Chapter II: Material and Methods

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A. Material

Swiss male albino mice (Mus musculus Linn.) were used for the present investigation. The breeding pairs of mice were obtained from Hindustan Antibiotics Ltd. Pune. The animal ethics committee approved the protocol of animal experiments. The mice were allowed to bred and reared in the air-conditioned animal house of the department (Registration no. CPCSEA/233). They were housed in aluminum cages having dimensions of 10” x 8” x 12” in group of 3 to 4 with rice husk bed. They were fed with Amrut Rat/Mice feed, which was obtained from Pranav Agro Industries Ltd. Sangli and water was given ad Libitum.

To asses the structure and function of sublingual glands in the absence of submandibular glands in male mice, the effect of sialoadenectomy on protein content, amylase content and glycoprotein content in male mice was examined. For this the animals were operated at the age of 20 days and they were sacrificed at the age of three months after operation.

1. Control Group

Twenty male offsprings were sham operated on 20\textsuperscript{th} day of their age. Sham-operated animals were subjected to identical procedures like sialoadenectomy, except that the glands were not removed. Operated mice were maintained in animal house with proper care and were sacrificed by cervical dislocation at the age of three months.
2. Sialoadenectomised group

Sialoadenectomy is the removal of submandibular glands from both sides. Twenty days old twenty male mice were selected for the study of sublingual gland structure and function after sialoadenectomy.

Sialoadenectomy was carried out under mild ether anesthesia. Operations were carried out in between 8 a.m. to 10 a.m. Operated mice were maintained in animal house with proper care and were sacrificed by cervical dislocation at the age of three months and then sublingual glands were dissected out and were subjected to the following investigations,

1. Histology of sublingual gland by HE technique.
2. Electron microscopy of sublingual gland.
3. Estimation of amylase activity
5. Electrophoretic separation of proteins.
7. Estimation of sialic acid.
B. Methods

- Preparation of Homogenates

From each group mice were weighed and sacrificed between 9.00 am to 10.00 am by cervical dislocation. Their sublingual glands from both sides were dissected, pooled and weighed. The sublingual gland from one side were used to prepare homogenates in different homogenizing media as per the requirements of the respective methods viz, estimation of proteins, amylase, fucose and sialic acid. Sublingual glands from other side were used for histology, histochemistry and electrophoresis.

1. Histology:

For histological study the sublingual glands were fixed in 2% calcium acetate formaldehyde (CAF) for 24 hours. The glands were washed in running tap water for 24 hours, dehydrated through alcoholic grades, cleared in xylene and embedded in paraffin wax. The sections were cut at a thickness of 7µ and stained with Hematoxyline and Eosin (H/E).

- Standard HE technique:

  i. After deparaffinization, sections were brought to distilled water.
  
  ii. Stained with hematoxylene for 8 minutes.
  
  iii. Washed under running tap water for 5 minutes.
  
  iv. Transferred in ascending series of alcoholic grades.
v. Stained with alcoholic eosin (prepared in 90% alcohol) after staining washed in 90% alcohol and dehydrated in absolute alcohol.

vi. Cleared in xylene and mounted in DPX.

2. Transmission Electron Microscopy (TEM):

Transmission electron microscopy (TEM) is a microscopy technique whereby a beam of electrons is transmitted through an ultra thin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of electrons transmitted through the specimen; the image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor such as a CCD camera.

- To observe under TEM, the specimen to be used must be cut into ultra thin slice with the help of broken glass or diamond shape knife by microtome.
- The sections are stained with heavy metals as lead or uranium to increase the contrast between internal structures i.e. a beam of electrons is passed through this specimen.
- A condenser lens focuses the beam of electrons on to the sample. The beam strikes the specimen and parts of beam are transmitted.
- The transmitted portion is focused by the objective lens into image. The image strikes the phosphor image screen and the light is generated that is allowed to view.
The darker areas of the image represent the areas of sample that fewer electrons were transmitted through. This represents thick or denser portion of the sections; whereas the lighter area represents the area of the sample where more electrons were transmitted and is thinner, less dense portion.

The resolution power of TEM is 0.1µm. TEM cannot visualize the surface of unsectioned specimens, because electrons pass through the entire specimen.

