# CHAPTER - II

## MATERIALS AND METHODS

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With the objective of studying the effect of corticosterone administration on developing rat brain regions, the following three aspects were studied :-

(a) Studies were carried out on the effect of chronic corticosterone administration on GABA-related enzymes such as Glutamate decarboxylase (GAD), GABA-transaminase (GABA-T), glutamine synthetase (GS) and glutamate dehydrogenase (GDH) from rat brain regions such as the hippocampus, hypothalamus, striatum and cerebellum at 10, 20, 40 days postnatal and in adult ( $>$90 days). Since the information about developmental changes in atleast some of these regions with respect to GAD, GABA-T, GS and GDH were not available at that time, developmental pattern of these enzymes were studied initially, followed by an experiment to find out the suitable corticosterone dosage required in various brain regions in addition specificity of such response was studied by injecting other steroid hormones viz., testosterone and estrogen.

(b) Studies on the effect of biogenic amines by chronic corticosterone treatment in the above mentioned regions were carried out.

(i) A developmental profile of biogenic amines DA, NE and 5HT, their precursor p-tyr and tryptophan, and metabolites DOPAC, HVA and 5HIAA in the hippocampus, hypothalamus and striatum was studied at 10, 30, 50 days and in adult ( >90 days). A separate experiment was performed in order to avoid possible alterations in their levels due to the stress of injection in control animals of the corticosterone experiment.

(ii) The effect of chronic corticosterone treatment on these above mentioned paramters and regions was studied in 10, 30 and >90 days animals.

(iii) As the glucocorticoid effect on biogenic amines are known to change with the dose and time of treatment, studies on these parameters with acute corticosterone treatment were also conducted.

(iv) The trace amines (p- & m-tyramine and tryptamine) have been shown to play a neuromodulatory role in striatum with respect to DA and 5HT respectively. A developmental effect, with acute and chronic corticosterone treatment on these trace amines (p- & m-tyramine and tryptamine) was also carried out in the striatum.

(c)(i) As already mentioned glucocorticoids may play an important role in the process of myelination and also could affect the membrane associated factors such as receptor binding, enzyme activities ($Na^+$,$K^+$-ATPase), transport of glucose and neurotransmitters etc. Hence, effect of chronic corticosterone treatment on the brain regional lipids during development was studied. The lipids studied were cholesterol as for general membrane associated change and galactolipid, associated with myelin specific change. Phospholipid estimation was also performed at 20 days with steroid treatment in the hippocampus and the striatum.

As the normal level of these two lipid components is not known in the hippocampus and the striatum during development, information derived in this regard from the above said experiment itself, is discussed. This was possible because of the relative stability of these lipid components even with the stress conditions.

(ii) As changes in the cholesterol concentrations were observed with chronic steroid treatment, the rate of lipid synthesis in these regions at 20th postnatal day in the hippocampus and the striatum was studied using $^{14}C$-glucose as substrate.

(iii) A decreased $^{14}C$-glucose incorporation, contrary to the expectation, observed from the experiment led to the study of ketone body utilizing enzyme, BDH, in these brain regions with steroid treatment. In addition, blood ketone body and glucose level were also estimated.

An attempt is made to collate and correlate the changes occurring under steroid treatment from these three main approaches. The rationality for the parameters measured and age groups studied is mentioned at the beginning of the results and discussion part of each experiment.

## 2.1 Materials :

Reagent grade chemicals and solvents were used throughout and majority of the chemicals were obtained from Sigma Chemical Co., St. Louis, Missouri. Solvents were obtained locally (chiefly from BDH, India, Sarabhai M.Chemicals and E. Merck, India). Precoated TLC plates were obtained from Brinkmann Instruments, Riversdale, Ontario. The deuterated internal standards for mass spectrometric analysis, p-, m-t and tryptamine were kindly provided by Dr. Bruce Davis Psychiatric Res. Divn. Univ. Saskatchewan, Canada. Radioactive chemicals U-$^{14}$C glucose (Sp. Act. 166 mci/mmole) was obtained from Bhabha Atomic Research Centre, Trombay, Bombay, India, $^{14}$C-glutamic acid (Sp. Act. 0.2 mci/mmole) and $^{14}$C-GABA (Sp. Act. 1 mci/4.5 mmole) were obtained from Amersham Intl., U.K.

