# CHAPTER II

## MATERIAL AND METHODS

Material

For the present investigation, females of Indian wall lizard (Hemidactylus flaviviridis - Ruppel,) were selected.

Hemidactylus flaviviridis - Indian gecko H. flaviviridis is found usually on the walls of houses and buildings. This species was collected from the houses of Kolhapur city (Maharashtra) India. The skin colour is yellow-green and brownish spots are observed on dorsal side of the tips of digits. Dorsal side of the skin is rough and ventral side is smooth and covered with small epidermal scales.

Anatomy of the system -

Female reproductive system - The system consists of a pair of ovaries, oval in shape. Each ovary consists of number of follicles enclosed inside the ovarian capsule. The surface of ovary is smooth, with 3 lobes. Ovaries are white to yellowish in colour depending upon the vitellogenic oocytes. Each ovary is connected to the abdomen by means of a ligament. The fallopian tube emerges on the lateral side and opens in to the oviduct. The two oviducts finally open in cloaca. During hibernation the oviduct is slender, but in breeding period it is well developed. In breeding period one egg is found in each oviduct.

About 10 to 15 females were collected in each month and acclamatized to the laboratory conditions before they

were sacrificed. The animals sacrificed and dissected for ovaries and oviducts. The ovaries were fixed in different fixatives viz. calcium acetate formalin 10% buffered, formalin and Baker's fixative for the histochemical studies and processed for wax sectioning and frozen sectioning. The oviducts were serially cut into 4 pieces and were processed as ovaries.

Methods

To get an insight of the mucopolysaccarides the battery of histochemical techniques employed in the present investigation, a brief histochemical classification of mucosubstances and a review of the progress in these techniques is given in the subsequent pages, which is followed by details of the various techniques actually employed. A tabular presentation of the techniques, staining reactivities and their interpretations is appended at the end of the present chapter for ready reference.

The ovaries and oviducts were also studied for other histochemical techniques wherever needed e.g. lysosomal enzymes, proteins and calcium.

Histological techniques

The small pieces of the reproductive organs were fixed in the ice-cold calcium-acetate-formalin (CAF-2% calcium acetate in 10% formalin) fixatives for 24 hrs. The fixation of the tissues was followed by washing in chilled distilled water, in running

tap water, dehydration in alcohol, clearing in xylene and paraffine embedment. The sections were cut at 5 to 6-μ. Some sections were routinely stained by haematoxylene-eosin and Mallory's triple technique for histological observations.

1) Histochemical procedures for identification of Mucosubstances :

Before describing the histochemical techniques for the identification of mucosubstances, it will be worthwhile to note the detailed histochemical classification of mucosubstances. Spicer et al., (1965), suggested the histochemical classification of mucosubstances, in which the mucosubstances are named by (1) staining site in which they are found and (2) subtyping them as far as possible, as neutral mucosubstances, mucopolysaccharides, sulfomucins and sialomucins. It is suggested that further subdivisions of these subtyped mucosubstances can be achieved by means of the following histochemical reactions.

a) Affinity for basic dyes such as azure A.
b) Affinity for alcian blue,
c) Lability with respect to testicular hyaluronidase, and
d) Lability with respect to Vibrio cholerae neuraminidase.

Below is set-forth, a working histochemical classification of mucosubstances which is substantially that of Spicer et al., (1965).

## Histochemical classification of mucosubstances

I. Neutral mucosubstances :

Neutral glycoproteins, immunologically reactive glycoproteins, fucomucins, mannose-rich mucosubstances in epithelia and connective tissues. All react towards PAS and some towards periodic acid para-diamine procedure.

II. Acid mucosubstances :

(A) Sulfated :

1. Connective tissue mucopolysaccharides (Per-iodate unreactive).

   (a) Resistant to testicular hyaluronidase.

   i) Alcinophilic at or below 1.0 M $MgCl_2$ - Keratin sulfate, Heparin.

   ii) Alcianophilia in presence of 0.7 (or less) M $MgCl_2$ - - Dermatan sulfate.

   (b) Susceptible to testicular hyaluronidase.

   i) Alcohol resistant, affinity for 0.02% azure A at or below pH 2.0 - chondroitin sulfate in cartilage.

   ii) Alcohol resistant, affinity for 0.02% azure A at or below pH 4.0 - chondrotin sulfates in vascular tissues.

