CHAPTER - TWO

MATERIAL AND METHODS

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MATERIAL :

The present investigation was carried out on two annelids viz. the earthworm P. posthuma and leech H. granulosa.

Earthworms are perhaps the most familier annelids belonging to class - Oligochaeta and are found burrowing in the moist soil or mud, all over the world. The P. posthuma has a long narrow body, nearly circular throughout its length, the form of the body being well adapted to its burrowing habit. A full grown worm is about 150 mm. in length. The whole surface of the body is metamerically divided into 100 - 120 segments or metameres. This external segmentation of the body corresponds to an internal segmentation. In a mature worm, there is a prominent cylindrical band of glandular tissue known as clitellum or cingulum. The clitellum extends over three segments, 14^{th} to 16^{th} and forms important landmark on the external surface of the worm so that three distinct regions of the body; pre-clitellar, clitellar and post-clitellar can be distinguished. The earthworms live on the surface of layers of moist soil, as moisture is necessary condition for their life. In summer, when the upper surface of the earth becomes dry, they go deeper into the grounds in search of moisture and to avoid dessication. They burrow into the earth by eating the soil

through, and passing it out as pellets of mud which are commonly known as worm casts. The food of earthworms consists of decaying animal and vegetable matter contained in the soil. Earth worms are chiefly nocturnal in habits; they lie in burrows during the day, and come out at night for feeding. In rainy season, they may be seen coming out of burrows when the burrows are filled with water.

Leeches are found in temperate and warmer waters of the world. The Indian cattle leech, H. granulosa is found in fresh water ponds, lakes, tanks and slow flowing streams and belongs to class - Hirudinea The body of a leech is elongated and dorso-ventrally flattened, with the upper surface slightly convex than the lower. The size of the animal vary considerably as the body has the power of contraction and expansion to a great degree. When contracted the length may be 7-10 cms; when extended, the length may reach to 25 to 30 cms. At the two ends of the body, are anterior and posterior suckers mouth lies in the middle of the anterior sucker while anal aperture lies at the base of the posterior sucker dorsally. The dorsal surface is dark greenish and the ventral orange red, the two flanks having orange, yellow and black stripes. The whole body is metamerically segmented. In all there are thirtythree segments in the body. The external segmentation does not correspond to the internal segmentation, as each

segment shows superficial annulation. The leeches are ectoparasitic annelids found attached firmly to the outer parts of such animals as cattles, frogs, some times to human being that frequent the places. They are sanguivorous - blood sucking ectoparasites that suck the blood from the body of host. Leeches require at least two feedings in a year. Therefore, leeches can be kept alive in captivity for about six months.

These two annelids viz. P. posthuma and H. granulosa were selected for the present investigation, because they reflect wide variation in mode of life, uptake of food and dietary habits.

COLLECTION :

The earthworms, P. posthuma were collected from sugar cane fields around Karad and Kolhapur while leeches, H. granulosa were collected from the fresh water ponds near Karad and Kolhapur. The animals were collected periodically throughout the year (July 1987 to June 1988). The earthworms and leeches were used to study histology of the alimentary canal. The mucosubstances were studied histochemically in the different regions of the alimentary canal of earthworm and leech. For confirmation of the results the earthworms and leeches were collected again from July 1988 to June 1989.

METHODS :

HISTOLOGICAL :

Earthworms were collected and narcotised in 30% alcohol and then pieces were made in accordance with the external segmentation that correspond to the divisions of the alimentary canal. Some earthworms were fed on wet blotting paper to remove the soil from the alimentary canal. Such worms were kept for a couple of days in a clean moist cloth to clear the gut of blotting paper. Then the worms were narcotised, cut and fixed in Bouin's fluid for histological observations. Similarly, leeches were narcotised and cut in to pieces and immediately fixed in the Bouin's fluid. After fixation (24 hrs.) material was washed in running water, dehydrated through ethanol grades, cleared in xylene and embedded in paraffin wax. The serial sections were cut at 5 - 7 u and were stained with Haematoxylene - Eosin (H - E) technique for the study of histology of the alimentary canal.

