**Animal selected**

*Catla catla* (Hamilton, 1822), the Indian major carp is an economically important edible fish, having a great commercial value, occurs abundantly in fresh water tanks and ponds in and around Anantapur. Besides its wide availability and commercial importance in tropical countries this carp fish is known for its adaptability to laboratory conditions and appear to be suitable test animal to toxic studies (Sreenivasan and Swaminathan, 1967). Hence, this fish is selected as the ideal experimental model for the present investigation. Since this investigation is an ecotoxico physiological nature, a brief account of the biology of the fish involved may constitute a suitable preamble to this study.

**Biology of Carp, Catla catla**

Catla is endemic to the riverine systems in northern India, Indus plain and adjoining hills of Pakistan, Bangladesh, Nepal and Myanmar, and has been introduced later into almost all riverine systems, reservoirs and tanks all over India. The carp belongs exclusively to fresh water fishes of the family cyprinidae, sub-family cyprininae. The fish, commonly called as Catla in Assam, Bengal, Bihar and Uttarpradesh, Bhakur in Orissa, Thaila in Punjab, Bocha in Andra, Thoppameen in Madras and Karakatla in Malabar. Though its natural distribution extends only to the Godavari River in Andra Pradesh, it is now common in the Krishna and Cauvery rivers also. It is the fastest growing carp in India. Catla is a eurythermal species that grows best at temperatures between 25-32 °C. Characteristically the fish has a deep body with conspicuous head large upturned mouth, non fringed lips, devoid of barbels and a broad dorsal fin with 14 to 16 branched rays distinguish the adult fish from the advanced fingerlings.
**Systematic position of *Catla catla***

<table>
<thead>
<tr>
<th>Group</th>
<th>Chordate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Vertebrata</td>
</tr>
<tr>
<td>Subphylum</td>
<td>Gnathostomata</td>
</tr>
<tr>
<td>Super class</td>
<td>Pisces</td>
</tr>
<tr>
<td>Class</td>
<td>Osteichthyes</td>
</tr>
<tr>
<td>Subclass</td>
<td>Actinopterigii</td>
</tr>
<tr>
<td>Super order</td>
<td>Teleostei</td>
</tr>
<tr>
<td>Order</td>
<td>Cypriniformes</td>
</tr>
<tr>
<td>Family</td>
<td>Cyprinidae</td>
</tr>
<tr>
<td>Genus</td>
<td><em>Catla</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>Catla</em></td>
</tr>
</tbody>
</table>

The body is ordinarily dull silver white, but tends to be rather darkish in weedy waters. The fry, from half to one inch in size, are distinguished by the relatively large head with reddish gills, broad dorsal fin with greyish margin pale body color owing to scarcity of pigment spots; scales are pink or coppery in the centre and dorsal side and whitish below, dorsal side of the body is more convex, head is large with large rounded eyes, mouth is wide with prominent lips, dorsal fin is quite large, caudal fin is bilobed, airbladder is usually large and divided into an anterior and posterior parts.
The fish catla is a surface, column feeder with its upturned mouth and large gill rackers are adapted to feed on numerous organisms floating in water. Young ones from the time they begin feeding until they reach a length of about 15 to 20 millimeters, feed almost exclusively on water fleas and other animalcules in the water. *Catla catla* is reported to grow very quickly, even upto 3 to 4 inches per month. This phenomenal growth may often be due to extreme and perhaps uneconomical under stocking, in virgin waters. In normally stocked waters, a growth of 15 to 18 inches in the first year can ordinarily be expected, if under stocked, catla grows to over 20 inches in length in the first year it does not however attain normal maturity at that age. The fish in second year are ordinarily sexually ripe. 22 months old specimens measuring 18 inches in total length and weighing about 3 pounds are therefore ready to breed in the third season, after hatching.

