MATERIALS AND METHODS
Pesticides selected for study:

Commercial grades of endosulfan and chloropyrifos were selected as representatives of the two groups of pesticides organochlorides and organophosphates respectively for the comparative study of the effects of these pesticides on the fish. These pesticides were obtained from the local market. Pesticides from the same company were used throughout this investigation to avoid any possibility of variations in the concentration. The following are the details of the pesticide selected.

1. **Endosulphan**:

   - **Brand name**: Thiodan 35% (W/V)
   - **Company**: Rallis India Company, Bombay
   - **Physical state**: Dark brown liquid
   - **Chemical name**: 6,7,8,9,10- hexachloro- 1,5,5a,6a,9a hexahydro-6,9-methano-2,4,3-benzo (e)-dioxathiepin-3-oxide.
   - **Emperical formula**: \( \text{C}_3\text{H}_6\text{Cl}_6\text{O}_3\text{S} \)
   - **Structural formula**: ![Structural formula diagram]
Molecular weight : 406.95

Melting point: 70-100 C

Solubility:

Soluble in most organic solvents. In this investigation stock solutions were prepared in acetone for experimental purpose. The acetone used in the preparation of stock solution is biologically safe (Jagannatha Rao, 1981).

Active ingredient:

2.587 grams of pesticide contains 1 gram of active ingredient

Application:

It is a broad-spectrum insecticide and acaricide. It is highly efficient in controlling pests on all plantation crops. It is also widely used in the control of pests of agricultural crops, including paddy.

2. Chlorpyrifos:

Brand name : Dursban 20% (W/V)

Company : Motilal Company, Bombay

Physical state : Light yellow liquid

Chemical name : 0,0 - diethyl 0-3,5,6-trichloro-2-pyridyl phosphorothioate

Empirical formula : C₉H₁₁Cl₃No₃PS
Structural formula:

\[
\begin{align*}
\text{Cl} & \quad \text{Cl} \\
\text{S} & \quad \text{Cl} \\
\text{II} & \quad \text{P} \\
\text{C}_2\text{H}_5 & \quad \text{O} \\
\text{C}_2\text{H}_5 & \quad \text{O} \\
\text{Cl} & \quad \text{Cl}
\end{align*}
\]

Molecular weight : 350.5
Melting point: 41.5-43.5°C

**Solubility:**

Insoluble in water and soluble inorganic solvents. In this investigation stock solutions were prepared in acetone for experimental purpose.

**Active ingredient:**

5 grams of pesticide contains 1 gram of active ingredient.

**Application:**

It is well used against the control of pest of agricultural crops. Also it is used as insecticide particularly in mosquito control and in the control of household pests.

II  **Test species: Channa punctatus (Bloch)**

These are air-breathing fish which are also called as murrels. On the roof of the pharynx, they have a pair of cavities which have folded linings, richly supplied
PLATE

Chenna punctatus (Bloch.)
with blood vessels for taking in air. That enable the fish to survive out of water even for few hours, or to migrate from pool to pool. Therefore they are called as live fishes or Jiol-machi. In general three species of *Channa* are available in Andhra Pradesh.

1. *Channa marulius* or *Ophiocephalus marulius*: in Telugu it is called as poola matta
2. *Channa punctatus* or *Ophiocephalus punctatus*: in Telugu it is called variously as burada matta guidisa, garigallu, mittalu etc.
3. *Channa striatus* or *Ophiocephalus striatus*: in Telugu it is called as koramenu

Of these *Channa punctatus* (*Ophiocephalus punctatus*) is freshwater eurythermal teleost belonging to the order Channiformes. These fishes have long-body, cylindrical in front, slightly compressed from side to side towards the hind end. They are found throughout India, especially abundant in the southern districts of Andhra Pradesh. It is widely available in the tanks and paddy fields in and around Nellore district of Andhra Pradesh. *C. punctatus* is also called snake heads, as they have reptilian looking head with a jutting lower jaw, and a wide gap in the mouth which is armed with numerous teeth. The dorsal fin is soft rayed and runs from just behind the head, almost to the tail end. There is a similar long ventral fin. The fish also possess with a pair of pectoral fins, pelvic fins and a single caudal fin. The body is mottled with block markings in the form of irregular bands.
This fish lives in stagnant waters and comes to the surface at intervals to gulp in air, thereby exhibiting air breathing nature. Inside the gill chambers on each side are tiny pouches, which are well supplied with a network of fine blood vessels that take up oxygen. Snake heads can move on land by wriggling their bodies and by making rowing movements with their short broad pectoral fins. When the ponds in which they live dry up, they bury themselves in the mud to a depth of 1-2 feet.

