Manual of Histological Techniques

Salient Features

- Short and concise manual of different histological techniques and histological stains
- Apart from conventional histological techniques, this manual also includes modern histological techniques such as immunohistochemistry, cell block, immunofluorescence, in situ hybridization, fluorescent in situ hybridization (FISH) and molecular diagnostic procedures
- Written in a simple and lucid language for better understanding of the students
- Useful for students of MD (Pathology), DCP (Diploma in Clinical Pathology), BSc. BMLT and DMLT technicians as well as specialist/consultants, technicians and others working in histological laboratories.

Santosh Kumar Mondal passed MBBS from North Bengal Medical College, West Bengal, India, in 1994 and MD in Pathology from Institute of Postgraduate Medical Education and Research (ISKM Hospital), West Bengal, India, in 2000. He joined West Bengal Medical Education Service in 2003 as Demonstrator in Pathology and worked in different capacities since then. Presently, he is Associate Professor, Department of Pathology, Bankura Sammilani Medical College, West Bengal since January, 2011. He got post MD training on Oncopathology at Tata Memorial Hospital, Mumbai, Maharashtra, India, for 6 months. He acted as both external and internal examiner for different examinations such as MBBS; DMLT, etc. He has published 65 articles in different indexed national and international journals. Besides, he has been selected as reviewer of various reputed national and international journals such as Diagnostic Cytopathology.
Manual of
Histological Techniques

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Dedicated to

My parents,
Mr Nitai Chandra Mondal
and
Ms Jyotsna Mondal
The relentless progresses of medical technology have brought into its wake novel methods of investigations and treatments, rendering many older methods obsolete. Pathological investigations remain cornerstone of medical diagnosis. Histotechnology plays a pivotal role to make a diagnosis of surgical specimens.

With the advent of newer diagnostic techniques like molecular diagnostic methods, a quick but very reliable diagnosis is possible, even from small amount of tissue. In this manual, I have included the molecular techniques, immunohistochemistry, cell blocks, and immunofluorescence along with the conventional techniques. For students’ easy understanding; many figures, charts, diagrams and tables have been included. At the same time, the volume of the book has been restricted; so that students do not become overburdened during preparation of examination. Specialists and consultants who are working in a surgical pathology laboratory will also find it useful.

I am indebted to my parents Mr Nitai Chandra Mondal and Smt Jyotsna Mondal for their value-based guidance, blessings and constant support that I have received throughout my life. I thank my son Soumyadeep, brother Monotosh and wife Shampa, for their continuous encouragement during preparation of this manual. I also thank postgraduates of pathology (MD, PGT), especially Dr Saikat Mandal and Dr Debasish Bhattacharya, for their help during preparation of this book. I am thankful to my friend Dr Sanjib Pattari, for supplying a few special stained slides.

Despite my best efforts, some mistakes might have crept in. So, I request all readers to kindly bring it to my notice. Your constructive criticism, appreciation and suggestions are most welcome.

Santosh Kumar Mondal
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Figs 2A and B: PAS positive materials (mucin) are stained as magenta/bright red: (A) PAS: Appendix, low power; (B) PAS: Appendix, high power (Chapter 6)

Figs 3A and B: Alcian blue, intestine, low and high power. Alcian blue stains acid mucins as blue (Chapter 6)
INTRODUCTION

Carbohydrates are compounds of carbon, hydrogen and oxygen, the latter two usually in the proportion of water. The most important carbohydrates are sugar, starches, cellulose and gums. These are classified as mono-, di-, tri- and heterosaccharide. The word “carbohydrate” actually is descriptive of the 1:1 ratio of carbon molecules to water (hydrate).