Transmission Electron Microscopy technique:

- **Fixative:**
  i. 3% glutaraldehyde in 0.1M phosphate buffer pH- 7.2.
  ii. 1% Osmium tetroxide

- **Method:**
  i. The sublingual gland was cut into 1mm x 1mm size. Fixed in glutaraldehyde fixative for 24 hours.
  ii. Then the sublingual gland was washed in buffer for 15 minutes (2 changes) (Glutaraldehyde completely removed).
  iii. It was post fixed in 1% osmium tetroxide- 1 ½ - 2 hours.
  iv. Washed in buffer.
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❖ **Dehydration:**

Then the gland was dehydrated through alcohol grades.

i. 70% alcohol- 1 hrs
ii. 80% alcohol- 1 hrs
iii. 90% alcohol- 1 hrs
iv. 100% alcohol- 1 hrs (30 minutes 2 changes).

❖ **Clearing:**

After dehydration the gland was cleared in propylene oxide for 15 minutes (2 changes).

(Note- Excess time in propylene oxide will make the tissue brittle)

❖ **Infiltration:**

Resin was prepared freshly

10 ml- of Araldite Cy 212- Epoxy Resin
10 ml- Dodecenyl Succinic Anhydride (DDSA)
1 ml- Dibutylphthalate
0.4 ml- Dimethyl amino phenol (DMP)

i. The contents were mixed thoroughly in magnetic stirrer for 15 to 30 minutes. Followed by keeping in the oven at 60 °C till all air bubbles were removed.

ii. 1:1 solution of propylene oxide and epoxy resin was prepared.

iii. The gland was transferred to above solution and kept in rotator over night.
iv. The gland was transferred to fresh epoxy resin and kept in rotor for 6 hours.

**Embedding:**

The resin was poured into beam capsule/ flat embedding moulds. The labels were placed and the tissue was oriented under stereomicroscope and kept in the oven at 60 °C for 48 hours for polymerization.

**Sectioning:**

Selected area for ultra thin sections was marked out and the blocks were further trimmed. Ultra thin sections (800 Å) were cut on the ultramicrotome LEICA EM UC6 and the sections were collected on 300 mesh copper grids.

To ensure more contrast, the grids were stained with a double staining technique with 10% uranyl acetate for 30 minutes and lead citrate for 10 mins.

**Photography:**

The grids were observed on a transmission electron microscope Techani G2 at 60KV accelerating voltage and photographed for observation.
3. Estimation of Amylase activity (Jayaraman, 2000):

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

- **Procedure:**
  
  For estimation of amylase activity sublingual glands were weighed and homogenized in distilled water (1mg/ml distilled water). The homogenates were centrifuged at 3000 rpm for 10 minutes at 10°C. Supernatants were used for estimation of amylase activity. The additions for the estimation of amylase activity were made as follows, three readings were taken:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Blank (ml)</th>
<th>Sample (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>2.</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>3.</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Mix well and tubes were kept for 10 minutes at 37°C

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Amount</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.</td>
<td>Distilled water</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>5.</td>
<td>Sample</td>
<td>--</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Incubated for the 15 minutes at 37°C and added

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Amount</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.</td>
<td>2N NaOH</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>7.</td>
<td>1% Di-nitro-salicylic acid</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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.

 ключ

Calculations:

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

\[
\text{Amylase activity} = \frac{\mu g \text{ maltose from graph}}{\mu g \text{ maltose/mg Protein} \times \text{Amount of protein/mg tissue}} \times \text{dilution}
\]
4. Estimation of Protein (Lowry, 1951):

Homogenization of the sublingual gland was carried out using refrigerated glass mortar and pestle. Sublingual gland was crushed at the bottom of the mortar by instantaneous freezing and gradual thawing with cold distilled water (0.1 mg/ml distilled water). The perfectly uniform homogenates were centrifuged at 10°C at 3000rpm for 10 minutes. The supernatant was used for estimation of proteins.

