CHAPTER-II

MATERIAL

AND

METHODS
II MATERIAL AND METHODS

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1. MATERIAL:

A. Animals:

For present investigation male mice (*Mus musculus* Linn.) were used as an experimental animal model. Breeding pairs were obtained from Hindustan Antibiotics, Pune. They were housed and maintained in departmental animal house in separate cages under proper conditions of light, temperature and humidity. The animals were housed in aluminium cages having dimensions of 10”×8”×5” and allowed to live in groups of 3 to 4 per cage. They were supplied with Amrut Mice Feed (Pranav Agro Industries, Pvt. Ltd. Sangli.) and water *ad libitum*. The record of their age and body weight was maintained. Adult male mice of 5 to 6 month age weighing about 50 to 55 ± 2 gm and old mice of 16 to 18 month age weighing about 40 to 45 ± 2 gm were selected. (Old mice were selected after testing their fertility). Both adult and old male mice were divided into two groups viz. protective group and curative group. Protective groups were those which received 0.5 ml 0.9% saline, 0.5 ml 5% D-galactose, 0.5 ml 5% D-galactose along with centrophenoixine and 0.5 ml 5% D-galactose along with glycowithanolides for 20 days and sacrificed on 21\textsuperscript{st} day, while curative groups were those which received 0.5 ml 0.9% saline for 40 days, 0.5 ml 5% D-galactose alone for 20 days and followed by 0.5 ml saline or centrophenoixine or glycowithanolides alone for 20 days, then sacrificed on 41\textsuperscript{st} day. Accordingly both protective and curative groups of adult as well as old mice were further divided into four batches.

- **Batch A: Control**

Both adult and old male mice were received 0.5 ml, 0.9 % saline per day subcutaneously for 20 days and 40 days for protective and curative groups respectively. It is denoted as label ‘control’ and in electrophoretic plates as ‘C’. 
• **Batch B: D-galactose stressed**

Both adult and old male mice were received D-galactose to accelerate the aging process (Song *et al.*, 1999; Deshmukh *et al.*, 2006). The control batch for protective group received 0.5 ml 5% D-galactose per day subcutaneously for 20 days, while the control batch of curative group received 0.5 ml 5% D-galactose per day subcutaneously for 20 days and then received 0.5 ml 0.9% saline per day subcutaneously for next 20 days.

Protective batch denoted as Dg-treated and curative batch denoted as Dg → saline.

• **Batch C: Centrophenoxine treated**

Both adult and old male mice were received 0.5 ml 5% D-galactose along with Centrophenoxine (80 mg/kg body weight.) per day for 20 days (Patro and Sharma, 1984) for protective group, while curative group received 0.5 ml 5% D-galactose per day for 20 days and then 0.5 ml centrophenoxine (80 mg / kg body wt.) per day for further 20 days.

Protective batch is denoted as Dg + cent and curative batch is denoted as Dg → cent.

• **Batch D: Glycowithanolides (WSG) treated**

Protective group of adult and old male mice were received 0.5 ml D-galactose along with WSG (20 mg/kg body weight) per day for 20 days. (This dose was selected by Bhattacharya *et al.* (1997) for *Mus musculus* to study the effect of WSG on the brain). While curative groups received 0.5 ml D-galactose per day for 20 days and then dose of 0.5 ml WSG (20 mg/kg body weight) per day for further 20 days.

Protective batch is denoted as Dg + WSG and curative batch is denoted as Dg → WSG.

All drug administrations and sacrifice of the animals were done between 09.00 am and 12.00 am.
B. Plant Extract:

Fresh ashwagandha plants (*W. somnifera* Dunal) were obtained from Shri Prasad Nursery, Kognoli, Dist- Belgum (Karnataka State). Fresh green leaves were separated and washed properly with water and rinsed with distilled water. Washed leaves were blotted properly with blotting paper and kept for drying in the shade. Glycowithanolides from these leaves was separated as described by Bhattacharya *et al*, (1997). An aqueous concentrate of *W. somnifera* leaves was exhaustively extracted with chloroform to remove fatty materials and separates the withanolides. The aqueous solution was then spray dried. After spray drying a thick paste like extract was obtained and stored in glass bottle. It was kept refrigerated (4°C) for further use.