In order to understand different types of carbohydrates and their location in different parts of the body, let us know the classification of carbohydrates based on chemical nature:

- **Group I: Polysaccharides**, e.g. glycogen.
- **Group II: Mucopolysaccharides**:
  - Simple or sulfate free, e.g. hyaluronic acid in synovial fluid or umbilical cord.
  - Complex or sulfate containing, e.g. chondroitin sulfate, mucosulfate of gastric mucin, corpora amylacea, and mast cell granules.
- **Group III: Mucoproteins and glycoproteins**: These are protein-carbohydrate compounds having high protein or peptide within it.
  - Mucoprotein without sialic acid, e.g. serum mucoprotein, submaxillary and Brunner’s gland mucin, beta granules of anterior pituitary.
  - Mucoprotein containing sialic acid (N-acetylneuraminic acid), e.g. sialomucin or carboxylated mucin.
- **Group IV: Glycolipids**: These are carbohydrate lipid compounds and have fat residue within it, e.g. cerebrosides.
- **Group V: Phosphatides or Phospholipids**, e.g. lecithin, cephalin and sphingomyelin.

Mucins are hexosamine containing polysaccharides covalently bound to varying amounts of proteins. The original term of "mucin" was coined by an American worker named Carpenter in 1846. Subsequently different names of mucin follow, i.e. mucosubstances, mucopolysaccharides and glycosaminoglycans. Later on, Reid and Clamp in 1978 suggested a general term “glycoconjugates”, which again subdivided into proteoglycans or glycoaminoglycans and glycoproteins. To avoid confusion of terminologies we prefer to use the term mucin. The synthesis of mucin starts in the rough endoplasmic reticulum in the synthesizing cells and complete in the Golgi apparatus. For staining different types of staining techniques are employed. These are PAS (periodic acid-Schiff), Alcian blue, mucicarmine, aldehyde fuchsin, high iron diamine, etc.

Mucins are high molecular weight glycoproteins and are commonly found in epithelium of gastrointestinal tract, respiratory tract and reproductive tract. Mucins are composed of a central protein core with multiple chains of carbohydrates (polysaccharide) attached. Carbohydrate component of mucin accounts for 60–80% of total molecular weight. The protein core of mucin contains high contents of two amino acids—serine and threonine.

But there are other glycoproteins which share structural similarities with mucin (which is a high molecular weight glycoprotein) and are often confused with mucins. Proteoglycans are high molecular weight glycoconjugate complexes. These are found in abundance in connective tissues and in extracellular matrix. In the past proteoglycans were frequently referred to as connective tissue mucins. However,
protein core of proteoglycon is different and distinct from that of mucins.

- Histochemical reactivity is largely dependent upon the carbohydrate component of mucin. Some carbohydrate molecules do not carry electric charge as they do not have ionizable groups under normal conditions (e.g. glucose, mannose and galactose). In contrast to these monosaccharides, other monosaccharides may contain acidic groups or ions such as carboxyl (COOH) and sulfonic (SO₃⁻) groups which are capable of ionization to confer an overall negative charge on the molecule. The carboxylated monosaccharide N-acetyneuraminic acid is commonly known as sialic acid. The presence of these ionizable groups determine the chemical reaction with dyes of the stains.

- From histochemical perspective, mucins can be grouped into acid mucins and neutral mucins based on the presence of ions in their carbohydrate components. The charged or so called “acid” mucins contain carboxylate (COO⁻) or sulfonate (SO₃⁻) ions (anions). Both of these ionizable groups are ionized (acid groups/anions) at a physiologic pH to produce an overall negative charge on these mucins. But carbohydrate component of neutral mucins lack acidic groups, and hence they do not carry no net charge (neutral). The acid mucins are found widely in the epithelium of gastrointestinal tract and respiratory tract. The neutral mucins can be found in the gastric glands, Brunner’s glands of duodenum and prostatic epithelium.

- The special stains of acid mucin usually contain cationic dyes molecules (positively charged) at a specific pH. This applies to stains like alcian blue, mucicarmine and metachromatic dyes (Azure A or toluidine blue). The cationic dye molecules bind via electrostatic forces to the anionic carboxylated or sulfated polysaccharide chains of the mucin molecules.

**Mucins have some common characteristics:**
- They are soluble in alkaline solutions.
- They stain intensely with basic dyes.
- They are precipitated by acetic acid excepting gastric mucin.
- They are metachromatic in many of the cases, so they turn into red or reddish blue when stained with toluidine blue or thionin.

From histochemical standpoint, mucins can be subdivided into following groups:

**Acidic Mucin**

*Strongly Sulfated Mucin*
- Epithelial mucin: Seen in bronchial serous glands, lesser extent in intestinal goblet cells.

