CHAPTER - TWO

MATERIAL AND METHODS

A. MATERIAL :

For the present investigation entitled "Effect of Malathion and Sumithion on Histology and Histochemistry of Mucosubstances in the Gills of *Channa punctatus* the following materials were used.

I. PESTICIDES :

i) MALATHION (Carbophos, O, O dimethyl –S-1-2 dicarboethoxyethyl-dithiophosphate).

Malathion is one of the safest organophosphorous compounds because of its low mammalian toxicity, now marketed in the form of emulsion concentrate. It is also possible to use malathion in the form of dusts or suspensions prepared from a wettable powder. It is produced by the addition of dimethyl dithiophosphoric acid to maleic acid-ester in the presence of basic catalyst in various organic solvents or without solvents . The technical grade compound obtained by this method contains small amounts of impurities like trimethyldithiophosphate, dimethyl maleate and solvent which decomposes malathion very rapidly. Xylene is most often used as a solvent for malathion synthesis. Decomposition takes place even at very low temperature.

Pure malathion is a colourless liquid boiling at 120°C. At 20°C, it is sparingly soluble in water but highly soluble in most organic solvents with the exception of saturated hydrocarbons. Malathion on prolonged heating at 150°C is isomerised and goes over

to the thioloisomers.

Hydrolysis of malathion follows different paths in acid and alkaline media. In acid medium the main products of hydrolysis are dimethyldithiophospharic acid and the ester of mercapto succinic acid while in alkaline medium the salt of dimethyl dithiophospharic acid and ester of fumaric acid are formed.

Malathion on prolonged contact with iorn or materials containing iorn, breaks down and completely loses its insecticidal properties. Hence it is not kept in iorn container but in glass.

Malathion is a persistant general purpose insecticide particularly suitable for house hold, home, garden, vegetable and fruit pest control.

It is highly toxic to mites and number of insect species while being generally nonphyto toxic. Malathion has relatively low toxicity to higher animals . Chronic toxicity and inhalation effects are said to be low.

Malathion is used generally for the control of following crop pests.

CROPS	PESTS
Cotton	Aphids, mealy bugs, bollworms, mites
Paddy	Army worms, lice, cranefly, jassids.
Jute	Semilooper, hairy caterpillar.
Almonds	Aphids, spiders.
Apples	Mealy bugs.

CROPS	PESTS
Citrus	Mites and scales.
Grapes	Jassids.
Mango, Guava, Chikoo	Jassids.
Melons	Aphids.
Vegetables	Aphids, Jassids, catterpillar.
Cereale	Mites.

50% EC malathion (Commercial grade) was used for experimental studies.

ii. SUMITHION :

Sumithion is also an organophosphorous insecticide which is a clear liquid with an unpleasant odour. It's boiling point is 95°C. Its solubility in water is about 30 mg/L. It is highly souble in most of the organic solvents and is mixible in all proportions with methyl and ethyl alcohols, alkyl acetates, ketones and aromatic hydrocarbons. Its solubility in kerosene is about 45% and in petroleum and ether about 7%.

This compound must be stored in enameled, aluminium or glass containers. Iorn promotes decomposition of sumithion like that of most other organophosphorous compounds. It is compitable with most of pesticides except those of alkaline nature such as

Bordeaux mixture, nicotine sulphate and lime sulphur. The LD 50 for various experimental animals ranges from 142 to 1000 g/kg. The nature of action on animals is similar to that of methylparathion.

Sumithion is used for the control of following crop pests.

CROPS	PESTS
Paddy	Stemborer, swarming caterpillar, gall fly green and white jassid, climbing cutworm, caseworm, skipper grass hopper, earthead or guandhy bug.
Sugarcane	Pyrilla
Groundnut	Red hairy caterpillar, leafminer aphid, tobaccocaterpillar.
Cotton	Aphid, jassids, thrips, red cotton bug, white fly.
Tobacco	Aphid.
Cabbage	Prodenia or tobacco caterpillar.
Cauliflower, Bringal, Lady's finger, Vegetables	jassid, aphid red pumpkin beetle, thrips, white fly, lace - wing bug, dimond back moth caterpillar.
Apple	Deloliating beetle, scale, mealy bug, aphid
Coffee	Green scale.
Citrus	White fly, leafminer.

50% EC sumithion (Commercial gread) was used

for experimental studies.

