B.Sc. ZOOLOGY LAB MANUAL

3rd Semester

Prepared By
Biological Science Dept.
Zoology

MIDNAPORE CITY COLLEGE

PREFACE TO THE FIRST EDITION

This is the first edition of Lab Manual for UG Zoology third Semester. Hope this edition will help you during practical. This edition mainly tried to cover the whole syllabus. Some hard core instrument based topic are not present here that will be guided by responsive teachers at the time of practical.

ACKNOWLEDGEMENT

We are really thankful to our students, teachers and non teaching staffs to make this effort little bit complete.

Mainly thanks to Director and Principal Sir to motivate for making this lab manual.

CP5: Chordates (Lab)

List of Practical

1. Protochordata - *Balanoglossus*, *Herdmania*, *Branchiostoma*

2. Agnatha-*Petromyzon*, *Myxine*

3. Fishes- *Scoliodon*, *Sphyrna*, *Pristis*, *Torpedo*, *Chimaera*, *Mystus*, *Heteropneustes*, *Labeo*, *Exocoetus*, *Echeneis*, *Anguilla*, *Hippocampus*, *Tetrodon*/ *Diodon*, *Anabas*, Flat fish

4. Amphibia- *Necturus*, *Bufo*, *Hyla*, *Alytes*, *Axolotl*, *Tylototriton*

5. Reptilia- *Chelone*, *Trionyx*, *Hemidactylus*, *Varanus*, *Uromastix*, *Chamaeleon*, *Ophiosaurus*, *Draco*, *Bungarus*, *Vipera*, *Naja*, *Hydrophis*, *Zamenis*, *Crocodylus*. Key for Identification of poisonous and non-poisonous snakes

6. Mammalia: Bat (Insectivorous and Frugivorous), *Funambulus*

7. Pecten from Fowl head

8. Dissection of brain and pituitary of Tilapia

C6P: Animal Physiology: Controlling & Coordinating Systems Lab Credits 02 List of Practical

1. Recording of simple muscle twitch with electrical stimulation (or Virtual

2. Demonstration of the unconditioned reflex action (Deep tendon reflex such as knee jerk reflex)

3. Preparation of temporary mounts: Squamous epithelium, Striated muscle fibres and nerve cells

4. Study of permanent slides of Mammalian skin, Cartilage, Bone, Spinal cord, Nerve cell, Pituitary, Pancreas, Testis, Ovary, Adrenal, Thyroid and Parathyroid

5. Microtomy: Preparation of permanent slide of any five mammalian (Goat/white rat) tissues

C7P: Fundamentals of Biochemistry

1. Qualitative tests of functional groups in carbohydrates, proteins and lipids.

2. Paper chromatography of amino acids.

3. Quantitative estimation of Lowry Methods.

4. Demonstration of proteins separation by SDS-PAGE.

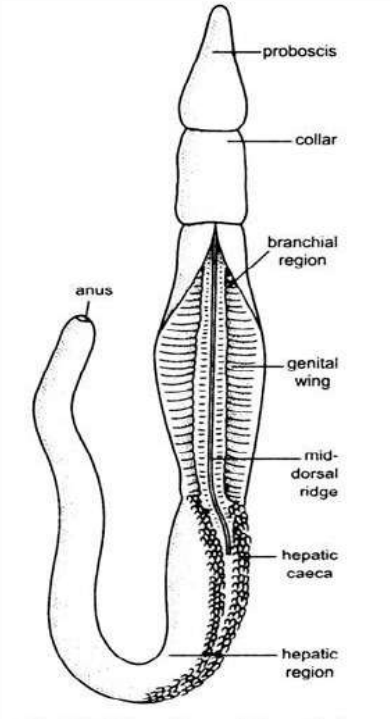
5. To study the enzymatic activity of Trypsin and Lipase.

6. To perform the Acid and Alkaline phosphatase assay from serum/ tissue.

CP5: Chordates (Lab)

HEMICHORDATA

Balanoglossus sp. (Acorn worm)



1. Vermiform body is divided into proboscis, collar and head.

2. Pharyngeal **clefts** may or may not be present.

Hence, the specimen belongs to the Phylum Hemichordata.

1. **Proboscis** is elongated.

2. Presence of numerous U-shaped pharyngeal gill slits.

3. Anus is terminal at the distal end.

Hence, the specimen belongs to the Class Enteropneusta.

1. Proboscis is conical in shape.

2. Anterior part of collar is funnel-like.

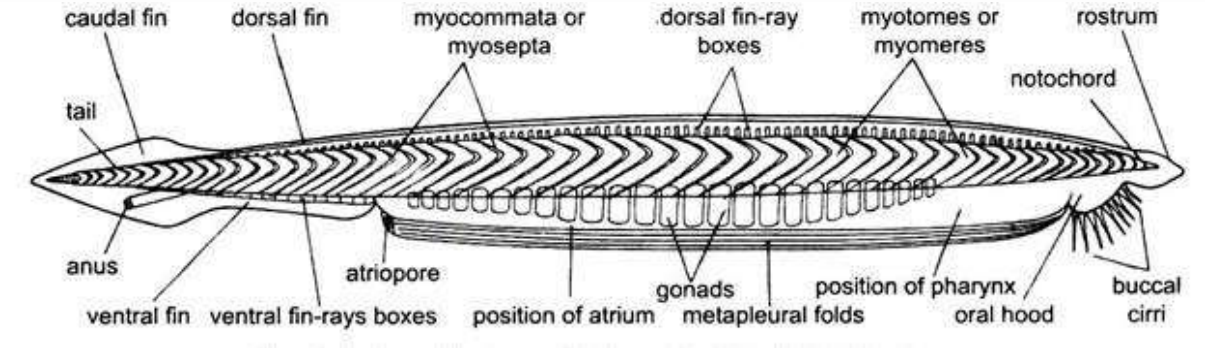
3. Trunk is divided into branchio-genital, hepatic and post hepatic regions.

4. Hepatic region is marked externally with irregular elevations.

Hence, the specimen seems to be *Balanoglossus sp.*

PROTOCHORDATA

Branchiostoma sp. (Amphioxus)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Body is divided into two parts, a trunk and a post anal tail.

2. Notochord extends through the entire length of the body.

3. Persistent numerous pharyngeal gill slits.

Hence, the specimen belongs to the Subphylum Cephalochordata.

1. Body is elongated, laterally compressed and fish-like.

2. Skin has no pigment, series of **myotomes** are visible.

3. Presence of buccal **cirri** around the oral hood.

4. Dorsal, caudal and ventral fins are continous.

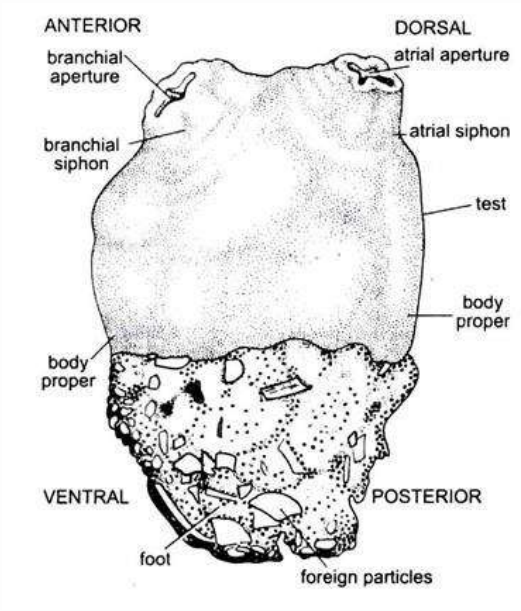
5. Anterior ventrolateral ridges of the body project as a pair of **metapleural** fold.

6. Presence of a ventral and median atriopore.

7. Anus is asymmetrically placed on the left side.

Hence, the specimen seems to be *Branchiostoma sp.*

Herdmania sp.



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Body is enclosed in a test.

2. Pharyngeal clefts open in an **atrium**.

Hence, the specimen belongs to the Subphylum Urochordata.

1. The test is permanent and thick.

2. Atrial aperture opens dorsally.

3. Mouth is terminal.

Hence, the specimen belongs to the Class Ascidiacea.

1. Includes both colonial and solitary animals.

2. Presence of folded pharyngeal baskets.

3. The complete absence of an abdomen.

Hence, the specimen belongs to the order Stolidobranchia.

1. The body has a flat purse like structure with length of 6-13 cms. Breadth is 4-7 cms.

2. The whole body is covered by thick leathery and translucent **tunic** or **test**.

3. The body has two projections- **atrial** and **branchial siphons.**

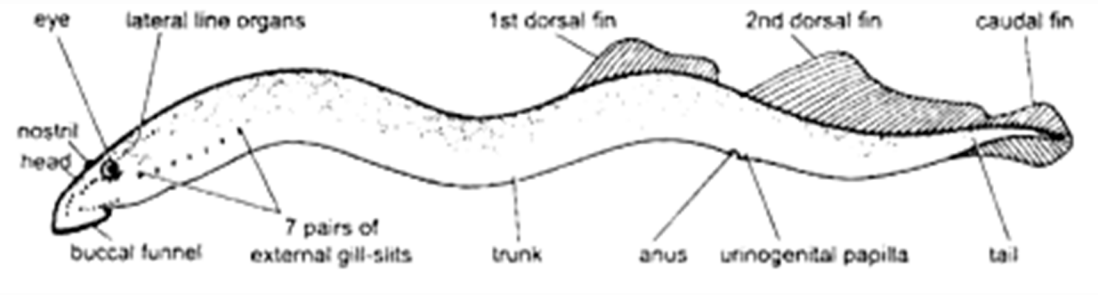
4**.** Branchial siphon has a branchial or incurrent aperture or mouth. The atrial siphon has atrial or excurrent aperture or cloaca. Each aperture is surrounded by four **lobes** or **lips**.

Hence, the specimen seems to be *Herdmania sp.*

VERTEBRATA

AGNATHA

Petromyzon sp. (Lamprey)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Jaws and paired fins are absent.

Hence, the specimen belongs to the Super Class Agnatha.

1. Mouth is suctorial and circular.

2. Gill slits are 1 to 16 pairs.

3. Nostril is single and median.

Hence, the specimen belongs to the Class Cyclostomata.

1. Presence of seven pairs of round gill openings.

2. Branchial basket is complete.

3. Dorsal fin may be one or two.

Hence, the specimen belongs to the Order Petromyzontia.

1. Body is smooth and eel-like.

2. Head and trunk are cylindrical but tail is laterally compressed.

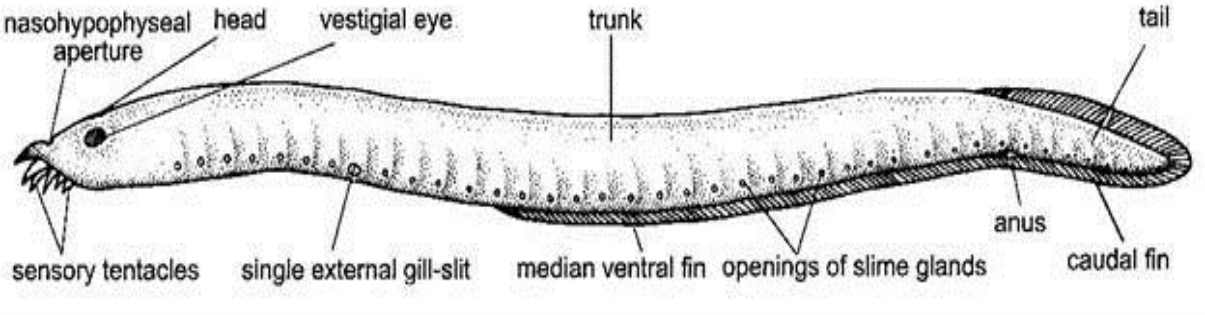
3. Presence of paired eyes covered by transparent skin.

4. Buccal funnel is beset with many horny teeth.

5. Two median dorsal dorsal fins are almost equal in dimensions.

Hence, the specimen seems to be *Petromyzon sp.*

Myxine sp. (Hag fish)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Jaws and paired fins are absent.

Hence, the specimen belongs to the Super Class Agnatha.

1. Mouth is suctorial and circular.

2. Gill slits are 1 to 16 pairs.

3. Nostril is single and median.

Hence, the specimen belongs to the Class Cyclostomata.

1. Mouth is terminal with four pair of tentacles.

2. Nostril is terminal.

3. Gill slits are 1 to 15 pairs.

Hence, the specimen belongs to the Order Myxinoidea.

1. Scale-less cylindrical body divided into head, trunk and tail.

2. Mouth is surrounded by lips.

3. Eyes are vestigeal.

4. Single external branchial aperture on both sides.

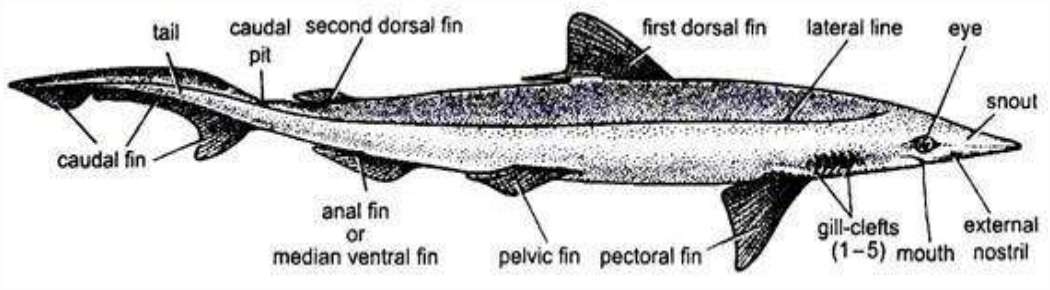
5. Down the sides of the body, mucous pores are present.

Hence, the specimen seems to be *Myxine sp.*

GNATHOSTOMATA

FISHES

Scoliodon sp. (Indian shark/Dogfish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.
2. Tail is **heterocercal.**
3. Presence of calcareous endoskeleton.
4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. Multiple gill-slits are protected by individual skin flap.
2. Male bears **clasper.**

Hence, the specimen belongs to the Subclass Selachii.

1. Gill-slits are on the lateral side of the body.
2. Pectoral fins are constricted at the base.

Hence, the specimen belongs to the Superorder Pleurotremata.

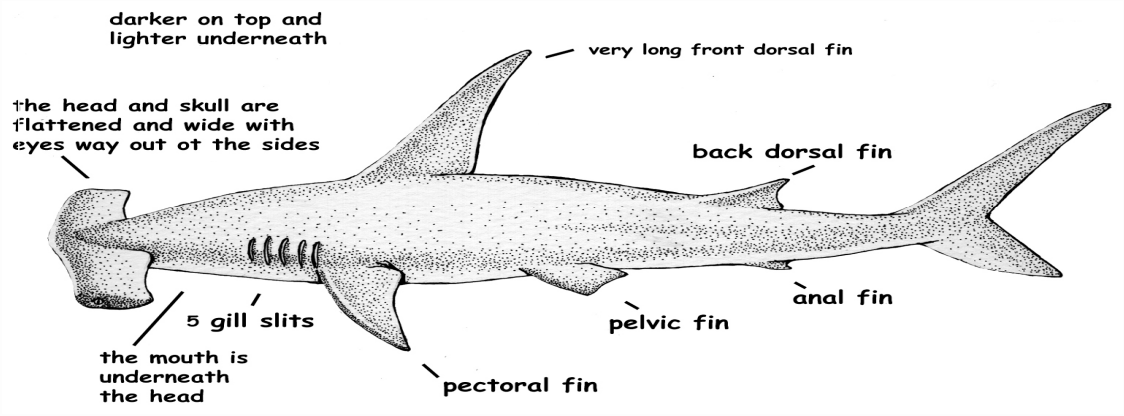
1. Presence of two dorsal fins with spines.
2. Presence of a single anal fin.
3. Presence of five gill-slits.

Hence, the specimen belongs to the Order Lamniformes.

1. Body is divisible into head thorax and abdomen.
2. Head is dorso-ventrally flattened but the rest of the body is laterally compressed.
3. Possess several sets of **homodont** teeth.
4. **Cloacal** aperture is longitudinal.

Hence, the specimen seems to be *Scoliodon sp.*

Sphyrna sp. (Hammer headed shark)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.
2. Tail is **heterocercal.**
3. Presence of calcareous endoskeleton.
4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. Multiple gill-slits are protected by individual skin flap.
2. Male bears **clasper.**

Hence, the specimen belongs to the Subclass Selachii.

1. Gill-slits are on the lateral side of the body.
2. Pectoral fins are constricted at the base.

Hence, the specimen belongs to the Superorder Pleurotremata.

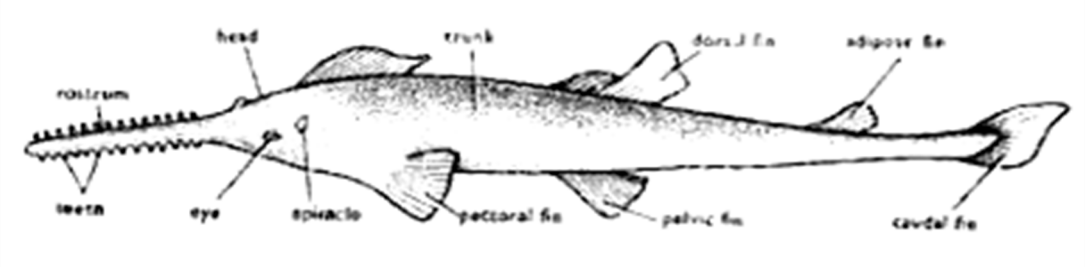
1. Presence of two dorsal fins with spines.
2. Presence of a single anal fin.
3. Presence of five gill-slits.

Hence, the specimen belongs to the Order Lamniformes.

1. **Malleate**-shaped head bearing eyes and nasal openings at the lateral extremities.
2. Eyes contain nictitating membrane.
3. **Cresent**-shaped ventrally located mouth.
4. Dorsal fins devoid of spines.

Hence, the specimen seems to be *Sphyrna sp.*

Pristis sp. (Saw fish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.
2. Tail is **heterocercal.**
3. Presence of calcareous endoskeleton.
4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. Multiple gill-slits are protected by individual skin flap.
2. Male bears **clasper.**

Hence, the specimen belongs to the Subclass Selachii.

1. Gill-slits are on the ventral side of the body.
2. Pectoral fins are enlarged but tail and other fins are reduced.

Hence, the specimen belongs to the Superorder Hypotremata.

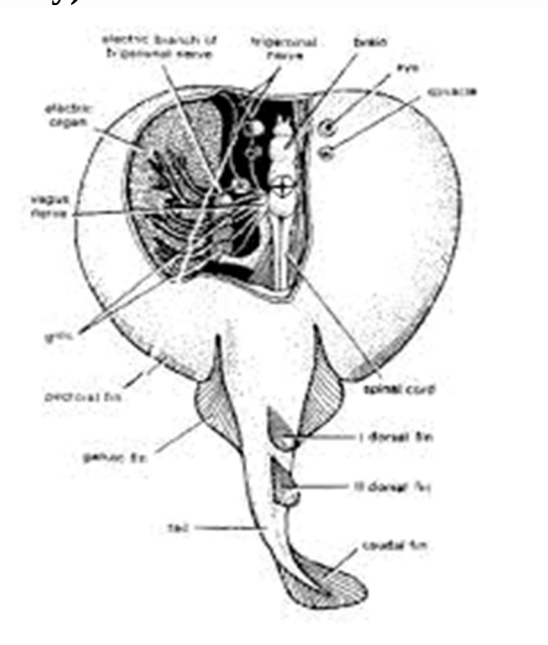
1. Body is fleshy and dorsoventrally compressed.
2. Eyes are on the dorsal surface.

Hence, the specimen belongs to the Order Rajiformes.

1. Anterior part of the body is dorsoventrally flattened while the posterior part is shark like.
2. **Snout** is prolonged into a long **rostrum** with a series of lateral tooth-like **denticles.**

Hence, the specimen seems to be *Pristis sp.*

Torpedo sp. (Electric Ray)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.
2. Tail is **heterocercal.**
3. Presence of calcareous endoskeleton.
4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. Multiple gill-slits are protected by individual skin flap.
2. Male bears **clasper.**

Hence, the specimen belongs to the Subclass Selachii.

1. Gill-slits are on the ventral side of the body.
2. Pectoral fins are enlarged but tail and other fins are reduced.

Hence, the specimen belongs to the Superorder Hypotremata.

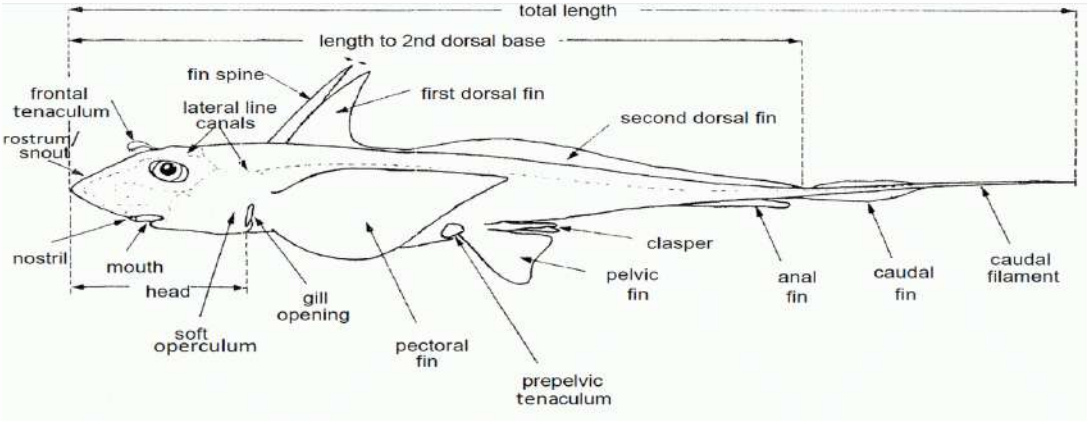
1. Presence of electric organ between head and pectoral fin.

Hence, the specimen belongs to the Order Torpediniformes.

1. Anterior end of the body is semi-circular.
2. Mouth is a slit-like aperture.
3. Tail is short and thick with two dorsal fins and a caudal fin.

Hence, the specimen seems to be *Torpedo sp.*

Hydrolagus sp. (Ghost shark) (Chimaera)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Presence of ventrally located subterminal mouth.
2. Tail is **heterocercal.**
3. Presence of calcareous endoskeleton.
4. Presence of 5-7 pairs of gill slits.

