Materials and Methods
1. COLLECTION AND MAINTENANCE OF RABBITS:

The albino rabbits were procured from M/s. E.L. Ghosh, Calcutta. They were acclimatized in individual cages in the animal house. The animals were fed animal feed, green leafy vegetables and water ad libitum.

2. SELECTION OF VANADIUM CONCENTRATION:

A preliminary experiment was performed on two extreme concentrations of vanadium (80 ppm and 5 ppm), on the basis of the information available in literature (Pomingo et al 1985) on rats, to evaluate its toxicity and lethality on rabbits. One group of rabbits (5 animals) were given drinking water containing 80 ppm vanadium as sodium metavananadate and all the animals died within 28 days of experimentation. The second group of rabbits (5 animals) received drinking water containing 5 ppm vanadium and the animals survived even after 5 months of vanadium exposure.
The third group of rabbits (5 animals) received drinking water without vanadium and were observed to be surviving even after 8 months of experiment.

Taking into consideration the above observations in which 80 ppm vanadium was highly toxic and lethal whereas 5 ppm was non-toxic and non-lethal, upto 8 months, three concentrations of vanadium i.e., 80 ppm, 40 ppm and 20 ppm were selected for further experimental studies to assess its effects on different blood parameters.

3. EXPERIMENTAL STUDIES TO EVALUATE THE VANADIUM EFFECT:

The study consists of two main groups (a) control groups, in which the rabbits were given drinking water without vanadium (b) the experimental groups, in which the rabbits were given drinking water containing different concentrations of vanadium (20, 40 and 80 ppm vanadium). Further, the study has been completed in two phases. In the first phase of study, the haematological parameters i.e., RBC and WBC count, haemoglobin concentration, packed cell volume and the absolute values such as MCV, MCH and MCHC were determined in the blood of control and experimental groups of rabbits. In the second phase of study, the plasma levels of different enzymes e.g., alkaline phosphatase, acid phosphatase, glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase, succinate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase and histidinase as well as plasma levels of
cholesterol and protein were estimated in controls and experimental group of rabbits.

The body weight and amount of drinking water utilized by each of the rabbits were also determined in both the groups of animals throughout the experimental periods in both the phases of study.

3.1: 20 ppm VANADIUM TREATMENT:

The experimental rabbits were given drinking water containing 20 ppm of vanadium throughout the experimental period. The control group of rabbits received the same drinking water without vanadium. The blood samples were collected from the ear vein of each rabbit on zero and every third day i.e. 0, 3, 6, 9, 12, 15, 18, 21, 24th day and then weekly i.e. on 31, 38, 45, 52, 59, 66, 73, 80, 87, 94, 101, 108, 115, 122, 129, 136, 143, 150, 157, 164 and 171st day. The study was completed in two phases. In the first phase of study, the blood of both the control and experimental group of rabbits, was used to study the haematological parameters. In the second phase of study, another group of control and experimental group of rabbits were used to collect the blood in a similar fashion in heparin and the plasma was separated. The plasma was used for assay of different enzymes, protein and cholesterol.

The body weight and the amount of water consumed by each rabbit of each group of animals were also recorded throughout the experimental period.
3.2: 40 ppm VANADIUM TREATMENT:

The control group of rabbits were given drinking water and the experimental group of rabbits were given the same water containing 40 ppm vanadium throughout the experimental period. The blood samples were collected from the ear vein of both the groups of rabbits on 0, 3, 6, 9, 12, 15, 18, 21 and 24th day and then weekly i.e. on 31, 38, 45, 52, 59, 66, 73, 80, 87, 94, 101, 108, 115, 122 and 129th day.

The study was completed in two phases. In the first phase of study, the blood of control and experimental groups of rabbits were analyzed for its haematological parameters. In the second phase of study, the blood of the other group of control and experimental rabbits were collected in heparin and the plasma was separated. The plasma was used for the study of enzymes, protein and cholesterol.

The body weight and the amount of drinking water utilized by each rabbit of both the groups were also determined throughout the experimental period.

3.3: 80 ppm VANADIUM TREATMENT:

The experimental group of rabbits were given drinking water containing 80 ppm of vanadium throughout the experimental period. The control group of rabbits received same drinking water without vanadium. The blood samples
were collected from the ear vein of each rabbits on 0, 3, 6, 9, 12, 15, 18, 21 and 24th day of experiment.

The study was completed in two phases like 20 and 40 ppm vanadium treated groups.

The body weight and the amount of water used by each rabbit of both the groups of animals were also recorded.

4. HEMATOLOGICAL METHODS FOLLOWED:


Principle: The blood is collected, added into acid and after leaving for 20 minutes sufficient alkali is added to neutralize the acid and to convert the acid-haematin into alkaline haematin.

Reagents:

1. 0.1 N HCl: 0.9 ml of concentrate hydrochloric acid was added to 100 ml of double distilled water.

2. 1 N NaOH: 4 g of sodium hydroxide was added to 100 ml of double distilled water.

3. Gibson and Harrison artificial haemoglobin standard was prepared by addition of 1.161 g (CrK(SO₄))₂H₂O, chromium potassium sulfate, 1.131 g (CoS₂O₄)(anhydrous) cobaltous sulfate and 0.18 ml of H₂SO₄ 69 mg, (K₂Cr₂O₇) potassium dichromate to 50 ml of double distilled water and the solution was heated to boiling. After
boiling for one minute, the solution was cooled and the volume was made up to 100 ml. The solution is equal in colour to a 1 in 100 dilution of blood containing 16.0 g \textsuperscript{Hb} per dl.

