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Cell Biology part

Cell biology is a branch of biology, which is mainly involved with the study of the cell, its types, structure, functions and its interaction with other cells. Cell biology is also called cytology.

Discovery of the cell

There are several discoveries that have changed the course of science and the world. Louis Pasteur discovered how heat can be used to kill pathogens in food, Nikola Tesla discovered alternating currents, and so on.

Similarly, the discovery of cells was a major breakthrough in the advancement of life science. Let us have a detailed overview of the cell discovery, who discovered cells and how were the cells discovered.

Who Discovered Cells?

Cells are the basic structural and functional unit of life. The term “cells” was first coined in 1665 by a British scientist Robert Hooke. He was the first person to study living things under a microscope and examined a thin slice of cork under a microscope and observed honeycomb-like structures. Robert Hooke called these structures as cells.

Soon after this Antonie Van Leuwenhoek made further discoveries by inventing his own microscope lenses that were more powerful than the microscopes of his time. He was the first person to observe human cells and bacteria under his microscope.

With the advancements in microscopes, more discoveries were made about cells. However, with the help of a light microscope, it became difficult to visualise the minute structures inside the cells. As a result, a more powerful microscope, known as the electron microscope was invented that made it easier to observe objects smaller than cells.

Discovery of Stem Cells

The stem cells were discovered by Martin Evans and Matt Kauffman. They identified, isolated and cultured the embryonic stem cells from a mouse blastocyst in 1981. Later, James Thomson and his subordinates isolated the embryonic stem cells from a human blastocyst. This helped the scientists to generate the building blocks of the human body in unlimited amounts. The discovery made it possible to have cell types for therapeutic purposes and new transplantation methods that were impossible in earlier times.

Landmarks in Discovery of Cells

Scientist	Discovery
Robert Hooke	Discovered cells
Anton Van Leuwenhoek	Discovered protozoa and bacteria
Robert Brown	Discovered cell nucleus
Albert Von Kolliker	Discovered mitochondria
Schleiden and Schwann	Proposed cell theory
Evans and Kauffman	Discovered Embryonic Stem Cells from mice
James Thomson	Discovered Embryonic Stem Cells from humans
Camillo Golgi	Golgi apparatus
Joseph Beinaime Caventou and Pierre Joseph Pelletier	Discovered chlorophyll

CELL STRUCTURE AND CELL DIVISION

GENERAL CONCEPTS

I. Heirarchy of body organization

A. Cells

B. Tissues (epithelium, muscle, connective, nervous)

C. Organs (stomach, heart, skin, lung, etc.)

D. Organ systems (digestive, respiratory, excretory, etc.)

E. Individual

II. Although there are approximately 200 different cell types in the body, cells are more alike than different. Specialization of function, (e.g., glandular cells for secretion or muscle cells for contraction) is really an emphasis of a function that all cells possess to some degree. In some cases, cells have become so specialized that some functions are lost altogether (e.g., cell proliferation).

III. Cells are the structural units of all living organisms.

A. Cells vary in size and shape according to location and function.

1. Cells widely in diameter, from the largest, the mature human ovum (120 microns) to the smallest, the red blood cell (7-8 microns).

2. Cells shapes.

a. **Spherical**. Cells in a fluid environment, e.g., blood cells or some nerve cells

b. **Squamous**. Flattened cells with a width much greater than height. Found at surfaces where rapid exchanges of gases or fluids occur.

c. **Cuboidal**. Cells shaped like a cube, roughly equal height and width. Often form tubes or tubules.

d. **Columnar**. Cells shaped like columns, much taller than they are wide. Often function in absorption.

e. **Pyramidal**. Cells shaped like a pyramid. Often found comprising spherical glandular structures.

f. **Stellate**. Star-shaped cells. Possess many slender processes for interaction with multiple cells such as neurons.

g. **Spindle-shaped**. Elongated shape with tapering ends.

B. Cells vary in internal structure depending upon their function.

1. Specialized cells possess abundant internal structures related to their specific function, e.g., contractile filaments in muscle cells or secretory granules in gland cells.

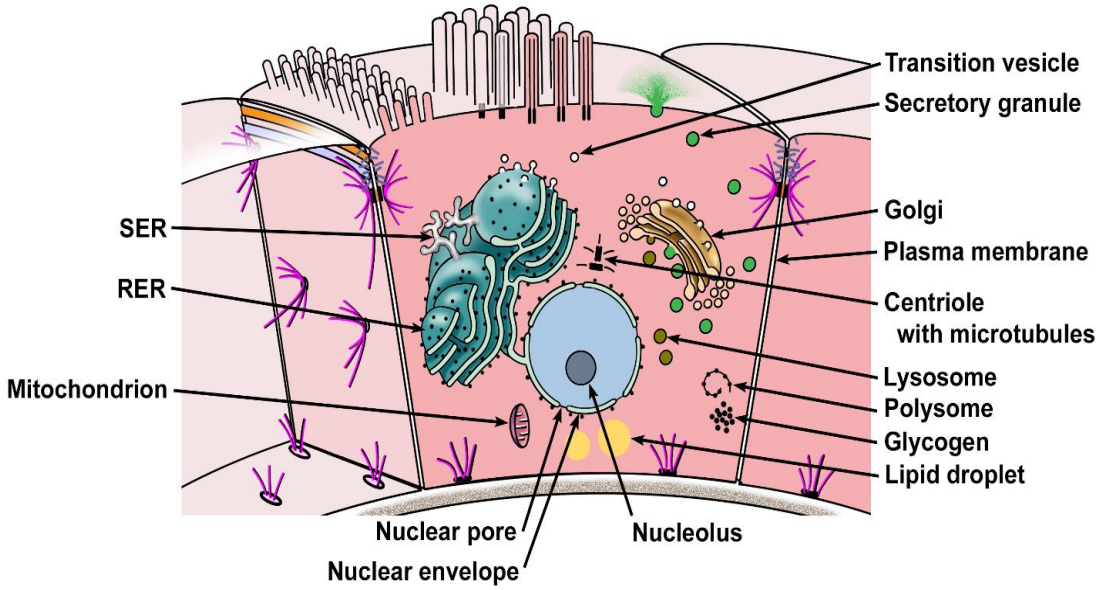
2. Cell polarity. Polarity is a feature of a cell which is exhibited when the organelles are not homogenously distributed in the cytoplasm. This distribution correlates with the function of the cell, e.g., secretion or absorption.

C. Cells vary in their life history, for example, rates of cell renewal.

IV. Major compartments of the cell

A. Cytoplasm. Composed of an aqueous matrix containing the internal structures of the cell, thus allowing for the cytosolic metabolic pathways (e.g., glycolysis) to function.

B. Nucleus



CELL STRUCTURES

Figure. Structural features of a typical cell.

CELL MEMBRANES

I. All membranes have a similar structure and are referred to as unit membranes. A specialized **unit membrane** forms the surface boundary of the cell and is called the **plasma membrane**. Other membranes are present within the cells, where they form mitochondria, endoplasmic reticulum, or nuclear envelope, for example. The structure of the unit membrane cannot be resolved with the light microscope; however, at high magnification with the electron microscope, it appears as a trilaminar dark-light-dark band.

A. Fluid mosaic model of membrane structure

1. **Phospholipid bilayer** consists of two leaflets of phospholipids.

a. The polar, phosphate head groups face the surfaces of the membrane.

b. The hydrocarbon tails form the hydrophobic core of the membrane.

2. Membrane proteins

a. **Integral membrane proteins** are proteins that extend into one or both of the phospholipid layers. Proteins that extend across both of the phospholipid layers are called transmembrane proteins.

b. **Peripheral membrane proteins** are either associated with the polar head groups of the phospholipids or with integral membrane proteins. They do not contact the hydrophobic core of the membrane.

3. **Glycocalyx** is composed of complex carbohydrates on the external surface of the plasma membrane. The carbohydrates are covalently attached to proteins or lipids.

II. Functions of the plasma membrane

A. Membrane transport

1. **Diffusion**

a. Passive diffusion

b. Facilitated diffusion. Utilizes transmembrane proteins to increase the permeability of the membrane to certain materials.

2. **Active transport**. Energy-requiring process of moving materials across the membrane.

3. **Vesicular transport**

a. **Endocytosis**. Internalization of small membrane vesicles formed from the plasma membrane

i. **Pinocytosis** ("cell drinking"). Uptake of fluid into the cell by a continuous process

ii. **Receptor-mediated endocytosis**. Requires receptor-ligand binding for vesicle formation and internalization

b. **Phagocytosis** ("cell eating"). Ingestion of large particles (e.g., bacteria) into the cell; prominent in some macrophages and white blood cells.

c. **Exocytosis**. Fusion of cytoplasmic vesicles with the plasma membrane and release of the vesicle contents to the outside of the cell

i. **Constitutive exocytosis**. Continuous process that renews the plasma membrane.

ii. **Regulated exocytosis**. Requires an extracellular signal for vesicle fusion and release (e.g., hormone secretion)

d. **Transcytosis**. Uptake of material on one side of a cell followed by transport and release from the opposite surface

B. Cell adhesion. Proteins provide cell-to-cell attachment and cell-to-extracellular matrix anchorage.

C. Intercellular communication. Transmembrane proteins assemble to form pores (gap junctions) between cells.

D. Signal transduction. Following interaction with extracellular signals, e.g., hormones and growth factors, receptor proteins initiate intracellular signaling pathways.

NUCLEUS

ı. Houses the DNA; produces ribosomes and messenger RNA

II. Components

A. **Nuclear envelope**

1. Composed of two unit membranes, **inner and outer nuclear membranes**, which are separated by the **perinuclear space**; outer membranes and space are continuous with those of the endoplasmic reticulum.

2. Outer membrane possesses ribosomes.

3. **Nuclear pores**. Perforations in the nuclear envelope, provide direct, bidirectional continuity between the contents of the nucleus and the cytoplasm.

a. Inner and outer nuclear membranes become continuous at the rim of the pore.

b. Pores are surrounded by an octet of proteins with a central granule comprising the **nuclear pore complex**.

4. **Nuclear lamina**. Intermediate filaments on the inner nuclear membrane provide support for the nuclear envelope.

B. **Nucleolus**

1. Site of ribosomal RNA (rRNA) synthesis and initial ribosome subunit assembly

2. Subdivisions of the nucleolus

a. **Nucleolar organizing centers (fibrillary centers)**. Pale staining regions containing DNA sequences that encode rRNA

b. **Pars fibrosa (dense fibrillar components)**. Electron dense fibrillar regions composed of rRNA transcripts

c. **Pars granulosa**. Granular-appearing regions composed of maturing ribosome particles

C. **Chromatin**

1. Composed of DNA plus protein, mostly histone protein

2. Chromatin exists in transcriptionally active and inactive states.

a. **Euchromatin**. Refers to the state of chromatin that is transcriptionally active, dispersed, and pale staining

b. **Heterochromatin**. Refers to the state of chromatin that is transcriptionally inactive, condensed, and dark staining

D. **Nucleoplasm**. Similar to cytoplasm, an aqueous matrix with cytoskeletal elements

ENDOPLASMIC RETICULUM

I. Intracellular system of membranes

II. **Rough endoplasmic reticulum (RER)**

A. Flattened membrane sacs; can occur singly or as multiple, parallel stacks

B. Continuous with the nuclear envelope

C. Possesses ribosomes on the cytoplasmic surface

D. Site of protein synthesis and some phospholipid synthesis

III. **Smooth endoplasmic reticulum (SER)**

A. Tubular membranous structures in a meshwork configuration that is continuous with rough endoplasmic reticulum; lack ribosomes

B. Highly specialized in muscle cells where it is called the **sarcoplasmic reticulum**

C. Functions

1. Synthesis of triglycerides, cholesterol, and steroid hormones

2. Detoxifies drugs

3. Stores and mobilizes calcium

RIBOSOMES

I. **Ribsomes** are composed of two subunits containing rRNA and proteins.

II. Site of translation of messenger RNA (mRNA) to produce protein

III. Distribution

A. Free in the cytoplasm. **Polysomes (polyribosomes)**, spiral clusters of ribosomes along a mRNA molecule; synthesize proteins for use in the cytoplasm, mitochondria, peroxisomes, and nucleus

B. Associated with membranes

1. Attached to the endoplasmic reticulum or outer nuclear membrane

2. Synthesize:

a. Proteins for incorporation into secretory granules for release outside the cell.

b. Hydrolytic enzymes in lysosomes.

c. Integral membrane proteins.

d. Proteins that function in the endoplasmic reticulum and Golgi apparatus

GOLGI APPARATUS

I. The **Golgi apparatus** is composed of flattened, membranous sacs **(Golgi cisterns)**, usually located near the nucleus. Has no structural continuity with the endoplasmic reticulum

II. Modifies proteins formed in the RER (post-translational modification), for example, glycosylation and phosphorylation, and packages them into vesicles

III. Well developed Golgi complex appears as a distinct, unstained region in the cytoplasm near the nucleus and, for that reason, is often referred to as a **"negative Golgi".**

IV. Components

A. **Transition/transfer vesicles** deliver proteins synthesized in the rough endoplasmic reticulum to the Golgi

B. Transition vesicles fuse with the ***cis* or forming face** (convex surface) of the first Golgi cistern.

C. Proteins move between cisterns in vesicles formed at the margins of the cisterns.

D. ***Trans* or maturing face** (concave surface), represented by the last Golgi cistern, is the site of final vesicle formation.

E. **Trans Golgi network**, located at the *trans* face, is the collection of newly formed vesicles that are then transported throughout the cell.