2. METHODS:

A. Preparation of Homogenates

From each batch 5 mice were weighed and sacrificed between 9.00 am to 12.00 am by cervical dislocation. Their submandibular and sublingual glands from one side were dissected, pooled and weighed. The homogenates of these glands were prepared in different homogenizing media as per the requirements of the respective methods for estimation of proteins, fucose, sialic acid and enzyme assay. Submandibular and sublingual glands from other side were used for histology, histochemistry and electrophoresis.

B. Histology of Salivary Glands:

For the histology of major salivary glands viz. submandibular and sublingual glands were fixed in 10% formaldehyde for 24 hours at 4°C. The glands were washed in running tap water for 24 hours, dehydrated through alcoholic grades, cleaned in xylene and embedded in paraffin. The sections were cut at a thickness of 7 μ and stained with Haematoxylene and Eosin (H/E).
C. Estimation of Total Proteins (Lowry et al, 1951)

- **Reagents:**
  
  i. Lowry’s A: 2% Na$_2$CO$_3$ in 0.1 N NaOH.
  
  ii. Lowry’s B: 0.5% CuSO$_4$ in 1% freshly prepared Na or K tartarate.
  
  iii. Lowry’s C: 50 ml of Lowry’s A + 1 ml of Lowry’s B (made freshly at the time of use).
  
  iv. Folin reagent: 90% Ciocalteau phenol reagent prepared in the laboratory. At the time of use it was diluted in water in 1:1 proportion.

- **Procedure:**

  The glands were weighed, freeze-dried and homogenized in prechilled glass mortar and pestle with distilled water (2 mg/ml) and centrifuged at 3000 rpm for 10 minutes. The supernatants were used for estimation of proteins and additions were made as follows.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Blank</th>
<th>Salivary sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distilled water</td>
<td>4 ml</td>
</tr>
<tr>
<td>2</td>
<td>Sample</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>Reagent C</td>
<td>5.5 ml</td>
</tr>
<tr>
<td>4</td>
<td>Folin reagent (Diluted 1:1)</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

The above tubes were shaken well and kept for 10 minutes.

The optical density was measured at 660 nm on spectronic 20 calorimeter (Milton Roy) adjusting to zero with blank.

The final color production is the result of bi-uret reaction of protein with copper ions in an alkaline medium and reduction of the phosphomolybdic phosphotungstic reagent by the tyrosine and tryptophan present in proteins.
• **Calculation:**
  The protein concentration per mg gland was determined using standard graph of bovine serum albumin (Hi – media).

**D. Estimation of Non-specific Esterase** (Bier, 1955)

• **Reagents:**
  i. 0.66 M phosphate buffer (pH 7.0)
  ii. 0.001 M p-nitrophenyl acetate in 0.66 M phosphate buffer (pH 7.0).
  iii. Stock solution: was prepared by adding 63 mg p-nitrophenyl acetate in 10 ml ethanol.
  iv. Working substrate: was prepared by diluting 0.1 ml of stock with 10 ml 0.66 M phosphate buffer.

• **Procedure:**
  The glands were weighed, freezed and homogenized in prechilled glass mortar and pestle with 0.66 M phosphate buffer pH 7.0 (2 mg/ml). The homogenates were centrifuged at 5000 rpm for 10 minutes at 10°C. Supernatants thus obtained were used as enzyme sources.

  For enzyme estimation following additions were made.
  i. 5 ml --- Ice cold distilled water
  ii. 2 ml --- Phosphate buffer (0.66 M, pH 7.0)
  iii. 1 ml --- Enzyme supernatant

  The tubes were shaken vigorously and centrifuged for 5 minutes at 2000 rpm. In the supernatants, 2 ml of working substrate solution was added. Readings were taken immediately and treated as activity at 0 hours. The aliquots were shaken vigorously and incubated at 20°C for 20 minutes and readings were taken at 400 nm against distilled water as a blank.
• Calculations:

