The object of applying histochemical methods is naturally to have clear understanding of the internal structure as well as the function of the structure concerned. Locally available house rats of the species *Rattus rattus* were brought periodically to the laboratory and were examined for nematode fauna. The nematode parasites selected for the histochemical studies belong to two different types, viz. *Trichuris muris* (Schrank, 1788) and *Ganagleterakis spumosa* (Schneider, 1866) Lane, 1914. The morphological study of these worms is given in the next chapter. The worms were collected from the colon, caecum and the rectum of the alimentary canal of the host. Proper care was taken while removing the worms especially in case of *Trichuris muris*, since a part of its body is embedded into the host tissue. They were recovered gradually to prevent them from distortion. During the course of collection, the number of *Ganagleterakis spumosa* was found to be more than that of *Trichuris muris*.

The worms were washed thoroughly first with normal saline and then with distilled water so as to get rid of completely any adhering host tissue or
mucus, then they were subjected to treatment of warm water (Temperature about 37°C) so as to make them straight. In case of the material used for the detection of enzymes, it was directly transferred to a suitable fixative in order to prevent the loss of enzyme activity. The male worms were separated from the female ones. The worms were preserved in 10% glycerine-alcohol and they were mounted in 20% glycerine-alcohol for morphological studies. Lactophenol was used for giving clarity. Before transferring to various fixatives for histological and histochemical studies, the parasites were cut into pieces of suitable size to ensure proper penetration of the fixative.

Various histochemical methods were followed as cited in Pearse (1968), Bird (1971), Humason (1973), and Culling and Dunn (1975). For most of the histochemical reactions, control sections were processed simultaneously.

**Histochemical methods for carbohydrates and proteins.**

The material was fixed in alcoholic Bouin's fluid and Carnoy's fluid for 24 hours separately. After fixation it was dehydrated in 70% alcohol
onwards, cleared in xylene and embedded in paraffin wax (58 - 60°C). Sections were cut at 5 microns. The material fixed in alcoholic Bouin's was washed several times with saturated solution of lithium carbonate in 70% alcohol to remove the yellow colour of picric acid. In case of the material fixed in alcoholic Bouin's and Carnoy, the former gave satisfactory results.

**Fixatives:**

**Alcoholic Bouin's fluid:**

- 80% alcohol ... ... 150 ml
- Picric acid ... ... 1 gm
- Formaldehyde ... ... 60 ml
- Glacial acetic acid 15 ml

**Carnoy's fluid:**

- Absolute alcohol ... 60 ml
- Chloroform ... ... 30 ml
- Glacial acetic acid 10 ml

**BEST'S CARMINE METHOD**

**Preparation of carmine stock solution:**

2 g. of carmine, 1 g. of Potassium carbonate and 5 g. of Potassium chloride were added to 60 ml of distilled water. It was then boiled gently for 5 minutes, cooled and filtered. To the filtrate added 20 ml of
ammonia (Sp. G. 0.91) and 12.5 ml of methyl alcohol. This solution was kept in refrigerator at 0° - 4°C.

**Best’s carmine solution:**

15 ml of stock solution was diluted with 12.5 ml of ammonia (Sp. G. 0.91) and 12.5 ml of methyl alcohol.

**Best’s Differentiator:**

- Absolute alcohol ... 8 ml
- Methyl alcohol ... 4 ml
- Distilled water ... 20 ml

**Preparation of Ehrlich’s alum haematoxylin:**

- Haematoxylin ... 6 g.
- Absolute alcohol 300 ml
- Distilled water 300 ml
- Glycerol 300 ml
- Glacial acetic acid 30 ml
- Potassium alum in excess.

The haematoxylin was dissolved in the alcohol and other components added in the order given. Finally, Potassium alum was added and the solution was shaken until there was a deposit of alum crystals at the bottom of the stock container. After preparation of stain it was kept in a loosely plugged bottle in warm, light place for ripening.
Procedure:

(1) Deparaffinized sections were brought to water;

(2) Stained in Ehrlich's haematoxylin for 5 minutes;

(3) Rinsed and differentiated rapidly in 1 percent acid alcohol;

(4) Rinsed in distilled water;

(5) Stained in Best's Carmine Solution for 30 minutes.

