MATERIAL AND METHODS
1. **Procurement of Material:**

Twenty-one day old immature male Wistar strain albino rats were used for the present study. This age of rats was selected because, the secretion of PRL from the hypophysis was low and negligible thereby eliminating the possibility of interference of endogenous PRL (Barrette, 1976) and at this period rats were highly sensitive to exogenous gonadotropin treatment (Shikata and Hall, 1967a, b; Sandler and Hall, 1968). The parents of the litters were obtained from Indian Institute of Science, Bangalore, and a rat colony was maintained in the laboratory at 27°C ± 2°C and 12 hrs light and 12 hrs darkness. They were fed on standard rat diet obtained from Hindustan Lever Ltd., Bombay, India and water was supplied ad libitum. The rats were kept in individual cages during the course of treatment.

The experimental group of rats received subcutaneous injections of prolactin (1.0 μg/gm body weight) dissolved in saline, daily for five days. The control group of rats received similar volume of physiological saline (Hafiez et al., 1972a, b).

The rats were sacrificed by decapitation on post-treatment day one and the tissues like testis, epididymis,
semenal vesicles, prostate gland, brain, liver, kidney, and gastrocnemius muscle were isolated with least injury and kept immediately in freezer compartment of refrigerator. In the case of bilateral organs, tissue minces were obtained from both the sides and in other cases the tissue minces were obtained from the entire organs and used for experimentation.

2. **Estimation of Tissue Somatic Index (TSI):**

The body weight of the rats was taken before decapitation and the complete tissues were isolated. These tissues were weighed accurately using single pan electrical balance (Sartorius, Germany). The tissue somatic indices of these tissues were determined using the formula:

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\text{TSI} = \left( \frac{\text{Weight of the organ}}{\text{Weight of the body}} \right) \times 100
\]

3. **Tissue gravimetric changes:**

Reproductive and non-reproductive organs were carefully isolated from the normal male albino rats of different age groups. At each age group, organs were isolated from six rats and mean value was taken with standard deviation. A graph was plotted between age of the rats on one side and organ weights on the other, and normal growth rates of these
organs were obtained. From these growth rate patterns of different organs of normal rats, the equivalent appropriate ages for PRL treated organs were obtained from their respective weights as represented in the graph.

4. **Estimation of Acetylcholinesterase (AchE), E.C.3.1.1.7 activity.**

Control and experimental rat tissues were isolated and 2% homogenates were prepared in ice-cold 0.25 M sucrose solution using glass mortar and pestle. The levels of AchE activity in these homogenates were determined by the method of Metcalf (1951). The incubation mixture of a total volume of 2.5 ml contains 8 μ moles of buffered substrate, and 0.5 ml of homogenate. The reaction mixture was incubated for 30 mins at 37°C and arrested by the addition of 2.0 ml of cold alkaline hydroxyl amine hydrochloride and 1.0 ml of (1%HCl: Distilled water. The contents were filtered and to the supernatant, 1 ml of ferric chloride reagent was added. Simultaneously zero time controls were maintained for each tissue by the addition of 2.0 ml of alkaline hydroxylamine hydrochloride and 1.0 ml (1:1) HCl: Distilled water before the addition of homogenate. The color developed was read at 540 mμ in Spectrophotometer (Elico, Hyd, India) and the enzyme activity was expressed in μ moles Acetylcholine chloride hydrolysed/gm dry wt/hr.
5. Estimation of Proteins:

Control and experimental animal tissues were homogenized separately in glass distilled water and these homogenates were centrifuged at 3000 rpm for 30 minutes. The protein content in the supernatant (Water-soluble proteins) and residual (Structural-Water insoluble) fractions of the homogenates were estimated by the method of Lowry et al. (1951). Bovine serum albumen was taken for the preparation of standard graph. The protein values were expressed in mg protein/gm dry wt of the tissue.

6. Estimation of Nucleic Acids:

The levels of nucleic acids in the tissues were estimated by the methods of Munro and Fleck (1966) as given by Glick (1966) and Giles and Myers (1965) for estimation of RNA and DNA respectively. The tissue slices below 100 mg weight were taken and homogenized separately in 5 ml of methanol and centrifuged at 3000 rpm for 10 minutes. The supernatant was discarded, 5 ml of methanol was added, mixed well, centrifuged and the supernatant was discarded. The residues were treated in a similar manner with ethanol, ethanol: ether (2:1) mixture, 5% (v/v) TCA, ether. Ultimately 5 ml of 5% PCA was added to the residues, mixed well and centrifuged. The supernatants were carefully collected into separate tubes. These supernatants were used for estimation of nucleic acids.
(a) Estimation of DNA:

1.0 ml of PCA extract (supernatant) was added to 2.5 ml of di-phenyl amine reagent, mixed well and heated in a hot water bath for 10 minutes and the contents were cooled, later made up to 5 ml with distilled water. The developed colour was read out at 560 mμ in spectrophotometer. DNA content determined by using the standard graph and expressed as mg DNA/gm dry wt of the tissue.