*Weakly Sulfated Mucin or Sulfomucin*
- Epithelial mucin: Colonic goblet cells
- Atypical mucin: They are not stained by usual mucin stains (e.g. PAS) but stained by alcian blue, e.g. tracheobronchial mucous glands.

*Carboxylated Sialomucin*
- Enzyme-labile: They are digested by enzyme sialidase, hence called labile. Examples are submandibular salivary glands, bronchial submucous glands, and goblet cells of small intestine.
- Enzyme resistant: These are resistant to denaturation by the enzyme sialidase. Also, they are PAS-negative unlike enzyme labile mucins. Examples are mucosal glands of large intestine, lesser extent in stomach and bronchus.

*Carboxylated Nonsulfated Uronic Acid Mucin*

Hyaluronic acid: Composed of N-acetyl-D-glucosamine and D-glucuronic acid. These are widely distributed connective tissue mucin and are also found in synovial fluid, synovial membrane, umbilical cord and early placenta (surface of placental syncytiotrophoblasts).

*Sulfated Sialomucin*

These mucins are a mixture of sulfomucins and sialomucins. Found in synovial sarcoma and prostatic carcinoma.

**Neutral Mucin**

As there is no acidic group or ion (carboxyl or sulfonic group), they are neutral. They are composed of different hexosamines. Mostly they are epithelial in origin, e.g. stomach, prostate, and Brunner’s gland of duodenum.

There are many types of carbohydrates (as we see in the above classification) of which glycogen and mucins are most important. Now let us know about different types of carbohydrates which may be present in our body. This will enable us to understand role of different types of carbohydrates in many diseases including cancer (Table 1).
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Table 1: Different types of carbohydrates in normal and abnormal conditions of body

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<td>1.</td>
<td>Glycogen</td>
<td>Liver, hair follicles, voluntary muscles, endometrial glands, mesothelial cells, megakaryocyte and umbilical cord</td>
</tr>
<tr>
<td>2.</td>
<td>Neutral mucin</td>
<td>Stomach, prostate, Brunner’s gland and duodenum</td>
</tr>
<tr>
<td>3.</td>
<td>Sialomucin (enzymelabile)</td>
<td>Submandibular salivary glands, bronchial submucosal glands, goblet cells of small intestine</td>
</tr>
<tr>
<td>4.</td>
<td>Sialomucin (enzyme-resistant)</td>
<td>Large intestine, lesser extent in stomach and bronchus</td>
</tr>
<tr>
<td>5.</td>
<td>Strongly sulfated epithelial mucin</td>
<td>Bronchial serous glands, lesser extent in stomach goblet cells</td>
</tr>
<tr>
<td>6.</td>
<td>Weakly sulfated epithelial mucin (sulphomucin)</td>
<td>Colonic goblet cells</td>
</tr>
<tr>
<td>7.</td>
<td>Sulfated sialomucin</td>
<td>Prostatic adenocarcinoma</td>
</tr>
<tr>
<td>8.</td>
<td>Hyaluronic acid</td>
<td>Synovial fluid/synovium, skin, umbilical cord and early placenta</td>
</tr>
<tr>
<td>9.</td>
<td>Keratan sulfate</td>
<td>Intervertebral disc and hyaline cartilage</td>
</tr>
<tr>
<td>10.</td>
<td>Heparin/Heparan sulfate</td>
<td>Mast cells and aorta respectively</td>
</tr>
<tr>
<td>11.</td>
<td>Chondroitin sulfate A</td>
<td>Hyaline cartilage</td>
</tr>
<tr>
<td>12.</td>
<td>Chondroitin sulfate B</td>
<td>Heart valves, skin</td>
</tr>
<tr>
<td>13.</td>
<td>Chondroitin sulfate C</td>
<td>Umbilical cord, skin</td>
</tr>
<tr>
<td>14.</td>
<td>Cellulose</td>
<td>May be present abnormally in gastro-intestinal tract and skin</td>
</tr>
<tr>
<td>15.</td>
<td>Chitin</td>
<td>Hydatid cysts of liver, lung and brain</td>
</tr>
</tbody>
</table>

Table 1: Different types of carbohydrates in normal and abnormal conditions of body

CHOICE OF STAINING METHOD

Mucin can be demonstrated in paraffin, frozen and celloidin sections. Though there are so many stains, Southgate’s mucicarmine was most popular in the past. Metachromatic stains are also very good as they also stain many types of carbohydrates.

