor binding assays.

Scintillation cocktails

Toluene-based cocktails are the most efficient, but will not accept aqueous samples, because toluene and water are immiscible and massive quenching results. Cocktails based on 1,4-dioxane and naphthalene that can accommodate up to 20 % (v/v) water can be used, but because of toxicity, they are not in use.

Emulsifier-based cocktails are the most frequently used for counting aqueous samples. They contain the emulsifier, Triton X-100 and can accept up to 50% water (v/v), however, phase transitions occur from single phase to two-phase or gel formation as the water content increases. Accurate counting cannot be done if samples are in the two-phase state. Many ready-made cocktails are sold with precise instructions regarding sample preparation.

Volume of cocktail

The efficiency of scintillation counting varies with sample volume. Therefore care should be taken that sample vials in a given series of counts contain the same volume of sample and that all instrument calibration is done using the same volume as for experimental sample.

Overcoming major color quenching

Color quenching can be removed by bleaching samples before counting. Bleaching agents such as H_2O_2 can give rise to chemiluminescence in some scintillation cocktails. Other color removal agents are activated charcoal and Fuller's earth.

Tissue solubilizers

Solid samples such as plant and animal tissue may be best counted after solubilization by quaternary amines such as NCS solubilizer or Soluene (a mixture of quaternary ammonium bases in toluene). These solutions are highly toxic and great care is required. The sample is added to the counting vial containing a small amount of solubilizer and allowed to digest. When digestion is complete, scintillation cocktail is added and the sample counted. Chemiluminescence is a problem with tissue solubilizers.

Combustion methods

An alternative to bleaching of colored samples or digestion of tissues is the use of combustion techniques. Samples are combusted in oxygen contained in a combustion apparatus. Samples containing ^{14}C would be combusted to $^{14}CO_2$ which is collected in a trapping agent such as NaOH and then counted; ^{3}H-containing samples are converted to $3H_2O$ for counting.

Methods based on exposure of photographic emulsions- Autoradiography

Autoradiography is a means of detecting location of radioactive compounds with a photographic emulsion. Ionizing radiation acts on a photographic emulsion to produce a latent image just as photography with visible light. To produce a photograph, one needs a radiation source, an object to be imaged, and a photographic emulsion. the sample containing the radioactive material is placed in close contact with the photographic emulsion.

The emulsion may consist of a large number of silver halide crystals embedded in a solid phase, such as gelatin. As energy from the radioactive material is dissipated in the emulsion, electrons are produced and the silver halide is reduced to metallic silver (Ag^0) which appears as black deposits of silver grains. The metallic silver acts as a catalyst for further reduction of Ag halide in its vicinity. Upon development to remove any remaining silver halide, a permanent image of the location of the original radioactive event is obtained.

Autoradiography is very sensitive and has been used in a wide variety of biological experiments, such as to locate the distribution of radioactivity in biological specimens of different types. The site of localization of a radiolabeled drug throughout the body of an experimental animal can be determined by placing whole body sections of the animal in close contact with a sensitive emulsion such as X-ray plate. After a period of exposure, the plate upon development will show an image of the sections in tissues and organs in which radioactivity was present.

