CHAPTER - TWO

MATERIAL AND METHODS

I. Nomenclature of complex carbohydrates :

The complex carbohydrates have variously been defined in the past. The word " mucus " was used for such complex carbohydrates from the 18th century (Hoffbauer, 1734; Darwin, 1778; Lorleberg, 1789; Henle, 1790). Mucus was considered as a slimy, vicid and tenaceous substance of the animal body. The intestinal mucus was precipitated by acetic acid at cold temperaterature (Gmelin, 1826). Presence of carbohydrate in the mucin was identified by Eichwald (1865). He defined mucin as " a conjugated single compound of a moiety with properties of protein and a moiety released under certain conditions as a sugar ".

Hammarsten (1895) used the term " Glycoproteide " for acidic mucin from the submaxillary glands. He also introduced the term " Mucoid " for carbohydrate containing proteins. Hammarsten (1895) and Mörner (1894) isolated mucoids from ovarian cyst, cartilage, cornea, egg white etc. The mucoids from these sources were considered different from " true mucins " of submaxillary glands. Levene (1925) considered " Mucoproteins " as glycoproteins and " Mucins " and " Mucoids " as chondroitin sulfuric acids and mucoitin sulfuric acids.

Meyer (1938) coined the term " Mucopolysaccharides " for carbohydrate-protein complexes. According to Curran (1964) " Mucopolysaccharides " are heteropolysaccharides with high molecular weight like hyaluronic acid. A large number of publications in recent years include different terms such as mucins, mucoids, mucopolysaccharides mucoproteins, glycoproteins, glycosaminoglycans, proteoglycans, mucosubstances etc. Such

terminologies made it difficult to understand the chemistry of these complex carbohydrates (Jeanloz, 1960). Sometimes confusion arises because the same substance is named by many terms such as " chondritin sulfate " which is also known as " β-heparin ", " dermatan sulfate " and " dermatoidin sulfate " (Hirst, 1962).

Meyer (1938) introduced the term " Mucopolysaccharides " to describe polysaccharides of animal origin either pure or conjugated with proteins through salt-linkages. Meyer (1953) introduced the terms " Mucoproteins " and " Mucoids " in his original schemes. Mucoproteins result from dissociable ionic binding between mucopolysaccharides and proteins, mucoids contain more than 4 % hexosamine and glycoproteins contain less than 4 % hexosamine. Mucoids were further distinguished as neutral mucoids (blood group substances, serum mucoids), acidic mucoids (submaxillary gland mucin, sialic acid containing mucoids) and insoluble mucoids (Ovomucin). The " glycoproteins " include ovalbumin, serum albumin, serum globulin etc.

According to Gottschalk (1960) the term " Mucoprotein" be used for the substances composed of proteins to which are attached small side chains of carbohydrates. At present such compounds are also known as " Glycoproteins ". The prefix "muco" has changed its original meaning of " derived from mucus " to " hexosamine - containing ", " protein-containing"(Stacey & Barker, 1962) and " carbohydrate-containing " (Meyer, 1955). "Glycosamino-glycans" is another term used for protein-carbohydrate complexes (Jeanloz, 1960). This term is well accepted by Scott et al. (1964) and Scott and Dorling (1965).

Balaz (1967) defined protein-polysaccharide complexes as " proteoglycans " in which more polysaccharide chains are covalenly bound to one or more protein chains. Hunt (1970) opined that in " Glycoproteins " the protein part predominates, whereas in "Mucopolysaccharides", the polysaccharide part predominates. He further described that these terms are rather unsatisfactory in that they fail to refer directly the protein part of the complex, while prefix " muco " or " glyco " may be held to imply mucinous, viscous and slimy properties which are not necessarily the characteristics of the materials in natural state.

In last two to three decades carbohydrate histochemistry has advanced because of new techniques and stains. As a result of this more and more information is available to the histochemists. Spicer et al. (1965) classified the carbohydrate - rich tissue components and introduced the term " Mucosubstances " for them. They distinguished epithelial and connective tissue mucosubstances as neutral mucosubstances and acidic mucosubstances. The latter group was further classified as sulfomucins, sialomucins, hyaluronic acid etc. Their classification and terminology are based on 1) histological sites and 2) affinity of mucosubstances to :

i) basic dyes such as Azure A ,

ii) alcian blue,

iii) extinction values of alcianophilia in graded concentrations of $MgCl_2$,

iv) lability or resistance towards hyaluronidase and

v) lability or resistance towards sialidase (neuraminidase), the enzyme isolated and purified from Vibrio cholerae.

