CHAPTER TWO

MATERIAL AND METHODS

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A) MATERIAL.

For the present investigation entitled **"Studies on Mucosubstances in the Gall Bladder of some Birds and Mammals"**, ten species of vertebrates (Birds - four, and Mammals - six) were used. While selecting the animals, care was taken to select the animals differing in their feeding habits. Mostly, the adult animals were selected, however, developing fetus of man, guinea-pig and rabbit were also used. To confirm the observations, three to four animals of each species (without considering the sex of the animals) were employed. The gall bladders of human fetus were kindly supplied by Dr.Mane from Satara. Most of the birds and mammals were collected locally, while the guinea pigs were purchased from animal husbandry suppliers at Bombay.

For the present investigation, adults of the following species of birds and mammals and fetus of man, guinea pig and rabbit were used:

I) Aves:

1. Numida meleagris (Guinea fowl),
2. Anas strepera (Duck)
3. Acridotheres tristis (Indian myna),
4. Larus argentatus (Sea gull),

II) Mammals:

5. Sus indicus (A common village pig, Indian boar),
6. Cavia porcellus (Guinea pig),
7. Bos indicus (Ox),

8. Lepus reficaudatus (Hare)

9. Megaderma lyra (Blood sucking bat),

10. Homo sapiens (man).

B) METHODS.

Most of the live animals were killed by decapitation and the gall bladder was dissected out along with the part of liver. Some of the animals were ether anaesthetized and then killed and the gall bladder was dissected out. The gall bladder was then fixed immediately in cold (4°C), 2% calcium acetate in 10% neutral formalin (CAF fixative) for 24 hours.

The tissues were then washed thoroughly in running tap water for about 12 hours. These tissues were then dehydrated through different grades of alochol, such as 30%, 50%, 70%, 90% and absolute alcohol, keeping for about 30 minutes in each grade. These were finally transferred to xylene for clearance. The tissues were then embedded in paraffin (M.P. 58° to 60°C) according to usual manner and blocks were made. The sections were cut at 4 to 5 µM and spread on albuminised slides.

The sections were de-paraffinised, hydrated and brought to distilled water. Some of the sections were stained with hematoxylin-eosin (H-E) for histological observations and the remaining sections were subjected to various histochemical techniques for characterization of mucosubstances.

a) Hematoxylin-Eosin Method (H-E):

1. After dewaxing and hydration, sections were brought to distilled water.
2. Stained with hematoxylin (nuclear stain) for 2 to 3 minutes.
3. Washed in running water for 4 to 5 minutes.
4. Dehydrated in 30%, 50%, 70% alcoholic grades.
5. Stained with 70% alcoholic eosin (cytoplasmic stain) for 10 minutes.
6. Dehydrated in 90% and absolute alcohol, cleared in xylene.
7. Mounted in DPX.

b) Histochemical Methods:

There are several histochemical techniques used for the demonstration of mucosubstances in animal tissues. In addition to the mannuals of histochemistry, there are several contributions by Spicer (1963), Curran (1964), Spicer and Henson (1967), Spicer et al. (1967), Leppi (1968), Nalavade (1975) and Nalavade and Varute (1971, 1972-a, -b, -c , 1973-a, -b, 1976-a, -b, 1977), which contribute greatly towards a survey of the literature on the subject of the histochemistry of the mucosubstances. The specificity of different methods can be enhanced by the use of chemical reactions such as blockade of reactive groups and their restoration, control of pH of the basic dyes, sequential staining procedures, critical electrolyte concetration and selective removal of the moieties by acid hydrolysis or enzyme digestions. Thus, non-specific histological and histochemical methods can be supplemented with the modified and specific ones for the better understanding of the chemical composition of the cellular components. The terminology suggested by Spicer et al. (1965)

for carbohydrate rich tissue components is followed in the present investigation. The methods employed in the present investigation for detection of histochemically identifiable mucosubstances are described hereafter in some greater details.

I) Neutral Mucosubstances:

A) Periodic Acid Schiff Reaction (PAS):
(McManus, 1946; Hotchkiss, 1948)

Solutions required:

1. 0.5% periodic acid,
2. Schiff's reagent,
3. 0.5% sodium or potassium meta-bi-sulphite.

