MATERIALS
AND
METHODS
Fig 3.1 :- Albino Rat.
3. MATERIALS AND METHODS:

3.1 Materials

Male albino rats of Charles Foster strain, (Fig. 3.1) weighing 150± 20 gm. were obtained from the Central Animal House of J.N. Medical College, A.M.U., Aligarh. They were fed commercial diet (Hindustan Lever Laboratory, Bombay) and water ad libitum and maintained in normal laboratory conditions with light dark cycle of 12:12 hrs.

3.2 Methods

3.2.1 Experimental Design

Animals were divide into four groups of six rats each. Group I, II and III were the experimental group whereas rats of group IV served as control. Group - I : Freshly prepared dose of sodium selenite (0.7 mg./kg. b.wt.) was administered intraperitoneally (i.p.) daily for 7 days.

Group - II : The animals of group II were administered intraperitoneally, sodium selenite (0.7 mg./kg. b.wt) in combination with lead acetate (8 mg./kg. b.wt) for 7 days.

Group - III : Lead acetate (8 mg./kg. b.wt) was administered intraperitoneally daily for 7 days to animals of this group.
Fig 3.2 :- Dorsal view of rat brain.
Group - IV: Animals of this group served as control and an equal volume of physiological saline was given concurrently.

3.3 Dissection of brain into discrete regions:

Cervical dislocation.

For biochemical studies, where perfusion of the rat brain was not required, the animals were killed by cervical dislocation - one of the most acceptable methods of euthanasia.

The control as well as experimental rats were grasped at their neck near the base of skull, with the thumb and forefinger of one hand, handlimbs and tail with the other. A swift but controlled motion separated the cervical vertebrae from the base of skull. This resulted in instantaneous loss of consciousness and loss of all vital signs within a few minutes.

3.4. Neurobiochemical Studies

The rats were killed after drug administration and the brain (Fig-3.2) was removed immediately and placed on ice. Thereafter brain was dissected out into cerebrum, cerebellum, brain stem and spinal cord according to the method of McEwen and Praff (1970) (Fig 3.3). Different CNS regions were then cleaned with normal saline to remove the blood clottings and weighed accurately to the nearest milligram. Each part was separately homogenized.
Fig 3.3 :- Dissected brain regions of rat CNS (McEwen and Praff, 1970) Where A→Cerebrum, B→ Cerebellum, C→ Brain Stem, D→Spinal Cord.
in chloroform: methanol (2:1 v/v), but for the estimation of enzyme activity, the brain was homogenized in their respective buffer to give 10% (w/v) homogenate. The various neurochemical parameters were studied according to the following methods.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Methods</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Cellular components</strong></td>
<td></td>
</tr>
<tr>
<td>Total lipids</td>
<td>Woodman and Price (1972)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Zlatis et. al., (1954)</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>Marinetti (1962)</td>
</tr>
<tr>
<td>Gangliosides</td>
<td>Pollet et. al., (1978)</td>
</tr>
<tr>
<td>Proteins</td>
<td>Lowry et. al., (1951)</td>
</tr>
<tr>
<td><strong>Nucleic Acids</strong></td>
<td></td>
</tr>
<tr>
<td>i) DNA</td>
<td>Burton (1956)</td>
</tr>
<tr>
<td>ii) RNA</td>
<td>Dische (1955)</td>
</tr>
<tr>
<td><strong>2. Free Radical system</strong></td>
<td></td>
</tr>
<tr>
<td>Lipid Peroxidation</td>
<td>Utley et al., (1967)</td>
</tr>
<tr>
<td>Superoxide Dismutase</td>
<td>Marklund and Marklund (1974)</td>
</tr>
<tr>
<td><strong>3. Antioxidant enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>Glutathione Reductase (GR)</td>
<td>Hazelton and Lang (1985)</td>
</tr>
<tr>
<td>Glutathione Peroxidase (GSHPx)</td>
<td>Lawrence and Burk (1976)</td>
</tr>
<tr>
<td></td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>Aebi and Sutter (1974)</td>
</tr>
<tr>
<td><strong>4. Antioxidant Substances</strong></td>
<td></td>
</tr>
<tr>
<td>Total Sulphydryl groups (T-SH)</td>
<td>Sedlack and Lindsay (1968)</td>
</tr>
<tr>
<td>Free Sulphydryl groups (GSH)</td>
<td>Sedlack and Lindsay (1968)</td>
</tr>
<tr>
<td>Protein bound sulfhydryl</td>
<td>Sedlack and Lindsay (1966)</td>
</tr>
<tr>
<td>Glutathione oxidized (GSSG)</td>
<td>Folbergrova et.al., (1979)</td>
</tr>
<tr>
<td>GSSG/GSH Ratio</td>
<td></td>
</tr>
<tr>
<td><strong>5. Neurotransmitter systems</strong></td>
<td></td>
</tr>
<tr>
<td>(i) Cholinergic</td>
<td></td>
</tr>
<tr>
<td>Acetylcholinesterase (AChE)</td>
<td>Ellman et.al., (1961)</td>
</tr>
<tr>
<td>(ii) Catecholaminergic</td>
<td></td>
</tr>
<tr>
<td>Monoamine oxidase (MAO)</td>
<td>Tabor et.al., (1953)</td>
</tr>
<tr>
<td>Monoamines</td>
<td>Welch and Welch (1959)</td>
</tr>
</tbody>
</table>