The nomenclature suggested by Spicer et al. (1965) is followed in the present dissertation. Inspite of the several definitions given above, still some of the investigators introduce some new terminologies e.g. "Neatral saccharides" for neutral mucosubstances, " Sialosaccharides " for sialomucins, " Sulfosaccharides " for sulfomucins etc. The existing literature in various histochemical journals indicates that the term introduced by Spicer et al. (1965) i.e. " Mucosubstances " is favoured by several investigators.

II. A brief survey of the advances in histochemical techniques for detection of mucosubstances :

Some of the reactions involving the use of anilin dyes employed in the past, were infact histochemical, their significance was often unappreciated. A reaction in this category was the staining of amyloid with methyl violet first described by Cornil (1875). List (1885) used Bismark brown to stain "mucin" and alcoholic solution of this dye was used by Hardie and Wesbrook (1895) to stain water soluble mucoproteins. Best's carmine method is emperical one but was found to stain glycogen, mucin, fibrin and mast cell granules (Best, 1906; Mallory, 1938; Bensley, 1939).

Chromic acid was introduced by Bauer (1933) as oxidizing agent. It oxidizes glycol groups to aldehydes which react with Schiff's reagent. This method gave staining for glycogen, galactogen, starch, cellulose, chitin, thyroid colloid and epithelial mucins. Later on it was found that over oxidation results in the formation of carboxyl groups from aldehyde groups.

Periodic acid is an oxidizing agent which was employed by Malprade (1928, 1934) for the oxidation of glycol groups to aldehydes. This is mild oxidizing agent which does not oxidize aldehydes to acids. It was used to demonstrate polysaccharides by Jackson and Hudson (1937). Its use in histochemical method together with Schiff's reagent was described by Mc Manus (1946) for demonstration of mucins and by Schabadasch (1947) for glycogen. At present PAS reaction is one of the important histochemical techniques widely used for several types of mucosubstances.

Mc Manus and Cason (1950) used acetic anhydride - pyridine as acetylation mixture for blockade of PAS reaction of carbohydrates. Ozello et al. (1958) showed deacetylation with weak alkali (0.1 N KOH) restores PAS reaction. This allowed the differentiation of 1:2 glycol groups of carbohydrates and 1:2 glycol groups of amino alcohols.

Glycogen gives PAS and Best's carmine positive reactions which can be abolished by amylase digestion. Lillie and Greco (1947) introduced the use of 1 % malt diastase for digestion of glycogen. Then negative results are obtained with PAS and Best's carmine. Some investigators use saliva, α-amylase, β-amylase or taka - diastase for digestion of glycogen.

Hale (1946) introduced colloidal iron method based on the affinity of colloidal iron for sulfate groups, uronic acid of sulfated mucopolysaccharides and carboxyl groups of hyaluronic acid. Iron is subsequently rendered blue with acidified potassium ferrocyanide (Perl's prussian blue reaction). This method was then modified by several investigators. Selective staining for

sulfate and carboxyl group containing polysaccharides can be obtained if dialysed colloidal iron is used. Curran (1961) and Curran and Crane (1962)observed that this method gave results closely parallel to those obtained with alcian blue and metachromasia. Ritter and Oleson (1950) and Mowry (1958, 1963) used colloidal iron in combination with PAS to distinguish acidic mucopolysaccharides from neutral polysaccharides. At present colloidal iron method is adopted for visualization of acidic mucopolysaccharides in ultrastructural studies.

Aldehyde fuchsin was introduced by Gomori (1950). The stain is prepared by adding paraldehyde to acidified solution of basic fuchsin. Originally this method was used to stain elastic fibers. This method was then modified by Halmi (1952) and Gabe (1953). Halmi and Davies (1953) found that most of the metachromatic substances were aldehyde fuchsin positive. Abul Haj and Rinehart (1952-1953) and Scott and Clayton (1953) suggested that the dye has affinities for sulfated mucopolysaccharides. Spicer and Meyer (1960) reported that sulfated polysaccharides are stronly stained than non-sulfated polysaccharides with this method.

