حيوان 5

(Histochemistry and physiology)

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الفصل الدراسي الأول

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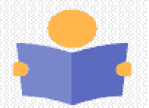

نص للقراءة والدراسة

أسئلة للتفكير والتقييم

رابط خارجي

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Part 1

Lecture 1

Historical Review of Histochemistry

Histo = histology: chemistry = chemical reactions

The branch of science concerned with the qualitative and quantitative assessment of chemical compounds in a cell/tissue using stains/dyes and microscopy.

Histochemistry/cytochemistry is as old as histology itself. In early 19th century, histochemical research to study chemical components of biological structure in combination with chemistry and biology started first in botany in France (Raspail,1825).

He stained starch in plant tissues blue with potassium iodide solution under the light microscope and demonstrated its localization microscopically Then, he published an essay on microscopic chemistry for the first time (Raspail, 1825)

Later, from the 1840's to 1870's, histochemistry in zoology and medicine was developed mainly as biological chemistry, together with histology (Lehmann,1842).

Among these zoologists, anatomists and pathologists who were at that time interested in analyzing chemical constituents in animal tissues

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including human, Miescher (1874) was the first to introduce cell fractionation to analyze nucleic acids in nuclei of leukocytes.

During these times in the 19th century, this new field was called as “microchemie” in French or “Mikrochemie” in German, which meant microchemistry in English. Microchemistry was, in other words, microscopic chemistry or chemical microscopy and meant to observe chemical reactions in situ under microscopy.

In early 20th century, aniline dyes were frequently used to stain tissues in anatomy and pathology.

Histologists and pathologists were much interested in new dyes and less interested in histochemistry at that time. When Lison (1936) published his famous book entitled “Histochemie Animale”, many histologists were again interested in histochemistry.

Lison classified histochemical techniques into two categories, “méthodes extra situm” or “méthodes extractives” and “méthodes histochimiques in situ” or “méthodes topochimique” in French.

He proclaimed Histochemistry to be the new science. Then, many histologists, anatomists, pathologists, physiologists started to study this new field and published many original papers and books dealing with histochemistry and cytochemistry. Innumerable literature, as well as textbooks and handbooks, are available from the 1950’s to 1990’s.

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Nagata (1995) proposed that the field should be designated General Histo-cytochemistry just like we have General Histology. One of the foremost histochemists in Nigeria is the Late Prof. Caxton-Martins.

Histochemical and Histological Methods

The purpose of histochemical and histological methods is to provide as exact a picture of living tissue as possible. This is exceedingly difficult as any intervention, such as taking a biopsy and preparing it for microscopic examination, effects changes in tissue structure and reactivity. Interpretation of the result therefore requires an understanding of the effects of a11 aspects of the intervention. One of the primaries aims of this book is to give a theoretical background for such understanding.

The discipline of histochemistry lies on the boundary between histology and biochemistry. The principal aim of histochemistry is to obtain information regarding the chemical composition and localization of the components of the tissue. As the microscope is the most important single instrument used in histochemistry, the reaction products must be directly visible or be made so. They will in general be colored.

Aim of Histological Methods. The chief aim of these methods is to visualize and differentiate between tissue components, not to determine the chemical composition. A division between qualitative and quantitative histochemistry is widely recognized.

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1. Qualitative histochemistry is concerned with the occurrence and localization of histochemical demonstrable components.

2. Quantitative histochemistry also assesses the amount of the individual chemical components.

This book is mainly concerned with qualitative histochemistry. Distinction is

sometimes made between histochemistry and cytochemistry, depending on whether

tissues or individual cells are being examined.

The Histochemical Reaction

In principle all reactions that ultimately produce colored products on tissue sections may be considered as histochemical. In such a reaction it is necessary to understand both the chemical and the histological aspects of the method. The chemical aspects comprise a description of the mechanisms involved, the specificity or selectivity, and the sensitivity.

The histological aspects require that the precision with which localization is achieved should be assessed. For a reaction to be designated as histochemical, an account of both chemical and histological aspects must be given. We have adopted the following headings throughout this book.

1- Mechanism

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The following sub-headings have been used: reactive groups, reagents, reaction

products, reaction equation, and sources of error.

2- Selectivity

As tissues contain a vast range of different compounds, it is essential to know which of these may react in the chosen method. If only one chemically well-defined compound reacts, the method is specific. In the great majority of cases the method will, however, demonstrate a restricted group of chemical compounds and may then be designated selective. Very few methods are specific while the remainder are selective. In several cases the selectivity of a method may be

increased by inactivating or removing certain reactive groups. These interventions are called respectively blocking and extraction.

3- Sensitivity (Detection Limit)

In the assessment of a method, it would be useful to know what amount of material and how much of the demonstrable chemical entity within the material are required for the reaction product to be perceived. In quantitative chemistry this can be expressed as the limit 0/ detection.

In an ideal world it would be possible to cite the absolute amount of the chemical entity in question that reftects the limit of detection for or sensitivity of the technique applied. In histochemistry the number of variables involved is so large that this is only rarely possible. In this book

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we have therefore chosen to use the term in the relative sense to indicate the sensitivity of one method compared to others used for demonstrating the same chemical entity. In this way the need forabsolute figures is avoided.1 The Scope of Histochemistry 5

It is important to appreciate that relative sensitivity is not a constant property of one method. All steps before and after staining, the exact technical details of staining, the optical system used, the nature of the precipitate, and the substantivity of the reaction product influence the relative potential of one technique to demonstrate a given chemical entity. (The term substantivity refers to the ability of the reaction product to attach itself or "stick" to various tissue components, notably proteins. A high substantivity implies low solubility in lipids.)

4 Localization

This is the central histological consideration which refers to the location of reaction products. Changes in the location of the chemical compound under examination may take place during fixation, dehydration, embedding, sectioning, and during or after the histochemical reaction itself.

The compound, one wishes to demonstrate, the intermediate products during the histochemical reaction or the final product may all diffuse. This may result in the compound or reaction product being completely removed from the tissue, diffusely deposited throughout or even specifically bound to other areas away from its site of origin.

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According to Grimelius (1968), an assessment of the localization of the reaction

product may be made by:

1. The use 0f consecutive sections. The histochemical reaction is performed on one of two adjacent thin sections (1-2fLm). The second section is then stained using a general oversight method (Sect.31.2) or a second histochemical procedure whose reaction product distribution is weIl established.

2. Double-staining technique. Two different histochemical reactions are performed consecutively on the same section. It is essential that the two methods used do not affect each other qualitatively or quantitatively. The assessment is made by taking a photograph after the first reaction has been performed and comparing the result with that obtained after the second. If possible, the test should be repeated with the reactions in reverse order.

3. Restaining technique. A photograph is taken after the first reaction as above.

The section is then destined, a second reaction performed, and the results compared Interactions between the two reactions should be checked for as with the double staining technique. Comparison is facilitated if thin sections are used.

This is a valuable method as it is often possible to perform three or more reactions consecutively with destainings inserted between. Unfortunately,

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the approach is limited by the almost unavoidable damage to the section during destaining.

4. Differential count technique.

In this method the number of cells demonstrated by different reactions is compared.

5 Controls

For the majority of histochemical methods, control reactions are required in order to discriminate between true positive reactions and non-specific reactions. Thefollowing control reactions are usually necessary:

1. A positive control reaction performed on a section from another block of the same tissue or another tissue containing the chemical substance under consideration. The substance should be at a concentration close to the relative limit of detection (eliminates false negative reaction).

2. A negative control reaction performed on a positive control tissue block, a consecutive section, or both. Here the control sections are subjected to the same treatment as the test section but a key reagent in the reaction sequence is omitted (eliminates a false positive reaction).

3. Blocking and/or extraction procedures. These assist in determining the selectivity or specificity of the histochemical reaction.

Histopathology- Definition it is a branch of pathology which deals with the study of disease in a tissue section.

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The tissue undergoes a series of steps before it reaches the examiners desk to be thoroughly examined microscopically to arrive at a particular diagnosis. To achieve this it is important that the tissue must be prepared in such a manner that it is sufficiently thick or thin to be examined microscopically and all the structures in a tissue may be differentiated.

The objective of the subsequent discussions will be to acquaint the staff with their responsibility, the basic details of tissue handling, processing and staining.

The term histochemistry means study of chemical nature of the tissue components by histological methods.

The cell is the single structural unit of all tissues. The study of cell is called **cytology.**

A tissue is a group of cells specialized and differentiated to perform a specialized function. Collection of different type of cells forms an organ.

Type of material obtained in laboratory

The human tissue comes from the surgery and the autopsy room from surgery two types of tissue are obtained.

As biopsy- A small piece of lesions or tumor which in sent for diagnosis before final removal of the lesion or the tumor (Incisional biopsy).

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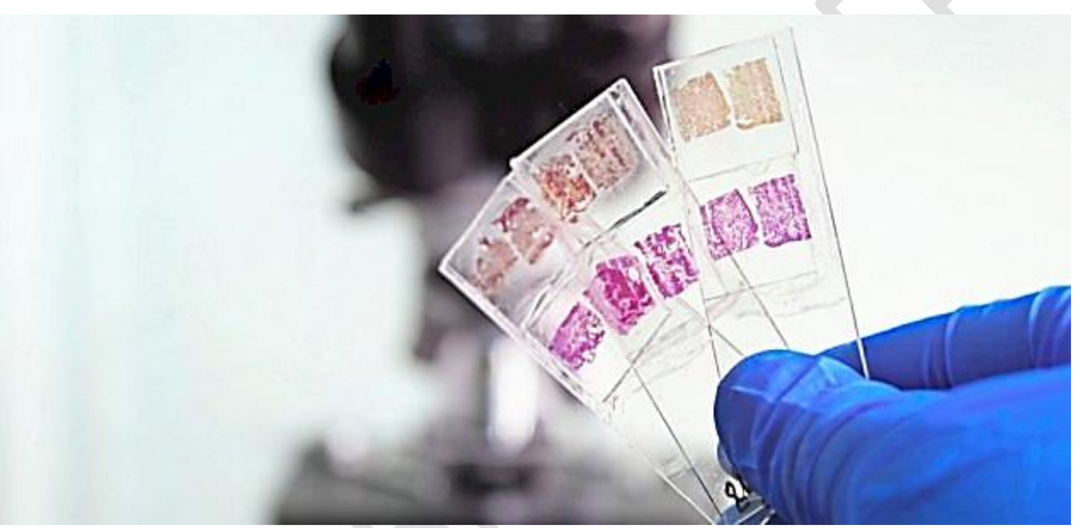
1. If the whole of the tumor or lesion is sent for examination and diagnosis by the pathologist, it is called excisional biopsy.

2. Tissues from the autopsy are sent for the study of disease and its course, for the advancement of medicine.

Types of Histological preparation



The histological specimen can be prepared as

1. Whole mount 2. Sections

3. Smears.

1. **Whole mounts**- These are preparation entire animal eg. fungus, parasite. These preparations should be no more than 0.2-0.5 mm in thickness.

2. **Sections-** The majority of the preparations in histology are sections.

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The tissue is cut in about 3-5 mm thick pieces processed and 5 microns thick sections are cut on a microtome. These are then stained and permanently mounted.

Microtomes are special instruments which have automatic mechanism for cutting very thin sections. To cut the sections on the microtome; the tissue must be made hard enough to not get crushed. There are 2 methods of hardening the tissues. One is by freezing them and the other is by embedding them in a hard material such at paraffin wax or gelatin.

3. **Smears**- Smears are made from blood, bone marrow or any fluid such as pleural or ascitic fluid. These are immediately fixed in alcohol to presence the cellular structures are then stained. Smears are also made by crushing soft tissue between two slides or an impression smear in made by pressing a clean slide in contact with the moist surface of a tissue. By doing this, the cells are imprinted on the slide and these may be stained for cytological examination.

Responsibility of a technician

The technician is responsible for

1. Specimen preservation.
2. Specimen labeling, logging and identification.
3. Preparation of the specimen to facilitate their gross and microscopy.

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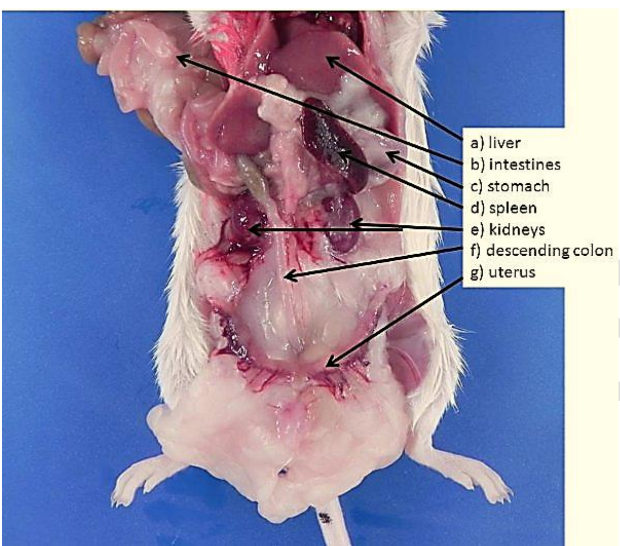
4. Record keeping.



To obtain these aims the following point need consideration.

1. As soon as the specimen is received in the laboratory, check if the specimen is properly labeled with the name, age, Hospital Registration No. and the nature of tissue to be examined and the requisition form is also duly filled.

2. Also check if the specimen is in proper fixative. Fixative should be fifteen to twenty times the volume of the specimen add fixative if not present in sufficient amount.

3. Check if the financial matters have been taken care off.

4. Make the entries in biopsy register and give the specimen a pathology number called the accession number. Note this number

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carefully on the requisition form as well as the container. This number will accompany the specimen everywhere.

5. If the specimen is large inform the pathologist who will make cut in the specimen so that proper fixation is done. Container should be appropriate to hold the specimen without distorting it.

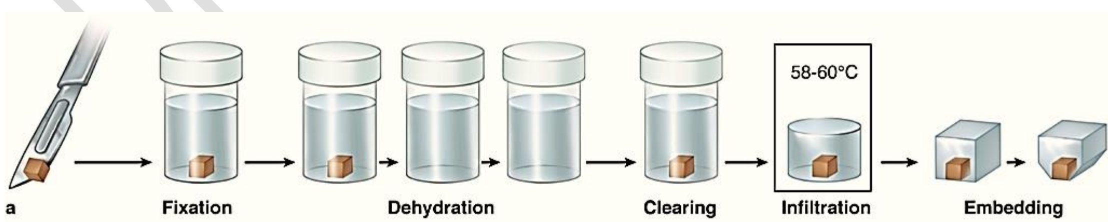
6. Blocks of tissues taken for processing should be left in 10% formalin at 60°C till processing. These would be fixed in 2 hours.

7. Slides should be released for recording after consultation with the pathologist.

8. Specimens should be kept in their marked container and discarded after checking with pathologist.

9. Block must be stored at their proper number the same day. Note the blocks have to be kept preserved for life long. Slides should be stored in their proper number after 3 days. It gives time for the slides to be properly dried.

Fixation



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Definition It is a complex series of chemical events which brings about changes in the various chemical constituents of cell like hardening, however the cell morphology and structural detail is preserved.

Unless a tissue is fixed soon after the removal from the body it will undergo degenerative changes due to autolysis and putrefaction so that the morphology of the individual cell will be lost.

Principle of fixation- The fixative brings about crosslinking of proteins which produces denaturation or coagulation of proteins so that the semifluid state is converted into semisolid state; so that it maintains everything in vivo in relation to each other. Thus, semisolid state facilitate easy manipulation of tissue.

Aims and Effects of fixation

If a fresh tissue in kept as such at room, temperature it will become liquefied with a foul odour mainly due to action of bacteria i.e. putrefaction and autolysis so the first and fore most aim of fixation is

1. To preserve the tissue in as if like manner as possible.
2. To prevent postmortem changes like autolysis and putrefaction.

Autolysis is the lysis or dissolution of cells by enzymatic action probably as a result of rupture of lysosomes.

Putrefaction The breakdown of tissue by bacterial action often with formation of gas.

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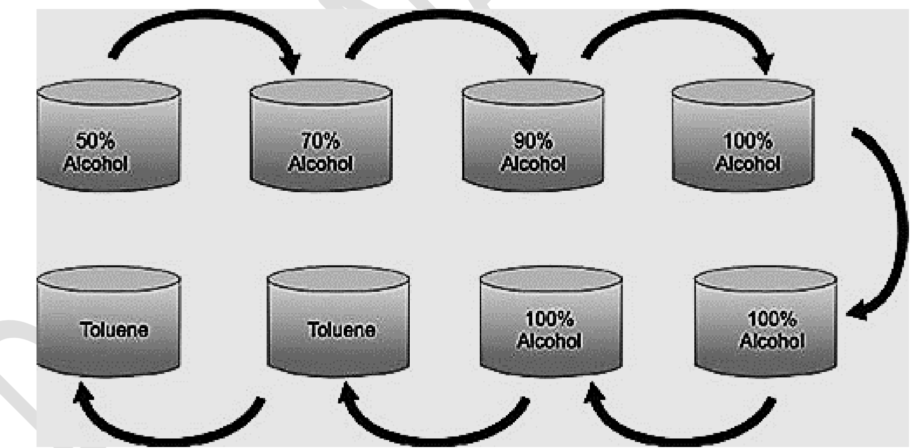
3. Preservation of chemical compounds and microanatomic constituents so that further histochemistry is possible.

4. Hardening: the hardening effect of fixatives allows easy manipulation of soft tissue like brain, intestines etc.

5. Solidification: Converts the normal semifluid consistency of cells (gel) to an irreversible semisolid consistency (solid).

6. Optical differentiation - it alters to varying degrees the refractive indices of the various components of cells and tissues so that unstained components are more easily visualized than when unfixed.

7. Effects of staining - certain fixatives like formaldehyde intensifies the staining character of tissue especially with hematoxylin.



Properties of fixatives

1. Coagulation and precipitation as described above.

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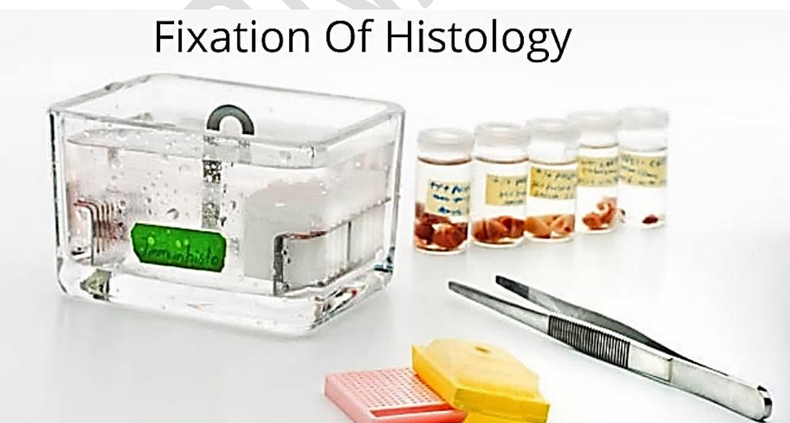
2. Penetration Fixation is done by immersing the tissue in fluid containing the fixative. Faster a fixative can penetrate the tissue better it is penetration power depends upon the molecular weight e.g. formalin fixes faster than osimic acid.

3. Solubility of fixatives - All fixatives should be soluble in a suitable solvent, preferably in water so that adequate concentrations can be prepared.

4. Concentration - It is important that the concentration of fixative is isotonic or hypotonic

5. Reaction - Most fixatives are acidic. It may help in fixation but can affect staining so has to be neutralized e.g. formalin is neutralized by adding of calcium carbonate.



Amount of fixative

The fixative should be at least 15-20 times the bulk of tissue. For museum specimens the volume of fixative is > 50 times.

Note: If the specimen is large then see that the sections are made to make slices which have a thickness of 1.5 cm so that fixative can penetrate the tissue easily

Reagents employed as fixatives (simple fixatives)

I. **Formaldehyde** - Formaldehyde is a gas but is soluble in water to the extent of 37-40% w/v. This solution of formaldehyde in water is called formalin or full-strength formalin. Formalin is one of the commonly used fixatives in all laboratories since it is cheap penetrates rapidly and does not over harden the tissues.

- It preserves the proteins by forming cross linkage with them and the tissue component.
- It denatures the proteins.
- Glycogen is partially preserved hence formalin is not a fixative choice for carbohydrates.
- Some enzymes can be demonstrated in formalin fixed tissues.
- It neither preserves nor destroys fat. Complex lipids are fixed but has no effect on neutral fat. After formalin fixation fat may be demonstrated in frozen section. Pure formalin is not a satisfactory fixative as it overhardens the tissue. A 10% dilution in water (tap or distilled) is satisfactory.

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Since it oxidizes to formic acid if kept standing for long period so it should be neutralized by phosphates or calcium carbonate otherwise it tends to form artifact; a brown pigment in tissues. To remove this pigment picric alcohol or saturated alcoholic sodium hydroxide may be used. Concentrated formalin should never be neutralized as there is a great danger of explosion.

The commercial formalin becomes cloudy on standing especially when stored in a cool place due to formation of precipitate of paraformaldehyde which can be filtered.

Formalin on prolonged exposure can cause either dermatitis its vapor may damage the nasal mucosa and cause sinusitis.

Time required for fixation.

At room temperature - 12 hours For small biopsies - 4-6 hours At 65°C fixation occurs in - 2 hours

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II. Alcohol (Ethyl Alcohol)

Absolute alcohol alone has very little place in routine fixation for histopathology.

- It acts as a reducing agent, become oxidized to acetaldehyde and then to acetic acid.
- It is slow to penetrate, hardens and shrinks the tissue.
- Alcohol penetrates rapidly in presence of other fixative hence in combination e.g. Carnoy's fixative is used to increase the speed of tissue processing.
- Ethanol preserves some proteins in relatively undenatured state so that it can be used for immunofluorescence or some histochemical methods to detect certain enzymes.
- It is a fat solvent hence it dissolves fats and lipids
- Methyl alcohol is used for fixing blood and bone marrow smears.

III. Acetone:

Cold acetone is sometimes used as a fixative for the histochemical demonstration of some tissue enzymes like phosphatases and lipases. Its mode of action as fixative is similar to that of alcohol

IV. Mercuric Chloride ($HgCl_2$)

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Mercuric chloride is a very good salt employed in fixing but is rarely used alone because it causes shrinkage of the tissue.

- It brings about precipitation of the proteins which are required to be removed before staining by using potassium iodide in which they are soluble.
- The size (thickness) of the tissue to be fixed in mercuric chloride is important, since if the tissue is more than 4 mm, then it hardens the tissue at the periphery whereas the centre remains soft & under fixed.
- It penetrates rapidly without destroying lipids.
- It neither fixes nor destroys carbohydrates. Treatment of the tissue with mercuric chloride brings out more brilliant staining with most of the dyes.
- Tissues fixed with mercuric chloride containing fixatives contain black precipitates of mercury which are removed by treating with 0.5% iodide solution in 70% ethanol for 5-10 minutes, sections are rinsed in water, decolorized for 5 minutes in 5% sodium thiosulphate and washed in running water.

V. Picric acid

It produces marked cells shrinkage hence it is not used alone.

It has to be stored in a damp place because of its explosive nature it is preferably stored under a layer of water.

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Advantage It penetrates well and fixes rapidly.

It precipitates proteins and combines with them to form picrates some of the picrates are water-soluble so must be treated with alcohol before further processing where the tissue comes into contact with water.

Note: All the tissues fixed in picric acid containing fixatives should be thoroughly washed to remove the yellow discolouration to ensure proper staining of tissue sections.

If the fixative is not removed by washing thoroughly with time even the embedded tissue looses its staining quality.

VI. Potassium dichromate

It fixes the cytoplasm without precipitation. Valuable in mixtures for the fixation of lipids especially phospholipids. Used for fixing phosphatides and mitochondria.

Note - Thorough washing of the tissue fixed in dichromate is required to avoid forming an oxide in alcohol which cannot be removed later.

VII. Osmium tetroxide

It is a strong oxidizing agent and brings about fixation by forming cross links with proteins.

- It gives excellent preservation of details of a cell, therefore exclusively used for electron microscopy.
- It fixes fat e.g. myelin.

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• It also demonstrates fat when 0.5-2% aqueous solution is used it gives a black colour to fat.

VIII. Acetic acid - It causes the cells to swell hence can never be used alone but should be used with fixatives causing cell shrinkage

IX. Glutaraldehyde - It is used alone or in combination with osmium tetroxide for electron microscopy.

Compound fixatives

Some fixatives are made by combining one or more fixative so that the disadvantage of one are reduced by use of another fixative.

All these compound fixatives have their own advantages and disadvantages. They should be used judiciously.

Choice of fixative - The choice of fixative depends on the treatment a tissue is going to receive after fixation e.g. what is the chemical structure that needs to be stained? If fat is to be demonstrated the formalin fixed tissue is better. For demonstration of glycogen formalin should never be used instead alcohol should be the choice of fixative.

Preparation of the specimen for fixation

1. For achieving good fixation, it is important that the fixative penetrates the tissue well hence the tissue section should be > 4mm thick, so that fixation fluid penetrates from the periphery to the centre of the tissue. For fixation of large organs perfusion method is used i.e. fixative

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is injected through the blood vessels into the organ. For hollow viscera fixative is injected into the cavity e.g. urinary bladder, eyeball etc.

2. Ratio of volume of fixative to the specimen should be 1:20.

3. Time necessary for fixation is important routinely 10% aqueous formalin at room temperature takes 12 hours to fix the tissue. At higher temperature i.e. 60-65°C the time for fixation is reduced to 2 hours.

Fixatives are divided into three main groups

A. **Microanatomical fixatives** - such fixatives preserve the anatomy of the tissue.

B. **Cytological fixatives** - such fixation are used to preserve intracellular structures or inclusion.

C. **Histochemical fixatives**: Fixative used to preserve he chemical nature of the tissue for it to be demonstrated further. Freeze drying technique is best suited for this purpose.

Microanatomical fixatives

1. **10% (v/v) formalin in 0.9% sodium chloride (normal saline).** This has been the routine fixative of choice for many years, but this has now been replaced by buffered formal or by formal calcium acetate

2. **Buffered formation**

(a) Formalin 10ml

(b) Acid sodium phosphate - 0.4 gm

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(c) (monohydrate) Anhydrous disodium - 0.65 gm Phosphate

(d) Water to 100 ml

- Best overall fixative

3. Formal calcium (Lillie: 1965)

(a) Formalin: 10 ml

(b) Calcium acetate 2.0 gm

(c) Water to 100 ml

• Specific features

- They have a near neutral pH
- Formalin pigment (acid formaldehyde haematin) is not formed.

4. Buffered formal sucrose (Holt and Hicks, 1961)

(a) Formalin : 10ml

(b) Sucrose : 7.5 gm

(c) M/15 phosphate to 100 ml buffer (pH 7.4)

• Specific features

- This is an excellent fixative for the preservation of fine structure phospholipids and some enzymes.
- It is recommended for combined cytochemistry and electron microscopic studies.
- It should be used cold (4°C) on fresh tissue.

5. Alcoholic formalin

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Formalin 10 ml

70-95% alcohol 90 ml

6. Acetic alcoholic formalin

Formalin 5.0ml

Glacial acetic acid 5.0 ml

Alcohol 70% 90.0 ml

7. Formalin ammonium bromide Formalin 15.0ml

Distilled water 85.0 ml

Ammonia bromide 2.0 gm

• Specific features : Preservation of neurological tissues especially when gold and silver impregnation is employed

8. Heidenhain Susa

(a) Mercuric chloride 4.5gm

(b) Sodium chloride 0.5 gm

(c) Trichloroacetic acid 2.0 gm

(d) Acetic acid 4.0 ml

(e) Distilled water to 100 ml

• Specific features

- Excellent fixative for routine biopsy work

- Allows brilliant staining with good cytological detail

- Gives rapid and even penetration with minimum shrinkage

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- Tissue left in its for over 24 hours becomes bleached and excessively hardened.
- Tissue should be treated with iodine to remove mercury pigment

9. Zenker's fluid

(a) Mercuric chloride 5gm

(b) Potassium dichromate 2.5 gm

(c) Sodium sulphate 1.0 gm

(d) Distilled water to 100 ml

(e) Add immediately before use : Glacial acetic acid : 5 ml

- Specific features
- Good routine fixative
- Give fairly rapid and even penetration
- It is not stable after the addition of acetic acid hence acetic acid (or formalin) should be added just before use
- Washing of tissue in running water is necessary to remove excess dichromate

10. Zenker formal (Helly's fluid)

(a) Mercuric chloride - 5 gm

(b) Potassium dichromate 2.5 gm

(c) Sodium sulphate 1.0 gm

(d) Distilled water to 100 ml

(e) Add formalin immediately before use 5 ml

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- Specific features

- It is excellent microanatomical fixative
- Excellent fixative for bone marrow spleen and blood containing organs
- As with Zenker's fluid it is necessary to remove excess dichromate and mercuric pigment

11. B5 stock solution

Mercuric chloride 12 gm, Sodium acetate 2.5gm, Distilled water 200ml,

B5 Working solution

B5 stock solution 20ml

Formalin (40% w/v formaldehyde) 2 ml

- Specific Features

- B5 is widely advocated for fixation of lymph node biopsies both to improve the cytological details and to enhance immunoreactivity with anti-immunoglobulin antiserum used in phenotyping of B cell neoplasm.