Chemical reaction:

Oxidation of vicinal hydroxyls to dialdehydes by periodate and formation of coloured complexes with Schiff's reagent.

Procedure:

1. After dewaxing and hydration, sections were brought to distilled water.
2. Oxidized with 0.5% periodic acid for 10 min.
3. Washed with distilled water.
4. Treated with Schiff's reagent for 10 minutes.
5. Rinsed three times (total 6 min.) with 0.5% sodium meta-bi-sulphite.
6. Washed in distilled water, followed by alcoholic dehydration, cleared in xylene and mounted in Canada balsam.

Result:

Pericdate reactive, hexose containing mucosubstances stain pink-magenta.

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

Solutions required:

1. 0.5% periodic acid.
2. Schiff's reagent,
3. 0.5% sodium or potassium meta-bi-sulphite,
4. 5% phenylhydrazine hydrochloride.

Chemical reaction:

Phynylhydrazine selectively blocks periodate engendered dialdehydes in mucosubstances leaving unblocked dialdehydes in periodate reactive mucosubstances available to subsequent Schiff staining.

Procedure:

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 min.
4. Washed with distilled water.
5. Immersed in Schiff's reagent for 10 min.
6. Rinsed three times (total 6 min.) with 0.5% sodium meta-bi-sulphite.
7. Washed, dehydrated, cleared routinely and mounted in Canada balsam.

Result:

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

C) Sulfation-azure A
(Pearse, 1960)

Solutions required:

1. Mixture of dilute sulphuric acid and acetic acid,
2. Azure A pH 1.5 (0.02% Azure A in 50 ml buffer, 30 ml 0.1 N HCl + 20 ml 0.1M KH_2PO_4)

Chemical reaction:

Induction of sulfate groups in neutral mucosubstances.

Procedure:

1. After dewaxing and hydration, sections were brought to distilled water.
2. The sections were treated with a mixture of sulphuric acid and acetic acid for 30 minutes.
3. Washed in distilled water for 5 minutes.
4. Stained with Azure A pH 1.5.
5. Washed in distilled water and observed under microscope.
6. Dehydrated, cleared and mounted on DPX.

Result:

The neutral mucosubstances which exhibit orthochromatic blue staining with Azure A at lower pH (1.5) exhibit metachromatic pink or red staining after sulfation.

II) Glycogen:

A) Diastase digestion-PAS (or α-amylase-PAS).
(Lillie, 1954; Lison, 1960)

Solutions required:

1. 0.5% periodic acid,
2. Schiff's reagent,
3. 0.5% sodium or potassium meta-bi-sulphite,

4. 0.1% malt diastase or α-amylase in 0.2 M phosphate buffer at pH 6.0.

Chemical reaction:

Hydrolyses and removes glycogen.

Procedure:

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 or α-amylase in 0.2 M phosphate buffer at pH 6.0.
3. Washed in distilled water.
4. Processed as in I-A for PAS.

Result:

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

III) Acid Mucosubstances:

A) Alcian Blue (AB) at pH 1.0:
(Lev and Spicer, 1964)

Solution required:
1. 1% AB in 0.1 N HCl

Chemical Reaction:

Probably formation of alcian blue complexes with sulfate groups.

Procedures:

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

Result:

Only sulfated mucosubstances stain intense blue.

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

Solutions required:

1. 3% acetic acid,
2. 1% AB in 3% acetic acid (pH 2.5)

Chemical reaction:

Probably formation of alcian blue complexes with carboxyls and sulfate groups.

Procedure:

1. After dewaxing and hydration, sections were brought to distilled water.
2. Rinsed in 3% acetic acid.
3. Stained with AB pH 2.5 (1% AB in 3% acetic acid) for 30 min.
4. Rinsed in 3% acetic acid.
5. Washed in running water for 5 min.
6. Dehydrated, cleared and mounted as usual.

Result:

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

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

Solutions required:

1. 29% Ferric chloride solution.
2. Stock colloidal iron 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.

3. Glacial acetic acid.
4. Working coloidal iron solution :
   - Glacial acetic acid : 5 ml.
   - Distilled water : 15 ml.
   - Stock colloidal iron solution. : 20 ml.
5. 12% acetic acid,
6. 2% HCl
7. 2% Potassium ferrocynide.

