CHAPTER-III
MATERIAL AND METHODS

Chemicals

Cadmium chloride (CdCl₂·H₂O) was obtained from E. Merck (Germany), carrier-free radioisotopic (¹⁰⁹Cd) cadmium chloride from Amersham, U.K., calcium trisodium diethylene triamine-pentaacetate from Heyl and Co., Germany and glibenclamide from Hoechst Pharmaceutical Ltd., India. All other chemicals were of "Extra pure" or "Analar" quality and procured from Sigma (USA), E. Merck (Germany), BDH, Glaxo or S. Merck (India).

Instruments

Ultraviolet and visible spectrophotometer (Bausch and Lomb, Spectronic 21), atomic absorption spectrophotometer (Perkin-Elmer 5000), gamma counter (LKB-1280 Wallac, Ultra-
Chemical Formula of the Compounds

1-((p-(2-(Chloro-o-anisamido)ethyl)phenyl)sulfonyl)-3-cyclohexylurea (Glibenclamide, Hoechst Pharmaceutical Ltd., India)

Calcium trisodium diethylenetriaminepentaacetate (Ditripentat, CaNa$_3$DTPA, Heyl, Germany)

trans-1,2-Diaminocyclohexanetetraacetic acid (CDTA, Sigma, U.S.A.)
Sodium diethyldithiocarbamate (trihydrate) (NaDDC, E. Merck, Germany).

\[
\text{CH}_3\text{CH}_2\text{N-C-S-Na}\cdot 3\text{H}_2\text{O}
\]

\[
\text{CH}_3\text{CH}_2\text{S}
\]

Sodium diethyldithiocarbamate (trihydrate) (NaDDC, E. Merck, Germany).

\[
\text{H}_2\text{N-CH}_2\text{-CH}_2\text{-NH-CH}_2\text{-CH}_2\text{-NH-CH}_2\text{-CH}_2\text{-NH}_2
\]

Triethylenetetramine (TETA, Lab-Chem, India)
gamma), hematofluorometer (Aviv ZPP meter) and refrigerated centrifuge (Remi K-24) were used.

**Animals and Diets**

Male or female albino rats (120-160 g) of Industrial Toxicology Research Centre's colony were used. They were maintained on standard pellet diet (Hindustan Lever Ltd., India) or synthetic diet (Table 1) and water *ad libitum* in an air conditioned room. The metal contents (ppm dry weight) of the standard pellet diet were - Cu 10.0, Mn 55.0, Co 5.0, Zn 45.0 and Fe 70.0.

**Table 1: Chemical Composition of Synthetic Diet.**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Percent ingredients of diet</th>
<th>Normal</th>
<th>Low protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(9%)</td>
<td>(5.5%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Normal</td>
<td>Low protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>21.0</td>
<td>9.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>10.0</td>
<td>15.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>18.65</td>
<td>20.65</td>
<td>24.15</td>
</tr>
<tr>
<td>Dextrin</td>
<td>20.0</td>
<td>25.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Potato starch</td>
<td>20.0</td>
<td>20.0</td>
<td>25.0</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>Refined groundnut oil</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Salt mixture a</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Vitamin mixture b</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>
1,000 g of salt mixture consisted of calcium carbonate 68.6 g, calcium citrate 308.3 g, calcium biphosphate 112.8 g, magnesium carbonate 35.2 g, magnesium sulfate (anhydrous) 38.3 g, potassium chloride 124.7 g, dibasic potassium phosphate 218.8 g, sodium chloride 77.1 g, and 16.2 g from the following mixture (cupric sulfate 0.48 g, ferric ammonium citrate 94.33 g, manganese sulfate 1.24 g, ammonium alum 0.57 g, potassium iodide 0.25 g, sodium fluoride/3.13 g = 100 g).

1,000 g of vitamin mixture consisted of thiamine HCl 600.0 mg, riboflavin 600.0 mg, pyridoxine HCl 700.0 mg, nicotinic acid 3.0 g, D-calcium pentothenate 1.6 g, folic acid 200.0 mg, D-biotin 20.0 mg, cyanocobalamine (vitamin B-12) 1.0 mg, retinol acetate (to provide 400,000 IU vitamin A activity) 137.6 mg, dl-α-tocopherol acetate (to provide 5,000 IU vitamin E activity) 5.0 g, cholecalciferol (Vitamin D₃) 2.5 mg, menaquinone (Vitamin K) 5.0 mg, sucrose to make up.