F. The **cisternal progression model** is an alternate theory by which the initial *cis* face cistern is formed by the fusion of transition vesicles. Thereafter, the cistern and the proteins it contains moves as a unit towards the *trans* face. Upon

reaching the trans face, the cistern breaks up into vesicles thereby contributing to the trans Golgi network

v. Fates of Golgi vesicles from the *trans* Golgi network

A. Fuse with plasma membrane, thereby supplying new lipids and proteins to the membrane

B. Form Golgi hydrolase vesicles ("pre-lysosomes")

C. Form secretory granules

D. Return to the endoplasmic reticulum or Golgi

LYSOSOMES

i. **Lysosomes** are membrane-bound vesicles that serve as sites of intracellular digestion.

ii. Abundant in cells with high phagocytic activity (macrophages, neutrophils)

iii. Formation and function

A. Pre-lysosomal vesicles form at the *trans* Golgi network; contain multiple (>40) inactive hydrolytic enzymes (e.g., proteases, lipases and nucleases)

B. Fuse with endosomes, phagosomes, or autophagosomes, which is followed by a decrease in the luminal pH that activates the hydrolases

C. Hydrolases degrade the contents of the lysosome; undigestible materials are retained in **residual bodies** as **lipofuscin pigment**.

SECRETORY GRANULES

i. **Secretory granules** are derived from the *trans* Golgi network.

II. Contain highly concentrated secretory product in single membrane-enclosed structures

III. Transported to the cell surface and fuse with the plasma membrane either in a continuous,
constitutive mode or in a regulated fashion that requires an external signal

IV. **Zymogen granules**. Secretory granules that contain enzymes,

MITOCHONDRIA

I. **Mitochondria** (singular, mitochondrion) are sites of ATP production in the cell and are self-replicating.

II. Spherical to ovoid shape, 1-10µm; may be dispersed throughout the cytoplasm or clustered (e.g., at the poles of the nucleus)

III. Composed of **inner and outer unit membranes**. The inner membrane is highly folded to form **cristae**, which provide increased surface area.

A. Most mitochondria possess cristae that are shaped like thin shelves that project into the matrix.

B. Mitochondria in steroid hormone-secreting cells have cristae that are tubular.

IV. **Intercristal space**. Located between the cristae and is occupied by **matrix**

V. Contain the enzymes for ATP production. Krebs cycle enzymes are located in the matrix whereas those for the electron transport system are located in the inner membrane.

VI. Matrix contains mitochondrial DNA, RNA, and electron-dense **calcium-containing granules** that, along with the smooth endoplasmic reticulum, provide calcium storage and buffering.

PEROXISOMES

I. **Peroxisomes** are membrane-bound vesicles containing oxidative enzymes (e.g., catalase and beta-oxidation enzymes)

II. Carry out fatty acid oxidation and detoxification of alcohol.

LIPID DROPLETS

I. **Lipid droplets** consist of accumulations of cholesterol and triglycerides in the cytoplasm and are not surrounded by a membrane.

II. Can occur as numerous small accumulations or as a single large droplet, as in adipose cells

GLYCOGEN GRANULES

I. **Glycogen granules** occur in small clusters or in accumulations as in liver cells and appear highly electron dense; not surrounded by a membrane; stain magenta with the periodic acid-Schiff (PAS) reaction for carbohydrate

II. Storage form of glucose

III. Present in all tissues but highest in liver and striated muscle

PIGMENT

I. **Lipofuscin pigment**

A. Composed primarily of lipid-containing residues of lysosomal digestion.

B. Contained within membrane-bound vesicles **(residual bodies)** derived from late-stage lysosomes

II. **Melanin**

A. Primarily synthesized by **melanocytes** in the skin but can occur in other cell types

B. **Melanosome**. Membrane-bound vesicle in the cytoplasm containing the melanin pigment

C. Main pigment responsible for hair and skin color

CYTOSKELETON

I. Gives shape and support for the cell, provides cell motility, and facilitates intracellular transport

II. Classification

A. **Microfilaments**. 4-6 nm filaments composed of actin; function in support of the plasma membrane, cell movement and extension of cellular processes

B. **Intermediate filaments**. Structurally and chemically heterogeneous among different cell types; 8-10nm filaments that are cell-type specific; function in structural support

C. **Microtubules**. 18-20 nm tubules composed of cc and p tubulin; multiple functions within the cell. Microtubules form a number of structures in the cell including:

1. **Centriole**. A short rod-like structure composed of nine sets of three microtubules; centrioles occur in pairs near the nucleus and are oriented at right angles to each other. The pair is called a **diplosome** and the region of the cytoplasm where the pair is located is called the **centrosome** or the **microtubule organizing center**.

2. **Basal body**. Structurally similar to a centriole; located at the base of each cilium and flagellum, providing support and serving as the source of the microtubule core of these structures.

3. **Axoneme**. Forms the core of cilia and flagella and provides for the movement of these structures; the axoneme consists of a column of nine pairs of microtubules surrounding two central unpaired microtubules. Each of the paired microtubules is continuous with two of the microtubules of the basal body.

4. **Mitotic spindle**. Individual microtubules that extend from the centrioles to the kinetochore of chromatids during cell division

CELL CYCLE and CELL DIVISION

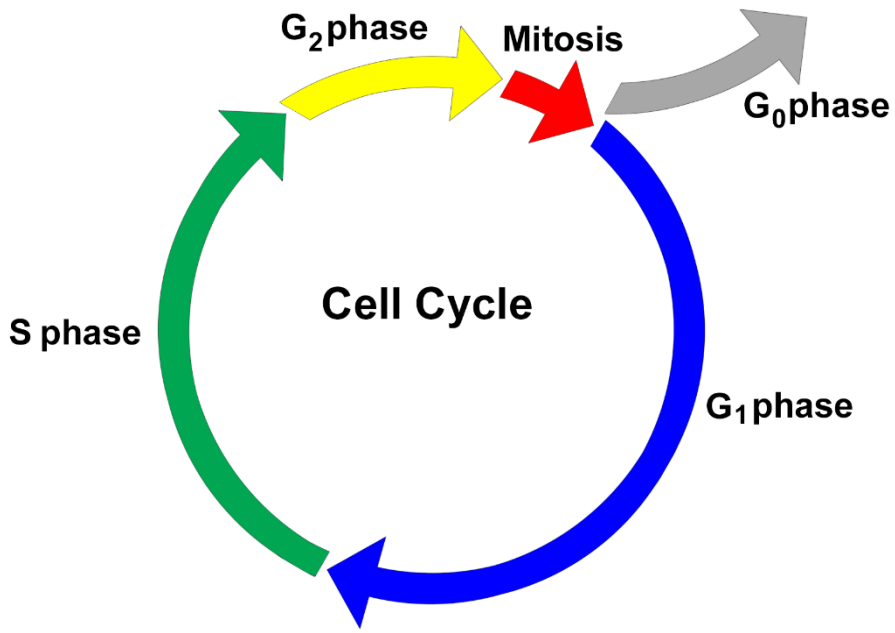


Figure. The cell cycle

I. The cell cycle is the period that extends from the time a cell comes into existence, as a result of cell division, until it completes its own cell division.

II. Humans have 23 pairs of chromosomes, 22 pairs of autosomes and one pair of sex chromosomes, XX (females) or XY (males). The full complement of 26 pairs of chromosomes is designated 2N.

III. Phases of the cell cycle

A. **Interphase**. The longest phase of the cell cycle

1. **Go Phase**. Special G_1 phase for quiescent (non-dividing) cells

2. **G_1 Phase**. Initial period of cell growth

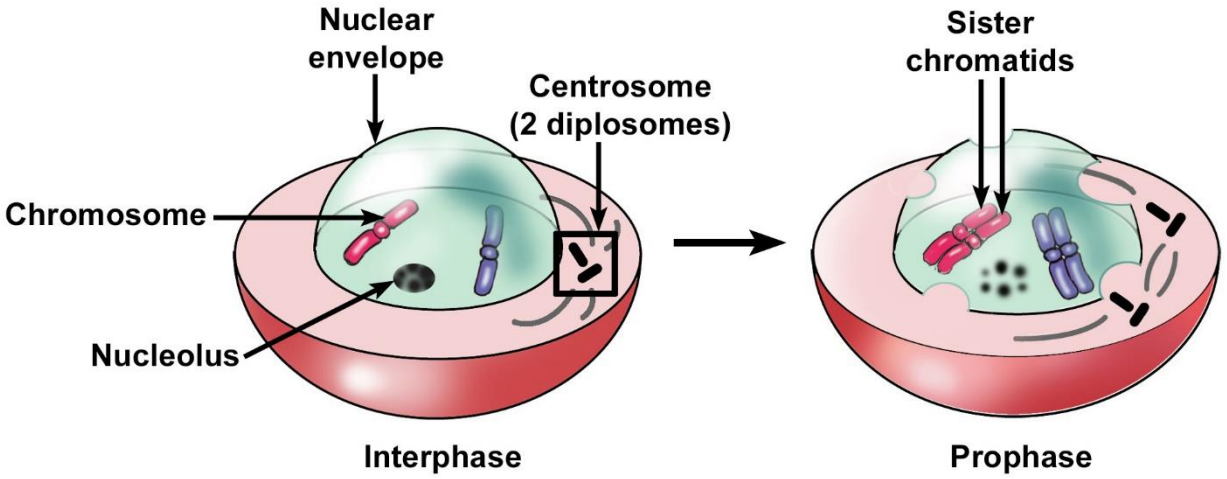
3. **S Phase**. Period of DNA synthesis, chromosome duplication, and duplication of the centrioles

4. **G_2 Phase**. Preparation for cell division, which leads to M phase (mitosis)

B. **Mitosis (M phase)**

1. Mitosis is the process whereby cells divide to produce two identical daughter cells. Mitosis is the portion of the cell cycle during which chromosomal segregation and cell division occurs.

2. Phases



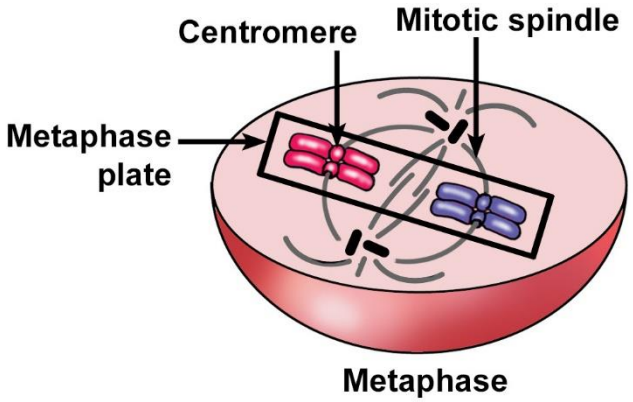
a. **Prophase**

i. The initial phase of mitosis, follows the G2 phase of interphase.

ii. Replicated DNA condenses into paired, visible **chromosomes***;* each member of the pair is called a **chromatid**. Chromatids are attached to each other at a junction point called the **centromere** and are called sister chromatids. Adjacent to the centromere, a protein complex called the **kinetochore** forms, providing an attachment point for the developing microtubules of the mitotic spindle.

iii. The two **centrioles** in the centrosome move apart and migrate toward opposite poles of the cell. The microtubules of the **mitotic spindle** begin to form from each centrosome.

iv. The nuclear envelope disintegrates and the nucleolus disappears. This late stage of prophase is sometimes referred to as prometaphase.



b. **Metaphase**

i. Centrosomes have migrated to opposite poles of the cell

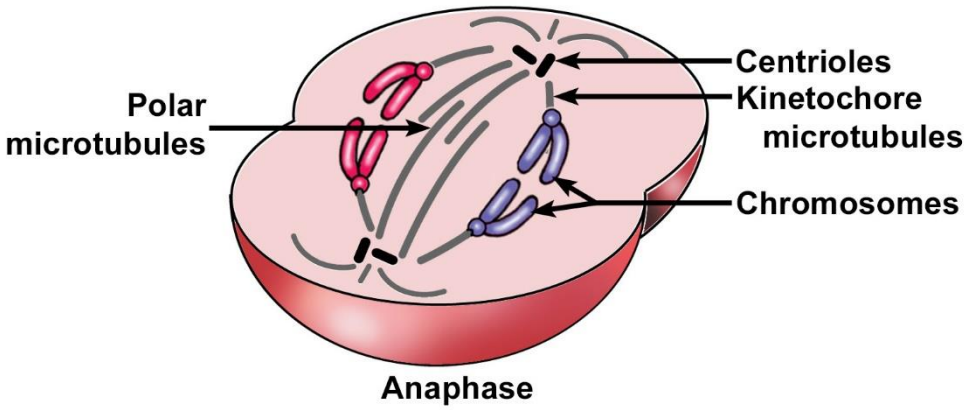
ii. The mitotic spindle is completed, consisting of three types of microtubules.

