MATERIAL AND METHODS

MATERIAL

Cypraea arebica arebica is the gastropod - prosobranch selected for the present investigation. The animal is readily available at the reocky shore of the Arebeian Sea. According to Miss L.H.Hymen (1967) its systematic position in the phylum mollusca is in mesogastropod family cypraeacea. The snail is also referred commonly as 'Porcelain shell'.

COLLECTION SITE, HABITS AND HABITAT.

Animals for investigation were collected in the month of August 1984 from the rocky & sandy shore of Padmagad - a place about a kilometer distance from jetty of Malvan, Sindhudurga, Maharashtra, India. Being intertidal form the animals were collected immediately after lowest tide on black moon day. During collection it was observed that the animals were not seen easily. They were either hidden in the crevices or underneeth surfaces of the rocks. They were firmly anchored themselves with their foot to the rock surface. In an undisturbed condition the snails moved over rocks scrapping off the surface accumulation with the help of the redula. Scraping marks were observed. Thus cypraea is among the grazer and scrapper. It habitually dwell among coral reefs from which they scrape algae, sponges, and possibly the baby molluscs and other small animals. (Kay, 1960).

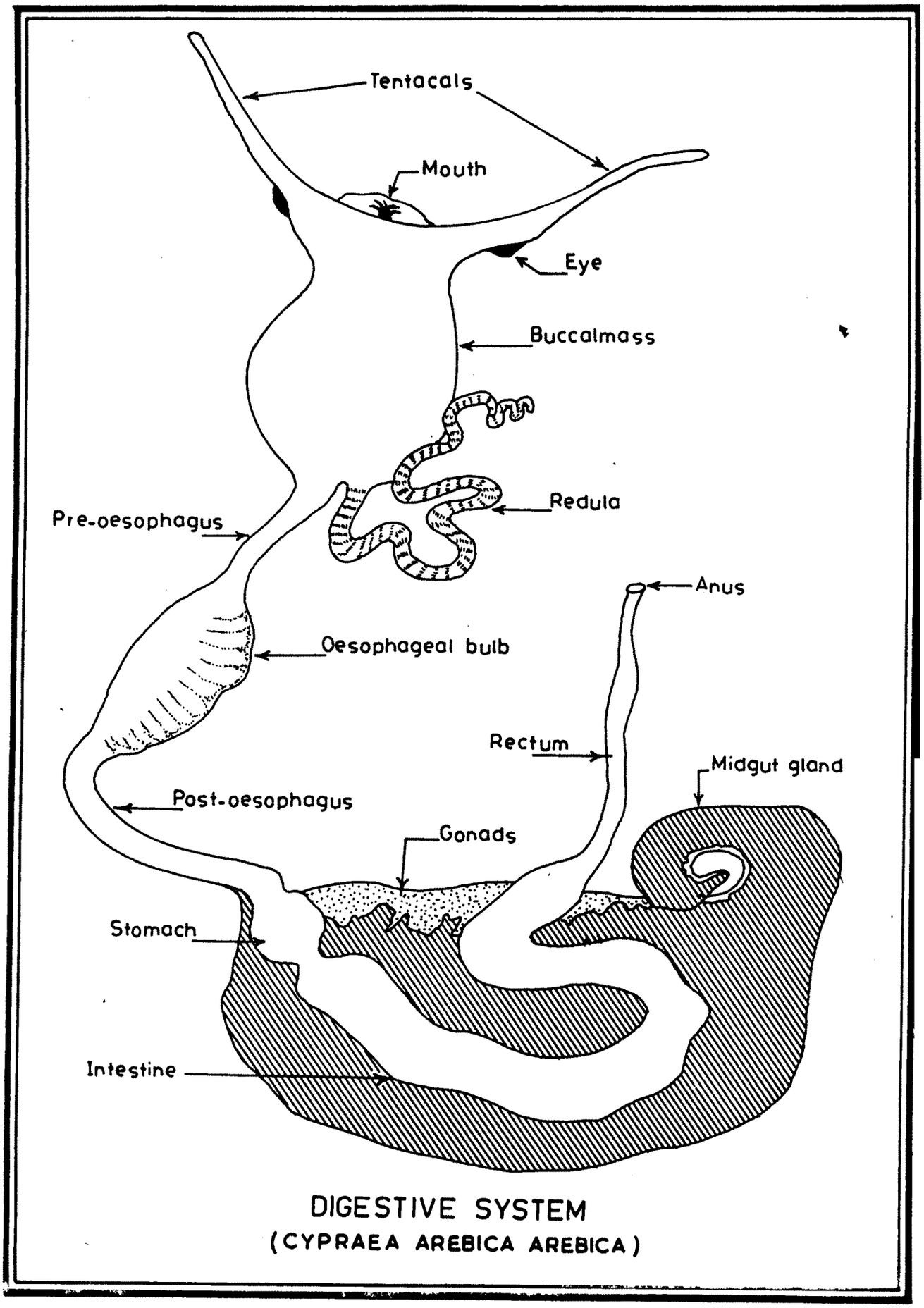
Living snails were sacrificed, dissected and

