PART I

CHAPTER III

MATERIALS AND METHODS
# MATERIALS AND METHODS

## THE SPECIMENS AND THEIR NUMBER USED IN THE PRESENT STUDY

<table>
<thead>
<tr>
<th>CLASS</th>
<th>SPECIMENS</th>
<th>NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHONDRICHTHYES</td>
<td>SHARK</td>
<td>4</td>
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<tr>
<td>OSTEICHTHYES</td>
<td>OPHIOCEPHALUS PUNCTATUS (LATA)</td>
<td>15</td>
</tr>
<tr>
<td>AMPHIBIA</td>
<td>(a) TOAD</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(b) FROG</td>
<td>5</td>
</tr>
<tr>
<td>REPTILIA</td>
<td>(a) TURTLE</td>
<td>7</td>
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<tr>
<td></td>
<td>(b) LIZARD</td>
<td>3</td>
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<tr>
<td>AVES</td>
<td>(a) PIGEON</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>(b) COCK</td>
<td>7</td>
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<tr>
<td></td>
<td>(c) SPARROW</td>
<td>8</td>
</tr>
<tr>
<td>MAMMALIA</td>
<td>(a) MOLE</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(b) GUINEA PIG</td>
<td>6</td>
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<tr>
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<td>(c) ALBINO RAT</td>
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<tr>
<td></td>
<td>(d) RABBIT</td>
<td>6</td>
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<tr>
<td></td>
<td>(e) PIG</td>
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<td></td>
<td>(f) GOAT</td>
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</tr>
<tr>
<td></td>
<td>(g) CAT</td>
<td>4</td>
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<tr>
<td></td>
<td>(h) DOG</td>
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<tr>
<td></td>
<td>(i) MAN</td>
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</table>
In the specimens of vertebrates mentioned, different aspects of the testis were studied under the following headings:

1. An anatomical study on the testis of vertebrates in respect of the following gross features:
   (i) SITUATION, (ii) SHAPE, (iii) SIZE,
   (iv) CONSISTENCY, (v) ABSOLUTE WEIGHT,
   (vi) RELATIVE WEIGHT IN PROPORTION TO BODY WEIGHT,
   (vii) SPECIFIC GRAVITY OF TESTICULAR TISSUE.

2. A histological study on the testis of the same animals in respect of the following structural elements:
   (i) TUNICA ALBUGINEA, SEPTA, OR TRABECULAE AND LOBULES.
   (ii) SEMINIFEROUS TUBULES, THEIR PATTERN, ARRANGEMENTS OF SPERMATOGENIC CELLS AND SPERMATOZOA, TUNICA PROPRIA ET BASEMENT MEMBRANE.
   (iii) INTERTUBULAR SPACE, INTERSTITIAL TISSUE.

3. A micrometric study on the following:
   (i) THICKNESS OF TUNICA ALBUGINEA, AND TUNICA PROPRIA ET BASEMENT MEMBRANE.
   (ii) WIDTH OF THE INTERTUBULAR SPACE.
   (iii) DIAMETERS OF SEMINIFEROUS TUBULES.
   (iv) NUMBER OF SEMINIFEROUS TUBULES FOUND IN THE EQUATOR OF ONE FIELD UNDER THE SAME LOW POWER OF MICROSCOPE.
SOURCES OF THE SPECIMENS:

The SHARKS, the origin of which was the Bay of Bengal, were procured by arrangement with a fisherman of the local market. OPHIOCEPHALUS PUNCTATUS (LATA), TOADS, FROGS, LIZARDS, TURTLES, PIGEONS, COCKS, SPARRROWS, GOATS, PIGS, CATS, DOGS, MOLES, GUINEA PIGS, ALBINO RATS, RABBITS were all of local origin and procured alive. All the animals under observation were apparently healthy and well-built. Of the total fourteen Albino rats utilised in this study, eight were littered in the department. In the human series, the testes of fresh post-mortem specimens of the adults were obtained within 12 to 48 hours and a few only within 72 hours of death from the Mortuary adjoining the department. As relatively rapid changes occur in the testes due to various systematic diseases, the testes from only the individuals of accidental death or sudden death having no apparent sign of wasting diseases, excessive edema or haemorrhage, were obtained at necropsy.

