CHAPTER II

MATERIALS AND METHODS
I. EXPERIMENTAL ANIMAL

(i) Collection and Maintenance of *Heteropneustes fossilis*

Specimens of teleost fish *Heteropneustes fossilis* (Cat fish) locally known as Singhan were collected from local nursery. The average length and weight were 14.5±2cm and 15±2gm respectively. Fishes were acclimatized in the laboratory conditions in glass aquaria of size 36"x18"x18" for two weeks prior to experimentation. The fishes were fed with commercial fish food regularly. In another set of experiment, fishes were fed with the larvae of Silk Moth to increase the cholesterol level (Jhingran, 1988) for two months. The water of the aquaria was changed on every alternate day.

(ii) Systematic Position of Fish (Datta Munshi and Choudhary, 1996).

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Chordata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-Phylum</td>
<td>Vertebrata</td>
</tr>
<tr>
<td>Division</td>
<td>Gnathostomata</td>
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<tr>
<td>Superclass</td>
<td>Pisces</td>
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<td>Class</td>
<td>Teleostomi</td>
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<tr>
<td>Order</td>
<td>Cypriniforms</td>
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<td>Suborder</td>
<td>Siluroidis</td>
</tr>
<tr>
<td>Family</td>
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</tr>
<tr>
<td>Genus</td>
<td><em>Heteropneustes</em></td>
</tr>
<tr>
<td>Species</td>
<td><em>fossilis</em></td>
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</table>

(iii) Local Names of the Experimental Fish

<table>
<thead>
<tr>
<th>Language</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hindi</td>
<td>Singi</td>
</tr>
<tr>
<td>Marathi</td>
<td>Bitchuka Machi</td>
</tr>
<tr>
<td>Telgu</td>
<td>Ingilayee</td>
</tr>
<tr>
<td>Tamil</td>
<td>Thelimeen</td>
</tr>
<tr>
<td>Malayalam</td>
<td>Kahree-meen</td>
</tr>
<tr>
<td>Oriya</td>
<td>Singee</td>
</tr>
<tr>
<td>Punjab</td>
<td>Noorie, Nalakhi, Nulli</td>
</tr>
<tr>
<td>Assam</td>
<td>Singi, Shini</td>
</tr>
<tr>
<td>Madhya Pradesh</td>
<td>Singhi</td>
</tr>
</tbody>
</table>
Mysore - Chelu-meenu
Eastern U.P. - Singhi
Bengal - Singh

(iv) Distribution

In India this fish is widely available in fresh water and wetlands of Haryana, Uttar Pradesh, Bihar, West Bengal, Orissa and Madhya Pradesh. In south east asian countries, it is found in Pakistan, Nepal, Sri Lanka, Bangladesh and Thailand.

(v) Structure

Body Profile: Elongated, compressed with rounded abdomen.

Eyes: Small, lateral in the anterior part of the head not visible from ventral surface.

Head: Moderate sized, greatly depressed, covered with thin skin. Width of head is shorter than its length. Snout flat, mouth terminal, transverse and narrow, lips are fleshy and papillated. Jaws are subequal, upper being slightly longer.

Teeth: Teeth are villiform in broad bands on jaws end in two oval patches on palate. Vomerine teeth in a pyriform patch, on either side covering anteriorly, widely divergent posteriorly.

Barbels: 4 pairs, one pair of maxillary, nasal and two of mandibular. The maxillary extends beyond the pectoral fin, nearer to the pelvic fin.

Branchiostegal membrane: Membranes separated by a deep notch, not united with isthmus.

Respiratory sacs: Gill chambers with accessory respiratory air sacs extending backwards into caudal region.
Fins: Rayed, dorsal short and without any spine inserted above the tip of pectoral fin or in anterior third of the body. Adipose dorsal absent. Pectoral fins with a strong Spine Serrated along inner edge with a few serrations at its anterior end. It is 2/3 as long as head. Ventral fins reaches upto the third or fourth anal ray. Anal fin long and caudal fins are separated by a distinct notch. Caudal fin is almost rounded.

Lateral line: Present, complete and simple.

