Materials & Methods
Animal selected

Cyprinus carpio (Linnaeus) is an economically important edible fish having great commercial value, occurring abundant in the freshwater tanks and ponds in and around Anantapur. Besides its wide availability and commercial importance, this fish is known for its adaptability to laboratory conditions and suitability to toxic studies (Sreenivasan and Swaminathan, 1967). Hence, this fish is selected as the experimental model for the present investigation. A brief account on the biology of it may constitute a suitable preamble.

Systematic position of Cyprinus carpio

Group : Chordata
Phylum : Vertebrata
Subphylum : Gnathostomata
Super class : Pisces
Class : Osteichthyes
Sub class : Actinopterygii
Super order : Teleostei
Order : Cypriniformes
Family : Cyprinidae
Genus : Cyprinus
Species : carpio

Biology of Carp

Cyprinus carpio commonly known as common carp belongs to the family Cyprinidae. It is extensively cultured throughout the world. It is an exotic
species and native of the temperate regions of Asia, especially China (Gunther, 1968). It is introduced into Indian waters in the year 1939 from Ceylon (Alikunhi, 1957). It is a very divergent species having many subspecies or strains and the one used for this investigation is of Dangtok strain. It is non-predatory and has a non-elongated body with an abdomen conspicuously larger than the rest of its body. There is a pair of barbles to both sides of the mouth. The fins are yellowish to red but become red during the breeding season. The fish is known for its rapid growth and attains sexual maturity at the end of the first year. The maximum length of which it grows is about 76 cm (approximately 6.8 Kg by weight). As the smaller fish are considered to be more sensitive to heavy metals than larger ones (Anderson and Weber, 1975), small sized fish weighing around 10 g were selected for the present investigation.

**Metallic salts selected**

Lead and Chromium salts are available in various salt forms like chlorides, sulphates, nitrates and acetates etc., which are more or less soluble in water. The degree of solubility of each salt depends upon the hardness of water. As the lead nitrate and chromium chloride are more soluble in the laboratory water, pure salts of chromium chloride and lead nitrate with molecular formulae Pb(NO₃)₂ and CrCl₃ having molecular weights 331.1 and 122.9 respectively are used in this investigation. Every 1.6 gms of lead nitrate contains 1 gm of lead and 2.36 gms of chromium chloride contains 1 gm of chromium.
Water quality

Since it is known that the water chemistry influence bioassessment 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 experimentation

<table>
<thead>
<tr>
<th>Factor</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.4 to 7.6</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>6 - 7 ml / litre</td>
</tr>
<tr>
<td>Salinity</td>
<td>0.191 ml / litre</td>
</tr>
<tr>
<td>Alkalinity</td>
<td>87 ppm (as CaCO₃)</td>
</tr>
<tr>
<td>Chlorinity</td>
<td>0.111 gm / litre</td>
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<tr>
<td>Sodium</td>
<td>1.22 m. moles / litre</td>
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<tr>
<td>Potassium</td>
<td>30.5 m. moles / litre</td>
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<tr>
<td>Calcium</td>
<td>4.31 m. moles / litre</td>
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<tr>
<td>Hardness of Water</td>
<td>160 ppm (as CaCO₃)</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>2.09 mg / litre</td>
</tr>
<tr>
<td>Oxygen % saturation</td>
<td>8</td>
</tr>
</tbody>
</table>

Procurement and maintenance of fish

*Cyprinus carpio* were collected from the Department of Fisheries, Anantapur, Andhra Pradesh, and were immediately transported in big fish containers to the laboratory. Then they were released in large cement tanks contained of dechlorinated tap water. The fish were fed with commercial fish pellets having around 40% protein content, chopped sheep liver once in two days, and allowed to acclimatize for 15 days. Then the fish were separated
into the batch of having the size 10±2 gm (fingerlings) and were maintained in static water without any flow. Water was renewed everyday to provide freshwater rich in oxygen. During experimentation water was aerated once a day to prevent hypoxic conditions. As the level of toxicity is reported to vary with the interference of various extrinsic and intrinsic factors like temperature, salinity, pH, hardness of water, exposure period, density of the animals, size, sex etc., (Sivaramakrishna et al., 1991), precautions were taken throughout this investigation to control all these factors as far as possible. As a part of it, water from the same source has been used for the maintenance of fish. The animals were starved for 24 hours prior to each experimentation, to avoid any influence of differential feeding. The size of the animals selected also maintained strictly throughout the investigation.