Catla attains maturity in its second year, performing a spawning migration during the monsoon season towards the upper stretches of rivers, where males and females congregate and breed in shallow marginal areas. The spawning season coincides with the south-west monsoon in north-eastern India and Bangladesh, which lasts between May and August and in north India and Pakistan from June to September. Its fecundity generally varies from 100 000-200 000/kg BW, depending on fish length and weight. The resultant seed are brought by water flow to the downstream areas where they are caught by seed collectors.

In India, Catla breeds in rivers during the rainy months from June to August. It does not breed in ordinary ponds though in certain ponds in Bengal and Bihar, known as bundle type tanks closely alien to the minor irrigation tanks of South India, it is known to breed during the south-west monsoon, after sudden heavy rain. Early fish are available in large numbers during june-july in Assam, Bengal and Bihar, and during July-August in Orissa, Punjab and Andra are collected in millions and stocked in nursery ponds.

As smaller fishes are considered to be more sensitive to heavy metals than larger ones (Anderson and Weber, 1975), small sized fingerling fish weighing about 10±2 gms were selected for the present investigation.
Materials and Methods

**WATER QUALITY**

Since, it is known that the water chemistry influence bioassesment of toxicity of a chemical substance, it is essential to maintain uniform water quality. The composition of the water used for the maintenance of fish is given below.

**Physico, chemical factors of water used for the experiment:**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.5 to 7.7</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>6-8 ml / liter</td>
</tr>
<tr>
<td>Salinity</td>
<td>0.193 ml / liter</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>88 ppm (as Caco$_3$)</td>
</tr>
<tr>
<td>Chlorinity</td>
<td>0.112 gm / liter</td>
</tr>
<tr>
<td>Sodium</td>
<td>1.23 m. moles / liter</td>
</tr>
<tr>
<td>Potassium</td>
<td>30.6 m. moles / liter</td>
</tr>
<tr>
<td>Calcium</td>
<td>4.32 m. moles / liter</td>
</tr>
<tr>
<td>Hardness of water</td>
<td>160 ppm (as Caco$_3$)</td>
</tr>
<tr>
<td>Carbondioxide</td>
<td>2.09 mg / liter</td>
</tr>
<tr>
<td>Oxygen % saturation</td>
<td>8</td>
</tr>
</tbody>
</table>

**Metallic salt selected**

Chromium salts are available in various salts forms like chlorides, sulphates, nitrates and acetates etc., which are more or less soluble in water. The degree of solubility depends upon the hardness and temperature of water. As the chromium chloride and potassium dichromate are more soluble in laboratory water, pure salts of chromium chloride and potassium dichromate with molecular formula CrCl$_3$ and K$_2$Cr$_2$O$_7$ with molecular weights 122.9 and 294.18 respectively are used in this investigation.
**Procurement and maintenance of fish**

*Catla catla* were collected from the department of fisheries, Anantapur, Andra Pradesh, and were immediately transported in big fish containers to the laboratory. Then they were released into large cement tanks contained of chlorinated tap water. The fish were fed with commercial fish pellets having around 40% protein content, and allowed to acclimatize for 15 days. Then the fish were isolated into batches having weight of 10±2gms were maintained in static water without any flow. Water was renewed every day to provide fresh water rich in oxygen. During experimentation water was aerated once a day to prevent hypoxic conditions. As the level of toxicity reported to vary with the interference of extrinsic and intrinsic factors like temperature, salinity, $P^H$, hardness of water, exposure period, density of the animals, size, sex etc., (Sivaramakrishna et al., 1991), and precautions were taken throughout this investigation to control all these factors as far as possible. As part of it, water from the same source has been used for maintenance of fish. The animals were starved for 24 hours prior to experimentation, to avoid any influence of differential feeding. The size of the animals selected also maintained strictly throughout the investigation.