Recently PAS is getting popularity which also stains most of the carbohydrates/mucin excepting sialomucin and strongly sulfated mucin. Alcian blue and aldehyde fuchsin are sometimes preferred because of ease in staining technique. High iron diamine staining is not used nowadays (Figs 1A to D and Table 2).

❖ **PAS stain**: Stains glycogen, neutral mucin and carbohydrate portions of glycoproteins and glycolipids. This technique is perhaps the most versatile and widely used mucin stain.

❖ **PAS stain with diastase pretreatment**: Glycogen is digested, not stained by PAS but stains glycoproteins, glycolipids and glycomucins.

❖ **Alcian blue (pH 2.5)**: Best stain for acid mucins (strongly sulfated) which is produced by mesenchymal cells.

❖ **Combined Alcian blue-PAS stains**: best general mucin stain and considered as “pan” mucin stain. This combination is also useful for studying inflammatory and metaplastic conditions of gastrointestinal tract. For example, intestinal metaplasia in stomach (differentiates goblet cells of intestine and gastric mucosa).

❖ **Mucicarmine stain**: It stains strongly sulfated or acid mucin, other acidic mucin, hyaluronic acid. But neutral mucins are negative or weakly positive. Mucicarmine is commonly used but relatively insensitive stain for epithelial mucin. Mucicarmine is one of the oldest techniques and is replaced by other sensitive techniques.

METACHROMASIA AND MUCIN

Ranvier, Cornil, Jurgens and several other scientists discovered metachromasia in 1875 by using several different dyes like dahlia and cyanine. But the term...
‘metachromasia’ was first used by Ackroyd in 1876. The dyes which show metachromasia exist in orthochromatic form (normal form). When they bind to certain substances (chromotropes), these dyes are converted to polymeric (metachromatic) form. This is because the negative charges of certain chromotropes attract many positively-charged polar groups on the metachromatic dyes to polymerise and polymeric (metachromatic) form is found.

Due to this polymerization, there is a shift of absorption towards the shorter wavelength of light. Normally, toluidine blue dye exists in the blue monomeric form and when they stain nonchromatropes, they polymerize and give purple to red color. Other metachromatic stains are thionin, Azure A, methylene blue, and few fluorochromes like acridine orange, etc.

The carbohydrates or mucins which contain acidic groups or negative charges (both sulfated and carboxylated mucins) show metachromasia with metachromatic stains/dyes. The neutral mucins, as they do not have acidic groups (carboxylate or sulfonate), do not show this property (Table 3).

### Carbohydrates

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Type of carbohydrate</th>
<th>PAS</th>
<th>AB at pH 2.5</th>
<th>AB at pH 0.2</th>
<th>Aldehyde Fuchsin</th>
<th>High iron diamine</th>
<th>Diastase digestion</th>
<th>Sialidase digestion</th>
<th>Metachromasia</th>
<th>Grocott–hexamine silver</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Glycogen</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2.</td>
<td>Neutral mucin</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>3.</td>
<td>Sialomucine (enzyme labile)</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>4.</td>
<td>Sialomucin (enzyme resistant)</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5.</td>
<td>Strongly sulfated mucin (epithelial)</td>
<td>–</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>6.</td>
<td>Weakly sulfated mucin (epithelial)</td>
<td>V</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>7.</td>
<td>Sulfated sialomucin</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>8.</td>
<td>Chitin</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>9.</td>
<td>Cellulose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Abbreviations: PAS, periodic acid-Schiff; AB, alcian blue; “+”, positive; “–”, negative; V, variable

### Choice of Fixative

Fixatives containing picric acid or alcohol are preferred for demonstration of glycogen, e.g. Bouin’s fluid or Rossman’s solution and 80% alcohol, the original method was done by Best in 1906 in celloidin embedding after alcohol fixation. It was thought that celloidin is essential to prevent diffusion of glycogen from the tissues. But later on Lillie (1947) and Vallance–Owen (1948) proved that glycogen is not lost in running water, at least for 24 hours if tissues are properly fixed.