Hence, the specimen belongs to the Class Chondrichthyes.

1. The tail is long and thin and they move by sweeping movements of the large pectoral fins.
2. The erectile spine in front of the dorsal fin is sometimes venomous.
3. The mouth is a small aperture surrounded by **lips**, giving the head a **parrot-like** appearance.

Hence, the specimen belongs to the Subclass Holocephali.

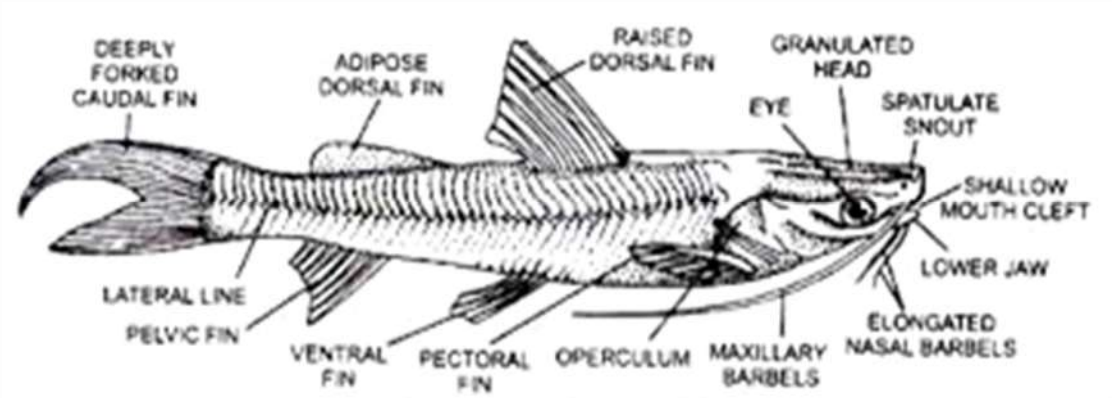
1. Elongated, soft bodies, with a bulky head and a single gill-opening.
2. Have a **venomous spine** in front of the dorsal fin.
3. Male chimaeras have retractable sexual appendages on the forehead and in front of the pelvic fins.
4. Their upper jaws are fused with their skulls having three pairs of large permanent grinding tooth plates.
5. Have gill covers or **opercula** like bony fishes.

Hence, the specimen belongs to the Order Chimaeriformes.

1. Have a smooth and scaleless skin that is a silvery-bronze color, often with sparkling shades of gold, blue, and green.
2. Dark edges outline both the caudal and dorsal fins, whereas the pectoral fins have a transparent outline.
3. Pectoral fins are large and triangular, and extend straight out from the sides of their bodies like airplane wings.
4. It has a duckbill-shaped snout and a rabbit-like face.
5. Have large, emerald green eyes, which are able to reflect light.

Hence, the specimen seems to be *Hydrolagus sp.*

Mystus sp. (Tangra)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

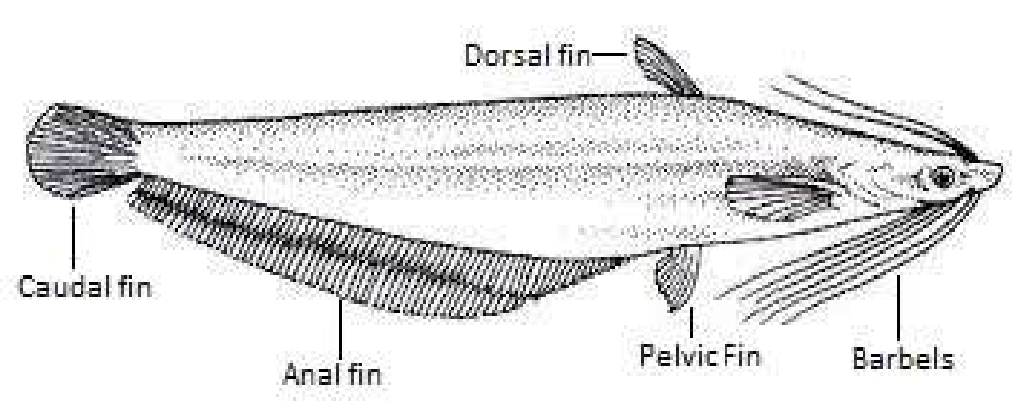
1. Pectoral fin with a spine.
2. Barbels present.
3. Scales absent.

Hence, the specimen belongs to the Order Siluriformes.

1. Presence of **adipose** dorsal fin.
2. Body is blackish above and slivery on the sides.
3. Snout is distinctly long with four pairs of barbells.
4. Presence of light and dark coloured longitudinal bands on bodywall.

Hence, the specimen seems to be *Mystus sp.*

Heteropneustes sp. (Singhi)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

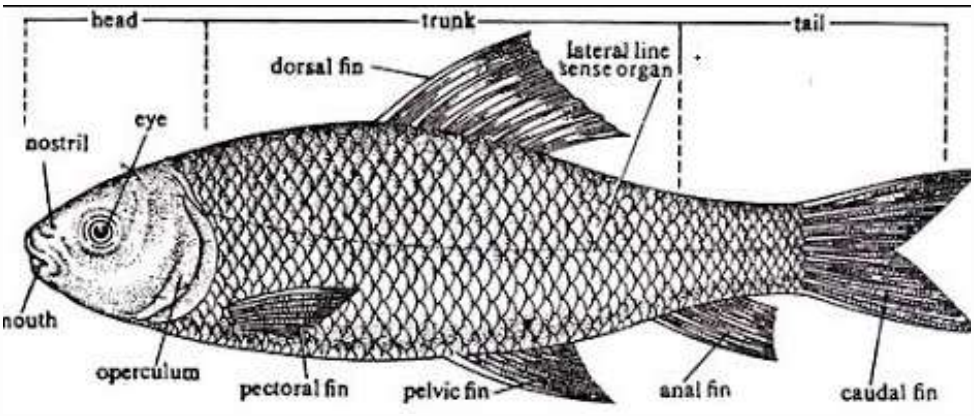
1. Pectoral fin with a spine.
2. Barbels present.
3. Scales absent.

Hence, the specimen belongs to the Order Siluriformes.

1. Skin is scaleless.
2. Head is flat with four pairs of **barbells.**
3. First ray of pelvic fin is modified into a serrated spine.
4. Dorsal fin is very small.
5. Caudal fin is separated by a notch from extended anal fin.

Hence, the specimen seems to be *Heteropneustes sp.*

Labeo sp. (Rohu)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

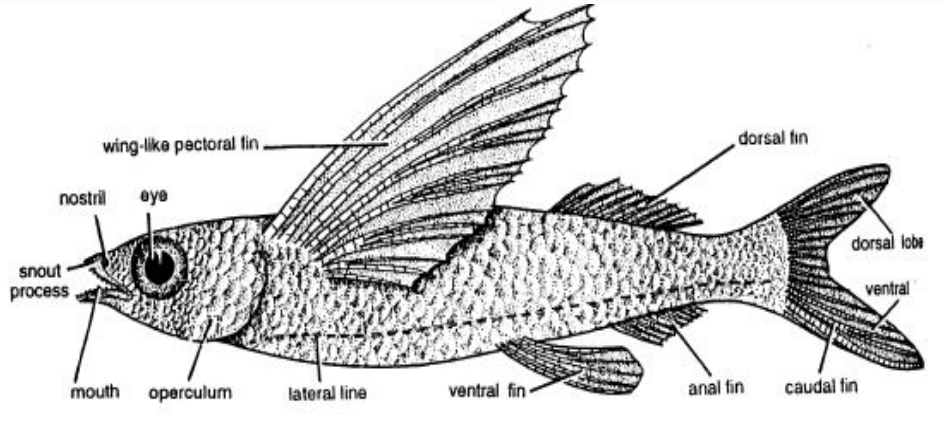
1. No scale on the head, head is covered by plates.
2. No teeth in the jaws.
3. Single large dorsal fin.
4. Fins supported by soft fin rays.
5. Lateral line distinct.

Hence, the specimen belongs to the Order Cypriniformes.

1. Body covered with large cycloid scales, no scale on the head.
2. Head prominent with blunt snout projecting beyond the narrow mouth.
3. Lips thick and horny covering the jaw, having inferior transverse fold.
4. Barbels are very short or absent.
5. Body colour is bluish-brown on the dorsal aspect, and silvery-white with reddish-orange tinge on the ventral aspect.

Hence, the specimen seems to be *Labeo sp.*

Exocoetus sp. (Flying fish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

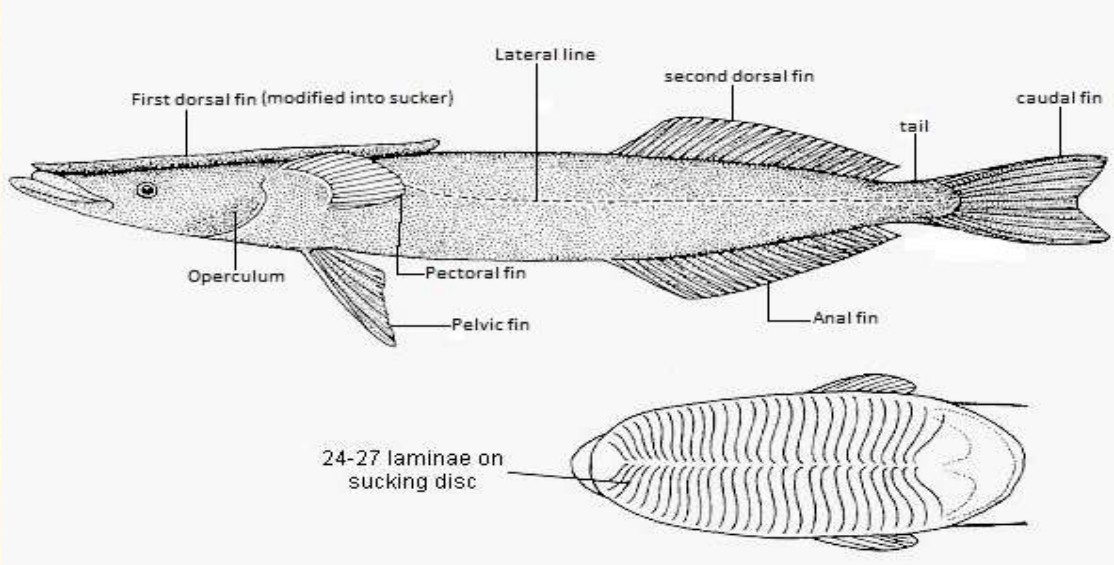
1. Actually spindle like long body.
2. Pectoral fins are very big and modified into flying structure.
3. Pelvic fins are at the abdominal region.

Hence, the specimen belongs to the Order Beloniformes.

1. Pectoral fins are enormously elongated to form wing-like structures. They serve as parachute to sustain the fish in its gliding leap.
2. Lateral line is located low on the body.
3. Pectoral fin with black spots.
4. Tail is hypobatic and ventral lobe of the tail fin is much elongated and help in skipping over the water.

Hence, the specimen seems to be *Exocoetus sp.*

Echeneis sp. (Sucker fish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

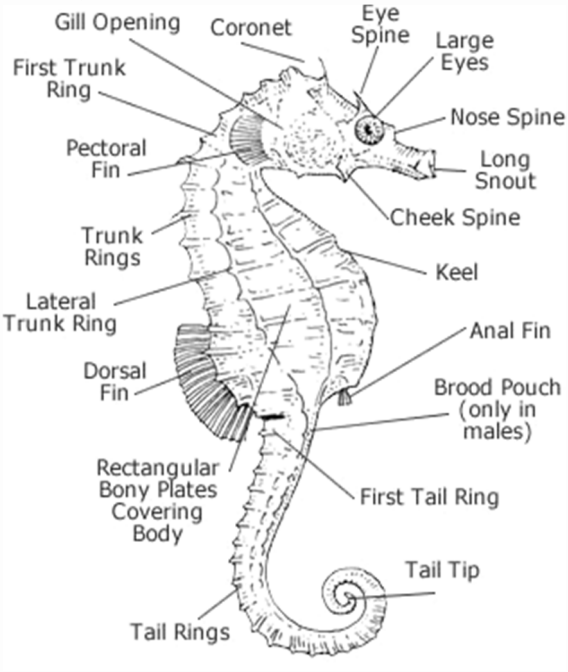
1. Body covered by cycloid scales.
2. Swim bladder absent.
3. First dorsal fin modified into sucker on the dorsal side of the head.
4. Fins are without rays.

Hence, the specimen belongs to the Order Echeniformes.

1. First dorsal fin modified into sucker.
2. Dorsal and anal fins without rays.
3. Dorsal and anal fins are identical and opposite to each other.

Hence, the specimen seems to be *Echeneis sp.*

Hippocampus sp. (Sea horse)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

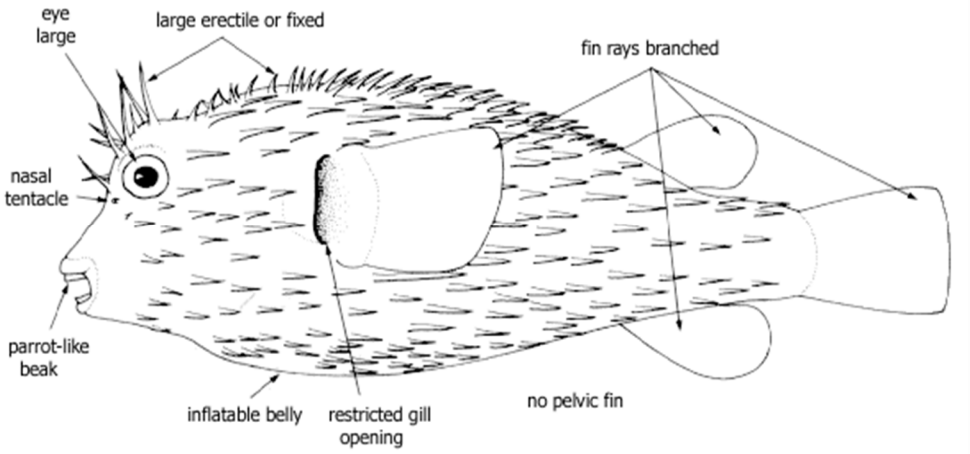
1. Body is covered by ring like bony plates.
2. Snout tubular with suctorial mouth.

Hence, the specimen belongs to the Order Solenichthyes.

1. Head is at right angle to the body axis.
2. Pectoral fin is transparent behind the operculum.
3. Tail is long and **prehensile.**
4. Presence of **brood pouch** on the belly (in male)/an anal fin on the belly (in female).

Hence, the specimen seems to be *Hippocampus sp.*

Diodon sp. (Porcupine fish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

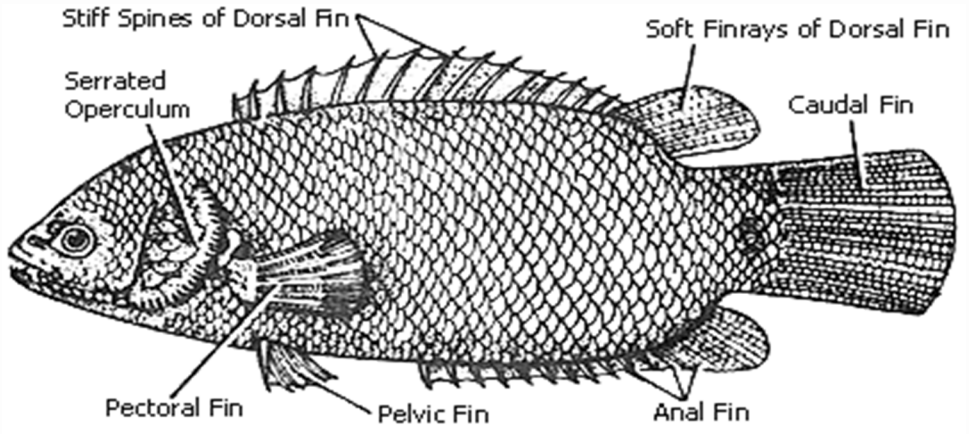
1. Range from nearly square or triangular, globose to laterally compressed.
2. The bones of the jaw are modified and fused into a sort of "beak"; visible sutures divide the beaks into "teeth".

Hence, the specimen belongs to the Order Tetraodontiformes.

1. Jaws are aided by powerful muscles, and many species also have pharyngeal teeth to further process prey items.
2. They lack swim bladders and spines, and are propelled by their very tall dorsal and anal fins.
3. The caudal peduncle is absent and the caudal fin is reduced to a stiff rudder-like structure.

Hence, the specimen seems to be *Diodon sp.*

Anabus sp. (Koi fish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

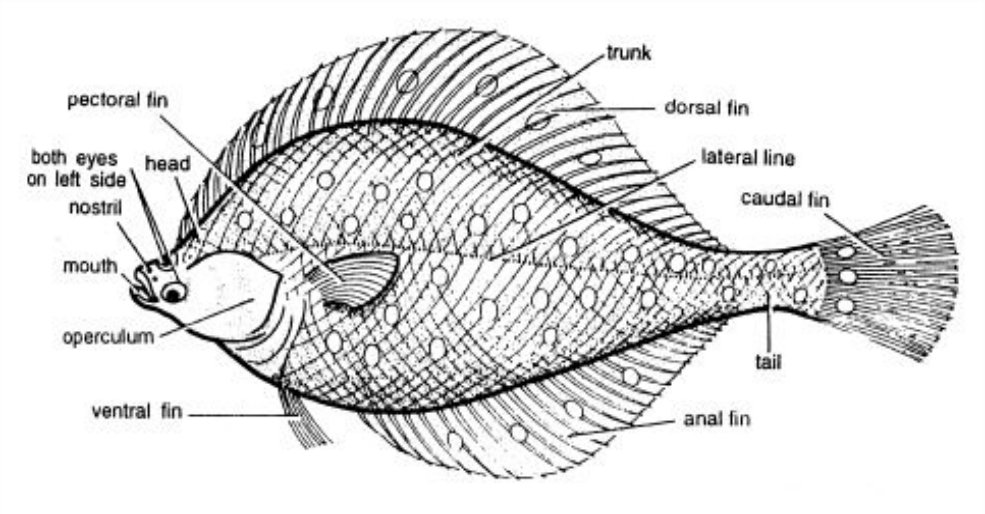
1. Dorsal and anal fins are both with spiny and soft rays.
2. Dorsal fins are two in number.
3. Body is covered by **ctenoid** scales.

Hence, the specimen belongs to the Order Perciformes.

1. Dorsal and anal fins are divided and supported by anterior spiny and posterior soft rays.
2. Small spiny projections are present along the posterior edge of operculum.

Hence, the specimen seems to be *Anabus sp.*

Pleuronectes sp. (Flat fish)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Gills are covered by an **operculum.**
2. Tail is **homocercal.**
3. Presence of bony endoskeleton.
4. Mouth is terminal.

Hence, the specimen belongs to the Class Osteichthyes.

1. Fins have basal support projecting outside the body wall.
2. Fins are supported by fin rays.

Hence, the specimen belongs to the Subclass Actinopterygii.

1. Scales are covered by **epidermis** or absent.
2. Gills are fully filamentous.
3. Presence of swim bladder.

Hence, the specimen belongs to the Super Order Teleostei.

1. Body highly compressed, somewhat rounded on eyed side and flat on blind side.
2. Asymmetrical body, with both eyes lying on the same side of the head in the adult fish.
3. Dorsal and anal fins with long bases.

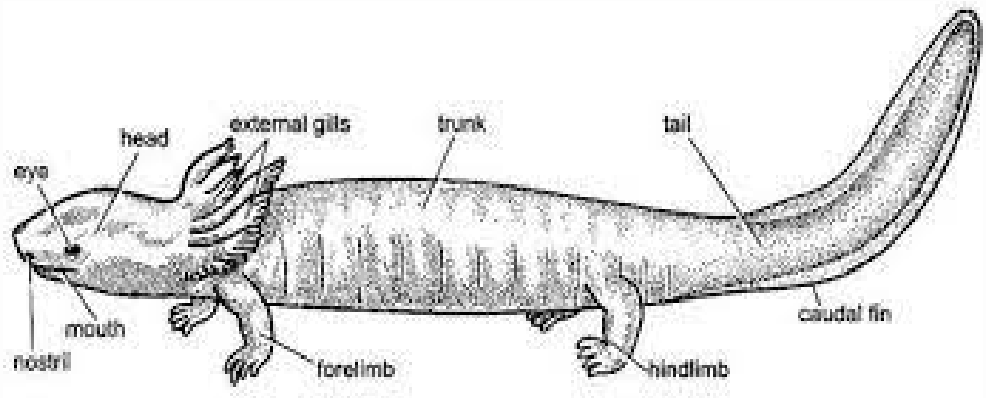
Hence, the specimen belongs to the Order Pleuronectiformes.

1. The surface of the fish facing away from the sea floor is pigmented.
2. Protruding eyes above body surface.
3. Presence of six or seven branchiostegal rays.
4. Adults having small body cavity; swim bladder absent.
5. Scales **cycloid, ctenoid** or **tuberculate**.

Hence, the specimen seems to be *Pleuronectes sp.*

AMPHIBIANS

Necturus sp. (Mud puppy)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. **Tetrapod** animal with 4-5 toes or less; few are limbless.
2. Skin is naked, soft and glandular.

Hence, the specimen belongs to the Class Amphibia.

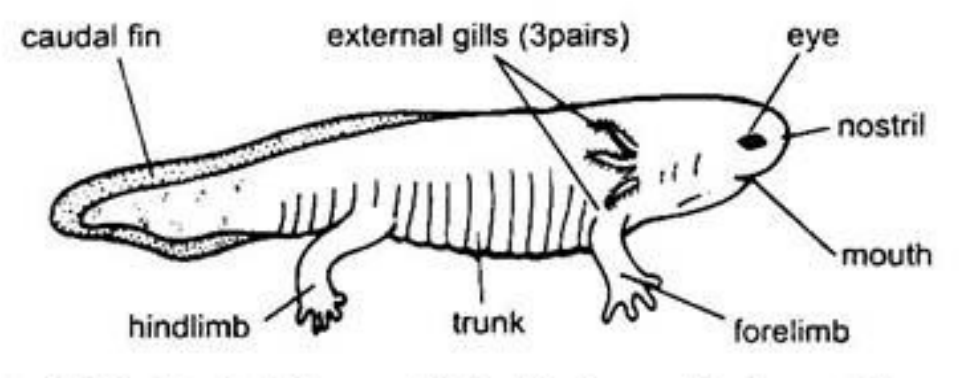
1. Presence of a well developed tail.
2. Two pairs of limbs are equal but weak.
3. Adults are with or without external gills and gill slits.

