Materials & Methods
ANIMAL SELECTED:

*Catla catla* (Ham) is an edible fish widely distributed in inland waters of India and reared abundantly in the freshwater tanks and ponds for commercial purpose. Large population of fish eaters prefers this fish. Besides its wide availability and commercial importance this is also known for its adaptability to laboratory conditions and suitability to toxicity studies. Hence, this fish was selected as experimental animal.

**Classification:** Systematic position of *Catla catla.*

- **Phylum:** Chordata
- **Sub phylum:** Vertebrata
- **Division:** Gnathostomata
- **Super class:** Pisces
- **Class:** Osteichthyes
- **Sub class:** Actinopterygii
- **Super order:** Teleostei
- **Order:** Cypriniformes
- **Genus:** *Catla*
- **Species:** *C. catla*

**HISTORY AND BIOLOGY OF *CATLA CATLA***:

Hamilton (1822) described *Catla catla* as *Cyprinus catla.*

Velenciennes (1842) proposed a separate genus for *Cyprinus catla*...
and placed it in the new genus *Catla*. The species thereafter came to be known as *Catla buchanani* by subsequent workers. Raj (1916) renamed the species as *Catla catla*. It is commonly known as *Catla* and is the most valuable edible fish found all over India. The head of the fish is enormously large with wide mouth and prominent lower lip. Body is deep giving the dorsal profile a more convex look than the ventral. Eyes are located in front of head. Barbies are absent. Dorsal fin commences in advance of the ventral and pectoral fin extended much to the pelvic fin. Anal and caudal fin is covered with large conspicuous scales. Lateral line is central and continuous till the base of caudal fin and has 40-43 scales on it. The fish is greyish on back and flank silvery white below. Fins are dark. It is principally a surface feeder but may feed thoroughly at the column of water. It mostly consumes plankton and vegetable debris like decaying vegetation. The principal food consists of higher algae (Viz., Myzophyceae, Chlorophyceae), rotifer, crustaceans, insects and zooplanktons.

**PROCUREMENT AND MAINTENANCE OF FISH:**

The fish *Catla catla* weighing 6 ± 2g were collected from Karnataka state Fisheries Department, Dharwad (India) and initially stored in large cement tank previously treated with potassium permanganate. Water was renewed once in a month. Fish were fed with rice bran and oil cake powder mixed in the ratio of 2:1. Before
experiments fish were caught and acclimated to laboratory conditions for the minimum of fifteen days in plastic trough with dechlorinated tap water.

**FACTORS INFLUENCING PESTICIDE TOXICITY:**

In nature effect of a pesticide on the biota is influenced by number of factors like flow of water, temperature, density of fish and water quality. These factors, which are likely to contribute variation in toxicological investigation, were approximately nullified to a satisfactory level.

**Flow of water**

Burke and Ferguson (1969) reported significant difference in the toxicity of pesticides between static and flowing water in fish. Therefore, the experiments of the present investigation were conducted in the static media as suggested by Doudoroff et al (1951).

**Temperature**

According to Macek et al (1969) pesticide toxicity increases with the rise in temperature of the medium. Therefore, throughout the investigation the temperature of the water was maintained invariably at $28 \pm 2 ^{\circ} C$.

**Density of fish**

Increase in fish density enhances the toxicity of the pesticide (Holden, 1970). Hence, constant ratio of fish biomass to the water volume was maintained by taking one fish per one liter of water.
Water quality

Water quality determines the compatibility of water for fish rearing and also influences the toxicity of chemical (Pickering & Anderson, 1966). Therefore, care was taken to maintain uniformity of water quality used, which is as follows,

- Temperature : 28 ± 2°C
- pH : 7 ± 0.2 at 26.3°C
- Dissolved oxygen : 5.7 ± 0.9 mg/l
- Carbon dioxide : 7.3 ± 0.4 mg/l
- Calcium : 16.04 ± 1.2 mg/l
- Magnesium : 0.8 ± 0.1 mg/l
- Nitrate : 9.14 ± 0.13 μg/l
- Phosphate : 0.401 ± 0.001 μg/l
- Chlorinity : 7.2 ± 0.4 mg/l
- Total hardness : 43.4 ± 3.4 mg/l
- Total alkalinity : 25.2 ± 1.3 mg/l

