SEMESTER I
CORE COURSE 3

ZOPALT3: ENDOCRINOLOGY

1. **Handling, sexing, numbering and maintenance of rat**

The common laboratory rat *Rattus norvegicus* is an excellent experimental animal for several reasons: abundance of literature published pertaining to them, ease of handling, high fertility rate, short gestation period, low maintenance and disease model for various human disorders and diseases.

Manual restraint:

- Before opening the cage observe the animals within.
- Nervous or young rats can escape quickly.
- Rats will not stay on top of the wire-bar lid of the cage. Always have a hand on them.
- Rats should not be held by their tail as their skin is fragile and can easily strip from the underlying tissue.
- Rats should not be scruffed by the loose skin on the back of the neck.

Three methods are commonly used:

1. **“V” grip**
    - With your dominant hand, slide your index and middle finger along both sides of the head as far as possible and grasp the head with your knuckles on the jaw bones.
    - Place your thumb and remaining fingers under both forelimbs to grasp the thorax.
    - If possible, you can hold the lower body with your other hand or rest the rat on your chest for the comfort of the animal. This is especially important for larger or pregnant animals.
2. **Cross leg**
    - With your dominant hand, position your thumb and middle finger on each elbow.

- Push on both elbows to cross the two front paws. With your remaining fingers support the thorax of the rat.
- If possible, you can hold the lower body with your other hand or rest the rat on your chest for the comfort of the animal. This is especially important for larger or pregnant animals.

3. **Towel method**
    - If you have nervous or aggressive animals, you can wrap it in a towel. This method has the advantage of controlling the hind limbs which prevents potential scratching.

Restraint devices: Several restraint devices are available in various sizes and materials (e.g., Plexiglas, plastic) and can be used when performing different techniques such as injections or blood collection. The restrainer should be small enough so that the animal cannot turn around yet allow the animal to rest comfortably and breathe normally. Observe animals to ensure that they do not overheat and never leave an animal in a restrainer unattended.

IDENTIFICATION

Rat can be identified by the following methods:

1. Cage cards

2. Temporary markings

3. Ear punching/notching

4. Ear tags

5. Tail tattoo

6. Micro-tattooing

7. Electronic identification with microchips

SEX DETERMINATION

- Sexing of mice is based upon ano-genital distance
- Males have a greater distance between the anus and urogenital opening than females.

- An opposite sex comparison is advisable initially.
- The testicles can be retracted into the abdomen; therefore, it may be easier to sex a mature male by holding its head up vertically. The genital papilla is more prominent in males than females

SAMPLING FOR GENOTYPING

Rats can be genotyped by the following methods:

1. Fecal pellet

2. Buccal epithelial cell

3. Ear punching

4. Tail snipping

2. General survey of endocrine glands in rat

1. Hypothalamus

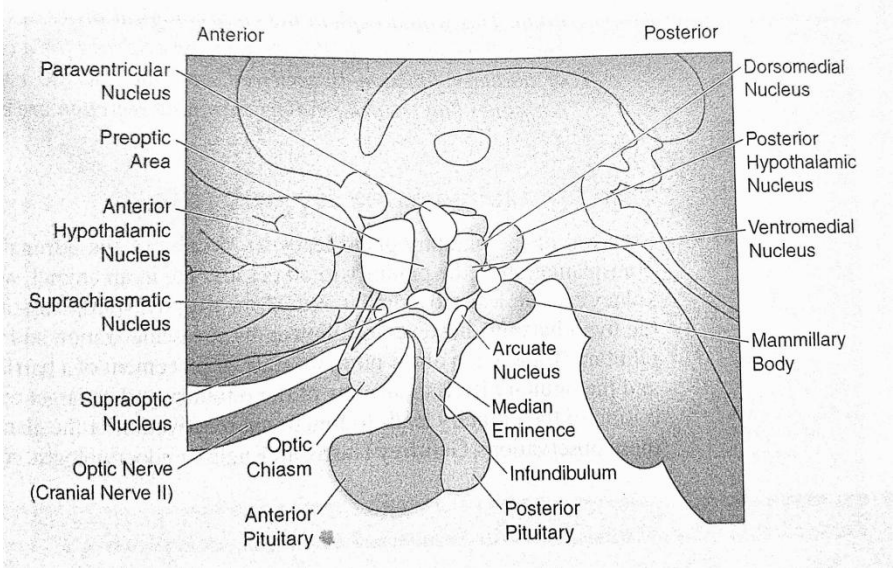


Fig: Hypothalamus

Comments:

i. The hypothalamus is located at the base of the brain just below the thalamus and above the Pituitary Gland**.**

ii. The hypothalamus secretes hormones that stimulate or suppress the release of hormones in the pituitary gland, hence also called "Master of the Master Gland".

iii. The structure of the hypothalamus is made up of three main regions: Anterior, Middle and the Posterior Region.
iv. Anterior Region is also called Supraoptic Region, consists of Supraoptic and Paraventricular Nuclei.
v. Anterior region regulates the temperature and maintains the circadian rhythm.
vi. Middle region is also known as Tuberal Region, it contains Ventromedial nuclei and Arcuate nuclei.
vii. Middle region's nuclei regulate the appetite and growth hormone secretion respectively.
viii. Posterior region is also known as Mammilary Region and it hosts Hypothalamic nuclei and Mammilary Nuclei.
ix. Hypothalamus serves as the link between the endocrine and the nervous system.

2. Pituitary Gland

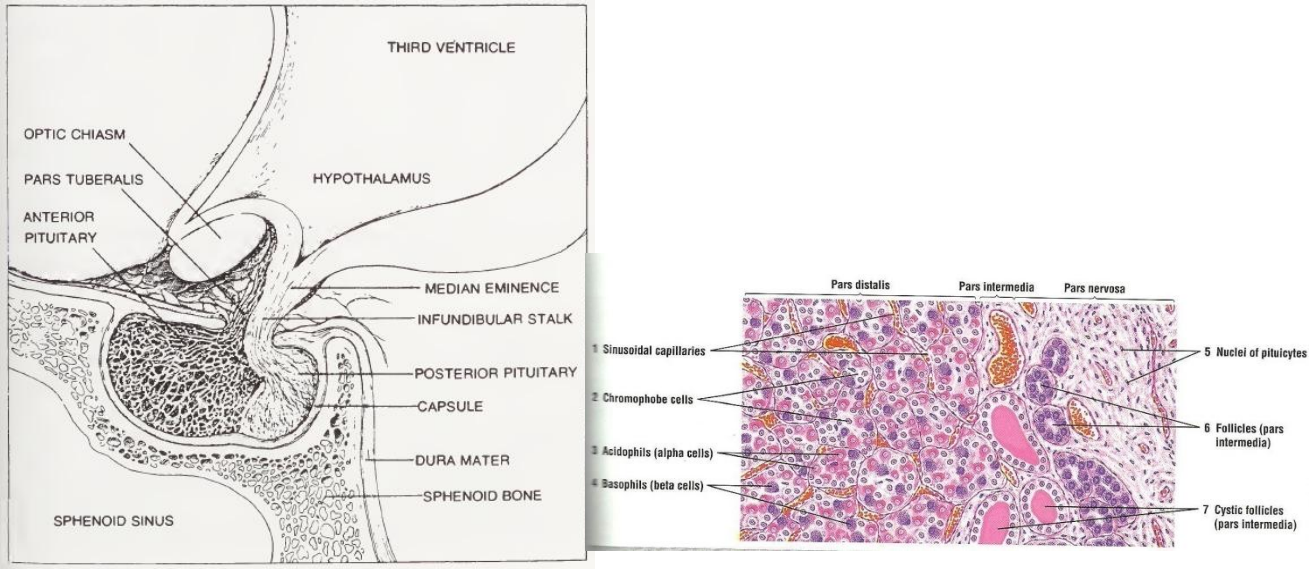


Fig: T.S. of the Pituitary Gland

Comments:

i. Pituitary Gland is also known as the Master Gland, as it controls three major glands: Thyroid, Adrenal and the Gonads.
ii. The Pituitary broadly consists of two parts: Adenohypophysis and Neurohypophysis.
iii. Adenohypophysis is also called the Anterior Lobe of the Pituitary, it consists of Pars Tuberalis, Pars Distalis and Pars Intermedia.
iv. Anterior Pituitary is composed of winding cords of epithelial cells flanked by vascular sinusoids and has the Acidophils, Basophils and Chromophores.
v. Acidophils have cytoplasm that stains red or orange, Basophils have cytoplasm that stains a bluish colour and Chromophores have poorly stained cytoplasm.
vi. Pars Nervosa is also known as the Posterior Lobe of the Pituitary, it consists of Infundibulum, Median Eminence and the Pars Nervosa.

vii. The neurohypophysis contains abundant capillaries and herring bodies, many of these capillaries are fenestrated (contain holes), facilitating delivery of hormones into blood.

viii. Adenohypophysis releases growth hormone (GH), prolactin (PRL) or luteotropic hormone (LTH) and adrenocorticotropin (ACTH), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and thyroid-stimulating hormone (TSH).

ix. Neurohypophysis doesn't produce any hormone, instead it stores and release Oxytocin and Vasopressin (Anti Diuretic Hormone) released by the Hypothalamic Neurons.