Hence, the specimen belongs to the Order Urodela.

1. Head is broad and flattened, trunk is elongated and depressed and tail is laterally compressed with fin.
2. Presence of three pairs of bushy external gills and two pairs of gill slits.
3. Eyes are without eyelids.
4. All limbs bear 4 digits.

Hence, the specimen seems to be *Necturus sp.*

Axolotl larva



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. **Tetrapod** animal with 4-5 toes or less; few are limbless.
2. Skin is naked, soft and glandular.

Hence, the specimen belongs to the Class Amphibia.

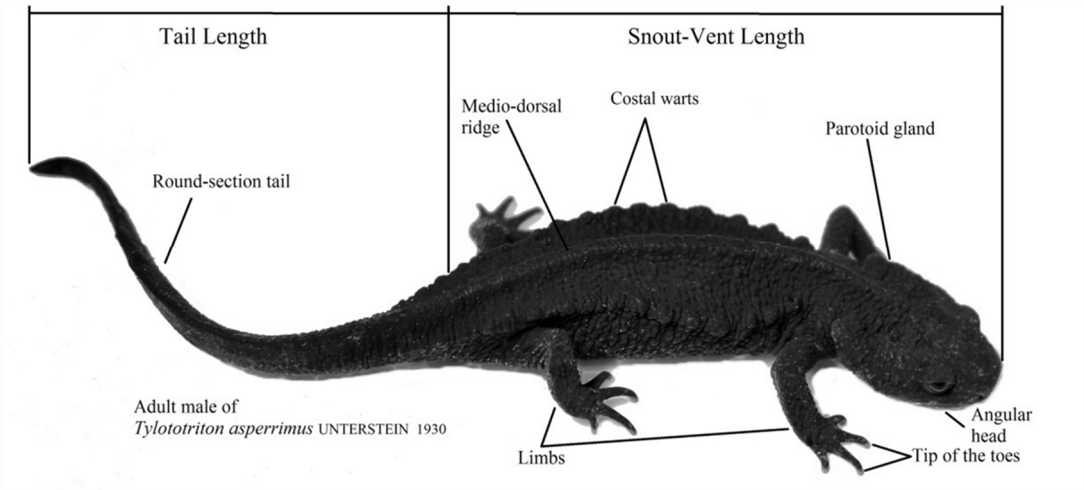
1. Presence of a well developed tail.
2. Two pairs of limbs are equal but weak.
3. Adults are with or without external gills and gill slits.

Hence, the specimen belongs to the Order Urodela.

1. 3 pairs of external bushy gills.
2. Broad terminal mouth.
3. Weakly developed limbs.
4. Ill developed eyes with eyelids.
5. Tail with tail fin.

Hence, the specimen seems to be Axolotl larva of *Ambystoma sp*.

Tylototriton sp. (Himalayan Newt)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.

2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. **Tetrapod** animal with 4-5 toes or less; few are limbless.

2. Skin is naked, soft and glandular.

Hence, the specimen belongs to the Class Amphibia.

1. Presence of a well developed tail.

2. Two pairs of limbs are equal but weak.

3. Adults are with or without external gills and gill slits.

Hence, the specimen belongs to the Order Urodela.

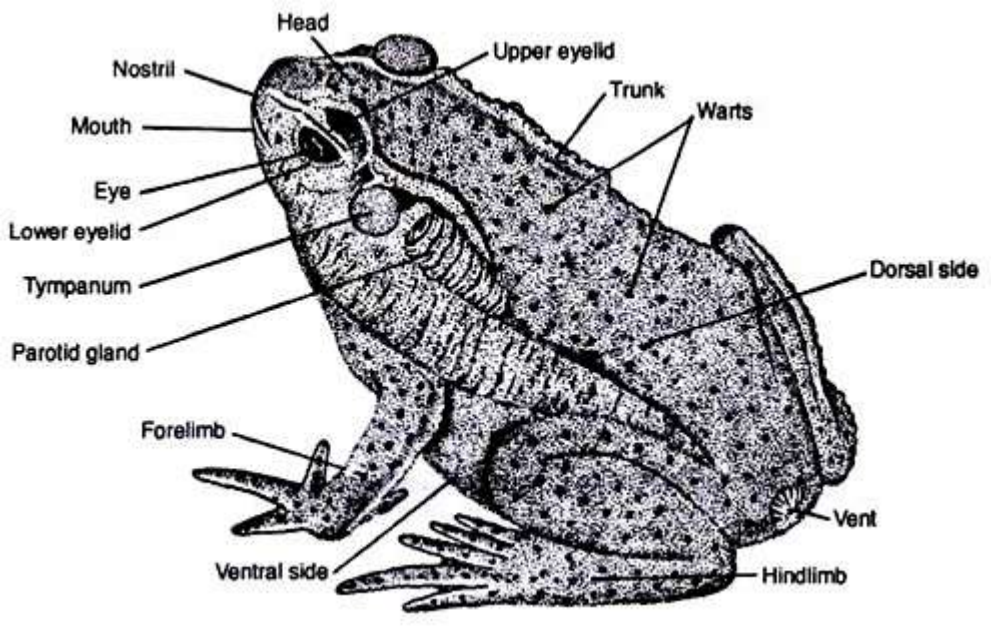
1. Head and trunk bear dorso-lateral ridges and beads respectively.

2. Presence of a mid dorsal vertebral ridge.

3. Tail is laterally compressed with fin.

Hence, the specimen seems to be *Tylototriton sp.*

Bufo sp. (Toad)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.

2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. **Tetrapod** animal with 4-5 toes or less; few are limbless.

2. Skin is naked, soft and glandular.

Hence, the specimen belongs to the Class Amphibia.

1. Four legged, **squat**-bodied, tailless creature.

2. Presence of eyelids, **nictitating membrane** and **tympanum.**

Hence, the specimen belongs to the Order Anura.

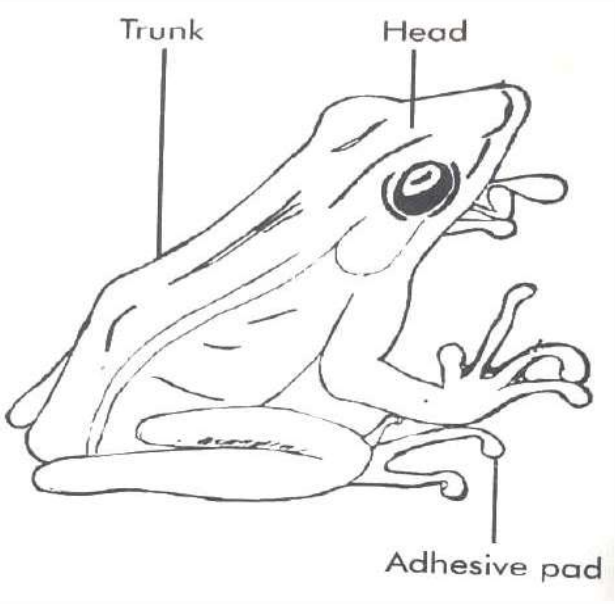
1. Body is divided into head and trunk.

2. Presence of **warts** on skin.

3. Presence of **parotid** glands behind the tympanum.

Hence, the specimen seems to be *Bufo sp.*

Hyla sp. (Tree frog)



1. Presence of hollow, dorsal, tubular nerve cord.

2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.

2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. **Tetrapod** animal with 4-5 toes or less; few are limbless.

2. Skin is naked, soft and glandular.

Hence, the specimen belongs to the Class Amphibia.

1. Four legged, **squat**-bodied, tailless creature.

2. Presence of eyelids, **nictitating membrane** and **tympanum.**

Hence, the specimen belongs to the Order Anura.

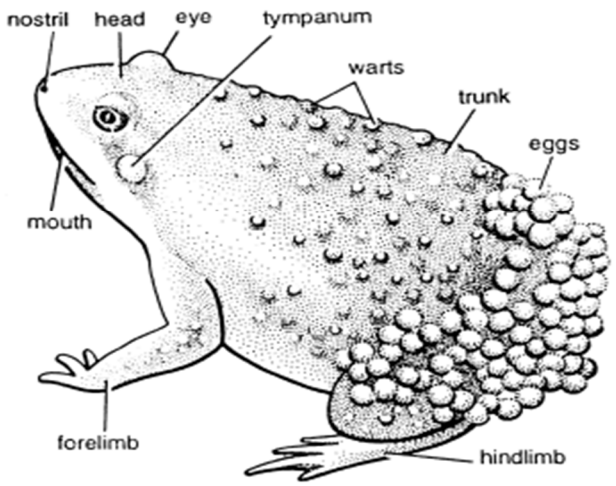
1. Apex of all toes contains adhesive pads.

2. Digits are webbed in hind-limbs.

3. Skin of belly contains hygroscopic glands.

Hence, the specimen seems to be *Hyla sp.*

Alytes sp. (Midwife toad)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. **Tetrapod** animal with 4-5 toes or less; few are limbless.
2. Skin is naked, soft and glandular.

Hence, the specimen belongs to the Class Amphibia.

1. Four legged, **squat**-bodied, tailless creature.
2. Presence of eyelids, **nictitating membrane** and **tympanum.**

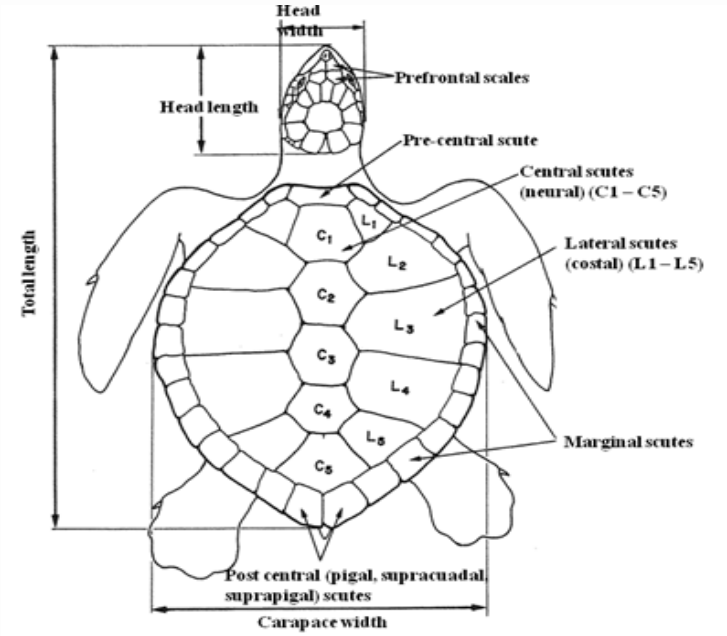
Hence, the specimen belongs to the Order Anura.

1. It is broad and stocky and has a large head with prominent eyes, the pupils being vertical slits.
2. The skin is mostly smooth with a few small warts and granules and a row of large warts down either side.
3. The parotid glands are small and there are additional glands in the under arm and ankle regions.

Hence, the specimen seems to be *Alytes sp.*

REPTILES

Chelone sp. (Turtle)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Skull roof is solid.
2. No temporal fossa on skull.

Hence, the specimen belongs to the Subclass Anapsida.

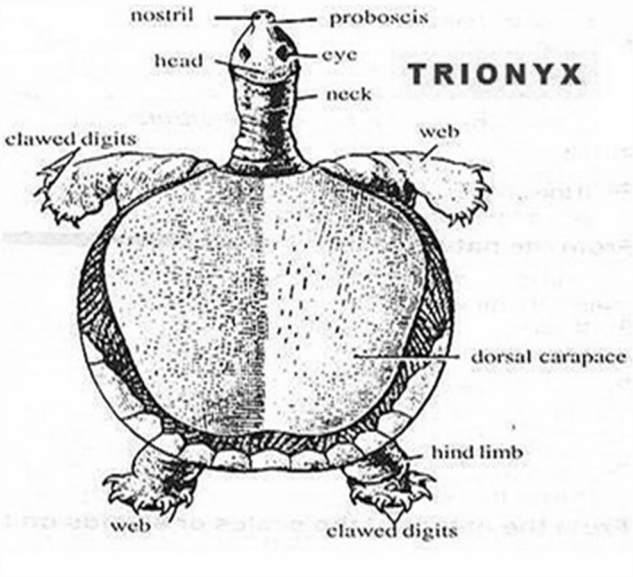
1. Presence of rounded dorsal **carapace** and flat ventral **plastron**.
2. Jaws have horny investment.
3. Limbs are paddle like.
4. Cloacal aperture is longitudinal.

Hence, the specimen belongs to the Order Chelonia.

1. Five rows of bony plates, median **neurals**, paired **costals** and **marginals**, constitute the carapace.
2. All digits of hindlimbs and only the first digit of forelimbs are clawed.

Hence, the specimen seems to be *Chelone sp.*

Trionyx sp. (Soft shelled turtle)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Skull roof is solid.
2. No temporal fossa on skull.

Hence, the specimen belongs to the Subclass Anapsida.

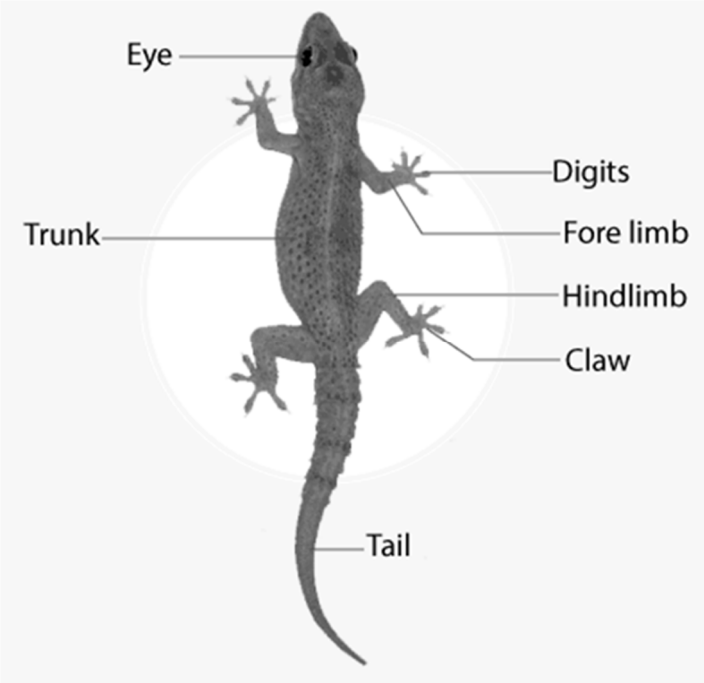
1. The upper shell, or carapace, can be tall and rounded, can be flat, or can be some shape in between.
2. The upper shell connects to the lower shell by way of a bony bridge.

Hence, the specimen belongs to the Order Testudines.

1. Females can reach up to 33 cm in carapace length, while the smaller males reach 27 cm, but however have longer tails than the females.
2. Has webbed feet for swimming.
3. They are called "softshell" because their carapace lacks horny scutes (scales); the carapace is leathery and pliable, particularly at the sides.
4. The throat is mottled and there may be small, dark bars on the lips.

Hence, the specimen seems to be *Trionyx sp.*

Hemidactylus sp. (House lizard)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Body is covered by minute scales.
2. Triangular head bears eyes, nostrils and external ear openings.
3. Each digit has adhesive lamellae on the ventral side.

Hence, the specimen seems to be *Hemidactylus sp.*

Varanus sp. (Water Monitor)

1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

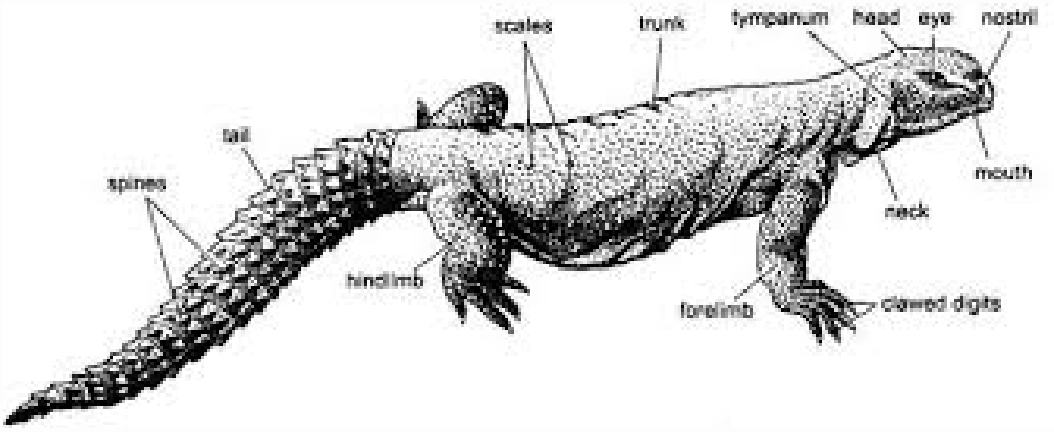
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Body is divisible into conical head, long neck, large trunk and elongated tail.
2. Snout is convex terminally.
3. Nostrils are oblique slits.
4. Pleurodont type of teeth are sharp and recurved.

Hence, the specimen seems to be *Varanus sp.*

Uromastyx sp. (Spiny tailed lizard)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

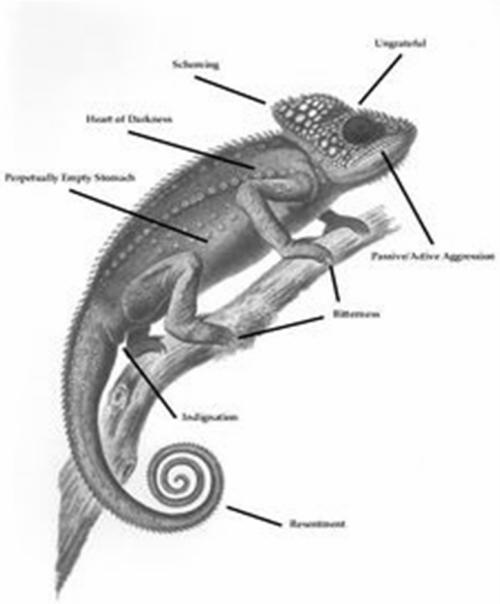
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Size ranges from 25 cm to 91 cm.
2. Tail is spiked, muscular and heavy.
3. Jaw surrounded by small teeth.

Hence, the specimen seems to be *Uromastyx sp.*

Chameleo sp. (Chameleon)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

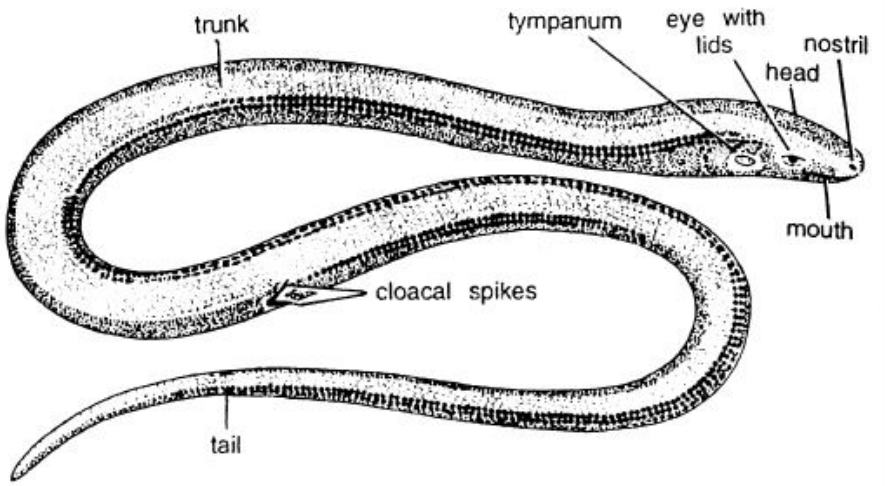
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Laterally compressed head bears large eyes and backwardly directed hood.
2. Tongue is very long, projectile and club shaped.
3. Trunk bears a row of spines along the mid-dorsal line.
4. **Syndactyl** digits are **zygodactylous**.
5. Tail is prehensile.

Hence, the specimen seems to be *Chameleo sp.*

Ophiosaurus sp. (Snake lizard)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

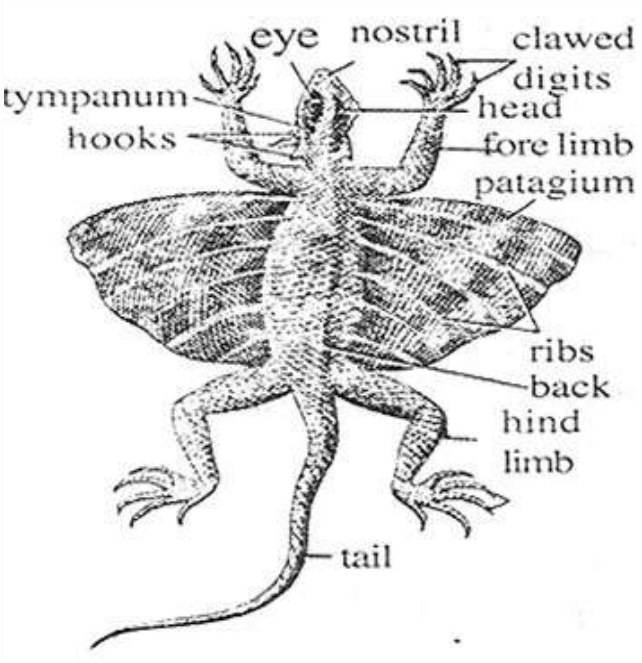
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Have no legs.
2. Their head shapes, movable eyelids, and external ear openings identify them as lizards.
3. Have very small, stub-like legs near their rear vents; these are vestigial organs.
4. Reach lengths of up to 1.2 metres, but about two-thirds of this is the tail.

