# CHAPTER TWO

## MATERIAL AND METHODS

MATERIAL

Albino rats (Rattus norvegicus) of both the sexes were used for this present investigation. Rats were obtained from Hindustan Antibiotics, Pune and maintained in the laboratory in individual cages. The rats were supplied with Gold Mohur rat feed (Lipton India Limited) and water ad libitum.

All the animals (normal, control and treated) were sacrificed by cervical dislocation. The stomach and duodenum of rats were dissected out and opened along the greater curvature of stomach and mesentry of duodenum. Then they were examined under occular magnifier and stereoscopic microscope. The morphological observations were made by fixing the stomach and duodenum in wax tray containing 0.9 % saline chilled to 4°C. Tissues were fixed in respective fixatives to study histology and histochemistry. Bioassays of protein, β-glucuronidase and electrophoretic seperations were carried out in tissue homogenates.

METHODS

I. Whole Mount Preparation of Brunner's Glands

Duodenums of rats were processed according to the method used by Landboe-Christensen (1944b). It involved washing of the duodenums (3 cm long from pyloroduodenal junction) which were split opened and pinned into a small wax tray in cold running water till the mucous secretion from Brunner's glands was stopped.

The tissues were cleared in 3 % acetic acid, washed again

in water and stained for 1 day in 1 % solution of haematoxylin. The extra stain was removed in 3 % acetic acid. The muscularis mucosa and externa were carefully removed so that Brunner's glands could be observed directly.

## II. Histology

To study the histological structure of the Brunner's glands, the duodenums were fixed in 10 % neutral buffered formalin for 24 hrs at 4°C. The tissues were washed in running tap water for 24 hrs, dehydrated through alcohol grades, cleared in xylene and embedded in paraffin. The sections were cut at a thickness of 7 μ on a rotary microtome (Spencer type). The sections were mounted on albuminzed glass slides and routinely stained with haematoxylin and eosin (H-E).

## III. Histochemistry

### A. Glycoprotein

Polysaccharides are classified as glycogen and muco-polysaccharides. The mucopolysaccharides are further classified into neutral mucopolysaccharides and acidic mucopolysaccharides (Spicer et al., 1965). Neutral glycoproteins, immunologically reactive glycoproteins, fucomucins and manose rich muco-substances are neutral mucopolysaccharides and all are periodate reactive. Sulfated mucosubstances and carboxyl mucosubstances are called as acid mucosubstances. Some of them are periodate reactive and some are periodate non-reactive (Roberts, 1977). But 1 % alcian blue (pH 2.5 - 2.7) stains both the types of acid mucopolysaccharides. Alcian blue (0.5 %) in pH 1.0 stains only

sulfated mucosubstances (Spicer et al., 1967 and Pearse, 1968).

a. Periodic Acid-Schiff Reaction

The periodic acid-Schiff reaction includes two steps of reaction -

i) Oxidation by periodic acid: Periodic acid is an oxidizing agent that is known for its ability to oxidize 1, 2 glycols to form dialdehydes. Many other oxidizing agents could be theoritically used as well, and some have been actually substituted for periodic acid. However, other oxidizing agents are not convenient to use, because the aldehydes are further oxidized to carboxyl groups.

ii) Aldehydes are demonstrated with Schiff reagent and tissue sections are given several rinse in 0.5 % aqueous solution of sodium metabisulfide as reducing agent. The PAS-procedure colors glycogen and this can be used to demonstrate glycogen by diastase digestion using control. To demonstrate mucosubstances in tissues, periodic acid-Schiff technique was first introduced by McManus (1946) followed by Hotchkiss (1948).