Method:

Test: 0.05 ml of blood was added into 4 ml of 0.1 N HCl and was immediately mixed well. After leaving for 20 minutes, 0.95 ml of a NaCl was added and mixed well by repeated inversions. After leaving for two minutes, the colour developed in the test sample was read at 540 nm in specol (Carl Zeiss).

Standard: Gibson and Harrison artificial standard was used as standard.

Blank: Double distilled water was used as blank solution.

Calculation:

If the optical density of test is \( T \) and that of standard is \( S \), and the standard is equivalent to 16 g of haemoglobin per 100 ml, the haemoglobin of test solution will be:

\[
\frac{T}{S} 
\]

4.2. Determination of Packed Cell Volume (PCV)

(Dacie and Lewis 1977).

Wintrobe's macro method (1929 and 1930) was followed for estimating the packed cell volume of blood, using EDTA as anticoagulant. The packed cell volume was determined
by centrifuging a sample of blood at 3000 rpm for 20 minutes in Wintrobe tubes. The height of the column of red cells so packed was taken as packed cell volume.

4.3. Red blood cell count (Dacie and Lewis, 1977): Red blood cell was counted in Howksley improved Neubauer Counting chamber. Blood was diluted 1:200 times with red cell diluting fluid, which is isotonic with blood and prevents its coagulation.

Apparatus and Reagent:

1. Improved Neubauer Counting Chamber.
2. Red Cell Pipette.
3. Red cell diluting fluid (formal citrate solution):
   1% Formalin (10 ml 1% 40% Formaldehyde) in 31.3 g trisodium citrate/litre double distilled water.

Calculations: In the Neubauer Counting Chamber:

\[ \frac{2}{3} \times \text{the number of red cells counted in 80 small squares.} \]

The area of each square is \(1/400 \text{ mm}^2\), and the depth of the chamber is \(1/10 \text{ mm}\). The volume of fluid over small square is therefore \(1/400 \times 1/10 = 1/4000 \text{ mm}^3\). \( \\frac{2}{3} \) cells are counted in \(50/4000 \text{ mm}^3\) of diluted blood. \(1 \text{ mm}^3\) of diluted blood contains \(8 \times 4000/80\) cells. Since blood is diluted 1 in 200, \(1 \text{ mm}^3\) of blood contains \(8 \times 4000 \times 200/80\) cells, that is \(2 \times 10000\) cells.
4.4. White blood cell count (Dacie and Lewis 1977):
The leucocytes were enumerated on the same principle as the RBC with following modifications:
1. As the number of leucocytes were in thousands/cu.mm of blood, the blood was diluted only 20 times.
2. The leucocytes were made more distinct by the addition of colouring material (gentian violet) in the diluting fluid.

Apparatus and Reagent:
1. Improved Neubauer Counting Chamber
2. White blood cell diluting fluid: 2% (20 ml/l) acetic acid coloured pale violet with gentian violet.

4.5. Absolute Values (Haematological Indices) (Dacie and Lewis, 1977):
4.5.1 Mean Cell Volume (MCV): This is the average volume of a single red cell expressed in femtolitres (fl). It was calculated as follows:

\[
MCV = \frac{\text{Volume of packed cells in } ml \text{ per litre of blood}}{\text{Red cells in millions per cu. mm of blood}}
\]

4.5.2 Mean Cell Haemoglobin (MCH) (Dacie and Lewis 1977):
It is the average amount of haemoglobin contained in a single red cell and is expressed in picograms (pg). It was calculated as follows:

\[
MCH = \frac{\text{Haemoglobin in } \mu g \text{ per litre of blood}}{\text{Red cells in millions per cu. mm of blood}}
\]
4.5.3. Mean Corpuscular Haemoglobin content (MCHC) (Cacle and Lewis, 1977). It indicates the percentage saturation of red cell with haemoglobin and is expressed in g/dl. This was calculated as below:

\[
\text{MCHC} = \frac{\text{Hemoglobin in g per 100 ml of blood}}{\text{Volume of packed cells in ml per 100 ml of blood}} \times 100
\]

5. BIOCHEMICAL METHODS FOLLOWED:

The blood samples were collected in heparinized tubes and were centrifuged at 3,000 rpm for 20 minutes. The plasma so collected was used as the source of enzymes, cholesterol, protein and immunoglobulins.

5.1. Protein Estimation (Lowery et al 1951):

5.1.1. Preparation of samples for protein estimation:

To 0.1 ml plasma, 4 ml of 10% trichloroacetic acid was added to precipitate the proteins. The mixture was allowed to stand for 10 minutes and then centrifuged for 15 minutes at 3,000 rpm. The supernatant was discarded. The precipitate was washed with ethanol-ether (3:1) mixture, twice. The resulting precipitate was dissolved in 4 ml of 1 N sodium hydroxide and was used for protein estimation.

5.1.2. Procedure:

Principle: Protein reacts with the Folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of the alkaline copper with the protein and the
reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Materials:

1. 0.1 N sodium hydroxide: 1 g sodium hydroxide was dissolved in 250 ml of distilled water.

2. 2% alkaline sodium carbonate solution: 2 g sodium carbonate was dissolved in 100 ml of 0.1 N sodium hydroxide.

3. 1% sodium potassium tartrate solution: 1 g sodium potassium tartrate was dissolved in 100 ml of distilled water.

4. Copper sulfate-Sodium potassium tartrate solution: 50 mg of copper sulfate was dissolved in 10 ml of 1% sodium potassium tartrate solution (3). This solution was always prepared freshly.

5. Alkaline copper sulfate solution: 50 ml of sodium carbonate solution (2) and 1 ml of copper sulfate sodium potassium tartrate solution (4) were mixed before use.