PESTICIDE SELECTED FOR THE STUDY:

For the present study technical grade endosulfan supplied by Rallis India Ltd, Mumbai, was used. Thionyl Chloride produces endosulfan with the hydrolyzed Diels-Alder Adduct of Hexachloro cyclopenta diene and (2)-but-2-enylene di acetate. It is used in a formulated form as broad-spectrum contact and stomach insecticide.
mainly in agriculture and in some countries in public health. It is used to control insects such as the Colorado potato beetle, flea beetle, cabbage worm, peach tree borer and tarnished plant bug as well as several species of aphid and leaf hopper (Canada National Research Council, 1975).

**PREPARATION OF STOCK SOLUTION:**

Stock solution of technical grade endosulfan was prepared by dissolving 10 mg of endosulfan in 10 ml of analytical grade acetone to get 1000 µg/ml of stock. Acetone used as solvent is known to be non-toxic up to relatively high concentration (Jaganatha Rao, 1981). Desired concentration of endosulfan was drawn from the stock.

**METHODS:**

**TOXICITY EVALUATION:**

The percent mortality of fish in different concentrations of endosulfan was determined at 96 hours exposure. For this the experimental fish were divided into batches of ten each, and were exposed to different concentrations of endosulfan ranging from 2 µg/l to 5 µg/l. This range was obtained on trial and error basis. Toxicity evaluation was carried out in static water (Doudoroff *et al.*, 1951) and mortality rate in all the concentrations of endosulfan after 96 hours was observed and recorded. A batch of fish maintained along side in freshwater medium without endosulfan served as control. The
The experiment was repeated thrice for accuracy. The LC\textsubscript{50} value was derived following probit method (Finney, 1971) and Dragstedt-Beheren’s equation (Carpenter, 1975) as mentioned by Bhargava and Rawat (1999), which is,

$$\log \text{LC}_{50} = \log A + \frac{50 - a \log B}{a - b} - \frac{A}{a - b}$$

Where,

- $A$ = Concentration of pesticide, whose concentration is just below 50% mortality.
- $B$ = Concentration of pesticide, whose concentration is just above 50% mortality.
- $a$ = Percent mortality observed below 50% mortality.
- $b$ = Percent mortality observed above 50% mortality.

The mean LC\textsubscript{50} value was calculated from the values obtained from the above three methods namely percent, probit mortality and Dragstedt and Beheren’s method.

**FIXATION OF LETHAL AND SUBLETHAL CONCENTRATIONS:**

Taking into consideration the fact that the effect of a pesticide on fish becomes consistent with in 96 hours of exposure LC\textsubscript{50} / 96 hours of endosulfan was taken as lethal concentration to study the behavioural, physiological and biochemical responses of the fish, *Catla catla*. However, knowledge on the concentration of toxicant that
kills 50% of the test animals in a fixed period of time could become insufficient to assess various responses of the animal to toxicant (Nobbs & Pearu, 1976). Further, studies on acute toxicity have significant limitations such as the occurrence of adaptation of test animal to the imposed toxicity (Stockner & Anita, 1976). Hence, Perkin (1979) also viewed the need for sublethal studies because distinct changes involving a sequence of events in the responses of test animal could occur in sublethal concentration. So, one-tenth of the 96 hours LC$_{50}$ was taken as the sublethal concentration of endosulfan for further studies.

**FIXATION OF EXPOSURE PERIODS:**

Since the duration of exposure is having a great influence on the toxicity of a pesticide on an organism. The effect of lethal and sublethal concentration of endosulfan were studied at different periods of exposure in order to understand the influence of time over toxicity. In the lethal 1, 2, 3, 4 days and in the sublethal 1, 5, 10 and 15 days were chosen to observe the short-term and long-term effects of endosulfan on the fish, *Catla catla*.