The optical densities were converted to µ moles of p-nitrophenol from the p-nitrophenol standard curve. The non-specific esterase activity in terms of p-nitrophenol in µ mols / mg protein was calculated as –

\[ \text{µmols p-nitrophenol / mg protein} = \frac{\text{µ mols p-nitrophenol from graph} \times \text{dilution}}{\text{Amount of protein / mg tissue}} \]

E. Histochemical Demonstration of Esterase (Burstone, 1962).

Esterases are a very diverse group of enzymes and have most significant enzymatic property is that their natural substrates are esters of carboxylic acids. Many esterases have low substrate specificities and are referred to as non-specific esterase. Substituted naphthol AS-D acetate has been used as substrate with excellent results (Burstone, 1957a, 1957b and Gossner, 1958). Because of the highly insoluble nature of the final reactions, very sharp localization of enzymes is possible.

Presence of non-specific esterase was studied by using α naphthol AS–D acetate substrate as below.

a. Fixation:

Salivary glands fixed in 2% cold formal calcium for 24 hours and fixed frozen cryostat sections were used.

b. Incubation medium:

In 50 ml container placed 5.0 mg α–naphthol AS-D acetate. Added 0.5 ml acetone to dissolve the substrate. Diluted with 25 ml distilled water and 25 ml 0.2 M tris butter, pH 7.1. Dissolved 40 mg fast Garnet GBC and filtered into couplin jar. Eserine 10^{-5} M added to inhibit choline esterase activity.

• Procedure:

i. Fixed frozen cryostat sections were incubated for 2 hours.

ii. Washed in cold distilled water.
iii. Mounted with PVP (Poly Vinyl Pyrrolidone).

• Results:
Esterase activity was represented by an intense red dye.

F. Kinetics Study of Esterase:

In kinetic study, Km and Vmax were studied in submandibular and sublingual salivary glands of adult and old male mice. In kinetic study only effect of the substrate concentration on enzyme activity was studied.

• Effects of substrate concentration on esterase activity

For the determination of Km and Vmax values of esterase, the rate of hydrolysis of paranitro-phenylacetate by the enzyme was measured using different concentrations of p-nitrophenyl acetate. Different concentrations of p-nitrophenyl acetate were prepared in phosphate buffer 0.66 M, pH 7.0. The pH was adjusted with 1 N HCl. The concentrations of p-nitrophenyl acetate were taken as below.

1) 0.315 mg 2) 0.63 mg 3) 0.945 mg 4) 1.26 mg 5) 1.575 mg
6) 1.89 mg 7) 2.20 mg 8) 2.52 mg 9) 2.835 mg 10) 3.15 mg

With the help of optical densities obtained at various concentrations of p-nitrophenyl acetate for the enzyme esterase, the esterase activities were calculated from standard graph. The graph was plotted as enzyme activity V/s substrate concentration. The km and Vmax values of esterase were obtained from this graph as described by Eisenthal and Cornish Bowden, (1974).

G. Estimation of Amylase activity (Jayaraman, 1988)

• Reagents:
  i. Phosphate buffer (0.1 N pH 6.7).
  ii. 0.5% starch solution.
  iii. 1% NaCl.
  iv. 2 N NaOH.
  v. 1% Di-nitro-salicylic acid.
**Procedure:**

The additions for the estimation of amylase were made as follows, three readings were taken:

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Phosphate buffer (0.1 N pH 6.7)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>2.</td>
<td>0.5% starch solution</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>3.</td>
<td>1% NaCl</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Mixed well and kept for 10 minutes at 37°C and then added

| 4.     | Water | 1.5 ml | 1.0 ml |
| 5.     | Sample | ---- | 0.5 ml |

Incubated for the 15 minutes at 37°C and added

| 6.     | 2 N NaOH | 0.5 ml | 0.5 ml |
| 7.     | 1% Di-nitro-salicylic acid | 0.5 ml | 0.5 ml |

Mixed well and tubes were heated in the boiling water bath for 5 minutes. Cooled at room temperature and readings were taken at 520 nm against the blank.