**Objectives**

To determine the toxic effects of both trivalent and hexavalent chromium on

1. Acute toxicity tests to evaluate LC50 values
2. Chronic toxicity tests by exposing to sub lethal concentration
3. Hematological studies-RBC and WBC count
4. Carbohydrate metabolism: Estimation of blood glucose, Estimation of liver and muscle glycogen, Estimation of total carbohydrates, SDH and LDH activity, lactate and pyruvate levels in gills, kidney, liver and muscle
5. Protein metabolism: Estimation of total, soluble and structural proteins in gills, kidney, liver and muscle
6. Histopathological studies
**EXPERIMENTAL DESIGN**

200 fishes were divided into two batches, each batch again divided into 5 groups and each group contains 20 fishes. Batch-1 was exposed for sublethal concentration (1/10 of LC50) of trivalent chromium and Batch-2 was exposed for sublethal concentration of hexavalent chromium. The I-group of two batches was controlled unexposed.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Fishes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-Group</td>
<td>control</td>
<td>20 fishes (unexposed group)</td>
</tr>
<tr>
<td>II-Group</td>
<td>exposed for 1 day</td>
<td>20 fishes</td>
</tr>
<tr>
<td>III-Group</td>
<td>exposed for 8 days</td>
<td>20 fishes</td>
</tr>
<tr>
<td>IV-Group</td>
<td>exposed for 16 days</td>
<td>20 fishes</td>
</tr>
<tr>
<td>V-Group</td>
<td>exposed for 32 days</td>
<td>20 fishes</td>
</tr>
</tbody>
</table>

After the completion of stipulated exposure period, blood was collected from caudal region of the fish for hematological studies, and then the fish were sacrificed and isolated four tissues liver, kidney, muscle and gills under laboratory conditions for biochemical analysis and histopathological studies. DMR (Duncan’s Multiple Range Test) had been employed for the statistical analysis of the data and P value (level of significant) is significant at <0.05.

**METHODS**

**EVALUATION OF TRIVALENT AND HEXAVALENT CHROMIUM TOXICITY**

Lethal concentration (LC50) of chromium chloride (trivalent chromium) to fish *Catla catla* was determined by “Probit method” of Finney (1971).

The percent mortality of the fish in different trivalent chromium concentrations was determined immediately after 96 hours of exposure. For this, the experimental fish were divided into batches of thirty each and were exposed to different concentrations of trivalent chromium and each batch to one...
concentration, ranging from 54 to 66 mg/l these ranges were obtained on trial and
error basis. Mortality rate was observed in all the concentrations of chromium after
96 hours of exposure period. A batch of fish separately maintained along side in
fresh water served as controls. The experiment was repeated thrice. The mortality
value observed at each concentration, obtained from the mean of three repetitions
was converted as percent mortality. LC50s were obtained from the percent
mortality versus concentration and probit mortality versus concentration curves.
Dragstedt and Behren’s method was employed as given by Carpenter (1975). As
per this method, animals were exposed to log 2 concentration of chromium for 96
hours. The percent mortality at each concentration was derived from the
cumulative mortality value. Using these values LC50 were calculated by adopting
the formula:

$$\log \text{LC50} = \log A + 50 - \frac{a}{b} - a \times \log 2$$

Where,

$A =$ Concentration of metal that has a percent mortality immediately below 50%

$a =$ Percent mortality observed immediately below 50%

$b =$ Percent mortality observed immediately above 50%

Finally, the mean of the LC50s obtained from percent and probit mortality curves
and Dragsted and Behren’s method was taken for further studies. The LC50/96 hrs
of hexavalent chromium ($K_2Cr_2O_7$) to Catla catla is 100mg/l (Cr as 35.40 mg/l),
was already evaluated and it has been taken from the reference, International
Acute Effects of Hexavalent Chromium on Survival, Oxygen Consumption,
Hematological Parameters and Some Biochemical Profiles of the Indian Major
Carp, Labeo rohita by S.S.Vutukuru: Chromium induced alterations in some
Environ. Contam. Toxicol. 2003, 70, 118-123.
Materials and Methods