3. Pineal Gland

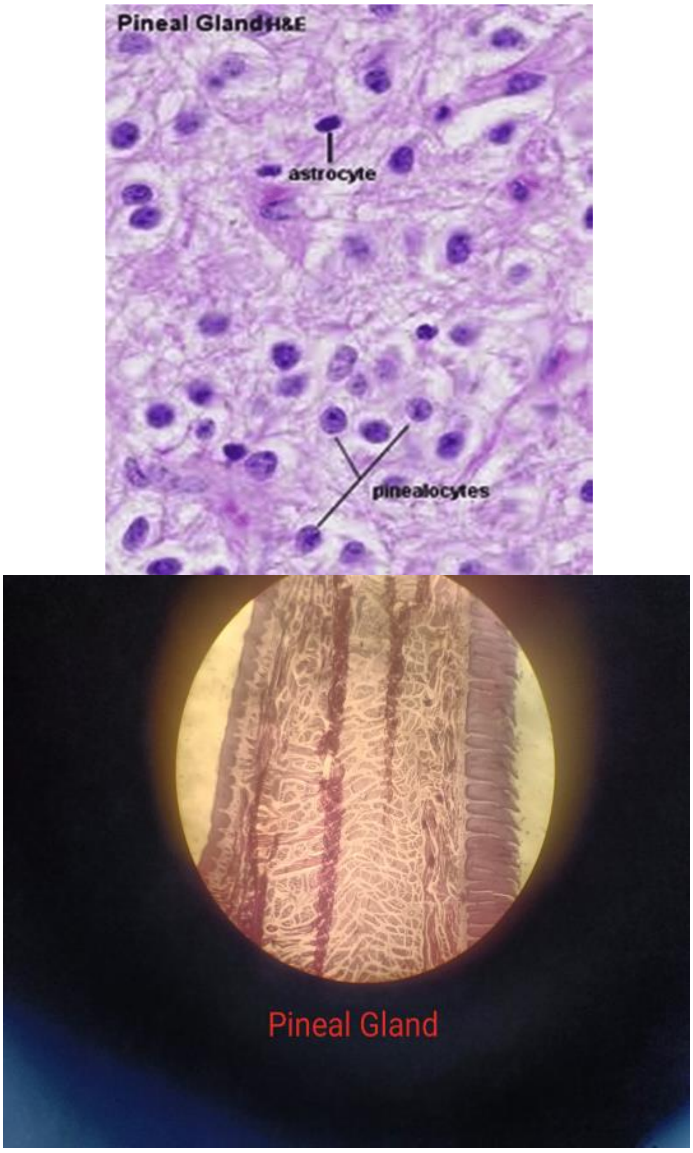


Fig: T.S. of the Pineal Gland

Comments:

i. The pineal gland, or epiphysis is a small cone-like structure that comprises a part of the diencephalon.
ii. The gland has several functions, the most important of which is maintaining the body's circadian rhythm and regulating the sleep-wake cycle.
iii. It consists of Capsule, Trabeculae and the Parenchyma, consisting of Pinealocytes, Peptidergic Neurons and the Astrocytes.
iv. Pinealocytes forms the majority of the histological structure and consists of the highly modified neurons, arranged in the cords and clusters.
v. The calcareous bodies in the Pineal Gland have been regarded as signs of aging and degeneration.
vi. The chief secretion of the Pineal Gland includes the Melatonin Hormone, which is important for the regulation of Diurnal Rhythm and regulation of the reproductive system development.
vii. Pineal glands tissue is surrounded by the blood vessel, indicating the gland's reliance on rich blood supply for its endocrine activity.
viii. Nerve fibre in some section reflects the connection with the CNS and its role in neuro endocrine secretion regulation.

4. Thyroid Gland

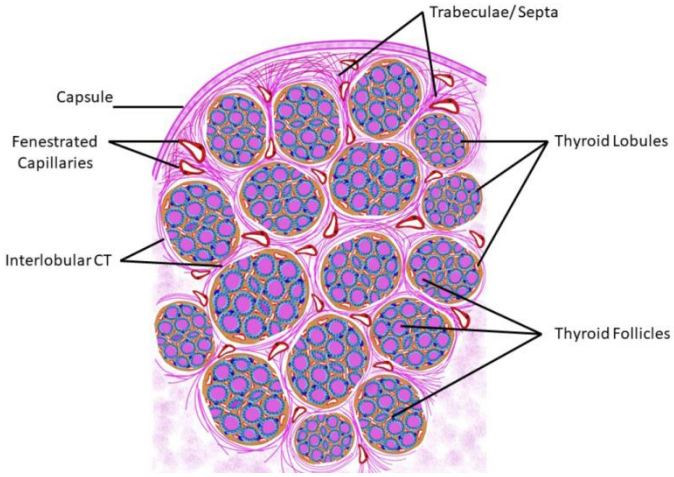


Fig: T.S. of Thyroid Gland

Comments:

i. Thyroid gland consists of thyroid follicles of various sizes, separated by connective tissue strands.
ii. It consists of right and left lobes connected across to the ventral side of trachea by isthmus.
iii. It is supplied with blood vessels and nerves, also it is innervated from the sympathetic nerves.
iv. It is composed of follicular and interfollicular zones.
v. Follicles are surrounded by single layered cubical or rounded epithelial cells.
vi. Lumen of follicle consists of viscous liquid called as thyroid colloid.

vii. Interfollicular zone consist of connective tissues, nerve fibres blood vessels and large number of nuclei.
viii. Lumen of each follicle is filled with the gel-like mass called colloid.
ix. It is mostly the protein thyroglobulin (pink) and bound thyroid hormones (triiodothyronine and tetraiodothyronine (or thyroxin) (C15 H11O4N14) which contains thyroxine amino acid and 65% of iodine.)

5. Adrenal Gland

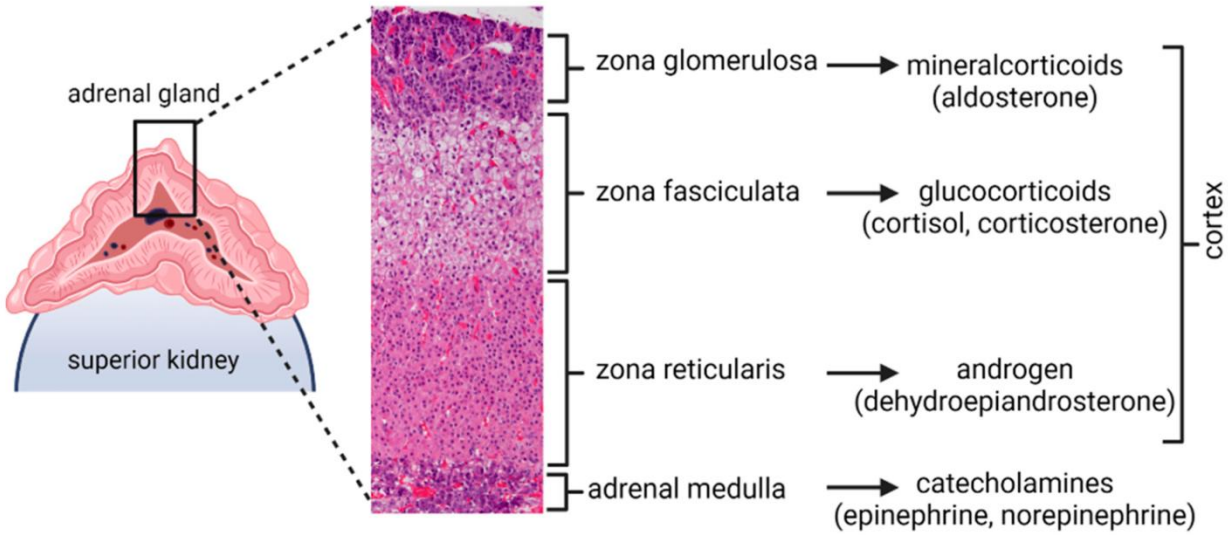


Fig: T.S. of the Adrenal Gland

Comments:

i. Adrenal glands are small, triangular-shaped glands located on top of both kidneys.
ii. Adrenal glands produce hormones that help regulates metabolism, immune system, blood pressure, response to stress and other essential functions.
iii. Adrenal glands are composed of two parts — the cortex and the medulla.
iv. The adrenal cortex is the outer region and also the largest part of an adrenal gland. It is divided into three separate zones: zona glomerulosa, zona fasciculata and zona reticularis.
v. The adrenal medulla is located inside the adrenal cortex in the centre of an adrenal gland.
vi. The adrenal cortex and adrenal medulla are enveloped in an adipose capsule that forms a protective layer around an adrenal gland.
vii. Zona Glomerulosa is composed of columnar cells regulating the mineral, water balance and fat-carbohydrate metabolism.
viii. Zona Faciculata composed of compressed cell, secreting controlling carbohydrate metabolism
ix. Zona reticularis consisting of pigmented, reticular cells, secreting sex hormones.
x. Adrenal medulla, releases, epinephrine and norepinephrine hormones.