METHODS

Evaluation of Lead and Chromium toxicity

The percent mortality of the fish in different lead and 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 lead and chromium, each batch to one concentration ranging from 12 to 24 mg/L for lead and 22 to 34 mg/L for chromium. These ranges were obtained on trial and error basis. Mortality rate was observed in all the concentrations of lead and chromium immediately after 96 hour exposure period. A batch of fish separately maintained along side in freshwater 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 also employed as given by Carpenter (1975). As per this method, animals were exposed to log 2 concentrations of lead and chromium for 96 hours. The percent mortality at each concentration was derived from the cumulative mortality value. Using these values LC50s were calculated by adopting the formula:

\[
\text{Log } LC_{50} = \text{Log } A+50-a/b - a \times \log 2
\]

Where,

- \( A \) = Concentration of the 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.

**Fixation of sublethal concentrations**

Based on the fact that the effect of a metal on fish become consistent within 96 hour of exposure (Eisler, 1977), LC50s/96 hours of lead and 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 significant 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 one tenth of the 96h LC$_{50}$s i.e., 0.72 mg/l and 2.49 mg/l respectively for lead and chromium were taken as the sublethal concentrations 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 (Radhakrishaniah and Busappa, 1986), the effects of the sublethal concentrations of lead and chromium on the fish were studied at different periods of exposure in order to understand the influence of time over toxicity. Thus 1, 7, 15 and 30 days were chosen to observe the short-term and long-term effects of chromium and lead on *Cyprinus carpio*.

**General experimentation procedure for biochemical studies**

The biochemical studies in this investigation were carried out in the gills, brain, liver and muscle of the fish at 1, 7, 15 and 30 days on exposure to the sublethal concentrations of lead and chromium. Selection of the gills, brain, liver and muscle was to understand the difference in the effects of lead and chromium on respiration and osmoregulatory (gill), nervous integration (brain), metabolic (liver) and locomotor (muscle) activities of the fish. Prior to each estimation, groups of fish were exposed to the respective sublethal concentration of lead and chromium and were maintained upto the stipulated period of exposure. At the end of it, the fish were 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 microbeakers containing fish...
ringer solution. The fish ringer was prepared as per the composition given by Ekberg (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 entire process was carried out in a sterilized cold room with temperature maintained at 15±1°C. Each experiment was carried out in the organs from six animals at each exposure period and the mean of the six is taken into consideration. Similar studies made in animals from normal medium served as controls.

In addition, for the estimation of blood glucose, blood was collected from the heart with a sterilized hypodermal syringe. The insertion of syringe needle into the heart was exactly in the middle line, 0.5 to 1.0 cm cranially from the posterior margin of the opercular cover and directed dorsocaudally at an angle of 45°. Care was taken to see that blood was not haemolised. It was collected in vials coated with 3% sodium oxalate solution as an anticoagulant.

Some aspects of carbohydrate metabolism

The levels of blood glucose, liver and muscle glycogen content, the activity of glycogen phosphorylase, the levels of pyruvate and lactate and the activities of succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) were estimated under this study.

Estimation of blood glucose

The level of blood glucose was estimated in Cyprinus carpio subjected to the sublethal concentrations of lead and chromium. The blood from the cardial region of each fish was collected. Prior to the collection of blood
samples, the microbeakers were cleaned thoroughly and rinsed with the anti-coagulant solution. Into each microbeaker 0.5 ml of blood was drawn with a fine 1 ml of tuberculin micro-syringe and glucose content in the blood sample was determined by the colorimetric micro-method as described by Mendel et al., (1954) as follows.