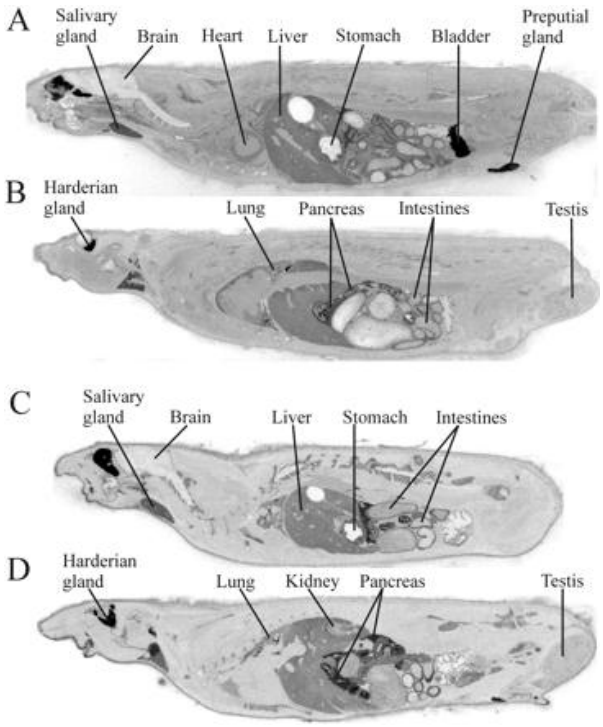
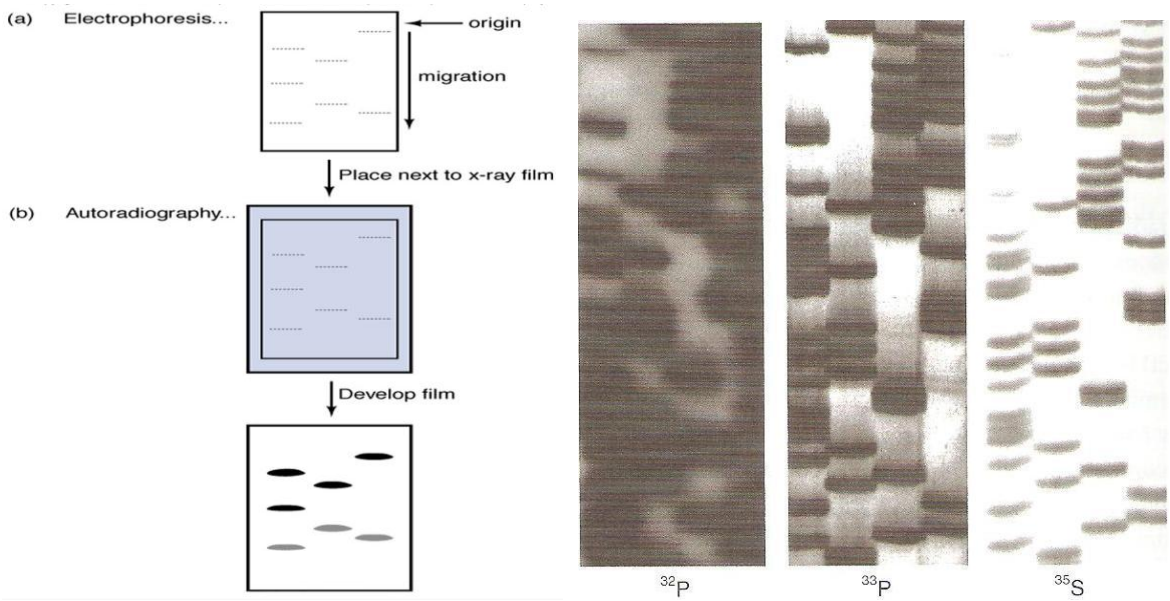


Fig. 11.4. Whole body autoradiography in a rat sacrificed at 15 min (A and B) and 60 min (C and D) after I.V. administration of [^{14}C] Ethanol. Dark spots indicate sites of radioactivity

Radioactive metabolites isolated and separated by chromatography or electrophoresis can be located on the chromatograph or the electrophoregram and the radioactive spots can subsequently be recovered for counting and identification.

Molecular biologists use X-ray film for autoradiography. In the Fig. 11.5, the researcher electrophoresed some radioactive DNA fragments on a gel and then placed the gel in contact with X-ray film in the dark for few hours or even days. The radioactive emissions from the bands of DNA reduced the film to produce dark bands. In effect, the DNA bands took a picture of themselves which is why the technique is called **autoradiography**.



(A) (B)

Fig. 11.5. (A) Steps in autoradiography. (B) Three autoradiographs showing the use of different isotopes in DNA sequencing. The isotope with the highest energy (^{32}P) leads to the poorest resolution because the radiation spreads out further, making DNA bands appear thicker. The lowest energy radiation (^{35}S) gives the best resolution (Source: Cunningham et al. 2002)

Important aspects of autoradiography

In general, weak β-emitters (^{3}H, ^{14}C and ^{35}S) are most suitable for autoradiography, particularly for cell and tissue localization experiments. This is because, as a result of the low energy of the negatrons, the ionizing track of the isotope will be short and a discrete image will result. This is particularly important when radioactivity associated with subcellular organelles is being located. For this reason, ^{3}H is the best radioisotope since its energy will all be quickly dissipated within the emulsion.