❖ Reagents:-
  i. Reagent A :- 2% Na$_2$CO$_3$ in 0.1 N NaOH
  ii. Reagent B :- 0.5% CuSO$_4$ in 1% Na-K tartarate
  iii. Reagent C :- 50 ml A + 1ml B (This was prepared at the time of use)
  iv. Reagent D :- Folin Ciocalteau Phenol reagent
  v. Standard protein solution:- Bovine Serum Albumin (1mg in 10ml)

❖ Folin Ciocalteau-Phenol Reagent

100 gm sodium tungstate Na$_2$WO$_4$·2H$_2$O and 25 gm sodium molybdate Na$_2$MoO$_4$·2H$_2$O were dissolved in 700 ml distilled water. 100 ml HCl and 50 ml 85% phosphoric acid were added and the mixture was refluxed for 10 hours in glass apparatus. There after added 150gm of lithium sulfate followed by 50ml distilled water and few drops of Br$_2$. Boiled for 15 minutes to remove excess bromine, cooled diluted upto 1000ml and then filtered.
The reagent was golden yellow colour, stored in refrigerator. This was stock solution and was diluted with equal volume of water just before use.

Procedure:

0.5ml supernatant was taken in test tube, that test tube was labeled as sample. Standard tube was prepared using bovine serum albumin. Additions were made as follows

<table>
<thead>
<tr>
<th></th>
<th>Sample</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>0.5ml</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>----</td>
<td>0.5ml</td>
<td>----</td>
</tr>
<tr>
<td>Distilled water</td>
<td>----</td>
<td>----</td>
<td>0.5ml</td>
</tr>
<tr>
<td>Reagent C</td>
<td>5ml</td>
<td>5ml</td>
<td>5ml</td>
</tr>
</tbody>
</table>

Mix well and stand for 10 minutes at room temperature

<table>
<thead>
<tr>
<th></th>
<th>Sample</th>
<th>Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent D (Phenol reagent)</td>
<td>0.5ml</td>
<td>0.5ml</td>
<td>0.5ml</td>
</tr>
</tbody>
</table>

Mix well and wait for 30 minutes

Readings were taken at 660nm on Spectronic 20 colorimeter adjusting against blank to zero.
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❖ **Reactions**

This method is about 10 times more sensitive than the biuret method. The reagent, called Folin-Ciocalteau reagent, is quite complex and contains phosphomolybdic acid and tungstate. The aromatic acids, tyrosine and tryptophan, present in proteins react with these and produce dark blue colour.

❖ **Calculations:** The Protein concentration from sample was calculated by using standard graph of protein.

5. **Electrophoretic Separation of Proteins (Laemmli, 1970):**

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, describes a technique widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility (a function of the length of a polypeptide chain and its charge). In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis.
Polyacrylamide gel (PAG) had been known as a potential embedding medium for sectioning tissues as early as 1964, and two independent groups employed PAG in electrophoresis in 1959 (Davis, 1959 and Raymond, 1959). It possesses several electrophoretically desirable features that make it a versatile medium. It is a synthetic, thermo-stable, transparent, strong, chemically relatively inert gel, and can be prepared with a wide range of average pore sizes (Rüchel, 1978) The pore size of a gel is determined by two factors, the total amount of acrylamide present (%T) (T = Total concentration of acrylamide and bisacrylamide monomer) and the amount of cross-linker (%C) (C = bisacrylamide concentration). Pore size decreases with increasing %T; with cross-linking, 5% C gives the smallest pore size. Any increase or decrease in %C from 5% increases the pore size, as pore size with respect to %C is a parabolic function with vertex as 5% C. This appears to be because of non-homogeneous bundling of polymer strands within the gel.

**Preparation of Stock Buffers:**

**i. 4X Stacking gel buffer (0.5M Tris-HCl pH 6.8):**

60.5gm Tris base was dissolved in 850ml of H₂O and pH to 6.8 was adjusted with 6M HCl. Again the pH to 6.8 was readjusted at room temperature then distilled water was added to 1000ml and the buffer was stored at 4 °C.
ii. 4X Resolving (Separating) buffer (1.5 M Tris- HCl pH 8.8):

181.5gm of Tris base was dissolved in 850ml of H₂O and pH to 8.8 was adjusted with 6M HCl. The solution was cooled to room temperature and pH to 8.8 was readjusted. Then distilled water was added to 1000ml and was stored at 4°C.

iii. 10X Running buffer (500 ml):

1.520gm of Tris base, 7.240gm of glycine and 500mg of SDS was dissolved in 500ml distilled water. The pH was 8.3 and no adjustment of pH is required. The running buffer was stored at room temperature.