Chemical reaction:

Probably formation of complexes between cationic colloidal ferric aggregates and carboxyls, sulfate and phosphate esters.

Procedures:

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 teperature.
4. Rinsed in 12% acetic acid.
5. The sections were treated with freshly prepared mixture of equal volumes of 2% HCl and 2% potassium ferrocynide for 20 min.
6. Washed with running water for 5 min.

7. Dehydrated, cleared and mounted as usual.

Result:

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

IV) Distinction between Neutral and Acidic Mucosubstances:

A) AB pH 1.0-PAS Sequential Staining Technique: (Spicer, 1965: Spicer et al., 1967)

Solutions required

1. 1% AB in 0.1 N HCl (pH 1.0),
2. 0.5% periodic acid,
3. Schiff's agent,
4. 0.5% sodium or potassium meta-bi-sulphite.

Chemical reactions:

Addition of results by single methods.

Procedure:

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 min.
3. Sections were blotted on puffless filter paper.
4. Processed as in I-A for PAS.

Result:

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

B) AB pH 2.5-PAS Sequential Staining Technique:
(Mowry and Winkler, 1956; Mowry, 1963).

Solutions required:

1. 3% acetic acid,
2. 1% AB in 3% acetic acid (pH 2.5)
3. 0.5% periodic acid,
4. Schiff's reagent,
5. 0.5% sodium of potassium meta-bi-sulphite.

Chemical reaction:

Addition of results by single methods.

Procedure:

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 min.
4. Rinsed in 3% acetic acid.
5. Washed in distilled water for 5 min.
6. Procssed as in I-A for PAS.

Result:

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 colur magenta.

C) Colloidal Iron-PAS Sequential Staining Technique:
(Ritter and Olesen, 1950; Mowry, 1963)

Solutions required:

1. 29% ferric chloride solution,
2. Stock colloidal iron solution,

3. Glacial acetic acid,
4. Working colloidal iron solution,
5. 12% acetic acid
6. 2% HCl
7. 2% potassium ferrocynide
8. 0.5% periodic acid
9. Schiff's reagent
10. 0.5% sodium or potassium meta-bi-sulphite.

Chemical reaction:

Addition of results by single method.

Procedure:

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 min. at room temperature.
4. Rinsed with 12% acetic acid.
5. Treated with freshly prepared mixture of equal volume of 2% HCl and 2% potassium ferrocynide for 20 min.
6. Washed with running water for 5 min.
7. Processed as in I-A for PAS.

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.

V) Distinction between Sulfomucins and Carboxymucins:

A) Aldehyde Fuchsin (AF):
(Gomori, 1950; Halmi and Davies, 1953)

Chemicals required:

1. 70% alcohol,
2. Conc.HCl
3. Paraldehyde,
4. Basic fuchsin,
5. AF staining solution (the staining solution was prepared by dissolving 0.5 gm. of dry crystals in 70% alcohol).

Preparations 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 gm. of basic fuchsin was added and the solution was let to boil for one min., then cooled and filtered. To the filtrate, 2 ml. of Conc.HCl and 2 ml. of paraldehyde were added. The solution was left stoppered at room temperature. When the solution had lost its reddish colour, usually after 3-4 days, it was filtered and the the filtrate was discarded. The precipitate was dried on the filter paper at 60°C).

Chemical reaction:

Formation of salt complexes between cationic staining entity and sulfated and carboxyl groups.

Procedure:

1. After dewaxing and hydration, sections were brought to distilled water.
2. Rinsed in 70% alcohol.

3. Stain with AF staining solution for 30 min.
4. Rinsed in 70% alcohol.
5. Dehydrated in 90% and absolute alcohol, cleared in xylene and mounted as usual.

Result:

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

B) Aldehyde Fuchsin-AB (AF-AB pH 2.5)
Sequential Staining Techniques:
(Spicer and Meyer, 1960)

1. 70% alcohol,
2. Conc.HCl
3. Paraldehyde,
4. AF staining solution,
5. 3% acetic acid,
6. 1% AB in 3% acetic acid.

Chemical reaction:

Formation of salt complexes between cationic staining entity and sulfate and carboxyl groups.