**Calculations:**

The optical densities were converted in to µg of maltose from the standard graph. Amylase activity in terms of µg maltose per mg salivary proteins was calculated.

\[
\text{Amylase activity (µg maltose/mg protein)} = \frac{\text{µg maltose from graph}}{\text{Amount of protein / mg tissue}} \times \text{dilution}
\]
H. Histochemical Demonstration of Amylase by Substrate Film Method. (Smith & Frommer, 1973)

Substrate film method involves the use of films of high molecular weight enzyme substrates and useful for studying enzymes that are unable to hydrolyze low molecular weight substrates. These techniques require the enzymes to be somewhat soluble. Tissue sections, (fixed frozen sections) were placed on a thin film of substrate. The enzyme will diffuse from the section into film and hydrolyzing it. The substrate film is stained to visualize the remaining substrate. Unstained “holes” correspond to the sites where the enzyme is located in the tissue.

- **Tissue fixation:**

  Submandibular and sublingual glands were fixed in 2% formal-calcium for 24 hours at 10°C; fixed frozen cryostate sections of 6 µ thickness were used.

- **Preparation of substrate film:**

  5 gm of hydrolyzed starch powder was dissolved in 100 ml borate buffer (0.02 M boric acid and 0.01 M sodium hydroxide). The suspension was heated with continuous stirring; temperature approaching 100°C. Thoroughly degassed the solution and allowed the starch solution to reach 70°C and the slides to reach 22°C. The slides were dipped into the starch solution for 1 minute, setted the slides on end at 22°C. The slides were used within 2 to 4 hours and they remained usable for 7 to 10 days.

- **Procedure:**
  
  i. Sections were placed on starch substrate film slide and incubated in moist chamber at 37°C for up to 45 minutes.

  ii. Fixed starch film and tissue sections for 1 hour in mixture of methanol, acetic acid and distilled water (in a ratio of 5:1:5)

  iii. Rinsed in running tap water for 1 minute.

  iv. Stained in Lugol’s iodine solution for 1 minute.
v. Wiped off starch film from unused side of the slide.

• Result:
Areas on the starch film corresponding to site of amylase in the tissue sections remain unstained.

I. Kinetic Study of Amylase:
In kinetic study, Km and Vmax were studied in submandibular and sublingual salivary glands of adult and old male mice. In kinetic study, only effect of the substrate concentration on enzyme activity was investigated.

• Effects of substrate concentration on Enzyme activity:
For determination of the values of Km and Vmax of amylase, the rate of hydrolysis of the starch by the enzyme was measured using starch of different concentrations as below:

1) 0.1%  2) 0.2%  3) 0.3%  4) 0.4%  5) 0.5%
6) 0.6%  7) 0.7%  8) 0.8%  9) 0.9%  10) 1.0%

Using above concentrations of the substrate and keeping incubation time 15 minutes, temperature 37°C constant and pH 6.7, the enzyme activity was estimated. The amylase activity was calculated for per mg tissue proteins. The graph was plotted as enzyme activity V/s substrate concentration. The km and Vmax values of amylase were obtained from this graph as described by Eisenthal and Cornish Bowden, (1974).

J. Estimation of Fucose: (Dische & Shettles 1948)

• Reagents:
  i.  0.02% standard α-D (+) fucose in distilled water.
  ii. Mixture of cold H₂SO₄ and distilled water (6:1).
  iii. 3% cystein hydrochloride in distilled water.

• Procedure:
For estimation of fucose, submandibular glands and sublingual glands were weighed and homogenized in distilled water (2 mg/ml
distilled water). The homogenates were centrifuged at 5000 rpm for 10 minutes at 10°C. Supernatants were used for estimation of fucose. Additions were done as below.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Standard</th>
<th>Blank</th>
<th>Sample</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sample</td>
<td>----</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>2.</td>
<td>Standard</td>
<td>1.0 ml</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>α-D(+) fucose(0.02%)</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>3.</td>
<td>Distilled water</td>
<td>----</td>
<td>1.0 ml</td>
<td>----</td>
</tr>
<tr>
<td>4.</td>
<td>Cold H$_2$SO$_4$</td>
<td>4.5 ml</td>
<td>4.5 ml</td>
<td>4.5 ml</td>
</tr>
<tr>
<td>5.</td>
<td>3% cystein hydrochloride</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

The tubes were shaken vigorously and placed at 20°C for 10 minutes. Then tubes were capped with glass bulbs and placed in vigorously boiling water bath exactly for 3 minutes. Then added

| 5.     | 3% cystein hydrochloride | 0.1 ml | 0.1 ml | 0.1 ml | ---- |

Mixed well and kept them at room temperature for two hours. The absorbance was determined at 400 nm and 430 nm adjusting calorimeter to zero with blank.

