Materials and Methods
MATERIALS

Animal Selected:

Cyprinus carpio (Linnaeus) is an economically important edible fish having great commercial value, occurs abundantly in the freshwater tanks and ponds in and around Anantapur. Besides its wide availability and commercial value, 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 animal for the present investigation.

Since this investigation is ecobiochemical in nature, a brief account on the biology of the crap may constitute a suitable preamble. Cyprinus carpio belongs to the family cyprinidae and is commonly known as 'common carp'. 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 very variable species having many subspecies or strains; the strain used in this investigation is of Dankok strain. It is a non-predatory and has a nonelongated body with an abdomen conspicuously bigger in size over the rest of the body.
There are a pair of barbles at the sides of the mouth. The fins are yellowish to red but become bright red during breeding season. The fish is known for its fast growth and attains sexual maturity at the end of the first year. The maximum length to which it grows is about 76 cm (approximately 6.8 Kg by weight). However, as the very small fish are more sensitive to heavy metals than the large ones and there are very few studies on this size group (Anderson and Weber, 1975; Chapman, 1978), the fingerlings of the fish having the size of about 25 mm in length (approximately 300 ± 50 mg by weight) are selected for the present investigation.

Metallic Salts Selected:

Aluminium and copper are the two metals selected to study their effects on fish fingerlings. They are available in different salt forms like chlorides, sulphates, nitrates, acetates etc., and these salts are more or less soluble in natural waters. However, the degree of solubility of each salt varies depending on the hardness of water. The water available in the laboratory, where the present investigation was carried out, has a total hardness of 100 ± 5 mg/l as CaCO₃. In this water aluminium chloride and copper nitrate are
relatively more soluble than the other salt forms. Hence, a pure salt of aluminium chloride having the molecular formula $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ and molecular weight 241.45 and a pure salt of copper nitrate having the molecular formula $\text{Cu(NO}_3\text{)}_2 \cdot 3\text{H}_2\text{O}$ and molecular weight 241.60 are used in this investigation. 8.95 grams of aluminium chloride contains one gram of aluminium and 3.80 grams of copper nitrate contains one gram of copper.

**Maintenance of Fish:**

The fish fingerlings weighing 300 ± 50 mg were collected from local fish farms and were brought to the laboratory. They were maintained in large glass aquaria at room temperature (28 ± 1°C) and exposed to natural photoperiod. The water in the aquaria was renewed once a day to provide freshwater rich in oxygen. The fish were fed daily with groundnut cake milled with rice bran and were adapted to laboratory conditions for ten days before being subjected to experimentation.

Since the effects of heavy metals on organisms are known to modify by various extrinsic and intrinsic factors like temperature, salinity, pH, hardness of water, size and sex of the organism etc., (Anderson and Weber, 1975; Sullivan, 1977; Lehnberg and Theede, 1979;
Chakoumakos et al., 1979; Klockner, 1979; Arillo et al., 1982; Lakshman and Nambison, 1983; Ravera, 1984), precautions were taken throughout this investigation to control all these factors as far as possible. To this end, water from the same source has been used for the maintenance of the fish and for experimentation. The water used in this investigation has pH range of $7.6 \pm 0.2$ and total hardness $100 \pm 5$ mg/l CaCO$_3$. The temperature of the water was maintained strictly at $28 \pm 1^\circ$C. Chlorinity and dissolved oxygen content were checked frequently during the period of investigation, and were found to be within the range $0.08 \pm 0.003\%$ and $5.79 \pm 0.4$ mg/l respectively. Similarly, the feeding schedules have been maintained throughout the investigation, and the animals were starved for 24 hours prior to each estimation so as to eliminate the possibility, if any, of differential feeding influencing estimations. Also the range of variation in size is minimized by selecting the fingerlings within the weight range of $300 \pm 50$ mg. Precautionary measures have also been taken in handling the animals with utmost care to avoid any fatality and laboratory diuresis. During experimentation, water in aquaria containing normal and metal-exposed animals was
aerated periodically to prevent anoxia or hypoxia (Khorram and Knight, 1977).

