CHAPTER TWO

MATERIAL AND METHODS
## 2. MATERIAL AND METHODS

### 2.1. MATERIAL

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### 2.2. METHODS

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2.1. MATERIAL:

2.1.1. Selection of animals:

To study the effects of Butea leaf extract the Swiss inbred male albino rats (Hafkine Biopharmaceuticals Ltd., Bombay) were selected as experimental animals. The selected rats were 3-4 months old weighing 250 to 300 g.

2.1.2. Maintenance of rat colony:

The white rat colony was maintained in the animal house of the department. Separate rooms were reserved for record and surgery, case cleaning and storage of food. The rooms in which the experimental animals were kept, were maintained at about 24°C. Exhaust fans were attached to the rooms to decrease odours and moisture generated by rats. All the rooms were protected against the entrance of wild rats, cockroaches and any other animals which may carry infections.

The rats were maintained in the animal cages. All the cages were thoroughly cleaned every alternate day with disinfectants, air dried and then rats were transferred to them. The cages were arranged on clean racks which were also cleaned every alternate day. An accurate weighing balance having capacity to weigh 1 to 500 g was maintained and rats were weighed at regular intervals. For experimental observations hereafter described, rats of a particular weight were taken. The rats were supplied with water ad libitum. Dry food pellets supplied by Hindustan Lever Co. Ltd., Bombay, were used as food for rats.

2.1.3. Plant material:

The leaves of Butea monosperma were collected from the Shivaji University campus, Kolhapur. Leaves were shed dried and powdered.
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2.2.1.a) Method of extraction:

The powdered leaves (60 mesh) were taken in soxhlate and extracted with adequate amounts of ethyl alcohol. The soxhlation was run for 10 to 12 hours. The extractives thus obtained were concentrated by vacuum distillation under reduced pressure and controlled temperature (40°C). The ethanolic extract was then taken in adequate amounts of water. The thick syrupy mass was filtered through Whatman No.1 filter paper. Insoluble portion was removed and water soluble portion was collected. In the next step water was removed under reduced pressure and controlled temperature (75-80°C). The concentrated water soluble extract was dried in a porcelain dish in a vacuum desiccator. One kilogram of air dried leaves of Butea monosperma gave 150 g at alcoholic extract. The alcoholic extract gave 60 g of water soluble portion.

2.2.1.b) Preparation and administration of dose:

One gram of ethanolic water soluble portion was dissolved in 20 ml of mammalian saline (0.9%). Thus 1 ml of saline contained 50 mg of Butea monosperma leaf extract. It was always stored at low temperature in freeze (0° to 2° C). After every fifteen days the extract was prepared freshly in order to maintain the potency of the extract and to avoid the contamination.

Male albino rats were divided into two groups - control and experimental. Each of group consisted of 30 rats. Each animal from experimental group daily received plant extract intraperitoneally (400 mg/kg body weight), while that from control group received equal quantity of saline by the same route. The experiment was conducted for ninety days of duration.
For each parameter to be studied different sets of experiment were planned. Each time after every fifteen days of interval five animals from each group were sacrificed by cervical dislocation. All the reproductive organs were dissected out and blotted dry of blood and weighed. Tissues were processed as per requirements for different parameters described separately hereafter. Small pieces of tissue were fixed in Bouin's fluid for histology, in Carnoy's fluid for Feulgen technique, in CAF for mucopolysaccharides and in Baker's fixative for enzyme histochemistry.

2.2.2.1) Histology:

Small pieces of tissues were fixed in Bouin's fluid for 24 hours, tissues were well washed in running water, dehydrated through ethanol grades, cleared in xylene and embedded in paraffin wax. The sections were cut at 5-6 µm. These sections were stained with routine Haematoxyline-Eosin (H-E) technique for the study of histological alterations in the testes and other organs of reproduction caused due to induced aspermatogenesis.

The spermatogenesis follows a seminiferous epithelial wave (Perry et al., 1951), which constitutes a series of cell associations. Two main classifications have been established for the spermatogenesis in rat for these cell associations, which are known as LC method (Leblond and Clermont, 1952) and RG method (Roosen-Runge and Giesel, 1950). The LC method subdivided the process of spermatogenesis into fourteen different stages, while RG method subdivided it into eight stages. A very high resolution optical microscopy is essential to observe clear cut differences in these stages. So instead of the 14 or 8 stages used in LC method and RG method, it appeared more practical to reduce these phases for convenience to
four phases. This classification can be very useful in describing the alterations induced by *Butea monosperma* leaf extract. The four phases designated as stages $P_1$ through $P_4$ were defined and correlated to LC method and RG method. The chronological order was maintained according to LC method. The detailed description of each phase and its correlation with LC method and RG method is given in the Chapter III which deals with alteration in testis.

