2

Material and Methods
Material & Methods

The investigations on involvement of Zn in obesity, non-insulin dependent diabetes mellitus and hypercholesterolemia were carried out in two steps:

Epidemiological studies on human population and experimental studies on mice (Lacca strain)

2.1 Epidemiological Studies on Human Population

For epidemiological studies, the healthy human volunteers were randomly recruited from the population of Chandigarh and diseased volunteers from outpatient department (O.P.D.), General Hospital, Sector -16, Chandigarh and Endocrinology Department, Post-Graduate Institute of Medical Education and Research (P.G.I.M.E.R.), Chandigarh. Their name, age, weight, height, occupation, food habits and family history were recorded on a proforma given below:

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Date:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name:</td>
<td>Sex: M / F</td>
</tr>
<tr>
<td>Height: cm</td>
<td>Weight: Kg.</td>
</tr>
<tr>
<td>Food Habits: Veg./Non-Veg./Occasional Non-Veg.</td>
<td></td>
</tr>
<tr>
<td>Family History: 1. Address: ____________________</td>
<td></td>
</tr>
<tr>
<td>2. ____________________</td>
<td></td>
</tr>
<tr>
<td>3. ____________________</td>
<td></td>
</tr>
<tr>
<td>Blood Glucose: F PP</td>
<td></td>
</tr>
<tr>
<td>Blood Cholesterol:</td>
<td></td>
</tr>
</tbody>
</table>
Material & Methods

The trace metals status in volunteers was assessed by analysing hair, urine and blood serum employing standard techniques given below:

2.1.1 Collection of hair samples

A small lock of hair, very close to scalp from each of the volunteer was cut from occipital region with stainless steel scissors and stored in clean, polythene bags for further processing.

2.1.2 Collection of blood samples

5 ml of postprandial blood from the antecubital superficial vein of the volunteers was collected in thoroughly washed, autoclaved and dried centrifuge tubes.

2.1.3 Collection of urine sample

The volunteers were asked to collect their morning mid stream urine samples in small, sterilized glass vials which they deposited the next morning.

The hair, blood and urine samples of the volunteers were numbered corresponding to the serial no. given to them in the proforma.

2.1.4 Processing of hair samples

To estimate the trace metal contents (Zn and Cu) in hair, they were thoroughly cleaned to eliminate external environmental contamination in order to avoid discrepancies of any kind. For this, each sample was cleared of the nits and lice, if any. Then it was soaked in a mild detergent for overnight to get rid of oil, dust or any other particles sticking to the hair. The hair were then washed in tap water, soaked in 0.1% EDTA solution for 20 min. to chelate the trace metals sticking to the surface due to environmental contamination. Washed thoroughly again in deionized triple distilled
water. Thereafter, they were washed in absolute alcohol, acetone and finally in ether. They were then dried in oven at 60 °C and stored carefully in polythene bags.

2.1.5 Preparation of serum:

The freshly drawn blood was collected in a thoroughly washed and dried graduated centrifuge tubes. The blood filled tubes were kept in a slanting position and allowed to clot at room temperature. Thereafter the blood was chilled in the refrigerator and centrifuged. The supernatant as serum was collected in washed and sterilized microcentrifuge tubes and used for bioassays.

2.1.6 Assessment of General Obesity in Humans:

In order to assess the obesity, the desirable body weight for the height was calculated by regression analysis using \( y = a + bx \) model of standard weight for height tables of men and women of Indian population developed by Indian council of Medical Research, New Delhi. Excess or less body weight in volunteers was calculated using as:

\[
\text{Percent excess Body wt} = \frac{\text{Observed body Weight} - \text{Desirable Body Weight}}{\text{Desirable Body Weight}} \times 100
\]

Depending upon the percent excess or less body wt. than desirable body for height, the volunteers were divided into four groups.
Material & Methods

(i). **Lean**: The volunteers were considered lean whose body weight was less than -10% of desirable body weight for height.

(ii). **Normal**: The volunteers whose body weight was between -10% and +10% of the desirable body weight for the height were designated as normal.

(iii). **Overweight**: The volunteers were designated as overweight who had their body weights between +10% and +20% above desirable body weight for height.

(iv). **Obese**: The volunteers having body wt. above +20% of desirable weight for height were considered obese.