6. Pancreas

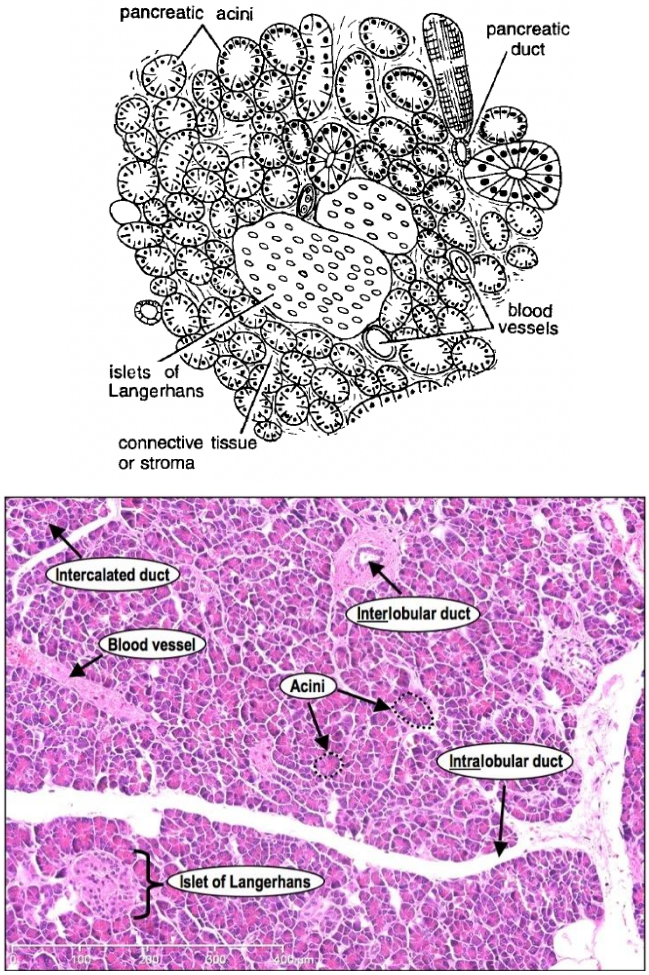


Fig: T.S. of the Pancreas

Comments:

i. Pancreas is a very important digestive gland. T.S. passing through pancreas shows that it is composed of various alveoli or acini.

ii. It is a compound tubulo-alveolar racemose gland consisting of both exocrine and endocrine parts.

iii. Each pancreatic lobe contains 10-20 secretory cells which are nucleated. The central part has narrow to wide lumen. The pancreatic duct, large artery and vein are also seen in the section.

iv. Several scattered groups of cells called as islets of Langerhans, which are somewhat paler, are distinctly seen.

v. Acini and islets of Langerhans are very clearly seen. The wall of each acinus is made up of columnar or pyramidal cells.

vi. Each cell contains a central nucleus and course granules. Each acinus has wide lumen.

vii. The region of islet of Langerhans reveals 3 or 4 kinds of cells -α, β and undifferentiated cells.

viii. Alpha cells (α-cells) which secrete a hormone called as glucagon. It increases blood sugar level in the body and its deficiency causes hypoglycemia.

ix. Beta cells (β -cells) secrete another hormone, insulin, which plays an important role in carbohydrate metabolism. Its deficiency causes diabetes. It regulates blood sugar level.

7. Testis

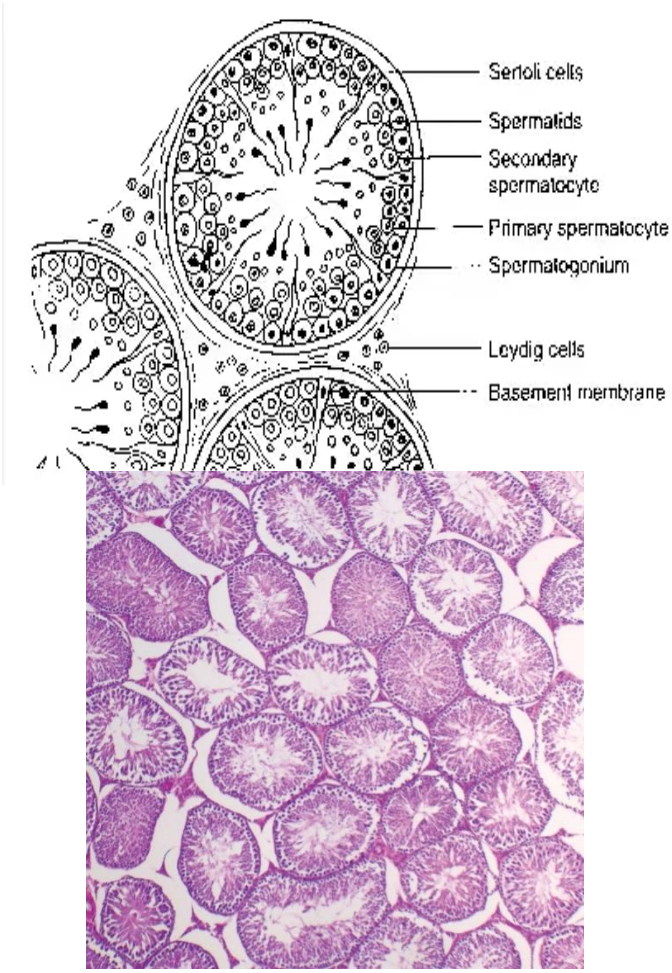


Fig: T.S. of the Testis

Comments:

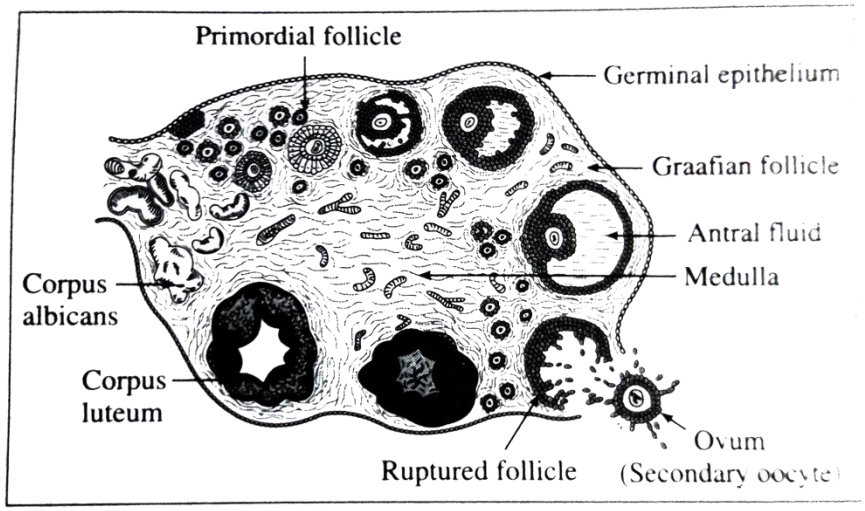
i. The testis is externally covered by fibrous connective tissue called Tunica albuginea. It is covered internally by Tunica vasculosa which is formed by capillaries.

ii. Externally it is covered by the incomplete peritoneal covering called as Tunica vaginalis.

iii. The Transverse section shows the presence of seminiferous tubules lined by cuboidal germinal epithelial cells. The germ cell undergoes the process of spermatogenesis.

iv. The transverse section also reveals different stages of spermatogenesis like spermatogonia, primary and the secondary spermatocyte, spermatids and sperm.
v. Few large pyramidal cells present between germinal cells are known as Sertoli cells or Nurse cells.
vi. The function of Sertoli cells is to provide Nourishment of the sperm till maturation.
vii. Between the seminiferous tubules, are the present cluster of polygonal cells known as Interstitial cells or Leydig cells.
viii. The Leydig cells secrete male sex hormone Testosterone or Androgen hormone.

8. Ovary



T.S. of Ovary

Comments:

i. The ovarian mesothelium is a layer of simple epithelium that covers the surface of ovary.
ii. The tunica albuginea is a whitish capsule of dense irregular connective tissue located deep to the ovarian mesothelium.
iii. Underlying germinal epithelium give rise to the oogonia.
iv. Ovary is differentiated into the two regions: Cortex and the Medulla.
v. The ovarian cortex consists of ovarian follicles surrounded by dense irregular connective tissue.
vi. Ovarian follicles in the cortex consists of oocytes in the various stages of development.
vii. Follicle cells secrete estrogen which helps in the maturation of oocytes. Mature graafian follicle contains secondary oocyte.
viii. Secondary oocyte is surrounded by the zona pellucida and a layer of corona radiata cells, Corona radiator is surrounded by mass of cells called discus proligerous, surrounded by another layer of liquor follicle, and then by membrane granulosa

ix. At the condition of LH surge, ovulation takes place, releasing the secondary oocyte from graafian follicle.
x. After ovulation corpus luteum is formed, which secretes progesterone.