After 45 days of treatment due to many alterations in various cell types, the identification of different spermatogenic cells become very difficult and so also the classification of tubules as $P_1$ through $P_4$. To solve this difficulty it was proposed further to classify the various damaged seminiferous tubules into four types on the basis of the degree of damage (alterations) observed and enhancement in degeneration. These are then designated as A, B, C and D type tubules. The detailed description of each tubule is given in the Chapter III which deals with alterations caused in testis due to *Butea* leaf extract administration.

2.2.2.b) Feulgen Technique:

In this investigation chromatin was demonstrated histochemically in fixed tissue sections by the Feulgen Method (Feulgen, 1923; Feulgen and Rosenbeck, 1924).

i) Fixative: Tissues were fixed in Cornoy's fixative which was prepared by adding ethanol, chloroform and acetic acid in the proportion of 160:30:10.

ii) Reagents:

1) Schiff's reagent - It is prepared according to the method of de Tomasi (1936). Reagent was kept in refrigerator. Before using it was allowed to reach room temperature.
2) Rinsing solution - 0.5% aqueous potassium metabisulfite prepared fresh from a 10% stock solution.

iii) Procedure:
1) Small pieces of tissues were fixed in Cernoy's fixative for 18-21 hrs.
2) Kept in double distilled alcohol for one hour.
3) Cleared in Xylene and paraffin sections of 5-6 μ were obtained.
4) After dewaxing and hydration sections were brought to water.
5) Rinsed in cold 1 N HCl.
6) Sections were hydrolysed in preheated 1 N HCl for 7 minutes at 60°C.
7) Washed with distilled water.
8) Immersed in Schiff's reagent for 10 minutes.
9) The slides were washed in three changes of two minutes each in rinsing solution.
10) Washed thoroughly under tap water.
11) Dehydrated through different grades of alcohol.
12) Cleared in Xylol and mounted in D.P.X.

iv) Results:
Nucleic acid sites stained redish pink.

2.2.3. Enzymes:

Biochemical and histochemical studies on four enzymes viz. β-glucuronidase, acid phosphatase, nonspecific esterase and alkaline phosphatase were carried out in reproductive tract by employing following methods.
2.2.3.1. β-glucuronidase (EC 3.2.1.3) :

I) Biochemical assay :

The activity of the enzyme was determined in the extract by the method of Fishman (1965, 1967).

a) Preparation of sample : The homogenation of the tissues was carried out in glass mortar and pestle which were previously well washed, rinsed in distilled water, dried and kept in the ice box of a refrigerator nearly for 5 hours. Such a homogenation has two advantages. (i) during homogenation no loss of enzyme activity occurs due to low temperature and the temperature increase due to the friction of the mortar and pestle does not exceed beyond 12° C at the end of the homogenation and (ii) during homogenation when the tissue is first crushed at the bottom of the mortar it instantaneously freezes and then gradually thaws which helps in breaking the lysosomes. When the tissue was thawing 2.00 ml of chilled acetate buffer (0.1 M, pH 4.5) were added and homogenation was carried out to completion. When a perfectly uniform suspension was formed the homogenate was transferred to a calibrated flask and further dilution was done by adding the necessary quantity of the acetate buffer. Care was taken to see that the final concentration was about 1 % (w/v) or even less. Throughout the work the concentration of the homogenate of the tissue was kept practically constant.

b) Purification of the sample : The partial purification of the enzyme was done by the method of Varute and More (1971), which was a modification of the early steps of purification of Calf liver β-glucuronidase described by Bernfeld et al. (1953). The partially purified fraction contained about 9-93 % of total β-glucuronidase activity of crude homogenate.
c) Preparation of reagents:

i) Phenolphthalein mono β-D-glucosiduronic acid solution
0.01 M (Sigma, stored at 4°C).

ii) Acetate buffer: (0.1 M, pH 4.5).

iii) Trichloroacetic acid: 5% (w/v).

iv) Alkaline glycine buffer: (0.1 M) as suggested by Fishman (1965), pH was adjusted as recommended by Fishman (1967).

v) Phenolphthalein standard solution (1 mg/ml): A standard curve was plotted as recommended by Fishman (1965).