WEIGHTS OF THE ANIMALS

Weights were taken in shirks without any difficulty, in Lizards, Pigeons, Cocks, Sparrows, Moles, Guinea Pigs, Albino rats, Rabbits, Cats and Dogs under ether anesthesia, in Toads and Frogs while stunned or pithed, in Ophiocephalus punctatus (Latas) while dead after taking them out of the preserving water, in Turtles in dorsal position to make them immobile,
and in human subjects just before post-mortem examination for medico-legal purposes started.

**EXPOSURE OF THE TESTIS**

After measurements of weights with or without anaesthesia as the case may be, the testes were properly exposed for the purpose of study in situ in respect of their actual position, shape, size and relations as well as for taking photographs of the organs in situ in selective cases. The method of exposure of the testes varied a little in different vertebrates as mentioned below.

**IN FISHES:**

Abdominal cavity was opened by a longitudinal median incision all through the ventral abdominal wall with a sharp scalpel and the margins of the incised wall were retracted sufficiently by artery forceps. In large sharks, additional transverse incisions in one or both halves of the wall or removal of a portion of the same on either side were necessary for the purpose of proper exposure. Retraction upwards of the bulky lobes of liver that practically overlapped all other structures, was imperative. For better exposure surrounding viscera were also sacrificed in many instances.

**IN TOADS AND FROGS**

The distal ends of the limbs were fixed to a wooden board
by nailing in proper position; the abdominal and thoracic cavities were opened by a cruciate incision on the ventral wall of the abdomen and thorax. The surrounding viscera obstructing proper view of the testes required either removal or retraction.

**IN TURTLES**

The ventral shells were cut off and removed; superimposed viscera, chiefly the coils of intestine and the surrounding viscera were either removed or retracted to bring the testes in proper view.

**IN LIZARDS**

The abdominal and thoracic cavities were opened by a longitudinal median incision on the ventral abdominal wall. The surrounding viscera were retracted to bring the testes in proper view.

**IN AVES, PIGEON, COCKS AND SPARROWS**

The abdomino-thoracic cavity was opened by longitudinal median incision. The removal of ventro-lateral thoracic wall was necessary in every case for the purpose of photography. The surrounding viscera, chiefly the intestine, gizzard and liver were removed for proper exposure of the testes.

**IN PIGS, GOATS, CATS AND DOGS**

A bold longitudinal median incision was made on the
ventral abdominal wall from where two slightly oblique incisions one on either side were made following the vas deferens and spermatic cords along the inguinal canal and extended right upto the scrotum. The spermatic cord, the testis with its appendages were brought into proper view.

**IN MOLES, GUINEA PIGS, ALBINO RATS AND RABBITS**

The animals were placed in dorsal position on a wooden board and kept stretched with thread strings tied to their limbs and to the pegs that were driven at four corners of the board. The abdominal and thoracic cavities were opened by a longitudinal median incision on the ventral abdominal wall. The dorsal abdominal wall was brought into proper view by the removal of the superimposed structures.

**IN HUMAN SUBJECTS**

Abdominal and thoracic cavities were opened by a bold longitudinal median incision from the chin to the symphysis pubis. Two divergent slightly oblique incisions, one on either side from the lower part of the median incision were extended on to the scrotum along the line of inguinal canal, and by retraction of the margins of the incised skin and fascia, the spermatic cord and tunica vaginalis were brought in view. The tunica vaginalis was incised and the testes with their appendages were exposed properly.
COLLECTION OF TESTIS

In every case, after observations in situ for shape, size, colour, level of right and left testes, any abnormality in the testes, such as presence of tumour or cyst, the testes with their appendages were made free from the surrounding structures with a sharp scalpel or scissors and removed practically en masse. The mass was now placed on a small tray and dissected cautiously so that even in smallest testis as in Ophioccephalus punctatus (Latas) and lizards, nothing but the testis with epididymis and vas deferens was retained for intimate gross study of the organ with its appendages. The testis was now separated from the epididymis and the vas deferens. The loose fascia and the fatty body (corpus adiposum) attached to the testis of some animals, were also carefully removed. Thus the testis alone was isolated and collected for further study.