**Processing of tissues**

The control and experimental animals were sacrificed by decapitation or cervical dislocation at appropriate intervals. The blood was collected from the heart in double oxalate (ammonium oxalate and potassium oxalate) coated vials.

Liver, kidney, brain, spleen or pancreas were removed free of extraneous material, blotted and stored at -4°C till
the estimation of metals, enzymes and other parameters. Liver and kidney were homogenized in 0.25 M sucrose using Potter-Elvehjem homogenizer fitted with teflon pestle to obtain 10% (w/v) homogenate.

**Estimation of non-enzyme parameters**

**Blood glucose**

Blood glucose was estimated by the method of Folin and Wu (1965). 0.2 ml blood was diluted with 3.2 ml of distilled water; 0.3 ml of 2/3 N \( \text{H}_2\text{SO}_4 \) and 0.3 ml of sodium tungstate were added and centrifuged. 1 ml of supernatant was transferred to Folin-Wu sugar tube, 1 ml of alkaline copper sulfate solution* was added and tubes were kept in boiling water bath for 8 min, cooled in running water without shaking and 1 ml of phosphomolybdic acid reagent** was added. After about 1 min, it was made up to 15 ml with water. Absorbance was read at 420 nm and glucose content expressed as mg/100 ml.

*Alkaline copper sulfate solution*

40 g of pure anhydrous sodium carbonate was dissolved in 400 ml of water, 7.5 g tartaric acid followed by 4.5 g of crystalline copper sulfate were added, mixed and diluted to 1 litre.

**Phosphomolybdic acid reagent**

To 35 g of molybdic acid and 5 g of sodium tungstate, 200 ml of 10% NaOH and 200 ml of water were added, boiled for 20 min., cooled and diluted to 350 ml. 125 ml of conc. phosphoric acid (85%) was added and diluted to 500 ml.
Liver Glycogen

Glycogen content was estimated by the phenol-sulphuric acid method of Montgomery (1957). About 1 g of liver, immediately after killing, was transferred to a weighed tube containing 3 ml of 30% KOH solution. The tubes were quickly weighed and kept in a boiling water bath for about 30 min. After the dissolution of the tissue, 0.5 ml of saturated sodium sulfate solution was added. The glycogen was precipitated by addition of 1.2 volume of 95% ethanol, contents were mixed thoroughly and kept in a boiling water bath till boiling, cooled and centrifuged. The supernatant was decanted and the tubes were kept in boiling water bath for a few min. to expel the adhering alcohol. The residue was dissolved in 2 ml of distilled water and reprecipitated with 2.5 ml of 95% ethanol, centrifuged, supernatant discarded and alcohol removed as above. The precipitate was redissolved in water and diluted to a known volume (10 ml) and centrifuged to remove insoluble material.

To 2 ml of clear solution, 0.1 ml of 80% phenol and 5 ml conc. H$_2$SO$_4$ were added, mixed properly and allowed to stand for 30 min at room temperature. The yellow orange colour was read at 490 nm. Standard glycogen solution was processed identically for comparison and glycogen expressed as mg/g, fresh tissue.
Metallothionein

Metallothionein (MT) was estimated according to the method of Eaton and Toal (1982). Tissue was homogenized in 4 volume of 10 mM Tris-HCl buffer (pH 7.4) and centrifuged at 10,000 x g for 10 min and the supernatant was heated for 2 min in a boiling water bath, cooled and centrifuged again at 10,000 x g for 2 min to remove precipitated proteins. 0.2 ml of supernatant was transferred to 1.5 ml polyethylene microcentrifuge tube and 0.2 ml of radioisotopic cadmium chloride ($^{109}$CdCl$_2$, 2µg Cd/ml in tris-HCl buffer, activity 1.0 µCi/ml) was added and mixed. After 10 min, 0.1 ml of bovine haemoglobin (2% in tris-HCl buffer) was added, mixed and tubes were heated in a boiling water bath for 2 min, cooled and centrifuged. 0.1 ml of haemoglobin solution was again added and the heating, cooling and centrifuging were repeated. 0.2 ml of clear supernatant was transferred to a gamma counting tube and radioactivity was measured on a gamma counter. Blank tube with buffer in place of the tissue supernatant and another tube for total activity with buffer in place of tissue supernatant and haemoglobin, were run with each assay. The amount of MT represented as µg MT/g, tissue.