- **Calculations:**
  1. O.D. of the control at 400 nm − O.D. of the control at 430 nm = $X$
  2. O.D. of sample at 400 nm − O.D. of sample at 430 nm = $Y$
  3. Actual O.D. of sample, $A = Y − X$
  4. O.D. of standard at 400 nm − O.D. of standard at 430 nm = $Z$
  5. Actual O.D. of standard, $B = Z − X$
• Formula:

\[ \mu g \text{ fucose/mg. Proteins} = \frac{\text{O.D. of sample A}}{\text{O.D. of standard B}} \times \text{Amount of standard} \]

**Estimation of Sialic Acid**: (Warren 1959)

• **Reagents:**
  
  i. N-acetyleneuraminic acid (0.001%)
  
  ii. 0.1 N H\textsubscript{2}SO\textsubscript{4}.
  
  iii. 0.2 M sodium metaperiodate in 9 M phosphoric acid.
  
  iv. 10% sodium arsenite in a solution of 0.5 M sodium sulfate and 0.1 N H\textsubscript{2}SO\textsubscript{4}
  
  v. 0.6% thiobarbituric acid in 0.5 M sodium sulfate.
  
  vi. Cyclohexanone.

• **Procedure:**

For estimation of sialic acid submandibular and sublingual salivary glands were homogenized in distilled water (2 mg / ml distilled water). Sulphuric acid was added to the homogenates to a final concentration of 0.1 M and centrifuged at 5000 rpm at 10°C for 10 minutes and then heated at 80°C for one hour to release bound sialic acids. Aliquots were assayed in triplicates by thiobarbituric acid methods (Warren, 1959). Test tubes were labeled as blank, standard and sample, additions were carried out as follows:

<table>
<thead>
<tr>
<th>Sr.No</th>
<th>Standard</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Sample</td>
<td>---</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>2.</td>
<td>N-acetyleneuraminic acid (0.001%)</td>
<td>0.2 ml</td>
<td>---</td>
</tr>
<tr>
<td>3.</td>
<td>0.1 N H\textsubscript{2}SO\textsubscript{4}</td>
<td>---</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>4.</td>
<td>Periodate solution</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

41
Mixed well and allowed to stand at room temperature for 20
minutes and then added.

<table>
<thead>
<tr>
<th></th>
<th>10% sodium arsenite</th>
<th>1.0 ml</th>
<th>1.0 ml</th>
<th>1.0 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.</td>
<td>Shaken till yellow brown color disappeared and then added</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>0.6% thiobarbituric acid</th>
<th>3.0 ml</th>
<th>3.0 ml</th>
<th>3.0 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.</td>
<td>Mixed the content vigorously by capping and shaking.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It was heated in boiling water bath for 5 minutes and then added

<table>
<thead>
<tr>
<th></th>
<th>cyclohexanone</th>
<th>4.3 ml</th>
<th>4.3 ml</th>
<th>4.3 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>Shaken till red color come to organic phase, centrifuged for about 3 minute at 1000 rpm. The clear top of cyclohexanone phase was transferred to cuvettes. The optical density was determined 549 nm against blank.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Reaction:**

Sialic acid can usually be liberated by very mild acid hydrolysis from sample because of their glycosidic bonds. Complete release of this sugar can be accomplished by the use of 0.1 N H$_2$SO$_4$ at 80°C for one hour. This reaction is specific for the determination of sialic acid.

**Calculations:**

\[
\text{µg sialic acid/ mg protein} = \frac{\text{OD of sample} \times \text{amount of standard}}{\text{OD of standard} \times \text{amount of sample}} \times \text{proteins/mg tissue}
\]

**K. Histochemical Demonstration of Glycoproteins:**

Glycoproteins are the complex proteins having short and branched carbohydrate chains, usually with no indication of specific repeating units. The carbohydrate complexes of salivary glands are the glycoproteins, consists of at least two of the following components viz; hexoses, fucose and sialic acid. Glycoproteins are of two types, neutral
glycoproteins and acid glycoproteins. Acid glycoproteins classified as sulphomucins and sialomucins.