**EXPERIMENTAL DESIGN:**

After the determination of 96 h LC$_{50}$ further studies in this investigation were carried out on the gill, muscle and liver at 1, 2, 3 and 4 days of exposure to lethal and 1, 5, 10 and 15 days of exposure to the
sublethal concentration of endosulfan. Selection of the gill, muscle, and liver in fish was to understand the difference in the effects of endosulfan in different tissues. Prior to each experiment, fishes were exposed to their respective lethal and sublethal concentration of endosulfan and were maintained in these concentrations up to the stipulated period of exposure. At the end of exposure the fish were stunned to death and the target organs were dissected out from each animal using sterilized instruments. The organs were weighed accurately on a sartorius electrical semi-microbalance and transferred into ice-jacketed microbeakers containing fish ringer solution. The fish ringer was prepared as per the composition given by Ekenberg (1958). An equilibration time of 15 minutes was allowed to the organs to regain normalcy from a state of shock, if any, due to the handling and dissecting procedures. The experimentation was repeated for six times and results were analyzed.

**BIOACCUMULATION:**

**A) PREPARATION OF TISSUE EXTRACT FOR ACCUMULATION STUDIES:**

Endosulfan residues were extracted from the tissue samples with n-hexane and isopropanol (3:1) in a warring blender. Isopropanol was removed from the extract by washing the hexane layer several times with water. The hexane layer was dried over
anhydrous sodium sulphate and chromatographed over alumina (acidic) and the column was eluted with n- hexane and acetone (9:1). Elute was evaporated to dryness in rotary evaporator and the volume was made up to 3 ml.

B) GAS LIQUID CHROMATOGRAPHY QUANTIFICATION OF ENDOSULFAN RESIDUE IN THE TISSUES:

Endosulfan residues were quantified using Gas Liquid Chromatography (GLC) instrument (NUCON, 5700) equipped with FID system following the method suggested by Verma (1989) with slight modification. The column used was OV-101 on chromosorb 80/100 meshes. The operational parameters of GLC were injector temperature 275°C, detector temperature 275°C, oven temperature 250°C, nitrogen flow rate 30 ml/ min, hydrogen flow rate 30 ml/ min and oxygen flow rate 300 ml/min. The volume of sample injected was 2 μl. The relative retention time of technical grade endosulfan was noted and matched with the tissue extract of the experimental fish. Standard curves based on the 10 cm of peak height were used for quantification of endosulfan residue in different tissues.

BEHAVIOURAL STUDIES:

Changes in behavioural pattern on exposure to endosulfan were observed both in control and treated fish (Median lethal and sublethal) as described by Murthy (1987).
WHOLE ANIMAL OXYGEN CONSUMPTION:

The rate of whole animal oxygen consumption was estimated following Winkler's Iodometric method as described by Welsh and Smith (1953) and the apparatus setup was the same as described by Saroja (1959). The difference in the oxygen content of the initial and final samples was taken as the amount of oxygen consumed by the fish during the period of experimentation. The oxygen consumed by the normal and endosulfan exposed fish was determined. After experimentation, the fish were individually weighed and their unit metabolism was calculated and expressed as mg of O$_2$ consumed / gm wet wt / h.

HAEMATOLOGY:

COLLECTION OF BLOOD:

Blood was collected by the method of Steuke and Schoegttger (1970) by severing the caudal peduncle. Before considering the blood for haematological observation, a drop was placed on a clean slide and examined for the presence of parasites if any. Blood free from any infection was used to study the haematological parameters to avoid variation, the samples were taken at a particular time during early hours of the day. Ethylene diamine tetra acetic acid (EDTA) was used as an anticoagulant.
A) DETERMINATION OF RED BLOOD CORPUSCLE (RBC) COUNT:

RBC count was determined with a Neubauer crystalline counting chamber as described by Davidson and Henry (1969). Blood was collected in a vial rinsed with 2% ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. The blood was sucked upto 0.5 mark on the RBC pipette and immediately RBC diluting fluid (Hayem's fluid) was drawn up to 101 mark and the pipette was rotated between the thumb and the forefinger to facilitate adequate mixing of the solution (dilution 1:200).

The counting chamber and the cover glass were cleaned thoroughly and the cover glass was placed in position over the ruled area. The fluid from the stem of the pipette was expelled as it contains only the diluting fluid. The pipette was then held at an angle of 45 degrees with the tip of the pipette at the junction of the edge of cover glass and the counting chamber. A drop of blood was placed from the tip of the pipette on the central platform near the edge of the cover slip, so that the drop was sucked up between the central platform and the cover slip by the capillary force. The cells were allowed to settle for two or three minutes. The ruled area of the counting chamber was focused under the microscope and the numbers of RBC's were counted in 80 small squares (4 squares of 16 at the four corners and
one of 16 at the center). The cells touching the upper and left hand lines were counted. The cells touching the lower and the right hand lines were omitted.