(6) Differentiated in Best's Differentiator for 1 minute.

(7) Washed in 80 percent alcohol.

(8) Dehydrated in absolute alcohol, cleared in xylene and mounted in DPX.

Results:

Nuclei - dark blue;

Glycogen - red.

Control:

Control was prepared using human saliva. A little water was added to the saliva and it was spread on the control section. The slide was kept at 37°C for 45 minutes. In this period two changes of saliva were
given, then the slide was washed with distilled water. This saliva treated slide was processed along with the sample slides and treated from step two onwards.

Results:

Control sections were negative for glycogen.

**PERIODIC ACID - SCHIFF METHOD**

(McManus, 1947).

**Preparation of Schiff's reagent**: (de Tomasi, 1936):

1 g. of basic fuchsin was dissolved in 200 ml. of boiling distilled water, shaken for 5 minutes and cooled to 50°C. It was then filtered and added to the filtrate 20 ml. of N-HCl, cooled to 25°C and added 1 g. of Sodium metabisulphite. This solution was kept in the dark for 24 hours. To the solution added 2 g. of activated charcoal, stirred, filtered and stored in the dark at 0°C - 4°C. Before use it was allowed to reach room temperature.

**Procedure**:

1. Deparaffinized sections were brought to water;

2. Oxidized for 10 minutes in 0.5 percent aqueous periodic acid;
(3) Washed in running water for 5 minutes;
(4) Treated with Schiff's reagent for 30 minutes;
(5) Rinsed quickly in three successive baths of freshly prepared bisulphite solution for one minute each (Bisulphite solution consists of 10 ml N-HCL, 10 ml. 10 percent. Sodium metabisulphite and 200 ml of distilled water);
(6) Washed in running tapwater for 5 minutes;
(7) Dehydrated, cleared and mounted in DPX.

Control:

Control slides were prepared as in Best's Carmine method. Control slides were placed alongwith the sample slides and were treated for Periodic acid onwards.

Results:

Glycogen stained deep red;
on other substances purplish red;
control sections negative for glycogen.

PERIODIC ACID PHENYLHYDRAZINE Schiff Method
(Spicer, 1961)

(1) Deparaffinized sections were brought to water;
(2) Oxidized in 1 percent Periodic acid for 10 minutes;
(3) Washed in running water for 5 minutes;

(4) Aldehydes were blocked in 0.5 percent aqueous phenylhydrazine hydrochloride for 30 minutes;

(5) Washed in water;

(6) Immersed in Schiff reagent for 15 minutes;

(7) Washed in water for 5 minutes;

(8) Dehydrated, cleared and mounted in DPX.

Results:

Phenylhydrazine blocks the staining of neutral polysaccharides and glycoproteins; some sialo-acid and sialo-sulphated glycoproteins stain in red.

**STANDARD ALCIAN BLUE METHOD (pH 2.5) FOR ACID GROUPS (STEEDMAN, 1950):**

(1) Deparaffinized sections were brought to water;

(2) Stained in freshly filtered 1 percent, Alcian blue 8 G X in 3 percent acetic acid (pH 2.5) for 30 minutes;

(3) Washed in water;

(4) Dehydrated, cleared and mounted in DPX.

Results:

Acid mucopolysaccharides - blue green.
ALCIAN BLUE (pH 1.0) FOR SULPHATE GROUPS

(1) Sections were brought to water;
(2) Stained in 1 percent Alcian blue 8 G X 0.1 N hydrochloric acid for 30 minutes. Rinised briefly in 0.1 N HCl.
(3) Blotted dry with fine filter paper;
(4) Dehydrated in alcohol, cleared in xylene and mounted in DPX.

Results:
Sulphated mucosubstances stained blue.