Procedure

- Prepare working solution just before use
- Fix small pieces of tissue (7x7x2.5mm) for 1-6 hours at room temperature
- Process routinely to paraffin.

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12. Bouin's fluid

(a) Saturated aqueous picric acid 75ml

(b) Formalin 25ml

(c) Glacial acetic acid 5 ml

- Specific features

- Penetrates rapidly and evenly and causes little shrinkage

- Excellent fixative for testicular and intestinal biopsies because it gives very good nuclear details, in testes is used for oligospermia and infertility studies

- Good fixative for glycogen

- It is necessary to remove excess picric acid by alcohol treatment

13. Gender's fluid - better fixative for glycogen.

(a) Saturated picric acid in 95% v/v/ alcohol 80ml

(b) Formalin 15ml

(c) Glacial acetic acid 5ml

Cytological fixatives

Subdivided into

(A) Nuclear fixatives

(B) Cytoplasmic fixatives

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A. Nuclear fixatives: As the name suggests it gives good nuclear fixation. This group includes

1. Carnoy's fluid.

(a) Absolute alcohol 60ml

(b) Chloroform 30ml

(c) Glacial acetic acid 10 ml

• Specific features

- It penetrates very rapidly and gives excellent nuclear fixation.
- Good fixative for carbohydrates.
- Nissil substance and glycogen are preserved.
- It causes considerable shrinkage.
- It dissolves most of the cytoplasmic elements. Fixation is usually complete in 1-2 hours.

For small pieces 2-3 mm thick only 15 minutes in needed for fixation.

2. Clarke's fluid

(a) Absolute alcohol 75 ml

(b) Glacial acetic acid 25 ml.

• Specific features

- Rapid, good nuclear fixation and good preservation of cytoplasmic elements.

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- It in excellent for smear or cover slip preparation of cell cultures or chromosomal analysis.

3. Newcomer's fluid.

(a) Isopropranolol 60 ml

(b) Propionic acid 40ml

(c) Petroleum ether 10 ml.

(d) Acetone 10 ml.

(e) Dioxane 10 ml.

- Specific features

- Devised for fixation of chromosomes

- It fixes and preserves mucopolysacharides. Fixation in complete in 12-18 hours.

(b) Cytoplasmic Fixatives

(1) Champy's fluid

(a) 3g/dl Potassium dichromate 7ml.

(b) 1% (V/V) chromic acid 7 ml.

(c) 2gm/dl osmium tetraoxide 4 ml.

- Specific features

- This fixative cannot be kept hence prepared fresh.

- It preserves the mitochondrial fat and lipids.

- Penetration is poor and uneven.

- Tissue must be washed overnight after fixation.

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(2) Formal saline and formal Calcium

Fixation in formal saline followed by postchromatization gives good cytoplasmic fixation.

Histochemical fixatives

For a most of the histochemical methods. It is best to use cryostat. Sections are rapidly frozen or freeze dried. Usually, such sections are used unfixed but if delay is inevitable then vapor fixatives are used.

Vapor fixatives

1. Formaldehyde- Vapour is obtained by heating paraformaldehyde at temperature between 50° and 80°C. Blocks of tissue require 3-5 hours whereas section require ½- 1 hours.

2. Acetaldehyde- Vapour at 80°C for 1-4 hours.

3. Glutaraldehyde- 50% aqueous solution at 80°C for 2 min to 4 hours.

4. Acrolein /chromyl chloride- used at 37°C for 1-2 hours

Other more commonly used fixatives are (1) formal saline (2) Cold acetone Immersing in acetone at 0-4°C is widely used for fixation of tissues intended to study enzymes esp. phosphates. (3) Absolute alcohol for 24 hours.

Secondary fixation - Following fixation in formalin it is sometimes useful to submit the tissue to second fixative eg. mercuric chloride for 4

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hours. It provided firmer texture to the tissues and gives brilliance to the staining.

Post chromatin- It is the treatment and tissues with 3% potassium dichromate following normal fixation. Post chromatization is carried out either before processing, when tissue is for left for 6-8 days in dichromate solution or after processing when the sections are immersed in dichromate solution, In for 12-24 hours, in both the states washing well in running water is essential. This technique is used a mordant to tissues.

Washing out- After the use of certain fixative it in urgent that the tissues be thoroughly washed in running water to remove the fixative entirely. Washing should be carried out ideally for 24 hours.

Tissues treated with potassium dichromate, osmium tetroxide and picric acid particularly need to be washed thoroughly with water prior to treatment with alcohol (for dehydration).

Lecture 3

Decalcification

Specific Objective - The aim of the study is to ensure staining of hard bony lesions so that the study of pathological lesions is possible.

Definition : Decalcification is a process of complete removal of calcium salt from the tissues like bone and teeth and other calcified tissues following fixation.

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Decalcification is done to assure that the specimen is soft enough to allow cutting with the microtome knife. Unless the tissues in completely decalcified the sections will be torn and ragged and may damage the cutting edge of microtome knife.

The steps of decalcification

1. To ensure adequate fixation and complete removal of the calcium it is important that the slices are 4-5 mm thick. Calcified tissue needs 2-3 hours only, for complete decalcification to be achieved so it in necessary to check the decalcification after 2-3 hours.

2. Fixative of choice for bone or bone marrow is Zenker formal or Bouin's fluid. Unfixed tissue tends be damaged 4 times greater during decalcification than a properly fixed tissue.

Decalcification

Decalcification is affected by one of the following methods.

(a) Dissolution of calcium by a dilute mineral acid.

(b) Removal of calcium by used of dilute mineral and along with ion exchange resin to keep the decalcifying fluid free of calcium.

(c) Using Chelating agents EDTA.

(d) Electrolytic removal of calcium ions from tissue by use of electric current.

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The Criteria of a good decalcifying agents' area.

1. Complete removal of calcium.
2. Absence of damage to tissue cells or fibers.
3. Subsequent staining not altered.
4. Short time required for decalcification.

Removal of calcium by mineral acids - Acid decalcifies subdivided into- Strong acid, weak acid.

Strong acid - eg. Nitric and hydrochloric acid.

Nitric acid- 5-10% aqueous solution used.

They decalcify vary rapidly but if used for longer than 24-48 hrs. cause deterioration of stainability specially of the nucleus

Hydrochloric acid - 5-10% aqueous solution decalcification slower than nitric acid but still rapid. Fairly good nuclear staining.

Weak acid e.g. formic, acetic and picric acid of these formic acids is extensively used as acid decalcifier. 5-10% aqueous solution or with additives like formalin or buffer are used.

Formic acid

1. Brings out fairly rapid decalcification.
2. Nuclear staining in better.

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3. But requires neutralization and thorough washing prior to dehydration.

Aqueous nitric acid

Nitric acid 5-10 ml

Distilled water to 100 ml.

Procedure

1. Place calcified specimen in large quantities of nitric acid solution until decalcification is complete (change solution daily for best results).
2. Washing running water for 30 minutes
3. Neutralize for a period of at least 5 hours in 10% formalin to which excess of calcium or magnesium carbonate has been added.
4. Wash in running water over night
5. Dehydrate, clear and impregnate in paraffin or process as desired.

Note: Overexposure to nitric acid impairs nuclear staining. Nitric acid is the solution of choice for decalcifying temporal bones.

Perenyi's fluid

10% nitric acid 40.0ml

Absolute alcohol 30.0 ml.

0.5% chromic acid. 30.0 ml.

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Note all these ingredients may be kept in stock and should be mixed immediately before use. This solution may acquire of blue violet tinge after a short while but this will have no effect in the decalcifying property.

It is slow for decalcifying hard bone but excellent fluid for small deposits of calcium eg. calcified arteries, coin lesions and calcified glands. Also good for human globe which contains calcium due to pathological conditions. There is little hardening of tissue but excellent morphologic detail is preserved.

Formalin Nitric acid

Formalin 10 ml

Distilled water 80 ml

Nitric acid 10ml

Nitric acid causes serious deterioration of nuclear stainability which partially inhibited by formaldehyde. Old nitric acid also tends to develop yellow discolouration which may be prevented by stabilization with 1% urea.

Aqueous formic acid

90% formic acid 5-10 ml

Distilled water to 100 ml.

Gooding and Stelwart's fluid.

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90% formic acid 5-10ml.

Formalin 5ml

Distilled water to 100 ml.

Evans and Krajian fluid

20% aqueous trisodium citrate 65 ml

90% formic acid 35 ml

This solution has a pH of - 2-3

Formic acid sodium citrate method Procedure

1. Place calcified specimen in large quantities of formic acid-sodium citrate solution until decalcification is complete (change solution daily for best results).

2. Wash in running water for 4-8 hours

3. Dehydrate, clear and impregnate with paraffin or process as desired.

This technique gives better staining results then nitric acid method, since formic acid and sodium citrate are less harsh on the cellular properties. Therefore even with over exposure of tissue in this solution after decalcification has been complete, causes little loss of staining qualities. This method of choice for all orbital decalcification including the globe.

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Surface decalcification- The surface of the block to be decalcified is trimmed with scalpel. The block is then placed in acid solution at 1% hydrochloric acid face downwards so that acid bathes the cut surface for 15- 60 min. As penetration and decalcification is only sufficient for a few sections be cut the block shall be carefully oriented in microtome to avoid wastage of decalcified tissue.

Decalcification of Bone marrow biopsy.

Tissue after fixation in Bouin's or Zenker's fixative is decalcified for 2½ hours followed by an hour of washing. The tissue in then dehydrated beginning with alcohol.

Use of Ion exchange resins

Ion exchange resins in decalcifying fluids are used to remove calcium ion from the fluid. Therefore, ensuring a rapid rate of solubility of calcium from tissue and reduction in time of decalcification. The resins an ammoniated salt of sulfonated resin along with various concentrations of formic acid are used.

The resin in layered on the bottom of a container to a depth of = ½ inch, the specimen is allowed to rest in it.

After use, the resin may be regenerated by washing twice with dilute N/10 HCL followed by three washes in distilled water. Use of Ion exchange resin has advantage of (ii) faster decalcification (ii) tissue preservation and

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(iii) cellular details better preserved.

Chelating agents

Chelating agents are organic compounds which have the power of binding certain metals. Ethylene-diamine-tetra-acetic acid, disodium salt called Versenate has the power of capturing metallic ions. This is a slow process but has little or no effect on other tissue elements. Some enzymes are still active after EDTA decalcification.

Versenate 10 gm.

Distilled water 100 ml

(pH 5.5 to 6.5)

Time 7-21 days.

Electrolytic method

This is based on the principle of attracting calcium ions to a negative electrode in to addition to the solution. Decalcifying solution HCL (Conc.)

80ml Formic acid 90% 100 ml

Distilled water 1000 ml.

Decalcify with electrolyte apparatus with the above-mentioned decalcifying fluid. This method has no added advantage over any other method.

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Neutralization: It has been said that following immersion in mineral acids, tissues should be deacidified or neutralized, before washing by treatment with alkali. This may be affected by treatment overnight in 5% lithium or sodium sulphate.

Washing: Through washing of the tissue before processing is essential to remove acid (or alkali if neutralized has been carried out) which would otherwise interfere with staining)

Determination of end point of decalcification

1. Flexibility method

Bending, needling or by use of scalpel if it bends easily that means decalcification is complete. Unreliable, causes damage and distortion of tissue.

2. X-ray method

Best method for determining complete decalcification but very costly. Tissue fixed in mercuric chloride containing fixatives cannot be tested as they will be radio opaque.

3. Chemical Method

It is done to detect calcium in the decalcifying fluid when no further calcium is detected, decalcification in considered complete.

Procedure

Take 5 ml of decalcifying fluid from the bottom of container which has been in contact with the tissue for 6-12 hrs. Add 5 ml each of 5%

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ammonium oxalate and 5% ammonium hydroxide. Mix and let it stand for 15-30 min. A cloudy solution caused by calcium oxalate indicates that specimen is not thoroughly decalcified. Absence of turbidity indicates completeness of decalcification.

Treatment of hard tissues

Keratin and chitin are softened by use of concentrated sulphuric and with that aid of heat keratin is completely dissolved from the tissue sections. But much tissue distortion will also occur.

For softening of chitin full procedure gives a satisfactory result.

1. Fix the specimen in fixative of choice.
2. Place the specimen in following solution until complete dechitinized.

Change the solution every two days for best results.

Mercuric chloride - 4 gm Chromic acid- 0.5gm Nitric acid (Conc.) - 10.0ml Ethyl alcohol 95% - 50.0 ml Distilled water - 200.0ml

3. Washing running water for 3 hours
4. Dehydrate, clear and impregnate with paraffin.

Prenyi's fluid

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Immersing hard tissues in this solutions for 12-24 hours will make sectioning easier and excellent preparation of calcified arteries, thyroid and calcified glands is possible.

Lendrum's technique

It is very useful for tissues which became hard at the time of fixation. Following washing out of the fixative, tissue is immersed in a 4% aqueous solution of phenol for 1-3 days.

Wax blocks - The treatment of wax embedded block of hard tissue may be done by soaking in soap water overnight.

Lecture 4 TISSUE PROCESSING

Specific objective - The tissue processing is the heart of any tissue section which will be cut adequately only if the tissue is properly preserved and processed. The study of this topic is to understand the coarse and fine details of tissue processing so that excellent sections are obtained.

Definition - The term tissue processing refers to treatment of the tissue necessary to impregnate it into a solid medium so that the tissue is rendered sufficiently firm yet elastic for the tissue sections of desirable thickness to be cut on microtome.

This is not the only technique employed for tissue sections. Sections can also be produced by means of cryostat or freezing microtome on frozen tissues.

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The fixed impregnated tissues have an advantage that they can be more easily stored and reproducibility of sections at a later date is easier.

Before proceeding on tissue processing as soon as the tissue is received it in very important that the tissue be properly labeled so as to avoid any confusion regarding duplication of same name or giving a wrong diagnosis to the patient.

The labeling has to be a full proof system.

The label should remain throughout the entire processing and later as

permanent record keeping. To ensure this most laboratories have a numbering system for each specimen. As soon as the specimen is received it is given a specific individual number, which is also recorded in the register with the details like patient's name, name of the doctor referring it, nature of tissue is noted.

- Labeling should not be done using ordinary ink as it gets dissolved in the reagent used during processing.

- Thin white card with a soft lead pencil, typed or printed labels are satisfactory. To ensure that the label remains with their correct specimens' tissues processing baskets can be used. These are small, perforated metal containers in which the tissue and labels are placed. these containers can be transferred as such from reagent to reagent.

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Alternatively, use of tissue trek system in which the tissue identity is written on the cassette and retained as permanent record during sectioning and storage of tissue blocks.

Principle of tissue processing - The tissue is embedded in a solid medium by the help of first removing the tissue water which is then replaced by any solid medium such as paraffin wax so that the tissue is rendered firm enough to enable thin sections to be cut, at the same time, the tissue is soft (not so hard) to enable microtome knife to cut the sections.

The embedding medium has to thoroughly permeate the tissue in fluid form so that it solidifies without any damage to the tissue. The most satisfactory embedding medium used in routine histology is paraffin wax. Most of the tissue fixatives are aqueous fixatives so before the tissue can be embedded in paraffin wax it is necessary that the water and some of the lipid tissue fluids be removed completely by a variety of compounds through a process called dehydration.

Prior to paraffin wax embedding and impregnation the tissue must be subjected to the following steps:

1. Fixation
2. Dehydration
3. Clearing - with a substance which is totally miscible with both the dehydrating agent which precedes it and embedding agent which follows it.

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4. Embedding

All these 4 processes depend upon complete impregnation of the tissue by the agent like paraffin wax being used.

Before going into the details of these 4 stages it is important to understand the factors which influence the rate and efficiency of tissue impregnation

Factors influencing the rate of impregnation

A tissue immersed in fluid interchange occurs between tissue fluid and surrounding fluid. The process continues through all stages of processing from fixation to final impregnation.

Agitation - Tissue placed in liquid is agitated so that the fluid immediately in contact with the surface of tissue which is mixed by tissue fluid is replaced by the fresh immersing liquid.

This can be achieved by a pumping system which removes and replaces fluid at selected intervals or by rotation and vertical oscillation method. Efficient agitation reduces the processing time by 25-30% with improved impregnation of the tissue.

Heat - Heat increases the rate of penetration.

Viscosity - Larger the molecule the higher is the viscosity slower is the rate of penetration.

Ultrasonic: Use of ultrasonics increases the penetration rate.

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Vacuum: Use of reduced pressure in well known in the impregnation of tissue by molten paraffin wax. It hastens the process. Use of vacuum during dehydration and clearing has little advantage except removal of air bubble trapped within the tissue.

STEPS OF PARAFFIN WAX EMBEDDING

Fixation - Usually tissue that is received at the laboratory is already fixed but before proceeding further check if the fixation is complete.

Dehydration - After fixation in aqueous solvent the delicate tissue needs to be dehydrated slowly starting in 50% ethyl alcohol. The other routine tissue specimen may be put in 70% alcohol. A higher concentration of alcohol initially is in inadvisable because this may cause very rapid removal of water may produce cell shrinkage. An exception to this is in case of Heidenhain's Susa fixed tissue where it may be placed directly in 95% alcohol. Tissue transferred from alcoholic based fixative like Carnoy's fixative may be placed in higher grades of alcohol or even in absolute alcohol.

For routine biopsy and postmortem tissue of 4-7 mm thickness 70%, 90% and absolute alcohol (2-3 changes for 2-4 hours each) are sufficient to give reasonably satisfactory result.

Use of solid dehydrants

Anhydrous copper sulphate is used in higher grade of dehydrating alcohols. A layer 1-2.5 cm thick is placed at the bottom of a dehydrating

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vessel or beaker and is covered with 2 or 3 filter papers to prevent contamination of the tissues. Anhydrous copper sulphate is white, it removes water from alcohol which in turn has been diluted upon absorption of water from the tissues. The change of colour of copper sulphate from white to blue indicates that both alcohol and water should be changed. Use of copper sulphate enhances the process of dehydration and also prolongs the life of alcohol.

Other dehydrating agents

1. Acetone - It is clear, colorless volatile inflammable fluid.

- It has a rapid action in dehydrating the tissue but produces shrinkage and distortion and subsequent brittleness to the tissue.
- Low cost is also an advantage.

Acetone usually dehydrates within 20-30 minutes, but four changes of acetone should be used, it is preferable to use acetone after low strength of alcohol so that distortion of the tissue is less.

2. Dioxane - It dehydrates and clears at the same time. It is miscible with paraffin and with water and alcohol, tissue from dioxane can be transferred straight to paraffin.

- There is less shrinkage of tissues
- Tissues can be left in dioxane without danger of hardening for longer period of time.

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Disadvantage: It is more expensive than alcohol.

* It is toxic to man

3. Isopropyl alcohol

- It is miscible with water and other organic solvents
- It does not harden the tissue like alcohol
- It is expensive

Clearing

Definition - Clearing means appearance of tissue after it has been treated by the fluid chosen to remove the dehydrating agent.

Most of these tissues have similar refractive index to that of protein therefore the tissue is left translucent.

Clearing agent is required when the dehydrating agent is not miscible with the impregnating medium. It is essential for a clearing agent to be miscible both in dehydrating agent as well as embedding agent.

Commonly used clearing agents are as follows:

1. **Xylene** - It has a rapid action. Biopsy specimens of 3-4 mm thickness are cleared in 2-4 hours.

Immersion time must not be prolonged otherwise the tissue become brittle.

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2. **Toluene and Benzene** are similar in properties to xylene but are less damaging to the tissues on prolonged exposure.

3. **Chloroform** - It is slower in action, but it causes less brittleness therefore tissue can be left in it overnight.

- It does not affect the refractive index of the tissue is not rendered translucent.
- It is expensive.
- It is inflammable.

4. **Carbon tetrachloride** - It has similar properties to chloroform but is cheaper.

5. **Cedar wood oil** (Histological): It is good for treatment of delicate tissues as it has the least hardening effect.

- It is very slow in action.
- It is very expensive.

Care should be taken not to confuse it with cedar wood oil (microscopic) used with oil immersion lens.

Techniques of clearing: If the tissue is being cleared in chloroform or carbon tetrachloride it may be left overnight. In automatic tissue processor three changes of one hour each are usually satisfactory.

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In Xylene, benzene or toluene one change after 30-60 minutes is satisfactory to give a clear translucent appearance to the tissue.

Impregnation

Definition - It is the complete removal of clearing reagents by substitution of paraffin or any such similar media.

Impregnation with wax

Impregnation with paraffin wax takes place in an oven heated to 56-60°C depending upon the melting point of the wax in use.

Frequent check of the temperature of paraffin baths is required since temperature 5°C above the melting point of the paraffin will cause tissue shrinkage and hardening.

Properties of paraffin wax

1. Easy to prepare large number of tissue blocks in comparatively short time.
2. Minimum supervision is required
3. It is cheaper than other impregnating media
4. During staining there is very little difficulty than other media.

Points to be remembered during use of paraffin wax

1. It should be free from dust, grit and other foreign matter.

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2. It should not contain water, which causes it to crystallize and turn it white.

3. The wax has to be filtered before use by use of ordinary filter paper.

4. Higher melting point waxes are hard to ribbon.

For impregnation the wax oven has to be kept at high temperature, making the tissue hard, too low melting point wax may not be hard enough to support the tissue during cutting. If the wax is overheated and remains in that state for a long time, it tends to crystallize and become useless.

Paraplast - This is mixture of highly purified paraffin and several plastic polymers.

It has greater elasticity than normal paraffin wax, therefore, the results are superior.

It ribbons well allowing almost wrinkle free serial sections to be cut with ease at 4-micron thickness. It should not be used for thin-walled structures as it prevents complete expansion of the specimen.

Bioloid - Good embedding medium in which thin-walled structures can be sectioned satisfactorily.

Technique of impregnation: The tissue is transferred from clearing agent to molten paraffin wax. The amount of wax should be 25-50 times the volume of tissue. The tissue must be submitted to 3 changes in wax.

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The temperature of the wax bath should be 2-3°C above the melting point of wax.

Time of impregnation

Depends on the following 3 factors

1. The size and type of tissue
2. The clearing agent employed
3. The use of vacuum embedding oven.

1. Size and type of tissue:

The thicker the tissue the longer will be the time required for wax to penetrate to the center in addition a thick tissue has more of clearing agent so more changes of wax are necessary to remove it. If even small amounts of clearing agents remain with the wax this will cause crystallization and produce crumbling of the sections during cutting.

The type of tissue is also important since bone, skin, CNS needs twice as long as soft tissue like liver or kindly.

Tissue like muscle and fibrous tissue tends to overharden and become brittle in wax bath so the time for impregnation must be kept to a minimum. The reduction of time can be achieved by using vacuum embedding medium.

2. Clearing agent employed

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Some clearing agents are more rapidly and easily cleared than other e.g. Xylene, benzene and toluene are easiest to remove, and one change of wax is normally sufficient; whereas for chloroform and carbon tetrachloride 2-3 changes are needed.

3. Use of vacuum embedding oven

With the use of normal paraffin oven, 2 changes of paraffin wax for a period of 4 hours are needed but by using vacuum embedding oven this time may be halved

Embedding - It is the orientation of tissue in melted paraffin which when solidified provides a firm medium for keeping intact all parts of the tissue when sections are cut.

Types of moulds

a) **Leuckhart's L pieces** - These are two 'L' which are resting metal usually brass, which are resting on a flat metal or glass plate.

1. L-moulds or embedding box

b) **Compound embedding units** - consists of square shaped brass or metal plates in a series of interlocking plates.

c) **Others like plastic embedding blocks** (tissue Tek system)

Techniques of casting

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1. Molten paraffin wax which is heated at a temperature 2-3° above the melting point is poured into the mould to an adequate depth so as to cover the thickest tissue block.

2. The wax touching the mould will quickly form a thin semi solid layers, Now introduce the tissue with a prewarmed forceps to prevent the wax to stick to it. The tissue is pressed in this semisolid wax to orient it at the bottom of mould in a correct plane.

3. Fix the label in position by pressing one edge against solidifying wax usually sides of the mould are preferred.

4. As soon as a film of solid wax is formed on the surface, the whole block with mould are submerged in cold water at 20°C. If this is not done there will be crystallization of wax, using ice water to do initial cooling will also cause the block to crack.

5. When blocks are set hard they are removed from mould.

The tissue surface towards the mould base is from where the sections are to be cut this surface should be trimmed lightly with a scalpel so as to expose the tissue.

Following points must be taken care off during casting.

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1. Paraffin should not be allowed to cool around the tissue to be blocked for this before introducing the tissue in the mould it should be kept in heated wax or in cassette placed over thermostatic hot plate.

2. To prevent excess of wax solidifying on the bottom of the block during winter prewarmed moulds may be used.

3. The cutting surface of the tissue should be facing at the bottom of the mould.

4. If 2 or more tissues have to be casted remember to keep them both at the same depth.

5. If small biopsy fragments have to be casted, the largest piece should be first blocked and other pieces should be as near it as possible.

6. All four corners of the block should be in one horizontal plane.

7. The tissue should have atleast 2 mm wax around its edges.

8. Smear mineral or machine oil on the inner surface of the mould for facilitating easy removal of block.

9. Whitish areas around tissue in block denotes crystalization which may be due to moisture or due to incomplete removal of clearing agent.

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Most tissue sections are cut from the largest area but some tissue needs special mention.

1. Tissue of tubular nature are cut transversely so should be embedded vertically.

2. Skin is cut in a plane at right angles to the surface so should be embedded at right angles to the bottom.

3. Muscle biopsy should be sectioned in both transverse and longitudinal planes.

Automatic tissue processor

It has 2 advantages

1. Transferring the tissue mechanically from one reagent to another can be done both by day and night.

2. Reduces processing time by the action of continuous agitation.

3. This eliminates the possibility of human errors of leaving the tissue for long time in one solution due to forgetfulness.

VARIOUS PARTS OF THE MACHINE ARE AS FOLLOWS

(a) **Tissue containers** - These are also the cassettes. The tissue to be processed is placed in an appropriate container, together with a label and the lid snapped on. These containers are placed in the tissue basket in which they remain throughout the whole process.

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(b) **Beakers and wax baths** - Most machines are equipped with ten beakers and 2 wax baths thermostatically controlled at 56°C + 4°C. The beakers are filled with appropriate fluids and wax is placed in the wax baths after ensuring that main switch is on, so as to keep the wax in molten state.

(c) **Stirring mechanism** - The basket is attached to the arms of the machine on which one arm is designed in such a manner so as to bring about the rotation of the basket nearly at the rate of one revolution per minute.

(d) **Timing mechanism** - Timer is meant to keep the tissue in different reagents and wax for an optimum time. If kept for longer or shorter period than necessary, tissue will not be adequately processed.

Points to noted

1. Fluid and wax beakers must be filled up to appropriate mark and located in their correct position in the machine.

2. Any spillage of the fluid should be wiped away.

3. Accumulations of wax must be removed from beaker, covers, lids and surrounding areas.

4. Wax bath thermostats should be set at satisfactory levels usually 2-3°C above the melting point of wax.

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5. Particular attention should be paid to fastening the processing baskets on the crousel type of machines, if the baskets are shed they will remain in one particular regent for a long period till it gets noticed.

6. Timing should be set with utmost care when loading the machine.

7. Paraffin wax baths should be checked to ensure that the wax is molten.

Automated processing schedule

1. 80% alcohol (holding point) 1 hours
2. 95% alcohol 2 hours
3. 95% alcohol 1 hour
4. 100% alcohol 1 hour
5. 100% alcohol 1 hour
6. 100% alcohol 1 hour
7. Chloroform 1 hour
8. Chloroform 1 hour
9. Chloroform 1 hour
10. Paraffin wax 2 hours
11. Paraffin wax 2 hours
12. Paraffin wax 2 hours

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Note: Keep watch on paraffin temperature

- Tissue should not be left in any solution for a longtime
- Frequent filtration and changes of solution are needed

Schedule for hard & delicate tissues

1. 80% alcohol two changes 1 hour each
2. 95% alcohol two changes 1 hour each
3. Absolute alcohol three changes 1 hour each
4. Absolute alcohol and xylene equal parts 1 hour
5. Cedar wood oil 2 changes 2 hours each
6. Cedar wood oil 1 change 1 hour
7. Paraffin wax one change 2 hours
8. Paraffin wax four changes 1 hour each

Vacuum the last paraffin change. Embed and cool quickly. Cut as desired.

Processing schedule for skin

1. 70% alcohol 1 hour
2. 95% alcohol 2 hours
3. Absolute alcohol 2 hours
4. Absolute alcohol 3 hours

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5. Cedar wood oil over night

6. Xylene 20 minutes

7. Wax I3 hours

8. Wax II over night

Embedding wax local preparation in laboratory. Method (A)

1. Paraffin wax (Candle) 1 Kg.

2. Bees Wax 25gm Melt and filter

Method (B)

1. Paraffin wax1kg

2. Liquid paraffin 5-10 ml

Melt and filter. Find the ratio by experiment according to the melting point desired.