The numbers of RBC’s per sq mm were calculated as follows-

\[
\text{The area of a small square} = 1/400 \text{ sq mm}
\]

\[
\text{The depth of the counting chamber} = 1/10 \text{ mm}
\]

Therefore the volume of a small square is

\[
1/400 \times 1/10 = 1/4000 \text{ Cu.mm.}
\]

The dilution of the blood is \( = 1/200 \)

Total RBC = \( n/80 \times 4000/1 \times 200/1 \)

\( n \) = number of cells counted in 80 small squares

**B) DETERMINATION OF WHITE BLOOD CORPUSCLES (WBC) COUNT:**

WBC count was done as per the procedure described by Donald Hunter and Bonford (1963). Blood was collected in a vial containing 2% EDTA as an anticoagulant. The blood was drawn up to 0.5 mark of WBC pipette and immediately the diluting fluid was drawn up to the 101 mark above the bulb (the dilution fluid consists of 1.5 ml of glacial acetic acid and 1 ml of aqueous gentian violet solution and made up to 100 ml with distilled water). The solution was mixed thoroughly by shaking gently. It was allowed to stand for 2 to 3 minutes. The Neubauer’s counting chamber and cover glass were
cleaned and the cover glass was placed over the ruled area. Excess solution was expelled and a drop of fluid was allowed to flow under the cover slip by holding the pipette at an angle of 40°. It was allowed to stand for 2 to 3 minutes and the WBC’s were counted in the four corner square millimeters. The numbers of WBC’s per cubic millimeter were calculated.

C) ESTIMATION OF HAEMOGLOBIN:

Haemoglobin (Hb) content in the blood was estimated by Cyanmethaeglobin method as described by Dacie and Lewis (1961). According to this method, haemoglobin is converted into cyanmethaeglobin by the addition of potassium ferricyanide. The colour of cyanmethaeglobin is read in a photoelectric colorimeter at 540 nm against a standard solution, since cyanide has the maximum affinity for haemoglobin.

20 ml of blood was transferred with the help of a haemoglobin pipette into a test tube containing 5 ml of Drabkin’s solution. The test tubes were vigorously shaked and readings were taken in a photoelectric colorimeter at 540 nm. The reagent blank is adjusted to zero.

D) DETERMINATION OF PACKED CELL VOLUME (PCV) OR HAEMATOCRIT VALUE:

Packed cell volume was determined by Micro haematocrit method of Schalm et al., 1975.
The heparinised blood was filled up to the mark 100 of the haematocrit tube with the help of Pasteur pipette and centrifuged at 3000 rpm for 30 minutes. The relative volume of the height of the RBC’s packed at the bottom of the haematocrit tube was recorded as packed cell volume in terms of percentage of total blood column taken in the haematocrit tube.

E) DETERMINATION OF MEAN CORPUSCULAR VOLUME (MCV):

Mean corpuscular volume indicates the average size of the red blood cell in a given sample of blood. MCV was calculated by the following formula and represented in cubic microns.

\[
MCV = \frac{PCV \times 10}{\text{RBC count (In millions per Cumm)}}
\]

F) DETERMINATION OF MEAN CORPUSCULAR HAEMOGLOBIN (MCH):

Mean corpuscular haemoglobin (MCH) represents the average weight of the haemoglobin contained in each red blood cell in a given volume of the blood. MCH is influenced by the size of the cell and concentration of the haemoglobin.

MCH was calculated by the following formula and expressed in picograms.

\[
MCH = \frac{\text{Haemoglobin (grams/deciliter)}}{\text{RBC count}}
\]
G) MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION (MCHC):

Mean corpuscular haemoglobin concentration reflects the average concentration of the haemoglobin in the Red blood cells in a given volume of the blood (unlike MCH, MCHC is not influenced by the size of the cell).

MCHC was obtained by the following formula and expressed in terms of percentage

$$\text{MCHC} = \frac{\text{Hemoglobin (grams / deciliter)}}{\text{Packed cell volume}}$$

IONS AND ASSOCIATED ATPases:

The levels of sodium, potassium and calcium ions and the activities of $\text{Na}^+$- $\text{K}^+$ ATPase, $\text{Mg}^{2+}$ ATPase, and $\text{Ca}^{2+}$ ATPases were estimated in gill, liver and muscle of fishes under this study.

