MATERIALS AND METHODS
### MATERIALS

#### ANIMAL SELECTED

Classification and Biology of the animal

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*Pila globosa* is an edible freshwater snail occurring abundantly in the freshwater ponds, irrigating canals and in rice fields of South India. It is also known as "apple snail". A tick spiral and yellowish brown or black coloured shell enclose the body of Pila. Pila moves with the help of foot situated on the ventral side of the body. It is a herbivore feeds mainly on aquatic succulent vegetation. Its sessile nature prevents it from migrating away from the source of pollution and individuals must therefore either adapt to the increasing concentrations of pollutants or perish. Therefore it is considered as an ideal experimental animal in bioassay studies and serves as one of the bio-indicators of pollution (Lavie and Eviatar, 1988).

#### Reasons for selection of animal

The reasons that have prompted the author to select the snail, *Pila globosa*, as an experimental animal for the present study are as follows:
1. This animal once abundantly present in irrigation canals, tanks and ponds of Anantapur district is gradually decreasing in number due to increase in water contamination by the dumping of sewage and industrial and agricultural effluents. This may lead imbalance in food chain.

2. *Pila globosa* also served as a protein food source for poor in this region. Therefore, the possibility of biotransfer of toxicants may occur through this animal to human beings.

3. Besides, this snail is known for its ability to adapt to laboratory conditions and suitability to toxicological studies (Szeer and Plotr, 1986). Hence, to analyse the response patterns to nickel this animal is selected as an experimental model in the present investigation.

**TOXICANT SELECTED**

Nickel is available in different salt forms like nickel chloride, nickel carbonate, nickel sulphate, nickel acetate etc., and these salts are more or less soluble in water. Solubility rate of each salt varies depending upon the hardness of water. Nickel chloride is relatively more soluble in water than the other salt forms. Pure salt of nickel chloride (LOBA make) having the molecular formula \( \text{NiCl}_2 \cdot 6\text{H}_2\text{O} \) and molecular weight 273.71 is used in this investigation to study the effect of nickel on the freshwater snails. Every 4.049 gms of nickel chloride contains one gram nickel.

**Maintenance of snails**

The snails were collected from local irrigating canals and ponds in and around Anantapur. As the present study includes the influence of size of snails on the toxicity of nickel, two different size groups of them were selected. An
allometric relation between the total body weight and the soft parts weight was earlier reported in freshwater molluscs (Madanmohan Das, 1983). Based on this report the total body weight including that of shell was taken as a measure of the body size of the animal. Two size groups representing small and large having the total body weight 20±2 g and 40±2g respectively were used in this present investigation. All the two size groups of animals were maintained in laboratory in large aquaria; and the water in aquaria was renewed once a day to provide freshwater rich in oxygen. The snails were fed ad libitum with pieces of hydrilla (water plant) for ten days before using them for experimentation. As the effect of metals are known to vary by the factors like temperature, salinity, pH, hardness of water, size, sex and density of animals etc., (Arillo et al., 1982; Moore and Ramamoorty, 1984; Victoriamma and Radhakrishnaiah, 1986;), care was taken to control those factors as far as possible throughout this investigation. To this effect, water flow from the same source has been used for the maintenance of animals and also for experimentation. The water used has a pH range of 7.6±0.2 and total hardness 100±5 mg/l CaCO₃. Temperature, chlorinity and dissolved oxygen content of water were checked periodically during the course of investigation and were maintained at 28° C±0.5° C, 0.08±0.003 % and 5.79±0.4 ml/l. respectively. All the animals used for experimentation were maintained in static water without any flow. Water was aerated once a day to prevent influence of hypoxia on experimental animals, if any (Khorram and Knight, 1977). The size of animals selected was also maintained strictly throughout the investigation.
METHODS