2.1.7 **Body Mass Index** :

The excess body weight than the desirable body weight as an indicator of degree of obesity being an arbitrary assumption, the measurements of body mass index that has direct relationship to the total body fat (Benn, 1971) was calculated for each volunteer using weight - height relationship (Bray, 1992) i.e.

\[
B.M.I = \frac{W}{H^2}
\]

where \( W \) = weight of the body in kg.

\( H \) = Height in m.

The body mass indices of volunteers were arranged in an ascending manner along with their percent excess or less of body wt. for height, and they were classified as lean, normal, overweight and obese:
Material & Methods

Lean - Volunteers with B.M.I. \(<20\text{ kg/m}^2\)

Normal - Volunteers with B.M.I. \(20\leq 25\text{ kg/m}^2\)

Overweight - Volunteers with B.M.I. \(25<27\text{ kg/m}^2\)

Obese - Volunteers with B.M.I. \(>27\text{ kg/m}^2\)

2.2. Experimental Studies on Mice

48 male albino mice of Lacca strain were purchased from Central Animal House of Panjab University, Chandigarh and were maintained in a separate well aerated and lighted room allotted to the department of Zoology of the Panjab University for maintaining experimental animals. The mice were divided into four groups, 12 mice in each group and six mice per cage. They were kept in perspex cages with stainless steel grills. They were maintained at room temperature varied between 25-32°C.

2.2.1 Ingredients of the diet:

The mice during the experimental period were fed on a semi-synthetic basal diet containing ingredients as shown in Table-1 (Orgebincrist, 1971) which was prepared in the laboratory. Vitamins and minerals constituted 0.5% and 3.5% of the total dry diet respectively.

2.2.1.a Preparation of the basal diet:

The water soluble vitamins and mineral mixture were thoroughly ground and mixed with sucrose, while the fat soluble vitamins were dissolved in corn oil before casein, sucrose, oil and cellulose were mixed together. Agar that served as the binding agent in diet, was dissolved in 200 ml of cold water with constant stirring on water bath.
maintained at 60°C. The agar solution was allowed to cool to 40°C. Thereafter, the mixture of all ingredients was added slowly with gentle stirring. The dough thus formed was poured into the containers. These containers were then placed in the refrigerator for about 30 min. to harden the wet fresh diet. On solidification of the diet, it was cut into small pellets of half inch square each. The pellets were then stored in sterilized bottles with tight lids and stored in refrigerator.
## Table 1

### Composition of Diet for Mice

<table>
<thead>
<tr>
<th>Total Diet</th>
<th>(g/100 g)</th>
<th>* Vitamin Mixture</th>
<th>(mg/kg)</th>
<th>** Mineral Mixture</th>
<th>(g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>30</td>
<td>Ascorbic acid</td>
<td>500</td>
<td>CaH$_2$PO$_4$</td>
<td>25.8</td>
</tr>
<tr>
<td>Agar</td>
<td>1.5</td>
<td>Biotin</td>
<td>4</td>
<td>CoCl$_3$</td>
<td>0.04</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5</td>
<td>Calcium pentothenate</td>
<td>320</td>
<td>CuCl$_2$</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Choline chloride</td>
<td>2500</td>
<td>FeSO$_4$.7H$_2$O</td>
<td>0.60</td>
</tr>
<tr>
<td>Cellulose</td>
<td>8</td>
<td>Folic acid</td>
<td>10</td>
<td>MnSO$_4$.H$_2$O</td>
<td>0.31</td>
</tr>
<tr>
<td>Sucrose</td>
<td>51.5</td>
<td>Inositol</td>
<td>1000</td>
<td>MgSO$_4$.H$_2$O</td>
<td>4.05</td>
</tr>
<tr>
<td>*Vitamin Mixture</td>
<td>0.5</td>
<td>Nicotinic acid</td>
<td>300</td>
<td>KCl</td>
<td>3.43</td>
</tr>
<tr>
<td>** Mineral Mixture</td>
<td>3.5</td>
<td>Pyridoxine HCl</td>
<td>80</td>
<td>Na$_2$CO$_3$</td>
<td>1.15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Riboflavin</td>
<td>120</td>
<td>NaF</td>
<td>0.008</td>
</tr>
<tr>
<td>Water</td>
<td>200</td>
<td>Thiamin HCl</td>
<td>200</td>
<td>ZnSO$_4$.7H$_2$O</td>
<td>0.088</td>
</tr>
<tr>
<td>α-tocopherol acetate (E) Cyanocobalamin</td>
<td>60</td>
<td>0.40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total diet</td>
<td>1000</td>
<td>Retinol</td>
<td>0.3</td>
<td>Orgacalciferol</td>
<td>0.0031</td>
</tr>
</tbody>
</table>
2.2.1. b Modification of Diet for Experiment:

