MATERIALS AND METHODS
EXPERIMENTAL DESIGN

Species: Albino rats

Pesticide: Chlorpyrifos Technical (95.30%) was obtained from Nagarjuna Agri. Chem Limited, Ravulapalem Mandal, East Godavari District, A.P., India.

Concentration selected: Tenth fold ($1/10^{th}$) lower concentration of LD$_{50}$ was selected for sublethal treatment to the experimental rats.

Course of study: Single, double and multiple doses with 48 hours interval.

Route of administration: Oral

Tissues selected: Heart, liver, kidney, muscle, intestine, testes and blood.

Pesticide stock solution: Stock solution of chlorpyrifos was prepared in acetone. Working pesticide test solutions were prepared by diluting the stock solution with distilled water.

Selection of sublethal treatment to the experimental model: As the acute oral LD$_{50}$ value of chlorpyrifos was determined, tenth fold lower ($1/10^{th}$) concentration was selected as sublethal to study the effect of chlorpyrifos. Healthy adult albino rats of same age (100±10 days) and weight (200±10 g) were divided into four groups having ten animals each. The second, third and fourth groups of animals were termed as experimental animals. To the animals of second group single dose of pesticide (i.e. on 1$^{st}$ day) was administered orally by gavage method. To the third group of animals double doses were given i.e. on 1$^{st}$ and 3$^{rd}$ day. Similarly multiple doses i.e., 1$^{st}$, 3$^{rd}$, 5$^{th}$ and 7$^{th}$ day were given to the fourth group of animals. The first group of animals was considered as controls.

Isolation of tissues: The control and experimental animals after the stipulated period (i.e. on 9$^{th}$ day) were sacrificed and the tissues were isolated, cleaned in physiological saline and processed immediately for microscopic analysis.
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The tissues were also quickly isolated under ice cold conditions and stored in deep freezer at -80°C for biochemical analysis.

Programme of the Present Study

The experimental design has the following parameters for investigation.

Selection of Experimental Model: The present study was carried out in the albino rats. This animal was chosen for the study because of the following reasons.

1. Rats considered as an animal model of studies (McEwen and Stephenson, 1979).

2. Albino rats are relatively small, can be handled easily and require less feed.

3. The rats are preferred because of the plethora of toxicity data that exists for rodents (Bruce, 1985).

4. The laboratory rats close to the field rats in its effects to the pesticides sprayed on the field.

5. Extrapolation of the rats to the human being (man) is usually done for risk assessment (Moser, 1990) for a wide variety of compounds (Groten et al., 1997).

6. Acute toxicity test was conducted as it throws light on how the animal responds to a single, double, multiple dose of a particular pesticide.

7. To study the effect of chlorpyrifos in the albino rats.

8. To identify the effects of chlorpyrifos by the potential target tissues namely heart, liver, kidney, muscle and intestine selected for different parameters.
9. The choice of the animal model to be used in toxicity studies appears critical as different tissues may be the target of the drug’s toxic effect in different animal species (Roncaglioni et al., 1982).

**Materials and Methods**

**Procurement of experimental animals**

Healthy wistar strain albino rats of the same age group 100±10 days and weight 200±10 grams were selected as experimental animals for the present study. The rats were collected from Indian Institute of Science (I.I.Sc.), Bangalore. Prior to experimentation the animals were acclimatized according to the instructions given by Behringer (1973).

**Maintenance of animals**

The rats were maintained at laboratory conditions in the animal house at 25±2°C with a photoperiod of 12hrs light and 12hrs darkness throughout the course of the present study. The rats were fed with standard pellet diet supplied by Sai Durga feeds and foods, Bangalore and water *ad libitum*.

**Pesticide selected**

Chlorpyrifos, an organophosphate insecticide was selected for the present investigation.

Chlorpyrifos O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate with 95.30% purity was used as the test chemical for the present study. Technical grade chlorpyrifos was obtained from Nagarjuna Agri. Chem Limited, Ravulapalem Mandal, East Godavari District, A.P., India. Chlorpyrifos has a wide applicability and safety compared to other compounds of its class. Hence this pesticide was selected for the present study.
The following are the specifications of chlorpyrifos used in the present study.