Lecture 5 SECTION CUTTING

Specific objective

1. To ensure good section cutting

2. To overcome troubleshooters during section cutting.

3. To familiarize the staff with the equipment used for section cutting.

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Introduction: To master in the art of good section cutting it is required

1. To have a thorough knowledge of the equipment used.
2. Quality of equipment
3. Quality of processing the tissue.

Microtome Knives: The knife is probably the greatest single factor in producing good sections.

Types of microtome knives : Microtome knives are classified by the manner in which they are ground and seen in their cross section.

1. Plane wedge
2. Plano concave
3. Biconcave
4. Tool edge

Plane wedge: It is used for paraffin and frozen sections.

Planoconcave: used for celloidin section since the blade is thin it will vibrate when for used for other harder materials.

Biconcave: It is recommended for paraffin section cutting on rocking and sledge type of microtome.

Tool edge: This is used with a heavy microtome for cutting very hard tissues like undecalcified bone.

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General description

In the description of knives, the expressions “Heel” and “Toe” are used to indicate to indicate which end of the cutting edge is referred to. The heel of the knife is the angle formed by the cutting edge and the end of the knife nearest to handle. The “toe” of knife is the angle formed by the cutting edge and the end of the knife farthest from the handle.

Sharpening of microtome knives

The cutting edge of an ideal microtome knife is a straight line formed by intersection of 2 planes, the cutting facets. The angle between the planes is called the bevel angle and is greater than the wedge angle between the sides of knife. The standard microtome knife has a wedge angle of approximately 15º and bevel angel varying between 27 and 32º.

Honing

Definition - Grinding of knife on a hone to restore straight cutting edge and correct bevel.

There are various types of hones

1. Belgian black vein or Belgian yellow

It is a yellow stone ½ inch thick and is backed with a black stone of same thickness. Only yellow side should be used for honing. It is the best hone. It is quite a fast hone and may be used for coarse grinding and finishing.

2. Arkansas – Not very fast.

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3. **Aloxide** – Fairly fast but coarse and not good for finishing a knife.

4. **Carborundum** – These hones can be obtained in a variety of grades only the finest of which should be used that too for coarse work.

5. **Plate glass** – May be used as a hone by applying an abrasive such as aluminium oxide to the surface and then using in the same way as ordinary hone.

The advantage of such a hone is that it can be used for all types of honing by changing the abrasive powder.

Lubricants for hone

1. Soap water
2. Liquid paraffin
3. Castor oil
4. Clove oil

Method of honing

1. The hone is placed on a bench on a nonskid surface.

2. A small quantity of light lubricant oil is poured on the centre of the hone and lightly smeared over the surface.

3. The knife complete with handle and backing sheath is laid on the hone with the cutting edge facing away from the operator, and the heel in the centre of the nearest end of hone. Correct positioning of the fingers is

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achieved by holding the handle of the knife between the thumb and forefinger with the cutting edge facing away from the operator (so that the thumb in on the back). When the knife is on the hone the tips of finger and thumb of other hand rest on the other end of knife ensuring even pressure along the whole edge of knife during honing.

4. The knife is pushed forward diagonally from heel to toe, turned over on its back and moved across the hone until the heel is in the center with the cutting edge leading, and then brought back diagonally. It is turned to its original position, thus completing figure of 8 movement.

5. The process is continued until all jagged edges have been removed.

The knife is ready for stropping.

Stropping

Definition: It is the process of polishing an already fairly sharp edge. It removes burrs formed during honing.

Fine quality leather is used leather strops may be either flexible / hanging or rigid. In stropping usually firm surface is preferred. Action is reverse of honing toe to heel direction of stropping is also opposite.

Assessment of the sharpened knife edge.

Examine the edge the knife by reflected light and under microscope to assess the honing and stropping.

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Care of the knife

1. Keep the knife covered in the box when not in use.
2. Oil the knife to prevent corrosion.
3. Always clean knife with xylol rag before and after use.
4. It should always be stropped before use.
5. Knife should be sharpened as and when required.

Automatic microtome knife sharpeners

There are many automatic knife sharpeners available Shandon type is most commonly used which consists of a glass plate on which fairly coarse abrasive powder like alumina powder is applied. First matting is done followed by lapping to remove all finer scratches.

In all stages of use of abrasive powder care must be taken to remove by thorough washing any traces of abrasive powder from both knife and plate.

Microtomes

These are mechanical devices for cutting uniform sections of tissue of appropriate thickness. All microtomes other than those used for producing ultra-thin sections for election microscopy depend upon the motion of a screw thread in order to advance the tissue block on knife at a regulated number of microns.

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Motion of screws can be direct or through system of gears or levers to magnify the movement.

Types of microtomes

1. Hand microtomes – limited for use in botanical sections
2. Rocking microtome
3. Rotary microtome
4. Freezing microtome
5. Base sledge microtome
6. Vibrating knife microtome

Size of Knives

110 mm Knife - Frozen sections

120 & 185 mm - Routine paraffin block

Paraffin section cutting

Equipment required

1. Microtome
2. Water bath preferably thermostatically controlled.
3. Hot plate or drying oven thermostatically controlled.
4. Fine pointed forceps.

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5. Small hair brush.

6. Seeker

7. Scalpel

8. Clear cloth or paper towel.

9. Slide rack

10. Clean glass slides

11. Section adhesive

12. Fluff less blotting paper

13. Ice cubes

14. Diamond marker pencil

Water bath. Thermostatically controlled for paraffin wax of melting point 56ºC, a water temperature of 45ºC is sufficient ordinary distilled water is satisfactory; addition of a trace of detergent to water is beneficial in flattening of sections.

Hot plate or drying oven

Drying of sections at around the melting point of wax is satisfactory Brush, seeker, forceps – needed to remove folds and creases in sections after floating out.

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Slides - Majority of sections fit comfortably on a 76 x 25 x 1.2 mm slide. Diamond pencil – needed to write the identification details like name or specific number.

Section adhesives

An adhesive is a substance which can be smeared on to the slides so that the sections stick well to the slides.

Most of the tissue sections which are adequately thin and thoroughly dried without any air bubble trapped under them do not require an adhesive, as in case of routine H and E staining, but for histochemical methods requiring alkaline solutions eg ammonia tend to remove sections from slide for such cases adhesive is required. Also adhesive is required for tissues like brain, spinal cord, blood clot, decalcified tissues which have a tendency to detach themselves from the slide. Tissue impregnated with ester wax also require section adhesive.

Types of adhesives

Albumin Gelatin Starch Cellulose

Sodium silicate Resin

Poly L Lysine

Adhesives are either added to water bath or smeared thinly on slide.

1. Agar – add 50 ml of melted agar to water bath
2. Gelatin – Add 30 ml of melted gelatin to water bath

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3. Mayer's glycerol albumin – This is the most popular adhesive for routine use

1. Fresh egg white 50 ml

2. Glycerol 50 ml

3. Sodium salicylate 1 ml

Mix and agitate the ingredients filter through coarse filter paper smear fluid over the slide. This fluid may be diluted 1:20 with distilled water and section floated on the fluid, while manipulating the albuminized slide under water in the floatation bath to pick up the section, avoid dipping the entire slide as the albumin may wash off.

Section cutting of paraffin embedded tissue Fixing of block

1. Fix the block in the block holder on the microtome knife in such as position that it will be clear of the knife when it is in position, block may be fixed directly, or it may be fixed to a metal carrier which in turn is fixed to the microtome.

2. Insert the appropriate knife in the knife holder and screw it tightly in position. Adjust if required. The clearance angle should be set at 3-4 degree and angle of slope should be set permanently at 90 degrees. It is important to tighten the knife clamp screw securely and block clamp screws most also be firm.

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The exposed ends of the knife must all the times be protected by magnetic or clip on knife guards to avoid any accidents.

3. Trimming of tissue block: Move the block forward so that he wax block is almost touching the knife. To trim away any surplus wax and to expose a suitable area of tissue for sectioning, the section thickness adjusters are set at 15 microns.

4. On exposing a suitable area of tissue, the section thickness is set to the appropriate level for routine purposes to 4-6 microns.

5. Apply ice to the surface of the block for a few seconds and wipe the surface of block free of water. This step is optional but makes sections cut easily.

6. Note that the whole surface of the block will move parallel to the edge of the knife in order to ensure a straight ribbon of sections.

7. The microtome is now moved in an easy rhythm with right hand operating the microtome and left hand holding the sections away from the knife. The ribbon is formed due to the slight heat generated during cutting, which causes the edges of the sections to adhere. If difficulty is experienced in forming the ribbon it is sometimes overcome by rubbing one of the edges of the block with finger.

8. During cutting the paraffin wax embedded sections become slightly compressed and creased. Before being attached to slides the creases must be removed and the section flattened. This is achieved by

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floating them on warm water. Thermostatically controlled water baths are now available with the inside coated black. These baths are controlled at a temperature 4-6ºC below the melting point of paraffin wax. It is easy to see creases if the inside of water bath is black.

9. The action in floating out must be smooth with the trailing end of ribbon making contact with water first to obtain flat sections with correct orientation, floating out with the shiny surface towards the water is essential. When the ribbon has come to rest on water the remaining wrinkles and folds are removed by teasing apart by using forceps or seeker.

10. Picking up sections – The ribbon of sections floating on water is split into individual or groups of sections by use of forceps or seekers. Picking up a section on slide is achieved by immersing the slide lightly smeared with adhesive vertically to three fourths of its length bringing the section in contact with the slide. On lifting the slide vertically from the water, the section will flatten on to the slide. The sections are then blotted lightly with moistened blotting paper to remove excess water and to increase contact between section and slide. For delicate tissues or when several ribbons of sections are placed on the slide, omit the blotting instead keep the slide in upright position for several minutes to drain.

11. Drying of section: Sections are then kept in incubator with a temperature 5-6ºC above the melting point of wax i.e. at 60ºC for 20- 60

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minutes. It is better to overheat than underheat. If the sections are not well dried, they may come off during staining.

The sections should not be allowed to dry without a good contact with the slide, such sections will come off during staining.

SOME USEFUL HINTS IN SECTION CUTTING

Methods of removing bubbles trapped beneath the sections

Bubbles may get trapped under a section while in the tissue flotation bath. These need to remove before the section are picked on the slides this may be done by:

1. With the edge of slide.
2. Can be teased out with bent dissecting needle.
3. Place the sections on slide and run 2% alcohol under them. Any fold or bubbles are removed.

To cut a tissue which has a tendency to crumble or fragment while cutting.

With the mouth open and sounding a soft long drawn 'H' thus 'h h h h h ' exhale gently on to the section as it leaves the knife and cut very slowly. This also helps to reduce the effect of static electricity.

If sections fragment due to large amount of blood in tissue, the block should be coated with celloidin between sections. The surface of the block should be wiped dry and painted with a camel hairbrush which has been

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dipped in 1% Celloidin. After allowing few seconds for the Celloidin to dry a section is cut in usual way.

It must be remembered that when floating the sections to remove the creases, the celloidin layer must be uppermost, and the water should be a little hotter than usual to counteract the effect of celloidin. Following drying in usual way, the celloidin is removed with equal parts of ether and alcohol before removing wax with xylol.

Serial sectioning

Serial sectioning may be needed to study the track of some structures or to find the extent of a lesions.

Sections are collected from the very first cut that includes any tissue. Ribbons of ten - 1-10, 11-20, 11-20, 21-30 so on are picked up and mounted on the slides.

Step sections

This is an alternative for serial sections and for the same reason.

Sections are taken at periodic level through the block. The request is made for every nth section for a total of 'n' sections.

Intermittent sections or sections on either side of each step sections for a total of 'n' sections.

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Cooling block and knife. In general, keep ice cubes ready at hand and cool the surface of block and knife before cutting, always dry and block and knife and block after the application of knife.

Trouble shooting for poor sections

There are times when proper section cannot be cut. Main reasons are either:

4. Faults occurring during section cutting or
5. Faults due to poor processing.

Below are given the various defects, reasons for the defect and the remedy for the same.

Faults in cutting

1. Fault - Tear or scratch across the section or splitting of ribbon.

   Cause - Jagged knife edge, Dirt or hair on knife edge.

   Remedy- Sharpen the knife, Clean the knife

2. Fault - Tear or scratch across part of section,

   Cause - Calcium, Carbon, or Suture etc., in the tissue or wax

   Remedy- Examine block under magnifying glass. If calcium is present, decalcify block. Remove suture from the tissue with scalpel point. If dust is in wax - Re-embed

3. Fault – Holes in the section.

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Cause - Air bubbles in the tissue or wax

A piece of hard material in tissue

A soft piece of tissue in block

Remedy- Re-embed

Remove hard material if possible

Reprocess specimen

4. Fault - Cracks across the section parallel to knife

Cause - A blunt knife, Knife tilt too small., Block too hard for thickness of specimen Remedy- Sharpen knife , Adjust tilt

Warm block slightly or re-embed in soft wax.

5. Fault - Section shows thin and thick horizontal lines (chatters) Cause - A loose knife, A loose block A blunt knife, extremely hard tissue

Remedy- Tighten knife and/or block

Sharpen the knife

Soften the tissue if possible or embed in harden wax.

6. Fault - section cut thick and thin alternative

Cause - Knife tilt is too great and is compressing the block Remedy Adjust tilt.

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7. Fault - Section compress at one end. Cause - Blunt spot on the knife

A soft spot in the wax, due to presence of clearing agent Remedy- Move block along the knife or sharp knife.

Re infiltrate tissue and re-embed

8. Fault - Section curves to one end.

Cause - Edge of block is not parallel to knife.

A dull spot on knife.

Remedy- Trim edges

Move block along knife or sharpen knife.

9. Fault - Section curl as the they are cut Cause - Blunt knife

Sections too thick Too much tilt to knife

Remedy- Sharpen knife

Adjust microtome Correct the tilt

10. Fault - Sections lift from knife on upward travel of block Cause - Blunt knife

Too much tilt to knife

A buildup of wax debris behind knife A greasy knife.

Remedy- Sharpen knife

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Correct the tilt Clean the knife

11. Fault - Knife bites deeply into block Cause - A loose knife

A loose block

Remedy- Tighten the knife and block

12. Fault - The block no longer feeds towards knife Cause - Forward feed mechanism had expired

Remedy- Release the safety locking catch, man back off feed mechanism and readjust knife holder

13. Fault - Sections crumble on cutting Cause - Knife is blunt

Wax is too soft; has crystallized due to slow cooling or contamination with water or clearing agent.

Defective processing e.g. incomplete fixation, dehydration, clearing or embedding.

Remedy- Sharpen knife.

Re-embed and block with fresh wax Reprocess

14. Fault - Failure of block to ribbon

Cause - Block not parallel to ribbon

Paraffin too hard. Knife tilted too much Sections too thick

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Remedy- Correct the alignment

Re-embed Correct the tilt

Adjust the section thickness

Fault due to poor processing

1. Fault - The tissue is shrunken away from wax Cause - Insufficient dehydration Remedy- Reprocess

2. Fault - The tissue is too soft when block is trimmed Cause - Insufficient fixation

Remedy- Reprocess

3. Fault - Specimen crumbles and drops out of the wax leaving a rim of wax as a section

Cause - Insufficient infiltration

Overheated paraffin bath causing tissue to become hard and brittle

Remedy- Re infiltrate and re-embed

Service the paraffin bath

4. Fault - Tissue is dried out or mummified

Cause - Mechanical failure of tissue processing machine or a basket was out of balance and hung up.

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Remedy- Place the specimen in the following rehydration solution for 18-24 hrs.

Sodium Carbonate - 1.0 gm Dist. Water - 70.0 ml Absolute ethyl alcohol - 30.0 ml Re hydrate the reprocess

Lecture 6 STAINING

The sections, as they are prepared, are colorless and different components cannot be appreciated. Staining them by different colored dyes, having affinities of specific components of tissues, makes identification and study of their morphology possible. Certain terminologies used in the following account are given below.

Basophilic: Substances stained with basic dyes

Acidophilic: Substances stained by acid dyes

Vital staining: Staining of structures in living cells, either in the body (in vivo) or in a laboratory preparation (in vitro). e.g. Janus green is taken up by living cells and stains the mitochondria.

Metachromatic staining: There are certain basic dyes belonging to aniline group that will differentiate particular tissue components by staining them a different color to that of original dye. The phenomenon is known as metachromasia. The tissue elements reacting in this manner are said to be exhibiting metachromasia.

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The generally accepted explanation of this phenomenon is that change in color is due to polymerization. Sulfated substances are highly metachromatic e.g. Mast cell granules. These contain Heparin which is highly sulfated.

Some of the common metachromatic dyes are :

Methylene blue   Methyl violent

Thionin   Crystal violent

Toluidine blue

Thionin and toluidine blue dyes are commonly used for quick staining of frozen selection using their metachromatic property to stain nucleus and cytoplasm differently.

Tissue components often demonstrated by metachromatic stains : Amyloid material, Mast cell granules Mucin, Cartilage

Direct staining: Application of simple dye to stain the tissue in varying shades of colors.

Indirect staining: It means use of mordant of facilitate a particular staining method or the use of accentuator to improve either the selectivity or the intensity of stain.

Progressive staining: Stain applied to the tissue in strict sequence and for specific times. The stain is not washed out or decolorized because

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there is no overstaining of tissue constituents. Staining is controlled by frequent observation under microscope

Regressive staining: Tissue is first overstained and then the excess stain is removed from all but the structures to be demonstrated. This process is called differentiation and should always be controlled under microscope.

Decolorization: Partial or complete removal of stain from tissue sections. When the colour is removed selectively (usually with microscopic control) it is called differentiation. In case decolorization is to restain the selection with some other stain, acid alcohol treatment is the method of choice.

Differentiation

In regressive staining differentiation is the removal of washing out of the excess stain until the colour is retained only in the tissue components to be studies.

Impregnation

It is the deposition of salts of heavy metals on or around cells, tissue constituents etc. It has followed characteristics

1. Structures demonstrated are opaque and black
2. The colouring matter is particulate
3. The deposit is on or around but not in the element so demonstrated.

Histochemical staining

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Staining which is used to indicate the chemical composition of the tissue or cellular elements.

Counter stains:

A counter stain is the application to the original stain, usually nuclear, of one or more dyes that by contrast will bring out difference between the various cells and tissues. A heavy counterstain is to be avoided lest it mask the nuclear stain. It can be done either by using dilute stain or cutting down the staining time. Some counterstains which are acidic may lighten or remove the nuclear stains.

Mordants:

Substance that causes certain staining reactions to take place by forming a link between the tissue and the stain. The link is referred as lake. Without it, dye is not capable of binding to and staining the tissue.

e.g. Ammonium and Potassium alum for haematoxylin.

Accentuators:

These are substances that causes an increase in the selectively or in the staining power of dye. Thus they lead to more intense staining.

e.g. Phenol in Carbol fuchsin, KOH in Mehtylene blue.

Leuco compounds:

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Conversion of a dye into a colourless compound by the destruction of its chromophore. Prefix leuco is applied to it, e.g. leucofuchsin used in PAS stain.

Dyes used in staining

Dyes are classified in various ways:

1. According to source	2. Affinity to tissues	3. Chemical composition
a. Natural	a. Acidophilic	a. Thiazines
b. Synthetic	b. Basophilic	b. Azo-dyes
	c. Rosailins	

Synthetic dyes have greater staining capacity, much greater spectrum of colours.

Natural dyes

These are very few in numbers. They are mainly two in common use.

1. **Hematoxylin:** This is the most popular dye used as a nuclear stain.

It is derived from the log tree mainly found in Mexico. It develops staining property after oxidation. It is a weak dye and to make it give sharp stain a mordant is needed

2. **Carmine:** It is a scarlet dye made from the ground bodies of cochineal beetles.

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Synthetic dyes

Most of these are in Aniline base and derived from coal tar. These aniline dyes offer wide range of colour and action. These aniline dyes offer wide range of colour and action. Chemical composition may be basic, acidic, amphoteric (neutral). According to these characters stain different components of tissue.

Basic dyes

These are cationic dyes and stain nuclei, basophilic granules or bacteria.

Acidic dyes

These are anionic dyes and stain mainly cytoplasm, eosinophilic granules.

Theories of staining

Physical theories:

1. Simple solubility e.g. Fat stains are effective because the stain is more soluble in fat than in 70% alcohol.

2. Adsorption: This is a property by which a large body attracts to itself minute particles from a surrounding medium.

Chemical theories

It is generally true that acid dyes stain basic elements (Cytoplasm) and basic dyes stain acidophilic material (nucleus) however this far from

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being complete truth, Indeed hematoxylin, which is an acid dye, does not stain the cytoplasm, but (in the presence of mordant) is one of the most widely used nuclear stains.

Staining of paraffin section

The most common method of histological study is to prepare thin sections (3-5 micron) from paraffin embedded tissues. These are then suitably stained and mounted in a medium of proper refractive index for study and storage. Commonest mountants used are resinous substances of refractive index close to that of glass. These are soluble in xylol. Hence sections are dehydrated and cleared in xylol and mounted. Mounting in aqueous mounting media is done directly after staining for sections which cannot be subjected to dehydrating and clearing agents.

The basic steps in staining and mounting paraffin sections are as follows:

1. Deparaffinization
2. Hydration
3. Removal of mercury pigments wherever needed
4. Staining
5. Dehydration and clearing
6. Mounting

1. Deparaffinization

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Removal of wax is done with xylol. It is essential to remove the wax completely, otherwise subsequent stages will not be possible. At least 2 to 3 changes in xylol are given for suitable length of time. Sections of this stage should appear clear and transparent. Presence of any patches indicates the presence of wax and sections should be kept longer in the xylol.

2. Hydration

Most of the stains used are aqueous or dilute alcoholic solutions. Hence it is essential to bring the section to water before the stains are applied. The hydration is done with graded alcohols from higher concentration to lower concentration. Alcohol and acetone are miscible with xylol. First change is made to absolute alcohol or acetone followed by 90%, 70% alcohol and finally distilled water.

Sections now should appear opaque. Presence of any clear areas are indicative of the presence of xylol. To remove this xylol, sections should be returned to absolute alcohol and rehydrated.

3. Removal of mercury pigments wherever needed

In case mercury containing fixatives e.g. Zenker, Susa etc are used, mercury pigments are precipitated on the sections. It has to be removed before staining is done. This is brought about by treatment with iodine solutions which changes mercury to an iodine compound. This in turn is converted to tetrathionate by thiosulphate, which is readily soluble in

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water. The slides are placed in running water to wash out all extraneous chemicals.

4. Staining

Various staining procedures are applied from this hydrated stage. The most common stain applied for histological study is Haemotoxylin and Eosin. Various types of haemotoxylin formulations are used.

Certain of the stains use strong chemicals e.g. ammonia. Sections tend to float off the slides in such stains. This can be prevented by coating the sections by a thin layer of celloidin. For these sections are returned to absolute alcohol and then dipped in a dilute solution of celloidin and finally hardened in 70% alcohol.

Washing and rinsing of tissue sections is a necessary part of most staining techniques. It eliminates carrying over of one dye solution to the next. Excess dye, mordants, or other reagents might react unfavourably or precipitate when placed in the fluid employed in the next step.

5. Dehydration and clearing

Dehydration is done is graded alcohols or acetones from 70% to absolute alcohol or acetone. Dehydrating alcohol and acetones can remove some of the stains. Time has to be suitably modified to minimize fading of stains.

Since alcohol and acetone are miscible in xylol, it is used for clearing the sections. Any sections from which water has not been completely

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removed would give a milky appearance after the first xylol. Such sections should be returned to absolute alcohol and the process repeated. Mounting is done after 2nd or 3rd xylol.

6. Cover slipping and mounting

Make quite sure that the sections are quite clear. Do not let the section go dry before mounting

1. Hold the slide between the thumb and the forefinger of one hand and wipe with a clean cloth both ends of the slides. Look for the engraved number to make sure the side the sections is present.

2. Clean carefully around the section and lay on a clean blotting paper with section uppermost along with appropriate coverslip which has already been polished.

3. Place a drop of mountant on the slide over coverslip. Amount of mountant should be just enough. Invert the slide over the coverslip and lower it so that it just adheres to the cover slip quickly turn the

slide over, then lay it on a flat surface to allow the mountant to spread. Do not press or push the slide at all. It can damage the section.

4. After the mountant has spread to the edge of the coverslip wipe around it for neatness. If proper care has been taken there should be no air bubbles. If many are present, slide should be returned to the xylol to

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remove the coverslip. It will slip off and remounting is done. No attempt should be made to pull the coverslip. Slight warming of the slide from below will make the small air bubbles to escape from the slide of the coverslip.

5. Coverslip should be in the center of the slide with neatly written label on one slide.

A good knowledge of various mountants and the coverslips is necessary for proper selection of the procedure.

Mountants

Histological sections which need to be examined for any length of time or to be stored, must be mounted under a coverslip.

There are two types of mounting media :

1. Aqueous media - Used for material which is unstained, stained for fat, or metachroamtically stained.
2. Resinous media - For routine staining.

Aqueous media

There are used for mounting sections from distilled water when the stains would be decolorised or removed by alcohol and xylene, as would be the case with most of fat stains (Sudan methods). Some stains, e.g. methyl violent, tend to diffuse into medium after mounting. This can be avoided by using Highman's medium. Aqueous mountants require addition of

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bacteriostatic agents such as phenol, crystal of thymol or sodium merthiolate to prevent the growth of fungi.

Permanent seal - After mounting the cover slip can be ringed by clear nail polish for storage.

Following are some of the commonly used aqueous mounting media: For formulation see the appendix.

1. Apathy's medium (R.I- 1.52)

A very useful medium for mounting sections for fluorescent microscopy.

2. Farrant's medium (R.I. 1.43) Recommended for fat stains.

3. Glycerine jelly (R.I. 1.47)

An excellent routine mountant for fat stains.

4. Highman's medium (R.I. 1.52)

Recommended with the metachromatic dyes especially methyl violent.

Resinous mounting media

Natural or synthetic resins dissolved in benzene, toluene or xylene. These are purchased readymade. In case they become too viscous they may have to be diluted with xylene. Following are some of these media.

1. Canada balsam - Natural resin (R.I. - 1.52)

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It is used as 60% resin by weight in xylene. H.&E stained slides are fairly well preserved but basic aniline dyes tend to fade and Prussian blue is slowly bleached. Slides take few months to dry.

2. **D.P.X. (R.I. 1.52)**

Polystyrene resin dissolved in xylene as a 20% solution. It is most commonly used.

3. There are many other synthetic resins sold under various trade names e.g. Coverbond (R.I. 1.53), H.S.R. (Harlew synthetic Resin), Histoclad (R.I. - 1.54), Permount (R.I. 1.54), Pro-Texx (R.I. 1.495).

Criteria of acceptable mounting media

1. Refractive index should be as close as possible to that of glass i.e.
2. It should not cause stain to diffuse or fade.
3. It should not crack or appear granular on setting.
4. It should be dry to a nonsticky consistency and harden relatively quickly.
5. It should not shrink back from edge of cover-glass.
6. It should be free flowing and free from air bubbles.

Cover glasses used in histopathology

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Care has to be exercised in selecting cover glasses for mounting, these are available in variable sizes and thickness and are supplied usually in 10 gm packings.

Following sizes are commonly available 22 x 22 mm   25 x 50mm

22 x 30 mm Circular

22 x 40 mm

Cover glass should preferably be the No. 1 thickness (0.13 - 0.16 mm), but never more than No. 1 ½ thickness (0.16 - 0.19 mm).

Hematoxylin

Haematoxylin as supplied has no staining properties until it has been ripened by oxidation into haematin. This ripening is achieved by two methods:

1. Exposure of prepared solutions to the air for periods up to 6-8 weeks, preferably in sunlight

or

2. Addition of an oxidizing agent such as sodium iodate, potassium permaganate or mercuric oxide.

In this ripening process Haemtoxylin (C16 HI & O6) loses two hydrogen atoms to become Haematin (C16 H12).

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Sufficient Haematoxylin should be left unoxidized in solution, so that natural oxidation can continue. It prolongs shelf life of the stain.

Blueing

Alum Haematoxylin stains nuclei and red color, which is converted to blue black color, when the section is washed in weak alkali. Tap water is usually alkaline enough to produce this color change.

Following may be used for rapid blueing of the sections.

1. 1% Lithium carbonate.
2. 2% Ammonia (Ammonia Water).
3. Scott's water

Sod. or Pot. Carbonate 2 to 3 gm Magnesium sulphate 20 gm Dist. water 1000 ml

There are many formulations for preparing haematoxylin stains. Use of many is a matter of personal preference of whether progressive or regressive staining is being used. In situations where haemotoxylin staining is followed by acidic stains, Iron haemotoxylin is preferred as it resists decolourisation by these counter stains. Various formulations differ mainly in regards to mordant and the shorter oxidiser used.