Method

i) Tissues were fixed in 10 % neutral buffered formalin for 24 hrs at 4°C, they were washed, dehydrated through alcohol grades, cleared in xylene and paraffin blocks were prepared. The sections were cut on rotary microtome (Spencer type) at 6 to 7 μ of thickness.

ii) After dewaxing and dehydration, the sections were brought to distilled water.

iii) Sections were oxidized with 0.5 % periodic acid for 10 minutes.

iv) Sections were washed with distilled water and treated with Schiff reagent for 10 minutes followed by rinsing, 3 times in 0.5 % sodium metabisulfide.

v) Sections were washed thoroughly in distilled water, dehydrated through alcohol grades, cleared in xylene and mounted in D.P. X (Distrene diphenyl Pthalate Xylene).

b. Enzyme Digestion Technique - Malt diastase.

This technique is used as an aid in identifying the sites of glycogen in tissue sections. Lillie and Greco (1947) introduced the use of malt diastase or ptylin for the digestion of glycogen in tissue sections. Originally they recommended the use of 1 % solution of enzyme preparation. Commenting on their method, McManus and Saunders (1950) reported that a 1 % solution of diastase at pH 6.8 removes all PAS-positive materials from tissue sections and usually the sections from slides. Lillie later recommended that a 1/1000 solution of malt diastase (pH 5 to 8) be used rather than 1 % solution. The malt diastase in which α-amylase is the principle hydrolytic enzyme (Pearse, 1960) and take diastase (Myrback and Neumuller, 1950) hydrolyze starch, glycogen and certain degradation products originating from these polysaccharides. This hydrolysis is accomplished (in vitro) by cleavage of the glucosidic 1, 4 linkage of starch or glycogen to yield sugars.

Method

i) Tissues were fixed in 10 % neutral buffered formalin for 24 hrs at 4°C, washed under running tap water for more than 6 hrs, dehydrated and embedded in paraffin. Tissue sections were cut at 7 to 8 μ thickness.

ii) Sections were deparaffinized in xylene, dehydrated through alcohol grades and brought to distilled water.

iii) Sections were treated with malt diastase solution for four hrs. (malt diastase solution was prepared as follows - 1. Buffered neutral saline - Sodium chloride 8.0 gm + sodium phosphate dibasic 1.3 gm. + sodium phosphate monobasic 0.8 gm. + distilled water 100 ml.
2. Malt diastase solution - malt diastase 0-1 gm. + 100 ml buffered neutral saline 1).

iv) Sections were washed with distilled water and processed for PAS-reaction, as described earlier (III A : a).

c. Alcian Blue Staining Method

Alcian blue 8, the textile dye, was adopted as histochemical stain specific for 'mucins' by Steedman (1950). The specificity was improved by Mowry (1956) by using higher concentration of dye at a lower pH. Alcian blue is unique in three ways : i) it does not have affinity for nucleic acids ii) it stains carboxyl groups at pH substantially below their pk value iii) it has high solubility even in the presence of high concentration of salts. Spicer (1960) in his comparison of affinity to alcian blue and

azure A observed that alcian blue exhibited greater resistance to acid and alkali decoloration than that of azure A. This property of alcian blue led Spicer to suggest that alcian blue is not held by a salt type linkage like that of which is apparently involved in azure A binding. Numerous studies have been made to ascertain the specificity of alcian blue for acid mucopolysaccharides (Vialli, 1951; Lison, 1954). Spicer (1960) studied the effect of pH on alcian blue affinity and observed that dilute solutions of the dye show a strong affinity for mucins from pH 1.0 to 3.0, but almost none at pH 3.0 to 4.0.

Method

i) Tissues were fixed in 10 % neutral buffered formalin for 24 hrs. at 4°C; they were washed, dehydrated through alcohol grades, cleared in xylene and paraffin blocks were prepared. Sections were cut at 6 to 7 µ thickness.

ii) Sections were deparaffinized and brought to distilled water.

iii) They were treated with 3 % acetic acid for 5 minutes and then stained with alcian blue for 30 minutes (1 % alcian blue in 3 % acetic acid pH 2.5).

iv) Sections were washed in running tap water for 5 minutes, dehydrated through graded alcohols, cleared in xylene and mounted in D.P.X.

