Chapter 3

MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS AND GENERATION OF HYPOXIA

Live specimens of *Clarias batrachus* (80-100 g, 16-20 cm) *Heteropneustes fossilis* (80-90 g 20-24 cm), *Cyprinus carpio* (80-100 g, 14-16 cm) and *Mystus seenghala* (70-80 g 20-24 cm) were procured from a local market and were acclimatized at normoxia (7.2±0.3 mg/L, DO), at least for a month in tanks of 100 L capacity filled with 25 L of water at 25±3°C. They were fed once a day with processed feed of goat liver or flesh and soybean powder. Feeding was stopped 48 h before the start of experiment.

All the fishes held for 12 hrs duration of experimentally provoked hypoxia at three different levels:

(i) 65%-40% Oxygen saturation or 5.0±0.3 mg/l to 3.5±0.3 mg/l O₂ (Slight Hypoxia)

(ii) 40%-20% Oxygen saturation or 3.5±0.3 mg/l to 1.5±0.1 mg/l O₂ (Moderate Hypoxia) and

(iii) Below 20% Oxygen air saturation or ≤1.5±0.1 mg/l O₂ (Severe Hypoxia)

Three separate experiments were carried out in the closed respirometer (without access to air-breath) for collection of different tissues. Decrease in dissolved oxygen (DO) was accomplished by bubbling nitrogen directly into the water of the experimental tank, or into
the reservoir that supplied water to the respirometer. DO probe (WTW, CellOx 325) and pH meter (pH electrode; WTW, SenTix® 41-3) were installed to record dissolved oxygen (DO) and temperature.

At the end of the hypoxia period the fish were anaesthetized with MS222 (3-aminobenzoic-acid ethyl-ester methanesulfonate salt, Sigma, USA) to reduce stress effects due to handling. The anaesthetic was injected slowly into the respirometer to expose the fish to a final concentration of 85 mg l\(^{-1}\) for 10 min.

### 3.2 ENZYME ASSAY

#### Sample preparation:

The tissues were homogenized (10%) in a medium of 50 mM Tris HCl medium for one minute in triple distilled water (TDW) with potter-Elvehjem homogenizer using a Teflon-coated pestle under ice-cold condition for all biochemical indices estimation. The details of reaction mixture used are described under each assay system. Spectrophotometer measurements were made using UV-Vis spectrophotometer with silica cuvettes (1 cm light-path, 3 ml) against enzyme blank containing all the reactants at room temperature.

#### 3.2.1 Lactate dehydrogenase Assay

Lactate dehydrogenase (LDH, EC 1.1.1.27) activity in the cell free extracts of muscle, liver, heart and brain was measured by a NADH linked optical assay following the method of Horecker and Kornberg (1948).
3.2.1 Pyruvate Dehydrogenase Assay

Reagents

- Sodium Pyruvate - 50 mM
- NADH - 2.4 mM
- KCl - 0.1 mM
- Tris-HCl, pH 7.4 - 0.2 M

Method:

Prepare the reaction mixture (3.0 ml) in quartz cuvettes of 1.0 cm light path by adding 50 μl of 2.4 mM NADH, 0.1 ml of 50 mM sodium pyruvate, 1.0 ml of 0.2 M TrisHCl buffer (pH 7.4), 1.0 ml of 0.1M KCl, 50 μl of cell free extracts and 0.8 ml of distilled water. Then add the supernatant (enzyme source) in cuvette and immediately take the reading i.e. change in optical density at 340 nm at 30 seconds interval by monitoring the rate of oxidation of NADH to NAD⁺ (or reduction of pyruvate to lactate). The reaction velocity is determined by increase in absorbance at 340nm resulting from the oxidation of NADH. One unit causes the oxidation of one micromole of NADH per minute at 25°C and pH 7.4, under the specified condition.

3.2.2 Malate dehydrogenase Assay

Malate dehydrogenase (MDH; E.C. 1.1.1.37) activity was determined by conversion of oxaloacetate to malate (Somero and Childress 1980).