different organs of the digestive tract were seprated and were treated for histochemical investigation which is described in the later part of this chapter.

GENERAL PLAN OF THE DIGESTIVE SYSTEM OF THE MARINE SNAIL CYPRAEA AREBICA AREBICA

The digestive system of the Cypraea arebica arebica is peculiar in its organisation especially at the anterior half of the system. It consists of mouth, buccal mass, redula, pre-oesophagus, oesophageal gland, post-oesophagus, stomach, intestine, rectum, anus and midgut gland. The month is located at the base of the siphonal canal of the shell. It appears as vertical slit due to retracted probosics in the laboratory. From the lateral margin of the mouth there arises a pair of tubular tentacals, bearing pair of sessile eyes at the level of mouth opening, one on each tentacle.

Mouth leads into voluminious highly muscular sac the buccal mass. Posterior corner of buccal mass sends externally a ribbon like redula. The redula is closely associated with buccal mass into a redular sac, like a closely coiled watch spring. The ribbon taper at its free end. While the broad end is within the lumen of the buccal cavity. Throughout the length of redula ribbon there are several horizontal rows of redular teeth. Each row consist of 7 teeth, one central, two laterals and two marginals on each side of the central teeth. Teeth



DIGESTIVE SYSTEM
(CYPRAEA AREBICA AREBICA)

organization is prominent towards the broad end which lies in buccal cavity. The redulary action is controlled by strong muscles of buccal mass. In transverse section the dorsal edge is much thicker than the ventral one. Thin ventral margin of buccal cavity is drawn into number of folds.

From the posterior tip of the buccal mass a narrow tubular pre-oesophagus takes an upturn towards mouth opening. Diffused glandular mass covers the pre-oesophagus on its dorsal surface. The lumen of the pre-oesophagus shows vertical folds.

After a short run the pre oesophagus enlarges into a roughly triangular sac called oesophageal bulb. Buccal mass, pre-oesophagus and oesophageal bulb forms a compact mass at the anterior end of the alimentary tract which may be called buccal complex. Tubular tall vertical folds in the lumen from dorsal and lateral wall is a pecularity of the oesophageal bulb as far as its inner core is considered. However the fold at the ventral surface of the oesophageal gland are smaller in their height.

Posterior oesophagus takes its origin from the tapering end of the oesophageal bulb and proceeds from buccal complex posteriorly towards the glandular mass of the digestive gland. Internally it is drawn into 10 to 15 principal folds which app ears to homologous

with intestinal villi of higher chordates. Each of the principal fold also shows two to four subsidiary folds.

Expanded knob like distal part of the posterior oesophagus is small stomach. Internally the wall shows folded nature. The folds are of shorter height than that of posterior oesophagus.

Intestine get its distinction from stomach as thin walled wide tube travelles through dirty green coloured midgut gland as a single spire like a watch spring and then comes out at the dorsal aspect of the mantle wall as rectum. Here it is thick walled comparing from the region of midgut gland. Anteriorly at the free edge of mantle the rectum opens as anus. It takes its cource along the margin of ctenidium. A line drawn figure explains the position and orientation of the different organs.