The experiment on mice was carried out in two phases. During phase -I, the mice were fed on a basal diet containing varying concentration of Zn keeping all other ingredient constant as given in table-1. During phase -II, the basal diet was supplemented with Cu keeping Zn in amount equal to that of phase -I.

2.2.1. c Diet of mice in Phase -I of the Experiment:

Following four diets were prepared for the phase-I by adding Zn as ZnSO₄·7H₂O in the basal diet.

1. **Group ZS-I**: Contained 20 mg of Zn per kg diet (control group).
2. **Group ZS-II**: Contained 40 mg of Zn/kg diet.
3. **Group ZS-III**: Contained 60 mg of Zn/kg diet.
4. **Group ZS-IV**: Contained 80 mg of Zn/kg diet.

2.2.1. d Diet of mice in Phase-II of the Experiment:

For the second phase of the experiment Cu concentration was increased by adding CuCl₂ in the mineral mixture in the same ratio as that of Zn as ZnSO₄·7H₂O in the corresponding diet group so that the final Zn: Cu ratio remained constant. According following four diets were prepared:

1. **Group CS –I**: Contained 48 mg of Cu and 20 mg of Zn/kg basal diet.
2. **Group CS-II**: Contained 96 mg of Cu and 40 mg of Zn/kg basal diet.
3. **Group CS-III**: Contained 144 mg of Cu and 60 mg of Zn/kg basal diet.
4. **Group CS-IV**: Contained 192 mg of Cu and 80 mg of Zn/kg basal diet.
2.2.1.e Dietary treatment:

Phase-I:

All the eight cages containing 6 mice each were divided into four groups, ZS-I to ZS-IV, each group with 12 mice. They were fed on a diet containing 20 mg Zn (ZS-I), 40 mg Zn (ZS-II), 60 mg Zn (ZS-III) and 80 mg Zn (ZS-IV) per kg diet for a period of 8 weeks, respectively.

After the termination of dietary period of phase-I, six mice from each group were used for the bioassays while the remaining 6 mice in each were employed for phase -II of the experiment.

Phase -II:

The mice of ZS-I to ZS-IV were fed on copper supplemented diet during the phase-II of the experiment for a period of another 8 weeks. The ZS-I mice were fed on CS-I; ZS-II on CS-II; ZS-III on CS-III and ZS-IV on CS-IV diets and were designated as CS-I, CS-II, CS-III and CS-IV groups in phase-II, respectively. These mice were sacrificed after the end of second phase of dietary treatment.

2.2.1.f Feeding method:

During the phase-I and II of the experiment, the mice of each group were provided ad libitum with the respective food pellets as described previously. These pellets were weighed and given twice a day - at 9 A.M. and 4.30 P.M. The left over food was weighed each time prior to adding a batch of freshly weighed pellets in order to keep the record of daily food intake. The distilled water was made available to them
freely. Nothing more than semi-synthetic diet and double distilled water was made available to the animals during either the phase-I or phase-II of the experiment.

2.2.1.g Food intake:

The record of food intake was maintained on daily basis. However, the net food intake was calculated on weekly basis by following method:

\[
\text{Food Intake per week} \times \frac{100}{\text{mean body wt.} \times 7} = \frac{\text{Daily food intake}}{100 \text{ g body wt.}}
\]

\[
= \frac{\text{wet food intake/day}}{100 \text{ g body wt.}}
\]

The dry food intake was calculated as:

\[
\left( \frac{\text{wet food intake/day}}{100 \text{ g body wt.}} \right) - \left( \text{Total water content in wet diet} \right)
\]

\[
= \frac{\text{Dry food intake/day}}{100 \text{ g body wt.}}
\]

2.2.2 Gain In Body weight:

Body weight gain or loss of mice was recorded on weekly basis. It was calculated as:

\[
\frac{\text{Final body wt.} - \text{initial body wt.}}{\text{Initial body wt.}} \times 100 = \% \text{ gain or loss of body wt.}
\]
2.3. BIO-ASSAYS

After the end of phase-I and phase-II of the experiment, the mice of each group were sacrificed using ether as anesthesia. The blood, skin, liver and abdominal muscles were taken from each mouse and processed for histological, histochemical, trace metals' and biochemical studies.