3. Study of vaginal smear preparation in rat

Preparation of vaginal smear in rat/mouse

In mammals, other that of primates, the reproductive cycle is known as **estrous cycle.** Rats and mice have an estrous cycle of 4 to 5 days and the cycle is divided roughly into four stages:

(a) **Estrous:** This is the period of heat during which ovulation occurs. This period lasts for 9-15 hours under the influence of follicle stimulating hormone (FSH) and estrogen. The female is receptive to the male only during this period; therefore, ovulation and fertilisation are well coordinated. The uterus becomes enlarged and the vaginal mucosa proliferates and the vaginal epithelium becomes squamous and

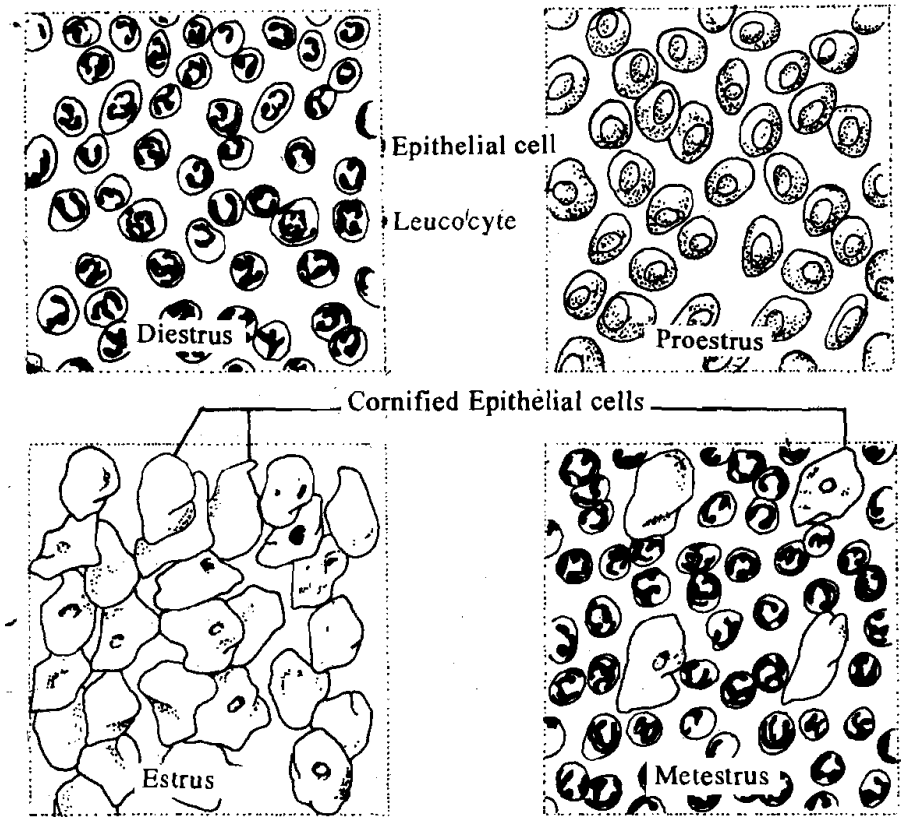
cornified. A vaginal smear taken during this period shows **squamous cells** indicative of estrous. Subsequent events depend on sexual contact with male.

2. **Metetrous:** In the absence of copulation this stage occurs shortly after ovulation and lasts for 10 to 14 hours. A small corpus luteum is formed and some progesterone is secreted. A vaginal smear taken at this stage shows **lecuocytes with some cornified cells.**

3. Diestrous: This stage lasts for 60 to 70 hours. The corpora lutea regress during this period and vaginal smear contains**only leucocytes.**

4. Proestrous: Lasts for about 12 hours. It precedes the next estrus. Degeneration of old corpora lutea continues but new follicles mature rapidly. The uterus becomes distended again and the vaginal smear contains individual **nucleated epithelial cells**.

All these changes in vaginal lining are shown

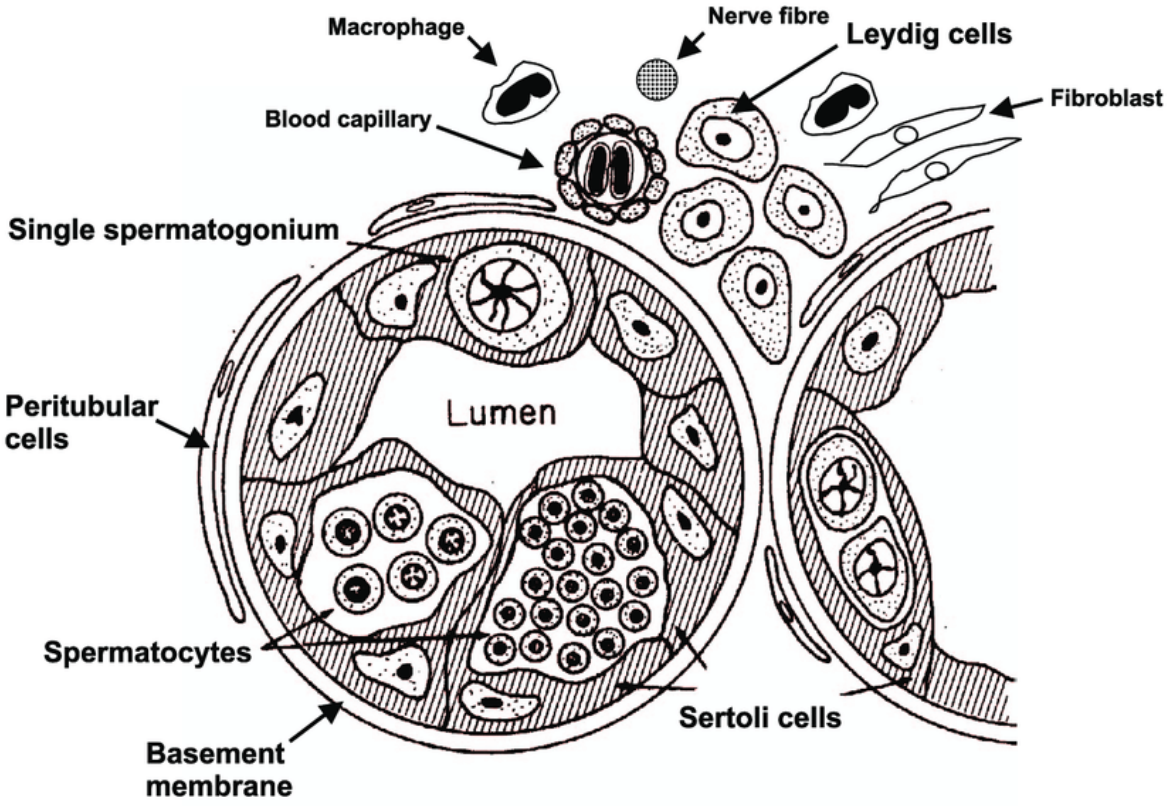


4. Study of the following using permanent slides:
   a. Endocrine glands and reproductive organs of rat
   b. Gonads (testis and ovary from fish to birds)
   c. Thyroid of fish (pharyngeal and ectopic) and reptile
   d. Adrenal homologues (interrenal and chromaffin tissues) in fish and reptile
   e. Cell types pituitary
   f. Hypothalamo-neurohypophsial system

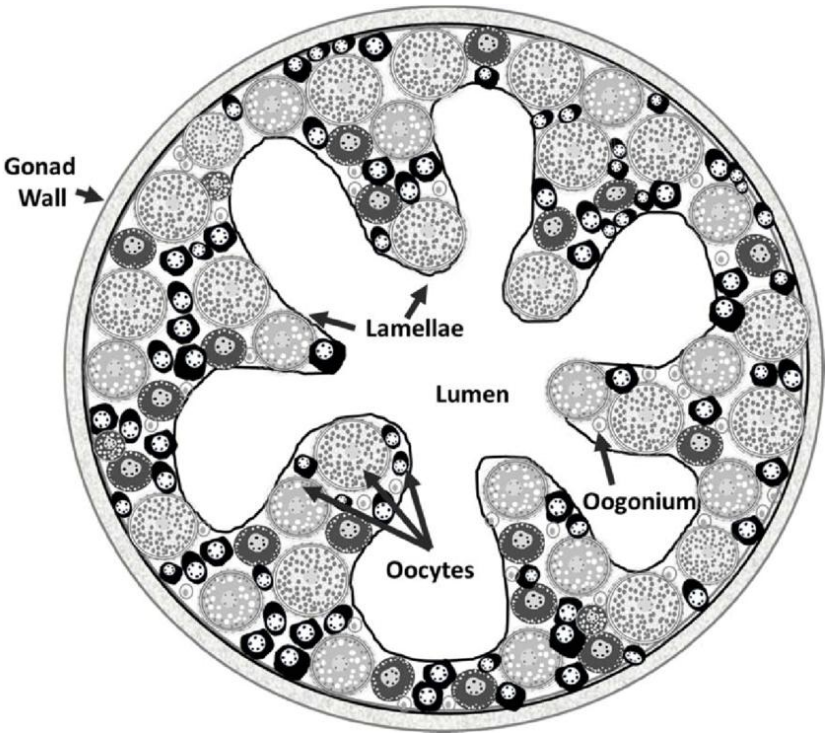
Testis and Ovary (Fish)