METHODS

I. HISTOLOGICAL TECHNIQUES

Small pieces of digestive tract organs were fixed in the ice-cold calcium acetate formalin (CAF-2% in 10% formalin) fixative for 24 hours. The fixation of the tissues was followed by washing in chilled distilled water, in running tap water, dehydration in alcohol, clearing in xylene and paraffin embedment. The sections were cut at 5 to 6 in some sections were routinely

stained by haematoxylene eosin and Mallory's triple techniques for histological observations.

II. HISTOCHEMICAL TECHNIQUES

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 teachiques, methylation, saponification, acid hydrolysis and enzyme digestion tests. Thus, the nonspecific histochemical methods can be supplemented 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 digestive tract organs of *Cypraea arebica arebica* were employed.

FIXATION AND POST-FIXATION PROCEDURES

The different tissues of the digestive tract organs were quickly cut into smaller pieces and immediately immersed in ice-cold solution (4° C) of 2% calcium acelate

in 10% formalin (CAF). After prolonged fixation (24 hours), 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 m. The section were subjected to various histochemical techniques hereafter described for the detection of mucosubstances.

I NEUTRAL MUCOSUBSTANCES

A PERIODIC ACID - SCHIFT 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 canada balsam.

RESULT

Periodic reactive, hexose containing mucosubstances stain pin 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 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 DIASTASE DIGESTION - PAS
(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 5 minutes.
6. Dehydrated cleared and mounted as usual.

RESULT

Weakly acidic sulfated mucosubtances, 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 HCL (PH 1.0)
3. Blotted on puffless filter paper.
4. Dehydrated quickly, cleared and mounted as usual.

RESULT

Only sulfomucins stain intense blue.

III DISTINCTION BETWEEN NEUTRAL AND ACIDIC MUCOSUBSTANCES

A AB pH2.5 - PAS sequential staining technique

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 megenta.

B AB PH 1.0 - PAS SEQUENTIAL STAINING TECHNIQUE

(Spicer, 1965; Spicer *et al.*, 1967)

1. After dewaxing and hydration, section were brought to distilled water.
2. Stained with 1 % AB in 0.1 HCL (pH 1.0) for 30 minutes.
3. Sections were blotted on puffless filter paper.
4. Processed as in I-A for PAS, staining techniques.

RESULT

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

IV DISTINCTION BETWEEN SULFOMUCIN AND CARBOXYMUCINS

A ALDEHYDE FUCHSIN (AF)

(Gomori, 1950, Halmi and Devies, 1953)

PREPARATION OF AF CRYSTALS

The crystals of AF were prepared according to the method suggested by Camerson and Steal (1959). To 200ml boilding distilled water, 1 gm. of basic fuchsin was added and solution was allowed to boil for one minute, then cooled and filtered. To the filterate, 2 ml. of concentrated HCl and 2 ml of paraaldehyde were added. The solution was left stoppered at room temperature. When the solution had lost its reddish colour, usually after 3 to 4 days, it was filtered and the filterate was discarded. 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. Rinced 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 stain light purple. Some elastic fibers also stain intense purple.

B ALDEHYDE FUCHSIN - [redacted] AF-AB(pH 2-5)

SEQUENTIAL STAINING TECHNIQUE

(Spicer and Meyer, 1960)

1. After dewaxing and dehydration, 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 (pH2-5) for 30 minutes.
8. Rinsed in 3 % acetic acid.
9. Washed in running water for 4 minutes.
10. Dehydrated, cleared and mounted as [redacted] usual.

RESULT

Sulfated mucosubstances, stain purple, non-sufated mucosubstances like sailic acid and hyaluronic acid stain blue.

C CRITICAL ELECTROLYTE CONCENTRATION TECHNIQUE USING AB at pH 5.6 WITH INCREASED CONCENTRATION OF MgCl2 (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 Mgcl2 was added and a series of increasing concentrations of Mg ++ were prepared such as 0.0 M, 0.1M, 0.2M, 0.4M, 0.5M, 0.6M,).8M and 1.0M.