ALCIAN BLUE PERIODIC ACID METHOD (pH 2.5)
(Mowry, 1963)

(1) Deparaffinized sections were brought to water;
(2) Rinsed briefly in 3 percent acetic acid;
(3) Stained for 2 hours in 1 percent Alcian blue 8 G X in 3 percent acetic acid.
(4) Rinsed briefly in 3 percent acetic acid and then in distilled water;
(5) Oxidized for 10 minutes in 1 percent aqueous periodic acid;
(6) Washed in running water for 5 minutes;
(7) Immersed in Schiff's reagent for 10 minutes;
(8) Washed in running water for 5 minutes;
(9) Dehydrated in alcohol, cleared in Xylene and mounted in DPX.

Results:

Acid mucosubstances - blue.
Nonalcinophilic components - red.

ALDEHYDE-FUCHSIN METHOD (Halmi and Davies, 1953)

Preparation of solution:

0.5 g. of basic fuchsin was dissolved in 100 ml of 60 percent ethanol; 1 ml of paraaldehyde and 1.5 ml of HCl were added to this solution. Ripened for 24 hours at room temperature and stored in refrigerator.

Method:

(1) Sections were brought to water;
(2) Rinsed in 70 percent alcohol;
(3) Stained for 30 minutes;
(4) Rinsed in 70 percent alcohol.
(5) Dehydrated in alcohol, cleared in xylene and mounted in DPX.

Result:

Sulphated mucosubstances stained moderately;
Non-sulphated acidic mucosubstances stained weakly.
MERCURY BROMOPHENOL BLUE METHOD (Bonhag, 1955)

Reagent:

\[ \text{HgCl}_2 \quad \ldots \quad \ldots \quad 1 \text{ g.} \]

Bromophenol blue \ldots \quad 0.05 \text{ g.}

2 percent glacial acetic acid \ldots \quad \ldots \quad 100 \text{ ml.}

Procedure:

1. Deparaffinized sections were brought to water;
2. Stained for 15 minutes in the reagent;
3. Rinsed in 0.5 percent acetic acid for 5 minutes;
4. Sections were transferred to tertiary butyl alcohol.
5. Cleared in xylene and mounted in DPX.

Result:

Proteins - deep blue.

MILLON REACTION (Baker modification)

Preparation of the reagent:

10 g. of \( \text{HgSO}_4 \) added to 100 ml of 10 percent \( \text{H}_2\text{SO}_4 \) and heated until dissolved, the volume made upto 200 ml.

0.5 ml of 0.25 percent \( \text{NaN}_2 \) was then added to the above solution.

Fresh reagent was prepared for each batch of sections.
Procedure:
1. Sections were brought to water;
2. Sections were placed in small beaker containing the reagent and boiled gently;
3. The solution was allowed to come to room temperature;
4. Sections washed three times in distilled water (2 minutes each wash);
5. Dehydrated, cleared and mounted in DPX.

Result:
Tyrosine containing proteins stained red.

NINHYDRIN - SCHIFF METHOD
(Yasuma and Itchikaw, 1953)

1. Deparaffinized sections were brought to water;
2. Treated with 0.5 percent ninhydrin in absolute alcohol for 20 hours at 37°C;
3. Washed in running water for 2 minutes;
4. Immersed in Schiff reagent for 30 minutes;
5. Washed in running water for 5 minutes;
6. Dehydrated, cleared and mounted in DPX.

Result:
proteins - Pinkish red for NH₂ group.
FERRIC FERRICYANIDE METHOD
(Chevremont and Frederic, 1943)

Reagent:

3 parts of 1.35 percent ferric chloride was added to 1 part of freshly prepared 0.1 percent potassium ferricyanide. The pH adjusted to 2.4.

Procedure:

1. Deparaffinized sections were brought to water;
2. Treated in three changes of the ferric ferricyanide reagent for a total period of 30 minutes;
3. Washed in distilled water;
4. Dehydrated, cleared and mounted in DPX.

Result:

Protein for sulphydryl group - blue.