d) Enzyme reaction: For each assay 3 test tubes were taken, to each of which were added 0.1 ml of the substrate solution, 0.8 ml acetate buffer and 0.1 ml of tissue homogenate. A single control test tube contained 0.9 ml of acetate buffer and 0.1 ml of tissue homogenate. The tubes were shaken gently to mix the aliquots properly, stoppered and incubation was carried out in water bath previously adjusted to 35.5°C for 21 hours. This incubation time was kept unchanged throughout the work.

e) Colour production and spectrophotometric measurements:

At the end of the incubation, the tubes were immersed for one minute in a boiling water bath to stop the enzymatic reaction. To each of the tubes, 1.5 ml of distilled water was added and tubes were centrifuged for 5 minutes at 2000 g. Two ml of supernatant was pipetted into clean dry test tube and 2.5 ml of the alkaline glycine buffer and 1.0 ml trichloroacetic acid solution were added. Then distilled water was added to make up the volume to 6.0 ml. Thorough mixing was done by shaking the tubes. The optical density was read at 540 μm on a spectronic 20 (Bosch and Lomb). Care was taken to see that pH of the final mixture was between 10.20 and 10.24. Matched tubes were used for calorimetric readings.
f) **Calculations**: The optical density was converted to micrograms of phenolphthalein from the calibrated phenolphthalein standard curve. The \( \beta \)-glucuronidase activity was expressed in terms of Fishman units (FU). The \( \beta \)-glucuronidase activity in Fishman units was calculated using the following formula:

\[
\text{FU/g sample} = \frac{\mu g \ \text{Phenolphthalein per tube} \times \text{dil x 2.5}}{\text{Incubation in hr x wt in g of sample x 0.1 x 2}}
\]

II) **Histochemical technique**:

a) **Fixation**: Tissues were fixed in calcium formol (4% w/v formaline and 1% calcium chloride) at 4°C for 24 hours. This fixative was found to give best localisation with minimal enzyme inactivation and diffusion artefacts of the end product of the enzyme reaction. Therefore all the further work was done with formol calcium fixed tissues. Fixation time (24 hours) was kept unchanged throughout the course of entire work.

b) **Sectioning**: Fixed tissues were blotted carefully on filter paper and transferred to Holt's gum sucrose (0.88 M sucrose solution containing 1% gum acacia) which was also previously chilled at 4°C (Holt, 1959; Hayashi and Fishman, 1961a). The tissues initially floated at the surface of gum sucrose solution and after some time sank to the bottom. The tissues were kept in Holt's gum sucrose for 24 hours at 4°C. The tissues were blotted on a puffless filter paper, without any washing and sections were cut at 5-8 μm on a Lipshaw cryostat (-20°C). The sections were received in chilled distilled water and only free floating sections were selected for further processing.

c) **Staining technique**: Localisation of the enzyme was demonstrated by post-coupling technique employing naphthol-AS-BI-\( \beta \)-D-glucosiduronic acid as substrate (Fishman and Goldman, 1965).
1) Free floating sections were selected.
2) The sections were then treated with 50 % ethanol at 0°C. Such ethanol treatment has been shown to improve the localization of β-glucuronidase by Varute (1970).
3) The stock substrate solution of naphthol-AS-BI-β-D-glucosiduronic acid (Sigma, 2 x 10^-4 M) was prepared by dissolving 11 mg of the substrate in 1 ml of 0.05 M NaHCO₃ and then diluted to 100 ml with 0.1 M acetate buffer (pH 4.5). The stock solution was preserved in refrigerator. Working solution was prepared by double dilution of stock solution.
4) Sections were incubated for 3 hours in working substrate solution at 37°C.
5) The incubated sections were washed in chilled distilled water and then coupled with fast Garnet GBC (Gurr) for 5 minutes at 0°C.
6) Sections were washed with cold distilled water and mounted in 1 % aqueous Polyvinyl pyrrolidone (PVP) on clean glass slides.
7) Photomicrography was carried out within a week.

Results: Sites of β-glucuronidase activities stained pink to magenta.

2.2.3.2. Acid Phosphatase (Orthophosphoric-monoester-phosphohydrolase, E.C. 3.1.3.2):

I) Biochemical assay:

The activity of the enzyme was determined according to the method described by Linhardt and Walter (1965).

a) Preparation of sample: Homogenates were prepared in the same manner as described for β-glucuronidase except in place
of acetate buffer sodium citrate buffer (0.05 M, pH 4.8) was used.

b) Purification of sample: The partial purification of the enzyme was done by the method of Varute and More (1971) which was a modification of the early steps of purification of acid phosphatase from rat-liver as described by Igarashi and Hollander (1968). The partially purified extract contained about 90% of total activity of acid phosphatase of crude homogenates.

c) Preparation of reagents:

1) 0.05 M P-nitrophenyl phosphatase in sodium citrate buffer (Sigma 0.1 M, pH 4.8) was prepared and stored at 4°C.