Periodic Acid Schiff reagent gives positive pink reaction to all the neutral glycoproteins and most of the acid glycoproteins (Mc Manus, 1946; Hotchkiss, 1948). Alcian blue at pH 2.5 stains mainly sialomucin, whereas at pH 1.0 stains sulphated glycoproteins (Steedeman, 1950; Scott and Dorling, 1965).

- **Tissue fixation:**

  Submandibular and sublingual glands were fixed in 2% formal calcium for 24 hours at 4°C. The glands were washed in running tap water for 24 hours, dehydrated through alcoholic grades, cleaned in xylene and embedded in paraffin. The sections of 7µ thickness subjected to the following staining techniques for histochemical localization of glycoproteins.

  a. **Periodic Acid–Schiff Reaction (PAS):** (Mc Manus, 1946; Hotchkiss, 1948):

     Periodic Acid–Schiff Reaction (PAS) method was used for the identification of glycoproteins. A positive staining reaction is given by all neutral glycoproteins and acid mucopolysaccharides (Mowry, 1956)

     i. After dewaxing and hydration, sections were brought to distilled water.

     ii. Sections were oxidized with 1% periodic acid for 10 minutes.

     iii. Sections were washed in distilled water for 5 minutes.

     iv. Treated with Schiff’s reagent for 10 minutes.

     v. Rinsed three to four times in 0.5% sodium meta-bi-sulfate (total 6 minutes).

     vi. Washed in distilled water followed by alcoholic dehydration.

     vii. Cleaned in xylene and mounted in D.P.X (Destrene Diphenyl Pthalate Xylene).
Result:
Periodic reactive mucosubstances were stained pink magenta.

b. Alcian blue (AB) at pH 1.0 (Mowry, 1956)

Presence of acidic mucosubstances containing sulphate group was studied by staining sections with Alcian blue (AB) at pH 1.0.

i. After dewaxing and hydration sections were brought to distilled water.

ii. Rinsed in 0.1 N HCl.

iii. Stained with AB (1% AB in 0.1 N HCl) for one hour.

iv. Rinsed in 0.1N HCl.

v. Washed in running tap water for 5 minutes.

vi. Dehydrated through alcohol grades.

vii. Cleaned in xylene and mounted in D.P.X.

Result:
Only sulphated mucosubstances stain selectively. The most strongly acid substances stain moderately i.e. acidic mucosubstances stain blue.

c. Alcian blue (AB) at pH 2.5 (Mowry, 1956).

Presence of acidic mucosubstances was studied by staining the sections with Alcian Blue (AB) at pH 2.5.

i. After dewaxing and hydration sections were brought to distilled water.

ii. Rinsed in 3% acetic acid.

iii. Stained with AB (1% in 3% acetic acid pH 2.5) for one hour.

iv. Rinsed in 3% acetic acid.

v. Washed in running tap water for 5 minutes.

vi. Dehydrated through alcohol grades.

vii. Cleaned in xylene and mounted in D.P.X.

Result: Acid mucosubstances stain blue.
d. PAS-Sodium borohydride technique for O-acetylated and non-acetylated sialic acid (Culling et al, 1976)

Presence of O-acetylated sialic acid was studied by staining the sections with Thionin-Sciff-Sodium Borohydride staining technique.

i. After dewaxing and hydration sections were brought to water.

ii. Oxidized in 1% periodic acid for 30 minutes.

iii. Washed in running water for 10 minutes.

iv. Placed in thionin-schiff reagent for 30 minutes.

v. Washed in running water for 10 minutes.

vi. Oxidized with 1% periodic acid.

vii. Washed in running water for 10 minutes.

viii. Treated with borohydride solution for 30 minutes.

ix. Rinsed in 70% ethanol.

x. Treated with 0.5% potassium hydroxide in 70% ethanol for 30 minutes.

xi. Washed gently in tap water.

xii. Placed in fresh 1% periodic acid for 10 minutes.

xiii. Washed gently in running water for 10 minutes.

xiv. Placed in standard Schiff’s reagent for 30 minutes.

xv. Washed gently in running water for 10 minutes.

xvi. Dehydrated, cleared and mounted with D.P.X.