### 3.5 Brain Lipids

**3.5.1 Extraction of Lipids from Discrete Brain Areas:**

Different parts of the brain (50-200mg) were homogenized in a glass homogenizer to a final volume of 6ml chloroform-methanol (2:1, v/v) according
Fig 3.4: Sintered glass funnel (G-4).
to the method of Folch et al., (1951). This method was partly modified in our
laboratory (Islam et al., 1980) for isolation of lipids from discrete areas of
the brain. Each homogenate was shaken periodically for an hour and filtered
through sintered glass funnel (G-4) under vacuum (Fig-3.4). The residue of
each test tube was again homogenized with fresh 2ml chloroform-methanol
mixture (2:1) and filtered. The test tubes were rinsed with fresh chloroform-
methanol mixture (2:1) and again filtered. The final volume of each extract
was made upto 10ml with fresh chloroform-methanol mixture (2:1). Thereafter,
25 ml of normal saline solution was added to the extract in each test tube
(4:1 v/v). This was shaken vigorously on cyclomixer and placed at-20 °C in a
deep freeze overnight for complete separation of the two layers. The junction
of the two layers in each test tube was marked. The upper layer was used for
the estimation of gangliosides and desired amount of the layer of each test
tube was collected in stoppered tube with the help of syringe (Fig. 3.5) and
stored at -20 °C for 24 hours. The test tube in which the two layers were
separated, were dried and the volume of the lower layer of each test tube was
measured. This extract was used for the estimation of total lipids,
phospholipids and cholesterol.

3.5.2 Estimation of Total Lipids

Total lipids were estimated according to the method of Woodman and
Price (1972) as follows:
Fig 3.5 :- Special syringe with a long needle.
Principle :- Colour was developed with the help of colouring reagent (phospho-Vanillin) in the presence of H$_2$SO$_4$ and O.D. was read at 540nm. H$_2$SO$_4$ acts upon the double bonds on lipids to produce carbonium ion which simultaneously reacts with phosphate ester of vanillin to form a coloured complex.

Chemical and Reagents-

1. **Standard Solution** :- Standard solution of 0.5mg brain lipids/ml of chloroform-methanol mixture (2:1) was prepared by diluting 1.0 ml refrigerated stock solution (50 mg brain lipids /10ml chloroform-methanol) in a 10ml standard flask and made up the volume to 10.0ml with chloroform-methanol mixture (2:1,v/v).

2. Colouring reagent :- 6.0gm potassium dihydrogen orthophosphate and 0.3gm vanillin were dissolved by heating in a 100ml volumetric flask and the volume was made upto 100.0ml with double distilled water (DDW)

3. Conc. Sulphuric acid

4. Brain lipids :- Lipids were isolated from the rat brain by the method of Folch et.al., (1951), as described in the extraction of lipids from discrete brain areas. The extract was vacuum dried at 40 °C and dried lipids were stored at 0-5 °C.

Procedure:- 0.1ml of duplicate brain extracts and a duplicate set of standard with 50 to 500 µg of brain lipids were taken in 18 x 150mm corning test tubes. 2.5ml conc. sulphuric acid was added to each test tube and heated in boiling
water bath for 20 mins. After cooling, 5.0ml colouring reagent was added and absorption was read at 530nm exactly after 10mins. against reagent blank.

A calibration curve with different concentration (100-600µg) of standard brain lipids (extracted from rat brain) was prepared by the above procedure. The values of the standard curve were plotted by least square method.

**Calculation**: The concentration of total lipids in brain samples were calculated by the following formula:

\[
\text{Total lipids (mg/g fresh weight)} = \frac{C \times V}{V_t \times W_t}
\]

Where,

- \(C\) = Concentration of lipids in µg in 0.1 ml brain extract
- \(V\) = Total volume of lower layer
- \(V_t\) = Volume taken for estimation
- \(W_t\) = Fresh weight of the tissue in mg

The above formula was also used for calculating the concentrations of the phospholipids and cholesterol.

### 3.5.3 Estimation of phospholipids

Phosphate of the phospholipids was estimated by the method of Marinetti (1962).
**Principle** - Organic phosphorus of phospholipids was converted to inorganic phosphorus by digesting the lipids with perchloric acid. When acid hydrolysate is treated with molybdate, it forms phosphomolybdic acid with inorganic phosphorus. Thus phosphomolybdic acid is reduced by addition of 1,2,4-aminonaphthal sulphonic acid (ANSA) reagent to produce a blue coloured complex. The intensity of the blue colour which is proportional to the amount of inorganic phosphorus present, was read at 700nm. Inorganic phosphate content was multiplied by 25 to a lecithin equivalent to organic phospholipids.

**Chemicals and Reagents:**

1. **Standard Solution** - A standard of 0.01mg inorganic phosphate /ml was prepared by diluting 5.0 ml refrigerated stock solution (0.439gm KH$_2$PO$_4$/500ml DDW) in a 100ml standard flask with DDW.

2. Perchloric acid 70% AR

3. 2.5% Ammonium molybdate solution

4. Reducing reagent - 3.0gm of sodium bisulphite, 0.6gm Sodium sulphite and 0.05gm recrystallised 1-amino-2-naphthol-4-sulphonic acid (ANSA) were dissolved in 25ml DDW. A slight yellow solution thus obtained was stored in amber coloured bottle. The colour is stable for a week at room temperature.

5. Recrystallization of ANSA - 15.0gm sodium metabisulphite, 1.0gm sodium sulphite (anhydrous) and 1.5gm crude ANSA were dissolved in 100ml DDW by heating on the boiling water bath. Hot solution was filtered through the filter paper. 1.0ml conc HCl was added in the filtrate and stirred. Precipitate was filtered with suction pump and washed with about 30ml double distilled water.
and finally alcohol till washing is colourless. This purified ANSA was dried in
oven at 100 °C for 1 hr. with least possible exposure to light and was
transferred to amber coloured bottle.

Procedure :- 0.2 ml of duplicate brain lipid extract (lower layer) was placed in
18 x 150mm corning test tube and all the solvent were dried up by heating on
a boiling water bath. 1.0ml AR grade 70% perchloric acid to the samples and
heated on a digestor for 30mins. or until the samples became clear. Thereafter
5ml ammonium molybdate, 0.2ml reducing agent and 7.0ml DDW were added
with vigorous shaking after each addition. The test tubes were heated on
boiling water bath for 7 mins. A calibration curve was prepared with 1µg to 8µg
of phosphorus and 1.0ml perchloric acid alone.

