CHAPTER TWO

MATERIAL AND METHODS

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I. Material:

For the study of histopathology and mucosubstances found in different pathological conditions of prostate as proposed in plan of present work in the introductory chapter, the tissue samples were collected from Krishna Medical and Research Institute (Karad) during the period from 1985 to May, 1989.

a) Collection of Tissue Samples:

The tissues of prostate were obtained after operation from the patients admitted for suspected disorders of prostate gland. Tissue pieces were selected in consultation with the expert pathologists at Krishna Medical and Research Institute (Karad).

II. Methods:

i) Chemicals:

All chemicals and stains in the present investigation used were reagent grade and were obtained from E.Merk & Company (U.S.A.) and BDH.

ii) Fixation:

The tissues were collected from the patients after biopsy and after prostectomy and immediately blotted on a piece of Watman

No. 1 filter paper to remove traces of blood and immersed in the fixatives described below. Size of the tissue block was taken so as to include the entire affected part of the prostate.

A) 10% neutral formalin -

i)	Formaldehyde solution (40 % HCHO by assay)	100 ml
ii)	Distilled water	900 ml
	(pH of this solution was maintained between 6.8 and 7.0 by adding phosphate buffer solution).	

B) Calcium acetate formol (CAF) -

i)	Calcium acetate	2.0 gm
ii)	4% formalin	100 ml
iii)	This solution was diluted to 1000 ml by adding glass distilled water	

Both the fixatives were preserved in freeze.

iii) Processing of Tissues:

After fixation for 18 to 24 hours at 4°c, the tissue blocks were washed in running tap water. Subsequently these tissue blocks were dehydrated through graded alcohol by keeping them in different grades for not less than 30 minutes, giving one or two changes. By this process the tissues were dehydrated completely. After dehydration the tissue blocks were transferred to xylene for about 8 to 10 minutes. Then they were transferred to xylene: 60°c to 62°c wax

(1:1) for about 30 minutes for cold impregnation. Then the tissues were kept in the melted wax (60°c to 62°c paraffin wax - BDH) in the oven adjusted at 62°c. After keeping them for 2 to 3 hours, paraffin blocks were prepared. After cooling, the blocks were trimmed and sections of 5 to 7 microns were cut on rotary microtome.The paraffin sections were spread on the albumin-coated slides.

iv) Staining:

(a) For histological differentiation routine technique of hematoxyline-eosine was used.

(b) Histochemical Procedures Actually Employed for Identification of Mucosubstances:

For visualization of mucosubstances, there are series of histochemical methods evolved by different workers in this field. The specificity of differential 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 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 musosubstances 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 human prostate under different pathological conditions, were employed.

I. 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 per cent 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 per cent Sodium-meta-bi-sulphite.
6) Washed in distilled water, followed by alcohlic dehydration, cleared in xylene and mounted in DPX.

Results: 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 0.5 per cent Sodium meta-bi-sulphite.

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

Results: Periodate reactive acid mucosubstances are selectively stained. Periodate engendered dialdehydes are blocked.

C) Diastase Digestion - PAS technique for identification of glycogen. (Lillie, 1964; 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 or α-amylase in 0.2 M phosphate buffer at pH 6.0.

3) Washed in distilled water.

4) Processed as I-A for PAS staining procedure.

Results: 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 per cent 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.

Results: Weakly acidic sulphated mucosubstances, hyaluronic acid and sialomucins stain dark blue. Strongly acidic sulfated mucine 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.

Results: Only sulfomucine stain intense blue.

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

Stock Solution: To the boiling 250 ml of distilled water, 4.4 ml of 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 acetic acid: 5 ml.

Distilled water: 15 ml.

Stock, colloidal

Iron Solution: 20 ml.

Procedure:

1) After dewaxing and hydration sections were brought to distilled water.
2) Rinsed in 12 per cent acetic acid.
3) Treated with freshly prepared working colloidal iron solution for 60 minutes at room temperature.
4) Rinsed in 12 per cent acetic acid.
5) The sections were treated with a freshly prepared mixture of equal volumes of 2% HCl and 2% potassium ferrocyanide, for 20 minutes.
6) Washed with running water for 5 minutes.
7) Dehydrated, cleared and mounted as usual.

Results: Sites of acidic mucosubstances are prusian 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 Acid 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.

Results: 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 Procedure 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.

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

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 Steel (1959).

To 200 ml boiling distilled water, 1 gm of basic fuchsin was added and the solution was allowed to boil for one minute; 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 3-4 days, it was filtered and the filtrate 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) Rinsed in 70% alcohol.

3) Stained with AF staining solution for 30 minutes.