2) 0.1 N NaOH.

d) Enzyme reaction: To each test tube were added 1 ml substrate buffer solution and 0.2 ml aliquot of well suspended tissue homogenate. A mixture was allowed to equilibrate for 5-10 minutes. Control test tube contained 1.0 ml substrate buffer solution. The tubes were shaken gently, stoppered and incubated in water bath previously adjusted to 37.5°C for 30 minutes. The incubation interval was kept constant throughout the work.

e) Colour production and spectrophotometric measurements:
At the end of the incubation, the reactions were stopped by adding 4.0 ml of 0.1 M NaOH and tubes were centrifuged for 5 minutes at 2000 g. The optical density was read at 400 μm on spectronic 20, using control as a reference.

f) Calculations: The optical density was converted to μmoles of P-nitrophenol from the formula suggested by Linnhardt and Walter (1965) for tissue samples.

\[
\text{Acid phosphatase activity in P-nitrophenol μmol/g.} = \frac{0.2 \times \text{wt of sample in g}}{\text{OD. x 2.76 x dilution}}
\]
II) **Histochemical technique:**

a) Fixation, and b) sectioning were done as described for \( \beta \)-glucuronidase.

c) **Staining technique:** Localization of the enzyme was demonstrated by naphthol-AS-TR phosphate method (Barka, 1960 and Barka et al., 1962).

l) **Solutions:**

i) **Michaelis' Veronal acetate buffer (pH 5):** Stock solution was prepared by adding 9.714 g of sodium acetate and 14.714 g of sodium barbiturate to 500 ml distilled water kept in refrigerator.

ii) **Pararosaniline solution:** 1.0 g pararosaniline hydrochloride, 20.00 ml distilled water and 5.0 ml HCl (conc.). First the pararosaniline was dissolved in water and HCl was added with gentle warming. The solution was allowed to cool, filtered and stored in refrigerator.

iii) 4 % sodium nitrate:

iv) 100 mg naphthol-AS-TR phosphate (Sigma) was dissolved in 10 ml of dimethyl formamide (Fisher reagent Na D-119) and it was stored in refrigerator.

v) **Substrate solution:** It was prepared by adding 0.8 ml pararosaniline, 0.8 ml sodium nitrite, 5.0 ml veronal acetate buffer, 1.0 naphthol-AS-TR phosphate and 20 ml distilled water. First pararosaniline and sodium nitrite were mixed together and mixture was added to the solution of naphthol-AS-TR phosphate and pH was adjusted at 5.0 with 1 N NaOH, final volume was brought to 20 ml with the addition of distilled water.
2) Procedure:

i) Free floating sections were selected.

ii) The sections were incubated in the above substrate solution at 37.5° C for 30 minutes.

iii) Incubated sections were washed in cold distilled water.

iv) Sections were mounted in 1% PVP.

Results: Sites of acid phosphatase activities stained red.

2.2.3.3. Esterase (Nonspecific, EC 3.1.1):

I) Biochemical assay:

The activity of the enzyme was determined according to the method described by Bier (1955).

a) Preparation of sample: Homogenates of the tissues were prepared in the same manner as described for β-glucuronidase, except in place of acetate buffer phosphate buffer (0.66 M, pH 7) was used.

b) Purification of sample: The partial purification of the enzyme was done by the method of Varute and More (1971) which was a modification of the early steps of esterase purification as described by Bier (1955).

c) Preparation of reagent:

1) Stock substrate solution: 0.001 M P-nitrophenyl acetate was prepared by adding 63 mg P-nitrophenyl acetate (Sigma) in 10 ml ethanol. The working substrate solution was prepared by diluting 0.1 ml of stock solution to 10 ml.

