CHAPTER II

MATERIAL AND METHODS
Female albino rats of Wistar strain from an inbred colony, were used for the experiments. They were maintained in a well ventilated animal house with free access to water and standard pellet diet (Hindustan Lever Limited, India). Vaginal smears were examined daily, for at least four consecutive cycles and the animals exhibiting a regular 3-day cycles only were selected for the study. All the animals were maintained under uniform laboratory conditions throughout the experimental period.

EXPERIMENTAL DESIGN

The adult female rats (90-120g body weight; 100-130 days old) were ovariectomised 14 days prior to experimentation and they were divided into:

Group - I : Control (treated with vehicle only)
Group - II : Treated with Estradiol - 17β (0.5 μg/rat/day/7 days)
Group - III : Treated with Progesterone (2.5 mg/rat/day/7 days).

Each group consisted of 5 rats. Estradiol-17β and progesterone were obtained from the British Drug House, Poole, England. The hormones were dissolved in propylene glycol and administered intramuscularly. The controls received only the vehicle.
(1) **Enovid-17β:**

0.5 mg/mouse/day was administered intramuscularly for 7 consecutive days. This dose was found optimum to augment uterine weight and other physiological responses in females (Se, et al., 1973).

(2) **Premarin:**

2 mg/mouse/day was administered intramuscularly for 7 consecutive days. This dose was found to be effective in eliciting physiological progestational responses (Bowie and Aldén, 1969; Nohutçu and Karim, 1973).

**VAGINAL SWABS:**

Vaginal swabs were examined by the method described by Long and Evans (1923). One or two drops of distilled water were introduced carefully into the vagina by means of a specially made Pasteur pipette, and the water was sucked in quickly back into the pipette by releasing pressure on the rubber bulb of the pipette. The sample was then fixed out on the microscope.
slide while wet. Wherever necessary, the smear was stained with eosin and hematoxylin, to favour clear identification of the cells. The characteristic cell types found during the different stages of estrous cycle were:

(a) Proestrus: Deciliated cuboidal epithelial cells.
(b) Estrus: Cornified epithelial cells.
(c) Metestrus: Cornified cells plus leukocytes.
(d) Diestrus: Leukocytes plus a few epithelial cells.

EQUIPMENT

Bilateral ovarioectomy was performed following the procedure of Saxe et al., (1944). The animal was anaesthetised with ether and a single transverse incision across the midline was made in the skin on the ventral side, just 2 cm above the vaginal opening. The incision was shifted from one side to the other, in order to visualize the ovary. A small puncture was then made over the site of the ovary which could easily be seen through the abdominal wall, embedded in a pad of fat. The tip of a pair of fine forceps was introduced, grasping the pad of fat around the ovary, care being taken not to rupture the capsule of the ovary itself. The tip of the oviduct was then cut by a pair of artery forceps, and the ovary was removed by a single cut with a bent scissors. On the completion of the
operation, the skin incision was closed by one or two interrupted sutures.

The animals were sacrificed by cervical dislocation, 24 hrs after the last injection of the respective hormone.

The brains were quickly removed and according to the guidelines of Cleasman and Iversen (1966), the cerebral hemispheres, diencephalon, hypothalamus, pituitary, cerebellum, and medulla oblongata with pons were dissected out, and weighed accurately to the nearest milligram on a torsion balance and frozen at -80°C until further assay was carried out. However, all the assays were completed within a week. Pre-weighed tissues were homogenized with acid butanol or 30% trichloroacetic acid for the extraction of biogenic amines and tyrosine, respectively.

ANALYTICAL METHODS

CHEMICALS:

All the chemicals and reagents used in the experiments were of the analytical grade and obtained from British Drug House Ltd. (England and India); E. Merck (Germany); Sigma (U.S.A.).
Flora (Switzerland); Corporation for Mechanical Research (Los Angeles, U.S.A.); Roch-Light (Guisebrook, England); Loba Chemie (Austria) and Samrahai M. Chemicals (India).