Air bladder: Greatly reduced, consisting of two thin walled pyriform sacs enclosed in incomplete bony capsules. Sacs united by a transverse tube which is connected with oesophagus through a slender tube.

Colour: Dark leaden brown.

Since the pectoral spine is capable of inflicting wound which is very painful and hence people afraid of catching it without breaking the pectoral spines. It is considered to be very nourishing and tasty fish (Qureshi and Qureshi, 1986).

(vi) Food and feeding habits

Das and Moitra (1963) have divided fresh water fishes into three groups according to the niche they occupy in the water. These are surface feeders, mid or column feeders and bottom feeders. Heteropneustes fossilis is a bottom feeder, omnivore fish. That is depend mainly on bottom organisms like insects, worms, copepods, ostracods, debris, and algae (Khanna, 1991).
Heteropneustes fossilis (Bloch.)
II. AMINO ACIDS

For experiments, amino acid reference collection (BDH) was used, containing the following amino acids:

1. DL-Alanine
2. DL-α-Amino butyric acid
3. L-Arginine Monohydro chloride
4. DL-Aspartic acid
5. L-Cysteine hydrochloride
6. L-Cystine
7. 3(3,4-Dihydroxy phenyl) DL-Alanine (DOPA)
8. L-Glutamic acid
9. Glycine
10. L-Histidine monohydrochloride
11. L-Hydroxy proline
12. L-Leucine
13. DL-Isoleucine
14. DL-Norleucine
15. L-Lysine monohydrochloride
16. DL-Methionine
17. DL-Ornithine monohydrochloride
18. DL-β-phenylalanine
19. L-Proline
20. DL-Serine
21. DL-Threonine
22. DL-Tryptophan
23. L-Tyrosine
24. DL-Valine

For each set of experiments, three different doses (dose I, II and III) of each of all amino acids were administered into the blood of fish through caudal vein. The average blood volume in the fish is 3% of the bodyweight (Khanna, 1991). In the present study, the average weight of the experimental fish is 15 gms, the blood volume thus calculated is 0.45ml. Three doses of each individual amino acid has been administered into the blood through caudal vein i.e. 40 μl by using
microsyringe. Prior to amino acid administration, fishes were kept on starvation for 48 hrs. The concentrations (dose I, II & III) of amino acids (m.mol/15gm. body weight) have been prepared (Table-A) so that even after their normal use by the cells, amino acids are still available in the target organs to influence oxygen uptake and cholesterol biosynthesis. After five to six hours of administration of amino acids, fishes were sacrificed, their liver, kidney and blood were removed and processed for the estimation of oxygen consumption and cholesterol contents.

Table-A : The doses of individual amino acids used in the study

<table>
<thead>
<tr>
<th>Name of the amino acids</th>
<th>Doses of amino acids (m.mol/15gm body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose I</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.0133</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.0079</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.0037</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.0301</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.0164</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.0020</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.0012</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.0027</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.0018</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.0006</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.0005</td>
</tr>
<tr>
<td>β-Phenylalanine</td>
<td>0.0009</td>
</tr>
<tr>
<td>Serine</td>
<td>0.0030</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.0023</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.00097</td>
</tr>
<tr>
<td>Valine</td>
<td>0.0029</td>
</tr>
</tbody>
</table>
The list of doses I, II and III of the individual amino acids have been prepared on the basis of literature survey (Coulson and Hernandez, 1983). Still there are amino acids which are present in the experimental tissues but their quantity have not been reported e.g. cystine, cysteine, tryptophan and aminobutyric acid. On the other hand, there are a few amino acids which have not been reported to be present in the experimental tissues viz. proline, hydroxyproline, DOPA and Norleucine. These amino acids are available in the BDH amino acid reference collection. The doses of these amino acids have been prepared on the basis of average value of all the amino acids administered into the fish since no basis of dose making have been observed during literature survey.

METHODS

[A] ESTIMATION OF OXYGEN CONSUMPTION

Warburg constant volume manometry technique has been applied for the estimation of oxygen uptake in fish liver, kidney and blood.