(a). **Astral microtubles** surround the centrosome

(b). **Kinetochore microtubules** attach to the kinetochore of the sister chromatids and direct chromatid movement to the center of the cell where they align to from the **metaphase (equatorial) plate.**

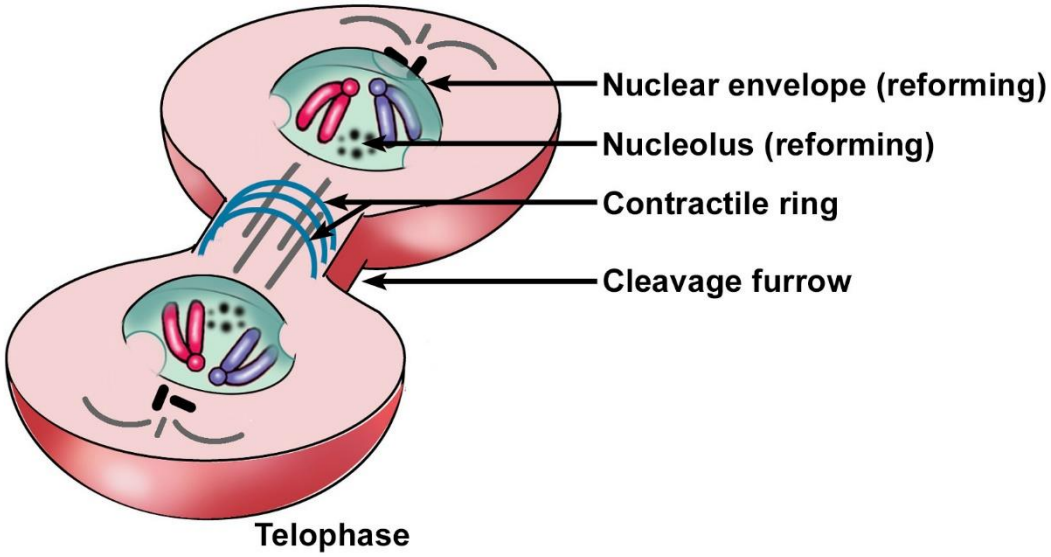
(c). **Polar microtubules** overlap with opposing microtubules around the metaphase plate and provide the force to drive the spindle poles apart.

c. **Anaphase**



i. Begins abruptly as sister chromatids separate with each moving towards an opposite pole of the cell. The movement of the chromatids occurs by the pulling forces of the kinetochore microtubules and the pushing force of the polar microtubules to separate the spindle poles.

ii. The end of anaphase is marked by the segregation of an identical set of chromosomes at each spindle pole.



d. **Telophase**

i. Vesicular fragments of the original nuclear envelope that remain in the cytoplasm after prophase reform the nuclear envelope around the chromosomes at each pole. The chromosomal DNA extends and uncoils, losing its stainability.

ii. The nucleolus reforms.

iii. The cytoplasm divides **(cytokinesis)**, forming two identical daughter cells.

(a). A **cleavage furrow** forms marking the eventual separation site of the daughter cells

(b). Separation is accomplished by a **contractile ring** of actin and myosin filaments. Interaction between the filaments tightens the ring and eventually pinches the cell into two daughter cells.

IV. Meiosis

A. This cell division is restricted to the germinal cells in the gonads (ovary and testis) for the production of gametes (ovum and sperm), respectively.

B. Results in reduction of the chromosome number by one-half (1N, haploid). Fusion of the gametes to form a zygote reconstitutes the diploid (2N) number of chromosomes present in somatic cells.

C. Entails two successive cell divisions, **meiosis I (reductional division)**, reducing chromosome number by one-half, followed by **meiosis II (equational division)**, reducing the DNA content by one-half.

D. Results in four, dissimilar daughter cells

E. Divisions of meiosis

1. Meiosis I (reductional division)

a. Prophase. Prophase is more protracted than in mitosis, lasting days to years. It consists of five stages and is marked by the close association of homologous chromosomes (synapsis), allowing exchange of homologous regions between maternal and paternal chromosomes. This exchange process, crossing over, increases genetic diversity. Stages of prophase are:

i. **Leptotene**. Replicated DNA condenses into visible, chromosomes consisting of paired, sister chromatids.

ii. **Zygotene**. Synapsis occurs resulting in the close alignment of homologous pairs of replicated chromosomes. The pairs are tightly bound to each other by protein bridges, the synaptonemal complex, forming bivalents consisting of four chromatids.

iii. **Pachytene**. Crossing over occurs in which fragments of homologous regions of maternal and paternal chromosomes are exchanged. Sites of crossing over, called chiasmata, resemble an "X" and form connections between paired homologous chromosomes. Crossing over leads to greater genetic diversity in the gametes.

iv. **Diplotene**. Chromosomes further condense and the synaptonemal complex breaks down, leading to a separation of homologous chromosomes.

v. **Diakinesis**. Chromsomes condense to their maximum thickness, the nuclear envelope disappears and the nucleolus fragments.

b. Metaphase. Paired homologous chromosomes, which are still joined by chiasmata, align at the metaphase plate. These pairs, consisting of four chromatids, are called bivalents.

c. Anaphase. Chiasmata break and, unlike mitosis, homologous chromosomes (consisting of attached sister chromatids) separate, thus halving the number of chromosomes from 2N (diploid) to 1N (haploid). Anaphase ends with the

segregation of one duplicated chromosome of each homologous pair to each spindle pole.

d. Telophase. Begins by the arrival of one duplicated chromosome of each homologous pair at each spindle pole.

i. Cytokinesis. Cleavage furrow develops, indicating the initial formation of the daughter cells.

ii. Contractile ring forms and its activity result in the formation of two daughter cells, each with a single set of chromosomes (haploid, 1N).

iii. Chromosomal DNA may decondense somewhat but quickly recondenses.

iv. Cells rapidly progress into prophase of meiosis II without passing through a second S phase period.

2. Meiosis II (equational division)

a. Stages

i. Prophase. The events of prophase, Meiosis II parallel those of mitosis.

ii. Metaphase. In each daughter cell, the haploid set of replicated chromosomes (sister chromatids) align at the metaphase plate.

iii. Anaphase. Sister chromatids separate and each is drawn towards the opposite pole of the cell. Thus, the number of chromosomes does not change (equational division), while the DNA content is halved.

iv. Telophase. Begins with the arrival of a single set of chromosomes at each spindle pole, reformation of the nuclear envelope, decondensation of the chromosomal DNA and the reappearance of the nucleolus. A cleavage furrow develops indicating the initial formation of the daughter cells (cytokenesis).

b. Produces gametes, ovum or sperm, each containing one set of chromosomes (haploid, 1N). However, while the nuclear events producing ova (oogenesis) and sperm (spermatogenesis) are similar, cytoplasmic stages are different between these two processes. In males, a single germ cell produces four sperm; in females, only a single ovum is formed.

COMPARISON OF MEIOSIS AND MITOSIS

I. Mitosis occurs in tissues where cell renewal is required and produces two identical, diploid daughter cells. Meiosis occurs only in the ovary and testis and results in gamete, egg and sperm, production. In meiosis in the male, a single germ cell produces four, dissimilar, haploid daughter cells (sperm); in females, a single germ cell usually produces only a single haploid daughter cell (ovum) plus three non-functional satellite cells.

II. Mitosis consists of a single cell division, while meiosis entails two successive cell divisions, meiosis I (reductional division), reducing the chromosome number by one-half, followed by meiosis II (equational division), reducing the DNA content by one-half.

III. Stages of mitosis and meiosis I

A. Interphase. Interphase is similar in meiosis and mitosis. The first stage of interphase, the G1 stage, is seen here. For diagrammatic purposes, chromosomes are shown in a condensed state.

B. Prophase. Prophase of meiosis I is much longer than in mitosis allowing for crossing over to occur during meiosis.

C. Metaphase. Some differences exist in the alignment of chromosomes at the equatorial plate, but this phase is essentially similar in both processes.

D. Anaphase. In mitosis, sister chromatids of each chromosome separate and move toward the spindle poles, resulting in diploid cells. In contrast, during anaphase

of meiosis I, chromatids remain attached and one duplicated chromosome of each homologous pair is pulled to each of the opposite spindle poles. This process halves the number of chromosomes resulting in haploid daughter cells.

E. Telophase. Cytokinesis is similar for both mitosis and meiosis. Mitosis results in the formation of two identical daughter cells, each with two sets of homologous chromosomes (diploid, 2N). Meiosis I results in the formation of two dissimilar daughter cells, each with a single set of chromosomes (haploid, 1N).

IV. Meiosis II

A. A second division, paralleling meiosis II, does not occur in Mitosis.

B. Meiosis II parallels mitosis except:

1. Cells enter prophase of meiosis II rapidly without any intervening DNA synthesis.

2. At metaphase, a haploid set of replicated chromosomes (sister chromatids) align at the metaphase plate.

3. Cytokinesis produces gametes, ovum or sperm, each containing one set of chromosomes (haploid, 1N)

Histochemistry Part

INTRODUCTION TO HISTOCHEMISTRY

Histochemistry can be defined as the chemistry of tissue components and its relation to tissue morphology.

Relation of Histochemistry to Other Disciplines:

The histochemist must have a good background in histology. A sound knowledge of inorganic, organic, and biological chemistry is also essential in order to appreciate the chemical nature of the tissue components being studied, and to understand the chemical reactions that form the bases of the histochemical methods.

A primary difference between the disciplines of histology and histochemistry is that histology is primarily concerned with the study of microscopic morphology while histochemistry is concerned with the chemistry of cells and tissues related to their morphology. Histology is therefore primarily subserved by empirical staining methods while histochemistry is based on specific chemical reactions.

Biochemical methods may yield highly quantitative information concerning a particular organ or tissue, while histochemistry may yield primarily qualitative information concerning a particular group of cells within the same tissue.

For example, a biochemical assay for a particular enzyme in the liver may show only that it was present in a relatively low concentration. A histochemical procedure may actually reveal a high concentration of the enzyme in the Kupffer cells and a total absence of the enzyme in the hepatocytes.

It is possible with histochemical methods to study cells and tissues that are too small to be studied by other methods. Histochemical methods can be applied to small

surgical biopsies or very small tissues, such as the **islets of Langerhans**, that cannot easily be isolated from the surrounding tissues.

Tissue Fixation

Purpose of fixation:

The tissue section to be observed under the microscope should be as similar in appearance to the original living tissue as possible. The way the tissue is handled from the time it is removed from the body until it is ready for viewing under the microscope affects the preservation of its original detail. A piece of tissue that is left in the air following its removal will dry, resulting in gross distortion of its microscopic appearance. If it is placed in water, it will swell; if placed in a strong salt solution, it will shrink. Any of these mistreatments would cause serious distortion of the histology.

Fixation is the first, and perhaps the most important, step in preparation of tissue for histochemical study. Fixation guards against four different forces that tend to alter the morphology of tissues and cells.

Prevention of autolysis:

Removing a tissue from its organism separates it from its source of oxygen and nutrition, which sets into motion certain degradative chemical processes. Most of the postmortem changes are probably due to the action of certain hydrolytic enzymes. Many hydrolases are always present in tissues under normal conditions but are neatly packed away in small organelles called lysosomes (these hydrolytic enzymes are therefore called lysosomal enzymes). Immediately following cellular death, the lysosomes seem to rupture and spill their enzymes. These enzymes are then free to begin disrupting the integrity of the cells.

Proper fixation prevents autolysis by two mechanisms. The lysosomal enzymes may be inactivated by the fixative, and the tissue components (upon which they would act) may be chemically altered so as to become unsusceptible to the enzymes.

Insolubilization of tissues:

Fixation of tissue renders insoluble certain tissue components. Lipids are the most difficult component to fix; they are nearly always dissolved during dehydration and clearing. No one fixative will insolubilize all tissue components. (In the case of bone, for example, it is highly desirable to remove the mineral salts that are responsible for hardness. Adecalcification step is therefore added in processing bone).

Protection from putrefaction:

Fixation serves to protect the tissues from damage by microorganisms. This process is called putrefaction.

Protection from damage:

Fixation helps to protect the tissue from sustaining damage by subsequent preparative operations, such as dehydration, embedding, sectioning, and amounting. Fixation stabilizes the protein skeleton of the cell, giving the cells some structural support to resist deformation or crushing.

Aspects of timing:

There is an inevitable time lag between the moment a tissue is separated from its source of nutrients and oxygen, and the moment of it is first exposed to the fixative. When this time lag is critical, it may be necessary to perfuse the animal, or at least the organ of interest, with the fixative through the blood vascular system. In this way, the tissue is exposed to the fixative at the moment when its nutrients and

oxygen supply are cut off. Perfusion may be necessary when tissue with high metabolic rates is to be studied or when a fixative that penetrates slowly is used.

When a block of fresh tissue is placed in a fixative, the tissue at the surfaces of the block will be exposed to the fixative immediately. However, the tissue near the center will not be exposed until the fixative has had time to diffuse through to the center. The size of the tissue block and the rate at which fixative diffuses through the tissue must both be taken into consideration for proper fixation.

The aim in fixing a tissue for enzyme histochemistry is to preserve both enzyme activity and morphology as faithfully as possible.

Specific fixatives:

It is of great interest for the histochemist to know as accurately as possible what chemical reactions take place between the tissue component and the fixative.

1) Formaldehyde:

Formaldehyde exists as a 40% solution in water. This 40% solution must be taken as 100%, e.g., to prepare a 10% solution of formalin, 10 parts of 40% formaldehyde are added to 90 parts of water.

Solution of formalin are acidic due to the formation of small amounts of formic acid. For all histochemical techniques it is necessary to bring the pH of formalin to neutral. This can be done by using either a buffer (sodium phosphate), or by placing calcium carbonate at the bottom of the container. Fixation in acid formalin for a long time produces formalin pigment, and difficulty may be met with in making acid dyes, such as eosin, correctly.

The following formaldehyde solutions are amongest those used in histochemistry and histology: (a) 10% formal saline (b) 10% formalin (aqueous), (c) neutral formalin, (d)

10% formal-calcium, (e) 10% formalin in alcohol (f) formol- sucrose and (g) formaldehyde vapour.

Formaldehyde is widely used in histochemistry. It is a good fixative for tissues in which lipids are to be demonstrated, as lipids are chemically unaltered by formaldehyde fixation, Phospholipids are well preserved by formaldehyde fixation when calcium has been added to the fixative.

In enzyme histochemistry, most hydrolytic enzymes are well preserved with fixation in formal calcium at $4^{o}C$. The enzyme activity is reduced if the fixation time is extended, or if it is carried out at room temperature. Formaldehyde vapour is recommended for the fixation of freeze dried tissues and produces excellent demonstration of muco-substances, glycogen, proteins and nucleic acids.

The chemical actions of formalin on tissue components:

The reactions of formalin with tissue proteins are numerous and complex since it can combine with a number of different groups of substances which become precipitated in the cells and tissues. But many of the combinations are reversible by the simple process of washing, whereas others are irreversible.