HISTOCHEMICAL METHODS FOR MUCOSUBSTANCES :

Narcotised earthworms and leeches were cut into pieces and immediately fixed in cold ($4^{o}c$) solution of 2% calcium acetate in 10% formalin (CAF fixative). After fixation for 24

hrs., the pieces were well washed in running water, dehydrated through ethanol grades, cleared in xylene and embedded in paraffin wax. The sections were cut at 5 - 7 um and stained with various histochemical methods described hereafter for the localization of mucosubstances.

The various histochemical techniques with their merits and demerits for the mucosubstance localization have been reviewed by Spicer (1963), Curran (1964), Barka and Anderson (1965), Lillie (1965), Thompson (1966), Spicer and Henson (1967), Spicer et. al. (1967) and Pearse (1968).

For the present study the following series of techniques for visulization of mucosubstances in the alimentary tract of P. posthuma and H. granulosa were employed.

A. NEUTRAL MUCOSUBSTANCES :

I) Periodic Acid Schiff reaction (PAS) (Mc Manus, 1946; Hotchkiss, 1948) :

1. After dewaxing and hydration, sections were 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 10 minutes.
5. Rinsed three times (total 6 minutes) with 0.5% sodium meta-bi-sulphite.
6. Washed in distilled water, followed by alcoholic dehydration, cleared in xylene and mounted in canda balsam/DPX.

Results :

Periodate reactive, hexose containing mucosubstances stain pink-magenta.

II) Phenylhydrazine-PAS (Spicer, 1965; Spicer et. al. 1967) :

1. After dewaxing and hydration sections were brought to distilled water.
2. Oxidized with 0.5% periodic acid for 10 minutes.
3. Followed by treatment with 5% phenylhydrazine for 30 minutes.
4. Washed with distilled water.
5. Immersed in Schiff's reagent for 10 minutes.
6. Rinsed three time (total 6 minutes) with 0.5% sodium meta-bi-sulphite.
7. Washed, dehydrated, cleared routinely and mounted in canda balsam/DPX.

Results :

Periodate reactive acid mucosubstances were selectively stained, periodate engendered dialdehydes are blocked.

III) Diastase digestion-PAS technique for identification of glycogen (Lillie, 1954; Lison, 1960) :

1. After dewaxing and hydration sections were brought to distilled water.
2. Incubated for one hour at 37°C in the following medium : 0.1% malt diastase in 0.2 M phosphate buffer at pH 6.0.
3. Washed in distilled water.
4. Processed as in A-I for PAS.

Results :

Loss of PAS reactivity or reduction in the staining intensity indicates the presence of glycogen.

B. ACID MUCOSUBSTANCES :

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

1. After dewaxing hydration, sections were brought to distilled water.

2. Rinsed in 3% acetic acid.
3. Stained with AB (1% AB in 3% acetic acid pH 2.5) for 30 minutes.
4. Rinsed in 3% acetic acid.
5. Washed in running water for 5 minutes.
6. Dehydrated, cleared and mounted as usual.

Results :

Weakly acidic sulfated mucosubstances, hyaluronic acids and sialomucins stain dark blue. Strongly acidic sulfated mucosubstances are stained weakly or not at all.

II) Alcian blue (AB) at pH 1.0 (Lev and Spicer, 1964) :

1. After dewaxing and hydration sections were brought to distilled water.
2. Stained for 30 minutes in 1% AB in 0.1N N HCl (pH 1.0).
3. Blotted slides on Puffless filter paper.
4. Dehydrated quickly, cleared and mounted as usual.

Results :

Only sulfated mucosubstances stain intense blue.

III) Colloidal Iron (C.I) (Hale, 1946; Rinehart and Abul Haj, 1951; Mowry, 1961, 1963) :

1. After dewaxing and hydration sections were brought to distilled water.
2. Rinsed in 12% acetic acid.
3. Treated with freshly prepared working colloidal iron solution for 60 minutes at room temperature.
4. Rinsed in 12% acetic acid.
5. The sections were treated with freshly prepared mixture of equal volume of 2% HCl. and 2% potassium ferrocyanide for 20 min.
6. Washed with running water for 5 minutes.
7. Dehydrated, cleared and mounted as usual.

Results :

Sites of acidic mucosubstances are prussian blue. The results obtained with this method are very much identical to those obtained with AB pH 2.5 procedure.