Steedman (1950) introduced alcian blue 8 GS a pthalocyanin dye for selective staining of acidic mucins. Mowry (1956) recommended its use with acetic acid at pH 2.5 to 3.0 for acid mucopolysaccharides, while Lev and Spicer (1964) used it at pH 1.0 with 0.1 N HCl for selective staining of sulfomucins. Alcian blue was used in combination with PAS (Mowry and Winkler, 1956) for distinction between acidic and neutral polysaccharides and Spicer and Meyer (1960) used a sequence of aldehyde fuchsinalcian

blue pH 2.5 to distinguish sulfomucins from carboxymucins.

When certain tissue elements are stained by a cationic dye such as toluidine blue, the blue colour of the dye is changed to pink, red or violet. This change in colour is called as metachromasia. Michaelis and Granick (1945) put forth the concept of formation of monomeric form of the dye to polymer after reacting with negatively charged acidic groups. Though toluidine blue frequently been used for metachromatic staining (Kramer and Windrum, 1954) others have used cresyl violet, methyl violet, gallocyanin, celestine blue and gentian violet. Recently Spicer (1962) and Spicer et al. (1967) showed an influence of pH on metachromasia based on which various acidic mucosubstances can be distinguished. They reported sulfomucins exhibit metachromasia from pH 0.5 to 2.0, sialomucins from pH 2.5 to 3.5 and hyaluronic acid above pH 4.5.

A technique involving artificial induction of sulfate groups in neutral mucosubstances rendering non-metachromatic substances to exhibit metachromasia was introduced by Bignardi (1940). Moore and Schoenberg (1957) induced sulfate groups in neutral polysaccharides by treating the sections with a mixture of sulfuric acid and acetic acid.

Scott et al. (1964) and Scott and Dorling (1965) established critical electrolyte concentration method for characterization of mucosubstances, on the basis of the graded concentration of $MgCl_2$ in alcian blue stain at pH 5.6. With this method, carboxymucins are not stained by the addition of 0.1 M Mg^{++}, weakly sulfated mucosubstances stain below 0.2 M Mg^{++} and various sulfomucins are selectively stained with alcian blue solution

containing 0.2 M and higher concentration of Mg^{++}. Various sulfomucins lose their alcianophilia at different concentrations of Mg^{++}.

Methylation of acidic polysaccharides was found to block or abolish alcianophilia (Fisher and Lillie, 1954) depending upon the presence of carboxyl and sulfate groups. Sulfate groups were entirely desulfated by methylation (Kantor and Schubert, 1957). Spicer and Lillie (1959) found that the treatment of methylated sections with alcoholic solution of alkali (1 % KOH in 70 % alcohol) resulted in the restoration of alcianophilia of only carboxymucins. Alcianophilia of sulfomucins is abolished by methylation and not restored following saponification.

Sialidase (neuraminidase) was found to remove sialic acid from the mucosubstance (Spicer and Warren, 1960) which results in the loss of alcianophilia. Quintarelli et al. (1961) reported that the effects of sialidase can be reproduced by acid hydrolysis. Testicular hyaluronidase hydrolyses hyaluronic acid, chondroitin sulfate A and C from the oligosaccharides (Weismann et al., 1952; Schultte and Greiling, 1955). On the other hand, Streptococcal hyaluronidase- digestion hydrolyses only hyaluronic acid (Schultte and Greiling, 1955; Linker et al., 1956; Meyer et al., 1957). Quintarelli(1963) used pepsin digestion to remove proteins which mask the carbohydrates.

In addition to the aforementioned histochemical techniques for characterization of mucosubstances several other methods have recently been developed with the use of fluorochromes (fluorescence microscopy) and radioisotopes (autoradiography).

III. Material :

For the present investigation adult males of the following species of mammals were used.

Order	Species	Common name
Chiroptera	Rhinolophus luctus beddomei	Horseshoe bat
Lagomorpha	Lepus cuniculus	Rabbit
Rodentia	Rattus rattus	House rat
	Rattus norvegicus	White rat
	Funambulus pennanti	Indian palm squirrel
Artiodactyla	Bubalus bubalus	He buffalo

The following is a brief account of their locality, feeding habits and some peculier features of the mammals under present investigation. The general information of these animals is given by Goodwin(1964) and Moris (1965).