The most important reaction of formalin is the transformation of a compound containing a reactive hydrogen atom into a hydroxyl compound.

$$RH + CH_2O \rightleftharpoons R.\ CH_2(OH)$$

The hydroxyl compound is also reactive and it may condense with a further H atom to form a methylene bridge (CH_2) that links the protein molecules:

$$R.\ CH_2(OH) + HR^1 \rightleftharpoons R - CH_2 - R^1 + H_2O$$

These methylene bridge are readily ruptured by hydrolysis. Methylene bridges be formed between two similar groups such as NH_2, or between NH_2 and peptide (CONH) or between NH_2 and NH.

Between pH 6 and 8, formalin reacts with keratin (the essential protein constituent of hair and skin) without affecting the S-S links of cysteine. In more alkaline solutions it is considered to reduce S-S to two SH groups, and subsequently to react with these forming, in some cases, a methylene bridge (S- CH_2-S) in place of the original disulphide link.

The groups particularly involved in the fixation of proteins are amino, imino, amido, peptide, hydroxyl, carboxyl as well as the sulphur-containing proteins (SH) and aromatic ring.

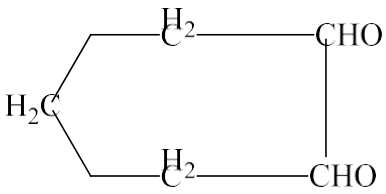
The knowledge of the action of formalin on tissues was primarily obtained from tanning and wool industries; collagen and reticulin are the two well-known substances in this connection.

2) Glutaraldehyde and acrolein:

Electron microscopical research and enzymes histochemistry needed the introduction of histochemical technique of certain aldehydes other than the formaldehyde among them are glutaraldehyde and acrolein.

Acrolein (acrylic aldehyde) H_2C = CHCHO is a bifunctional aldehyde used in the tanning industry which is capable of introducing more cross-links than formaldehyde. It is used in electron microscopy research but it is little used as histochemical fixative as it is unstable at alkaline pH levels.

Glutaraldehyde $(CH_2)_3$CHOCHO is represented by the following structural formula:



It is an efficient cross-linking bifunctional agent. In histochemical technique, cold glutaraldehyde is used to demonstrate hydrolytic enzymes in crystal sections. It is a fast acting aldehyde fixative for very short time, i.e. for an 8 micron cryostat sections. It is a fast acting aldehyde fixative for very short time, i.e. for an 8 micron cryostat section, 30 seconds to one minute is usually sufficient. It is not recommended to fix the tissues as blocks in glutaraldehyde for the long time taken to penetrate to the centers of the tissues.

The activities of phosphatases and esterases are well conserved after fixation in glutaraldehyde. Additionally, glutaraldehyde is very extensively applied in electron microscopy because of its ability to preserve ultrasturcture.

3) Alcohol:

It is more often used in histochemistry, especially for fixation of cryostat section to demonstrate enzymes. The enzymes are almost unaffected by cold alcohol (4C) with the exception of esterases. For demonstrating glycogen, alcohol is occasionally used as 80 percent solution. This, while being an ideal fixative for glycogen, has drawbacks form a morphological point of view. When used in compound fixatives, however, alcohol will give acceptable results. It will precipitate proteins, and will remove lipids. It also makes the freezing of tissues and the subsequent sectioning difficult.

The mode of action has not been understood by investigators for whom dehydration was the chief mechanism involved. The alcohol produces reticulation of the cytoplasm, destruction of the Golgi apparatus and mitochondria, extraction of lipids and dissolution of several secretory granules. Alcohol is rarely used on its own in

histology because of the damage it causes by shrinkage and excessive hardening of the block. The following are the most common fixatives which contain alcohol: formol alcohol, Carnoy, Clarke's, Gendre's fluid, acetic alcohol formalin (AAF) and Wolman's fixative.

4) Acetone:

It has frequently been used as a fixative for tissues to be processed to paraffin wax, and for hydrolytic enzyme methods. The acetone is used at 0-4^{o}C and may be used in histochemistry as a fixative of cryostat sections. Acetone is a fairly rapid fixative but does cause shrinkage of the tissue. It is frequently employed as the first step in the freeze-substitution technique. At -70^{o}C it will neither remove much lipid material from the tissue, nor fix the tissues. Whereas it will be substituted for the ice in the tissues. It is rarely used in any compound fixatives, certainly not in any of the standard fixatives. At 4^{o}C, it can fix cryostat sections (5-20 microns) in 1 hour or less also small pieces of tissues overnight.

5) Picric acid:

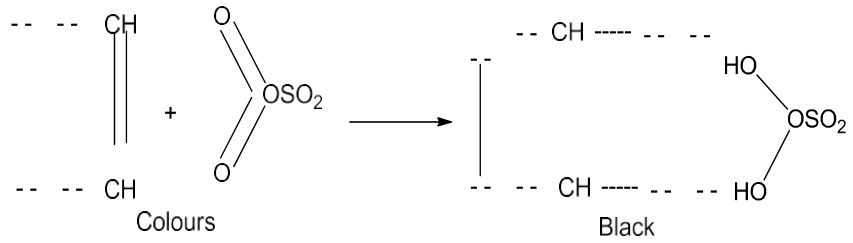
It is one of the most commonly used fixative agents. It is a rapid fixative that hardens the block well, without causing much shrinkage. It is used in many compound fixative as Bouin's and Gendre's fluids. Any fixative containing this acid is recommended for the demonstration of glycogen. Tissue fixed in picric acid must be washed well in either alcohol or water until the yellow colour of the block is removed.

6) Osmiun tetroxide (OSO_4):

This non-coagulant fixative is familiar to cytologists. Its qualities as a fixative of cytoplasmic structures have been well established. It is in constant use in electron

microscopy specially in the demonstration of Golgi apparatus and mitochondria where it is used in 1% buffered solution. This fixative is very damaging to enzymes and is rarely used in histochemistry and histology due to its poor penetrating properties.

OSO_4 can be used to render lipid insoluble. It has been assumed that unsaturated fats reduce OSO_4 with the formation of black compounds containing osmium or its hydroxide; and this has been suggested as being due to the oxidation of the double bonds between adjacent carbon atoms.



7) Mercury- containing fixatives:

These are rarely employed in histochemistry. Mercuric chloride has a rapid but uneven penetration and only thin blocks of tissues should be fixed. In produces a poor-result with glycogen. If the tissues are over fixed, the blocks become hard and sectioning becomes difficult. Mercuric chloride causes also a great deal of shrinkage and is therefore rarely used alone. It is usually used in a compound fixative with formaldehyde or acetic acid. All tissues fixed in mercuric- containing fixatives will have a mercury precipitate, which must be removed by treatment with dilute iodine, followed by immersion in 3 percent sodium thiosulphate to remove the iodine crystals. Sodium thiosulphate is then eliminated from the tissue by careful washing in distilled water.

In general, mercury behaves like other metallic ions in combining with the acid groups of proteins, especially carboxyl and hydroxyl, and the phosphoric acid of nucleoproteins. Some workers were able to prepare a fraction of serum albumin which contained one SH group per molecule. When this was allowed to reach with a

mercury salt the resulting protein substance was found to contain ½ an atom of mercury per albumin molecule. The reaction which takes place can be illustrated as follows:

$RSH + HgCl_2 \rightleftharpoons RS\text{-}Hg\text{-}Cl + HCl$

(protein mercaptide)

$RS\text{-}Hg\text{-}Cl + R.SH \rightleftharpoons (RS)_2Hg + HCl$

The second reaction is a slow one, and both reactions are reversible, but while the second can be reversed by any reaction which forms an undissociated mercury complex, the first is only reversed by reagents (e.g., cysteine) which form equally stable mercury derivatives.

8) Chromium:

Chromium fixatives have the property of forming certain complexes with water of the type Cr-O-Cr, and these complexes combine with the reactive groups of adjacent protein chains forming other complexes similar to those produced under the effect of formalin.

Generally, it has been established that cytological and histochemical fixatives containing chromium must be adjusted at a pH lower than 2.9. However, chromium fixatives give good results in case of glycogen, lipids nucleic acids and mitochondria fixation and preservation.

Preparation of tissue sections for histochemical studies:

Among the various methods for the preparation of frozen sections for histochemical studies are the following:

1) Freezing microtome:

Tissue blocks are usually frozen directly on the microtome stage. The method is affected by extrinsic factors such as the temperature and humidity of the room. These difficulties can be largely overcome with the cryostat.

2) Cryostat technique:

This technique differs from the previous cold microtome method in the fact that in this case, the microtome, the knife and the tissue block are all at the same temperature (-12 : 22^{o}C). However, a cryostat means a devise for maintaining cold.

The first cryostat was developed in Denmark by Lang (1938) for studies in quantitative histochemistry.

Generally, two methods for obtaining and maintaining cold in cryostats could be considered. These are:

Mechanical refrigeration and (2) carbon dioxide gas in cylinders. The first was used for maintenance of cold in the microtome chamber, and the second, whenever rapid processing, became necessary for freezing the tissue blocks on their brass holders. The atmosphere in the chamber is kept dry by means of a bag of silica gel (removed at weekly intervals).

Optimal conditions for cutting: Thornburg and Mengers (1957) pointed out that in frozen tissue, water could be regarded as the embedding medium and sectioning, therefore, as a matter of cutting ice. These authors found that the optimum values for the temperature of the knife, the chamber and the tissue block varied from one tissue

to another. However, cutting was usually possible within a wide range of temperatures.

Tissue temperature:

As is the case with ordinary freezing microtomes, when the block temperature falls below -45^{o}C, the tissues are brittle and friable and cannot be cut. At higher temperatures up to -5^{o}, thin serial sections could be obtained. With isothermic machines (knife and tissue temperature identical), cutting is possible between -30 and -10.

Chamber temperature:

With the cold environment cold knife machine chamber temperatures between 0^{o} and -10^{o} were found best. Below- 10^{o} the quality of the sections began to deteriorate.

Handling of sections after cutting:

With all the cold methods, handling of the tissue sections after cutting is very critical. A number of different techniques have been evolved for this purpose by many authors. The most widely used of these methods are the following:

1. Pick up the sections on warm or cold slide or coverslip.

2. Immerse in cold medium (Particularly in enzyme histochemistry).
3. Transfer into warm or cold test reagent solution (especially in case of protein and organic histochemistry).

4) Freeze-dry subsequent chemical or biochemical analysis.

3) Freeze-drying technique:

The basic principle of freeze- drying is the rapid freezing of tissues at about -160^{o}C and their subsequent desiccation in vacuum at somewhat higher temperature until

almost all of their water content has been removed. The frozen tissues are then dried in vacuum for a variable period. The dried tissues allowed to reach room temperature are vacuum- embedded in paraffin or some other wax and cut in the usual manner. Freeze-drying gives the best morphological preservation with the least chemical alteration of the tissues.

4) Freeze-substitution technique:

Freeze-substitution is a process in which very small pieces are quickly frozen (quenched), dehydrated at low temperature by dissolving the ice in a polar solvent and embedded in paraffin or carbowax, the technique has been employed in connection with many histochemical procedures, e.g., glycogen, mucopolysaccharides, mucoproteins and nucleic acids.

Tissue blocks are quickly frozen in isopentane chilled with liquid nitrogen.

CARBOHYDRATES

Carbohydrates are organic compounds formed of C, H and O with the latter two in the same ratio by which they are present in water. The carbohydrates are widely distributed in both animal and plant tissues. In plants they are produced by photosynthesis and include the starches. In animal cells the most important carbohydrates are galactose, glucose and glycogen. Carbohydrates taken into the body are transformed into simple sugars before being absorbed. These sugars are oxidized in the tissues and are the most important source of energy which can be used for different body functions. Some carbohydrates have highly specific functions in vital processes (e.g., ribose in the nucleoprotein of the cells, galactose in certain fats, and the lactose of milk).

Classification of carbohydrates:

Carbohydrates may be classified as monosaccharides, disaccharides and polysaccharides. The first two are known as sugars because of their sweet taste. They are soluble in water and alcohol and easily pass through semipermeable membranes. The polysaccharides, on the contrary, from colloidal solutions with water, do not crystallize and do not pass across living membranes.

1) Monosaccharides are simple sugars which cannot be hydrolyzed into a simpler form. The general formula is $C_n(H_2O)_n$. The simple sugars may be subdivided as trioses, tetroses, pentoses, hexoses, or heptoses depending upon whether the aldehyde or ketone groups are present. The most important of the monosaccharides in the cells are pentoses and hexoses. These are found usually combined with proteins and lipids. The pentoses are one of the main components of nuclear chromatin. Among the pentoses, ribose ($C_5H_{10}O_5$) and deoxyribose ($C_5H_{10}O_4$) intervene in the constitution of nucleic acids. The hexoses are represented by glucose ($C_6H_{12}O_6$) which is mainly involved in the energetic changes of the cell. Other examples of hexoses are galactose and fructose.

(2) Disaccharides are the result of condensation of two molecules of monosaccharides with the loss of one molecule of water:

$$2C_6H_{12}O_6 \xrightarrow{-H_2O} C_{12}H_{22}H_{11}$$

of the disaccharides, the most important are sucrose (cane sugar) and maltose (malt sugar) in plants and lactose (milk sugar) in animals.

Maltose is built up of two molecules of glucose; lactose is built up of glucose and galactose, and sucrose is formed of glucose and fructose. Thus, the

disaccharides yield on hydrolysis two molecules of the same or of different monosaccharides.

(3) Polysaccharides are formed by the condensation of many molecules of monosaccharides with a corresponding loss of water molecules:

$$n\ c_6H_{12}O_6 \xrightarrow{-n\ H_2O} (C_6H_{10}O_5)_n$$

Of the polysaccharides, the most important are starch and cellulose in plants and glycogen in animals:

Starch forms the reserve substance in plant cells and is synthesized from CO_2 and H_2O by means of chlorophyll. Starch $(C_6H_{10}O_5)_n$ is formed of an □-glucosidic chain. Such a compound, yielding only glucose on hydrolysis, is called a glucosan. Natural starch gives a blue colour with iodine solution.