## 2.2 Animals and treatment schedule :

Rats of Charles-Foster strain bred and maintained under standard laboratory conditions were used in the present study. Rats were given diet and water *ad libitum*. Corticosterone acetate suspended in 0.1% carboxymethylcellulose was injected subcutaneously in neck folds for 3 days beginning from 7, 17, 37 and > 87 days. The animals were sacrificed, 24 h after the last injection in the case of chronic study and 2 h after the single injection for acute study, on 10th, 30th and (>)90th days. Littermate controls were injected with vehicle only throughout the study.

For experiments on biogenic amines, the rats were of Wistar strain obtained from Charles River, Ontario, Canada and maintained under standard laboratory conditions. This part of the experiment was carried out in the laboratory of Prof. A.V. Jurio at University of Saskatchewan, Canada.

## 2.3 Dose of steroid treatment :

For chronic studies, corticosterone acetate equivalent to 40 mg/kg body weight prepared as mentioned above was used. This dosage was arrived at due to the considerations given below :-

(a) from the dose-response experiment performed as a pilot study on the GS activity.

(b) study by Patel *et al.* (1983b) have shown a similar response by corticosterone treatment at 40 mg/kg body weight.

(c) another study has shown that even a treatment 50 mg/kg body weight corticosterone would elevate the circulatory corticosterone level similar to that found under certain stress conditions (Dickinson <u>et al</u>, 1985) in the first 24 h after treatment.

(d) furthermore, the purpose of this study was not to elucidate the regulatory aspects of glucocorticoid on these parameters during development, but merely to study the changes associated with a consistently higher level of circulating corticosterone concentration. Due to unavoidable circumstances, plasma levels of corticosterone could not be measured, although such estimations were desirable.

<u>Dissection of brain regions and preparation of homogenates :-</u>

On specified days, rats were sacrificed between 8.00-10.00 a.m. After the removal of brain, regions such as the hypothalamus, hippocampus, striatum and cerebellum were separated out on glass plate cooled over liquid nitrogen, according to the method of Glowinski and Iversen (1966). The tissues were stored in eppendorf vials kept in liquid nitrogen till further processing.

On the day enzyme assays were done, tissue homogenate was prepared. Weighed tissue was homogenized in a Potter-Elvehjam homogeniser at 0-4°C temperature in 10 volumes of 0.02M potassium phosphate buffer, pH 7.0 containing 0.25%

Triton X-100. Homogenate thus prepared was used for assaying GAD, GS and GABA-T; for GDH assay homogenate was centrifuged in Sorval-RC5B at 8000 xg for 10 min and the supernatant was used for further analysis. A 100 µl aliquot from homogenate was diluted to 1 ml with distilled water and an aliquot from this was used for protein estimation.

## 2.4 Biochemical Estimation :

### 2.4.1 GAD assay : (Roberts and Simonson, 1963)

The method involves estimation of $^{14}CO_2$ liberated from 1-$^{14}C$ glutamic acid by the activity of GAD. In brief, 200 µl of the assay mixture containing 100 mM of phosphate buffer, pH 6.8, 4 µmoles of L-glutamic acid containing 1-$^{14}C$ glutamic acid (40,000 CPM), 0.5 mM of pyridoxal phosphate and 0.1 µM 2-aminoethylisothiouronium bromide hydrobromide (AET) was taken in a small tube and preincubated for 10 min. Reaction was started by adding 25 µl of 10% tissue homogenate and the tubes were closed with airtight special rubber stopping through which a plastic well containing 200 ul of hyamine base was suspended. At the end of 60 min of incubation at 37°C the reaction was terminated by injecting 0.2 ml of 20 N $H_2SO_4$ into the assay system by a syringe and allowing it to stand for 30 minutes for equilibration. Hyamine base from cups were then transferred into a scintillation vial by the addition of 2 ml ethanol (100%) and counted in scintillation fluid (made of 4 g PPO, 400 mg POPOP dissolved in 1000 ml Toluene) for $^{14}C$-$CO_2$ in

Beckman LS 7000 scintillation counter with automatic quench correction. 200 µl of the assay system was counted for standards and for enzyme blank $H_2SO_4$ was added prior to incubation. One unit of enzyme activity is defined as amount of enzyme catalysing to produce 1 µmole of $CO_2$ per hour.

2.4.2 GS (Patel et al, 1983a).

$^{14}C$-glutamine formed enzymatically from 1-$^{14}C$ glutamic acid was measured under assay conditions.

