MATERIALS AND METHODS

Fish:

Live specimens of the fish, *Anabas testudineus* (length 5.0±1.5cm; weight 25±5gm) were collected from ponds nearby Singramau, Jaunpur (U.P.) during the month September to January and were acclimatized with laboratory condition for 15 days before experiment were set. They were fed on every alternate days with dried Tokyu food granules. The fishes were kept at controlled room temperature (24±1ºC) in plastic aquaria containing continually aerated de-chlorinated tap water with dissolved oxygen contents. Feeding was, stopped 24h before the experiments began, and the fish were not fed during the experiments.

2,4-Dichlorophenoxyacetic acid:

**Herbicide**: The herbicide 2, 4-Dichlorophenoxyacetic acid (2, 4-D ) is used in this study

**2,4-Dichlorophenoxyacetic acid toxicity to fish**:

24 h renewal bioassay test following APHA, AWWA, WPCF (1980) was performed to determine LC$_{50}$-96h values of 2,4-Dichlorophenoxyacetic acid using the Spearman Karber method (Byron and Brown, 1970).

A group of 10 fishes were kept in different aquaria, each containing 10 litres of de-chlorinated tap water/test solutions during the experiment. Fresh test solutions having desired concentration of 2,4-Dichlorophenoxyacetic acid were made one hour prior to their renewal. During renewal of test solutions in the aquaria, due care was taken to keep the fishes undisturbed and to avoid handling stress. To remove test solution from the aquaria siphoning method was applied. When tap water/test solutions reached to a minimum quantity, sufficient to keep the fishes undisturbed, the aquaria were simultaneously filled by gradual addition of fresh test solution. The process of siphoning out and simultaneous addition of fresh tap water/test solution, to the aquaria was continued for some period to ensure their complete replacement.

The mortality of fishes, to calculate the LC$_{50}$ and the reaction of fishes to the 2,4-Dichlorophenoxyacetic acid at different intervals were regularly recorded. The fishes were
considered dead, if these did not react to gently prodding and showed lack of respiratory movements. Dead fishes, if any, detected during the experiment, were immediately removed from the aquaria.

The LC$_{50}$ values of 2,4-Dichlorophenoxyacetic acid to the fish were estimated using Spearman-Karber methods (Bryon and Brown) for precise calculations than the probit and logit often used in toxicological investigation for the purpose. The experiments were repeated three times to determine the reproducibility.

**Experimental design:**

Acclimated *Anabas testudineus* in batches of ten irrespective of their sex were subjected to lethal concentration of 600.6 mg/L and sub lethal concentration of 60.6 mg/L. Similar conditions excepting the addition of 2,4-D were also maintained in control tanks. Feeding was continued throughout the tenure of experiment. Gills and labyrinthine organ of *Anabas testudineus* were excised, rinsed in physiological saline and were fixed in 10% neutral formalin and aqueous Bouin’s fluid at 6 hour (h), 12 h, 1 day (d), 2d, 3d, 4d of lethal exposure and 6 h, 12 h, 1 d, 2d, 3d, 4d, 5d, 6d, 7d, 8d, 9d, 10d, 15d, 20d, 30d, 45d and 60d of sub-lethal exposure of 2,4-D.

**Cross section:**

Standard method of dehydration, clearing and embedding were used; paraffin sections were cut at 5 μm and were mounted on alcohol washed clean slides.

**HISTOLOGICAL ORGANIZATION:**

**Fixative:**

10% neutral formaline (Lillie, 1954), Helly’s fluid (Helly, 1903) and aqueous Boun’s fluid (Bouin, 1897).

**Stain:**

Sections were stained with Ehrlich’s haematoxylin/eosin (H/E) (Ehrlich, 1886), Verhoeff’s elastin stain (VHE) (Lillie, 1954) and Papanicolaou’s stain (PS) (Gurr, 1958).

**CARBOHYDRATE HISTOCHEMISTRY:**

**Fixative:**
10% neutral formalin (Lillie, 1954) aqueous Bouin’s fluid (Bouin, 1897) and alcoholic Bown’s fluid (Gatenby & Beams, 1950).