• Results:

PAS positive materials (including non-acetylated sialic acid) stained blue, while O-acetylated sialic acid were stained red. Sites with a mixture of both were stained purple.
L. Electrophoretic Separation of Glycoproteins.

(Ornstein, 1964; Davis, 1964)

The electrophoresis means the migration of charged particles under the influence of an electric field. It is single phase system and depends upon the relative mobility of ions under identical electrical conditions. The temperature and the current in the system are kept constant during the electrophoresis run.

Polyacrylamide gel electrophoresis is widely accepted, which is formed by the polymerization of the two monomers, acrylamide ($\text{CH}_2\text{CH-C} \text{-NH}_2$) and cross linking agent $\text{N,N-methylene bisacrylamide CH}_2\text{CH}_2\text{CH-CO-NH-CH}_2\text{NH CO-H-CH}_2$. The pore size of the gel decreases with increasing proportions of the bisacrylamide with a limiting value of 5% giving minimum pore size. Polymerization of the gel was achieved using ammonium per sulfate as catalyst. TEMED ($\text{N,N,N’N’ tetramethyl ethylene diamine}$) is used as an indicator. The PAGE was carried out in vertical slab gel (Andrew, 1981).

In this apparatus the gel was polymerized between two glass plates (140 mm × 160 mm × 3 mm) in a separate mould. The front plate has a slot of 20 mm deep cut in it and two plates were held apart with Lucite spacers 6 mm wide and 2 mm thick. The plates were clamped together and sealed round the edges with melted 1.5 percent agar solution to ensure a leak free seal. The acrylamide solution was poured in and the sample slot former (comb) was inserted. After polymerization comb was removed to leave a series of sample wells. The lower Lucite spacer was also removed, and the mould was then fitted into the main apparatus with the lower end resting on the plate support and clamped into position with the slot in the glass plate aligned with the slot in the side of the upper electrode chamber. A liquid tight seal was again made with the aid of melted 1.5% agar. Enough buffer solution was then added to the upper
chamber to flow across the slots and cover the top of the gel. The lower chamber was filled with buffer, the samples were applied and the apparatus was then ready for electrophoresis. The major advantage of slab gel is that a number of sample slots can be formed within a single slab so that several samples can be run side by side under identical conditions.

- **Preparation of the sample:**

  For electrophoretic study, sample (50 mg salivary glands / ml) was prepared in distilled water. Sample was centrifuged at 5000 rpm for 10 minutes at 10°C. The supernatants thus obtained were used for the preparation of sample dye.

- **Preparation of sample dye:**

  0.5 ml supernatant + 0.3 ml glycerol + 50 mg sucrose + 0.001% bromophenol blue. The higher density of the sample was achieved by the addition of glycerol and sucrose. Bromophenol blue was used as a tracking dye. A total 20 µg protein was loaded per well with Hamilton micro syringe.

- **Preparation of gel and buffer:**

  i.  **Separation gel buffer (0.07M, pH 7.5)**

      Tris (Hydroxyl methyl) 0.856 gm
      1 M HCl 0.06 ml

      Dissolved up to 100 ml distilled water.

  ii.  **5% PAG**

      Acrylamide 4.75 gm
      Bisacrylamide 0.25 gm
      TEMED 0.05 ml
      Ammonium per sulfate 50.00 mg

      Dissolved in 100 ml separation gel buffer pH 7.5

  iii.  **Electrode chamber buffer (pH 7.0)**
Na – Diethyl Barbituric acid 5.500 mg
TRIS (Hydroxymethyl) 1.0 mg
Dissolved up to 1000 ml distilled water and pH was adjusted with 1 M HCl

- **Electric supply:**
  During the operation of electrophoresis the voltage was kept constant at 150 V. and 3 mA current / well was employed. During first 5 minutes only 2 mA/well current was employed, to avoid diffusion. The separation time was about one hour. Electrophoresis was stopped when the dye reached at the tip leaving 2 inches distance from the base. The mobility was from cathode to anode.