B. β-Glucuronidase

The enzyme β-glucuronidase could be histochemically demonstrated using different substrates. Friedenwald and Becker (1948) demonstrated β-glucuronidase using naphthyl

glucuronide and 8-hydroxyquinoline glucuronides as substrates. Rath and Otto (1966) used 8-hydroxyquinoline as substrate with simultaneous coupling with fast black K salt. Bulmer (1966) followed the method of Rath and Otto using different coupling salts. But Bulmer felt that better results could be obtained with naphthol compounds. Hayashi et al. (1964) demonstrated β-glucuronidase using naphthol AS-BI-β-D-glucuronide as substrate and hexazotized pararosanilin as the simultaneous coupling agent. A more widely used method is of Fishman and Goldman (1965) where naphthol AS-BI-β-D-glucuronide is used as substrate and fast Garnet GBC salt as coupling agent.

Post-coupling technique employing Naphthol AS-BI-β-D-Glucuronic Acid as substrate for the demonstration of β-glucuronidase (Fishman and Goldman, 1965)

i) The stock substrate solution of naphthol AS-BI-β-D-glucuronic acid (Sigma, Batch Nos. 88C-3884; 53F-3893) $2 \times 10^{-4}$ M was prepared by dissolving 11 mg. of the substrate in 0.1 ml of 0.05 M $NaHCO_3$ and then diluting it to 100 ml. with 0.1 M acetate buffer pH 4.5. The stock solution was preserved in the refrigerator at 4°C. The stock solution was diluted with acetate buffer to obtain a solution $1 \times 10^{-4}$.

ii) For the histochemical demonstration of β-glucuronidase, the duodenums were fixed in 5 % neutral buffered formalin for 24 hrs. at 4°C and then transferred to Holt's 0.88 M sucrose solution containing 1 % gum acacia, which was

previously chilled to 4°C (Holt, 1959; Hayashi and Fishman, 1961). The tissues were kept in gum sucrose for 24 hrs. at 4°C.

iii) The sections were cut at 6 to 8 μ on Lipshaw microtome at -20°C. The sections were collected in distilled water cooled to 4°C. Free floating sections were selected for the demonstration of enzyme.

iv) The selected sections were incubated in the substrate medium at 37°C for 60 to 90 minutes.

v) The incubated sections were washed in cold distilled water and then coupled with fast Garnet GBC diazonium salt (Sigma). The coupling solution was prepared by dissolving 0.2 gm. of the salt in 100 CC. of 0.01 M phosphate buffer at pH 7.4 or distilled water and then filtered. It readily couples with liberated naphthol AS-BI and leaves no background staining so that stained product can easily be observed under microscope.

vi) After staining, the sections were transferred to cold distilled water, washed well and mounted in 1 % aqueous polyvinylpyrolidone on clean glass slides.

IV) Biochemistry

A) Isolation of Brunner's Glands (Smits et al., 1982)

The rats were killed by cervical dislocation, their duodenums were quickly removed and were washed in chilled distilled water after the gross observation. The tissues were frozen at -15°C on microscope slides with the mucosal face upwards and the mucosa

was removed by scraping the frozen tissue with a razor/blade. Microscopic examination of the scrapped tissues revealed that the villi and the major part of the crypts were removed and that only muscularis externa and the submucosal layer including the Brunner's glands were left.

Only those area of the tissues where Brunner's glands were densely packed were taken for the bioassay of protein, β-glucuronidase and electrophoretic study.

B) Preparation of Homogenate

Homogenation of the tissue was carried out using glass mortor and pestle. The glass mortor and pestle were well washed, rinsed in glass distilled water, dried and kept in a refrigerator atleast for 5 hrs at -4°C. Homogenation of this type has two advantages : 1) during homogenation no loss of enzyme activity occured as temperature due to friction of the mortor and pestle did not exceed beyond 10-12°C at the end of homogenation, 2) during homogenation when the tissue was initially crushed at the bottom of the mortor, it instantaneously freezed and then gradually thawed which helped in breaking the lysosomes, where this enzyme β-glucuronidase is partly localized. When the tissue was thawing, two ml of chilled glass distilled water was added. When a perfectly uniform suspension was formed, the hamogenate was transferred to a calibrated flask/jar and further dilution was done by adding the necessary quantity of water. Care was taken to have the final concentration of the homogenate about 1 mg/ml. Such homogenates were used for biochemical estimation.