C. DISTINCTION BETWEEN NEUTRAL AND ACIDIC MUCOSUBSTANCES:

I) AB pH. 2.5 - PAS (Mowry and Winkler, 1956; Mowry, 1963) :

1. After dewaxing and hydration sections were brought to distilled water.
2. Rinsed briefly 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 A-I for PAS.

Results :

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

II) 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 A-I for PAS.

Results :

Only sulfomucins are stained blue or blue-purple. Nonsulfated and only periodate reactive mucosubstances are stained pink-magenta.

III) Colloidal Iron-PAS (Ritter and Oleson, 1950; Mowry, 1963) :

1. After dewaxing and hydration, sections were brought to distilled water.
2. Rinsed briefly in 1.2% acetic acid.
3. Treated with freshly prepared colloidal iron working solution for 60 minutes at room temperature.
4. Rinsed with 1.2% acetic acid.
5. Treated with freshly prepared mixture of equal volumes of 2% HCl and 2% potassium ferrocyanide for 20 minutes.
6. Washed in running water for 5 minutes.
7. Processed as in A-I for PAS.

Results :

Acidic mucosubstances colour blue or blue-purple and neutral mucosubstances colour pink-magenta. Results are mostly

similar to those of AB pH 2.5-PAS.

D. DISTINCTION BETWEEN SULFOMUCINS AND CARBOXYMUCINS :

I) Aldehyde fuschin (AF) (Gomori, 1950; Halmi and Davies, 1953):

1. After dewaxing and hydration, sections were brought to distilled water.
2. Rinsed in 70% alcohol.
3. Stained with AF staining solution for 30 minutes.
4. Rinsed in 70% alcohol.
5. Dehydrated in 90% and absolute alcohol, cleared in xylene and mounted as usual.

Results :

Sulfated mucosubstances are stained dark-purple, sialomucins and hyaluronic acids stain light-purple, some elastic fibres also stain intense purple.

II) Aldehyde Fuschin-AB pH 2.5 (Spicer and Meyer, 1960) :

1. After dewaxing and hydration, sections were brought to distilled water.

2. Rinsed in 70% alcohol.
3. Stained in AF staining solution for 30 minutes.
4. Rinsed in 70% alcohol.
5. Washed in running water for 5 minutes.
6. Rinsed in 3% acetic acid.
7. Stained with AB (pH 2.5) for 30 minutes.
8. Rinsed in 3% acetic acid.
9. Washed in running water for 5 minutes.
10. Dehydrated, cleared and mounted as usual.

Results :

Sulfated mucosubstances stain purple, nonsulfated mucosubstances like sialic acid and hyaluronic acid stain blue.

III Critical electrolyte concentration technique using AB at pH 5.6 with increased concentration of $Mgcl_2$ (Scott et. al., 1964; Scott and Dorling, 1965) :

1. Eight slides after dewaxing and hydration were brought to distilled water.
2. Each slide was stained for 30 minutes in staining solutions : 00.0M, 0.1M, 0.2M, 0.4M, 0.5M, 0.6M, 0.8M and 1.0M.
3. Washed in running water for 5 minutes.

4. Dehydrated, cleared and mounted as usual.

Results :

Generally carboxymucins like sialic acid and hyaluronic acid are not stained at or above 0.1 M Mg^{++} concentrations. Sulfomucins are selectively stained at and above 0.2 M Mg concentration. Various sulfomucins loose their alcianophilia at different levels of Mg^{++} concentration.

IV) Azure a metachromatic staining technique at controlled pH levels (Wislocki et. al., 1947; Spicer, 1960; Spicer et. al., 1967 and Pearse, 1968) :

1. After dewaxing and hydration, sections were brought to distilled water.
2. Stained with azure A at desired pH for 30 minutes.
3. Quickly washed in distilled water.
4. Wet sections were observed under microscope.
5. Dehydrated in alcohol and observed under microscope.
6. Cleared in xylene and mounted as usual.

Results :

Strongly sulfated mucosubstances exhibited

metachromasia below pH 1.5, Sialomucins generally stain metachromatically between pH 2.5 to 3.5. Some protein masked sulfomucins and hyaluronic acid exhibited metachromatia at and above pH 4.5. Generally the metachromasia of sulfomucins resist alcohol dehydration.

