CHAPTER III

MATERIALS AND METHODS
EXPERIMENTAL

3.1 Animals

Female albino rats from Industrial Toxicology Research Centre (ITRC) colony weighing approximately 120 gms were housed in an air-conditioned room. Rats were maintained on ad libitum pellet diet (Hindustan Lever Laboratory Animal Feeds, India). Pellet composition conforming to the nutritional standards recommended by the United States National Research Council Publication No. 990, "National requirement of Laboratory Animals" and tap water.

3.1.1 Treatment

3.1.1.1 Studies on the kinetics of nickel binding bioligands in hepatic and renal cytosol of radioactive nickel (63NiCl₂) treated rats

Animals were divided into two groups indicating the treatment.

Group I (Control group)

Normal rats received 0.9% NaCl/kg body weight and killed at 6, 16, 24 and 48 hrs time intervals.
Group II (Experimental group)

Normal rats received radioactive nickel (II) chloride ($^{63}$Ni, $400 \mu$Ci) as $50 \mu$ mole Ni$^{2+}/2$ ml/kg body weight subcutaneously.

Radioactive Nickel ($^{63}$Ni) and its measurement

The desired nickel concentration was prepared by adding $^{63}$NiCl$_2$ (carrier free) to cold nickel chloride which was then diluted to isotonic (0.9% sodium chloride) saline solution. The tissue samples were cleaned out of extraneous material, blotted and known amounts of tissue was digested with nitric acid into colourless solution. Now to this colourless solution added 10 ml of aquasol universal LSC cocktail (obtained from New England Nuclear Corporation, Boston, U.S.A.). After this, radioactive $^{63}$Ni was counted on LKB Rack Beta 1215 Liquid Scintillation Spectrometer.

3.1.1.2 Evaluation of a novel chelating agent for the selective removal of nickel from the nickel poisoned rats

This study have been carried out in three groups:

Group I: Evaluation of the effective dose of chelating drugs

Normal rat received 0.9% NaCl/kg body weight and served as control.

The chelating drugs, i.e. cyclam, cyclam 'S', TETA and glutathione were administered to rats pretreated with the
500 μ mole NiCl₂/kg body weight at three different doses (250 μ mole, 500 μ mole and 1000 μ mole/kg body weight) immediately after nickel administration through the same route.

The same set of experiment was repeated for the estimation of nickel, copper, iron, zinc and manganese.

**Group II: Prolonged effect of chelating drugs against severe nickel intoxication**

Four groups of rats received 1000 μ mole of each chelating drugs (cyclam, cyclam 's', TETA and glutathione) followed by the 500 μ mole NiCl₂ as NiCl₂6H₂O/kg body weight, on 3rd and 6th day again same rats received 500 μ mole of nickel and watched the mortality upto day 9.

**Group III: Cation transport study**

The model is shown in Chapter V. The transport of nickel of different concentration (0.025, 0.00625, 0.000625 M nickel) was estimated by Atomic Absorption Spectrometry in outer control compartment of distilled water. The control sample contained only chloroform.

**3.1.1.3 Toxicity of chelating drugs**

This study was carried out in two phases:

**Phase 1**

Twentyone groups of rats received 500 μ mole/2 ml/kg
body weight, subcutaneously, of seven chelating drugs, namely, Cyclam, TETA, Glutathione, EDTA, CDTA, DTPA and HEDTA and were killed at 16, 24 and 72 hrs after treatment.

The control group received 0.9% NaCl/kg body weight. The liver and kidney were taken for the estimation of copper, zinc, manganese and iron.

**Phase 2**

To see the response of different chelating drugs towards the changes in the levels of glutathione, glutathione-S-transferase, phosphoglucomutase, lactate dehydrogenase and glutamate oxaloacetate transaminase, the animals were treated as described in Phase 1. Animals were killed and liver and kidney were taken out and processed for the enzyme estimations.

3.1.1.4 Reversal of nickel induced alterations by chelating drugs

**Phase 1**

Eight groups of normal rats received 250 μ mole/kg body weight nickel as NiCl₂ 6H₂O, subcutaneously. First group served as control and to the others seven group, chelating drugs (500 μ mole/kg body weight) were administered, namely, Cyclam, TETA, GSH, EDTA, CDTA, DTPA and HEDTA, in the ratio of 1:2 with nickel (250 μ mole), through the same route. Animals of all the groups were killed after 16 hrs of the
treatment. Liver and kidney were processed for copper, zinc, iron and manganese estimation.