#### GLYCOCEN

It is a simple polysaccharide which contains D-glucose units in branched or straight chains. Glycogen has two main forms—alpha and beta. It, in colloidal solution, is found in the cytoplasm of certain cells. Glycogen is derived from sugar and it breaks down into sugar within one hour of death. So tissue containing glycogen either should be fixed in fixatives or it should freeze as death of tissue results in breakdown into sugar.

### Table 2: Different types of carbohydrates and their staining pattern

### Table 3: Mucin expression in different malignancies/tumors

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Type of malignancy</th>
<th>Neutral mucin</th>
<th>Acid mucin (strongly sulfated)</th>
<th>Glycogen (PAS positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Gastrointestinal carcinoma</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>2.</td>
<td>Ovarian carcinoma</td>
<td>–/–</td>
<td>+/–</td>
<td>–/–</td>
</tr>
<tr>
<td>3.</td>
<td>Renal cell carcinoma</td>
<td>–/+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4.</td>
<td>Endometrial carcinoma</td>
<td>–/–</td>
<td>+/–</td>
<td>–/–</td>
</tr>
<tr>
<td>5.</td>
<td>Mucoepidermoid carcinoma</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>6.</td>
<td>Liposarcoma</td>
<td>––</td>
<td>+</td>
<td>––</td>
</tr>
<tr>
<td>7.</td>
<td>Rhabdomyosarcoma</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8.</td>
<td>Osteogenic sarcoma</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>9.</td>
<td>Smooth muscle tumors</td>
<td>–</td>
<td>+/–</td>
<td>–/–</td>
</tr>
<tr>
<td>10.</td>
<td>Neurogenic tumors</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>11.</td>
<td>Colloid carcinoma</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>12.</td>
<td>Seminoma</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>13.</td>
<td>Ewing’s sarcoma</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>
In routine practice, formol saline or other aqueous fixatives (e.g., formalin) give adequate result. But freeze drying should be used for histochemical studies. This freeze drying technique is superior to other methods in glycogen preservation and it almost preserves 100% glycogen. This method also prevents streaming of the intracytoplasmic glycogen to one pole of the cells (polarization).

Embedding Medium

Although celloidin was used as embedding medium in the original method by Best, paraffin wax is also equally good. But frozen sections are not suitable for glycogen demonstration.

USE OF ENZYMES

- **Diastase**: This is most commonly used enzyme for glycogen digestion, as it is cheap, stable and easy to use. It may be obtained from malt or saliva. Malt diastase is available commercially and is more reliable than saliva, but salivary diastase is routine as it is easily available. Disatase removes some other things (e.g., ribonucleic acid) besides glycogen, but as these things are not PAS positive, this does not pose a threat.

- **Amylase**: This can be alpha amylase or beta amylase. Alpha amylase is extracted from hog pancreas (also available in some organisms like *B. subtilis* and *Aspergillus oryzae*). Alpha amylase digests both branched and straight chains of glycogen. Beta amylase is derived from sweet potato or barley. It digests only straight chains of glycogen.

**PERIODIC ACID-SCHIFF REACTION AND STAIN**

Periodic acid is a very strong oxidizing agent and under the controlled condition of the staining reaction, it reacts with the aldehyde group of the carbohydrates. This periodic acid cleaves the carbon-carbon bond in amino or alkyl amino derivatives or 1.2-glycols to form aldehydes. These aldehydes will react with fuchsin-sulfurous acid and this product combines the basic pararo-saniline to give a positive result (magenta colored compound). This compound is chemically alkyl sulfate (Figs 2A and B).

Any substance that fulfills the following criteria will give a positive result during PAS reaction (Hotchkiss, 1948).
- The substance must not diffuse away during fixation
- It must produce an oxidation product which is not diffusible
- The substance must have the 1.2-glycol grouping or the equivalent amino or alkyl-amino derivative or the oxidation product CHOH-CO
- Sufficient concentration of the substance must be present in the tissue to give a positive reaction (magenta color).