2.3.1 Preparation of Blood Plasma:

The left brachial artery of the mouse was punctured and the blood was collected in a graduated centrifuge tube having known amount of anti-coagulant (3.8% of sodium citrate in double distilled water) taking care that R.B.C.s did not haemolyze. Immediately, thereafter the blood was centrifuged at 2500 rpm for 10-15 min. The clear supernatant was separated and the residue was discarded. The blood plasma was stored in deep freezer at a temperature of – 25°C.

2.3.2 Histochemical Studies

The skin was separated from the freshly dissected mice from abdominal region of the ventral side and the abdominal muscles lying immediate beneath the skin were separated from the main body, washed in ringer solution and fixed in formal calcium for 24 hrs.

After fixation, the tissues were transferred to dichromate calcium for post chroming, first at room temperature for 6-7 h. and then at 60°C for 18 h. Thereafter, the tissues were washed in running water and embedded in 25% gelatin solution at 37°C for 24 h. The tissues were taken out after embedding, the blocks were made in fresh 25% gelatin solution and stored in cold formal calcium in refrigerator. The frozen
sections were cut at 10\(\mu\)m on cryostat (Minotome-International Equipment Company, U.S.A.).

These gelatin sections were stained in a saturated section of Sudan black-B in 70% alcohol, differentiated in 70% alcohol, washed in distilled water and mounted in glycerine jelly. Lipids were stained blue black. This colouration depends upon the solubility of Sudan black-B in fat itself (Baker 1956).

2.3.3 Tissue Preparation for Electron Microscopic Studies:

Acinar cells of pancreas were studied under transmission electron microscope (T.E.M.). For this study, pancreas from the freshly killed mice was taken out and rinsed in ringer solution and fixed in gluteraldehyde for 2 h. The tissue was washed in phosphate buffer saline (P.B.S.). It was then fixed in 1% osmic acid, in phosphate buffer, for post fixation. After 1 h, the tissue was washed in P.B.S. and dehydrated in 30% graded concentration of acetone.

After dehydration of tissue in acetone, the tissue was kept in the polymerization medium and acetone (V:V/1:3, V:V/1:1, V:V/3:1), finally into pure polymerization media for 48 hours at 60\(^\circ\)C. The blocks were prepared in polymerization medium and the sections were cut (of the order of 600 \(\mu\)m) with glass knife. These sections were stained in sodium citrate and uranyl citrate and studied under transmission electron microscope (Jeol TEMSCAN 100 CX II, Jeol Ltd., Tokyo, Japan) at Institute of Pathology, Safdarjung Hospital Campus, New Delhi.
2.3.4 BIOCHEMICAL ANALYSIS

Blood serum of human volunteers and blood plasma of mice was employed for the estimation for glucose and cholesterol. The triglycerides concentration was estimated in blood plasma of mice only. Following techniques were employed:

2.3.4.a Plasma / Serum Glucose

This test was performed by the method of GOD/POD with the help of the reagents supplied in a kit by SPAN Diagnostics Ltd. (Trinder, 1969).

Reagents

1. Glucose enzyme reagent.
2. Glucose standard, 100 mg%.
3. Phenol reagent.

Principle

In a single reagent system, glucose oxidase converts glucose to gluconic acid and hydrogen peroxide. The peroxide in the presence of horse radish peroxidase (H.R.P.) forms a coloured complex of hydroxyl benzoate and 4 aminophenazone. The intensity of the colour formed is proportional to the glucose level.

Preparation of working glucose reagent

Quantitatively transferred the contents of one vial of glucose enzyme reagent to a black plastic bottle after reconstituting it to 50 ml with de-ionized water. Added 2.5 ml of phenol reagent to this reconstituted glucose reagent and mixed well which was labelled as 'Working Glucose Reagent'.
Procedure

Following solutions were added in the test tubes.