Hence, the specimen seems to be *Ophiosaurus sp.*

Draco sp. (Flying lizard)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

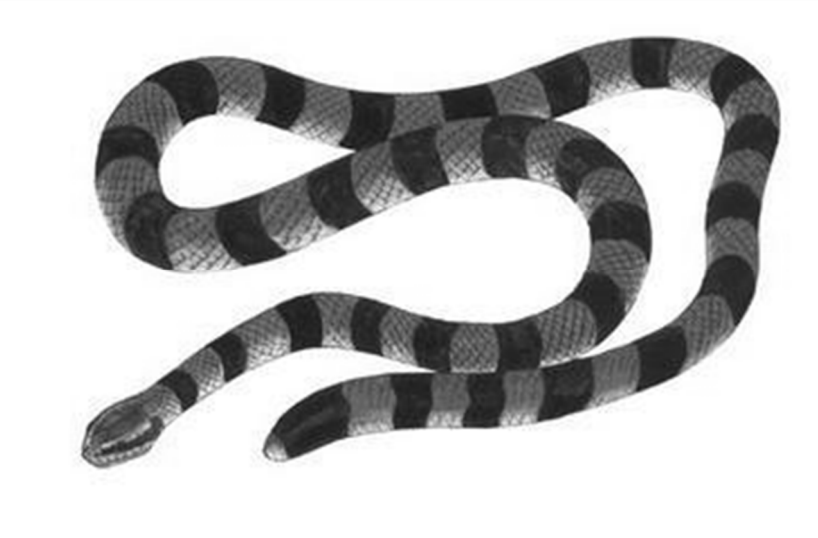
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Lateral side of the body bear semicircular, thin, membranous fold of skin, supported by 5-6 extended ribs.
2. **Gular** pouch hangs down below the neck.
3. Teeth are **heterodont**.

Hence, the specimen seems to be *Draco sp.*

Bangarus sp. (Common Krait)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

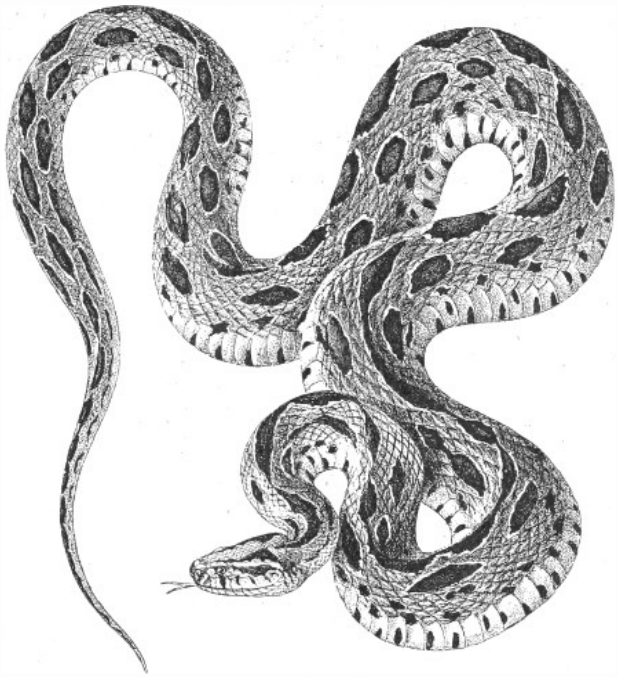
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Body has alternate light and dark coloured bands/patches.
2. Dorsomedian scales are hexagonal in shape.
3. Subcaudial shields are in a single row.

Hence, the specimen seems to be *Bangarus sp.*

Vipera sp. (Pitless Viper)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

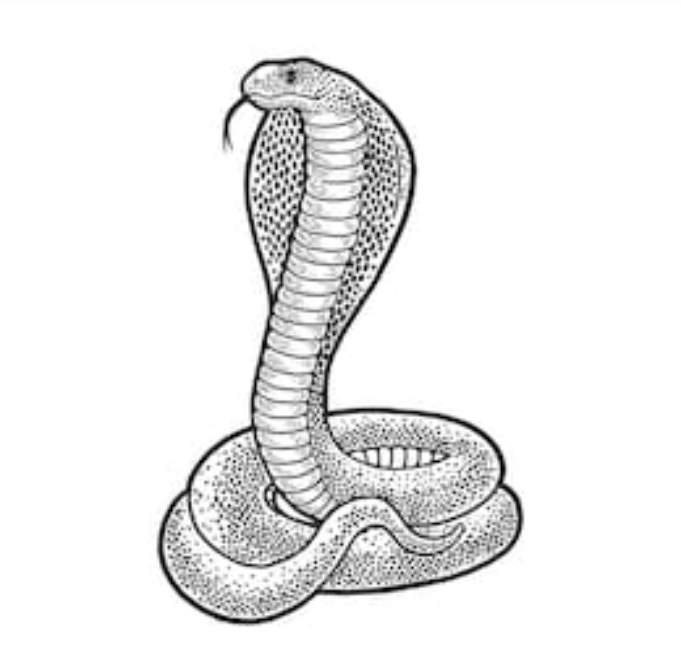
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Large triangular head is covered by small scales.
2. Nostrils are lateral, oblique slits and are very large.
3. Ventral plates are large and completely cover the belly.

Hence, the specimen seems to be *Vipera sp.*

Naja sp. (Indian Cobra)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

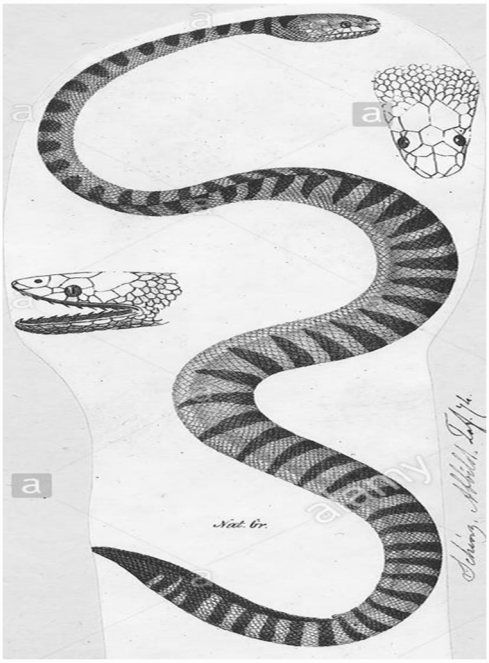
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Body is covered by oblique scales.
2. Neck region is dilated to form hood which has **binocellate** mark.
3. The third **supralabial** shield touches eye and nasal shields.
4. **Subcaudal** shields are in double row.

Hence, the specimen seems to be *Naja sp.*

Hydrophis sp. (Sea snake)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

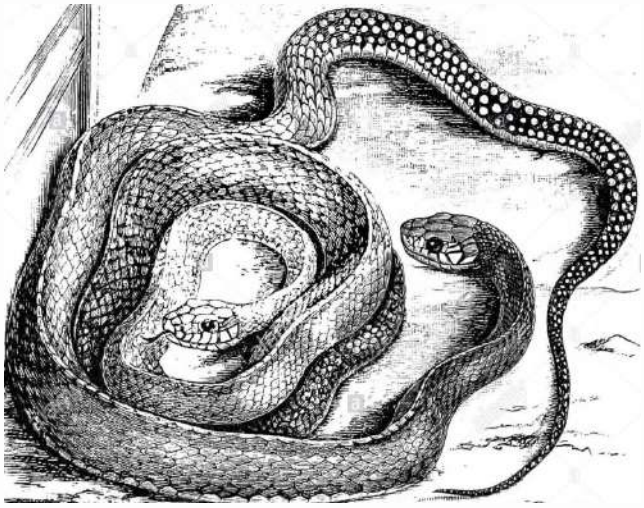
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Tail is laterally compressed and ore-like.
2. Head is small and shielded above.
3. Eyes are with rounded pupil.
4. About 14-18 maxillary teeth are present behind the poison fangs.

Hence, the specimen seems to be *Hydrophis sp.*

Zamenis sp. (Aesculapian Snake)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Presence of two **temporal** fossae on each side of the skull.

Hence, the specimen belongs to the Subclass Lepidosauria.

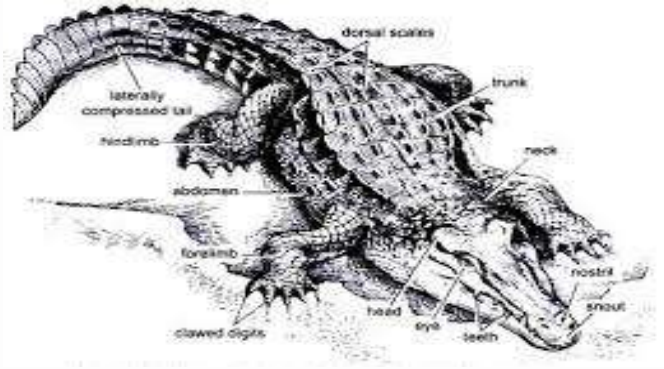
1. Body is covered by scales or shields.
2. Cloacal aperture is transverse.
3. The **quadrate** is movably articulated with skull.
4. The males have paired copulatory organs.

Hence, the specimen belongs to the Order Squamata.

1. Adults are usually from 110 cm to 160 cm in total length (including tail), but can grow to 200 cm, with the record size being 225 cm.
2. It is dark, long, slender, and typically bronzy in color, with smooth scales that give it a metallic sheen.
3. Males grow significantly longer than females.
4. Scale arrangement includes 23 dorsal scale rows at midbody, 211-250 ventral scales, a divided anal scale, and 60-91 paired subcaudal scales.
5. Ventral scales are sharply angled where the underside meets the side of the body.

Hence, the specimen seems to be *Zamenis sp.*

Crocodylus sp. (Crocodile)



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is divisible into head, neck, trunk and tail.
2. Presence of scales, shields, plates or **scutes** as **exoskeletal** structures.
3. **Pentadactyl** limbs are provided with claws.

Hence, the specimen belongs to the Class Reptilia.

1. Crocodylomorphs were and are oviparous, laying eggs in a nest.
2. Adult size varies widely, from about 55 cm long to much larger dimensions.

Hence, the specimen belongs to the superorder Crocodylomorpha.

1. Range in size from 1–1.5 m to 7 m.
2. Sexually dimorphic, with males much larger than females.
3. Have solidly built lizard-like bodies with elongated, flattened snouts and laterally compressed tails.
4. The eyes, ears and nostrils of crocodilians are at the top of the head.

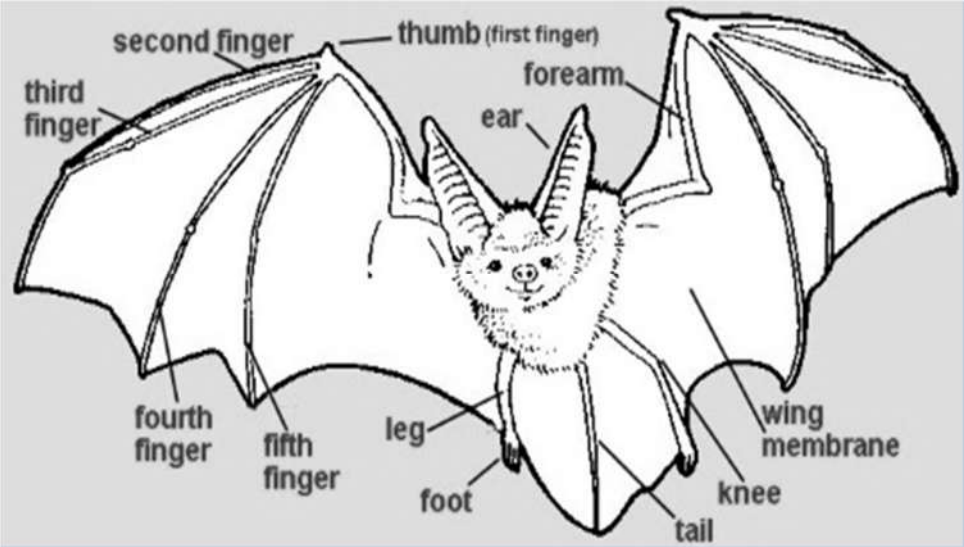
Hence, the specimen belongs to the Order Crocodilia.

1. Adults are dark olive to grey or brown.
2. The head is rough without any ridges and large scutes around the neck that is well separated from the back.
3. Scutes usually form four, rarely six longitudinal series and 16 or 17 transverse series.
4. The limbs have keeled scales with serrated fringes on outer edges, and outer toes are extensively webbed.
5. The snout is slightly longer than broad with 19 upper teeth on each side.
6. The nasal bones separate the premaxilla above.

Hence, the specimen seems to be *Crocodylus sp.*

MAMMALS

Microchiropteran (Insectivorous) Bat



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is covered by hairs.
2. Presence of mammary gland and socketed teeth.

Hence, the specimen belongs to the Class Mammalia.

1. Presence of well developed **pinna**.
2. Mammary gland possesses **teat**.
3. Presence of **cusps** on **molar** teeth.

Hence, the specimen belongs to the Subclass Theria.

1. Anal and urinogenital openings are separate.
2. **Vagina** is single.

Hence, the specimen belongs to the Infraclass Eutheria.

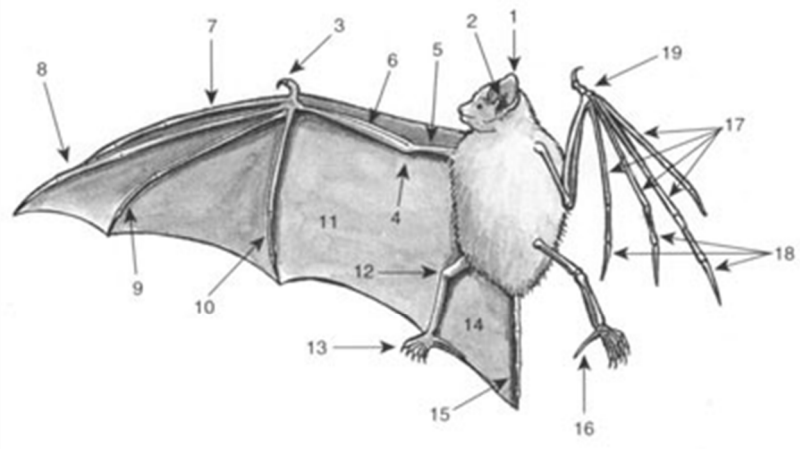
1. Forelimbs support a thin **patagium**.
2. **Polex** is directed forward and terminates in a curved claw.

Hence, the specimen belongs to the Order Chiroptera.

1. The **muzzle** is short.
2. The tail is enclosed in the **interfemoral** membrane.

Hence, the specimen seems to be Microchiropteran Bat.

Megachiropteran (Frugivorous) Bat/Flying Fox



1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is covered by hairs.
2. Presence of mammary gland and socketed teeth.

Hence, the specimen belongs to the Class Mammalia.

1. Presence of well developed **pinna**.
2. Mammary gland possesses **teat**.
3. Presence of **cusps** on **molar** teeth.

Hence, the specimen belongs to the Subclass Theria.

1. Anal and urinogenital openings are separate.
2. **Vagina** is single.

Hence, the specimen belongs to the Infraclass Eutheria.

1. Forelimbs support a thin **patagium**.
2. **Polex** is directed forward and terminates in a curved claw.

Hence, the specimen belongs to the Order Chiroptera.

1. **Muzzle** is elongated.
2. The second digits are armed with claws.

Hence, the specimen seems to be Megachiropteran Bat.

Funambulus sp. (Squirrel)

1. Presence of hollow, dorsal, tubular nerve cord.
2. Presence of **notochord**, pharyngeal gill slits and post anal tail during some stages in the **ontogeny**.

Hence, the specimen belongs to the Phylum Chordata.

1. Presence of **cranium** and **vertebral column**.

Hence, the specimen belongs to the Subphylum Vertebrata.

1. Mouth is covered by functional jaws.
2. Presence of paired appendages.

Hence, the specimen belongs to the Superclass Gnathostomata.

1. Body is covered by hairs.
2. Presence of mammary gland and socketed teeth.

Hence, the specimen belongs to the Class Mammalia.

1. Presence of well developed **pinna**.
2. Mammary gland possesses **teat**.
3. Presence of **cusps** on **molar** teeth.

Hence, the specimen belongs to the Subclass Theria.

1. Anal and urinogenital openings are separate.
2. **Vagina** is single.

Hence, the specimen belongs to the Infraclass Eutheria.

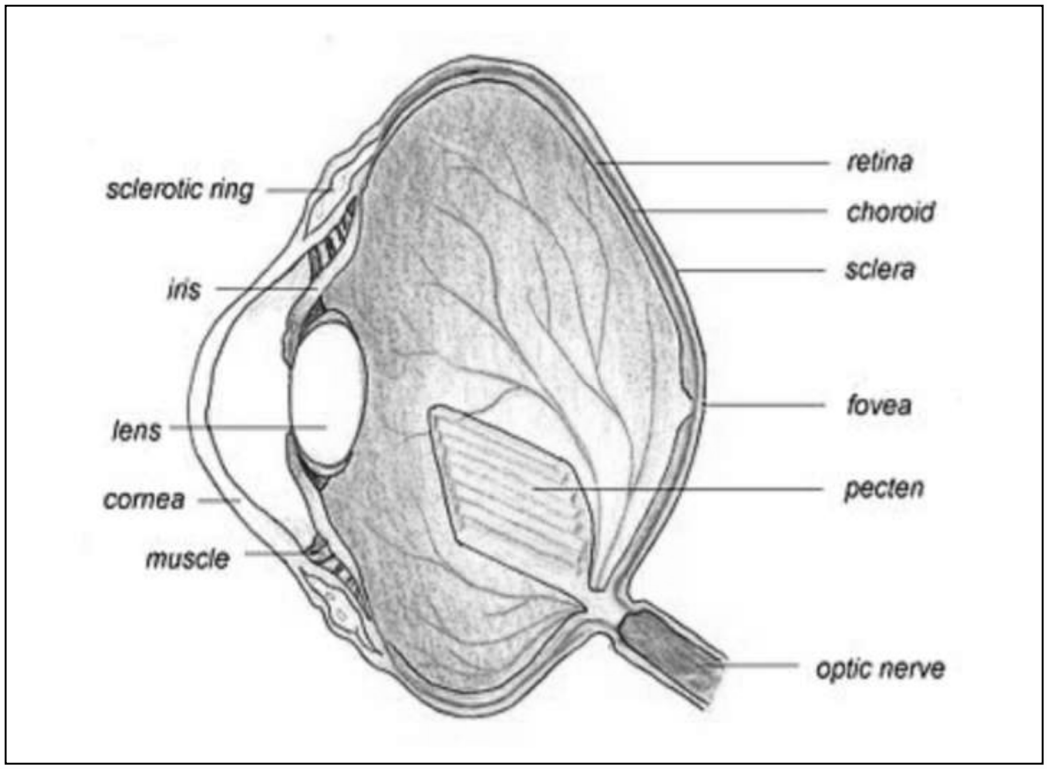
1. **Incisors** are chisel-shaped.
2. **Diastema** is observed.

Hence, the specimen belongs to the Order Rodentia.

1. Dorsal side of the body has three white and grey stripes.
2. Tail is long and bushy.
3. Presence of two pairs of mammary glands.

Hence, the specimen seems to be *Funambulus sp.*

PECTEN FROM FOWL HEAD (EYE)



DISSECTION OF BRAIN AND PITUITARY OF TILAPIA

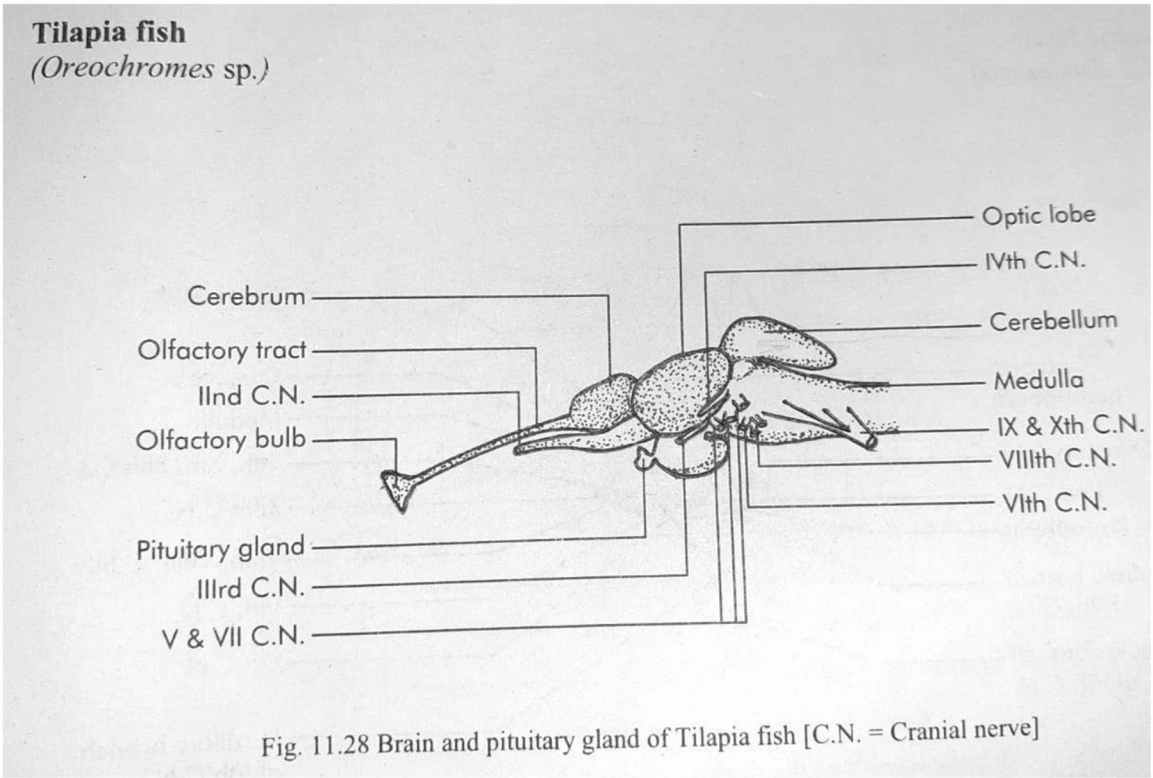


Fig. 11.28 Brain and pituitary gland of Tilapia fish [C.N. = Cranial nerve]

C6P: Animal Physiology: Controlling & Coordinating Systems

1. Recording of simple muscle twitch with electrical stimulation (or Virtual)

When an action potential travels down the motor neuron, it will result in a contraction of all of the muscle fibers associated with that motor neuron. The contraction generated by a single action potential is called a **muscle twitch**. A single muscle twitch has three components. The **latent period,** or lag phase, the **contraction phase,** and the **relaxation phase**. The latent period is a short delay (1-2 msec) from the time when the action potential reaches the muscle until tension can be observed in the muscle. This is the time required for calcium to diffuse out of the SR, bind to troponin, the movement of tropomyosin off of the active sites, formation of cross bridges, and taking up any slack that may be in the muscle. The contraction phase is when the muscle is generating tension and is associated with cycling of the cross bridges, and the relaxation phase is the time for the muscle to return to its normal length. The length of the twitch varies between different muscle types and could be as short as 10 ms (milliseconds) or as long as 100 ms (more on this later).

