Acknowledgments

First and Foremost I want to pay my gratitude to most magnificent God who enlightens my heart with spiritual light to find the true path of life.

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I am greatly indebted to Prof. Irfan Ahmed, Chairman, Department of Zoology for providing me infrastructure support.

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Whatever we do in this world reflects the image of our parents. If today I’ll be able to achieve anything whether socially or scientifically, its whole credit solely goes to the blessings of my great parents and other family members particularly my Bajjo, Mrs. Rehana Kazmi who provided me rock-like support during my acute moments of crisis. For now Thanks everybody.
Fishes constitute a major vertebrate group and a predominant component of aquatic fauna. The body surface of all multicellular organisms including fishes is protected by epithelial lining which serves as a physical barrier between the internal milieu and the external environment. Integument, gill and GI tract constitute a large surface area to serve as contact surface for the passage of solute, water and also possible invasion of pathogens. The occurrence of a thick and prominent mucus layer over the epithelial surface of these organs, in addition to others, contribute a great deal to lubrication for smooth passage, interface for the passage of gases and ions, defense against predators, protection against any frictional and mechanical injury and invasion of the environmental pathogens. It is largely because of the latter reason that the anatomical location of mucosa-associated lymphoid tissue (MALT) has been subdivided into gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT) and gill-associated lymphoid tissue (GIALT) (Esteban 2012).

Hence, mucus, an innocuous but complex slimy secretion contained predominantly in the cells called goblet cells and spread over various epithelial surfaces has assumed considerable biological significance due to its wide distribution in variety of vital organs. In view of its diverse functional nature, the study on mucus has of late, gained considerable momentum amongst clinicians, biochemists, histologists, physical chemists and biologists. This prompted Sir Frances Avery Jones and Reid (1978) highlighting the significance of mucus to observe that it is a field of research that offers great potential to clinicians and biologists because “we cannot do without mucus, irritating though it may be if there is too much or too little and predictably fatal if absent altogether in many system of the body where it plays a vital role”. It is, therefore, essential to have an overview of the physic-chemical and biological characteristic features of mucus.

1. Detailed Account of Mucus:

a. Nomenclature:

There has been great deal of confusion over the nomenclature with reference to mucus amongst the clinicians and physiologists with the vague and indeterminate
terminologies such as mucoid, mucoprotein, mucosubstance, mucopolysaccharides, mucin etc. which seems to have been used rather interchangeably (Sturla et al 2001). The term mucin has also been used very widely but inconsistently which according to some authors may refer to glycoproteins which have been assigned to MVC gene family (Lai et al 2009). In addition to mucin, mucus secretion consists of lipids, salts, proteins, macromolecules and cellular debris (Lai et al 2009).

It has been documented in the literature that mucus layer is in a dynamic process of being continuously secreted, shed and discarded or digested and this recycling spans from minute to hours. The mucus flow rate is about 5 mm/min and the mucus layer in renewed roughly every 20 minutes (Ali and Pearson 2007). Continuous secretion and clearance by peristaltic forces lead to quick turnover times which vary from organ to organ. The viscosity of mucus which is 1000-10,000 times greater than that of water ensures that neither viruses nor hydrophilic macromolecules are capable of penetrating the fluid of this viscosity. The characteristic slipperiness or stickiness of the mucus is largely due to its high water content coupled with the presence of high-molecular weight macromolecules which have gel-forming ability which may vary in different organisms. For example, in most vertebrates including fishes, it is glycoprotein (mucins), polysaccharides in algae and higher plants and mucopolysaccharides in invertebrates. All these macromolecules in mucus have the ability to swell in water, interact and provide three-dimensional configuration to water.

b. Broad Composition:

The initial efforts to isolate and characterize the constituents of mucus proved quite difficult largely due to inability to obtain mucus in a tolerably pure state. The conventional electrophoresis and ion-exchange methods remained unsuccessful because the mucus due to its biocolloidal and gel-like structure forming visco-elastic network did not lend itself to electrophoretic separation. However, with the emergence of gas-liquid chromatography, it became possible to isolate the components of mucus and subject it to detailed study (Schrager 1970).

Broadly mucus consists of 95% water and remaining 5% is made up of organic and inorganic constituents. The organic constituents are typically made-up of carbohydrates, proteins and lipids either in free or conjugated forms called glycoconjugates. The relative proportion of the various organic and inorganic constituents of mucus show considerable variations amongst the total 5% proportion, of which glycoconjugate constitute the major chunk 0.5 – 5% followed by 1% inorganic salts, 0.5 – 1% is free protein and lipids. The inorganic constituents are salts and other dialysable components. Among the free components lipids are fatty acids, phospholipids and cholesterol whereas the proteins include innate immune components such as complement, c-reactive proteins, antibodies,
immunoglobulins, antimicrobial peptides (AMP), enzymes like lysozymes, acid and alkaline phosphatases, proteases, hemolysin, agglutinin and lectins.

Reid and Clamp (1978) have described glycoconjugate as the polymeric substance consisting of carbohydrate which is covalently linked to materials, usually lipid or protein (but not nucleic acid). It has been further elaborated that the glycoconjugate linkage formed between carbohydrate and protein is of two types i.e. proteoglycans and glycoproteins and it is the latter which forms the principal constituent of mucus and imparts its typical physiochemical characteristics. The difference between the two protein glycoconjugates is that the proteoglycans is made up long unbranched carbohydrate chains (glycosaminoglycan) most of which have a repeating disaccharide structure and hence contain stretches consisting of hexuronic acid. The mucus glycoproteins, by contrast, are made up of relatively small carbohydrate units (about 10 monosaccharide residues hence called oligosaccharide units), usually branched, little or no repeating structure and without hexuronic acid. The mucus glycoprotein is of extremely high molecular weight and more than 50% of this molecule weight consists of carbohydrate. The structural organization is such that a polypeptide chain forms a central core to which oligosaccharide units are embedded along its length attached to almost every third amino acid. This projected oligosaccharide unit from the polypeptide chain gives the appearance of a porcupine or a lamp or a test tube brush. The acid groups sialic acid and sulphate are attached terminally to these bristles which protect the polypeptide core from the action of proteolytic enzymes.

