CHAPTER – V
Classification of Polysaccharides:

The histochemical classification as given by Pearse (1968, Pg 294):

1. **Polysaccharides**:

   Polysaccharides consisting of one type of units:

   (a) **Glycogen** - This is the only naturally occurring member of this group remaining in animal tissue after aqueous fixation and paraffin embedding. It is composed of chains of glucopyranose (D-glucose) units. They are readily hydrolysed to glucose by boiling with dilute acids.

   (b) **Starch** - Main carbohydrates of plants, made up of one linear molecule called amylose and the other a branched molecule called amylopectin. The amylose-amylopectin ratio varies from case to case. Though made up of 2 glucans, on hydrolysis starch yields only D-glucose.

   (c) **Cellulose** - Main constituent of the cell walls of plants. It yields only glucose on hydrolysis.
11. Mucopolysaccharides (MPS):

These are polysaccharides containing hexosamine as one component and occurring either free or as esters of sulphuric acid.

(a) **The neutral MPS** - contain hexose as their second carbohydrate component. They do not carry reactive acid radical.

(b) **Acid mucopolysaccharides (AMPS)** - They contain hexoronic acid as their second carbohydrate component. There are two types of AMPS as classified by Spicer et al. (1965).

1. **Sulfomucins**:
   
   (i) Strongly acidic, periodate unreactive.
   
   (ii) Strongly acidic with sulphate esters on vic-glycols.
   
   (iii) Weekly acidic, periodate reactive.

2. **Sialomucins**:
   
   (i) Digestible with sialidase rapidly and slowly.
   
   (ii) Resistant to sialidase.

**Chitin**:

The simplest of neutral MPS, consisting of acetyl glucosamine residue joined together in pairs by B-1, 4-glycosidic linkages. It is widely distributed in the animal kingdom, especially in invertebrates.
Hyaluronic acid:

AMPS composed of N-acetyl-D-glucose amine and D-glucose acid units which occurs probably as a freely disassociated compound in vitreous humour and in synovial fluids.

Heparin:

A complex MPS composed of glucosamine glucoronic acid and sulphuric acid.

Fixation of carbohydrates:

The most important factors in the fixation of carbohydrates is the choice of a fixative and the method of fixation. Improper fixation and a wrong choice of fixative will result in the loss of cellular relationship. Thus, the fixative chosen for histochemical work must also preserve a good histological picture and stabilise the chemical composition with respect to localisation. A good staining depends on the use of a good fixative and also helps in the interpretation of the reaction results.

Carbohydrates are not affected by fixatives but are entangled in the coagulant (Baker, 1958). Alcohols precipitate carbohydrates, but the reactions are reversible and the reverse carbohydrates may get lost during subsequent processing (Szirmai, 1963). Partridge (1948) found considerable loss of mucopolysaccharides during processing.
through aqueous media. Finar (1960) found glycogen to be soluble in water. Dempsey and Wislocki (1946) found that certain fixatives affect the protein composition of the cell.

Advantages and disadvantages of certain fixatives:

Alcohol:

Alcohol precipitates polysaccharides from the tissue and leaves them chemically unaltered. Ross (1953) and Szirmai (1963) studied various fixatives and found that alcohol causes the greatest shrinkage, which in turn causes significant displacement of compounds. According to McManus and Mowry (1960) cold alcohol causes least shrinkage.

Formalin:

Formalin causes the least shrinkage of tissue, it penetrates better and presents a better histological picture. It fixes proteins well but reacts with proteins. It increases basophilia which may affect mucopolysaccharide staining. Formalin prepared from formaldehyde is acidic due to the presence of formic acid and has a pH range between 3 and 4.6 (Baker, 1958). This acidity has to be minimised as it may reduce PAS staining (Hale, 1955). Formalin is stored above a layer of calcium or magnesium carbonate. The pH levels observed range between 6.3 or 7.4 for calcium carbonate and 6.5 to 8.2 for the latter. During processing the pH value
drops to 5.7 to 6.0 (Lillie, 1965). The addition of calcium acetate at 2% maintains a pH approximity at 7.0. This is better than Baker's calcium formalin (Baker, 1944). Effect of formalin has been studied by Hale (1955). Kramer and Windrum (1955), and Conklin (1963). In the present work calcium acetate formalin has been used as it was found to give the best results.

Staining:

Many methods are available for the study of mucopolysaccharides, but the specificity can be questioned. However, the specificity can be supplemented by other reactions such as acetylation, methylation and digestion. The use of basic and metachromatic dyes is well known in mucopolysaccharide histochemistry.

In the present work, Periodic Acid Schiff Method (PAS), Alcian Blue (AB) and metachromasia methods are used along with necessary supplementary reactions.

Periodic Acid-Schiff Reaction (PAS):

This method is one of the best known and ranks first in mucopolysaccharide histochemistry. Many publications have appeared on the merits and demerits of this method. (1) Oxidation of periodic acid or other suitable oxidising
agents. On oxidation, laboratory mucopolysaccharides yield aldehyde groups.

(2) Coloration: The aldehydes formed in oxidation, form coloured complex with Schiff's reagent.

The oxidation yields groups rather than compounds. The reactive groups are α-glycols, α-amino-alcohols and α-alkyl-
amino-alcohols. The PAS technique was developed by McManus (1946), Lillie (1947) and Hotchkiss (1948). The methods differ in the use of the oxidants. McManus used 0.5% aqueous periodic acid.

Oxidants:

A number of oxidants were suggested by various authors: Periodic acid (McManus, 1946; Lillie, 1947; Hotchkiss, 1948), lead tetracetate (Glegg et al., 1952; Shimzu and Kamamota, 1952; Jordan and McManus, 1952), and Chromic acid (Bauler, 1933). In this study, periodic acid has been used.

The aldehydes formed after periodic oxidation can be detected by any aldehyde reaction feasible in organic chemistry. Many reagents have been used to detect aldehydes but Schiff's is the most widely accepted. In histochemical tests, reactive groups stained with Schiff's have been proved to be mostly aldehydes (Gomori, 1952; Glegg et al., 1952 and
The chemistry, formation and reaction of Schiff's have been reviewed by Kasten (1960).

Methods for preparing Schiff's reagent have been recommended by various workers. Some of the important workers are De Tomasi (1936). Barger and Delamater (1948). Lillie (1951), Atkinson (1952), Itikawa and Ogura (1954). and Kasten (1959). A good Schiff's reagent is one which is specific for aldehydes and is sensitive to small amounts of aldehydes. Impurities in the dye reduce sensitivity (De Tomasi, 1936). The reagents deteriorate with age. Ely and Ross (1949), Atkinson (1952) and Longley (1952) have pointed out that concentration of sulphuric acid ($H_2SO_4$) affects sensitivity. Sulphurous acid is produced by bubbling Sulphur dioxide ($SO_2$) through the solution or by interaction of a sulphur compound with hydrochloric acid (HCl) to produce $SO_2$ which reacts with water to form sulphurous acid. A straw coloured solution is formed which is purified by activated charcoal as suggested by Coleman (1938). Schiff's reagent gets spoilt and turns pinkish after usage. In this case, fresh Schiff's should be prepared. In the present work, Schiff's was prepared according to the De Tomasi (1936) method.

Periodic acid is prepared by dissolving acid crystals in water (McManus, 1946), while Hotchkiss (1948) used alcohol. 
Hale (1957) pointed out that it takes two hours with alcoholic periodic acid to produce as much oxidation as would be produced by aqueous solution in five minutes. Concentration of periodic acid is important, as high concentration produces pseudo-reactions. Time of oxidation and temperature are important factors. Prolonged oxidation results in over oxidation (Dempsey et al. 1950). Rate of oxidation is increased with increasing temperature. Pearse (1958, Pg 315) advocates the use of periodic acids at room temperature. Hotchkiss (1948) introduced a reducing rinse but according to McManus (1946), Lillie (1951, 1952) and Pearse (1968 Pg 316) the reducing rinse should be omitted. Slides were treated with 0.5% sodium metabisulphite to remove excess Schiff's.