Evaluation of nickel toxicity

The percent mortality of small and large size groups of snails in different nickel concentrations was determined immediately after 96 hours of exposure. For this, the two sizes of experimental animals were divided into batches of twenty-four each and were exposed to different concentrations of nickel, each batch to one concentration, ranging from 75 to 200 mg/l for small size, 150 to 275 mg/l for large size groups of animals. These ranges were obtained on the trail and error basis. Mortality rate was observed in each concentration of nickel immediately after 96 hours exposure. A batch of animals in each size group maintained alongside in freshwater with 0.1 ml of hydrochloric acid per litre to nullify chloride effect served as controls. The experiments were repeated thrice for accuracy. The mortality rate at each concentration, derived from the mean of the three, was converted as present mortality values. From this, the probit mortality value was obtained (Finney, 1971). As the evaluation of toxicity of metal to an aquatic organism is by the determination of its LC$_{50}$, the percent mortality and probit mortality values of the two groups were plotted separately against nickel concentration, and LC$_{50}$s were derived from the two curves. For subsequent verification of the LC$_{50}$s obtained by graphical methods, Dragstedt a Behren’s method as given by Carpenter (1975) was employed. As per this method the small and large sized animals were exposed to log.2 concentrations of nickel, 50, 100, 200, 400, mg/l for 96 hours.

The percent mortality values were calculated from the cumulative mortality, with them LC$_{50}$s were obtained by adopting the following formula.

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

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

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

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

Thus, the mean \( LC_{50}/96 \) hours for each size group of snails was obtained through percent and probit mortality curves and Dragstedt and Behren's method, as described earlier (Koppar et al., 1993).

Fixation of lethal and sublethal concentrations

Based on previous studies, the effects of heavy metals on molluscs become constant within 96 hours of exposure (Sreedevi, 1988; Umadevi, 1996). Hence, \( LC_{50}/96 \) hours of nickel was fixed as lethal concentration. But, as two different \( LC_{50}s \) were obtained separately for small and large size groups of snails the average of two, approximately 150 mg/l was taken as the lethal concentration for both the size groups of snails to compare the impact of acute concentration of nickel on the physiological, biochemical and histological aspects of these animals in relation to body size. The knowledge on the concentration of a toxicant which kills 50 \% of the test animals in a fixed period of time is insufficient to assess various responses of animals to the 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 Anita, 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. So, about one
tenth of the lethal concentration i.e., 15 mg/l, was taken as the sublethal concentration of nickel for two size groups of snails to carry out all the further studies included in this investigation. Like lethal, only one sublethal concentration was used for two size groups of snails to assess the influence of body size on nickel toxicity.

Fixation of exposure periods

Since the duration of exposure is having a great influence on the toxicity of a metal in an organism (Radhakrishnaiah and Busappa, 1986), the effects of lethal and sublethal concentrations of nickel on small and large size groups of snails were studied at different periods of exposure in order to understand the influence of time over toxicity. As the lethal concentration taken for study is greater than the 96 h LC₅₀ for small animals, they may not survive up to 96 hours in that concentration. Hence, shorter duration of exposures i.e., 12, 24, 36, and 48 hours, were chosen to observe the effects of the lethal concentration; whereas 5, 10, 20 and 30 days were selected to study the effects of sublethal concentration. The results obtained at these exposure periods may give an insight on some specific events of responses of these animals to acute and chronic nickel stress on short-term and long-term exposures.

GENERAL EXPERIMENTAL PROCEDURE FOR FURTHER STUDIES:

Further studies in this investigation were carried in the mantle, hepatopancreas and foot of the two size groups of snails at 12, 24, 36, and 48 hours of exposure to the lethal and 5, 10, 20 and 30 days of exposure to the sublethal concentrations of nickel. Each experiment was carried 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 snails were deshelled and the required organs were dissected out from each animal using sterilized instruments. The organs were weighed separately to the nearest milligram on an electrical semi microbalance and transferred into icejacketed microbeakers containing the ringer solution. An equilibration time of 15 minutes was allowed to the organs to enable them to regain normalcy 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° ± 1 C°.