Calculation :- The absorption is a linear function of the phosphorus content
and the amount in unknown sample can calculated by direct proportion with
the absorbance obtained for the standard. The amount of phospholipid was
calculated by multiplying with a factor of 25.

3.5.4 Estimation of cholesterol

Cholesterol was estimated according to the method of Zlatis
et. al., (1954).

Principle:- Cholesterol when dissolved with acetic acid in the presence of
FeCl₃.H₂SO₄ reagents get dehydrogenated to 3,5 cholestadiene or
2,4 cholestadiene which simultaneously polymerises and react with FeCl₃ to
form a violet colour complex which is measured spectrophotometrically at 570nm.

Chemicals and Reagents :

1. **Standard solution**: For stock solution, 10mg cholesterol was dissolved in 10ml chloroform. The working standard was prepared by diluting 1.0ml of the stock solution to 25ml chloroform, so that 1ml of this solution contains 0.04mg cholesterol.

2. Glacial acetic acid

3. FeCl₃

**Procedure**: 0.05ml of different parts of brain extract in duplicate were taken in 18 x 150mm test tubes and placed in boiling water bath until the solvent was completely evaporated. The dried extracts were dissolved in 3.0ml of glacial acetic acid. Then 2.0ml of working FeCl₃ was added to the test tubes. The contents were thoroughly mixed. The tubes were kept in dark for 30 minutes and the O.D. was then measured at 570nm. Reagent blank and standard cholesterol solution were also run simultaneously.

**Calculation**: The cholesterol concentration in brain samples were calculated by the following method.

\[
\text{Cholesterol (mg/g tissue weight)} = \frac{C \times V \times V_t \times W_t}{V_1 \times W_t}
\]

Where,

\[
C = \text{Concentration in } \mu\text{g in 0.05ml brain extract.}
\]
V = Total volume of lower layer.

V_t = Volume taken for estimation.

W_f = Fresh weight of the tissue in mg.

3.5.5 Estimation of gangliosides

Gangliosides were estimated according to the method of Pollet et al., (1978).

Principle: Bound sialic acids in the gangliosides were isolated carefully without their degradation. When samples were heated at 100 °C in a boiling water bath for 30 minutes in resorcinol reagent, the pentose sugar moieties break up and make a coloured complex with resorcinol reagent. This coloured complex is extracted in organic solvent (butylacetate and n-butanol : 85:15 v/v). Organic phase was taken and absorbance was measured at 580nm.

Chemicals and Reagents:

1. Standard solution: A standard solution of 100µg N-acetyl neuraminic acid /ml DDW was made by diluting 1.0ml of the refrigerated stock solution (10.0mg N-acetyl neuraminic acid dissolved in 10ml DDW) to the final volume of 10.0ml with DDW.

2. Resorcinol Reagent: This was prepared by mixing 10.0ml of 3% resorcinol solution in DDW, 80ml Conc. HCl, 0.25ml 0.1 M Copper sulphate and DDW upto 100ml.
Procedure: To 2.0 ml of the upper layer of the lipid extract, 2.0 ml of resorcinol solution was added. The test tubes were heated on boiling water bath for 30 mins. After cooling 5.0 ml of a mixture of n-butyl acetate-n-butanol (85:15, v/v) was added to each tube. The tubes were shaken thoroughly and kept for 15 min. to separate the organic phase. About 3-4 ml of the organic phase was taken and absorbance was measured at 580 nm against a reagent blank. A standard curve with different concentrations of N-acetyl neuraminic acid (5-30 μg) having 2.0 ml final volume of DDW was prepared by the similar procedure.

Calculation: The gangliosides concentration in different brain parts was calculated by the following formula

\[
\text{Ganglioside (mg/g fresh wt.)} = \frac{C \times V}{V_t \times W_t}
\]

Where,

- \(C\) = Concentration in μg in 2.0 ml extract
- \(V\) = Total volume of the upper layer
- \(V_t\) = Volume taken for estimation
- \(W_t\) = Fresh weight of the tissue in mg.

3.6 Estimation of Rate of Lipid Peroxidation

The amount of malonaldehyde formed /30 mins during lipid peroxidation was estimated according to the method of Utley et. al., (1967)
Principle: TBA reacts with lipid peroxide, hydroperoxides and oxygen labile double bond to form the colour adducts.

Chemicals and Reagents:

1. 0.15 m Potassium chloride: 2.2368 g KCl dissolved in 200 ml. DDW

2. 10% (w/v) Trichloroacetic acid (TCA): 10.0 gm TCA dissolved in 100 ml. DDW

3. 0.67% 2-Thiobarbituric acid (TBA): This was prepared by dissolving 0.67 gm. of TBA in 25-50 ml. DDW by adding two pellets of NaOH. The pH of the solution was adjusted to 7.2 with the help of 1 N HCl and the volume was made upto 100 ml. with DDW.

Procedure: Different parts of the brain were homogenized (10% w/v) in chilled 0.15 M KCl. 1 ml. of each homogenate was taken in a 25 ml. conical flask and incubated at 37±1 °C in a metabolic shaker (120 strokes / min., amplitude 1 cm.) for 2 hours. Thereafter 1.0 ml. of the same homogenate was pipetted in centrifuge tube and protein was precipitated by adding 1.0 ml. of 10% TCA. After incubating 1.0 ml. of 10% TCA was added to each sample and both incubated as well as non-incubated samples were centrifuged at 3000 r.p.m. for 10 min. 1 ml. of the clear supernatant was mixed with 1.0 ml of 0.67% TBA and 1.0 ml DDW and the tubes were placed in a boiling water bath for 10 min., cooled and absorbance was measured at 535 nm.