Cellulose is the main constituent of most plant cell-walls and also enters in the formation of a series of structures which form part of the supporting skeleton of plants. It gives no colour with iodine and is not soluble in ordinary solvents.

Classification of Polysaccharides

The polysaccharides occur in different forms in the body cells. Their chemical nature and physiological role differ widely in different cells, but they resemble each other in containing sugar, and they are identified histochemically by the reactions of this sugar moiety. Furthermore, some of these substances have additional reactions due to the chemical or physiochemical features of their molecules.

The polysaccharides are classified into the following groups:

I) Simple polysaccharides:

Glycogen is the simple polysaccharide of the animal body. It is often called animal starch. It is a branched structure with straight chain units.

Glycogen is an important reserve of energy in the body. It is found in numerous tissues and organs, but the greatest proportion is contained in the liver cells and muscle fibers.

Glycogen is fairly soluble in water (15-20%) and may be dissolved in the protoplasm. It is difficult to demonstrate it in the living cell, but it can be precipitated with various fixatives, and can be demonstrated histochemically by the iodine reaction which gives a reddish brown colour with glycogen. In addition, glycogen gives a deep violet colour with periodic acid Schiff's reaction (PAS) and exhibits a dark red colour with best carmine staining.

To verify the presence of glycogen, a control is used. Material is subjected to the action of diastase or salivary amylase before staining. In this case, a negative result is obtained. This is due to the fact that glycogen has been removed by the diastase or amylase.

Sometimes, glycogen is not uniformly distributed in the cytoplasm of fixed cells particularly in the liver cells where it appears accumulated in certain regions of the cells, whereas the other regions remain almost empty. This phenomenon is referred to as “glycogen flight”. It is explained as being due to the fact that the fixatives used for the preservation of glycogen sweep this material in front of them until it becomes pressed against the part of the cell membrane opposite to the direction of diffusion.

Glycogen in the liver cells:

Liver cells are regarded to play an essential role in glycogen metabolism. They are the chief glycogen stores in the body, liver glycogen has two main sources. The first is

the glucose carried from the alimentary canal as the end product of carbohydrate digestion. This glucose passes to the liver by the hepatic portal circulation, and becomes polymerized in the liver cells into glycogen. The second source is the lactic acid produced in the muscle cells as a result of glycolysis (breakdown of glycogen) during muscle work. Lactic acid passes from the muscle cells to the liver cells where it also undergoes polymerization into glycogen. The hepatic portal vein which carries glucose to the liver is broken down into a large number of small vessels lying at the peripheral regions of the hepatic lobules. Hence, glucose diffuses from these vessels to the hepatic cells in the liver lobules. The peripheral cells of the lobule are the first to receive this supply and therefore they become loaded with glycogen. Glucose continues to diffuse into the hepatic lobules, but the rate of diffusion is markedly decreased in the inner regions of the lobules and thus the glycogen content is seen to be much less in the inner lobular cells than in the outer ones. Diffusion of the remaining carbohydrate material continues inwards until it reaches the central vein in the hepatic vein which ultimately leaves the liver and pours into the heart. Thus, it is clear that a considerable proportion of the glucose which reaches the liver is stored in the liver cells in the form of glycogen and the other proportion reaches the blood circulation to be distributed to the body cells where it is oxidized to give energy.

II) Mucoid substances:

Mucoids constitute the second group of polysaccharides. They are built up of sugar units in which a hydroxyl group is substituted by an amino group. These components are known as amino sugars or glucosamines.
The mucoids include three main classes:

1) Mucopolysaccharides
2) Mucoproteins and
3) Glycoproteins

1) Mucopolysaccharides:

The mucopolysaccharides occur naturally unassociated with proteins. The mucopolysaccharides which are associated with the structure of animal tissues are analogous to the cellulose of the plant cells. Examples are **hyaluronic acid and the chondroitin sulphates,** which are characterized by their content of amino sugars and urinic acids. The mucopolysaccharides which contain and acid as sulphuric acid or uronic acid linked with the carbohydrate units are identified as acid mucopolysaccharides. Other mucopolysaccharides, which do not contain acid groups are known as neutral mucopolysaccharides.

A) Neutral mucopolysaccharides:

The neutral forms of mucopolysacharides vary greatly in the degree of hydration, and thus they form moderately viscous fluids, jellies and solids of varying degrees of hardness as mucus, jelly of the umbilical cord and cartilage. A number of these substances occur as extracellular products such as the mucous of the glandular secretions or the matrix of the connective tissue and cartilage. The neutral mucopolysac- charides are also involved in the determination of the blood groups.

One of the well-known and widely distributed example of neutral mucopolysaccharides in **Chitin** which is the simplest neutral mucopolysaccharides. It exists mainly in the exoskeleton of insects and other arthropods. In the plant kingdom, the occurrence of chitin appears to be limited to fungi. Although the term chitin is used to mean the exoskeleton of many invertebrates, yet such exoskeletons contain less than 50% actual chitin and the remainder substance consists of protein or protein and calcium carbonate.

Chitin is characterized by being of the most insoluble organic materials but can be dissolved by hot HCl or H_2SO_4. Chitin is similar in chemical structure to the cellulose found in the plant cells, but it is not dissolved by cupric ammonium hydroxide as cellulose. Its structural similarity to cellulose is that both consist of long chains of monosaccharide units, but differ in the fact that cellulose units are glucose, whereas the units of chitin are glucosamine.

In other words, chitin consists only of N-acetyl-D- glucosamine units joined together in pairs by B (1-4) glucosidic linkages. Therefore, chitin is essentially cellulose in which the hydroxyl groups on C_2 carbon atoms are replaced by- $NHCOCH_2$. Chitin stains reddish purple following chromic acid oxidation and stains with Schiff's reagent and Congo red.

B) Acid mucopolysaccharides:

These substances are limited largely to animal sources, they are components of all epithelial mucins of the alimentary canal, and they occur in the majority of duct mucins also. They are characterized by the presence of an acid which is glucuronic acid as their second carbohydrate component. The acid mucopolysaccharides are classified into:

(a) Simple acid mucopolysaccharides:

The best known example of this group is hyaluronic acid which consists of the amino sugar glucosamine + glucuronic acid .Hyaluronic acid exists in a highly polymerized condition and acts as a barrier against the spread of external fluids or infections in the tissues. Hyaluronic acid is also found in the egg membranes. It is acted on by the enzyme hyaluronidase which causes its depolymerization. This enzyme is found in bacteria and in poisonous secretions as scorpion and snake venoms. It also occurs in

the testis and sperms. Hence, during fertilization, this enzyme acts to dissolve part of the egg membrane, and thus the sperm gains entrance into the egg.

Hyaluronic acid is also a part of the connective tissue system and hence it is distributed widely in the animal body. **Besides,** it exists in the aqueous humour and vitreous humour of the eye, in the synovial fluids of the joints and in the pleural cavity as well as in the umbilical cord.

The reactive group of hyaluronic acid is carboxyl group that stains with Alcian blue.

(b) Complex acid mucopolysaccharides:

These are substances consisting of glucosamine + glucuronic acid + sulphuric or phosphoric acid. Examples are heparin and chondroitin sulphate.

Heparin: is a complex acid mucopolysaccharide composed of glucuronic acid and sulphuric acid. It is an anticoagulant substance which prevents blood clotting in the tissues.

Chondroitin sulphate: is one of the main polysaccharide components of mammalian connective tissues and of cartilage. There are still differences of opinion as to its constitution but the general view regards the repeating unit as a disaccharide composed of sulphated N-acetylgalactosamine joined to glucuronic acid by a B-1,3 glycosidic linkage.

(2) **Mucoproteins:** are substances in which heoxamine- containing polysaccharide occurs in a firm chemical union with a peptide and the hexosamine content of the whole exceeds 4 percent of the total weight. Hexose and hexosamine are the usual carbohydrate components of mucoproteins, though uronic acid occur in some examples. The mucoproteins of the plasma are characterized by the presence of an acetyl hexosamino (N-acetyl glucosamine) and a hexose (mannose or galactose) in their polysaccharide portion. In addition, a methyl pentose (L-fucose) and the sialic

acids are commonly found in these conjugated proteins. Mucoproteins include the mucoids of the sub-maxillary gland and a certain fraction of the gonadotrophins. Mucoproteins are PAS-positive but are negative with the toluidine blue technique. Also they give positive result with protein methods.

(3) Glycoproteins: are distinguished from mucoproteins on basis of their hexosamine content of less than 4 percent. This division, is of service to biochemists and it may usefully be followed in histochemistry.

There is apparently no lower limit for hexosamine content which separates glycoproteins from simple proteins.

Simple proteins containing hexoses without hexosamine have not been found in nature. Examples of glycoproteins are ova- albumin and serum albumin. Both exocrine and endocrine glands secrete glycoproteins especially in the alimenary canal. Glycoproteins show position PAS reaction and are stained by protein methods.

III) Glycolipids:

The cerebrosides, represented chiefly by phrenosin kerasin and gangliosides, are the principal members of this group. They are present in the tissues of the central nervous system. These substances contain no phosphoric acid. By hydrolysis they yield sphingosine, long chain fatty acids and sugar usually glucose or galactose. Glycolipids are soluble in pyridine and hot alcohol but they are insoluble in water. They show a positive PAS reaction and are stained by lipid methods.

IV) Ascorbic acid:

Ascorbic acid is a strong reducing agent. It is regarded as a derivative of the hexose L-gulofuranose. The acid silver nitrate technique of Bourne is used in demonstrating the ascorbic acid in tissues, Vitamin C has the power to reduce silver nitrate solutions

without the action of light or heat. However ascorbic acid may be present either in reduced or oxidized form, and it is only the former that will give a positive reaction with acid silver nitrate.

Histochemical Demonstration of carbohydrates

1) Periodic acid-Schiff reaction:

Periodic acid (HIO_4) is a strong oxidizing agent which was employed for the chemical oxidation of glycols (CHOH.CHOH) and for amino-substituted alcohols ($CH_2OH.CHNH_2$) such as those which occur in the amino- acids serine, threonine and hydroxylysine. Periodic acid is an oxidant which breaks the C-C bonds in various structures, where these are present as 1:2 glycol groups (CHOH.CHOH), converting them into dialdehydes (CHO.CHO). The particular property of periodic acid is that it does not further oxidize the resulting aldehydes and these can, therefore, be localized by combination with Schiff's reagent to give a substituted dye which is red in colour. This is why periodic acid is superior to other reagents ($KMnO_4$, H_2O_2) commonly used in histochemistry for the oxidation of C-C bonds. It should be noted that the red dye stuff formed by the union of fuchsin sulphurous acid with dialdehyde is a new compound and not, as previously supposed, reoxidized fuchsin.

The amount of colour developed by the reaction is dependent on the amount of reactive glycol structure present in the tissues. The reactive groups concerned are those of the hexose sugars glucose, galactose, and mannose and of the methylpentose sugar positive results should be given by any substance which fulfils the following criteria:

(a) contains the 1:2 glycol grouping or the equivalent amino or alkylamino derivative, or the oxidation product CHOH-CO.

(b) does not diffuse away in the course of tissue fixation, (c) gives an oxidation product which is not diffusible, and (d) is present in sufficient concentration to give a detectable final colour.

The naturally-occurring animal substances which give a positive result in practice are monosccharides, polysac- charides, mucoproteins, glycoproteins, phosphorylated sugars, cerebrosides and inositol-containing lipids. After the use of ordinary aqueous fixatives only substances of high molecular weight remain in the tissues insufficient quantity to give a positive result. Such substances are polysaccharides, hyaluronic acid, mucoproteins and mucins.

Further consideration of the PAS reaction:

In this technique, sections are first treated with periodic acid and then stained with Schiff's reagent. This reagent is prepared by dissolving basic fuchsin in water to which is then added HCl and sodium or potassium metabisulphite. The solution is at first dark red, but under the effect of the sulphur dioxide liberated, it becomes transformed into a colourless fluid known as the leucofuchsin. When this colourless fluid becomes in contact with any aldehyde substance, a dark purple compound is formed.

Treatment of carbohydrates, in general, with periodic acid causes the oxidation of the glycol groups (HCOH-HCOH) carried on C_2 and C_3 in the carbohydrate substances. As a result, aldehyde groups (-HCO-HCO) are liberated. These react with the leuco-fuchsin giving a magenta or dark purple colour.

In carrying out the PAS technique it is usual to employ the double hydrate of periodic acid. A modification was employed in which periodic acid was produced from 1 percent sodium periodate by the action of dilute nitric acid. This method has the

advantage that a fresh periodic acid solution is produced from the stable periodate at the time of use.

Two solvents (water and ethyl alcohol) have so far been used for periodic acid in histochemistry. The use of 70% ethyl alcohol buffered with N.sodium acetate is also recommended. Five to ten minutes is sufficient for oxidation in 0.5% HIO_4: further oxidation is not required. The time of oxidation should be restricted to 5 minutes or less.

In the histochemical use of the PAS reaction, nothing higher than room temperature should be employed. The use of reducing rinse was used by Hotchkiss (1948) for the purpose of removing periodate or iodate remaining combined in the tissue after the periodic acid bath, since periodates and iodates restore the colour of Schiffs reagent. Hotchkiss rinse contains KI and $Na_2S_2O_3$ in 8% alcohol with 0.02 NHCl, it acts as an iodide-thiosulphate solution. It reduces both iodates and periodates when in the acid state,70% alcohol is used as a reducer or dist. H_2O is used to remove the trapped periodate and iodate.

Schiff bath is followed by two or three washes in sulphite water before dehydration and mounting are carried. After the last-sulphite, washing in running water for 5-10 minutes enhanced the final colour considerably.

Mucoproteins and neutral mucopolysaccharides acquire a deep purplish red colour, whereas glycoproteins are usually paler red or pink.