**Metachromasia**

The colouring of different tissues by a single dye, into colours different from that of dye solution, is known as metachromasia. There are many theories that explain the mechanism of metachromasia and these have been reviewed by Schubert and Hamerman (1956). Metachromasia is produced by polymerisation of the dye molecule. This is an accepted concept and was introduced by Michaelis and Graik (1945). Sylven (1954) pointed out that metachromasia is produced in two stages:
(1) The dye molecule combines with the anionic group of chromotropes (the capacity of a dye to colour tissue differently is known as chromotrope).

(2) More dye molecules are deposited at the point of combination in a linear order. A minimum distance of 5Å between adjacent molecules is a pre-requisite for metachromasia and the degree of metachromasia depends on this distance.

Many dyes are used such as methylene blue, Azure A and Toluidine blue. In this work, Azure A has been used.

The biggest problem is the survival of metachromasia after alcoholic dehydration. Kelly (1955, 1958) published beautiful papers on metachromatic staining of acid mucopolysaccharides. In the present work, dehydration is done according to Spicer (1960).

The solvent used for preparing dye solution plays an important role in staining. Water, alcohol and buffer mixtures are used as solvents.

The use of buffer as solvents has two main advantages. It minimises over staining and hence improves staining. Secondly, it helps in characterizing the nature of the anions of the chromotropes. In buffer solutions, the acid substances of the
various tissue elements ionise at different pH levels and the anions liberated combine with the cations of the dye and hence staining is effected. A substance therefore gets finally ionised and hence becomes intensely stained at a certain pH level, while another does so at another pH level, and hence the two substances can be distinguished.

Chondroitin sulphates are so strongly acidic that, they are virtually ionised at all pH levels, even, as low as pH 1 (with 0.1 N HCl). Most carboxyl groups ionise at pH 4 and above, while the phosphate groups between pH 3.5 to 3.8. Thus, a structure which is metachromatically stained below pH 2.5 consists of sulphate group, at 3.5 to 4.0 consists of phosphate and sulphate groups, while above 4.0 consists of all the three groups. Such a method of distinction has been termed the pH signature test (Dempsey, 1947).

In the present work, the buffer mixture suggested by Spicer (1968, Pg 295) has been used i.e. phosphate citrate buffer is used at pH 1.5, 2.0, 3.0, 4.0 and 4.5 for the Azure A solutions.

The production of metachromasia depends to a large extent on the ratio of the dye to the chromotrope, and if insufficient dye is present, metachromasia will not be produced. The usual concentration of the dye is of the order
of 0.1% but even 0.01% is adequate (Kramer and Windrum, 1955). The minimum concentration has been put down by Schubert and Hamerman (1956) to be 0.001%. Below this concentration, salts will exert their inhibitory action on the metachromatich production. In the present work, the concentration of Azure A is 0.002% as suggested by Spicer (1967, Pg 295).

**Alcian blue:**

Alcian blue as a stain for mucins was first introduced by Steedman (1950). According to Vialli (1951) the dye is specific for mucopolysaccharides and he obtained identical results with Toluidine blue. Lison (1954) concluded that AB is specific for mucopolysaccharide. Rizzoli (1955) also supported this view. Further Mowry (1956) elaborated the use of the dye and found that a better combination and a better selectivity are obtained at a low pH and at low dye concentration. He recommended 0.05% to 0.1% of AB in 3% acetic acid at a pH of 2.7 to 3 for 30 minutes, while Wagner and Shapiro (1957) found the specificity at a higher pH values. Spicer (1960) studied the effect of pH on AB staining and concluded that AB at a concentration of 0.04% had a strong affinity for mucins between pH 1.0 and 3.0, but not at pH 4.0.

Mowry (1960) modified his own technique because he observed that new dye samples were more soluble, more stable and slow to react. He suggested staining in AB for 1 to 2
hours in 0.5% to 2.0% of AB in 3% acetic acid or in 0.1 N HCl in citrate buffer of pH 2.2 to 2.4 respectively. Lev and Spicer (1964) reported that staining with 1% AB at pH 1.0 would selectively stain sulphate group but not carboxyl groups provided they were blotted dry and not rinsed in water.

**Combined AB-PAS:**

This combined staining is meant for simultaneous demonstration of acid and neutral mucopolysaccharide and was devised by Mowry and Winkler (1956). Staining results in contrasting colours. Blue indicates exclusively acid mucopolysaccharides and additive colours suggest carbohydrates having both acidic and hydroxyl groups. The predominancy of colour like red purple or blue purple suggest more neutral or more acidic groups respectively.

**Blocking reaction**

These reactions block the reactivity of active groups to the methods specific for their demonstration so that they do not result in coloration. Their performance is two fold. Firstly, they increase selectivity of the method and secondly they confirm the presence of a particular group. According to Casselman (1959) there are two types of blocking reactions:

1. Reversible, where the blocked reactivity of a group is restored by another chemical reaction.
(2) Irreversible, where the reactive group is destroyed (Monne and Slatterback, 1950).

**Acetylation and Deacetylation:**

This was introduced by McManus and Cason (1950) to confirm the nature of a carbohydrate which gives a positive PAS reaction. Acetylation blocks hydroxyl and amino-groups by esterification. The blocked groups do not react with Schiff's after periodic oxidation. Deacetylation restores the hydroxyl and α-glycol groups but amino groups are not restored. The PAS positive staining after deacetylation is due to α-glycols. Acetylation is carried out in a mixture of acetic anhydride and pyridine at room temperature for 45 minutes. Deacetylation is carried out in 0.1 N Potassium hydroxide (KOH) for 45 minutes at room temperature. Lillie (1951) states that many hydroxyl bearing substances resist 45 minutes acetylation but yield to prolonged treatment. He suggested 1 to 24 hours acetylation at room temperature.

**Methylation and Demethylation:**

Fraenkel-Conrat and Olcott (1945) used methanol with Hydrochloric acid (HCl) to block carboxyl groups. Fisher and Lillie (1954) showed that it could block sulphate, phosphate and α-glycol groups too, because it blocked metachromatic, nuclear and PAS staining. Methylation esterifies the carboxyl groups while sulphate groups are split off from the molecules.
Methylation is generally carried out with 1 N HCl in methanol at 60°C for four hours (Spicer and Lillie, 1959 and Lillie, 1965). Methylation when carried out at 37°C showed results different from that carried out at 60°C. He called the former mild and the latter active methylation. Lillie's (1958) demethylation restored the staining of pepsinogen granules. Demethylation is carried out in a mixture of 1 N KOH and 70% alcohol for 20 minutes. According to Spicer and Lillie (1959), this reversal can only be effected above a pH of 3-4 and not below. By this technique of methylation and demethylation, it is possible to characterise the acid groups in tissues.

In the present work, methylation and demethylation method was carried out according to Spicer and Lillie (1959) and Lillie (1965).

**Diastase Digestion Test:**

Saliva as an enzyme for the identification of glycogen in tissue sections was first used by Bauer (1933). It was popularised by Bensely (1939), who asserted that since no staining method was exclusive for glycogen, the study of the distribution of glycogen could not be complete without the use of saliva digestion. The enzyme amylase present in the saliva hydrolyses glycogen into simple sugar maltose, which dissolves out of the tissue sections, so that the latter reacts no more to the methods designed for the demonstration of glycogen. MPS
and other related substances are not acted upon so that, they still stain and are thus distinguished from glycogen. Lillie and Greco (1947) introduced the use of malt diastase and ptyalin. Of the two, they claimed that malt diastase was better because of aesthetic and sanitary reasons (Gomori, 1952). Saliva test requires a large amount of human saliva (Lillie and Greco, 1947), and because of highly variable activity of saliva (Casselman, 1959), Malt diastase has now taken the place of saliva, inspite of the attempt of Harter (1948) to improve the saliva method by using diluted saliva buffered at pH 4.5 and pH 8.