2) Phosphate buffer (pH 7.0, 0.66 M).

d) Enzyme reaction: To each test tube 5.0 ml ice cold water, 2.0 ml phosphate buffer, 1.0 ml homogenate and 2.0 ml working substrate solution were added. Tubes were shaken vigorously and
centrifuged for 5 minutes at 2000 g. Readings of assay tube were taken at '0' hour as reference control. Again tubes were vigorously shaken and kept for 20 minutes for incubation at 15° C. Tubes were again centrifuged and readings were taken at 400 mμ. Both control and incubated mixtures were measured against distilled water as blank. Reading at '0' hour was treated as control.

e) Calculations: The optical density was converted into micromoles of P-nitrophenol from the calibrated P-nitrophenol standard curve. Esterase activity was calculated in P-nitrophenol μmol/g units according to the following formula:

\[
\text{μmol P-nitrophenol from graph x dil} = \frac{\text{μmol P-nitrophenol/g Enzyme}}{\text{unit/g}}
\]

II) Histochemical technique:

a) Fixation, and b) Sectioning were done as described for β-glucuronidase.

c) Staining technique: Localisation of the enzyme was demonstrated by Holt's 5-Bromoindoxyl acetate method for esterase according to Shnitka and Seligman (1961).

1) Substrate solution: 2 ml Tris-HCl buffer (0.1 M, pH 8.3), 5.0 ml NaCl (11.7 %), 2 M), 1.0 ml redox buffer and 2.0 ml distilled water were mixed rapidly. The above mixture was poured into a small beaker containing 1.3 mg 5-Bromo-indoxyl acetate in 0.1 ml acetone.

2) Free floating sections were selected.
3) Sections were incubated for 45 minutes at 37° C.
4) Washed with chilled distilled water.
5) Mounted in 1% PVP.

Results: Site of esterase activity in the form of discrete granules or droplets appeared as blue precipitates of indigo.
2.2.3.4. **Alkaline phosphatase** (Orthophosphoric monoester phosphohydrolase E.C. 3.1.3.1):

I) **Biochemistry assay**:

The activity of the enzyme was determined according to the method described by Linhardt and Walter (1965).

a) **Preparation of sample**: Homogenation of the tissues was carried out in the same manner as described for β-glucuronidase, except in place of acetate buffer, glycine buffer (0.05 M, pH 10.5) was used.

b) **Preparation of reagents**:

1) 0.05 M *P*-nitrophenol phosphate (Sigma) in glycine buffer (5.5 x 10^{-3} M, pH 10.5) 375 mg glycine, 10 mg MgCl₂, 6 H₂O and 16 mg *P*-nitrophenyl phosphate were dissolved in 42 ml of 0.1 N NaOH and diluted to 100 ml with distilled water stored in freeze.

2) 0.02 N NaOH.

c) **Enzyme reaction**: To each test tube 1.0 ml of substrate buffer was added and kept for equilibration at 37°C for 10 minutes. Then 0.1 ml sample was added and the tubes were shaken and kept for incubation for 30 minutes at 37°C. After incubation the reaction was terminated by adding 10 ml 0.02 N NaOH. A control tube contained 1.0 ml substrate, after the addition of the NaOH to the control tube, 0.1 ml sample was added. All the tube were centrifuged and the reading was taken against control at 400 μm.

d) **Calculations**: Alkaline phosphatase activity was directly measured by using the formula suggested by Linhardt and Walter (1965) for tissue samples.

\[
\frac{0.5 \times 11.82 \times \text{dilution}}{0.1 \times \text{wt of tissue in g}} = \text{Alkaline phosphatase activity in P-nitrophenol μmol/g}
\]
II) Histochemical technique:

a) Fixation, and (b) sectioning were carried out as per the procedure described for β-glucuronidase.

c) Staining technique: Localization of the enzyme activity was demonstrated by the naphthol-AS-MX phosphate method (Burstone, 1958; modified by Barka, 1962).

1) Substrate solution: 5 mg naphthol-AS-MX phosphate (Sigma) was dissolved in 0.5 ml of N, N-dimethyl formamide, 25 ml veronal acetate buffer (pH 9.2) was added and to this solution fast blue RR 25 mg was added and then mixture was filtered.

2) Free floating sections were taken.

3) Sections were incubated at room temperature for 30 minutes.

4) Sections were washed in cold distilled water.

5) Mounted in 1% PVP.

Results: Sites of alkaline phosphatase activity are stained blue.

2.2.4. Mucosubstances:

Histochemical technique:

The reproductive organs were dissected out and immediately fixed in cold (4°C) solution of 2% calcium acetate in 10% formalin (CAF-fixative). After fixation for 24 hours, the tissues were well washed firstly in cold water and then in running water, dehydrated through ethanol grades, cleared in xylene and embedded in paraffin wax. The sections were cut at 5-6 μ and stained with various histochemical methods for the identification of mucosubstances.

The various histochemical techniques with their merits and demerits for the mucosubstances localization have been reviewed by

For the present investigation, the following series of techniques for visualization of mucosubstances in the reproductive tissues of rat were employed, which are briefed in Table No.1.