METHODS
Evaluation of Aluminium and Copper Toxicity:
The experimental fingerlings were divided into groups of thirty each, of which seven groups were exposed to the media containing seven different concentrations of aluminium ranging from 38 to 50 mg/l and another seven groups to seven different concentrations of copper ranging from 0.08 to 0.2 mg/l. Two groups of fingerlings were also been maintained alongside in normal water containing 0.1 ml of hydrochloric acid per litre in one group and 0.1 ml of nitric acid per litre in another. All the animals were maintained under the static system of water flow. The seven different concentrations of aluminium and copper were determined on trial and error basis. On exposure to different metal concentrations, the mortality of the fingerlings in each concentration was noted immediately after 96 hours. The experiment was repeated thrice for concurrent results. The mean mortality values were converted into per cent mortality values and from these the probit mortality values were derived (Finney, 1971). LC$_{50}$s of aluminium and copper were determined from the
per cent mortality verses concentration and probit mortality verses concentration curves. For subsequent verification of the LC₅₀'s obtained by graphical methods, the method of Dragesstedt and Behren's as given by Carpenter (1975) was employed. As per this method the fingerlings were exposed to log₂ concentrations of aluminium (32 and 64 mg/l) and copper (0.1, 0.2 and 0.4 mg/l) for 96 hours and the per cent mortality was calculated from the cumulative mortality. The formula employed for calculating the LC₅₀'s is as follows:

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

Where, 'A' is the concentration of the metal which has a per cent mortality immediately below 50 per cent; 'a' is the per cent mortality observed immediately above 50 per cent. Finally, the mean LC₅₀'s were derived by taking the LC₅₀'s obtained through the per cent and probit mortality curves as well as through Dragesstedt and Behren's method.

**Fixation of Lethal and Sublethal Concentrations:**

Keeping in view that the effect of a metal on fish may become consistent with in 96 hours of exposure, LC₅₀/96 h of aluminium and copper were taken as lethal concentrations to study a few biochemical responses of
the fingerlings to these metals. However, the knowledge on a toxicant at lethal concentration could be insufficient to assess various responses of the animal to the toxicant (Sprague, 1971; Olla, 1974; Nobbs and Pearu, 1976; Hoppenheit, 1977). Further, the studies on acute toxicity have serious limitations, like the possibility of ignoring the occurrence of adaptation of the test animal to the imposed toxic stress (Stockner and Antia, 1976). Perkins (1979) also viewed the need for sublethal toxic studies which prove to be of most practical because distinct changes involving sequence of events in the responses of the test animal could occur in sublethal concentrations. So, about one fifth of the LC$_{50}$/96 h of aluminium (9.0 mg/l) and copper (0.03 mg/l) were taken as sublethal concentrations to carry out the further studies included in this investigation.

**Fixation of Exposure Periods:**

As the period of exposure is having great influence on the toxic effects of any metal on an organism (Radhakrishnaiah and Busappa, 1986), the lethal and sublethal effects of aluminium and copper on fingerlings were studied at different periods of exposure. Thus, in lethal, 1, 2 and 3 days, and in sublethal, 1, 7 and 15 days were chosen as exposure periods to
observe the short-term and long-term effects in a sequential way at the respective concentrations.

**General Experimental Procedure for Further Studies:**

Further studies were carried out on groups of fingerlings, 6 to 10 in each group, on exposure to lethal and sublethal concentrations of aluminium and copper for 1, 2 and 3 days and 1, 7 and 15 days, as the case may be. A mean of six samples were taken into consideration for each experiment. Similar studies made in normal fingerlings served as controls. Prior to each estimation, the fingerlings were weighed separately to the nearest milligram in an electrical semi-microbalance and transferred into separate micro-beakers 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 fingerlings to enable them to regain normalcy from a state of shock, if any, due to handling. The entire process was carried out in a sterilized cold room with temperature maintained at 15 ± 1°C.

**Effects of Aluminium and Copper on Carbohydrates:**

Glucose and glycogen levels and the activity of glycogen phosphorylase (with AMP) were estimated in the whole animals under this study.
Estimation of Glucose:

Glucose levels in the fingerlings were estimated using the colorimetric method as described by Nelson and Somogyi (1952). 5% homogenates of fingerlings were prepared in pure distilled water. To 0.1 ml of each tissue extract, 3.9 ml of deproteinizing solution (5% zinc sulphate and 0.3 N sodium hydroxide solution in 1:1 ratio) was added. The mixture was centrifused at 300 rpm for 10 minutes. To 1 ml of the supernatant, 1 ml of alkaline copper reagent was added, the mixture was shaken vigorously and boiled in a boiling water bath exactly for 20 minutes. Then it was cooled and added 1 ml of arsennomolybdate colour reagent. The entire solution was made to 10 ml with distilled water and the optical density of the colour developed was measure in a spectrophotometer at a wave length of 540 nm. A blank and glucose standards were also run simultaneously. Glucose in the fish is expressed as mg/g wet wt.

Estimation of Glycogen:

Glycogen levels in the fingerlings were estimated using anthrone reagent method as described by Caroll et al., (1956). The fingerlings were digested with 3 ml of hot 30% potassium hydroxide (Hassid and Abraham,
1957). 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 2,500 rpm. Decanted 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.