SPECIFIC GRAVITY OR DENSITY

The specific gravity of the testicular tissue was determined by the application of Archimedes' principles as follows:

A 25 c.c. glass cylinder having 125 divisions was placed on a table in front of the observer against light. Distilled water was put into the cylinder upto a certain mark. The whole organ in case of a small testis or in large one only a piece from it, the weight of which in either case previously
had been measured in a chemical balance in metric system, was put now into the cylinder and the difference of water displacement was carefully noted by observation of the present level of water. Subsequently, by mathematical calculation, the water displacement was determined and the specific gravity found out as follows:

\[ \frac{\text{Wt. of the testis or a piece of it in grm.}}{\text{Displacement of water in c.c.}} = \text{Specific gravity or Density.} \]

**WEIGHT OF TESTIS**

The isolated testis free of its appendages as well as loose fascia and fat was weighed in a chemical balance and corrected upto the nearest milligram in every case and recorded accordingly.

**DIMENSIONS (LENGTH, BREADTH AND DORSO-VENTRAL DIAMETER OF TESTIS)**

After the weight and water displacement had been determined as mentioned above, the testis was placed flat on a porcelain smooth table. With the help of a graduated caliper, the length, breadth and dorso-ventral diameter of the organ were measured. The caliper reading was compared with that in a centimeter scale and the verified reading was noted immediately and under separate headings one after the other. As the breadth (side-to-side) and dorso-ventral diameter
often varied considerably in different sites due to shape of
the organ, 3 measurements from different sites for both breadth
and dorso-ventral diameter were taken in every case and the
mean average was calculated and recorded. As regards length,
the longest distance between the poles of the organ was taken
into consideration and recorded as length.

HISTOLOGICAL PREPARATIONS

After the weight and dimensions of the testis had been
taken, the organ was immediately put into the desired fixa-
tive solution and kept in it for 15 to 20 minutes for some
hardening to occur. It seemed quite necessary to avoid any
laceration of tissue while cutting into pieces with a sharp
safety-razor blade. Approximately 8 mm. to 1 cm. thick pieces
were made. The pieces included those of longitudinal and
transverse sections with a portion of the tunica albuginea
in them. The pieces were put into small phials or sample
tubes containing the same kind of fixative solution into
which the organ was first put for hardening purpose. Quantity
of the fixative solution was between 10 to 20 times the volume
of the tissue pieces.

Specimen number, name of fixative, date and time were
written on a piece of leucoplast with glass pencil and labe-
bled on the sample phial or tube for proper identification.
Fixatives used and the time kept in them are noted as follows:

1. Zenker-formol fixative - chiefly used and kept in it for total 18 to 24 hours ordinarily, but in case of Ophioccephalus punctatus (Latas) for 12 to 18 hours only.

2. Formo-saline fixative - used in moderately large number of cases and kept in it for 24 to 48 hours only.

3. Sanfelices fixative - used in some cases of human subjects only and kept in it for 18 to 24 hours only.

4. Bouin's fluid - used only in some cases of amphibians and kept in it for 6 to 12 hours only.

5. Carnoy's fluid - used only in some cases of albino rats and kept in it for a total period of 1 hour only.

Amongst the fixatives tried, Zenker-formol and Formo-saline were finally selected as both of them proved to be equally good.

DEHYDRATION, CLEARING, BLOCKING AND SECTIONING

After fixation, the tissue piece was washed slowly in running tap water for a period varying from 6 to 12 hours (except those fixed in Carnoy's fluid in which case the
tissue was put straightway into 95% Alcohol and the acetic acid washed off in several changes in this 95% alcohol). After washing, the tissue was dehydrated in graded alcohol starting from 50%, 60%, 70%, 80%, 90%, 95% and absolute alcohol (2 changes), cleared in cedar wood oil and in some cases in xylene. Then the tissue was impregnated with soft paraffin 52°C with 2 to 3 changes for 1 hour in each change, and with hard paraffin 60°C for 1 hour and finally embedded in hard paraffin 60 to 62°C, and blocks so made were labelled properly. Serial sections were cut with rotary microtome at 5 to 8 μ thickness; additional sections from some of the blocks of albino rats were cut at 20, 30, and 40, particularly for revealing intraglandular vessels.