Such glycoproteins are of high molecular weight (0.5 – 20 MDa) have protein backbone to which carbohydrate side chains are attached which make them highly glycosylated consisting of 80% carbohydrate moiety. The carbohydrate component of mucus glycoprotein is largely made up of galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine, sialic acid (N-acetylneuraminic acid) and traces of mannose and sulphate (Bansil and Turner 2006). The protein core of the mucin comprising remaining 20% (~ 200 – 500 Kda) is largely made up of serine, threonine and proline (STP) which constitute nearly 60% of the amino acid and the rest such as cysteine are derived from globular proteins. The linkage between the carbohydrate and protein in mucus glycoprotein is termed ‘O-glycosidic’ because the linkage is through an oxygen atom. This type of linkage is susceptible to cleavage by alkali except when the amino acid involved is terminal. Physicochemical properties of mucus may certainly be dependent on both protein as well as carbohydrate component of the mucus glycoprotein. However, since glycoproteins are largely composed of carbohydrate which is in immediate contact with the environment, the glycoprotein chemistry will be greatly dependent on the chemistry of oligosaccharide units particularly the peripheral sugar which are in terminal position. It is important to mention here that several other types of glycoproteins other than mucus glycoprotein may be found in the sites such as blood which is termed plasma glycoproteins. However, these glycoproteins will be different from the mucus glycoprotein in as much as that it may have less than 25% carbohydrate component,
have N-glycosidic linkage and may not have the predominance of serine, threonine and protein (STP) as found in mucus glycoproteins.

c. Source(s) of mucus secretion:

As stated earlier, mucus is largely produced by goblet cells which are abundantly present in all epidermal surfaces but other cell types such as sacciform cells (Sadovy et al 2005) and acidophilic granular cells/ serous goblet cells (Whitear 1986, Pickering and Fletcher 1987) may also contribute to mucus secretion.

d. Variations in mucus composition:

Considerable variations have been reported in mucus composition from the generalized picture described above. For instance the mucus in leech skin is highly glycosylated containing 80% carbohydrate and other 20% protein which in addition to typical threonine and serine may have glycine in place of proline. Similarly, there may be additional presence of glucose and mannose which are generally not reported in the carbohydrate moiety of other mucus. Similarly, certain fishes like *H. fossilis*, *C. batrachus* and mullets have been found to have high proportion of lipids in mucus (Lewis 1970, Venkaiah and Lakshmipathi 2000).

e. Acidic and neutral glycoproteins:

The glycoproteins (GPs) elaborated by the branchial mucous cells have been studied by a range of histochemical procedures. Broadly, there are two types of glycoproteins present in the mucous cells, one is neutral and the other is acidic glycoconjugate and the latter is either carboxylated or sulphated type also referred to as sialo- and sulphomucin respectively. These different chemical moieties in mucus glycoproteins can be identified histochemically using PAS which stains neutral glycoprotein with characteristic magenta shade and alcian blue pH 2.5 (AB 2.5) which stains both acidic glycoproteins as blue. Exclusive sulphated moiety of acid glycoprotein can be identified by alcian blue at pH 1.0 (AB 1.0) which excludes the staining of carboxylated acid glycoproteins. Both acid moieties in the same section / cell can be visualized distinctly histochemically by the combined staining with aldehyde fuchsin (AF) –AB (pH 2.5) which stain carboxylated acid glycoproteins purple and sulphated moiety as blue. This can also be done by another alternative approach i.e. methylation followed by AB 2.5 stain which suppresses the carboxylated component and specifically stains sulphated moiety as blue. However, when methylation is followed by saponification and stain with AB (pH 2.5), the carboxylated moiety is stained positive whereas sulphated component remains suppressed. The histochemical techniques have been very extensively used by biologists to localize neutral and acidic glycoprotein in mucus cells.
Some workers have further characterized the GPs with oxidisable vicinal diols by further confirming with the acetylation before PAS to block the oxidation of 1, 2 glycol groups by the periodic acid which would yield the negative result with PAS i.e. no magenta colour will appear. This is further confirmed by the sequential process of acetylation, saponification, PAS to restore 1,2 glycol groups which react with periodic acid and yield magenta colour. Also, it is established by α amylase digestion before PAS reactions which yields magenta colour. For the selective visualization of sialic acid (carboxylated GP) and some of their chain variants at C7 and / or C9, a technique named PA*S (selective periodic acid Schiff reaction) which involves oxidation with 0.4 mM periodic acid in 1M HCl for 1h at 4°C yielding magenta colour is used. The selectivity of this reaction is based on the fact that there is an increase in the rate of oxidation of sialic acid residues together with the decrease in the rate of the oxidation of neutral sugars. Further, to localize sialic acid with vicinal diols i.e. carboxylated and neutral GPs, PAPS (periodic acid oxidation-phenylhydrazine-Schiff) involving oxidation with 1% periodic acid for 2h at RT followed by treatment with 0.5 % phenylhydrazine for 2h and incubation with pararosaniline Schiff for 4h to yield magenta colour which confirms the presence of both these GPs together. For the differentiation of mucins that allows single section identification of side chain O-acylated, and nonacylated, sialic acids in contrasting colors, KOH/PA*S method (saponification-selective periodic acid Schiff reaction) has been used which involves saponification with 0.5% in 70% ethanol for 30 min at RT to deacetylatesialic acid residues followed by PA*S method yielding contrasting colour. Saponification-selective periodic acid- borohydride reduction-periodic Schiff reaction (KOH/PA*/Bh/PAS) method has been used to characterize the neutral sugar whereas another method named PA/Bh/KOH/PAS (Periodic acid- borohydride reduction-saponification-periodic Schiff reaction) is employed to visualize sialic acids with O-acyl substituents at C7, C8 and C9 and O-acyl sugars which yield PAS positive results.