Cytoplasmic stains

- **EOSIN (AFIP)** Eosin 1% stock

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Dissolve 1gm of eosin Y water soluble in 20ml of distilled water and 80ml of 95% alcohol.

Eosin working

Stock Eosin - 1 Part

Alcohol 80% - 3 Parts

Add 0.5ml of acetic acid just before use per 100 ml

- Eosin Phloxine (AFIP)

Eosin B 1% and distilled water Eosin Phloxine working

Stock eosin 1% in distilled water - 100ml Stock Phloxine 1% in distilled water - 10ml Alcohol 95% 780ml

Acetic acid 4 ml

Working solution to changed weekly

- Nuclear fast red (Kernechtrot) Aluminium sulphate 5 gms

Distilled water 100 ml Heat and dissolve and cool

nuclear fast red 0.1gm

Dissolved with the aid of heat, cool and filter. Add a crystal of thymol.

Nuclear stains

- Hematoxylin Ehrlich's

2% haematoxylin in alcohol 100 ml

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3% ammonium or potassium alum in distilled water 100ml Mix the two above and add the following in order

Glycerol 100 ml Acetic aid 10ml

Keep the bottle loosely plugged Let it ripen for 1-3 months

- Hematoxylin Harris

10% ammonium or potassium alum in distilled water 100 ml 10% alcoholic hematoxylin 10 ml

Bring the alum to boiling point and add the haematoxylin solution carefully till the solution is deep red. Add 0.5 gm of red oxide of mercury, solution becomes become deep purple. Promptly remove the flame and plunge into ice cold water. This is the most important part. Leave over night at room temperature and filter. Add 2 or 4 ml of acetic acid before use per 100 ml in the stain.

Note: In place of red oxide of mercury 0.177 gm of potassium permanganate can be used but should added after cooling the solution and never while boiling.

- Hematoxylin phosphotungstic acid Mallory's Haematoxylin 1.0gm

Phosphotungstic acid 20gm Distilled water 1000 ml

Dissolve haematoxylin and phosphotungstic acid separately in distilled water with the aid of heat, when cool, combine the solution and make

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upto 1 litre with distilled water. Let it stand for 5-6 weeks before use. Staining time - 12-24 hours

Note : Quick ripening may be done by adding 0.177 gm of potassium permanganate. However the results are not so good.

- Weigert's iron Hematoxylin

A. Haematoxyln 1gm Alcohol 100 ml

Let it ripen for a week

B. 30% solution of ferric chloride 4ml Distilled water 100 ml Hydrochroloric acid (conc.) 1 ml

Immediately before use mix equal parts of A&B add B to A and not vice versa.

Staining time 20-30 minutes

- Mayer's Hematoxylin Hematoxylin 1gm

Distilled water 1000ml

Heat distilled water to 55 to 60°C and add hematoxylin rotate till dissolved.

Ammonium or potassium alum 50 gms Sodium iodate 0.20gm (to be weighed exactly) Add the above in order given

Citric acid 1gm Chloral hydrate 50.0 gm

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Above must added in order given. Allow to stand overnight before use. Solution is stable for 6-8 weeks. Staining time 6-8 minutes to increase after a month.

Some basic rules for staining

1. Keep stains and solutions covered when not in use.

2. After the slides are removed from oven these should be cooled before being put in xylene.

3. Filter stains before use.

4. Once the slides have been put in the xylene to remove paraffin they should not be allowed to dry out. Particular care must be taken not to let the sections dry at the time of mounting as the xylene easily evaporates and if the section dried before mounting preparation would become useless.

5. Care should be taken that level of any solution used during staining is such as to cover the slides.

6. Drain the slides well and blot the bottom on filter paper before putting into the next solution. This is particularly necessary in transferring from 95% to absolute alcohol and absolute alcohol in xylol.

7. Xylol used to remove paraffin should not get mixed up with the clearing xylol. It also should be frequently changed as it tends to get saturated.

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8. If for blueing an alkali e.g ammonia has been used, it should be well washed out. Failure to do that will lead to disagreeably hazy blue colour of nuclei.

Haematoxylin and Eosin staining

Procedure

- Deparaffinize in hot air oven.
- Hydrate the section.

i) 3 dips in xylene (2 Min. each)

ii) 3 dips in acetone / alcohol (2 Min. each)

iii) In running tap water for 5 Minutes.

- Mayer's haemotoxylin for 15 minutes.
- Wash in running tap water for 20 minutes
- Counter stain with eosin for 2 minutes
- Dehydrate the section in 95% and absolute alcohol/ acetone 2 changes (2minutes each).
- Clear in xylene 3 changes (2 minutes each)
- Mount in DPX

Results

Nucleus - blue

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Cytoplasm and background - pink

Causes of poor quality of staining

1. Poor or inadequate fixation of tissue.
2. Over or under-ripened Haematoxylin.
3. Overused or worked out Haematoxylin.
4. Over or under differentiation of haematoxylin
5. Insufficient blueing following differentiation.
6. Failure to wash blueing agent out of section before counter staining with eosin (especially when ammonia is used).
7. Insufficient differentiation of eosin during washing or dehydration.
8. Insufficient dehydration and clearing of sections.
9. Contamination of stains.

CHAPTER 7: SPECIAL STAINING VAN GIESON METHOD

Aim: Staining of the connective tissue.

Principle: In the routine staining method collagen, elastic fibers and smooth muscle appear pink or reddish in color.

In the Van Gieson stain, collagen and most reticulin stain selectively with acid aniline dyes (acid fuchsin). Picric acid acts as counter stain for

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muscle and cytoplasm and form complex with the dyes. This complex has special affinity for collagen.

Reagents

1. Solution A

(a) Haematoxylin 1.0 gm

(b) Alcohol 95% 100 ml

2. Solution B

(a) 29% (w/v) ferric chloride 4 ml

(b) Conc. Hydrochloric acid 1.0 ml

(c) Distilled water 95.0 ml

3. Weight's iron heamatoxylin solution :mix equal quantities of solution A and solution B. Colour of this reagent should appear violet black.

4. Van Gieson's solution

(a) Saturated aqueous picric acid – 10 ml

(b) 1% (m/v) acid fuchsin – 1.5ml It should be freshly prepared

Procedure

(1) Deparaffinize with xylene

(2) Hydration take sections to water

(3) Stain with Weigert's haematoxylin for 20-40 minutes

(4) Wash in distilled water

(5) Van Gieson stain 1-3 min

(6) Rinse well in distilled water

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(7) Dehydrate in absolute alcohol (2 changes)

(8) Clear in xylene (2 changes)

(9) Mount in DPX

Results

1. Collagen Red
2. Muscle and Cornified epithelium Yellow

(B) Nuclei Blue to Black

GOMORI'S METHOD FOR RETICULUM

Aim – Demonstration of reticulum fibers by silver nitrate method.

Principle: In the connective tissue, reticulin appears as a fibrillary extracellular framework. Reticular fibers have low natural affinity for silver salts and require pretreatment with heavy metal solutions like ferric ammonium sulphate to enhance the selectivity of impregnation Silver in alkaline solution is in a state readily able to precipitate as metallic silver. Upon treatment with a reducing agent, silver taken up by the tissue in unreduced form is, converted to metallic silver which is deposited at the sensitized site.

Reagents

1. Ammoniacal silver solution

To 10 ml of 10% silver nitrate solution add 2.5 ml of 10% aqueous solution of potassium hydroxide, add 28% ammonium hydroxide drop by

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drop while shaking the container continuously until the precipitate in completely dissolved. Add again 4 drops of silver nitrate solution for every 10 ml of silver nitrate used. Make the solution with distilled water to twice its volume use acid clean glassware

2. 0.5% potassium permanganate – Potassium permanganate –0.5 gm Distilled water 100 ml

3. 2% potassium metabisulphite

Potassium metabisulphate 2.0 gm Distilled water 100 ml

4. 2% ferric ammonium sulfate solution

Ferric ammonium sulphate 2 gm Distilled water 100 ml

5. 20% formation solution

Formaldehyde 20 ml Distilled water 80 ml

6. 0.2% gold chloride solution

Gold chloride solution 1% - 10 ml Distilled water 40 ml

7. 2% sodium thiosulphate solution

sodium thiosulphate 2 gm Distilled water 100 ml

Procedure

1. Deparaffinze and hydrate to distilled water
2. Oxidize in potassium permagnate solution for 1 minute
3. Rinse well in tap water – 2 min
4. Differentiate with potassium metabisulphite solution for 1 minute.
5. Wash in tap water for 2 min
6. Sensitize in Ferric ammonium sulphate solution for 1 min.

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7. Wash in tap water for 2 minutes follow with two changes of distilled water 30 seconds each.

8. Impregnate in the silver solution for 1 minute

9. Rinse in distilled water for 20 seconds

10. Reduce in formalin solution for 3 minutes

11. Wash in tap water for 3 minutes

12. Tone in gold chloride solution for 10 minutes

13. Rinse in distilled water

14. Reduce in potassium metabisulfite solution for 1 minute

15. Fix in sodium thiosulfate solution for 1 minute.

16. Wash in tap water for 2 minutes.

17. Dehydrate in 95% alcohol, absolute alcohol and clear in xylene 2 changes

18. Mount in DPX.

Results

Reticulin fibers – black Background – grey

McManus FOR GLYCOGEN (PAS)

Aim – Staining and identification of the various types of carbohydrates (polysaccharides & mucopolysaccharides).

Principle: Tissue structures like liver & heart, striated muscles are studied by Periodic acid Schiff stain. Periodic acid reacts with aldehyde group of the carbohydrates and afterwards reaction with the Schiff's reagent produces a red or purple red color.

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Reagents

1. 0.5% w/v periodic acid solution

2. Schiff's reagent

(a) Dissolve 1.0 gm of basic fuchsin in □100 ml of boiling distilled water cool to about 60 degrees and filter.

(b) Add 20 ml of 0.1 N hydrochloric acid, cool further and add 1.0 gm of sodium metabisulphite and mix well.

(c) Keep in the dark for 24-48 hours. When the solution becomes straw coloured, add 300 mg of activated charcoal, shake vigorously, filter and store.

3. 1 N Hydrochloric acid

4. 0.1 gm of light green in 100 ml of 0.1% (v/v) acetic acid.

5. Harris haematoxylin stain

Procedure

1. Deparaffinize and hydrate to distilled water.

2. Oxidize in periodic acid solution for 5 minutes

3. Rinse in distilled water

4. Schiff's regent solution for 15 minutes.

5. Wash in running water for 10 minutes for pink colour to develop.

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6. Harris haematoxylin for 6 minutes or light green counter stain for a few seconds.

7. Wash in running water.

8. Differentiate in 1% acid alcohol solution 3-10 quick dips.

9. Wash in running water.

10. Dip in ammonia water to blue the sections

11. Wash in running water for 10 minutes

12. Dehydrate in 95% alcohol, absolute alcohol, clear in xylene two changes each.

13. Mount in DPX.

Results

With hematoxylin counterstain

1. Nuclei – blue

2. Glycogen, mucin, hyaluronic acid, reticulin, colloid droplets, amyloid infiltration, thrombi. – purple red

3. Fungi – Red

4. Background – pale green (with light green counter staining).

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Biological Molecules

Carbohydrates, Proteins, Lipids, and Nucleic Acids

Organic Molecules

- Always contain Carbon (C) and Hydrogen (H)
- Carbon is missing four electrons
- Capable of forming 4 covalent bonds
- Carbon can bind with hydrogen, nitrogen, oxygen, and itself!
- Forms long chains, branched, rings, etc