Calculation: Lipid peroxidation was calculated using the following formula:
\[ X = \frac{\text{OD} \times 30 \times 1000 \times 1000 \times 1000 \times 3 \times 2}{1.56 \times 100000 \times 100 \times 180} \]

or \[ X = \frac{\text{OD} \times 10}{1.56} \]

Where,

\[ X = \text{nanomoles of malondialdehyde formed /30 min.} \]

\[ \text{OD} = \text{Change of optical density at zero hour and 2 hour incubation of the sample.} \]

### 3.7 Estimation of Superoxide Dismutase (SOD)

Superoxide dismutase was measured by the method of Marklund and Marklund (1974).

**Principle:** Depends upon autoxidation of pyrogallol

\[ \text{Pyrogallol + O}_2 \xrightarrow{\text{Autoxidation}} \text{Oxidation products + O}_2 \quad (i) \]

\[ 2\text{O}_2 + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{O}_2 + \text{H}_2\text{O}_2 \quad (ii) \]

**Chemicals and Reagents:**

1. 0.05 m. Tris-succinate buffer (pH - 8.2): 606 mg. tris is dissolved in approximately 60 ml DDW. To this, 39.3 mg. EDTA was added and the pH was adjusted to 8.2 with succinic acid solution. The volume was then made to 100 ml with DDW.

2. Succinic acid solution (0.05 m.): 590 gm. succinic acid was dissolved in 100 ml DDW.
3. Pyrogallol solution (8 mM.) - 100 mg. pyrogallol was dissolved in 100 ml DDW. This solution was prepared fresh on the day of experiment.

Procedure: Different saline cleaned CNS parts were homogenized (10% w/v) in chilled 0.15 M KCl and centrifuged in cold at 10,000 rpm for 15 min. A 0.05 ml of clear supernatant was added to 2.85 ml of 0.05 mM Tris-succinate buffer (pH 8.2), mixed well and incubated at 25°C for 20 min. The reaction was started by adding 0.1 ml of 8 mM pyrogallol solution. The contents were shaken well and change in O.D. per min. was immediately recorded for 3 min at 420 nm. A reference set, consisting of 0.05 ml of DDW instead of the sample solution (Clear supernatant was also run similarly).

Calculation: -

\[ SOD = \frac{(A_{/ \text{min. ref}} - A_{/ \text{min. sample}}) \times 30}{(A_{/ \text{min. ref}}/2 \times 0.05 \times 1)} \] Unit/10 mg tissue

Where,

\[ A_{/ \text{min. ref.}} = \text{change of O.D. per min. in reference set} \]

\[ A_{/ \text{min. sample}} = \text{change of O.D. per min. in sample set} \]

Activity Unit: - One unit of the enzyme is defined as the amount of enzyme which causes a 50% inhibition of pyrogallol autoxidation under assay conditions.
3.8  Nucleic Acids

**Principle**: The procedure for the determination of nucleic acids is based on the finding that nucleic acids can be separated from other tissue compounds by their preferential solubility in hot TCA or perchloric acid. The isolated nucleic acids are then quantitated by means of spectrophotometric reactions involving the pentose compound of the nucleic acids.

### 3.8.1 Isolation of Nucleic Acids

Nucleic acids were isolated following the method of Searchy and Macinnis (1970). Different brain regions were weighed and homogenized in 5.0ml of 0.5N perchloric acid. The homogenates were heated at 90 °C in boiling water bath for 10min., cooled and centrifuged at 3,000 rpm for 10min. Supernatants were taken in graduated test tubes and the volume was maintained upto 5.0ml with 0.5N perchloric acid. This extract was used in the estimation of DNA and RNA.

### 3.8.2 Estimation of DNA

DNA was estimated following the method of Burton (1956)

**Principle**: Deoxoyribose is converted into highly reactive hydroxy vulaldehyde, which react with diphenylamine (DPA) to give a blue coloured complex.

Deoxoyribose sugar + DNA \[\text{-------}\] > hydroxy vulaldehyde.
Chemical and Reagents:-

1. Diphenylamine reagent: 1.5 gm. diphenylamine was dissolved in about 50 - 60 ml glacial acetic acid. 1.5 ml. conc. H$_2$SO$_4$ was added to it and the final volume was made upto 100 ml. with glacial acetic acid.

Procedure :- 2.0 ml of the perchloric acid supernatant of nucleic acid extract was taken in a test tube. To this 4.0 ml. diphenylamine (1.5% in CH$_3$COOH-H$_2$SO$_4$) reagent was added and the tubes were heated on boiling water bath for 15 min. After cooling, the colour intensity was measured at 600 nm against a blank sample (2.0 ml. DDW in place of supernatant). A standard curve was prepared by using standard solution in 0.5 NHClO$_4$ (100-600 μg) according to the procedure described. The values were plotted by the least square method.

Calculation :-

\[
\text{DNA} = \frac{C \times V}{V_t \times W_t}
\]

Where,

\[
\begin{align*}
C &= \text{Conc. in mg. (in 20 ml. extract)} \\
V &= \text{Total volume of the extract (4.0 ml.)} \\
V_t &= \text{Volume taken for the estimation} \\
W_t &= \text{Fresh weight of the brain in mg.}
\end{align*}
\]

DNA in the reaction product was calculated using the standard curve of DNA that was run simultaneously with the test sample. Results were expressed as mg. DNA/gm. fresh tissue weight.
3.8.3 Estimation of RNA

RNA was estimated by the method of Dische (1955).

**Principle** :- Pentose sugars are converted to furfural derivatives by heating with conc. HCl. In the presence of FeCl₃ solution the furfural derivatives react with orcinol and produce a green coloured complex.

**Chemicals and Reagents :-**

1. Orcinol reagent :- 33.0 mg. Ferric Chloride was dissolved in about 50 ml. conc. HCl. 3.5 ml 6% orcinol (dissolved in absolute alcohol) was mixed with it, and the volume was made upto 100 ml. with HCl.