A good way to understand the many factors that affect muscle response is to isolate a muscle and observe it during its simplest contraction: the single muscle twitch. When an electrical stimulus is applied to a muscle directly or through the nerve supplying the muscle, it causes the cell membrane (sarcolemma) to depolarize.

Equipment:

- double pithed frogs
- frog board or tray, pins
- figure 1
- muscle bath
- Ringer's solution (13g of NaCl, 0.28g of KCl, 0.3g of $CaCl_2 2H_2O$, 0.4g of $NaHCO_3$ for 2L of distilled water)
- glass probe
- apparatus to record muscle contraction: recorder with 2 pens, amplifier, timer, transducer
- thread, plasticine
- stimulator
- timer
- ruler
- weights

I. PREPARATION OF MUSCLE

1. At first taka one frog and do the double-pithed. The frog's entire central nervous system brain and spinal cord) has been destroyed by pithing. As a result they cannot feel pain.

2. Remove the skin from one leg by first cutting the skin as high as possible around the thigh. Roll the loosened skin back a short distance (Figure 1a). Grasp it with one hand and the frog thigh with the other. Peel the skin completely off the leg by pulling it quickly (Figure 1b).

3. Use figure 1c to help locate the gastrocnemius muscle. Keep the frog's tissues moist with Ringer's solution during the remainder of the exercise.

4. Carefully insert a glass probe to separate the thigh muscles and to expose the sciatic nerve (Figure 1c). Do not touch sciatic nerve with metal instruments and avoid unnecessary pulling and handling of this nerve.

5. Cut the sciatic nerve as proximally as you can to the body.

6. Using a glass probe, gently ease the nerve from its location and lay it on the surface of the gastrocnemius muscle.

7. Cut the thigh muscles first near their origin on the pelvic girdle and then, just above the knee (Figure 1d; be careful not to cut the gastrocnemius muscle or the sciatic nerve!).

8. Use the probe again to separate and free the gastrocnemius muscle from the other muscle of the frog's leg.

9. Locate the Achilles tendon (Figure 1 c&e). Tie a piece of thread about 15 cm long (6 inches) around its lowest end. Cut the tendon at the point between the thread and the animal's heel bone.

10. Lift and move the gastrocnemius muscle to one side. Cut the other muscles of the frog's lower leg and the bone (tibiofibula) just below the knee.

11. Cut the femur bone to about 2.5cm (1 inch; Figure 1e).

12. Push a straight pin through the knee joint, and secure the pin in the lucite plug of the muscle bath to anchor the knee. Fill the bath half way with Ringer's solution.

13. Run the thread, which is tied to the tendon through the pulley and attach its end to the transducer lever.

14. Adjust the thread tension by moving the transducer up or down, until the lever is horizontal. Make sure that the thread is aligned at a 90 degree angle to the transducer lever.

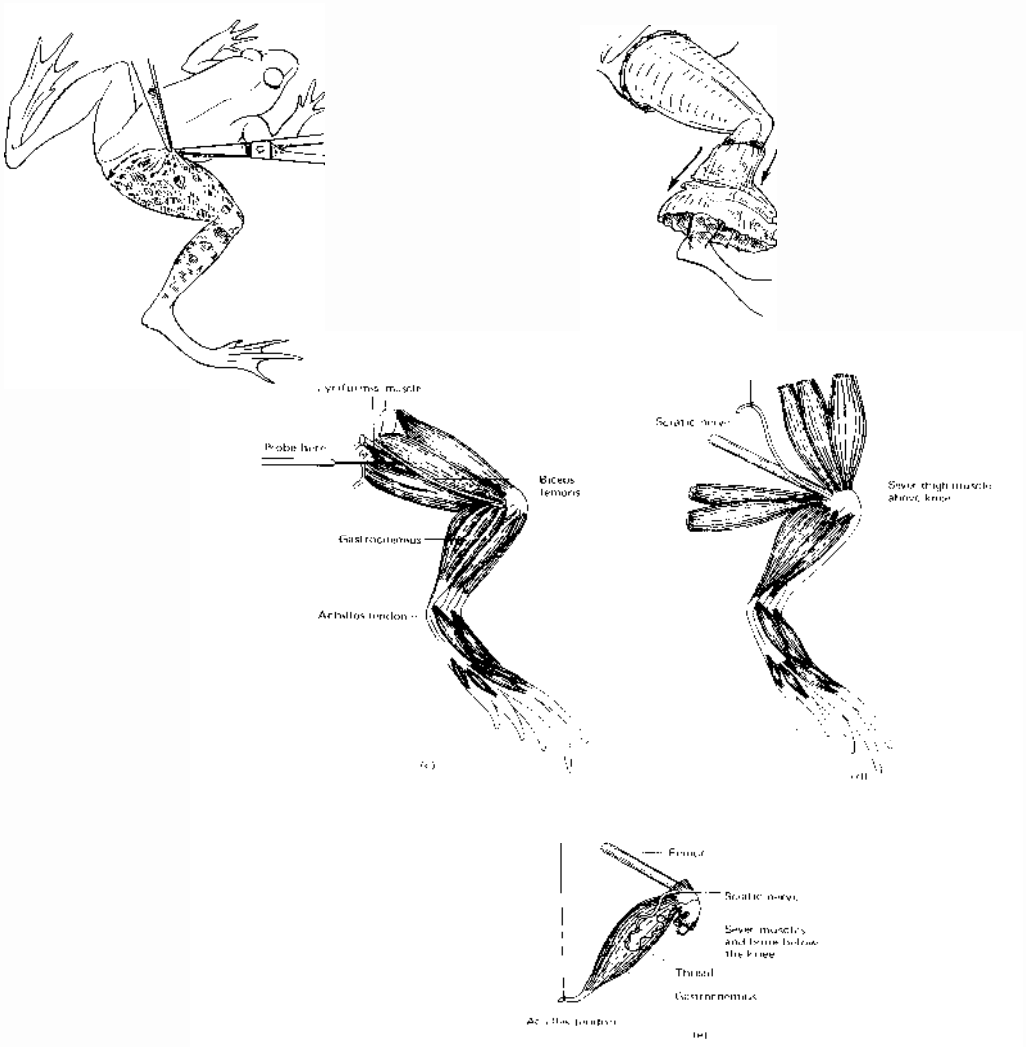


Figure 1: Preparation of the frog gastrocnemius muscle.

15. Set the amplifier switch to input, lower gently the lever with your finger and make sure that you have a deflection on your chart recorder when the distal extremity of the lever has been lowered by 2mm. To amplify your signal, turn the gain clockwise.

    To move the position of the signal on the chart paper without changing its amplitude, just turn the offset dial.

16. Using the glass probe, place the nerve over the electrodes. Keep the electrodes out of the Ringer's solution. Keep the nerve moist at all times by pouring Ringer's solution over it between recordings and remove the nerve from the electrode when not in use.

II. GRADED RESPONSE OF MUSCLE

After studying the response of skeletal muscle to stimuli of increasing intensity and the minimal and maximal stimuli could be determined from muscle of specimen.

EXERCISE A.

1. Set stimulator mode switch to single shot, and duration control to 1.2 msec.

2. Set chart recorder speed to 1-2.5 mm/sec and make sure that the pen touches the paper.

3. Set the voltage dial of the stimulator to its lowest setting and deliver one single stimulus to the muscle by pressing the pulse switch.

4. Administer single stimuli to the muscle at approximately 2-5 seconds intervals, increasing the voltage of the stimulation slightly until a contraction is obtained (shown by a spike on the paper). The voltage at which this contraction occurs is called the threshold (= minimal) stimulus. Write the value of the threshold in table 1. All stimuli applied prior to this point are termed subthreshold (subminimal) stimuli because at those voltages no contraction was elicited.

5. Continue to turn up the voltage in small increments, obtaining greater and greater muscle contraction until an increase in voltage no longer results in a greater contraction. The lowest voltage that produces the maximal contraction is called the maximal stimulus. Record its value in Table 1. Any stimulus greater than maximal is said to be supramaximal.

6. Keep the recording in lab report. Label the chart clearly, and record chart speed and direction, as well as the intensity of the stimuli applied to the muscle. Indicate on the chart recording the subminimal, minimal, maximal and supramaximal stimuli.

Table 1: Values of the minimal and maximal stimuli (in volts)

Minimal stimulus	Maximal stimulus

III. ISOLATED MUSCLE CONTRACTION (= MUSCLE TWITCH)

The response of a muscle to a single brief threshold stimulus is called a muscle twitch. There are three phases in a muscle twitch:

1) the latent period: it follows the stimulation, the excitation-contraction coupling is occurring and no response is seen on the myogram;

2) the period of contraction; and

3) the period of relaxation.

A twitch may be strong or weak depending on the number of motor units activated. A twitch may be fast or slow depending of which muscle is stimulated: for example the extraocular muscle has a fast twitch, but the soleus has a slow twitch.

EXERCISE B.

1. Set stimulator mode switch to single shot, and duration control to 1.2 msec.

2. Set chart recorder speed to 50 mm/sec and make sure that the pen touches the paper.

3. Set the voltage dial of the stimulator above the maximal stimulus found in exercise A and deliver one single stimulus to the muscle by pressing the pulse switch.

4. Make 3 recordings of a single muscle twitch.

5. Keep your recording. You will have to include it (or a photocopy of it) in your lab report. Label the chart clearly, and record chart speed and direction, as well as the intensity of the stimuli applied to the muscle. Indicate on the chart recording the latent period, the period of contraction and the period of relaxation.

IV. WA

VE SUMMATION, TETANUS and FATIGUE

If a skeletal muscle is stimulated with a rapid series of stimuli of the same intensity before it has a chance to relax completely, the response to the second stimuli will be greater than to the first. This phenomenon is called wave summation. Stimulation of a skeletal muscle at an even higher frequency will produce a "fusion" of the summated twitches. This is called tetanus. In incomplete tetanus, the contraction shows small relaxation curves; in complete tetanus the graph of contraction is apparently smooth.

EXERCISE C

1. Set chart recorder speed to 1-2.5 mm/sec.

2. Set stimulator intensity to a voltage sufficient to cause maximal contraction (this means **above** the maximal stimulus).

3. Set the repeat mode of the stimulator at a frequency of 1/sec.

4. Stimulate the muscle for 10 to 15 seconds.

5. Stop stimulation and allow the muscle to return to baseline (approx. 15-20 sec)

6. Repeat 3, 4, and 5 with frequencies of 2/sec, 5/sec., 10/sec., and in steps of 5 up until the muscle reaches a sustained contraction called tetanus.

7. Set the repeat mode of the stimulator to a frequency HIGHER than the one that caused tetanus.

8. Apply the stimuli. If fatigue does occur, record for 5 to 10 seconds.

9. DO NOT DAMAGE THE MUSCLE .If fatigue does not occur, continue to increase the stimuli frequency in steps of 5/sec (apply for 10-15 seconds allowing the muscle to rest between increments as instructed above) until fatigue is observed.

10. Stop stimulation and allow the muscle to rest for 10 minutes before doing the next experiment (do not leave the nerve on the electrodes, keep muscle and nerve moistened with Ringer's solution).

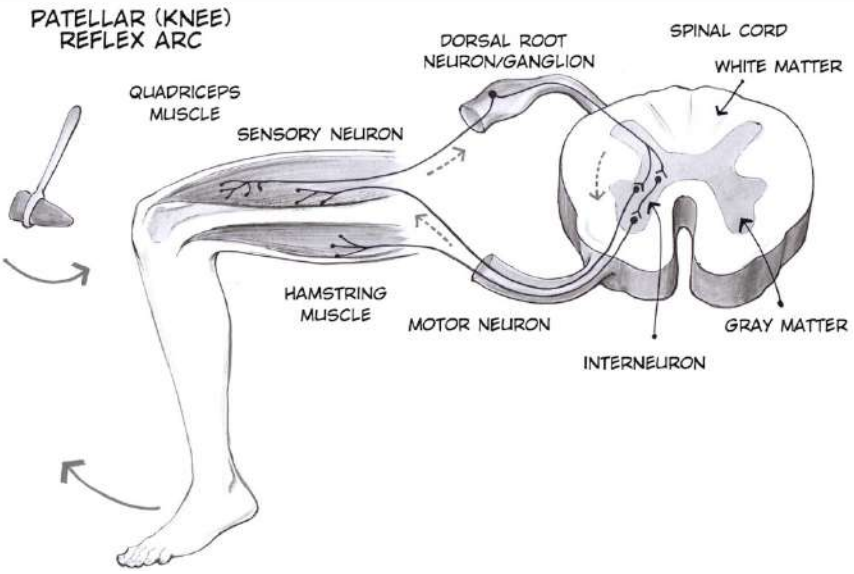
11. Keep recording.

12. Label the chart clearly and indicate individual twitches, wave summation, incomplete tetanus, tetanus and fatigue. Record chart speed and direction, as well as the intensity and frequency of the stimuli applied to the muscle.

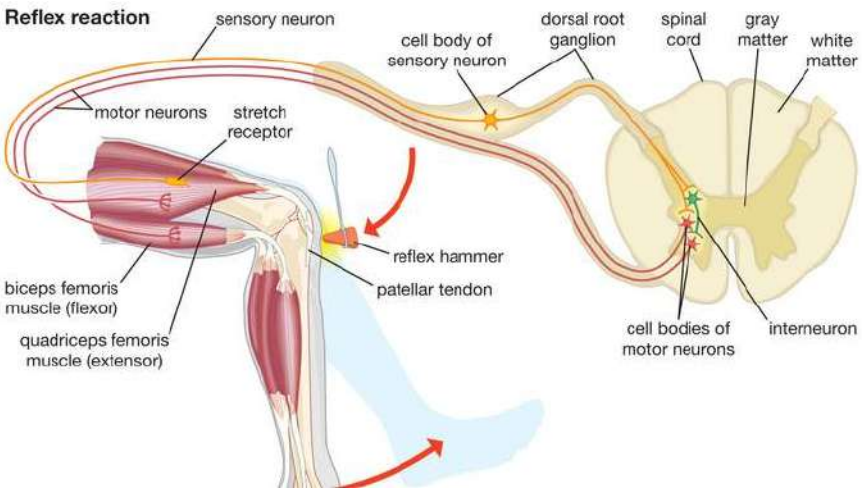
2. Demonstration of the unconditioned reflex action (Deep tendon reflex such as knee jerk reflex)

Reflexes require no thought. They are automatic, fast, and of huge importance to a human's ability to successfully respond to their environment. Despite the magnificent information-processing power of the billions of neurons in our brain, we need a lot of stuff to be done automatically. Without reflexes, our brains would be overloaded with worrying about constantly updating the position of our unstable bodies to keep us upright. .

Knee-jerk reflex, also called patellar reflex, sudden kicking movement of the lower leg in response to a sharp tap on the patellar tendon, which lies just below the kneecap. One of the several positions that a subject may take for the test is to sit with knees bent and with one leg crossed over the other so that the upper foot hangs clear of the floor. The sharp tap on the tendon slightly stretches the quadriceps, the complex of muscles at the front of the upper leg. In reaction these muscles contract, and the contraction tends to straighten the leg in a kicking motion. Exaggeration or absence of the reaction suggests that there may be damage to the central nervous system. The knee jerk can also be helpful in recognizing thyroid disease.



There is only one connection (a synapse) needed for the information from the sensory neuron to get to the motor neuron and cause a muscle contraction. Because of this single synapse, this can happen very fast. In a young, healthy person, it takes 15-30 milliseconds for the stretch stimulus to produce a muscle contraction, by comparison, it takes 5-10 times that long to blink your eye in response to a stimulus, or 150-300 milliseconds. This is super useful for correcting your muscle length in response to rapid changes such as a slip or trip. These situations require very fast corrections to prevent falling and injury. If you had to consciously flex your leg in response to the leg stretch (a reaction) it would be much slower than the 15-30 seconds of reflex.



3.Preparation of temporary mounts: Squamous epithelium, Striated muscle fibres and nerve cells

Aim

Preparation of temporary slide of animal tissues and their study.

Principle

Group of similar type of cells that perform a specific function, is called a tissue. Tissues are organised in a specific proportion and pattern to form different organs. There are four basic types of tissues : (i) Epithelial, (ii) Connective, (iii) Muscular and (iv) Nervous. The epithelium or epithelial tissue provides a covering or lining for some parts of the body. Connective tissues have special function of linking and supporting other tissues or organs of the body. Muscular tissue plays an active role in all movements of the body. Nervous tissue controls the body's responsiveness to changing conditions within and outside the body. Requirement: Live material/concerned tissue, beakers, glass slides, coverslips, watch glasses, dropping bottle, dropper, required stain, glycerine, NaCl solution (0.9% w/v), needle, forceps, brush, toothpick, water, wash-bottle, dissecting tray, microscope.

I. EPITHELIUM OR EPITHELIAL TISSUE

Procedure

• Rinse your mouth well with water.

• Gently scrap the inside of your cheek with the broad end of a clean toothpick. Discard this material.

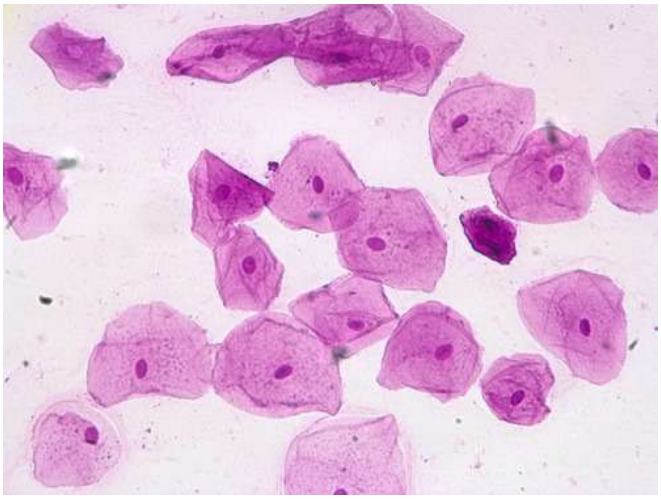
• Scrap again, and spread these cells gently on a clean slide. Add a drop of 0.9% NaCl solution or physiological saline and a drop of methylene blue with the help of a dropper. • After two minutes, remove the excess stain and saline using the edge of a filter paper. Now, put a drop of glycerine on the cells.

• Place a coverslip over the tissue and gently press it with the back of a pencil to spread the cells.

• Examine the slide under the low power of microscope.

• Draw a labelled diagram of your preparation.

Observations



Discussion

Epithelial tissue (epithelium) forms the covering tissue of the body. It covers the body surface and lines the body cavities and hollow visceral organs. It may be single or multi-layered. The lower most layer normally rests upon a non-cellular basement membrane. It is protective/sensory/absorptive/and secretory in nature and also helps in exchange and movement of materials inside the body.

CONNECTIVE TISSUE

Connective tissue is the tissue that connects, separates and supports all other types of tissues in the body. Like all tissue types, it consists of cells surrounded by a compartment of fluid called the extracellular matrix (ECM). However connective tissue differs from other types in that its cells are loosely, rather than tightly, packed within the ECM. Based on the cells present and the ECM structure, there are two types of connective tissue:

Connective tissue proper; further divided into loose and dense connective tissues

Specialised connective tissue; reticular, blood, bone, cartilage and adipose tissues

Blood is the specialized connective tissue within the circulatory system that transports blood cells and dissolved substances throughout the body via blood vessels.

Procedure

• Clean the slides so that it become free from grease, finger prints, etc.

• Clean the tip of your middle finger with rectified spirit and prick with a sterilised needle or lancet available at the medical store.

• When a drop of blood appears on the finger tip, wipe it away with cotton dipped in rectified spirit. • Press the finger tip to get the next drop of blood and touch it with the clean surface of slide (placed on working-table) (Fig 5.1 (a)) about 1 cm away from the right side edge (this be named as the first slide).

• Hold the narrow edge of another slide (2nd slide) at about 450 angle to the 1st slide and to the left of the drop of blood (Fig. 5.1(b)).

• Pull to the right until the 2nd slide touches the blood. Wait for 2-3 seconds till the blood spreads along the line of contact. Now push the 2nd slide towards the left in a steady but brisk movement. Take care to keep the edge pressed uniformly against the surface of the 1st slide. Keep pushing until the other end of the slide is reached (Fig. 5.1 (c)). This method spreads the blood thinly (also called a blood film) over the surface of the slide but does not run over the cells and crushes them. You may make 3 or 4 such film (smear) preparations.

• Once the uniform smear is made, air dry the slide for about 10 minutes.

• Mark with a wax pencil the region of the smear that is to be stained.

• Cover the region marked with wax pencil with few drops of Leishman's/ Geimsa/Wright's stain. Leave the stain over the smear for 2 to 3 minutes. Now, add an equal amount of distilled water gently with a dropper and leave it for 2 to 4 minutes. Repeat this process till its colour becomes light violet. Air dry the slide thoroughly and mount with a cover slip, using a drop of glycerine.

• Observe the slide under a compound microscope first under low magnification and then at higher magnification.

Observations and Results

A. RBCs (erythrocytes) are biconcave, circular and non-nucleated. The various types of white blood corpuscles (WBC's), the eosinophils, the basophils and neutrophils are also observed. Also observe the blood platelets

RBCs are biconcave as the central part is lighter.

B. Shape of WBC is irregular.

Mainly two types of WBC are found-

(a) Granulocytes: Granules are found in these cells. These cells are of the following types:

• Eosinophils or Acidophils: Its nucleus is trilobed and twisted into an 'S' shape.

Basophils – Its nucleus is bilobed.