PERFORMIC ACID - SCHIFF METHOD
(Pearse, 1951)

Reagent:

4 ml of 30 percent (100 vol.) hydrogen peroxide was added to 40 ml of 96 percent formic acid. 0.5 ml of concentrated $\text{H}_2\text{SO}_4$ was added to this solution. The mixture was allowed to stand for one hour before use.
and used within 24 hours. Schiff's reagent was prepared as in PAS method.

Procedure:

1. Deparaffinized sections were brought to water;
2. Treated with Performic acid for 20 minutes;
3. Washed in water for 5 minutes;
4. Treated with Schiff reagent for 1 hour;
5. Washed in running tap water for 10 minutes;
6. Dehydrated, cleared and mounted in DPX.

Result:

Protein for disulphide group - Pink.

**HISTOCHEMICAL METHOD FOR LIPIDS**

The material was fixed in formal calcium for 12 hours. It was then transferred to dichromate calcium (Potassium dichromate 5 g.; Calcium chloride 1 g. and distilled water 100 ml) for 18 hours at room temperature and further 24 hours at 60°C, the material was then washed thoroughly in running water and finally in distilled water, dehydrated in alcohol, cleared in xylene and embedded in paraffin was (60 - 62°C). Sections were cut at 7 microns.
Fixative - Formal calcium:

40 percent Formaldehyde ... 10 ml.
Calcium chloride ... ... 2 g.
Distilled water ... ... 100 ml.

The formaldehyde was neutralized by adding a small piece of chalk.

SUDAN BLACK B METHOD (McManus, 1946)

Procedure:

1. Deparaffinized sections were brought to 70 percent alcohol;
2. Stained for 30 minutes in saturated Sudan black B in 70 percent alcohol;
3. Rinsed quickly in 6 70 percent alcohol.
4. Washed in running water;
5. Mounted in glycerine jelly.

Result:

Lipids stained black.

HISTOCHEMICAL METHODS FOR ENZYMES

The material was fixed in chilled acetone and kept in refrigerator for 24 hours. After that it was dehydrated in 2 changes of acetone (at room temperature) each of 2 hours, cleared in benzene (2 changes each at 45 minutes) and embedded immediately in paraffin wax (56 - 58°C). Sections cut at 7 microns.
GOMORI'S TECHNIQUE FOR ACID PHOSPHATASE

Preparation of 1 M acetate buffer:

1 M acetate buffer was prepared by adding 50 ml of 6 percent acetic acid to 100 ml of 13.6 percent sodium acetate (Trihydrate).

Substrate:

1 M acetate buffer, pH ... 3 parts,
5 percent lead nitrate ... 1 part,
Distilled water ... ... 6 parts,
2 percent sodium glycerophosphate ... 3 parts.

The mixture was shaken well and allowed to stand for a few hours. Filtered and diluted 1:3 with distilled water.

Method:

1. Deparaffinized sections including controls were brought to water;
2. Sections were incubated in substrate at 37°C for 1 hour;
3. Rinsed rapidly in distilled water;
4. Treated with 1 percent ammonium sulphide for 2 minutes (control sections were not treated with this step).;
5. Washed in water for 2 - 3 minutes;

Result:
Structures possessing acid phosphatase activity - Brown to black.

**GOMORI'S TECHNIQUE FOR ALKALINE PHOSPHATASE**

**Special reagents required:**

**Substrate:**

2 percent sodium glycerophosphate 25 ml
2 percent sodium barbitone 25 ml
Distilled water ... ... 50 ml
2 percent calcium chloride 5 ml
2 percent magnesium sulphate 2 ml

Fresh substrate was prepared for each batch of section.

**Method:**

1. Deparaffinized section along with controls were brought to water;
2. Sections were incubated in the substrate for 2 hours at 37°C;
3. Rinsed rapidly in distilled water;
4. Treated with 2 percent cabalt nitrate for 2 minutes;
5. Excess of cobalt nitrate was washed out for 1 minute;
6. Treated with 1 percent yellow ammonium sulphide; for 1 minute (control sections not immersed here);
7. Washed in water for 2 - 3 minutes;
8. Dehydrated in alcohol, cleared in benzene and mounted in DPX.

Result:

Structures possessing alkaline phosphatase activity ... Brown to black.