C) Estimation of Protein

The total protein was estimated according to Lowry et al. (1951). The homogenate of tissue (1 mg/ml) was centrifuged for 10 minutes at 2000 X g.

Reagents

i) Reagent A - 2 % $Na_2CO_3$ in 1 N NaOH.

ii) Reagent B - 0.5 % $CuSO_4 5H_2O$ in 1 % $Na^+$, $K^+$ tartarate.

iii) Reagent C - To be prepared at the time of use.
(50 ml A + 1 ml B).

iv) Folin-Ciocalteu Phenol Reagent - Dissolve 100 gm sodium tungstate ($Na_2WO_4.2H_2O$) and 25 gm sodium molybdate ($Na_2MoO_4.2H_2O$) in 700 ml water. Add 100 ml HCl and 50 ml 85 % phosphoric acid and reflux the mixture for 10 hrs. in a glass apparatus. Add 150 gm. of lithium sulphate, 50 ml water, and a few drops of $Br_2$. Boil for 15 minutes to remove excess bromine, cool, dilute and then filter. The reagent should be golden yellow in colour and should be stored in the refrigerator. If the reagent acquires a greenish tint, it is unsatisfactory for use, but may be regenerated by boiling with a few drop of $Br_2$. Care must be taken to boil away all the excess bromine.

Estimation

Protein estimation was carried out in three replicates for each sample.

| Additions | Blank | Unknown |
|---|---|---|
| i) Chilled distilled water | 1.5 ml | 1 ml |

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| | | |
|---|---|---|
| ii) homogenate | - | 0.5 ml |
| iii) Reagent C | 3 ml | 3 ml |
| Wait for 15 to 20 minutes | | |
| iv) Phenol reagent | 0.5 ml | 0.5 ml |

Reaction

The final colour production is a result of biuret reaction of protein with copper ions in an alkaline medium and reduction of the phosphomolybdic phosphotungstic reagent by the tyrosine and tryptophan present in treated protein.

The readings were taken after 60 minutes at 660 nm on a spectronic 20 (Bosch and Lomb) spectrophotometer.

Calculation

Using the standard graph of Bovine Serum Albumin the mg. protein was read corresponding to the O.D. of the sample.

$$\frac{\text{Corresponding Protein to the O.D. of Sample}}{\text{Sample (in mg)}} \times 1000 = \text{mg protein per gm. tissue.}$$

D) Bioassay of β-glucuronidase (EC 3.2.1.31)

The homogenate of tissue (1 mg/ml) was made in distilled water and centrifuged for 10 minutes at 2000 X g. The suspension was directly taken for enzyme assays. β-glucuronidase activity was determined with phenolphthalein β-D-glucuronic acid as substrate (Fishman, 1965, 1967).

Reagents

1) Substrate solution : 0.001 M phenolphthalein β-D-glucuronic

acid was prepared in distilled water and kept in 4°C.
(5 mg phenolphthalein β-D-glucuronic acid in 10 ml distilled water).

2) Acetate buffer : (0.1 M, pH 4.5)
(Dissolve 5.79 gm. sodium acetate $3H_2O$ in distilled water, add 3.25 ml glacial acetic acid and make upto 1000 ml).

3) Trichloro acetic acid TCA : 5 % (W/V) in distilled water.

4) Alkaline Glycine Solution : (0.1 M)
(Dissolve 16.3 gm. glycine and 12.65 gm. NaCl in distilled water, add 12 ml NaOH (gm/ml $H_2O$) and make it to 1000 ml. Adjust the pH of this solution by the addition of glycine or NaOH, so that a mixture of 2.5 ml of this solution, 1 ml of TCA 5 %, 1 ml of acetate buffer, pH 4.5 and 1.5 ml of distilled water has a pH between 10.2 and 10.45).