1. The figure shows T.S. of a testis and an ovary of fish.
2. The interstitial compartment is separated from the lobular compartment by an acellular basement membrane that surrounds the germinal epithelium consisting of Sertoli cells and germ cells.
3. The germinal epithelium is organised in functional units called spermatocysts.
4. Each spermatocyst is formed by Sertoli cells that surround one clone of germ cells at the same stage of differentiation.
5. The interstitium is constituted by steroidogenic Leydig cells, peritubular myoid-like cells that form an incomplete layer over the surface of lobules, blood capillaries, scattered nerve fibres, fibroblasts and macrophages.
6. In most fishes, the ovaries are bi-lobed, although one lobe may be much larger than the other.
7. When sectioned transversely, they usually appear circular in shape and consist of a central lumen (cavity) bounded by ovarian tissue.

8. This tissue consists a mixture of oogonia, oocytes (eggs) and stromal (supportive) tissues, including connective tissue and blood vessels.



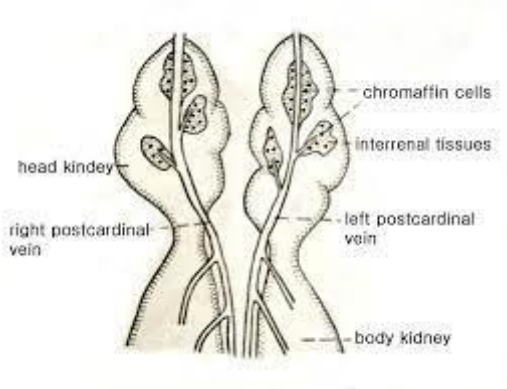
T.S. Testis of Fish



T.S. Ovary of Fish

Adrenal homologues (interrenal and chromaffin tissues)

1. Homologous tissue to the adrenal cortex of mammals.
2. Distributed in the head kidney and surrounds the posterior cardinal vein and its branches.
3. Histochemical characteristics of the cortical cells in the adrenal gland.
4. Secretion of corticosteroids.
5. Cortisol is the main in teleosts.
6. Also cortisone and corticosterone secreted.
7. Still unclear whether or not aldosterone is secreted
8. Aldosterone is the main mineralocorticoid in terrestrial vertebrates.
9. Chromaffin cells are closely related to the interrenal gland.
10. They secrete adrenaline and noradrenaline.



5. Demonstration of frog metamorphosis by models and charts

Amphibian Metamorphosis

Amphibian metamorphosis is associated with morphological changes that prepare an aquatic organism for a primarily terrestrial existence. In urodeles (salamanders), these changes include the resorption of the tail fin, the destruction of the external gills, and a change in skin structure. In anurans (frogs and toads), the metamorphic changes are more dramatic, with almost every organ subject to modification. The changes in amphibian metamorphosis are initiated by thyroid hormones such as thyroxine (T4) and triiodothyronine (T3) that travel through the blood to reach all the organs of the larva. When the larval organs encounter these thyroid hormones, they can respond in any of four ways: growth, death, remodeling, and respecification.

Growth of New Structures

The hormone tri-iodothyronine induces certain adult-specific organs to form. The limbs of the adult frog emerge from specific sites on the metamorphosing tadpole, and in the eye, both nictitating membranes and eyelids emerge. Moreover, T3 induces the proliferation and differentiation of new neurons to serve these organs. As the limbs grow out from the body

axis, new neurons proliferate and differentiate in the spinal cord. These neurons send axons to the newly formed limb musculature. Blocking T3 activity prevents these neurons from forming and causes paralysis of the limbs. One readily observed consequence of anuran metamorphosis is the movement of the eyes to the front of the head from their originally lateral position. In the tadpole, the right eye innervates the left side of the brain, and vice versa; there are no ipsilateral (same-side) projections of the retinal neurons. During metamorphosis, however, ipsilateral pathways emerge, enabling input from both eyes to reach the same area of the brain. In *Xenopus,* these new neuronal pathways result not from the remodeling of existing neurons, but from the formation of new neurons that differentiate in response to thyroid hormones.

Cell Death during Metamorphosis

The hormone T3 also induces certain larval-specific structures to die. Thus, T3 causes the degeneration of the paddle-like tail and the oxygen- procuring gills that were important for larval (but not adult) movement and respiration. Recent evidence suggests that the first part of tail resorption is caused by suicide, but that the last remnants of the tadpole tail must be killed off by other means. When tadpole muscle cells were injected with a dominant negative T3 receptor (and therefore could not respond to T3), the muscle cells survived, indicating that T3 told them to kill themselves by apoptosis. This was confirmed by the demonstration that the apoptosis-inducing enzyme caspase-9 is important in causing cell death in the tadpole muscle cells. However, later in metamorphosis, the tail muscles are destroyed by phagocytosis. Death also comes to the tadpole's red blood cells. During metamorphosis, tadpole hemoglobin is changed into adult hemoglobin, which binds oxygen more slowly and releases it more rapidly. The red blood cells carrying the tadpole hemoglobin have a different shape than the adult red blood cells, and these larval red blood cells are specifically digested by macrophages in the liver and spleen.

Remodeling during Metamorphosis

Among frogs andtoads, certain larval structures are remodeled for adultneeds. Thus, the larval intestine, with its numerous coils for digesting plant material, is converted into a shorterintestine for a carnivorous diet. It has been demonstrated that the new cells of theadult intestine are derived from functioning cells of the larvalintestine. Much of the nervous system is remodeled as neuronsgrow and innervate new targets. The lateral line systemof the tadpole (which allows the tadpole tosense water movement and helps it to hear)degenerates, and the ears undergo further differentiation. The middleear develops, as does the tympanic membranecharacteristic of frog and toad outer ears.Tadpoles experience a brief period of

deafnessas the neurons change targets. Thus, the anuran nervous system undergoes enormous restructuringassome neurons die, others are born, and others change theirspecificity.

The shape of the anuran skull also changes significantlyas practically every structural component of the head isremodeled. Themost obvious change is that new bone is being made. Thetadpole skull is primarily neural crest-derived cartilage;the adult skull is primarily neural crest-derived bone.

Biochemical Respecification

In addition to the obvious morphological changes, important biochemical transformations occur during metamorphosis as T3 induces a new set of proteins in existing cells. One of the most dramatic biochemical changes occurs in the liver. Tadpoles, like most freshwater fish, are ammonotelic—that is, they excrete ammonia. Like most terrestrial vertebrates, many adult frogsare ureotelic: they excrete urea, whichrequires less water than ammonia excretion. During metamorphosis,the liver begins to synthesize the enzymes necessaryto create urea from carbon dioxide and ammonia. T3 may regulate this change by inducing a setof transcription factors that specifically activates expressionof the urea-cycle genes while suppressing the genesresponsible for ammonia synthesis.

Hormonal control of amphibian metamorphosis

The metamorphic changes of frog development arebrought about by (1) the secretion of the hormone thyroxine(T4) into the blood by the thyroid gland; (2) the conversionof T4 into the more active hormone, tri-iodothyronine(T3) by the target tissues; and (3) the degradation of T3 inthe target tissues. T3 binds to the nuclear **thyroidhormone receptors (TRs)** with much higher affinitythan does T4, and causes these receptors to become transcriptionalactivators of gene expression. Thus, the levelsof both T3 and TRs in the target tissues are essential for producingthe metamorphic response in each tissue.The concentration of T3 in each tissue is regulated by theconcentration of T4 in the blood and by two critical intracellularenzymes that remove iodine atoms from T4 and T3.Type II deiodinase removes an iodine atom from the outer

ring of the precursor hormone (T4) to convert it into themore active hormone T3. **Type III deiodinase** removes aniodine atom from the inner ring of T3 to convert it into aninactive compound that will eventually be metabolized totyrosine. There are two types of thyroid hormone receptors. In*Xenopus,* **thyroid hormone receptor α (TR α)** is widely distributedthroughout all tissues and is present even beforethe organism has a thyroid gland. **Thyroid hormone receptor β (TR β),** however, is the product of a gene that is directlyactivated by thyroid hormones. **TRβ** levels are very lowbefore the advent of

metamorphosis; as the levels of thyroidhormone increase during metamorphosis, so do theintracellular levels of **TR β.**