1. Rhinolophus luctus beddomei (Horeseshoe bat) :

It is a rare species. It is a large microchiropteran bat with long and wooly fur and well developed nasal leaflets. It is insectivorous and lives alone or in pairs during breeding season. Two male specimens were collected from the Panhala hills (Dist. Kolhapur) for the present investigation on 13.8.1987 and 6.9.1987.

2. Lepus cuniculus (Rabbit) :

The true rabbit, in the wild, is a hardy, fast-breeding creature. It is greyish-brown in colour. It is about sixteen to

eighteen inches long with moderately large ears. Domestic rabbits were derived from the stock of true rabbits. It lives underground communal burrows, or warrens. These are connected by well-worn runways to the feeding grounds. Hundreds of rabbits may dwell together in a colony. Three adult male specimens were collected from Wai (Dist.Satara) for the present investigation on 26-10.1987 and 10.11.1987.

3. Rattus rattus (House rat) :

It is also called as black rat. It is small and less heavily built than white rat. It is more or less nocturnal in its habits. It lives in burrows near human dwellings and godowns of grains. It is a good swimmer and a better climber. Breeding continues throughout the year in favourable conditions, but reaches a peak in the spring. Fifteen male specimens were collected and used in the present investigation on 17.1.1988 and 25.2.1988.

4. Rattus norvegicus (White rat) :

It is also called as a Brown rat and the albino mutant of it is called as white rat. It is larger and more heavily built than the black rat. It is a better burrower and usually nests underground. Breeding season continues throughout the year in favourable conditions. Two adult male specimens were purchased and used for the present investigation on 15.12.1987 and 20.12.1987.

5. Funambulus pennanti (Indian palm squirrel) :

Indian palm squirrel is a small sized animal with the three strips on the back from the neck to the rump. It is commonly found in grooves and gardens and often enters dwelling houses. In

movement, it is quick and jerky. It eats nuts, seeds and fruits. It builds a bulky nest of twigs and grass in the branches of trees. Two adult male specimens were collected from Tasgaon (Dist.Sangli) for the present investigation on 15.2.1988.

6. Bubalus bubalus (He buffalo) :

The commonly known water-buffalo has extensively been domesticated in many parts of southern Asia including India but still exists in the wild state in many regions of Asia. A large, stockily built animal, reaches almost 5 to 6 feet in height at the shoulder. The horns are black, back-swept and may reach 78 inches in length. In domestic breeds the horns are shorter than the wild animals. The domestic breeds are very similar to the wild buffalos in general appearance and are equally powerful but considerably more docile. The testes and Cowper's glands from three male buffalos were purchased from the slaughter house at Kolhapur for the present investigations on 28.5.1988, 6.6.1988 and 15.6.1988.

IV. Processing of the tissues :

The bulbourethral or Cowper's glands were dissected out from decapited animals and immediately fixed in cold (4° C) solution of 2 % calcium acetate in 10 % formalin (CAF). Together with Cowper's glands, testes of the same animals were also fixed for the identification of the sexual status of the given animal. After prolonged fixation (24 h.) the tissues were well washed in chilled distilled water followed by washing in running tap water. After dehydration in ethanol grades, clearing in xylene and paraffin embedment, sections were cut at 4 to 5 µm. Some of the sections of Cowper's glands and testes were stained with

haematoxylin - eosin (H - E) and Mallory's Triple staining (M-T) for histological observations, while the remaining sections of the Cowper's glands were subjected to the histochemical methods described below for characterization of mucosubstances.

V. Histochemical methods :

Several histochemical methods with detail steps, merits and demerits for characterization of mucosubstances have been described in detail by Spicer (1963), Barka and Anderson (1965), Lillie (1965), Thompson (1966), Spicer and Henson (1967), Spicer et al. (1967), Leppi (1968), Pearse (1968), Nalavade (1975) and Nalavade and Varute (1973). After dewaxing in xylene and hydration in ethanol grades (absolute alcohol, 90 %, 70 %, 50 % and distilled water), the sections were subjected to staining with various histochemical methods described below for the identification of mucosubstances in the Cowper's glands.

Periodiate reactive, hexose-containing mucosubstances with vic-glycols or their derivatives were detected by PAS reaction (Mc Manus, 1946). Sections were exposed to 10 min. with 0.5 % periodic acid and after rinsing with distilled water they were stained with schiff's reagent. With this method both neutral and acidic mucosubstances exhibit pink or magenta staining.