**Estimation of Glycogen Phosphorylase Activity (1-4-glucan orthophosphate glucosyl transferase, EC:2.4.1.1) with AMP:**

Glycogen phosphorylase activity (with AMP) in the fingerlings was estimated using the method described by Cori et al., (1955). A 5% homogenate (W/V) was prepared in 0.1 M sodium fluoride and 0.037 M ethyl diaminetetrachloride solutions adjusted to 6.4 pH as recommended by Guillory and Mommaerts (1962). The homogenate centrifuged at 1,500 rpm for 15 minutes,
thus extracting the enzyme into the supernatant. The supernatant was diluted four times with 0.03 M cysteine and 0.015 M β-glycerophosphate buffer (pH 6.5). The phosphorylase activity in it was estimated with the cofactor AMP. The incubation mixture consisted of 0.2 ml of 2% glycogen and 0.4 ml of diluted enzyme. It was incubated at 37°C 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 30 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) at a wavelength of 660 nm and the activity is expressed as μM Pi liberated/mg protein/h.

Effects of Aluminium and Copper on Proteins:

Soluble, structural and total proteins, levels of free amino acids and the activity of protease were estimated in whole animals under this study.

Estimation of Soluble, Structural and Total Proteins:

The soluble, structural and total protein contents in the fingerlings were estimated using the Folin phenol reagent method as described by Lowry et al., (1951). A 1% homogenate (W/V) was prepared in ice cold 0.25 M sucrose solution. For soluble and
structural protein contents, 1 ml of homogenate was taken and centrifuged at 3,000 rpm for 10 minutes. The supernatant was separated and to both the supernatant (soluble proteins) and residue (structural proteins) 3 ml of 10% trichloroacetic acid was added and again centrifuged at 3,000 rpm for 10 minutes. The supernatants were discarded and the residues were taken for experimentation. For total protein content, 1 ml of homogenate was taken, to it 3 ml of 10% trichloroacetic acid was added and centrifuged at 3,000 rpm for 10 minutes. Discarded the supernatant and the residue was taken for experimentation. All the residues were dissolved in 5 ml of 0.1 N sodium hydroxide. 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 to them. Finally, the optical density of the colour developed was read in a spectrophotometer at a wavelength of 600 nm. A mixture of 4 ml of reagent-D and 0.4 ml of foline 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.

**Estimation of Free Amino Acids:**

Free amino acid levels in the fingerlings were
estimated by the ninhydrin method as described by Moore and Stein (1954). A 5% homogenates of fingerlings (W/V) was prepared in 10% trichloroacetic acid and centrifuged at 1000 x g for 15 minutes. To 0.2 ml of supernatant, 2.0 ml of 2% ninhydrin reagent, dissolved in 2-methoxy ethanol, was added and the contents were boiled for exactly 6 minutes. The contents 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 575 nm against reagent blank. Amino acid standards were also run similarly. The free amino acid levels are expressed as mg amino acid nitrogen/g wet wt.

Estimation of Protease Activity:

Protease activity in the fingerlings was estimated using the method as described by Davis and Smith (1955) with some modification. A 1% homogenate (W/V) was prepared in ice cold distilled water. The homogenate was centrifuged at 1000 x g for 15 minutes and the supernatant was separated and used for enzyme assay. The reaction mixture in a volume of 3.0 ml contained 1 ml of enzyme source and 2 ml of 1% buffered casein. The reaction mixture was incubated at 37°C for 30 min-
utes. The reaction was stopped by the addition of 2.0 ml of 10% trichloroacetic acid. Similarly, controls were prepared by treating with 2.0 ml of 10% trichloroacetic acid prior to the addition of enzyme. The contents of both samples and controls were filtered through whatman No.1 filter paper and aliquots were taken for estimation of amino acids by the method of Moore and Stein (1954). To the 0.5 ml of above filtrate, 2.0 ml of ninhydrin reagent was added and heated in a boiling water bath for exactly 6 minutes, and the contents were cooled under the tap water. The final volume was made to 10 ml with distilled water and the colour developed was measured at 575 nm against a reagent blank in a spectrophotometer. Amino acid standards were prepared alongside for comparison. The protease activity is expressed as μM amino acids released/mg protein/h.

Effects of Aluminium and Copper on Lipids:

Total lipids, levels of free fatty acids and the activity of lipase were estimated in whole animals under this study.

Estimation of Total Lipids:

Total lipid content in the fingerlings was estimated using the method of Folch et al., (1957). The
animals were homoginated in a 2:1 chloroform and methanol mixture, using 20 ml of the mixture per gram wet weight of the animal. The homogenate was centrifuged at 2,500 rpm for 5 minutes and the supernatant was collected into a corning centrifuge tube, the weight of which has previously been determined accurately in an electrical semimicrobalance. To each centrifuge tube, normal saline (9 g NaCl in one litre of distilled water) was added at the rate of 2.0 ml normal saline to every 1 ml of the homogenate. The contents were shaken well and again centrifuged at 2,500 rpm 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 an electrical semimicrobalance. The difference between the initial and final weights of centrifuge tube represents the amount of total lipids present in the sample. The total lipids are expressed as mg units/g wet wt.