EXTRACTION OF BIOMIC AMINES FROM BRAIN TISSUE:

A modified method of Hamriuch and Donner (1973) was used for the extraction of biogenic amines from the brain tissue. This procedure describes the simultaneous extraction and fluorometric measurement of 5-hydroxy indole acetic acid (5-HIAA); serotonin; Dop; Dopamine; Epinephrine, Noradipine and norepinephrine acid (NFA). The assay is modified from previously published methods (Chang, 1964; Ansell and Lesser, 1968; Wachtel et al., 1969; Addin, rose and Vesnay, 1943; Wachtel and vesnay, 1963 Spera and Rosen, 1972; and Johnson et al., 1973).

REFERENCES:

(1) Standard solutions:

Adrenaline bitartrate, L-Noradrenaline, Dopamine hydrochloride and serotonin creatinine sulphate were used. The equivalent of 5-8 mg free-base of each amine was dissolved in 20 ml of 0.01M hydrochloric acid and stored in a deep freeze.
On the day of the experiment 0.5 ml of this solution was diluted to 25 ml with distilled water to a final concentration of 5.0 μg/ml.

The stock solution of Dopa contains 30 mg depe/100 ml of 0.5 N acetic acid and can be stored at 4°C. Working standards were prepared to contain 1-200 mg depe/ml. The stock was diluted with 0.5 N acetic acid.

Stock solutions of homovanillic acid and 5-hydroxyindole acetic acid were prepared in 0.01 N hydrochloric acid (5 mg/20 ml of 0.01 N HCl).

1) Butanol-

Butanol of 500 ml n-butanol were washed successively with 20 ml in sodium hydroxide solution, 20 ml in hydrochloric acid and 4 x 20 ml distilled water, in a separating funnel fitted with a polytetrafluoroethylene stopcock and then saturated with sodium chloride.

2) Acid-butanol (20 ml HCl):

One litre of n-butanol washed and salt saturated was shaken with 0.35 ml concentrated hydrochloric acid (1.18 gm/ml) then 1.5 gm potassium metabisulphite and 0.1 gm ethylene diamine tetracacetate (EDTA) were added and shaken thoroughly.
(6) **n-Heptane**

n-heptane was redistilled, GL grade.

(5) **Tris-Hydrochloric acid buffer, pH 7.0 (10 ml)**

98.25 ml Tris solution and 11.75 ml of 0.01N hydrochloric acid were mixed thoroughly and the volume was made up to 200 ml with distilled water.

(6) **Tris-Hydrochloric acid buffer, pH 8.5 (0.5 M)**

Tris-HCl buffer (0.5M) containing 0.15M HCl and 15 ml sodium metabisulphite, adjusted to pH 8.5.

(7) **Sodium metabisulphite, 1L each**

1.63 gm of sodium metabisulphite dissolved in one litre of distilled water.

(8) **HEA 0.12 M**

4.847 gm of ethylene diamine tetra-acetic acid was dissolved in 100 ml of distilled water.

(9) **Alumina**

500 gm of chromatographic grade alumina was boiled in one litre 3N hydrochloric acid for 30 min, then washed with 30 x 100 ml
distilled water or until the pH of the washings had risen to between 4 and 5. Finally it was left to dry overnight at room temperature and then heated at 200°C for 2 hrs.

(10) Ammonium hydroxide, 2M:

49.87 ml of 20% ammonium hydroxide was made up to 500 ml with distilled water.

(11) Potassium ferricyanide (0.03 M):

0.63 gm of potassium ferricyanide was dissolved in 100 ml of distilled water.

(12) Cysteine (0.15 M):

0.1 gm of cysteine was dissolved in 100 ml distilled water.

(13) n-Butanol:

Batches of 500 ml n-butanol were saturated with distilled water and sodium chloride.

(14) Borate buffer, pH 10 (0.25 M):

Boric acid (31.4 gm) was dissolved in 1 litre distilled water and 35 ml 1M sodium hydroxide was added. This was then
saturated with n-butanol and sodium chloride and adjusted to pH 10, if necessary.

(15)  *Carbazole-Pthalaldehyde reagent*

30 mg of carba-zole-phthalaldehyde reagent was dissolved in 100 ml of 1M hydrochloric acid. This solution was prepared 4-6 hrs before use.