6. Folin and Ciocalteau's phenol reagent: The commercial reagent was diluted with distilled water in the ratio of 1:2 (v/v) on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric acid and hydrochloric acid.
7. **Standard protein solution:** 20 mg of bovine serum albumin (Sigma) was dissolved in 100 ml of normal saline and stored in refrigerator at 4°C.

**Method:**

0.1 ml protein sample was taken in a test tube containing 2.75 ml distilled water. 2.5 ml of the freshly prepared alkaline copper sulfate solution was added, mixed thoroughly and kept at room temperature for 15 minutes. To the above, 0.25 ml of Pollin and Clocalteau's reagent was added rapidly with immediate mixing. The solution was incubated for 30 minutes at 37°C and the optical density of the solution was read against a blank in a specol (Carl Zeiss) at 750 nm. Blank was prepared similarly except the protein was replaced by 1 N NaOH.

**Standard graph** was prepared by using different concentrations of bovine serum albumin and processing the standard sample as in the case of test samples. The protein concentration of the test sample was calculated with reference to the standard graph.

5.2 **Alkaline phosphatase:**

(Orthophosphoric monoester phosphohydrolase)

(Alkaline optimum: i.e., pH 7.1-7.4. (Kind & King, 1954)).

**Principle:**

Phenol released by enzymatic hydrolysis from phenyl phosphate under defined conditions of time, temperature and pH is estimated colorimetrically.
Reagent:

1. **Substrate: (0.01 M disodium phenyl phosphate):**
   0.254 g of disodium phenyl phosphate was dissolved in 100 ml of distilled water.

2. **Buffer (pH 9.9):** 0.536 g anhydrous sodium carbonate and 0.336 g of sodium bicarbonate were dissolved in 100 ml of distilled water and pH was maintained.

3. **Stock phenol standard solution (1 mg/ml):** 0.1 g of pure crystalline phenol was dissolved per 100 ml in 0.1 N HCl. The solution was kept 4°C in a brown bottle.

4. **Working phenol standard solution (1 mg/100 ml):** 1 ml of stock phenol standard solution was diluted to 100 ml with distilled water.

5. **0.5 N NaOH:** 2 g of sodium hydroxide was dissolved in 100 ml of distilled water.

6. **0.5 N sodium bicarbonate:** 4.2 g of sodium bicarbonate was dissolved in 100 ml of distilled water.

7. **4-Aminophenol:** 8.6 g of 4-Aminophenol was dissolved in 100 ml of distilled water and kept in a brown bottle.

8. **Potassium ferricyanide:** 2.4 g of potassium ferricyanide was dissolved in 100 ml of distilled water.
Procedure:

Test: In a test tube, 1 ml of buffer and 1 ml of substrate was added and kept in incubator at 37°C for 10 min. Further 0.1 ml of plasma was added to the above mixture and incubated for 15 minutes at 37°C. The reaction was stopped by the addition of 0.8 ml of 0.5 N NaOH.

Control: In a test tube 1 ml of buffer was added to 1 ml of substrate and kept at 37°C for 10 min. 0.8 ml of 0.5 N NaOH was added to the above mixture and incubated at 37°C for 15 minutes followed by the addition of 0.1 ml plasma.

Standard: To 1.1 ml of buffer, 1 ml of working phenol standard (1 mg/100 ml) was added along with 0.8 ml of 0.5 N NaOH and then incubated at 37°C for 15 minutes.

Blank: In a test tube 1.1 ml of buffer, 1 ml of water, 0.9 ml of 0.5 N NaOH were added and incubated at 37°C for 15 minutes.

To all the tubes, 1.2 ml of 0.5 N sodium bicarbonate, 1 ml of 4- aminoantipyrine and 1 ml of potassium ferricyanide were added with thorough mixing.

The reddish brown colours so formed were immediately read at 510 nm in specol. The amount of phenol present was determined as below:
The amount of phenol present in the standard tube is 10 μg. Thus the phenol produced in the test solution in 15 minutes is $\frac{T-0}{3-0} \times 10 \mu g$. Hence, 100 ml of plasma would liberate $\frac{C}{S-D} \times 10$ mg of phenol. King Armstrong unit is the production of 1 mg of phenol in 15 minutes under the conditions of test and the activity was expressed as K.A. units/100 ml plasma.

5.3 Acid phosphatase
(orthophosphoric-monooester phosphohydrolase)
(Acid optimum) (i.e. No. 3.1.3.2.) (Kind and King 1954):

Reagent:
All are same as in alkaline phosphatase except citrate buffer.

1. Buffer (pH 4.9) 4.2 g of crystalline citric acid was dissolved in 37.6 ml of 1 N NaOH and was made up to 100 ml with distilled water.

Procedure:

Test: In a test tube, 1 ml of citrate buffer, 1 ml of substrate was added and kept at 37°C for 10 min. Further, 0.2 ml of plasma was added to the above mixture and incubated at 37°C for 1 hour. Reaction was stopped by the addition of 1 ml of 0.5 % NaOH.

Control: In a test tube, 1 ml of buffer and 1 ml of substrate was added and kept at 37°C for 10 min. 1 ml of 0.5 % NaOH was added to the above mixture.
and incubated at 37°C for 1 hour followed by the addition of 0.2 ml of plasma.

**Standard:** In a test tube, 1.2 ml of buffer was added to 1 ml of working phenol standard solution (1 mg/100 ml) and 1 ml of 0.5 % NaOH and incubated at 37°C for 1 hour.

**Blank:** In a test tube, 1.2 ml of buffer was added to 1 ml of water and 1 ml of 0.5 % NaOH and incubated at 37°C for 1 hour.

To all the above tubes, 1 ml of 0.5 % sodium bicarbonate, 1 ml of 4-aminoantipyrine solution and 1 ml of potassium ferricyanide solution were added with thorough mixing. The reddish-brown colour so developed was immediately read at 510 nm in spectol. The amount of phenol present was determined as below:

The amount of phenol present in the standard tube is 10 μg. Thus the phenol in the test is \( \frac{T-C}{C-N} \times 10 \) μg.