**Procedure** :- 2.0 ml of the supernatant of nucleic acid was taken in test tubes. 4.0 ml of the orcinol reagent was added to it. Test tubes were heated on boiling water bath for 15 min., cooled and the absorbance was read at 660 nm against a reagent blank.

**Calculation :-** Same as for DNA

RNA content in the samples was calculated using the standard curve. The results were expressed as mg. RNA/gm fresh tissue weight.

3.9 Estimation Of Protein

Protein estimation was done by the method of Lowry et. al., (1951).
Principle: This method is based on colour reactions of amino acids tryptophan and tyrosine with Folin phenol reagent. By the reaction of these amino acids with phosphomolybdic acid and phosphotungstic acid (Present in Folin's reagent) a blue colour is formed. The colour is the result of reduction of phosphomolybdic acid and biuret reaction of proteins with Cu\(^{2+}\) ions in alkaline medium. O.D was read at 625 nm.

Chemicals and Reagents:

1. Standard Solution: A standard solution of 1.0 mg. BSA/ml. was prepared. Stock standard was diluted ten times to get the working standard of 100 μg/ml.

2. Copper Reagent:
   - Reagent A: 4.0 % Sodium Carbonate in DDW
   - Reagent B: 2.0 % Copper sulphate in DDW.
   - Reagent C: 4.0 % (w/v) Sodium potassium tartrate in DDW.
   - Alkaline Copper Reagent: 1.0 ml. of reagent B + 1.0 ml of reagent C + 48 ml of reagent A were mixed in the same sequence.

3. Folin - Ciocalteau Phenol Reagent

2N solution obtained commercially was diluted 1:1 with double distilled water before use.
Procedure: Residue left in the test tubes after taking the supernatant for nucleic acid estimation, was dissolved in 5.0 ml. DDW. 0.1 ml. of the aliquot was taken in the test tubes from this solution and the volume was maintained upto 1.0 ml with DDW. To this, 5.0 ml. of Copper reagent was added and shaken thoroughly on a cyclo-mixer. After 10 min., 1.0 ml of Folin-Ciocalteau reagent was added. Blue colour is developed. O.D. is read at 625 nm exactly after 30 minutes. Standard protein solution (BSA 20 - 100 µg) and blank were also run simultaneously.

Calculation: Protein in the sample were calculated using the standard curve of BSA and the results were expressed as mg/g weight of wet tissue.

3.10 Estimation of Total Sulfhydryl Group (T-SH)

Total sulfhydryl group was estimated according to the method of Sedlack and Lindsay (1968).

Principle: 5-5’ dithiobis - 2 nitrobenzoic acid (DTNB) is reduced by -SH groups of glutathione (GSH) in alkaline medium to produce 1 mol. of 2 nitro-5 mercaptobenzoic acid per mole of -SH group. The anion (2 nitro-5 mercaptobenzoic acid) has an intense yellow colour which is used to measure -SH group at 412 nm.

Chemicals and Reagents:

1. Standard Solution - A standard solution of 2 x 10^-3 M of GSH was prepared by dissolving 6.146 mg GSH in 10 ml of 0.02 M EDTA.
2. 0.15 M KCl
3. 0.2 M tris buffer in 0.02 M EDTA, pH 8.2
4. 0.01 M DTNB: 0.01 M solution of DTNB was prepared by dissolving 99 mg DTNB in 25 ml of absolute methanol.
5. Absolute methanol

**Procedure**: Various parts of the brain were homogenized in chilled 0.15 M KCl and the volume was adjusted to give a 10% w/v homogenate. 1.5 ml 0.2 M tris EDTA buffer and 0.1 ml DTNB were added. The mixture was shaken and made to 10 ml with 8.3 ml of absolute methanol. The reaction mixture was centrifuged at 6000 rpm for 5 min. in cold. The absorbance of the clear supernatant was read in cold. The absorbance was read at 412 nm. A calibration curve with different concentrations of GSH (61.46-491.68) was obtained according to the same procedure as described above.

**Calculation**: Total -SH group in the samples were calculated using the calibration curve and the results were expressed as μ moles / g tissue.

### 3.11. Estimation of Free Sulfhydryl Groups (GSH)

Free sulfhydryl group was estimated by the method Ellman (1959) as modified by Sedlak and Lindsay (1968)

**Principle**: Same as for total sulfhydryl group estimation.

**Chemicals and Reagents**: -
1. Standard Solution: A standard solution of $2 \times 10^{-3}$ M of GSH was prepared by dissolving 6.146 mg of GSH in 10 ml of 0.02 M EDTA

2. 0.015 M EDTA

3. 10% TCA

4. 0.4 M tris buffer in 0.2 M EDTA, pH-8.9

5. 0.01 M DTNB

**Procedure:** Brains parts were homogenised (10% w/v) in chilled 0.015 M KCl. 1 ml brain (10%) homogenate was doproteinized by adding 1 ml of 10% TCA and centrifuged at 6000 rpm for 5 min. 0.5 ml aliquot from clear supernatant was mixed with 0.5 ml DDW. Thereafter 2 ml, 0.4 M tris buffer and 0.1 ml DTNB were added to it with proper stirring. The absorbance was read within 5 mins. of the addition of DTNB at 412 nm. A calibration curve with different concentrations of GSH (61.46–491.68 μg) was drawn by the procedure described above.

**Calculation:** Free –SH (GSH reduced) in the samples were calculated using the standard curve of GSH and the results were expressed as μ moles / g tissue.

### 3.12 Estimation of Protein bound Sulfhydryl Groups

The estimation of protein bound sulfhydryl group was done according to the method of Sedlack and Lindsay (1968)
Procedure: According to Sedlak and Lindsay (1968),

\[
\text{Total SH} - \text{Free SH} = \text{Protein SH}
\]

Calculation: The results were expressed as μ moles of protein SH / g tissue.