**ACCUMULATION**

Nickel accumulated in the mantle, hepatopancreas and foot of small and large size groups of snails was estimated separately with atomic absorption spectrophotometer (Varian Techtron model 1000). The organs were first digested by wet digestion method as described by Humpries (1956). To begin with, the organs were kept in a hot air oven at 85°C for 24 hours to remove water. Then each organ was transferred into 300 ml kjeldhal flask and 4.0 ml of perchloric acid, sufficient nitric acid (about 7.0 ml per every gram material) and 5.0 ml of concentrated sulphuric acid were added to ensure complete oxidation of organic matter. After the addition of these acids the contents were mixed gently at low heat for 3–5 minutes until the first appearance of dense brown fumes. Then the flask was removed from the heater for 5 minutes to subside the reaction. The flask was replaced on the heater and digestion continued slowly at low heat until the appearance of dense white fumes of sulphuric acid. The digestion was continued for 5 to 10 minutes, 1 to 2 minutes at increased temperature, after the appearance of dense white fumes. Then the digestive was cooled and the colourless liquid was neutralized by the addition of 20 % sodium hydroxide. The entire digestive was diluted to 50 ml with double distilled water.
The diluted digestive was fed directly into the atomic absorption spectrophotometer by using nickel cathode lamp and nickel content was measured at the wavelength of 232 nm. The nickel standards were also fed similarly. Nickel present in the organs is expressed as µ g/g wet wt of the tissue.

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 mantle, hepatopancreas, foot of two size groups of snails 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°C as 0.265 µ moles per 1.0 ml of air saturated water. Then the water was removed form the reaction vessel and 2.0 ml of 0.05 M phosphate buffer (pH 5.5) was added. After 5 minutes of stabilization, 50 mg of the tissue in the form of thin slices 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 µ moles/g wet wt /5 Mts.

Estimation of pyruvate

The level of pyruvate in the organs was estimated using the method of Friedman and Hangen (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. 1.0ml of the supernatant was taken and to it 1.0ml of 0.001 M 2,4-Di nitrophyl hydrazine and 3 ml of 0.4 N sodium hydroxide were 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

The level of 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% trichloro acetic acid and centrifuged at 3000 rpm 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 % 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
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 0.24 M 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 0.4 M phosphate buffer (pH 7.7), 0.2 ml of 0.2 M sodium succinate, 1.0 ml of 0.004 M 2-(P-indophenol)-3-P nitrophenyl-tetrazolium chloride (INT), 0.1 ml of 0.005 M phenazime 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 formazan formed was extracted into 6.0 ml of toluene over night 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 formazan/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 0.25–M 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 1.0 ml
of 0.04 M phosphate buffer (pH 7.4), 0.5 ml of 0.1 M lithium lactate, 1.0 ml of
0.0001 M nicotinamide adenine dinucleotide (NAD), 1.0 ml of 0.004 M 2-(P-
indophenol)-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT) and 0.5 ml of
5 % enzyme preparation. The mixture was incubated at 37° C for 30 minutes and
then added 6.0 ml of glacial acetic acid to stop the reaction. The formozan
formed was extracted into 6.0 ml of toluene over night at 0° C; the optical density
of the colour developed was measured in a spectrophotometer at a wavelength
of 495 nm. A blank using 0.5ml of distilled water and a control by taking 0.5 ml of
boiled enzyme were also run similarly. INT standards were prepared along side
for comparison. The enzyme activity is expressed as μ M formozan/mg protein/h.

SOME ASPECTS OF PROTEIN METABOLISM

The levels of soluble proteins, structural proteins, free aminoacids,
ammonia and urea, and the activities of proteases, alanine and aspertate
aminotransferases, glutamate dehydrogenase and arginase were estimated in
the mantle, hepatopancreas and foot of the two size groups of snails exposed to
the lethal and sublethal concentrations of nickel, as well as in controls.

Estimation of soluble and structural proteins

The level of soluble and structural 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 0.25-M ice-cold sucrose solution. For
soluble and structural proteins, 1 ml of homogenate was taken and centrifuged
at 3000 rpm 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
3000 rpm. The two residues were dissolved in 5 ml of 0.1 N sodium hydroxide.
To 1ml of each of these solutions, 4 ml of reagent–D (mixture of 2 % sodium

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carbonate and 0.5 % copper sulphate in 5:1 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 0.04 ml of folin 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 of the organ.

