Chapter II
MATERIALS AND METHODS

For the present experimental project, a mammalian species, male Sprague Dawley albino rats (100-150gm body weight) were selected, as it is easily available, economic and has a high percentage of survivality in the laboratory condition, with a high reproductive potentiality.

The experimental male albino rats were collected from the animal house at random. All the rats were provided with standard diet gold mohur laboratory animal feed (Lipton feed) and water ad libitum and kept under laboratory condition at room temperature of 25°C±2. Experimental rats were divided into three sets and grouped as follows. Five rats were observed under each group.

Set I- Control:

A: Received 1ml of liquid paraffin termed as control.

Set II- Malignant:

B: Received 10 mg 20-methylcholanthrene (MCA) in 1ml liquid paraffin intra-peritoneally (IP) and killed on 15th day.

C: Received MCA as in group B killed on 30th day.

D: Received MCA as in group B killed on 60th day.
Set III - Chemotherapy treated:

E: Received cisplatin (4mg/kg/bw/wk.ip) after 7 days of MCA administration for 4 weeks expanded to 60th day.

F and G: Received cisplatin as in E with oral dose of vitamin C (200mg/day) and E (400mg/alt day) expanded to 45th (F) and 60th (G) day respectively.

Rats were killed by pithing and required organs were dissected out immediately.

The carcinogenic agent MCA was chosen, as it was one of the most potent chemical carcinogen known and can be secured in a state of highest purity. For injection 10mg powdered (sigma) MCA was dissolved in 1 ml of liquid paraffin, as it was insoluble in water.

Anticancer drug cisplatin (cis dichlorodiamine platinum - II, CDDP) and vitamin C and E were from David-Bull laboratories, USA and Emerck respectively. Hi Media chemicals were used in this investigation unless otherwise specified.

Estimation of different biochemical analysis, viz. element analysis, enzyme estimation, histopathological observation using light and scanning electron microscopy (SEM) were done by standard methods described below.
1. Analysis of tissue elements by wet ashing method (oxidation procedure of Kahnke 1966):

This method describes the determination of Zn in tissue using a wet ashing (oxidation) procedure. As little as 0.2 μg Zn/gm of tissue can be detected with the procedure. This method is also applicable to the determination of other elements.

Simple oxidation is accomplished by boiling formalinized tissue sample in water and a mixture of HNO₃ and HClO₄ until a clear solution appeared. A final dilution with deionized water is performed and the sample is then ready for analysis.

Procedure: About 5 gm of accurately weighed formalinized tissue was placed in a 125 ml erlenmeyer flask, to it 25 ml of deionized water and a few glass beads were added and finally 10 ml of a 1:2 mixture of concentrated HNO₃ and HClO₄ was added to the tissue and boiled till the solution was clear. The volume of the digested solution was measured and diluted to 100 ml with deionized water and mixed.

The final dilution with deionized water (100 ml) could be adjusted to ensure that the concentration fall with a suitable absorbance range.

Analysis: The Concentration of elements was determined by Atomic Absorption Spectrophotometer (Parkin-Elmer 3280) in standard conditions. Section standard were prepared by diluting the stock
standard solution with deionized water. Deionized water was also used as blank solution.

2. Estimation of Lactate dehydrogenase (LDH) activity (King, 1965): The LDH activity of parotid, submaxillary and oesophagus of rat was measured by the method of King (1965).

Principle: The measurement of the enzyme activity was based on the principle of conversion of lactate to pyruvate in presence of NAD.

\[
\text{LDH} \\
\text{Lactic acid} + \text{NAD} \rightarrow \text{Pyruvic acid} + \text{NADH} + \text{H}^+ 
\]

The pyruvic acid that formed will react with reduced NAD colour reagent to form the corresponding phenyl-hydrazone, which in alkaline medium, gives golden brown colour, which can be measured by spectrocolorimeter.

Reagents required:

1. Glycine buffer 0.1 N (Sorenson)

2. 0.1 N Sodium hydroxide (NaOH)

3. 0.4 N Sodium hydroxide (NaOH)

4. Buffer substrate: 4 gm of lithium lactate was dissolved in 125 ml of glycine buffer (reagent-1) and 75 ml of 0.1 N NaOH
(reagent-2) solution in a glass stoppered bottle. The reagent was stored at 4°C.