(16)  *Acetate buffer, pH 5.5:*

pH of 1M sodium acetate was adjusted to 5.5 with 1M acetic acid.

(17)  *Lodine reagent for Iodine:*

0.125% iodine in 0.5% potassium iodide solution.

(18)  *Thiosulphate solution (0.01M):*

1.104 gms of thiosulphate was dissolved in one litre of distilled water.

(19)  *Alkaline acetate:

5% sodium hydroxide containing 2 mg/ml of acetic acid.
(20) **HCl solution, pH 6.5 (0.1N):**

3.73 g HCl was dissolved in 200 ml of 1M sodium acetate and adjusted to pH 6.5 with 1M sodium hydroxide. The total volume was adjusted to one litre, and portions of approximately 1 ml were frozen until required.

(21) **Iodine solution 0.2M for褪色:**

This was prepared in absolute ethanol by dissolving 12.7 g iodine/litre ethanol for dopamine extraction.

(22) **Alkaline sulphite:**

Immediately before use, 4.3 ml 2N sodium hydroxide was mixed with 0.5 ml sodium sulphite solution. The latter solution (90 g hydrated salt/100 ml) was stored as frozen portions of approximately 1 ml.

(23) **Formic acid (sp.gr. 1.18) Analytical:**

Concentrations of

(a) 0.01 N HCl
(b) 0.1 N HCl
(c) 1.0 N HCl
(d) 10.0 N HCl and
(e) Molar HCl were prepared.
(26) Acetic acid (Glanzal, Analy, Redistilled):
Concentrations of (a) NH and
(b) 0.2M acetic acid were prepared.

(29) Sodium hydroxide:
Concentrations of (a) NH (50 gms in 100 ml distilled water);
(b) NH (200 gms in 100 ml distilled water);
(c) 100 (400 gms in 100 ml distilled water)
were prepared.

INTERACTION OF MAPPYLLIC ACID, 1-HEXANETHIOL ACETIC ACID
AND ENDOTHELIAL BRAIN TISSUE:

Tissue samples were homogenized in cold acidified (10m
HCl) butanol to give a final tissue concentration of 25-75 mg/ml.
The samples were centrifuged for 30 mins at 6000g and the super-
natant was added to a centrifuge tube containing 10 ml of a-
heptane. 1.5 ml of 0.01M HCl and known amount of internal
standards were added to the samples (0.5-2.0 μg). The samples
were shaken for 5 mins to extract the amines and the tubes were
centrifuged for 10 mins at 1000g. A 10 ml aliquot of the organic
phase, containing the amines was then added to a 15 ml centrifuge
tube containing 1.5 ml of 10 mM Tris-SECl buffer (pH 7.4) and the
5-ΕΚΑΑ and ETA were extracted into the Tris-SECl buffer by being
shaken for 5 min. After centrifugation of the tubes for 5 min at 1000g, the organic phase was aspirated and decanted, and the 
9-HEA and HVA in the aqueous phase were estimated. If the 
aqueous phase appeared cloudy after aspiration the tubes were 
centrifuged again until the sample appeared clear.

A 1.0 ml aliquot of the acid phase, containing the anines, 
was added to the 15 ml centrifuge tube containing 200 mg of alumina 
(prepared as described by Aronov and Saguy, 1980) and 1.5 ml of 
0.2 M Tris-Cl buffer (pH 8.5) containing 0.2M HCl and 13 ml 
sodium metabisulphite. The tubes were shaken for 2 hrs, to 
adsorb the catecholamines, then centrifuged for 2 min at 1000g 
and a 2 ml portion of the supernatant fluid was transferred 
to a 15 ml centrifuge tube containing 6.0 ml of n-hexane (salt 
and water saturated), 1.0 gm of NaCl and 2.0 ml of 0.2M borate 
buffer (pH 10). The mixture was shaken for 5 hrs, then centri-
fuged and 5 ml was transferred to another 15ml tube containing 
10 ml of n-hexane and 0.4 ml of 0.1M HCl. After 5 more hrs of 
shaking followed by centrifugation, 0.2 ml of the acid phase 
was removed for the assay of norepinephrine.
The remainder of the supernatant liquid over the alumina (from the previous step) was aspirated and discarded and the adsorbent washed by shaking for 5 min with 3 ml of distilled water then centrifuged at 2000g for 5 min. The wash liquid was removed by aspiration and the inside of the tubes were blotted with strips of filter paper. The alumina was shaken for 5 min with 1.0 ml of 0.2F acetic acid to elute the catecholamines. A portion (1.0 ml) of the supernatant liquid was transferred to a separate tube for the assay of epinephrine, norepinephrine and dopamine as described by Chang (1964). A second portion (1.0 ml) of the supernatant was taken for the assay of CA as described by Johnson, Gold and Clenet (1973).