4) Rinsed in 70% alcohol.

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

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

B) Mild-methylation - AB pH 2.5

C) Active-metehylation - AB pH 2.5

(Fisher and Lillie, 1964, Spicer, 1960)

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 (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 under running water.

6) Stained with AB pH 2.5 as in II-A.

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

Results: Generally mild-methylation abolishes the basophilia of carboxy-mucins by esterification while, active-methylation hydrolyses most of sulfate esters.

D) Mild-methylation Saponification - AB pH 2.5

E) 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 in II-A. After washing, dehydration and clearing the sections were mounted in DPX.

Results: Restoration of the basophilia after saponification indicates the presence of carboxyl groups but failure of restoration of the basophilia indicates the presence of the sulfate esters.

V. Acid Hydrolysis - AB pH 2.5 (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 hrs.

3) Washed in distilled water for 5 minutes.

4) Stained with AB pH 2.5.

5) Washed in distilled water, dehydrated, cleared and mounted as usual.

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

A bird's eye view of the various histochemical techniques employed for the mucosubstances in the present investigation along with the chemical reactions involved in the staining and the histochemical interpretations of the staining with the literature is given in Table-A.

TABLE-A

Histochemical Methods employed for visualizing mucosubstances.

No.	Histochemical method	Chemical reaction involved	Histochemical Results	Reference
1	Periodic acid Schiff reaction (PAS).	Oxidation of vicinyl hydroxyls to dialdehydes by periodates and formation of coloured complexes with Schiff's reagent.	All polysaccharides and mucosubstances colour magenta to pink.	McManus (1946).
2	Diastase digestion-PAS	Hydrolyzes and removes glycogen.	Loss of PAS reactivity in sites containing glycogen	Lillie (1954) Lison (1960)
3	Periodic acid-Phenylhydrazine-Schiff	Phenylhydrazine blocks periodates engendered dialdehydes in mucosubstances leaving unblocked dialdehydes in periodate reactive mucosubstances available to subsequent Schiff's reagent.	Periodate reactive acidic mucosubstances stain presumably are those in which acid groups are proximal to vicinyl glycols.	Spicer (1965), Spicer et al. (1967).
4	Alcian blue pH 1.0	Probable formation of alcian blue complexes with sulphate groups.	Weakly and strongly acidic sulfomucins are selectively stained.	Lev and Spicer (1964).
5	Alcian blue pH 2.5.	Probable formation of alcian blue complex with carboxyls and some sulfate groups.	Sialomucins and weakly acidic sulfomucins stain blue; the most strongly acidic sulfomucins stain weakly or not at all.	Lev & Spicer (1964).
6	Colloidal iron	Probable formation of complexes between cationic colloidal ferric aggregates and carboxyls, sulfated and phosphate esters.	Non-sulfated acid mucosubstances and some sulfated mucosubstances colour blue.	Hale (1946), Richart and Abul-Haj (1951), Mowry (1961, 1963).
7	A.B. pH 1.0-PAS	Addition of results by single method.	Sulfomucins stain blue or blue-purple. Neutral and non-sulfated periodate reactive mucosubstances stain pink to magenta.	Spicicer (1965) Spicer etal. (1967)

TABLE-A contd.

No.	Histochemical method	Chmical reaction involved	Histochemical Results	Reference
8	AB pH 2.5-PAS.	Addition of resuslts by single method.	Alcian blue reactive, periodate unreactive and mucosubstances stain blue, Alcian blue and PAS-reactive mucosubstances colour blue-purple. Neutral mucosubstances colour pink-magenta.	Mowry and Winker (1956).
9	Aldehyde-Fuschin (A.F.).	Formation of salt complexes between cationic staining entity and sulfate and carboxyl groups.	Sulfomucins stain dark purple, sialomucins and non-sulfated acidic mucosubstances stain light purple.	Gomori(1950), Halmi & Davies (1955)
10	Mily methylation AB pH 2.5.	Esterification of carboxyl groups.	Generally mild methylation abolishes the alcianophilia of carboxyl mucins.	Fisher and Lillie (1954), Spicer (1960).
11	Mild methylation-Saponification-AB. pH 2.5	Restoration of carboxyl groups.	Restoration of the alcianophilia after saponification of metylated sections, indicates the presence of carboxyl groups.	Spicer and Lillie (1959), Spicer (1960)
12	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)
13	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.	Spicer and Lillie (1959), Spicer (1960)
14	Acid hydrolysis-AB pH 2.5 or azure-A pH 2.5	Removes sialic acids from mucosubstances	Complete or partial loss of alcianophilia or metachromatia inicates the probable presence of sialomucins.	Quintarelli et al. (1961).