_C. punctatus_ has been chosen as an experimental animal because it forms an important protein source of diet, and has a great commercial value. In rural villages it is given to pregnant women, since it is said to have medicinal value. Besides, the fish is abundantly available in almost all the seasons in and around Nellore district of Andhra Pradesh. Owing to its economic, commercial and medicinal importance the fish is selected for the present study.

II Procurement and Maintenance of fish:

_Channa punctatus_ were collected from the unpolluted ponds in and around Rapur, Nellore District, Andhra Pradesh, and were immediately transported in big 20 l fish containers to the laboratory, each with 50 fish. Then they were released in large cement tanks with sufficient dechlorinated tap water. The fish were fed with commercial fish pellets _ad libitum_ having around 50% protein content daily and 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 20g ± 2gm and were
maintained in static water without any flow (Doudoroff et al., 1951). Water was renewed every day to provide freshwater rich in oxygen. During experimentation water was aerated once a day to prevent hypoxic conditions, if any (Khorram and Knight, 1977). 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 maintenance of the fish. The water has pH 7.5 ± 0.1 and total hardness 100 ± 5 mg/l CaCO₃. Temperature of water was maintained at 28° + 0.5°C. Chlorinity and dissolved oxygen were maintained within the range 0.08 ± 0.003% and 5.8 ± 0.4 mg/l respectively. The animals were starved for 24 hours prior to each estimation, to avoid any influence of differential feeding. The size of the animals selected also maintained strictly throughout the investigation.

III Methods

Evaluation of Toxicity

Percent mortality of the fish in different pesticide concentrations was determined immediately every after 24, 48, 72 and 96 hours of exposure. For this, the experimental animals were divided batches of thirty each and exposed to different concentrations of pesticides, each batch to one concentration, ranging
from 0.005 ppm to 0.04 ppm of chlorpyrifos and 0.0005 to 0.00225 ppm of endosulfan. These ranges were obtained on the trial and error basis. Mortality rate was observed in each concentration of pesticides immediately after 24, 48, 72 and 96 hours of exposure. A batch of animals maintained alongside in freshwater served as controls. The experiments were repeated twice for accuracy. The mortality rate at each concentration, derived from the mean of the three, was converted as percent mortality value, from this, the probit mortality value was obtained (Finney, 1971). As the evaluation of toxicity of pesticides to an aquatic organism is by the determination of its LC$_{50}$, the percent mortality values as well as probit mortality values were plotted separately against pesticide concentration, and LC$_{50}$s at 24, 48, 72 and 96 hours were derived from these two curves. For subsequent verification of the LC$_{50}$s obtained by graphical methods, Dragestedt and Behren’s method as given by Carpenter (1975) was employed. As per this method the animals were exposed to Log.2 concentrations of pesticides for the same exposure periods. The percent mortality values were calculated from the cumulative mortalities, with them LC$_{50}$s were derived by adopting the following formula:

\[
\log \text{LC}_{50} = \log A + \frac{a - b}{b - a} \times \log 2
\]
Where

\[ A = \text{Concentration of the pesticide which has a percent mortality immediately below 50\%} \]

\[ a = \text{Percent mortality observed immediately below 50\%} \]

\[ b = \text{Percent mortality observed immediately above 50\%} \]

Finally, the LC50s / 24, 48, 72 and 96 h of the pesticides, chloropyrifos and endosulfan, were obtained by taking the mean of LC50s derived from percent and probit mortality curves and Dragstedt and Behren's method as described earlier (Koppa et al., 1993).