**Generic name** : Chlorpyrifos

**Chemical name** : O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) Phosphorothioate

**Synonym(s)** : Phosphorothioic acid O,O-diethyl

**Registered trade name(s)** : Dowco 179; ENT 27311; Dursban; Lorsban; Pyrinex; DMS-0971

**Chemical formula** : C_{8}H_{11}Cl_{3}NO_{3}PS

**Chemical structure** : ![Chemical Structure Image]

**Identification numbers** :

- CAS Registry : 2921-88-2
- NIOSH RTECS : TF6300000
- EPA Hazardous Waste : 059101
- OHM / TADS : 7800025
- DOT/UN/NA/IMCO : NA 2783 Chlorpyrifos
- HSDB : 389

**Molecular Weight** : 350.57

**Color** : White granular crystals

White to tan

Amber solid cake with amber oil

Colorless crystals
Physical state: Crystalline solid
Melting point: 41-42°C
Boiling point: Decomposes at approximately 160°C
Density at 43.5°C: 1.398 g/cm³
Odor: Mild mercaptan
Solubility:
Water at 20°C: 0.7 mg/L
Water at 25°C: 2 mg/L
Organic solvent (s): 79% w/w in iso octane
43% w/w in methanol
Readily soluble in other organic solvents
Partition coefficients:
Log $K_{ow}$: 4.82
Log $K_{oc}$: 3.73
Vapor pressure at 20°C: $1.87 \times 10^{-5}$ mm Hg
Vapor pressure at 25°C: $1.87 \times 10^{-5}$ mm Hg
Henry’s law constant at 25°C: $1.23 \times 10^{-4}$ atm-m³/mol
Conversion factors (25°C):
1 ppm = 14.3 mg/m³
1 mg/m³ = 0.070 ppm
Flammability limits at 25°C: No data

(CAS = Chemical Abstracts Services; DOT/UN/NA/IMCO = Department of Transportation/United Nations/North America / International Maritime Dangerous Goods Code; EPA = Environmental Protection Agency, HSDB = Hazardous Substance Data Bank; NIOSH = National Institute for Occupational Safety and Health; OHM/TADS = Oil and Hazardous Materials / Technical Assistance Data System; RTECS = Registry of Toxic Effects of Chemical Substances).
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1. TOXICITY EVALUATION OF CHLORPYRIFOS

Lethal dose of chlorpyrifos was determined by “Probit method” of Finney (1971). Rats were treated with different concentrations of chlorpyrifos by oral intubation. Dose and mortality were noted and a graph was plotted between chlorpyrifos concentration and probit kill. LD₅₀ was the dose at which 50% of the test animals were killed.

Since the toxicity of a chemical depends upon many biotic and abiotic factors. The general conditions such as temperature, humidity, food and water supply etc., were maintained constant to the maximum possible extent during experimentation.

Sub acute (Tolerable) Daily Dose Evaluation

After determining the LD₅₀ dose, sub acute oral doses ranging from \( \frac{1}{5} \) to \( \frac{1}{10} \) LD₅₀ were administered to five batches of rats having ten animals in each batch. \( \frac{1}{10} \) LD₅₀ was selected as sub lethal and administered to rats as single, double and multiple doses with an interval of 48 hrs for biochemical and histopathological studies. After stipulated time the animals were sacrificed and different tissues were isolated for further investigations.

2.0 HAEMATOLOGY

2.1 Red blood corpuscle (RBC) count

RBC count was made with a Neubauer crystalline counting chamber as described by Davidson and Henry (1969). The blood was collected in a vial containing 2% ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. The blood was drawn up to 0.5 marks in RBC pipette and immediately the diluting fluid was drawn up to the mark 101 (thus the dilution is 1:200). The solution was mixed well by shaking gently. It was allowed to stand for 2 or 3 minutes. The counting chamber and cover glass were cleansed and the cover glass was placed over the ruled area. Again the solution was mixed gently and stem ful of solution was expelled and a drop of fluid was allowed to flow
under the cover slip holding the pipette at an angle of 40°, it was allowed to stand for 2 to 3 minutes to allow RBC to settle. Afterwards the ruled area of the counting chamber was focused under the microscope and the number of RBC's were counted in five small squares of the RBC column under high power and the number of RBC per cumm were calculated accordingly.