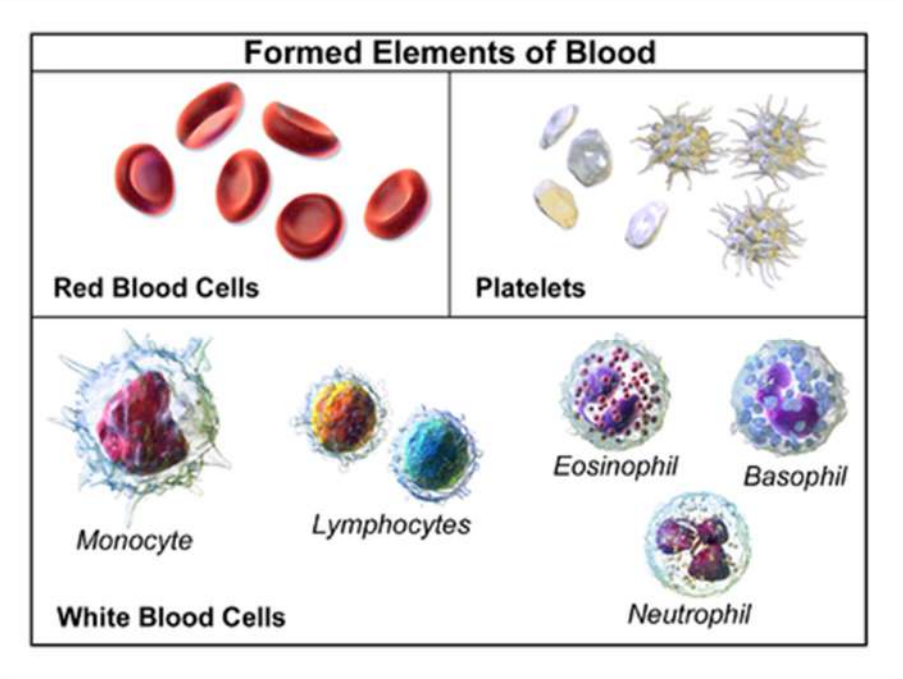
• Neutrophils – Its nucleus is multilobed (2-5 lobes).

(b) Agranulocytes: Its cytoplasm is without granules and nuclei are nonlobed. These are of the following types.

• Monocytes – These are large in size with kidney shaped nucleus.

• Lymphocytes – They are slightly bigger than RBC with spherical nucleus.

C. Platelets have no nuclei. They appear in clusters as violet granules.



Discussion

Different types of corpuscles present in the blood perform different functions. Haemoglobin present in the RBCs help in exchange of oxygen and carbon dioxide. Monocytes and lymphocytes participate in destroying harmful microorganisms that invade our body. Platelets help in blood clotting activity which to a certain extent prevents blood loss during injury. The total counts of RBCs and WBCs and the differential counts of various WBCs are of great medical significance for diagnostic purpose.

III MUSCULAR TISSUE

A. Striated Muscle Fibres Procedure

(a) Taking out the tissue

• Place a preserved cockroach/frog or other available animal in a dissecting tray containing water.

• Cut open the animal to expose its thigh region. (As an alternate preserved sample of striated muscle can be provided)

• Take a small piece of muscle from this region and tease it on a slide with the help of needles to get a few thinnest possible fibres.

• Wash it in water in a petridish, changing the water 2-3 times to remove the preservative, as it may interfere with staining.

(b) Staining and mounting

• Add a few drops of methylene blue to stain the muscle fibres.

• After staining, put the muscle fibres on a slide and tease it further, if necessary, with needles so that the muscle fibres are well separated.

• Blot out the excess of water and stain.

• Add a drop of glycerine on the slide and with the help of a needle gently put the coverslip and avoid the entry of air bubbles.

• Press the coverslip gently with a needle to spread the glycerine and the muscles properly.

• Examine the slide under the microscope. Observation Look for the following features in the muscle fibre

• Muscle fibres are elongated, cylindrical and multinucleated (syncytium).

• These fibres are enclosed in a membrane called sarcolemma.

• Several dark and light bands are alternately arranged perpendicularly to the long axis of the fibre. Presence of these bands alternately produce striations, hence these muscles are called striated muscles

Discussion

Striated muscles constitute the main component of musculature of our body, primarily attached to bones via tendons, hence are also called skeletal muscles.

Their contractions are voluntary in nature and thus are not controlled by autonomic nervous system.

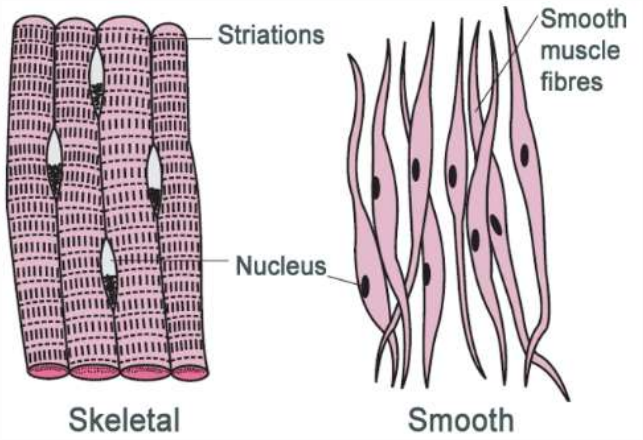
B. Smooth Muscle Fibre

Observation

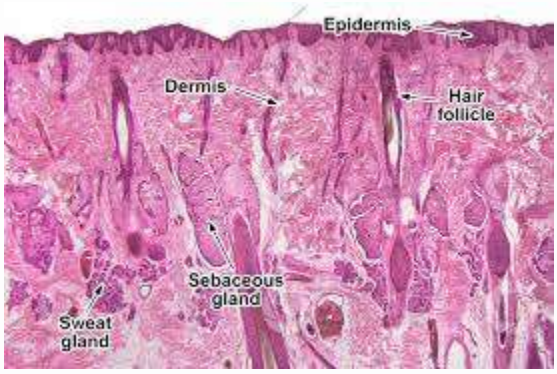
(i) Smooth muscle cells are extremely long and spindle shaped. They have an oval nucleus located centrally in the cytoplasm .

(ii) Sarcolemma is absent.

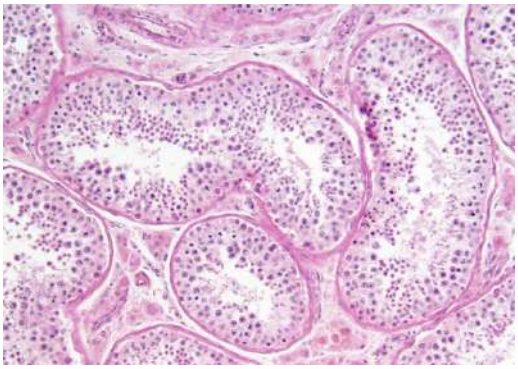
(iii) Since these fibres do not show dark and light bands, they are called unstriated muscles.



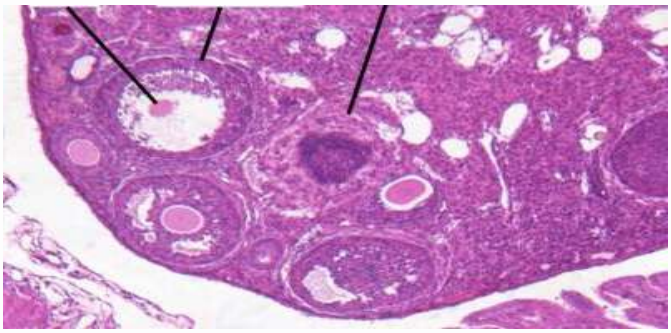
Study of permanent slides



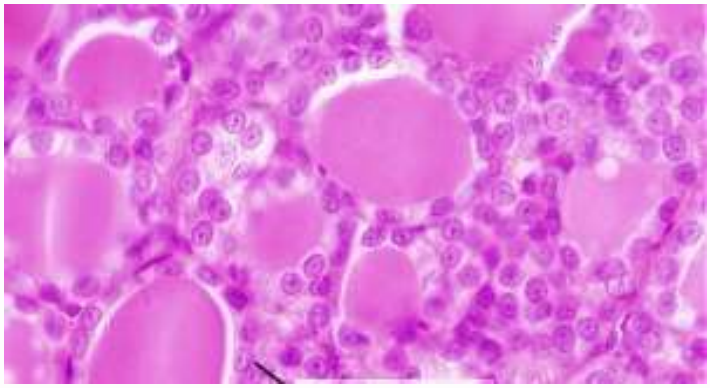
- The **skin** of **mammals** is constructed of two layers, a superficial nonvascular epidermis and an inner layer, the dermis, or corium. ...
- The dermis lies beneath the epidermis and nourishes it. ...
- Hair is derived from an invagination (pocketing) of the epidermis termed a follicle. ...
- Most **mammals** have three distinct kinds of hairs.



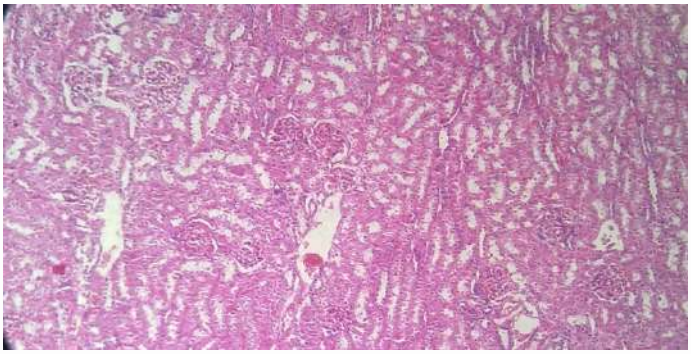
IDENTIFYING CHARACTERS	INFERENCES
1. Presence of outer thick capsules made up of fibrous connective tissue – tunica albuginea. 2. Presence of large no. Of rounded seminiferous tubules, leydig cells and blood cells. 3. Presence of spermatogonia, primary and secondary spermatocytes.	Hence the specimen seems to be the T.S of mammalian Testes.



IDENTIFYING CHARACTERS	INFERENCES
1. Presence of superficial layer of cubical cells- germinal epithelium. 2. Presence of graphian follicle. 3. Presence of fibrous and vascular connective tissue forming stroma.	Hence the specimen seems to be the T.S of mammalian Ovary.



IDENTIFYING CHARACTERS	INFERENCES
1. Presence of two layered capsules (outer and inner) made up of fibro elastic connective tissue . 2. Each lobule is made up of large no. of follicles supported by thin basement membrane. 3. The cavity of follicle is field with viscous fluid called colloid.	Hence the specimen seems to be the T.S of mammalian Thyroid gland.



IDENTIFYING CHARACTERS	INFERENCES
1. Presence of granular outer cortex and striated medulla. 2. Presenece of closely packed uriniferous tubules. 3. Presence of Bowman's capsule and Glomerulus	Hence the specimen seems to be the T.S of mammalian Kidney.

Microtomy: Preparation of permanent slide of any five mammalian (Goat/white rat) tissues

MICROTECHNIQUES :-

The microtechniques for tissue preparation consist from many steps to study the cellular structure which forming the body of the living human or animals, which can't see by naked eyes, but can see by using Light microscope or Electronic microscope.

There are three types of methods using for microscopic preparations are:

1- The paraffin Technique.
2- The celloidin technique.
3- The freezing technique.

THE PARAFFIN TECHNIQUE:

In this technique using paraffin wax, following are the steps used to work histological sections and are summarized as follows:

Obtaining of the specimen. Fixation. Washing. Dehydration. Clearing. Impregnation or Infiltration. Embedding. Trimming. Sectioning. Mounting. Rehydration.Staining. Cover slipper.

Obtaining of the specimen: First the specimen (animal) is collected and the tissue of interest is taken.

Fixation: The first step in the preparation of tissue using 10% formalin solution. The purpose from using formalin to maintaining the tissue and its contents to the state it was in the body living or near from that and the fixation process through chemical reactions and interactions physical between the active groups of the fixative substance and effective groups of chemicals in tissue (Carbohydrate - protein - fat - enzymes - metal salts-dyes). And the fixation process made upon stop fragmentation and disintegration process for bacteria and fungi activity, as well as stop the autolysis process of the tissue by enzymes .

Washing : The sample must be washed before and after fixation by using tap water current for 24 hours, to remove the remainder of the formalin from the specimen.

Dehydration: Dehydration by using series spiraling of ethyl alcohol beginning with (50%, 60%,70%, 80%, 90%, 90%, 100% and 100%) for 30 mins for each concentration, to gradual removal of water from the tissue to prevent tissue shrinking.

Clearing: Replacement of alcohol in tissue by clearing fluid like (Xylene, benzene, or acetone) for twice and ½ hour for each solution.

Impregnation or Infiltration: The tissue specimen infiltration by putting the tissue specimens in paraffin wax on (58 - 60°C) for two times and 2 hours for each step. It provides a strong support to prepare them for cutting with microtome, and helps to save them in normal conditions for a long time without any harm. 7- Embedding: - The specimens embedding within paraffin wax for 24 hour, to made a template so that the specimen is surrounded by paraffin wax to supported.

Trimming:- After preparation of wax templates preferably trimmed with a sharp blade so that the specimen be in a position Suitable for cutting edges so that they become parallel and can be applied to the edge of the knife microtome.

Sectioning:-Paraffin block are cut by microtome using metal knife, into thin sections ~ 6µ.

Mounting: - The sections spread on the hot plate and mounted on glass slides, it is using a thin smear from the Mayer's egg albumin to fix the sections strip on glass slides.

Rehydration:- Rehydration by alcohol descending series beginning by (100%, 90%, 80%, 70%, 60% and 50%). For 30 mins for each concentration.

Staining:- Finally staining the specimens by different stains, like H&E, PAS, Van Gieson's and Verhoff's stains to differentiate their different components.

Cover slipper:- The histological slides were dried by a hot plate at (40 °C) for 2 hours. Mounting is the process of adding the amount of (Canada balsam) and then putting the cover slide on the sections strip on glass slides and cover it.

Haematoxylin and Eosin Staining

These are two stains used in the examination of thin slices of biological tissue. Contrast is created by the stains where Haematoxylin turns the nuclei blue while eosin turns the cytoplasm as well as other parts pink or red.

Procedure

1- A rehydrated section is stained in a solution of haematoxylin for 20 to 40 minutes

2- The section is then washed in tap water for about 3 minutes until it turns blue,

3- The section is the differentiated in 70percent ethanol that contains 1 percent of HCL for about 5 seconds to remove excess dye and allow the nuclear to emerge,

This is then washed in tap water,

5- Stain with eosin for 10 minutes,

6- Then wash for about 1 to 5 minutes in tap water,

7- Dehydration, clear and mount on a rack

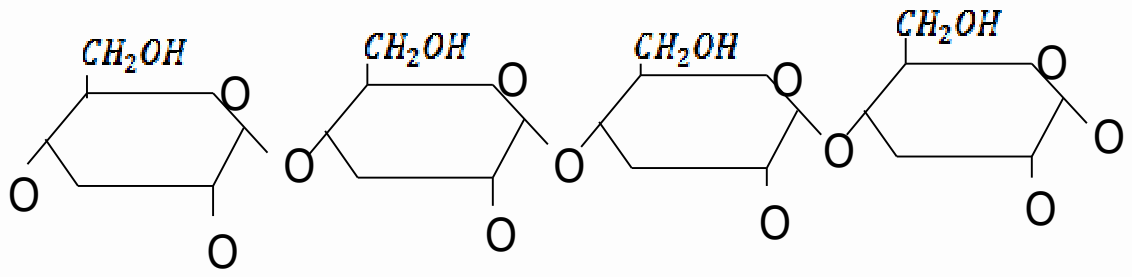
C7P: Fundamentals of Biochemistry Lab

Qualitative Analysis of Carbohydrates

A carbohydrate is a hydrophilic organic molecule with a 2:1 ratio of hydrogen and oxygen, and a general formula $C_x(H_2O)y$, where x and y are whole numbers. In the sugar glucose, for example, x=y=6 and the formula is, therefore $C_6H_{12}O_6$. Different kinds of carbohydrates along with their salient, features are tabulated below.

MONOSACCHARIDES: All monosaccharides have the molecular formula $C_6H_{12}O_6$, although they differ in the arrangement of H and OH groups in space (called isomers 1 steroisomers).

DISACCHARIDE: Monosaccharides combined in pairs from disaccharide(sucrose: glucose+ fructose; lactose: glucose+ galactose, maltose: glucose+ glucose). The C-O-C bond that holds monosaccharide in disaccharides are called glycoside bonds which can be broken by hydrolysis.
Ex – sucrose, lactose etc.



STARCH

POLYSACCHARIDES: often glucose can form long chain polymers called polysaccharide. Cellulose and starch are plant products while glycogen is made by animals, includinf humans. A cellulose molecule (found in wood, cotton etc) consist of a few thousand glucose monomers joined together with every other one inverted relative to the next.

Materials Required:

1) Glassware
2) Test tubes
3) Test tube holder
4) Water bath
5) Spatula
6) Dropper

Reagents Required:

1) Molisch's Reagent
2) Iodine solution
3) Fehling's reagent A
4) Fehling's reagent B
5) Benedict's qualitative reagent
6) Barfoed's reagent
7) Seliwanoff 's reagent
8) Phenylhydrazine hydrochloride
9) Sodium acetate
10) Glacial acetic acid
11) Glucose, fructose
12) Microscope

Procedure:

1**) Molisch's Test:**

In a test tube, add 2 ml of the test carbohydrate solution and 2 drops of α-naphthol solution. Carefully incline the tube and pour dropwise conc. H_2SO_4, using a dropper,along the sides of the tube. Observe the violet colour at the junction of the two liquids.

2) Fehling's Test:

In a test tube, add 2 ml of the test carbohydrate solution and add equal volumes of Fehling A & Fehling B and place it in a boiling water bath for few minutes.. When the contenst of the test tube comes to boiling, mix them together and observe any change in color or precipitate formation. The production of yellow 'or brownish-red precipitate of cuprous oxide indicates the presence of reducing sugars in the given sample.

3) Benedict's Test:

In the test tube with 2 ml of Benedict's reagent, add 5-6 drops of the test carbohydrate solution and mix well. Place the test tube in a boiling water bath for 5 minutes and observe any change in color or precipitate formation. Cool the solution. Observe the colour change from blue to green, yellow, orange or red depending upon the amount of reducing sugar present in the test sample.

4) Barfoed's Test:

To 2 mL of the test solution add about 2-3 mL of Barfoed's reagent. Mix it well and boil it for one minute in the water bath and allow to stand for a few minutes. Formation of a red

precipitate of cuprous oxide in the bottom and along the sides of the test tube immediately, only monosaccharides answer this test. Since Barfoed's reagent is slightly acidic, This test is specific for monosaccharides.

5) Seliwanoff's Test:

To 2 mL of Seliwanoff 's reagent, add two drops of test solution. The mixure is heated to just boiling. A cherry red condensation product will be observed indicating the presence of ketoses in the test sample. There will be no significant change in colour produced for aldose sugar.

6) Iodine Test:

Add 2 drops of iodine solution to about 2 mL of the carbohydrate containing test solution. A blue-black colour is observed which is indicative of presence of polysaccharides.

7) Osazone Test:

To 0.5 g of phenylhydrazine hydrochloride add 0.1 gram of sodium acetate and ten drops of glacial acetic acid. Add 5 mL of test solution to this mixture and heat under boiling water bath for about half an hour. Cool the solution slowly and examine the crystals under a microscope. Needle-shaped yellow osazone crystals will be observed for glucose and fructose, whereas lactosazone shows mushroom shaped and maltose produces flower-shaped crystals.

No.	Test	Observation	Inference	Reaction
1	**Molisch's Test** 2-3 drops of beta-naphthol solution are added to 2ml of the test solution. Very gently add 1ml of Conc. H_2SO_4 along the side of the test tube..	A deep violet coloration is produced at the junction of two layers.	Presence of carbohydrates.	This is due to the formation of an unstable condensation product of beta-naphthol with furfural (produced by the dehydration of the carbohydrate).
2	**Iodine test**	Blue colour is observed.	Presence of polysaccharide.	Iodine forms coloured adsorption complexes with

	4-5 drops of iodine solution are added to 1ml of the test solution and contents are mixed gently.			polysaccharides.
3	**Fehling's test** About 2 ml of sugar solution is added to about 2 ml of Fehling's solution taken in a test-tube. It is then boiled for 10 min	A red precipitate is formed	Presence of reducing sugar	This is due to the formation of cuprous oxide by the reducing action of the sugar.
4	**Benedict's test** To 5 ml of Benedict's solution, add 1ml of the test solution and shake each tube. Place the tube in a boiling water bath and heat for 3 minutes. Remove the tubes from the heat and allow them to cool.	Formation of a green, red, or yellow precipitate	Presence of reducing sugars	If the saccharide is a reducing sugar it will reduce Copper [Cu] (11) ions to Cu(1) oxide, a red precipitate
5	**Barfoed's test** To 2 ml of the solution to be tested added 2 ml of freshly prepared Barfoed's reagent. Place test tubes into a boiling water bath and heat for 3 minutes. Allow to cool.	A deep blue colour is formed with a red ppt. settling down at the bottom or sides of the test tube.	Presence of reducing sugars. Appearance of a red ppt as a thin film at the bottom of the test tube within 3-5 min. is indicative of reducing mono-saccharide. If the ppt formation takes more time, then it is a reducing	If the saccharide is a reducing sugar it will reduce Cu (11) ions to Cu(1) oxide

			disaccharide.	
6	**Seliwanoff test** To 3ml of of Seliwanoff's reagent, add 1ml of the test solution. Boil in water bath for 2 minutes.	A cherry red colored precipitate within 5 minutes is obtained. A faint red colour produced	Presence of ketoses [Sucrose gives a positive ketohexose test] Presence of aldoses	When reacted with Seliwanoff reagent, ketoses react within 2 minutes forming a cherry red condensation product Aldopentoses react slowly, forming the coloured condensation product.
7	**Osazone Test** Two two ml of the test solution, add 3ml of phenyl hydrazine hydrochloride solution and mix. Keep in a boiling water bath for 30mts. Cool the solution and observe the crystals under microscope.	Formation of beautiful yellow crystals of osazone Needle shaped crystals Hedgehog crystals Sunflower shaped crystals	Glucose/fructose Presence of lactose Presence of maltose	Reducing sugars forms ozazone on treating with phenylhydrazine

QUALITATIVE TEST FOR PROTEINS

Introduction

Proteins are sequences of amino acids, its contain C, H, O, and Nitrogen, Common structure: Central C, with a H, amino group (NH2), and an acid group (COOH), and a side group, proteins made up of about 20 different amino acids; Unique Side Groups, Differ in size, shape, electrical charge. Proteins are probably the most important class of biochemical molecules, although of course lipids and carbohydrates are also essential for life. Proteins are the basis for the major structural components of animal and human tissue. It can be hydrolyzed by acids, bases or specific enzymes. Amino acids are organic compounds that contain amino and carboxyl groups. The R- in the above formula stands for different chemical groups (may be aliphatic, aromatic or heterocyclic) and this determines the characteristics of the amino acids. The color tests have frequently been used for qualitative detection of amino acids. Not all amino acids contain the same reactive groups. For this reason the various color tests yield reactions varying in intensity and type of color according to the nature of groups contained in the particular amino acid under examination.