Electron microscopy can then be used to locate the image in the developed film. For location within whole organisms or tissues, either ^{14}C or ^{3}H is suitable; more energetic isotopes, such as ^{32}P are less suitable because their higher energy negatrons produce much longer track lengths and result in less discrete images that are not sufficiently discriminatory for microscopic location.

Conversely, for location of DNA bands in an electrophoretic gel, ^{32}P is useful. In this case, low energy ^{3}H negatrons would largely dissipate their energy within the gel before they reach the film, thereby reducing sensitivity to a low level. However, the more energetic ^{32}P will

leave the gel and produce a strong image on the film. If very thin gels are prepared, then ^{35}S or ^{14}C can be detected with high resolution, for example in DNA sequencing gels where ^{35}S is used as the label as an aid to rapid DNA sequencing.

Choice of emulsion and film

A variety of emulsions is available with different packaging densities of silver halide crystals. Care must be taken to choose an emulsion suitable for the purpose of the experiment, since the sensitivity of the emulsion will affect the resolution obtained. Manufacturer's literature must be consulted if one is in doubt.

X-ray film is generally suitable for macroscopic samples such as whole-body sections of small mammals, chromatographs or electrophoregraphs. When light or electron microscopic detection of the location of the image in the emulsion is required, (cellular and subcelllar localization of radioactivity), very sensitive films are necessary, as a very close apposition of sample and film is needed. In this case, a **stripping film technique** can be used in which the film is supplied already attached to a support. It is stripped from this and applied directly to the sample. Alternatively, liquid emulsions are prepared by melting strips of emulsion at $60^{o}C$. Then, either the emulsion is poured onto the sample or the sample, attached to a support, is dipped into the emulsion. The emulsion is then allowed to set before drying it. This method is often referred to as **dipping-film** method and is preferred when very thin films are required.

Background

Accidental exposure to light, chemicals in the sample, natural background radioactivity (particularly ^{40}K in glass) and even pressure applied during handling and storage of films will cause a background fog i.e., latent image on the developed film. This can be problematic, especially in high resolution work such as in microscopy, and care must be taken at all times to minimize this effect. Background fog will always increase during exposure time, which should therefore always be kept to a minimum. A trial and error is often involved in arriving at the most suitable time of exposure.

Direct autoradiography

In direct autoradiography, the X-ray film or emulsion is placed as close as possible to the sample and exposed at any convenient temperature. Quantitative images are produced until saturation is reached. The approach provides high resolution but limited sensitivity; isotopes of energy equal to, or higher than ^{14}C (E_{max} =0.156 MeV) are required.

Fluorography

Many of the currently popular methods in molecular biology involve separation of macromolecules or fractions of molecules by gel electrophoresis. The separated

macromolecules or fractions form bands in the electrophoregraph that must be located. This is often achieved by radiolabeling the macromolecules with ^{3}H or ^{14}C and subjecting the gel to autoradiography. Because these are weak β-emitters, much of their energy is lost in the gel and long exposure times are necessary even when very high specific activity sources are used. However, if a fluor (e.g., PPO or sodium silicate) is infiltrated into the gel, and the gel dried and then placed in contact with a preflashed film, sensitivity can be increased by several orders of magnitude. This is because the negatrons emitted from the isotope will cause the flour to become excited and emit light, which will react with the film. In this case, the use of both ionizing radiation and excitation effects of radioactivity in fluorography become relevant.

Intensifying screens

When ^{32}P-labeled or γ-isotope-labeled samples such as ^{32}P-DNA or ^{125}I-labeled protein fractions in gels are to be located, the opposite problem to that presented by low-energy isotopes prevails. These much more penetrating particles and rays cause little reaction with the film as they penetrate right through it, producing a poor image. The image can be greatly improved by placing, on the other side of the film, a **thick intensifying screen** consisting of a solid phosphor. Negatrons penetrating the film cause the phosphor to fluoresce and emit light which superimposes its image on the film. There is therefore an increase in sensitivity but a parallel reduction in resolution due to the spread of light emanating from the screen.

Preflashing

The response of photographic emulsion to radiation is not linear and usually involves a slow initial lag phase followed by a linear phase. Sensitivity of films may be increased by preflashing. This involves a millisecond light flash prior to bringing the sample into juxtaposition with the film and is often used where high sensitivity is required or if results are to be quantified.