Number of cells X dilution factor X depth factor

Area counted

2.2 Estimation of haemoglobin concentration (Hb)

The hemoglobin concentration was estimated by Acid - haematin method (Sahli, 1962). N/10 hydrochloric acid was taken up to 20 marks in a graduated tube. Blood was collected directly from the eyeball up to 20 cu mm in the Hb pipette and the outer side was wiped out and this was transferred into the graduated tube containing N/10 hydrochloric acid.

Pipette was rinsed two or three times with dilute hydrochloric acid. It was allowed to stand for 10 to 20 minutes after thorough mixing. Then N/10 HCl was added drop by drop, mixing between each addition until the blood color matched with the standard color. And then the results were read from the scale on the graduated tube and the Hb concentration was expressed in grams percent.

2.3 Estimation of packed cell volume (PCV)

PCV estimated by micro hematocrit method (Schalm et al., 1975).

The blood was drawn into capillary tubes containing the anticoagulant, by capillary action to 2/3 of their length. The tubes were tapped to permit blood to flow towards end and to provide sufficient space to prevent outflow when the opposite ends were sealed. The outside of the capillary tubes were wiped free of blood and the index finger was placed over the moist ends to hold the column of the blood in place as the opposite dry ends were forced through the capillary column.
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into the sealing material to form a tight plug. The capillary tubes were placed in the centrifuge with the sealed ends pointing outward and centrifuged at 12,000 rpm for 5 minutes. PCV was determined by rolling the capillary tubes a reader card until the top of the plasma column was aligned with 100% line and the bottom of the packed erythrocytes was on the zero line. The line that crossed the top of the packed erythrocyte column represented the PVC in percent.

2.4 **Mean corpuscular volume (MCV)**

MCV expresses the average volume of the red blood cells. For obtaining the mean corpuscular volume, the packed cell volume is divided by red blood cell count and the result is multiplied by 10. MCV is expressed in cubic microns (cμm).

2.5 **Mean corpuscular hemoglobin (MCH)**

MCH represents the average weight of hemoglobin contained in each cell. MCH is influenced by the size of the cell and concentration of hemoglobin. For getting MCH the Hb concentration is usually divided by red blood cell count and the result is multiplied by 10 and is expressed as pictograms (pg).

2.6 **Mean corpuscular hemoglobin concentration (MCHC)**

MCHC refers to the average concentration of the Hb in the red blood cells. In contrast to MCH, MCHC is not influenced by the size of the cell. For getting MCHC the hemoglobin is divided by packed cell volume and the result is multiplied by 100. The MCHC value is expressed in terms of percentage.

2.7 **White blood corpuscles (WBC) count**

Blood is drawn from the vial into WBC pipette up to 0.5 marks and immediately the diluting fluid is drawn up to 11 marks. The solution is mixed
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thoroughly by shaking gently. The rest of the procedure is the same as described by Davidson and Henry (1969) for RBC count. In case of WBC, count was made in bigger squares of the chamber. The WBC count was expressed in cu mm.

2.8 Differential leukocyte count

A drop of blood was placed on a clean glass slide about 1-2 cm from one end with the help of a spreading slide placed at an angle of 45° approximately. The drop of blood was spread out quickly along the line of control of the spreader with the slide. The slide was placed flat on two glass rods over a sink and was covered with Leishman stain. The stain was diluted by the drop by drop addition of buffered water and stained for a period of 5-7 minutes. The stain was drained and washed with water and air dried and observed under microscope.

Counting was started under high power oil immersion objective from the edge of the smear moving the smear towards center. Leucocytes were identified and the movement was repeated till a total 100 cells were counted. The values of different morphological types were expressed as the percentage.

3A. PROTEIN METABOLISM

3A.1 Estimation of Total proteins

The total protein content was estimated by the method of Lowry et al. (1951).