**Fixation of Sublethal concentrations:**

The knowledge on the concentration of a toxicant which kills 50\% of the test animals in a fixed period of time, however, is insufficient to assess various responses of the animals to that toxicant (Nobbs and Pearu, 1976; Hoppenheit, 1977). Further, studies on acute toxicity could have serious limitations like the possibility of ignoring the occurrence of adaptation of the test animal to the imposed toxicity (Stockner and Antia 1976; Hoppenheit, 1977). Hence, Perkin (1979) viewed the need for the sublethal studies because distinct changes involving sequence of events in the response of the test animal could occur in the sublethal concentrations. There are good member of reports on the effects of lethal concentrations of pesticides on freshwater fishes, but in nature the concentrations
present in water bodies are mostly sublethal. So, keeping in view that the effects of pesticides on fishes become consistent within 48 hours of exposure (Finney, 1964), about one fifth of the LC50/48h concentration of chlorpyrifos and endosulfan 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 compound (Radhakrishnaiah and Busappa, 1986), the effect of sublethal concentrations of pesticides on *C. punctatus* was studied at different periods of exposure in order to understand the influence of time over toxicity. So from shorter duration of exposure to longer duration, 1, 15 and 30 days were selected to study the effects of the sublethal concentrations. The result obtained at these exposure periods may give an insight on some specific events of responses of the fish to chronic pesticide stress at short term and long term exposures.

General experimental procedure for further studies:

Further studies in this investigation were carried in the gills, kidney, muscle and liver at 1, 15 and 30 days of exposure to the sublethal concentrations of chlorpyrifos and endosulfan. 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 the normal animals served as controls. Prior to each measurement, the fishes were dissected and the required organs were
taken out using sterilized instruments. The organs were weighed separately to the nearest milligram by an electrical semi-micro-balance and transferred into ice jacketed micro-beakers containing the ringer solution. An equilibration time of 15 minutes was allowed to the organs to enable them to regain normally 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 $15 \pm 1^\circ C$.

**SOME ASPECTS OF ENERGETICS:**

Tissue oxygen consumption, levels of pyruvate and lactate and activities of succinate dehydrogenase (SDH) and lactate dehydrogenase (LDH) were measured/estimated under this study.

**Tissue oxygen consumption:**

The rate of oxygen consumption of the organs of the fish was measured by following their rate of oxygen uptake in a Gilson 5/6 oxygraph. An oxygen electrode was prepared by adding a drop of potassium chloride to the probe and covered tightly with a teflon membrane using an “O” ring. This electrode was inserted into the water jacketed cell and calibrated with air saturated water taking oxygen concentration at $25^\circ C$ as $0.256 \mu$ moles per 1.0 ml of air saturated water. Then the water was removed from the reaction vessel and 2.0 ml of 0.05 M phosphate buffer (pH 5.5) was added. The reaction vessel was placed on a magnetic stirrer and covered with dark cloth. The rate of oxygen depletion from
the reaction medium was followed, and the rate of oxygen consumption is expressed as \( \mu \) moles/g wet wt/5 mts.

**Estimation of Pyruvate:**

Pyruvate in the organs was estimated using the method of Friedmann and Hagen (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 0.001 M 2,4-dinitrophenyl hydrazine and 3 ml of 0.4 N sodium hydroxide was 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 alongside for comparison. The pyruvate content in the organs is expressed as mg pyruvate/g wet wt of the organ.

**Estimation of Lactate:**

Lactate in the organs was estimated using the method of Barker and Summerson (1941) as modified by Huckabee (1961). 5% homogenates (W/V) were prepared in cold 10% trichloroacetic acid and centrifuged at 3000 rpm for 15 minutes. The supernatant was used for the estimation of lactate. To 1.0 ml of supernatant 20% copper sulphate was added and the mixture was made to 10.0 ml with distilled water. Then 1.0 g 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. When 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 a boiling water bath for 90 seconds, cooled and the optical density of the colour developed was measured in spectrophotometer at a wavelength of 560 nm against reagent blank. Lactate standards were prepared alongside for comparison. The lactate content present in different organs is expressed as mg lactate/g wet wt.