The hydrolytic enzyme actually useful in the digestion test is an α-amylase. Malt diastase are also present such as α-amylase (Pearse 1972, Pg 1034) ribonuclease, an enzyme often capable of destroying the metachromasia of nucleus pulposus and cartilage matrix (Lillie, 1949). These enzymes are mostly contaminants but fortunately they do not interfere with the specific use of diastase in identifying glycogen (Barka and Anderson, 1963).

The various factors that should be taken into consideration in the digestion test are:

1. Concentration of the enzyme usually between 0.1% to 1%.
2. Solvent buffered solution of pH 5 to pH 8 or distilled water.
The temperature of the incubating medium (Lillie, 1954, Pg 275 and Pearse, 1972, Pg 1016).

These various factors, used by different investigators, have been tabulated by Hale (1957). Pearse (1972) stated that with diastase it is necessary to use the enzyme either in a buffer or in a saline. Both of which have the tendency to increase its ribonuclease activity. Solutions made in distilled water remove glycogen adequately even within as short a period as 30 minutes at room temperature.

It is imperative that sections must be coated with colloidion before enzyme digestion. The enzyme, being protein in nature, cannot diffuse through colloidion and the results of digestion will be irregular. This substance must be removed from colloidion-embedded materials (Gomori, 1952). However, the step can be performed after the digestion test and before the staining, but this is unnecessary (Beckett and Bourne, 1958).

It is essential to carry out control experiments by exposing sections to the buffered solvent alone. This of course is not needed if the enzyme is dissolved in water only. Another control should be carried out to test the activity of the enzyme by exposing a section of the material fixed and processed in the same manner and known to contain glycogen.
Thus, digestion test using malt diastase if, carried out correctly is useful in studying the distribution of glycogen in tissue sections. A structure that gives a positive reaction to glycogen demonstrating technique, but a negative reaction after diastase digestion, is certain to be glycogen.

The method followed in the present work is the one advocated by Lillie (1954) using 0.1% malt diastase (1:50 U.S.P. No.8511, Constantine, Favria, Italy), dissolved in 0.2 M phosphate buffer of pH 6.0 for one hour at 37°C. The addition of sodium chloride is, however, not made. The diastase solution is filtered before use to remove starch granules.

**Pepsin Digestion**

Proteins can inhibit the development of the metachromasia of a MPS. The use of proteolytic enzymes would free the latter from the influence of the former and so metachromasia would be produced (Kelley, 1955; Kramer and Windrum, 1955).

There are at least three possible ways in which the inhibition can occur. One is that cationic centres of proteins may act as the cations of salts and compete with the dye cation for the anionic groups of the chromotrope. A second is that the metachromatic staining of tissue may fail to occur due to binding of protein and polysaccharides in such a way
that the anionic groups are sterically masked. A third is the actual formation of a covalent link, such as an ester bond between anionic group of the polysaccharide and the hydroxyl group of the protein (Schubert and Hamerman, 1956).

The enzyme pepsin has been employed by a number of investigators. Pearse (1972, Pg 1037) recommended the use of crystalline pepsin 2 mg in each ml of N/50 Hydrochloric acid at pH 1.6 at 37°C for 2-3 hours.

In the present study, the recommendation given by Pearse was followed. The incubation was done for three hours and the staining was carried out in Azure A pH 3.0 and in PAS according to method suggested by Spicer (1960).

Sulphation:

Esters of sulphuric acid of high molecular weight give a metachromatic reaction. It is thought that in introducing a sulphate group to the non-metachromatic neutral mucopolysaccharides, metachromasia can be introduced. The sulphate groups can be introduced by esterificating the hydroxyl groups with sulphuric acid (H₂SO₄). Neutral muco polysaccharides stained positively with PAS. Sometimes, they show a weak staining which sometimes becomes unidentifiable, when such compounds are sulphated, they show a metachromatic staining providing a good confirmatory test for PAS positive
material (Kramer and Windrum, 1954). The sulphation technique was developed by Kramer and Windrum (1954). Detachment of sections from slides during sulphation is commonly met with.

To overcome this, Moore and Schoenberg (1957) suggested a method in which deparaffinised sections were brought down to alcohol and then dried at 37°C for several hours. The dried sections were then immersed in a mixture of equal volumes of concentrated sulphuric acid and glacial acetic acid at room temperature for few minutes.

The sections were rinsed in glacial acetic acid for 2-3 minutes and washed in running water for 10 minutes. Lewis and Grillo (1959) supported the results, but suggested that the reaction should be carried out at room temperature.

He pointed out that neutral pH of metachromatic dye introduced much orthochromic background staining of non-sulphated substances and suggested the use of methylene blue at pH 2 to 3. Alcian blue and Toluidine blue also give good contrasts in this same pH range. In this study, the method of Moore and Schöenberg is followed, only staining is done with Azure A at pH 3.0 to pH 4.5.
Plasmal Reaction:

Schiff's reagent reacts with aldehydes to stain the tissues. Aldehydes may occur in a free form, like those not formed due to periodic oxidation. Such aldehydes will interfere with the PAS staining and render interpretation difficult. The aldehydes are mainly from:

(1) Acetyl phosphatides, and
(2) Unsaturated fatty acids.

(1) Acetyl phosphatides:

It is a fatty aldehyde joined to glycerol by actual bonds. The aldehyde is released on breaking of the bond. The breakage can be quickened by mercuric chloride oxidation. The liberated aldehyde is termed 'Plasmal' the acetyl phosphatide as 'Plasmalogen' and the reaction as 'Plasmal reaction'. The phosphatides being soluble in fat solvent are removed during alcohol dehydration. Aldehydes can then remain only in frozen sections or in materials fixed with mercuric chloride.

(2) Unsaturated fatty acids:

In this case, the aldehyde liberation is due to oxidation by atmospheric oxygen of double bonds in unsaturated fatty acid radical. This is affected only negligibly by mercuric chloride. The reaction takes place on exposure of sections, as a pseudoplasmal reaction to atmospheric oxygen and is mostly
termed as pseudoplasmal reaction. Thus

(i) A positive PAS reaction with Schiff's without prior periodic oxidation is due to unsaturated fatty acid aldehydes.

(ii) A positive PAS reaction after mercuric chloride oxidation is due to plasmalogen.

(iii) A positive PAS reaction after periodic oxidation is due to carbohydrates.

In this work, the plasmal reaction is carried out to distinguish the above mentioned three reactions.

**Acid Hydrolysis:**

The acid hydrolysis technique is particularly useful in the study of sialomucins. Since very early it was noted that sialic acid could be easily split off from the main mucin molecule by heating the mucin in a faintly acid solution. When bovine submaxillary gland mucin was treated at pH 1.0 and 80°C, sialic acid, and sialic acid only was released from the submaxillary mucoprotein (Gottachalk, 1957). Similar results were obtained when the mucin was treated with cholera vibro and influenza virus Neuraminadase (Gottachalk 1958). Quintarelli et al. (1961a) applied these findings to histochemistry. They subjected sections of bovine submaxillary and rat sublingual gland to treatment in various buffers at various pH level for different lengths of time at different
temperatures. They discovered that in the case of bovine submaxillary gland, an exposure of 4 hours at 70°C in a buffer of pH 2.5, completely prevented AB staining. In case of the rat sublingual gland a period of 6 hours, were required. They also noted that the staining results after acid hydrolysis and after neuraminidase treatment were comparable.

