CHAPTER - II

MATERIALS AND METHODS

SECTION A : Selection of animals and their maintenance:

1. Mature female rats:

The mature female rats weighing 120-125 g. were collected from a local strain. The animals were housed in a well ventilated room of 12 hours dark and 12 hours light and were supplied with a standard laboratory diet. The composition of the diet was as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage</th>
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</thead>
<tbody>
<tr>
<td>Protein (Casein)</td>
<td>18%</td>
</tr>
<tr>
<td>Carbohydrate (Equal parts of arrowroot starch and sucrose)</td>
<td>71%</td>
</tr>
<tr>
<td>Fat (Groundnut oil)</td>
<td>7%</td>
</tr>
<tr>
<td>Salt mixture (1)</td>
<td>4%</td>
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</tbody>
</table>

The vitamins were mixed with the diet according to Williams and his coworkers (2).

The animals were maintained on the above diet for a week to be accustomed with the new environment. The animals which showed estrous on every fourth day were selected for experiment.

2. Immature female rats:

The immature female rats weighing 30-35 g. were selected for the present investigation. The animals were maintained in a similar way like those of
the mature female rats. When the animals reached 40-45 g, they were used for the experiment.

SECTION B: Methods employed in histochemical studies:

1. Staining of neutral lipid in tissues:

Neutral lipid was localised in formol-fixed frozen sections according to Kay and Whitehead (3).

Preparation of the staining solution:

An equal amount of Sudan III and IV were mixed well and then taken in a clean stoppered bottle. The container was then filled with Herxheimer's mixture (equal parts of acetone and 70% alcohol) and shaken well. The mixture was preserved for a few days before use. During experiment the supernatant was pipetted off in a closed specimen tube to prevent the evaporation of acetone and the alcohol.

Method:

The tissues were removed immediately after killing the animal and fixed in 10% neutral formol for 24 hrs.

Frozen sections were cut at 15 μ. The sections were mounted on clean slides and stained as follows:

1) The slide was rinsed in distilled water.
2) It was then rinsed in 70% alcohol.
3) The slide was then dipped in the specimen tube containing Sudan solution for 1 min.
4) The slide was rinsed in 70% alcohol to remove excess stain.
5) It was then washed in distilled water to remove the alcohol.
6) Finally the slide was mounted in glycerine jelly.
Result:
Neutral lipid was demonstrated by brick red colour.

2. Staining of ascorbic acid in tissues:

Ascorbic acid content of tissues was localised by silver method as described by Bacchus (4).

Method:
1) The tissues within a short while of removal was kept in 5% AgNO₃ soln., the pH of which was adjusted to 2-2.5 by conc. HNO₃.
2) The container was covered by black paper so that light could not enter inside the tube.
3) The tissues were incubated for 45 mins. at 56°C.
4) Silver nitrate solution was poured off and the tissue pieces were washed with distilled water for 10-15 mins. in two changes.
5) Distilled water was then poured off and the tissue pieces were kept in 5% sodium thiosulphate soln. for 45 mins. at 56°C.
6) Sodium thiosulphate was washed out thoroughly with distilled water.
7) The tissues were placed in dioxan for 24 hrs. in two changes for dehydration.
8) The tissues were embedded in paraffin.
9) The tissues were sectioned at 6 μ, deparaffinised and mounted in DPX.

Result:
Ascorbic acid was demonstrated as black precipitate.

3. Localisation of SDH activity in the tissue:

Histochemical localisation of SDH activity in the tissue was determined by following the method of Wegmann and Tordet-Coridroit (5).
Preparation of substrate solution:

1) Sodium succinate, 6.75% (0.2M) ... 1 ml.
2) Nitro BT, 0.5 mg/ml. ... 1 ml.
3) Phosphate buffer, 0.1M (pH 7.4) ... 2 ml.

Procedure:

1) Fresh frozen sections of tissue samples were cut at 20 μm immediately after killing the animal and the sections were placed on to the cover slips.
2) The sections were incubated in the substrate medium at 37°C for a time period of 45 minutes.
3) Following incubation the sections were rinsed in distilled water and then fixed in 10% neutral formol for 5 minutes.
4) The sections were again rinsed in distilled water and finally they were mounted in Apatty.