**Estimation of Free amino acids**

Free amino acid levels in the organs were estimated by the ninhydrin method as described by Moor 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 proteases 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 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. The reaction was stopped by adding 2 ml
of 2 % ninhydrin reagent. 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 diluent (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 was also run similarly. Amino acid standards were prepared along side for comparison. The protease activity is expressed as μ M 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) aminotransferase activities.

Activities of alanine and aspartate amino transferases (AIAT and AAT) in the organs were estimated using the method of Reitman and Frankel (1957). A 5 % homogenate (W/V) was prepared in 0.25 M 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 amino transferase activity) consisted of 0.5 ml of 0.2 M alanine, 0.5 ml of 0.005 M 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 second set (for aspartate amino transferase activity) consisted of 0.5 ml of 0.2 M aspartic acid, 0.5 ml of 0.005 M 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 mixtures were incubated at 37° C for 30 minutes and then the action was stopped by the addition of 1 ml of 0.001 M 2,4–dinitrophenyl hydrazine (Ketone reagent). Finally, the reaction mixture was 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 was also run similarly. Pyruvate and oxaloacetate standards were prepared alongside for comparison. The alanine amino transferase activity is expressed as μ M pyruvate formed/mg protein/ h and the aspartate amino transferase activity as μ M 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 0.25–M 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 0.25–M sucrose at 2° C to 4° C for 24 hours. The incubation mixture in a final volume of 2.0 ml contained 40 μ M of sodium glutamate, 100 μ M of sodium phosphate buffer (pH 7.4), 0.1 μ M of NAD (nicotinamide adenine dinucleotide) and 4.0 μ M of INT (2–P–indophenol -3– P–nitriphenyl -5– phenyl tetrazolium 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 toluene over night 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 μ M 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% acetyl-trimethyl ammonium bromide. The homogenates were centrifuged at 3000 rpm 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-arginase, 50 μ moles of sodium glycinate buffer (9.5 pH), 0.5 μ moles of MnCl₂ and 200 μg 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 is expressed as μ moles of urea formed/mg protein/h.

Estimation of Ammonia

The level of 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 sulfate standards were run alongside for comparison. The ammonia content is expressed as μ M/g wet wt of the organ.
Estimation of Urea

The level of 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 centrifuged 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% diacetylmonoxime 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 regent blank. Standards of different urea concentrations were run simultaneously. The urea content is expressed as μM/g wet wt of the organ.

HISTOLOGY

The histological sections of the mantle, hepatopancreas and foot of both size groups of snails were taken by adopting the procedure as described by Humason (1972). The tissues were isolated from control and nickel treated animals and were gently rinsed with physiological saline solution (0.9% NaCl) to remove mucus and other debris adhering to them. They were fixed in Bouin’s fluide (75 ml saturated aqueous picric acid, 25 ml 40% formaldehyde and 5 ml glacial acetic acid) for 24 hours. The fixative was removed by washing through running tap water for overnight. Then the tissues were processed for dehydration. Ethylalcohol was used as the dehydrating agent, as it is the most suitable and economical besides its hardening effect. The alcoholic transfer schedules were so arranged as to utilize both dehydration and hardening effect. The tissues were passed through successive series containing 30%, 50%, 70%, 80%, 90%, 95%, and absolute alcohol’s. Then the tissues were cleared in
methylbenzoate and embedded in paraffin wax. Sections of 5 μ thickness were cut using "SIPCON" rotator microtome. The sections were stained with Harris hematoxylin (Harris, 1900) and counter stained with eosin, dissolved in 95% alcohol. After dehydration and cleaning, the sections were mounted in canadabalsam. Photomicrographs of the section preparations were taken using Olympus (PM–6 model) photomicrographing equipment.

Statistical Analysis

All the results obtained in the 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 those values in tables, the significance between controls and experimental was calculated by using those values and are represented in the respective tables.