5. **Nicotinamide adenine dinucleotide** (NAD) solution: 10 mg of NAD was dissolved with 2 ml of double distilled water and stored at 4°C. Fresh solution was prepared at regular interval.

6. **1 N Hydrochloric acid** (HCl) solution

7. **2,4-dinitrophenyl hydrazine reagent** (DNPH): 200 mg of 2,4-dinitrophenyl hydrazine was dissolved with hot 1 N HCl acid and made upto 1 litre with this acid. The reagent was then transferred to a glass stoppered bottle and stored at 4°C.

8. **Standard Sodium pyruvate solution**: 11 mg of Sodium pyruvate was dissolved in 100 ml buffered substrate (reagent 4). The reagent was then transferred into a glass stoppered bottle and stored at 4°C. This reagent contains 1 ml of pyruvate per ml of buffered substrate.

9. **Reduced nicotinamide adenine dinucleotide** (NADH) solution: 71 mg of NADH was dissolved in 100 ml buffered substrate (reagent 4). The reagent was then transferred into a glass stoppered bottle and stored at 4°C. This reagent contains 1 µmole of NADH per ml of buffered substrate.

Procedure: Fresh tissues of parotid, submaxillary and oesophageus were weighed and homogenized separately with ice cold redistilled
water in the proportion of 1:10. The homogenized liquid were centrifuged at 2000 r.p.m. for 20 minutes and the supernatant extracts were siphoned off. 1 ml of tissue homogenate was taken in a centrifuge tube and kept in an ice bath.

1 ml of buffered substrate (reagent 4) and 0.1 ml of tissue homogenate were taken in each of the two test tubes. 0.2 ml of double distilled water was then added to one of two test tubes and was marked as blank (B). Both the tubes were then incubated at 37°C for 15 minutes. 0.2 ml of NAD solution (reagent 3) was added to the other test tube marked as test (T) and mixed. Both the tubes (i.e. blank and test) were then placed again in the incubator. Exactly 15 minutes after addition of NAD solution (reagent 5), 1 ml of 2,4-dinitrophenyl hydrazine (reagent 7) was added to each tube, mixed and incubated for another 15 minutes. Both the tubes were then taken out from the incubator and 10 ml of 0.4 N NaOH (reagent 3) was added to each of the tube. Within 1 to 5 minutes of adding 0.4 N NaOH solution absorbance was measured in a spectrocolorimeter (Systronix model) at 440 nm. The result of LDH activity was obtained by extrapolation from the standard calibration curve.

Isolation of Isoenzyme of LDH (Davis 1964): LDH Isoenzymes were separated by polyacrylamide gel electrophoresis following the procedure of Davis (1964). Electrophoresis was carried out in small glass tubes in a vertical dimension and polyacrylamide gels (5.5% acrylamide gel cross linked with 0.14% bis- acrylamide) was made in the glass tubes.
Calibration:

A standard calibration curve was set by taking NADH\(_2\), Pyruvate solution, buffered substrate, NAD solution and distilled water in different quantity in a series of test tubes as shown below in the chart.

When all the tubes were ready, they were incubated for 15 minutes at 37°C. Exactly after 15 minutes of incubation, 1 ml of DNPH was added and were incubated for further 15 minutes at 37°C. All the tubes were then taken out from the incubator and 10 ml of 0.4N NaOH was added and mixed. The absorbance was measured in spectrocolorimeter.

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FIG. 1. CALIBRATION CURVE WITH REGRESSION LINE FOR LDH
Reagent:

Solution A: 48 ml of 1 N HCl was taken in a 100 ml volumetric flask. 36.6 gm of tris and 0.23 ml of tetra methyl ethylene diamine (TEMED) was added into the flask and dissolved by adding double distilled water. The volume was finally made upto the mark with double distilled water.

Solution B: 28 gm of acrylamide and 0.73 gm of bis acrylamide were dissolved with double distilled water in a 100 ml volumetric flask and the volume was made upto the mark with double distilled water.

Solution C: 48 ml of 1N HCl was taken in a 100 ml volumetric flask. Then 5.98 gm Tris and 0.46 ml TEMED were added to the flask and dissolved by adding double distilled water. The volume was made upto the mark with double distilled water.