11. SELECTION OF FISH :

For the present investigation the fish Channa punctatus was selected keeping in mind its availability in local river (Krishna river) around the Karad. Being one of the sturdy fish it was considered suitable to study the effects of above two pesticides on gills to this fish.

SYSTEMATIC POSITION :-

hylum	:	Chordata
Group	:	Craniata
Subphylum	:	Gnathostoma
Division	:	Pisces
Class	:	Teleostomi
Sub class	:	Actinopterygii
Order	:	Channiformes
Family	:	Channidae
Genus	:	Channa
Species	:	punctatus (Bloch)

This is commonly called snake-headed fish because of its snake like head and elongated body. It's head is depressed and is covered with large scales. Body anteriorly cylindrical and posteriorly compressed. Dorsal and anal fins are single and long. It has accessory respiratory organs and can take bubbles of air from surface of water to supplement their oxygen requirements. These organs enable this fish to survive out of water for few hours or migrate from one pool to

other.

B. METHODS :

The present investigation was carried out from June 87 to April 88. For the present study live and healthy fishes collected by fisherman from river, Krishna at Karad were used. While selecting the fishes care was taken to select the fishes of approximately uniform size. These fishes were then transported to the laboratory in natural water and transfered to large glass aquaria of hundred litre capacity, filled with fresh, chlorine free tap water. These were then allowed to rest for about 10 days in the above condition to bring them to their normal physiological conditions, after stress and strain of catch and transport. During this period , they were well fed twice daily, once in the morning and once in the evening until 48 hours before they were used for experimental study. The well acclimatised fishes only, were then used for actual toxicological experiments.

For the experimental study the fishes were transferred to glass aquaria of size 60 x 30 X 25 cm. and about 50 litre capacity containing different concentrations of malathion and sumithion . The aquaria were kept opened and the fishes were kept starved during experimentation. The room temperature and temperature of water in aquaria was noted. The water was analised for

pH, and dissolved oxygen (DO) before and after the addition of pesticides and the results were expressed graphically.

I. DETERMINATION OF LC 50 :

The well acclimatized fishes from the stock were divided into 5 batches containing 10 healthy fishes per batch for each pesticide. They were then transferred to the glass aquaria containing 4 ppm, 8 ppm, 10 ppm, and 12 ppm malathion and 10 ppm, 15 ppm, 20 ppm, 25 ppm and 30 ppm sumithion. The fishes were exposed to the respective concentration for a definite period and for each concentration the mortality was observed for 48 hours. All the experiments were started in the morning to facilitate observations on behavioural changes of fishes during the first 12 hours of exposure. The behavioural changes of the fishes were keenly observed and the number of deaths of fishes were recorded after 12, 24, 48, 72 and 96 hours for each concentration of both the pesticides. The LC 50 values were calculated by plotting the readings on the graph paper. While plotting the graph the concentration of pesticide was taken on X-axis and the percent mortality on Y-axis.

II. PREPARATION OF SLIDES FOR HISTOLOGICAL, HISTOPATHOLOGICAL AND HISTOCHEMICAL OBSERVATIONS :

The control as well as fishes exposed to different concentrations of malathion and sumithion

(overturned) were taken out of aquaria. Each fish was sacrificed and pinned in wax tray and it's gills were dissected out and were immediately fixed 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 alcohol 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 deparaffinized, hydrated and brought to distilled water. Some of the sections of control fish were stained with heamatoxylin-eosin (H-E) for histological observations and the adjacent sections were subjected to various histochemical techniques for characterization of mucosubstances. In exposed fishes some sections were stained with hematoxylin-eosin (H-E) for histopathological observations and remaining sections were subjected to various histochemical techniques for characterization of mucosubstances and to compare them with that of control fish.

a). HEMATOXYLIN-EOSIN METHOD (H-E):-

The following procedure was employed.

1. Deparaffinized sections after hydration were brought

to distilled water.

2. Treated with hematoxylin (nuclear stain) for 2 to 3 minutes (Here freshly prepared Harri's hematoxylin was used as it gives best results within short time).
3. Kept in distilled water for about 10 to 15 minutes.
4. Dehydrated through different alcoholic grades up to 70% alcohol.
5. Stained with 70% alcoholic eosin (cytoplasmic stain) for 10 minutes.
6. Followed by 90% and 100% alcoholic grades and transferred to xylene for about 20 minutes.
7. Mounted in Canada balsam.