Quantification

Typically, autoradiography is usually used to locate rather than to quantify radioactivity. However, it is possible to obtain quantitative data directly from autoradiographs by using a densitometer, which records the intensity of the image. This in turn is related to the amount of radioactivity in the original sample. There are many varieties of densitometers available and the choice depends on the objective of the experiment. Quantification is not reliable at low or high levels of exposure because of the lag phase or saturation, respectively. However, preflashing combined with fluorography or intensifying screens obviated the problem for small amounts of radioactivity from previous experience. In this case, all photons contribute equally to the image of the pre-exposed film.

Practical aspects of counting radioactivity

A) Counter characteristics

BACKGROUND COUNT

Radiation counters of all types register a count event every few seconds, even in the absence of radioactive material in the equipment. Where do these come from? Radioactivity is all around us – air, ground, and ocean. The reading is not constant but keeps going up and down because decay process is random. Source of background radiation in counters

- Cosmic rays
- Radon decay products
- Nearby X-ray generators
- Circuit noises

Cosmic rays are fast-moving particles from space that enter the earth's atmosphere, along with their decay products. Since the atmosphere absorbs some of these particles, the rate of detection of cosmic rays increases with increasing altitude. If you were to take a Geiger counter on a jet flight, you would observe a marked increase in count rate while at high altitude.

Another radiation source comes not from above us, but from below. The earth's crust contains radioactive elements, such as uranium-238 (^{238}U). ^{238}U has a long half-life, but its decay products do not. One of these products is radon gas (^{222}Rn). As a result of the long uranium half-life, there is a nearly steady production of radon, which itself decays with a short half-life of 3.8 days. Since radon is a gas, it diffuses out of the soil into the air, and can collect in low-level enclosed areas, such as basements. Radon decays to a series of products including polonium, lead, and bismuth, which precipitate out of air onto dust particles since they are solids unlike gaseous radon.

Background radiation may be considerably reduced by lead shielding but its value must always be recorded and accounted for in all experiments. Some commercial instruments have automatic background subtraction facility.

DEAD TIME

At very high count rates in G-M counting, counts are lost due to dead time of the G-M tube. Correction tables are available to correct for lost counts. Dead time is not a problem in scintillation counting.

Geometry

When samples with end-window ionization counter, such as G-M tube, are compared, it is important to standardize the position of the sample in relation to the tube, otherwise, the fraction of the emitted radiation entering the tube may vary and hence the observed count will also vary.

B) Sample and isotope characteristics

SELF ABSORPTION

Self-absorption is primarily a problem with low energy β-emitters, where radiation is absorbed by the sample itself. It is often encountered with particulate samples, or samples stuck to a membrane filter. It can be reduced by digesting or solubilizing the particulate samples prior to counting. Self absorption is a major problem with G-M counting and significantly reduces sensitivity and reliability.

Statistics of Counting

Emission of radioactivity is a random process. This can be demonstrated readily by making repeated measurements of the activity of a long-lived isotope, each, for an identical period of time. The resulting individual counts will not be the same but will vary over a range of values, with clustering near the center of the range. If a sufficiently large number of such measurements are made and the data are plotted, a normal distribution curve will be obtained.

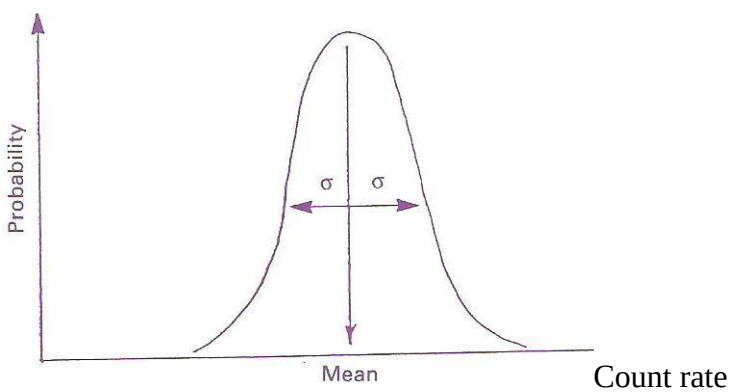


Fig. 11.6. The distribution of count rates around a mean, showing standard deviation, σ.

Because of this, a large number of counts, that is, counting more than a few times, are needed to establish a mean. The mean is then compared to the expectation, that is, the true count. However, the accuracy of this mean will depend on the spread or standard deviation (σ) for that data. The Empirical Rule states that, for a normal distribution such as that shown in Fig.