Solution D: 10 mg of acrylamide and 2.5 gm of bis-acrylamide were dissolved with water in a 100 ml volumetric flask and then volume was made upto the mark with double distilled water.

Solution E: 4 mg of riboflavin was added in double distilled water in a 100 ml volumetric flask and the volume was made upto the mark.
Solution F: 40 gm of sucrose was dissolved with double distilled water in a 100 ml volumetric flask and then volume was made upto the mark with double distilled water.

Solution G: 0.14 gm ammonium persulphate was dissolved with double distilled water in a volumetric flask and then volume was made upto the mark with double distilled water.

Solution H (Electrode buffer): 0.6 gm of Tris and 2.88 gm of glycine were taken in a 1 litre volumetric flask and dissolved in distilled water and volume was made upto the mark. pH was checked at 8.3.

Solution I (Staining mixture): The staining solution was prepared by adding 0.47 ml of NAD\textsuperscript{+} solution (8 mM), 0.78 ml of Nitroblue tetrasolium solution (N.B.T.) (4mM), 0.2 ml of phenazine methosulphate (P.M.S.) solution (8mM), 1.0 ml sodium lactate (5.35M) and 2.5 ml of water to 5.0 ml of Tris-HCl buffer (0.05M, pH 7.4).

Preparation of gel column: Glass tubes of diameter 0.5 and 7 cm long were used for the gel preparation. The tubes were thoroughly washed in chromic acid, detergent solution and finally rinsed several times with double distilled water.

The gel column were prepared in segments, first the running gel and then spacer gel above it.
a. Running gel preparation: In a electrophoretic glass tube, 1.275 ml of solution A, 2.55 ml of solution B and 1.275 ml of double distilled water were added and mixed thoroughly. The 5.1 ml of solution G was added and mixed properly. The above solution contains the acrylamide concentration of 5.5 percent.

The glass tubes were placed in a vertical plane by placing them with rubber stopper in one end. 1.7 ml of the above prepared gel solution was poured carefully into each tubes and 2-3 drops of double distilled water were added carefully above the gel solution with a syringe. The tubes were then placed under fluorescent light to polymerize. The water i.e. layered over it after polymerization was drained off by inverting the tubes.

b: Spacer gel preparation: In a electrophoretic glass tube, spacer gel solution was prepared by mixing of 0.45 ml of solution C, 0.9 ml of solution D; 0.45 ml of solution E and 1.8 ml of solution F.

After the running gel layer was polymerized the spacer gel solution was prepared as above and 0.1 ml of the spacer gel solution was poured over the running gel with the help of a pipette, 2-3 drops of double distilled water was added above it and again photopolymerized under fluorescent light. After polymerization of the gel, the water, that accumulated above the gel was removed as mentioned before.

Then the gel tube were placed carefully into the electrophoresis
chamber and 0.1ml of tissue extract was added on the gel column of each tube. Both the upper and lower chambers of electrophoresis tank were filled with tri glycine buffer (solution H) and allowed to run the current at the rate of 2mA (100 volt) for about 15 minutes. Then after 15 minutes the current was increased to 4mA.

Exactly after 90 minutes of electrophoresis at 4mA the gels were removed from the gel tube by running a jet of water by the sides of the gel inside the tube. The gels were then incubated in the staining mixture (solution I) at 37°C for 40 minutes in dark. The gels were then washed in double distilled water and fixed in 50:40:1 of double distilled water, methanol and glacial acetic acid (v/v) respectively. The gels were preserved and subsequently scanned with a linear transport device attached to the Backman spectrophotometer Acta III at a scanning rate of 2 cm/min with chart speed of 1 cm/min at 620 nm.


Principle: Glucose-6-phosphatase activity was measured following the procedure of Hers (1964; 1966) by estimating the liberated inorganic phosphate by the method of Fiske and Subbarow (1925). The measurement of the enzyme activity was based on the principle of release of inorganic phosphate from glucose-6 phosphate. The reaction is linear with time for at least 60 minutes in the presence of EDTA.
The required experimental organs were immediately removed to an ice bath and weighed. The organs were homogenized separately with ice cold redistilled water in the proportion of 9 volume of water per gm of tissue. The homogenate was kept ice cold.