2.2.5. Lipids:

**Biochemical assay:**

1) **Introduction**: The quantification of lipids has been reviewed by Stahl (1969), whereas Skipski and Barclay (1969) have reviewed in detail the various methods employed for quantitative analysis of neutral lipids and Skipski et al. (1964) have reviewed the techniques of phospholipid determination. The use of thin layer chromatography and subsequent quantitation of lipid components give a reliable information on lipid composition and also quantity of each component.

   a) **Chemicals**: All solvents in the present investigation were reagent grade and were obtained from E. Merk and B.D.H. unless otherwise indicated.

   b) **Reference lipids**: The following main reference compounds were used: Lysophosphatidyl choline (LPC), Spingomyelin (SPG), Phosphatidyl choline (PC), Phosphatidyl inositol (PI), (All from Bovine Brain extract type I), Phosphatidyl serine (PS) (from Bovine Brain extract type III) and Phosphatidyl ethanolamine (PE) (Cephalin), these were obtained from Sigma (U.S.A.) Cholesterol (CHO) and cholesterol ester (CE) were also obtained from Sigma.

   c) **Extraction of lipids**: The extraction and purification of lipids were carried out by using Folch's improved method (Folch et al., 1957).
The experimental animals were sacrificed by decapitation and testis and accessory glands of reproduction were dissected out and carefully weighed. The tissues were homogenized with 20 volumes of chloroform:methanol (2:1 v/v) at room temperature. The homogenates were allowed to stand for 2-4 hours at 4° C, and then filtered through the cintered funnel into a glass stoppered container. The precipitate was rehomogenized with 10 ml of chloroform:methanol mixture, and then filtered through cintered glass funnel. Both the filterates were pooled together and resultant mixture was shaken well with enough quantity of glass distilled water. Extracts were allowed to partition into two distinct phases by keeping the container in Freeze at 4° C for 5-6 hours. The upper phase which generally contained major part of the nonlipid contaminants was removed as completely as possible with a fine tipped pipette. The lower phase which mainly contained lipid fractions was transferred qualitatively through sodium sulphate to remove water completely from the lipid sample, then more chloroform was added to remove any lipid fraction from the sodium sulphate. Then it is transferred quantitatively into a glass stoppered container and evaporated under vacuum at 40° C. The lipid sample thus obtained was weighed and preserved in desiccator under vacuo at 20° C for further use.

d) **Thin layer chromatography (TLC)**: Different components of lipids were separated by thin layer chromatography (TLC) on silica gel coated plates.

I) **Preparation of plates**: 20 g of silica gel G (about 200 mesh, containing calcium sulphate as a binder, E. Merck, Germany) and 20 g of silica gel H (about 200 mesh, without binder, England) were slurried with 40 ml of distilled water for neutral
and phospholipids respectively. The slurry was transferred to the applicator immediately and applied to the plates (20 x 20 cm), setting of the adjustable applicator being done at 0.25 mm. The plates were activated by heating in an oven at 110° C for 60 minutes, cooled and preserved in dessicator for further use.

II) Application of sample: The edges of the plates were trimmed off excess silica gel. To each chromato plate, lipid extracted from tissues and a mixture of reference lipids of known composition dissolved in chloroform were applied with Hamilton microsyringe, 2.5-3.0 cm from the edge of the plates. The amount of standard compounds ranged from 5-25 µg whereas the total lipid tissue extracts applied ranges from 0.5 to 1.0 mg.

III) Quantification of neutral lipids:

a) Development of chromatogram: The chromatographic chambers (length, 25 cm, height 30 cm and width 10 cm) were prepared 20 minutes before the insertion of the plates. The chambers were lined on three sides with Whatman No.3 filter paper wetted with developing solvent. One step development system was followed. The plates were developed in Hexane (B.P. 65-70° C) : Diethyl-ether:acetic acid (85:15:2, v/v), as solvent system as recommended by Gloster and Fletcher (1956). The solvent system was allowed to move approximately 13.5 cm from the bottom of the plate (approximately 40-45 minutes). The plates were dried at room temperature.

