II MATERIAL AND METHODS

1. MATERIAL :

A stylommatophoran pulmonate garden slug, Semperula maculata was collected from the agricultural fields of Kadamwadi, near Kolhapur, Maharashtra, India, regularly in every month from August, 1994 to August, 1995. The slugs were brought to the laboratory and were kept in plastic troughs which contained moist soil collected from the site of animal collection. The troughs were covered with mosquito curtain cloth. They were fed by Coccinia grandis, Piper beetle, bread, etc. The food was provided daily in the evening only and the water was always available to the slugs.

The slugs were kept in eight separate troughs forming the separate groups. Each group contained about 10 to 20 slugs. These groups were given symbols as follows :

1) **OTAB Group** = Slugs with optic tentacles ablated.

2) **OTAB + OTEI** Group = Slugs with optic tentacles ablated and injected the extract of optic tentacles.

3) **OTIN + OTEI** Group = Slugs with intact optic tentacles and injected the extract of optic tentacles.

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4) **OTAB + CGEI** Group = Slugs with optic tentacles ablated and injected the extract of cerebral ganglia.

5) **OTIN + CGEI** Group = Slugs with intact optic tentacles and injected the extract of cerebral ganglia.

6) **OTAB + OVEI** Group = Slugs with optic tentacles ablated and injected the extract of ovotestis.

7) **OTIN + OVEI** Group = Slugs with intact optic tentacles and injected the extract of ovotestis.

8) **Control Group** = Laboratory acclimatized slugs were served as controls.

The optic tentacles of slugs belonging to 1, 2, 4, and 6 groups were destalked by their bilateral ablation. The slugs of groups 2 and 3 were injected with the extract of optic tentacles. The slugs of groups 4 and 5 were injected with the extract of cerebral ganglia. The slugs of groups 6 and 7 were injected with the extract of ovotestis. The control slugs for these experiments were given injections of molluscan saline (mollus-scan ringer) in which the extracts of organs were prepared.

The extracts of optic tentacles, cerebral ganglia and ovotestis were prepared in the molluscan saline as recommended by Lockwood (1961). The concentration of tissue homogenate was kept constant at the 0.5 mg per ml. of the supernatant fluid.

Every day 0.5 ml extract was injected by means of tuberculin (hypodermic syringe) No.10 to each slug of respective group. Such five injections of the extract of each organ were given to the slugs of respective group.

A batch of five slugs belonging to all the eight groups were sacrificed on 5th, 10th, 15th, 20th and 25th day after the experiments. The slugs were decapitated on these days and their three male accessory sex organs (ASOs) - prostate gland, dart gland and penial complex (The anotomical details and positions of these male ASOs are shown in the Plate No.1) were removed and used for various histological, histochemical and biochemical techniques in order to observe the changes in the poly-saccharides, proteins and cholesterol contents in the above ASOs, in order to assess the effects of neurohormones elaborated by the optic tentacles and cerebral ganglia and of hormones elaborated by the ovotestis.

The detailed information on the histological, histochemical and biochemical methods along with their interpretations is given below.

2. METHODS :

(I) Histological Technique :

The small pieces of male ASOs including the prostate gland, the dart gland and the penis were fixed in the ice-cold calcium acetate formalin (CAF - 2% in 10% formalin) fixative for 24 hrs.