A) ESTIMATION OF SODIUM, POTASSIUM AND CALCIUM IONS:

The weighed organs were wet ashed in 50:50 (V/V) concentrated perchloric acid and nitric acid (Dall, 1967). After keeping the wet ash solutions for half an hour, until the organs were completely dissolved, they were evaporated at 100\(^\circ\)C to 200\(^\circ\)C temperature. The residues were dissolved in glass with distilled water and made up to 10 ml. It was filtered through whatman No.1 filter paper. Further, appropriate dilutions were made prior to estimations.
and the sodium, potassium and calcium ions were estimated with the help of flame photometer (Elico Pvt. Ltd., Model CL –22A). Standard solutions of sodium, potassium and calcium were prepared by using analar grade chemicals. The values are expressed as μM/g wet wt of the organ.

**B) Na\(^+\)- K\(^+\), Mg\(^{2+}\) AND Ca\(^{2+}\) ATPase ACTIVITIES (ATPase PHOSPHORYLASE EC. 3.6.1.3.):**

Na\(^+\)- K\(^+\), Mg\(^{2+}\) and Ca\(^{2+}\) ATPase activities were estimated separately in the organs by the method described by Watson and Beamish, (1981) with slight modification. 1% tissue homogenate (W/V) were prepared in ice-cold 0.25 M sucrose solution containing 5 mM EDTA (Prepared in 40 mM tris-HCl buffer at pH 7.5) and 0.01 M imidazole. The homogenates were centrifuged at 2500 rpm for 10 minutes and the supernatants were taken as crude enzyme extract for the assay of the ATPase enzyme activities.

After due standardization of enzyme kinetic parameters, three sets of incubation mixtures were prepared. In a total volume of 2 ml, the first set consisted of 100 mM disodium adenosine triphosphate (Prepared in 20 mM tris-HCl buffer at pH 7.5), 100 mM NaCl, 20 mM KCl, 3 mM MgCl and 0.3 ml of enzyme extract. The second set consisted of 100 mM disodium ATP (Prepared in 2 mM tris-HCl buffer at pH 7.5), 100 mM NaCl, 20 mM KCl, 3 mM MgCl, 1 mM ouabain (potent inhibitor of Na\(^+\)-K\(^+\) ATPase) and 0.3 ml of enzyme extract and
the third set consisted of 100 mM disodium ATP (Prepared in 20 mM tris-HCl buffer at pH 7.8), 5 mM CaCl₂ and 0.3 ml of enzyme extract. All the three incubation sets were incubated at 37°C for exactly 15 minutes and then the reaction was arrested by adding 2 ml of cold 10% TCA. The inorganic phosphates liberated were estimated by the method of Fiske and Subba Rao (1925). The absorbance was measured at 660 nm. Endogenous blanks were prepared to find out the endogenous inorganic phosphates. Another blank was prepared without using the co-factor to deduct the sodium salt stimulated activity as the co-factor used was a disodium salt of ATP.

The first set gives the total ATPase activities of Na⁺-K⁺ and Mg²⁺, whereas the second set gives only the Mg²⁺ ATPase activity as ouabain inhibits Na⁺-K⁺ stimulated ATPase. Hence, the Na⁺-K⁺ activity was derived by subtracting the Mg²⁺ ATPase from total of Na⁺-K⁺ and Mg²⁺, ATPase activities. The third set directly gives the Ca²⁺ ATPase activity. All these three ATPase activities are expressed as μM Pi liberated/mg protein/h.