**Fixation of sublethal concentrations**

Based on the fact that the effect of a metal on fish becomes consistent within 96 hour of exposure (Eisler, 1977), LC$_{50/96}$ hours of trivalent and hexavalent chromium are considered as lethal concentrations. However, knowledge on the concentration of a 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 and Pearu, 1976; Hoppenheit, 1977). Further, studies on acute toxicity have limitations such as the occurrence of adaptation of test animal to the imposed toxicity (Stockner and Anita, 1976; Hoppenheit, 1977). Hence, Perkin (1979) also viewed the need for sublethal studies because distinct changes involving sequence of events in the responses of test animal could occur in sublethal concentration. So, about 1/10th of the 96 h LC$_{50}$ lethal concentration was taken as sublethal concentration i.e., 59.68mg/l, 100 mg/l(Cr as 35.40mg/lit) were the lethal concentrations, 5.96 mg/l of trivalent chromium and 10 mg/l(Cr as 3.54 mg/lit) of hexavalent chromium respectively was taken as the sublethal concentration for further studies.

**Fixation of exposure periods**

Since the duration of exposure is having a great influence on the toxicity of a metal in an organism (Radhakrishnaiah and Busappa, 1986), the effects of sublethal concentrations of trivalent and hexavalent chromium on the fish were studied at different periods of exposure in order to understand the influence of time over toxicity. Thus 1, 8, 16 and 32 days were chosen to observe the short term and long term effects of trivalent and hexavalent chromium on the fish *Catla catla*.

**General experimentation procedure for biochemical studies:**

The biochemical studies in this investigation were carried out in the gills, kidney, liver and muscle of the fish at 1, 8, 16 and 32 days on exposure to the sublethal concentrations of trivalent and hexavalent chromium. Selection of the
tissues such as the gills, kidney, liver and muscle was to understand the difference in the effects of trivalent and hexavalent chromium on osmoregulatory (gill and kidney) and non osmoregulatory (liver and muscle) organs of the fish. Prior to estimation, groups of fish were exposed to the respective sublethal concentration of trivalent and hexavalent chromium was maintained upto stipulated period of exposure. At the end of it, the healthy fishes were taken out and blood was collected from incision at the caudal vein region into the heparinized capillary tubes for hematological studies, and then the fishes were sacrificed, stunned to death and the required organs were dissected out from each animal using sterilized instruments. The organs were weighed accurately on an electrical semi-microbalance and transferred into ice jacketed micro beakers containing fish ringer solution. The fish ringer was prepared as per the composition given by Ekberg (1958). An equilibrium 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 biochemical analysis of each experiment was carried out in the organs from six animals at each exposure period and the mean of six is taken into consideration. Similar studies made in animals from normal medium served as controls.

**Haematology**

*Catla catla* exposed to sublethal concentrations for 1, 8, 16 and 32 days of trivalent and hexavalent chromium, at the end of each exposure period the healthy fishes were taken out, the blood from the control and treated fingerling was collected from incision at the caudal vein region into the heparinized capillary tubes for hematological studies. The treated and control blood samples were used to estimate the hematological parameters.

**Red blood corpuscle (RBC) count**

RBC count was made with a Neubauer crystalline counting chamber as described by Samuel (1977). The RBC number was determined in *Catla catla* at different sublethal exposure periods like 1, 8, 16 and 32 days including control medium. The RBC number was determined in 6 individual fish at each exposure period.
period including control medium. The red blood cell number was made with Nuebauer Crystalline counting chamber. The blood collected was diluted with Hayem’s fluid (5gm of sodium sulphate, 1gm of sodium chloride and 0.5 gm of mercuric chloride dissolved in 200 ml of distilled water) and the RBC number was represented in millions per cubic millimeter (mm3). The Hayem’s fluid was taken upto ‘0’ mark in the RBC pipette and it was mixed thoroughly by rotating the pipette and the mixture was allowed to stand about 2-3 minutes for uniform mixing. The counting chamber and cover slip were cleaned and the coverslip was placed over portified area. Again the solution was mixed gently and the stemful of solution was expelled and a drop of fluid is allowed to flow under coverslip by handling the pipette at an angle of 90. It was allowed to stand for 2-3 minutes till the RBC are settled. Afterwards, the portified area of the counting chamber was focused under the microscope and the RBC are counted in five small squares of the RBC columns ( the RBC were counted in the outer four corner squares and the central square ) under high power and the number of RBC per cubic millimeter (mm3) are calculated using the following formula.

\[
\text{Number of cells} \times \text{Dilution factor (200)} \times \text{Depth factor} / \text{Area counted} = \text{millions/mm}^3
\]

**WHITE BLOOD CORPUSCLES (WBC) COUNT**

WBC count was estimated by Samuel (1977).