2) The alcian blue method:

The Alcian blue is considered the most specific dye available for acid mucopolysaccharides. The use of Alcian blue as a specific stain for mucins was introduced by Steedman (1950) who showed that the dye possessed great

advantages over other dyes for the staining of mucin: the important advantages are the rapidity and-ease of application. Alcian blue is a copper phthalocyanin dye which gives a blue colour.

Alcian blue does not distinguish chondroitin sulphuric acid complexes from mucoitin sulphuric acid complexes, and it therefore stains cartilage and mucin equally. In practice, it stains the acid mucopolysaccharides of epithelial and connective tissue mucin, but does not stain the majority of mucoprotein. Its selectivity of mucin depends on the use of short staining ; with longer staining nearly every tissue component becomes coloured. Lison used 0.5% Alcian blue in 0.5% acetic acid for 30 minutes, and Mowry 0.1% of the dye in 3% acetic acid for 30 minutes at pH 2.7 to 3.0. In fixed tissues and paraffin sections Alcian blue in acid solution stains connective tissues mucins and most epithelial mucins with usually negligible staining of the background proteins. In fresh tissues some of the nuclei stain even if the time of staining is kept to minimum.

By the use of a strong (red) counter stain the staining of connective tissues by Alcian blue can be overlaid, leaving only the connective tissue mucins stained blue.

Methods for Histochemical Demonstration of Carbohydrates

I) Carbohydrates: PAS reaction:

Preparation of solution (1), periodic acid 1%:

- Periodic 1 g
- Distilled water 100 ml

Preparation of solution (2), Schiff's reagent:

Dissolve 1 g of basic fuchsin in 200 ml of distilled water and boil. Shake the solution for 5 minute allow to cool to 50^{o}C. Filter and add to the filterate 20 ml N-hydrochloric acid. Cool to 25^{o}C and add 1 g of sodium metabisulphite. Store the

solution in the dark overnight, then add 2 g of activated charcoal and shake for 1 minute. Filter and store the filterate at 5°C in a dark bottle.

Note:

Always allow the aliquot of solution to reach room temperature before use and discard it after use to avoid contamination of stock solution.

1) Staining method:

1. Bring sections (all types) to water.
2. Oxidize in periodic acid (solution 1) for 5 minutes.
3. Wash in running tap water for 3 minutes, then in distilled water for 1 minute.
4. Stain sections for 15minutes with Schiff's reagent (solution 2).
5. Wash for 10 minutes in running tap water, and then counter stain in haematoxylin.
6. Wash in tap running water for 5 minutes.
7. Differentiate, if necessary, in 1 percent acid alcohol.
8. Wash for 5 minutes in tap water, dehydrate through the usual alcohol series; clear in xylene and mount in DPX.

Results: PAS –positive material : magenta. Nuclei : blue.

2) Glycogen: Best's Carmine method (Best, 1905):

(Paraffin, frozen and freeze dried sections).

Preparations of solutions:

1) Best's carmine stock solution:

–	Carmine	2 g
–	Potassium carbonate	1 g

–	Potassium chloride	5 g
–	Distilled water	60 ml

Boil the solution gently for 5 min., allow to cool, then filter. Then add 20 ml of ammonia (880).

2) Best's carmine staining solution:

Stock solution	12 ml
Ammonia (880)	18 ml
Methyl alcohol	18 ml

3) Best's differentiator:

Absolute alcohol	8 ml
Methyl alcohol	4 ml
Distilled water	10 ml

Method:

1. Bring sections from xylene to 70 percent alcohol, and then transfer to 1% celloidin for 5 minutes.
2. Wash sections in tap water.
3. Stain nucleus in alum haematoxylin for 10-15 minutes.

4. Wash in running tap water.
5. Stain in Best's carmine staining solution (solution 2) for 30 minutes.
6. Differentiate sections in 2 changes of solution (3) for 20 seconds.
7. Wash section briefly in 90 percent alcohol, then transfer to absolute alcohol.
8. Clear in xylene and mount in DPX or Canada balsam.

Results:

Glycogen will show the characteristic red stain. Nuclei are stained in blue.

3) Alcian blue-PAS method (Mowry, 1956):

It is a good method for differentiating between the acid and neutral mucopolysaccharides.

Staining solution

(1) Alcian blue pH 2.5.

(2) Schiff's reagent.

Staining method:

1. Bring sections to water, and then stain in Alcian Blue solution for 5 minutes.
2. Wash in distilled water.
3. Oxidize for 5 minutes in 1 percent periodic acid.

4. Wash well in distilled water.

5. Stain with Schiff's reagent for 8 minutes.

6. Wash thoroughly in running tap water for 10 min, and counter stain lightly, if desired, in Mayer's haematoxylin.

7. Differentiate in 1% acid alcohol.

8. Dehydrate, through alcohol series, clear in xylene, and mount in DPX.

Results:

acid mucopolysaccharides: blue

neutral mucopolysac-charides: red. Mixtures: purple.

4) Acid mucosubstances: Alcian Blue (Stedman, 1950 modified).

Staining solutions:

Alcian blue pH 2.5:

– Alcian blue 1 g
– 3 percent acetic acid 100 ml

Alcian blue pH 0.2:

Alcian blue 1 g

10 percent sulphuric acid 100 ml

Staining method:

1. Bring sections (all types, freeze dried recommended) to water.
2. Stain for 5 minutes in Alcian Blue solution of choice.
3. Wash briefly in distilled water, and counterstain in Mayer's carmalum for 2 minutes.
4. Wash in tap water, dehydrate through alcohol series, clearin xylene and mount in DPX.

Results:

Alcian Blue pH 0.2: Strongly sulphated acid mucosubstances acquire a blue colour.
Alcian Blue pH 2.5: most acid mucosubstances appear blue.

Notes:

Alcian Blue stains should be filtered before use, and counter staining should always be light.

5) Silver method for ascorbic acid (Bacchas, 1950) Method:

1. Place tissues within a short while of removal in a dark vial containing 5 percent silver nitrate at pH 2 – 2.5.
2. Incubate at 56^{o}C for 45-60 minutes.
3. Wash in distilled water for 10-15 minutes.

4. Treat for 1 hour in 5 percent sodium thiosulphate.

5. Dehydrate in dioxan.

6. Embed in paraffin wax.

7. Cut sections 6-10 □ thick and mount on slides.

8. Bring to water, and counterstain with haemalum.

9. Wash, dehydrate through grades of alcohol, clear in xylene and mount in Canada balsam.

Results:

Ascorbic acid: black.

NUCLEIC ACIDS

Nucleic acids are present in both animals and plant tissues. They control the replication of living substances. DNA is mainly present in the nucleus, it combines with proteins forming the chromosomes. DNA is the hereditary material, it has the instructions for building proteins that determine the characteristics of all organisms. On the other hand, RNA is mainly present in the cytoplasm and small amount occurs in the nucleus. The main function of RNA is associated with protein synthesis.

Nucleic acids are complex organic polymers composed of repeating units called nucleotides. Nucleotide = N base – Pentose sugar – a molecule of phosphoric acid.

DNA Structure:

Components of DNA:

According to Watson and Crick (1953), DNA is a macromolecule assembled from small organic molecules called nucleotides. DNA is a long molecule of constant diameter and made up of two long thin strands that are twisted together forming a double helix. Each strand is a linear assembly of nucleotides, may be 40 million are present along each strand.

DNA molecule contains only four kinds of nucleotides, the building blocks of nucleic acids. A nucleotide consists of a five-carbon sugar called deoxyribose, a phosphate group, and one of the following nitrogen-containing base:

Adenine	Guanine	Thymine	Cytosine
(A)	(G)	(T)	(C)

Each type of nucleotide in DNA has its component parts joined together in such the same way as the others. T and C are smaller, single ring structures (called pyrimidines). A and G are larger, double ring structures (called purines).

The four kinds of nucleotide bases making up a DNA molecule differ in relative amounts from species to species. Besides, the amount of adenine always equals the amount of thymine (A = T), and the amount of guanine equals the amount of cytosine (G = C). Therefore DNA is a polymer of four kinds of nucleotides with phosphate group attached to C_3 and C_5 of the sugar pentose and the nitrogen base linked is attached to C_1.

They realized that in certain orientations, A and T could become linked by two hydrogen bonds, and G and C would become linked by three. Suppose there were two strands of nucleotides, with their bases facing each other. The hydrogen bonds could easily bridge the gap between them, like rungs of a ladder.

DNA Replication:

Assembly of nucleotide strands:

Until then, no one had any idea of how the hereditary material is replicated (that is duplicated) prior to cell division. The Watson-Crick model suggested at once how this might be done.

Hydrogen bonds hold together the two nucleotide strands making up the DNA double helix, and those weak bonds are readily broken. Enzymes acting on given region of the DNA molecule can cause one strand to unwind from the other, leaving bases exposed in the unwound region.

As replication proceeds, each parent strand is twisted into a double helix with its new, partner strand is twisted into a double helix with its new, partner strand. Because the parent strand is conserved, each “new” DNA molecule is really half- old, half-new. That is why the process is called semicon- servation replication.

A closer look at replication:

Origin and direction of replication. Where does replication of DNA molecule actually begin? The two strands of the double helix start to unwind at one or more distinct sites, each being a short, specific base sequence called the "origin". A viral or bacterial DNA molecule usually has one origin; a eukaryotic DNA molecule has many. Unwinding usually proceeds simultaneously in the both directions away from an origin. Strand assembly occurs behind each "fork" that continues to advance as the double helix is being unwound.

Energy and enzymes for replication. A DNA double helix does not unwind all by itself during replication. It takes a battery of enzyme and other proteins to unwind the molecule, keep the two strands separate behind the replication forks, and assemble a new strand on each one. Even while one DNA region is being unwound, enzymes are winding up the replicated regions.

DNA polymerases are major replication enzymes. They govern nucleotide assembly on a parent strand.

Where does the energy come from to drive replication? They are triphosphates, meaning they have three phosphate groups attached, not one. Triphosphates readily give up phosphate groups and so transfer energy to specific reactions. During replication, DNA polymerases use the energy released when two of the phosphate groups are split away. That energy drives the addition of nucleotides to growing DNA strand. The unwinding process runs on energy provided by ATP.

Biological Significance of Nucleic Acids: Role of DNA in genetics:

It has been suggested that the genetic or hereditary material of the cell must have 2 separated functions. It must be capable of self-duplication and of initiating certain actions that are ultimately expressed in a given cell structure or function. DNA must therefore be capable both of duplicating itself and of providing the necessary

information of protein synthesis. The structure of DNA makes it easy for a certain molecular sequence of base pairs to be duplicated.

Inter-relation between DNA and RNA:

It has been determined that both the nuclear and cytoplasmic characteristics of a cell are determined by the genetic material of the nucleus, namely the chromosomes. In other word, the synthesis of specific cellular protein, enzymes and cellular structures is determined by the information in the genes. The question is, how are the instructions in the chromosomes transmitted to the recipient protein-synthesizing mechanism in the cytoplasm? The answer is as follows:

The nucleus has, of course, some of the same reaction activities as the cytoplasm. It can apparently make its own energy in the form of ATP, for it seems to have many of the glycolytic enzymes. It can synthesize various small molecules, and it has various enzymes to perform different vital functions. This means that the nucleus has its own complement of enzymes; in some cases the same types as found in the cytoplasm, in other cases different; but anyhow these enzymes must be present in the nucleus of the daughter cells. The DNA of the chromosomes has specification for the synthesis of these enzymes. But most of the enzymatic material, and much of the cellular synthetic mechanism is the cytoplasm and the information for the duplication of the material is also in the chromosomes. There is no direct production of protein by genetic DNA, but there is an intermediary substance which constitutes a link between the genetic information and the specific protein. This substance is the RNA which has the ability of controlling the synthesis.

Role of RNA:

It is clear from the above conclusion that the means of information between DNA in the nucleus and the synthesizing machinery in the nucleus and cytoplasm, is tied up

with the metabolism of the other nucleic acid namely RNA. In the cells of the mammalian tissues, about 20% is in the dense nucleolar material (the nucleolus contains no DNA, though it may have some DNA material concentrated at its borders). The rest of the RNA includes certain fraction which occurs in the nucleoplasm some of it being intimately connected with the chromatin material and the remaining fraction is in the form small particles, namely the ribosomes which are found in the cytoplasm.

Different experimental procedures have led to the hypothesis that RNA is synthesized in the nucleus; and that the completed RNA molecules then move out into the cytoplasm. But it is still not clear whether all the RNA of the cell is synthesized in the nucleus or synthesized in the cytoplasm.

Types of RNA:

Studies showed that there are **3** main types of RNA. These types are:

1) **Ribosomal RNA.**
2) **Messenger RNA (informational RNA).**
3) **Transfer RNA.**

These different types are similar in the presence of A, G, C and U, usually appear single stranded.

Protein synthesis in brief:

Along molecule of messenger RNA is formed from DNA, it moves to a site where there are ribosomes and where it becomes attached. Here, it is in the midst of a pool of 20 amino-acids, each of which carries a distinguishing label of transfer RNA. The messenger RNA calls out the first 3-letter at the end of its molecule. The 3 letter end of transfer RNA that is attached to a particular amino acid and fits the 3- letter word on the messenger RNA, responds to the call and becomes attached to the messenger

RNA at this site e.g. ACC of proline and CCU for glycine, etc. then the next word along the molecule of messenger RNA is called out and the 3-letter word of this messenger RNA and the amino acid attached to the transfer RNA become linked with the amino acid that has just been attached to the messenger RNA by its transfer RNA. The process continues so that one by one the amino acid specified by the code along the messenger RNA are linked both to the messenger RNA and together in the proper order. After their work is done and the macromolecule of protein is completed, the messenger and the transfer RNA are probably broken down.

Evidences of the role of RNA in protein synthesis:

1) RNA is found in large amounts in the cells which are reproducing actively as those of the embryos and tumors. It is also rich in viruses, yeasts and bacteria which are known to possess the power of autoduplication. These structures also possess large amounts of proteins.