- **Gel staining:**
  a. **Total proteins staining method:** (Chrambach et al, 1967)
    i. Slab gel was immersed in 12.5% TCA for 1 to 3 hours before staining.
    ii. Washed with distilled water.
    iii. Gel was placed in Coomassie Brilliant Blue R 250.
        (1 gm Coomassie Blue R 250 dissolved in 500 ml methanol + 100 ml acetic acid + 400 ml H₂O) for one hour.
    iv. Gel was placed for destaining (500 ml methanol + 100 ml acetic acid + 400 H₂O) until excess stain was removed.
  b. **Glycoprotein staining by AB pH 1.0:**(Zacharius et al, 1969)
    The most widely used specific glycoproteins staining method the AB pH 1.0 was carried out as follows.
    i. Gel slab was immersed in 100 ml 12.5% TCA for one to three hours.
    ii. Gel slab was washed with cold distilled water.
    iii. Gel slab was placed in Alcian blue pH 1.0 (1%AB in 0.1N HCl)
    iv. Washed frequently with water until excess stain was removed.
• Gel fixation and storage:
  Gel slab was fixed and stored in 5% glacial acetic acid.

• Gel photography:
  The stained gel slab was photographed to record the observations.

3. STATISTICAL METHODS : (Ficher, 1936; Snedecor, 1946; Wills, 1949)

A. \( \overline{X} = \) Arithmetic mean of \( X \) independent variables.
\[
\overline{X} = \frac{\sum X}{N}
\]
Where \( N = \) number of variables

B. Standard Deviation = S.D.
\[
S.D. = \sqrt{\frac{\sum (X - \overline{X})^2}{N - 1}}
\]

C. Statistical significance:
\[
S = \sqrt{\frac{(N_1 - 1)S_1^2 + (N_2 - 1)S_2^2}{N_1 + N_2 - 2}}
\]
Where, \( N_1 = \) Number of observations of first group.
\( N_2 = \) Number of observation of second group.
\( S_1 = \) Standard deviation (SD) of first group.
\( S_2 = \) Standard deviation (SD) of second group.
D. Student ‘t’ test:

\[ t = \frac{\bar{X}_1 - \bar{X}_2}{S} \sqrt{\frac{N_1 \times N_2}{N_1 + N_2}} \]

Where, \( \bar{X}_1 \) = Mean of first group.
\( \bar{X}_2 \) = Mean of second group.

The probability ‘P’ of obtained ‘t’ value was tallied from the tabulated ‘t’ value from Fischer’s table at the probability level 0.1, 0.02, 0.05, 0.01, 0.001 at the respective degrees of freedom. In the present work the degree of freedom was ‘eight’. If the calculated value was higher than the tabulated value at the probability level 0.01, 0.05, 0.001 then the difference was accepted as significant. The ‘P’ values are significant according to the following conversions.

- \( P > 0.01 \) = The difference is said to be non-significant.
- \( P > 0.05 \) = The difference is said to be non-significant.
- \( P > 0.1 \) = The difference is said to be non-significant.
- \( P < 0.05 \) = The difference is said to be almost significant.
- \( P < 0.02 \) = The difference is said to be significant.
- \( P < 0.01 \) = The difference is said to be significant.
- \( P < 0.001 \) = The difference is said to be highly significant.
### 4. LIST OF CHEMICALS

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of the chemical</th>
<th>Batch No.</th>
<th>Source</th>
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<tbody>
<tr>
<td>1</td>
<td>D – galactose</td>
<td>RM 101</td>
<td>Hi – Media</td>
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<tr>
<td>2</td>
<td>centrophenoxine</td>
<td>G - 8773</td>
<td>Sigma</td>
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<td>Chloroform (AR)</td>
<td>LB 189406</td>
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<td>SRL</td>
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<tr>
<td>7</td>
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<td>s.d.fine</td>
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<td>Spectrochem</td>
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<td>Sodium arsenite</td>
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<td>S.Qualigen</td>
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<td>N,N,N,N’-tetramethylene diamide (TEMED)</td>
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