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```
    H   H   H   H   H   H   H   H   H   H
    |   |   |   |   |   |   |   |   |   |
H — C — C — C — C — C — C — C — C — C — C — H
    |   |   |   |   |   |   |   |   |   |
    H   H   H   H   H   H   H   H   H   H
```

Organic Molecules

Proteins, Carbohydrates, Lipids etc are macromolecules

Many molecules joined together

Monomer: Simple molecules

Polymer: Large molecules formed by combining monomers

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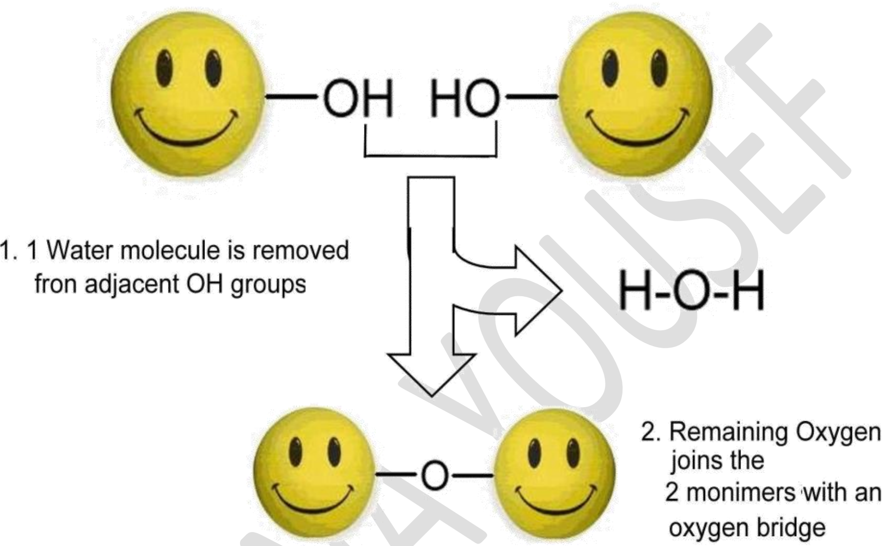
Polymer	Monomer
carbohydrate (e.g., starch)	monosaccharide
protein	amino acid
nucleic acid	nucleotide

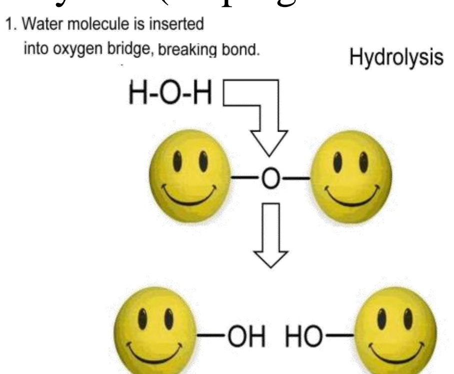
Polymer Formation: - Making big molecules from small molecules

- Requires water!
- Dehydration Synthesis
- Water is produced as monomers are combined together



Polymer Breakdown: -Breaking big molecules into small molecules

- Requires water!
- Hydrolysis
- Water breaks up the bonds in another molecule
- Requires enzymes (helping molecules)



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Carbohydrates

Sugars (glucose, sucrose, starch)

Functions:

- **Short term energy supply**

Glucose produces ATP energy

- **Short term energy storage**

Glycogen is stored in the liver and muscles

- **Structure**

Plant cell walls, insect exoskeletons

- **Cell Membrane markers**

Cell "identity tags"

- **All carbs have the formula Cn(H2O)n**

Monosaccharides

Monosaccharides have 3-7 carbon atoms

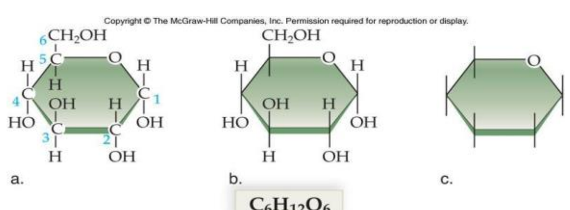
Ex) Glucose, ribose, galactose, fructose

Only one unit molecule

Pentose = 5 carbons

Hexose = 6 carbons

"ose" = carbohydrate



Disaccharides

Two molecules together

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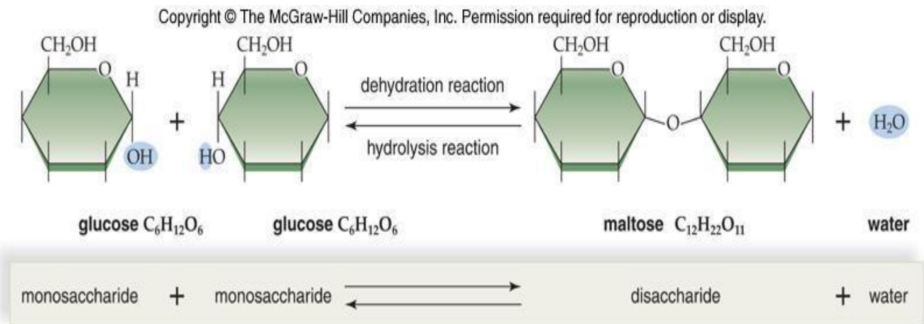
Ex) Maltose, lactose

Formed from dehydration synthesis



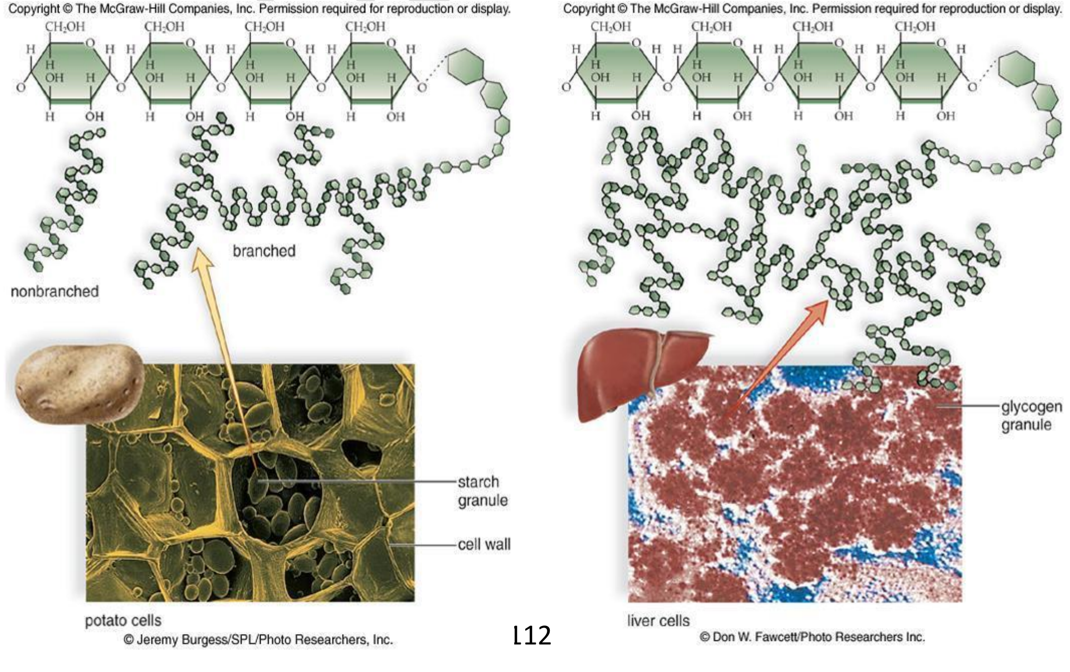
Polysaccharides

Many molecules together, Repeating glucose subunits

Examples: 1- **Starch**

Glucose storage in plants, Straight chains with little branching.

2-**Glycogen**; Glucose storage in animals, many side chains.



3-Cellulose: In plant cell walls

No branching – alternating oxygen positions for more structure

Cannot be digested - fiber

Carbohydrate Histochemistry

A. Demonstration of Homopolysaccharide

.1 Starch

The presence of starch in tissues can be determined by an iodine test.

Iodine Test

Principle: Reaction of iodine with the amylose in starch results in the formation of a polyiodide chain which gives deep blue color.

.2 Glycogen

In animals, glycogen is the major storage form. It is a highly branched polymer of D-glucose units and is mostly found in the liver and the muscles.

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Carmine Method

Principle: Carminic acid reacts with the hydroxyl group of glycogens that results in red color glycogen.

.3 Cellulose and Chitin

The presence of these can be determined by **Calcofluor white staining method.**

Principle: Calcofluor white is a fluorochrome stain. It is non-specific in nature and stains cellulose and chitin by binding with it in the tissue environment.

B. Demonstration of Heteropolysaccharides

Heteropolysaccharides are also called as heteroglycans. Mainly, it includes glycosaminoglycans (example- hyaluronate, chondroitin sulfate, and keratin sulfate) and peptidoglycan.

1-Glycosaminoglycans

Also known as mucopolysaccharides or proteoglycans, are linear molecules containing uronic acid and sulfated groups that make it highly acidic. These mucosubstances (acidic and non-sulfated/sulfated) can be demonstrated by various methods:

Hale's colloidal iron method

Principle: At very low pH, carboxyl and sulfate-containing substances absorb the colloidal ferric ions. Prussian blue staining reaction then stains the absorbed ferric substance in blue.

Periodic-acid-Schiff Reaction

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Principle: The free hydroxyl group is oxidized by periodic acid to aldehyde. which after reaction with Schiff's reagent shows carbohydrates of tissue in purple color. Carbohydrates that are stained by this method are Sulphomucins, Proteoglycan, Glycogen, Glycolipids, etc.

Alcian blue

Principle: Alcian blue is a basic dye which mainly stains acidic mucosubstances that are carboxylated and sulfated by forming a salt bridge with them.

Iron diamine method

Principle: Diamine stain the O-sulfate esters by oxidizing itself in the reaction with ferric chloride.

C. Demonstration of Glycoproteins

Glycoproteins are branched molecules containing sialic acid and fucose groups. The presence of sialic acid at the free end of the glycoproteins makes it a negatively charged compound. Most of the glycoproteins compose the integral membrane protein, where they have an essential role in cell-cell interaction.

Methods of demonstration: All the method which are involved in the demonstration of Glycosaminoglycans (GAGs) can also be used to demonstrate Glycoproteins; such as PAS (Periodic acid Schiff) reaction, alcian blue, and Cuprolinic blue staining method.

Proteins

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Functions:

Structure: Keratin and collagen

Movement: Actin and myosin

Enzymes: Speed up chemical reactions

Transport: Hemoglobin to carry oxygen in blood, proteins across cell membrane

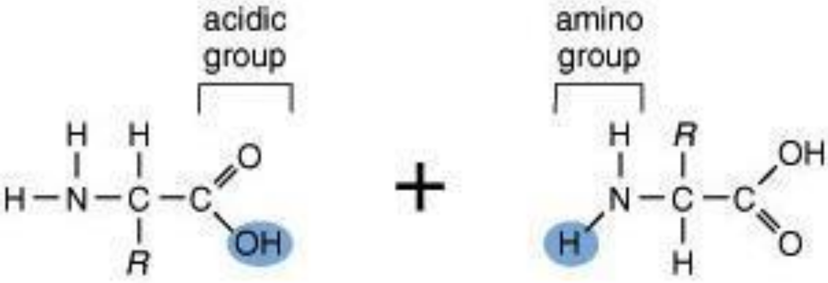
Antibodies: Fight disease

Hormones: Maintain cell function – insulin

Structure of Proteins:

Made of Amino Acids

Amine (NH3); Acid (COOH)



20 different amino acids have different R groups

-Amino acids undergo dehydration synthesis to form

Dipeptides (2 amino acids)

Polypeptides (~3-20 amino acids)

Proteins (many amino acids)

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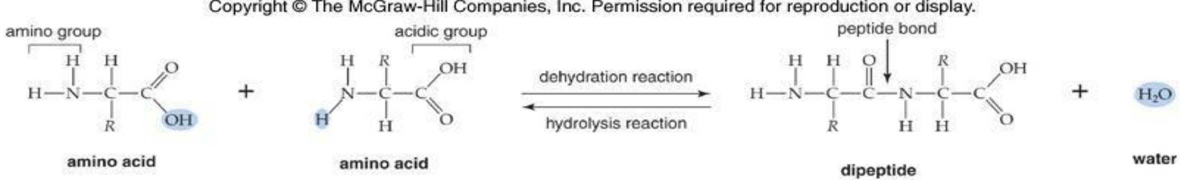
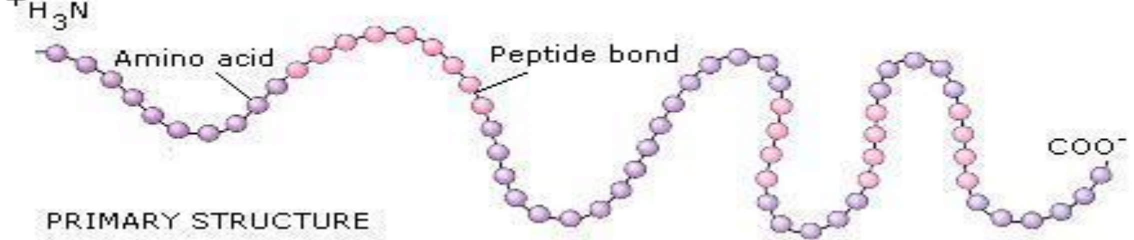
Peptide bond is formed (polar)



Structure of Proteins (4 Levels)

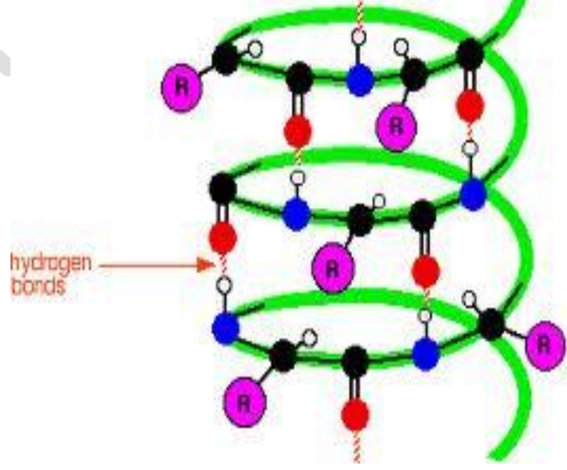
1-Primary Structure

Linear sequence of amino acids



2-Secondary Structure

- Hydrogen bonding between amino acids Causes folding
- Alpha helix and beta sheets



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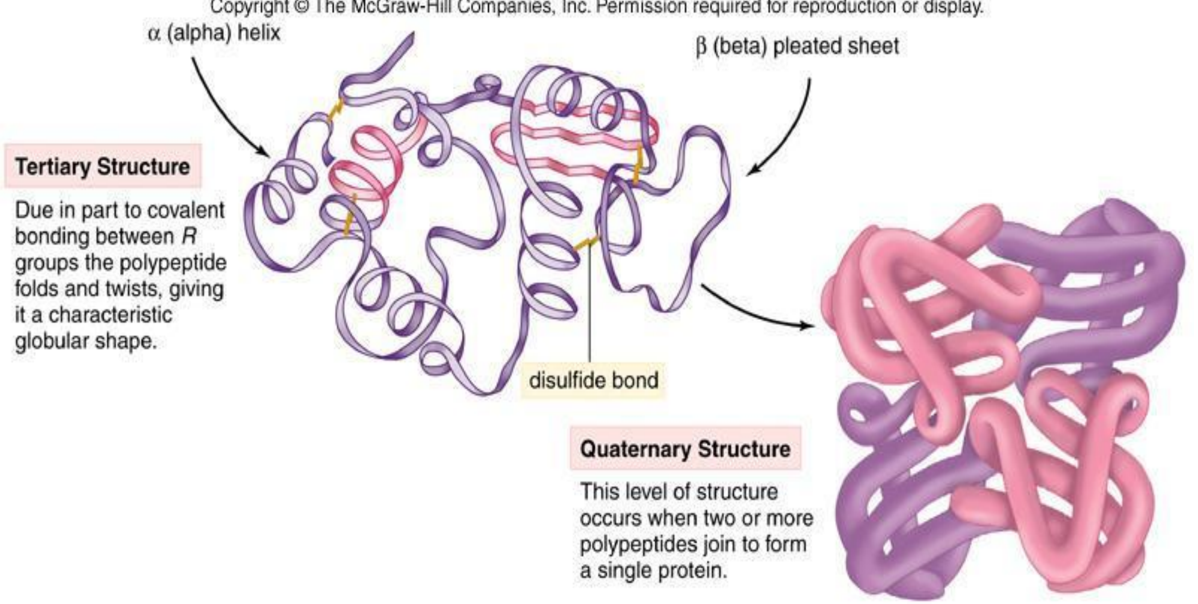
3-Tertiary Structure: 3D arrangement of amino acid chain

Caused by covalent, ionic, and hydrogen bonding between R groups

Precise shape = specific function

4-Quaternary Structure

More than one polypeptide chain grouped together



Denaturing Proteins

Cause protein to lose shape = not function

pH, temperature, chemicals and heavy metals disrupt bonds

Ex) Heating an egg, adding vinegar to milk

Illustration of proteins histochemical is only done when one or more amino acids are very high in their composition in the protein structure. But when the protein to be demonstrated is

mixed with other proteins and is in less concentration, their demonstration is done by enzyme activity. Some proteins are identified either by their tissue location or physio-chemical properties.

- There are mainly two principals involved in the illustration of protein:
- Reaction with amino acids, which after covalent bonding with a dye, highlights the protein.
- Reaction of charged dyes with a net charge on the protein (either positive or negative
- Basic proteins like histones, myoglobin, and ribosomal basic proteins are also stained and localized by staining with Anionic dyes.
- Depending upon the type of amino acid to be demonstrated in the protein, there are various methods involved, for ex- Tryptophan can be demonstrated by dimethylaminobenzaldehyde (DMAB) which is followed by coupling to diazonium salt. In this test, the blue color of the solution specifically shows the presence of tryptophan in the sample.

Test for proteins

.1 Biuret test

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The presence of protein in the test sample is often determined by the Biuret test. This test specifically checks the presence of the peptide bond. Two or more peptide bonds presence gives a positive test result.

Principle: Alkaline $CuSO_4$ reacts with the protein present in the sample, forming a complex that gives a violet-colored product.

.2 Ninhydrin test

This method is useful to detect amino acids, peptides, and proteins in the test sample. Ninhydrin reacts with a α-amino group of amino acids and proteins.

Principle: Ninhydrin is a very strong oxidative agent. Reaction with a α-amino group causes oxidative deamination of the amino acid, which gives a reduced form of ninhydrin, CO_2, and ammonia. Then, the reduced ninhydrin reacts with liberated ammonia and other ninhydrin, which results in the formation of a blue- colored complex

Tests for amino acids and lower peptides:

.1 Xanthoproteic Reaction

This test is used to check the presence of protein having aromatic amino acid. Principle: The Reaction of aromatic amino acids of the protein with the nitric acid on heating gives a yellow color because of the nitration of benzene ring.

The colored product changes to orange when alkali is added to the solution.

.2 Sakaguchi test (Arginine reaction)

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This method tests the presence of arginine in the test sample.

Principle: The reagent used in this method is comprised of α-naphthol and one drop of sodium hypochlorite (sometimes also called as Sakaguchi reagent). α- naphthol reacts with the guanidyl group of arginine and the obtained product is oxidized by sodium hypochlorite. The oxidation results in the red color of the compound.

.3 Hopkin's Cole test (Tryptophan reaction)

This method tests the presence of tryptophan in the sample.

Principle: This method is based on the reaction of tryptophan present in the sample with glyoxylic acid in the presence of conc. H2So4, that gives purple color to the solution.

Lipids

Fats, Oils, and Waxes

1- **Long term energy storage**: Pack energy into a small space
2- **insulation and Padding**: Protect organs
3- **Structure:** Cell membranes
4- **Chemical Messengers**: Steroids

All lipids do not dissolve in water = hydrophobic

Types

1-Fatty Acids: Chain of carbons ending in COOH

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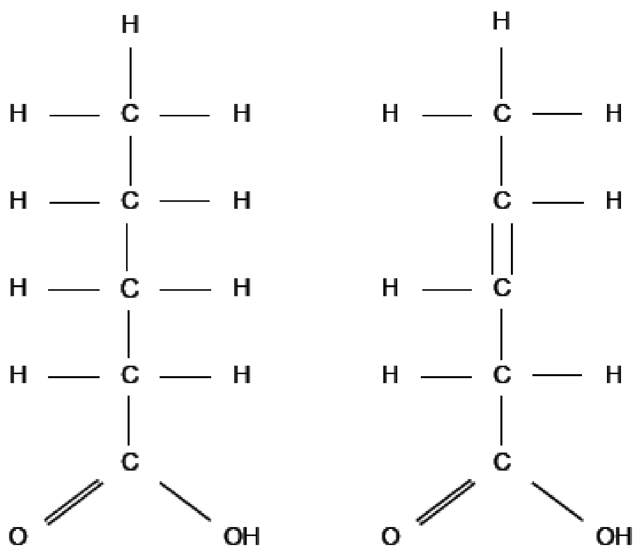
Saturated Fatty Acids: Solid at room temperature, Bad for health

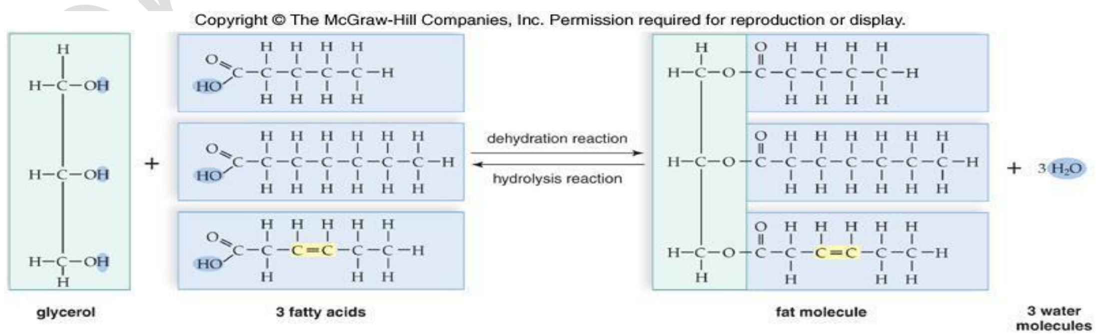
Unsaturated Fatty Acids: Contain double bonds, Liquid at room temperature.



2-Triclycerides: Neutral Fats

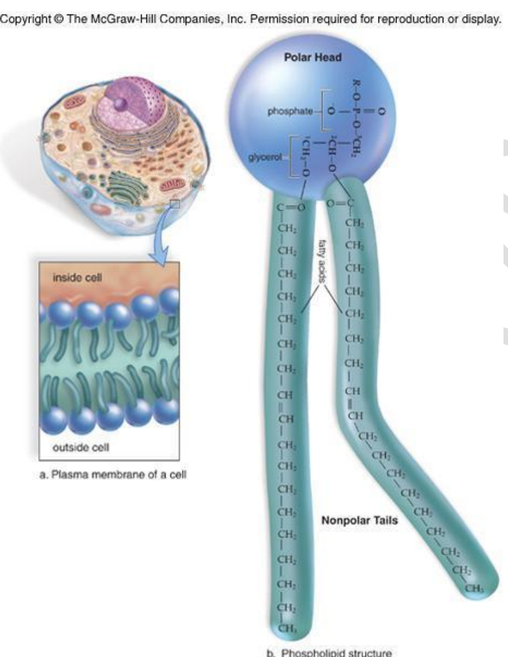
Glycerol + 3 Fatty Acids

Can be saturated or unsaturated



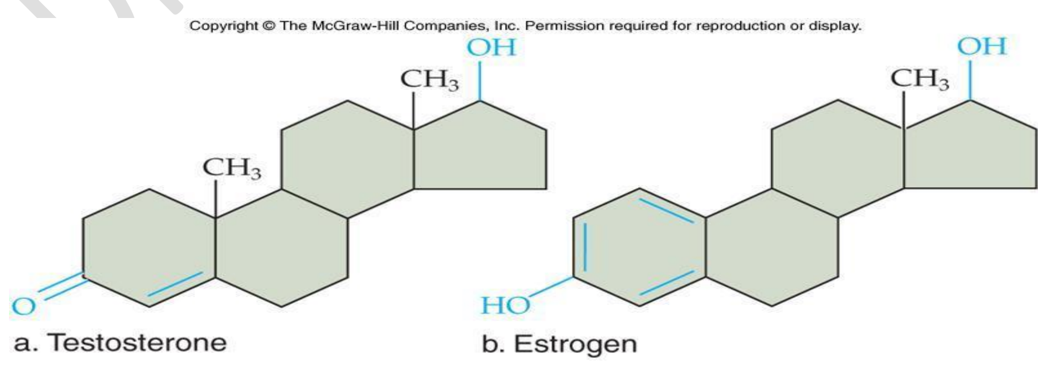
3-Phospholipids: Found in cell membrane

- Same structure as triglyceride but one fatty acid is replaced with a phosphate group (polar)
- Hydrophilic phosphate head, hydrophobic fatty acid tail.



4-Steroids: Ringed structures made from cholesterol

- Chemical messengers and form hormones, Ex) Cholesterol, Testosterone, Estrogen.



Lipid staining technique

is used for demonstrating intracellular lipids in various tissue sections. It involves the use of disparate Lysochromes (Lipid soluble dyes) like Sudan Black B, Nile red, and Oil Red O, etc. The dyes are selected depending on the type of lipid to be studied.

Principle

For this technique, the dye is more soluble in the lipid, which allows it to be more demonstrated than in the vehicular solvent. The dyes used in this technique are all interchangeable, which means that they can be substituted for each other for the staining process.

Demonstration of all types of Lipids (including hydrocarbons and higher alcohols)

Oil Red O Method

Principle: Staining with Oil Red O involves the principle of solubility of these

dyes in lipids than in the usual hydroalcoholic solvent.

Osmium Tetroxide method

Principle: Osmium tetroxide is fat-soluble and when it reacts with fats, it gets associated with the lipid head and forms a black colored reduction compound.

Demonstration of Hydrophobic or Hydrophilic Lipids

Bromine-Sudan Black method

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Principle: It is a basic dye and it reacts with the acidic group of lipids. This reaction results in the formation of a black colored product.

Marchi Method

Principle: The oxidation-reduction reaction of osmium tetroxide with lipid droplets results in the formation of a black colored product.

Demonstration of Hydrophobic Lipids (storage lipids)

Hydrophobic lipids include waxes, lipofuscin, free fatty acids, cholesterols, and triglycerides.

Nile Blue Method

Principle: Nile blue is the composition of two dyes that is blue oxazine and red oxazone (an oxidation product of oxazine). Oxazone is a lysochrome which reacts with the lipids to give a red to pink color.

Demonstration of Heterophasic Lipids

Heterophasic lipids are also called amphipathic lipids. This group includes phospholipids, glycosphingolipids, and cerebrosides. Histochemical techniques to demonstrate these lipids are: Nile Blue Method and PAS Method.

Demonstration of Phospholipids

OTAN Method

Principle: Osmium-α-naphthylamine chelates with the hydrophilic lipid present in the tissue that gives it orange to red color.

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Chromatin-acid hematin Method

Principle: Reaction of divalent chromate with the phospholipids form a chelated product. This product, when it reacts with acid Hematin solution, forms a dark blue to black colored product.

Nucleic acids

DNA and RNA

DNA: - Deoxyribonucleic acid

- Stores genetic information
- Codes for the order of amino acids in proteins
- Made of nucleotides
- 5 carbon sugar (deoxyribose)
- Phosphate
- Nitrogenous bases

Adenine (A)

Thymine (T)

Cytosine (C)

Guanine (G)

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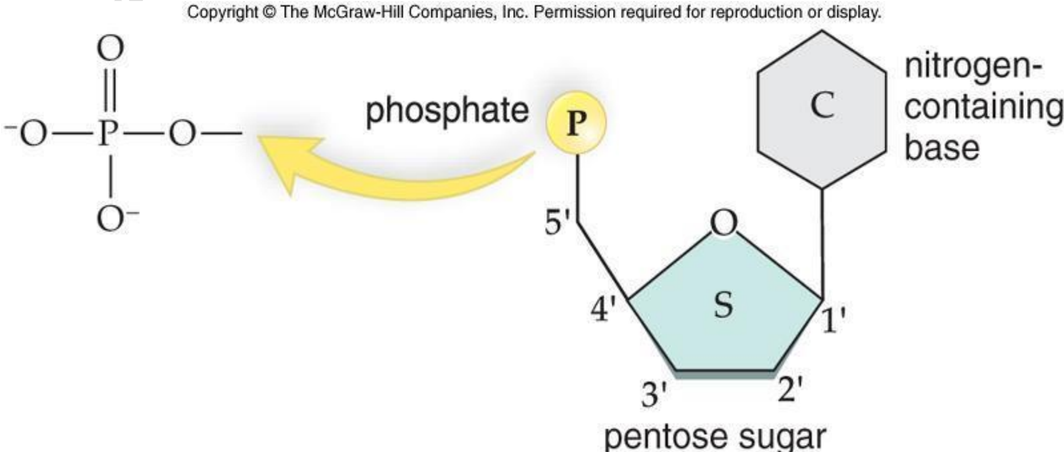
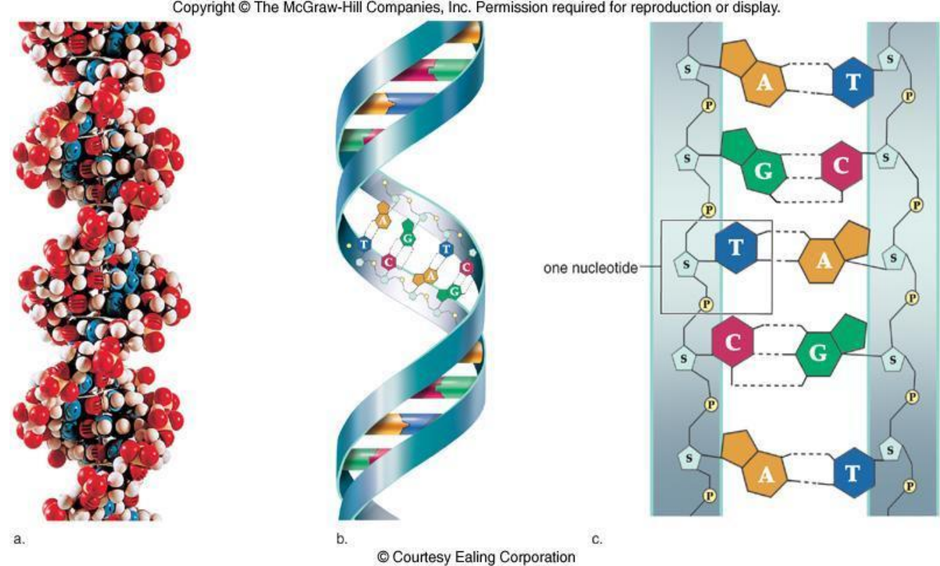


Nucleotide structure

- The sugar and phosphate bond to form a backbone
- Bases stick out and hydrogen bond with a second strand – antiparallel
- Strands wind around in a double helix



RNA: Ribonucleic acid

- Helps in protein synthesis
- Made of nucleotides
- 5 carbon sugar (ribose)
- Phosphate
- Nitrogenous bases

Adenine (A)

Uracil (U)

Cytosine (C)

Guanine (G)

- Single stranded

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TABLE 2.1 DNA Structure Compared to RNA Structure

	DNA	RNA
Sugar	Deoxyribose	Ribose
Bases	Adenine, guanine, thymine, cytosine	Adenine, guanine, uracil, cytosine
Strands	Double stranded with base pairing	Single stranded
Helix	Yes	No

Various methods that are involved in staining of Nucleic acids are as follows:

Feulgen's nuclear reaction: Most widely used method involves the principle of hydrolysis of DNA by HCl which exposes the deoxyriboses. Then Fuchsine reacts with the aldehyde group which colors the DNA in red.

S-Bromo-2′ -Deoxyuridine Method: BrdU is incorporated in DNA and for its visualization, BrdU specific monoclonal antibodies are used. Mainly used for the visualization of DNA in cultured cells, smears, and chromosomal spreads.

In-situ Hybridization: This method involves the melting of double-stranded nucleic acid and then hybridizing them with DNA or RNA probes having radioactive elements like 125I or 3H or non-radioactive elements like biotin for visualization of nucleic acid.

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- RNA is mainly stained by using basic dyes such as Toluidine Blue and methylene blue.Dyes that are used to stain both DNA and RNA include, Methyl green pyronin stain which is just used to observe the presence of nucleic acid; and Acridine orange which stains DNA in yellow-green and RNA in red-orange color.

A morphological technique that serves to demonstrate the activity of enzymes present on tissues.

Principle:

The visualization is based on the action of the enzyme on a specific substrate. Following this reaction, an insoluble product develops providing the location of enzyme. If the product is not stained, a metal precipitation technique or coupling with azoic dye and tetrazolium salt can be performed.

Methods to study the enzyme histochemistry depend upon the class of enzyme.

General principles of enzyme histochemical techniques

- Enzyme histochemistry combines the biochemical analysis of enzyme activity with information on its topographical localization.

- In a dehydrogenase reaction, enzyme substrates like sodium succinate or sodium L-lactate are oxidized and a stoichiometric color indicator tetranitrotetrazolium chloride blue (TNBT) is reduced to black or blue formazan. The formazan immediately binds to local protein and permits the precise

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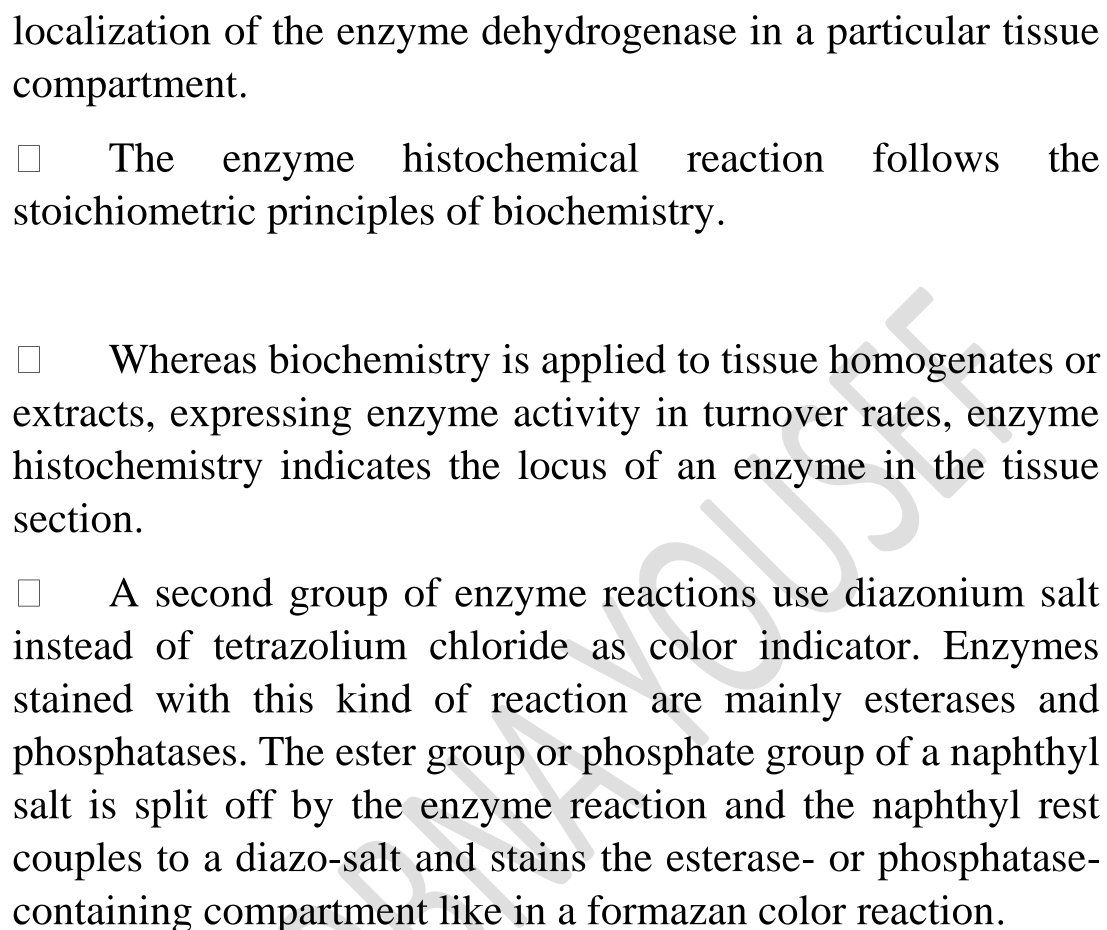
localization of the enzyme dehydrogenase in a particular tissue compartment.