From each micro-beaker 0.1 ml of blood was taken into a test tube and to it 1.9 ml of deprotenising 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**

Glycogen content in the liver and muscle was estimated using the anthrone reagent method as described by Caroll et al., (1956). Since glycogen concentration in muscle is known to vary in different regions of body (Amano et al., 1953; Faser et al., 1956) care was taken in dissecting out this sample from the same region of body of the fish i.e., the anterodorsolateral 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. Decanted the supernatant and 10.0 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 wavelength of 620 nm. A blank and glucose standards were also run similarly. The glycogen content is expressed as mg/gm wet of the organ.

**Estimation of glycogen phosphorylase (1-4-glucan:orthophosphate glucosyl transferase, EC 2.4.1.1) activity in the liver**

Total phosphorylase activity in the liver was estimated using the method described by Sutherland (1955). A 5% homogenate (w/v) was prepared in 0.1 M sodium fluoride solution (pH 6.5). The homogenate was centrifuged at 1500 rpm for 15 minutes, thus extracting enzyme into the supernatant. The supernatant was diluted four times with the cold sodium fluoride solution. The incubation mixture consisted of 0.2 ml of 2% glycogen and 0.4 ml of diluted enzyme. This mixture was incubated at 37°C and the reaction was started by adding 0.2 ml of 0.016 M glucose-6-phosphate and 0.004 M adenosine-5-monophosphate (1:1 ratio). The reaction was stopped after 15 minutes by adding 5 ml of 10% trichloroacetic acid. A blank was also run similarly. Finally, the inorganic phosphates liberated were estimated by the method of Fiske and Subba Row (1925). For this, to an aliquot of the above reaction mixture 4.5 ml of 0.44% ammonium molybdate and 0.2 ml of 1-amino-2-naphthol-4-sulphonic acid (ANSA) were added. The contents were
mixed well and heated in boiling water for 10 minutes. After cooling, the volume was made to 10 ml with distilled water and the colour developed was measured in a spectrophotometer at a wavelength of 660 nm and the activity is expressed as µM Pi liberated/mg protein/h, using the phosphate standards.

Estimation of glycogen phosphorylase activity in the muscle

Phosphorylase activity in the muscle was estimated using the method described by Cori et al., (1955). For each sample, a 5% homogenate (w/v) was prepared in 0.1 M sodium fluoride and 0.037 M ethyldiamine tetrachloride solution, adjusted to 6.5 pH. The homogenate was centrifuged at 1500 rpm for 15 minutes, thus extracting the enzyme into the supernatant. The supernatant was diluted four times with 0.03 M cystein and 0.015 M β-glycerophosphate buffer (pH 6.5). The incubation mixture consisted of 0.2 ml of 2% glycogen and 0.4 ml of diluted enzyme. The mixture was incubated at 37°C for a period of 20 minutes and the reaction was started by adding 0.2 ml of 0.016 M glucose-1-phosphate and 0.004 M adenosine-5-monophosphate (1:1 ratio). The reaction was stopped after 15 minutes by adding 10% trichloroacetic acid. A blank was also run similarly. Finally, the inorganic phosphates liberated were estimated by the method of Fiske and Subba Row (1925) at a wavelength 660 nm and the activity is expressed as µM Pi liberated/mg protein/h.

Estimation 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 1.0 ml of 0.001 M 2,4-dinitrophenyl hydrazine and 3 ml of 0.4 N sodium hydroxide were added. After 10 minutes, the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 540 nm against the reagent blank. Pyruvate standards were prepared along side for comparison. The pyruvate content in the organ is expressed as mg pyruvate/gm wt of the organ.

**Estimation of Lactate**

Lactate level in the organs was 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 the estimation of lactate. To 1.0 ml of supernatant, 1.0 ml of 20% copper sulphate was added and the mixture was made up to 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 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 both for 90 seconds, cooled and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 560 nm against a reagent blank.
Lactate standards were prepared alongside for comparison. The lactate content is expressed as mg lactate/gm wet wt of the organ.