Result:

The granules of blue formazone indicated the site of enzyme activity. No enzyme activity was observed when the tissue sections were incubated in the succinate-free medium.

4. Localisation of G-6-PD activity in tissue sections:

Histochemical demonstration of G-6-PD activity in the tissues of rat was performed according to Wegmann and Gerzeli (6).

Preparation of reagents:

1) Preparation of stock solution:
   
   i) Tris HCl buffer, 0.2M (pH 7.4) ... 5 ml.
   ii) MgCl₂, 0.1M ... 0.8 ml.
   iii) Distilled water ... 9.2 ml.
   iv) NADP ... 5 mg.
   v) Nitro BT ... 2 mg.
The stock solution was preserved in the refrigerator without adding NADP and nitro BT.

2) **Preparation of working solution:**

1.52 mg. glucose-6-phosphate (sodium salt) was added to 5 ml. of stock solution.

**Technique:**

1) Fresh frozen sections of tissues were made at 20 μm immediately after killing the animal and the sections were placed on coverslips.

2) The sections were incubated in the substrate solution at 37°C for a time period of 30 minutes.

3) Following incubation the sections were rinsed in distilled water and then fixed in 10% neutral formol for 5 mins.

4) After fixation the sections were rinsed with distilled water and mounted in Apathy.

**Result:**

Blue coloration demonstrated the cytoplasmic localisation of the enzyme activity. No enzyme activity was noticed when the tissue sections were incubated in glucose-6-phosphate-free medium.

5) **Localisation of Δ^5-3β-OHD activity in the tissue sections:**

Histochemical localisation of Δ^5-3β-OHD activity was carried out according to Deane and coworkers (7).

**Preparation of substrate medium:**

1) Dehydroepiandrosterone ... 1 x 10^{-5}M

2) Propylene glycol ... 1M
3) Phosphate buffer $\ldots \quad 5 \times 10^{-2} \text{M} \text{ (pH 7.1)}$

4) Nitro BT $\ldots \quad 1.5 \times 10^{-4} \text{M}$

5) NAD $\ldots \quad 5.5 \times 10^{-5} \text{M}$

The control medium contained no dehydroepiandrosterone.

**Technique:**

1) Fresh frozen sections of 20 \(\mu\)m were placed on coverslips.

2) The sections were washed in cold acetone and then they were rinsed in cold phosphate buffer (pH 7.1).

3) The sections were incubated aerobically in the substrate medium at 37°C for a time period of 30 minutes.

4) Following incubation the sections were rinsed in distilled water.

5) After this, the sections were fixed in 10% neutral formalin and then again rinsed in distilled water.

6) The sections were mounted in Apathy.

**Result:**

The crystals of blue formazone indicated the site of enzyme activity.

**SECTION C : Methods employed in biochemical studies:**

1. **Biochemical estimation of total ascorbic acid content in the tissue:**

   The biochemical determination of total ascorbic acid was performed according to Roe and Kuether (8).

**Preparation of reagents:**

1) Distilled water was used for the preparation of reagents.
2) 5% metaphosphoric acid - 10% acetic acid solution:

5 g. of metaphosphoric acid in 80 ml. of distilled water was dissolved and 10 ml. of glacial acetic acid was added to the solution and the volume was made to 100 ml. with distilled water.

3) Dinitrophenylhydrazine-thiourea reagent:

2 g. of 2,4-dinitrophenylhydrazine was dissolved in 100 ml. of approximately 9N H₂SO₄. 4 g. of reagent grade thiourea was added to the mixture and shaken occasionally until dissolved. The solution was filtered and kept in a refrigerator. The reagent was freshly prepared once monthly.

Mercuric chloride test was performed to check the presence of active reducing agent.

4) Bromine: Concentrated bromine in glass ampule was used for oxidation of ascorbic acid to dehydroascorbic acid.

5) 85% H₂SO₄: To 100 ml. of distilled water was added 900 ml. of conc. H₂SO₄ (sp.gr. 1.84).

Extraction:

Representative samples of tissues were weighed carefully in a chemical balance. The sample was ground in a mortar with acid-washed sea sand using 5% metaphosphoric - 10% acetic acid solution.