Reagents

- HEPES - 40 mM
- MgCl₂ - 20 mM
- Oxaloacetate (OAA) - 0.4 mM
NADH - 0.15 mM
KCl (pH 8.0 at 15°C) - 100 mM

Method:

MDH activity determined in a medium containing 40 mM HEPES, 20 mM MgCl₂, 0.4 mM oxaloacetate (OAA), 0.15 mM NADH, 100 mM KCl (pH 8.0 at 15°C). The reactions were initiated by the addition of oxaloacetate (OAA). The increase in absorbance is monitored at 340 nm at 30 seconds interval by monitoring the rate of oxidation of NADH to NAD⁺ for 3 min. in a UV-visible double beam spectrophotometer. Enzyme activity is expressed as μmole min⁻¹ mg protein⁻¹. The molar extinction coefficient of NADH at 340 nm (6.22×10³ M⁻¹ cm⁻¹) was used for calculating the enzyme activity.

3.2.3 Acetylcholinesterase Assay

The assay of Acetylcholinesterase (AChE 3.1.1.7) was done by the method described by Ellman et al. (1961). The increase in absorbance is monitored at 412 nm for 3 min. in a UV-visible double beam spectrophotometer.

Reagents

- Sodium phosphate buffer (pH 8.0) - 100 mM
- Sodium phosphate buffer (pH 7.5) - 10 mM
- Sodium bi carbonate - 15 mg
- DTNB - 5.0 mM
- Acetylthiocholine iodide (ATI) - 5.0 mM
**Method:**

The reaction mixture (3.0 ml) contains 1.5 ml of 100 mM sodium phosphate buffer (pH 8.0), 0.3 ml of 5 mM DTNB [5, 50-dithiobis-(nitrobenzoic acid) prepared in 10 mM sodium phosphate buffer (pH 7.5) containing 15 mg sodium bicarbonate added per 10 ml of solution], 0.3 ml of 5 mM acetylthiocholine iodide (ATI), 0.1 ml of 10% homogenate and 0.8 ml of distilled water. The increase in absorbance is monitored at 412 nm and 28°C for 3 min in a UV-visible double beam spectrophotometer with quartz cuvettes (1.0 cm light path) against distilled water as blank. Simultaneously, use two blanks also. One blank containing phosphate buffer, DTNB and ATI but not enzyme protein to determine the spontaneous hydrolysis of ATI, and the second blank containing phosphate buffer, DTNB and enzyme protein but no substrate (ATI) to correct for any non-AChE-dependent formation of thionitrobenzoic acid (TNB). One unit of AChE activity is expressed as nanomoles of substrate hydrolyzed/min/mg protein under specified experimental conditions. The extinction coefficient of the yellow anion (1.36×10⁴ M⁻¹ cm⁻¹) is employed for calculating the enzyme activity.

### 3.3 PROTEIN ESTIMATION AND SDS-PAGE ELECTROPHORESIS

#### 3.3.1 Protein estimation

Protein concentration of different tissues were estimated by Folin-phenol method (Lowery et al., 1951).
Reagents

1. **Reagent A**
   
   \[
   \text{Na}_2\text{CO}_3 \quad - \quad 2.0 \text{ g}
   \]
   
   \[
   \text{NaOH} \quad - \quad 10 \text{ g}
   \]
   
   \[
   \text{NaK tartrate} \quad - \quad 0.1 \text{ g}
   \]

2. **Reagent B**
   
   \[
   \text{CuSO}_4 \quad - \quad 0.5 \text{ g}
   \]

   Dissolved in 500 ml of distilled water

3. **Reagent C**
   
   Reagent A = 10 ml
   
   Reagent B = 0.2 ml

   Prepare freshly before use.

4. One part Folin’s reagent was mixed with one part double distilled water.

5. **Standard:** Protein BSA (Bovine Serum Albumin).

Method:

1. 75 µl of the sample was mixed with 750 µl of the reagent C and was shaken well.

2. It was allowed to stand for 10-30 min.

3. 75 µl of Folin’s reagent was added and mixed.
4. Allowed to stand for 20 min (not more than 2 hrs.) at room temperature.