iv. 2X SDS-PAGE sample buffer:

Preparation of sample buffer was done by mixing

- 2.0ml of 4X stacking gel buffer
- 1.6ml of glycerol
- 3.2ml of 10% SDS
- 0.8ml of 2-β mercaptoethanol
- 0.4ml of 1% bromophenol blue
v. 30% (2.6% C) Acrylamide stock solution:

29.22gm of acrylamide and 0.78gm of bisacrylamide was added to 100ml of distilled water. The stock solution was filtered through Whatman filter paper and stored at 4 °C.

vi. 10% SDS stock solution:

10gm of SDS was dissolved in 80ml of distilled water and then volume was make upto 100 ml.

vii. 10% ammonium persulphate solution (Fresh):

10gm of ammonium persulphate was dissolved in 100ml of distilled water

viii. N, N, N', N'- tetramethylethlenediamine (TEMED): Used as supplied.

ix. Preparation of 10% separation and 4% stacking gel:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>10% Acrylamide</th>
<th>10% Separation gel (100ml)</th>
<th>4% Stacking gel (10ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>30% Acrylamide</td>
<td>33.33 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>2.</td>
<td>Resolving gel buffer</td>
<td>25.00 ml</td>
<td>----</td>
</tr>
<tr>
<td>3.</td>
<td>Stacking gel buffer</td>
<td>----</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>4.</td>
<td>Distilled water</td>
<td>39.87 ml</td>
<td>6 ml</td>
</tr>
<tr>
<td>5.</td>
<td>10% SDS</td>
<td>1.0 ml</td>
<td>100 µl</td>
</tr>
<tr>
<td>6.</td>
<td>10% Ammonium persulphate</td>
<td>0.75 ml</td>
<td>50 µl</td>
</tr>
<tr>
<td>7.</td>
<td>TEMED</td>
<td>0.05 ml</td>
<td>10 µl</td>
</tr>
</tbody>
</table>
x. Preparation of Sample:

Tissue was weighed and freezed. After freezing the homogenization of the tissue was carried out using refrigerated glass mortar and pestle. Tissue was crushed at the bottom of the mortar for instaneous freezing and gradual thawing with chilled distilled water. The concentration of homogenate was 50mg/ml of distilled water. Then the homogenate was centrifuged at 10°C for 10 minutes at 3000 rpm. The supernatant was used for electrophoresis.

xi. Sample dye:

1ml of supernatant + 1ml of sample buffer were mixed with each other and 30 µl of sample dye was loaded in each well with the help of micro syringe.

xii. Loading Gel:

a. Loading separation gel:

After proper sealing of the glass plates with agarose gel or sealing the glass plates with the help of agarose gel, the addition were made for 10% separation gel as per table. Swirling the flask and emergence of bubbles was avoided during additions. Finally the mixture was pipetted out and poured into sandwich of glass plates (0.4 x 19 x 17 cm) the level of about 4cm to of top. The gel was overlaid by applying about 0.3 ml of distilled water. The gel was allowed to set for half hour. A sharp liquid gel interface was visible when gel polymerizes.
b. Loading of stacking gel:

Gel sandwich was then filled with 4% stacking gel. The comb was inserted avoiding any bubble below the teeth of comb, as oxygen inhibits polymerization and may cause a local distortion in gel surface at bottom of wells. The gel was allowed to set at least for half hour.

The comb was removed carefully without disturbing any well divider. The wells were rinsed with distilled water and entire water was drained off by inverting casting stand.

The gel was fixed to electrophoresis unit (Bangalore GeNei) fixing upper and lower chamber. The upper and lower chambers were filled with the running buffer; the sandwich gel was allowed to immerse in buffer. 30 µl of sample dye was loaded in each well, with the help of Hamilton micro syringe.

xiii. Electric supply:

Finally the unit was connected to power supply. Voltage (150V) was kept constant during entire electrophoresis running. Electrophoresis was allowed to run in refrigerator at 10°C. Since glass has much higher thermal conductivity cooling is required. When dye front reached the tip of gel leaving distance 5mm the current / electric supply was stopped.
xiv. **Gel staining:**

Gel was kept in petri dish containing staining mixture and incubated at 60°C for 2 hours.