Reagent Required:

1. 0.1 M Glucose-6-phosphate: pH 6.5: Glucose-6-phosphate disodium salt (Sigma, USA 0.188 gm) was measured and dissolved in 5 ml of redistilled water, the pH was adjusted to 6.5.

2. 0.002 M EDTA solution in 0.1 M glucose-6 phosphate

3. 10% Trichloro acetic acid solution (T.C.A.)

4. Standard phosphate solution: 0.351 gm of pure monopotassium phosphate was quantitatively transferred to 1 litre volumetric flask and dissolved in a minimum amount distilled water, 10 ml of 10N sulphuric acid was added and the volume was made upto the mark with distilled water. This solution contained 0.4 mg of phosphorous per 5 ml and was stable indefinitely.

5. 10 N Sulphuric acid
6. **Molybdate II reagent**: 25 mg of ammonium molybdate was transferred to a 1 litre volumetric flask and dissolved in about 200 ml of distilled water. 300 ml of 10 N sulphuric acid was added and the volume made upto the mark with distilled water.

7. **15% Sodium bisulphite solution**

8. **20% Sodium sulphite solution**

9. **Aminonaphthol sulphonic acid**: 195 ml of 15% Sodium bisulphite solution was taken in a glass stoppered cylinder. 0.5 gm of 1,2,4-aminonaphthol sulphonic acid was added, followed by 5 ml of 20% Sodium sulphite solution. The cylinder was stoppered and shaken till the powder dissolved. 1 ml of sodium sulphite solution was added in order to facilitate solution. The solution was stored in a dark glass bottle in the cold. It was stable for about 4 weeks.

10. **Working phosphate standard solution**: 5 ml of standard phosphate solution containing 0.4 mg of phosphorous was transferred to a 50 ml volumetric flask, and made upto the mark with 10% trichloro acetic acid. 5 ml of this diluted standard contained 0.04 mg of phosphorous.

11. **0.1 M acetate buffer, pH 5.0**:

   a. 0.1 M acetic acid
b. 0.1 M sodium acetate:

0.1 M acetate buffer was prepared by 3 volume of 0.1 M acetic acid with 7 volume of 0.1 M sodium acetate solution.

Procedure:

Test: Six dry clean test tubes were taken and 0.002 M EDTA in 0.1 M glucose-6 phosphate solution was placed in each. The tubes were divided into two sets, three in each set. 0.1 ml of tissue homogenate of parotid, submaxillary and oesophagus was added separately to the three tubes of the first set. The tubes of the first set were mixed and incubated at 37°C for one hour. At the end of this time, the reaction was stopped by addition of 9.8 ml of 10% TCA and the tubes kept in ice.

To the tubes of the second set, 0.1 ml of tissue homogenate of parotid, submaxillary and oesophagus was added accordingly and placed in the ice bath immediately after addition of 9.8 ml of 10% TCA.

Tubes of both sets were centrifuged and the phosphate content of the supernatant fluids were determined following the procedure as described below. The difference in phosphate concentration between the tubes of same organ of two sets is the amount of phosphate released during one hour incubation at 37°C.
Control: A control set of experiment was run simultaneously. 0.1 ml of the homogenate of parotid, submaxillary and oesophagus were mixed with 0.1 ml of 0.1 M acetate buffer in three tubes separately. The three tubes were incubated at 37°C for 5 minutes. 0.1 ml of 0.002 M EDTA solution in 0.1 M glucose-6 phosphate was added and the mixture was incubated for one hour. Reaction was stopped by the addition of 9.8 ml of 10% TCA and the tubes were placed in an ice bath. Another set of three tubes were similarly prepared without incubation after addition of 0.002 M EDTA in glucose-6 phosphate. The amount of inorganic phosphate released during the one hour incubation was estimated following the same procedure described below.)

The actual phosphate released due to glucose-6 phosphatase activity was the difference between the phosphate released in the test and phosphate released in the control runs. The glucose-6 phosphatase activity is calculated in terms of micromoles of phosphate released per minute per gm of experimental tissue.

Estimation of inorganic phosphate (Fiske and Subbarow, 1925):

5 ml of supernatant was transferred to a stoppered graduated cylinder. 1 ml of molybdate II reagent was added and mixed. 0.4 ml of aminonapthol-sulphonic acid was added, mixed again, and the solution was diluted to 10 ml with distilled water. The colour was allowed to develop for 5 minutes.