Lectins are carbohydrates binding proteins other than enzymes and proteins and are considered reliable tools to characterize glycoconjugates in tissues. A battery of several lectins have been used to study the binding on the tissue sections to characterize glycoconjugates in gills, skin, GIT and other tissues. Hence, histochemical methods have proved to be valuable tools for localization and characterization of high molecular weight glycoproteins in teleostean mucus which may reflect functional characteristics.

2. Functional Overview of Mucus with Specific Reference to Osmoregulation:

The trident mucus functions include lubrication, water proofing and indeed protection. Even though mucus overlays the epithelial surfaces of gill, skin and GIT but it plays a more critical role in case of integument and gills which is the first line of defense against the microbial exposure. In case of GIT, mucus functions as the ‘skin of the gut’ where it acts
more effectively than the skin of the body to provide protection to gastric mucosa against powerful acid and proteolytic enzymes.

Fish skin mucus serves as a repository of a variety of biologically active substances. Due to relatively impermeant nature of mucus to pathogens, it traps and immobilizes these microbes which get constantly removed due to flowing water current thus preventing their stable colonization. In fish mucus, the various active immune components and defense molecules with biocidal activity include variety of proteolytic enzymes, complement, C-reactive proteins, immunoglobulins, crinotoxins, pheromones and lectins. Among the important enzymes are lysozyme, acid and alkaline phosphatase, cathepsins, superoxide dismutase (SOD), estras, trypsin and metalloproteases. The documented antimicrobial peptides in fish mucus are the alpha-helical amphipathetic peptide which is very common in addition to others such as pleurocidins, pisadins, pardoxin, hipposin, oncorhyncin III & II. Among the commonly occurring proteins are lectoferrins, histones and ribosomal proteins whereas the important immunoglobulins include IgM molecule as a predominant isotype in addition to polymeric immunoglobulin receptor. The fish lectin, agglutinins may confer a limited amount of immunity against infection by pathogens. Compliment brings about cell lysis of activation of immunoglobulins and other materials. Interestingly, mucus provides a renewable surface and whatever is attached to mucus is rapidly lost. The stress-mediated release of mucus due to heavy metals presence helps to bind these heavy metals to mucus which is removed as shed mucus to prevent its entry into the fish.

The involvement of mucus in osmoregulation has been suggested in some earlier experiments in eel Anguilla anguilla where wiping of fish skin mucus compromised the osmoregulatory ability (Baldwin 1948, Negus 1963). Hypophysectomy of these fishes reduced the number of goblet cells and replacement with prolactin, an important osmoregulatory hormone in fresh water fish, reversed this effect. Abundance of goblet cells on fish integument and gills may correlate with environmental salinity (Johnson 1973 and Laurent 1984). That sulpho- and sialomucin of fish mucus have been very intimately linked with the maintenance of homeostatic balance in divergent salinities (Clamp et al 1978). Hence, there is strongly suggestive evidence that fish mucus in integument, gill and GIT plays an important role in osmoregulatory adjustment of teleosts.

3. Rationale of the Present Study:

The fresh water stenohaline catfish H. fossilis and C. batrachus are important components of capture and culture fishery of the Indian subcontinent and are preferred consumer’s choice. These catfishes survive up to a salinity of 35% seawater or 1.1% NaCl (Parwez et al 1979 and Parwez et al, 2001). They actively osmoregulate up to 10% SW and their survival up to its maximum salinity tolerance limit is achieved through passive tissue tolerance. The basic osmoregulatory mechanism of H. fossilis in high salinities as well as in deionized water involves the coordinated participation of the major target organs i.e. gill, skin, gut and
kidney (Goswami et al 1983, Parwez et al 1984, Sherwani and Parwez 2008). The actions of these target organs are hormonally-mediated process with prolactin from adenohypophysis and cortisol and arginine vasotocin (AVT) from adrenal cortex and pars nervosa respectively playing the major regulatory roles (Parwez and Goswami 1985, Sherwani and Parwez 2008). However, despite the fact that mucus covers at least three major osmoregulatory target organs i.e. skin, gills and gut and is known to be a multifunctional entity, its biochemical nature and possible role in these osmoregulatory target organs have not been studied. Moreover, no detailed account with reference to cellular and other histological features of the above three important osmoregulatory target organs i.e. skin, gill and gut is documented in literature. Clearly, such an account is extremely important to understand the role of mucus vis-à-vis these three target organs in the overall perspective of osmoregulatory adjustment. Hence, the objectives of the present study are as follows:

4. Objectives of the Present Study:

(I) To study the detailed organizational arrangement with particular reference to morphology, histology and cellular details of skin, gill and GI tract of catfish, *H. fossilis* and also *C. batrachus* on selected target organs.

(II) To map the relative abundance of mucous cells in different regions of integument and antero-posterior gill lamellae of *H. fossilis* and *C. batrachus*. Also, to study the effects of handling stress on the population of mucous cells in the above two catfishes.

(III) To localize and characterize the glycoprotein moieties of mucous cells in all the three target organs using a wide array of histochemical stains.

(IV) To study the monthly and annual variations and salinity induced changes in the abundance and size of mucus-secreting cells with particular reference to the changes in its glycoconjugate moiety and to explore the physiological significance of the specific glycoproteins.

(V) To identify and localize the chloride cells on the branchial epithelium by light microscopy, histochemically, immuno-histochemically and through ultrastructural studies using SEM and TEM approaches and to study salinity induced changes in chloride cells population.

5. Expected Outcome of the Study:

The information thus generated will help us to build up a comprehensive overview of better and more precise understanding of the morphology, histology and cellular composition of skin, gill and GI tract, the important osmoregulatory target organs in fishes. It would also
provide an insight into the biochemical composition of mucus glycoproteins, a major component of overlying mucus on the above organs, its seasonal profile and salinity and stress induced changes in these catfishes. The consortium of such an information in conjunction with the pre-existing documented information on these aspects will greatly enhance our current understanding of osmoregulatory adjustment in teleost in general and in tropical freshwater stenohaline fishes in particular.