Procedure:

1. After dewaxing and hydration, sections were brought to distilled water.
2. Rinsed in 70% alcohol.
3. Stained in AF staining solution for 30 min.
4. Rinsed in 70% alcohol.
5. Washed in running water for 5 min.
6. Rinsed in 3% acetic acid.

7. Stained with AB (pH 2.5) for 30 min.

8. Rinsed in 3% acetic acid.

9. Washed in running water for 5 min.

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 concentration of $MgCl_2$:

(Scott et al., 1964; Scott and Dorling, 1965; Stoward, 1967).

Solutions required:

1. 0.1% alcian blue in 0.05 M sodium acetate/acetic acid buffer at pH 5.6.

2. AB solutions as above with $MgCl_2$ graded concentrations from 0.1 M to 1.0 M.

Chemical reaction:

Alcian blue forms complexes with sulfate groups. Different sulfomucins vary in the critical electrolyte concentration at which alcianophilia is lost.

Procedure:

1. Eight dewaxed slides, after hydration, were brought to distilled water.

2. Each slide stained for 30 min. in staining solutions such as 0.1 M, 0.2 M, etc. respectively.

3. Washed in running water for 5 min.

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^{++} concentration. Sulfomucins are selectively stained at and above 0.2 M Mg^{++} concentration. Various sulfomucins lose their alcianophilia at different levels of Mg^{++} concentration.

D) Azure A Metachromatic Staining Techniques at controlled pH levels: (Wislocki et al., 1947; Spicer, 1960; Spicer et al., 1967; Pearse, 1968).

Solutions required:

1. 0.02% Azure A in 0.5 N HCl (pH 0.5),
2) 0.02% Azure A in 0.1 N HCl (pH 1.0),
3. 0.02% Azure A in 50 ml. buffer (30 ml.) 0.1 N HCl + 20 ml. 0.1 M KH_2PO_4 (pH 1.5),
4. 0.02% Azure A in 50 ml. of buffer (20 ml. 0.1 N HCl + 30 ml. 0.1 M KH_2PO_4) (pH 2.0),
5. 0.02% Azure A in 48 ml. distilled water + 2 ml. 0.1 M citric acid (pH 2.5),
6. 0.2% Azure A in 48 ml. distilled water + 1.65 ml. 0.1 M citric acid + 0.35 ml. 0.2 M Na_2HPO_4 (pH 3.0),
7. 0.02% Azure A in 48 ml. dstilled water + 1.4 ml. 0.1 M citric acid + 0.6 ml 0.2 M Na_2HPO_4 (pH 3.5),
8. 0.02% Azure A in 48 ml. distilled water + 1.25 ml. 0.1 M citric acid + 0.75 ml. 0.2 M Na_2HPO_4 (pH 4.0),
9. 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 4.5),
10. 0.02% Azure A in 48 ml. distilled water + 1.0 ml. 0.1 M citric acid + 1.0 ml. 0.2 M Na_2HPO_4 (pH 5.0).

Chemical reaction.

Formation of blue orthochromatic or purple to red metachromatic complexes with the extinction values indicating the degree of acidity of the polymer.

Procedures:

1. After dewaxing and hydration, secretions were brought to distilled water.
2. Stained with Azure A at desired pH for 30 min.
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.

Result:

Strongly sulfated mucosubstances exibit metachromasia below pH 1.5, sialomucins generally stain metachromically between pH 2.5 and 3.5. Some protein masked sulfomucins and hyaluronic acid exhibit metachromasia at and above pH 4.5. Generally, the metachromasia of sulfomucins resists alcohol dehydration.

D) Mild Methylation - AB pH 2.5

E) Active Methylation - AB pH 2.5
(Fisher and Lillie, 1954; Spicer, 1960)

Solutions required:

1. Absolute methanol,
2. 0.1 N HCl in absolute methanol,
3. 3% acetic acid,
4. 1% AB in 3% acetic acid (pH 2.5).

Chemical reaction (Mild Methylation):

Esterificatoion of carboxyl groups.

Chemical reaction (Active Methylation):

Carboxyl groups are esterified. Sulfomucins are desulfated.

Procedure:

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 (pre-heated) for 4 hrs. 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 min. washing in running water.
6. Stain with AB pH 2.5 as in III-B.
7. After washing, dehydration and clearing, sections were mounted as usual.