The assay mixture (0.5 ml) containing final concentration of 100 mM imidazole buffer, pH 7.2, 50 mM glutamate, 12.5 mM $MgCl_2$, 20 mM mercaptoethanol, 10 mM ATP, 1 mM ouabain, 4 mM ammonium chloride, 13 mM phosphocreatine, 1.3 units of creatine phosphokinase and 1 mg enzyme protein (100 µl of 10% homogenate) was incubated for 20 min at 37°C. For blank boiled enzyme was added. The reaction was stopped by the addition of 1 ml ice cold distilled water and then charged immediately onto a column of Dowex-1-8 acetate form (0.7 cm i.d. x 3.9 cm). $^{14}C$-glutamine was eluted with 2 ml water wash and counted using scintillation fluid containing : 4 gm BBOT in 1 litre of solution of Triton X-100 in Toluene (2:3 v/v). One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmole of glutamine per hour.

2.4.3 GABA-transaminase : (Dye and Taberner, 1975)

This method involves coupling of GABA-T with succinic-semi-aldehyde dehydrogenase reaction giving labelled succinate which was separated from labelled GABA by Dowex-50H$^+$ column.

In brief, 1 ml of assay mixture containing 0.05 M sodium borate buffer (pH 8.6), mercaptoethanol 0.01 M, pyridoxal-phosphate 0.5 mM, sodium succinate 0.02 mM; 2-ketoglutaric acid 9 mM, U-$^{14}$C GABA 4 mM; and 100 µl of 10% homogenate was incubated for one hour at 37°C. The reaction was stopped by adding 0.05 ml of 10% perchloric acid (PCA) (added before starting the reaction in the case of blank). After allowing it to stand for 10 min, samples were centrifuged for 10 min in cold and the supernatant charged onto a column (2 x 0.5 cm) of Dowex-50H$^+$ form; labelled succinic acid was eluted with 2 ml water and counted as mentioned earlier. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 µmole of succinate per hour.

2.4.4 GDH : (Lai and Clark, 1976).

Spectrophotometric reduction of optical density at 340 nm for NADH converted to NAD by the enzyme GDH was measured. 1 ml of assay mixture containing 100 mM Tris buffer, pH 7.7, 162 mM ammonium acetate, 1 mM EDTA, 0.167 mM NADH, 17 mM ADP, 10 mM 2-oxoglutarate, 100 µg of enzyme protein were taken in the cuvettes. The reaction was started by the addition of NADH and blank was without substrate. Reduction ~~was~~ in optical density due to the conversion of NADH to NAD was monitered over

75 mM $NaH_2PO_4$, 1 mM sodium octylsulphate, 0.5 mM disodium EDTA, 12% acetonitrile (pH 2.8, adjusted with 85% phosphoric acid), and the flow rate of the mobile phase was kept at 1 ml $min^{-1}$. ATL-5 glassy carbon electrode and RE-1 reference electrode (BAS, West Lafayette Ind. USA) were used for electrochemical detections and the oxidation potential was set at 0.93V. A working standard solution of all the compounds to be measured was prepared over a range of 5 ng to 50 ng 50 $\mu l^{-1}$ and this was run at the beginning of the experiments and from it a correction factor for each of the compounds with respect to internal standard (ISP) was determined. The minimum detection limits were 50 pg for DA, NE, DOPAC, HVA, 3 MT and ISP and 100 pg for 5-HT and 5HIAA. The detection limits for p-tyrosine and tryptophan were 1 ng.

Calculation : The ratio of the peak height of the internal standard to the peak height of each compound was calculated from the working standard solution run ($K_1$). The ratio of the peak height of each compound to that of the internal standard in the biological samples ($K_2$) was also calculated. The concentration of each compound in the biological sample was then calculated from the equation :

$$K_1 \times K_2 \times \text{ng of I.S.} \times \text{Dilution factor} \times \frac{1}{\text{tissue wt. (g)}}$$

$$= \text{concentration of the compound in ng/g tissue weight.}$$

$K_1$ = Ratio of the peak height of the internal standard to that of the compound in the standard.

$K_2$ = Ratio of the peak height of the compound to that of the internal standard in the biological sample.

I.S = Internal Standard.