b) Detection and Identification of spots: 40% H₂SO₄ spray (Privett and Blank, 1962; Skipski et al., 1963), Dichromate sulfuric acid spray (Blank and Schauplein, 1964), Iodine vapour (Sims and Larose, 1962), 2-7 Dichlorofluorescein spray in 0.2% in ethanol (Mangold and Maline, 1960; Mangold, 1961) were used for general detection of all lipid components.
Spots on chromatogram of tissue extracts were identified according to their positions with respect to reference compound. Identification of individual neutral lipid components was carried out by using specific chemical tests directly on the plates. The detection of the cholesterol and cholesterol ester was further confirmed by employing antimony tri-chloride spray (Weicker, 1959), whereas that of esterified fatty acids was confirmed by Hydroxyl-amine ferric chloride spray (Weicker, 1959).

c) Elution of neutral lipids: The elution of lipids from silica gel scrappings was performed separately for different classes of lipids. To testtubes containing silica gel with free fatty acids (FFA), triglycerides (TG), and Diglycerides (DG), 5 ml of diethyl ether was added. The tubes were vigorously shaken for ten minutes at room temperature and were centrifuged for 15 minutes (300 g), diethyl ether was removed with pipettes and elution step was repeated with another 5 ml portion of diethyl ether in a similar manner. Both the elutes were pooled and filtered through a cinked glass funnel. To the silica gel, 3 ml of diethyl ether was added and again the tubes were vigorously shaken. The process was repeated, (chloroform: methanol, 4:1, v/v, elution mixture also gives better results).

Cholesterol esters (CE), Cholesterol (CHO) and Monoglycerides (MG) were eluted by a mixture of chloroform and methanol in proportion of 4:1 (v/v). The elution procedure was repeated twice as in case of FFA, DG and TG. All the three elutes were combined.

1) Analysis of MG, DG and TG in the elutes (Viogue and Holman, 1962): Lipid elutes were chemically analysed. Each test-tube with elutes was treated as follows -
1) 0.1 ml of sodium hydroxide and 0.1 ml of hydroxylamine hydrochloride were added. Sodium chloride which precipitated prevented bumping during the boiling of diethyl ether.

ii) The solutions were evaporated to dryness in a water bath (65°-70° C) and last traces of solvents were eliminated.

iii) The tubes were cooled under tap water.

iv) 5 ml of feric-perchlorate was added.

The colour obtained was measured after 30 minutes at 520 µm on Spectronic 20 against ethanol solutions containing graded known amounts of methylpalmitate which were treated as standard for colour development.

2) Analysis of CHO and CE (Abell et al., 1952): The elutes from the CE and CHO scrappings were evaporated to dryness. Before that CE was converted into free CHO. After cooling to room temperature 6 ml of modified Libermann-Burchard reagent was added first to the empty tube and then at regular intervals to the other samples. The tubes were tightly corked, shaken and returned to the water bath. After 30 minutes the optical density of each sample was read against the blank on Spectronic 20 at 620 µm.

3) Analysis of FFA by titration (Skipski et al., 1968): The elutes of FFA were evaporated to dryness. The contents of the tube was redissolved in 5 ml of mixture of diethyl ether and 95 % ethanol (1:1, v/v), cresol red 0.005 % in water was used as indicator. Then the fatty acids were titrated with 0.005 - 0.01 N NaOH prepared in 79 % ethanol. The titer of NaOH was determined for each experiment by 5 mm H₂SO₄.

For the confirmation of results the assay of individual neutral lipids was carried out in three different sets of tissues.
IV) **Quantification of phospholipids**

The basic procedure of TLC of phospholipids (PL) originates from the work of Schlemmer (1961) and Wagner *et al.* (1961). These two laboratories introduced the use of Silica gel G and the solvent system, chloroform:methanol:water. With neutral chromatographic plates, many a time phosphatidyl serine (PS) is not properly separated. Under such circumstances the use of basic plates has been recommended by Skipski *et al.* (1962). Therefore the separation of phospholipids was carried out on silica gel H plates. Rest of the procedure was carried out as described earlier.

a) **Development of chromatogram**

One dimensional one step TLC was followed for the separation of PL. The chromatographic chambers were lined on 3 sides with Whatman No.3 filter paper wetted with developing solvent. The section of suitable solvent system was made by taking several trials with solvent systems recommended by various workers (Schlemmer, 1961; Wagner *et al.*, 1961; Dittmer and Laster, 1964; Ways and Nanahan, 1964; Skipski, 1964, 1967).

