

### **2.1 EXPERIMENTAL ANIMALS**

Healthy, young albino rats were selected and utilized for experimental work. Albino rats, both male and female 60-80 days old with mean body weight 250 gm were selected as experimental animals. The animals brought from Banaras and were acclimatized at laboratory conditions. They were fed proper animal food (Animal food care, Mumbai) and inspected regularly for any disease or parasitism. These animals were breed and reared in the best atmosphere. The rat were separated according to age and weight and brought up in the best healthy and hygienic conditions. The temperature of the cages was maintained 32°C ( $\pm 1^\circ\text{C}$ ) and the humidity was 80% ( $\pm 5\%$ ). Water was provided ad-libitum.

### **2.2 HERBICIDES**

- (i) 2,4-D, Technical grade was procured from Chemical Indian Limited, Haryana. The purity of 2,4-D was 80%.
- (ii) Butoxone (2,4-DB), Technical grade was obtained as a gift from M/s Ralis India Limited. The purity of Butoxone was 85%.

### **2.3 PURIFICATION OF HERBICIDES**

Purification of 2,4-D was done by column chromatography. The glass column was packed with silica and industrial product 2,4-D was treated with hexane as one of the solvent systems to remove the impurities and obtained the purest form of the compound for present

study. Hexane gave the best separation. From the vacuum dried compound fraction solutions were prepared.

#### **2.4 DETERMINATION OF SUBLETHAL CONCENTRATION OF BUTOXONE AND 2,4-D TOXICITY**

For parental application, 2,4-D and Butoxone were dissolved in distilled water. To calculate LD<sub>100</sub>, LD<sub>50</sub> and sublethal concentration, solutions of different amount of the 2,4-D and Butaxone were prepared in alcohol and water and given according to the body weight of the animal.

Eight Albino rats were taken and divided into two groups of 4 rats, each group consists of 2 males and 2 females. For determination of LD<sub>100</sub> 750 mg 2,4-D/Kg body weight in 1 ml of distilled water was infused intraperitoneal to the rats of first group. To the rats of second group, a solution of 700 mg Butoxone/Kg body weight in 1 ml of distilled water was infused intraperitoneally. None of the so treated rats were survived after 96 hrs (LD<sub>100</sub>).

The experiment was repeated with other 8 albino rats divided similarly into two groups and herbicidal doses administered were 375 mg 2,4-D/Kg body weight and 350 mg Butoxone/Kg body weight. The treated rats were observed for 96 hrs and it was found that 50% rats of each group died and rest 50% survived (LD<sub>50</sub>).

The experiment was repeated with other group of 8 rats divided similarly into two groups. Herbicidal doses administered were 185 mg

2,4-D/kg body weight and 175 mg butoxone/kg body weight separately to two groups of rats. All experimental rats survived for 96 hrs (LD<sub>0</sub>).

Above experiment was conducted with fresh batch of 8 albino rats divided as earlier groups. Doses of 130 mg 2,4-D/kg body weight and 100 mg butoxone/kg body weight, were administered intraperitoneally to the experimental rats and were observed upto 6 weeks. All the rats survived and were found active. Thus, these doses i.e. 130 mg/kg body weight of 2,4-D and 100 mg/kg body weight of butoxone were found to be sublethal for the experimental albino rats. The results are presented in table 2.1 and 2.2 for the determination of LD<sub>100</sub>, LD<sub>50</sub>, LD<sub>0</sub> and sublethal doses.

## **2.5 BIOCHEMICAL STUDIES**

To investigate the effect of 2,4-D and Butoxone, healthy rats of both sex upto the age of 60-80 days and weighing between 200-250 gm were selected. Rats were divided into many groups, with 4 rats in each group. 2,4-D and Butoxone both were given parentally i.e. intraperitoneally. For 2,4-D 5 mg/ml and for Butoxone 10 mg/ml were given every day to the rats of different groups. After the injection of toxicant the rats were watched carefully for any changes in their behaviour.

The various biochemical parameters were studied at intervals of 1<sup>st</sup> day, 5<sup>th</sup> day, 15<sup>th</sup> day, 25<sup>th</sup> day, 35<sup>th</sup> day, 75<sup>th</sup> day, 115<sup>th</sup> day and 145<sup>th</sup> day. Blood was obtained from retro-orbital plexus and centrifuged in a capillary, 1% sodium citrate solution was used as an anticoagulant. After centrifugation at 5000 rpm for 10 min, serum was collected, marked and refrigerated.