- The enzyme histochemical reaction follows the stoichiometric principles of biochemistry.



- Whereas biochemistry is applied to tissue homogenates or extracts, expressing enzyme activity in turnover rates, enzyme histochemistry indicates the locus of an enzyme in the tissue section.

- A second group of enzyme reactions use diazonium salt instead of tetrazolium chloride as color indicator. Enzymes stained with this kind of reaction are mainly esterases and phosphatases. The ester group or phosphate group of a naphthyl salt is split off by the enzyme reaction and the naphthyl rest couples to a diazo-salt and stains the esterase- or phosphatase-containing compartment like in a formazan color reaction.

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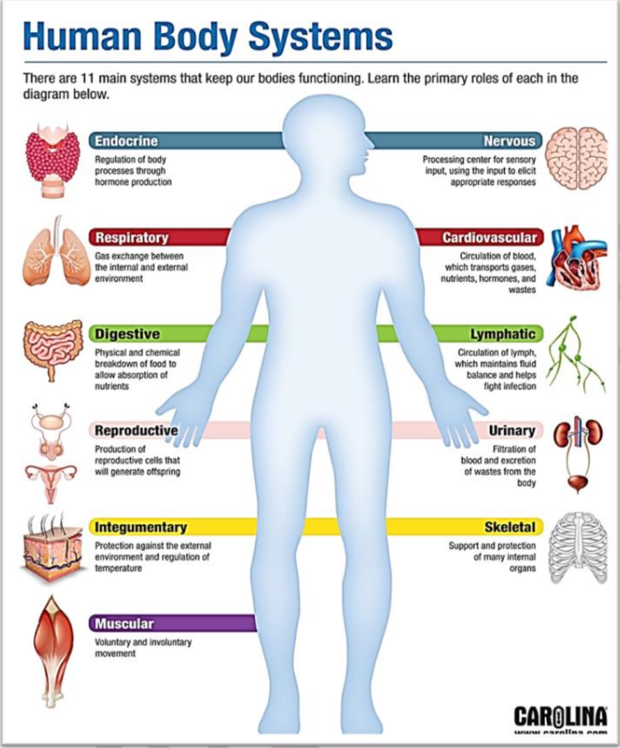
Part II: Physiology



Physiology content:

- An introduction of general physiology.
- Nutrition and Digestion.
- Absorption.
- Metabolism.
- Excretion.
- Respiration.
- Circulation system, Blood and Lymph.
- Reproductive system.
- Nervous System.
- Endocrine System and hormone’s function.

Physiology and life processes

Physiology tells us how our bodies work structurally and functionally (**figure 35**).

The most important life processes of human:

Metabolism: includes catabolism and anabolism that provides energy and body components.

Excitability: ability to sense changes in and around us.

Conductivity: ability to carry the effects of stimulus from part of a cell to another.

Contractility: ability to contract in response to stimulus.

Growth

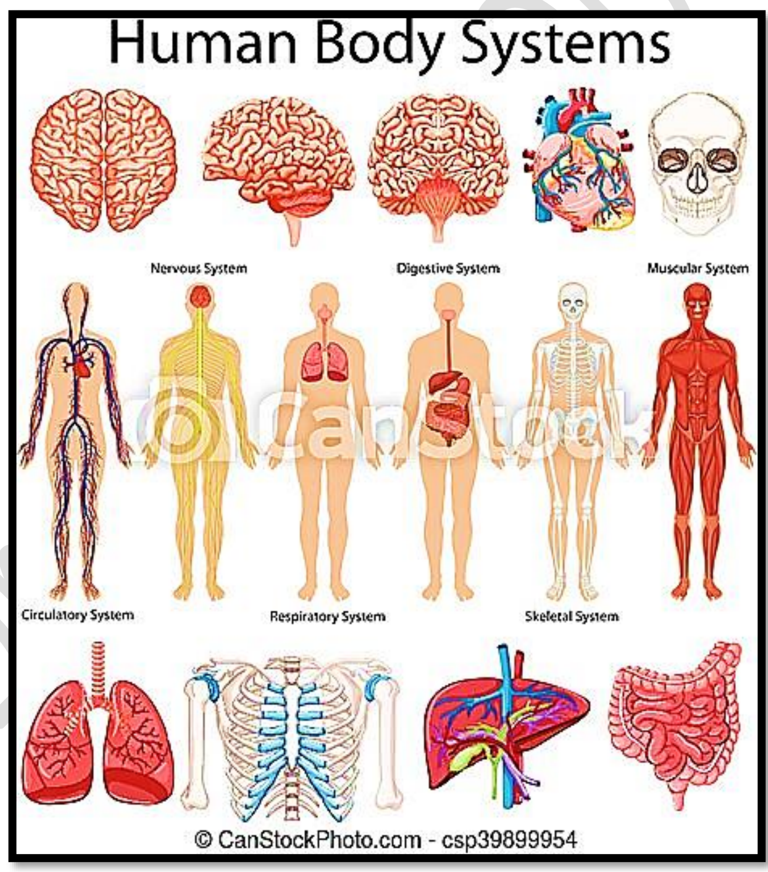


Figure 35: Components of body system

Homeostasis

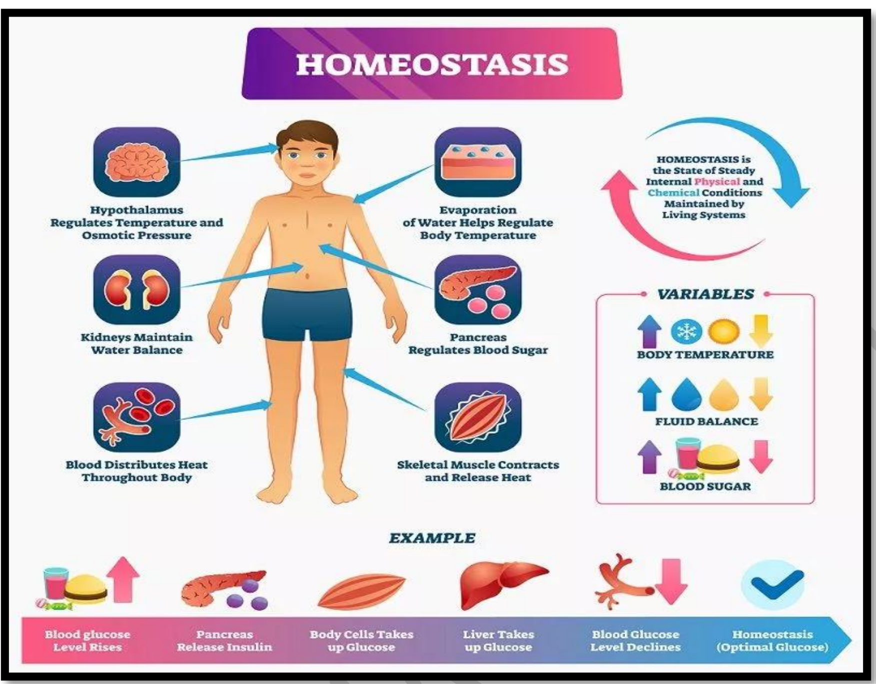


Figure 36: Role of body system in maintaining homeostasis

Nervous system:

Information from the external environment, also acts through electrical signals to control rapid responses for higher functions such as concentration, memory, and creativity.

Endocrine system:

Acts by hormones secreted into the blood to control processes that require duration rather than speed, such as metabolic activity, water, and electrolytes balances.

Circulatory system:

Transports nutrients, oxygen, CO_2, wastes, electrolytes and hormones through the body.

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Respiratory system:

Obtains oxygen and eliminates CO_2 to the external environment; helps regulate pH by adjusting the rate of removal of acid-forming carbon dioxide.

Urinary system:

Important in regulating the volume, electrolyte composition, and pH of the internal environment; removes waste and excess water, salt, acid and other electrolytes from the plasma and eliminate them into the urine.

Digestive system:

Obtains nutrients, water and electrolytes from the external environment and transfers them into the plasma; eliminates undigested food residues to the external environment.

Muscular and skeletal system:

Supports and protects body parts and allows body movements, heat generated by muscular contraction are important in temperature regulation, calcium stored in the bones.

Immune system:

Defense against foreign invaders and cancer cells; tissue repair.

Integumentary system:

Keeps internal fluids in and foreign materials out serves as a protective barrier between the external environment and the remainder of the body, temperature regulation (**figure 37**).

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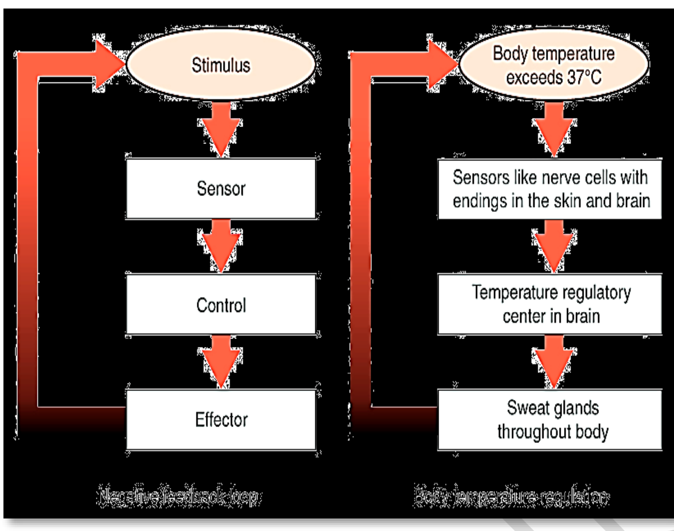

figure 37: Temperature regulation process

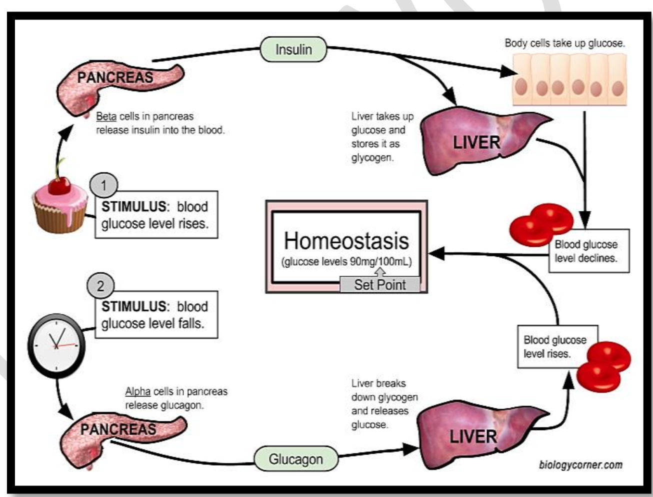

Figure 38: Example of a negative feedback

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https://www.youtube.com/watch?v=9E58qPRX5XY&ab_channel=Dr.MohamedKhairat

Digestive System

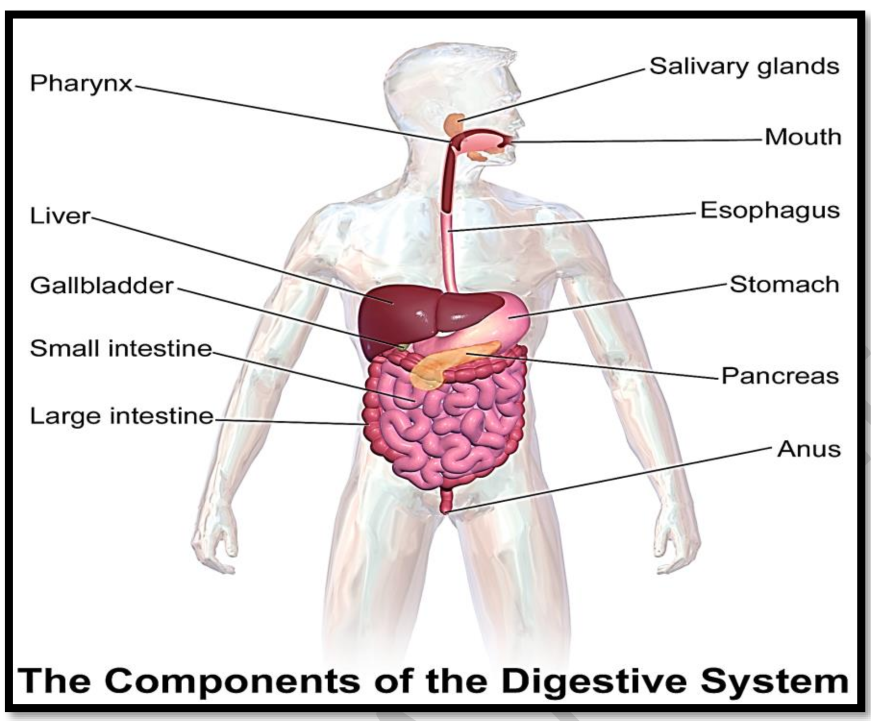

Figure 39: Digestive system

The digestive system breaks down food and delivers nutrients to every cell in the body via the bloodstream. The digestive tract starts at the mouth and ends at the anus. Organs that are part of the digestive system include the stomach, small intestine, pancreas, liver and large intestine (bowel).

Digestive System Function:(figure 39)

Acquires nutrients from environment

Anabolism: Uses raw materials to synthesize essential compounds.

Catabolism: Decomposes substances to provide energy cells need to function.

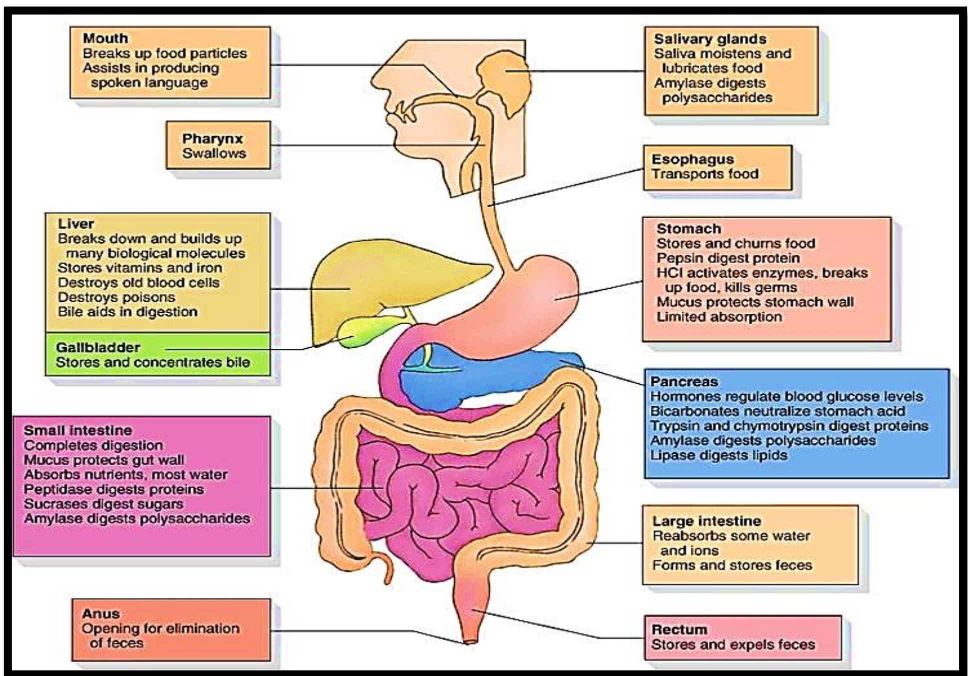

Figure 40: Actions of Digestive (GI) Tract

1- Ingestion

Occurs when material enters via the mouth.

2- Mechanical Processing

Crushing / Shearing – makes material easier to move through the tract.

3- Digestion

Chemical breakdown of food into small organic compounds for absorption.

4- Secretion

Release of water acids, buffers, enzymes & salts by epithelium of GI tract and glandular organs.

5- Absorption

Movement of organic substrates, electrolytes, vitamins & water across digestive epithelium.

6- Excretion

Removal of waste products from body fluids.

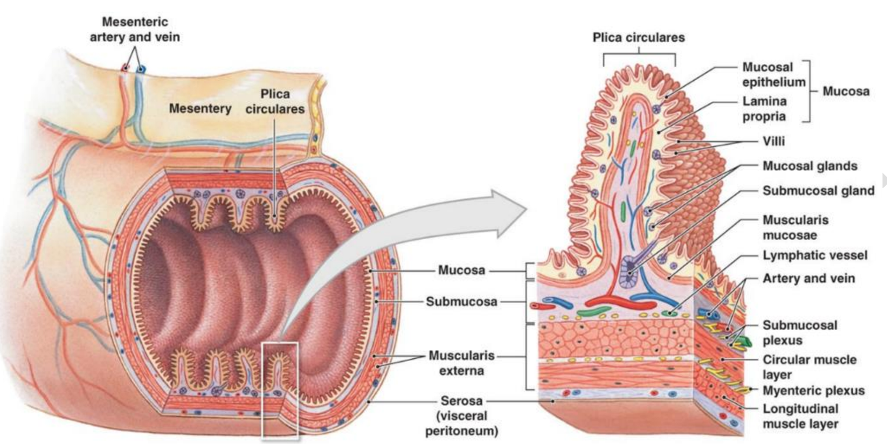

Figure 41: Histological Structure of the Digestive (GI) Tract

The mouth

The mouth is the beginning of the digestive tract; and in fact, digestion starts here when taking the first bite of food. Chewing breaks the food into pieces that are more easily digested, while saliva mixes with food to begin the process of breaking it down into a form your body can absorb and use (**figure 42**).

Functions of Oral Cavity

- Sensory analysis of material before swallowing
- Mechanical processing: Through actions of teeth,tongue, and palatal surfaces
- Lubrication: Mixing with mucus and salivary gland secretions
- Limited digestion of carbohydrates and lipids

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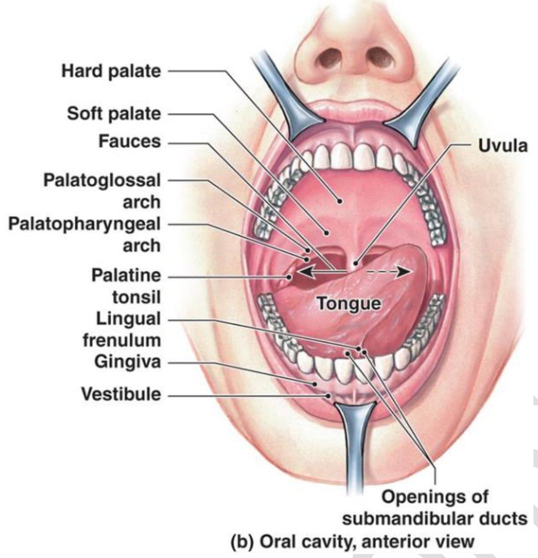

Figure 42: Mouth

Esophagus

Located in your throat near your trachea, the esophagus receives food from your mouth when you swallow. By means of a series of muscular contractions called peristalsis, the esophagus delivers food to your stomach.

A hollow muscular tube About 25 cm (10 in.) long and 2 cm (0.80 in.) wide Conveys solid food and liquids to the stomach. Begins posterior to cricoid cartilage is innervated by fibers from the esophageal plexus (**figure 43**).

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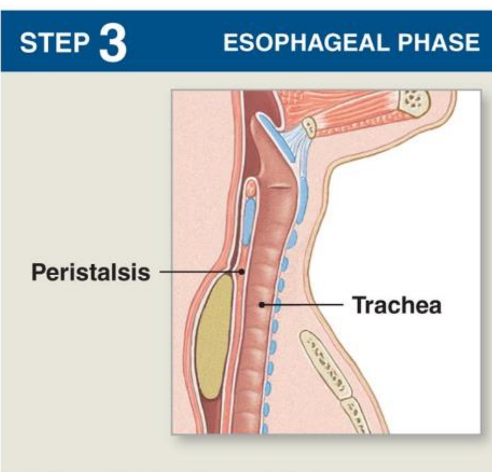

Figure 43: Esophagus

The stomach

The stomach is a hollow organ, or "container," that holds food while it is being mixed with enzymes that continue the process of breaking down food into a usable form. Cells in the lining of the stomach secrete strong acid and powerful enzymes that are responsible for the breakdown process. When the contents of the stomach are sufficiently processed, they are released into the small intestine (**figure 44**).

Stomach Function

Major Functions of the Stomach

-Storage of ingested food.

-Mechanical breakdown of ingested food.

-Disruption of chemical bonds in food material by acid and enzymes.

-Production of intrinsic factor, a glycoprotein required for absorption of vitamin B12 in small intestine.

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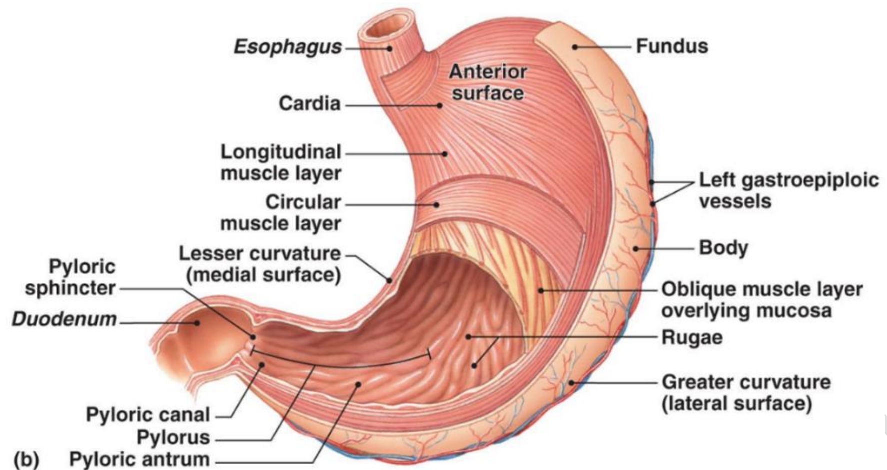

Figure 44: Gastric Anatomy

Digestion in the Stomach

Stomach performs preliminary digestion of proteins by pepsin

- Some digestion of carbohydrates (by salivary amylase)
- Lipids (by lingual lipase)

Stomach contents

- Become more fluid
- PH approaches 2.0
- Pepsin activity increases
- Protein disassembly begins

Although digestion occurs in the stomach, nutrients are not absorbed there

Small Intestine

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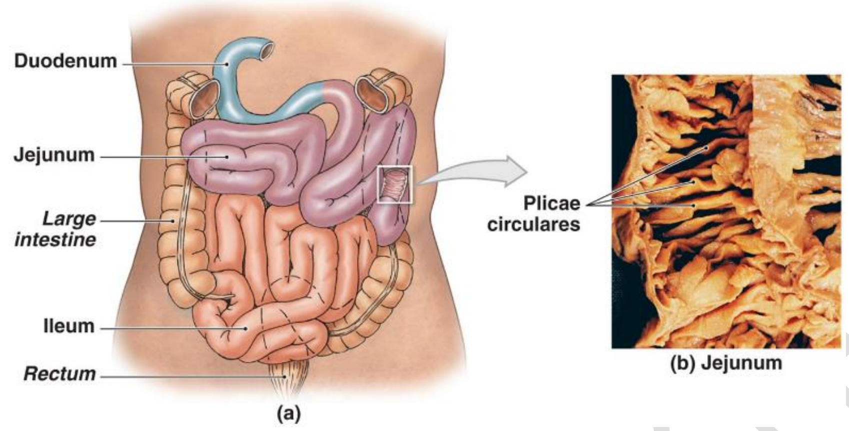

figure 45: Small intestine

Made up of three segments — the duodenum, jejunum, and ileum — the small intestine is a 22-foot-long \muscular tube that breaks down food using enzymes released by the pancreas and bile from the liver. Peristalsis also is at work in this organ, moving food through and mixing it with digestive secretions from the pancreas and liver. The duodenum is largely responsible for the continuous breaking-down process, with the jejunum and ileum mainly responsible for absorption of nutrients into the bloodstream (**figure 45).**

The Duodenum

- The segment of small intestine closest to stomach
- 25 cm long
- "Mixing bowl" that receives chyme from stomach and digestive secretions from pancreas and liver

Functions of the duodenum

- To receive chyme from stomach
- To neutralize acids before they can damage the absorptive surfaces of the small intestine

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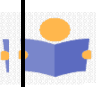

The Jejunum

- Is the middle segment of small intestine

- 2.5 meters (8.2 ft) long

- Is the location of most: Chemical digestion - Nutrient absorption

- Has few plicae circulares

- Small villi

The Ileum

- The final segment of small intestine

- 3.5 meters (11.48 ft) long

- Ends at the ileocecal valve, a sphincter that controls flow of material from the ileum into the large intestine

Intestinal Secretions

- Watery intestinal juice

- 1.8 liters per day enter intestinal lumen

- Moisten chyme

- Assist in buffering acids

- Keep digestive enzymes and products of digestion in solution

Intestinal Movements

- Chyme arrives in duodenum
- Weak peristaltic contractions move it slowly toward jejunum
- Not under CNS control

Parasympathetic stimulation accelerates local peristalsis

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Pancreas (figure 46):

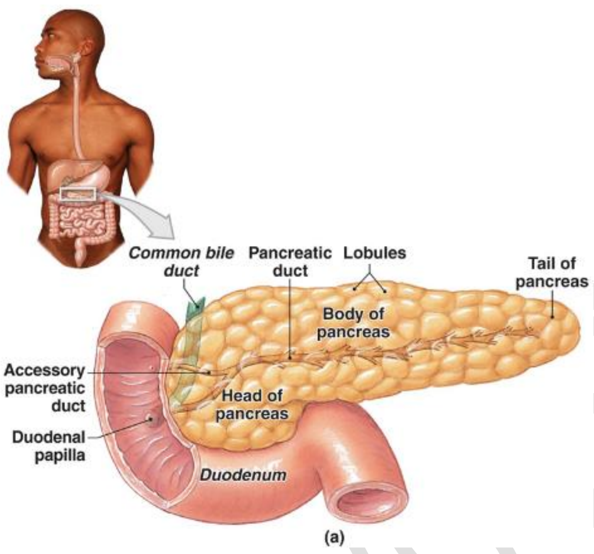

Figure 46: Pancreas

- Lies posterior to stomach from duodenum toward spleen.
- Is bound to posterior wall of abdominal cavity.
- Is wrapped in thin, connective tissue capsule.

Functions of the Pancreas

Endocrine cells of the pancreatic islets:

Secrete insulin and glucagon into bloodstream. Insulin is the chief hormone for metabolizing sugar.

Exocrine cells: Acinar cells and epithelial cells of duct system secrete pancreatic juice.

Pancreatic Enzymes

- **Pancreatic alpha-amylase:** A carbohydrase.

Breaks down starches Similar to salivary amylase.

- **Pancreatic lipase**

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Breaks down complex lipids.

Releases products (e.g., fatty acids) that are easily absorbed.

- **Nucleases**

Break down nucleic acids.

- **Proteolytic enzymes**

Break certain proteins apart.

Proteases break large protein complexes.

Peptidases break small peptides into amino acids.

70% of all pancreatic enzyme production.

Secreted as inactive proenzymes.

Activated after reaching small intestine.

Liver (figure 47):

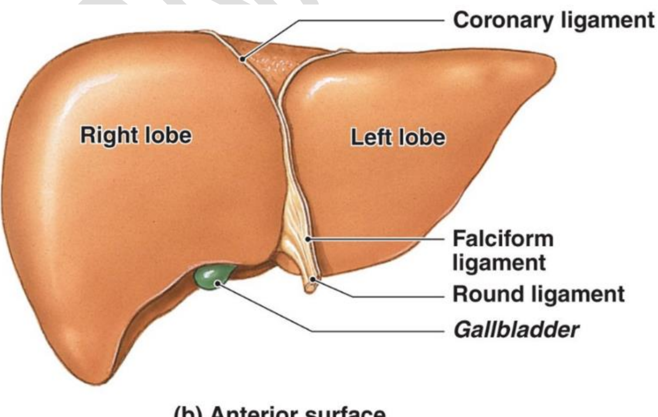

Figure 47: Liver

The liver has multiple functions, but its main function within the digestive system is to process the nutrients absorbed from the

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small intestine. Bile from the liver secreted into the small intestine also plays an important role in digesting fat.

In addition, the liver is the body's chemical "factory." It takes the raw materials absorbed by the intestine and makes all the various chemicals the body needs to function. The liver also detoxifies potentially harmful chemicals. It breaks down and secretes many drugs.

Liver function

The Physiology of the Liver

Metabolic regulation

- Hematological regulation
- Bile production

Metabolic Regulation

The liver regulates:

- Composition of circulating blood.
- Nutrient metabolism (carbohydrate, lipid & amino acid).
- Waste product removal.
- Vitamin Storage (A, D, E & K).
- Nutrient storage (iron).
- Drug inactivation.

Composition of Circulating Blood

- All blood leaving absorptive surfaces of digestive tract enters hepatic portal system flows into the liver
- Liver cells extract nutrients or toxins from blood before they reach systemic circulation through hepatic veins
- Liver removes and stores excess nutrients

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- Corrects nutrient deficiencies by mobilizing stored reserves or performing synthetic activities

The Functions of Bile

- Dietary lipids are not water soluble
- Mechanical processing in stomach creates large drops containing lipids
- Pancreatic lipase is not lipid soluble interacts only at surface of lipid droplet
- Bile salts break droplets apart (emulsification)
- Increases surface area exposed to enzymatic attack
- Creates tiny emulsion droplets coated with bile salts

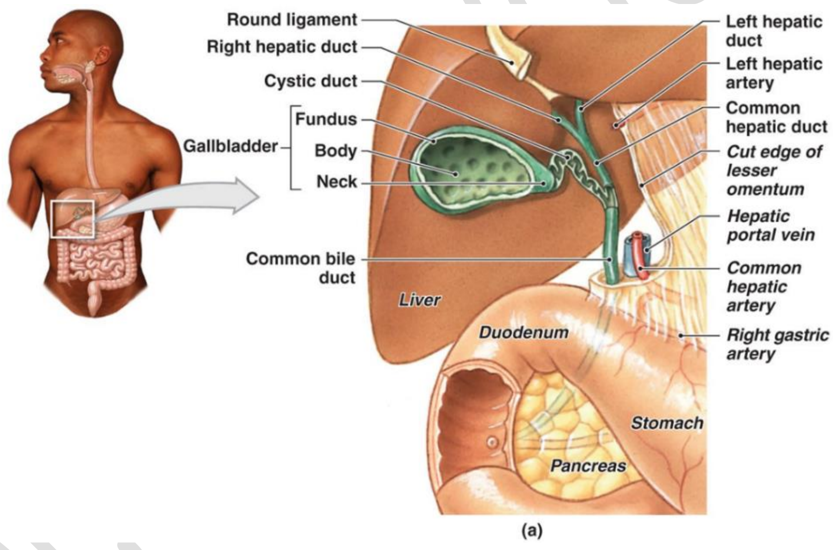

Figure 48: Gallbladder

Gallbladder (figure 48):

- Is a pear-shaped, muscular sac.
- The gallbladder stores and concentrates bile, and then releases it into the duodenum to help absorb and digest fats.

Coordination of Secretion & Absorption

Intestinal Absorption

- It takes about 5 hours for materials to pass from duodenum to end of ileum.
- Movements of the mucosa increases absorptive effectiveness, stir and mix intestinal contents, constantly change environment around epithelial cells

Large Intestine (figure 49)

- Is horseshoe shaped.
- Extends from end of ileum to anus.
- Lies inferior to stomach and liver.
- Frames the small intestine.
- Also called large bowel.
- Is about 1.5 meters (4.9 ft) long and 7.5 cm (3 in.) wide.

Large Intestine Functions

- Reabsorption of water.
- Compaction of intestinal contents into feces.
- Absorption of important vitamins produced by bacteria.
- Reabsorption of bile salts in the cecum transported in blood to liver.
- Storage of fecal material prior to defecation.

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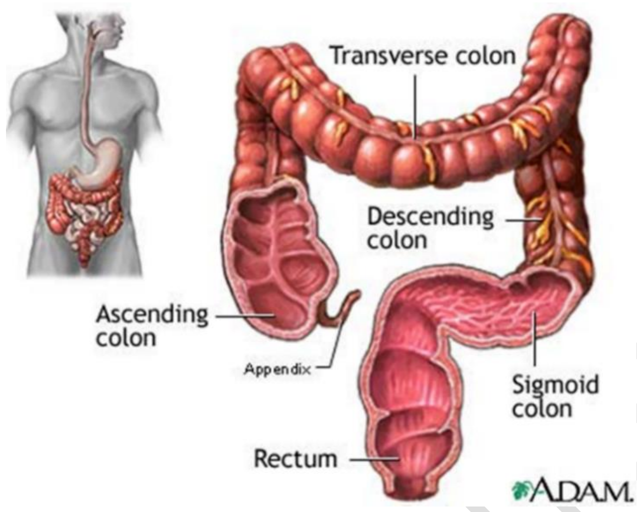

Figure 49: Large intestine

Digestion

Digestive enzymes break molecular bonds in large organic molecules. Carbohydrates, proteins, lipids, and nucleic acids in a process called hydrolysis (**figure 50**).

- Are divided into classes by targets:

Carbohydrates break bonds between simple sugars.

Proteases break bonds between amino acids.

Lipases separate fatty acids from glycerides.

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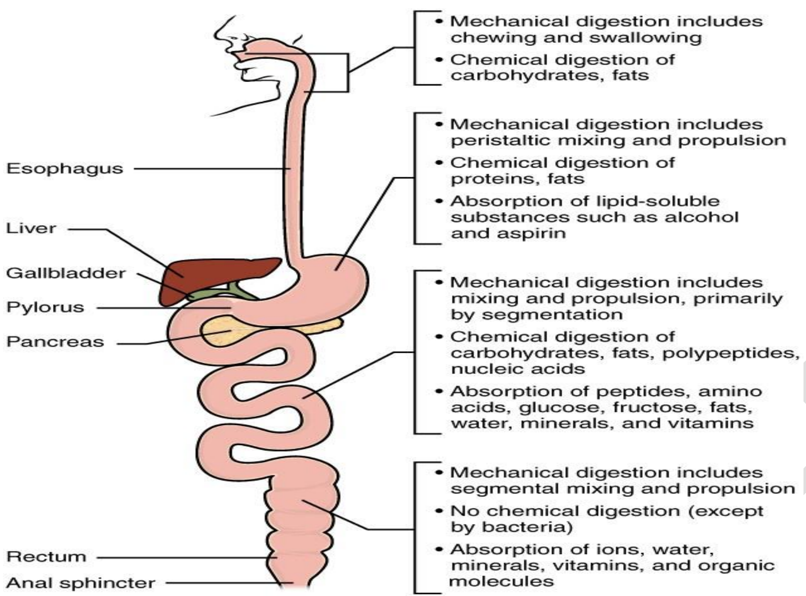

Figure 50: Chemical digestion and absorption

Carbohydrate Digestion

Glucose, galactose, and fructose are the three monosaccharides that are commonly consumed and are readily absorbed. Your digestive system is also able to break down the disaccharide sucrose (regular table sugar: glucose + fructose), lactose (milk sugar: glucose + galactose), and maltose (grain sugar: glucose + glucose), and the polysaccharides glycogen and starch (chains of monosaccharides).

Protein Digestion

Proteins are polymers composed of amino acids linked by peptide bonds to form long chains; The digestion of proteins begins in the stomach. When protein-rich foods enter the stomach, they are greeted by a mixture of the enzyme pepsin and hydrochloric acid (HCl; 0.5 percent). The latter produces an environmental pH of1.5–3.5 that denatures proteins within food. Pepsin cuts proteins into smaller polypeptides and their constituent amino

acids. When the food-gastric juice mixture (chyme) enters the small intestine, the pancreas releases sodium bicarbonate to neutralize the HCl. This helps to protect the lining of the intestine. The small intestine also releases digestive hormones, including secretin and CCK, also, the cells of the brush border of the small intestine secrete enzymes such as aminopeptidase and dipeptidase, which stimulate digestive processes which to break down peptide chains. This results in molecules small enough to enter the bloodstream.

Lipid Digestion

A healthy diet limits lipid intake to 35 percent of total calorie intake. The most common dietary lipids are triglycerides, which are made up of a glycerol molecule bound to three fatty acid chains. Small amounts of dietary cholesterol and phospholipids are also consumed. The three lipases responsible for lipid digestion are lingual lipase, gastric lipase, and pancreatic lipase.

Nucleic Acid Digestion

The nucleic acids DNA and RNA are found in most of the foods you eat. Two types of pancreatic nuclease are responsible for their digestion: deoxyribonuclease, which digests DNA, and ribonuclease, which digests RNA. The nucleotides produced by this digestion are further broken down by two intestinal brush border enzymes (nucleosidase and phosphatase) into pentoses, phosphates, and nitrogenous bases, which can be absorbed through the alimentary canal wall.

Absorption

The mechanical and digestive processes have one goal: to convert food into molecules small enough to be absorbed by the epithelial cells of the intestinal villi. The absorptive capacity of the