(b) Estimation of RNA:

1.0 ml PCA extract was added to 5 ml of orcinol colour reagent, mixed well, and heated in a hot water bath for 30 minutes cooled and the developed colour was read out at 660 mμ in spectrometer. RNA content was determined by using standard graph and expressed as mg RNA/gm dry wt of the tissue.

7. Estimation of protease activity:

Protease activity was estimated by the method of Moore and Stein (1957) with slight modification. The assay mixture in a final volume of 2.5 ml contained 100 μM of potassium phosphate buffer (pH 7.0), 10 mg of heat denatured haemoglobin and 1 ml of 5% hemogenate prepared in ice-cold distilled water. The reaction mixture was incubated at 37°C.
for one hour and stopped by the addition of 2 ml of 10% TCA. Zero time controls (ZTC) were treated with 2.0 ml TCA prior to the addition of homogenate. The incubated samples and ZTC were filtered and free amino acid levels were estimated in the filtrates as follows.

0.5 ml of filtrate was added to 2.0 ml of phenhydrazine and kept in boiling water bath for 6.5 minutes and then cooled. The solution was made up to 10 ml by distilled water in each tube. The colour developed was read out at 570 m/μ in spectrophotometer. All the samples were correlated for ZTC. The protease activity was expressed in mg tyrosine equivalents/gm dry wt/hr.

8. Estimation of free amino acids:

Total free amino acids were estimated by the method of Moore and Stein (1954) as given by Colowick and Kaplan (1957). 5% (w/v) tissue homogenates were prepared in 10% TCA and centrifuged at 3000 rpm for 10 minutes. The amino acids content in the filtrates were estimated as described above.

9. Estimation of Total Carbohydrates:

Total carbohydrate content was estimated by the method of Caroll at al (1956). All the tissues were ground separately
in 10% TCA and centrifuged at 3000 rpm for 15 minutes. 1 ml of TCA filtrate was added to 5 ml of anthrone reagent and boiled for 15 minutes. The tubes were cooled and the colour was read out at 620 m/μ in spectrophotometer. The total carbohydrate content was determined by using standard graph and expressed in mg/gm dry wt.

10. Assay of succinate dehydrogenase (SDH) (succinate: acceptor oxidoreductase E.C.: 1.3.99.1); Glutamate dehydrogenase (GDI) (L-glutamate NAD (P) oxidoreductase E.C.: 1.4.1.3); Lactate dehydrogenase (LDH) (L-Lactate NAD oxidoreductase E.C.: 1.1.1.27).

The activity levels of SDH (by the method of Nachlas et al., 1960) GDH (by the method of Lee and Lardy, 1965) LDH (by the method of Srikantan and Krishnamurthy as modified by Reddianna and Govindappa, 1978) were estimated. 2% (w/v) homogenates of all the tissues were prepared in ice-cold 0.25 M sucrose solution and centrifuged at 3000 rpm for 15 minutes. The supernatant fraction was used for enzyme assay.

The reaction mixture in a final volume of 2 ml contains 40 μM of substrate (sodium succinate for SDH, sodium lactate for LDH, and sodium L-glutamate for GDH), 0.1 μM of NAD (for LDH and GDH only), 100 μM of 7.4 pH buffer and 2 μM
of INT (2 - 4 Iodophenyl - 3(4 - nitrophenyl)-5-phenyl tetrazolium chloride). The reaction was initiated by the addition of 0.5 ml of tissue extracts. The incubation was carried out for 30 minutes at 37°C and stopped by the addition of 5.0 ml glacial acetic acid. The formazan formed was extracted overnight in cold (10°C) with 5 ml of toluene. The intensity of colour was read out at 495 m/μ against toluene blank. The activities of SDH, GDH and LDH were expressed by μ moles of formazan formed/g dry wt/hr.

11. Estimation of Acid phosphatase (ACP), (E.C. 3.1.3.1) and Alkaline phosphatase (ALP (E.C. 3.1.3.2) activity levels:

ACP and ALP activity levels were estimated by the method of Shinowara, Jones and Reinhart (1942) method as modified by Fiske and Subba Row (1925). 4%(w/v) homogenates were prepared in ice-cold 0.25 M sucrose solution and centrifuged at 300 rpm for 15 minutes. The supernatant fractions were used for enzyme assay.