Phase 2

To see the effect of chelating drugs on the various biochemical parameters, namely, hepatic and renal glutathione, glutathione-S-transferase and phosphoglucomutase and serum lactate dehydrogenase and serum glutamate oxaloacetate transaminase, animals were treated as described in Phase 1. Animals were killed after 16 hrs of treatment and liver, kidney and serum were processed for various enzymatic estimations.

3.1.1.5 Pharmacokinetic studies on the mobilization of nickel by chelating drugs from the liver and kidney of nickel treated rats

Kinetic study (16, 24 and 72 hrs) have been carried out on the groups of animals received cyclam, TETA, GSH, EDTA, CDTA, DTPA and HEDTA (500 μ mole/kg body weight) in the rats pretreated with nickel (250 μ mole/kg body weight as NiCl₂·6H₂O), subcutaneously in the ratio of 1:2:metal:chelating drugs. The animals were killed at 16, 24 and 72 hrs after treatment. Liver and kidney were processed for nickel estimation.

3.1.2 Tissue preparation

Animals were kept on fasting for 24 hrs and sacrificed by decapitation or cervical dislocation for specific
SCHEME FOR SUBCELLULAR FRACTIONATION

Homogenate (10%, w/v)

↓

Centrifugation at 800 x g x 10 min

Post nuclear fraction

↓

Centrifugation at 1200 x g x 20 min

Post-nuclear fraction (Pellet)

↓

Centrifugation at 1200 x g x 20 min

Post-mitochondrial

supernatant

↓

Resuspended carefully in 0.25 M sucrose solution and again centrifuged at 1200 x g x 20 min.

Supernatant

↓

Centrifugation at 105,000 x g x 60 min in ultracentrifuge

Post-microsomal

supernatant

↓

Resuspended carefully in 0.25 M sucrose and centrifuged at 10,500 x g x 60 min

Supernatant

Cytosol fraction

↓

Microsomal fraction.

Fig. III.1

(Witschi and Aldridge, 1968)
studies at different time intervals. Blood samples were collected and plasma was separated by centrifugation. Livers were rapidly excised out, washed, weighed and homogenized in ice-cold, 0.25 M sucrose unless otherwise stated. The subcellular fractionations were carried out according to the procedure described by Witschi and Aldridge (1968) (Fig. III.1).

3.2 ESTIMATION OF ENZYMATIC AND NON-ENZYMATIC PARAMETERS

3.2.1 Phosphoglucomutase

The activity was determined by V.A. Najjar (1955). Specific activity/µg glucose-6-phosphate formed per minute per milligram protein.

**Assay**

The reaction mixture contained:

<table>
<thead>
<tr>
<th></th>
<th>Experimental</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄ (0.006 M)</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>Glucose-1-phosphate (0.02 M, pH 7.5)</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
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<tr>
<td>GDW (pH = 7.5)</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Cystein-HCl (0.1 M, pH 7.5)</td>
<td>0.1 ml</td>
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Incubated at 30°C for 3 min then add enzyme PMS (5% supernatant) 0.1 ml in experimental tubes. Incubated for 5 min at 30°C then add 1 ml of H₂SO₄ (5N) in both experimental and control tubes. Now added 0.1 ml enzymes in controls. Finally, added 2.5 ml distilled water, kept in boiling water
bath for 3 minutes then allowed to cool. Centrifuged properly for 10 minutes. After centrifugation, estimated the enzyme as following:

- Ammonium molybdate (0.5%) 2.5 ml
- Supernatant 2.0 ml
- ANSA (1-Amino-2-napthol-4, sulphonic acid) 0.2 ml
- ... 4.7 ml

Mixed properly then centrifuged and read at 660 nm after 10 minutes.