During the present investigation the solvent system containing chloroform:methanol:acetic acid:water, in proportion 100:50:9:5 (v/v) was used as it was found to be suitable for the separation of phospholipid components from reproductive organs.

b) **Detection and Identification**

Detection of PL on dried plates was made by exposing the plates to iodine vapour (Sims and Larose, 1962). Ninhydrin (0.2 % in acetone; Lytidine, 9:1, v/v) recommended by Skipski *et al.* (1962), Dragendorff reagent (Wagner *et al.*, 1961) for choline phospholipids; P-benzoquinone for PE, Ammonium silver nitrate spray and mercuric
oxide-barium acetate spray for PI; Colox benzidine spray for SPG (Bischel and Austin, 1963; Skipski et al., 1967) were used for confirmation of the individual phospholipid components. Details of these sprays and their diagnostic importance in the TLC are described by Marinetti (1962), Krebs et al. (1967) and Skipski and Barclay (1969).

c) Elution of Phospholipids:

Each PL component was eluted from the silica gel by suspending the powder in the eluting solvent by vigorously shaking the tube. First and second elutions were performed with the solvent mixture, chloroform:methanol:acetic acid:water (110:50:9:5, v/v). Third elution with methanol and the fourth with methanol:acetic acid:water (94:6:5, v/v) was performed. All elutes were pooled and taken directly into the digestion flasks. All the elutes prior to digestion were evaporated to approximately to 2 ml.

d) Phosphorus determination of PL components:

Bartlett's method (Bartlett, 1959) modified by Marinetti (1962) was followed for the estimation of lipid phosphorus. Samples eluted from TLC were digested with 0.9 ml of 70% perchloric acid. Digestion was carried out for 15-25 minutes on a medium gas flame in a sand bath, till the solution became clear. Tubes were cooled under tap water. 7.0 ml of distilled water, 1.5 ml of 2.5% ammonium molybdate and 0.2 ml of ANSA (Amino-Napthol-Sulfonic-Acid) described by Bartlett (1959) were added. Then the tubes were placed in boiling water for exactly 7 minutes. The tubes were removed and allowed to cool for 20 minutes. The optical densities were determined at 830 μm on Spectronic 20. Blank and standards were also run simultaneously.
e) **Phosphorus determination from Total Lipids**:

For the determination of lipid phosphorus from total lipid, Marinetti's semimicrophosphorus method was followed (Marinetti, 1962). Digestion was carried out with 0.5 ml of 70% perchloric acid and two drops of nitric acid for 15 minutes. After the flasks were cooled, 7 ml of distilled water were added. To each flask were then added 1.0 ml of 2.5% ammonium molybdate and 1.0 ml of Elon reagent. The solutions were mixed and left at room temperature for 30 minutes. The optical density of each solution was determined at 330 nm on Spectronic 20.

The values of PL and its components were calculated in terms of mg/g wet weight of the tissues by multiplying the phosphorus value obtained by a factor of 25.

2.2.6. **Protein**:

Biochemical assay of proteins was carried out according to the method described by Lowry et al. (1951).

a) **Preparation of reagents**:

1) Standard protein solution - 13 mg bovine albumen dissolved in 250 ml distilled water and made alkaline.

2) Lowry's A solution: 2% sodium carbonate in 0.01 M NaOH.

3) Lowry's B₁ solution: 2% sodium citrate in distilled water.

4) Lowry's B₂ solution: 1% copper sulphate in distilled water.

5) Lowry's C solution: This solution was prepared just before use and used within 15 minutes. To 1.0 ml of Lowry's B₁ solution was added 1 ml of Lowry's B₂ solution and 100 ml of Lowry's A solution. All solutions were mixed well.

6) Folin's phenol reagent.
b) **Preparation of sample** :

Homogenation of tissues was carried out in the same manner to that described for bioassay technique of enzymes, except in place of buffer only chilled distilled water was used for the preparation of homogenate and dilution.

c) **Procedure** :

For the bioassay of proteins seven test tubes were taken. In 5 tubes 0.00, 0.2, 0.4, 0.6 and 0.8 ml of tissue homogenates were added. Two control test tubes contained 0.2 and 0.4 ml standard protein solution. To each of the tube, distilled water was added to adjust the volume to 1.5 ml, the 3 ml of Lowry’s C solution were added to each tube. The solutions in the tubes were mixed well and kept for 15 minutes to form copper protein complex. After 15 minutes 0.5 ml of Folin’s Phenol reagent was added to each tube and allowed to develop colour for one hour at room temperature. After one hour optical density was read at 660 nm on Spectronic 20.

d) **Calculations** :

The proteins were calculated in terms of mg/g wet weight of tissue using following formula:

\[
\frac{\text{O.D. of sample} \times \text{standard protein} \times \text{dilution}}{\text{O.D. of standard} \times \text{weight of sample}} = \frac{\text{mg protein/g wet}}{\text{weight of tissue}}.
\]