Result:

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

F) Mild Methylation - Saponification - AB pH 2.5 and

G) Active Methylation - Saponification - AB pH 2.5:
(Spicer and Lillie, 1959; Spicer, 1960)

Solutions required:

1. Absolute methanol,
2. 0.1 N HCl in absolute methanol,
3. 3% acetic acid,
4. 1% AB in 3% acetic acid (pH 2.5),

5. 1% KOH in 70% alcohol.

Chemical reaction:

Mild Methylation - Saponification

- Restoration of carboxyl groups,

Active Methylation - Saponification

- Restoration of carboxyl groups.

Sulfomucins are hydrolytically removed during active methylation and are not restored following subsequent saponification.

Procedure:

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 min. After washing briefly with distilled water, they were stained with AB pH 2.5 as in III-B. After washing, dehydration and clearing, the sections were mounted as usual.

Result:

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

H) Acid Hydrolysis - AB pH 2.5:
(Quintarelli et al., 1961)

Solutions required:

1. 0.1 N HCl,

2. 3% Acetic acid,

3. 1% AB in 3% acetic acid.

Chemicals reaction:

Removes sialic acid from mucosubstances.

Procedure:

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 with AB pH 2.5
5. Dehydrated, cleared and mounted as usual.

Results:

Complete or partial loss of alcianophilia or metachrcmasia indicates the probable presence of sialomucins.

VI) Enzyme Digestion Tests:

A) Sialidase Digestion:
(Spicer and Warren, 1960)

Solution required:

1. Sialidase solution in 0.1 M sodium acetate at pH 5.3 containing 0.04 M $CaCl_2$,
2. 3% acetic acid,
3. 1% AB in 3% acetic acid.

Chemical reactions:

Removal of sialic acid from mucosubstances.

Procedure:

1. After dewaxing and hydration, sections were brought to distilled water.
2. The slides were placed on glass rods, close to the surface of water in petridish 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 containing 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.

Result:

Complete or partial loss of alcianophilia or metachromasia indicated the presence of sialic acid.

B) Hyaluronidase Digestion:
(Barka and Anderson, 1965; Spicer et al., 1967)

Solutions required:

1. 0.05% hyaluronidase (testicular, Sigma) in freshly prepared buffer at pH 5.5 (94 ml. 0.1 M KH_2PO_4 + 6 ml. 0.1 M Na_2HPO_4),

2. 3% acetic acid,

3. 1% AB in 3% acetic acid.

Chemical reaction:

Depolymerization of hyaluronic acid, chondroitin sulfate A and C.

Procedure:

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

2. Sections were incubated at 37°C for 6 hrs. in 0.05% hyaluronidase in freshly prepared buffer at pH 5.5 (94 ml. 0.1 M KH_2PO_4 + 6 ml. 0.1 M Na_2HPO_4). Control sections were incubated only in buffer.

3. Washed in running water for 5 min.

4. Stained with AB pH 2.5.

5. Dehydrated, cleared and mounted as usual.

Result:

Complete or partial loss of alcianophilia or metachromasia indicates the presence of hyaluronic acid, chondroitin sulfate A and C.

C) Pepsin Digestion:
(Pearse, 1960; Spicer, 1960; Quintarelli, 1963; Thompson, 1966)

Solution required:

1. 0.1% pepsin in 0.1 N HCl,

2. 3% acetic acid,

3. 1% AB in 3% acetic acid.

Chemical Reaction:

Hydrolysis of internal peptide bonds as well as those of the terminal amino acids of proteins.

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 hrs.

3. Washed thoroughly in running water.

4. Stained with AB pH 2.5,

5. Dehydrated, cleared and mounted as usual.

Result:

Protein masked mucosubstances (PAS-positive but AB, C.I. and Azure A negative) stained with basophilic dye after removal of protein masking.

A summary of histochemical techniques employed in the present investigation, chemical reactions involved in the staining and their interpretations are presented in Table-1 (on the following page).

TABLE NO.1 : Histochemical methods employed for vizualizing mucosubstances.