For extraction of amines other than CA, a 1.0 ml portion from the previous step was taken and 0.2 ml of 0.1F HCl was added to it to adjust the pH to 0.1. Then 0.1 ml of 0.1F iodine reagent (0.1F iodine in ethanol) was added to oxidized catecholamines. Exactly after 1 min, oxidation was stopped by adding 0.2 ml of freshly prepared alkaline sulfite and the solution was
allowed to stand for 3 hrs. The pH of the solution was adjusted to 5.4 with 0.3 ml of 5N acetic acid. Then the fluorescence was read in a spectrophotofluorimeter.

For the extraction of 5-hiaa, the second 1.0 ml portion of the supernatant fluid from the previous step was taken and mixed thoroughly with 1.0 ml of acetate buffer (pH 5.3). To this, 0.5 ml of iodine reagent was added and mixed. Exactly within a minute 0.5 ml of thiosulphate solution was added and mixed again. This was followed by the addition of 1.0 ml of alkaline acetate. The sample was placed in a closed-view cabinet (Camag) under 'long' wave-length light (330-500 nm) for 15 mins and then the fluorescence was measured.

Reagent blanks were prepared in the same order.

PHOTOMETRIC ANALYSIS:

Fluorescence was read on a Amiles-Swan Spectrophotofluorometer (American Instrument Co.). The wave-lengths reported below (excitation/emission) are uncorrected. The 5-hydroxy-indole acetic acid was estimated in the Tri-tris-buffer by measuring native fluorescence of the indole ring at 395/380 nm. Fluorescence was stable in Tri-tris-buffer for at least four hours. After
The sample had been treated for S-DNAA, 1 ml of the tri-buffer
was transferred to another tube for the estimation of homo-
vanilllic acid by the procedure of Andén et al. (1943). The pH
of the medium was first increased by the addition of 0.1 ml of
1 N ammonium hydroxide. Potassium ferricyanide (0.01 ml) was then
added to oxidize the homovanilllic acid. After 4 hrs the oxida-
tion was stopped by the addition of 0.02 ml of cysteine (0.1 M)
and fluorescence was read within one hour at 310/450 nm. Reagents
blanks were prepared in the same order.

A fluorescent derivative of acetic acid was prepared by
heating a 0.2 ml aliquot of the final acid extract for 10 min
with 0.4 ml of ortho-phthalaldehyd reagent. After being cooled
in tap water, the samples were extracted briefly with 0.6 ml
of chloroform to reduce the blank fluorescence. Fluorescence
was read at 380/470 nm.

Data samples were read in a quartz cuvette with activa-
tion and emission maxima of 365 and 405 nm respectively after
placing the samples in a chromato-view cabinet at long wave-
length.
Fluorescence of epinephrine was read immediately with activation and emission wave lengths at 410 and 500 nm respectively. Norepinephrine was determined after heating the above mixture for 2 min in boiling water bath, cooling and reading the fluorescence at 365/488 nm. Dopamine was measured by heating this solution for a further 4 min at 100°C, cooling, and then reading the fluorescence at 315/375 nm.