Later Quintarelli (1961b) found that salivary gland sections lost their basophilia to the same extent after treatment with neuraminidase, after methylation for 4 hours at 60°C and after acid hydrolysis (0.1 N HCl in water) at 60°C for 4 hours. Thus mild acid hydrolysis can be used as a tool in the histochemical study of sialomucins.

In the present work, the acid hydrolysis was carried out in 0.1 N HCl in water at 60°C for 4 hours.

Lipid Extraction:

To assure that lipids do not interfere with the PAS reaction, they must first be removed from the tissues. Several methods have been suggested for lipid extraction (Baker, 1944; Lovern, 1955; Barka and Anderson, 1963, Pg 114; Pearse, 1968, Pg 398).

Since the purpose of employing the extraction technique in the present study is the total removal of lipids, the
technique can safely be performed on formaldehyde fixed tissues, especially so, when it is also known that lipid solvents exert a damaging effect on fresh tissues (Baker, 1944). This damage would certainly affect the PAS reaction (staining picture). The use of calcium acetic formalin instead of the recommended weak Bouins is resorted to because of two reasons. Firstly this is the fixative used in the present study in conjunction with the PAS method. Secondly the use of two fixatives, the fixing action of which is different on proteins (precipitant and coagulation) which may lead to different results (Cain, 1949).

Extraction on tissue sections instead of on fresh tissues have been carried out by the investigators (Dixon and Herbertson, 1950; Schiebler, 1952; Koihikawa, 1957 and Seaman, 1958).

In the present work, tissue sections were extracted in pyridine for 24 hours at 60°C.
MATERIALS AND METHODS

Carbohydrates:

Fixation:

Earthworms were dissected, the blood glands were removed and fixed in calcium acetate formalin at room temperature.

Preparation of Fixative:

To 10 ml of 40% formaldehyde solution, 90 ml of distilled water was added. In this, 2 gms of calcium acetate was dissolved. A little calcium carbonate was added to the above solution to maintain a pH of 7. After fixation, the glands were washed under running tap water (overnight). They were then dehydrated via different grades of alcohol: 30%, 50%, 70% and 90% each for one hour duration and absolute alcohol for two hours (the last with one change). The complete washing and dehydration was done at room temperature. The glands were then transferred to vials containing xylene, giving two changes of xylene at one hour interval. The glands were kept for cold infiltration of wax for two hours. Then onto hot infiltration of wax for two hours with two changes of molten paraffin wax (E. Merck, 52°C to 54°C).

Blocks were prepared, trimmed and kept overnight. Sections were cut at 7 μ on the Spencer's rotary microtome. A
very thin smear of adhesive (egg albumin) was used on the slides on which sections were mounted. The stretching of sections was done by flooding the slides with distilled water and placing them on a hot plate. Excess water was drained off and the slides were kept to dry and then stored in the slide boxes.

METHODS:

I. Periodic Acid Schiff Reaction (PAS):

The Schiff's reagent was prepared according to the method of De Tomasi (1936). One gram of Basic Fuchsin was dissolved in 200 ml of boiling distilled water. The solution was stirred for five minutes and cooled to exactly 50°C. It was filtered and to the filtrate 20 ml of 1 N HCl was added. This solution was cooled to 25°C and one gram of anhydrous sodium metabisulphite was dissolved in it. The solution was then kept in the dark overnight (18-20 hours). Two grams of activated charcoal was added and the resulting suspension was shaken for two-three minutes and then filtered. The filtrate was stored in dark bottles and kept in the refrigerator (4°C). Before using, the Schiff's reagent was allowed to attain room temperature.

Theory:

The reaction is based on the fact that aqueous periodic
acid will oxidise 1, 2 glycol groups in tissues that consists of or contains CHO group to produce aldehyde that are coloured by Schiff's reagent.

1) Periodic acid solution 0.5% (Spicer, 1967, Pg 300). Periodic acid was dissolved in 100 ml of distilled water.
2) Schiff's reagent.
3) 0.5% sodium metabisulphite.

PAS : (McManus, 1948, Pg 99):

1) Sections were deparaffinised in xylene and hydrated through different grades of alcohol, and brought down to distilled water.
2) They were oxidised for fifteen minutes in 0.5% aqueous periodic acid solution.
3) Rinsed in distilled water.
4) Then the sections were immersed in Schiff's reagent for fifteen minutes.
5) They were rinsed in three changes of (two minutes each) 0.5% sodium metabisulphite solution.
6) They were washed in running tap water for fifteen minutes.
7) They were finally dehydrated through different grades of alcohol, cleared in xylene and mounted in Canada balsam.
**Expected result**: Hexose containing mucosubstances stain in various shades of purplish red. Glycogen stain deeply.

II. Diastase + PAS: (Lillie, 1954, Pg 275):

1) Sections were deparaffinised and brought down to water through alcohol grades.
2) They were treated with 0.1% Diastase (PJV Japan) (preheated) dissolved in 0.2 M phosphate buffer at pH 6 for one hour at 37°C. Control sections were exposed only to the buffer solution (37°C).
3) Sections were washed in water for five minutes.
4) They were then stained for PAS as in (I) from step 2 onwards).

**Expected result**: Positive result indicates the material was not glycogen.

III. Acetylation + PAS: (Lillie, 1954, Pg 161):

- Acetic anhydride 20 ml
- Anhydrous pyridine 30 ml

1) Sections were brought down to water through xylol and alcohol.
2) They were treated with acetic anhydride-pyridine solution for 45 minutes at room temperature.
3) They were washed thoroughly in running tap water.
4) They were then stained for PAS as in (1) (steps 2 to 7).
5) They were dehydrated in alcohol, cleared in xylene and mounted in Canada balsam.

Expected result: Negative result confirms the carbohydrate nature of the reacting group.

IV. Deacetylation + PAS: (Lillie, 1954, Pg 161):

1) Sections were brought down to water through xylol and alcohol grades.
2) They were subjected to the acetylation process as given in (III) (step 2).
3) They were washed thoroughly in running tap water.
4) They were treated in 0.1 N potassium hydroxide solution for 45 minutes.
5) They were washed thoroughly in running tap water.
6) They were then stained for PAS as in (1) (steps 2 to 7).
7) They were dehydrated in alcohol, cleared in xylene and mounted in Canada balsam.

Expected result: Positive result confirms carbohydrate nature of the reacting groups.
V. **Pyridine Extraction** (Baker, 1946b, Pg 441):

1) Sections were brought down to absolute alcohol.
2) Treated with pyridine for one hour at room temperature.
3) Treated with pyridine for 24 hours at 60°C.
4) Rinsed in absolute alcohol.
5) Brought down to water via graded alcohol.
6) Washed in tap water.
7) Stained for PAS as in (I) and mounted in Canada balsam.

**Expected result**: Positive results indicate presence of mucosubstances.

VI. **Plasmal reaction**: (Danielli, 1949, Pg 67):

1) Sections were brought down to water.
2) Oxidised for 15 minutes in (a) 1% aqueous HgCl₂.
   (b) 0.1 N HCl.
3) Washed in tap water for 5 minutes.
4) Stained for PAS as in (I) (step 2 onwards).

**Expected result**: Acetal lipids stain reddish purple.

VII. **Direct Schiffs**:

1) Sections were brought down to water.
2) Stained in Schiff's reagent as in (1) (step 4 onwards).