2% homogenates were prepared in 10 % TCA and centrifuged at 1000xg for 15 minutes. The supernatant was discarded and the residue was dissolved in a known amount of 1N sodium hydroxide. From this 0.2ml was taken and 4 ml of alkaline copper reagent and 0.4ml of folin phenol reagent (1:1 folin phenol: distilled water) was added. The contents were allowed to stand for 30 minutes at room temperature and the developed color was read at 600 nm in a spectrophotometer against a reagent blank.
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The amount of total proteins present in the sample was calculated by using bovine albumin standard and the values were expressed as mg/g wet weight of tissue.

3A.2 Estimation of Free amino acids (FAA)

Free amino acids content was estimated by the method of Moore and Stein (1954) as described by Colowick and Kaplan (1957).

5% homogenates of different tissues were prepared in 10% TCA and centrifuged for 15 minutes at 1000xg. To 0.25ml of the supernatant 2ml of ninhydrin reagent was added and kept in boiling water bath for 6.5 minutes and then cooled. The contents were made up to 10ml with distilled water. The intensity of the color developed was read at 570nm in a spectrophotometer against a reagent blank.

The total free amino acids content was expressed as μmoles of tyrosine equivalents /g wet weight of the tissue.

3A.3 Estimation of Protease activity

Protease activity was estimated by the method of Moore and Stein (1954) considering the amount of free amino acids liberated from the protein substances as a measure of proteolytic activity.

4% w/v homogenates were prepared in cold distilled water. The homogenates were centrifuged at 1000rpm for 10 minutes. The supernatant was used as enzyme source. The reaction mixture in a volume of 2 ml contained 100 μ moles of phosphate buffer (pH 7.4), 20 mg of heat denatured hemoglobin as substrate and 0.5ml of the supernatant. The contents were incubated at 37°C for 30 minutes and the reaction was stopped by the addition of 2 ml of 10% TCA. Zero time controls were conducted by adding 2 ml of 10% TCA prior to the addition of enzyme source. The contents of the
samples were filtered and the free amino acid level was determined in the filtrates. To 0.5 ml of aliquot of the filtrate, 2 ml of ninhydrin reagent was added. The contents were heated in boiling water bath for 5 minutes and cooled. The volume was made up to 10 ml with distilled water and read at 570 nm against a reagent blank in a spectrophotometer. All the samples were corrected with zero time controls.

The proteolytic activity was expressed as μ moles of tyrosine equivalents / mg protein / hr.

3A.4 Estimation of Aspartate aminotransferase (AST)

The activity of aspartate aminotransferase (AST) was assayed by the colorimetric method of Reitman and Frankel (1957) as described by Bergmeyer and Bernt (1965).

2 % w/v tissue homogenates of the selected tissues were prepared in 0.25 M ice cold sucrose solution. The homogenates were centrifuged at 1000xg for 15 minutes and supernatant was used for the enzyme assay. The incubation mixture of 2.0 ml contained 100 μ moles of phosphate buffer (Na₂HPO₄ + NaH₂PO₄) (pH 7.4), 100μmoles of L-aspartatic acid, 2 μ moles of α-keto glutarate and 0.5 ml of supernatant as enzyme source. After incubation for 30 minutes at 37°C, the reaction was stopped by the addition of 1 ml of ketone reagent (0.001 M, 2,4-dinitrophenyl hydrazine solution in 1 N HCl) and the contents were allowed to stay at laboratory temperature for 20 minutes. After 20 minutes of 10 ml of 0.4 N NaOH was added. The developed color was read at 545 nm in a spectrophotometer against a reagent blank.

The enzyme activity was expressed as μ moles of pyruvate formed / mg protein / hr.
3A.5 Estimation of Alanine aminotransferase (AIAT)

The activity of alanine aminotransferase (AIAT) was assayed by the colorimetric method of Reitman and Frankel (1957) as described by Bergmeyer and Bernt (1965).

The incubation mixture of 2 ml contained 100 µ moles of DL-alanine, 100 µ moles of phosphate buffer (pH 7.4), 2 µ moles of α-ketoglutarate and 0.5 ml of the supernatant of the homogenate 2% w/v prepared in 0.25 M ice-cold sucrose solution, as enzyme source. The reaction mixture was incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 1.0 ml of 2, 4-dinitrophenyl hydrazine solution prepared in 1 N HCl (ketone reagent). The color was developed by the addition of NaOH as described above for AST. The optical density was measured at 545 nm in a spectrophotometer against a reagent blank.