Millon's reaction

Principle

The reaction is due to the presence of the hydroxyphenyl group, C_6H_5OH in the amino acid molecule; and any phenolic compound which is unsubstituted in the 3,5 positions such as tyrosine, phenol and thymol will give the reaction. Solutions of nitric acid containing mercuric nitrate reacts with phenols, producing red colors or yellow precipitates which react with nitric acid to form red solution. The reaction probably depends on the formation of a nitro compound; which then reacts with phenol.

Procedure

Add 3 to 4 drops of Millon's reagent to 5 ml of test solution. Mix and bring the mixture gradually to a boiling point by heating over a small flame. Development of red color is due to the presence of protein. Excess of reagent should however be avoided since it may produce a yellow color which is not a positive reaction.

Xanthoproteic reaction

Principle

This reaction is due to the presence in the amino acid molecule of the phenyl group $–C_6H_5$, with which the nitric acid forms certain nitro modifications. The particular amino acids which are of especial importance in this connection are those of tyrosine and tryptophan. Phenylalanine does not respond to this test as it is ordinarily preformed.

Materials

1. Conc. HNO_3
2. Ammonium hydroxide
3. Sodium hydroxide

Method

Add 1 ml of conc. Nitric acid to 2 to 3 ml of test solution in a test tube. A white precipitate forms, which upon heating turns yellow and finally dissolves, imparting to the solution a yellow color, cool the solution and carefully add ammonium hydroxide or sodium hydroxide in excess. Note that the yellow color deepens into an orange.

Biuret test

Principle

The Biuret test is given by those substances whose molecules contain two cabamyl ($-CONH_2$) groups joined either directly or through a single atom of nitrogen or carbon. Similar substances which contain- $CSNH_2$,

$-C(NH)NH_2$, or $– CH_2NH_2$ in place of the $–CONH_2$ group also respond to the test. It follows from this fact that substance which are non-protein in character but which contain the necessary groups will respond to the biuret test.

Protein responds positively since there are pairs of CONH groups in the molecule. A copper coordination complex with the ring structure is probably produced. Short chain polypeptides give a pinkish violet color, longer one including proteins a more purple blue. The amino acid histidine gives a pink color, which depends on the peptide linkage, does not vary greatly in intensity from protein to protein. Several procedure based on this method have been suggested for quantitative determination of milk proteins, but it is not in general use in dairy research.

Materials

1. 10% NaOH
2. 0.5% $CuSO_4$

Method

To 2 to 3 ml of test solution in a test tube add an equal volume of 10% sodium hydroxide solution, mix thoroughly, and add a 0.5% copper sulphate solution drop by drop, mixing between drops, until a purplish-violet or pinkish-violet color is produced. The color depends upon the nature of the protein, proteoses and peptones give a decided pink; the color produced with gelatin is not far removed from a blue.

Ninhydrin reaction

Principle

This test gives positive results with proteins, peptones, peptides, amino acids and other primary amines, including ammonia. Proline and hydroxyproline give yellow color with ninhydrin, while other acids give blue to purple color.

Materials

1. 0.1% Ninhydrin
2. pH paper

Method

To 5 ml of dilute test solution, which must be approximately between pH 5 and pH 7 (a few drops of pyridine or a few crystals of sodium acetate may be used to adjust the pH), add 0.5 ml of 0.1 % ninhydrin, heat to boiling for one to two minutes, and allow to cool. A blue color develops if the test is positive.

Add 5ml of the alkaline solution to 1 ml of the test solution. Mix thoroughly and allow to stand at room temperature for 10 mins. Add 0.5 ml diluted Folin-Ciocalteau reagent rapidly with immediate mixing. Observe for development of color after 30 mins. Development of characteristic blue color indicates presence of indolyl or phenol group.

Sakaguchi test

Principle

Arginine and other guanidyl derivatives (glycocyamine, methylgyanidine etc) react with hypo bromide and alpha napthol to give a red colored product.

Materials

1. Sodium hydroxide solution (40%)
2. Alpha napthol solution (1% in alcohol)
3. Bromine water (a few drops of bromine in 100 ml distilled water)

Method

Mix 1 ml of sodium hydroxide with 3 ml of test solution and add 2 drops of alpha napthol. Mix thoroughly and add 4 to 5 drops of bromine water. Note the color formed. Formation of a red color indicates presence of guanidine group. This is a very sensitive and specific test.

Nitroprusside test

Principle

Sodium nitroprusside reacts with compounds containing sulphahydryl groups produce an intensely red but somewhat unstable color.

Materials

1. Sulphur amino acids (1.0% cystine, cysteine and methionine)
2. Sodium nitroprusside (2% prepared fresh)
3. Ammonium hydroxide

Method

Mix 0.5 ml of a fresh solution of sodium nitropruside with 2 ml of the test solution and add 0.5 ml of ammonium hydroxide.

General Test for Lipid

Lipid, any of a diverse group of organic compounds including fats, oils, hormones, and certain components of membranes that are grouped together because they do not interact appreciably with water. One type of lipid, the triglycerides, is sequestered as fat in adipose cells, which serve as the energy-storage depot for organisms and also provide thermal insulation. Some lipids such as steroid hormones serve as chemical messengers between cells, tissues, and organs, and others communicate signals between biochemical

systems within a single cell. The membranes of cells and organelles (structures within cells) are microscopically thin structures formed from two layers of phospholipid molecules. Membranes function to separate individual cells from their environments and to compartmentalize the cell interior into structures that carry out special functions. So important is this compartmentalizing function that membranes, and the lipids that form them, must have been essential to the origin of life itself.

1. Sudan III test Procedure:
Take 0.5 ml ether or chloroform in a test tube and add 0.5 ml sample—drop by drop till the sample is fully dissolves. Add one drop of Sudan III reagent.

Observation:
Red colour appears.

Inference:
The sample contains fat.

2. Acrolein test:

Procedure:
Take 0.5gm powdered sodium bi-sulphate ($NaHSO_4$) or potassium bi-sulphate ($KHSO_4$) in a clean dry test tube, add 3 to 4 drops of sample. Mix thoroughly and heat.

Observation:
An irritating smell of acrolein is felt.

Inference:

Sample contains fat.

Solubility Test for Lipid:

Procedure:
Take five test tubes marking A, B, C, D, E. Put 5 ml—water, absolute alcohol, ether, chloroform and benzene one in each test tube. Add 3 to 4 drops of sample in each test tube, shake thoroughly, allow to stand.

Observation:
(1) In test tube A drops of oils are seen floating on the surface of water,

(2) In test tube B oil drops settle at the bottom of alcohol,

(3) In test tubes C, D and E the sample is mixed.

Inference:
The sample contains fat, as it is not soluble in water (test tube A) but soluble only in organic solvents (test tube B, C, D, E) and sinks to the bottom in alcohol (B).

Emulsification Test for Lipid:

Procedure:

Take 3 ml sample in a test tube, add 2 drops of oleic acid, shake well. Add 2 drops of the mixture to another test tube containing 3 ml 10% caustic soda.

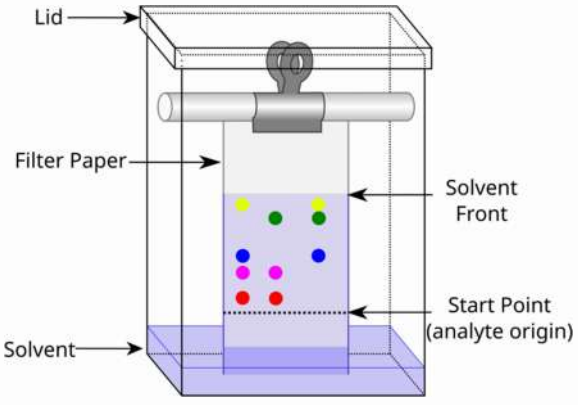
Observation:
Emulsion is formed (acid neutralize alkali forming soap).

Inference:
The test sample contains fat.

Paper Chromatography of Amino Acids

Introduction

Chromatography is a collective term for a set of analytical techniques used to separate mixtures. Chroma means color and graph means to write or draw. Paper chromatography is an analytical technique used to separate mixtures of chemicals (sometimes colored pigments) using a partitioning method. The paper in this method is called the stationary phase because it does not move and serves as a substrate or surface for the separation. Analytes (substances being analyzed) are separated from each other based on a differential affinity to a solvent. The solvent dissolves and carries the analytes along the matrix of the stationary phase. Since the solvent moves through a wicking action, it is called the mobile phase.

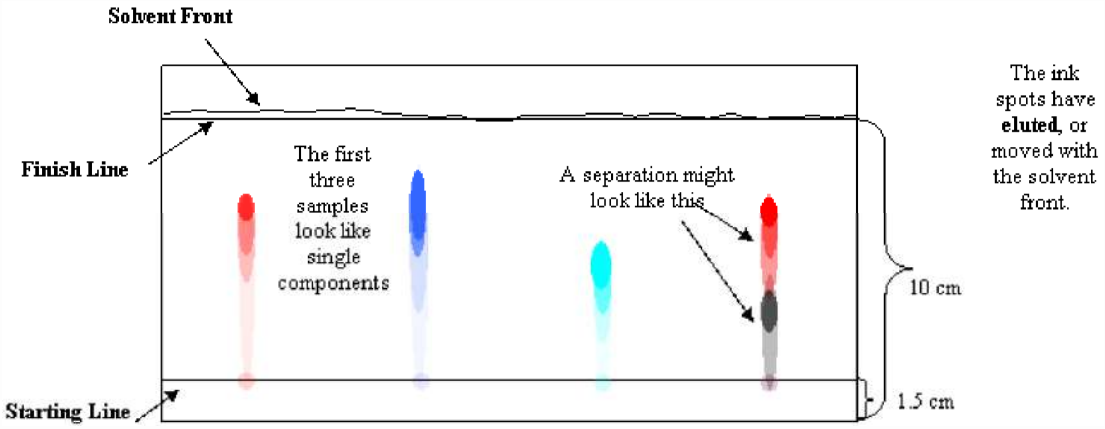


The figure above shows a chamber containing mobile phase and a prepared paper stationary phase. A line drawn at the bottom edge of the paper is the starting point/line. The starting line and sample dots must be above the level of the mobile phase when the paper is placed inside the chamber. If the starting line is below the liquid level, the samples will wash out into the mobile phase rather than elute up the stationary phase. Another line is drawn about 10 cm above the bottom edge of the paper. This is the finish line. Its location was chosen for this

experiment because when the eluting solution reaches that line, any inks that are mixtures should be clearly separated.

When the solvent front reaches the finish line, the paper should be removed immediately from contact with the mobile phase.

The figure below shows a typical paper chromatogram of the separation of pen inks. There are a few difficulties commonly encountered in the elution process. One problem is that spots tend the spread out as they elute, and can bleed into each other as they proceed up the paper. This can be confusing when interpreting the chromatogram. To avoid this problem, space the spots of sample far apart and make repeated, tiny applications of sample to prevent spreading. Another problem is an uneven solvent front. This can happen if the beaker is nudged – if the mobile phase sloshes inside, the elution trails may travel diagonally, which makes interpretation very difficult. This can also happen if the two edges of the chromatogram are allowed to touch when they are stapled or taped together to form a cylinder.



A component with a given solubility travels along with the mobile phase at one rate, regardless of what other components are present in the sample. If the red part of purple ink travels at the same rate as pure red ink, and both stop in the same place, the two should be the same red ink. The two red spots should have the same Retention Factor, Rf. The Rf is the distance, D, traveled by the spot divided by the distance traveled by the eluting solution, or Solvent Front, F.

Rf= frac DF

Comparing the Rf values allows the confirmation of a component in multiple samples because unique components have unique Rf values.

Experimental Procedure

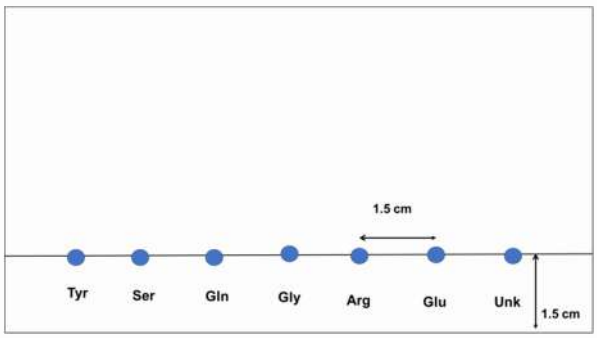
Materials and Equipment

Materials: chromatography paper, amino acids (1% solutions): tyrosine, serine, glutamine, glycine, arginine, glutamic acid and unknown, eluting solution (isopropyl alcohol, 0.5 M NH4OH; 0.2% ninhydrin spray).

Equipment: 600-mL beaker, pencil, ruler, evaporating dish, toothpicks, hair dryer, stapler and paper towels.

Part A: Preparation of Chromatography Paper

1. Wash your hands thoroughly to remove excess oils from your skin. Obtain a ruler and a piece of chromatography paper from the fume hood. Handle the paper only on the edges to avoid leaving fingerprints, as these may hinder the elution process.
2. Place the chromatography paper on a sheet of clean notebook paper or paper towel to avoid picking up dirt or contaminants from the bench top. Orient the paper into a "landscape" position and write your name on the top edge of the paper in one corner. Using a pencil and ruler to measure accurately, draw a straight line across the paper, about 1.5 cm above the bottom edge. This is the starting line. At the top of the paper draw a straight line across the paper about 2.0 cm from the top, this is finish line.
3. On the starting line, measure in from one side about 2.5 cm and lightly draw a small "X" centered on the starting line. Draw six more, 1.5 cm apart.
4. In the center of each X, make a small spot of amino acid sample in this order: tyrosine, serine, glutamine, glycine, arginine, glutamic acid and unknown.
5. When you have finished, you should have something that looks like ?????? below.
6. Go back over each amino acid spot a second time to ensure there is enough amino acid in the spot.
7. Gently curl the paper into a cylinder, with the spots on the outside. Staple the ends together near the top and bottom, taking care that the two edges of the paper do not touch. If they do touch, the eluent will creep on a diagonal, and the spots will run together, or not in straight lines.



Part B: Acquisition of Chromatogram

1. Take a 600-mL beaker and pour 10-mL of 0.5 M NH_4OH and 20 mL of isopropyl alcohol (eluting solution) into the beaker. Obtain your evaporating dish and use it to cover the beaker.
2. Gently place the paper cylinder into the beaker and cover the top with the plastic wrap. Remember that the spots must be above the liquid level for the experiment to work. Watch the eluent creep up the paper until it is about 2 cm from the top. It will take about 45-60 minutes for the solvent front to reach the finish line.
3. When the solvent front reaches the finish line, remove the paper from the beaker, being careful to touch only the top. Let excess eluent drip into the beaker. Gently remove the staple and lay the chromatogram on a piece of paper towel. Use a hair dryer to dry the chromatogram completely. Pour the eluting solution in the organic waste container under the fume hood.
4. Working in the fume hood, spray the chromatogram lightly with the ninhydrin solution. Dry the sprayed chromatogram with a hair dryer, distinct colored spots will appear as a result of the ninhydrin reacting with the amino acids.
5. CAUTION: Use the ninhydrin inside the fume hood. Do not breathe the fumes or get spray on your skin.

Part C: Interpretation of Chromatogram

1. Circle around each color spot.
2. Use a ruler and draw a plus sign in the center of each spot. Measure the distance from the starting line to each plus sign. Record this distance for each spot on your lab report. These are the DD values, in cm.
3. Measure the distance between the starting line and the finish line or, the farthest up that the solvent front reached. Record this distance. This is the FF value, in cm.
4. Calculate the retention factor (Rf) for each spot and record the values in your lab report.
5. You and your lab partner will hand in your lab reports at the same time, with the paper chromatogram stapled to one of the lab reports.

Quantitative estimation of Lowry Methods.

INTRODUCTION:

Measurement of the quantity of the protein present in the solution is common and fundamental methods used in all laboratories. Generally these are of two types-

a. Direct spectophotometric method
b. Colorimetric method

Colorimetric method such as Biuret method, Lowery method, Bradford method etc are used extensively. These are the destructive method of protein analysis because the protein being measure cannot be recovered after such analysis. In this colorimetric method protein concentrations are measured by taking absorbance of visible wed length of light.

PRINCIPLE:

The "Lowery or Folin-Ciocalteau method" combines the copper reaction of the biuret method and the Folin-Ciocalteau reagent which reacts with tyrosine residues in proteins. When alkaline copper sulphate solution containing Sodium-Potassium Tartarate reacts with a protein solution, the copper ions forms a co-ordination complex with four NH peptide bond groups. In addition the phosphor molybdate present in the Folin-Ciocalteau reagent is reduced by tyrosine and tryptophan (aromatic amino acid) present in the protein, produce a dark blue/ purple colour complex, with maximum absorbance at 700nm. The intensity of the colour depends on the amount of these aromatic amino acids present which thus very with the different proteins present in the test sample.

CHEMICALS:

1. Alkaline sodium carbonate solution(20g/liter Na_2Co_3 in 0.1 mol/ltr NaOH)
2. Copper sulphate sodium Patasium tartarare solution(5g/ltr $CuSo_4$,$5H_2O$ in 10g/ltr Na-K tartarate).
3. Alkaline solution, prepared freshly by using 50ml of solution1 and 1ml of solution 2.
4. Folin-ciocalteau reagent(containing solution of sodium tungstate and sodium molybolate in phosphoric and hydrochloric acids and it is commercially available). Diluted the commercially available reagent with an equal of water just before use.
5. Standard sample preparation:- BSA standard solution are prepared by dissolved BSA in DH_2O. The concentration of new stock sample is 0.1mg/ml.
6. At first 0.1ml, 0.2ml, 0.3ml, 0.4ml, 0.5ml, 0.6ml, 0.7ml, 0.8ml, 0.9ml of protein sample is taken in dry test tube from 2nd stock or diluted stock solution of protein(BSA). Then the volume make upto 1ml by adding distilled water in test tube and prepared different concentration of protein solution(20, 40, 60, 80μg/ml).

OTHER REAGENTS:

i. Test tube
ii. Test tube rack
iii. Tissue paper
iv. Graph paper
v. Reagent bottle
vi. Beakers
vii. Measuring cylinders
viii. Posteur pipettes
ix. Notepad
x. Calculator

PROCEDURE:

1. previously prepared BSA stock solutions are used(10μg/ml, 20μg/ml, 30μg/ml, 40μg/ml, 50μg/ml, 60μg/ml, 70μg/ml, 80μg/ml, 90μg/ml).
2. 1ml distilled water is taken in a dry test tube and marked as "Blanked" there is no protein solution is present.
3. 1ml alkaline solution is added into each and every test tube.
4. Solution are incubated for 15 min at room temperature.
5. 0.1ml Folin reagent is added into each test tube.
6. Next the complete mixture are allowed for incubation at room temperature for 30 min. This incubation should be in dark.
7. Last the OD are measure in colorimeter a 700nm and graph is plotted based on the OD value.

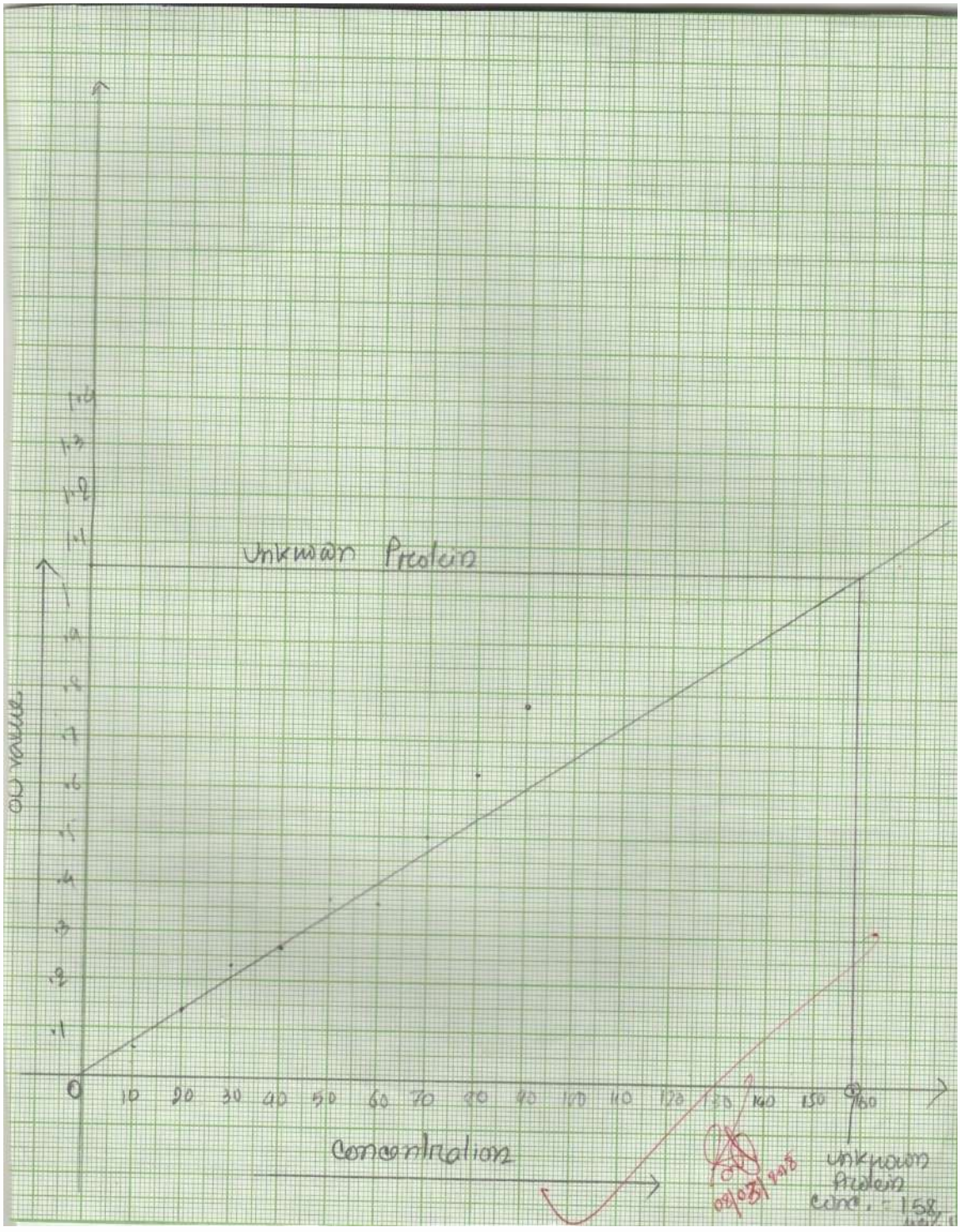
PREPARATION OF BSA STANDARD CURVE:

SAMPLE NO.	BSA CONCENTRATION (μg)	BSA TAKEN (μl)	DISTILLED WATER (μl)	OD VALUE	CORRECT OD VALUE
1.	10	100	900	0.07	0.06
2.	20	200	800	0.15	0.14
3.	30	300	700	0.24	0.23
4.	40	400	600	0.28	0.27
5.	50	500	500	0.37	0.36
6.	60	600	400	0.38	0.37
7.	70	700	300	0.51	0.50
8.	80	800	200	0.64	0.63

9.	90	900	100	0.78	0.77
BLANK	0	0	1000	0.01	

SAMPLE	PROTEIN TAKEN (μl)	DISTILLED WATER (μl)	OD VALUE	CORRECTED OD VALUE
UNKNOWM	400	600	1.06	1.05

CALCULATION: According to the graph the concentration of unknown sample is 158μg/ml.