**Expected result**: Negative staining indicates absence of naturally occurring aldehydes.

**VIII. Alcian Blue (AB)**: (Mowry, 1960, Pg 323):

1) Two sets of sections were deparaffinised in xylol and hydrated through different grades of alcohol.
2) They were rinsed in Set A - 3% acetic acid and Set B in 1N HCl (one minute)
3) They were stained in Alcian Blue, Set A - 1% in 3% acetic acid (pH 2.5) and Set B - 1% in 1N HCl (pH 1) for one hour.
4) They were rinsed in water.
5) They were dehydrated in alcohol.
6) Finally, they were cleared in xylene and mounted in Canada balsam.

**Expected result**: Acid mucopolysaccharides stain clear blue green.

**IX. Alcian Blue + PAS method**: (Spicer, 1967, Pg 296):

1) Sections were taken through xylene and graded alcohols to water.
2) They were stained for AB (pH 2.5) as in (VIII) (steps 2-4).

3) They were then stained for PAS as in (I) (from step 2 onwards).

**Expected result**: Alcianophilic mucosubstances blue. Periodic alcianophilic components blue purple, Periodate reactive nonalcianophilic compounds red.

X. **Methylation + AB**: (Lillie, 1954, Pg 163):

Methylation reagent:

i. Concentrated hydrochloric acid 0.4 ml

ii. Absolute anhydrous methanol 50.0 ml

1) Sections were deparaffinised in xylol and brought down to absolute alcohol.

2) They were rinsed in absolute methanol.

3) They were placed in preheated acidified methanol solutions, for four hours at 60°C. Control sections were treated in absolute anhydrous methanol for the same duration and temperature as the test section.

4) Rinsed in two changes of absolute alcohol, two changes of 90% and two changes of 70% alcohol.

5) Washed under running tap water for 5 minutes.

6) Rinsed all sections in two changes of 3% acetic acid.
7) Stained all sections by standard pH 2.5 alcian blue technique.

8) They were dehydrated, cleared in xylene and mounted in Canada balsam.

Expected result: Blocks AB staining of sulphated and non-sulphated mucopolysaccharides.

XI. Demethylation + AB: (Spicer, 1967, Pg 302):

Saponification solution - 1% KOH in 70% ethanol.

1) Sections were subjected to the methylation process as in (X) (steps 1 to 5).

2) Sections were taken up to 70% alcohol.

3) They were treated in 1% potassium hydroxide in 70% ethanol at room temperature for 30 minutes.

4) They were rinsed in 70% alcohol.

5) They were then brought down to water through alcohol grades.

6) They were washed in running tap water.

7) They were rinsed in two changes of 3% acetic acid.

8) They were stained with alcian blue (pH 2.5).

9) They were dehydrated, cleared and mounted in Canada balsam.
Expected result: Stains non-sulphated mucopolysaccharides blue green.

XII. Acid Hydrolysis: (Quintarelli, 1963, Pg 339):

1) Sections were brought down to water.
2) Sections were treated with 0.1 N HCl in water for four hours at 60°C (HCl preheated at 60°C).
3) They were washed in tap water.
4) Stained with AB as in (VIII).
5) Dehydrated and mounted in Canada balsam.

Expected result: Negative result indicates presence of sialic acid.

XIII. Aldehyde Fuchsin (AF): (Gomori, 1950, Pg 665):

1) Sections were deparaffinised and hydrated through alcohol grades up to water.
2) They were rinsed in 70% alcohol.
3) They were stained for 30 minutes in aldehyde fuchsin solution (Halmi's). (Dissolved 0.5 ml of basic fuchsin in 100 ml of 70% alcohol. To this 1.5 ml of Conc. HCl and 1 ml of paraldehyde was added. It was allowed to ripen for 48 hours at room temperature and stored at 4°C).
4) They were rinsed in 70% alcohol.
5) They were quickly dehydrated in 90% and absolute alcohol.
6) Finally, they were cleared in xylene and mounted in Canada balsam.

**Expected result**: Sulphated mucosubstances stain strongly. Non-sulphated acidic mucosubstances stain weakly.

**XIV. Azure A**: (Spicer, 1967, Pg 295):

1) Sections were deparaffinised and brought down to distilled water through different grades of alcohol.
2) They were then stained for 30 minutes with 0.002% Azure A in buffer, at various pH (1.5, 2.0, 3.0, 4.0 and 4.5).

**pH 1.5 = 0.002% Azure in buffer**
(30 ml of N/10 HCl + 20 ml of M/10 KH₂PO₄)

**pH 2.0 = 0.002% Azure in buffer**
(20 ml of N/10 HCl + 30 ml of M/10 KH₂PO₄)

**pH 3.0 = 48 ml of 0.002% Azure in distilled water**
1.65 ml of M/10 citric acid + 0.35 ml of M/5 Na₂HPO₄.

**pH 4.0 = 48 ml of 0.002% Azure in distilled water**
1.25 ml of M/10 citric acid + 0.75 ml of M/5 Na₂HPO₄.
pH 4.5 = 48 ml of 0.002% Azure in distilled water +
1.10 ml of M/10 citric acid + 0.90 ml of M/5 Na₂HPO₄.

3) They were rinsed in distilled water.
4) They were dehydrated in graded alcohol, cleared in xylene
    and mounted in Canada balsam.

Sections were examined for wet metachromasia, before
dehydrating through alcohols for final mounting.

Expected result: Below pH 2.0 only sulphated mucosubstances
retain the dye in dehydrated sections. Above pH 3.0 mostly
sialomucins stain metachromatically.

XV. Pepsin digestion: (Pearse, 1972, Pg 1377):

1) Sections were hydrated through alcohol grades after
deparaffinisation in xylene.
2) They were treated in 0.2% pepsin (1:300 B.P. No.8514
    Constantine Favria, Italy) in 0.02 N HCl pH 1.6 for three
    hours at 37°C.
3) They were washed in running tap water.
4) They were then stained for PAS as in (1) (step 2 to 7)
    and in Azure A at pH 3.0 and pH 4.5 as in (XIV).
Expected result: Positive results indicates presence of mucosubstances.
OBSERVATIONS

Carbohydrates

1. PAS - The control and treated slides were positive towards PAS staining.

Control - The follicle wall or membrane and the wall of the duct stained magenta. The cytoplasm of the syncytial capsule stained a lighter shade of magenta. Plate VI - Fig.1.

Day 5 - There was an increase in the intensity of the staining. All the structures of the follicle staining deeply. Plate VI - Fig.2.

Day 10 - Very weak staining was observed. The follicle membrane stained light pink. The cytoplasm staining more lighter than the membrane. Plate VI - Fig.3.

Day 15 - The follicle membrane and the cytoplasm showed further decrease in the intensity of the staining. The follicle membrane stained very lightly. Plate VI - Fig.4.

Day 20 - The follicle membrane and the syncytial capsule stained dark pink indicating an increase in the PAS positive material. Plate VI - Fig.5.
Day 25 - The follicle membrane and the wall of the duct showed a further increase in the staining. The cytoplasm staining a darker shade of pink. Plate VI - Fig.6.

Day 30 - The follicle membrane and the cytoplasm of the syncytial capsule stained magenta. Indicating a further increase in the PAS positive material. Plate VI - Fig.7.

Day 35 - The follicle membrane, the duct and the cytoplasm of the capsule stained very deeply. The intensity of the magenta colour was much more than that of the control slide. Most of the follicle structures were obscured due to this deep staining. Plate VI - Fig.8.

II. Acetylation

Slides were subjected to acetylation to ascertain the nature of the PAS positive material. Negative results were obtained.

III. Deacetylation

Slides were also subjected to deacetylation to see the nature of the PAS positive material. Positive results were obtained.

The negative results obtained from acetylation and positive results obtained from deacetylation concludes the presence of is a polysaccharide material.
IV. Diastase digestion

Materials subjected to malt diastase digestion reduced the intensity of the PAS staining, otherwise the picture remained the same as in PAS alone. This established the presence of glycogen and some PAS positive material other than glycogen.