Estimation

Bioassay for β-glucuronidase was carried out in three replicates for each sample.

| Additions | Control | Unknown |
|---|---|---|
| i) Substrate | - | 0.1 ml |
| ii) Buffer | 0.9 ml | 0.8 ml |
| iii) Sample | 0.1 ml | 0.1 ml |

Incubate in water bath for about 17 hrs. at 37°C.
Immerse the tubes in boiling water and add 1.5 ml distilled water and then centrifuge for 5 minutes at 2000 g.

Pipette off 2 ml supernatent from each test tube and keep in respective tubes and then add

2.5 ml Alkaline glycine

1 ml 5 % TCA

0.5 ml distilled water

The liberated phenolphthalein develops a pink colour in the alkaline medium. The solutions were mixed well and after centrifugation resultant phenolphthalein from the supernatent was read at 540 nm on a spectronic 20 (Bösch and Lomb) spectrophotometer.

The µg phenolphthalein was calculated using the standard graph plotted, optical density vs Phenolphthalein.

Calculation

$$\frac{\mu g \text{ phenolphthalein X dilution X } 2.5}{\text{Incubation hrs. X wt in gm. X } 0.1 \text{ X } 2} = \text{FU/gm wetweight of tissue}$$

FU = Fishman units.

V. Electrophoresis

The term electrophoresis is used to describe the migration of charged particles under the influence of an electric field. It is single phase system and depends upon the relative mobilities of ions under identical electrical conditions. Zonal electrophoresis is the most frequently used form of electrophoresis and involves application of a sample as a small zone to a relatively large area of supporting medium which enables subse-

quent detection of the separated sample zones. A wide range of supporting media have been developed to eliminate difficulties caused by some media such as the absorptive effects of paper and molecular sieving effects of polyacrylamide gel.

The electrophoretic mobility of an ion is related to i) the ionic strength of the buffer rather than its molar concentration. ii) Voltage gradient applied across the supporting medium; which is quoted in volts per centimeter (i.e. the distance between electrodes). iii) The current generates heat which increase in the conductivity of the electrolyte solution and this further increases the current. This rise in temperature causes some evaporation of the buffer, which is, in small, held in the supporting medium results in an increase in its concentration and affecting the conductivity of buffer. Because of these effects it is necessary to control the temperature of the system in some way and to choose between the use of a fixed voltage or a fixed current. iv) In electroendosmosis, the buffer itself moves due to an electrophoretic effect and hence masks the movement of the solute to some extent. Electroendosmosis is caused by the presence of charged groups on the surfaces of some supporting media and this induce an opposite charge in the buffer solution.

A) Supporting Media

i) Filter paper has been an extremely useful and convenient medium, but the random structure of the paper, however, tends to result in irregularities in the separation patterns and its absorptive effects gives significant

degree of 'tailing' of the zones.

ii) Cellulose acetate membrane (CAM) was developed with the aim of reducing the polar nature of paper by acetylating the hydroxyl groups and producing a more regularly defined pore structure. For these reasons, it has considerable advantage over paper and show minimal absorptive effects with increased separation rate. But it shows electroendosmotic effect.

iii) The starch gel is prepared by heating a slurry of the starch in the appropriate buffer in a boiling water bath until the mixture becomes translucent. A vaccum is applied to the flask to remove air bubbles and the solution quickly poured into a neat tray and allowed to cool to form a gel about 5 mm thick. During electrophoresis, gel must be adequately cooled to prevent it from melting and separation may take about 6 hours at about 150 volts. These gels are extremely tragile and the method of hydrolysis is entirely arbitrary and it is difficult to reproduce the pore size in successive preparation.

iv) Polyacrylamide gel shows many advantages over starch gel as a medium for high resolution electrophoresis and because of its synthetic nature, its pore size can more easily be controlled. The gel is formed by polymerization of the two monomers, acrylamide and a cross linking agent, N, N methyl bis-acrylamide.