Hence 100 ml of plasma would liberate \( \frac{T-C}{C-N} \times 5 \) mg of phenol. The enzyme activity was expressed as K.E. units/100 ml plasma.

5.4 Glutamate-Oxaloacetate Transaminase:

(Aspartate aminotransferase; L-Aspartate:
L-Oxaloacetate aminotransferase E.C. 2.6.1.1)

(Reitman and Frankel, 1957 as described by Ernweyer and Herm, 1974):
Principle: L-Aspartate and 2-oxoglutarate react to form pyruvate according to equation:

(1) \[
\text{L-Aspartate} + \text{2-Oxoglutarate} \xrightarrow{\text{GOT}} \text{Oxaloacetate} + \text{L-Glutamate}
\]

\[
\text{decarboxylation} \xrightarrow{\text{Pyruvate}} \]

Pyruvate formed reacts with 2,4-dinitrophenyl hydrazine to give 2,4-dinitrophenyl hydrazone, which gave colour with alkali. Intensity of colour was determined colorimetrically.

Reagents:

1. Buffer/substrate (0.1 M phosphate buffer, pH 7.4, 0.1 M aspartate, 2 mM oxoglutarate). 1.5 dipotassium phosphate (K₂HPO₄), 0.2 g of monopotassium phosphate (KH₂PO₄), 30 mg of 2-oxoglutaric acid and 1.33 g of L-aspartic acid were dissolved in 70 ml of double distilled water; pH was adjusted to 7.4 with 0.4 N sodium hydroxide solution and the solution was made to 100 ml with distilled water.

2. Chromogen (1 mM 2,4-dinitrophenylhydrazine): 20 mg of 2,4-dinitrophenyl hydrazine was dissolved in 1 ml hydrochloric acid and made to 100 ml.

3. 0.4 N sodium hydroxide: 4 g of sodium hydroxide was dissolved in 250 ml of distilled water.

4. Standard pyruvate (2 ml): 22 mg of sodium pyruvate was dissolved in 100 ml of distilled water and stored at 4°C in a brown bottle.
Procedure:

Test: One ml of buffered substrate and 0.2 ml plasma were taken in a test tube and the reaction mixture was incubated for 60 minutes at 37°C. The reaction was stopped by adding 1 ml of 2,4-dinitrophenyl hydrazine solution.

Control: A control sample was prepared towards the end of the incubation period by taking 1 ml of buffered substrate and 1 ml of dinitrophenyl hydrazine solution in a test tube; 0.2 ml plasma was added to it.

Blank: One ml of buffered substrate, 0.2 ml distilled water and 1 ml of 2,4-dinitrophenyl hydrazine solution were mixed together.

Standard: Different volumes of standard pyruvate were taken in test tubes and made to 1.2 ml with buffered substrate. 1 ml of 2,4-dinitrophenyl hydrazine was added to each tube.

Tubes containing test, control, blank and standard solutions were allowed to stand at room temperature for 20 minutes. 10 ml 0.4 N sodium hydroxide solution was added to each of the above tubes and mixed. Optical density was measured against blank after 5 minutes at 545 nm in specol.
Evaluation: When the measurements were made at 549 nm in specific, the activity of GOT (U/l) was obtained by the following table:

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<th>U/l</th>
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5.5 Glutamate-Pyruvate Transaminase

(Alanine aminotransferase; L-Alanine: 2-oxoglutarate aminotransferase, I.C. 2.6.1.2):

(Reitman and Frankel, 1957 as described by Bergmeyer and Bernt, 1974)

Principle: L-Alanine and 2-oxoglutarate react to form pyruvate according to equation:

\[
\text{GPT} \quad \text{L-Alanine} + \text{2-Oxoglutarate} \rightarrow \text{Pyruvate} + \text{L-Glutamate}
\]

Pyruvate so formed reacts with 2,4-dinitrophenyl hydrazine to give 2,4-dinitrophenyl hydrazone which gives colour with alkali. Intensity of colour is determined colorimetrically.
Reagents:

All are same as in GGT except buffer-substrate solution.

1. Buffer/substrate solution (0.1 M phosphate buffer, pI 7.4, 0.2 M L alanine, 2 mM 2-oxoglutarate):

1.5 g dipotassium phosphate (KH₂PO₄), 30 mg of 2-oxoglutaric acid and 1.78 g alanine were dissolved in 70 ml of distilled water; pH was adjusted to 7.4 with 0.4 M sodium hydroxide solution and made to 100 ml with distilled water.

Procedure:

Test: One ml of buffered substrate and 0.2 ml plasma were taken in a test tube. The reaction mixture was incubated for 30 minutes at 37°C. The reaction was stopped by adding 1 ml of 2,4-dinitrophenyl hydrazine solution.

Control: Towards the end of incubation period, a control sample was prepared by mixing together 1 ml of buffer substrate, 1 ml of 2,4-dinitrophenyl hydrazine solution and 0.2 ml of plasma.

Blank: One ml of buffered substrate, 0.2 ml of distilled water and 1 ml of 2,4-dinitrophenyl hydrazine solution were mixed together.

Standard: Different volumes of standard pyruvate solutions were made to a final volume of 1.2 ml with buffer substrate. 1 ml of 2,4-dinitrophenyl hydrazine solution was then added.
Tubes containing test, control, blank and standard solutions were allowed to stand at room temperature for 10 minutes. 10 ml of 0.4 N sodium hydroxide solution was added to each of the above tubes and mixed. Optical density was measured against blank after 5 minutes at 545 nm in specol.