V. Direct Schiff's

Sections were treated with Schiff's reagent without oxidation with periodic acid. The results were negative. This was done to see if the staining was due to naturally occurring aldehydes.

VI. Plasmal reaction

Sections subjected to plasmal reaction also gave negative results.

VII. Pepsin digestion

Material was PAS positive, but the intensity of the staining was not as intense as that of the PAS staining.

VIII. Pyridine extraction

Slides were subjected to lipid extraction for twenty four hours at 60°C. The staining was less than the original PAS staining showing the presence of lipids and PAS positive material.
IX. Alcian Blue (AB)

The control and the treated slides of the blood gland follicles were positive towards AB staining. The staining was cytoplasmic and nuclear. The nuclei staining very faintly.

Control - The follicle membrane stained bluish green. The syncytial capsule of the follicle stained light blue. The nuclei also staining very lightly. Plate VII - Fig.1.

Day 5 - The follicle membrane, the wall of the duct stained a deep bluish green. The syncytial cytoplasm stained light blue. The overall staining was much more than the control slide. Plate VII - Fig.2.

Day 10 - The generalised staining of the follicles was light blue. Only the follicle membrane and wall of the duct showed a blue stain. Plate VII - Fig.3.

Day 15 - There was a further drop in the AB staining. The follicles staining very light blue, indicating loss of AB positive material. Plate VII - Fig.4.

Day 20 - Most of the follicles again showed the appearance of AB positive material. The follicle membrane and the syncytial protoplasm staining light bluish green. Plate VII - Fig.5.
Day 25 - There was an increase in the intensity of AB staining, all the structures of the follicles showing a darker bluish green stain. Plate VII - Fig.6.

Day 30 - The wall of duct and the membrane of the follicle staining a deeper bluish green. The syncytial capsule showed light coloured nuclei in the cytoplasm. Plate VII - Fig.7.

Day 35 - The follicle membrane, the duct and the syncytial capsule all stained deeply. The intensity of AB staining was the greatest here, much more than the staining of the control slide. All the structures showed bluish green stain. Plate VII - Fig.8.

X. Methylation

Control and treated slides were subjected to both mild and active methylation.

Mild methylation at 37°C had no effect on the AB staining of the follicles. But active methylation at 60°C for four hours totally abolished the alcianophilia.

XI. Demethylation

This restored the AB staining.
XII. Acid Hydrolysis (AH)

Both the control and treated slides were subjected to acid hydrolysis test at 60°C. Acid hydrolysis completely abolished the AB staining in the blood gland follicles, indicating the presence of sialomucin.

XIII. AB-PAS

The control and the treated slides were positive toward AB and PAS. The PAS staining was more prominent than the AB staining.

Control - The follicle membrane, the wall of the duct and developing follicles stained purplish blue. Indicating the presence of both AB and PAS material. The cytoplasm of the syncytial capsule and the nephridia stained pink.

Plate VII - Fig.1.

Day 5 - The follicle membrane and the wall of the duct stained a deeper purplish blue. The cytoplasm stained a darker pink or magenta.

Plate VII - Fig.2.

Day 10 - Most of the follicle structure, the membrane, the wall of the duct stained light purple. The cytoplasm of the syncytial capsule stained very light pink. The general pattern of staining was weak.

Plate VII - Fig.3.
Day 15 - There was a great decrease in the AB as well as the PAS staining. Very faint outline of the follicles and the nephridia were seen. Plate VIII - Fig.4.

Day 20 - There was a slight increase in the staining. Both the AB and PAS staining seen in membrane of the follicle, giving a light purplish colour. The cytoplasm of the capsule staining pink. Plate VIII - Fig.5.

Day 25 - There was a further increase in the staining. The membrane of the follicle and the wall of the duct staining purplish blue. The cytoplasm of the syncytial capsule stained pink. Plate VIII - Fig.6.

Day 30 - All the above structures stained more deeper than the day 25 slide. The magenta stain was seen in the cytoplasm of the capsule. The lumen of the capsule stained light pink. The membranes stained purplish blue. Plate VIII - Fig.7.

Day 35 - The membrane of the follicle and the wall of the duct stained dark purplish. The cytoplasm of the capsule stained magenta. Nephridia also stained magenta, while the membrane of the nephridia staining purplish blue. Plate VIII - Fig.8.
**XIV Azure A**

At lower pH of 1.5 there was no metachromatic staining, but metachromasia was seen at a pH of 4.5. Nuclear as well as cytoplasmic staining was observed.

Control - The membrane of the follicle the nuclei and the granules in the cytoplasm stained navy blue. The cytoplasm stained light blue.  
Plate IX - Fig.1.

Day 5 - The intensity of the navy blue stain in the follicle membrane and the duct was further increased. The syncytial capsule stained light blue, while the nuclei and granules stained dark blue. The lumen of the capsule stained very light blue.  
Plate IX - Fig.2.

Day 10 - The follicle membrane, the wall of the duct of the membrane of the nephridia all stained navy blue. Few granules and nuclei in the syncytial capsule stained purplish blue.  
Plate IX - Fig.3.

Day 15 - The follicle membrane and the wall of the duct stained lighter navy blue. The syncytial capsule stained light blue. Nuclear fragments seen in the cytoplasm stained navy blue. Very few granules were seen in the cytoplasm.  
Plate IX - Fig.4.
Day 20 - The follicle membrane stained navy blue. The syncytial capsule showed quite a few normal sized nuclei. The cytoplasm stained lighter shade of blue. Granules in the cytoplasm stained navy blue. Plate IX - Fig.5.

Day 25 - The syncytial capsule of the follicle show many blue stained nuclei in the cytoplasm. The nuclei appear to have regained their normal shape. Plate IX - Fig.6.

Day 30 - The follicle membrane, stained blue. The nuclei and granules in the light blue coloured cytoplasm stained blue. Plate IX - Fig.7.

Day 35 - There was an increase in the navy blue staining of the membrane, the wall of the duct and the numerous nuclei. The syncytial cytoplasm of the capsule stained darker shade of blue. The overall staining was much more than the control slide. Also most of the follicle structure regained their normal configuration. Plate IX - Fig.8.

Xv. Aldehyde Fuchsin (AF)

The slides did not stain with AF.
DISCUSSION : CARBOHYDRATES

In the blood glands, it is observed that PAS staining is present in all the regions of the gland indicated by a magenta coloration. It is well known that the PAS reaction is not solely due to the carbohydrates and it may yield coloration with other chemicals substances too, which yield aldehydes. Since coloration of aldehydes is produced as a result of periodic acid oxidation of glycols, amino alcohols and alkyl amino alcohols. These form the basis of the PAS reaction. The PAS reaction may also be due to the presence of carbohydrates, lipids and proteins.

Proteins :

Hotchkiss (1948) interpreted that polysaccharides combined with proteins would give a positive PAS reaction. Glegg et al. (1952) believed that the presence of serine and threonine at the end of the protein chain or hydroxy lysine at any position in the chain would give a PAS positive reactions to proteins due to the exposed α-amino alcohol groups. They however pointed out that hydroxy lysine was seldom found in proteins, while serine and threonine are infrequently found at the chain end and hence the staining is not due to proteins (Hotchkiss, 1948; Seamen, 1958). Nicolet and Shinn (1939) showed that a number of amino acids could be
converted to aldehydes by periodic acid through the oxidation of their \( \alpha \)-amino alcohol groups.