### **2.5.1 Determination of serum Glutamate oxalate Transaminase (SGOT)**

#### **Method**

SGPT was estimated by UV kinetic method using span Diagnostic Reagent kit.

#### **Principle**

$\alpha$ -Oxoglutarate + L - aspartate = L - glutamine + oxaloacetate

NDH + NADH<sub>2</sub> = L - malate + NAD

**Normal Values**

<b>Temperature</b>	<b>25°C</b>	<b>30°C</b>	<b>37°C</b>
Rat	Up to 18/ $\mu$ /L	Up to 25/ $\mu$ /L	Up to 37/ $\mu$ /L
Female rat	Up to 15/ $\mu$ /L	Up to 21/ $\mu$ /L	Up to 31/ $\mu$ /L

**Sample material**

Blood serum

**Reagent**

<b>Buffered substrate</b>	<b>Concentration in test</b>
Tris buffer (ph - 7.5)	80 mmol/L
Aspartate	240 mmol/L
$\alpha$ -Oxoglutarate	12.0 mmol/L
MDH	0.42 $\mu$ /ml
NADH <sub>2</sub>	0.18 mmol/L

**Stability of the reagent**

The reagent 1 and 2 are stable between 2°C and 8°C for 6 month. The reagent 3 and 4 should be prepared fresh for the test from the stock solution, reagent 5 should be prepared fresh which is stable between 0°C to 4°C for 5 days.

**Procedure**

Wave length : 340 nm  
Cuvette : 1 cm light path  
Temperature : 25°C, 30°C, 37°C

Pipette in to cuvette :

Buffered substrate	:	1.0 ml
NADH <sub>2</sub>	:	0.02 ml
MDH	:	0.01 ml
Mix and all sample	:	0.02 ml
Oxoglutarate	:	0.04 ml

Mix and read initial absorbance. Note readings after 1, 2, and 3 min. determine the mean absorbance change per min. (A/min)

### **Calculation**

SGOT : U/L = 1051 × A/Min.

## **2.5.2 Determination of Serum Glutamate Pyruvate Transaminase (SGPT)**

### **Method**

Estimation of SGPT was based on UV - kinetic using span diagnostic kit.

### **Principle**

GOT catalyses the following reaction:

L-oxoglutarate + alanine = L-glutamate + pyruvate + NADH<sub>2</sub> = Lactate + NAD

### **Normal value**

<b>Temperature</b>	<b>25°C</b>	<b>30°C</b>	<b>37°C</b>
Rat	Up to 27/μ/L	Up to 29/μ/L	Up to 40/μ/L
Female rat	Up to 17/μ/L	Up to 22/μ/L	Up to 31/μ/L

**Sample material**

Blood serum

**Reagent**

<b>Buffered substrate</b>	<b>Concentration in test</b>
Tris buffer + alanine (ph - 7.5)	Tris - 100 m mol/L and alanine - 500 m mol/L
L - oxoglutarate	12.0 m mol/L
LDH	1.2 $\mu$ /ml
NADH	0.18 m mol/L

**Stability of the reagent**

The reagent 1 and 2 are stable between 2.8<sup>o</sup>C for 6 month. The reagent 3 is prepared fresh from the stock solution. Reagent 4 is prepared fresh (stable between 0.4<sup>o</sup>C for 5 days).

**Procedure**

Wave length : 340 nm  
Cuvette : 1 cm light path  
Temperature : 25<sup>o</sup>C, 30<sup>o</sup>C, 37<sup>o</sup>C

**Pipette in to cuvette :**

Buffered substrate : 1.0 ml  
NADH : 0.02 ml  
LDH : 0.02 ml  
sample : 0.02 ml  
L - Oxoglutarate : 0.04 ml

**Calculation**

SGPT : U/L = 1051 x A/ Min.

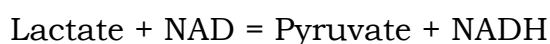


**Procedure limitation**

Specimen was diluted in normal saline and then the test was repeated. For calculations the dilution factor was taken into consideration.

**2.5.3 Determination of Serum Lactate Dehydrogenase**

Lactate dehydrogenase (LDH) catalyses the conversion of pyruvate to lactate with the oxidation of NADH.