Evaluation: when the measurements were made at 545 nm in specol, the activity of GNT (U/l) was obtained by the following table:

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<th>U/l</th>
</tr>
</thead>
<tbody>
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<td>0.100</td>
<td>12.00</td>
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<tr>
<td>0.125</td>
<td>17.00</td>
</tr>
</tbody>
</table>

2.6 Histaminase (Histidinolase) Karsen and Kemp (1964)

Principle: Histaminase acts on histamine forming the product imidazolescetaldehyde, H2O2 and NH3. The colourless orthodianisidine dye of the incubation mixture gets converted to a coloured compound during incubation period. The increase in O.D. during incubation is the measure of the histaminase activity and the activity is expressed in Provisional Units.

Provisional Units:

One Provisional Unit (PU) is defined equal to that which may produce an increase of 0.01 in O.D. at 470 nm after incubation for four hours at 37°C.
Reagents:
1. 0.1 M sodium phosphate buffer pH 7.2.
2. 0.2 M histamine dihydrochloride solution in distilled water.
3. Peroxidase solution: 4 mg of peroxidase (horse radish type I) was dissolved in 10 ml cold distilled water.
4. Ortho-dianisidine dye: 10 mg of ortho-dianisidine dye was dissolved in 10 ml of 96% alcohol.

Procedure:
Test: To a test tube 3.0 ml buffer, 0.15 ml histamine dihydrochloride, 0.15 ml peroxidase, 0.15 ml ortho-dianisidine dye and 0.1 ml plasma were added; and incubated for 4 hours at 37°C.

Control: To a test tube, 3.15 ml buffer, 0.15 ml peroxidase, 0.15 ml ortho-dianisidine dye and 0.10 ml plasma were added and incubated for 4 hours at 37°C.

At the end of fourth hour, the C.D. of the coloured solutions of both the tubes were measured and the activity of the histaminase was calculated with difference of the readings of test and control tubes measured at 470 nm and the values are expressed as provisional units.
5.7 Succinate dehydrogenase (Succinate:Acceptor Oxidoreductase) E.C. No. 1.3.99.1 (Nachlas et al 1960)

Principle:

\[ \text{Succinate} + \text{ETF} \xrightarrow{\text{SLO}} \text{Fumarate} + \text{ETF}^2H_2 \]

\[ \text{ETF}^2H_2 + \text{Phenazine methosulfate} \rightarrow \text{PAL} + \text{Reduced PMS} \]

Reduced PMS + tetrazolium soln \( \rightarrow \) formazan + Phenazine methosulfate.

ETF \text{H}_2\text{H}_2 \text{ formed in first reaction reacts with 2-}p\text{-iodophenyl 3-}p\text{-nitrophenyl 5-phenyl tetrazolium (INT) salt in the presence of PMS. PMS serves as an intermediate electron carrier between this dehydrogenase and INT. Addition of simple protein (gelatin) to the reaction mixture keeps formazan dispersed finely enough to permit determination of the colour density in aqueous solution.}

Reagents:

1. 0.2 M monopotassium solution: 2.721 g \( \text{K}_2\text{HPO}_4 \) dissolved in 100 ml of distilled water.

2. 0.2 M KOH solution: 1.002 g \( \text{KOH} \) dissolved in 100 ml of distilled water.

3. Phosphate buffer \( (pH \; 7.7) \): 50 ml of solution (1) and 44 ml of solution (2) were mixed and pH was adjusted to 7.7. Total volume was made to 100 ml with double distilled water.

4. 0.2 M sodium succinate solution: 0.540 g of sodium succinate dissolved in 10 ml of double distilled water.
5. Gelatin solution (0.1\%) 100 mg of gelatin dissolved in 100 ml of distilled water.

6. Stock INT solution (0.2%) 200 mg 2-p-iodophenyl 3-p-nitrophenyl-5-phenyl tetrazolium chloride dissolved in 100 ml of distilled water.

7. Working INT solution (for calibration curve) 1 ml of stock INT solution (6) diluted to 10 ml with distilled water.

8. Phenazine methosulphate solution (PMS): 90 mg of PMS was dissolved in 10 ml of distilled water. Solution stored at 5°C in brown bottle.

9. PMS (for calibration curve): 100 mg of PMS dissolved in 10 ml of 0.2 M phosphate buffer.

10. 0.25 N HCl: 0.935 ml concentrated hydrochloric acid diluted to 100 ml with distilled water.

11. NADH solution: 50 mg of reduced NADH (nicotinamide adenine dinucleotide) dissolved in 30 ml of cold distilled water.

Procedure:

Test: In a test tube 0.6 ml of phosphate buffer, 0.2 ml of sodium succinate, 0.4 ml of INT solution, 0.2 ml gelatin solution and 0.2 ml plasma were added and 0.2 ml PMS was immediately added to above. Incubation was done exactly for 15 minutes at 37°C. After incubation reaction was stopped by adding 0.2 ml of 0.25 N HCl with mixing.
Control: In a test tube 0.6 ml of phosphate buffer, 0.2 ml of sodium succinate, 0.4 ml of INT solution, 0.2 ml gelatin solution and 0.2 ml of 0.25 M HCl were added. 0.2 ml of plasma and 0.2 ml of PBS were added as above.

Blank: As control, except that equal volumes of water were taken in place of INT and plasma.

Standard graph was prepared by using different concentrations of INT as follows:

A series of test tubes containing 0.6 ml of phosphate buffer, 0.2 ml of gelatin, 0.2 ml of NAD, 0.2 ml of PBS and 0.2 ml of HCl were taken, to which INT in different concentrations, i.e. 0.08, 0.16, 0.24, 0.32 and 0.40 ml (i.e., 16, 32, 48, 64 and 80 ug respectively) was added. After 15 minutes of incubation at 37°C, OD was measured at 540 nm in specol.