Preparation of 1-Amino-2-napthol-4-sulphonic acid (ANSA) solution

i) Na$_2$S$_2$O$_5$ - 14.6% or NaHSO$_3$ - 15%

ii) Na$_2$SO$_3$ - 20%

Mixed 195.0 ml of NaHSO$_3$, 0.5 ml of 1-amino-2-napthol-4-sulphonic acid and 5.0 ml of Na$_2$SO$_3$. Heat and filter then finally stored in a dark coloured bottle. Solution remains stable for one month.

3.2.2 Glutathione-S-transferase (E.C. 3.5.1.18)

The activity was measured by the method of Habig et al. (1974). Specific activity was expressed as n moles of conjugate formed/min/mg protein using a molar extinction coefficient of 9.6.
The assay mixture contained:

- Phosphate buffer (0.1 M, pH 6.5) 2.90 ml
- Glutathione (1.0 mM) 0.05 ml
- 1-chloro-2,4-dinitrobenzene (CDNB) 1.0 mM 0.02 ml
- Enzyme (PMS) 0.10 ml
- 3.07 ml

Change in absorbance was noted at 15 seconds interval for two minutes at 340 nm against a suitable blank.

3.2.3 Estimation of total sulfhydryls (TSH)

Reaction mixture contained:

- Tris buffer (0.2 M, pH 8.2) 2.0 ml
- Homogenate (10% in 0.25 M sucrose) 1.0 ml
- DTNB (0.01 M) 0.2 ml
- Methanol (cold) 4.0 ml

Incubated for 30 minutes at room temperature. Centrifugation was performed at 400 x g at room temperature. Absorbance was measured at 412 nm against blank containing buffer, Methanol and DTNB.

3.2.4 Glutathione content

Glutathione was assayed by Ellman's method (1955) modified by Jollow et al. (1974). Equal amount of homogenate (10%) and sulphasalicylic acid (4%, w/v) is mixed and kept in cold for one hour. The solution was centrifuged at 3000 x g for 10 minutes at 4°C. Assay mixture contained:
Phosphate buffer (0.1 M, pH 7.4) 4.0 ml
Aliquot (supernatant) 0.20 ml
5-5'-dithiobis 2-nitrobenzoic acid (DTNB) in PO₄ buffer, 0.01 M (pH 7.4, 0.1 M) 0.20 ml

Incubation for 30 minutes at room temperature and then centrifuged. Absorbance was measured at 412 nm against blank containing phosphate buffer and DTNB. Results were expressed as μ mole GSH per gram fresh wet weight of the tissue.

3.2.5 Ceruloplasmin (Curzon and Vallet, 1960).

The reaction mixture contained:
Acetate buffer (0.2 M; pH 5.5) 2.0 ml
Serum 40.0 μ litre
Normal saline (1 M) 1.0 ml

Incubated for 5 minutes at 30°C
N,N'-dimethyl, p-phenylenediamine (DPD, 1.7 mM) 1.0 ml

Waited for 15 minutes for colour development. Kept the reaction mixture in ice cold water to stop the reaction.
Sodium azide (10 mM) 2.0 ml

Read at 550 nm specific activity was expressed in I.U.


The reaction mixture contained:
Phosphate buffer (0.08 M, pH 7.4) 1.40 ml
Serum

-\( \text{\textit{\textit{S}} \text{ rum}} \)

\[ \text{\textquoteleft{keto\textquoteright{glutarate} 2 mM} \quad 0.20 \text{ ml} \]

\[ \text{\textquoteleft{d\textquoteright{l-aspertate} 200 mM} \quad 0.50 \text{ ml} \]

Incubation at 37°C for 15 min. Reaction was stopped with the addition of 0.50 ml, 2,4-\( \text{d\textquoteright{initrophenylhydrasine} (1 mM) (DNPH)} \). Colour was developed by adding 0.4 N NaOH. Absorbance was read at 510 nm after 30 min. Enzyme activity was expressed as n moles of pyruvate/min/ml serum.

3.2.7 Lactate dehydrogenase (LDH) (L-Lactate: \( \text{NAD} \) oxidoreductase, E.C. 1.1.1.27) (Kornberg, 1955).

The reaction mixture contained in a final volume of

\[ \text{3 ml; phosphate buffer (0.1 M, pH 7.4)} \quad 2.70 \text{ ml} \]
\[ \text{Sodium pyruvate (0.01 M)} \quad 0.10 \text{ ml} \]
\[ \text{NADH (0.002 M)} \quad 0.10 \text{ ml} \]
\[ \text{Serum} \quad 0.10 \text{ ml} \]

The decrease in absorbance at 340 nm was read at a total of 3 min. The activity was expressed as n moles of NAD oxidised/min/ml serum.