The enzyme activity was expressed as µ moles of pyruvate formed / mg protein / hr.

3A.6 Estimation of Glutamate dehydrogenase (GDH)

The activity of GDH was assayed by the method of Lee and Lardy (1965).

3% w/v tissue homogenate was prepared in ice cold 0.25M sucrose solution and centrifuged at 1000xg for 15 minutes. The supernatant was used as enzyme source. The reaction mixture in a volume of 2ml contained 100 µ moles of phosphate buffer (pH 7.2), 4.0 µ moles of sodium glutamate, 0.1 µ moles of NAD, 4 µ moles of INT and 0.2 ml enzyme source. The reaction mixture was incubated at 37°C for 30 minutes and the reaction was stopped by adding 5 ml of glacial acetic acid. Zero-time controls were maintained by adding 5 ml of glacial acetic acid prior to the addition of homogenate. The
formazon formed was extracted overnight in 5 ml of cold toluene. The intensity of color developed was read at 495 nm against a reagent blank in a spectrophotometer.

The enzyme activity was expressed as μ moles of formazon formed / mg protein / hr.

3A.7 Estimation of Ammonia

Ammonia was estimated by the method of Bergmeyer (1965).

Homogenates of tissues were prepared in 10 % TCA medium. The homogenates were centrifuged at 1000xg for 15 minutes. To the clear supernatant 2 ml of 15% sodium hydroxide was added. To this 0.5 ml of Nessler’s reagent was added and the optical density of the color was read immediately at 495 nm in a spectrophotometer against a reagent blank.

Ammonia content was expressed as μ moles of ammonia/g wet weight of tissue.

3A.8 Estimation of urea

Urea was estimated by the diacetylmonoxime method as described by Natelson (1971).

Tissues were isolated and homogenates were prepared in 15% PCA. To 0.5ml of the supernatant, 1 ml of acid mix (1:3 sulfuric acid: phosphoric acid) and 0.5ml of 2% diacetylmonoxime were added and vortexed. The contents were boiled in a water bath for 30 minutes and cooled to the laboratory temperature immediately. The optical density of the samples was read at 480nm against the reagent blank in a spectrophotometer. Urea content was expressed as μmoles of urea/g wet weight of tissue.
3B. Sodium Dodecyl Sulphate-polyacrylamidegel electrophoresis

Reagents

Staining solution

0.2 gm of coomassie brilliant blue was dissolved in 30 ml of methanol. To this 10 ml of acetic acid added and finally the volume was made up to 100ml with distilled water.

Destaining solution

30ml of methanol, 10 ml of glacial acetic acid were mixed and volume was made up to 100 ml with distilled water.

Acrylamide solution (30%)

100 ml Acrylamide solution was prepared by dissolving 30 g of Acrylamide, 0.8 g N,N-methylene-bis-acrylamide in 70 ml of distilled water. The contents were then filtered and the solution was finally adjusted to 100 ml.

Sample loading buffer

1.2 ml of 0.5M Tris (pH 8.0), 2 ml of 10% SDS, 1 ml of 10% glycerol, 0.5 ml of β-mercaptoethanol, 0.001 g of bromophenol blue were taken in to a 10 ml reagent bottle, mixed well and volume was made up to 10 ml and solution was stored at 4°C. If necessary 80 μl of loading buffer was taken.

Staking gel buffer

3.93 g of Tris was dissolved in the 50 ml of double distilled water and the pH of the solution was adjusted to 6.8 using 1N HCl of final volume of the buffer was made up to 100 ml with distilled water.
Running buffer

59.93 gm of Tris was dissolved in 400 ml of distilled water and pH of the solution was adjusted to 8.8 using 1N HCl. Final volume of the buffer solution was made up to 500 ml with distilled water.

Tank buffer (pH 8.5)

6.005g of Tris, 28.8 gm of glycine and 1g of SDS was dissolved in 500 ml of distilled water and PH of the solution was adjusted to 8.8 with 2N HCl. Finally the volume of the buffer was made up to 1000ml.