3.13 Estimation of Oxidized Glutathione (GSSG)

GSSG was estimated by the method of Folbergrova et al., (1979)

Principle: The estimation of GSSG by enzymatic method is based on the reduction of GSSG in the presence of NADPH and glutathione reductase and the determination of the decrease in NADPH absorbance at 340 nm.

\[
\text{GSSG} + \text{NADPH} \xrightarrow{\text{GR}} \text{NADP}^+ + \text{GSH}
\]

Chemicals and Reagents:

i) EDTA (1.0 mM, 5.0 mM)

ii) 0.05 M N-Ethyl maleimide (NEM)

iii) TCA (12%, 5% soln)

iv) 100 mM Imidazole (glyoxaline) HCl buffer (pH 7.5)

v) 0.02% BSA

vi) 0.002 mM NADPH

Principle: The brain parts were homogenised in ice-cold 1.0 mM EDTA solution (10% w/v). An aliquot of the homogenate was immediately transferred to tubes containing 0.1 ml of 0.05 M N-Ethyl maleimide (NEM) and allowed to stand for 10 mins at 0°C after mixing. The proteins were precipitated by the
addition of ice-cold 12.0 % TCA giving a final concentration of approximately 5.0% TCA. After thorough mixing the suspension was centrifuged at 0 °C for 10 mins at 3000 rpm. The supernatant was extracted four times with solvent ether to remove TCA and NEM. Aliquots of supernatant were then used for GSSG analysis. The reaction mixture consisted of 0.05 ml of 100 mM imidazole (glyoxaline) HCl buffer (pH 7.5), 0.002 mM EDTA, 0.1 ml of 0.02% BSA, 0.1 ml cystolic supernatant (total volume 1.0 ml). The reaction was initiated by the addition of enzyme, and the final reading were made when the reaction was complete (5 - 10 mins). Standards of GSSG (15 mM solution range of 1.5 x 10^-10-----10^-6) was also run simultaneously.

**Calculation**: The GSSG content in the samples was calculated using the standard curve and the results were expressed in μ mol GSSG/g of wet tissue.

### 3.14 Estimation of Glutathione Reductase (GR)

Glutathione reductase was assayed by the method of Hazelton and Lang (1985).

**Principles**: Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) by NADPH to reduce glutathione (GSH) according to the following equation:

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \xrightarrow{\text{GR}} \text{NADP}^+ + \text{GSH}
\]
The activity of the enzyme was measured by following the decrease in optical density / minute at 340nm during oxidation of NADPH.

**Chemicals and Reagents :-**

i) 0.1 M Tris HCl buffer (pH - 8.0)

ii) 1.0 mM EDTA

iii) 13.0 mM GSSG

iv) 0.1 mM NADPH

**Procedure :-** The reaction mixture consisted of 0.1 ml of 1.0 mM NADPH, 0.2 ml of 3.0 mM GSSG, 0.1 ml of 1.0 mM EDTA, 2.5 ml of 0.1 M Tris - HCl buffer (pH - 8.0) and 0.1 ml tissue supernatant (10% w/v in Tris-HCl buffer, enzyme source) in a total volume of 3.0 ml. The reaction was initiated by the addition of tissue supernatant. Oxidation of NADPH was followed at 340nm. Reference reaction was also run simultaneously. Protein content in enzyme source was also determined (Lowry et al., 1951) Increase in optical density / minute was deduced.

**Calculation :-** Enzyme activity was calculated using the molar extinction coefficient of NADPH (6.22 x 10⁻³ M⁻¹ cm⁻¹) and results were expressed as n mole of NADPH oxidized per minute per mg protein.

### 3.15 Estimation of Catalase (CAT)

Catalase activity was assayed according to the method of Aebi and Sutter (1974)
Principle: It catalyzes the following reaction.

\[
2H_2O_2 \rightarrow 2H_2O + O_2
\]

In the UV range, \(H_2O_2\) shows a continual increase in absorption with decreasing wavelength and maximum at 240 nm. The decomposition of \(H_2O_2\) can be followed directly by the decrease in extinction of 240 nm (\(E_{240}= 40 \text{ cm}^2/\mu \text{ mole}\)). The difference in extinction (\(E_{240}\)) per unit time is a measure of the catalase activity.

Chemicals and Reagents:

i) 50 mM Phosphate buffer (pH - 7.0)

ii) 30 mM Hydrogen peroxide

0.34 ml of 30% \(H_2O_2\) was diluted to 100 ml with phosphate buffer.

Procedure:

Pipetted 3.0 ml of \(H_2O_2\) phosphate buffer into the cuvette. The required amounts of tissue supernatant (cytosolic fraction) as enzyme source was added and the contents were mixed thoroughly. The decrease in absorbance at 240 nm was recorded after every 30 seconds for 3 minutes.

Calculation:

Catalase activity in the samples was calculated using the standard curve and the results were expressed as Units/mg of proteins.

3.16 Estimation of glutathione peroxidase (GSHPx)

GSHPx was assayed according to the method of Lawrence and Burk (1976).
**Principles:** It measure the rate of GSH oxidation by H$_2$O$_2$ as catalyzed by the GSHPx present in the tissue supernatent. The substrate is maintained at a constant concentration by the addition of exogenous GSSG-R and NADPH which immediately converts any GSSG produced to the reduced form (GSH).

$$2\text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSHPx} \rightarrow \text{H}_2\text{O} + \text{GSSG} \rightarrow \text{GSSG-R} \rightarrow \text{GSH} + 2\text{NADP}^+$$

The rate of GSSG formation is then measured by following the decrease in absorption of the reaction mixture at 340nm as NADPH is converted to NADP$^+$.