The permenant slides of the controlled and exposed fishes were observed simultaneously under light microscope at different magnification for histological and histopathological observations in gill. The histological structure in controlled fish and well marked histopathological changes occured in the structure of gill of exposed fishes were recorded photomicrographically. The results were analysed and compared with the available data of other workers.

b). HISTOCHEMICAL METHODS :-

There are several histochemical techniques used for the demonstration of mucosubstances in animal

tissues . In addition to the manuals 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 concentration and selective removal of the moieties by acid hydrolysis or enzyme digestions. Thus nonspecific histological and histochemical methods can be supplemented with the modified and specific ones for the better understanding of the chemical composition of the celluler 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)

(Mc Manus, 1946; Hotchkiss, 1948)

Solutions required:

1. 0.5 % periodic acid, 2) Schiff's reagent, 3)0.5% sodium or potassium metabisulphite.

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 min.
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 :

Periodate 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 :

Phenylhydrazine 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 min.
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 time (total 5 min.) with 0.5% sodium meta-bi-sulphite.
7. Washed, dehydrated, cleared routinely and mounted in Canada balsm.

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 0.1N HCl + 20 ml 0.1 M 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 min.
3. Washed in distilled water for 5 min.
4. Stained with azure A pH 1.5
5. Washed in distilled water and observed under microscope. The results were recorded.
6. Dehydrated, cleared and mounted as usual.

Result :

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

II. Glycogen :

A. Diastase digestion-PAS (or α - amylase -PAS)

(Little, 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 remove 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 :

ss 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.

Procedure

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 (pH2.5).

Chemical reaction

Problably formation of alcian blue complexes with carboxyls and sulfate grous.

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 Abul-Haj, 1951; Mowry , 1961. 1963)

Solutions required

1. 29% Ferric chloride solution,

2. Stock collodial iron solution : To the boiling 250 ml. of distilled water, 4.4 ml. 29% ferric chloride solution was added with constant stiring. 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 colloidal iron solution :

Glacial acetic acid	5 ml.
Distilled water	15 ml.
Stock colloidal iorn solution.	20 ml.

5. 12 % acetic acid.

6. 2% HCl.

7. 2% potassium ferrocyanide.

Chemical reaction

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

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. Rinsed in 12% acetic acid.
5. The sections were treated with freshly prepared mixture of equal volumes of 2% HCL and 2% potassium ferrocyanide 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 much 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 0.1N HCl (pH 1.0), 2) 0.5% periodic acid, 3) Schiff's reagent, 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 or 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 (pH 2.5).
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. Processed 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 colour magenta.

C). Colloidal Iron PAS Sequential Staining Technique :

(Ritter and Oleasan, 1950 ; Mowry, 1963)

Solution required

1) 29% ferric chloride solution, 2) Stock colloidal iron solution, 3) Glacial acetic acid, 4) Working collodial iron solution, 5) 12% acetic acid, 6) 2% HCL, 7) 2% potassium ferrocyanide, 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 methods.

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 ferrocyanide for 20 min.
6. Washed with running water for 5 min.
7. Processed as in IA 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) Con. 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).

Preparation of AF Crystals : The crystals of AF were preapred 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 Con. 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 filtrate was discarded. The precipitate was dried on the filtre 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. Stained 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 Technique :—

(Spicer and Meyer, 1960)

Solution required

1) 70% alcohol, 2) Con. 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, nonsulfated mucosubstances like sialic acid and hyaluronic acid stain blue.

C. Critical Electrolyte Concentration Technique Using AB at 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. Dehydratd, 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 Technique 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) 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 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 distilled wter + 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) , 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 salt complexes with the extinction values indicating degree of acidity of the polymer.

Procedure

1. After dewaxing and hydration, sections were brought to distiled 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 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 resists alcohol dehydration.

E. Mild Methylation - AB pH 2.5

F. Active Methylation - AB pH 2.5

Fisher and Lillie, 1954; Spicer, 1960)

Solutions required

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

Chemical reaction (Mild methylation)

Esterification 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 couplin jars containing 0.1N 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 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.

G) Mild methylation - saponification -AB pH 2.5 and

H) 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 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% alochol 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.

Resutl : Pestoration of the basophilia after saponification indicates the presence of carboxymucins but failure of restoration of basophilia indicates the presence of the sulfomucins.