The total reaction mixture of 10 ml contains 9.5 ml of alkaline or acid buffered substrate and 0.5 ml of tissue supernatant. The reaction mixture was incubated at 37°C for 1 hr. The reaction was stopped by the addition of 2 ml of
30% TCA and mixed well and filtered. 1 ml of ammonium molybdate solution was added to 9 ml of filtrate followed by 0.4 ml of L- amino-naphthosulfonic acid reagent. The contents were made up to 10 ml with distilled water. The tubes were kept for 5 minutes and the colour was read at 660 m\(\mu\) in spectrophotometer. Simultaneously 37°C were maintained by adding 2 ml of 30% TCA solution prior to the addition of homogenate. Phosphate content was determined by using standard graph. The levels of phosphatase activities were expressed in mg phosphorus liberated per gm dry wt per hr.

12. Estimation of total lipids:

The total lipid content was estimated by gravimetric method. All the tissues were isolated and kept in hot air oven (110°C ± 2°C) for 2 days. The dry tissues were ground separately and the weight of dry tissue powders were carefully transferred into centrifuge tubes which were already weighed. 2.0 ml of methanol was added to each tube, stirred well, centrifuged and supernatant was discarded. The same process was repeated once again. Similarly the process was repeated with methanol:chloroform (2:1) mixture and ether separately. Finally the tubes were kept in hot air oven for one hour and the weight of the tubes with the residue was noticed down. This was repeated till 2 consequent values were coincided. The
difference between initial and final weights of tissue powders had given the total lipid content and was expressed in mg lipids per gm dry wt of the tissue.

13. Estimation of phospholipids:

The phospholipid content in the tissues was estimated by the method of Zilversmith and Davis (1950) and the liberated phosphorus was estimated by the method of Fiske and Subba Row (1925).

All the tissues (approximately 50 mg) were homogenized in 10% TCA separately and centrifuged at 3000 rpm for 15 minutes. The supernatants were discarded and to the residue 2 ml of 60% perchloric acid was added, mixed well and heated at 60°C. After decolourization of the contents, tubes were removed from the mantle heater and cooled. The contents were made up to 8 ml with glass distilled water and inorganic phosphorus content was estimated. Simultaneously inorganic phosphorus standards were carried out. The phospholipid content was expressed in mg phospholipids per gm dry wt of the tissue.

14. Estimation of total cholesterol:

Total cholesterol content was estimated by the method of Henly (1957). Approximately 50 mg of the tissue was taken and homogenized in 1 ml of 10% TCA. 9.0 ml of ferric chloride
acetic acid reagent was added to individual clean test
tubes and 1.0 ml of homogenate was added. The tubes were
kept in hot water baths for 10 minutes, cooled, and centri-
fuged at 3000 rpm for 10 minutes. 5 ml of supernatant was
transferred to separate tubes and 3.0 ml concentrated sul-
phuric acid was added and mixed well. The colour was read
at 560 m/µ in spectrophotometer. The total cholesterol con-
tent was determined by using standard graph and expressed
in mg cholesterol per gm dry wt of the tissue.

Kinetic Studies:

The mean values of enzyme activity levels of four
values were employed for all the tissues and plotted as 1/V
against 1/S where 'V' was the reaction velocity and 'S' the
substrate concentration as per the method of Lineweaver and
Burk (1934). The slopes, intercepts, Vmax and Km were calcu-
lated by the method of least squares and found to be coincided
with the values obtained from the Line Weaver-Burk Plots.

Validity of Analytical Procedures:

a. Aliquots of assay: Aliquots were selected such that in-
itial rates were approximated as nearly as possible, yet
providing sufficient product to fall in convenient range
for spectrophotometric measurement.
b. **Substrate requirement:** All the assays were made under the conditions following zero order kinetics.

c. **Lambert-Beer Law:** Almost all the products of the reactions were measured by colorimetric procedures in which the optical density (absorbance) of the resulting coloured complexes are proportional to the concentration of the reaction products.

d. **Assay of dehydrogenases using INT:**

The advantage of using tetrazolium salts as electron acceptors were:

1. the tetrazolium salts give stable coloured formazans on reduction.
2. they are highly soluble in aqueous solutions.
3. they can be reduced both aerobically and anaerobically.
4. they have high redox potential which makes the reduction easier, and
5. they are freely permeable through membranes.

Karmarkar *et al* (1959) reported that INT was superior to most of the tetrazolium salts as an electron acceptor for the assay of dehydrogenases.