A blank was prepared by similarly treating 5 ml of 10% TCA, 5 ml
of the working phosphate standard was taken in a third tube and treated in an identical fashion.

The tubes were then read in a Systronics spectrocolorimeter at 660 nm.

Calculation:

Since 5 ml of the solution taken represented only 0.05 ml of the original liver extract, the amount of phosphate present per ml of the extract may be calculated as follows.

\[
\text{Optical density of unknown} \times 0.04 \times 20 = \text{mg of phosphorus/ml}
\]

Optical density of standard

The recovery and accuracy in the determination of inorganic phosphate by the method of Fiske and Subbarow (1925) were tested.

Different volumes of the working phosphate solution were taken in different tubes and the volume made up to 10 ml with distilled water in each, 5 ml of solution was taken from each tube and the inorganic phosphorous estimated as described earlier. The amount of phosphorous present in 100 ml of the solution was calculated, and the results obtained compared with the amount of phosphorous actually present. This was repeated a number of times.
Reproducibility of results and recovery of inorganic Phosphorous by method of Fiske and Subbarow (1925).

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Reproducibility of results and recovery of inorganic Phosphorous by method of Fiske and Subbarow (1925).
The method was judged to be reliable as results were reproducible and recovery was found to vary between 98.75 and 100 percent.

4. Estimation of gamma-amylase activity (Rosenfeld, 1964):

Principle: The gamma amylase activity of parotid, submaxillary and oesophagus was measured following the procedure of Rosenfeld (1964). The gamma amylase was first isolated from the tissue with acetone, heated with ethanol and dialyzed in acetate buffer (pH 4.8). The enzyme activity was estimated in terms of glucose and protein by allowing the enzyme to act on glycogen solution.

Reagent required:

A. For extraction:

i. Acetone

ii. 0.1N acetate buffer (pH 4.8)

a. 0.1N acetic acid

b. 0.1N sodium acetate solution

iii. Starch suspension: 12.5 gm of soluble starch was mixed with 100 ml of 50% ethanol.
B. For Estimation:

**Reagent A** : Glycogen solution : 50 mg of glycogen was dissolved in redistilled water and diluted to a final volume of 100 ml to obtain 0.05 gm% of glycogen solution.

Procedure : Isolation (extraction) of gamma amylase :

1. Parotid, submaxillary glands and oesophagus were homogenized separately with 4 volumes of cold (-12°C) acetone. After filtration on the suction fitted Butchner funnel, the semi dry cake was blended with 4 volume of cold acetone and filtered. The filtered cakes pulverized and allowed to dry at room temperature.

2. About 3 gm of acetone extracted powder of experimental organs were treated separately with 10 volumes of cold 0.1N acetate buffer (pH 4.8) for 20 minutes at room temperature. The suspension was centrifuged.

3. To the supernatant equal volume of ethanol was added and kept for some time for precipitation. The precipitate was discarded after centrifugation.

4. To 10 volumes of supernatant, 1 ml of 12.5% starch suspension (50% ethanolic) was added and the mixture was agitated for 2 hours in a mechanical shaker.
5. After the completion of agitation, the starch was removed by centrifugation. To the supernatant equal volume of ethanol was added.

6. The solution was centrifuged and the precipitate was collected. The precipitate was dissolved in 0.1N acetate buffer (pH 4.8) and dialysed against 0.1N acetate buffer. The solution after dialysis was heated at $55^\circ C$ for 30 minutes and was then centrifuged.

7. The supernatant was further dialysed with stirring against 0.1N acetate buffer and the pure gamma amylase was thus isolated.

Estimation : 0.05% glycogen solution (Reagent A) was incubated with extracted enzyme for 20 hours at 37°C in the proportion of 2.5 mg of glycogen and 1 ml of enzyme solution in 0.1N acetate buffer (pH 4.8).