I. Acid Hydrolysis - AB pH 2.5

(Quintarelli et al. , 1961)

Solutions required

1)0.1N HCl, 3) 3% acetic acid, 3) 1% AB in 3% acetic acid.

Chemical reaction

Removes sialic acid from mucosubstances.

Procedure

1. After dewaxing and hydration, sections were brought to distilled water.
2. They were treated with 0.1N HCl at 60°C for 4 hrs.
3. Washed in running water for 5 minutes.
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 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% acitic acid.

Chemical reaction

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 surface of water in petridish kept at 37°C. Sections were covered with enough sialidase (Vibrio Cholerae, type V, Sigma) in 0.1 M sodium acetate at pH 5.3 containing 0.04 M $Cacl_2$. Control sections were covered with buffer only (0.1 M sodium acetate at pH 5.3 containing 0.04 M $Cacl_2$). Sections were incubated for 16 to 24 hrs.
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 aminoacids of proteins.

Procedure :—

1. After dewaxing and hydration, sections were brought to distilled water.
2. Digested in 0.1% pepsin in 0.1N HCl at 37° 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 bird's eye view of the various histochemical techniques employed in the present investigation along with the chemical reactions involved in the staining and the histochemical interpretation of staining reactions is given in Table No. 1.

Table No. 1 :- Histochemical methods employed for visualizing mucosubstances.

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 regent.	All polysaccharides and mucosubstances colour pink to magenta.
2.	Periodic acid phenylhydrazine Schiff (P-PAS)	Phenylhydrazine selectively blocks periodate engendered dialdehydes in mucosubstances leaving unblocked dialdehydes in periodate reactive mucousbstances available to subsequent Schiff staining.	Periodate reactive acidic mucosubstances stained red presumably are those in which acid groups are proximal to vicinal glycols.
3.	Sulfation - Azure A 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 remove glycogen.	Loss of PAS reactivity in sites containing glycogen.

NO.	HISTOCHEMICAL METHOD	CHEMICAL REACTIONS INVOLVED	HISTOCHEMICAL RESULT
5.	Alcian blue pH 1.0	Probably formation of alcian blue complexes with sulfate groups.	Weakly and stongly acidic sulfomucins are selectively stained.
6.	Alcian blue pH 2.5	Problably formation of alcian blue complexes with carboxyls and sulfate groups.	Only carboxymucins and weak sulfomucins are stained blue.
7.	Collodial Iron (C.I.)	Probably formation of complexes between cationic colloidal ferric agregates and carboxyls, sulfate and phosphate esters.	Non-sulfated acidic mucosubstances and some sulfated mucosubstances colour blue.
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.

NO.	HISTOCHEMICAL METHOD	CHEMICAL REACTIONS INVOLVED	HISTOCHEMICAL RESULT
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 ping-magenta.
10.	Collodial Iron-PAS	Addition of results by single methods	Collodial iron-reactive, periodate unreactive acid mucosubstances stain blue. Collodial 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

NO.	HISTOCHEMICAL METHOD	CHEMICAL REACTIONS INVOLVED	HISTOCHEMICAL RESULT
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. Sulfomucins stain selectively at and above 0.2 M Mg^{++} concentration.
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.

NO.	HISTOCHEMICAL METHOD	CHEMICAL REACTIONS INVOLVED	HISTOCHEMICAL RESULT
16.	Mild methylation-Saponification-AB pH 2.5	Restoration of carboxyl groups.	Restoration of alciano-philia 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 abolished alcianophilia of carboxymucins 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 oa alcianophilia indicates the presence of sulfate groups.

NO.	HISTOCHEMICAL METHOD	CHEMICAL REACTIONS INVOLVED	HISTOCHEMICAL RESULT
19.	Acid hydrolysis - AB pH 2.5	Removes sialic acid from mucosubstances.	Complete or partial loss of alcianophilia indicates the probable presence of sialomucins.
20.	Sialidase - AB pH 2.5	Removes sialic acid from mucosubstances.	Complete or partial loss of alcianophilia confirms the presence of sialomucins.
21.	Hyaluronidase - AB pH 2.5	Depolymerization of hyaluronic acid, chondroitin sulfate A and C.	Complete or partial loss of alcianophilia 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 aminoacides of proteins.	Protein masked mucosubstances stain with basophilic dyes after removal of protein masking.