Demonstration of proteins separation by SDS-PAGE.

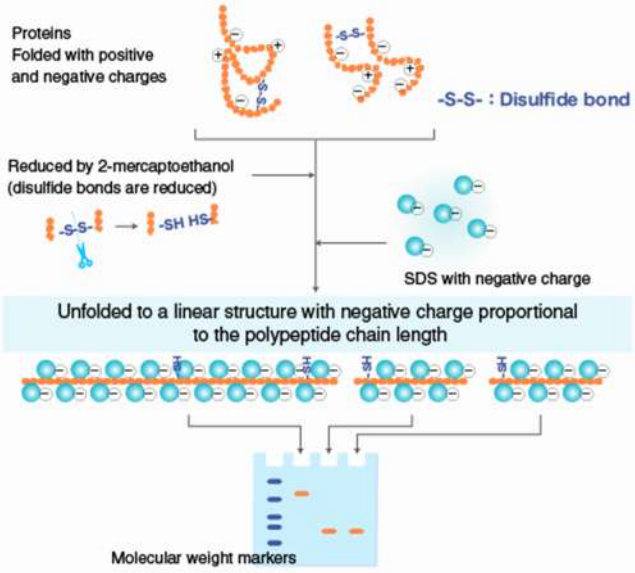
SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) is a technique used to separate the proteins according to their masses. Separation of macromolecules under the influence of the charge is called **electrophoresis**. The gel used in **SDA-PAGE** is polyacrylamide and agent which is used to linearize the proteins is SDS.

Principle of SDS-PAGE

Protein samples and ladder are loaded into wells in the gel and electric voltage is applied. A reducing agent such as mercaptoethanol or dithiothreitol (DTT) (in the presence of a detergent i.e. SDS) breaks down the disulfide bridges that are responsible for protein folding; and a detergent such as SDS imparts negative charge to the proteins thereby linearizing them into polypeptides. Polyacrylamide provides a matrix for the polypeptides to run. Polypeptides run towards the positive electrode (anode) through the gel when electric field is applied. Electrophoretic mobility of the proteins depends upon 3 factors:

- **Shape** – All the proteins are in the primary structure after the treatment with a reducing agent. So, shape doesn't affect the protein separation.
- **Charge** – All the proteins are negatively charge proportional to their molecular weight after treatment with SDS. So charge doesn't affect the separation.
- **Size**– proteins get separated solely on the basis of their molecular weight.

Smaller polypeptides move faster because they have to face less hindrance, larger ones move slower because of greater hindrance. Hence proteins get separated only on the basis of their mass.



Materials Required For SDS PAGE

Acrylamide solutions (for resolving & stacking gels).

Isopropanol / distilled water .

Gel loading buffer.

Running buffer.

Staining, destaining solutions.

Protein samples .

Molecular weight markers.

The equipment and supplies necessary for conducting SDS-PAGE includes:

An electrophoresis chamber and power supply.

Glass plates(a short and a top plate).

Casting frame .

Casting stand.

Combs .

Reagents

1. 30% Polyacrylamide solution(29g acrylamide+1g bisacrylamide in 50 mL of water, dissolve completely using a magnetic stirrer, make the volume upto 100mL). Keep the solution away from sunlight.
2. 1.5 M Tris, pH 8.8
3. 1 M Tris, pH 6.8
4. 10% SDS (10 g SDS in 100mL distilled water).
5. 10% ammonium persulfate (0.1 g in 1 ml water). It should be freshly prepared.
6. 10x SDS running buffer(pH ~8.3) - Take 60.6 g Tris base, 288g Glycine and 20g SDS in separate beakers and dissolve them using distilled water. When properly dissolved ,mix three of them and make upto 2L.(working standard is 1X buffer).

Resolving gel (10%)

dH_20	4.0 ml
30% acrylamide mix	3.3 ml
1.5M Tris pH8.8	2.5 ml
10% SDS	0.1 ml
10% ammonium persulfate	0.1 ml
TEMED	0.004ml

Stacking gel (5%)

dH_20	5.65 ml
30% acrylamide mix	1.65 ml
1.0M Tris pH 6.8	2.5 ml
10% SDS	0.1 ml
10% ammonium persulfate	0.1 ml
TEMED	0.004ml

Gel loading buffer:

To make 10 mL of 4X stock:

2.0 ml 1M Tris-HCl pH 6.8.
2. 0.8 g SDS.
3. 4.0 ml 100% glycerol.
0.4 ml 14.7 M β-mercaptoethanol.
1.0 ml 0.5 M EDTA.
8 mg Bromophenol Blue.

Staining solution:

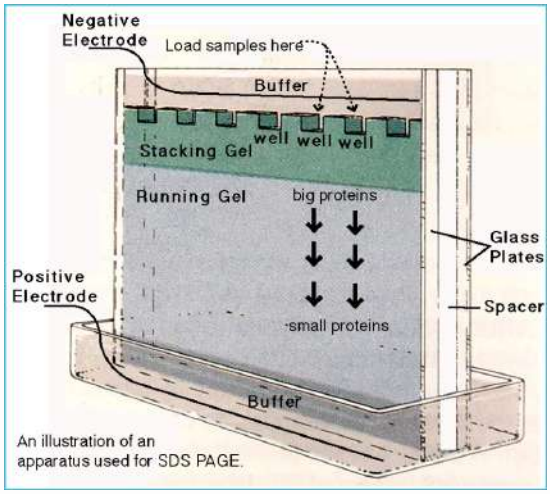
Weigh 0.25g of Coomassie Brilliant Blue R250 in a beaker. Add 90 ml methanol:water (1:1 v/v) and 10ml of Glacial acetic acid ,mix properly using a magnetic stirrer. (when properly mixed, filter the solution through a Whatman No. 1 filter to remove any particulate matter and store in appropriate bottles)

Destaining solution:

Mix 90 ml methanol:water (1:1 v/v) and 10ml of Glacial acetic acid using a magnetic stirrer and store in appropriate bottles.

Major steps of SDS-PAGE

Pouring of the resolving gel:



An illustration of an apparatus used for SDS PAGE.

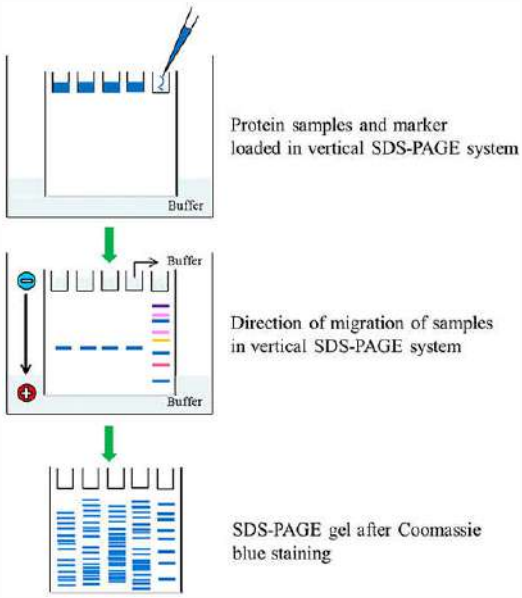
Resolving gel is poured between two glass plates (one is called short plate and the other one is tall plate), clipped together on a **casting frame** (Fig. 05) Bubbles are removed by adding a layer of isopropanol on the top of the gel. (The level of the gel is predetermined by placing the comb on the glass-plates and leaving approximates 1cm space below the comb. Use a pen to mark the level. Now pour the gel up to this mark.) The gel is then allowed to solidify. When the gel is solidified, remove the isopropanol by using a filter paper.

Pouring of the stacking gel:

When the resolving gel is solidified, stacking gel is loaded all the way to the top of the glass plates. Comb is placed just after loading. The gel is, then, allowed to polymerize (solidify). When stacking gel is solidified, comb is removed very carefully not damaging the well's shape.

Loading the ladder in wells

Add the ladder very carefully into the well which is on the extreme right using a micropipette. The samples are loaded into the other wells. Ladder is mostly pre-stained with the known molecular weight proteins.



Loading the ladder in wells

Loading the samples in wells

Samples are loaded in each well with equal amount of the proteins mixture using micropipette. Be careful while loading the samples. Make sure not to damage the size of the wells or not to pour the sample out of the well instead of pouring inside it. At this stage, sample of the proteins appears to be blue because of a dye (bromophenol) used while preparing the sample.

Running the gel by applying voltage

A voltage is applied after dipping the "sandwich of gel and glass plates" in running buffer. Turn of the voltage when the tracking dye has reached or crossed the gel. The gel is further proceeded for the subsequent analysis.

Subsequent analysis – Coomassie Blue Staining

The gel is rinsed with deionized water 3-5 times to remove SDS and buffer. It may create hindrance with the binding of the dye (0.1% Coomassie Blue) to the proteins. The gel is then dipped in Coomassie Blue stain (staining buffer) on a shaking incubator at room temperature. The invisible bands of the proteins beginning to appear within minutes but it takes approximately 1h for complete staining.

To perform the Acid and Alkaline phosphatase assay from serum/ tissue

Alkaline Phosphatase (ALP)

Inrtoduction:

Alkaline phosphatase (**ALP**, or **basic phosphatase)** is a homodimeric protein enzyme of 86 kilodaltons. Each monomer contains five cysteine residues, two zinc atoms and one magnesium atom crucial to its catalytic function, and it is optimally active at alkaline pH environments.

ALP has the physiological role of dephosphorylating compounds. The enzyme is found across a multitude of organisms, prokaryotes and eukaryotes alike, with the same general function but in different structural forms suitable to the environment they function in. Due to its widespread prevalence in these areas, its concentration in the bloodstream is used by diagnosticians as a biomarker in helping determine diagnoses such as hepatitis or osteomalacia.

Procedure

I. Tissue homogenate preparation

5%, weight/volume, tissue homogenate was prepared in chilled 0.25M sucrose solution using tissue homogenizer. Tissue homogenate solution were centrifuged in 10000 RPm and the clear supernatants were stored at -20°c until use.

II. Reagents:

1. 0.25M Sucrose: 4.278gm of sucrose in 50ml DH_2O
2. 0.1 M Magnesium chloride: 0.203 gm of $MgCl_2$ in 10 ml of DH_2O

3. 0.1 M Para-nitrophenyl phosphate:0.3712gm PNPP in 10 ml of DH_2O
4. 0.1 N NaOH: 0.08gm of NaOH in 20ml of DH_2O
5. Carbonate-bicarbonate buffer stock (pH 9.2): Sodium carbonate, anhydrous and Sodium bicarbonate.
   i. Prepare a 0.2-M solution of anhydrous sodium carbonate (2.2 g/100 mL).
   ii. Prepare a 0.2-M solution of sodium bicarbonate (1.68 g/100 mL).
   iii. Combine 4 ml of carbonate solution and 46 ml of bicarbonate solution (Total 50 ml)
   iv. Bring to 200 mL with H_2O.
   v. Final pH will be 9.2.

III. Reaction Mixture

The assay mixture comprised of

i. 0.2 ml bicarbonate buffer (0.2M),
ii. 0.1 ml of 0.1 M magnesium chloride,
iii. 0.1 ml tissue homogenate,
iv. 0.5 ml of distilled water
v. 0.1 ml of freshly prepared 0.1 M para-nitrophenyl phosphate.

The reaction mixture will be incubated in water bath at 37°c for 15 mins. and the reaction will be stopped by 1.0 ml of 0.1 N NaOH. OD 410nm will be recorded against blank.

Acid Phosphatase (ACP)

Introduction:

Acid phosphatase is a phosphatase, a type of enzyme, used to free attached phosphoryl groups from other molecules during digestion. It can be further classified as a phosphomonoesterase. Acid phosphatase is stored in lysosomes and functions when these fuse with endosomes, which are acidified while they function; therefore, it has an acid pH optimum. This enzyme is present in many animal and plant species. It is used as a cytogenetic marker to distinguish the two different lineages of Acute Lymphoblastic Leukemia(ALL) : B-ALL (a leukemia of B Lymphocytes) is Acid-Phosphatase negative , T-ALL (originating instead from T Lymphocytes) is acid-phosphatase positive .

Acid phosphatase catalyzes the following reaction at an optimal acidic pH (below 7):

Orthophosphoric monoester + H_2O → alcohol + H_3PO_4

Phosphatase enzymes are also used by soil microorganisms to access organically bound phosphate nutrients. An assay on the rates of activity of these enzymes may be used to ascertain biological demand for phosphates in the soil.

Procedure

I. Tissue homogenate preparation

5%, weight/volume, tissue homogenate was prepared in chilled 0.25M sucrose solution using tissue homogenizer. Tissue homogenate were centrifuged in 10000 RPm and the clear supernatants were stored at -20°c until use.

II. Reagents:

1. 0.25M Sucrose: 4.278gm of sucrose in 50ml DH_2O
2. 0.1 M Magnesium chloride: 0.203 gm of $MgCl_2$ in 10 ml of DH_2O
3. 0.1 M Para-nitrophenyl phosphate:0.3712gm PNPP in 10 ml of DH_2O
4. 0.1 N NaOH: 0.08gm of NaOH in 20ml of DH_2O
5. Sodium acetate buffer (0.2 M, pH 5.0) **:**Quantity (for 200ml)

i.	Sodium acetate trihydrate	5.443 g
ii.	Glacial acetic acid	1.2 mL
iii.	H_2O	198.8 mL

Combine the reagents and adjust the pH to 5.0 with 10 N NaOH.

III. Reaction Mixture:

The assay mixture comprised of

i. 0.2 ml acetate buffer (0.2 M, pH 5.0)
ii. 0.1 ml of 0.1 M magnesium chloride,
iii. 0.1 ml tissue homogenate,
iv. 0.5 ml of distilled water
v. 0.1 ml of freshly prepared 0.1 M para-nitrophenyl phosphate.

The reaction mixture will be incubated in water bath at 37°c for 15 mins. and the reaction will be stopped by 1.0 ml of 0.1 N NaOH. OD 410nm will be recorded against blank.

To study the enzymatic activity of Trypsin and Lipase

Effect of Bile Salt on Pancreatic Lipase Action

The major dietary lipids for humans are animal and plant triglycerides, sterols, and membrane phospholipids. Digestion of lipids occurs in the duodenum. Lipids are emulsified by the action of bile secreted from the gall bladder and hydrolysed by the action of lipases secreted from the pancreas. The site of action of lipase is the interface between the lipid droplets and the aqueous phase, so that the degree of emulsification plays an important role in establishing the active substrate concentration. Degradation is incomplete, triglycerides are broken down to a mixture of diglycerides, monoglycerides, glycerol and fatty acids. The reaction can be followed by noting the change of pH with time. The source of lipase used in this experiment is pancreatin, a crude extract of animal pancreas, containing amylase, trypsin, lipase, ribonuclease and protease. It is used internally as a digestive, and also in the preparation of predigested food.

Materials and apparatus

(1) Porcine pancreatin powder (Sigma)

(2) Fresh milk

(3) Phenol red solution. (0.8 mg/ml of the sodium salt in distilled water)

(4) 0.2 M Na_2CO_3

(5) 1 M HCI

(6) Sodium cholate solution. (Prepared by adding 10% NaOH slowly with stirring to a suspension of 10 g cholic acid in 150 ml distilled water, until the pH was 8.0 and the solids dissolved. The solution was made up to 200 ml with distilled water.)

(7) 37°C water bath.

Experimental procedure

(1) Pipette 10 ml of distilled water to 100 mg of porcine pancreatin to make a suspension of 10 mg/ml. Place the enzyme sample on ice.

(2) Neutralize fresh milk in the following way: to 40 ml of milk add 10 drops of the phenol red solution and 10 ml 0.2 M Na_2CO_3 to make it pink in colour.

(3) Draw 5 ml of the neutralized milk into each two tubes. To one of them add 0.2 ml 1 M HCI to make it yellow in colour. Place these two tubes aside as reference for the colour change.

(4) Set up the following tubes:

Tube Contents	T1	T2	C1	C2
Neutralized milk	5 ml	5 ml	5 ml	5 ml
Sodium cholate solution		1ml		1ml
Distilled water	1ml		1ml	

(5) Place the tubes in the 37°C water bath for equilibration. At intervals of 15 s, add 2 ml of the pancreatin suspension to T1 and T2, and 2 ml of distilled water to CI and C2. (Note: shake up the pancreatin suspension during pipetting.)

(6) Note the time required for the colour change and also note the odour of the tubes from time to time.

Results

i. The time required to notice a colour change in T1 is typically 4-6 min. Addition of cholate (T2) enhances the activity of lipase, thereby reducing the time required to about half.
ii. The observations in C1 and C2 should serve the purpose of demonstrating to the students that there is no intrinsic lipase activity in the sodium cholate solution added.
iii. The odour arising from the volatile short chain fatty acids typical of diary products should be noticed in T 1 and T 2.

Activation of Chymotrypsinogen to Chymotrypsin

The digestion of proteins is catalyzed by several proteolytic enzymes, such as pepsin of gastric origin, and trypsin, chymotrypsin, carboxypeptidases and elastase of pancreatic origin. These proteases display a certain selectivity towards the peptide bonds they cleave. Thus the digestive actions of these enzymes are complementary and, together with the aid of other intestinal enzymes, amino acids are formed. The specificity of some of the digestive proteases is given in Table 1. These proteases are derived from their respective inactive proenzymes, or zymogens. In this experiment, chymotrypsinogen is assayed first for intrinsic chymotryptic activity, then activated by adding a dilute trypsin solution, and reassayed.

Materials and apparatus

(1) Chymotrypsinogen (Sigma);

(2) Trypsin (Sigma)

(3) 1 mM N-succinyI-L-phenylalanine-p-nitroanilide (Prepared by dissolving 385.4 mg of the substrate in 20 ml methanol with warming. Make up the solution to 1 litre with 50 mM Tris HCI 20 mM $CaCI_2$ pH 7.6.)

(4) 1 mM HCI

(5) 200 mM Tris HCI, pH 7.6

(6) 50 mM Tris HC1, 20 mM $CaC1_2$, pH 7.6

(7) 30% acetic acid

(8) 37°C water bath

(9) Spectrophotometer (410 mm).

Experimental procedure

(1) Pipette 3 ml of 1 mM HC1 to 60 mg of chymotrypsinogen to make a solution of 20 mg/ml. Allow the protein to dissolve by itself. Agitate gently if necessary but do not shake. Place the enzyme solution on ice.

(2) Assay the chymotrypsinogen solution for chymotryptic activity as described in step 3 below.

(3) The chymotryptic activity of an enzyme sample is assayed in the following way: pipette 5 ml of 1 mM N-succinyl-L-phenylalanine-p-nitroanalide (in 50 mM Tris HC1, 20 mM CaC12, pH 7.6) into each of the two test-tubes (assay and blank) and equilibrate at 37°C. Add 1 ml of the enzyme sample to the assay tube and incubate at 37°C for 20 min. Then add 1 ml of 30% acetic acid to both the assay tube and the blank tube. Lastly add 1 ml of the enzyme solution to the blank tube. The colour developed is stable and you can read the absorbance at the end of all the incubations.

(4) Activate the chymotrypsinogen at room temperature in the following manner:

Tube contents	Activation Test	Mixture Control
Chymotrypsinogen (20 mg/ml in 1 mM HCI)	0.5 ml	-
1 mM HC1	-	0.5 ml
0.2 M Tris HCI, pH 7.6	0.4 ml	0.4 ml
Trypsin solution (1 mg/ml in 1 M HC1, freshly prepared)	0.1 ml	0.1 ml

Add the trypsin solution last and note the time. The control tube is to show that the increased chymotryptic activity noted afterwards is not due to the added trypsin.

(5) At time = 10 min, take an 0.2 ml aliquot of the activation mixture (test and control) to a tube containing 4.8 ml 1 mM HC1 on ice (x25 diluted). Immediately assay the chymotryptic activity of the diluted enzyme sample as described in step 3 above. Each enzyme sample should have its own blank.

(6) Read the absorbances of the chymotryptic assays at 410 nm against their own blanks.

Results

Typically, an absorbance of less than 0.1 is observed for the chymotrypsinogen sample before activation. After activation the value will increase to about 0.6.

Note that this drastic increase in chymotryptic activity is not due to the added trypsin since the control tube gives a value close to zero.

Sample Calculation Molar extinction coefficient of p-nitroaniline = 8800 litre mo1-1 cm -1 410 nm Raw data = A OD/20 min = 0.5 (say)

One unit of chymotrypsin generates one ~mol of p-nitroaniline per min.

Therefore, amount of chymotryptic activity 0.5 1 7 20 x ~ × ~ × 106 units (volume of final assay mixture = 7 ml)

Then determine how much protein is in the 1 ml enzyme sample used in the assay.

For the original chymotrypsinogen solution (20 mg/ml) 1 ml'-= 20 mg

For the x25 diluted activation mixture (10 mg/ml) 1 ml0.4 mg Express the chymotryptic activity of the enzyme sample as specific activity (ie in units per mg protein)