**Materials and Methods**

**A. Collection and Care of Fish:**

Live specimens of catfishes *Heteropenestus fossilis* (local name Singhi) and *Clarias batrachus* (local name Magur), weighing between 35 - 40 gm (approximately 6 cm Length) were obtained from local fish market of Aligarh. The fish were kept under laboratory conditions in glass aquaria (57 x 33 x 22 cm) containing stored dechlorinated tap water. They were acclimatized for 15 - 20 days in the laboratory prior to the initiation of experiments. During this period they were fed daily *Ad libitum* with minced meat and water in aquaria was changed daily by siphoning off and replenishing simultaneously with freshwater adjusted to laboratory temperature.
B. Artificial Sea Water:

Artificial sea water (SW) was prepared in dechlorinated TW according to Goswami et al (1983). Briefly, to prepare full strength SW (100%), the following salts (gm) were dissolved in 1 liter dechlorinated TW; NaCl, 400.8; KCl, 9.8; CaCl2 (fused), 10.1; MgCl2.6H2O, 52.7; Na2SO4, 27.8; NaHCO3, 2.5; and NaBr, 0.6. Further dilution (25% SW in the present study) was prepared by diluting 100% SW with dechlorinated TW.

C. Tissue Collection:

(a) **Skin**- A small skin piece just below the dorsal fin of approximately 1cm² was excised along with some underlying muscles.

(b) **Gills**- All the gills of both sides along with gill arches from anterior to posterior side were taken out.

(c) **Gastrointestinal Tract (GIT)**- The entire GIT was removed and small segments were cut from oesophagus, stomach (anterior, middle and posterior), intestine (duodenum, mid intestine and rectum).

D. Histology of Tissues:

(1) Fixation, rinsing, dehydration, clearing, infiltration, embedding and sectioning).

1. The skin and all gills were fixed in Carnoy’s and aqueous Bouin’s fluid (Appendix-I) for 2 and 24 hrs keeping them completely immersed in these fixatives.

2. After the optimum fixation, the tissues were rinsed with distilled water several times and then dehydrated with ascending alcohol grades of 30%, 50%, 70%, 90% and up
to 100%, keeping in each grade for 1-2 hrs and then transferred to xylene for clearing with 2-3 intermittent changes, until the tissues became completely transparent.

3. For wax infiltration, the dehydrated and cleared tissues were transferred in a mixture of xylene and wax (50% xylene + 50% wax) for 10 min in an oven maintained at 65°C to allow the xylene to come out of the tissue and the wax getting infiltrated into it followed by transfer to pure molten wax and kept in an oven for appropriate time depending upon the tissue giving two changes of fresh wax.

4. For block making, the molten paraffin wax (Qualigens, melting point 60-62°C) was poured into the cavity block which was smeared with glycerine and the tissue was embedded into it at the desired orientation.

5. When the wax solidified completely (approx. 30 min), the blocks were easily removed from the cavity block and trimmed according to the desired plane of sectioning.

6. Five micron thick sections were cut with the help of microtome (Leica RM 2125 RTS), stretched at a temperature of 40-45°C over the egg albumin coated slides to ensure sticking of the sections.

**E. Staning Procedure:**

(a) **General Histology:**

<table>
<thead>
<tr>
<th>S.N</th>
<th>Stains</th>
<th>Interpretation of staining reaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Harris Hematoxylin/</td>
<td>Nuclei (blue), cytoplasm (pink)</td>
<td>Humason</td>
</tr>
<tr>
<td></td>
<td>eosin</td>
<td></td>
<td>(1967)</td>
</tr>
<tr>
<td>2.</td>
<td>MassonsTrichrome</td>
<td>Nuclei (black), cytoplasm (red),</td>
<td>Masson</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen (blue)</td>
<td>(1929)</td>
</tr>
</tbody>
</table>

(i) **Harris Hematoxylinand Eosin, (Humason 1967)**

1. The slides were deparaffinized in xylene and hydrated through progressively decreasing alcoholic grades and transferred into distilled water.

2. The sections were stained with Harris alum Hematoxylin (Appendix-I) for 30 min, rinsed with DDW and destained in 0.1% HCl for 20 to 30 seconds.

3. This followed the extensive washing of the sections with running tap water until blue colour appeared.
4. The sections were dehydrate inascending alcohol grades (30% to 95%) and transferred in 0.2% eosin in 95% alcohol for counterstaining followed quickly by dehydration in 95% and 100% alcohol, cleared in xylene and mounted with DPX.

(ii) **Massons Trichrome**, *(Masson 1929)*

1. The deparaffinized slides were hydrated by the same steps as described above.

2. The sections were mordant in Bouin’s solution for 1 hr at 56°C, cooled and washed in running water until yellow color stops appearing and rinsed in DDW.

3. Now the sections were immersed in Weigert’s iron hematoxylin solution (Appendix-I) followed by DDW for 10 min each and again stained in Biebrich scarlet-acid fuchsin solution (Appendix-I) for 10-15 min and finally washed with DDW.

4. The sections were now put in Phosphomolybdic-phosphotungstic acid solution (Appendix-I) for 15 min and then stained with Aniline blue solution (Appendix-I) for 5 min.

5. Finally, these sections were rinsed with DDW and transferred in Glacial acetic acid for 5 min, dehydrated in alcohol grade, cleared in xylene and mounted with DPX.