Sr. No.	Histochemical Method	Chemical reactions involved	Histochemical result
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.
2.	Period acid phenylhydrazine Schiff (P-PAS)	Phenylhydrazine selectively blocks periodate engendered dialdehydes in mucosubstances leaving unblocked dialdehydes in periodate reactive mucosubstances available to subsequent Schiff staining.	Periodate reactive acidic mucosubstances stained red presumably are those in which acid groups are prximal to vicinal glycols.
3.	Sulfation - Azure A 1. pH 1.5	Sulfate groups are induced in neutral mucosubstances.	Only PAS reactive mucosubstances which exhibit orthochromatic blue staining with Azure A become metachromatic after sulfation.
4.	Diastase digestion - PAS	Hydrolyses and removes glycogen	Loss of PAS reactivity in sites containing glycogen.
5.	Alcian blue pH 1.0	Probably formation of alcian blue complexes with sulfate groups.	Weakly and strongly acidic sulfomucins are selectively stained.
6.	Alcian blue pH 2.5	Probably formation of alcian blue complexes with carboxyls and sulfate groups.	Only carboxymucins and weak sulfomucins are stained blue.
7.	Colloidal Iron (C.I.)	Probably formation of complexes between cationic colloidal ferric aggregates and carboxyls, sulfate and phosphate esters.	Non-sulfated acidic mucosubstances and some sulfated mucosubstances colour blue.

TABLE NO.1 (contd.)

Sr. No.	Histochemical Method	Chemical reactions involved	Histochemical result
8.	AB pH 2.5-PAS	Addition of results by single methods.	Alcian blue reactive periodate unreactive acid mucosubstances stain blue. Alcian blue and PAS-reactive substances colour purple-blue. Neutral mucosubstances colour pink-magenta.
9.	AB pH 1.0-PAS	Addition of results by single methods.	Sulfomucins stain blue or blue-purple. Neutral and non-sulfated periodate reactive mucosubstances stain pink-magenta.
10.	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.
11.	Aldehyde Fuchsin (AF)	Formation of salt complexes between cationic staining entity and sulfated and carboxyl groups.	Sulfated mucosubstances stain dark purple. Sialomucins and hyaluronic acid colour light purple.
12.	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.
13.	Alcian blue at pH 5.6 with graded concentration of $MgCl_2$.	Alcian blue forms complexes with sulfate groups. Different sulfomucins vary in the critical electrolyte concentration at which alcianophilia is lost.	Mucins stained at 0.1 M $MgCl_2$, but not at 0.2 M $MgCl_2$ are believed to contain carboxyl group. Sulfomucin stain selectively at and above 0.2 M Mg^{++} concentration.

TABLE NO.1 (contd.)

Sr. No.	Histochemical method	Chemical reactions involved	Histochemical result
14.	Azure A at controlled pH levels	Formation of blue orthochromatic or purple to 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.
15.	Mild-methylation - AB pH 2.5	Esterification of carboxyl groups	Generally, mild methylation blocks the alcianophilia of carboxymucins.
16.	Mild-methylation Saponification - AB pH 2.5	Restoration of carboxyl groups	Restoration of alcianophilia after saponification of methylated sections indicates the presence of carboxyl groups.
17.	Active-methylation - AB pH 2.5	Carboxyl groups are blocked by esterification and sulfate groups are hydrolytically removed.	Active methylation abolishes alcianophilia of carboxylmucins through esterification and of sulfomucins through hydrolytic removal of the sulfate groups.
18.	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 alcianophilia after subsequent saponification indicates the presence of carboxyl groups and loss of alcianophilia indicates the presence of sulfate groups.
19.	Acid hydrolysis - AB pH 2.5	Removes sialic acid from mucosubstances.	Complete or partial loss of alcianophilia indicates the probable presence of sialomucins.

TABLE NO.1 (concld.)

Sr. No.	Histochemical method	Chemical reactions involved	Histochemical result
20.	Sialidase - AB pH 2.5	Removes sialic acid from muco-substances.	Complete or partial loss of alciano-philia confirms the presence of sialomucins.
21	Hyaluronidase - AB pH 2.5	De-polymerization of hyaluronic acid, chondroitin sulfate A and C.	Complete or partial loss of alciano-philia indicates the probable presence of hyaluronic acid and chondroitin sulfate A and C.
22.	Pepsin digestion - AB pH 2.5	Hydrolysis of internal peptide bonds as well as those of the terminal aminoacids of proteins.	Protein masked mucosubstances stain with basophilic dyes after removal of protein-masking.