Reagents:

1. Kreb's ringer phosphate buffer solution:

This buffer solution has been used throughout the experiments to maintain isotonicity and pH. The composition of the solution as under -

NaCl (0.59%) - 100 parts,

KCl (1.15%) - 4 parts,
CaCl₂ (1.22%) - 3 parts,
MgSO₄.7H₂O (3.82%) - 1 part
Phosphate buffer (0.1M) - 20 parts
Phosphate buffer (pH 7.4) contains 17.8 gms Na₂HPO₄.2H₂O+20ml 1N HCl (diluted to 1 liter).

2. Brodie's fluid :

This fluid has been used in manometers with a specific gravity of 1.033, so that 10,000 mm are equivalent to 1 atmospheric pressure (760 mmHg). This fluid is prepared as follows (Hawk, 1976).

Dissolve 23 gms Sodium Chloride (BDH) and 5 gm Sodium Glycocholate (E. Merck) in 500 ml of distilled water. A few drops of alcoholic thymol solution was added as a preservative and 400 mg basic fuschin was added to colour the fluid.

3. Sodium chloride solution :

0.59 gm NaCl was dissolved in 100cc buffer solution (0.59%) used as isotonic solution for fish.

4. KOH solution

10 gm% aqueous KOH (BDH) solution has been prepared and used in the central cavity of the manometric flask for the absorption of CO₂, evolved during cellular metabolism.

Warburg Apparatus

The Warburg manometer is mainly a U tube. One end of the upper arm is open to the atmosphere. To the other end, a conical flask is attached through a three
WARBURG MANOMETER

2 way stopcock
Manometer
Respiratory nossel
side arm
Suspension of cells
Center wall (for KOH)

Brodie fluid
(with dye)
in manometer

Screw for regulating fluid in manometer

Stopper to fluid reservoir
way stop cock. By suitable manipulation of the stop cock, this arm can be left open to
the atmosphere or connected to the flask as a closed system.

In the lower curved portion of the U tube, there is an opening, to which a
rubber tube with glass stopper is connected. This rubber tube acts as an reservoir for
Brodie's fluid.

A pinch cock is placed in position on the reservoir rubber tube in such a way
that on applying pressure to the tube, the fluid level rises in the 'U' tube. The two
arms of the U tube are marked with millimeter (mm) scale. When both the arms are
left open to the atmosphere, the fluid level remains the same in both the arms of the
U tube and is then adjusted to 250 mm.

For estimation of oxygen uptake, the experimental tissue i.e. liver, kidney and
blood of the fish *Heteropneustes fossilis* has been taken in to the flask. By adjusting
the three way stop cock, the flask is connected to the side arm of the U tube. The
change in the fluid level in both the arms depend upon the reaction takes place in the
flask. If oxygen is released, the level goes down and if oxygen is absorbed, the level
rises in the closed arm indicating a change in the volume of air in the flask. By
adjusting the pinch cock on the reservoir, the fluid level in the closed arm is brought
to the original level i.e. 250 mm. As a result the pressure is also altered, which can
be ascertained by the difference in the fluid level of the two arms of the manometer U
tube (Jayaraman, 1985).

If gas is evolved during reaction, the volume of the flask and side arm of the
U tube also increase. Since, the volume is kept constant, pressure increases. In order
to balance the increased pressure in the closed arm, the fluid level in the open arm rises above the original level in such a way that -

Pressure due to released gas = Pressure exerted by the increased column of fluid

Procedure:

(1) Manometric measurement of cellular oxygen uptake:

By using the Warburg constant volume manometry technique, the oxygen consumption has been estimated (Umbreit et al., 1972). The manometry has been a useful tool for the measurement of tissue respiration in vitro since the time of Warburg. The early respirometers required accurate calibration, but more recent versions are designed to avoid the need for this.

This apparatus usually accommodate up to twenty vessels and hence enable a much greater number of experimental conditions to be explored. Manometric methods enable oxygen consumption to be measured for a large number of samples, at relatively constant pressure of O₂. Over extended incubation periods (30-120 min.) depending upon various incubation conditions. However, certain limitations in their use will be encountered. Because of the sensitive nature of manometry, the vessels must equilibrate completely with the surrounding bath before accurate reading can be taken. Any subsequent alteration of environmental conditions will inevitably lead to inaccurate readings. The requirement for equilibration demands that the cells be incubated for at least 10 minutes before the first reading is observed. The steps followed during the process are -
(a) Cleaning of Glasswares

All the glasswares were thoroughly cleaned with chromic acid. They were then washed twice with distilled water and dried. The greased parts were also cleaned with a cotton swab soaked in chromic acid.