V) Mild methylation - AB pH 2.5

VI) Active methylation - AB pH 2.5 (Fisher and Lillie, 1954; Spicer, 1960) :

1. After dewaxing and hydration, sections were brought to distilled water.
2. Rinsed in absolute methanol.
3. Sections were placed in coupling jars containing 0.1 N HCl in absolute methanol (preheated) for 4 hours at 37°C (mild methylation) and at 60°C (active methylation). Correspondingly the control sections were kept at 37°C and 60°C in methanol only (without HCl).
4. Rinsed in absolute methanol.
5. Followed by 5 minutes washing in running water.
6. Stained with AB pH 2.5 as in B-I.
7. After washing, dehydration and clearing sections were mounted as usual.

Results :

Generally mild methylation abolishes the basophilia of carboxymucins by esterification, while active methylation hydrolyses most of sulfate esters.

VII) Mild methylation - saponification - AB pH 2.5 :

VIII) Active methylation - saponification - AB pH 2.5 :
(Spicer and Lillie, 1959; Spicer, 1960) :

Sections were methylated separately at 37° and 60°C as above. After brief washing with distilled water, they were treated with 1% KOH in 70% alcohol for 20 minutes. After washing briefly with distilled water, they were stained with AB pH 2.5 as in B-I after washing, dehydration and clearing, the sections were mounted as usual.

Results :

Restoration of the basophilia after saponification indicates the presence of carboxymucins but failure of restoration of basophilia indicates the presence of the sulfate esters.

IX) Acid hydrolysis (Quintarelli et. al., 1961) :

1. After dewaxing and hydration, sections were brought to distilled water.
2. They were treated with 0.1 N HCl at 60°C for 4 hrs.
3. Washed in running water for 5 minutes.
4. Stained with AB pH 2.5 or azure A pH 3.0.
5. Dehydrated, cleared and mounted as usual.

Results :

Complete or partial loss of alcinophilia or metachromasia indicates the probable presence of sialomucins.

E. ENZYME DIGESTION TESTS :

I) Sialidase (Neuraminidase) digestion (Spicer and Warren, 1960) :

1. After dewaxing and hydration, sections were brought to distilled water.
2. The slides were placed on glass rods, close to surface of water in petridish kept at 37°C. Sections were covered with enough sialidase (Vibrio cholerae, type V, Sigma) in 0.1 M sodium acetate at pH 5.3

containing 0.04 M $CaCl_2$. Control sections were covered with buffer only (0.1 M Sodium acetate at pH 5.3 cantaining 0.04 M $CaCl_2$). Sections were incubated for 16 to 24 hours.

3. Rinsed with distilled water.
4. Stained with AB pH 2.5.
5. Dehydrated, cleared and mounted as usual.

Results :

Complete or partial loss of alcinophilia indicated the presence of sialic acid.

II) Hyaluronidase digestion (Barka and Anderson, 1965; Spicer et. al., 1967) :

1. After dewaxing and hydration, sections were brought to distilled water.
2. Sections were incubated at 37°C for 6 hours in 0.05% hyaluronidase (Testicular, Sigma) in freshly prepared buffer at pH 5.5 (94 ml. 0.1 M $KH_2 PO_4$ + 6 ml 0.1 M Na_2HPO4). Control sections were incubated only in buffer.
3. Washed in running water for 5 minutes.
4. Stained with AB pH 2.5.

5. Dehydrated, cleared and mounted as usual.

Results :

Complete or partial loss of alcinophilia indicates the presence of hyaluronic acid, chondrotin sulphate A and C.

III) Pepsin digestion (Pearse, 1960; Spicer, 1960; Quintarelli, 1963; Thompson, 1966) :

1. After dewaxing and hydration, sections were brought to distilled water.
2. Digested in 0.1% pepsin in 0.1 N HCl at 37°C for 4 hours.
3. Washed thoroughly in running water.
4. Stained with AB pH 2.5.
5. Dehydrated, cleared and mounted as usual.

Results :

Protein masked mucosubstances (PAS Positive but AB, CI and azure A negative) stained with basophilic dyes after removal of protein masking.