2. Epithelial sulfomucins (Testicular hyaluronidase resistant).

   (a) Periodate unreactive.

   i) Sulfate esters on vic-glycols.

ii) Sulfate esters not on vic-glycols.

I) Alcohol resistant affinity for 0.02% azure A at or below pH 2.0.

II) Alcohol resistant affinity for 0.02% azure A at or above pH 4.5.

(b) Periodate reactive (acid glycoproteins?)

i) Alcohol resistant, affinity for 0.02% azure A at or above pH 2.0.

ii) Weak or negligible, alcohol resistant affinity for 0.02% azure A at or above pH 4.5.

B) Non-sulfated -

1. Hexuronic acid rich mucopolysaccharides - hyaluronic acid, chondroitin.

2. Sialic acid rich mucosubstances.

(a) Connective tissue mucopolysaccharides containing sialic acid (?).

(b) Epithelial sialomucins - Acid glycoproteins.

i) Highly susceptible to Vibrio cholerae sialidase, periodate reactive and metachromatic.

ii) Slowly digestible with Vibrio cholerae sialidase.

a) Periodate reactive.

b) Periodate unreactive.

c) Resistant to Vibrio cholerae sialidase.

I) Rendered metachromatic and susceptible to enzyme by prior saponification.

II) Sialidase resistant after saponification.

a) Periodate reactive.

b) Periodate unreactive.

For visualization of mucosubstances there are series of histochemical methods evolved by different workers in this field. 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 the non-specific histochemical methods can be supplimented with the 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 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 visualization of mucosubstances in the different reproductive organs of female H. flaviviridis were employed.

Fixation and Post-fixation procedures :

The different tissues of the reproductive organs were

quickly cut into smaller pieces and immediately immersed in ice-cold solution (4°c) of 2% calcium acetate in 10% formalin (CAF). 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, clearing in xylene and paraffin embedment; the sections were cut at 5 to 6-μ. The sections were subjected to various histochemical techniques hereafter described for the detection of mucosubstances.

1. Neutral mucosubstances -

(A) Periodic acid-schiff-reaction (PAS)

(McManus, 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 D.P.X.

Result :- Periodate reactive, hexose containing mucosubstances stain pink magenta.

(B) 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 times (total 6 minutes) with .5% sodium metabisulphite.

7) Washed, dehydrated, cleared routinely and mounted in DPX.

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

(C) 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 I-A for PAS, staining procedure.

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

II-Acid mucosubstances :-

(A) 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 3% acetic acid pH 2.5) for 30 minutes.

4) Rinsed in 3% acetic acid.

5) Washed in running water for 3 minutes.

6) Dehydrated, cleared and mounted as usual.

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

(B) -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 0.1 N HCl (pH 1.0).

3) Blotted on a puffless filter paper.

4) Dehydrated quickly, cleared and mounted as usual.

Result - Only sulfomucins stain intense blue.

(C)- Colloidal Iron (CI) (Hale, 1946; Rinehart and Abul-Haj, 1951; Mowry, 1961; 1963).

Stock solution - To the boiling 250 ml of distilled water, 4.4 ml 29% ferric chloride solution was added with constant stirring. When the solution turned dark red it was allowed to cool and then dialysed against distilled water, using dialysing membrane.

Working solution :

Glacial acitic acid - 5 ml

Distilled water -15 ml

Stock collodal iron solution. -20 ml

Procedure - 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) The sections were treated with a freshly prepared mixture of equal volumes of 2% HCl and 2% potassium ferrocyanide for 20 minutes.

5) Washed with running water for 5 minutes.

6) Dehydrated, cleared, and mounted as usual.

Result : Sites of acidic mucosubstances are Prussian blue. The results obtained with this method are very much similar to those obtained with AB (pH 2.5) procedure.

III- Distinction between neutral and acidic mucosubstances.

A. AB pH 2.5 - PAS sequential staining technique.

(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 in I - A for PAS staining technique.

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.

B. - AB pH 1.0 - PAS sequential staining technique

(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 a puffless filter paper.

4) Processed as in I-A for PAS, staining technique.

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

C Colloidal Iron-PAS sequential staining technique

(Ritter and Oleson, 1950; Mowry, 1963).

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

2) Rinsed briefly in 12% acetic acid.

3) Treated with freshly prepared colloidal iron working solution for 60 minutes at room temperature.
4) Treated with 12% acetic acid.
5) Treated with freshly prepared mixture of equal volumes of HCl and 2% potassium ferrocyanide for 20 minutes.
6) Washed with running water for 5 minutes.
7) Processed as in I-A for PAS staining technique.