Stain (Table-1):

Sections were stained with schiff without oxidation for the demonstration of free aldehydes (Pearse, 1968) the periodic acid-schiff (PAS) method for polysaccharides (Mc Manus, 1946) diastase treatment followed by PAS to distinguish mucopolysaccharides (glycoproteins, Reid & Clamp, 1978) from glycogen (Lillie and Greco, 1947) alcian blue (AB) at pH 2.5 method to localize acid mucopolysaccharides (Steedman, 1950; Mowry, 1956) and alcian blue at pH 1.0 method for sulphated acid mucopolysaccharides (Lev & Spicer, 1964). To differentiate the neutral mucopolysaccharides from acid mucopolysaccharides, combined AB at pH 2.5/PAS and AB at pH 1.0/PAS techniques (Moury, 1963; Spicer and Henson, 1967; Johannes & Klessen, 1984) were employed. With these techniques, neutral mucopolysaccharides stain magenta, acid mucopolysaccharides stain blue or greenish blue and mixed neutral and acid mucopolysaccharides stain purple or violet (Table-1).

Blocking and conversion methods:

Sections were subjected to the following blocking and conversion techniques (Table-1)

Acetylation (Lillie, 1954):

Treatment of sections with 16ml acetic anhydride and 24ml anhydrous pyridine for 1h-24h at 25ºC or ½h-6h at 58ºC to block the hydroxyl groups.

Deacetylation (Lillie, 1954):

Sections subjected to prior acetylation are treated with 70ml alcohol, 10ml water and 20ml 28% ammonia water for 24h at 37ºC to hydrolyze the acetyl esters, unblocking the reactive hydroxyl group.

Mild Methylation (Spicer, 1960):

Treatment with preheated 99.2ml methanol and 0.8ml conc. hydrochloric acid solution for 4h at 37ºC to block the basophilia of carboxylated or non sulphated acid mucopolysaccharides.
**High temperature methylation (Spicer & Lillie, 1959):**

Treatment of the sections with preheated 99.2 ml methanol and 0.8 ml conc. hydrochloric acid solutions for 5 h at 60°C to block the basophilia of both carboxylated or non-sulphated acid mucopolysaccharides.

**Combined high temperature methylation/saponification (Spicer & Lillie, 1959):**

Sections subjected to prior high temperature methylation are treated with 1 gm potassium hydroxide, 70 ml ethanol and 30 ml water solutions for 30 minutes at room temperature to unblock the basophilia of carboxylated or non-sulphated acid mucopolysaccharides.
Table-1: A Summary of the histochemical techniques used to locate and differentiate carbohydrate moieties in gills and labyrinthine organs of *Anabas testudineus*.

<table>
<thead>
<tr>
<th>Histochemical techniques</th>
<th>Reaction</th>
<th>Chemical Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment/Schiff</td>
<td>Magenta</td>
<td>free aldehydes</td>
</tr>
<tr>
<td>Periodic acid/Schiff (PAS)</td>
<td>Magenta</td>
<td>Polysaccharides</td>
</tr>
<tr>
<td>Acetylation/PAS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Distase/PAS</td>
<td>-</td>
<td>Glycogen</td>
</tr>
<tr>
<td>Deacetylation/PAS</td>
<td>Magenta</td>
<td>-</td>
</tr>
<tr>
<td>Alcian Blue (AB)/PH 2.5</td>
<td>Greenish blue</td>
<td>Acid mucopolysaccharides</td>
</tr>
<tr>
<td>High Temperature methylation/AB PH 2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>High temp. methylation/ Saponification/AB pH 2.5</td>
<td>Greenish blue</td>
<td>Non-sulphated acid mucopolysaccharides</td>
</tr>
<tr>
<td>Mid Methylation/AB pH 2.5</td>
<td>Greenish blue</td>
<td>Sulphated acid mucopolysaccharides</td>
</tr>
<tr>
<td>Alcian blue pH 1.0</td>
<td>Greenish blue</td>
<td>Sulphated-acid mucopolysaccharides</td>
</tr>
<tr>
<td>Alcian blue pH 2.5/PAS</td>
<td>Magenta</td>
<td>Neutral Mucopolysaccharides</td>
</tr>
<tr>
<td></td>
<td>Greenish blue</td>
<td>Acid Mucopolysaccharides</td>
</tr>
<tr>
<td></td>
<td>Purple</td>
<td>Mixed acid and neutral mucopolysaccharides</td>
</tr>
</tbody>
</table>

*Symbols*: “-”, negative

**PROTEIN HISTOCHEMISTRY:**
Fixative:

10% neutral formalin (Lillie, 1954).