Lipids:

Lipids consist of many complex compounds. The lipids that can give a positive PAS reaction are unsaturated lipids, glycolipids and spingomyelins. Wolman (1950) reported a positive reaction with unsaturated lipids due to the oxidation of ethylene linkage to aldehydes. Later, he stated (Wolman, 1956) that unsaturated lipids could only be stained in frozen sections and they are lost during paraffin embedding. Lecithins was reported to give a PAS positive reaction only in fresh tissue (Chu, 1950). But the staining vanished if the tissues were treated with alcohol before or after staining. Spingomyelins are reported to be PAS positive by Wolman (1950) and Hale (1957). Diezel's (1954) findings contradicted the work of Wolman. Wolman (1956) later concluded that these substances would react positively with PAS on prolonged oxidation in a strongly acidic solution and that 1% periodic acid for 10 minutes would have no effect on their oxidation. In the present work, therefore, the PAS staining cannot be due to these compounds.

The best example of glycolipids are the cerebrosides and gangliosides found mainly in the neural tissue and ganglion cells. Due to the presence of glucose or galactose in them,
they are PAS positive (Hale, 1957; Casselman, 1959; Barka and Anderson, 1963). According to Hotchkiss (1948) cerebrosides would no longer be present if 70% alcoholic periodic acid solution is used for tissues fixed in an aqueous fixative. Leblond et al. (1957) could assign no significant role to lipids in the PAS reaction. Kuroki (1959) obtained a weak reaction with PAS staining of glycolipids fixed in aqueous formalin.

Evidence on the production of aldehydes from lipids and phospholipids by periodic acid oxidation of tissues sections cannot yet be considered conclusive (Seaman, 1958). Hack (cited by Seaman, 1958) showed that the following lipid substance in vitro react with Schiff's reagent after periodic acid oxidation of namely kerasin phrenosin (cerebrosides), phosphatidylethanolamines and gangliosides. But in paraffin sections, they are either no longer present or present in very small quantities that do not react (Pearse, 1968; pg 398).

In the present work, the fixed tissues during the process of paraffin embedding were kept overnight in 70% alcohol and in a saturated solution of paraffin in xylene, in tact an interval of four days elapsed from fixation to block making. In this condition, hardly any lipid could be expected to be present so as to react positively with PAS reaction.
However, lipid extraction methods were performed on the tissue sections to ensure that lipids could not be responsible for the PAS staining. Extraction of dehydrated tissue sections, instead of fresh tissue have been carried out by many investigators (Dixon and Herbertson, 1950; Schiebler, 1952; Seaman, 1958). In the present study, no reduction of PAS staining intensity was observed after extraction. La Bella (1958) showed that some lipids "pseudoplasmalogen" or native aldehydes as they are termed, resisted lipid extraction procedures and were positive to the PAS reaction. But they were also equally positive with Schiff's reagent after pretreatment with mercuric chloride. Therefore pretreatment with mercuric chloride and also with hydrochloric acid (Danielle, 1949) were performed but, yielded negative results, thus indicating that the extraction resistant materials could not be due to pseudoplasmalogen.

From all the above, it can be concluded that the PAS staining is not due to proteins, lipids or free aldehydes. It could be due to carbohydrates polysaccharides, mucopolysaccharides, mucoproteins and glycoproteins.

Carbohydrates:

Glycogen is the only naturally occurring polysaccharides in tissues which survives aqueous fixation and paraffin embedding (Pearse, 1968, Pg 70). Its presence is established
if PAS staining is abolished by digestion with diastase (Lillie and Greco, 1947) or with saliva (Bensely, 1939 and Bauer, 1963). It is true that these enzymes can abolish staining due to substances other than glycogen, but complete removal of glycogen after treatment with diastase has not been recorded (Pearse, 1960, Pg 266). Beckett and Bourne (1958) found that glycogen could be diastase resistant and attributed this resistance to the presence of protein around the glycogen particles. A confirmation of the presence of glycogen could be said by the fact that glycogen would require the full period for acetylation, that is, 24 hours at room temperature or 5 hours at 60°C (Lillie, 1954). The other confirmation is that of sulphation, using concentrated sulphuric acid which causes the hydrolysis of glycogen and thus no metachromasia results (Kramer and Windrum, 1954 and Lewis and Grillo, 1959).

Hence all the diastase resistant substance in the present work would therefore contain mucopolysaccharides, mucoproteins and glycoproteins.

The mucopolysaccharides are of two types:

1. Neutral,
2. Acidic.

The neutral mucopolysaccharides (NMPS) are always PAS
positive, but PAS positive staining of acid mucopolysaccharides (AMPS) is controversial. The AMPS have been reported by Gersh (1947); Hotchkiss (1948); Jorpes et al. (1948); McManus (1948); Arzac (1950); Wislocki (1950); Pearse (1952a) to be PAS positive.

There are other workers who have reported AMPS to be PAS negative (Meyer and Felling, 1950). Jeanioz and Forchielli (1951); Glegg et al. (1952, 1952b); Davies (1952) and Hale (1953). It has been shown that sites of high acid content were also strongly PAS positive (Harter, 1948; Lillie, 1950; and Montango et al., 1952), and yet the metachromatic staining could be removed by hyaluronidase without affecting the PAS coloration (Harter, 1948; Bunting, 1950 and Braden, 1952). It has therefore been suggested that AMP staining is associated with nonmetachromatic PAS positive substance (Davies, 1952 and Braden, 1955). The PAS positive substance might be NMPS.

According to Hoogwinkel and Smits (1957) hexuronic acid reacts with periodic acid but without yielding aldehyde groups, hence, will show no PAS staining. Similarly, chondroitin sulphate and hyaluronic acid are also PAS negative. Heparin in its fully sulphated form does not react with periodic acid thus it is PAS negative, but the least sulphated groups may give a PAS positive staining due to
adjacent hydroxyl groups, (Jorpes and Gardell, 1948; Lillie, 1950). According to Leblond (1950) heparin contains sulphated groups which would block aldehyde groups. The assumption that it may contain aldehyde groups is based on the staining of certain mast cells, however most mast cells are PAS negative. According to Leblond et al. (1957) they found that in their fraction (I) all acid mucopolysaccharides in the tissue extracts to be PAS negative. Fraction (II) which contain hexoses, fructoses, hexosamines and sialic acid are strongly PAS positive. They believed that once glycogen has been removed, only one class of materials gave a positive PAS reaction, these they referred to as carbohydrate protein complex. Pearse (1968, Pg 312) earlier assumed that the AMPS were capable of giving a positive, though weak reaction with PAS. Later, he ruled out any reaction with PAS.

Spicer (1963) observed that weakly acidic sulphated mucopolysaccharides (MPS) stain intensely with PAS. But these substances can be distinguished from the PAS positive NMPS by the fact that they stain purple in the AB-PAS procedure, show β-metachromasia with Azure A at pH 3.

Quintarelli (1963) stated that sialic acid can also be stained by the PAS technique. In the AB-PAS procedure, the sialomucins stain purple but the colour changes to red after
mild acid hydrolysis, which is a procedure for the removal of sialic acid.

Thus, where as the PAS staining indicates the presence chiefly of NMPS, it may also include such AMPS as sialomucins or weakly acidic sulphated mucin. The neutral ones cannot be stained by any of the basic stains or by any of the special stains, example AB is usually meant primarily for AMPS. Hence in the present study wherever in the AB-PAS procedure, the staining is given as if by PAS alone. Then such PAS staining if diastase resistant can be assumed to be due to carbohydrate protein complexes. If the PAS staining is diastase susceptible, it is assumed to be due to glycogen.