11.6, 68.2% of values obtained would lie ±1σ from the mean $\bar{x}$; and 95.5% of the data would lie ± 2σ from the mean, $\bar{x}$; and 99.5% of the data would lie ± 3σ from the mean $\bar{x}$, in other words, nearly all of the data will cover 3σ from the mean.

To compare if samples contain different amounts of radioactivity, we need to consider the counting statistics based on Poisson's distribution which describes the probability of a given number of events occurring in a fixed interval of time or space if these events occur with a known constant rate and they are independent, i.e., the occurrence of one event does not depend on the occurrence of another event,

From Poisson's distribution, the standard deviation of decay events is given by:

$$\sigma = \sqrt{\text{total counts taken}} \quad \ldots (1) \quad \text{or} \quad \sigma = \sqrt{\text{count rate} \times \text{time}} \quad \ldots. (2)$$

Therefore, to quote a figure with 95.5% certainty, that is, 2σ, we state a standard deviation of

$$\text{total counts} \pm 2\sqrt{\text{total counts}} \quad \ldots(3)$$

For example, if 1,600 counts are recorded, this can be expressed as 1,600 ± 80. There is therefore a 95.5 % chance that the true figure lies between 1,520 and 1,680 c.p.m.

When data are expressed as d.p.m. or c.p.m., again using 95.5% certainty, then

$$\text{error on count rate} = 2\sqrt{\text{count rate} \times \text{time}} \quad ..(4)$$

Using the same example, if the 1,600 counts were obtained in 1 min:

$$\text{error on count rate} = 2\sqrt{1{,}600 \times 1} = 80$$

and therefore, 1,600 c.p.m. ± 80 c.p.m.

If the 1,600 c.p.m. were obtained in 10 mins:

$$\text{error on count rate} = 2\sqrt{1{,}600 \times 10}$$

and therefore 1,600 ± 252 c.p.m.

Using the same example, if the 160 counts were obtained in 1 min

$$\text{error on count rate} = 2\sqrt{160 \times 1}$$

and therefore 160 c.p.m. ± 25 c.p.m.

Let's work these other simple examples also for a series of 1 min counts at a confidence level of 68.2%:

Counts = 100, $\sigma = \sqrt{100}$ = 10 therefore ± 10% ($^{10}/_{100}$) error at 68.2% certainty

Counts = 1,000, $\sigma = \sqrt{1000}$ = 31 therefore ± 3% ($^{31}/_{1000}$) error at 68.2% certainty

Counts = 10,000 $\sigma = \sqrt{10000}$ = 100 therefore ± 1% ($^{100}/_{10,000}$) error at 68.2% certainty

In summary, the counts per minute data become more accurate (i.e., error reduces) for higher counts and less accurate for longer counting times. It is common practice to count to 10,000 counts or to count for shorter times, whichever is quicker.

Worked example: Accuracy of counting

Question: A sample recording 564 c.p.m. was counted over 10 mins. What is the accuracy of the measurement for 95.5% confidence?

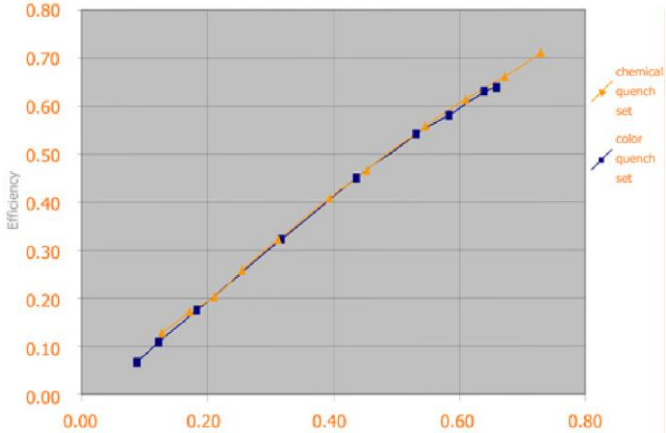
Solution

We apply equation 4: $\text{error on count rate} = 2\sqrt{\text{count rate} \times \text{time}}$

$$\text{error on count rate} = 2\sqrt{564 \times 10} = 150$$

So, the range in which the counts should fall within 95.5% confidence is 564 ±150 c.p.m.

Automatic Liquid Scintillation Beta Counter



SQP