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alimentary canal is almost endless. Each day, the alimentary canal processes up to 10 liters of food, liquids, and GI secretions, yet less than one liter enters the large intestine. Almost all ingested food, 80 percent of electrolytes, and 90 percent of water are absorbed in the small intestine. Although the entire small intestine is involved in the absorption of water and lipids, most absorption of carbohydrates and proteins occurs in the jejunum. Notably, bile salts and vitamin B12 are absorbed in the terminal ileum. By the time chyme passes from the ileum into the large intestine, it is essentially indigestible food residue (mainly plant fibers like cellulose), some water, and millions of bacteria.

Absorption can occur through five mechanisms: (1) active transport, (2) passive diffusion, (3) facilitated diffusion, (4) cotransport (or secondary active transport), and (5) endocytosis.

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https://www.youtube.com/watch?v=Og5xAdC8EUI&ab_channel=TED-Ed

https://www.youtube.com/watch?v=Ze1P9Mq2CRk&ab_channel=AhmedMansourAlzohairy

https://www.youtube.com/watch?v=4cV_Ic3Lxwg&ab_channel=pearlBiochemistery

The Excretory System

How Our Body Eliminates Cellular Wastes?

removes excess water, urea, carbon dioxide, and other wastes from our blood.

- Urinary System – remove excess water and urea

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- Lungs – remove carbon dioxide from the blood.
- Skin – excretes water, as sweat which contains some trace chemical wastes including urea.

The excretory system is responsible for maintaining body homeostasis by controlling fluid balances, and removal of waste products.

When cells break down proteins, they create nitrogenous waste called Urea; the excretory system removes this waste and other wastes created as a byproduct of digestion.

Urinary System

- After the blood gets pumped to the kidney, water and other solutes are filtered through the nephrons.
- Then the filtered waste and excess water reconvenes as Urine in the Ureter.
- After traveling through the Ureter, it is stored in the Bladder.
- When the Bladder is full it is then expelled by the Urethra.

The kidneys regulate the amount of water, salts, and other substances in the blood (**figure 51**).

The kidneys are fist-sized, bean shaped structures that remove nitrogenous wastes (urine) and excess salts from the blood.

The ureters are tubes that carry urine from the pelvis of the kidneys to the urinary bladder.

The urinary bladder temporarily stores for the urine until

it is released from the body.

The urethra is the tube that carries urine from the urinary bladder to the outside of the body.

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The outer end of the urethra is controlled by a circular muscle called a sphincter.

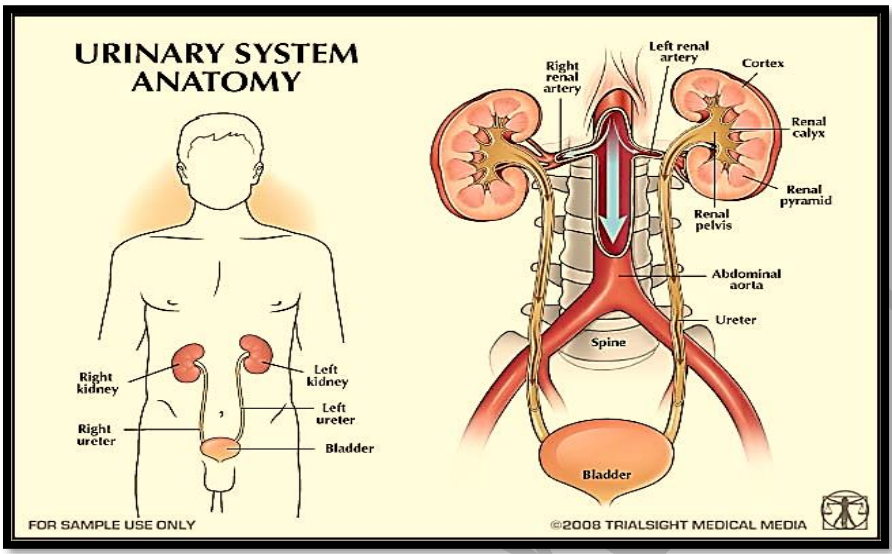

Figure 51: Urinary system

The Kidney

- Each kidney is composed of three sections: the outer (renal) cortex, the (renal) medulla (middle part) and the hollow inner (renal) pelvis.
- The cortex is where the blood is filtered.
- The medulla contains the collecting ducts which carry filtrate (filtered substances) to the pelvis.
- The pelvis is a hollow cavity where urine accumulates and drains into the ureter.

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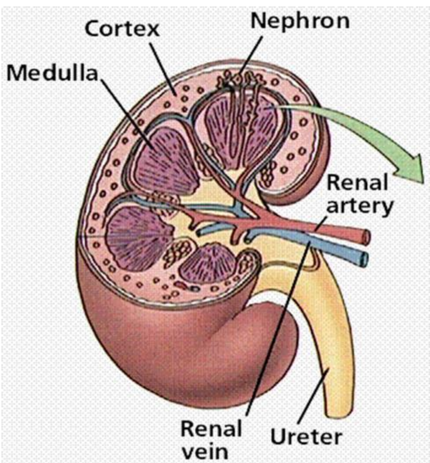

Figure 52: ANATOMY OF THE KIDNEYS

The medial surface of the kidney is concave with a hilum carrying renal nerves and blood vessels.

The renal parenchyma is divided into an outer cortex and inner medulla. Extensions of the cortex (renal columns) project toward the sinus, dividing the medulla into 6-10 renal pyramids. Each pyramid is conical with a blunt point called the papilla facing the sinus (**figure 52**).

The papilla is nestled into a cup called a minor calyx, which collects its urine. Two or three minor calyces merge to form a major calyx. The major calyces merge to form the renal pelvis.

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Nephrons

The filtering units of the kidneys is the nephrons. There are approximately one million nephrons in each kidney. The nephrons are located within the cortex and medulla of each kidney.

The tubes of the nephron are surrounded by cells and a network of blood vessels spreads throughout the tissue. Therefore, material that leaves the nephron enters the surrounding cells and returns to the bloodstream by a network of vessels.

Parts of the Nephron (figure 53)

Each nephron consists of the following parts:

1) glomerulus

2) Bowman's capsule

3) proximal tubule

4) loop of Henle

5) distal tubule

6) collecting duct

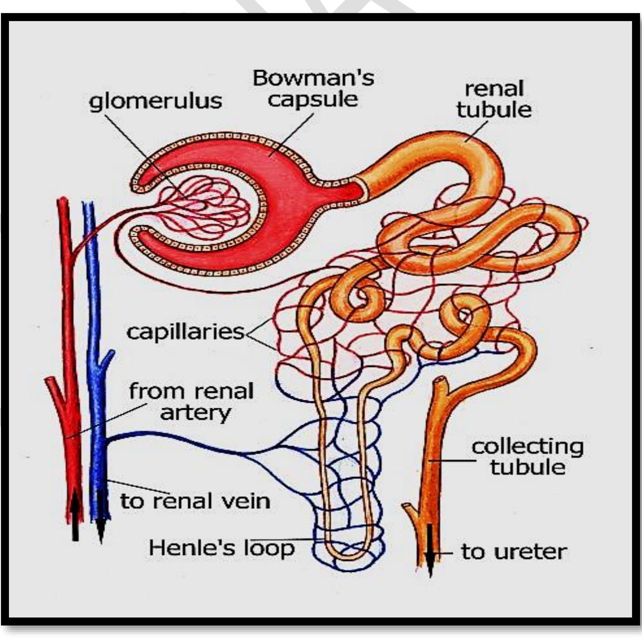

Figure 53: Parts of the Nephron

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The **glomerulus is** a mass of thin-walled capillaries.

The **Bowman's capsule** is a double-walled, cup-shaped structure.

The **proximal tubule** leads from the Bowman's capsule to the Loop of Henle.

The **loop of Henle** is a long loop which extends into the medulla.

The **distal tubule** connects the loop of Henle to the collecting duct.

Function of the Kidney

The principal function of the kidney is to filter blood to remove cellular waste products from the body. At any given time, 20 % of blood is in the kidneys. Humans can function with one kidney.

The kidney has other functions but it is usually associated with the excretion of cellular waste such as:

1) urea (a nitrogenous waste produced in the liver from the breakdown of protein. It is the main component of urine).

2) uric acid (usually produced from breakdown of DNA or RNA).

3) creatinine (waste product of muscle action).

- All these compounds have nitrogen as a major component.

- The kidneys are more than excretory organs.

- They are one of the major homeostatic organs of the body.

- They control blood pH

- Secrete erythropoietin (a hormone that stimulates red blood cell production)

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- Activate vitamin D production in the skin.

https://www.youtube.com/watch?v=OnA2HJXMLnY&ab channel=EngineerPassion

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The Circulatory System

(The Heart, Blood Vessels, Blood Types)

Circulatory systems generally have three main features:

- Fluid (blood or lymph) that transports materials.
- System of blood vessels.
- A heart to pump the fluid through the vessels.

Types of circulatory systems:

Animals that have a circulatory system have one of two kinds:

Open: fluid is circulated through an open body chamber.

Closed: fluid is circulated through blood vessels.

Open system

Arthropods and most mollusks have an open circulatory system. Hemolymph is contained in a body cavity, the hemocoel. A series of hearts circulates the fluid (**figure 54).**

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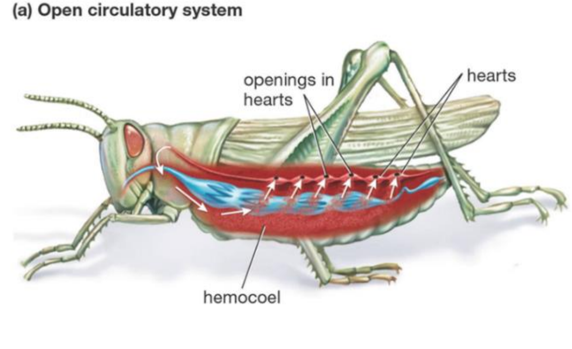

Figure 54: Open circulatory system

Closed system

Vertebrates, annelid worms, and a few mollusks have a closed circulatory system. Blood is moved through blood vessels by the heart's action (**figure 55**).

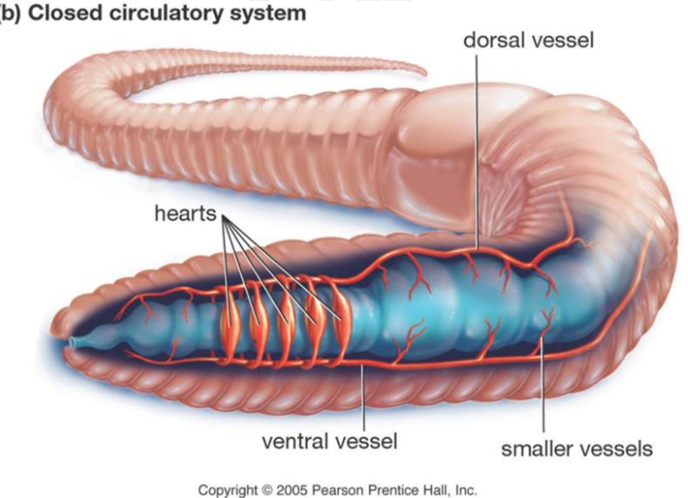

Figure 55: Closed circulatory system

Function

- Transport materials needed by cells (Oxygen- Glucose).
- Remove waste materials from cells (Carbon dioxide- Urea)

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Major Components

- **Blood:** Fluid that fills the circulatory system
- **Pump (heart):** Continuously circulates blood
- **Network of tubes** (blood vessels)

Arteries- blood away from heart

Veins- blood back to the heart

Capillaries- link Arteries with Veins

Blood Components:

Blood is made up of four major components. What do each of these do?

- Plasma: the liquid portion.
- Red blood cells (RBCs).
- White blood cells (WBCs).
- Platelets (PLT).

A-) Plasma

Liquid portion of the blood. Contains clotting factors, hormones, antibodies, dissolved gases, nutrients, and waste (**figure 56**).

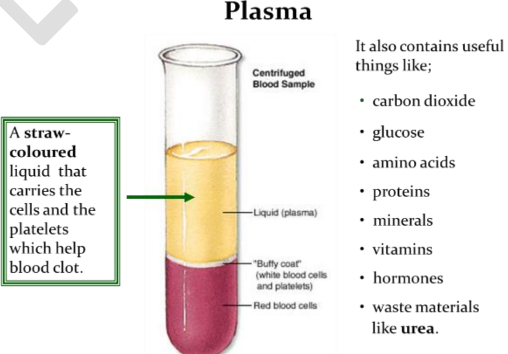

Figure 56: Plasma components

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B) Red Blood Cells

- A biconcave disc that is round and flat

- Carry hemoglobin and oxygen. Do not have a nucleus and live only about 120 days.

- Can change shape to an amazing extent, without breaking, as it squeezes single file through the capillaries.

- Cannot repair themselves.

- Make up about 99% of the blood’s cellular component.

- Red color is due to hemoglobin (**figure 57**).

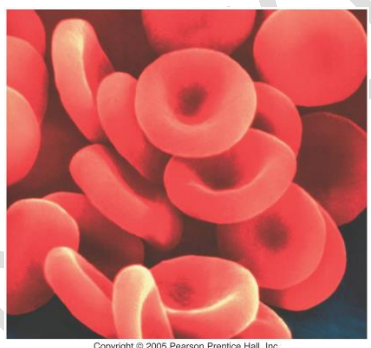

Figure 57: Red Blood Cells

Hemoglobin

Hemoglobin is a complex protein made up of four protein strands, plus iron-rich heme groups. Each hemoglobin molecule can carry four oxygen atoms. The presence of oxygen turns hemoglobin bright red (**figure 58**).

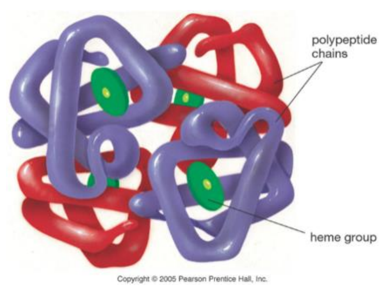

Figure 58: Hemoglobin

RBC lifespan

RBCs live about 4 months. Iron from hemoglobin is recycled in the liver and spleen. The hormone erythropoietin, made by the kidneys, stimulates the production of RBCs in red bone marrow (**figure 59**).

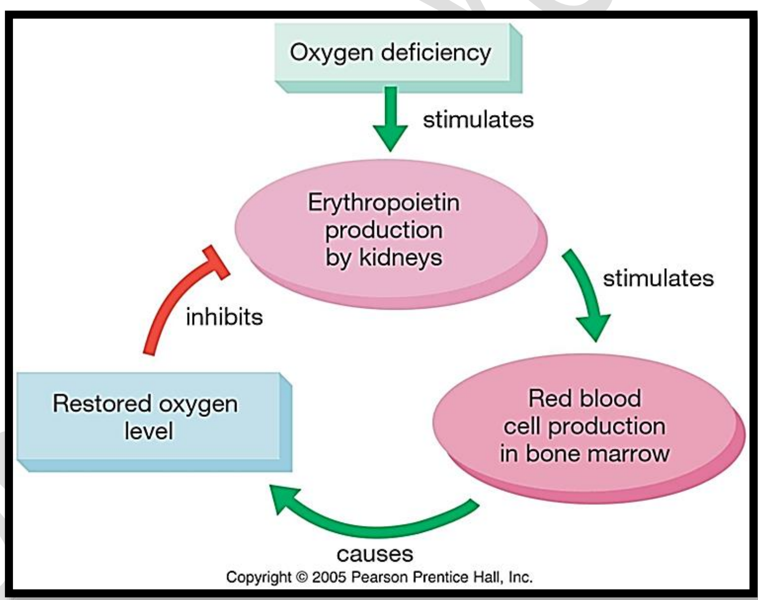

Figure 59: RBC lifespan

C) White Blood cells

- Fight infection and are formed in the bone marrow.

- White blood cells defend against disease by recognizing proteins that do not belong to the body.

-White cells can ooze through the walls of capillaries to patrol the tissues and reach the lymph system

-Have five types (neutrophils, lymphocytes, eosinophils, basophils, and monocytes) (**figure 60**).

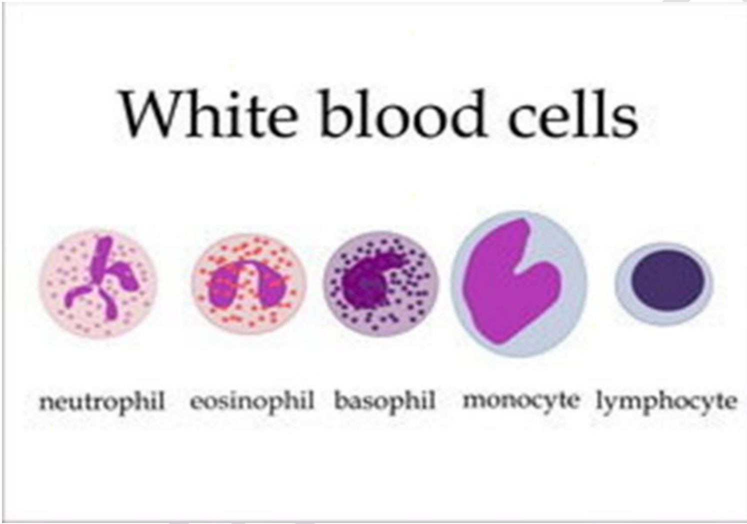

Figure 60: White blood cell types

the two main ones are the lymphocytes and the macrophages, macrophages 'eat' and digest micro-organisms, some lymphocytes fight disease by making antibodies to destroy invaders by dissolving them. other lymphocytes make antitoxins to break down poisons.

D) Platelets

Platelets are cell fragments used in blood clotting. Platelets are derived from megakaryocytes. Because they lack a nucleus, platelets have a short lifespan, usually about 10 days (**figure 61**).

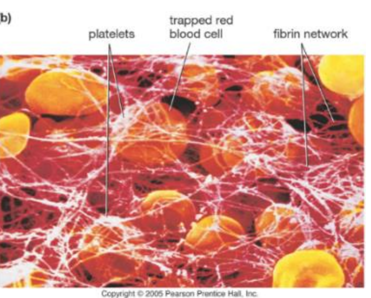

Figure 61: Platelets

Blood clotting

1- Platelets aggregate at the site of a wound.
2- Broken cells and platelets release chemicals to stimulate thrombin production.
3- Thrombin converts the protein fibrinogen into sticky fibrin, which binds the clot (**figure 62**).

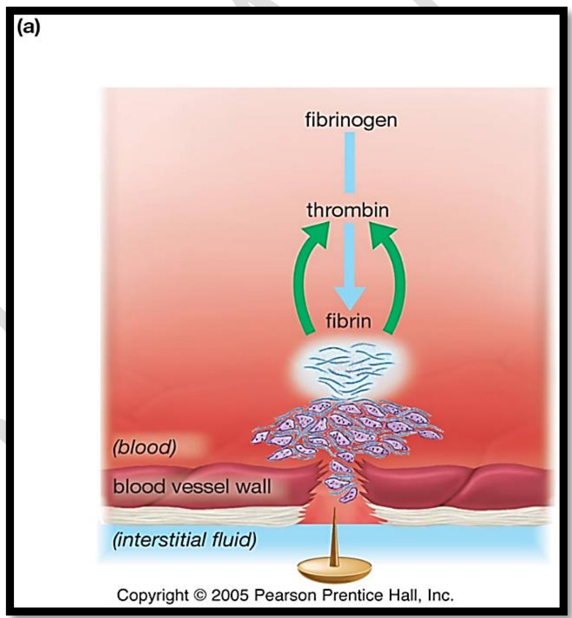

Figure 62: Blood clotting

Disorders of the Circulatory System

- Anemia - lack of iron in the blood, low RBC count.
- Leukemia - white blood cells proliferate wildly, causing anemia.
- Hemophilia - bleeder's disease, due to lack of fibrinogen in thrombocytes.
- Heart Murmur - abnormal heartbeat, caused by valve problems.
- Heart attack - blood vessels around the heart become blocked with plaque, also called myocardial infarction.

2-The Heart

The human heart has four chambers (Left and right ventricle - Left and right atrium). The left side of the heart pumps oxygenated blood to the body while the right side of the heart pumps deoxygenated blood to the lungs where oxygen can be absorbed by the hemoglobin carrying red blood cells (**figure 63**).

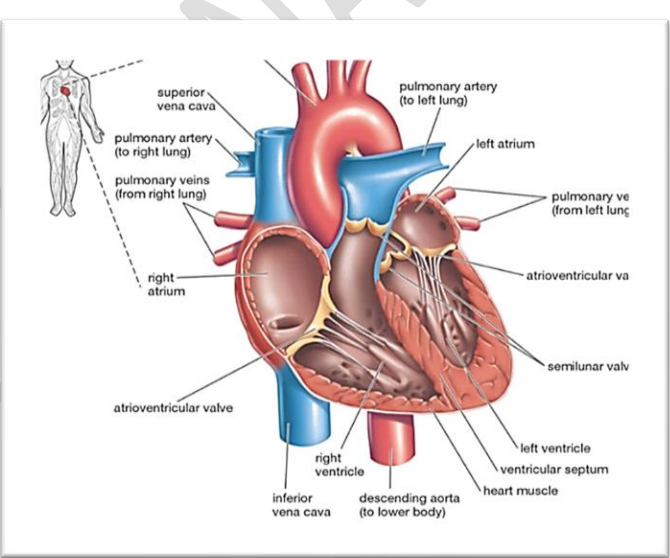

Figure 63: Heart

Functions of the Heart

- Generating blood pressure.
- Routing blood.
- Heart separates pulmonary and systemic circulations.
- Ensuring one-way blood flow.
- Heart valves ensure one-way flow.
- Regulating blood supply.

Size, Shape, Location of the Heart

Shape:

Apex: Blunt rounded point of cone.

Base: Flat part at opposite of end of cone.

- Size of a closed fist
- Located in thoracic cavity between tow lungs

External Anatomy

- **Four chambers**

2 atria

2 ventricles

- **Major veins**

Superior and inferior vena cava

Pulmonary veins

- **Major arteries**

Aorta

Pulmonary trunk

Heart Valves

- **Atrioventricular valves**

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Tricuspid

Bicuspid or mitral

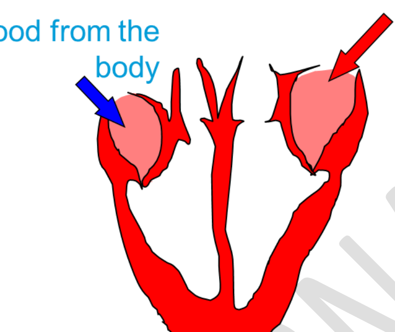
- **Semilunar valves**

Aortic

Pulmonary

Prevent blood from flowing back

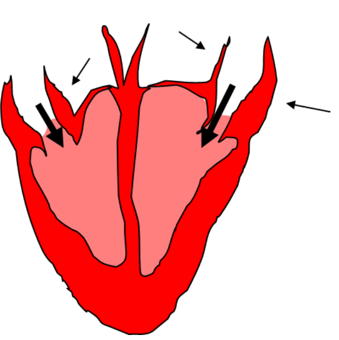
How does the Heart work? Step one



The heart beat begins when the heart muscles **relax** and blood flows into the atria.

STEP TWO

The atria then **contract** and the valves **open** to allow blood into the ventricles.



STEP THREE

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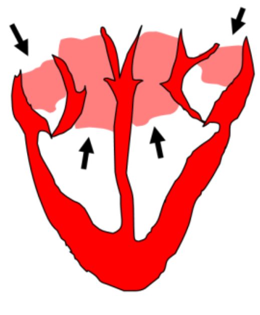

The valves **close** to stop blood flowing backwards.

The ventricles **contract** forcing the blood to leave the heart.

At the same time, the atria are **relaxing** and once again filling with blood

.

The cycle then repeats itself.

Circulation

After passing through the capillaries of the lungs, the blood which is now oxygenated returns to the heart in the pulmonary veins. The left atrium receives blood from the pulmonary vein. Blood passes through the mitral valve into the left ventricle. Contraction of the left ventricle pushes blood through the aortic semilunar valve into the aorta. Blood travels to all regions of the body where it feeds cells with oxygen picked up from the lungs and nutrients from the digestive tract. Deoxygenated blood returns from the rest of the body through the superior and inferior vena cava. Contraction of the right ventricle pushes blood through the pulmonary semilunar valve into the pulmonary arteries in which it travels to the lungs (**figure 64**).

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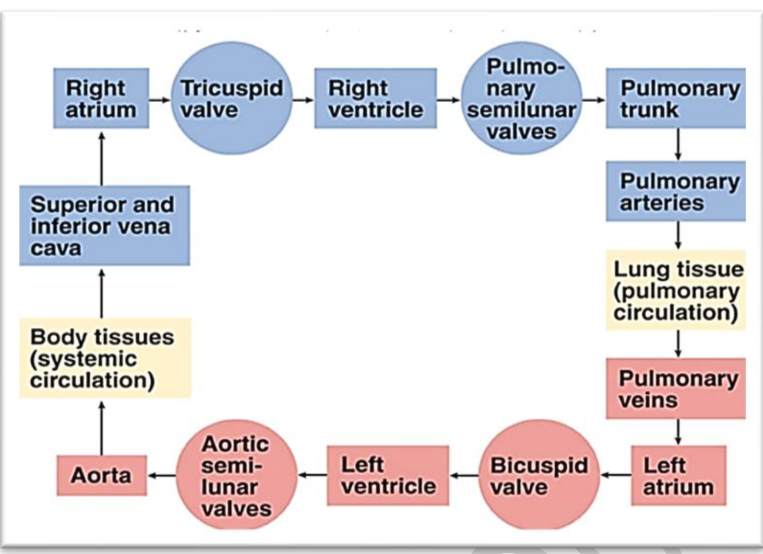

Figure 64: Blood circulation

Blood Pressure

Blood pressure is a measure of the force exerted by the blood on the wall of the arteries. An example is 120/80 (systolic pressure/diastolic pressure. Systolic pressure is the result of the contraction of the ventricles (normal 110-140). Diastolic pressure is during the ventricle relaxation (normal 70-90)

Blood Vessels: Blood vessels fall into three major classes:

- Arteries and arterioles carry blood away from the heart.
- Veins and venules carry blood to the heart.
- Capillaries allow exchange of nutrients, wastes and gases (**figure 65**).

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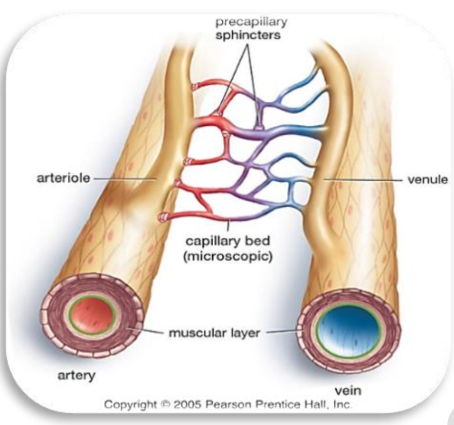

Figure 65: Blood vessels

The ARTERY

Arteries are thick-walled and lined with smooth muscle. How does the structure of an artery help with its function? **Figure 66.**

Arteries carry blood away from the heart.

the elastic fibres allow the artery to ***stretch*** under pressure

thick muscle and elastic fibres

the thick muscle can contract to ***push*** the blood along.

Figure 66: Arteries carry blood away from the heart.

The VEIN

Veins have thinner walls than arteries. Veins have fewer smooth muscle cells but do have valves. How do valves and the skeletal muscles help vein's function? **Figure 67**.

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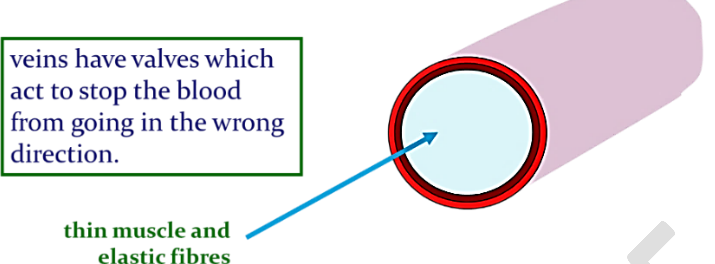

Figure 67: Veins carry blood towards from the heart

The CAPILLARY

Body tissues contain a vast network of thin capillaries. Capillary walls are only one cell thick, allowing exchange of gases, nutrients, and wastes, **figure 68**.

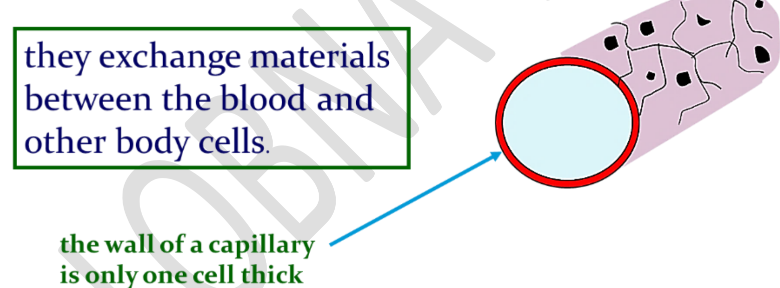

Figure 68: Capillaries link arteries with veins

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Respiratory System

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The Respiratory System is what controls breathing. It brings in the oxygen your body needs and gets rid of the carbon dioxide. If you didn't breathe you would pass out or die.

"Respiration" is used several different ways:

- Cellular respiration is the aerobic breakdown of glucose in the mitochondria to make ATP.
- Respiratory systems are the organs in animals that exchange gases with the environment.

Respiratory system function

Respiratory systems allow animals to move oxygen (needed for cellular respiration) into body tissues and remove carbon dioxide (waste product of cellular respiration) from cells.

Gas exchange by Diffusion

- Some animals simply allow gases to diffuse through their skins. These animals have a low metabolic rate. All of these are aquatic animals, **figure 69**.

Figure 69: Gas exchange by diffusion

Specialized structures (figure 70)

Structures specialized for gas exchange include:

- gills (aquatic animals).

- spiracles (terrestrial insects).
- lungs (most terrestrial vertebrates).

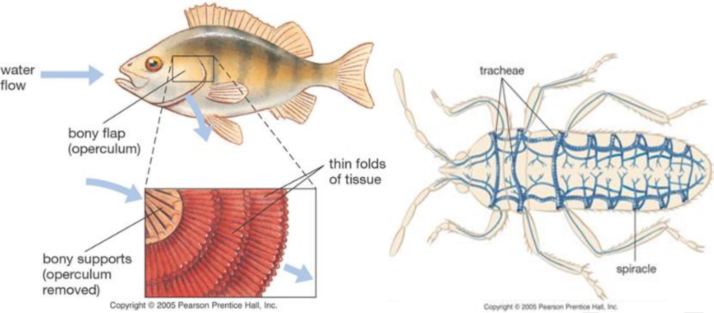

Figure 70: Examples for specialized structures for gas exchange

Human respiratory system (figure 71)

Parts of the respiratory system include:

-Trachea -Bronchi -Bronchioles -Alveoli

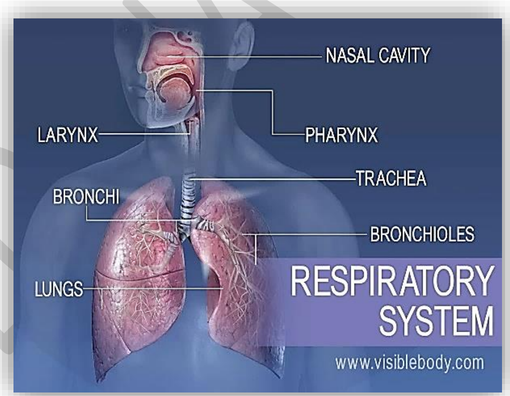

Figure 71: Respiratory system

The Nose and Mouth

This is where it all begins. This is where the oxygen first enters your body and where Carbon Dioxide leaves.