For the blockade of periodate engandered dialdehydes (derived from 1:2 glycol groups of hexose units), 5 % aqueous phenylhydrazine solution was used following periodic acid oxidation (Spicer, 1965; Spicer et al., 1967). Then the sections were stained with Schiff's reagent. Complete loss or reduction in PAS

staining indicates the presence of neutral mucosubstances rich in vic-glycols. Absence of positive staining or orthochromatic blue staining with 0.02 % Azure A at low pH (pH 1.5) but positive metachromatic purple or pink staining after induced sulfation (Moore and schoenberg, 1957; Pearse, 1960) confirms the presence of neutral mucosubstances.

Glycogen was detected by digesting the sections with 0.1 % malt-diastase or α-amylase in 0.2 M phosphate buffer at pH 6.0 (Lillie, 1954; Lison, 1960) followed by PAS. The presence of glycogen was inferred from loss or reduction in the intensity of PAS staining.

Mucosubstances bearing acidic groups were viasualized by staining the sections with 1 % alcian blue 8 GX - 300 (AB) in 3 % acetic acid at pH 2.5 for 30 min. (Mowry, 1956). Weakly acidic sulfomucins, hyaluronic acid and sialomucins are stained blue or blue-green with this procedure. Some of the sections were also stained with 1 % alcian blue in 0.1 N HCl at pH 1.0 according to the method developed by Lev and Spicer (1964) for selective staining of sulfomucins.

Acidic mucosubstances were also demonstrated by some additional histochemical methods such as colloidal iron (C.I.) (Hale, 1946, Mowry, 1963), 0.02 % Azure A buffered at pH 0.5 to 5.0 (Spicer et al., 1967) and aldehyde fuchsin (AF) (Gomori, 1950). For protein masked acidic mucosubstances, pepsin digestion (0.1 % pepsin in 0.1 N HCl) - AB pH 2.5 procedure (Quintarelli, 1963; Thompson, 1966) was used.

Sequential staining procedures such as AB pH 2.5 - PAS (Mowry and Winkler, 1956), AB pH 1.0 - PAS (Spicer et al., 1967) and C.I. - PAS (Ritter and Oleson, 1950; Mowry, 1963) were employed for the distinction between acidic and neutral muco-substances. In these histochemical methods, acidic mucosubstances are stained blue or blue-green, neutral mucosubstances remain pink or magenta coloured and their simultaneous occurrence gives purple-blue or blue-purple staining.

AF - AB pH 2.5 combined staining procedure (Spicer and Meyer, 1960) was employed to distinguish sulfomucins from carboxymucins (sialomucins and hyaluronic acid). In this sequential staining procedure, sulfomucins are stained purple and carboxymucins blue. Their simultaneous occurrence gives purple-blue or blue-purple staining.

Critical electrolyte concentration (CEC) technique using 0.1 % AB in 0.05 M acetate buffer with graded concentrations of $MgCl_2$ (from 0.0 to 1.0 M Mg^{++}) (Scott et al., 1964; Scott and Dorling, 1965) was employed. In CEC technique carboxymucins are not stained at and above 0.1 M Mg^{++} concentration. Sulfomucinc are selectively stained at and above 0.2 M Mg^{++} concentrations. Various sulfomucins lose their alcianophilia at different levels of Mg^{++} concentration.

For the confirmation of acidic groups of mucosubstances (carboxyl and sulfate), some sections were treated with methanol containing 0.1 N HCl for 4 hours at 37° C (mild methylation) and some sections were treated with the above methylation mixture for 4 hours at 60° C (active methylation). (Fisher and Lillie, 1954;

Spicer, 1960). These sections after rinsing with distilled water were stained with AB pH 2.5. Mild and active methylations block alcianophilia of non-sulfated carboxymucins, whereas generally active methylation eliminates alcianophilia of sulfomucins as a result of desulfation (Kantor and Schubert, 1957). Restoration of carboxyl depent alcianophilia was attempted by demethylation or saponification by treating the methylated sections with 1 % KOH in 70 % alcohol for 20 min. prior to AB pH 2.5 staining (Spicer and Lillie, 1959). This methylation - saponification procedure does not result in the restoration of alcianophilia of sulfomucins as the sulfate groups are hydrolytically removed.