**Estimation of Succinate dehydrogenase (Succinate:Acceptor oxido-reductase, EC : 1.3.99.1) activity (SDH):**

Succinate dehydrogenase activity in the organs was estimated using the colorimetric method of Nachlas et al., (1960). A 5% homogenate (W/V) was prepared in 0.25M 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 0.4M phosphate buffer (pH 7.7), 0.2 ml of 0.2M sodium succinate, 1.0 ml of 0.004M 2-(p-indophenol)-3-p-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 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 wave length of 495 nm. A blank taking 0.5-ml of distilled water and control-taking 0.5 ml of boiled enzyme was also run for comparison. The 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 Srikantan 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 1.0ml of 0.4M phosphate buffer (pH 7.4), 0.5 ml of 0.1M lithium lactate, 1.0 ml of 0.0001M nicotinamide adenine dinucleotide (NAD), 1.0 ml of 0.004M 2-(P-indophenol)-3-p-nitrophenyl-5-phenyltetrazolium chloride (INT) and 0.5 ml 5% enzyme preparation. The mixture was incubated at 37° C for 30 minutes and then adding 6.0 ml of glacial acetic acid stopped the reaction. 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 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 $\mu$M formozan/mg protein/h.

**SOME ASPECTS OF PROTEIN METABOLISM**

The levels of soluble, structural and total proteins, free amino acids, ammonia, urea and the activities of protease, alanine and aspartate aminotransferases, glutamate dehydrogenase, and arginase were estimated in the gills, kidney, muscle and liver of fish exposed to the sublethal concentrations of chlorophyrifos and endosulphan.

**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 0.25M ice-cold sucrose solution. For soluble and structural proteins, 1 ml of homogenate was taken and centrifuged at 3000rpm 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 3000rpm. 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 3000rpm. After discarding the supernatant, the residue was taken for experimentation. All the three residues were dissolved in 5 ml of 0.1N 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. 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 as a blank. Bovine albumin was used for the preparation of protein standards. The protein content is expressed as mg/g 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, 2.0 ml of 2% ninhydrin reagent was added and the contents were boiled for exactly 5 minutes. They were cooled under tap water and the volume was made to 10.0 ml with distilled water. The optical density of the colour developed was measured 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/g wet wt 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 2.0 ml of homogenate 0.5 ml of 1% casein and 2.0 ml of 0.1M phosphate buffer (pH 5.0) were added. The contents were mixed well and incubated at 30° C for 30 minutes. Adding 2 ml of 2% ninhydrin reagent stopped the reaction. 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 dilute (distilled water and n-propanol in 1:1 ratio). The optical density of the colour developed was measured in spectrophotometer at a wavelength of 570 nm. A blank taking 2.0 ml of distilled water and control-taking 2.0 ml of boiled enzyme were also run similarly. Amino acid standards were prepared alongside for comparison. The protease activity is expressed as 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) amminotransferase activities:

Activities of alanine and aspartate aminotransferases (AIAT and AAT) in the organs were estimated using the method of Reitman and Frankel (1957). A 5% homogenate (W/V) was prepared in 0.25M 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 0.2M alanine, 0.5 ml of 0.005M α-ketoglutaric and (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 0.2M aspartic acid, 0.5 ml of 0.005M 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 mixture 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-dinitrophenylhydrazine (Ketone reagent). Finally, the reaction mixtures were made to 10.0 ml with 0.4 N sodium hydroxide and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 545 nm. A blank taking 0.1 ml of distilled water and control taking 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 (L-glutamate: NAD oxalo-reductase EC 1.4.13) activity (GDH):

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
0.25M 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 2.0 ml contained 40 μM of sodium glutamate, 100 μM sodium phosphate buffer (pH 7.4), 0.1μM of NAD (nicotinamide adenine dinucleotide) and 4.0 μM of INT (2-p-indophenol-3-p-nitriophenyl-5-phenyltetrazolium chloride). The reaction was initiated by the addition of 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37°C for 30 minutes in a thermostatic water bath, and then the reaction was stopped by the addition of 5.0 ml of glacial acetic acid. The formozan formed was extracted into 5.0 ml of toluence 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 of 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity is expressed as μM formozan formed/mg protein/h.