**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-p-nitrophenyl-5-phenyltetrazolium chloride (INT), 0.1 ml of 0.005 M 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 6.0ml of glacial acetic acid. The formozan formed was extracted into 6.0 ml of toluene overnight at 0°C and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 495nm. 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 formozan/mg protein/h.

**Estimation of Lactate dehydrogenase (L-lactate NAD oxido reductase. EC 1.1.27) activity (LDH)**

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 0.25 M 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 lithium lactate, 0.1 ml of 0.0001 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 formozan formed was extracted into 6.0 ml of toluene overnight at 0°C. The optical density of the colour 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 $\mu$M formozan/mg protein/h.

**Ions and associated ATPases**

The levels of sodium, potassium and calcium ions and the activities of Na$^+$ - K$^+$ ATPase, Mg$^{2+}$ ATPase and Ca$^{2+}$ ATPase were estimated in the gills, brain, liver and muscle of fingerlings of *C. carpio* under this study.

**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 to 200°C temperature. The residues were dissolved in glass 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/gm wet wt of the organ.

**Na⁺ - K⁺, Mg²⁺ and Ca²⁺ ATPase activities (ATPase phosphorylase EC 3.6.1.3.)**

Na⁺ - K⁺, Mg²⁺ and Ca²⁺ ATPase activities were estimated separately in the organs by the method described by Watson and Beamish (1981) with slight modification. 1% tissue homogenates (W/V) were prepared in ice-cold 0.25M sucrose solution containing 5mM EDTA (prepared in 40mM tris-HCl buffer at pH 7.5) and 0.01M imidazole. The homogenates were centrifuged at 2500rpm for 10minutes and the supernatants were taken as crude enzyme extracts 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 2ml, the first set consisted of 100mM disodium potassium triphosphate (prepared in 20mM tris-HCl buffer at pH 7.5), 100mM NaCl, 20mM KCl, 3mM MgCl₂, 1mM ouabain (potent inhibitor of Na⁺ - K⁺ ATPase) and 0.3ml of enzyme extract and the third set consisted of 100mM disodium ATP (prepared in 20mM tris-HCl buffer at pH 7.8), 5mM CaCl₂ and 0.3ml of enzyme extract. All the three incubation sets were incubated at 37°C for exactly 15minutes and then the reaction was arrested by adding 2ml of cold 10% TCA. The inorganic phosphates liberated were estimated by the method of Fiske and Subba Row (1925). The absorbance was measured at 660nm. Endogenous blanks were prepared to
find out the endogenous inorganic phosphates. Another blank was prepared without using the cofactor to deduct the sodium salt stimulated activity as the cofactor used was a disodium salt of ATP.

The first set gives the total ATPase activity of Na⁺ - K⁺ and Mg²⁺, whereas the second set gives only the Mg²⁺ ATPase activity as ouabain inhibits Na⁺ - K⁺ activity. Thus the Na⁺-K⁺ activity was derived by subtracting the Mg²⁺ ATPase from the 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 aminoacids, ammonia and urea and the activities of protease, alanine and aspartate aminotransferases and glutamate dehydrogenase were estimated in the gills, brain, liver and muscle of fingerlings of *C. carpio* under this study.

Estimation of soluble, structural and total proteins

The soluble, structural and total proteins in the organs were estimated using the folin phenol reagent method as described by Lowry et al., (1951). A 1% homogenate (W/V) was prepared in 0.25 M ice cold sucrose solution. For soluble and structural proteins, 1 ml of 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% TCA 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% TCA was added and centrifuged at 3000 rpm. Discarded the supernatant and the residue was taken for experimentation. All the three residues were dissolved in 5 ml of 0.1 N sodium hydroxide, and to 1 ml 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 in a 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 for blank. Bovine albumin was used for the preparation of protein standards. The protein content is expressed as mg/gm wet wt of the organ.