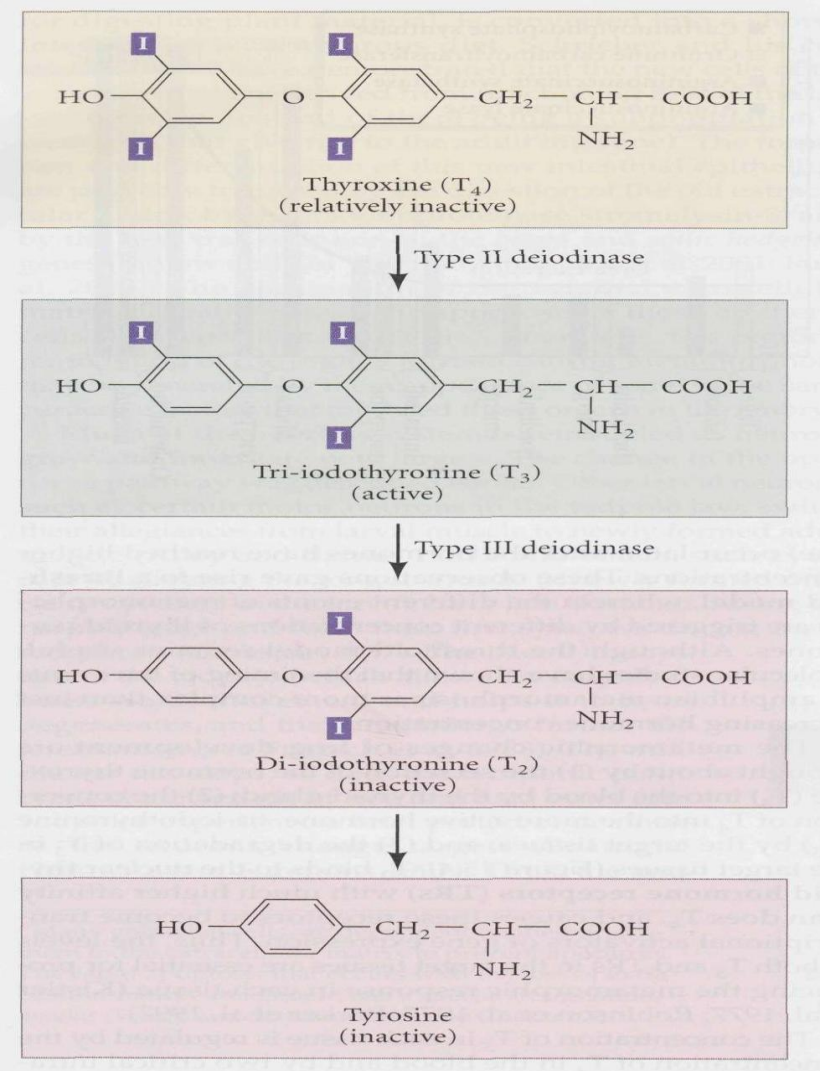


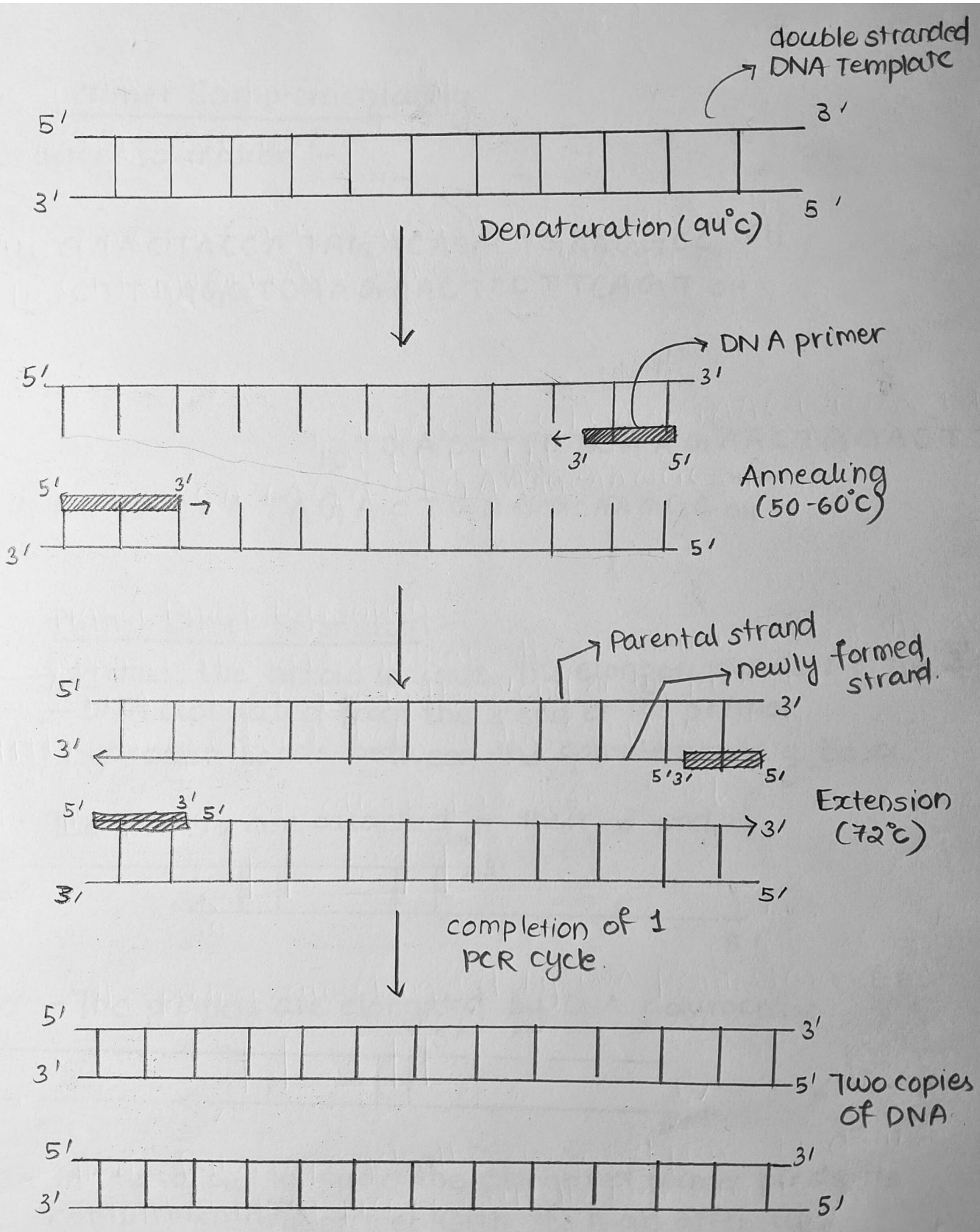
Fig. Metabolism of thyroxine (T4) and tri-iodothyronine (T3). T4 serves as a prohormone. It is converted in the peripheral tissues to the active hormoneT3 by deiodinase II. T3 can be inactivated by deiodinase III, which converts T3 into di-iodothyronine and then to tyrosine

The TRs do not work alone, however, but form dimers with the retinoid receptor, RXR. These dimers bind thyroid hormones and can effect transcription. The TR-RXR complex appears to be physically associated with appropriate promoters and enhancers even before it binds T3. In its unbound state, the TR-RXR is a transcriptional repressor, recruiting histone deacetylases to the region of these genes. However, when T3 is added to the complex, the T3-TR-RXR complex activates those same genes by recruiting histone acetyltransferases.

Metamorphosis is often divided into stages based on the concentration of thyroid hormones in circulation. During the first stage, **premetamorphosis,** the thyroid gland has begun to mature and is secreting low levels of T4 (and very low levels of T3). The initiation of T4 secretion may be brought about by corticotropin releasing hormone (CRH, which in mammals initiates the stress response). CRH may act directly on the frog pituitary, instructing it to release thyroid stimulating hormone (TSH), or it may act generally to make the body cells responsive to low amounts of T3.

The tissues that respond earliest to the thyroid hormones are those that express high levels of deiodinase II, and can thereby convert T4 directly into T3. As the thyroid matures to the stage of **prometamorphosis,** it secretes more thyroid hormones. However, many major changes (such as tail resorption, gill resorption, and intestinal remodeling) must wait until the **metamorphic climax** stage. At that time, the concentration of T4 rises dramatically, and TRβ levels peak inside the cells. Since one of the target genes of T3 is the TRβ gene, TRβ may be the principal receptor that mediates the metamorphic climax. In the tail, there is only a small amount of TRα during premetamorphosis, and deiodinase II is not detectable then. However, during prometamorphosis, the rising levels of thyroid hormones induce higher levels of TRβ. At metamorphic climax, deiodinase II is expressed, and the tail begins to be resorbed. In this way, the tail undergoes absorption only *after* the legs are functional (otherwise, the poor amphibian would have no means of locomotion). The wisdom of the frog is simple: never get rid of your tail before your legs are working. The frog brain also undergoes changes during metamorphosis, and one of the brain's functions is to downregulate metamorphosis once metamorphic climax has been reached. Thyroidhormones eventually induce a negative feedback loop, shutting down the pituitary cells that instruct the thyroid to secrete them. It has been shown that, at the climax of metamorphosis, deiodinase II expression is seen in those cells of the anterior pituitary that secrete thyrotropin, the hormone that activates thyroid hormone expression. The resulting T3 suppresses transcription of the thyrotropin gene, thereby initiating the negative feedback loop so that less thyroid hormone is made.