SDS- Polyacrylamide gel electrophoresis

The protein samples were separated by SDS- Polyacrylamide gel electrophoresis following the procedures of Laemeli (1970).

The solution was mixed well and degassed before adding ammonium persulphate. The contents were then poured in between two sealed glass plates containing 1 mm spacers to form a slab. These contents were over layered with 0.1 ml water saturated n-butanol and allowed to polymerize for 20 minutes at room temperature. After polymerization of running gel, butanol was removed by repeated washing and the traces of water were removed by wiping with filter paper strips.

The stacking gel solution was carefully over layered on running gel. Immediately a comb of required size was placed to form the wells. The stacking gel was allowed to polymerize for 20 minutes. After 20 minutes the comb was removed and the wells were washed with tank buffer (50M Tris HCl, 0.384M glycine, 0.1% SDS, pH 8.3). Protein samples were mixed with equal volumes of sample loading buffer (50M Tris HCl, PH 6.8, 2%SDS, 0.1% bromophenol blue, 10% glycerol, 100mM 2-mercaptoethanol) and kept in a boiling water bath for 5 minutes. The contents were then briefly centrifuges before loading the sample in to the wells. The electrophoresis was carried at 120 volts till the tracking dye reached the anode end of the gel. The gel was removed from the glass plate and the protein bands were stained with staining solution.
4.0 DETOXIFICATION ENZYMES

4.1. Estimation of xanthine oxidase

Xanthine oxidase activity was estimated by the dye reduction method of Srikanthan and Krishnamoorthy (1955). The assay mixture contained 100 mM sodium phosphate buffer (PH 7.4), 50 μ M of INT and the enzyme source. The reaction was initiated by the addition of enzyme source and incubated at 37°C for 30 minutes. The reaction was stopped by the addition of 5 ml of glacial acetic acid and the formazan formed overnight was extracted in toluene and read at 495nm against toluene blank. The activity was expressed as μM of formazan formed /mg protein / hour.

4.2. Estimation of superoxide dismutase

The activity of SOD was assayed by the reduction of nitro blue tetrazolium. Here the superoxide was produced by riboflavin mediated photochemical reaction system. Superoxide dismutase activity was determined according to the method of Beachamp and Fridovich (1971). Different tissues were homogenized in ice cold 50mM phosphate buffer (PH 7.0) containing 0.1 mM EDTA to give 5% homogenate (w/v). The homogenate were centrifuged at 10,000 rpm for 10 minutes at 0 °C in cold centrifuge. The supernatant was separated and used for enzyme assay. The reaction mixture contained 1.7 ml of phosphate buffer (PH 7.8), 150 ml EDTA (10 mM), 600 ml methionine (130 mM), 300 ml nitro blue tetrazolium (750mM) and the enzyme source. The reaction was initiated by the addition of riboflavin and the samples were placed under 15 watts fluorescence bulb for 30 minutes and the absorbance was taken at 560 nm against reagent blank kept in a dark place. A system, devoid of any superoxide radical scavenger was used as a positive control to compare the results.

The activity of the enzyme was expressed as units/mg protein.
4.3. Estimation of catalase activity

Catalase activity was measured by a slightly modified version of Aebi (1984) at room temperature. Different tissues were homogenized in ice-cold 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA to give 5% homogenate (w/v). The homogenates were centrifuged at 10,000 rpm for 10 minutes at 0 °C in cold centrifuge. The resulting supernatant was used as an enzyme source. 10 μl of 100% ethyl alcohol was added to 100 μl tissue extract and then placed in an ice bath for 30 min. After 30 min the tubes were kept at room temperature followed by the addition of 100 μl of Triton X-100 RS. In a cuvette containing 200 μl of phosphate buffer, 50μl of tissue extract and 250 μl of 0.066 M H₂O₂ (in phosphate buffer) was added and decrease in optical density was measured at 240 nm for 60 seconds in a UV spectrophotometer. The molar extinction coefficient of 43.6 με.m⁻¹ was used to determine Catalase activity.

One unit of activity is equal to the moles of H₂O₂ degraded/ mg protein/ min.