The entire blood gland was positive towards PAS staining. The control and the day 5 slides showed intense PAS positive staining, while the day 10, day 15 and day 20 experimental slides were still PAS positive but to a lesser degree, showing loss of PAS positive material (NMPS). From the day 25 onwards to day 35, the slides again showed intense PAS positive staining. This may be attributed to the maturation of the developing follicles or development of new follicles to replace the destroyed follicles (during the toxic effect of the herbicide Glyphosate from the day 5 to day 20). The intense PAS positive staining seen in the day 25 to day 35 slides, may show the recovery of PAS positive
material from the new developing follicles. This PAS positive staining is not due to glycogen, as sections treated with diastase showed diastase resistance staining of PAS. Absence of glycogen is further confirmed by acetylation and the re-establishment of stain after deacetylation. When acetylation was carried out for only 45 minutes there was a complete blocking of PAS positive staining. However, if glycogen was present, it would require the full period for acetylation, that is 24 hours at room temperature (Lillie, 1965).

In the earthworm, the blood gland is attached to the pharynx in the dorsolateral position within the folds of the pharynx and bathed in the body fluid. Though the follicles are loosely bound in a connective tissue membrane and the presence of PAS positive material may serve, to hold the cells in position and protect them from movements of the pharynx. According to Bradfield (1950), polysaccharides in significant amount confer a greater degree of plasticity on the connective tissue. Spicer et al. (1967) suggest the function of lubrication and protection due to neutral mucopolysaccharides.

Thus the PAS reaction in the blood gland could be said to be due to neutral mucopolysaccharides and sialomucins. This is further established by the fact that complete blocking of PAS positive material needs only a very short
duration of acetylation. There is a complete re-estab.
of PAS staining in the entire gland after deacetylation.
saponification. This indicates presence of hydroxyl groups.

At least three possible functions could be assigned to neutral polysaccharides in the gland:
1. They may function as reserve nutritive material.
2. They may provide a cytoskeleton for alkaline phosphatase (Schmidt and Thanhauser, 1943, Bern, 1951).
3. They may assist in the elaboration and discharge of secretory material.

In addition to NMPS, the gland also has AMPS as demonstrated by the positive staining towards Alcian blue (AB) (Steedman, 1950; Mowry, 1956). The control and day 5 slide showed intense AB positive staining showing the presence of AMPS. The day 10 to day 15 slides showed a decrease in AB staining. This may be the fact that AMPS material must be lost due to the destruction of the blood follicles which have been subjected to toxic effects of Glyphosate. The recovery of the AB staining in the experimental slides of day 20 to day 35 may be the rejuvenation of destroyed follicles or the maturation of developing follicles which would mean abundant amount of AMPS, hence the intense AB staining. The day 35 slide showed much more intense staining than the control slide.
Sulphated MPS are however absent as shown by the following co-related results. The cells of the blood glands were orthochromotic towards Azure A at low pH levels (Dempsely et al. 1947; Lillie, 1949; Hamerman and Schubert 1953; Kramer and Windrum, 1954). They were not stained by the aldehyde fuchsin (AF) procedure (Comori, 1950; Abul-Haj and Rinehart, 1952; Halmi and Davies, 1953; Scott and Clayton, 1953; Spicer and Meyer, 1960). Even as masked groups sulphated MPS are absent, because digestion with pepsin does not produce metachromasia (French and Benditt, 1953; Kelly, 1955; Morris and Krikos, 1958).

The AB staining was abolished by methylation (Fraenkel-Conrat and Olcott, 1945; Fisher and Lillie, 1954; Spicer, 1960) suggesting a process of desulphation and hence the presence of sulphate groups (Kantor and Shubert, 1957). This could not possibly be the case, as the above direct staining results have clearly indicated the absence of these groups. Lillie (1958) suggested the restoration of staining after saponification due to the presence of carboxyl groups. These groups are esterified by methylation and hence fail to stain, they are de-esterified by saponification and hence get restained. In the present case, the existence of hyaluronic acid, a substance rich in carboxyl group is thus ruled out.
Since neither sulphomucin nor hyaluronic acid is present, it is possible that MPS present is of sialomucin type that is a mucin containing sialic acid. It was shown that some sites with histochemically proven sialomucin apparently contained no uronic acids or sulphate esters, since they lacked basophilia after the removal of sialic acid. Spicer (1960, 1963) noted that mild methylation of certain epithelial mucins readily blocks that AB basophillia, while demethylation always restores it. Warren and Spicer (1961) substantiated these results and confirmed that mild methylation helps to distinguish sialomucins from other acid mucins such as chondroitin sulphates or hyaluronic acid. But Quintarelli (1963) observed that mild methylation failed to destroy basophilia in dog, sheep and pig submaxillary gland, while demethylation further suppressed tissue basophilia instead of restoring it, as expected. This was not the case in the present work. Again Spicer and Warren (1960) showed that metachromasia was produced in the mouse sublingual and laryngo-tracheal glands by sialic acid containing glycoproteins. But Quintarelli et al. (1961a, 1961b) and Quintarelli (1963) also noted that in a number of salivary glands that they studied, there was a complete lack of metachromasia even though these glands contained sialomucin. This is not the case in the present study, for staining with Azure A showed metachromasia. The procedure included the staining of control and experimental slides with Azure A at
various pHs. Azure A at pH 2 and 2.5 hardly showed any staining but the slides were positive at pH 3, 3.5, 4.0 and 4.5. As there is a positive metachromatic staining at pH 3.0 to pH 4.5 one may conclude that staining is due to sialomucin.

According to Gottachalk (1957) sialic acid could be easily split off from the rest of the mucin molecule by heating the mucin in a faintly acidic solution. Quintarelli et al. (1961b) applied this mild acid hydrolysis treatment to tissue sections and noticed that AB staining was abolished. Sialic acid was successfully split off and mild acid hydrolysis became an important histochemical procedure for the demonstration of sialic acid. Later, Quintarelli (1963) noted the loss of basophilia from sections due to acid hydrolysis and methylation was the same to some extent. He concluded that at 60°C, acid methanol also had hydrolysing effect on sialic acid. Hence acid hydrolysis was performed in the present work to establish the presence of sialomucin.

The various above observations reveal that the blood gland of the earthworm mainly consists of acid mucopolysaccharides in all regions. It is known that AMPS play an important role in protein synthesis. Immer (1961) stated that there is a correlation between the metabolism of
proteins and that of AMPS. Quintarelli (1963) noted that, sites where AMPS are present are also the sites of protein production.

Sialic acid in the blood gland of the treated worms also show a rise, a fall and a rise again, which is in agreement with the work of Ruth et al. (1957). She shows here that the cells involved in antibody production contain large amounts of carbohydrates. These carbohydrates are resistant to diastase digestion and are not extracted with organic solvents.

According to Matsuuchi, et al. (1981) sialic acid plays an important role in glycosylation of immunoglobulins. The glycosylation pattern of immunoglobulins can affect their binding specificities and influence the antigen-antibody interactions. This may be the case in the blood gland of the earthworm. For, it has been shown, that haemocytes in Annelids (Roett et al., 1956) are one of the immunological types. Fearon (1978); Hirsch et al. (1981) and Edwards et al. (1982) have shown that sialic acid plays an important part in regulating the complement 'C' reactions.

The presence of sialic acid in the follicles of the blood gland of the earthworm shown by subsequent staining
methods is a sure indicator of how the worm tries to combat the toxic effects of Glyphosate. Patel et al. (1992) working on rats exposed to lead found that there was a great increase in sialic acid in the serum of the rats, he also found an increase in sialoglyco conjugates in the brain, liver and the blood serum of rats exposed to lead. Also in this work on the blood glands, a similar increase in sialic acid was found.

The proliferation of new cells from the syncytial capsule into the lumen of the capsule, their intense staining after the recovery from toxic effects, shows that these glands may function as an immunological organ. Despont et al. (1975) have shown that sialic acid content and sialytransferase are indicator of T-cell maturation. The thymocytes contain lowest amount of sialic acid whereas T-cells and B-cells contain higher amount. This may be the case in the blood glands also.
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