**Chemicals and Reagents:**

i) 50 mM Potassium Phosphate buffer (pH - 7.0)

ii) 10 mM EDTA

372.24 mg EDTA was dissolved in 100ml double distilled water.

iii) 10 mM NaN$_3$

65.01 mg NaN$_3$ in 100 ml double distilled water

iv) 2 mM NADPH

166.68 mg NADPH in 100 ml DDW.

v) 10 mM GSH

307.32 mg GSH in 100ml DDW

vi) 2.5 mM H$_2$O$_2$

vii) Glutathione reductase (Purified enzyme)

**Procedure:** The reaction mixture consisted of 0.5 ml of 50 mM potassium phosphate buffer (pH - 7.0), 0.3 ml of EDTA (10 mM), 0.4 ml of NaN$_3$, etc.
(1.0 mM), 0.1 ml of NADPH (0.2 mM), GSSG-R (purified enzyme) (1.0 E.U.)
0.1 ml of GSH (1.0 mM), 0.2 ml of H₂O₂ (0.25 mM) and 0.9 ml of tissue
supernatant (10% w/v in phosphate buffer, total volume is 2.5 ml). All
ingredients except enzyme source (tissue supernatant) and H₂O₂ were
combined at the beginning of experiment. Tissue supernatant was added to
the above mixture and allowed to incubate for 5 mins. at room temperature.
The reaction was initiated by addition of H₂O₂. Optical density at 340 nm was
recorded for 5 minutes after 30 seconds interval. Blank reaction with enzyme
source replaced by distilled water were also carried out to find out the non-
enzymatic change, if any. Protein enzyme source was also determined.

Calculation :- Enzyme activity was calculated on the basis of molar
extinction coefficient for NADPH (6.22 x 10⁻³ M⁻¹ cm⁻¹). Results were
expressed as nmol NADPH oxidized / min / mg protein.

3.17 Estimation of Acetylcholinesterase

(AChE)

AchE was assayed according to the method of Ellman et al. (1961).

Principle :- AChE estimation is based on the measurement of the rate of
production of thiocholine as AChE is hydrolyzed. This is accompanied by the
continuous reaction of the thiol with 5,5' dithiobis -2 - nitrobenzoate ion to
produce the yellow anion of 5-thio - 2 - nitrobenzoic acid.

Chemicals and Reagents :-
1. Phosphate buffer (0.1 M, pH - 8.0)
2. DTNB Reagent

39.6 mg of 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was dissolved in 10 ml of 0.1 M phosphate buffer (pH - 8.0) and 15 mg of sodium bicarbonate was added to it.

3. Substrate: - 0.075 M acetylcholine iodine

4. Inhibitor: - $10^{-4}$ M eserine sulphate was dissolved in 0.1 M phosphate buffer (pH - 8.0)

**Procedure:**

Saline cleaned and accurately weighed tissue were homogenized in 0.1 M phosphate buffer (pH - 8.0) in a concentration of 10 mg / ml and centrifuged at 1500 rpm for 5 min. 0.4 ml of the supernatant was pipetted in a cuvette containing 2.6 ml of phosphate buffer. To this, 0.1 ml of DTNB reagent was added and mixed well. O.D. was read at 412 nm and absorbance of the suspension was set to zero. 0.02 ml of the substrate was added and the change in the absorbance were recorded from 5th to 10th min. at the interval of one minute. To determine non-specific esterase, 0.1 ml of eserine sulphate (inhibitor) was added to another cuvette containing 0.4 ml of homogenate supernatant, 2.5 ml of phosphate buffer and 0.1 ml DTNB reagent. The changes in absorbance were recorded as described above, after adding 0.02 ml of substrate. The rate of change of activity of the suspension with eserine was subtracted from that of the suspension without eserine. The enzyme activity is expressed as $\mu$ moles of substrate hydrolyzed per gm. tissue per minute.
Calculation :-

\[ R = \frac{A \times 1}{1.36 \times (10^{-4})} = \frac{5.74}{(400/3120) \times Co} \]

Where,

- \( R \) = Rate of enzyme activity in moles of substrate hydrolyzed/g tissue/min.
- \( A \) = change in absorbance per minute
- \( Co \) = Original concentration of tissue (mg/ml).

3.18 ESTIMATION OF MONOAMINE OXIDASE (MAO)

The activity of monoamine oxidase (MAO) was assayed following spectrophotometric method of Tabor et al. (1953).

Principle:- The benzylamine undergoes oxidative deamination in the presence of MAO and benzaldehyde is formed.

\[ \text{R-CH}_2\text{CH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{R-CH}_2\text{CHO} + \text{H}_2\text{O}_2 + \text{NH}_3 \]

Chemicals and Reagents :-

1. 0.5 M Phosphate buffer (pH - 7.2)
2. Perchloric acid, 10% (AR)
3. Substrate 0.1 M benzylamine hydrochloride

Procedure :- The reaction mixture in a final volume of 2 ml. consisted of 0.4 ml 0.5 M phosphate buffer (pH - 7.2), 0.1 ml of 0.1 M benzylamine hydrochloride and 0.2 ml of brain homogenate (10% w/v in phosphate buffer).
The reaction mixture was incubated at 37°C for 30 min. Centrifuging at 2,500 rpm for 10 min precipitated the proteins. The O.D. of benzaldehyde formed was read in the supernatant at 250 nm. against reagent blank treated similar to samples containing 0.2 ml of 0.44 M sucrose instead of the brain homogenate.

**Calculation :-**

\[
\text{Activity of MAO} = 15.385 \times \frac{\text{O.D.}}{\text{Protein Conc.}}
\]

Where,

\[
\text{O.D.} = \text{Change in O.D per minute}
\]

The enzyme activity is expressed as n moles of benzaldehyde formed / min / mg. protein.

### 3.19 Estimation of Monoamines

Monoamines, dopamine (DA), norepinephrine (NE), and Serotonin (5-HT) were extracted and estimated according to method of Welch and Welch (1969).

**Chemicals and Reagents :-**

1. n-Heptane (AR) was washed with one fifth volume of IN NaOH followed by a wash of IN HCl before use.
2. n - Butanol ( AR)
3. Peroxide free ether :- Diethyl ether was washed with saturated solution of ferrous sulphate to remove accumulated peroxides. Thereafter, washed with double distilled water (DDW) to remove the excess of sulphate.
4. Ethylenediamine: was redistilled and stored in dark bottle in the cold.