<table>
<thead>
<tr>
<th></th>
<th>Blank(B)</th>
<th>Standard(S)</th>
<th>Test (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum/Plasma</td>
<td>-</td>
<td>-</td>
<td>0.02</td>
</tr>
<tr>
<td>Glucose std. (100 mg/dl)</td>
<td>-</td>
<td>0.02 ml</td>
<td>-</td>
</tr>
<tr>
<td>Working glucose reagent</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>Deionized distilled water</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
<td>1.5 ml</td>
</tr>
</tbody>
</table>

The solutions were mixed well, incubated at 37°C for 15 minutes and colour intensity was measured at 510 nm against distilled water on spectrophotometer (UVIKON-860, Kontron, U.S.A.).

2.3.4.b Plasma / Serum Triglycerides

This was performed by the method of Gottfried and Rosenberg (1973). The details are given under "Assay of Triglycerides" in tissues.

2.3.4.c Plasma / Serum Cholesterol

Ferric chloride method of Chiamori and Henry (1959) was employed for the estimation of cholesterol. Details are given under "Assay of Cholesterol" in tissues.
Biochemical Analysis of Other Tissues

A known weight of shaven skin, abdominal muscles and liver (perfused with 0.9% saline) from different diet groups of mice were taken and processed for following estimations:-

i) Total lipids
ii) Phospholipids
iii) Triglycerides
iv) Cholesterol
v) Glycogen
vi) Super oxide - dismutase in P.M.S. fraction of liver

2.3.4.d Assay Of Total Lipids

Extraction

The lipids were extracted following the method of Folch et al. (1957). Accordingly 2:1 v/v mixture of chloroform - methanol was added to the known wt. of the tissue sample. It was then shaken vigorously. Kept the sample in test tubes overnight at 4°C. The resultant mixture was filtered through fat free filter paper. The filtrate thus obtained was washed twice with 0.2 volumes of normal saline (0.9%) for eliminating non-lipid substances. Each filtrate was kept at least for 2 hours. On the appearance of a biphasic system, the upper layer was removed. The lower layer, containing pure lipids was washed three times with upper phase solvents, chloroform: methanol: water (3:48:47) containing 0.29% sodium chloride. The lower phase was then dried in oven at 45°C to remove any traces of moisture. When a pure lipid extract was obtained in a condensed form, it was finally made to a single phase by the addition of methanol. The
resulting solution was diluted to 5 ml by the addition of chloroform: methanol (2:1) mixture.

**Estimation**

Fringes and Dunn's (1970) colorimetric method was employed for the estimation of total lipids. The colour was developed with the help of phosphovanillin reagent in the presence of concentrated sulphuric acid.

**Reagents**

1. Concentrated sulphuric acid.
2. Phosphovanillin reagent *
3. Olive oil standard (500 mg/100 ml of absolute alcohol).

**Procedure**

To 0.1 ml of lipids extract, 2 ml of concentrated sulphuric acid was added. After shaking, the tubes were kept in boiling water for 15 min. Thereafter, 0.2 ml was taken from each in different test tubes and 5 ml of phosphovanillin was added in each. To the standard olive oil with different concentrations, concentrated sulphuric acid and colouring reagents were added. The colour intensity was recorded on UVIKON spectrophotometer (UVIKON-860, Kontron, U.S.A.) at 540 nm. The amount of total lipid was calculated in mg/g of the fresh tissue weight.

*Appendix I*
2.3.4.e Assay of Phospholipids

Phospholipids were assayed by employing the method of Zilversmit and Davis (1950). The proteins were precipitated by trichloroacetic acid (T.C.A.) and the precipitate was digested with 60% perchloric acid. The inorganic phosphorous thus obtained was analysed with ammonium molybdate and 2-amino-4-napthosulphonic acid (ANSA) reagent to develop the blue colour.

Reagents

1. Trichloroacetic acid (T.C.A.) 10%
2. Perchloric acid (PCA) 60%
3. Ammonium molybdate 4%
4. ANSA reagent*
5. Potassium dihydrogen phosphate as standard (4.391g/l).
6. Working standard: The stock standard was diluted in the ratio of 1:100 (10 µg phosphorus/ml).

Estimation

To 0.2 ml of lipid extract, 3 ml of distilled water was added. To this, 1.5 ml of 10% T.C.A. was added, mixed well and centrifuged after 10 min. To the residue, 1 ml of 60% P.C.A. was added and was kept on a sand bath till the fluid became colourless. Thereafter, the volume was made to 7 ml with distilled water and 1 ml of ammonium molybdate was added. The contents of the tubes were shaken well and 1 ml of ANSA reagent was added in each.