**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). A 5% homogenate (W/V) was prepared in 10% TCA and centrifuged at 2000 rpm for 15 minutes. To 0.2 ml of supernatant 2 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 ml with distilled water. The optical density of the colour developed was measured in a 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/gm wet weight of the organ.
Estimation of protease activity

Protease activity in the organs was estimated using the ninhydrin method as described by Davis and Smith (1955). A 1% homogenate (W/V) was prepared in distilled water. To 2 ml of homogenate, 0.5 ml of 1% casein and 2 ml of 0.1 M phosphate buffer (pH 5) 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 dilutent (distilled water and n-propanol in 1:1 ratio). The optical density of the colour developed was measured in a spectrophotometer at a wavelength of 570 nm. A blank taking 2 ml of distilled water and control taking 2 ml of boiled enzyme were also run similarly. Amino acid standards were prepared alongside for comparison. The protease activity is expressed as μM amino acid nitrogen released/mg protein/h.

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

Activities of alanine and aspartate aminotransferases in the organs were estimated using the method of Reitman and Frankel (1957). 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 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, 0.5 ml of 0.005 M α-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 of 0.2 M aspartic acid, 0.5 ml of 0.005 M α-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 mixtures were incubated at 37°C for 30 minutes and then the reaction was stopped by the addition of 1 ml of 0.001 M 2,4-dinitrophenyl hydrazine (ketone reagent). Finally, the reaction mixtures were made to 10 ml with 0.4 N sodium hydroxide and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 545 nm. A blank taking 0.1 ml of distilled water and control 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 µM pyruvate formed/mg protein/h and the aspartate aminotransferase activity as µM oxaloacetate formed/mg protein/h.

**Estimation of glutamate dehydrogenase (GDH) (L-glutamate: NAD oxalo-reductase, EC: 1.4.13) activity**

GDH activity was estimated in the organs using the method of Lee and Lardy (1965) with slight modification. A 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 0.25M sucrose at 2°C to 4°C for 24 hours. The incubation mixture in a final volume of 2 ml contained 40 µM of sodium glutamate, 100 µM of sodium phosphate buffer (pH 7.4), 0.1 µM of NAD (nicotinamide adenine dinucleotide) and 4µM of INT (2-P-indophenol-3-P-nitrophenyl-5-phenyltetrazolium chloride). The reaction was initiated by the addition of 0.5ml
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 ml of glacial acetic acid. The formozan formed was extracted into
5 ml of toluene overnight at 5°C. The optical density of the colour developed
was measured in a spectrophotometer at a wavelength of 495 nm. A blank by
taking 0.5 ml of distilled water and control by 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 formozan formed/mg
protein/h.

**Estimation of ammonia**

Ammonia was estimated in the organs by the method of Bergmeyer
(1965) with a slight modification. A 5% tissue homogenate (W/V) was
prepared in cold distilled water and centrifuged at 2000 rpm for 15 minutes.
To 1 ml of the supernatant, 2 ml of 15% perchloric acid was added and
centrifuged again at 2000 rpm for 15 minutes. The supernatant was
neutralized with 2 ml of 15% sodium hydroxide. To this, 0.5 ml of Nessler's
reagent was added and the colour developed was read immediately in a
spectrophotometer at a wavelength of 495 nm against a reagent blank.
Ammonium sulphate standards were run alongside for comparison. The
ammonia content is expressed as μM/gm wet wt of the organ.

**Estimation of urea**

Urea was estimated in the organs by diacetylmonoxime method as
described by Natelson (1971). A 10% tissue homogenate (W/V) was prepared
in 15% perchloric acid and centrifuged at 2000 rpm for 15 minutes. To 1.5 ml
of supernatant, 1 ml of acid mix (3:1 orthophosphoric acid and concentrated sulphuric acid) was added and the contents were shaken well. To this 0.5 ml of 2% diacetylmonoxime was added and heated at 100°C in a boiling water bath for 30 minutes. The tubes were cooled and the colour developed was read in a spectrophotometer at a wavelength of 480 nm against a reagent blank. Standards of different urea concentrations were run simultaneously. The urea content is expressed as µM/gm wet wt of the organ.