6. **Demonstration of ELISA-based hormone assay**



[Fig:- Steps involved in PCR]

Primer Complementarity

- Primer dimer Formation :-

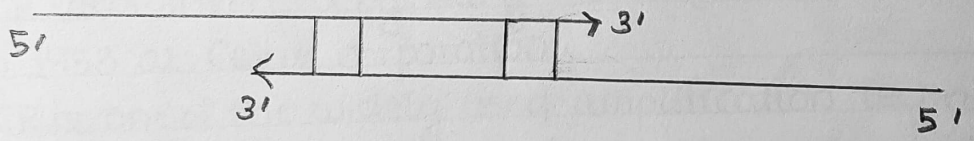
U. GAACTACCA TAGACACACTGAAGGGCC 3' OH
L. CTTGAGGTCAAGAACTCCTTCAGT OH

HO TGACTTCCTCAAGAACTGGACTTC
GAACTACCA TAGACTGAAGGCAAGGC OH

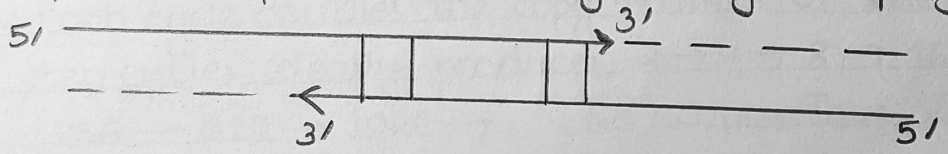
Primer Dimer Formation :-

→ Primer, the arrow indicates the elongation side i.e. the 3'end.
— — DNA elongated from the 3'end or the primer.
IIIII Hydrogen bonds between the complementary bases.

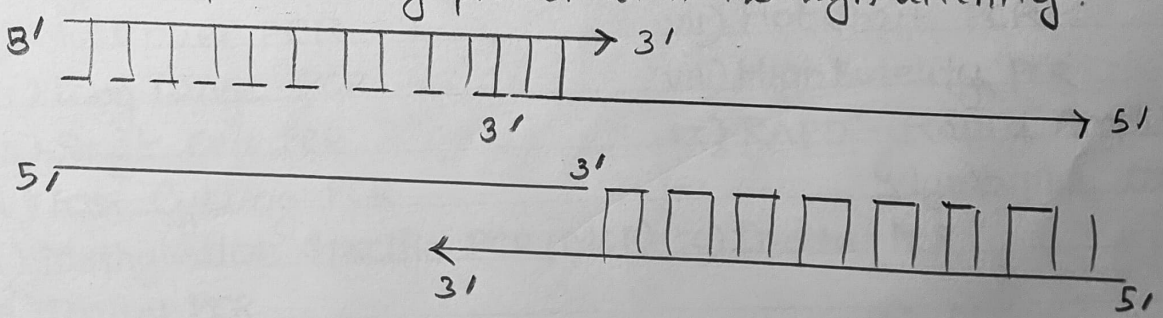
Step1 :- The primers are attached in their 3'-end.



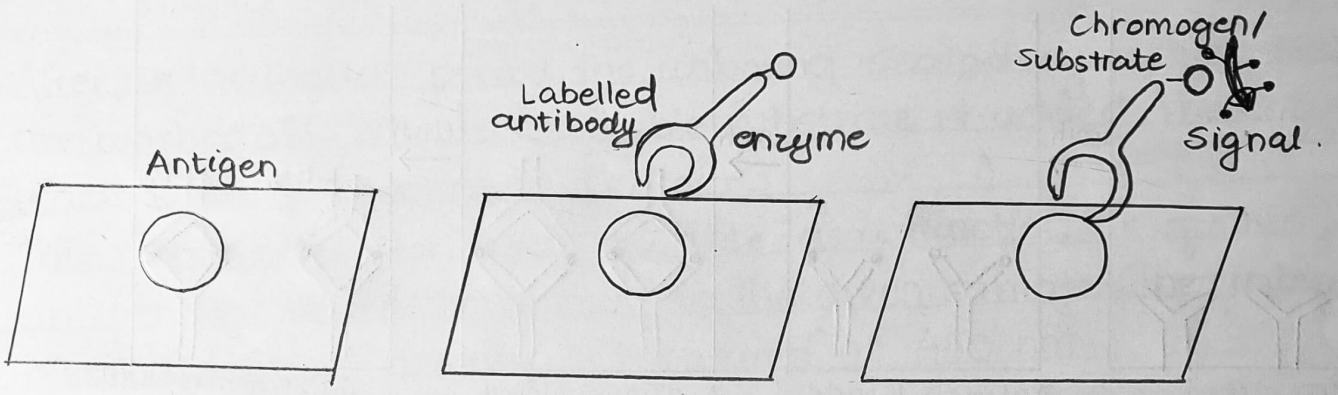
step 2 :- The primers are elongated by DNA polymerase.



step 3- In the following cycle the elongated primer binds its complementary primer with the high affinity.



i) Direct ELISA :-

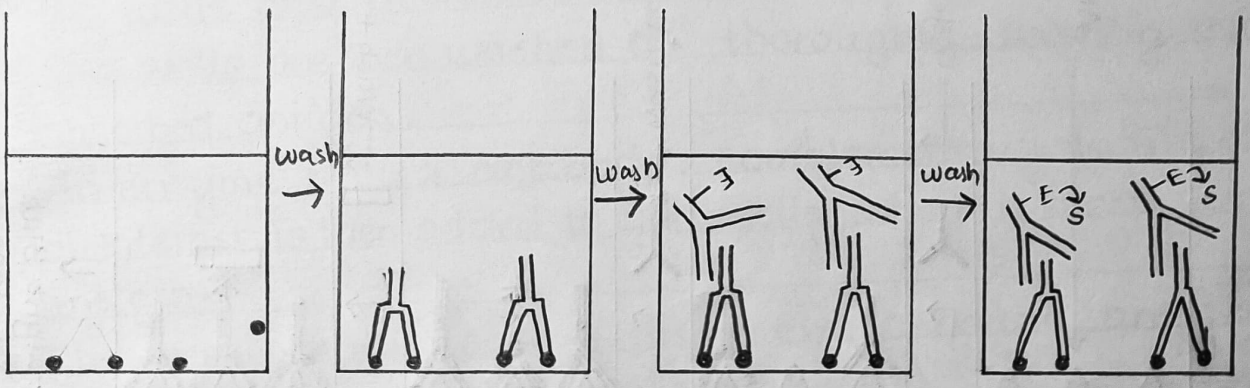


1. Antigen is coated by passive adsorption.

2. Antibody conjugated with enzyme is added and incubated with antigen and incubation.

3. Substrate / Chromophore is added and colour develops.

ii) Indirect ELISA :-



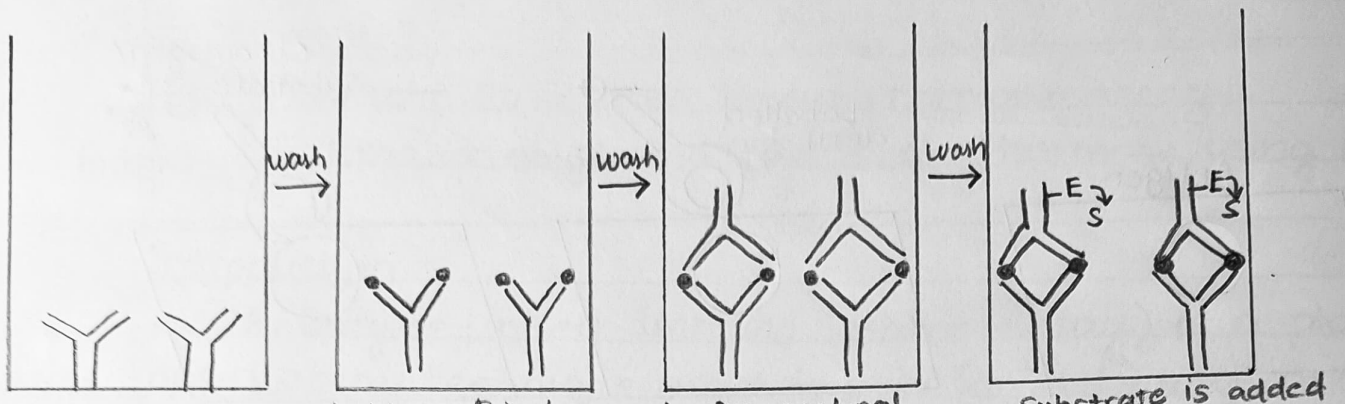
Antigen coated well

Specific antibody binds to antigen.