**GOMORI'S TECHNIQUE FOR LIPASE**

Preparation of 0.2 M Tris buffer pH 7.2:

0.2 M Tris (Hydroxymethyl) aminomethane; was prepared by dissolving 2.42 g. of Tris in 100 ml of distilled water, 25 ml of 0.2 M Tris + 45 ml of 0.1 N HCl were then diluted up to 100 ml of distilled water to make 0.2 M Tris buffer pH 7.2.

**Substrate:**

- 2 percent Tween 80 in water ... 5 ml
- 0.2 M Tris buffer pH 7.2 ... 20 ml
- 4 percent calcium chloride (anhydrous) ... 5 ml
- Distilled water ... 20 ml
Control:

Tween compound was left out of the substrate.

Method:

1. Deparaffinized sections along with controls were brought to water;
2. Incubated in substrate at 37°C for 6 hours.
3. Rinsed in distilled water;
4. Treated with 2 percent lead nitrate for 15 minutes;
5. Treated with 1 percent ammonium sulphide for 1 minute;

Result:

Sites of lipase activity - Brown
Control - negative for staining.

HOLT'S INDOXYLACETATE TECHNIQUE FOR ESTERASE

Special reagents required:

Oxidant:

- Potassium ferricyanide ... 210 mg
- Potassium ferrocyanide ... 155 mg
- Distilled water ... 100 ml.
Preparation of 0.2 M Tris - buffer, pH 8.3:

0.2 M Tris was prepared as shown in early method. 0.2 m Tris buffer pH 8.3 was prepared by taking 25 ml of 0.2 M Tris + 20 ml of 0.1 N HCl and making upto 100 ml with distilled water.

Substrate:

Tris buffer (0.2 m) at pH 8.3 ... 2 ml
Oxidant ... ... 1 ml
1 M calcium chloride ... ... 0.1 ml
2 M Sodium chloride ... ... 5 ml
Distilled water ... ... 2 ml

1.3 mg. of 5-bromoindoxyl acetate was dissolved in 1 ml of absolute ethanol in a small beaker, then added the above solution (separately mixed) agitating to mix.

Method:

1. Deparaffinized sections were brought to water;
2. Placed in substrate at 37°C for 1 hour;
3. Washed in distilled water;

Result:

Enzyme activity - Blue.
CATECHOL METHOD FOR POLYPHENOL OXIDASE
(Smyth, 1954)

The material was fixed in 70 percent alcohol for 24 hours. Dehydrated, cleared and embedded in Paraaffin wax (58 - 60°C). Sections cut at 7 microns.

Method:

1. Deparaffinized sections were brought to water;
2. Placed in 0.1 percent freshly prepared catechol for 1 hour at 40°C;
3. Washed in water for 15 minutes;
4. Dehydrated in alcohol, cleared in xylene and mounted in DPX.

Result:

Structures possessing enzyme activity - Reddish brown.

METHODS FOR NEUROSECRETION

The worms were fixed in aqueous Bouin's fluid for 24 hours. After fixation the material was washed with saturated solution of lithium carbonate in water to remove yellow colour, then it was dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraaffin wax (58 - 60°C). The sections were cut at 7 microns.
COPPER SULPHATE - SILVER NITRATE METHOD

(Betchaku, 1960).

1. Deparaffinized sections were brought to water;
2. Rinsed briefly in distilled water;
3. Treated with 7 percent $\text{Ag}_2\text{NO}_3$ for 48 hours at room temperature;
4. Immersed in 20 percent $\text{CuSO}_4$ for 48 hours at room temperature;
5. Rinsed in distilled water and placed in Bodian developer (Hydroquinone 1 g, Sodium sulphite 5 g. and distilled water 100 ml) for 5 minutes;
6. Washed in distilled water, dehydrated, cleared and mounted in DPX.

Result:

Nerve fibres - Yellow to brown
Nerve cells - Brown.

MALLORY'S TRIPLE STAIN METHOD

(Pantin, 1964).