(b) **Glycohistochemical stains:**

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Staining Techniques</th>
<th>Interpretation of staining reaction</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>AB (2.5)</td>
<td>Glycoconjugates with acidic groups (carboxylated and sulphated)</td>
<td>Mowry (1963)</td>
</tr>
<tr>
<td>2.</td>
<td>AB (1.0)</td>
<td>Glycoconjugates with O-sulphateesters</td>
<td>Lev and Spicer (1964)</td>
</tr>
<tr>
<td>3.</td>
<td>Active Methylation (AM)/AB(2.5)</td>
<td>Glycoconjugates with O-sulphateesters</td>
<td>Spicer and Warren (1960)</td>
</tr>
<tr>
<td>4.</td>
<td>AM/Saponification (KOH)/AB(2.5)</td>
<td>Glycoconjugates with carboxylated moiety</td>
<td>Spicer and Lillie (1960)</td>
</tr>
<tr>
<td>5.</td>
<td>PAS</td>
<td>Glycoconjugates with neutral moiety</td>
<td>McManus (1948)</td>
</tr>
<tr>
<td>6.</td>
<td>PAS/AB (2.5)</td>
<td>Glycoconjugates with neutral and acidic moiety</td>
<td>Mowry (1963)</td>
</tr>
<tr>
<td>7.</td>
<td>PAS/AB (1.0)</td>
<td>Glycoconjugates with neutral and sulphated moiety</td>
<td>Spicer et al. (1967)</td>
</tr>
<tr>
<td>8.</td>
<td>AF/ AB(2.5)</td>
<td>Glycoconjugates with carboxylated and sulphated moiety</td>
<td>Cameron and Steele (1959)</td>
</tr>
</tbody>
</table>
(i). Alcian Blue (pH 2.5) / AB (2.5), (Mowry 1963 see Pearse 1968)

1. The slides containing tissues were deparaffinized in xylene, hydrated in down grade alcohol series i.e. 100 to 30% and finally brought to water.

2. The slide were stained with 1% AB 8GX (Sigma) in 3% acetic acid for 30 min.

3. The sections were washed in running water for 5 min, dehydrated with ascending alcohol grades i.e. 30% to 100%, cleared in xylene and mounted in DPX.

(ii). Alcian Blue (pH 1.0)/ AB (1.0), (Lev and Spicer 1964)

1. The slides were hydrated and transferred to 1.0% alcian Blue 8GX in 0.1 N-HCl for 30 min.

2. These slides were blotted dry with fine filter paper, dehydrated in alcohol, cleared in xylene and mounted in DPX.

(iii). Active Methylation, AM/AB (pH 2.5), (Spicer and Warren 1960)

1. Brought the sections up to hydration and coated with a film of 0.5% celloidin and kept in pre-heated 0.8: 99.2 mixture of Conc.HCl and Methanol for 5 hrs at 60ºC to activate Methylation.

2. These slides were then washed in running water, rinsed in absolute ethanol and kept in equal part of ethanol and diethyl ether to remove celloidin film and washed again with water. The sections were stained with 1% AB 8GX (pH 2.5) for 30 min, washed with running water for 5 min, dehydrated with alcohol ascending series, cleared in xylene and mounted in DPX.

(iv). AM/Saponification, KOH / AB (pH 2.5), (Spicer and Lillie 1960)

1. After methylation as described above, the slides were placed in 70% ethanol.

2. For saponification, the slides were kept in 1% KOH made in 70% ethanol for 30 min. and treated with ethanol and diethyl ether in equal part for removal of the celloidin film.

3. Then these sections were washed with running water and stained with 1% AB 8GX (pH 2.05) for 30 min, again washed with running water, dehydrated in alcohol and finally mounted in DPX.

(v). Periodic acid Schiff’s (PAS) Method (pH 2.5), (McManus 1948, see Pearse 1968)
1. The slides were brought hydrated and transferred to 1% aqueous periodic acid for 15 min for oxidation, washed with running water for 5 min and stained with Schiff’s reagent (Appendix - I) for 15 min.

2. Then the tissue were washed with running tap water for 5 min, dehydrated in upgrade alcohol series i.e. 30 to 100% alcohol, cleared in xylene and mounted in DPX.

(vi). Alcian blue (pH 2.5) – Periodic acid Schiff’s, AB (2.5)-PAS, (Mowry 1963)

1. The sections were brought up to water and rinsed briefly with 3% aqueous acetic acid.
2. The sections were stained with 1% Alcian Blue 8GX (pH 2.5) made in 3% acetic acid for 2 hrs, rinsed briefly in water and then in 3% acetic acid, washed in running water and distilled water quickly.
3. These sections were then transferred for oxidation for 15 min in 1% periodic acid solution at room temperature, washed in running water for 5 min and subjected to the same steps as described above in para (v) for PAS staining.

(vii). Alcian blue (pH 1.0) – Periodic acid Schiff’s, PAS-AB (1.0), (Spicer et al 1967)

1. Step I is the same as described in AB/PAS pH 2.5 above.
2. The sections were stained for 2hrs in 1% AB 8GX in 0.1 N-HCl.
3. The tissues were blotted dry with fine filter paper, then oxidized with 1% periodic acid for 15 min and subjected to the same steps as described above in para (v) for PAS staining.

(viii). Aldehyde Fuchsin / Alcian Blue, AF/ AB(2.5), (Cameron and Steele 1959)

1. Deparaffinized and hydrated the sections, transferred in Alcian blue (pH 2.5) as described in para (i) above.
2. Then the slides were oxidized in Gomori’s solution (Appendix - I) for about 1 min, rinsed with distilled water for 5 min, bleached in sodium bisulphite solution until the permanganate colour is completely removed.
3. These slides were washed in running water for 5 min, transferred to 70% alcohol for 2 min and stained in Aldehyde fuchsin (Appendix I) reagent for 10 min at room temperature.
4. The back of slides were wiped off and rinsed in 95% alcohol until no more Aldehyde fuchsin stain comes out of the sections.
5. The slides were washed in absolute alcohol two times for 5 min each, cleared in xylene and mounted in DPX.

(c) **Staining Techniques used for localization of Chloride cells:**

<table>
<thead>
<tr>
<th>S.N</th>
<th>Stains</th>
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(i) **Acid Hematein Method, (Baker 1946 see Pearse 1968)**

1. The gills of *H. fossilis* were fixed in Formal calcium (Appendix-I) for 18 hrs and then transferred to dichromate calcium (Appendix-I) for 18 hrs at 22ºC and then into a fresh dichromate calcium for 25 hrs at 60ºC.