OD is measured against blank at 540 nm in specol and the activity was expressed as ug formazan formed/mg protein/15 minutes. Plasma protein was determined by the method of Lowry et al (1957).

5.8 Glutamate dehydrogenase (L-Glutamate: NAD oxidoreductase) \( \text{(E.C. 1.4.1.2)} \) (Nachlas et al, 1960).

Principles:

1) Glutamate + NAD \[ \rightarrow \text{oxoglutarate} + \text{NAD}^{+} + H^{+} + \text{H}_{2} \text{O} \]
2) Alan + Fructose \[ \rightarrow \] (reduced coenzyme)
3) Reduced PBS + NAD \[ \rightarrow \text{formazan} + \text{PBS} \]
NADH formed in the reaction (1) in the presence of PMS reacts with INT to form strongly coloured formazan (reduced tetrazolium salt) PMS serving as an intermediate electron and carrier between this dehydrogenase and INT.

Addition of simple protein (gelatin) into the reaction mixture keeps formazan dispersed finely enough to permit determination of the colour intensity in aqueous solution.

Reagents: All reagents are same as in succinate dehydrogenase except 0.2 M sodium glutamate instead of sodium succinate and 15 mM NAD solution.

1. 0.2 M sodium glutamate solution: 0.374 g of sodium glutamate was dissolved in 10 ml of double distilled water.

2. 15 mM NAD solution: 10.27 mg of NAD dissolved in 1 ml of 0.1 M phosphate buffer.

Procedure:

Test: In a test tube 0.56 ml of phosphate buffer, 0.2 ml of sodium glutamate, 0.4 ml of INT solution, 0.2 ml gelatin solution and 0.2 ml plasma were added.

Enzyme reaction was started by adding 0.04 ml of NAD solution and 0.2 ml of PMS subsequently.

Incubation was done for exactly 15 minutes at 37°C.

Reaction was stopped by adding 0.2 ml of 3.25 M HCl with mixing.

Control: In a test tube 0.56 ml of phosphate buffer,

0.2 ml of sodium glutamate, 0.4 ml of INT solution,
.2 ml of gelatin and 0.2 ml of 0.25 N HCl were added. 0.2 ml of plasma, 0.24 ml NAD solution and 0.2 ml of PBS were then added to this solution.

Blank: As control, except that 0.60 ml of distilled water was taken in place of INT and plasma. 0.D. was measured against blank at 540 nm in specol. The activity was expressed as µg formazan formed/mg protein/15 minutes. Plasma protein was determined by the method of Lowry et al (1957).

5.9 Lactate dehydrogenase

(L-Lactate NAD oxidoreductase E.C. 1.1.1.27)

(modified method of Nachlas et al 1960)

Principle:

1. \[
\text{Lactate} + \text{NAD}^+ \rightarrow \text{Pyruvate} + \text{NADH} + H^+
\]

2. \[
\text{NADH} + \text{PBS} + H^+ \rightarrow \text{NAD} + \text{Reduced PBS}
\]

3. \[
\text{Reduced NAD + Tetrazolium salt} \rightarrow \text{Formazan + PBS}
\]

NADH formed in the reaction (1) in the presence of PBS reacts with INT to form strongly coloured formazan (reduced tetrazolium salt) PBS serving as an intermediate electron carrier between this dehydrogenase INT.

Addition of simple protein (gelatin) into the reaction mixture keeps formazan dispersed finely enough to permit determination of the colour intensity in aqueous solution.
Reagents: All are same as in succinate dehydrogenase except buffer, sodium lactate and NAD.

1. 0.1 M sodium lactate (lactolin) solution: 0.10 ml of lactolin was mixed with water and solution was made up to 10 ml of double distilled water.

2. 0.001 M NAD solution: 7.0 mg of NAD was dissolved in 100 ml of double distilled water.

3. Buffer: 1. 0.25 M disodium phosphate solution;
   
   4.272 g of Na₂HPO₄ was dissolved in 100 ml of double distilled water.

   2. 0.25 monopotassium phosphate solution;
   
   6.528 g of KH₂PO₄ was dissolved in 100 ml of distilled water.

   Phosphate buffer (pH 7.4): mixed the solutions 1 & 2 and the pH was maintained to 7.4.

Procedure:

Test: In a test tube 0.56 ml of buffer, 0.2 ml of sodium lactate, 0.4 ml of ldH solution, 0.2 ml of gelatin solution and 0.2 ml of plasma were taken. Enzyme reaction was started by adding 0.04 ml of H₂O₂ solution and 0.2 ml of AS subsequently. Incubation was done exactly for 15 minutes at 37°C. Reaction was stopped by adding 0.2 ml of 0.25 N HCl with mixing.

Control: In a test tube 0.56 ml of buffer, 0.2 ml of sodium lactate, 0.4 ml of ldH solution, 0.2 ml of
gelatin solution and 0.2 ml of 0.25 M HCl were taken and mixed thoroughly. Further 0.2 ml of plasma, 0.04 ml of NAD solution and 0.2 ml of PMS were added to above solution.

Blank: As in control, except that 0.6 ml of distilled water is taken in place of INT and plasma. C.L. was measured against blank at 540 nm in specol. The values were expressed as μg formazan/mg protein/15 minutes. Plasma protein was determined by the method of Lowry et al (1957).

6.0 Determination of Total Cholesterol in Plasma
(Henly 1957) as described by Singh (1985).

Principle: Extraction and oxidation of cholesterol by an acidic solution of ferric chloride and subsequent addition of sulphuric acid to form a coloured complex has been used as the basis of this procedure.