The enzyme activity was measured by the following formula:

$$\text{Enzyme activity (gamma amylase) } = \frac{\text{Mg of glucose/ml} \times 100}{\text{Mg of protein/ml}}$$

Glucose and protein were estimated following the procedure of Nelson and Somogyi (Oser, 1965) and Lowry et al. (1951).
Estimation of protein (Lowry et al., 1951):

**Principle:** Protein of the experimental organs were estimated according to the method of Lowry et al. (1951). Proteins was first precipitated with a strong acid and brought into solution with NaOH. In presence of alkaline Na₂CO₃ and Rochelle salt, CuSO₄ · 5 H₂O reacted with protein to form protein copper compound which reduces the phosphomolybdic-phosphotungstic regent to give a final colour, which was to be measured in spectrocolorimeter.

**Reagent required:**

a. For extraction

i. 10% trichloro acetic acid (T.C.A.)

ii. Absolute alcohol

iii. Ether

iv. 1 N NaOH

b. For estimation

**Reagent A:** 2% Na₂CO₃ in 0.1N NaOH

**Reagent B:** 0.5% CuSO₄·5H₂O in 1% sodium potassium tartarate (Rochelle salt)
Reagent C: 50 ml of reagent B added to 1 ml of reagent C. The reagent C is unstable and therefore prepared freshly before the estimation.

Reagent D: Folin Ciocalteu phenol reagent. 1 N in acid (Sisco Research Lab. B. No 30215, analar grade).

Procedure:

Estimation: To final solution of enzyme and substrate, 10% TCA was added. The precipitated protein was brought into solution by adding NaOH. To 1 ml of protein solution, 5 ml of reagent C was added and mixed well. After 10 minutes 0.5 ml of reagent D was added, mixed immediately by shaking and kept for 30 minutes. The blue colour was read against a redistilled water blank at 760 nm and compared with the reading of standard solution.

Preparation of Standard Solution:

1. Stock: 100 mg of analar grade bovine serum albumin (Sigma chemical company B. No-51F-0321) was dissolved in 100 ml of all glass redistilled water to give a solution of 0.1%.

2. Working: From the standard stock solution, a series of working solution, containing 25 μg, 50 μg, 100 μg, 150 μg, 200 μg, 250 μg, 300 μg, 350 μ and 400 μg of albumin were
prepared by diluting the stock solution as shown in Table 3 and were used for the preparation of standard calibration curve

**Preparation of Standard Calibration Curve**: To 1 ml of the standard working solution 5 ml of reagent C was added, mixed and kept for 10 minutes, 0.5 ml of reagent D was added, mixed by shaking and allowed to stand at room temperature for 30 minutes. The blue colour was read against a redistilled water blank, treated in the same manner, at 760 nm in a systronic spectrocolorimeter.

The mean absorbance of nine replicates of each standard working solution was plotted against the strength (concentration), a linear graph was obtained, which was found to fit the regression line (Fig. 2) and was judged reliable.

Protein was calculated by plotting the unknown value (absorbance) from the regression calibration line.

Protein content in the present experiment was expressed in mg/ml.

6. **Estimation of Glucose**:

**Principle**: The glucose was estimated following the procedure of Nelson and Somogyi (Oser, 1965). The tissues was deproteinized by Zinc sulphate-Barium hydroxide procedure, which gave filtrate containing practically no reducing substance other than glucose.
Table 3

<table>
<thead>
<tr>
<th>Test</th>
<th>Strength of standard working solution (µg)</th>
<th>Water required</th>
<th>Final Volume</th>
</tr>
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<tr>
<td>1</td>
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</tr>
<tr>
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<tr>
<td>9</td>
<td>4.00</td>
<td>6.00</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Table 3: Test ml of stock albumin solution taken to make required strength of albumin.
FIG. 2. CALIBRATION CURVE WITH REGRESSION LINE FOR PROTEIN
The Zinc-barium filtrate was heated with an alkaline-copper reagent, treated with arseno-molybdate colour reagent and was read at 550 nm.