SOME ASPECTS OF PROTEIN METABOLISM:

The levels of soluble, structural and total proteins, free amino acids, ammonia and urea and the activities of protease, alanine and aspartate aminotransferases, glutamate dehydrogenase and glutamine were estimated in the gill, muscle, liver of fish under this study.
A) ESTIMATION OF SOLUBLE, STRUCTURAL AND TOTAL PROTEINS:

The soluble, structural and the total proteins in the organs were estimated using the folin-phenol reagent method as described by Lowry et al., (1951). 1% homogenate (W/V) was prepared in ice-cold 0.25 M sucrose solution. For soluble and structural proteins 1.0 ml of the homogenate was taken and centrifuged at 3000 rpm for 10 minutes. The supernatant was separated and to both the supernatant and residue 3 ml of 10% trichloroacetic acid was added and again centrifuged at 3000 rpm. The supernatants were discarded and the residues were taken for experimentation. For total proteins, 1 ml of homogenate was taken, to it 3 ml of 10% trichloroacetic acid was added and centrifuged at 3000 rpm. Supernatant was discarded and the residue was taken for experimentation. All the three residues were dissolved in 5 ml of 0.1 N sodium hydroxide and to 1ml of each of these solutions, 4 ml of reagent –D (mixture of 2% sodium carbonate and 0.5% copper sulphate in 50:1 ratio) was added. The samples were allowed to stay for 10 minutes, at the end of which 0.4 ml of folin-phenol reagent (Diluted with distilled water in 1:1 ratio before use) was added. Finally the optical density of the colour developed was measured using spectrophotometer at a wavelength of 600 nm. A mixture of 4 ml of reagent–D and 0.4 ml of folin-phenol reagent was
used as blank. Bovine albumin was used for the preparation of protein standards. The protein content is expressed as mg/g wet wt of the organ.

B) ESTIMATION OF FREE AMINO ACIDS:

Free amino acid levels in the organs were estimated by the ninhydrin method as described by Moore and Stein (1954). 5% organ homogenates (W/V) were prepared in 10% trichloroacetic acid and centrifuged at 2000 rpm for 15 minutes. To 0.2 ml of supernatant, 2.0 ml of ninhydrin reagent was added and the contents were boiled for exactly 5 minutes. They were cooled under tap water and the volume was made to 10.0 ml with distilled water. The optical density of the colour developed was measured using spectrophotometer at a wavelength of 570 nm. A blank using distilled water and amino acid standards were also run similarly. The free amino acid levels are expressed as mg amino acid nitrogen released/g wet wt of the organ.

C) ESTIMATION OF PROTEASE ACTIVITY:

Protease activity in the organs was estimated using the ninhydrin method as described by Davis and Smith (1955). 1% homogenate (W/V) was prepared in distilled water. To 2.0 ml of homogenate 0.5 ml of 1% casein and 2.0 ml of 0.1 M phosphate buffer (pH 5.0) were added. The contents were mixed well and incubated at 30°C for 30 minutes. The reaction was stopped by adding
2 ml of 2% ninhydrin reagent. Again the contents were mixed thoroughly and placed in a boiling water bath for 20 minutes. The solution was cooled and made to 10 ml with diluents (distilled water and n-propanol in 1:1 ratio). The optical density of the colour developed was measured using spectrophotometer at a wavelength of 570 nm. A blank taking 2.0 ml of distilled water and control taking 2.0 ml of boiled enzyme were also run similarly. Amino acid standards were prepared alongside for comparison. The protease activity is expressed as µ moles amino acid nitrogen released/mg protein/h.

**D) ESTIMATION OF ALANINE (DL-alanine: 2-oxoglutarate, EC 2.6.1.2) AND ASPARTATE (L-aspartate: 2-oxoglutarate, EC 2.6.1.1.) AMINOTRANSFERASE ACTIVITIES (AAT and A1AT):**

Activities of alanine and aspartate aminotransferase in the organs were estimated using method of Reitman and Frankel (1957). 5% homogenate (W/V) was prepared in 0.25 M ice-cold sucrose solution, centrifuged at 3000 rpm for 10 minutes and the supernatant was used as the source of enzyme. Two sets of incubation mixtures were prepared. The first set (for alanine aminotransferase activity) consisted of 0.5 ml of 0.2 M alanine and 0.5 ml of 0.005 M ketoglutaric acid (which was prepared in M/15 phosphate buffer and adjusted with 10% sodium hydroxide to 7.4 pH) and 0.1 ml of enzyme. The second set (for aspartate aminotransferase activity).
Consisted of 0.5 ml 0.2 M aspartic acid, 0.5 ml of 0.005 M ketoglutaric acid (which was prepared in M/15 phosphate buffer and adjusted with 10% sodium hydroxide to 7.4 pH) and 0.1 ml of enzyme. Mixtures were incubated at 37°C for 30 minutes and then the reaction was stopped by the addition of 1 ml 0.001 M 2, 4-dinitrophenylhydrazine (ketone reagent). Finally the reaction mixtures were made to 10.0 ml with 0.4 N sodium hydroxide and the optical density of the colour developed was measured using spectrophotometer at a wavelength of 545 nm. A blank taking 0.1 ml of distilled water and controls taking 0.1 ml of boiled enzyme were also run similarly. Pyruvate and oxaloacetate standards were prepared alongside for comparison. The alanine aminotransferase activity is expressed as μ moles pyruvate formed/mg protein/h and the aspartate aminotransferase activity as μ moles oxaloacetate formed/mg protein/h.