Reagents:
1. Glacial acetic acid A.R.
2. Ferric chloride: 0.05% solution. 50 mg of ferric chloride was dissolved in 100 ml acetic acid.
3. Sulphuric acid A.R.
4. Stock cholesterol standard: 100 mg in 100 ml of the purified acetic acid.
5. Cholesterol standard for use: 1 ml of stock standard was diluted to 25 ml with the ferric chloride-acetic acid reagent and was kept in a cool dark place.
6. Physiological saline: 0.9 g of A. R. grade sodium chloride was dissolved in 100 ml of distilled water.

Procedure:

Test: In a centrifuge tube 0.1 ml of plasma was added to 10 ml of ferric chloride-acetic acid reagent and was mixed well. The solution was kept for 15 minutes and then centrifuged for 15 minutes at 3000 rpm. 5 ml of clear supernatant was transferred to glass stoppered tube.

Standard: 0.1 ml of physiological saline was added to 10 ml of working cholesterol standard and 5 ml of it was taken.

Blank: Ferric chloride acetic acid reagent was used as blank.

To all the glass stoppered tubes, 3 ml of sulphuric acid was added and mixed by repeated inversions and left to stand for 30 minutes in a dark place. U.V. of unknown/standard were measured at 560 nm against blank. The values expressed as mg cholesterol/100 ml plasma.

Evaluations:

\[
\text{mg cholesterol per 100 ml plasma.} = \frac{\text{reading of unknown}}{100} \times 0.05 \times 0.2 \times \frac{\text{reading of standard}}{400} 
\]
6.1 Estimation of Immunoglobulin-G (IgG) in plasma of rabbits (Ouchterlony, 1962)

Principle: This technique is based on the ability of antibodies to form precipitin lines specifically with the antigen. Free diffusion of both the antigen and antibody take place in the agar gel and the resulting precipitin lines are normally visible to the naked eye.

Materials and Chemicals:

1. **Phosphate Buffer Saline (PBS)** (0.05 M sodium phosphate buffer pH 7.1): 0.8902 g Na₂HPO₄, 0.7802 g NaH₂PO₄, and 0.600 g sodium chloride were dissolved in 100 ml of distilled water.

2. **1% Noble Agar**: 100 mg noble agar was dissolved in 10 ml of PBS buffer and was heated directly on flame.

3. **1% Amido black solution**: 1 g of amido black in 10 ml of glacial acetic acid and was made up to 100 ml with 90% methanol.

4. **Destaining solution (2% acetic acid)**: 2 ml of acetic acid was made up to 100 ml with double distilled water.

Method: Glass slides were taken and cleaned with alcohol and placed on a horizontal level surface. Slides were pre-coated with a thin layer of agar solution to ensure good contact between the gel and glass slide. 3 ml of hot 1% agar solution was poured on the glass slides avoiding the
air bubble in the gel. The gel slide was transferred into humid chamber, after solidification. Wells of 3 mm diameter were punched with an interspace of 3 mm in agar gel using a template and a gel punch. The punched gel was aspirated out. 20 µl of anti-rabbit IgG (1:1 diluted) was loaded in the central well and rabbit plasma (20 µl of different dilutions each) were taken in peripheral wells and left over night. Bands were seen next day. Slides were washed with normal saline for five times, covered with wet whatman filter paper and was kept for drying overnight. The dried slides were then stained for 15 minutes with amido black stain. Then the slides were destained with 2% acetic acid until the bands were clear and then they were kept for drying overnight and were photographed.

6.2 Estimation of vanadium in different organs of rabbits:

(Hair and Gupta, 1977):

Materials and Chemicals:

1. TRBHA (N-(m-tolyl)-2-methoxybenzohydroxamic acid).
2. Chloroform (distilled).
3. 0.1% TRBHA solution: 0.1% TRBHA solution was prepared by dissolving 100 mg TRBHA in 100 ml of distilled chloroform.
4. Stock standard solution (10 µg V/ml): 0.2<95 g of ammonium metavanadate was dissolved in 25 ml of 4 H2SO4 and diluted to 1 litre with double distilled water.
5. Working standard solution (10 µg V/ml): 1 ml of stock standard solution (4) was diluted to 10 ml with double distilled water.

Method:

Digestion of tissue: Digestion was done by heating the weighed tissue with 5 g of K₂CO₃ and 10 ml of concentrated HNO₃ to obtain white ash. The ash so obtained was further heated with 25 ml of double distilled water, after complete heating again the ash is formed.

Estimation of vanadium: The ash from tissue of experimental rabbits so obtained was dissolved in double distilled water and the volume was made up to 25 ml. This ash solution was filtered and transferred to separating funnel. 2 drop of KMnO₄ was added to the above solution and shaken well. 5 ml of 0.1 M TETRA-CHCl₃ reagent was added and shaken well. Two layers were formed at this stage, upper one containing water and lower one containing TETRA-CHCl₃ reagent. The coloured TETRA-CHCl₃ layer was separated into a beaker containing H₂SO₄. This coloured solution was made up to 10 ml with CHCl₃ and readings were taken at 530 nm in specol against blank.

Standard graph for vanadium:

To the series of test tubes, 5, 10, 15, 20, 25, 30, 35 and 40 µg vanadium were taken in the form of ammonium metavanadate. The solution was made up to 5.0 ml with distilled water. To all the tubes, 5 ml of TETRA solution
was added, shaken well in separating funnel, and filtered through $\text{Na}_2\text{SO}_4$. The total volume was made up to 10 ml with chloroform and 0.1 ml was measured at 530 nm in spectol.

Control: Same as in experimental except tissue of control animals were taken.

Blank: Distilled CHCl₃ was used as blank. The values expressed as µg V/g tissue.

