C H A P T E R - II

MATERIAL AND METHODS

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Male mice were used for the present investigation. Mice were obtained from Hindustan Antibiotics, Pune and maintained in the animal house in separate cages. They were supplied with Gold-Mohur Rat Feed (Lipton India Limited), and water ad-libitum and were maintained under proper condition of light, temperature and humidity.

Animals were divided into three groups.

Juvenile group : (4 to 7week, weight 24 to 28 gm)

Adult group: (15 to 27 week, weight 36 to 41 gm)

Old group: (70 to 75 week, weight 55 to 62 gm)

A minimum of five mice from each group were sacrificed by cervical dislocation, liver was excised and subjected to homogenization.

METHODS :

Preparation of Cell Free Homogenate :

Homogenization of liver was carried out using glass morter and pestle. The glass morter and pestle were well washed,rinsed

with distilled water, dried and kept in a refrigerator at least 4 to 5 hrs at $4^{o}C$.

Liver was removed and dropped into a cold beaker containing homogenization medium (0,25 M sucrose containing 1 m M EDTA) blotted on filter paper, weighed and minced with cold scissors. It was crushed at the bottom of the morter with the help of pestle. Homogenate was fractionated by cold centrifugation, initially at low speed 3000 rpm for 10 min. at $10^{o}C$ i.e. sample A. This low speed was taken for estimation of acid phosphatase activity and also subjected to get following samples :

Sample B : In 9.8 ml low speed supernatant (Sample A), 0.2 ml Triton X-100 (10 %) was added for the liberation of bound acid phosphatase. Sample was used for the estimation of the enzyme activity.

Sample C : The sample A was centrifuged at high speed (12,000 rpm) for 10 minutes at $10^{o}C$. Supernatant was taken for the estimation of microsomal acid phosphatase activity.

Sample D : The pellet obtained in sample C was suspended in 9.8 ml ml 3 mM Tris HCl buffer pH 7.0 and 0.2 Ml Triton X-100 (10%) centrifuged for 10 minutes at high speed (12000 rpm), Supernatant was used for the determination of lysosomal acid phosphatase activity.

Assay of Acid Phosphatase :

Acid phosphatase activities were routinely measured at $37^{o}C$ for 30 minutes at pH 4.8, by the rate of liberation of p-nitrophenol from p-nitrophenyl phosphate (Linhardt and Walter, 1965). Assay mixture contained 5.5 X 10^{-3} M of p-nitrophenyl phosphate, 0.05 M citrate buffer pH 4.8 and 0.2 ml enzyme sample in a total volume of 1.2 ml. Reactions were stopped by the addition of 4 ml 0.1N NaOH.The absorbance of each sample was measured at 405 nm. Enzyme activity was defined as 1 μ mols of p-nitrophenol liberated per gm wet weight of the tissue, and per minute, per mg of protein.

Effect of pH on Enzyme Activity :

Estimations of the enzyme activities were carried out in o.05 M sodium citrate buffers of pH 4.0 to 6.2 with a difference of 0.2. A series of citrate buffers were prepared according to Gomori (1955) and Burstone (1962). The substrate concentration (5.5 X 10^{-3} M) was kept constant. The acid phosphatase activities/ gm wet weight of the tissue were plotted on the graph paper as activity against pH.

Effect of Temperature on Enzyme Activity:

To determine the effect of temperature on enzyme activity enzyme estimations were carried out as follows :

Sample tubes were prepared by adding 1 ml substrate buffer solution, and 0.2 ml enzyme sample. Sample tubes were incubated for each temperature such as 20^{o}, 30^{o}, 40^{o}, 50^{o}, 60^{o}, 70^{o}, 80^{o}, 90^{o} and 100^{o}C for 5'. Reactions were stopped by the addition of 4 ml 0.1 N NaOH. The readings were taken at 405 nm, using corresponding sample free controls.

Effect of Time on Enzyme Activity:

To determine the effect of time on enzyme activity enzyme estimations were carried out as follows :

Sample tubes were prepared by adding 1 ml substrate buffer solution and 0.2 ml enzyme sample. Sample tubes were incubated 37^{o}C at different time intervals, such as 10', 20', 30', 40',50', 60' and 70'. Reactions were stopped by the addition of 4 ml O.1N NaOH. The readings were taken at 405 nm, using corresponding sample free controls.

Effect of Substrate Concentration on Enzyme Activity :

To determine the values of Km and Vmax of acid phosphatase following p-nitrophenyl phosphate substrate concentrations were prepared.

i)	0.68×10^{-3} M	viii)	6.18×10^{-3}M
ii)	1.37×10^{-3} M	ix)	6.87×10^{-3}M
iii)	2.75×10^{-3} M	x)	7.55×10^{-3}M
iv)	3.43×10^{-3}M	xi)	8.25×10^{-3}M
v)	4.12×10^{-3}M	xii)	8.93×10^{-3}M
vi)	4.81×10^{-3}M	xiii)	9.62×10^{-3}M
vii)	5.5×10^{-3} M		

The experimental procedure followed the same protocol as before, keeping time, temperature and pH constant. The enzyme concentration was also kept constant, only the amount of substrate added varied the total volumes of reaction mixture was the same.