E) ESTIMATION OF GLUTAMINE DEHYDROGENASE (GDH) (L. Glutamate: NAD oxalo-reductase E.C. 1.4.1.) ACTIVITY:

GDH activity was estimated in the organ using the method of Lee and Lardy (1965) with slight modification. 5% homogenate (W/V) was prepared in 0.25 M ice-cold sucrose solution and centrifuged at 2500 rpm for 20 minutes at 2°C to remove cell debris. The clear cell-free extract was subjected to dialysis against 0.25 M
sucrose at 2°C to 4°C for 24 hours. The incubation mixture in a final volume of 2.0 ml contained 40 μ moles of sodium glutamate, 100 μ moles of NAD (nicotinamide adenine dinucleotide) and 4.0 μ moles of INT (2-p-Indophenol-3-p-nitrophenyl-5-phenyltetrazolium chloride). The reaction was initiated by the addition of 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37°C for 30 minutes in a thermostatic water bath and then the reaction was stopped by the addition of 5.0 ml of glacial acetic acid. The formozan formed was extracted into 5.0 ml of toluene overnight at 5°C. The optical density of the colour developed was measured using spectrophotometer at a wavelength of 495 nm. A blank by taking 0.5 ml of distilled water and control by taking of 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity is expressed as μM formozan formed /mg protein/h.

F) ESTIMATION OF GLUTAMINE:

Glutamine was estimated by acid hydrolysis method as described by Colowick and Kaplon (1967). 5% tissue homogenate were prepared in cold distilled water and centrifuged at 1000 rpm for 15 minutes. To 1 ml of the supernatant 0.2 ml of 10% sulphuric acid (H₂SO₄) was added and the contents were boiled for 10 minutes and then cooled. The contents were centrifuged and to the supernatant, 0.5
ml of 10% NaOH was added and the mixture was made up to 2 ml with distilled water. To this 0.4 ml of Nessler’s reagent was added and the colour developed was read against reagent blank at 495 nm using spectrophotometer. The glutamine content was expressed as μ moles/g wet weight of the tissue.

G) ESTIMATION OF AMMONIA:

Ammonia was estimated by the method of Bergmeyer (1965) with a slight modification. 5% tissue homogenates were prepared in cold distilled water and centrifuged at 1000 rpm for 15 minutes. To 1 ml of supernatant 2 ml of 15% perchloric acid (PCA) was added and centrifuged at 1000 rpm for 15 minutes. The supernatant was neutralized with 2 ml of 15% sodium hydroxide (NaOH). To this 0.5 ml of Nessler’s reagent was added and the colour developed was read immediately using spectrophotometer at 495 nm against blank. The ammonia content was expressed as μ moles/g wet weight of the tissue.

H) ESTIMATION OF UREA:

Urea was estimated by diacetylmonoxime method as described by Natelson (1971). 5% tissue homogenates were prepared in 15% PCA and centrifuged at 1000 rpm for 15 minutes. To 1.5 ml of supernatant, 1 ml of acid mix 3:1 ratio (orthophosphoric acid: conc H₂SO₄) was added and the contents were shaken well. To this 0.5 ml of 2% diacetylmonoxime was added and heated at 100°C in boiling
water bath for 30 minutes. The tubes were cooled and the colour developed was read against blank at 480 nm using spectrophotometer. The urea content was expressed as μ moles/g wet weight of the tissue.

STATISTICAL ANALYSIS

The data in all cases were subjected to analysis of variance and the means were compared by Duncan's new Multiple Range test (Duncan, 1955) at 0.05 % level.