Evaluations:

\[
\text{Concentration of standard} \times \frac{\text{C. T. of standard}}{\text{C. T. of unknown}} \times \frac{\text{Weight of tissue}}{\text{C. T. of standard}}
\]
STATISTICAL ANALYSIS OF DATA:

The analysis of variance is a powerful statistical method which refers to the homogeneity or heterogeneity in the whole set of data.

Changes on levels of haematological parameters, such as RBC, WBC, Haemoglobin, PCV, MCV, MCH and MCHC, and biochemical parameters, such as Alkaline and Acid phosphatase, Glutamate-oxaloacetate and Glutamate-pyruvate transaminases, Succinate, Glutamate and Lactate dehydrogenases, Histaminase and cholesterol, and body weight following 20 ppm, 40 ppm and 80 ppm of vanadium exposure were analysed by two way analysis of variance as described by Brunning and Fjntz in 1977 and significant levels were determined by student t-test according to Wallpole (1974).

1. Two way Analysis of Variance (ANOVA):

<table>
<thead>
<tr>
<th>Factor A</th>
<th>Factor B</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Y111</td>
<td>Y211</td>
<td></td>
</tr>
<tr>
<td>Y112</td>
<td>Y212</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y11n</td>
<td>Y21n</td>
<td>Yb1n</td>
</tr>
<tr>
<td>Y121</td>
<td>Y221</td>
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<tr>
<td>Y122</td>
<td>Y222</td>
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<tr>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y12n</td>
<td>Y22n</td>
<td>Yb2n</td>
</tr>
</tbody>
</table>

Total | T1 | T2 | Tb | T
Factor A = Treatment
B = Time point
n = number of replicate per group
Y = single observation

Preliminary computation:
1) Grand total: \[ \sum \sum \sum Y \]
2) Sum of squared observation: \[ \sum \sum \sum Y^2 \]
3) Sum of squared subgroup (cell), total divided by the sample size of the subgroup:
\[ \frac{\sum \sum (\sum Y)^2}{n} \]
4) Sum of squared column, total divided by the sample size of column:
\[ \frac{\sum (\sum \sum Y)^2}{b n} \]
5) Sum of squared row, total divided by the sample size of the row:
\[ \frac{\sum (\sum \sum Y)^2}{a n} \]
6) Grand total squared and divided by the total sample size = correction term \( C \):
\[ C = \frac{\sum \sum \sum Y^2}{ab n} \]
7) Sum of square total = $\sum \sum \sum y^2 - CT$
   $(SST)$

8) Sum of square subgroup:
   \[
   \frac{\sum \sum (\sum Y)^2 - CT}{n}
   \]

9) Sum of square A (SSA):
   \[
   \frac{\sum (\sum \sum Y)^2 - CT}{b n}
   \]

10) Sum of square B (SSB):
    \[
    \frac{\sum (\sum \sum Y)^2 - CT}{a n}
    \]

11) Sum of squares A x B (interaction; SSAB):
    = SS sub group - SSA - SSB

12) Sum of square within (Error SS; SSE)
    = SS total - SS sub group

For the data obtained during present investigation
an ANOVA table was prepared as follows:
<table>
<thead>
<tr>
<th>Source of Variance</th>
<th>Degree of Freedom (df)</th>
<th>Sum of Square (SS)</th>
<th>Mean Square (MS)</th>
<th>E</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor A</td>
<td>a-1</td>
<td>SSA</td>
<td>( \frac{SSA}{(a-1)} )</td>
<td>MS of A</td>
<td></td>
</tr>
<tr>
<td>Factor B</td>
<td>b-1</td>
<td>SSB</td>
<td>( \frac{SSB}{(b-1)} )</td>
<td>MS of B</td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>(a-1)(b-1)</td>
<td>SSAB</td>
<td>( \frac{SSAB}{(a-1)(b-1)} )</td>
<td>MS of A*B</td>
<td></td>
</tr>
<tr>
<td>Within group ab</td>
<td>(a-1)(b-1)(error=1)</td>
<td>SSE</td>
<td>( \frac{SSE}{ab(n-1)} )</td>
<td>MS of Error</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>(ab n-1)</td>
<td>SST</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

P (Probability of committing an error for rejecting or accepting the null hypothesis) refers to the level of significance of the calculated F; it was determined by comparing tabulated F value with standard table (Zar, 1974).

Tabulated F values were obtained by taking into consideration the degree of freedom of both numerator mean square as well as denominator mean square. Calculated F value when greater than tabulated value, at 0.05, 0.025, 0.01 and 0.001 level, data considered as significant and the null hypothesis was rejected.

Significant and insignificant ANOVA results did not clearly indicate which two sets of data (among whole data) differ or do not differ significantly from each other, and students' 't' test is a very useful test to evaluate the level of difference between a pair of sets of data.
t: *t* test:

The test is a frequent one in statistics. It is applicable to establish the significance of the difference between two means. The method followed is described by Calpou (1974):

**Formula of t:**

\[ t = \frac{M_1 - M_2}{(SE_1)^2 + (SE_2)^2} \text{ at } N - 2 \text{ df} \]

where:

- \( M_1 \) = 1st group mean
- \( M_2 \) = 2nd group mean
- \( SE_1 \) = 1st group standard error
- \( SE_2 \) = 2nd group standard error
- \( n_1 \) = Sample size of 1st group
- \( n_2 \) = Sample size of 2nd group

Significance of *t* value is determined by comparison of standard *t* value with calculated *t* value. Standard *t* value is determined from Fisher's table at 0.02, 0.05, 0.01 and 0.001 level on the basis of \((n_1 - 1) + (n_2 - 1)\) degree of freedom. When calculated *t* value was greater than tabulated *t* value, data was considered to be significant.