**PAS Technique**

- **Periodic acid solution (0.5%)**
  - Periodic acid: 1 g
  - Distilled water: 200 mL
- **Schiff’s reagent**: Dissolve 1 g basic fuchsin in 200 mL of boiling distilled water. When dissolved, cool it to 50–60°C. Add 2 g of potassium metabisulfite and mix

**Figs 2A and B**: PAS positive materials (mucin) are stained as magenta/bright red: (A) PAS: Appendix, low power; (B) PAS: Appendix, high power (For color version, see Plate 2)
it. Bring the mixture to room temperature and then add 2 mL of hydrochloric acid and mix it. Also add 2 g of activated charcoal and this chemical solution is kept at room temperature in a dark place overnight. Next morning, filter it (Whatman paper no. 1). The ideal solution after filtration should be pale yellow or clear. Store this solution at 4°C in dark container.

**Staining Method**

1. Deparaffinize two test sections and two positive control sections.
2. Treat one test section and one positive control section in diastase solution for 1 hour at 37°C.
3. Wash the sections in running tap water for 5–10 minutes.
4. Now stain all the sections with the desired staining technique for glycogen (e.g., PAS, alcian blue, mucicarmine, etc.).

**Result**

Presence of glycogen will be confirmed by loss of staining of glycogen after enzyme treatment, but the untreated sections will give positive staining reaction.

**Alcian Blue Staining**

Alcian blue is a water soluble copper thalocyanin. Although the exact staining mechanism is not known, it is presumed that alcian blue stains by salt linkage to acidic groups. Common alcian dyes are alcian blue 8GX, alcian yellow and a mixture of these two known as alcian green 2 GX (staining an emerald green color) and alcian green 3 BX (staining a blue green color). It has a high molecular weight (>1300) and one of the largest amongst the commonly used histologic dyes.

It stains acid mucin specifically but not the neutral mucin. The intensity of stain depends upon the ionization of tissue component in a particular pH. It gives best with the alcian blue dyes when in a particular pH; the tissue component is fully iodized into acid groups. This property may be advantageous to identify, to separate the different acid mucins by using alcian blue solutions of varying pH. As for example sulfate esters reacts at a lower pH, compared to carboxylated.

In general, a pH 2.5 solution of alcian blue is satisfactory. The optimum pH for different mucins is given below:

- Strongly sulfated mucin: at lower pH (pH ≤1).
- Weakly sulfated mucin: pH 2.5–1.0.
- Hyaluronic acid and N-acetyl sialomucin: pH 3.2–1.7.
- N-acetyl-O-acetyl sialomucin: pH 1.5.

Though neutral mucin is not normally stained with alcian blue dyes, it can be done by different ways (Figs 3A and B). These include:

- By employing acid esterification using a periodic acid hydrochromic acid sequence.
- Treating with an ether-sulfuric acid mixture to introduce sulfate groups (SO₄⁻⁻).
Over oxidization of neutral mucin glycol groups to form aldehydes which subsequently produce carboxylic acid or acid groups needed for alcian dyes.

Preparation of Stain
- Alcian blue: 1 g
- 10% sulfuric acid yielding a pH of 0.2: 100 mL.
  - Or 0.2 M hydrochloric acid (yielding a pH of 0.5): 100 mL.
  - Or 0.1 M HCl (yielding pH of 1): 100 mL.
  - Or 3% acetic acid (yielding pH of 0.25): 100 mL.
  - Or 0.5% acetic acid (yielding pH of 3.2): 100 mL.
Mix it to prepare the alcian blue solution. This solution should be filtered before use. Old solutions lose staining power.

Staining Method
1. Deparaffinise the sections and bring to water.
2. Stain in alcian blue solution for 10–20 minutes.
3. Rinse in distilled water (or omit and blot dry if the pH of staining is critical).
4. Counterstain with 0.5% aqueous neutral red for 2–3 minutes.
5. Rinse in water.
6. Dehydrate rapidly in 95% and absolute alcohol.
7. Clear in xylol and mount in DPX or HSR resin.

Results
- Acid mucins (and most sulfated mucopolysaccharide): Blue.
- Nuclei: Red.
- Other tissue constituents: Red.