Result - 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.

IV - Distinction between sulfomucins and carboxymucins :

A- Aldehyde fuchsin (AF)

(Gomori, 1950; Halmi and Davies, 1953).

Preparation of AF crystals - The crystals of AF were prepared according to the method suggested by Cameron and Steal (1959). To 200 ml boiling distilled water, 1 gram of basic Fuchsin was added and the solution was allowed to boil for one minute, then cooled and filtered. To the filterate, 2 ml of conc. HCl and 2 ml of paraldehyde were added. The solution was left in stoppered bottle at room temperature. When the solution had lost its reddish colour, usually after 3-4 days, it was filtered and the filterate was discorded. The precipitate was dried on the filter paper at 60°c.

Staining solution :- The staining solution was prepared by dissolving 0.5 gm of dry crystals in 70% alcohol.

Procedure :

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 with 70% alcohol.
5) Dehydrated in 90% and absolute alcohol, cleared and mounted as usual.

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

B. - Aldehyde fuchsin - AB (AF - AB pH 2.5) sequential staining technique (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.

Result - Sulfated mucosubstances stain purple, non-sulfated mucosubstances like sialic acid and hyaluronic acid stain blue.

C. Critical electrolyte concentration technique using AB at pH 5.6 with increased concentrations of $MgCl_2$.

(Scott et al., 1964; Scott and Dorling, 1965).

Staining solution : 0.1% AB was added to 0.05 M sodium acetate/acetic acid buffer at pH 5.6. Then more $MgCl_2$ was added and a series of increasing concentration of $Mg^{++}$ were prepared such as 0.0M, 0.1 M. 0.2M. 0.4M. 0.5M. 0.6M, 0.8M and 1.0M.

Procedure :

1) 8 dewaxed slides after hydration were brought to distilled water.

2) Each slide was stained for 30 minutes in staining solutions 0.0, 0.1, 0.2 etc. respectively.

3) Washed in running water for 5 minutes.

4) Dehydrated, cleared and mounted as usual.

Result : 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 $Mg^{++}$ concentrations. Various sulfomucins

lose their alcianophilia at different levels of $Mg^{++}$ concentration.

D.-Azure A. Metachromatic staining technique at controlled pH levels.

(Wislocki et al., 1947; Spicer, 1960; Spicer et al., 1967; Pearse, 1968).

Staining solutions :

pH 0.5 - 0.02% azure A in 0.5 N HCl.

pH 1.0 - 0.02% azure A in 0.1 N HCl.

pH 1.5 - 0.02% azure A in 50 ml buffer

(30 ml. 0.1 N HCl + 30 ml 0.1 M $KH_2$ PO4).

pH 2.0 - 0.02% azure A in 50 ml buffer.

(20 ml 0.1 N HCl + 30 ml 0.1 M $KH_2$ PO4).

pH 2.5 - 0.02% azure A in 48 ml distilled water + 2 ml 0.1 M citric acid.

pH 3.0 - 0.02% azure A in 48 ml distilled water + 1.65 ml 0.1 M citric acid + 0.35 ml 0.2 M $Na_2H$ $PO_4$.

pH 3.5 - 0.02% azure A in 48 ml distilled water + 1.4 ml 0.1 M citric acid + 0.6 ml 0.2 M $Na_2$ HPO4.

pH 4.0 - 0.02% azure A in 48 ml distilled water + 1.25 ml 0.1 M citric acid + 0.75 ml 0.2 M $Na_2HPO4$

pH 4.5 - 0.02% azure A in 48 ml distilled water + 1.1 ml 0.1 M citric acid + 0.9 ml 0.2 M $Na_2HPO_4$.

pH 5.0 - 0.02% azure A in 48 ml distilled water + 1.0 ml 0.1 M citric acid + 1.0 ml 0.2M $Na_2HPO_4$.

Procedure -

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 and the observations were recorded.

5) Dehydrated in alcohol and observed under microscope.

6) Cleared and mounted as usual.

Result: Strongly sulfated mucosubstances exhibit metachromasia below pH 1.5, Sialomucins generally stain metachromatically between pH 2.5 and 3.5; some protein masked sulfomucins and hyaluronic acid exhibit metachromasia at and above pH 4.5. Generally metachromasia of sulfomucins resists alcoholic dehydration.