5.0 HISTOLOGY

Histological examinations of the tissues were followed according to Humason (1972).

5A. Light Microscopy

Tissues were isolated from control and chlorpyrifos treated rats. They were gently rinsed with a physiological saline solution (0.9% NaCl) to remove blood and debris adhering to the tissues. They were fixed in 5% formalin for 24 hrs. The fixative was removed by washing through running tap water overnight. After dehydrating through a graded series of alcohols, the tissues were cleared in methyl benzoate, embedded in paraffin wax. Sections were cut at 6μ thickness and stained with hematoxylin (Harris, 1900) and counter stained with eosin (dissolved in 95% alcohol). After dehydration and clearing, sections were mounted with DPX and observed under microscope.
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5B. Transmission Electron Microscopy

Tissue samples were isolated from freshly sacrificed control and experimental rats. The tissues were gently rinsed in physiological solution (0.9% NaCl) to remove blood and debris adhering to the tissue. Tissues were transferred to vials and fixed in 2.5% gluteraldehyde in 0.05M phosphate buffer (pH 7.2) for 24 hr at 4°C and post fixed 0.5% aqueous osmium tetroxide in the same buffer for 2 hr. After the post fixation samples were dehydrated in a series of graded alcohol, infiltrated and embedded in Araldite 6005 resin. Both Semi thin and Ultra thin sections were cut with a glass knife on a Leica Ultra cut UCT-GA-D/E-1/00 ultra microtome, Semi thin of 200-300nm thick were stained with toludine blue and ultra thin sections (50-70 nm thickness) were mounted on grids. Then the Ultra thin sections were stained with saturated aqueous uranyl acetate and counter stained with 4% lead citrate. Observed at various magnifications (Bozzola and Russell, 1999) and electron micrographic photographs taken under Hitachi H-7500 model (from JAPAN) transmission electron microscope at Ruska Lab, College of Veterinary Sciences, Sri Venkateswara Veterinary University (SVVU), Rajendra Nagar, Hyderabad, A.P.

VALIDITY OF EXPERIMENTAL PROCEDURES

Procurement of chemicals

1. All the chemicals used in this study were of analar grade and were procured from the following companies.

   i) Sigma, ii) BDH, iii) E. Merck, iv) Loba, v) Merck, vi) Kochlite

2. Aliquots for assay

   Aliquots were selected such that initial rates were approximated as nearly as possible yet providing sufficient product to fall in a convenient range for Spectrophotometric measurement.
3. **Enzyme units**

Enzyme activities were expressed in standard units i.e., μ moles of product formed or substrate utilized per mg protein per minute.

4. **Substrate requirements**

All the enzyme assays were made under the conditions following zero order kinetics.

5. **Lambert – Beer’s Law**

Almost all the products of the reactions were measured by colorimetric procedures in which the optical density (absorbance) of the resulting colored complexes was proportional to the concentration of the reaction products.

6. **Enzyme nomenclature**

The nomenclature of the enzyme used in the present context was according to the report of the commission on enzyme of the International Union of Biochemistry.

7. **Assay of dehydrogenases by using INT**

The advantage of using tetrazolium salts as electron acceptors are:

1. Tetrazolium salts give a stable color on reduction.
2. They are highly soluble in aqueous solution.
3. They can be reduced both aerobically and anaerobically.
4. They have high redox potential which makes the reduction easier.
5. They are freely permeable through membrane.

Various tetrazolium salts receive electrons from various sites of electron transport system (Nachlas et al., 1960). This is due to the inherent .
difference in the redox potentials of tetrazolium salts. The introduction of P-Nitro-Phenyl group in \( \text{N}_2 \) phenyl region was observed to increase the efficiency of the dye by increasing its redox potential. Karmarker et al., (1959) reported INT was superior to most of tetrazolium salt as an electron acceptor for the assay of dehydrogenase.

8. Statistical treatment of the data

The mean, standard deviation (SD), percent change and one-way analysis of variance (ANOVA) (Steel and Torrie, 1960) were performed using the SPSS package programming techniques on “Intel Core 2 Duo Processor” personnel computer. Probability values less than 0.05 were considered significant (Snedecor and Cochran, 1968).