Some aspects of lipid metabolism

The levels of total lipids, free fatty acids, the activity of lipase, phospholipids and cholesterol were estimated in the gills, brain, liver and muscle of fingerlings of *C. carpio* under this study.

Estimation of total lipids

Total lipids were estimated separately in the organs using the method of Folch *et al.*, (1957). Each excised organ was weighed accurately to the nearest milligram in a Sartorius electrical semi-microbalance. The weight of the organs, used for the estimations, usually range between 150 to 200mg. Separate homogenates were prepared for these organs in 2:1 chloroform-methanol mixture, using 20ml of the mixture per gram weight of the organ. The homogenates were centrifuged at 2500rpm for 5 minutes and the supernatant was collected into a corning centrifuge tube, the weight of which was previously determined accurately in a Sartorius electrical semi-micro balance. To each centrifuge tube, normal saline (9g NaCl in one litre of distilled water) was added at the rate of 0.2ml to every 1ml of the homogenate. The contents were shaken well and again centrifuged at
2500rpm for about 10 minutes. Now the upper phase, which essentially consists of nonlipids, was removed completely and the lower phase, which essentially consists of lipids, was evaporated to dryness slowly at 60-65°C. After the completion of evaporation, the residue, left behind in the centrifuge tube, was weighed accurately in a Sartorius electrical semi-microbalance. The difference between the initial and final weights of centrifuge tubes represents the amount of total lipids present in the sample and this is expressed as mg units/gm wet wt of the organ.

**Estimation of free fatty acids (FFA)**

FFA were estimated separately in the organs using the method as described by Natelson (1971). The lipids were extracted separately from each of the excised organ, using the method of Folch et al., (1957) the details of which are given above. 2ml of chloroform-lipid extract (obtained from the above cited method) of each organ was evaporated to dryness slowly at 60-65°C. After that, the residue in each case was dissolved in 2ml of 95% ethanol. A drop of 0.1% phenolphthalein (prepared in alcohol) was added to these as an indicator. The contents were titrated against N/50 KOH until pink colour was developed. A microburette of 1ml capacity was used for the titration and 2ml of ethanol was used as blank.

The titration value of the blank was substracted from the titration value of the sample (unknown). By multiplying this value with 0.02, FFA content (in milli equivalents) in the samples was calculated. Finally, this value was multiplied with 277 (assuming that the average molecular weight of FFA is 277) and the same is expressed as mg units/gm wet wt of the organ.
Estimation of lipase activity

Lipase activity (Glyceroiester hydrolase) was estimated separately in the organs using the procedure described by Colowick and Caplan (1955). Each excised organ was accurately weighed to the nearest milligram in a Sartorius electrical semi-microbalance. A 1% homogenate (W/V) was prepared for each organ in 5ml of ice cold distilled water. The homogenate was centrifuged at 2500rpm for 5minutes. The supernatant was taken into a flask which is the enzyme source. The substrate was prepared by mixing 100ml of buffer (0.2M sodium acetate) with 50ml of Tween-20, 10ml of indicator (0.02% aqueous phenol red) and 90ml of distilled water. The pH of this mixture was adjusted to 7.2. 10ml of this substrate was added to the enzyme source and then the flask was stoppered and placed in a cold water bath at 20°C. After 10minutes, a drop of decyclic alcohol was added to the enzyme-substrate mixture, so as to prevent foaming and the solution was titrated against 0.02N sodium hydroxide till the reaching of the end point (rosy-red colour). The contents of the flask were thoroughly mixed during the titration. Blanks were prepared by boiling the supernatant and these blanks were also titrated as the regular samples. One ml of 0.02N sodium hydroxide to titrate the liberated acids in the assay was taken as equivalent to 100 lipase units and lipase activity is expressed as the number of units per gram wet weight of the organ.