The blood is mixed with a weak acid solution, it haemolyses the red blood cells leaving only WBC’s. As the number of WBC are usually more, blood samples are diluted in the thumb pipette taking blood upto 0.1 mark and diluted it upto 11 mark with the diluting fluid known as Shaw’s solution. It has two solutions as follows.

White Blood Cell count fluid: (Shaw’s solution, 1930)

**Solution A**

- Sodium chloride: 0.900 gm
- Neutral red: 0.025gm
- Distilled water: 100 ml
Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crystalline violet</td>
<td>12.0 mg</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>3.8 gm</td>
</tr>
<tr>
<td>Formaldehyde solution (40% w/v)</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Both the solutions were filtered and mixed in equal volume before starting the experiment. Thus hundred fold dilution of the original sample was obtained. The diluted blood was changed into Neubauer’s chamber taking the precautionary measures and the number is expressed in thousand’s per cubic millimeter using the following formula.

White Blood Cells / cu mm = Number of white blood cells counted × correction

Volume × correction for dilution

Some aspects of carbohydrate metabolism

The levels of blood glucose, liver and muscle glycogen content, total carbohydrate content in gills, kidney, liver and muscle, activities of succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH), the levels of pyruvate and lactate were estimated under this study.

Estimation of blood glucose

The level of blood glucose was estimated by Mendel et al., (1954) method in *Catla catla* exposed for 1, 8, 16 and 32 days of sublethal concentrations of trivalent and hexavalent chromium. The blood collected from the caudal incision into the heparinized capillary tubes drawn into micro beaker with a fine 1 ml of tuberculin micro syringe and the glucose content in the blood sample was determined as follows.
From each micro-beaker 0.1 ml of blood was taken into a test tube and to it 1.9 ml of deproteinising solution (5% trichloro acetic acid) (analar) containing 100 mg of silver sulphate (analar) was added to it. The mixture was centrifuged at 3000 rpm for about 10 minutes. Then 1.0 ml of supernatant was taken and to it was added 3.0 ml of concentrated sulphuric acid (analar). The mixture was shaken vigorously for about 5 minutes and then heated in a boiling water bath exactly for 6.5 minutes. Subsequently, the mixture was cooled in running tap water. Finally, the optical density of the pink colour developed was measured against blank at 520 nm. The level of blood glucose was calculated in mg of glucose/100ml.

**Estimation of glycogen**

Liver and muscle glycogen content was estimated using the anthrone reagent method described by Caroll et al., (1956).

Since glycogen concentration in muscle is known to vary in different regions of the body (Amano et al., 1953; Faser et al., 1956) care was taken in dissecting out this sample from the same region of the fish i.e., the antero dorsal region of the trunk. The organs were digested with 3.0 ml of hot 30% potassium hydroxide. The digestate was cooled and 3.75 ml of absolute ethanol was added to it. The entire mixture was kept overnight in a refrigerator. Then the mixture was centrifuged for 15 minutes at 2500 rpm. Decantated the supernatant and 10 ml of warm distilled water was added to the residue to dissolve the precipitated glycogen. To 0.2 ml of this, 1.8 ml of distilled water and 0.5 ml of 2% anthrone reagent dissolved in 72% concentrated sulphuric acid were added and heated in a boiling water bath exactly for 10 minutes. The mixture was cooled and the optical density of the colour developed was measured in a spectrophotometer at a wave length of 620 nm. A blank and glucose standards were also run similarly. The glycogen content is expressed as mg/g wet wt. of the organ.