2.4.6 Measurement of p-tyramine (p-TA), m-tyramine (m-TA) and tryptamine (T) :

The tissues were homogenised as described for bioamine separation by HPLC, and were added with tetra-deuterated p-TA, m-TA as internal standards. For the determination of tryptamine, tetra-deuterated tryptamine was added. The amines in the tissue homogenates were derivatized with 5-dimethylamino-1-napthalene sulphonyl chloride (dansyl chloride) extracted into toluene; ethylacetate (9:1), evaporated to a small volume and separated chromatographically on two different monodimensional systems. The estimations of the trace amines were carried out by a high resolution mass-spectrometric selected ion monitoring technique (Philips et al, 1974a for p-TA; 1974b for T and 1975 for m-TA; Juorio, 1982; Juorio and Kazakoff, 1984). A flow chart of the experimental detail is given in the following page.

Flow Chart for m- & p-TA Analysis.

Homogenise samples in 1.0 ml homogenising media

↓ Add

25 ng of $d_4$ std. p- & m-TA

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150 mg $Na_2CO_3$ ; 2 ml Acetone

↓ Shake well and add

2.5 ml of DNS chloride (8.0 mg/ml) shake and leave it overnight

↓ Extract dansylated TAs in (2 x 3 ml)

Toluene-Ethylacetate (9:1) dry and take up in Acetone-methanol (1:1).

↓

Apply onto TLC plates (Silica-G) and chromatograph in Toluene-ethyl acetate (5:2) for $1\frac{1}{2}$ hrs.

↓

Extract spots (rf $\sim$ 0.8) in acetone-methanol (1:1) dry and take up in acetone-toluene; apply to TLC plate and chromatograph in Toluene; Triethylamine; methanol (50:5:1)

↓ After $\frac{1}{2}$ h

Visualise m- & p-TA spots scrape into capillary tubes and extract in Ethylacetate (0.1 - 0.2 ml).

↓

Analyse extract on Mass Spectrophotometer.

## 2.4.7 Extraction of Lipids

The procedure of Folch et al. (1957) as modified by Suzuki (1965) was used for the extraction of lipids from rat tissues.

The tissue was homogenized with 19 volumes of cold chloroform : methanol (C:M) (2:1 v/v) mixture in a Potter-Elvejem homogenizer for 2-4 minutes at 200 rpm. The contents were filtered through sintered glass funnel. Twice residue was re-extracted with 10 volumes of chloroform:methanol (2:1 v/v) mixture and filtered again. The filtrates were pooled together. 0.2 volumes of glass distilled water was then added, mixed well by inverting the tube and then allowed to stand for phase separation at room temperature (25-30°C). The upper phase was collected with a pasture pipette and the lower phase was washed with 0.4 volumes of Folch's pure upper phase solvent (theoretical upper phase consists of chloroform:methanol:water in the proportion of 3:48:47 (v/v/v) ) and centrifuged at 3000 rpm for 10 minutes. The upper phase formed was collected and combined with the upper phase obtained earlier. The lower phase was used for the estimation of cholesterol, galactolipids and phospholipids.

### 2.4.7.1 Estimation of cholesterol

The method of Bowman and Wolf (1962) was used for the estimation of cholesterol. Aliquots (0.1-0.2 ml) of the lipid extract were taken in duplicates in test tubes and evaporated to dryness at 60°C in a water bath. To this 3 ml of distilled alcohol was added. 3 ml of iron reagent* was added while shaking the contents of the tube on a vortex shaker. The colour developed was read after 30 minutes at 540 nm in a Beckman spectrophotometer against a reagent blank which contained alcohol instead of the sample. A standard graph was prepared taking different concentrations of cholesterol (20-120 µg) and treating them similarly.

* Iron reagent - 2.5% of $FeCl_3$ in 87% orthophosphoric acid. For working reagent 8 ml was diluted to 100 ml with conc. $H_2SO_4$.

2.4.7.2 Estimation of galactolipids

The method of Svennerholm (1956) modified by Neskovic et al.(1972) was used for the estimation of galactolipids. Aliquots of the lipid extract (0.1-0.2 ml) were taken in test tubes and evaporated to dryness at 60°C in a water bath. To this, 0.3 ml of ethanol was added and the tubes were again kept in 60°C water bath for 3 minutes to dissolve the lipid in alcohol. After cooling to room temperature (30°C), 3 ml of orcinol reagent were added. The tubes were shaken well on the vortex mixer and heated at 80° for 20 minutes. The colour formed in the tubes was read at 505 nm in a Beckman spectrophotometer against a reagent blank which contains alcohol instead of the lipid extract. A standard graph was prepared using different concentrations of standard galactose (5-15 µg) and treating them similarly.