Estimation of phospholipids

Phospholipids were estimated separately in the organs using the method of Zilversmith and Davis (1950) with a slight modification in which lipid extract is used instead of TCA extract.
The lipids were extracted separately from the organs of fingerlings using the method of Folch et al., (1957). About 2ml aliquot of the lipid extract of each organ was evaporated to dryness at 60-65°C. The residue in each case was digested with 1ml of perchloric acid and the tubes were placed in a mantle heater, set at 80°C, for a period of 2 to 3 hours until the contents become clear and colourless. To this, as well as to the standards* and also to the blanks**, 1ml of 4% ammonium molybdate solution was added and allowed to mix. This was followed by the addition of 1ml of 1Amino-2Napthol-4Sulfonic Acid (ANSA)*** reagent and allowed to mix thoroughly. After thorough mixing, the volume in each case was made upto 10ml with distilled water, and after exactly 10minutes the intensity of the colour was read at 600nm against blank. The concentration of lipid phosphorous in the unknown was read from the standard curve and from this the concentration of phospholipids was calculated by multiplying the former with a common factor 25. The phospholipid content is expressed as mg units/gm wet wt of the organ.

* 10µg-100µgs of phosphorous + 1ml of perchloric acid. 1ml of 0.439% of potassium dihydrogen phosphate corresponds to 1mg of phosphorous.

** 1ml of perchloric acid.

*** ANSA reagent was prepared by mixing 30gms of sodium sulphite, 6gms of sodium bisulphate and 0.5gm of ANSA and made up the volume to 250ml with distilled water. The mixture was filtered after two or three hours and stored in a refrigerator in a dark coloured bottle. Just before use, 30ml of the reagent was diluted to 75ml with distilled water.
Estimation of cholesterol

Total cholesterol content was estimated separately in the organs using the method of Liebermann-Buchard reaction as described by Natelson (1971). Each organ was accurately weighed to the nearest milligram in a Sartorius electrical semi-microbalance. A 1% homogenate of each organ (W/V) was prepared in 4ml of 1N sulphuric acid. To this added exactly 4ml of chloroform from a burette. The tubes were then stoppered tightly and shaken vigorously but intermittently for 20 minutes. Following this, they were centrifuged at 3000rpm for 5 minutes, and the supernatant acid and the protein bottom at the interface were removed. A 2ml aliquot of chloroform was then taken from the remaining chloroform and 1ml of acetic anhydride mixture (100ml of acetic anhydride and 6.5ml of concentrated sulphuric acid were mixed just before use) was added to it. Colour was allowed to develop in this mixture in dark in a water bath set at 25°C to 26°C. After exactly 10 minutes, the colour thus developed was read in a spectrophotometer at 625nm wavelength against a reagent blank, prepared by mixing 1ml of acetic anhydride mixture and 2ml of chloroform. Cholesterol standards (10µg-500µg) were prepared in chloroform and the cholesterol content in the organs is expressed as mg units/gm wet wt of the organ.

Histology

The histological sections of the gills, brain, liver and muscle of fish were taken by adopting the procedure as described by Humason (1972). The tissues were isolated from control and lead and chromium treated animals and were gently rinsed with physiological saline solution (0.9% NaCl) to remove mucus and other debris adhering to them. They were fixed in Bouin’s
fluid (75 ml saturated aqueous picric acid, 25 ml 40% formaldehyde and 5 ml 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. Ethylalcohol 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 alcohol. Then the tissues were cleared in methylbenzoate and embedded in paraffin wax. Sections of 5µ thicknesses were cut using "SIPCON" rotator microtome. The sections were stained with Harris haematoxylin (Harris, 1900) and counter stained with eosin, dissolved in 95% alcohol. After dehydration and cleaning, the sections were mounted in canadabalsam. Photomicrographs of the section preparations were taken using Olympus (PM-6 model) photomicrographing equipment.

**Statistical Analysis**

All the results obtained in this investigation were subjected to statistical analysis. For this, the standard deviations were calculated and 't' values were derived between the controls and experimental. The levels of significance were noted down form the standard 't' table and represented in the respected tables.