**CALCULATIONS:**

The amine content of each tissue sample was calculated by comparing with known standards, based on modified method of Small and Seeman (1966). The amines were calculated using the following formula and expressed as pg/gm tissue.

\[
\frac{A-\text{blank}}{B-A} \times \frac{\text{concentration of standard}}{1000} \times \frac{X}{4} \times \frac{1000}{Y} = \text{pg/gm tissue.}
\]

A = 'fluorescent units' obtained for tissue homogenate alone.
B = 'fluorescent units' obtained for known standards.
X = volume of acid butanol used for homogenization.
Y = Weight of the tissue homogenized.
EQUATION OF METHOD:


The reaction between 1-nitroso-2-naphthol and tyrosine, under suitable conditions, yields a yellow product which fluoresces and has been utilized for the estimation of tyrosine. This fluorometric method does not distinguish between tyramine and tyrosine. However, the amount of tyramine normally present in tissues is negligible.

METHOD:

(1) **Standard solution:**

The equivalent of 5.0 mg of tyrosine was dissolved in 30 ml of 0.01 N hydrochloric acid and stored in deep freeze. On the day of the experiment 0.5 ml of this solution was diluted to 50 ml with distilled water to a final concentration of 250 μg/ml.

(2) **1-nitroso-2-naphthol:**

0.1 gm of 1-nitroso-2-naphthol was dissolved in 95% ethanol.

(3) **Nitric acid reagent:**

24.5 ml of 1:5 nitric acid was mixed with 0.5 ml of 2.5N sodium nitrite.
(4) **Triphenyltetrazolium chloride (TTC)**

30 gms of TTC was dissolved in 100 ml of distilled water.

**PROCEDURE**

Tissue was homogenized with 4 ml distilled water and 1 ml of 20% TTC was added. After 10 mins, the mixture was centrifuged. To 2 ml of the supernatant was added 1 ml of nitrosonaphthol reagent and 1 ml of the nitric acid reagent. The tubes were stoppered, shaken and placed in a water bath at 55°C for 30 mins. After cooling, 10 ml of ethylene dichloride redistilled (Analar) was added, and the tubes were shaken to extract the unchanged nitrosonaphthol reagent. After the tubes were centrifuged the supernatant aqueous layer was transferred to a cuvette and read in the Amisco-Beaman spectrophotofluorometer. The fluorescence of the tyrosine derivative, resulting from its activation at 400 nm was measured at 570 nm. Tissue blanks and internal standards were run simultaneously along with the test.

**CALCULATION**

Tyrosine content was estimated using the following formula and was expressed in μg/gm tissue.
\[
A = \text{blank concentration of standard} \quad \frac{X}{1000} = \frac{X}{Y} = \text{pg/\text{gm tissue}}
\]

\[
B = A \quad \frac{1000}{2}
\]

\[
X = \text{volume of water used for homogenization.}
\]

\[
Y = \text{weight of the tissue homogenized.}
\]

**Statistical Analysis**

The data were analyzed statistically and were expressed as mean \( \pm \) standard error of mean (S.E.M.). The standard errors were calculated by the procedure given by Gaitie (1966):

\[
(1) \quad \text{Standard error (S.E.)} = \frac{\sum X^2 \cdot \frac{\sum X}{n}^2}{n(n-1)}
\]

where \( X \) is the individual observation and 'n' is the number of observations.

\( (2) \) Students 't' test was used to compare the two means obtained between the controls and the experimental groups. 't' value was calculated by the following formula and compared by the table values at 5\% and 1\% levels of significance.

\[
t = \frac{X_1 - X_2}{S \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}
\]
\[ \sqrt{\frac{\sum X_i^2 - \left( \frac{\sum X_i}{n_1} \right)^2}{n_1 + n_2 - 2}} + \left[ \sum X_k^2 - \left( \frac{\sum X_k}{n_2} \right)^2 \right] \]

'\( n_1 \)' and '\( n_2 \)' denote the number of observations in the two classes being compared.

From the degrees of freedom the value of probability was obtained from the standard table given by Fisher and Yates (1963). If the calculated value was more than the table value, it was significant at that probability level.

For various biochemical parameters employed ± 10% of control values were considered as normal variations. Any change above ± 10% was taken as change due to hormone treatment. The following levels of significance were used: \( P = 0.001 \) as the level for the highly significant data, \( P = 0.01 \) and \( P = 0.05 \) as levels for significant data. Levels more than \( P = 0.05 \) were considered as non-significant data.