(b) Flask Constant

For each flask and its particular manometer, flask constant was determined. Flask constant is the calibration of the system to calculate the amount of gas utilized, as oxygen in the present investigation (in microlitres (μl) at 0°C and 760 mm Hg pressure). For calibration, mercury was used. First of all, an empty flask was weighed and then filled with mercury. Small air bubbles trapped at the sides or bottom of the flask have been removed by a bent capillary tube. A reference mark, about one centimeter above the flask, on the side arm of manometer was made with a diamond pencil. The flask was then seated on the manometer joint in such a way that the mercury reached the reference mark on manometer ground joint. The flask with mercury was then weighed and the difference in weight using a standard empty flask was calculated. The volume of the Warburg’s flask, up to the reference mark, was calculated by dividing the weight of mercury by its density at particular temperature recorded earlier. Similarly, the volume of the manometer from the reference mark up to 250 mm was calculated. The flask’s volume and manometer’s volume up to 250 mm were added. This sum, minus the volume of the tissue homogenate (5ml) and volume of KOH (0.2ml) in the central cavity of the flask for absorption of CO₂ gives Vg to be utilized in calculating the Flask Constant.
\[
\text{Flask Constant (N)} = \frac{V_g \frac{273}{T} + V_f}{P_o}
\]

where -

\(V_g\) = Volume of gas in the flask and connecting tube upto 250 mm on the closed arm of manometer (250 mm is the best reference point for the study of oxygen uptake).

\(V_f\) = Volume of fluid in the vessel.

\(P_o\) = Standard pressure (760 mm of mercury or 10,000 mm of Brodie’s fluid).

An example of calculation of Flask Constant (N) used in the present study is as under:

\(V_f\) = 5 ml homogenate + 0.2 ml KOH (10%)

\[= 5.2 \text{ ml}\]

\[= 5200 \mu l\]

\(V_g\) = Weight (in gms) of mercury filled in the flask and in the closed arm of the manometer tube upto 250 mm / Density of mercury at 27°C minus \(V_f\)

\[
\text{Weight of mercury} \quad V_g = \frac{\text{Weight of mercury}}{\text{Density of mercury}} = 5.2
\]

\(P_o = 10,000\)

\[
T = 273 + 27 = 300
\]

\[= 0.027\]

\[
\text{Density of mercury at 27°C} = 13.5389
\]

\[
\frac{336.4368}{13.5389} = \frac{5.2}{13.5389} = 0.38496 - 5.2
\]

\[= 19.6496 \text{ ml}\]

\[= 19549 \mu l\]
\[
N = \frac{19649 \times 273/300 + 5200 \times 0.027}{10,000}
\]
\[
N = \frac{17880.59 + 140.4}{10,000}
\]
\[
N = \frac{18020.99}{10,000}
\]
\[
N = 1.8020 \quad \text{(Flask Constant)}
\]

(c) Manometry

The U tubes of manometers have been filled with Brodie’s fluid upto 250 mm as the reference point which has been found suitable to study the oxygen uptake. The stop cock of the closed arm kept open while adjusting the Brodie’s fluid and closed after adjustment to the reference point (250mm). For all experiments, temperature was uniformly maintained at 28°C by constantly stirring of water through electrical device and thermostate of the Warburg apparatus. The pH was maintained at 7.4. The equilibrium time was 10 minutes for all experiments.

Initially the experiment was performed for 120 minutes. The most appropriate method for the estimation of oxygen uptake was found to be an average of the volume of oxygen consumed in 10, 20, 30, 40, 50 and 60 minutes. Readings were taken after every 10 minutes and calculated respectively for each 10 minutes interval. Control manometers were also kept simultaneously for comparison with experimental values. Oxygen consumption (QO₂) has been expressed throughout as μl (microliters) of Oxygen taken up per mg dry weight of tissue per hour.
(D) Thermobarometer (TMB)

The variation in the atmospheric pressure and temperature of water was corrected with the help of a Thermobarometer. Thermobarometer similar to the other manometers used with only one exception i.e. the flask contains only Kreb's ringer phosphate buffer (5ml) and KOH (0.2ml) in the reaction flask. The rise in the reading of the Thermobarometer is subtracted from the reading of reaction manometer, while the fall in the Thermobarometer is added to the reading of reaction or experimental manometer.