Glutathione (GSH)

Glutathione was assayed using the procedure of Ellman (1959) as modified by Jollow et al. (1974). 2 ml of 10% (w/v) liver or kidney homogenate and an equal volume of
sulphosalicylic acid (4%) were mixed and kept in cold for 1 hr, centrifuged at 3,000 x g for 10 min at 4°C. To 0.5 ml of the aliquot, 4 ml of 0.1 M phosphate buffer (pH 7.4) and 0.2 ml of 5,5'-dithiobis-2-nitrobenzoic acid (0.01 M DTNB in 0.1 M phosphate buffer) were added and kept at room temperature for 30 min. The absorbance was read at 412 nm against blank containing phosphate buffer and DTNB. 100 µg/ml of standard GSH in phosphate buffer was used for standard curve. Results expressed as µmole GSH/g, tissue.

**Blood zinc protoporphyrin (ZPP)**

Blood ZPP was determined in a drop of blood with the help of Aviv haematafluorometer using a calibrated glass slide (Grandjean, 1979).

**Protein**

Total protein was estimated in serum, liver and kidney using bovine serum albumin as standard according to the reported procedure (Lowry et al., 1951).

**Enzyme Assays**

**Alkaline phosphatase (ALP)** (Orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1)

The method of King and Armstrong (1964) was followed for the colorimetric estimation of ALP. The assay system consisted of 1.0 ml of 0.05 M sodium carbonate buffer (pH 10), 0.1 ml
of 0.01 M disodium phenyl phosphate and 0.1 ml of 1% (w/v) liver or kidney homogenate. The reaction mixture was incubated at 37° for 15 min and the reaction was terminated by the addition of 0.8 ml of 0.5 N NaOH. The colour was developed by the addition of 1.2 ml of 0.5 M sodium bicarbonate, 1.0 ml of 0.03 M 4-aminoantipyrine and 1 ml of 2.4% K₃Fe(CN)₆ to the reaction mixture which was read immediately at 510 nm. The activity expressed as n mole of phenol liberated/min/mg protein.

Acid phosphatase (AP) (Orthophosphoric-monoester phosphohydrolase, EC 3.1.3.2)

Enzyme activity was determined as described by King and Armstrong (1964). The assay system consisted of 1.0 ml of 0.01 M disodium phenyl phosphate, 1 ml of 0.2 M citrate buffer (pH 4.9) and 0.05 ml of 1% (w/v) liver or kidney homogenate. After incubation at 37° for 1 hr, the reaction was stopped by addition of 1.0 ml of 0.5 N NaOH. The colour was developed by addition of 1.0 ml of 0.5 M NaHCO₃ followed by 1 ml of 0.03M 4-aminoantipyrine and 1 ml of 2.4% K₃Fe(CN)₆ to the reaction mixture. The reddish brown colour was read immediately at 510 nm. The activity expressed as n mole of phenol liberated/min/mg protein.

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9)

Enzyme activity was determined using the method of
Swanson (1955). The assay system in a total of 0.5 ml consisted of 0.3 ml of maleate buffer (0.2 M maleic acid-NaOH, pH 6.5) and 0.1 ml of 10% liver or kidney homogenate. The reaction was started by the addition of 0.1 ml of 0.1 M glucose-6-phosphate. The tubes were incubated at 37° for 15 min. The reaction was terminated by the addition of 1.0 ml of 10% ice cold trichloroacetic acid, diluted with 1 ml of water and centrifuged. Suitable aliquot (0.5 ml) was taken for the estimation of inorganic phosphorous (Pi) according to the method of Fiske and SubbaRow (1957). The amount of Pi produced was calculated from standard curve for Pi prepared simultaneously. The activity expressed as n mole Pi liberated/min/mg protein.

**Fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate-1-phosphohydrolase, EC 3.1.3.11)**

Enzyme activity was measured as described by McGilvery (1955). The assay system consisting of 0.1 ml of 0.05 M fructose-1,6-diphosphate, 0.4 ml of borate buffer (0.05 M boric acid -NaOH, pH 9.5), 0.1 ml of 0.05 M magnesium sulfate and 0.1 ml of 0.005 M manganese chloride was kept at 37° for 5 min. 0.2 ml of 10% liver or kidney homogenate was added, followed immediately by 0.1 ml of cysteine (0.05 M cysteine-NaOH, pH 9.5) previously warmed to 37°. After 20 min of incubation at 37°, 1 ml of 0.1 M trichloroacetic acid was
added and tubes centrifuged. Suitable aliquot (0.5 ml) was taken for the estimation of inorganic phosphorous (Pi) (Fiske and Subbarow, 1957). The amount of Pi produced was calculated from standard curve and the activity expressed as nmole Pi liberated/min/mg protein.

**Glutamic oxaloacetic transaminase** (GOT) (L-Aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1)

The enzyme assay was carried out as described by Reitman and Frankel (1957). 1 ml of substrate (2 mM Ketoglutarate and 0.2 M dl-aspartate in 0.1 M phosphate buffer, pH 7.4) and 0.2 ml of serum, liver or kidney homogenate were incubated at 37° for 60 min the reaction was terminated by the addition of 1 ml of 2,4-dinitrophenylhydrazine (1 mM in 1N HCl) and allowed to stand at room temperature for 20 min, followed by addition of 10 ml of 0.4 N NaOH. After 30 min, absorbance was measured at 505 nm against water as blank. The activity expressed as nmole hydrazone formed/min/mg protein.

**Glutamic pyruvic transaminase** (GPT) (L-Alanine: 2-oxoglutarate aminotransferase, EC 2.6.1.2)

The procedure was essentially same as described above for GOT, except that 0.2 M dl-alanine instead of 0.2 M dl-aspartate was taken for the substrate and incubation time was reduced to 30 min. The enzyme activity expressed as nmole of hydrazone formed/min/mg protein.
Ribonuclease (EC 3.1.27.5)

The enzyme activity was determined as described by Kalnitsky et al. (1959). The assay system comprised of 1 ml of acetate buffer (0.1 M, pH 5.0), 0.2 ml of liver or kidney homogenate and 0.3 ml of distilled water. The reaction was initiated by addition of 0.5 ml of ribonucleic acid solution (1%, in acetate buffer) and was incubated at 37° for 30 min. The reaction was terminated by the addition of 1 ml of chilled uranyl acetate solution (0.75%, in 25% perchloric acid). The acidified samples were cooled in an ice bath and centrifuged. An aliquot of the supernatant was diluted 30 times with distilled water and absorbance read at 260 nm. The activity expressed in arbitrary unit; one unit will produce acid soluble oligonucleotides equivalent to a change in absorbance at 260 nm of 1. The specific activity expressed as unit/hr/mg protein.

Estimation of Metals

Measured samples of urine, faeces, blood, liver or kidney were digested in acid mixture (HNO₃:H₂SO₄:HClO₄::6:1:1) (Schroeder and Nason, 1971) and the carbon free residue dissolved in 5 ml of 5% HNO₃. The samples were read at 228.8, 213.9, 324.7 and 248.3 nm for Cd, Zn, Cu and Fe respectively on an atomic absorption spectrophotometer (Perkin-Elmer-5000) against suitable standards prepared identically. A mixture of acetylene and air was used as fuel.
For counting of radioisotopic cadmium ($^{109}$Cd), a weighed sample of liver or whole tissue was transferred to a gamma counting tube and the radioactivity was measured on a gamma counter (LKB-1280 Wallac, Ultragamma). The radioactivity of a suitable standard was measured simultaneously.

**Statistical Analysis**

Student's 't' test (Fischer, 1950) was applied to calculate the degree of statistical significance between two groups.