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- When the air comes into your nose it gets filtered by tiny hairs and it is moistened by the mucus that is in your nose.
- Your sinuses also help with your respiratory System. They help to moisten and heat the air that you breath.
- Air can also get into your body through your mouth/oral cavity, but air is not filtered as much when it enters in through your mouth (**figure 72**).

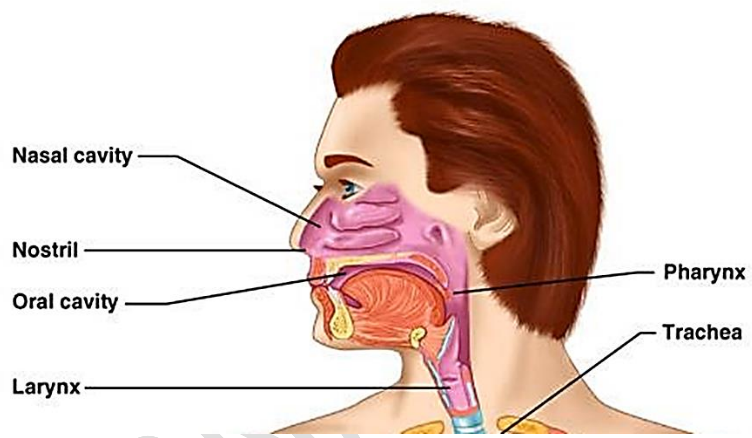

Figure 72: Nasal and oral cavity

The Pharynx and Trachea

Next, we will head down to your pharynx (throat) and your trachea (windpipe). This is where the air passes from your nose to your bronchi tubes and lungs, **figure 73**.

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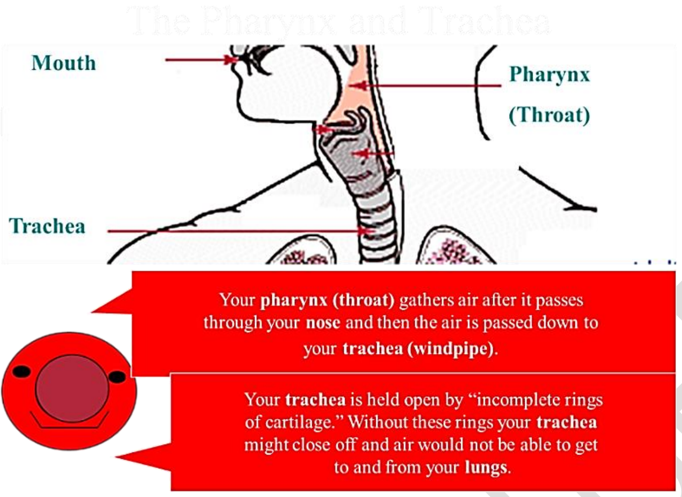

Figure 73: The Pharynx and Trachea

The Bronchi Tubes and Bronchiole

Your trachea (windpipe) splits up into two bronchi tubes. These two tubes keep splitting up and form your bronchiole. These bronchi tubes split up, like tree branches, and get smaller and smaller inside your lungs.

The air flows past your bronchi tubes and into your bronchiole. These tubes keep getting smaller and smaller until they finally end with small air sacs (called alveoli), **figure 74.**

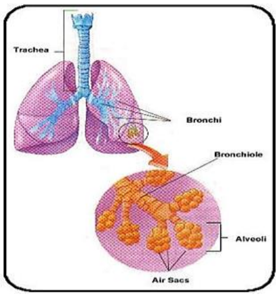

Figure74: Alveoli and Bronchi Picture

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The Alveoli and Capillary Network

Now we will head over to the alveoli and what happens when the air finally makes it down there, **figure 75**.

- Your alveoli are tiny air sacs that fill up with air/oxygen when you breath in.
- Your alveoli are surrounded by many tiny blood vessels called capillaries.
- The walls of your alveoli (and capillaries) are so thin that the oxygen or carbon dioxide can pass through them, traveling right into, or out of your blood stream.

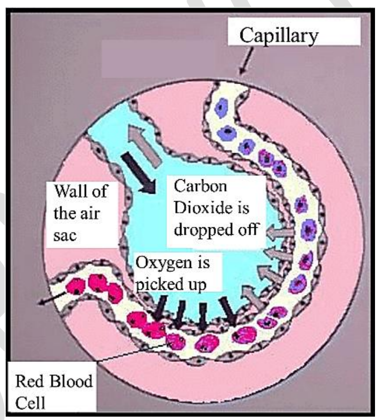

Figure 75: Alveoli Picture

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Human Reproduction ♀ ♂

Reproductive system

Both sexes have reproductive organs called GENITALS or GENITALIA

Female Reproductive System

Female reproductive organs are for reproduction, pregnancy, and childbirth, **figure 76**.

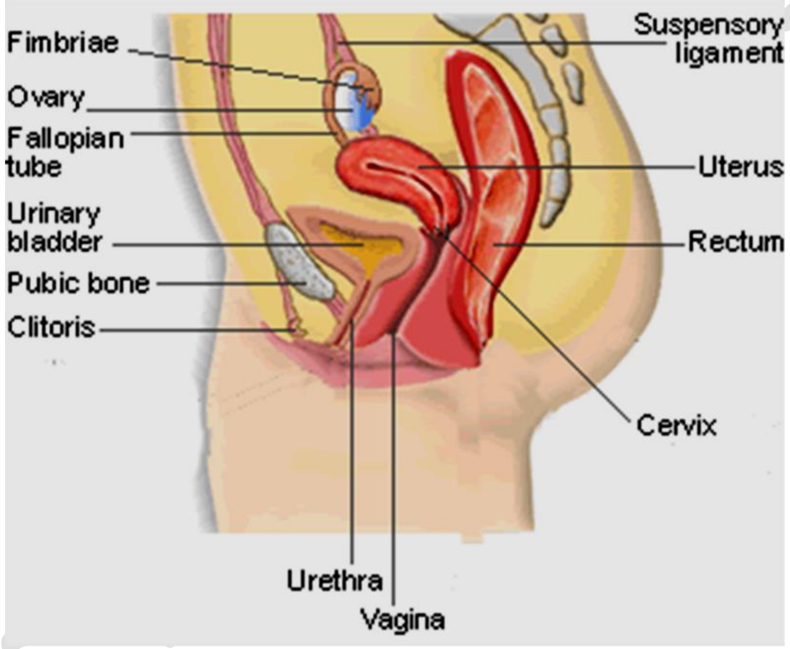

Figure 76: Internal Female Anatomy

Ovary (Ovaries)

Two solid egg-shaped structures. They are attached to the uterus by ligaments. Ovaries have two main functions:

1-store and release the ova or female egg cell. Some of the ova disappear; others are released after puberty.

2-produce female sex hormones ESTROGEN and PROGESTERONE

Ova

The female reproductive cell. They are the largest cells in the female body. (About the size of a grain of sand.) the female baby is born with all the ova she will ever have (about 200,000 in each ovary). About 400-500 ova mature and are released over a lifetime.

Estrogen

Estrogen is responsible for the secondary sex characteristics.

Progesterone

Progesterone builds up the lining of the uterus called the endometrium in preparation for the fertilized ovum.

Ovulation

When the egg is released from the ovary at the age of puberty. The ovum moves to the surface of the ovary. The ova fall into the fallopian tube and waits for fertilization. This happens every 28 days. It happens at about the 14th day of the cycle.

FALLOPIAN TUBES

(oviducts)

Two tubes attached on either side of the uterus. They are about four inches long (the size of a cooked spaghetti noodle). The oviducts carry egg cells toward the uterus and sperm cells toward the egg cell. Fertilization takes place in the upper third of the oviduct.

Uterus

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A hollow, muscular organ (shaped somewhat like an upside-down pear, about the size of a fist). The uterus is lined with endometrium. The uterus has one main function—to protect and nourish a fetus. The walls of the uterus can stretch to the size of a small watermelon. After childbirth the uterus shrinks back to the original shape in 6-8 weeks.

Cervix

The neck or opening of the uterus. A normal healthy cervix is the strongest muscle in the body. It dips down about half an inch into the vagina.

Vagina

It is an empty passageway leading from the vaginal opening to the uterus. It is only 3-4 inches long. The vaginal walls are made of many small folds of membrane that stretch greatly to accommodate a baby during birth.

Urethra: The opening to the bladder.

Male Reproductive System

Male reproductive organs are for reproduction and urination, **figure 77.**

Scrotum

A sac-like pouch located behind the penis that holds each testis and helps regulate temperature for sperm production.

Testicles or Testes

- The two testes are small organs that lie in the scrotum and produce sperm and the male hormone testosterone.
- The Testes are the male sex gland.

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- The Testes are outside the body because the male sperm that is manufactured in the testes need cooler-than-body temperature for normal growth and development.
- Loss of one does not impair the function of the other.
- Four to five billion sperm cells are produced each month.

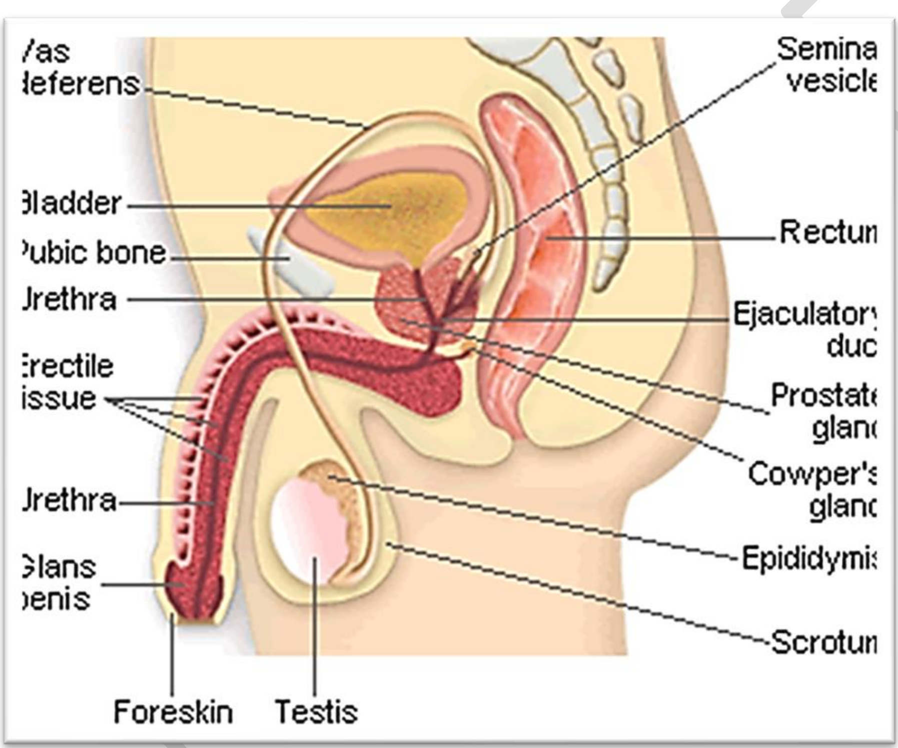

Figure 77: Male reproductive system

Testosterone

the male reproductive hormone made by the testes which causes the changes of puberty. This hormone causes secondary sex characteristics, production of sperm.

Epididymis

the structure that forms a mass over the back and upper part of each testis. Sperm are stored there for as long as six weeks while they ripen to maturity.

Cowper's Gland

two small pea-sized glands located beneath the prostate gland on both sides of the base of the penis. They secrete a clear, sticky fluid that helps to neutralize the acidity of the urethra.

Vas Deferens

two long, thin tubes that serve as a passageway for sperm and a place for sperm storage. The contraction of the vas deferens along with the action of the cilia help transport the sperm through the vas deferens.

Seminal Vesicles

two small glands that secrete a fluid that nourishes and enables the sperm to move.

Prostate Gland

surround the urethra beneath the bladder. The gland secretes an alkaline fluid that neutralizes the acid found in the male urethra and the female reproductive tract.

Without the action of the secretions of the prostate gland, many sperm would die, and fertilization of an ovum would be impossible.

Urethra

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- A dual-purpose tube that both semen and urine pass through to leave the body. Semen and urine never mix.
- Special muscles or sphincters surround the urethra.
- During urination, one sphincter will relax so that the pressure from the bladder will push urine out from the body.
- During ejaculation, another sphincter will relax so that semen can flow through the urethra to the outside of the body.

Penis

The male organ for sexual intercourse and urination.

Semen

A combination of fluid that is produced in the seminal vesicles, prostate gland, and Cowper's gland. This fluid nourishes and helps sperm move through the urethra.

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https://www.youtube.com/watch?v=lqV94iTsCHA&ab_channel=NucleusMedicalMedia

THE NERVOUS SYSTEM

Dr. LOBNA YOUSEF

Basic Structure and Function of the Nervous System

the nervous system probably includes the brain, the nervous tissue contained within the cranium, and the spinal cord, the extension of nervous tissue within the vertebral column. That suggests it is made of two organs—and you may not even think of the spinal cord as an organ—but the nervous system is a very complex structure. Within the brain, many different and separate regions are responsible for many different and separate functions, **figure 78.**

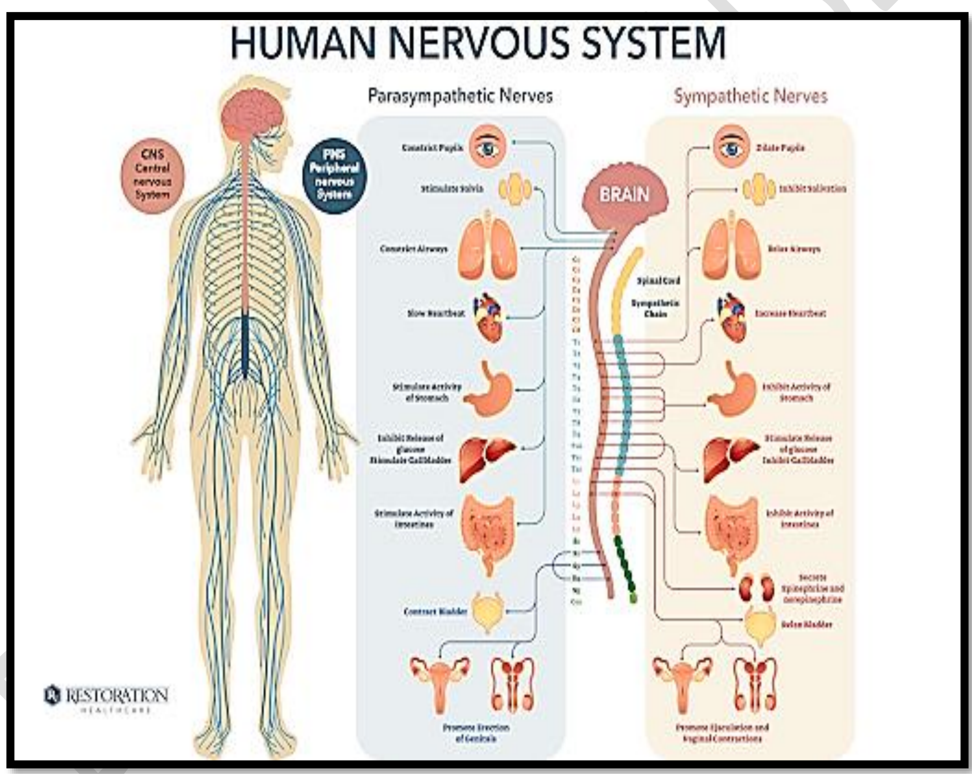

Figure 78: THE NERVOUS SYSTEM

The Central and Peripheral Nervous Systems

The nervous system can be divided into two major regions: the central and peripheral nervous systems.

The central nervous system (CNS) is the brain and spinal cord, and the peripheral nervous system (PNS) are referred to as ganglia and Nerves. The brain is contained within the cranial cavity of the skull, and the spinal cord is contained within the vertebral cavity of the vertebral column.

Basic Functions of the Nervous System

The nervous system is involved in receiving information about the environment around us (sensation) and generating responses to that information (motor responses).

The nervous system can be divided into regions that are responsible for sensation (sensory functions) and for the response (motor functions).

But there is a third function that needs to be included. Sensory input needs to be integrated with other sensations, as well as with memories, emotional state, or learning (cognition). Some regions of the nervous system are termed integration or association areas. The process of integration combines sensory perceptions and higher cognitive functions such as memories, learning, and emotion to produce a response.

Sensation:

The first major function of the nervous system is sensation—receiving information about the environment to gain input about what is happening outside the body (or, sometimes, within the body). The sensory functions of the nervous system register the presence of a change from homeostasis or a particular event in

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the environment, known as a stimulus. The senses we think of most are the “big five”: taste, smell, touch, sight, and hearing.

Response:

The nervous system produces a response based on the stimuli perceived by sensory structures. An obvious response would be the movement of muscles, such as withdrawing a hand from a hot stove.

Controlling the Body:

The nervous system can be divided into two parts mostly based on a functional difference in responses. The somatic nervous system (**SNS**) is responsible for conscious perception and voluntary motor responses. Voluntary motor response means the contraction of skeletal muscle,

The autonomic nervous system (**ANS**) is responsible for involuntary control of the body, usually for the sake of homeostasis (regulation of the internal environment). Sensory input for autonomic functions can be from sensory structures tuned to external or internal environmental stimuli, **figure 79.**

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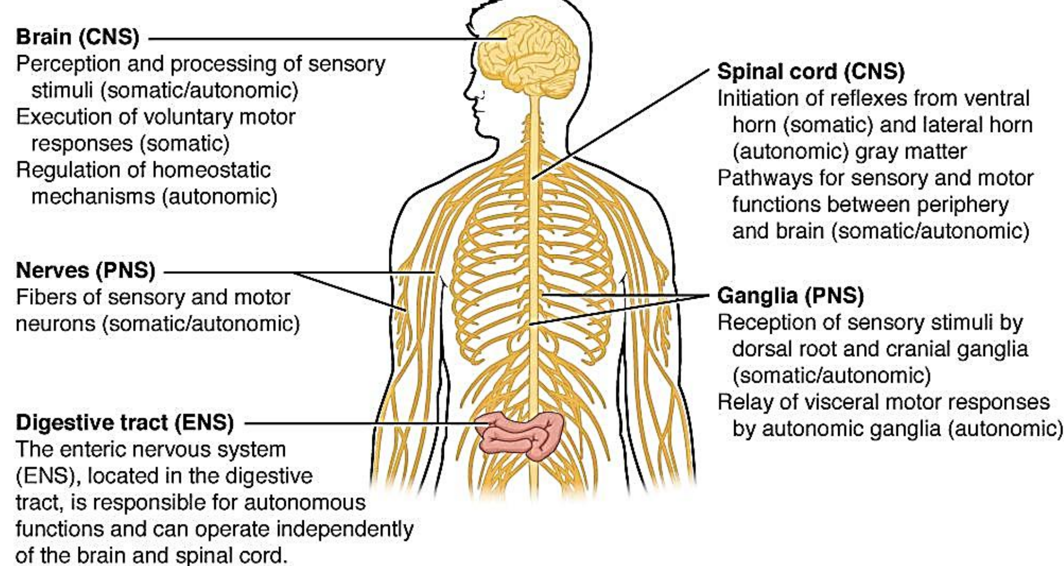

Figure 79: Somatic, Autonomic, and Enteric Structures of the Nervous System Somatic structures include the spinal nerves, both motor and sensory fibers, as well as the sensory ganglia (posterior root ganglia and cranial nerve ganglia). Autonomic structures are found in the nerves also but include the sympathetic and parasympathetic ganglia. The enteric nervous system includes the nervous tissue within the organs of the digestive tract.

Nervous Tissue:

Nervous tissue is composed of two types of cells, neurons, and glial cells. Neurons are the primary type of cell that most anyone associates with the nervous system.

They are responsible for the computation and communication that the nervous system provides.

Neurons:

Neurons are the cells considered to be the basis of nervous tissue. They are responsible for the electrical signals that

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communicate information about sensations, and that produce movements in response to those stimuli, **figure 80.**

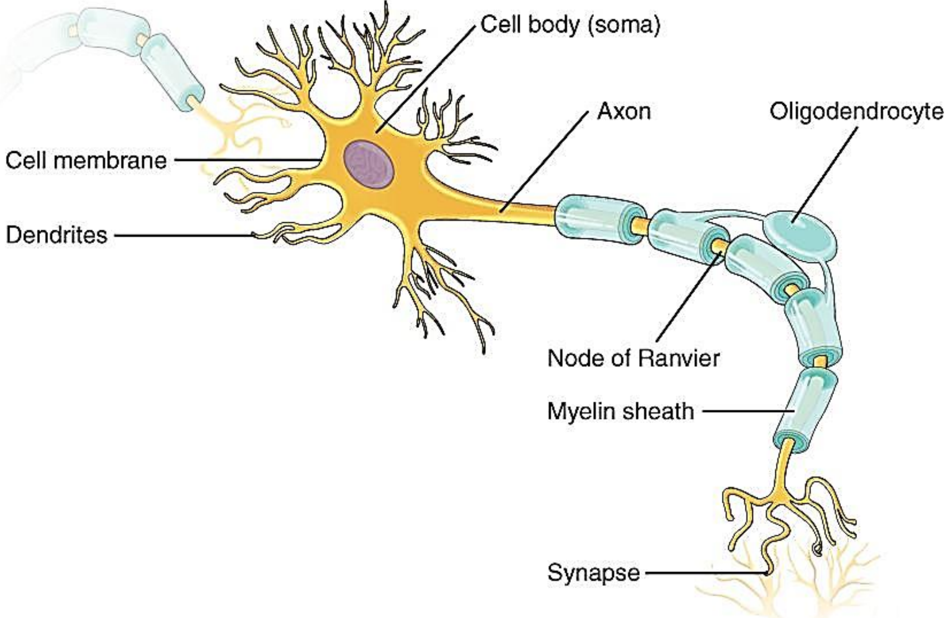

Figure 80: Parts of a Neuron

Structure: The major parts of the neuron are labeled on a multipolar neuron from the CNS. Where the axon emerges from the cell body, there is a special region referred to as the axon hillock. This is a tapering of the cell body toward the axon fiber. Within the axon hillock, the cytoplasm changes to a solution of limited components called axoplasm. Because the axon hillock represents the beginning of the axon, it is also referred to as the initial segment.

Glial Cells

Glial cells, or neuroglia or simply glia, are the other type of cell found in nervous tissue. They are supporting cells, and many functions are directed at helping neurons complete their function for communication, **figure 81**.

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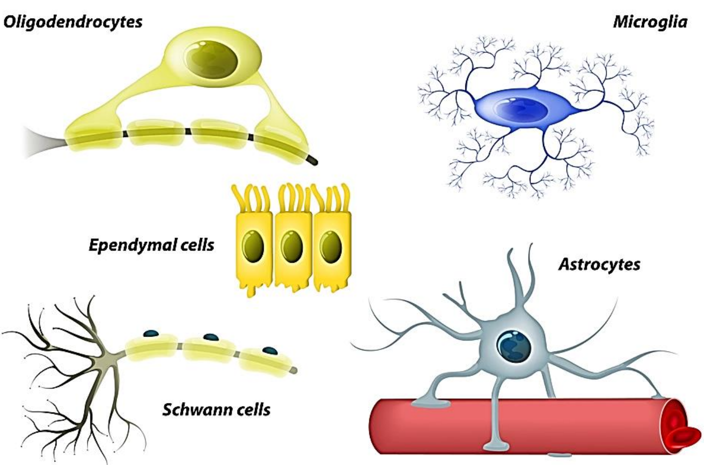

Figure 81: Glial Cells

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THE ENDOCRINE SYSTEM

Structures:

The endocrine system consists of cells, tissues, and organs that secrete hormones as a primary or secondary function. The endocrine gland is the major player in this system. The primary function of these ductless glands is to secrete their hormones directly into the surrounding fluid. The interstitial fluid and the blood vessels then transport the hormones throughout the body.

The endocrine system includes the pituitary, thyroid, parathyroid, adrenal, and pineal glands. Some of these glands have both endocrine and non-endocrine functions. For example, **the pancreas** contains cells that function in digestion as well as cells that secrete the hormones insulin and glucagon, which regulate blood glucose levels.

The hypothalamus, thymus, heart, kidneys, stomach, small intestine, liver, skin, female ovaries, and male testes are other organs that contain cells with endocrine function. Moreover, adipose tissue has long been known to produce hormones, and recent research has revealed that even bone tissue has endocrine functions, **figure 82.**

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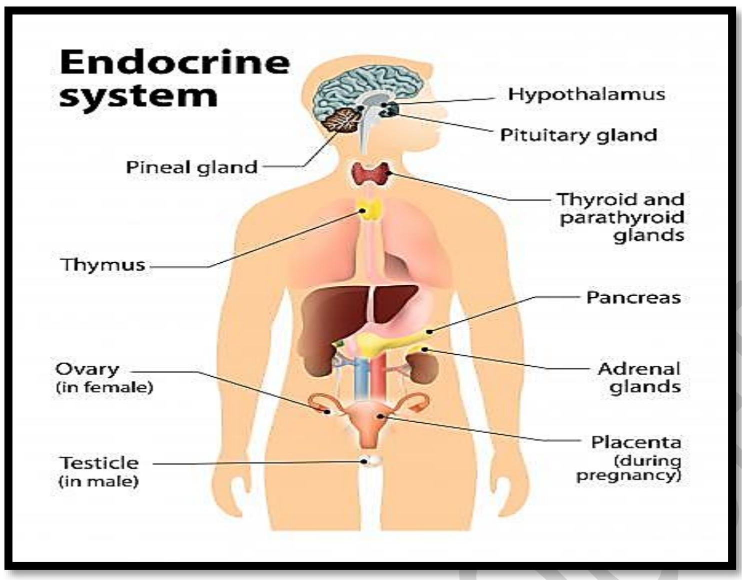

Figure 82: THE ENDOCRINE SYSTEM

Hormones:

Hormones are derived from amino acids or lipids. Amine hormones originate from the amino acids tryptophan or tyrosine. Larger amino acid hormones include peptides and protein hormones.

Steroid hormones are derived from cholesterol. Steroid hormones and thyroid hormones are lipid soluble.

All other amino acid– derived hormones are water soluble. Hydrophobic hormones can diffuse through the membrane and interact with an intracellular receptor. In contrast, hydrophilic hormones must interact with cell membrane receptors.

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The Pituitary Gland and Hypothalamus:

The hypothalamus–pituitary complex is in the diencephalon of the brain. The pituitary gland is divided into two distinct structures with different embryonic origins.

The posterior lobe houses the axon terminals of hypothalamic neurons. It stores and releases into the bloodstream **two** hypothalamic hormones: **oxytocin** and **antidiuretic hormone** (ADH).

The anterior lobe is connected to the hypothalamus148 by vasculature in the infundibulum and produces and secretes six hormones. Their secretion is regulated, however, by releasing and inhibiting hormones from the hypothalamus. **The six anterior pituitary hormones** are: growth hormone (GH), thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), follicle stimulating hormone (FSH), luteinizing hormone (LH), and prolactin (PRL).

The Thyroid Gland:

The thyroid gland is a butterfly-shaped organ located in the neck anterior to the trachea. Its hormones regulate basal metabolism, oxygen use, nutrient metabolism, the production of ATP, and calcium homeostasis.

The parathyroid glands:

The parathyroid glands are small structures located on the posterior thyroid gland that produce parathyroid hormone (PTH), which regulates blood calcium levels. Low blood calcium levels cause the production and secretion of PTH.

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The Adrenal Glands:

The adrenal glands, located superior to each kidney, consist of **two regions**: the adrenal cortex and adrenal medulla. The adrenal cortex—the outer layer of the gland—produces mineralocorticoids, glucocorticoids, and androgens. The adrenal medulla at the core of the gland produces epinephrine and norepinephrine.

The Pineal Gland:

The pineal gland is an endocrine structure of the diencephalon of the brain and is located inferior and posterior to the thalamus. It is made up of pinealocytes. These cells produce and secrete the hormone melatonin.

Gonadal and Placental Hormones:

The male and female reproductive system is regulated by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) produced by the anterior lobe of the pituitary gland in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus.

The Endocrine Pancreas:

The pancreas has both exocrine and endocrine\ functions. The pancreatic islet cell types include alpha cells, which produce glucagon; beta cells, which produce insulin; delta cells, which produce somatostatin; and PP cells, which produce pancreatic polypeptide.

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