Captions to Figures

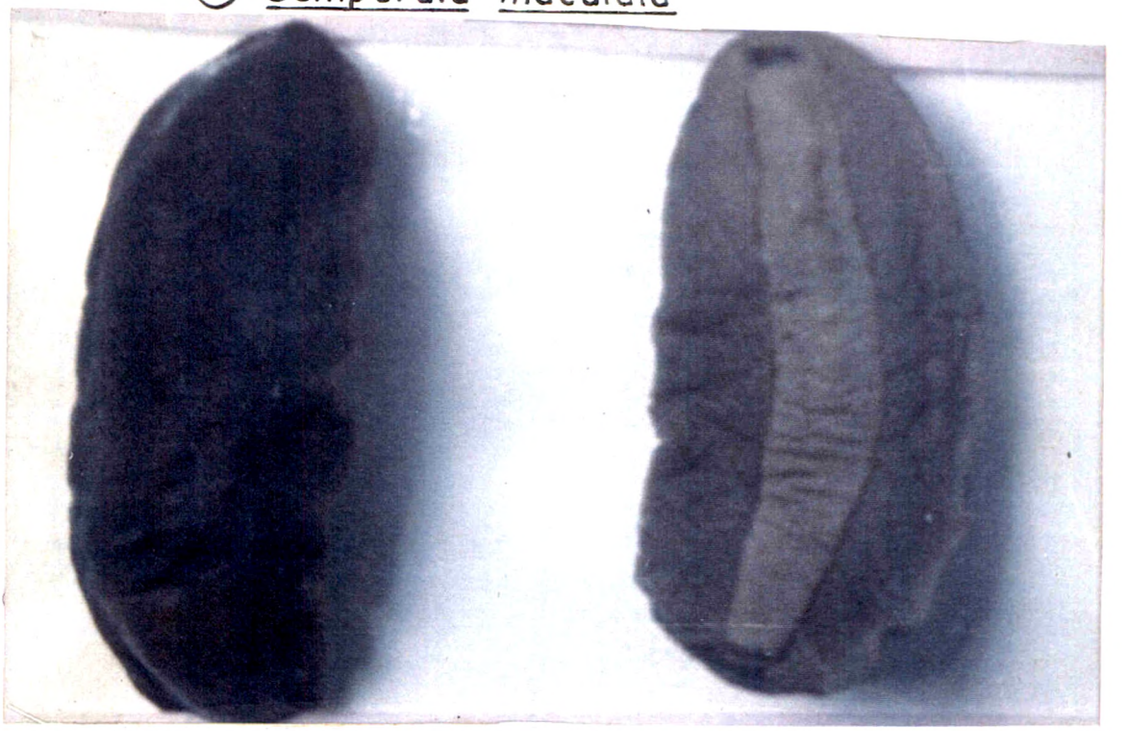
PLATE NO. 1

A) Pulmonate hermaphrodite slug Semperula maculata showing dorsal and ventral view of animal

B) Figure shows the reproductive system of S. maculata. Three labelled male accessory sex organs - 1) Prostate gland, 2) dart gland and 3) penis were selected for the present investigation.