*Appendix 1
To 1 ml of standard solution, 0.8 ml of 60% PCA was added and volume was made to 7 ml. Thereafter, 1 ml ammonium molybdate and 1 ml ANSA reagent was added. For blank, 6.2 ml distilled was added to 0.8 ml of 60% perchloric acid, 1 ml ammonium molybdate and 1 ml ANSA.

The intensity of the colour in all test tubes was read at 660 nm after 20 min. on UVIKON spectrophotometer (UVIKON-860, Kontron, U.S.A.). The lipid phosphorous thus obtained was multiplied by a factor of 25, to obtain the total phospholipids present in the aliquot.

2.3.4.f Assay of Triglycerides

The triglycerides' fraction was assayed by employing the method of Gottfried and Rosenberg (1973). It was extracted in the organic solvent and hydrolyzed by potassium hydroxide to produce glycerol and fatty acids. Glycerol was allowed to react with sodium metaperiodate to form formaldehyde in the presence of ammonium ions and which gave specific coloured complex.

Reagents

1. N-heptane
2. Isopropanol
3. Sulphuric acid (concentrated).
5. Sodium metaperiodate*
Material & Methods

6. Ammonium acetate*

7. Acetyl acetone reagent*

8. Triolein (100 mg/100 ml Isopropanol)

Extraction

Glass stoppered tubes were taken and following solutions were added accordingly.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Test sample</td>
<td>-</td>
<td>-</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>2. Standard</td>
<td>-</td>
<td>0.2 ml</td>
<td>-</td>
</tr>
<tr>
<td>3. Isopropanol</td>
<td>3.8 ml</td>
<td>3.6 ml</td>
<td>3.8 ml</td>
</tr>
<tr>
<td>4. n-Heptane</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>5. Distilled water</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>-</td>
</tr>
</tbody>
</table>

Thereafter, 1 ml of concentrated sulphuric acid was added and the mixture in each test tube was shaken vigorously.

Saponification, oxidation and colour development

To 0.8 ml of the upper layer (which was triglyceride extract), 4 ml of isopropanol was added followed by two drops of potassium hydroxide. After stoppering, the tubes were incubated at 60°C for 10 min. Thereafter, 0.4 ml of sodium metaperiodate reagent and 2 ml of acetyl acetone reagent were added in each. The mixture was mixed well, stoppered and was again incubated at 60°C for 10 min.
The intensity of the colour in all the test tubes was read at 425 nm after 20 min. on UVIKON spectrophotometer (UVIKON-860, Kontron, U.S.A.). A standard curve was plotted by using different concentrations of triolein. Finally the quantity of triglyceride was calculated in mg/g of fresh tissue weight.

2.3.4.g Assay of Cholesterol

Ferric chloride method of Chiamori and Henry (1959) was employed for the estimation of cholesterol in the lipid extract. This method is based on the fact that ferric chloride-acetic acid reagent precipitates the proteins and liberates the cholesterol that remains in supernatant. The cholesterol on reaction with concentrated sulphuric acid makes a chromophore (coloured complex).

Reagents

1. Ferric chloride - acetic acid reagent.*

2. Concentrated sulphuric acid.

3. Cholesterol standard (4mg/100 ml).

Procedure

To 9.9 ml of ferric chloride-acetic acid reagent in a centrifuge tube, added 0.1 ml of lipid extract. The tubes were shaken thoroughly and were kept at room temperature for 10 min. These were then incubated at 60°C for 2 min. with occasional shaking. After centrifuging, 5 ml of supernatant was taken and 3 ml of concentrated sulphuric acid was added. After keeping the tubes for 10 min. at room temperature, the colour

*Appendix 1
intensity was read at 560 nm. Finally the amount of cholesterol was calculated in mg/g fresh tissue wt.

2.3.4.h Assay of Glycogen

The methods of Heatley (1935) and Seifter et al. (1950) were adopted for the extraction and estimation of glycogen. The former is based upon the fact that carbohydrates are precipitated in the presence of excess alcohol. The precipitated glycogen can be separated by centrifugation. For the quantitative estimation of glycogen, Seifter’s method determines hexoses, aldopentoses and hexuronic acids, either free or present in polysaccharides with the help of anthrone reaction.