The proportion of the two monomeres and not their total concentration is the major factor in determing the pore size,

the later having more effect on the elasticity and transferency of gel. A minimum total concentration of approximately 20 g $l^{-1}$ is necessary for gel formation although concentrations in the region of 70 g $l^{-1}$ are frequently used. The pore size of the gel decreases with the increasing proportions of the bis-acrylamide with a limiting value of approximately 5 % of the total, giving minimum pore size (for glycoprotein electrophoresis, gel containing 5 % of the cross linking agent are often used).

Polymerization of gel may be achieved either by ultraviolet photoactivation with riboflavin or using ammonium per sulfate as a catalyst. It is necessary to include initiator for the reaction. TEMED (tetramethyl ethylene diamize) is being commonly used.

Polyacrylamide gel electrophoresis (PAGE) is performed either in cylindrical glass tubes or in flat bed. When PAGE is carried out in cylindrical glass tubes, it is called as disc electrophoresis method, vertical gels, either slabs or rods, are technically easier to use with multiphasic buffer systems. Ornstein (1964) and Davis (1964) introduced the so-called disc electrophoresis method. When a gel rod apparatus is used each sample is run on a separate rod, for an accurate comparision of different samples. Conditions must be identical in all rods throughout the experiment. The prime requirements for good and reproducible separations are that all the tubes must have identical dimensions, must be precisely vertical and should contain the same length of gel, and that the upper surfaces of the gels should be accurately flat so that identical running conditions

occur in all gels.

For the present investigation the disc electrophoresis on polyacrylamide gel was employed. The method employed was of Davis (1964), Ornstein (1964), Andrews (1981) and Osterman (1984). The apparatus used for conducting the electrophoresis was 'AIMIL' type (No.1760). The glass tubes used for gel preparation were having 5 mm dimension and 6 cm length.

B) Gel Preparation

5 % PAG

| | |
|---|---|
| Acrylamide | 4.75 gm. |
| Bis-acrylamide | 0.25 gm. |
| TEMED | 0.05 ml |
| Ammonium per sulphate | 0.05 gm. |

prepared in 100 ml electrophoresis buffer. No stacking gel was used.

C) Electrophoresis buffer (Homogenous buffer system)

Tris-borate buffer, pH 9 (0.1 M Tris, 0.025 M Boric acid).

D) Sucrose Dye

50 % sucrose and 0.25 % Bromophenol blue in distilled water.

E) Sample Dye

Four parts sample : 1 part sucrose dye. 100 µl of sample dye was loaded for a single gel rod.

F) Electric supply

The voltage was kept constant at 150 V during the operation of electrophoresis and 3 mA current per rod was employed. During the first five minutes only 2 mA per rod was employed to avoid

diffusion. The separation time was 25 to 30 minutes. Electrophoresis was stopped when the dye front reached the tip, leaving 5 mm distance, the mobility being cathode to anode.

G) Gel Staining

i) Staining procedures for proteins (Andrews, 1981)

The earliest protein stain Amido Black 10 B, also called as Naphthalene Black 10 B, Naphthol Blue Black B, or Buffalo Black is still being used to detect the protein bands on the gel. A more sensitive method is by staining with coomassie Brilliant Blue.

| Staining mixture | Staining time (hr.) | Destaining mixture |
|---|---|---|
| 1. Amido Black 10 B 1-10 g dissolved in 250 ml methanol, then add 100 ml acetic acid + 650 ml $H_2O$ | 0.5 - 2.0. Prefixing in 12.5 % TCA may be advantageous | 10 percent acetic acid. |
| 2. Coomassie Blue 1 gm dissolved in 500 ml methanol; then add 100 ml acetic acid + 400 ml $H_2O$ | 1.0 | 500 ml methanol + 100 ml acetic acid + 400 ml $H_2O$ |

Gels were stored in 7 % acetic acid.