**Estimation of total carbohydrates**

Total carbohydrate content was estimated by the method of Carroll et al., (1956). A 10% homogenate of the tissues were prepared in 10% Trichloro acetic acid (TCA). The contents were centrifuged at 1000g for 15 minutes. To 0.5 ml of
TCA filtrate 5 ml of anthrone reagent was added and boiled for 15 minutes. The tubes were cooled and the colour was read at 620 nm in a spectrophotometer using blank consisting of TCA and anthrone in the same proportion. The values were expressed as mg / gm wet wt of the tissue.

**ESTIMATION OF SUCCINATE DEHYDROGENASE (SUCCINATE: ACCEPTOR OXIDO REDUCTASE. EC 1.3.99.1) (SDH)**

Succinate dehydrogenase activity in the organs was estimated using the calorimetric method of Nachlas et al., (1960).

A 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 taken as the source of enzyme. The incubation mixture consisted of 0.2 ml of 0.4 M phosphate buffer (pH 7.7), 0.2 ml of 0.2 M sodium succinate, 1.0 ml of 0.004 M 2-(p-indophenol)-3-nitrophenyl-5-phenyltetrazolium chloride (INT), 0.1 ml of 0.005M phenazine methosulphate and 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37°C for 30 minutes and the reaction was stopped by adding 6.0 ml of glacial acetic acid. The formazone formed was extracted into 6.0 ml of toluene overnight at 0°C and the optical density of the color developed was measured in a spectrophotometer at a wave length of 495 nm. A blank taking 0.5 ml of distilled water and control taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity is expressed as µM formazone / mg protein / hr.

**ESTIMATION OF LACTATE DEHYDROGENASE (LDH) (L-LACTATE NAD OXIDO REDUCTASE. EC 1.1.27) ACTIVITY**

Lactate dehydrogenase activity in the organs was estimated using the method of Srikanthan and Krishnamoorthi (1955) as modified by Govindappa and Swami (1965).

A 5% homogenate (w/v) was prepared in 0.25M ice cold sucrose solution, centrifuged at 2500 rpm for 15 minutes and the supernatant was taken as the source of enzyme. The incubation mixture consisted of 1.0 ml of 0.4 M phosphate
buffer (pH 7.4), 0.5 ml of 0.1 M nicotinamide adenine dinucleotide (NAD), 1.0 ml of 0.004 M 2-(p-indophenol)-3-p-nitrophenyl 1,5-phenyltetrazolium chloride (INT) and 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37°C for 30 minutes and then the reaction was stopped by adding 6.0 ml of glacial acetic acid. The formazone formed was extracted into 6.0 ml of toluene overnight at 0°C. The optical density of the color developed was measured in a spectrophotometer at a wavelength of 495 nm. A blank using 0.5 ml of distilled water and a control taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity is expressed as µM formazone / mg protein /hr.

**Estimate of Pyruvate**

Pyruvate was estimated using the method of Friedman and Hangen (1942). A 5% homogenate (w/v) was prepared in 10% Trichloroacetic acid and centrifuged at 3000 rpm for 15 minutes. The supernatant was used for the estimation of pyruvate. 1.0 ml of supernatant was taken and to it 1.0ml of 0.001 M 2, 4 – dinitrophenylhydrazine and 3ml of 0.4N sodium hydroxide were added. After 10 minutes, the optical density of the colour developed was measured in a spectrophotometer at a wave length of 540 nm against the reagent blank. Pyruvate standards were prepared alongside for comparison. The pyruvate content in the organ is expressed as mg pyruvate/ gm wt of the tissue.