(E) Experimental procedure

Immediately after the dissection of both control and experimental fishes, liver, kidney and blood were removed. The liver and kidney homogenized in 40 ml of Kreb's ringer phosphate buffer solution and centrifuged for 2 minutes at 2000 rpm. 5 ml of the homogenate was kept in each manometer flask (4 control and 4 experimental flask) for the estimation of oxygen uptake.

(F) Observations

Observations of the experiments (Qo2) have been tabulated as follows -
Table Showing actual readings on the Warburg-manometer’s U tube for fish liver under the influence of amino acid Glutamic acid.

Reference point on manometer’s U tube 250 mm.
Temperature 28°C
pH 7.4

<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>Thermobarometer (TMB)</th>
<th>Control (i)</th>
<th>Control (ii)</th>
<th>Experimental (iii)</th>
<th>Experimental (iv)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Readings</td>
<td>Change</td>
<td>Reading</td>
<td>Change</td>
<td>TMB correction</td>
</tr>
<tr>
<td>10</td>
<td>251</td>
<td>+1</td>
<td>255</td>
<td>+5</td>
<td>-1</td>
</tr>
<tr>
<td>20</td>
<td>252</td>
<td>+2</td>
<td>256</td>
<td>+6</td>
<td>-2</td>
</tr>
<tr>
<td>30</td>
<td>254</td>
<td>+4</td>
<td>257</td>
<td>+7</td>
<td>-4</td>
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<tr>
<td>40</td>
<td>255</td>
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<tr>
<td>60</td>
<td>258</td>
<td>+8</td>
<td>259</td>
<td>+9</td>
<td>-8</td>
</tr>
</tbody>
</table>
### A. Control manometer

**Flask Constant 1.5766**

<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>TMB</th>
<th>Correction</th>
<th>Reaction flask</th>
<th>Correction</th>
<th>Net change</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>251</td>
<td>+1</td>
<td>255</td>
<td>+5</td>
<td>+4</td>
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<td>20</td>
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<td>60</td>
<td>258</td>
<td>+8</td>
<td>259</td>
<td>+9</td>
<td>+1</td>
</tr>
</tbody>
</table>

**Dry wt. of tissue 13.04mg**

### B. Experimental manometer

**Flask Constant 1.5695**

<table>
<thead>
<tr>
<th>Time in minutes</th>
<th>TMB</th>
<th>Correction</th>
<th>Reaction flask</th>
<th>Correction</th>
<th>Net change</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>251</td>
<td>+1</td>
<td>256</td>
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<td>258</td>
<td>+8</td>
<td>260</td>
<td>+10</td>
<td>+2</td>
</tr>
</tbody>
</table>

**Dry wt. of tissue 16.96mg**

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Model of calculations - Calculation for estimation of oxygen consumption applied is as follows:

- **Volume of Kreb’s ringer phosphate buffer solution = 40ml**
- **Volume of tissue homogenate in the reaction flask = 5ml**
- **Dry wt. of liver (A) = 13.04 mg**
- **Dry wt. of liver (B) = 16.96 mg**
Calculation for estimation of Oxygen Consumption in manometer A (control):

Net change in the open arm of manometer x Flask Constant
\[ Q_{O_2} = \frac{\text{Net change}}{\text{Dry weight of tissue liver}} \times 6 \]

4 x 1.5766 x 6
\[ Q_{O_2} \text{ for first 10 minutes} = \frac{2.9017}{13.04} \mu l \]

4 x 1.5766 x 6
\[ Q_{O_2} \text{ for next 10 minutes} = \frac{2.9017}{13.04} \mu l \]

3 x 1.5766 x 6
\[ Q_{O_2} \text{ for next 10 minutes} = \frac{2.1762}{13.04} \mu l \]