ii) Glycoprotein staining method of Zacharius et al. (1969)

| Staining procedure (Successive steps) | Time (min.) |
|---|---|
| 1. Immerse gels in 12.5 % TCA (25-50 ml per gel rod of diameter 5 mm). | 30 |

| | | |
|---|---|---|
| 2. | Rinse in $H_2O$ | 0.25 |
| 3. | Immerse in 1 % Periodic acid in 3 % aqueous acetic acid | 50 |
| 4. | Wash thoroughly with $H_2O$ (atleast six changes over 1 to 2 hr with stirring or overnight). | overnight |
| 5. | Transfer into fuchsin-sulphite stain in dark | 50 |
| 6. | Wash with three changes (10 min.each) of 0.5 % sodium metabisulphide (25-50 ml per gel rod) | 30 |
| 7. | Wash in frequent changes of water until excess stain is removed. | Overnight |
| 8. | Store in 5 % acetic acid | |

iii) Staining Procedure for β-glucuronidase

The gels were stained for β-glucuronidase using the histochemical method of Fishman and Goldman (1965) described earlier. The procedure was followed after making slight alterations from the histochemical method for the demonstration of β-glucuronidase.

| Staining mixture | Staining time (hrs) | Destaining mixture |
|---|---|---|
| Naphthol AS-BI-β-D-glucuronic acid in acetate buffer as substrate. Fast Garnet GBC diazonium salts as post coupling agent. | Gels prefixed in acetate buffer, 0.1 M, pH 4.5. Incubated at 37°C ($1\frac{1}{2}$ to 2 hrs.). Washed with distilled water and coupled with fast garnet GBC salt. | Frequent washing with cold distilled water. |

The gels were stored in 5 % acetic acid.

H) Gel photography and scanning

The photographs of gels were made to record the observations.

The scanning of gels were done using Shimadzu UV-240 model spectrophotometer. Gels stained for protein were scanned at 660 nm, whereas the gels of glycoprotein and β-glucuronidase were scanned at 540 nm.

VI. Chemicals

The chemicals used for the present investigation were obtained from different sources.

| Name of chemical | Batch No. | Source |
|---|---|---|
| 1. Amido Black 10 B | | Standard Fluka, England. |
| 2. Bromophenol Blue Indicator | | Romali, U.S.A. |
| 3. Coomassie Brilliant Blue G | | Koch-Light, England. |
| 4. Cysteamine HCl | 114F-3474 | Sigma, U.S.A. |
| 5. Fast Garnet GBC | | Sigma, U.S.A. |
| 6. Naphthol AS-BI-β-D-glucuronic acid | 88C-3884 | Sigma, U.S.A. |
| 7. N',N',N',N'-tetramethyl ethylene diamine (TEMED) | 83789 | Koch-Light, England. |
| 8. Phenolphthalein mono-β-glucuronic acid | 71F-7025<br>100F-7280 | Sigma, U.S.A. |

Other chemicals used were of analytical grade.

VII) Statistical Methods (Fisher, 1936; Snedecor, 1946 and Wilks, 1949).

1. $\bar{X}$ = Arithmatic mean of X, independent variable

$$\bar{X} = \frac{\leqslant X}{N} \qquad (N = \text{number of variables})$$

2. S = Standard deviation

$$S = \sqrt{\frac{\leqslant x^2 - \frac{(\leqslant x)^2}{N}}{N-1}}$$

3. S.E. = Standard Error for a mean of N variables

$$S.E. = \frac{S}{\sqrt{N}}$$

4. t = Student test

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{S_1^2}{N_1} + \frac{S_2^2}{N_2}}}$$

5. df = degree of freedom for a difference of two means of $N_1$ and $N_2$ variables.

6. Significance : The probability, $P_1$ of obtaining t-value at least as great as the calculated one for a given number of df. is found in Fisher's tables.

The P-values are signified according to the following conventions

$P = > 0.05$ The difference is said to be nonsignificant.

$P = \leq 0.05$ The difference is said to be almost significant.

$P = \leq 0.01$ The difference is said to be significant.

$P = \leq 0.001$ The difference is said to be highly significant.