**Estimation of Lactate**

Lactate levels in the organ estimated using the method of Barker and Summerson (1941) as modified by Huckabee (1961). A 5% homogenate (w/v) was prepared in cold 10% trichloroacetic acid and centrifuged at 3000 rpm for 15 min. The supernatant was used for estimation of lactate. To 1.0 ml of supernatant, 1.0 ml of 20% copper sulphate was added and the mixture was made upto 10.0 ml with distilled water. Then 1.0 gm of powdered calcium hydroxide was added, shaken vigorously and kept for an hour at room temperature with intermittent shaking. The contents were centrifuged at 3000 rpm for 10 minutes and to 1.0 ml of the supernatant, 0.5 ml of 4% copper sulphate was added followed by 6.0 ml of
Materials and Methods

concentrated sulphuric acid. The contents were mixed by lateral shaking, kept in boiling water bath for exactly 6.5 minutes and cooled. After the contents were sufficiently cooled, 0.1 ml of 1.5% P-hydrophenyl (prepared in 0.5% of sodium hydroxide) was added and the precipitate formed was kept at laboratory temperature for 30 minutes. Then the contents were placed in boiling water bath for 90 seconds, cooled and the optical density of the colour developed was measured in a spectrophotometer at a wave length of 560 nm against a reagent blank. Lactate standards were prepared alongside for comparison. The lactate content is expressed as mg / gm wet wt of the tissue.

Some aspects of Protein metabolism

The levels of total, soluble and structural proteins were estimated separately in different organs such as the gills, kidney, liver and muscle of the fish *Catla catla*.

Estimation of soluble, structural and total proteins:

The soluble, structural and total proteins were estimated using Folin phenol reagent method as described by Lowry et al., (1951).

A 1% homogenate (w/v) was prepared in ice cold 0.25M 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 used for soluble protein estimation and the residue was taken for structural protein estimation. To the supernatant 1.0 ml of 10% trichloro acetic acid (TCA) was added and centrifuged at 3000 rpm for 10 minutes. Discarded the supernatant and the residue was taken for soluble protein estimation. For total protein content 1.0 ml of homogenate was taken, to it 1.0 ml of 10% trichloro acetic acid was added and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded and the residue was taken for the estimation of total proteins. All the three residues were taken separately dissolved in 1 ml of 1N NaOH and to them 4.0 ml of CuSo4 reagent and 0.4 ml of Folin-ciocalteau reagent were added. The contents were stirred well and kept aside for 30 minutes at room temperature. Later the optical density of the solutions was read at 600 nm in spectrophotometer. A mixture of 1.0
Materials and Methods

ml of NaOH, 4.0 ml of CuSO4 reagent and 0.4 ml of Folin ciocalteau was used as blank. Bovin’s Serum albumin was used for the preparation of protein standards. The protein content is expressed as mg/gm wet wt of the tissue.

Histopathology

The histological sections of the gills, kidney, liver and muscle of fish were taken by adopting the procedure as described by Humason (1972).

The tissues were isolated and gently rinsed with physiological saline solution (0.9% NaCl) to remove mucus and other debris adhering them. They were fixed in Bouin’s fluid (75 ml saturated aqueous picric acid, 25 ml 40% formaldehyde and glacial acetic acid) for 24 hours. The fixative was removed by washing through running tap water for overnight. Then the tissues were processed for dehydration. Ethyl alcohol was used as the dehydrating agent, as it is the most suitable and economical besides its hardening effect. The alcoholic transfer schedules were so arranged as to utilize both dehydration and hardening effect. The tissues were passed through successive series containing 30%, 50%, 70%, 80%, 90%, 95% and absolute alcohols. Then the tissues were cleaned in methyl benzoate and embedded in paraffin wax. Sections of 5µ thickness were cut using “SIPCON” rotatory microtome. The sections were stained with Harris hematoxylin (Harris, 1900) and counter stained with eosin, dissolved in 95% alcohol. After dehydration and cleaning, the sections were mounted in Canada balsam. Photomicrographs of the section preparations were taken using Magnus photomicrography equipment.

Statistical Analysis

All the results obtained in this investigation were subjected to statistical analysis. For this, the data were fed to the computer, the standard deviation to each mean, and the percent change over the means of controls and experimental were derived. The significance of the data among controls and experimental were derived at 5% level using the DMR test, and is represented in the respective tables.