Estimation of Free Fatty Acids:

Free fatty acid levels in the fingerlings were
estimated using the method as described by Natelson (1971). The lipids were extracted separately from each fish, using the method of Folch et al., (1957). 2 ml of chloroform-lipid extract (obtained from the above cited method) was evaporated to dryness slowly at 60-65°C. After that, the residue in each case was dissolved in 2 ml of 95% ethanol. A drop of 0.1% phenolphthalein (in alcohol) was added to these as an indicator. The contents were titrated against N/50 KOH solution in water until pink colour was developed. A microburette of 1 ml capacity was used for the titration and 2 ml ethanol was used as blank. The titration value of the blank was subtracted from the titration value of the sample. 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 total fatty acids is 277, and the same is expressed as mg units/g wet wt.

Estimation of Lipase Activity:

Lipase activity in the fingerlings was estimated using the procedure described by Colowick and Caplan (1965). 1% homogenate (W/V) was prepared for each fish in 5 ml of ice-cold distilled water. The homo-
genate was centrifuged at 2,500 rpm for 5 minutes. The supernatant was taken into a flask and this is the enzyme source. The substrate was prepared by mixing 100 ml of buffer (0.2 M sodium acetate) with 50 ml of tween-20, 10 ml of indicator (0.02% aqueous phenol red) and 90 ml of distilled water. The pH of this mixture was adjusted to 7.2. 10 ml of this substrate was added to the enzyme source and then the flask was stoppered and placed in a coldwater bath at 20°C. After 10 minutes, a drop of decyclic alcohol was added to the enzyme-substrate mixture, so as to prevent foaming and the solution was titrated against 0.02 N sodium hydroxide till the reaching of end point (rosy-red colour). The contents of the flask were thoroughly mixed during titration. Blanks were prepared by boiling the supernatant and those blanks were also titrated as the regular samples. 1 ml of 0.02 N sodium hydroxide to titrate the liberated acids in the assay was taken as equivalent to 100 lipase units and the lipase activity is expressed as units/g wet wt.

Effects of Aluminium and Copper on Acetylcholine (Ach) and Acetylcholinesterase (AchE) activity:

Estimation of Acetylcholine:
Acetylcholine content in the fingerlings was estimated using the procedure described by Augustinsson (1957). The animals, after their sacrifice, were kept in hot water bath for 5 minutes to inactivate AchE activity and to release bound Ach. The animals were allowed to cool at room temperature and were homogenised in 2.0 ml of double distilled water. 2.0 ml of alkaline hydroxylamine hydrochloride (1 M hydroxylamine hydrochloride and 3.5 M sodium hydroxide in 1 : 1 ratio) was added to the homogenate. The mixture was centrifuged and 0.5 ml of ferric chloride solution was added to 2.5 ml of clear supernatant. The amount of Ach was measured in a spectrophotometer at 540 nm. A blank and Ach standards were also run similarly. Ach content is expressed as mg Ach/g wet wt.

Estimation of Acetylcholinesterase activity (Acetylcholine hydrolase: EC.3.1.1.7):

AchE activity in the fingerlings was estimated using the method of Metcalf (Glick, 1957). A 5% homogenate (W/V) of the fingerlings was prepared in 0.25 M ice-cold sucrose solution. 1 ml of buffer acetylcholine chloride solution (9 volumes of M/15 phosphate buffer pH 7.2 and 1 volume of 0.04 M acetylcholine chloride solution) was added to 0.1 ml of homogenate.
The mixture was incubated at 37°C for 30 minutes and then 2 ml of alkaline hydroxylamine solution (1 M hydroxylamine hydrochloride and 3.5 M sodium hydroxide solution in 1 : 1 ratio) was added to it. The mixture was vigorously shaken for one minute and to it 1 ml of diluted hydrochloric acid (concentrated hydrochloric acid and distilled water in 1 : 1 ratio) and 1 ml of 0.37 M ferric chloride solution were added and the optical density of the colour developed was measured in a spectrophotometer at a wave length of 540 nm. A blank taking 0.1 ml of distilled water and a control taking 0.1 ml of boiled enzyme were also prepared alongside for comparison. The amount of reacted acetylcholine was determined by subtracting the amount of unreacted acetylcholine by the help of standards. The enzyme activity is expressed as mg Ach hydrolysed/mg protein/h.

**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 from the standard 't' table and represented in the respective tables.