Some sections were subjected to acid hydrolysis (0.1 N HCl, 4 hr., 60° C) and then stained with AB pH 2.5 (Quintarelli et al., 1961). Complete or partial loss indicates the presence of sialomucins. The presence of sialomucins was confirmed by sialidase (neuraminidase, Sigma) digestion procedure (Spicer and Warren, 1960). Sections were digested at 37° C with sialidase incubation medium (one vial of enzyme in 0.5 M acetate buffer, pH 6.0 containing 1 % NaCl and 0.1 % $CaCl_2$). Control sections were incubated with buffer only. Then the sections were stained with AB pH 2.5. Complete loss or reduction in alcianophilia confirms the presence of sialomucins.

The effect of hyaluronidase (Testicular, Sigma) was studied according to the method described by Barka and Anderson (1965). Sections were incubated in hyaluronidase medium (0.05 % in 0.1 M phosphate buffer, pH 5.5) for 1-4 hours at 37° C followed by staining with AB pH 2.5. Complete or partial loss of staining indicates the probable presence of hyaluronic acid and

some sulfomucins (chondroitin sulfate A and C).

A bird's eye-view of the various histochemical techniques employed in the present investigation together with the chemical reactions involved and histochemical results is given in Table No.1.

VI. Procedure for castration and testosterone propionate administration in house rats :

The house rats were employed in the present investigation because castration and in some animals castration followed by androgen administration studies have been carried out in the slender loris, rabbit, white rat, guinea pig, barrow, sheep, cattle and rhesus moneys. Secondly, house rats can be obtained for such studies throughout the year. Thirdly, these rats are continuous breeders and hence one has not to wait for active breeding period which is restricted to few months of a year in seasonally breeding animals. Finally, castration studies are easy in these rats because of the external testes in the scrotal sacs.

Three groups (each group of three males) of rats were housed in separate cages. One group was used for castration studies, the second group for castration followed by androgen treatment and the third group of normal intact rats was used for effects of androgen treatment without any castration.

First group of rats was ether anesthetized and each rat was placed on operation board at half an hour time interval. The hairs on the scrotal sacs were clipped. The skin was cleaned with absolute ethanol. The first incision was made superficially through the skin of the scrotum. The second incision was made through the

TABLE 1. HISTOCHEMICAL METHODS EMPLOYED FOR VISUALIZING MUCOSUBSTANCES

Histochemical method	Chemical reaction involved	Histochemical result	References
1 Periodic acid Schiff's reaction (PAS)	Oxidation of vicinal hydroxyls to ialdehydes by periodate and formation of coloured complexes with Schiff's reagent.	All polysaccharides and mucosubstances colour pink to magenta.	Mc Manus(1946).
2 Periodic acid phenylhydrazine Schiff	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 pink presumably are those in which acid groups are proximal to vicinal glycols.	Spicer (1965); Spicer et.al. (1967).
3 α - amylase - digestion-PAS	Hydrolyses and removes glycogen.	Loss of PAS reactivity in sites containing glycogen.	Lillie (1954); Lison (1960).
4 Alcian blue, pH 2.5	Probably formation of alcian blue complexes with carboxyls and sulfate groups.	Sialomucins and weakly acidic sulfomucins stain blue; the most strongly acidic sulfomucins stain weakly or not at all.	Mowry (1956).
5 Alcian blue, pH 1.0	Probably formation of alcian blue complexes with sulfate groups.	Weakly and strongly acidic sulfomucins are selectively stained.	Lev and Spicer (1964).

	Histochemical method	Chemical reaction involved	Histochemical result	References
6	Colloidal Iron	Probably formation of complexes between cationic colloidal terric aggregates and carboxyls, sulfate and phosphate esters.	Non-sulfated acid mucosubstances and some sulfated mucosubstances colour blue.	Hale (1946); Mowry (1963).
7	AB pH 2.5-PAS	Addition of results by single methods.	Alcian blue reactive, periodate unreactive acid mucosubstances stain blue. Alcian blue and PAS reactive mucosubstances colour purple-blue. Neutral mucosubstances colour pink-magenta.	Mowry and Winkler (1956); Spicer et al. (1967)
8	AB pH 1.0-PAS	Addition of results by single methods.	Alcian blue reactive, periodate unreactive acid mucosubstances stain blue. Alcian blue and PAS reactive mucosubstances colour purple-blue. Neutral mucosubstances colour pink-magenta.	Spicer et al., (1967)
9	Colloidal Iron- PAS	Addition of results by single methods.	Colloidal iron reactive, periodate unreactive acid mucosubstances stain blue. Colloidal iron and PAS reactive mucosubstances colour purple-blue. Neutral mucosubstances colour pink-magenta.	Ritter and Oleson (1950); Mowry (1963).
10	Aldehyde fuc hsin (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.	Gomori (1950).