PLATE No. 1

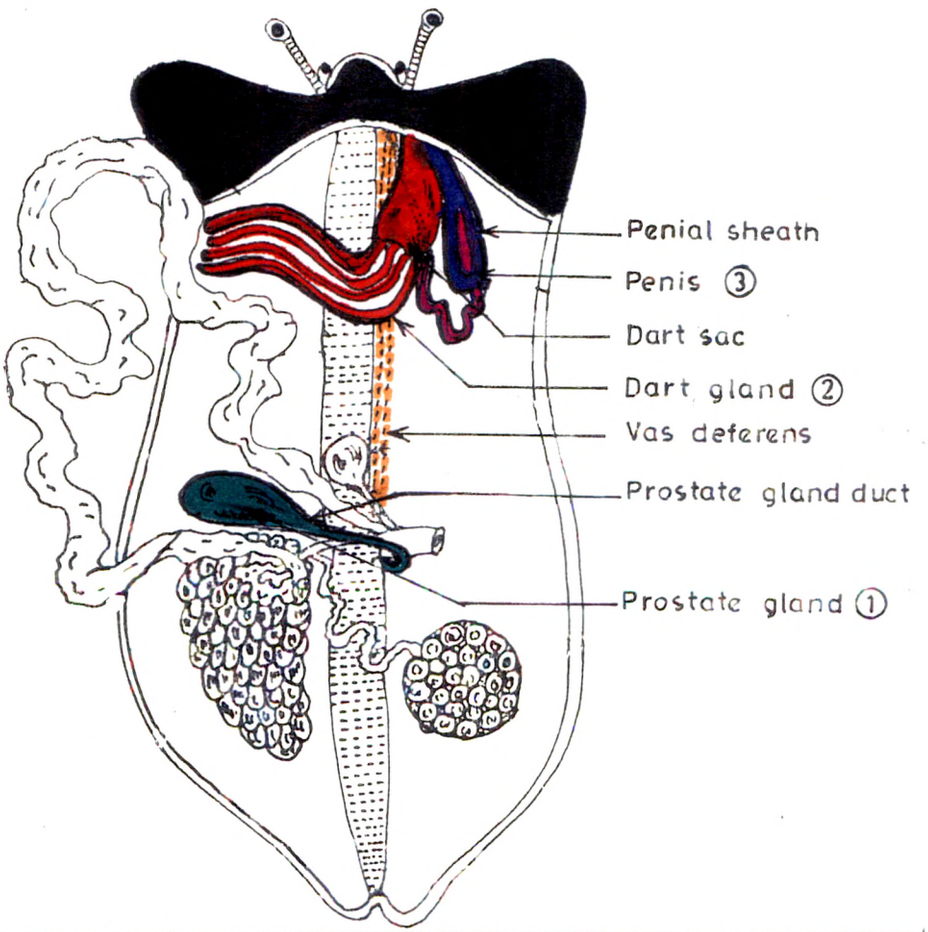
Ⓐ Semperula maculata



Dorsal view

Ventral view

Ⓑ Male Accessory Sex Organs



The fixation of the tissues were followed by washing in chilled distilled water and in running tap water, dehydration in alcohol, cleaning in xylene and paraffin embedment. The sections were cut at 5 to 6 μ. The histological technique Haematoxylene-Eosine was employed for the present investigation.

Hematoxylene-Eosin (H-E) Method :

1. The sections were dewaxed in xylene and brought to distilled water.
2. Sections were treated in haematoxylene for 15 seconds.
3. Differentiated in distilled water.
4. Dehydrated through 50% and 70% alcohol grades, 2-3 dips in each.
5. Stained in alcoholic eosin for 15 seconds.
6. Differentiated in 70% alcohol.
7. Quickly dehydrated through 90% and absolute alcohol.
8. Cleared in xylene and mounted in D.P.X.

Result : Nucleus-blue, cytoplasm pink.

(ii) Histochemical Techniques :

For visualization of various polysaccharides in the tissues a series of histochemical methods has been evolved by different investigators. The specificity of various methods can be enhanced by restoring the use of chemical reactions such as control of pH of basic dye, sequential staining techniques, methylation, saponification, acid hydrolysis and enzyme digestion tests. Thus, non-specific histochemical methods can be supplemented with histological and ancillary ones for the better understanding of the chemical composition of the cellular components. The various histochemical techniques with their merits and demerits for the mucopolysaccharide localization, have been reviewed by Spicer (1965), Barka and Anderson (1965), Thompson (1966), Spicer et al. (1967) and Pearse (1968).

For the histochemical observations of polysaccharides in the different male ASOs i.e. prostate gland, dart gland and penis were quickly cut into smaller pieces and immediately imersed in ice-cold solution (4°C) of 2% calcium acetate in 10% formalin. After prolonged fixation (24 hrs), the tissues were well washed in chilled distilled water, followed by washing in running tap water. After dehydration in alcohol, cleaning in xylene and paraffin embedment, the sections were cut at 5 to 6 μ. The sections were subjected to various important histochemical techniques hereafter described for detection of muco-polysaccharides.

A] Periodic acid-Schiff's reagent (PAS) :

(McManus, 1964; Hotchkiss, 1948)

1. After dewaxing and hydration sections wre brought to distilled water.
2. oxidized with 0.5% periodic acid for 10 minutes.
3. Washed with distilled water.
4. Treated with Schiff's reagent for 30 minutes.
5. Washed in distilled water, followed by alcoholic dehydration.
6. Cleared in xylene and mounted in D.P.X.