5. Acetate Buffer (2M, pH-6.8): 2M acetic acid was adjusted to pH 6.8 with 2N sodium hydroxide.

6. Phosphate Buffer (0.5 M, pH-7.3): 77 ml. of 0.5 M monobasic sodium phosphate (NaH$_2$PO$_4$) was mixed with 23 ml. of 0.5 M dibasic potassium phosphate (KH$_2$PO$_4$).

7. Sodium Thiosulphate (0.1N): 1.581 g of Sodium thiosulphate (Na$_2$S$_2$O$_3$ 5H$_2$O) was dissolved in 100 ml DDW and stored in dark bottles.

8. EDTA solution (10%): 10 g of ethylenediamine tetra-acetic acid (EDTA) was dissolved in 100 ml DDW.

9. Alkaline Sulphite - EDTA solution: 12.6 gm. Sodium sulphite (Na$_2$SO$_3$ 7H$_2$O) was dissolved in 100 ml 10% EDTA solution and diluted to 250 ml with 5N NaOH.

10. Alkaline Ascorbic Acid - Ethylenediamine Solution: 200 mg. of ascorbic acid was dissolved in 25 ml of 0.01 N HCl and added to a mixture of 0.5 ml ethylenediamine and 22.5 ml of 10 N NaOH. This was thoroughly mixed by shaking. This reagent was freshly prepared immediately before use.

11. Iodine Solution: 3.175 g iodine together with 12.5 g of sodium iodine were dissolved in 250 ml of DDW and stored in dark coloured bottle in cold.

12. Stock Solutions: The solutions of dopamine, norepinephrine and serotonin were prepared in 0.01 N HCl at a concentration of 100 μg/ml and stored in refrigerator.
13. Standard Solutions:- On the day of experiment, the stock solutions were diluted with 0.01 N HCl to get working standard solutions corresponding to 100 ng/ml of NE and 200 ng/ml of DA and 5-HT.

3.19.1 Extraction of Monoamines

The brain parts were homogenized in 1.5 ml. ice-cold 0.01 N HCl. 10 ml. of 10% EDTA was added to the homogenate and then transferred to glass stoppered bottles containing 2.5 ml. of n-butanol and 4g of NaCl. The bottles were shaken for 10 min. on a reciprocating shaker at about 250 excursions/min. and centrifuged at 3,000 rpm. for 10 min. in cold. The butanol layer was separated and transferred to another set of glass-stoppered bottles containing 40 ml. n-heptane. 1.5 ml. of 0.5M phosphate buffer (pH-7.3) was added to heptane: butanol mixture. The bottles were shaken again for 10 min. and centrifuged at 2000 rpm. for 8 mins. in cold. At this stage, it was necessary that the pH of phosphate buffer should not be allowed to drop below 7.0 for even if the pH dropped only to 6.5 - 6.8 the recovery was greatly reduced. The phosphate buffer (1.5 ml.) was drawn from butanol phase and transferred to clean 30 ml. bottle and was acidified with 3N HCl to pH 3.5 - 4. Thereafter 20 ml. of peroxide free ether was added and the bottles were shaken for 10 mins. and centrifuged at 2000 rpm. for 10 mins. in cold. The acidic aqueous layer was taken from bottom with 0.5 ml micropipette and three 0.5 ml. aliquots were collected separately in cold. These were used for the determination of DA, NE and 5-HT respectively.
3.19.2 Analysis of Monoamines

The samples of monoamines obtained after extraction, were analyzed using F-2000 fluorescence spectrofluorometer (Hitachi, Japan) according to the following procedures:

1) ESTIMATION OF 5-HT

0.5 ml. of the sample was mixed with 0.5 ml. of 6 N HCl and its native fluorescence was immediately read at 295/535 nm using 5 mm slit. Thereafter these tubes were used as blanks for DA analysis.

2) ESTIMATION OF NOREPINEPHRINE

To each 0.5 ml. extract, 0.5 ml. 2 M acetate buffer, 0.1 ml. iodine solution, 0.15 ml. 0.1 ml. sodium thiosulphate and 0.2 ml. alkaline ascorbic acid - ethylenediamine mixture were added in respective order, thereby making a total volume of 1.4 ml. Each reagent was added after an interval of 5 min. and the viscous mixture was thoroughly mixed by shaking. The fluorescence was recorded at 410/510 nm using 2 or 3 mm slit.

3) ESTIMATION OF DOPAMINE

0.5 ml. extract was mixed at 5 min. intervals with 0.5 ml. 2 M acetate buffer, 0.1 ml. iodine solution, 0.2 ml. alkaline sodium sulphite - EDTA solution and 0.2 ml. 1.1 glacial acetic acid- HCl mixture, respectively to make a final volume of 1.5 ml. The tubes were placed in boiling water bath for 45 mins. and then allowed to cool at room temperature. Fluorescence was read within 1-2 hr. at 335/380 nm using 2mm. slit.

Preparation of Standard Curve :- The standard curve for DA, NE and 5-HT were prepared by analyzing fluorometrically (as described
in "Analysis of Monoamines"), the standard amine solutions in the range of 0.1 ml - 0.7 ml corresponding to 10 ng - 70 ng of NE and 20 ng - 140 ng each of DA and 5-HT.

3.20 Statistical Analysis

The data obtained were statistically analyzed by student's 't' test method using the following formula:

\[ t = \frac{|d|}{s / n} \]

where,

- \( s \) = standard deviation
- \( n \) = no of observation
- \( d \) = mean of difference between control and experimental values

'p' values were obtained from student's 't' test table using the 't' values calculated to find significant changes.

'p' values of 0.005, 0.01 and 0.001 were regarded as significant.