2. The tissues were washed with distilled water and paraffin blocks were made as described earlier.

3. Five micron thick sections were cut, stretched and stuck on the slide, deparaffinized and brought to water.

4. These sections were transferred into mordant (dichromate-calcium) for 1 hr at 60ºC, washed with DDW thoroughly, and transferred to acid hematein solution (Appendix-I) for 5 hrs at 37ºC.

5. The sections were then rinsed with DDW, transferred to borax-ferricyanide-differentiator (Appendix-I) for 18 hrs at 37ºC, washed with DDW, dehydrated, cleared and mounted in DPX.

(ii) **Sudan Black B, (McManus 1946, see Pearse 1968)**

1. The tissues were fixed in a solution containing 1gm cobalt nitrate dissolved in 80ml DDW with 10ml of 10% CaCl₂ and 10ml of 40% formalin for 5-weeks and paraffin blocks were made as described earlier.

2. Five micron thick sections mounted on slide were brought to 70% alcohol, transferred in saturated Sudan Black B made in 70% alcohol for 30 min at room temperature.
3. They were then washed with 70% alcohol quickly for removing the excess dye and dehydrated with ascending series of alcohol, cleared in xylene and mounted in DPX.

(iii) Toluidine Blue Method, (Belanger and Hartnett 1960 see Pearse 1968)

Brought deparaffinized sections up to 30% alcohol and stained with 0.1% toluidine blue in 30% ethanol for 30 min, rinsed with 70% and 95% alcohol thoroughly, transferred in absolute alcohol for dehydration, cleared in xylene and mounted in DPX.

(iv) OZI Staining Method, (Garcia-Romeu and Masoni 1970)

1. The gills were excised from all fishes, cut in small pieces of 2 mm size and immersed in freshly mixed ChampyMaillet’s fixative(Appendix-I) for 16 hrs at room temperature.

2. These small pieces of tissues were washed with DDW for 5 - 10 times to remove the excess fixative, dehydrated with a series of ethanol and processed for making paraffin blocks as described earlier.

3. Five micromin thick sections were cut, taken on slides, deparaffinized and mounted in DPX.

F. Immunohistochemistry:

a. Tissue fixation:

The gill tissues were fixed for 8 – 10 hrs in 4% paraformaldehyde (Sigma) (Appendix-I) prepared in 0.1M phosphate buffer saline (PBS) (Appendix-I) for 8-10 hr. The tissues were sequentially transferred to PBS containing 10% and 20% sucrose for 2hrs each and finally to 30% sucrose where they could be stored up to 72 hrs at 4ºC. These processed tissues were embedded in tissue-tek OCT compound (Sakura USA) and the blocks were sectioned at 15 µm thickness at −25ºC on Cryostat (Leica CM-18.50) and the sections were mounted on the slides coated with poly-L-lysine (Sigma P 8920) and stored at −20ºC overnight or until use. The slides coated with poly-L-lysine were prepared by dispensing 25 µl of poly-L-lysine on a slide and spreading it evenly with finger.

b. Antibodies:

For immunolocalization of gill Na⁺-K⁺-ATPase, a monoclonal antibody specific for the α-subunit of chicken Na⁺/K⁺-ATPase α5 was used (Takeyasu et al 1988). The antibody (α5) developed by D.M. Fambrough (Johns Hopkins University MD, USA) was obtained from Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Science, Iowa City, IA 52242, USA, developed under the auspices of the National Institute of Child Health and Development (NICHD). The
antibody was purchased in the form of cell culture concentrate (0.1 ml) which is now in routine use for identification of chloride cells by way of their high Na⁺/K⁺-ATPase levels (Witters et al 1996 and Wilson et al 2000). The secondary antibody was generated against IgG of mouse in rabbit (Department of Zoology, Nagpur University campus, Nagpur, India).

c. Staining technique of immunohistochemistry:

Before use, the poly-L-lysincocated slides stored at −20°C were brought to room temperature for 1 hrs, a well around the sections was drawn with PAP pen and transferred to the humidity chamber. These tissues were then rinsed with PBS three time for 5 min each to wash out the fixative and were finally washed again with triton X 100(Appendix-I) for 20 min which is mild detergent, used to render the antigen accessible to the antibodies and also helps to expose the antigen sites and facilitates antigen-antibody reaction and greater signal to noise ratio. The slides were then transferred in blocking solution (50% PBS + 50% BSA) for 30 min, which is used to prevent non specific cross-reactivity of both primary and secondary antibody with the tissue. The slides were incubated in primary antibody at the antibody dilution of 1:1000 in PBS for 2hrs at room temperature. After incubation the slides were rinsed 3 times with PBS for 5 min each and exposed to secondary antibody (1:100 in blocking solution) (Appendix-I) at room temperature for 1 hr and then rinsed 3 times with PBS. The sections were now incubated in streptavidinperoxidase (Appendix-I) for 1 hr at room temperature and washed 2 times with PBS and DDW. Following this, DAB (Appendix-I) was applied on the sections for 5-7 min until reddish brown colour appears and washed the slides properly with DDW and mounted in glycerol gelatin at 60°C and visualized under microscope (Carl Zeiss model Axioskop 40 FL).

G. Visualization of Sections:

The sections were visualized under Zeiss microscope (Carl Zeiss model Axioskop 40 FL) at the suitable magnification and images were captured with Axiocam ICc3 camera and 4 sectionseach from 5fishes were quantified with Auto measure software (Rel. 4.8). For statistical analysis, the data were expressed as mean ± S.E. Analysis of data was carried out using GraphPadInStat software and one-way analysis of variance (ANOVA) followed by Tukey’s test to calculate the statistical significance of the data.