Reagent Required:

a. For extraction:

i. Barium hydroxide solution: 90 gm of Ba(OH)$_2$,8H$_2$O was dissolved in double distilled water and diluted to 2000 ml in a cylindrical flask. The solution was filtered and stored in a glass stoppered bottle. The solution was protected with a sodaline tube in the stopper.

ii. Zinc sulphate solution: 100 mg ZnSO$_4$ was dissolved in redistilled water and diluted to 2000 ml. Both the solution was checked by titrating Zinc-sulphate solution with Ba(OH)$_2$,8H$_2$O solution using phenolphthalein solution as indicator. 10 ml of Zinc-sulphate solution was diluted with 50 ml redistilled water and taken in a conical flask. 4 drops of phenolphthalein indicator was added to it and titrated with Barium hydroxide solution slowly with constant shaking, until one drop of alkaline turned the mixture pink. If it required more or less than 10.00±0.50 ml of barium hydroxide, the one or the other solution was diluted and checked again until the two solution were exactly equivalent.
b. For Estimation:

Reagent A: Alkaline copper reagent

Solution a: 50 gm of anhydrous sodium carbonate, 50 gm of sodium potassium tartarate, 40 gm of sodium bicarbonate and 400 gm of anhydrous sodium sulphate was dissolved in 1600 ml of double distilled water and diluted to 2 litres. The solution was mixed, filtered and stored at room temperature.

Solution b: 150 gm copper sulphate (CuSO_4·5H_2O) was dissolved in redistilled water and diluted to 1000 ml. 0.5 ml of concentrated sulphuric acid was added and mixed.

On the day of estimation of glucose, the alkaline copper reagent (Reagent A) was prepared freshly taking 4 ml of solution (b) in 100 ml volumetric flask and diluted upto the mark with solution a.

Reagent B: Arseno-Molybdate colour reagent: 100 gm ammonium molybdate was dissolved in 1800 ml of redistilled water. 84 ml of concentrated sulphuric acid was added to it with constant stirring. 12 gm of disodium orthoarsenate was dissolved in 100 ml of redistilled water and was added to the acidified molybdate solution with stirring. The mixture was then incubated at 37°C for 36 hours and stored in a glass stoppered amber coloured bottle.
Reagent C: Standard glucose solution:

i. Stock solution: A stock standard solution was prepared by dissolving 100 mg of anhydrous analar grade glucose solution and diluted to 100 ml with the benzoic acid solution.

ii. Standard working solution: From the standard stock solution, a series of working solutions containing 100 µg, 200 µg, 250 µg, 300 µg, 350 µg and 400 µg of glucose/ml was prepared by diluting the stock solution with redistilled water as shown in table 4.

Procedure:

Deproteinization: 1 ml of incubated gamma amylase glycogen solution was placed in a 50 ml flask, 9.5 ml of barium hydroxide solution was added to it and mixed by rotation. 9.5 ml of Zinc sulphate solution was then added and mixed by rotation. The mixture was shaken vigorously and filtered on a dry filter paper. The deproteinized filtrate was collected in a dry flask.

Determination of glucose: 0.5 ml of Barium Zinc filtrate was taken in a graduated (10 ml) test tube and 1 ml of reagent A was added and was mixed by tapping. The top of the tube was covered with a marble and placed in a boiling water bath in upright position for 10 minutes. The tube was then placed in a water bath for cooling at room temperature for 1 minute. 1 ml of reagent B was added, mixed and diluted to 10 ml with double distilled
Table 4

<table>
<thead>
<tr>
<th>Test</th>
<th>Strength of standard working solution μg/ml</th>
<th>Volume of stock standard solution (ml)</th>
<th>Volume of redistilled water (ml)</th>
<th>Total volume (ml)</th>
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<td>6.00</td>
<td>10.00</td>
</tr>
</tbody>
</table>
FIG. 3. CALIBRATION CURVE WITH REGRESSION LINE FOR GLUCOSE
water. The mixture was mixed by inversion and read against a redistilled water blank at 550 nm in spectrocolorimeter. The absorbance was compared with the standard solution of glucose of the regressed calibrated line and glucose/100 ml of tissue was calculated out.

**Preparation of Standard calibration line**: 0.5 ml of each standard working solution (Reagent C-II) were placed in graduated tube (10 ml) and numbered serially. To each tube 1 ml of reagent A was added and thereafter the same procedure was followed as described above, to develop the colour. The absorbance of standard solutions were measured and plotted against the concentration of glucose working solution to obtain the calibration line.

The calibration line obtained is presented in Fig. 3. It was judged reliable as it was found to fit the regression curve (Fig.3).

5. Sialic acid estimation (Aminoff, 1961):

The sialic acid level of glandular tissues and oesophagus was measured by the method of Aminoff (1961).