Effect of Inhibitor on Km. :

To determine the effect of inhibitor on the Km of the enzyme the activity of acid phosphatase was estimated using p-nitrophenyl phosphate of different concentrations containing 0.02 M L(+) Na Tartrate. Throughout the experimental procedure the time, temperature, pH, enzyme and inhibitor concentrations were kept constant. Concentrations of p-nitrophenyl phosphate were same as earlier.

Effect of Heat Inactivation on Enzvme Activity :

The effect of heat inactivation on enzyme activity was determined by following experiment.

0.2 ml samples in 0.5 ml 0.05 M sodium citrate buffer were treated at different temperatures 20°, 30°, 40°, 50°, 70°, 80°, 90° and 100°C for 10' minutes.

The sample tubes were prepared by the addition of 1 ml substrate (5.5 X 10^{-3} M p-nitrophenyl phosphate) buffer solution in treated samples. Tubes were incubated at optimal temperature of 37°C for 30 minutes. The reaction was stopped by the addition of 4 ml 0.1 N NaOH readings were taken at 405 nm, adjusting colorimeter with sample free controls.

Estimation of Proteins:

Protein was estimated according to Lowry et al. (1951) in the homogenates of liver samples using bovine serum albumin as a standard.

Determination of Lipid Peroxide Oxidation Rate in Liver Homogenate:

This method is based on ;the determination of malonic dialdehyde, an end product of lipid peroxide oxidation, which can react with thiobarbituric acid to yield a pink coloured trimethine complex exhibiting an absorption maximum at 530-532 nm.

10 mg freshly excized liver was taken in 10 ml cold (1°C) KCl. The tissue was crushed with the help of cold glass morter

and pestle. 2 ml homogenate was transferred into three test tubes labelled as 1, 2 and 3 and following addition were made. In second tube add 0.1 ml ascorbic acid solution and 0.1 ml Mohr salt solution, and in third test tube, transfer the same components as have been added to the second tube and, in addition, add 1 ml TCA (Trichloroacetic acid) solution.

All the tubes were incubated at $37^{o}C$ for 10 minutes and then 1 ml 40 % TCA was added in first and second tube. Test tubes were centrifuged for 10 minutes at 3000 rpm. 2ml of supernatant was transferred into three clean, dry test tubes, added 1 ml of thiobarbituric acid to each and tubes were placed in a boiling water bath for 10 minutes and then cooled in ice water to room temperature.

Controls were prepared as 2 ml of potassium chloride solution. 1 ml thiobarbituric acid solution were placed in boiling water bath for 10 minutes and then cooled in ice water to room temperatureand readings were taken at 532 nm.

Calculations were carried out by the equations :

$$X_1(X_2) = \frac{E_1\ (E_2)\ 3\ X\ 3.2\ X\ 6}{0.156\ X\ 2}$$

$$X_3 = \frac{E_3\ 3\ X\ 3.2}{0.156\ X\ 2}$$

E_1, E_2 and E_3 are the absorbance values measured respectively for the first, second and third tubes.

3.2 is the total volume of the samples taken for analysis.

X_1 = is the spontaneous lipid peroxide oxidation in the homogenates measured in nanomoles of malonic dialdehyde formed in the sample during an incubation period of one hr.

X_2 = is the rate of ascorbate - dependent nonenzymic peroxide oxidation of lipids measured in nanomoles of malonic dialdehyde formed in the sample.

X_3 = is the amount of malonic dialdehyde in the initial homogenate.

Statistical Methods (Fisher, 1936; Snedecor,1946 and Wilks,1949).

Group values were expressed as mean ± S.D. student 't' test was used to determine statistical significant difference. A, P value less than 0.05 was accepted as significant.

i) $\bar{X} = \frac{\Sigma X}{N}$ where $\bar{X}$ = arithmatic mean

N = number of observations

X = variable

ii) $S.D.(S) = \sqrt{\frac{\Sigma x^2}{N}}$ where X = deviation from mean

S.D.(S) = Standard deviation

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iii) $$S = \sqrt{\frac{(n_1-1)\ S_1^2 + (n_2-1)\ S_2^2}{n_2 + n_2 - 2}}$$

where S = Pulled standard deviation

S_1 = Standard deviation of first group

S_2 = Standard deviation of second group

iv) $$t = \frac{\bar{X}_1 - \bar{X}_2}{S} \times \sqrt{\frac{n_1 n_2}{n_1 + n_2}}$$

$\bar{X}_1$ = mean of first group

$\bar{X}_2$ = mean of second group.

t = Student 't' test.