PRECEDURE

1. 8 dewaxed slides after hydration were brought to distilled water.
2. Each slide was stained for 30 minutes in staining 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 carbomucins like sialic acid and hyaluronic acid are not stained at or above 0.1M Mg ++ concentrations. Sulfomucins are selectively stained at and above 0.2M Mg ++ concentrations. Various sulfomucins lose their alcinophilia at different levels of Mg ++ concentrations.

D AZURE A METACHROMATIC STAINING TECHNIQUE AT CONTROLLED pH LEVELS

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

STAINING SOLUTION

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

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

pH 1.5 - 0.02% azur A in 50ml. buffer.
(30ml 0.1 N Hcl + 30ml 0.1 M KH_2Po_4)

pH 2.0 - 0.02% azur A in 50ml. buffer.
(20ml 0.1 N Hcl + 30ml 0.1 M KH_2Po_4)

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

pH 3.0 - 0.02% azur A in 48ml of distilled water +
1.65 ml 0.1 M citric acid + .35ml 0.2 M
M Na_2HPo_4

pH 3.5 - 0.02% azur A in 48 ml distilled water +
1.4ml 0.1 M citric acid + 0.6ml 0.2 M
Na_2HPo_4

pH 4.0 - 0.02 azur A in 48 ml distilled water +
1.25ml 0.1 M citric acid + 0.75 ml 0.2 M
Na_2HPo_4

pH 4.5 - 0.02 azur A in 48 ml of distilled water +
1.1 ml 0.1 M citric acid + 0.9 ml 0.2 M
Na_2HPo_4

pH 5.0 - 0.02 azur A in 48 ml distilled water +
1.0 ml 0.1 M citric acid + 1.0ml 0.2 M
Na_2HPo_4

PROCEDURE

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

2. Stained with azur 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 the metachromasia of sulfomucins resist alcoholic dehydration.

E 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, section were brought to distilled water.
2. Rinsed in absolute methenol.
3. Sections were placed in couplin jars containing 0.1 N HCl in absolute methenol (preheated) for 4 hours at 37° C (mild methylation) and at 60°C (active methylation). Correspondingly, the control sections were kept at 37° and 60° C in methenol only(without HCL)
4. Rinsed in absolute alcohol.

5. Washed in running water for 5 minutes.

6. Stained with AB P^H 2.5 as in II-A.

7. After washing, dehydration and clearing sections were mounted in Canadabalsam.

RESULT

Generally mild methylation abolished the basophilia of carbomucins by esterification while active methylation hydrolyses most of the sulfate esters.

G. MILD METHYLATION - SAPONIFICATION- AB pH 2.5

H ACTIVE METHYLATION - SAPONIFICATION-AB pH2.5
(Spicer and Lillie, 1959, Spicer, 1960)

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 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 canada balsam.

RESULT

Restoration of the basophilia after saponification indicates the presence of the basophilia indicates the presence of the sulfate esters.

I ACID HYDROLYSIS
(Quintqrelli 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 four hours
3. Stained either with AB pH 2.5 or Azure A pH 3.0
4. Washed in running water for 5 minutes.
5. Denydrated, cleared and mounted as usual.

RESULT

Complete or partial loss of alcianophilia or metachromasia indicates probable presence of sialomucins.

V ENZYME DIGESTION TESTS

A SIALIDASE (Neuraminidase Vibrio Cholerae, Type V)

DIGESTION

(Spicer and Warren, 1960)

PROCEDURE

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 petridisn kept at 30°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 $CaCl_2$). Sections were incubated for 16 to 24 hours.
3. Rinsed with distilled water.
4. Stained 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 metachromasia indicates the presence of sialic acid.

B HYALURONIDASE DIGESTION

(Barke 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_2PO_4 + 6ml 0.1 M Na_2HPO_4). Control sections were incubated only in buffer.
3. Washed in running water for 5 minutes.
4. Stained with AB pH 2.5 or azure A pH 4.5
5. Dehydrated, cleared and mounted as usual.

RESULT

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

C PEPSIN DIGESTION

(Pearse, 1960; 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 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, and azure A negative stain with basophilic dyes after removal of protein masking.