Notes:
- For general demonstration of acid mucins, alcian blue dissolved in 3% acetic acid (pH 2.5) is the solution of choice.
- Counterstain with a weak solution of neutral red (0.1–0.5%). Otherwise it will mask the alcian blue staining.
- Staining time will vary as per strength of solution used. As for example, if a 1% solution is used for 5 minutes, 0.1% of that solution needs more time say 30 minutes.

Combined Alcian Blue – PAS Technique
This technique separates acid mucins and neutral mucins. In this technique, firstly all acid mucins are stained with alcian blue but these stained acid mucins which are also PAS-positive would not react in the subsequent PAS stain. Only the neutral mucins will be stained with subsequent PAS stain.

Staining Method
1. Step 1–3 above, followed by steps 4–10 of PAS staining.

Results
- Acid mucin: Blue
- Neutral mucins: Magenta.
Points to Remember

- Avoid Ehrlich’s hematoxylin as a counterstain as it stains certain types of mucin and hampers final staining method.
- Stain lightly with counterstain otherwise it will be difficult to distinguish the staining color of hematoxylin and alcian blue (both give blue color).

SOUTHGATE’S MUCICARMINE METHOD
(MAYER, 1896; MODIFIED BY SOUTHGATE, 1927)

Southgate’s modification of Mayer’s original method (which did not contain aluminium hydroxide) gives more consistent results. This stain demonstrates both gastric and epithelial mucin well.

Composition of Staining Solution

- Carmine: 1 g
- Aluminium hydroxide: 1 g
- 50% alcohol: 100 mL
- These constituents are mixed by shaking and then add aluminium chloride (anhydrous): 0.5 g.

Preparation

Boil the above mixture in water – bath for 2.5–3 minutes. Cool, filter and store at 4°C.

Staining Method

1. Deparaffinize histologic sections and bring to water.
2. Stain the nuclei with conventional hematoxylin (but not with Ehrlich).
3. Differentiate in acid alcohol and blue in tap water.
4. Stain with above staining solution for 20–30 minutes.
5. Rinse in distilled water.
6. Dehydrate in 95% and absolute alcohol.
7. Clear in xylene and mount in Canada balsam or DPX.

Results

- Mucin: Red
- Nuclei: Blue.

Points to Remember

- It is also useful to stain encapsulated fungi, e.g. Cryptococcus neoformans.

Best Carmine Method (Best 1906)

Composition of Staining Solution

- Carmine stock solution:
  - Carmine: 2 g
  - Potassium carbonate: 1 g
  - Potassium chloride: 5 g
  - Distilled water: 60 mL
  - These reagents in a 250 mL flask should be gently boiled for 3–5 minutes until the color deepens. The deeper color will give deeper stain of glycogen. Cool the mixture and add 20 mL of concentrated ammonia. Filter it and store in a refrigerator at 4°C (0–5°C) or in a dark container at 4°C. Discard after 6–8 weeks.
- Carmine working solution:
  - Carmine stock solution: 12 mL
  - Fresh concentrated ammonia: 18 mL
  - Methyl alcohol: 18 mL

Fig. 4: Mucicarmine stains strongly sulfated or acid mucin as red (For color version, see Plate 3)
Best’s differentiating fluid:
- Absolute alcohol: 20 mL
- Methyl alcohol: 10 mL
- Distilled water: 25 mL.

Staining Method

1. Dewax the histologic sections and bring to water.
2. Place in alum hematoxylin (Harris or Ehrlich) for 10–15 minutes to stain nuclei.
3. Rinse rapidly in 1% acid alcohol for differentiation to make the background clear.
4. Wash in running tap water to remove alcohol and blueing.
5. Stain with carmine working solution for 10–15 minutes.
6. Wash slide with Best’s differentiating fluid for celloidine section. Use methyl alcohol for paraffin sections.
7. Flood with fresh alcohol or acetone.
8. Clear in xylol and mount in DPX or Canada balsam.

Results
- Glycogen: Deep red
- Nuclei: Blue
- Some mucin, fibrin: Weak red
- Nuclei: Deep red
- Some mucin, fibrin: Weak red