**Reagent required**:

1. **Periodic acid**: (HIO₄) M wt 191.93 - 0.025 M in 0.125 N H₂SO₄ (pH 1.2) i.e., 4.82 gm/1000 ml 0.125 N H₂SO₄.
2. Sodium arsenite: 2% 0.5 N HCl in 2 gm of Sodium arsenite made upto 100 ml.

3. 0.5 N Hydrochloric acid

4. Thio barbituric acid: 0.1 N solution in water (adjust pH 9.0 with NaOH) keep well for one month. Thio barbituric acid’s molecular weight is 144.09.

5. Acid butanol: Containing 5% v/v 12 N HCl in 160 ml butanol.

6. 0.125 N H₂SO₄.

7. 0.1 N H₂SO₄.

Procedure: 10 mg fresh tissues of each parotid, submaxillary and oesophagus were homogenized separately with 1 ml of 0.1 N chilled H₂SO₄. Homogenized tissues were then incubated for 1 hour at 78-82°C. 0.5 ml of supernatant were drawn from the incubated liquid and 0.25 ml of periodic reagent were added to it. The solution was left for 30 minutes at 37°C. Then 0.2 ml of Sodium arsenite solution was added. A yellow colour was liberated from the solution, after 1 to 2 minute of addition of Sodium arsenite which indicated disappearance of iodine. Then 2 ml of Thio barbituric acid was added. The test tube was plugged with cotton wool and heated in a boiling water bath for 30 minutes.
Colour solution was then shaken vigorously with 5 ml acid butanol, decanted in a centrifuge tube and allowed it to centrifuge for 10 minutes at 1000 r.p.m. Upper layer was decanted and the reading was taken at 549 nm and 532 nm in spectrophotometer.

\[ \mu \text{ moles of } N \text{ acetylneuraminic} = 0.090 \times \text{OD}_{549} - 0.33 \times \text{OD}_{532}. \]

7. Ultrastructure under Scanning Electron Microscope (SEM):

The experimental tissues were dissected out quickly from the rat and were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 6 hours, washed in buffer overnight, post fixed in 1% osmium tetroxide for 1 hour and dehydrated through increasing concentration of acetone. Dehydrated specimens were prepared using acetone as the intermediate fluid and CO\textsubscript{2} as the transitional fluid. They were dried in an HCP-2 (Hitachi) critical point drier. The dried samples were secured horizontally to brass stubs with double sided sticky tabs, coated with gold in a fine coat Ion sputter JFC-1100 (Joel) and observed with JFM-35CF (Joel) Scanning Electron Microscope operated at 15 KV.

8. Histological preparation of tissues:

The tissues of parotid, submaxillary gland and oesophagus were dissected out quickly from the experimental rat and clear in
normal saline (0.15 ml/e). After clearing, the tissues were fixed in Carnoy's fluid for 20 hours then washed thoroughly in 60% alcohol to remove any trace of fixative and processed. Haemotoxyline and Eosine stains were used for routine histological preparation.

Statistical Analysis:

The experimental values obtained were evaluated by applying different statistical methods to determine the significance of the impact of chemotherapy on some specific elements and biochemical parameters.

The regression Analysis was performed to get the accuracy of the methods of biochemical estimation by using the linear regression equation:

\[ Y = a + b x \]

\[ b = \frac{(x_1 - x)(y_1 - y)}{(x_1 - x)^2} \]

\[ a = y - b x \]

Analysis of variance between control and treatment series and between experimental organs and treatment series was performed by Fisher's method.

\[ F = \frac{\text{variance between sample}}{\text{variance within sample}} \]
The critical difference (CD) was estimated by the equation:

\[ CD = t_{0.005} \sqrt{\frac{1}{EMS} \left( \frac{1}{K_1} + \frac{1}{K_2} \right)} \]

where \( K_1 \) = No. of observation in group A
\( K_2 \) = No. of observation in group B.

The data collected on various elements were subjected to correlation analysis by using correlation matrix:

\[ r = \frac{\text{Cov}(x,y)}{\sqrt{\text{Var}(x)\text{Var}(y)}} \]

All the statistical calculations were carried out by using the software Microstat and the graphs were generated using Harvard Graphics.