**Staining mixture:**

1gm Comassie Brilliant Blue R-250 dissolved in 500ml Methanol + 100ml Acetic acid + 400ml Distilled water.

xv. **Destaining:**

Several changes of destaining solution were given until the gel gets properly destained.

**Destaining mixture:**

500ml Methanol + 100ml Acetic acid + 400ml Distilled water.

xvi. **Gel fixation and storage:**

Gel slab was fixed and stored in 7% glacial acetic acid.

xvii. **Gel photography:**

The stained gel slab was photographed to record the observations.
6. **Electrophoretic Separation of Glycoproteins (Laemmli, 1970):**

SDS-PAGE for glycoproteins was carried out as described above for proteins.

i. **Glycoprotein staining by PAS:** (Packer, *et al.*, 1996)

The most widely used specific glycoproteins staining method the AB pH 1.0 was carried out as follows.

**Chemicals:**

1. Solution A: 1.0% (v/v) periodic acid in 3% acetic acid. Periodic acid is corrosive and volatile.
2. Solution B: 0.1% (w/v) Sodium metabisulfite in 10 mM HCl.
3. Schiff’s reagent.
4. Solution C: 50% (v/v) Ethanol.
5. Solution D: 0.5% (w/v) Sodium metabisulfite in 10 mM HCl.

Solutions A, B, and D should be made up freshly.

**Gel staining:**

1. The gels were soaked solution C for 30 min.
2. Washed in distilled water for 10 min. All of the ethanol removed from the gel.
3. The gels were incubated in solution A for 30 min.
4. Washed in distilled water for 6 × 5 min or 5 × 5 min and 1× overnight.
5. Then gels were washed in solution B for $2 \times 10$ min.

6. Incubated in Schiff's reagent for 1 h in the dark.

7. Then incubate in solution B for 1 h in the dark.

8. Washed with several times in solution D for a total of at least 2 h and leaved as long as overnight to ensure good color detection.

**ii. Gel fixation and storage:**

Gel slab was fixed and stored in 7% glacial acetic acid.

**iii. Gel photography:**

The stained gel slab was photographed to record the observations.

7. **Estimation of Sialic Acid:** *(Warren, 1959)*

   **Reagents:**

   i. N–acetylneuraminic acid (0.001%)

   ii. 0.1 N H$_2$SO$_4$.

   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$_2$SO$_4$.

   v. 0.6% thiobarbituric acid in 0.5 M sodium sulfate.

   vi. Cyclohexanone.
Procedure:

For estimation of sialic acid sublingual glands were homogenized in 0.1 N H$_2$SO$_4$ (1mg/ml) and heated at 80$^\circ$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-acetylneuraminic acid (0.001%)</td>
<td>0.2 ml</td>
<td>----</td>
</tr>
<tr>
<td>3.</td>
<td>0.1 N H$_2$SO$_4$</td>
<td>----</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>4.</td>
<td>Periodate solution</td>
<td>0.1 ml</td>
<td>0.1ml</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Standard</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.</td>
<td>10% sodium arsenite</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

Shaken till yellow brown color disappeared and then added.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Standard</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.</td>
<td>0.6% thiobarbituric acid</td>
<td>3.0 ml</td>
<td>3.0 ml</td>
</tr>
</tbody>
</table>

Mixed the content vigorously by capping and shaking.

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

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Standard</th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>Cyclohexanone</td>
<td>4.3 ml</td>
<td>4.3 ml</td>
</tr>
</tbody>
</table>

The tubes were shaken twice and then centrifuged for 3 minutes at 1000 rpm. The clear upper cyclohexanone phase red which was transferred to cuvettes. The optical density was determined at 549 nm against blank.
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- **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:**

  $\frac{\mu g \text{ sialic acid/mg protein}}{\text{O. D. of sample} \times \text{amount of standard}} = \frac{\text{O. D. of standard} \times \text{amount of sample}}{\text{proteins/mg tissue}}$

8. **Estimation of Fucose: (Dische and Shettles, 1948)**

- **Reagents:**
  
  i. 0.02% standard α-D (+) fucose in distilled water.
  
  ii. Mixture of cold H$_2$SO$_4$ and distilled water (6:1).
  
  iii. 3% Cystein hydrochloride in distilled water.