2) RNA is abundant in the cells and tissues which are characterized by their marked ability of protein synthesis, e.g., pancreatic cells, and pepsin-secreting cells in the gastric mucosa, liver cells, nerve cells (being located in Nissl's bodies) and young oocytes. On the other hand, cells that do not actively produce proteins contain small amounts of RNA, e.g., heart tissues, muscle cells and kidney cells.

In silk worms, silk glands which only function to produce protein silk, are very rich in RNA.

3) Experimentally, it was found that when testosterone was injected, both RNA and protein markedly increased in the cells of the seminal vesicles.

4) In the field of autoradiography, it was shown by the use of S 35-labelled amino acids in the tissues of mice, rats and rabbits, that the incorporation of the amino acid

is highest in the protein-producing glands as the pancreas, gastric mucosa and neurons.

5) From the biochemical point of view, it was found in embryo development that synthesis of RNA always proceeds protein synthesis.

6) In microorganisms, the inhibition of RNA synthesis in Entamoeba was found to lead to inhibition of protein synthesis but the reverse is not true.

The effect of ribonuclease in such cases was explained by the fact that it units with RNA forming a compound which soon breaks down in the cell. This point is of great importance in the therapy of tumours. As well known growth of tumours is strongly rated to protein synthesis which is dependent on synthesis of RNA.

Action of reagents on DNA:

Irreversible reduction in the viscosity of DNA solutions are caused by acid and alkalies. This may be caused by rupturing of the hydrogen bonds between amine and hydroxyl groups of adjacent bases, and these groups are thus freed to react titrations. The viscosity of DNA solutions can also be irreversibly decreased by guanidine, urea, and phenol in relatively high concentrations.

The nitrogen and the sulphur mustards are known to cause abnormalities in mitosis as well as a high incidence of mutations. These compounds, in higher concentrations, may inhibit growth, and in low concentrations, they reduce the viscosity of DNA solutions.

It is worth mentioning that those radiations in the ultraviolet which induce mutations most readily are of the same wave length as those which characterize the absorption spectra of nucleic acids. This further supports the idea that alterations in nucleic acid structure are involved in the causation of mutations.

Action of ribonucleases and deoxyribonucleases on nucleic acids:

The highly polymerized nucleic acids or polynucleotides as they exist in nature are broken down into smaller component by the action of specific enzymes. Examples are the enzymes ribonuclease (Rnase) and deoxyribonuclease (Dnase) which split RNA and DNA respectively.

Histochemical Demonstration of DNA and RNA

A. General Reactions for Nucleic Acids

1) Reaction for organic phosphate:

The phosphate radical is demonstrated in the DNA of the nucleus by hydrolysis and subsequent fixation of the released PO_4 groups with ammonium molybdate. The resulting phosphomolybdate is reduced to a blue compound with benzidine (instead of the more usually employed stannous chloride).

2) Reactions for purines and pyrimidines:

The tetrazonium reaction after benzoylation or acetylation is the only reaction which has been used for the histochemical demonstration of purines and pyrimidines. It was first used for the demonstration of cytoplasmic ribonucleoprotein in tumour cells and staining chromatin in the chromosomes.

3) Reactions for deoxyribose and ribose:

In 1994, turchini and his associates developed a method for the detection of DNA and RNA depending on mild acid hydrolysis followed by reaction with phenyl (or methyl) trihydroxyfluorone.

The method has the advantage that distinct and different colours are given by the two nucleic acids in plant tissues, however the results in animal tissues are less

satisfactory. Using the methyl substituted fluorine, DNA stained violet to blue black and RNA yellow to red.

B. Reactions Specific for Nucleic acids

1) The Feulgen (Feulgen-Schiff) reactions for DNA (Feulgen and Rossenback, 1924):

In 1924, Feulgen and Rossenback introduced a reaction for demonstrating thymonucleic acid (DNA). The reaction\hydrolysis (with N-HCl at 60^{o}C) which could release aldehyde groups from the deoxypentose sugar of DNA. Following hydrolysis, the tissues are washed and then transferred to Schiff's reagent which reacts with the exposed aldehyde groups to produce a purple dye in the nuclear chromatin alone. The optimum times of acid hydrolysis depend on the fixative employed. Prolonged hydrolysis leads to a decrease in staining which is probably caused by chemical alteration, depolymerization and extraction of DNA. A gentle acid hydrolysis will transform deoxyribose into hydroxyl-aevulinic aldehyde ($HOH_2.CO.CH_2.CHO$); this labile aldehyde is responsible for the purple colour given with Schiff's reagent in the Feulgen test. The specificity of the test, or more strictly speaking its specificity for the localization of DNA, becomes the object of increasing doubt. It may be well to state here that its specificity for DNA is able to survive and that it may be used with confidence for the purpose. On the other hand, RNA takes no part in Feulgen reaction.

Preparation of solutions:

1) N-HCl

- Hydrochloric acid, (conc.) 8.5 ml
- Distilled water 91.2 ml

This solution should be preheated to 60^{o}C.

2) Schiff's reagent (See PAS reaction)

3) Bisulphite solution

10 percent potassium metabisulphite 5 ml N-Hydrochloric acid 5 ml

Distilled water 90 ml

Method:

1) Bring sections (all types) to water and rinse them in N-HCl at room temperature for 1 minute.
2) Transfer sections to N-HCl at 60^{o}C.
3) Wash sections in N-HCl at room temperature for 1 minute.
4) Treat sections with Schiff's reagent for 45 minutes.
5) Rinse sections in 3 changes of bisulphite solution (2 minutes in each).
6) Wash well in distilled water.
7) Counterstain, if required, in 1 percent light Green for two minutes.
8) Dehydrate through alcohol series, clear in xylene and mount in Canada Balsam or DPX.

Result:

DNA: appears reddish purple; Cytoplasm: green.

2) Hibiscus method for DNA (El-Aaser, 1982)

Hibiscus subdariffa is a nuclear stain and was found to have a wide application on different types of cells such as liver, pancreas, intestine, bladder, kidney cells & blood film. The stain works at an acid pH; 2.0. chromatin stains red when treated with hibiscus extract & turns blue when it is followed by lead nitrate.

Reagents:

10% Hibiscus subdariffa (Karkadeh), N Hydrochloric acid, 1% lead nitrate.

Method:

1) Cells are first treated by HCl pH 2.0 for 10-20 min. depending upon the type of tissue and the fixative used.
2) Transfer sections to 10% Hibiscus extract stain.

3) Rinse with water then acidify with one drop conc HCl, in order to precipitate lead as lead chloride.
4) Treat with 1% lead nitrate, rinse with water, counterstain with eosin, dehydrate and mount.

Results: nucleus: dark blue, cytoplasm: light red.

This stain has many advantages – It gives two stable colours, either red which might be counterstained with light green (cytoplasm) or DNA appears blue after treating the section with 1% lead nitrate which could be counterstained with eosin.

Notice that: the site of binding of the Hibiscus with DNA is different form that of Feulgen's reaction. The mechanism, the nature of binding to nuclear chromatin and the specificity of the stain is not fully understood.

3) Methyl green-pyronin for DNA and RNA (Modified by Kurnick,1955):

This technique is used for demonstrating both DNA & RNA. It was first published by Pappenheim (1899) and modified by Unna (1902), and often referred to as the Unna Pappenheim Stain. Several attempts have since been made to render the method more specific.

Methyl Green is an impure dye containing methyl violet. To obtain satisfactory results with this method it is necessary to wash Methyl Green (2 percent aqueous solution) six or more washings with equal parts of chloroform in order to remove all the violet. When treated in this way, Methyl Green seems to be specific for DNA at a slightly acid pH. Methyl Green is a basic dye, and its affinity for DNA is not fully understood. However, Kurnick (1955) suggested that binding of Methyl Green to DNA involves two sites, two amino groups on the dye combining with two phosphoric acid groups of the DNA. If the dye is used at a slightly acid pH.

Pyronin is also a basic dye. It has the affinity to stain RNA yielding a pink colour.

Pyronin Y, is less specific and a controlled staining time must be used, and the pH of the staining solution is critical. When using pyronin solution, it is always advisable to use a control section which has been subjected to ribonuclease digestion. Pyronin Y obtained commercially varies, and certain batches fail to give satisfactory results, thus it is necessary to compare each batch on its arrival against a known good batch of dye. The chemical basis of the method is not fully understood.

Preparation of solutions:

1) **Methyl Green:** 2 gm methyl green in 100 ml distilled water.

Dissolve the Methyl Green in the distilled water by stirring well. Pour the solution into a separating funnel. Add

100 ml chloroform and shake well; pour off contaminated chloroform and repeat until no more violet colour is extracted.

2) **Pyronin Y:** 2 g

3) Staining solution:

- Methyl Green 7.5 ml
- Pyronin Y 12.5 ml
- Acetate buffer, pH 4.8 30.8 ml

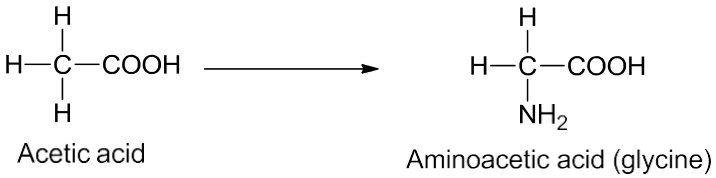
Method:

1) Bring sections (all types, freeze dried recommended) to water and stain for 4-10 minutes in Methyl Green Pyronin solution.
2) Plot dry, then rinse rapidly in absolute acetone in xylene.
3) Wash rapidly in 10 percent acetone in xylene.
4) Pass rapidly in 50 percent acetone in xylene.
5) Rinse in xylene and transfer to fresh xylene and mount in DPX.

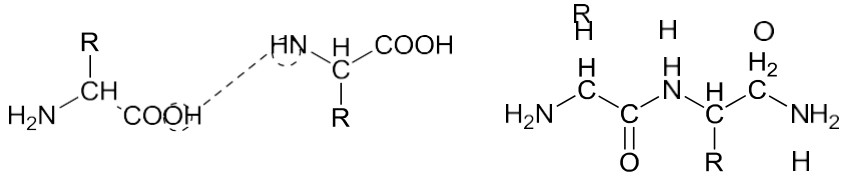
Results: DNA : green ; RNA: red.

PROTEINS

Proteins are organic substances of high molecular weight. They are formed by a number of amino acids which are derived from aliphatic acids such as acetic acid (CH_3COOH) by replacement of one hydrogen atom by the amino group ($-NH_2$) as shown by the simplest of these compounds, namely amino acetic acid or glycine ($CH_2.NH_2.COOH$)).



One of the most important characteristics of the amino acids in their capacity of combining with each other to form long chains. This property is due to the presence of the carboxyl group (-COOH) and the basic amino group [$-NH_2$] in each molecule. Such substances, which contain at the same time an acid group and a basic group are called amphoteric. Proteins are amphoteric, i.e., they possess both acid and basic properties, depending upon the reaction of the solution. They usually form colloidal solutions (emulsoids) in water. Separation from solution is accomplished by precipitation in the presence of electrolyte at low temperatures with alcohol at varying H^+ concentrations. Physical techniques using centrifugal methods or electrophoresis may also be used.



(R and R` represent different group or residue for each amino acid).

The condensation of amino acids occurs in such a way that the acid group of one molecule combines with the basic group of another molecule with the loss of one

molecule of H_2O. When the amino group ($-NH_2$) of one amino acid joints the carboxyl group (-COOH) of another one, a dipeptide results, and when a number of such linkage occur, a polypeptide is formed. A large number of amino acids may be united into a polypeptide. About 25 amino acids are known to exist in nature besides other ones which exist in the different proteins. 8 amino acids are essential to human bodies but cells cannot build them and they must be obtained indirectly from food.

Proteins are ultimately built up of C, H, O, N and in some cases with traces of sulphur, iron, copper and phosphorus. One the other hand, amino acids could be obtained from proteins by hydrolysis under the effect of enzymes or by boiling with strong acids or bases.

Classification of proteins:

From the biochemical point of view, proteins are divided into 3 main categories:

I. **Simple proteins:** These are proteins that yield on hydrolysis amino acids only.

II. **Conjugated proteins:** Which yield on hydrolysis other substances of non-protein nature. The conjugated proteins include proteins in which a simple protein is combined with another substance called prosthetic group. Examples of this group are:

1) **Nucleoproteins:** In which the prosthetic group is the nucleic acids. Example: nuclein, nucleohistone from nuclei- rich material (gland).

2) **Glycoproteins and mucoproteins:** as mucin found in various cells and tissues. They consist of proteins and carbohydrates.

3) **Lecithoprotiens: consisting of lecithin and protein e.g. blood fibrinogen.**

4) **Chromoproteins:** include a number of coloured substances as the respiratory pigments haemoglobin (ironporphyrene haeme + globin) and haemocyanin (copper + protein) as well as number of respiratory enzymes as cytochromes and flavoproteins.

III) Derived proteins: These include compounds of coagulated proteins and the partially soluble proteins as proteoses, peptones and polypeptides. This category was originally devised to include the artificially synthesized proteins. Those proteins which may be isolated after the removal of the non-protein prostetic groups of conjugated proteins might also be included here rather than among the simple proteins (e.g., protamines and histones from nucleoproteins or from haemoglobin).

Simple proteins:

The simple proteins are classified into various groups according to their solubilities. These include:

1) Albumins: Soluble in water and coagulated by heat, e.g., serum albumin.

2) Globulins: Insoluble in water, but soluble in various dilute salt solutions, e.g., serum globulins.

3) Histones: Soluble in water, but insoluble in dilute solutions of ammonia, e.g., nucleohistones of thymus gland.

4) Protamines: Soluble in water, and coagulable by heat.

5) Albuminoids: Insoluble in all natural solvents, but soluble in acids and alkalies. This group includes mainly collagen, reticulin, elastin and keratin which are usually know as scleroproteins.