Result : Periodic reactive, hexose containing mucosubstances stain pink magenta.

B] Alcian blue (AB) at pH 2.5 : (Mowry, 1956)

1. After dewaxing and hydration, sections were brought to distilled water.
2. Rinsed in 3% acetic acid.
3. Stained with AB (1% AB in 8% acetic acid, pH 2.5) for 30 minutes.

4. Rinsed in 3% acetic acid.

5. Washed in running tap water for 5 minutes.

6. Dehydrated, cleared and mounted in D.P.X.

Result : Weakly acidic, sulfated mucosubstances, hyaluronic acids and sialomucins stain dark blue, strongly acidic i.e. sulfated mucins are stained weakly or not at all.

C] Alcian blue (AB) at pH 1 : (Lev and Spicer, 1964)

1. After dewaxing and hydration, sections were brought to distilled water.
2. Stained for 30 minutes in 1% AB in 0.1 N HCl (pH 1).
3. Blotted on puffless filter paper.
4. Dehydrated quickly, cleared and mounted in D.P.X.

Result : Only sulfomucins stain intense blue.

D] AB pH 2.5-PAS : (Mowry and Winkler,1956; Mowry,1963)

1. After dewaxing and hydration, sections were brought to distilled water.
2. Rinsed in 3% acetic acid.
3. Stained with 1% AB in 3% acetic acid (pH 2.5) for 30 minutes.

4. Rinsed in 3% acetic acid.
5. Washed in distilled water for 5 minutes.
6. Processed as in 'B' for PAS staining techniques.

Result : Alcian blue reactive, periodate unreactive acid mucosubstances stain blue, alcian blue and PAS reactive mucosubstances stain blue purple and PAS reactive but alcian blue unreactive mucosubstances colour magenta.

E] AB pH 1.0 - PAS : (Spicer, 1965; Spicer et al., 1967)

1. After dewaxing and hydration, sections were brought to distilled water.
2. Stained with 1% AB in 0.1 N HCl (pH 1.0) for 30 minutes.
3. Sections were blotted on puffless filter paper.
4. Processed as in 'B' for PAS staining technique.

Result : Only sulfomucins are stained blue or blue purple. Non-sulfated and only periodate reactive mucosubstances are stained pink magenta.

F] Saliva-digestion-PAS : (Spicer et al., 1967)

1. Sections were brought to distilled water.
2. Incubated for 1 hour at 37°C in own saliva.
3. Washed in distilled water.
4. Processed as in 'B' for PAS staining procedure.

Result : Glycogen containing sites were not stained by PAS. Thus the loss of PAS reactivity or reduction in the staining intensity indicates presence of the glycogen.

After staining histological and histochemical preparations were observed under microscope and the photomicrographs of some of the sections were taken.

iii) BIOCHEMICAL TECHNIQUES :

A] Glycogen : (Carrol et al., 1956)

Procedure : The tissues with known weights were digested by using 30% KOH solution, 0.5 ml saturated sodium sulphate and ethanol. After centrifugation precipitation of digested tissue was dissolved in distilled water and diluted to 50 ml in volumetric flask. 2 ml of this diluted solution was again diluted to 100 ml. 5 ml of this diluted solution was taken in a test-tube. In second tube, 5 ml standard glycogen solution (concentration 1 mg per ml) was taken. In the third tube, 5 ml distilled water was taken for the blank preparation.