Extraction

Fresh tissue of known weight was fixed in alcohol to stop enzymatic activity. It was then digested in 30% potassium hydroxide in a stoppered test tube in a water bath. 0.2 ml of saturated sodium sulphate solution was added to it. Glycogen was precipitated with an excess of absolute alcohol. The process of precipitation with alcohol was repeated thrice to ensure complete removal of unwanted proteins. To the precipitate, 5 ml of distilled water was added with gentle warming and diluted to 10 ml with more distilled water.

Estimation

Quantitative estimation of glycogen was made by colorimetric method of Seifter et al. (1950). The anthrone reaction is the basis of a rapid and convenient method for the determination of hexoses, aldopentoses and hexuronic acids, either free or present in polysaccharides. The blue green solution shows an absorption maxima at 620 nm.
Material & Methods

Reagents

1. Anthrone*
2. Glucose (0.1 g/l)

Procedure

To 1 ml of protein free carbohydrate solution, 4 ml of anthrone reagent was added and mixed it rapidly. The tubes were covered with glass marbles and heated for 10 min. in a boiling water bath. Thereafter, they were cooled and the colour intensity was read at 620 nm against a reagent blank. The colours were compared against glucose as standard. Finally, the amount of glycogen was calculated in mg/g of fresh tissue weight.

2.3.4.i Assay of Super Oxide Dismutase (S.O.D.)

Preparation of post mitochondrial supernatant (P.M.S.) fraction :-

A known wt. of perfused liver was homogenized in known quantity of 0.25 M sucrose in ice. Then the contents were centrifuged differentially at 10,000 rpm at 4°C in cold centrifuge (Remi, C-30, India). The supernatant was stored in freezer and pellet was discarded.

The supernatant was then used for the protein estimation and SOD estimation.

Protein estimation in P. M. S. Fraction

The method of Lowry et al. (1951) was followed. Protein reacts with the Folin - Ciocalteau reagent to form coloured complex. The colour produced is due to the

*Appendix I
combination of biuret reaction and the reduction of phosphomolybdate by aromatic amino-acids.

Reagents

1. Folin - Ciocalteau reagent*
2. Lowry reagent*

Procedure

To 0.1 ml of the protein fraction, 0.9 ml of distilled water and 5 ml of Lowry reagent were added. They were mixed well and allowed to stand for 10 min. Then 0.5 ml of Folin-Ciocalteau reagent was added to the mixture. The contents were shaken vigorously and allowed to stand for 30 min. for maximum development of the colour. The colour intensity was then measured at 700 nm on UVIKON spectrophotometer (UVIKON-860, Kontron, U.S.A.).

A standard curve was plotted by using known but different concentrations of bovine serum albumin (Sigma). Finally the amount of protein (0.1N NaOH extractable protein) was calculated in mg/g of fresh tissue weight.

Estimation of S.O.D.

Principle

SOD was estimated by the method of Kono (1978). This method is based on the principle of the inhibitory effect of SOD on the reduction of nitroblue tetrazolium (N.B.T.)
Material & Methods

dye by superoxide anions which are generated by the photoxidation of hydraxylamine hydrochloride (NH₂OH.HCl) at 560 nm.

Reagents

Solution A: 0.1 mM EDTA containing 50 mM sodium carbonate solution, pH = 10.

Solution B: 96 µM of NBT in solution A (2 mg NBT/25ml of distilled water).

Solution C: 0.6% Triton - X -100 in solution A.

Solution D: 20 mm Hydroxylamine hydrochloride (14 mg/10ml in double distilled water), pH adjusted to 6.0 by 0.1 N NaOH.

Procedure

To the test cuvette the following solutions were added successively: 1.3 ml of solution A, 0.5 ml of solution B and 0.1 ml of solution C. Reaction was initiated by the addition of 0.1 ml solution D. to the reaction mixture and the rate of N.B.T. reduction in the absence of the enzyme source was recorded for about 20 seconds. Following small aliquots of P.M.S. from liver (25-400 µg protein) were added to the test cuvette as well as the reference cuvette, which did not contain solution D. Finally, percentage inhibition rate of N.B.T. reduction was noted and one unit of enzyme was expressed as inverse of the amounts of P.M.S. protein (mg.) required to inhibit rate of N.B.T. by 50%.
2.3.5 ESTIMATION OF ZINC AND COPPER

The concentration of Zn and Cu were measured in the tissues of the human volunteers and the mice on which the experiments were conducted. These tissues were hair, blood serum and urine of human volunteers and hair, blood plasma, shaven skin, and liver of experimental mice groups. They were processed as follows:

2.3.5.a Hair:

50 mg of hair samples were taken in a crucible and digested in 3:1 v/v concentration of nitric acid and perchloric acid in a sand bath till a white ash was obtained. The ash was dissolved in 6 ml of 10 mM nitric acid.