From the structural point of view, the simple proteins are classified into:

A) Fibrous proteins: They can also be called structural proteins as they are concerned primarily with the maintenance of morphology on both the grow and microscopic levels. Contain the collagens, reticulins, elastin, keratin and fibrin, such proteins are insoluble in aqueous media and highly resistant to most animal proteases.

B) Globular proteins: As globulins, histones, protamines and albumins which are soluble in aqueous media and many of them have been crystallized. However, proteins which have received marked interest form the histochemical point of view are the following:

1) Histones:

These are proteins characterized with a strong alkaline reaction. Some of them unite with nucleic acid to form nucleohistones which are found in large amounts in the tissues of certain organs such as the pancreas and thymus gland.

Histones are known as basic proteins since they are composed essentially of the amino acids arginine, histidine and lysine.

2) Protamines:

They also have strong alkaline reaction. To this group belong the proteins found in the spermatozoa of many fishes.

Protamines also combine with nucleic acids forming nucleoproteins which are the chief constituents of the chromosomes.

3) Collagen and reticulin:

Collagen consists of bundles of coarse non-branched fibers which are red stained with van Gieson, faintly pink with PAS and yellow or brown with silver impregnation techniques.

Reticulin. On the other hand, consists of fine branching fibres which are unstained or faintly stained with van Gieson and black by silver impregnation.

As regards the relation between these two substances. Some authors observed that when certain connective tissue fibers occurred singly they gave the staining reactions of reticulin, but that on joining into a bundle of fibers, they stained as collagen. For these reasons, these authors regarded collagen and reticulin to be identical. Dublin (1946), however, regarded reticulin as the precursor of collagen.

On the other hand, Lillie (1947), using periodic acid Schiff, succeeded in obtaining a deep staining of the reticulin network of a variety of organs, whereas collagen was only faint pink. This new evidence was taken as an indication that collagen and reticulin are not chemically identical.

In addition, Glegg (1953) showed that reticulin contains galactose, glucose and ribose in large quantities, whereas collagen contains very small amounts of these substances.

Physical characters of collagen and reticulin:

Collagen is regarded as a scleroprotein which exists in the soluble form with is individual molecules widely separated. When fibers appear in this solution, they do so by arrangements of the molecules into micellar form i.e., into aggregation of molecules oriented in a particular manner. According to Astbury (1940), the X-ray diffraction studies indicate that collagen fibers are composed of fully extended chains of carbon and nitrogen atoms held together by lateral bonds of electrovalent nature. These linkages though comparatively strong, yet easily penetrated by ions.

Electron microscope studies showed that collagen fibers were crossly striated by 650 A^{o} cross-striations. They are similar in this respect to the striated muscle fibers.

It has been found that whereas collagen is composed of bundles of regularly arranged fibrils, reticulins have a membranous structure in which the fibrils occur in a disorderly manner. They concluded that reticulin differs from collagen in 4 respects:

1) In having an abundant carbohydrate-rich matrix.

2) In having randomly arranged fibrils.

3) In its membranous structure.

4) In failing to yield gelatin on boiling.

Biochemical features of collagen and reticulin:

As regards the amino-acid composition of collagen and the product of its partial hydrolysis (gelatin), they show a high content, particularly of glycine, Proline and hydroxyproline. The relative amino acid contents of reticulin are nearly the same as those of collagen except for proline and hydroxyproline in which reticulin is somewhat deficient.

Three form of collagen have been described:

i) One form referred to as acid-soluble collagen and is extractable with dilute acetic acid.

ii) The second form can be extracted from the skin with a weak alkaline phosphate buffer or means of neutral salts. It is therefore called either alkali-soluble or neutral salt- soluble collagen.

iii) The third form known as the insoluble collagen which constitutes the bulk of tissue collagen.

The neutral salt-soluble collagen is regarded as the precursor of the insoluble form. Many authors believe that during early development, neutral salt-soluble collagen is secreted by fibroblasts and is transformed into collagen fibrils. Reticulin was found by

Windrum (1955) to contain fatty acid closely bound to protein and therefore it resists extraction with trichloacetic acid. They also contain small amounts of phospholipids.

Effect of temperature on collagen and reticulin:

It was found that collagen fibers undergo marked shrinkage on heating in the presence of H_2O. In such case, they markedly lose resistance to trypsin and also most of their optical character. French (1945) found that formalin fixation increases the temperature at which collagen undergoes thermal shortening from $65^{O}C$ about $90^{O}C$.

4) Elastic tissue:

Elastins are branching fibers of varying size and diameters. They form an important constituent in arterial walls, tendons, elastic cartilage and dermis of the skin.

Elastin tissue is easily distinguishable by a large number of staining procedures. Orcein is widely used in elastin staining. It was recently found that orcein is a mixture of closely related amphoteric dyes which could be separated into 4 principal coloured fraction. Fractions I and II were cationic. Fraction III anionic and fraction IV partly anionic and partly electroneutral. When these were used to stain formalin-fixed elastic tissue. Fractions I, II and III stained certain fibers throughout the pH range up to 9.5, whereas fraction II stained the nuclei blue. Elastins stain with Congo red dye, aldehyde fuchsin technique and weakly stained with PAS reagent.

Elastin differs chemically from collagen in some important respects. Chiefly in possessing far less arginine, histidine and lysine and more leucine and valine. It contains no tryptophan but has slightly more tyrosine than collagen.

Elastic tissues contain more lipid than collagen. They are attached easily by pepsin and to a small extent by trypsin. Elastic fibers from all sources are remarkably insoluble in inorganic or organic solvents differing in this respect form collagen which

dissolves for instance in 2% acid. This difference is explained by the fact that elastin contains 90% of non-polar amino acids and collagen only 50%.

With advancing agent, marked changes occur in the elastic fibers. These take the form of longitudinal splitting breaking into fragments and ultimately into granules. These changes are associated with chemical changes in the amino acid content and in the calcium of the elastic fibers.

5) Keratin:

Keratin is a fibrous protein occurs in hair, nail, hooves and epidermis. It is characterized by its high content of the basic amino acids arginine, lysine and histidine and of the sulphur-containing amino acid cysteine. Keratin in hair differs from that found elsewhere in its high values for cysteine and lower values for cysteine. It has a strong affinity for both basic and acidic dyes and is impervious to the action of pepsin and trypsin. Keratin can be demonstrated by performic acid- methylene blue/alcian blue technique.

Process of keratinization:

One of the principal changes which takes place during cornification in normal human skin is the oxidation of the SH groups in the lower strata to form SS groups in the stratum corneum. It is also found that the keratinization of skin into nails and hairs is accompanied by an increase of cysteine and a decrease of methionine. The histidine, lysine and arginine ratio are the same in both cases.

Bonting (1950) found that adult skin contained only 60% of the total sulphur present in skin form young animals. During maturation, i.e., during progress from infancy to the adult structure the cysteine decreased to 44%.Bonting considered that the cysteine lost from the skin was transferred to the keratin of the adult hair.

General Identification of Proteins

The classical methods which have been employed for the demonstration of proteins are:

1. **Millon's reaction for tyrosine.**
2. **The diazonium reaction for tryptophan and histidine,**
3. **The xanthoproteic reaction for phenolic compound.**
4. **The Saguchi reaction for arginine.**
5. **The nitroprusside test for sulphydryl groups.**

A positive reaction indicates the presence of protein since free amino-acids do not occur in tissue preparations from animal sources. All these reactions except the last can be applied to fixed tissue sections, but only two (the Millon and the diazonium reactions) are essentially suitable for the demonstration of proteins.

1) The Millon reaction:

) is based on the presence in the protein molecule of the hydroxyl-phenyl group. Positive result can be given by any compound not substituted in the meta – position, but such compounds are not found free in tissue; and the only known amino-acid containing the hydroxyl-phenyl group is tyrosine.

The original Millon's reagent was made by digesting mercury in nitric acid and dilution the resulting solution with water. For the histochemical use the reagent was modified for use in the cold, and employed in the demonstration of mitochondria in

freeze-dried sections. If it is used for paraffin sections the application of moderate heat is essential and the warm reagent must be washed off with dilute nitric acid as soon as the maximum colour has developed. The reaction proceeds in two stages, first, a nitrosophenol is produced by the substitution of No of H ortho or meta to the hydroxyl of the phenol. Secondly, Hg^{2+} is incorporated into a new ring. By chelation, which includes the nitrose group. the new complex is red in colour.

2) The diazonium reaction:

Diazonium compounds are prepared by the action of nitrous acid in the cold on the salts of primary aromatic amines; sulphanilic acid (p-aminobenzene sulphonic acid) is commonly used for this purpose.

The resulting compounds, acting in alkaline aqueous solutions as diazonium hydroxides, combine with the phenol group of tyrosine, the indole group of tryptophan and the heterocyclic imidazole group of histidine to give coloured products. Other amino-acids found in proteins are known to be able to react with diazonium salts to give products which may be coloured in some instances.

A method for the simple demonstration of proteins in tissue sections, making use of the above principle is the **"coupled tetrazonium reaction".**

3) Nitroprusside methods for SH:

All the modification of the nitroprusside reaction depend on the production of a purplish-red colour when SH groups come into contact with sodium nitroprusside in the presence of hydroxyl ions (usually NH_4OH) and saturated ammonium sulphate. This method was found to be useful for substances possessing free SH groups such as cysteine and glutathione. The majority of workers have employed frozen sections of fresh material in their work, others, however, applied the method to the study of

alcohol or formalin-fixed sections, also after brief treatment with 2 percent trichlor-acetic acid to release SH groups from the tissue proteins. It was suggested that the red color of the positive reaction must be stabilized by previous treatment with 5% zinc acetate for few seconds.

Methods for Histochemical Demonstration of Proteins

1) The Millon reaction:

Preparation of the reagent:

Add 10 g. $HgSO_4$ to 100 ml. 10% H_2SO_4 and heat until dissolved. Make up to 200 ml. Add 0.5% $NaNO_2$.

Method:

1) Bring sections (formalin; paraffin) through 50% alcohol to water.
2) Place sections in a small beaker containing the reagent.
3) Warm gently to the boiling point, and then stop heating.
4) Allow the solutions to come to laboratory temperature.
5) Wash in three baths of distilled water (2 minutes for each washing).
6) Mount in glycerine jelly, or dehydrate, clear and mount in DPX or other suitable synthetic medium.

Result: Proteins containing tyrosine are stained red, pink or yellowish-red.

2) Mercury-bromphenol blue method for proteins:

(Carnoy formalin; etc. avoid osmium).

Preparation of solutions:

Two alternatives have been employed: (1) 1%. Alcoholic bromphenol blue saturated with $HgCl_2$ and 0.05% bromphenol blue in2% aqueous acetic acid. The second of this is recommended.

Method:

1. Paraffin sections adhering to slides are brought to water.
2. Stain in one of the two alternative solutions for 2 hours at room temperature.
3. Differentiate in 0.5% acetic acid for 5 minutes.
4. Transfer sections directly into tertiary alcohol.
5. Clear in xylene and mount in a suitable synthetic medium.

Result: Proteins, deep blue.

3) Diazotization method for tyrosine (Lillie, 1957). (Formalin, etc., hot chloroform / methanol; paraffin sections).

4) Method:

1. Paraffin sections, dewaxed and brought into water.

2. Treat for 16 hrs at 3°C in a mixture containing 6.9 g $NaNO_2$, 5.8 ml acetic acid, distilled water to 100 ml.

3. Wash in 3 changes of ice-cold dist. Water (5 seconds each).

4. Treat for 1 hour at 3°C with a mixture containing 1 g sulphanilic acid, 1 g KOH. 2 g urea and 100 ml 70% alcohol.

5. Pass through 3 washes of 0.1N HCl (5 minutes in each).

6. Wash in running water for 10 minutes.

7. Dehydrate in absolute alcohol, clear and mount in neutral synthetic resin.

Result: Protein containing tyrosine stain purplish-red to pink.

5) The coupled tetrazonium reaction: (Formalin, alcohol, freeze-dried; paraffin sections). **Method:**

1. Bring sections to water and remove mercury if necessary.

2. Place sections in freshly tetrazotized benzidine at 4°C for 15 minutes. alternatively one can use a 0.2% aqueous solution of Fast blue B salt in tris buffer pH 9.2, for 5 minutes at room temperature.

3. Wash in water and in three changes of veronal acetate buffer at pH 9.2 (2 minutes in each change).

4. Immerse in a saturated (1 g in 50 ml) solution of H-acid in veronal acetate buffer at pH 9.2 for 15 minutes.

5. Wash in water for 3 minutes, dehydrate in alcohol, clear in xylene and mount in balsam or DPX.

Result: The majority of tissue components stain in shades of reddish brown; the coupled tetrazonium reaction is preceded by mild heat and benzoylation; positive structures now stain either in shades of deep reddish brown or, in the case of collagen, in purplish red. These colours are quite stable.

6) Disulphides: Performic acid-alcian blue method

(Adams & Sloper, 1956)

(Freeze-dried, paraffin and cryostat sections).

Preparation of solutions:

1) Oxidizing solution: Performic acid:

98% formic acid	40 ml
100 vol. hydrogen peroxide	4 ml
Sulphuric acid	0.5 ml

The oxidizing solution must be prepared fresh daily, and should be allowed to stand for 1 hour before use.

2) Staining solution:

Alcian blue	1 g
98% sulphuric acid	1.7 ml
Distilled water	47.2 ml

Method:

1) Bring sections to water and remove excess water by blotting.
2) Transfer to oxidizing solution for 5 min, then wash for 10 min in tap water.
3) Dry sections by gently heating to 60^{o}C till just dry.

4) Rinse sections in tap water, then stain for 1h in Alcian blue solution at room temperature.
5) Wash well in running tap water, and counterstain if desired.

6) Wash in water, dehydrate in alc. series, clear in xylene and mount in DPX.

Result: Disulphides are stained dark blue.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