Enzyme linked antibody binds to specific antibody

Substrate is added and converted by enzyme into coloured product, the rate of colour formation is proportional to the amount of specific antibody

iii) Sandwich ELISA :-

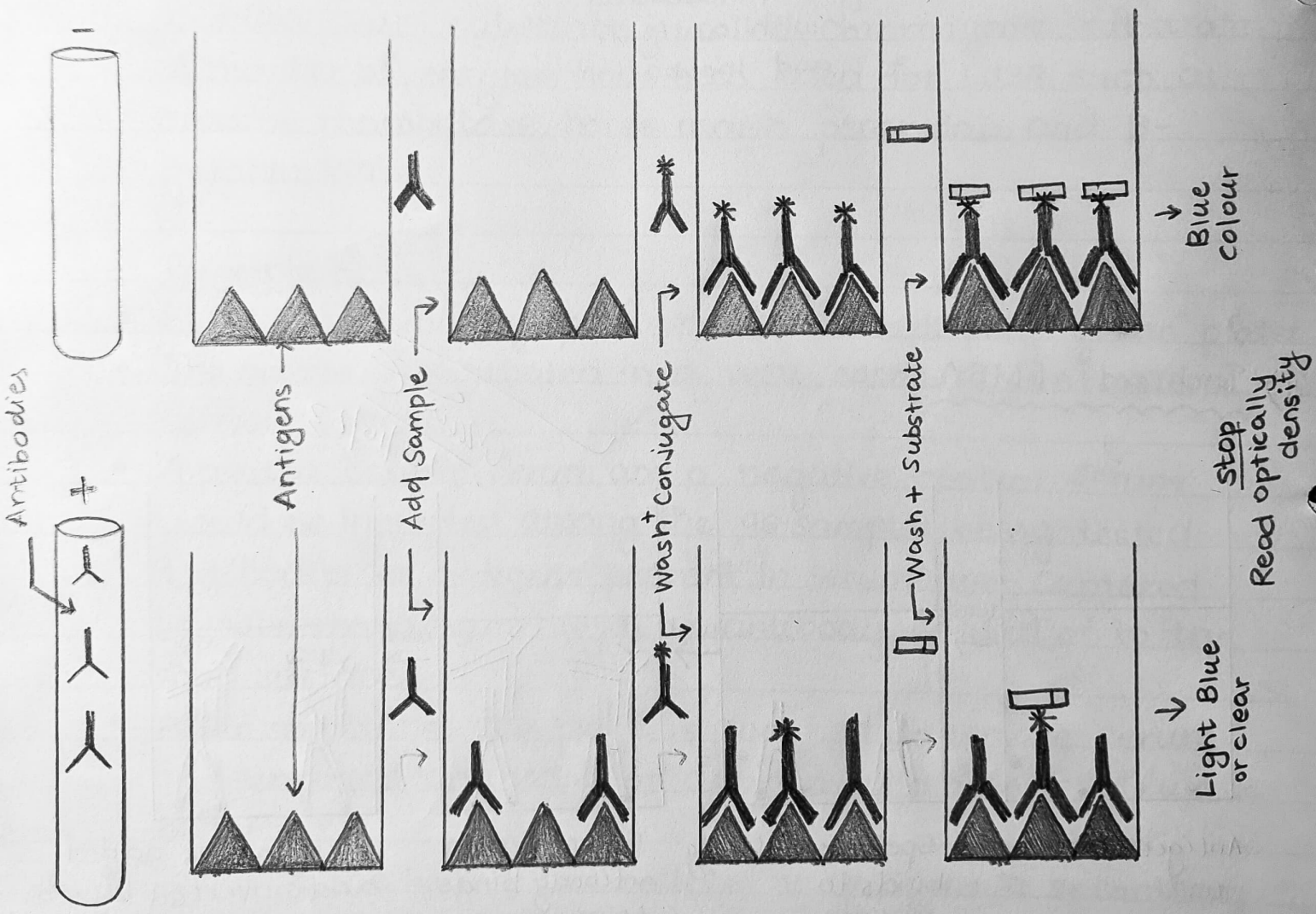


Monoclonal antibody coated well

Antigen Binds to Antibody

A 2° monoclonal antibody, linked to enzyme binds to immobilized antigen.

Substrate is added and converted by enzyme into colored product.



iv) Competitive ELISA

ELISA

Experiment - 5

Aim :- To study Elisa based Immuno hormone assay.
Estimation of plasma level of any hormone using ELISA.

Introduction :-

ELISA (Enzyme linked Immuno Sorbant Assay) is a plate based assay technique, which is used for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. An enzyme conjugated with an antibody reacts with colourless substrate to generate a coloured product. Such substrate is called chromogenic substrate A number of enzymes have been used for ELISA such as alkaline phosphatase, horse radish peroxidase and β-galactosidase.

Principle :-

- "ELISA" are typically performed in 96-well polystyrene plates.
- The serum is incubated in a well, each well contains a different serum.
- A positive control Serum and a negative control serum would be included among the 96 samples being tested antibodies or antigens present in serum are captured by corresponding antigen or antibody coated on to the solid surface.
- After sometime, the plate is washed to remove serum and unbound antibodies or antigen with a series of wash buffer.
- To detect the bound antibodies or antigens, a secondary antibody that attached to an enzyme such as peroxidase or alkaline phosphatase are added to each well.

- After an incubation period, the unbound secondary antibodies are washed off. When a suitable substrate is added, the enzyme reacts with it to produce a colour.
- This colour produced is measurable as a function or quantity of antigens or antibodies present in the given sample. The intensity of colour/optical density is measured at 450 nm.
- The intensity of the colour gives an indication of the amount of antigen or antibody.

* ELISA can be divided into two types :-

A. Quantitative ELISA :- It reflects the concentration of the target muscle in a sample.

B. Qualitative ELISA :- It provides a simple positive or negative result for a sample.

Four Main Types of "ELISA" :-

i) Direct ELISA :-

→ Simplest ELISA technique
→ The antigen in the sample is first immobilized to the wall of the wells of a microtitre plate.
→ The wells are then washed off thoroughly, leaving only the absorbed antigen.
→ An enzyme linked antibody, complementary to the antigen of interest, is then added to the wells, where it binds to the antigen.
→ The well is again washed. This leaves a bound antigen-antibody complex on the surface of well.
→ A substrate is then added, which will be converted by the enzyme-linked with antibodies into a detectable product.
→ This method is quicker and simpler than the other ELISA methods.

ii) Indirect ELISA :-

- Antibody can be detected or quantitatively determined by Indirect ELISA.
- A complementary antibody (primary antibody) is ~~washed~~ away the presence of antibody bound to the antigen de then added, which binds to the antigen forming a complex.
- After any free primary antibody is washed away, the presence of antibody bound to the antigen is detected by adding an enzyme linked secondary antibody, which binds to the primary antibody.
- And free secondary antibody then is washed away and a substrate for the enzyme is added.
- The amount of coloured reaction products that form is measured by specialized spectrophotometric plate readers which can measure the absorbance of all of the wall.

The indirect ELISA is used to detect the presence of antibody against HIV.

iii) Sandwich ELISA :-

- An antigen can be detected or measured by a sandwich ELISA.
- In this, the antibody (rather than the antigen) is immobilized on a microtiter well.
- The sample containing antigen is added to the well, which binds to the antibody.
- Finally, a second different antibody to the antigen is added.
- This antibody is enzyme linked.
- After any free second antibody is removed by washing substrate (s) for enzyme linked with antibody is added and the coloured reaction is product (p) is measured.

The extent of reaction is directly proportional to the amount of antigen present.

iv) Competitive ELISA :-

→ This is perhaps the most complex of all ELISA types.
→ It involves the use of inhibitor antigen, so competitive ELISA is also known as inhibitor ELISA.
→ In competitive ELISA, the inhibitor antigen and the antigen of interest compete for binding to the primary antibody i.e. two antigens to compete with each other for binding to antibodies.
→ The unlabeled primary antibody is first incubated with the sample containing antigen of interest, leading to the formation of antigen antibody complex.
→ Since the antibody is excessive compared with the antigen, So there are free antibodies left.
→ The antigen antibody mixture is added to the plate coated with inhibitor antigen that can also bind to the primary antibody.
→ The free antibody in the mixture binds to the inhibitor antigen on the plate, while the antigen-antibody complexes in the mixture do not react and are therefore washed away.
→ The enzyme labeled secondary antibody is added to the plate and binds to the primary antibody bound to the inhibitor antigen on the plate.
→ Finally, a substrate is added to react with the enzyme and emit a visible signal for detection.