Stain (Table-2):

Sections were stained with mercury-bromo-phenol blue (Hg-BPB) method to detect total proteins (Pearse, 1985); acid selochrome cyanine technique for basic proteins (Pearse, 1985); ninhydrin-Schiff technique for the demonstration of protein-bound amino (–NH₂) groups (Yasuma and Itchikawa, 1953); Millon reaction for tyrosine (Baker, 1956); dimethylamino benzaldehyde-nitrite (DMAB-nitrite) method for cysteine-bound sulphydriyl (–SH) group (Barnnet and Seligman, 1952); performic acid alcin blue method for cysteine-bound disulphide (–SS) group (Adams and Sloper, 1955) and Sakaguchi reaction for arginine (Baker, 1947), (Table-2).

Deamination (–NH₂ groups):

Treatment with fresh nitrous acid solution (1gm solution nitrite in 30 ml 3% sulphuric acid) for 48h at 0°C–5°C in dark followed by 4h treatment at 60°C in (a) water and (b) absolute ethanol.

Performic acid oxidation (tryptophan):

Treatment of sections with freshly prepared performic acid (4ml of 30% (100 Vol.) hydrogen peroxide, 0.5ml conc. Sulphuric acid, 40ml of 98% formic acid) for 15-60 minutes at room temperature.

Iodination (tyrosine and tryptophan):

Treatment of sections with a mixture of 30ml of Gram’s iodine (1gm iodine, 2gm potassium iodide and 30 ml water) and 2 ml 3% ammonia at pH 10.0 for 24h at room temperature.

Maleimide block (–SH groups):

Treatment of sections with 0.1M N-ethyl maleimide in 0.1M phosphate buffer at pH 7.4 for 4h at 37°C followed by washing in 1% acetic acid and then in tap water.

Iodine Oxidation (–SH groups):
Treatment of sections with 38mg iodine 33mg potassium iodide and 100ml water titrated to pH 3.2 with 0.01N hydrochloric acid before use 4h at room temperature.

Table-2: A Summary of the histochemical techniques used to locate and differentiate protein moieties in gills and labyrinthine organs of *Anabas testudineus*.

<table>
<thead>
<tr>
<th>Histochemical techniques</th>
<th>Reaction</th>
<th>Chemical Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercury-bromophenol blue</td>
<td>Blue</td>
<td>General proteins</td>
</tr>
<tr>
<td>Acid solochrome cyanine</td>
<td>Red</td>
<td>Basic proteins</td>
</tr>
<tr>
<td></td>
<td>Blue</td>
<td>Nuclear proteins</td>
</tr>
<tr>
<td>Ninhydrin-Schiff</td>
<td>Purple</td>
<td>Protein bound ~NH2 groups</td>
</tr>
<tr>
<td>Deamination/ninhydri-Schiff</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dimethylaminobenzaldehyde (DMAB) nitrite</td>
<td>Blue</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Iodination/DMAB-nitrite</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Million reaction</td>
<td>Red</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Iodination/Millon reaction</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dihydroxy-dinaphthyl-disulphide (DDD) reaction</td>
<td>Blue or Red</td>
<td>Cysteine bound sulphhydr (-SH group)</td>
</tr>
<tr>
<td>Maleimide block/DDD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Iodine oxidation/DDD</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Performic acid-alcian blue (PFAAB)</td>
<td>Blue</td>
<td>Cystein bound disulphide (-SS) groups</td>
</tr>
<tr>
<td>Thioglycollate reduction/PFAAB</td>
<td>Red</td>
<td>Arginine</td>
</tr>
<tr>
<td>Sakaguchi reaction</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenylglyoxal/Sakaguchi reaction</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Symbols-* “-”, negative.

**Thioglycolate reduction (−SS group):**

Treatment of sections covered with 0.5% celloidin, with freshly prepared 0.5M thioglycollic acid titrated to pH 8.0 with 0.1N sodium hydroxide for 4h at 37°C and then washed in tap-water rinsed briefly in 1% acetic acid and washed again in water.
Phenylglyoxal block (arginine):

Treatment of sections with 1.5% phenylglyoxal monohydrate in 125m mole bicarbonate (p 7.9) for 30-60 minutes at room temperature.