Estimation of Arginase Activity (L-Arginase Ureohydrolase E.C.3.5.3.1):

Arginase activity was estimated following the method of Campbell (1961) with slight modification. Tissue homogenates were prepared in cold 0.1% acetyltrimethyl ammonium bromide. The homogenates were centrifuged at 3000rpm for 10 minutes. The supernatant was used for the enzyme assay. The reaction
mixture in a final volume of 2.0 ml contained 20 μ moles of L-arginine, 50 μ moles of sodium glycinate buffer (9.5 pH), 0.5 μ moles of MnCl₂ and 200 μ g of protein as the enzyme source. The reaction mixture was incubated at 37°C for 30 minutes. After incubation, the reaction was stopped by the addition of 4.0 ml of 0.5 M perchloric acid. The urea thus liberated was estimated by the method of Natelson (1971). The enzyme activity was expressed as μ moles of urea 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 1.0 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 2.0 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 μ M/g wet wt of the organ.

**Estimation of Urea:**

Urea was estimated in the organs by diacetylmonoxime method as described by Natelson (1971). 10% tissue homogenate (W/V) was prepared in
15% perchloric acid and centrifused at 2000 rpm for 15 minutes. To 1.5 ml of supernatant 1.0 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% diacetylmoxine 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 μ M/g 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, liver, kidney and muscle of fishes under this study.

Estimation of total lipids:

Total lipids were estimated separately in the organs using the method of Floch et al., (1957). Each excised organ was weighed accurately to the nearest milligram in a Sartorius electrical semi-micro balance. The weight of the organs, used for the estimations, usually range between 150 to 200 mg. Separate homogenates were prepared for each organ in 2:1 chloroform-methanol mixture, using 20 ml of the mixture per gram weight of the organ. The homogenates were centrifuged at 2500 rpm for 5 minutes and the supernatant was collected into a
coming centrifuge tube, the weight of which was previously determined accurately. To each centrifuge tube, normal saline (9 g NaCl in one litre of distilled water) was added at the rate of 0.2 ml to every 1 ml of the homogenate. The contents were shaken well and again centrifuged at 2500 rpm for about 10 minutes. Now the upper phase, which essentially consists of non-lipids, 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. 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/g 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 Floch et al., (1957) the details of which are given above. 2 ml 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 2 ml 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 1 ml capacity was used for the titration and 2 ml of ethanol was used as blank.

The titration value of the blank was subtracted from the titration value of the sample (unknown). By multiplying this value with 0.02, FFA content (in mill 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/g wet wt of the organ.

**Estimation of lipase activity:**

Lipase activity (glycerolester 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 5 ml of ice-cold distilled water. The homogenate was centrifuged at 2500 rpm for 5 minutes. The supernatant was taken into flask, which 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 cold-water 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 0.02N sodium hydroxide till reaching 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 using the method of Folch et al., (1957). About 2 ml aliquot of the lipid extract of each organ was evaporated to dryness at 60-65 °C. The residue in each case was digested with 1 ml 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**, 1 ml of 4% ammonium molybdate solution was added and allowed to mix. This was followed by the addition of 1 ml of 1 amino-2 naphthol-4 sulfonic acid (ANSA)** reagent and allowed to mix thoroughly. After thorough mixing, the volume in each case was made up to 10 ml with distilled water, and after exactly
10 minutes the intensity of the colour was read at 600 nm 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/g wet weight of the organ.

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

** 1 ml of perchloric acid.

*** This ANSA reagent was prepared by mixing 30 gms of sodium sulphite, 6 gms of sodium bisulphite and 0.5 gms of ANSA and made up the volume to 250 ml 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, 30 ml of the reagent was diluted to 75 ml 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 4 ml of 1N sulphuric acid. To this added exactly 4 ml 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 3000 rpm for 5 minutes, and the supernatant acid and the protein bottom at the interface were removed. A 2 ml
A aliquot of chloroform was then taken from the remaining chloroform and 1 ml of acetic anhydride mixture (100 ml of acetic anhydride and 6.5 ml 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 625 nm wavelength against a reagent blank, prepared by mixing 1 ml of acetic anhydride mixture and 2 ml of chloroform. Cholesterol standards (10 μg-500 μg) were prepared in chloroform and the cholesterol content in the organs is expressed as mg units/gm wet weight of the organ.

**Statistical Analysis:**

All the results obtained in this investigation were subjected to statistical analysis. For this, the data were fed to the computer and ‘t’ values were derived at 5% level. As it becomes bulky to give all these values in the tables, the significance between controls and experimental was calculated by using these values and are represented in the respective tables.