2 x 1.5766 x 6
\[ Q_{O_2} \text{ for next 10 minutes} = \frac{1.4508}{13.04} \mu l \]

2 x 1.5766 x 6
\[ Q_{O_2} \text{ for next 10 minutes} = \frac{1.4508}{13.04} \mu l \]

1 x 1.5766 x 6
\[ Q_{O_2} \text{ for next 10 minutes} = \frac{0.7254}{13.04} \mu l \]

\[ \frac{2.9017 + 2.9017 + 2.1762 + 1.4508 + 1.4508 + 0.7254}{6} = 11.6066 \]
\[ \frac{\text{Average of 60 minutes}}{6} = 11.6066 \]
\[ \frac{\text{Average QO}_2 \text{ for 10 minutes}}{6} = 1.9344 \mu l \]
Calculation for estimation of oxygen consumption in manometer B (control):

\[
5 \times 1.5695 \times 6
\]

Qo₂ for first 10 minutes = \[\frac{2.7762 \mu l}{16.96}\]

\[
5 \times 1.5695 \times 6
\]

Qo₂ for next 10 minutes = \[\frac{2.7762 \mu l}{16.96}\]

\[
4 \times 1.5695 \times 6
\]

Qo₂ for next 10 minutes = \[\frac{2.2209 \mu l}{16.96}\]

\[
3 \times 1.5695 \times 6
\]

Qo₂ for next 10 minutes = \[\frac{1.6657 \mu l}{16.96}\]

\[
3 \times 1.5695 \times 6
\]

Qo₂ for next 10 minutes = \[\frac{1.6657 \mu l}{16.96}\]

\[
2 \times 1.5695 \times 6
\]

Qo₂ for next 10 minutes = \[\frac{1.1104 \mu l}{16.96}\]

\[
\frac{2.7762 + 2.7762 + 2.2209 + 1.6657 + 1.6657 + 1.1104}{6}
\]

Average Qo₂ for 10 minutes = \[\frac{12.2151}{6}\]

Average Qo₂ for 10 minutes = \[\frac{2.0358 \mu l}{6}\]

In the present investigation, the average value of Qo₂ in control and in reaction flasks have been recorded respectively and mentioned in all the tables. In every table each value of oxygen consumption is an average of four values.
II. CHOLESTEROL ESTIMATION

For estimation of cholesterol contents in fish liver, kidney and blood, the Liebermann- Burchard technique has been applied (Plummer, 1985).

Principle:

Acetic anhydride reacts with cholesterol in a chloroform solution to produce a characteristic blue-green colour. The exact nature of the chromophore is not known but the reaction probably include esterification of the hydroxyl group on the position 3 as well as other rearrangements in the molecule.

\[
\text{Cholesterol} + \underset{H_2SO_4}{\xrightarrow{HC-Cl}} \underset{H_3COO}{\text{H}_3C\text{-CO}} \xrightarrow{H_3C\text{-CO}_{2}} \text{Cholesteryl acetate}
\]

Reagents:

1. Chloroform: Chloroform (Ranbaxy) with molecular weight 119.38 has been used.

2. Alcohol: Acetone mixture: Alcohol: Acetone in 1:1 ratio has been used.

3. Acetic anhydride-sulphuric acid reagent: Place 15 ml of Acetic anhydride (BDH) in a glass stoppered cylinder and chill in ice water. When cold, add 1ml of concentrated sulfuric acid, a little at a time, with mixing and cooling during the
addition. Shake the contents vigorously for a few moments and return to the ice to keep cold during use.

Estimation of Cholesterol:

After determining the dry weight of the tissue (liver, kidney and blood), the dry residue was then washed ten times with chloroform and poured into a graduated tube. Blood is mixed with an Alcohol-Acetone mixture which removes cholesterol and other lipids and precipitates protein. The organic solvent is removed by evaporation on a boiling water bath and dry residue dissolved in chloroform. The volume of cholesterol solution was made up to 5ml by adding chloroform. The estimation of cholesterol was done by Liebermann-Burchard reaction method. A mixture of Acetic anhydride and concentrated sulfuric acid (8:1) was freshly prepared and one ml of this mixture was added to each graduated tube and covered with glass ball. For the development of colour, tubes were kept for 30 minutes. Percent transmission recorded for each tube by using Digital Spectrophotometer (166 Systronics) at 680nm and calibrated from a standard graph, prepared earlier with known concentrations of cholesterol.
Cholesterol concentration (mg/5 ml)
Standard curve for cholesterol
III. DETECTION OF FREE AMINO ACIDS:

Separation of free amino acids in the experimental tissues (liver, kidney and blood) have been done by Paper Chromatography.