Three test-tubes were kept in ice-cold water (- 16° to - 20°C) for 20-30 minutes. Then 10 ml anthrone reagent was added to each tube. After cooling, the readings were immediately read on spectrophotometer (Bausch-Lomb spectronic -20) at 620 m. The % transmittance was read. The percentage of glycogen was calculated by using following formula.

mg. of glycogen/100 mg. of tissue

$$= \frac{\text{\% transmittance of standard}}{\text{\% transmittance of unknown}} \times \frac{\text{Amount of standard}}{\text{Amount of unknown}} \times 100$$

B] Proteins (Mayer, 1961) :

Procedure : The tissues of prostate gland, dart gland and penis were accurately weighed and taken for the digestion in micro-kjeldahl's flask. The tissues were digested by adding 1 ml diluted (1:1) H_2SO_4. to this solution some cleaned glass beds were added to avoid bumping action of the solution. It was digested over a microburner untill excess water had been driven off, the solution was darkened and white fumes appeared. After some time when the tube was nearly full of dense fumes, the mouth of the tube was covered with a watch glass and the flame was reduced. the solution started boiling gently which was continued for 2 to 3 minutes more. Then the burner was removed and the solution was allowed to cool for few minutes. Afterwards 0.5 ml saturated potassium per sulfate solution was added to the tube contents drop by drop and was allowed to boil until the clear solution was formed.

When the digestion mixture was cooled, the tube content was quantitatively taken with 2 to 3 washings of distilled water in a large protein tube marked at 35 ml and 50 ml. Finally the digested solution was diluted up to 35 ml and was set aside until the standard and blank solutions were

ready, since in this determination it is important that colour development be carried out in both unknown and standard as nearly identical conditions as possible.

A standard was prepared in a second large protein tube graduated at 35 ml and 50 ml. Similar to that used for the preparation of unknown. To this tube 3 ml standard solution of ammonium sulfate, containing 0.15 mg of nitrogen was transferred. To this solution 1 ml. diluted H_2SO_4 (1:1) and 0.5 ml saturated potassium persulfate were added and tube contents were diluted with distilled water upto the 35 ml mark.

In third graduated protein tube, 1 ml diluted H_2SO_4 (1:1) and 0.5 ml saturated potassium persulfate were added and then diluted with distilled water up to the 35 ml mark.

When all the tubes were ready, each tube was nesslerized by adding 15 ml of Nessler's reagent. The tube contents were shaken by gentle rotatory motion. The tubes were kept for 10 minutes after addition and mixing of Nessler's reagent to permit maximum colour development and colorimetric readings were taken within the next 10 minutes. Prolonged standing leads to the development of a turbidity which renders the colorimetric reading difficult. The readings were taken using 520 μ filter in a Bousch and Lomb Spectrons - 20 spectro-photometer, setting the colorimeter to

100% transmittance with the blank. The amount of % Total nitrogen (TN) was calculated by using the formula :

$$\% \text{ Total Nitrogen (TN)} = \frac{\% \text{ Transmittance of standard}}{\% \text{ Transmittance of unknown}} \times \frac{\text{Amount of standard}}{\text{Amount of unknown}} \times 100$$

The remaining pieces of the same organs were utilised for the determination of non-protein nitrogen values. Here method is the same with some modifications in the digestion of unknown solution only. Then by substrating non-protein nitrogen values the proteinous nitrogen was obtained. The proteins were calculated by multiplying the proteinous nitrogen by a conversion factor (6.25).

C] Cholesterol : (Zovolsky's method, 1968)

Procedure : Homogenate of ovotestis, prostrate gland, dart gland, and penis was made in molluscan saline and it was diluted to obtain the appropriate dilution about 1%. Three test-tubes were taken. In the first test-tube, 0.1 ml homogenate and 2 ml colour reagent (31 ml 7% sulfosalicilic acid + 57 ml acetic unhydride + 12 ml conc. sulphuric acid which was stored at -4°C in refrigerator), were added. In second test-tube, 0.1 ml freshly prepared 0.2% standard cholesterol solution (prepared in glycial acetic acid) was

taken and 2 ml colour reagent was added. In third test-tube 0.1 ml distilled water was diluted with 2 ml of colour reagent.

Three tubes wre kept for 10 minutes at room temperature and percent transmittance was measured at 650 μ on spectrophoto-meter and the percentage of cholesterol was calculated by using following formula,

$$\text{Cholesterol (mg/100 mg)} = \frac{T}{S} \times 200$$

where, T = Percent transmittance of unknown

and S = Percent transmittance of standard.

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