5. The intensity of blue colour was measured colorimetrically at 650 nm.

**Calculation:**

\[
\text{mg protein/gm wet tissue} = \frac{O.D.\text{test}}{O.D.\text{Standard}} \times \text{Conc. of Standard} \times \frac{1}{\text{Sample}} = \text{Unit}
\]

**3.3.2 SDS-PAGE of Protein**

**Preparation of Reagent:**

**1. Preparation of 10% Separating Gel (10 ml)**

(a) 30% Acrylamide mix - 3.3 ml

(b) 4x tris (pH 8.8) - 2.5 ml
(c) distilled water - 4.0 ml
(d) 10% APS - 50 µl
(e) 10% SDS - 100 µl
(f) TEMED - 5 µl

(29.2% acrylamide+0.8% bisacrylamide)

**2. Preparation of 4% Stacking Gel**

(a) 30% bisacrylamide - 3.3 ml
(b) 4x tris (pH6 6.8) - 2.5 ml
(c) distilled water - 3.0 ml
(d) 10% APS - 50 µl
(e) 10% SDS - 25 µl
(f) TEMED - 2.5 µl

(3) 2X Sample Buffer:
(a) 0.5M Tris (pH 6.5) - 2.5 ml
(b) Glycerol (100%) - 2.0 ml
(c) SDS (10%) - 4.0 ml
(d) β-mercaptoethanol - 0.8 ml
(e) Bromophenol blue - 300 µl
(f) Distilled water - 400 µl to 10 ml

(4) Separating gel overlaying solution:
- n- or butanol - 50 ml
- Distilled water - 50 ml

(5) 10% SDS:
- SDS (AR) - 10 g
- Distilled water - 100 ml

(6) Tank Buffer:
- Tris base - 6.05 g
- Glycine - 28.50 g
- 10% SDS - 10 ml
- Distilled water - 1000 ml

(7) Staining Solution:
- Comassie blue (R-250) - 0.3 g
- Methanol (AR) - 80 ml
- Glacial Acetic Acid - 20 ml
- Distilled water - 100 ml

Dissolve dye in methanol for 60 min on a shaker then add GAA & water. Filter the dye solution through Wattman filter paper and store in brown bottle.
This solution can be reused about 10 times.

(8) **Destaining solution:**

<table>
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<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>100 ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>300 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Procedure:**

The SDS-PAGE was carried out according to Laemmli (1970) in Mini-PROTEAN Tetra System of BIO-RAD using a 5% (w/v) separating gel. After electrophoresis the gels were stained with coomassie blue R-250 for Visualization of the proteins. Molecular of the protein bands was determined with reference to standards (Genei Marker, PMW).

**Staining the Polyacrylamide Gels for Visualisation of Protein Bands:**

Stained with Coomassie Brilliant Blue R250 (CBBR250).

**Fixing Solution:**

1. 40% methanol, 10% acetic acid in double distilled water.
2. Staining solution and
3. Destaining solution as above.

The gel, after electrophoresis, was incubated in 10 volumes of fixing solution for 20 min on a gyratory shaker.

The gel was stained in 5 volumes of 4 hr to overnight on the shaker. Stain was removed; gel was rinsed with DDW and was then left in 20 volumes destaining solution for 2-3 hrs. The destaining solution was replaced 2/3 hours interval, until the background was clear.

The gel was preserved in 7% acetic acid in DW.
3.4 HEMATOLOGIC PARAMETERS

Fish were anaesthetized prior the collection of blood samples to reduce the handling stress during normoxia. Heparinized blood was used for erythrocyte counts, haemoglobin estimation and haematocrit (Hct) evaluation. Erythrocyte count was made with the help of Neubaur’s haemocytometer using standard diluents. Haemoglobin was estimated by the method of Blaxhall and Daisley (1973). [Hct] was determined following centrifugation of microhematocrit capillary tube filled with blood, at 10,000 rpm for 5 min (Assendelft and England 1982). Erythrocytic indices like mean corpuscular volume (MCV) mean corpuscular haemoglobin (MCH) Mean cell haemoglobin concentration (MCHC) was measured by Wells and Weber (1991).

3.5 INTERMEDIATE METABOLITES

Blood and tissues were treated in 3 volumes of ice-cold 6% perchloric acid (PCA) homogenized in an ice cold bath and centrifuged at 4 °C. Extracts were used for glucose and lactate determinations. Sigma kits procedures n. 635 and 826-UV were used respectively.