The values obtained for galactose were multiplied by 4.7 to obtain the galactolipid content of the sample.

2.4.7.3 Separation of various phospholipids

The method of Horrocks and Sun (1972) was used for separation of phospholipids by thin layer chromatography (TLC). Glass plates (20 x 20 x 0.25 cm) were cleaned with sodium carbonate powder and washed with water first and then with glass distilled water and dried at room temperature. Then the plates were coated to a thickness of 0.5 mm with silica gel 'G' (a slurry of the gel was prepared by taking 35 g of silica gel 'G' in 67 ml of 0.01M sodium carbonate. This can be used to coat 5 plates). The coated plates were air dried at room temperature and then activated for 1 hr at 110°C in an oven before use. The lipid extract containing 20-40 µg of phosphorus was spotted at the lower left corner of the silica gel plate. The plates were developed to a height of 13-15 cm in a glass chamber presaturated for one hour with C:M:$NH_4OH$ (65:25:4). It took one hour to develop. The plates were removed and air dried at room temperature. The second TLC run was made at right angles to the first in a solvent system containing chloroform:methanol:acetone:acetic acid:water (75:15:30:15:7.5), allowing it to run to a height of 10-12 cm. After this, the plates were removed and air dried for 10 minutes and the spots were visualised by exposing the plates to iodine vapours.

The various phospholipid spots were scrapped into test tubes and phosphorus estimated by the method of Bartlett (1959). For blanks silica gel from unspotted area was processed.

### 2.4.7.4 Estimation of phospholipids

Lipid phosphorus was estimated by the method of Bartlett (1959). Aliquots (0.1-0.2 ml) of the lipid extract were taken in duplicates in test tubes and evaporated to dryness at 60°C in a water bath. One ml of 60% perchloric acid was added to each tube along with a small piece of percelain. The tubes were heated for 30 minutes in a sand bath maintained at 230°C. After the completion of digestion, as judged by the colourless appearance of the solution, the tubes were cooled to room temperature (27-30°C) and volume made upto 1 ml with perchloric acid. To this was added 8.1 ml of distilled water, 0.5 ml of molybdate reagent[1] and 0.4 ml of ANSA reagent[2]. The tubes were shaken well and kept in a boiling water bath for 8 minutes. They were then cooled to room temperature (27-30°C) and the colour obtained was read at 820 nm in a Beckman spectrophotometer against a reagent blank containing only perchloric acid.

A standard graph was prepared by taking different concentrations of phosphorus standard (1-8 μg). The values obtained for phosphorus were multiplied by 25 to obtain the phospholipid content of the sample.

| | | |
|---|---|---|
| 1 - Molybdate reagent | : | 2.5% of ammonium molybdate in 3N sulfuric acid. |
| 2 - ANSA reagent | : | 0.25% of 1-amino-2-Naphthol-4-sulfonic acid (ANSA) in 97.5 ml of 15% sodium bisulphite + 2.5 ml of 20% sodium sulphite. |

### 2.4.8 Estimation of protein

The method of Lowry *et al*. (1951) was used for the estimation of protein. 0.2 ml of 1-10 diluted synaptosomal preparation was taken in a test tube. To this 0.8 ml of 0.1N NaOH and 5 ml of Lowry's 'C' reagent* was added and allowed to stand for 15 minutes. After this 0.5 ml of 1N Folin ciocalteau reagent was added and the contents in the tube were mixed well on the vortex mixer. Colour developed in the next 30 minutes was read at 750 nm in a spectrophotometer against a blank containing distilled water instead of the sample. Different concentrations (20-100 µg) of standard protein (bovine serum albumin) was taken and processed as above to prepare a standard graph.

* Lowry 'C' reagent : 2.0 ml of 1% $CuSO_4$ (dissolved in 1% Na-K-tartarate) was mixed with 100 ml of 2% $Na_2CO_3$ prepared in 0.1N NaOH.

### 2.4.9 Studies on incorporation of labelled precursors into lipids *in vivo*

For studies on *in vivo* incorporation of labelled glucose, (U-$^{14}$C)-glucose (Sp. Act. 255 mci/mmole) 40 µci/100g body weight was injected intraperitoneally and animals were killed one hour after injection of labelled glucose; the brain regions - hippocampus and the striatum were removed and used for extraction of lipids as given above.