After extraction the mixture was centrifuged and the supernatant was taken in a hard glass test tube.

Oxidation:

A very small drop of concentrated bromine was added to the tube containing the extract. The tube was shaken and kept for 10 mins. for complete oxidation of ascorbic acid to dehydro form. The solution was decanted from the excess liquid bromine into a test tube and a current of air was passed through the solution.

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until the bromine colour is completely removed. The air was bubbled through a
tap before admission to the solution containing bromine in order to avoid
volume change.

Colour development:

2 ml. of each tissue extract after bromine oxidation was taken in the test
tubes. 0.5 ml. of dinitrophenylhydrazine-thiourea reagent was added in the
test tubes. Then the mixture was incubated at 37°C for 5 hrs. Then the test
tubes were removed and placed in a beaker of ice water containing sufficient
quantities of ice. 2.5 ml. of 85% H₂SO₄ was run into the tubes very slowly
for 1 min. The tubes were shaken thoroughly to obtain complete mixing. The
tubes were kept in room temperature for about 30 mins. for colour development.
The reading was taken in a photoelectric colorimeter against a reagent blank
prepared simultaneously.

Preparation of calibration curve:

A calibration curve was prepared from a series of aliquots of ascorbic
acid solution having known concentration. The ascorbic acid contents of the tissue
samples were then calculated by referring the colorimetric readings to the
calibration curve.

2. Estimation of total cholesterol content in tissue:

Total cholesterol contents of the tissue samples were determined by a
modified Sperry and Webb procedure (9).

Extraction:

Tissue sample was weighed carefully and homogenised in chloroform-ethanol
(2:1) mixture. The content was transferred to separating funnel. Then the
Procedure:

The aliquot was evaporated to dryness in water bath. Two drops of 33% alcoholic KOH was added to the centrifuge tube. 1 ml. of alcohol-acetone (1:1) solution was added to the mixture. The contents were mixed and incubated at 60°C for half an hour with occasional shaking. 2 drops of phenolphthalein was added to the content and it was then neutralised with 15% acetic acid (about 6 drops). 2 ml. of alcohol-acetone (1:1) solution was then added to the neutralised solution. This was followed by the addition of 1 ml. of 0.5% digitonin solution in alcohol-acetone (1:1) mixture. The mixture was incubated at least for 3 hrs. at 25°C or overnight at room temperature. Then the cholesterol-digitonide complex was precipitated by centrifuging at 3500 RPM for 15-20 mins. The supernatant was decanted and discarded. To the precipitate was added 3 ml. of anhydrous ether by means of a syringe with care so that the ether mixes well with the precipitate. The mixture was centrifuged for 10 mins. The ether was drained off and discarded. Precipitate was dried with heat and air. To the precipitate was added 0.5 ml. of glacial acetic acid. The tube was stoppered and incubated at 60°C until the precipitate was dissolved. 3 ml. of chloroform was added and the mixture was brought to 35°C.

Preparation of colour reagent:

1 ml. of conc. $\text{H}_2\text{SO}_4$ was added to 9 ml. of acetic anhydride previously cooled in an ice-salt bath. Reagent was stable upto 1 hr.

Development of colour:

To the dissolved cholesterol in chloroform at 35°C was added 1 ml. of colour reagent. The contents were mixed well. Exactly after 10 minutes the
tubes were removed and put into ice-salt bath. The reading was taken in a photoelectric colorimeter after 10 minutes.

Blank experiment:

0.5 ml. of glacial acetic acid and 3 ml. of chloroform was mixed and to the mixture 1 ml. of colour reagent was added.

Standard:

Cholesterol was dissolved in chloroform so that each ml. contained 0.1 mg. of cholesterol. Standards were set up in duplicate from 0.1 mg. to 0.6 mg. cholesterol. Readings were taken in the same photoelectric colorimeter at 620 m\(\mu\). The colorimetric readings were plotted against different concentrations of cholesterol. Thus a standard curve was obtained.

Calculation:

Cholesterol contents of tissue samples were determined by extrapolating the experimental colorimetric readings in the standard curve.

REFERENCES