E - Mild methylation - AB pH 2.5

F - Active methylation - AB pH 2.5

(Fisher and Lillie, 1954; Spicer, 1960).

Procedure -

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

2) Rinsed in absolute methanol.

3) Sections were placed in coupl-in 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 II-A.

7) After washing, dehydration and clearing, sections were mounted in D.P.X.

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

G - Mild Methylation - saponification - AB pH 2.5

G - Active methylation - saponification - AB pH 2.5

(Spicer and Lillie, 1959,; Spicer, 1960).

Sections were methylated separately at 37°c 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 II-A . After washing, dehydration and clearing, the sections were mounted in DPX.

Result : Restoration of the bosophilia after soponification indicates the presence of carboxyl groups but failure of

restoration of the bosophilia indicates the presence of the sulfate esters.

I-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 hours.

3) Washed in running water for 5 minutes.

4) Stained either with AB pH 2.5 or azure A pH 3.0

5) Dehydrated, cleared and mounted as usual.

Result - Complete or partial loss of alcianophilia or metachromatia indicates probable presence of sialomucins.

V - Enzyme digestion tests :

A - Pepsin digestion

(Pearse, 1969, Spicer, 1960, Quintarelli, 1963, Thompson 1966).

Procedure -

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 throughly in running water.

4) Stained with AB pH 2.5, CI or azure A pH 1.5, 3.0 and 4.5.

5) Dehydrated, cleared and mounted as usual.

Result - Protein masked mucosubstances (PAS positive but AB, CI and azure A negative) stain with basophilic dyes after removal of protein masking.

A bird's eye view of the various histochemical techniques employed in the present investigation alongwith the chemica l reactions involved in the staining and the histochemical interpretations of the staining reactions with the literature is given in Table 1.

For the histochemical demonstration of lysosomal enzymes the standard methods were used. For β-glucuronidase activities post-coupling method by Seligaman et al., (1954), was employed and for acid phosphatase activities simultaneous coupling method by Barka (1960, 1962) was used. For calcium demonstration Alizarin red S method (McGee-Russell, 1958) and for demonstration of yolk globules Sudan black B, Chiffelle, and Putt, 1951) method were employed. For enzyme and yolk studies fresh frozen sections were utilized.

Table

Histochemical Methods employed for visualizing Mucosubsta nces

| Histochemical Method | Chemical reaction involved | Histochemical result | References |
|---|---|---|---|
| 1) Periodic acid schiff's reaction (PAS) | Oxidation of vicinal hydroxyls to dialdehydes by periodate and formation of coloured complexes with schiff's reagent. | All polysaccharides and mucosubstances colour pink to magenta. | McManus(1946) Hotchkiss (1948). |
| 2) Periodic acid phenylhydrazine Schiff | Phenylhydrazine selectively blocks periodate engendered dialdenhydes in mucosubstances, leaving unblocked dialdehydes in periodate reactive muco-substances available to subse-quent Schiff staining. | Periodate reactive acidic mucosubstances stained red presumably are those in which acid groups are proximal to vicinal glycols. | Spicer (1965) Spicer et al. (1967). |
| 3) Diastasedigestion PAS. | Hydrolyses and removes glycogen. | Loss of PAS reactivity in sites containing glycogen. | Lillie (1954) Lison (1960) |
| 4) Alcin blue pH 2.5. | Probably formation of alcian blue complexes with carboxyls and sulfate groups. | Sialomucins and weakly acidic sulfomucins stain; the most strongly acidic sulfomucins stain weakly or not at all. | Mowry (1956). |

| Histochemical Method | Chemical reaction involved | Histochemical result | References |
|---|---|---|---|
| 5) Alcian blue, pH 1.00. | Probably formation of alcian blue complexes with sulfate groups. | Weakly and strongly acidic sulfomucins are selectively stained. | Lev and Spicer (1964). |
| 6) Colloidal Iron. | Probably formation of complexes between cationic colloidal ferric aggregates and carboxyls, sulfate and phosphate esters. | Non-sulfated acid muco-substances and some sulfated mucosubstances colour blue. | Hale (1957) Rinehart and Abyl-Haj (1951) Mowry (1961,62) |
| 7) AB pH 2.5-PAS. | Addition of results by single methods | Alcian blue reactive periodate unreactive acid mucosubstances stain blue Alcian blue and PAS reacti-ve mucosubstances colour purple blue. Neutral muco-substances colour pink magenta. | Mowry and Winkler 1956. |