3.2.8 Estimation of protein

Protein content of supernatant and homogenate fractions was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard. Protein estimated was expressed as mg protein/ml homogenate or supernatant.
The protein in these fractions was precipitated by using equal volume of 10% Trichloroacetic acid and keeping it overnight in cold and then centrifuged at 3000 rpm for 15 minutes. After this, residue was dissolved in suitable amount of 1-N-NaOH by keeping it hot water bath and then diluted to a suitable volume and finally assayed as given by Piscator and Petterson (1977).

**Assay method**

- **Protein solution**: 0.5 ml
- **Distilled water**: 0.5 ml
- **Alkaline copper reagent**: 5.0 ml

Ten minutes allowed for complex formation.

- **Folin's reagent (0.5 N)**: 1.0 ml

Leave it for 30 minutes and then absorbance was read at 750 nm against a suitable blank.

**Alkaline copper reagent**

This reagent is prepared by mixing of equal volume of the two solutions:

1) 8% NaCO₃

2) 0.06% CuSO₄·5H₂O

0.012% Na-K tartrate

mixed to 100 ml

3.2.9 **Estimation of trace element in tissues**

Metals were estimated by digesting tissues in digestion mixture to colourless solution by the method of Schroeder
and Nason (1971) and expressed as metal $\mu$g/gm wet weight of tissue.

**METHOD**

**Tissue digestion**

1.0 gm tissue was mixed with 10 ml of digestion mixture prepared by mixing nitric acid, perchloric acid and sulphuric acid in proportion of 6:1:1 and kept for overnight. Then dissolved tissue was heated at low temperature until the solution becomes colourless. Solution was diluted upto desired concentrations. The estimations of different metals were performed on Atomic Absorption Spectrophotometer 5000 Perkin Elmer.

**Chemicals**

Nickel chloride ($\text{NiCl}_2\cdot6\text{H}_2\text{O}$) was obtained from Fluka AG, Switzerland. Radioactive nickel (with specific activity 5.5 m $\text{Ci}/\text{mg}$) and aquasol universal L.S.C. cocktail were purchased from New England Nuclear Corporation, Boston, U.S.A., Sephadex G-75 used in the study of nickel binding proteins was obtained from Pharmacia, Fine Chemicals, Uppasala, Sweden. Glucose-1-phosphate, cysteine-hydrochloride, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 1-amino 2-naphthol4-sulphonic acid (ANSA), 1,2-cyclohexylenediaminetetraacetic acid (CDTA), Ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), N-hydroxyethylenediaminetetraacetic acid (HEDTA), triethylenetetramine (TETA) were purchased from
Biochemical Unit, Sigma, U.S.A. 1,4,8,11-tetraazacyclotetradecane (Cyclam) was purchased from Research Chemicals and Materials Unit, Alfa Products, U.S.A.

Besides these, other chemicals such as sodium chloride, nitric acid, perchloric acid, sulphuric acid and chloroform, sodium metasulphate, sodium bisulphite, trichloroacetic acid, etc. used in the studies, were either BDH (AR) or E. Merck extrapure grade.

3.2.11 Instruments

Ultraviolet and visible absorption spectra were recorded on spectronic 2000 and spectronic 21 spectrophotometer obtained from Bausch and Lomb. Metal estimations were performed using Perkin Elmer Model 5000 Atomic Absorption Spectrophotometer. Refrigerated Centrifuge (Indian Equipment Corporation) and Ultracentrifuge were used for cellular fractionation.

Besides these instruments, other instruments as Shaking Water Bath (Scientronic) Digital pH meter (Electronic Corporation of India Ltd.), Electronic Balance Mettler $H_{54}$ AR (Neo Pharma Instruments Corporation) and Homogenizer (Remi) were also used.

3.2.12 Statistical Analysis

The test described by Fischer (1950) was employed to calculate the statistical significance between control and experimental values. $P$ values less than 0.05 were considered to be significant.