STAINS

In every case, the following stains were used:

1. Weigert's Iron-Haemotoxylin and eosin - for cells and connective tissue.
2. Mallory-Heidenhain's stain - for muscles and connective tissue and blood vessels.
3. Van Gieson's stain - for cells and connective tissue.
4. Bielschowsky's silver technique - for reticular tissue and for defining the outline of the seminiferous tubules.
5. Weigert's Resorcin - Fuchsin stain - for elastic tissue.
6. PA-Schiff Haematoxylin in some instances only - for cells and for defining the outline of the seminiferous tubules.

7. Ehrlich's Haematoxylin and eosin - in a few cases at the initial period of work - for cells and connective tissue.

8. ORCEIN stain ) only in limited instances for

9. Verhoeff's stain ) elastic tissue.

10. In 3 cases of albino rats, intra-vital staining with Trypan blue injected subcutaneously 1 c.c. daily for 7 consecutive days was used.

11. In 2 cases, India ink was injected through the heart and the descending thoracic aorta, for intra-vital staining.

MICROMETRY

Measurements were taken with the help of Eye Piece Micrometer and Stage micrometer (German made, Leitz). Eye Piece Micrometer measurement was adjusted to Microns by multiplying with Vernier Factor obtained from different necessary magnifications of the object and the one-millimeter scale fractioned into one thousand Microns.

All counts were made under low power with eyepiece No. 8.
and objective No. 10 i.e., at 80 times magnification of the object, except in case of the measurement of tunica propria cum basement membrane in which counts were made under high power with eyepiece No. 8 and objective No. 40 i.e., at 320 times magnification of the object. Adjustment of the marks in eyepiece was done with those of the stage micrometer scale as follows:

Under low power: - 1 division i.e., 1 small division of stage micrometer is 10 u, and 1 division of eyepiece is 15 u i.e., 10 small divisions of eyepiece micrometer corresponds to 15 small divisions of stage micrometer, i.e., 1 division is to be multiplied by 150 to get the result in micron:

Under high power: - 1 division of stage micrometer is 10 u and 1 small division i.e., 1 division of eyepiece is 4.17 u i.e., 1 division is to be multiplied by 41.7 to get the result in micron.

As far as practicable only those seminiferous tubules cut in cross section and appearing more or less round as judged by their approximation to a circle, were measured. Cross sections of three tubules of different sizes, small, medium and large, in a field were measured by means of an ocular micrometer. From each of these three cross sections
3 measurements, one in vertical direction of the eyepiece scale and the other in transverse direction by rotating the eyepiece clockwise at 90° angle, and the 3rd in oblique direction by rotating the eyepiece scale clockwise to another 45° angle, were measured and the mean calculated and recorded. Therefore, in three seminiferous tubules in one field $3 \times 3 = 9$ measurements were made to avoid any flaw.

In case of intertubular space - 3 spaces were selected, small, medium and large by eye estimation and one measurement in each was made and recorded.

In case of tunica albuginea, 3 sites were selected at random and the measurements were made and recorded.

In case of basement membrane and tunica propria, both together were measured in 3 different tubules and recorded.

Under low power with eyepiece No. 8 and objective No. 10 (80 times magnification). The cross sections of seminiferous tubules irrespective of their sizes but lying in the equatorial line (imagining the eyepiece scale as the equator) of a single field, were counted, and recorded. If more than one-half of a cross section could be visualised within the field, it was included in the count, but if less than one-half was visualised, it was excluded from the count. This was followed in each and every case.
PHOTOGRAPHY AND MICROPHOTOGRAPHY

All photographic exposures were taken with the help of a focar lens No. 2 attached to the Camera lens covered with hood at a distance of 113/4" under illumination with ordinary bulbs of 150 watts and 100 watts placed close to and from above the Camera lens, Camera being fitted on low stand. No attempt, whatsoever, was made to touch the print with a view to retaining naturality of the objects. Only in some cases exposures were taken from a distance of 2.8 feet without focar lens. Exposures for all photographs and microphotographs, both black and white and coloured were taken in the department by the author himself with the help of Petri Camera and Olympic Microscope with microphotographic attachments. Selected area or areas of stained slides were microphotographed.