### 2.4.10 Estimation of BDH activity

The tissues were homogenized in 10 volumes of homogenizing fluid containing 0.1M Tris-HCl buffer, pH 7.4. The 10%

homogenate was sonicated using Branson Sonicator at 40 KHz for one minute at 15 sec. intervals in ice cold condition and used further for BDH activity according to Williamson et al. (1971). Accordingly, BDH activity was determined by measurement of the acetoacetate formed on incubation of the homogenate with DL-3-hydroxybutyrate in the presence of $NAD^+$. The incubation mixture (final volume 2.5 mM) consisted of Tris-HCl buffer 5 mM; pH 8.5, $NAD^+$ 0.5 mM, BOHB 0.5 mM, nicotinamide 12.5 mM and enzyme sample (0.5 ml). At 10 and 20' of incubation at 37°C, 1 ml of assay mixture was added with 0.1 ml of 30% PCA to terminate the reaction and neutralized with 0.3 ml of 10% KOH. Neutralized assay mixture was centrifuged at 3,000 rpm for 10' and 0.5 ml of the supernatant used for acetoacetate estimation by Walker's method (1954).

To 0.5 ml of the supernatant, 0.5 ml of 0.5 M sodium acetate buffer, pH 5.2 and 3 ml of chilled Diazo reagent was added (Diazo reagent was prepared by adding 3 mg of $NaNO_2$ to 20 ml p-nitroaniline; chilled in ice bath and added with 7 ml of 0.2 M sodium acetate buffer to bring the pH to 4.0). After 30 min of incubation at room temperature the reaction was stopped by adding 1 ml of 5N HCl. The product was extracted in 4 ml of ethyl acetate and read at 440 nm on the spectrophotometer. Blanks contained 0.5 ml distilled water. A standard of acetoacetate in the range of 2-10 µg was prepared with each batch of estimations.

One unit of enzyme activity is defined as the amount of enzyme required to produce 1 μmole of acetoacetate per hour.

2.4.11 Determination of blood ketone bodies and glucose

Approximately 0.2 ml of blood from decapitated rats was collected in heparinized Kahn tubes. 2 vol. of 6% (w/v) $HClO_4$ was added and the precipitated protein was removed by centrifugation and the supernatant solution was neutralized with 20% (w/v) KOH. Treatment of the blood sample was carried out at 0°C to prevent spontaneous breakdown of acetoacetate to acetate and $CO_2$. After the solution had stood for 1 hr at 0°C the $KClO_4$ precipitate was sedimented by centrifugation and 0.5 ml of the supernatant (after making the final volume to 1 ml with distilled water) was taken for the determination of β-hydroxybutyrate (BOHB) by the enzymatic method of Williamson and Mellanby (1974) and the remaining 0.5 ml supernatant for glucose estimation by glucose oxidase method (Dahlquist and Norstrom, 1966).

2.4.12 Estimation of BOHB

The method involves measurement of NADH formation on the oxidation of 3-hydroxybutyrate in the sample by 3-hydroxybutyrate dehydrogenase in the presence of $NAD^+$. Briefly, 0.5 ml of the supernatant was added with 0.5 ml of assay mixture containing Hydrazine-Tris buffer 0.1M, pH 8.5, NAD 0.4 mM and BDH ~ 50 MU (5 μl) in spectrophotometer cuvettes and increase in O.D. till 20' was read at 340 nm; the results are expressed as µmoles of 3-hydroxybutyrate/ml blood. Hydrazine-tris (pH 8.5) was prepared by mixing 1 ml hydrazine hydrate, 20 mg EDTA-$Na_2H_2.2\ H_2O$ and 5 ml N HCl and diluted to 20 ml with Tris buffer (0.1 M, pH 8.5).

2.4.13 Estimation of glucose

0.5 ml of supernatant was diluted to 1 ml with distilled water and 1.0 ml of Tris-glucose oxidase (TGO, prepared by dissolving 20 mg of glucose oxidase (15,000 U/g), 0.5 ml peroxidase (1 mg/ml in water : ~ 50 U/mg) and 2 ml of O-dianisidine 2HCl (10 µg/ml in water) in 80 ml of 0.5 M Tris-HCl buffer pH 7.0 and volume made upto 100 ml with water) was incubated for 10 min at 37°C. Reaction was terminated with 1.0 ml of 66% conc. $H_2SO_4$. The colour was read at 560 nm against a blank containing Tris buffer alone. Glucose standards in the range of 20-100 µg/ml were also processed simultaneously.

Statistics : The significance of difference between control and **hormone** treated samples were tested by Student's 't' test.