Principle:

The basic principle was first described by Consden, et al. (1944). In this technique a small drop of solution containing the mixture of compounds is used. It is desired to separate is evaporated to dryness on a strip of filter paper and a suitable solvent is allowed to flow slowly along the filter paper over this spot, either aided by gravity (descending) or by capillary alone (ascending). The substances in the initial spot are extracted by the flowing solvent and carried along the filter paper to an extent which appears related to their distribution between the free and bound solvent phase of the filter paper. Under the proper conditions, each substance present has been carried away from the initial spot to a characteristic extent and localized in a relatively small area on the filter paper.

Reagents:

1. 80% Ethanol: 80 ml dehydrated alcohol was mixed with 20 ml distilled water.

2. Chloroform: The Chloroform (Ranbaxy) with molecular weight-119.38.

3. N-Butanol: Acetic acid: Water: Acetic acid: water in 3:1:1 ratio has been used as solvent.

4. Spray reagent (0.25% ninhydrin): 250 mg ninhydrin dissolved in 100 ml distilled water.
Procedure:

The adult healthy specimens of *Heteropneustes fossilis* were collected locally from Sagar lake and kept in the laboratory aquarium. Qualitative analysis of the various tissue homogenate was done by descending paper chromatography on whatman paper No.1. The papers were kept in an atmosphere, saturated with the solvent system for twenty four hours before use. The solvent system found most suitable for this purpose was n-Butanol Acetic acid water in the proportion of 4:1:1 by volume. The papers were run for twelve to fifteen hours at the room temperature. Aqueous Ninhydrin solution (0.25%) was utilised throughout as a developer. The papers were dried at the temperature 80°C in chromatography chamber.

\[
\text{Ninhydrin} + \text{Amino acid} \rightarrow \text{Product}
\]

The tissue homogenates were prepared from four different specimens to observe any variation in the amino acid contents. The extracts were prepared by the mildest method available (Awapara, 1948). The tissue was homogenized in 80% ethanol and then centrifuged. The supernatent was mixed with 3 volumes of chloroform and again centrifuged. The upper aqueous layer was removed and concentrated. B.D.H. standard amino acids were used for identification. Rf values
were calculated for n-Butanol : Acetic acid : Water (4:1:1) as solvent system. Rs values were calculated with respect to alanine. The results were confirmed with standard Rf and Rs values (Table-B) for each amino acid. This is done by running the homogenates along with the standard amino acids. Free amino acids of the liver, kidney and blood thus separated and summarised in table-III.

**Table-B showing Rf and Rs for each amino acid**

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Rf values</th>
<th>Rs values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>0.14</td>
<td>0.53</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.26</td>
<td>-</td>
</tr>
<tr>
<td>Serine</td>
<td>0.12</td>
<td>0.46</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.20</td>
<td>0.77</td>
</tr>
<tr>
<td>Valine</td>
<td>0.51</td>
<td>1.93</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.44</td>
<td>1.68</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.027</td>
<td>0.10</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.022</td>
<td>0.08</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.66</td>
<td>0.25</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.62</td>
<td>0.24</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.11</td>
<td>0.42</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.17</td>
<td>0.66</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.085</td>
<td>0.32</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.32</td>
<td>1.21</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.38</td>
<td>1.45</td>
</tr>
<tr>
<td>α-aminobutyric acid</td>
<td>0.40</td>
<td>1.54</td>
</tr>
</tbody>
</table>
STATISTICAL ANALYSIS

Experimental data was processed for Arithmetic mean, Percent Change, Standard Deviation, Standard Error, Coefficient of Variation and Student 't' test have been calculated statistically and results were summarised in the tables (Elhance, 1987).