H. Electron Microscopy:

(i) Scanning Electron Microscopy (SEM)

Anesthetized fishes with MS222, were sacrificed and gills and skin were excised as detailed in section B. Dissected tissues (size 1cm) were prefixed in a Karnovasky’s
fixative (2.5% Glutaraldehyde and 2% paraformaldehyde (Sigma) dissolved in 0.1 M PBS (pH 7.2) (Appendix-I) at 4°C for 10 hrs, then rinsed three times with 0.1M PBS and postfixed in 1% osmium tetroxide (Sigma) in 0.1M PBS (pH 7.2) for 2 hr at 4°C. The tissues were washed with buffer 3 times for 15 min each at 4°C followed by dehydration through graded series of methanol at 4°C (25%, 50%, 70%, 80%, 90%, 95% and 100%), air dried and finally transferred in hexamethyldisilazane (HMDS) for 15 min at room temperature. Then specimens were critical-point dried using liquid CO₂ in a critical-point drier (Hitachi HCP-2), mounted on the aluminium stub and sputter coated with a gold palladium complex of 35 nm thick film, kept for 4 min in a vacuum evaporator (Electron microscopy sciences EMS150R) and examined under SEM (Zeiss EVO HD25).

(ii) Transmission Electron Microscopy (TEM)

The gills and skin (size- 1mm) were fixed in Karnovsky’s fixative (2.5% Glutaraldehyde and 2% paraformaldehyde dissolved in 0.1 M PBS pH 7.2) for 10 hrs at 4°C, washed 3-4 times with 0.1 M PBS and postfixed in 1% osmium tetraoxide (Sigma) in 0.1 M PBS for 3 hrs at 4°C. The tissues were rinsed thoroughly with PBS buffer to remove excess fixative, followed by dehydration through a graded series of ethanol (10%, 25%, 50%, 75%, 90%, 95%) at 4°C for 10 min each and then allowed to warm to room temperature. This was followed by four changes of absolute ethanol for 15 min each, cleared in toluene for 30 min and embedded in Epon 812 and polymerized at 60°C overnight. Ultrathin sections (1 μm) were cut with diamond knife using ultratome (Leica EM UC6), mounted on grids, double stained with 2% uranyl acetate in 50% ethanol for 10-15 min followed by lead citrate for 5-10 min and examined at 75 kV under Transmission Electron Microscope (JEOL JEM-2100F).

Background

Fishes constitute a major vertebrate group and a predominant component of aquatic fauna. The body surface of all multicellular organisms including fishes, is protected by epithelial lining which serves as a physical barrier between the internal milieu and the external environment. Integument, gill and GI tract constitute a large surface area to serve as contact surface for the passage of solute, water and also possible invasion of pathogens. The occurrence of a thick and prominent mucus layer over the epithelial surface of these organs, in addition to others, contribute a great deal to, lubrication for smooth passage, interface for the passage of gases and ions, defense against predators, protection against any frictional and mechanical injury and invasion of the environmental pathogens. Hence, mucus, an innocuous but complex slimy secretion contained predominantly in the cells called goblet cells and spread over various epithelial surfaces has assumed considerable biological significance due to its wide distribution in variety of vital organs. In view of its diverse
functional nature, the study on mucus has of late, gained considerable momentum amongst clinicians, biochemists, histologists, physical chemists and biologists.

Broadly mucus consists of 95% water and remaining 5% is made up of organic and inorganic constituents. The organic constituents are typically made-up of carbohydrates, proteins and lipids either in free or conjugated forms called glycoconjugates. The mucus glycoprotein is of extremely high molecular weight and more than 50% of this molecule weight consists of carbohydrate. The structural organization is such that a polypeptide chain forms a central core to which oligosaccharide units are embedded along its length attached to almost every third amino acid. The trident mucus functions include lubrication, water proofing and indeed protection. Even though mucus overlays the epithelial surfaces of gill, skin and GIT but it plays a more critical role in case of integument and gills which is the first line of defense against the microbial exposure. In case of GIT, mucus functions as the ‘skin of the gut’ where it acts more effectively than the skin of the body to provide protection to gastric mucosa against powerful acid and proteolytic enzymes.

The involvement of mucus in osmoregulation has been suggested in some earlier experiments in eel *Anguilla anguilla* where wiping of fish skin mucus compromised the osmoregulatory ability. Hypophysectomy of these fishes reduced the number of goblet cells and replacement with prolactin, an important osmoregulatory hormone in fresh water fish, reversed this effect. Abundance of goblet cells on fish integument and gills may correlate with environmental salinity. That sulpho- and sialomucin of fish mucus have been very intimately linked with the maintenance of homeostatic balance in divergent salinities. Hence, there is strongly suggestive evidence that fish mucus in integument, gill and GIT plays an important role in osmoregulatory adjustment of teleosts. The fresh water stenohaline catfish *H. fossilis* and *C. batrachus* are important components of capture and culture fishery of the Indian subcontinent and are preferred consumer’s choice. The basic osmoregulatory mechanism of *H. fossilis* in high salinities as well as in deionized water involves the coordinated participation of the major target organs i.e. gill, skin, gut and kidney. However, despite the fact that mucus covers at least three major osmoregulatory target organs i.e. skin, gills and gut and is known to be a multifunctional entity, its biochemical nature and possible role in these osmoregulatory target organs have not been studied. Moreover, no detailed account with reference to cellular and other histological features of the above three important osmoregulatory target organs i.e. skin, gill and gut is documented in literature. Clearly, such an account is extremely important to understand the role of mucus vis-à-vis these three target organs in the overall perspective of osmoregulatory adjustment. Hence, the present study is designed to fill this long standing gap in our knowledge to study the distribution of mucus in three important osmoregulatory target organs i.e. skin, gills and GIT, its glycoptotein components and temporal variations and functional role in osmoregulatory processes of these two important Indian catfishes *H. fossilis* and *C. batrachus*.
The information thus generated will help us to build up a comprehensive overview of better and more precise understanding of the morphology, histology and cellular composition of skin, gill and GI tract, the important osmoregulatory target organs in fishes. The consortium of such information in conjunction with the pre-existing documented information on these aspects will greatly enhance our current understanding of the role of mucus in osmoregulatory adjustment in teleost in general and in tropical freshwater stenohaline fishes in particular.