Products so formed are coupled with 2, 4 - dinitrophenylhydrazine (2,4 - DNPH) to give the corresponding hydrazone, which gives brown colour in alkaline medium and is measured colorimetrically with blue filter.

**Reagent**

The diagnostic kit contained six reagents:

- Reagent 1 : Buffered lactate substrate, PH 10.0
- Reagent 2 (A) : NAD for test
- Reagent 2 (B) : NAD for graph
- Reagent 3 : DNPH colour reagent
- Reagent 4 : NADH
- Reagent 5 : sodium hydroxide , 4 N
- Reagent 6 : working pyruvate standard, 1 m m

### **Preparation of working solution**

For the estimation of LDH, reagent 2 (A) and 2 (B) were mixed with 0.3 ml and 1.4 ml of distilled water to prepare solution 1 (A) for test, and 1 (B) for graph respectively. Reagent 4 was diluted with 1.2 ml of reagent 1 (buffered substrate) and reagent 5 (sodium hydroxide) was diluted 10 times with distilled water. Serum was diluted 5 times with normal saline. Reagent 1, 3 and 6 are prepared and brought at room temperature before use.

### **Procedure**

Dry and clean test tubes, marked with 'C' for control and 'T' for test. 1 ml of reagent 1 was mixed in both the tubes, 0.1 ml of dilute serum was added in tube 'T' and 0.2 ml of distilled water was added to tube 'C', mixed well and incubated at 37°C for 5 minute. 0.2 ml of solution 1 (A) was mixed in tube 'T', incubated at 37°C for 15 minutes. 0.1 ml of reagent 3 was mixed in both the tubes and 0.1 ml diluted serum was added in tube 'C' and incubated at 37°C for 15 minutes. 10 ml of solution 1 was added in both the tubes, mixed well by inversion, and allowed to stand at room temperature for 5 minutes. O.D. of test and standard was measured against distilled water on a colorimeter, using blue filter. Reagent 6 working pyruvate standard was used for standard graph.

**Calculation**

The net O.D. of test ( $T_n$ ) was calculated as follows:

$$T_n = \text{O.D. Test} - \text{O.D. control}$$

Net O.D. of Test ( $T_n$ ) was marked on Y - axis of the standard curve and extrapolated to the corresponding enzyme activity (IU/litre) on X - axis.

**2.6 STATISTICAL ANALYSIS**

Results for enzymes activity in rats have been showed as mean + SD (standard deviation) or as mean  $\pm$  SEM (standard error of mean). The variation present in a set of data was analysed using one- way analysis of variance (ANOVA) by least significant difference at 99.5 (0.05), 99.9% ( $P < 0.001$ ) confidence level:

**Mean**

$$\bar{X} = \frac{1}{n} \sum_{i=1}^n x_i$$

$\bar{X}$  = Arithmetic mean

n = Number of observation

$x_i$  = Values of observation

**Standard Deviation**

$$\sigma = \frac{\sqrt{\sum d^2}}{n}$$

$\sigma$  = Standard Deviation

d = Deviation

n = Number of observation

**Standard Error**

$$\text{S. E. of } \bar{X} = \frac{s}{\sqrt{n}}$$

$\bar{X}$  = Arithmetic mean

S = Standard deviation

n = Number of observation

## **2.7 HISTOLOGICAL STUDY**

### **2.7.1 Tissue Fixation and Fixatives**

For the histopathological studies of thyroid, pancreas, liver, testis and ovaries, the tissues were fixed in Cornoy's fixatives for 24 hrs (Pearse, 1985).

#### **Cornoy's fluid**

Absolute Alcohol	:	60 ml
Chloroform	:	30 ml
Glacial acetic acid	:	10 ml

Cornoy's fluid penetrates very fast and gives an excellent nuclear fixation with preservation of glucogen and Nissl's substance.

After fixation, the material was thoroughly washed with 70% alcohol and dehydrated gradually in 70%, 90% of alcohol and with two changes of absolute alcohol. The material was cleaned in xylene, cleaned material was transferred to molten paraffin wax and three changes were given to the material. The tissue was embedded in the wax and paraffin blocks were prepared. Sections were cut at 6  $\mu$  with the help of microtome.

### **2.7.2 Staining Techniqnes**

Sections were stained with double staining techniques. In double staining techniques hematoxylin and eosin were used. Hematoxylin is a nuclear stain and eosin is a cytoplasmic stain.