	Histochemical method	Chemical reaction involved	Histochemical result	References
11	AF-AB pH 2.5	Formation of salt complexes between cationic staining entity and sulfate and carboxyl groups.	Sulfomucins stain purple or blue-purple, sialomucins and other non-sulfated acidic mucosubstances stain blue.	Spicer and Meyer (1960).
12	Alcian blue at pH 5.6 with graded concentrations of $MgCl_2$	Alcian blue complexes with sulfate groups. Different sulfomucins vary in the critical electrolyte concentration at which alcianophilia is lost.	Non sulfated acidic mucosubstances are not stained at an above 0.1 M Mg^{++} concentration. Sulfomucins stain selectively at and above 0.2 M Mg^{++} concentration.	Scott et al. (1964); Scott and Dorling (1965).
13	Azure A at controlled pH levels.	Formation of blue orthochromatic or pruple 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, sialomusins stains 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	Spicer et al. (1967).
14	Sulfation-Azure A pH 1.5	Sulfate groups are induced in neutral mucosubstances.	The neutral mucosubstances which exhibit orthochromatic blue staining with azure A at lower pH (1.5), become metachromatic pink or red after sulfation.	Pearse (1960).
15	Mild-methylation - AB pH 2.5	Esterification of carboxyl groups.	Generally mild methylation blocks the alcianophilia of carboxy mucins.	Fisher and Lillie (1954); Spicer (1960).

	Histochemical method	Chemical reaction involved	Histochemical result	References
16	Mild methylation - saponification - AB pH 2.5	Restoration of carboxyl groups	Restoration of the alcianophilia after saponification of methylated sections, indicates the presence of carboxyl groups.	Spicer and Lillie(1959).
17	Active methylation- AB pH 2.5	Carboxyl groups are esterified. Sulfomucins are desulfated.	Active methylation blocks alcianophilia of carboxymucins through esterification and of sulfomucins through hydrolytic removal of the sulfate groups.	Fisher and Lillie (1954); Spicer(1960).
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 the alcianophilia after subsequent saponification indicates the presence of carboxyl groups and loss of alcianophilia indicates the presence of sulfate groups.	Spicer and Lillie (1959).
19	Acid hydrolysis AB pH 2.5	Removes sialic acids from mucosubstances.	Complete or partial loss of alcianophilia indicates the probable presence of sialomucins.	Quintarelli et al. (1961).
20	Sialidase (neuraminidase) AB pH 2.5	Removes sialic acids from mucosubstances.	Complete or partial loss of alcianophilia confirms the presence of sialomucins.	Spicer and Warren (1960).

	Histochemical method	Chemical reaction involved	Histochemical result	References
21	Hyaluronidase AB pH	Depolymerization of hyaluronic acid, chondroitin sulfate A and C.	Complete or partial loss of alcianophilia indicates the probable presence of hyaluronic acid, chondritin sulfate A and C.	Barka and Anderson (1965)
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 alcian-blue after removal of protein masking.	Quintarelli (1963); Thompson (1966)

transparent tunica vaginalis. The tunica vaginalis and vas deference were ligated by sterilized nylon thread and testis was dissected out. Similar operation was done on the other scrotum to remove the testis. Then the skin of the incision region was stitched by C-shaped needle with sterilized nylon thread. In the same manner a group of three rats was castrated bilaterally and caged separately for 10 days.

Second group of rats was castrated in the similar manner and in such animals, testosterone propionate (40 mg/100 g body wt.) was injected in the thigh muscles. These rats were housed in separate cages.

In the third group of rats (without castration), testosterone propionate injection (40 mg/100 g body wt.) was given and the animals were kept in separate cages. The rats of the first, second and third group were sacrified after 10 days by decapitation, the Cowper's glands were dissected out and used for histological and histochemical studies.