| Histochemical Method | Chemical reaction involved | Histochemical result | References |
|---|---|---|---|
| 8) AB pH 1.0 - PAS | Addition of results by single method. | Sulfomucins stain blue or blue-purple. Neutral and non-sulfated periodate reactive muco-substances stain pink magenta. | Spicer (1965) Spicer *et al.* (1967). |
| 9) Colloidal Iron PAS | Addition of results by single methods. | Colloidal iron reactive periodate unreactive acid mucosubstances stain blue. Colloidal iron and PAS reactive mucosubstances colour purple-blue neutral mucosubstances colour pink magenta. | Ritter and Oleson (1950) Mowry (1963). |
| 10) Aldehyde fuchin (AF) | Formation of salt complexes between cationic staining entity and sulfated and carboxyl groups. | Sulfated mucosubstances stain dark purple, sialo-mucins and hyaluronic acid colour light purple. | Gomori (1950) Halmi and Davies (1953). |

| Histochemical Method | Chemical reaction involved | Histochemical result | References |
|---|---|---|---|
| 11) AF-AB pH 2.5 | Formation of salt complexes between cationic staining entity and sulfate and carboxyl groups. | Sulfomucins stain purple or blue-purple, sialomucins and other non-sulfated acidic mucosubstances stain blue. | Spicer and Meyer (1960) |
| 12) Alcian blue at pH 5.6 with graded concentrations of $MgCl_2$ | Alcian blue complexes with sulfate groups. Different sulfomucins vary in the critical electrolyte concentration at which alcianophilia is lost. | Non-sulfated acidic mucosubstances are not stained at and above 0.1 M $Mg^{++}$ concentration. Sulfomucins stain selectively at and above 0.2 M $Mg^{++}$ concentration. | Scott *et al.* (1964) Scott and Dorling (1965). |
| 13) Azure A or toluidine blue at controlled pH levels. | Formation of blue orthochromatic or purple or red metachromatic salt complexes with the extinction values indicating degree of acidity of the polymer. | Strongly sulfated mucosubstances stain purple red at pH 0.5 to 1.5 sialomucins stain purple-red at pH 2.5 to 3.5 hyaluronic acid and weakly acidic mucosubstances stain purple at pH 4.5 to 5.0. | Wislocki *et al.* (1947). Spicer (1960). Pearse (1968). |

| Histochemical method | Chemical reaction involved | Histochemical result | References |
|---|---|---|---|
| 14) Mild methylation AB pH 2.5 | Esterification of carboxyl groups. | Generally mild methylation abolishes the alcianophilia of carboxymucins. | Fisher and Lillie(1954). Spicer(1960). |
| 15) Mild methylation saponification AB pH 2.5 | Restoration of carboxyl groups. | Restoration of the alcianophilia after saponification of methylated sections, indicates the presence of carboxyl groups. | Spicer and Lillie(1959) Spicer(1960) |
| 16) Active methylation AB pH 2.5. | Carboxyl groups are esterified sulfomucins are desulfated. | Active methylation abolishes alcianophilia of carboxymucins through esterification and of sulfomucins through hydrolytic removal of the sulfate groups. | Fisher and Lillie(1954). Spicer(1960). |

| Histochemical Method | Chemical reaction involved | Histochemical result | References |
|---|---|---|---|
| 17) Active methylation saponification AB pH 2.5 | Restoration of carboxyl groups. Sulfomucins are hydrolytically removed during active methylation are not restored following subsequent saponification. | Restoration of the alcianophilia after subsequent saponification indicates the presence of carboxyl groups and loss of alcianophilia indicates the presence of sulfate groups. | Spicer and Lillie(1959). Spicer(1960). |
| 18) Acid hydrolysis AB pH 2.5 or Azure A pH 3.0 | Removes sialic acids from mucosubstances. | Complete or partial loss of alcianophilia or metachromasia indicates the probable presence of sialomucins. | Quintarelli et al,(1961). |
| 19) Pepsin digestion AB pH 1.0, 2.5 or azure A pH 1.5, 3.0 and 4.5. | Hydrolysis of internal peptide bonds as well as those of the the terminal amino acids of proteins. | Protien masked mucosubstances stain with basophilic dyes after removal of protein masking. | Pearse(1960). Spicer(1960). Quintarelli (1963). Thompson(1966). |