- **Procedure:**

  For estimation of fucose sublingual glands were weighed and homogenized in distilled water (2 mg/ml distilled water). The homogenate was centrifuged at 5000 rpm for 10 minutes at 10°C. Supernatant was 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 α-D (+) fucose (0.02%)</td>
<td>1.0 ml</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 H₂SO₄</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>----</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:

Mixed well and kept them at room temperature for two hours. The absorbance was determined at 400 nm and 427 nm adjusting colorimeter to zero with blank.

**Calculations:**

i. O.D. of the control at 400 nm – O.D. of the control at 427 nm = X

ii. O.D. of sample at 400 nm – O.D. of sample at 427 nm = Y

iii. Actual O.D. of sample, A = Y – X

iv. O.D. of standard at 400 nm – O.D. of standard at 427 nm = Z

v. Actual O.D. of standard, B = Z – X
9. Histochemical Demonstration of Glycoproteins:

Glycoproteins are proteins which carry covalently-bonded sugar units. The carbohydrate portions of glycoprotein are often called glycans which generally occur as oligosaccharides, and usually constitute 2-30% of the total weight of the molecule. However, some glycoproteins contain 50-60% or more carbohydrate.

The oligosaccharides almost always contain the hexose, N-acetylhexosamines and often terminate with sialic acids or L-Fucose residues. Although the oligosaccharides are often branched, they rarely contain more than 15 glycosyl residues. In its widest sense the term glycoprotein also covers polymers such as peptidoglycans and proteoglycans. Glycoproteins are of two types, neutral glycoproteins and acid glycoproteins. Acid glycoproteins classified as sulphomucins and sialomucins. In addition carbohydrates may be sulphated at variable degree.
Periodic Acid Schiff reagent gives dark pink to magenta colour 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:**

Sublingual glands were fixed in 2% calcium acetate formaldehyde for 24 hours. The glands were washed in running tap water for 24 hours, dehydrated through alcoholic grades, cleared 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, 1964; 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 gradual 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 and counter stained with hematoxylene for 10 minutes followed by alcoholic dehydration.

vii. Cleared in xylene and mounted in D.P.X (Destrene Diphenyl Pthalate Xylene).

❖ Result:

Periodic reactive mucosubstances were stained in pink magenta colour.

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, pH-1) for one hour.

iv. Rinsed in 0.1N HCl.

v. Washed in running tap water for 5 minutes.

vi. Counter stained with hematoxylene for 10 mins. Washed in distill water.

vii. Dehydrated through alcohol grades.

viii. Cleared in xylene and mounted in D.P.X.

❖ Result:

Only sulphated mucosubstances stain selectively.
c. Alcian blue (AB) at pH 2.5 (Mowry, 1956).

Presence of acidic mucosubstances was studied by staining 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% AB 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. Counter stained with hematoxylene for 10 mins. Washed in distilled water.
vii. Dehydrated through alcohol grades.
viii. Cleared in xylene and mount in D.P.X.

❖ Results:

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 Schiff-Sodium Borohydride staining technique.

i. After dewaxing and hydration sections are 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 (freshly prepared) 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 in xylene and mounted with D.P.X.

Results:

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

C. Statistical analysis:

Statistical analysis were done by using analysis software for One Way Analysis of Variance for Correlated and Independent Samples and Tukeys Post hoc test were applied.
### D. List of chemicals:

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of Chemicals</th>
<th>Batch no.</th>
<th>Source</th>
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<tbody>
<tr>
<td>1.</td>
<td>Acetic Acid</td>
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<tr>
<td>2.</td>
<td>Alcian Blue</td>
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</tr>
<tr>
<td>3.</td>
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<td>4.</td>
<td>Bis-acrylamide</td>
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<tr>
<td>5.</td>
<td>Bovine Albumen Fraction</td>
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<td>Bromophenol blue</td>
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<td>8.</td>
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<tr>
<td>9.</td>
<td>Comassie Brilliant Blue</td>
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<td>Folin Ciocalteau phenol reagent</td>
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<td>LOBA chemicals</td>
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1. Introduction

2. Material and Methods
   a. Histology of sublingual gland-HE technique
   b. Mucous acinar cell count
   c. Transmission electron microscopy

3. Results

4. Discussion