2.3.5.b Serum / Plasma:

0.1 ml of serum/plasma sample was dried first at 60°C in oven, then digested in a crucible, with 3:1 v/v nitric acid and perchloric acid on a sand bath till a white ash was obtained. The ash was dissolved in 6 ml of 10 mM nitric acid and collected in cleaned, sterilized vials.

2.3.5.c Urine:

3 ml of urine sample was taken and digested it in 3:1 v/v nitric acid and perchloric acid on a sand bath till a white ash was formed. Again ash was dissolved in 6 ml of 10 mM nitric acid and collected in clean, sterilized vials.

2.3.5.d Mice tissues:

50 mg of shaven skin, perfused liver samples of the sacrificed mice were taken separately and digested in 3:1 v/v nitric acid and perchloric acid on a sand bath till a
white ash was formed. This ash was dissolved in 6 ml of 10m M nitric acid and stored in clean, sterilized vials.

The digested material of serum, hair, urine of human volunteers and liver, skin, hair and blood plasma of mice were used for the estimation of the Zn and Cu concentrations in them on atomic absorption spectrophotometer (AAS 4139, Electronic Corporation of India Ltd., Hyderabad) on 213.9 nm and 324.8 nm respectively.
2.4 ANALYSIS OF DATA

The data collected during this study were subjected to statistical analysis.

2.4.1 Standard deviation (SD):

It is the square root of the average of square of difference between the observed and mean value:

\[ S.D = \sqrt{\frac{\text{mean} - \text{Individual value}}{n - 1}} \]

\[ = \frac{\sqrt{\sum(x - \bar{x})^2}}{n - 1} \]

2.4.2 Standard error:

It is the measure of confidence with which statistical result is interpreted. S.E. =

\[ S.E. = \frac{S.D.}{\sqrt{n}} \]

2.4.3 Karl Pearson's Co-efficient of Correlation:

It is denoted by the symbol of \( r \) and is used universally for describing the degree of correlation (i.e. co-variance) between two variables:

\[ r = \frac{\sum xy}{n\sigma_x\sigma_y} \]

where \( \sigma_x \) = Standard deviation of series x

\( \sigma_y \) = Standard deviation of series y
Material & Methods

\[ N = \text{Number of pairs of observations} \]

\[ r = \text{The co-efficient of correlation} \]

Or by the direct method

\[ r = \frac{\sum xy - Nx\bar{y}}{\sqrt{\sum x^2 - N\bar{x}^2}\sqrt{\sum y^2 - N\bar{y}^2}} \]

2.4.4 Student's t-Test

\[ t = \frac{\text{Mean}_1 - \text{Mean}_2}{\sqrt{(S.E.)_1^2 + (S.E.)_2^2}} \]

Degree of freedom : \( n_1 + n_2 - 2 \)

\( \text{Mean}_1 \) = mean value of control data.

\( \text{Mean}_2 \) = mean value of experimental data.

\( S.E.\_1 \) = Standard error of control data.

\( S.E.\_2 \) = Standard error of experimental data.

Students t test on coefficient of correlation (r) =

\[ t = \frac{r\sqrt{n}}{1 - r^2} \]

Degree of freedom = \( n_1 + n_2 - 2 \)

\( r \) = co-efficient of correlation

\( n \) = no. of observations
2.4.5 Significance

The 't' values were used to identify the significance by employing Ipsen and Fiegl (1970) formula for significant test. The probability 'p' for obtaining 't' value for a degree of freedom (df) was determined by comparing the 't' value with probability for a given degree of freedom. 'p' values were considered significant to the following conventions:-

- p > 0.05 Insignificant
- p < 0.05 Almost significant
- p < 0.01 Significant
- p < 0.001 Highly significant