Special reagent required:

Aniline blue - Orange G mixture:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline blue</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Orange G</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
Method:

1. Deparaffinized sections were brought to water;
2. Stained in 1 percent aqueous acid fuchsin for 2 minutes;
3. Rinsed in distilled water;
4. Transferred to 1 percent aqueous phosphotungstic acid for two minutes (Differentiating and Mordant solution);
5. Rinsed in distilled water;
6. Stained in aniline blue - Orange G mixture for 5 minutes,
7. Washed in water until no more colour was observed in water;
8. Dehydrated and differentiated in 95 percent alcohol, followed by absolute alcohol;
9. Cleared in xylene and mounted in DPX.

Results:

Nerve fibres and neurosecretory cells in various shades of violet -
Nuclei - red.
ALDEHYDE FUCHSIN STAINING TECHNIQUE

Staining solution:

Basic fuchsin ... 1 g.
70 percent alcohol ... 200 ml
concentrated HCl ... 2 ml
Paraldehyde (Fresh) ... 2 ml

The fuchsin was dissolved in the alcohol and the hydrochloric acid and paraldehyde were then added. The mixture was shaken well and allowed to stand at room temperature until it was a deep purple (48 hours). The solution was stored in the refrigerator when not in use.

Oxidant:

Potassium permangmate ... 0.15 g.
Conc. sulphuric acid ... 0.1 ml.
Distilled water ... 50 ml.

Mordant:

Phosphotungstic acid ... 4 g.
Phosphomolybdic acid ... 1 g.
Distilled water ... 100 ml.
Counterstain: Halmi's mixture:

- Distilled water ... 100 ml
- Light green ... 0.4 g.
- Orange G ... 1.0 g.
- Chromotrope 2 R ... 0.5 g.
- Glacial acetic acid 1.0 ml.

Method:

1. Deparaffinized sections were brought to water;
2. Oxidized for one minute in acid permanginate;
3. Rinsed in distilled water;
4. Decolourized in 2.5 percent sodium bisulphite;
5. Passed through rinses of distilled water to 70 percent alcohol and then to aldehyde fuchsin, stained for 10 minutes;
6. Differentiated for 30 seconds in 0.5 percent acid alcohol;
7. Passed through rinses of 70 percent alcohols and distilled water;
8. Transferred to mordant 10 minutes;
9. Rinsed in distilled water;
10. Counterstained for 30 minutes in Halmi's mixture;
11. Rinsed in 0.2 percent acetic acid and in 95 percent alcohol;
12. Dehydrated rapidly through absolute alcohol, cleared in xylene and mounted in DPX.
Results:

Cytoplasm ... light green,
Nuclei ... Orange;
Neurosecretion ... dark purple.

BARGMANN'S CHROME HAEMATOXYLIN METHOD

Preparation of stain:

1 percent aqueous haematoxylin ... 50 ml
3 percent aqueous chrome-alum ... 50 ml
5 percent aqueous potassium dichromate
5 percent aqueous H₂SO₄ ... ... 1 ml.

The mixture was allowed to ripen for 48 hours and filtered before use.

Method:

1. Sections brought down to water;
2. Placed in a solution of Bouin's fixative, containing 3 percent chromealum for 24 hours at 37°C;
3. Washed in running tap water to remove yellow colour;
4. Oxidized for 3 minutes in a mixture of 2.5 percent KMnO₄ (1 part), 5 percent Conc H₂SO₄ (1 part) distilled water (6 parts);
5. Washed in distilled water;  
6. Bleached in 1 percent Oxalic acid for 1 minute.  
7. Washed in running tap water for 5 minutes;  
8. Stained for 10 minutes;  
9. Differentiated for 30 seconds in 0.5 percent acid alcohol and washed in running tap water for 3 minutes;  
10. Stained for 2 minutes in 0.5 percent aqueous phloxine;  
11. Rinsed in 5 percent aqueous phosphofungastic acid and then in running tap water for 5 minutes;  
12. Differentiated in alcohols, cleared and mounted in DPX.

**Results:**

Neurosecretory substance - deep purple  
Nuclei - Purple.