**The organization of the thesis components**

This thesis is divided into four distinct chapters which are preceded by an introductory preface on the significance and other important aspects of the mucus. The first chapter deals with the effects of handling stress on the skin and gill mucous cells and mapping of the relative abundance of mucous cells in different locational regions of skin and the gills. The next three chapters focuses on the general histological organization and role of mucus in integument, gills and GIT of these catfishes. The detailed protocol of the common methodology used in each chapter has been elaborately described under separate section captioned ‘Materials and Methods’ and the specific ‘Experimental Protocol’ is included in each chapter. The chapter-wise salient findings are described below.

*(Chapter I)*

**Handling stress and mucous cells region-based distribution:**

This chapter deals with two important aspects to study the effects of experimental handling on the mucous cells population and also to ascertain the distributional pattern of mucous cells in different regions of the integument and different gills of the catfishes *H. fossilis* and *C. batrachus*.

The fish and other aquatic vertebrates are subjected to greater variety of stressors compared to their terrestrial counterparts which will directly affect their homeostatic mechanism. The focal theme of the present investigation is to explore the role of overlying mucus layer in three important osmoregulatory organs i.e. skin, gills and the GIT. This mucus is being secreted chiefly by the goblet cells / mucous cells in these organs. The sampling protocol, normally followed, is to net out fishes from aquaria, sacrifice them and excise the target tissues for histological and other investigations. However, considering the basic tenets of stress physiology, one wonders if this simple innocuous protocol of sampling is not exerting...
any stress on to the fishes and particularly on the mucous cells which are known to play a significant role in defense and protection of fishes against unfavourable conditions and invading pathogens. If the sampling protocol followed by us is stressful, then, any data generated based on this protocol of sampling will be ‘grossly suspect’ until the issue of perceived stressful conditions are settled. This, therefore, greatly underlines the need for establishing a baseline data based on the protocol which may yield data on “unstressed/normal” conditions to arrive at meaningful conclusions. In the present study, we were seized of the fact that even a simple act of netting the catfish from the aquarium for sampling purpose may prove to be a stressful situation which might obscure the ultimate objective of the study. Hence, the twin objectives of the present chapter were: (i) to establish whether the sampling protocol followed by different investigators to study the mucous cells in fishes not affect the normal histological and physiological status of these cells? and (ii) secondly, whether the mucous cells are uniformly distributed all over the skin with no region or gill - number based variations.

Our findings have shown that routine experimental handling of catfishes has caused total disappearance of mucous cells in the skin of both the catfishes which, however, was on somewhat expected lines. But what surprised us most was the acuteness of response which seems to have been more accentuated since both these catfishes are devoid of scales on the skin surface and, therefore, were in direct contact with the handling implement i.e. the nylon net which was used to take out these fishes from aquarium during the experimental sampling. However, the possible underlying mechanism which brings about such complete disappearance of mucous cells following handling stress in both catfishes has not been investigated because it is not under the focal theme of the present study. Based on our long experience of having worked with these fishes, we found both these catfishes are extremely hardy and quite capable of withstanding simultaneously performed surgical manipulations like hypophysectomy, gonadectomy and pinealectomy on the same individual with almost negligible mortality despite such heavy surgical trauma. Also, in one of our earlier studies, we have established conclusively that the repeated handling stress caused in the present protocol does not activate HPI axis since plasma cortisol levels do not show any significant increase in *H. fossilis*. What, then, is causing the complete disappearance of skin mucous cells is an interesting proposition for further investigation. Another observation in the present study is that gill mucous cells seem unaffected by the same handling stress which brings about the complete disappearance of skin mucous cells. To the best of our knowledge, there is no other report in literature on any fish species where such varied response to the same stimulus and in the same species has been observed. At this point, it is believed that the reason of variations in such a response could be physical rather than metabolic. Hence, the skin of both these catfishes which are subjected to touch stimuli have responded by sudden and complete exudation of mucus and the ultimate loss of its mucous cells. However, since gills are internalized by opercular covering and hence, quite unlike skin, not in direct physical touch with the implement of netting, have shown the normal
distribution of mucous cells all over gill epithelium even following the handling stress. The observation in the present study that anesthesia MS222 (Tricaine methanesulphonate) provide the complete resting / basal level conditions as reflected in the normal histopathology of skin mucous cells and obviate stress-induced changes is interesting. Anaesthesia has been frequently applied in aquaculture and live animals experimentation to subdue neuromotor control. Hence, this approach is ideally suited to collect samples from unstressed fish and to generate baseline data.

We have also observed highly significant region-based variations in the density of integumentary mucous cells in both catfishes. Interestingly, while the profiles of region based variations are different in both catfishes but the highest abundance of mucous cells in both these catfishes is recorded in the same region i.e. below the dorsal fin. Further, the degree of inter-region variations is more marked in C. batrachus which has recorded a near total absence of mucous cells in the caudal region whereas in H. fossilis these cells are present in good noticeable number even in the anal region which recorded the minimum population of these cells in H. fossilis. The possible reasons of the maximum abundance of mucous cells in and around dorsal fin region in both the catfishes could possibly be due to its protective strategy against predators. In both these catfishes, external body contours and undulating swimming movements are more or less identical and dorsal region below the pectoral fin is not as much subjected to gyrating body movements as the other parts of the body and hence may serve as the ideal site for the predator for grasping. Abundant mucus covering over this region due to greater number of mucous cells may well be an adaptational response against such adversities. The total lack distributional variations in the abundance of mucous cells populations in different gills located antero-posteriorly in both catfishes is exactly the same as observed in the handling response to gills in both these catfishes. At this point, we may not be able to offer any other plausible explanation for such a lack of distributional variation in gills except to reiterate the same logic of the internalized existence of gills unlike integument and complete lack of any chance of tactile stimulus acting directly on the gill surface.

In conclusion, it may be stated that the findings of the present chapter have demonstrated comprehensively that the handling stress brings about the total disappearance of mucous cells in the skin but not in gills and that there is a region-based distributional variation of mucous cells in the integument but not the gills of both catfishes. These findings may be of practical significance to those involved in the study of fish integumentary biology and fish toxicology using histology and histopathology as the end markers.

(Chapter II)