MATERIALS AND METHODS
The description of the materials and methods used in this study is under the following headings:

[A] Materials used for collection of the data
[B] Methodology used to acquire the data
[C] Statistical analysis of the data

[A] MATERIALS USED FOR COLLECTION OF DATA:

Materials used in this study has been broadly classified under two different headings as follows:

i) ANIMALS:

Male Wistar rats, weighing in the range of 200-300 gm were used. Rats were chosen as experimental animals for the following reasons:

(1) Easy availability.
(2) Ease of handling.
(3) Sufficient literature were available.

All the rats used in this study were obtained from the Central Animal House Facility, Jawaharlal Nehru University. The animals were brought from the animal house to the lab, kept in semitransparent polyethylene cages and were maintained under 12:12 dark:light cycle. Food and water was provided ad libitum to all the animals.

ii) CHEMICALS, SOLVENTS AND BIOCHEMICALS:

All the solutions were prepared in glass double distilled water. The list of chemicals, solvents and the biochemicals used and the name of the supplier is as follows:
CHEMICAL

5-Hydroxyquinoline
Acetic Acid
Acetone
Acetylthiocholine iodide
Adenosine deaminase
Adenosine monophosphate
Adenosine triphosphate (ATP)
Ammonia solution (20%)
Ammonium Molybdate
ANS AL
Butylated-hydroxytoluene
Chloroform
Clorgyline
D-Glucose
Diphenyl hexatriene
Deprenyl
DiSodium Hydrogen Phosphate
DTNB
Ficoll
Folin's reagent
Glucose-6-phosphate
Glucose-6-phosphate dehydrogenase
Glycylglycine
HEPES
Kynuramine dihydrobromide
Magnesium Chloride
Mannitol
Methanol
NAD
NADP
Perchloric Acid
Potassium dihydrogen Phosphate
Potassium salt of EDTA
Silica Gel G
Sodium Carbonate
Sodium Chloride
Sodium Dihydrogen Phosphate
Sodium glycerophosphate
Sodium Pyruvate
Sodium hydroxide
Sucrose
Tetrahydrofuran
Trichloroaceticacid
Tris-HCl
Triton-X
Zinc Sulphate

SUPPLIER

Sigma Chemicals, U.S.A.
Qualigens, India.
Qualigens, India.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
S.R.L., India.
S.R.L., India.
Sigma Chemicals, U.S.A.
Merck, India.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
Qualigens, India.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
B.D.H., India.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
S.R.L., India.
S.R.L., India.
Sigma Chemicals, U.S.A.
Pharmacia, U.S.A.
S.R.L., India.
S.R.L., India.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
Sigma Chemicals, U.S.A.
Loba Chemie, India.
Qualigens, India.
S.R.L., India.
S.R.L., India.
Qualigens, India.
Qualigens, India.
B.D.H., India
Merck, India.
Qualigens, India.
Qualigens, India.
Qualigens, India.
Qualigens, India.
Sigma Chemicals, U.S.A.
B.D.H., India.
Qualigens, India.
B.D.H., India.
S.R.L., India.
Sigma Chemicals, U.S.A.
B.D.H., India.
B) METHODOLOGY USED TO ACQUIRE DATA:

Methodology used in this study has been divided into two main topics as follows:

METHODOLOGY USED FOR REM SLEEP DEPRIVATION:

In this study, a very simple, non-surgical, non-chemical methodology was used for selective REM sleep deprivation. This technique, known as the "Flower pot or the water tank technique", was originally developed by Jouvet (1972) and has been found to be highly effective technique for selective REM sleep deprivation without a significant loss of NREM sleep.

PRINCIPLE:

The principle of this method as follows:

The animal (rat in this study) is placed on a small platform (an inverted flower pot) surrounded by water. This allows the animal to sit and sleep (only NREM sleep) but no REM sleep is possible because the platform is very small and the animal is unable to maintain the necessary posture due to the postural atonia. At the onset of REM the animal is awakened to avoid falling in water or due to its coming in contact with water. Hence the animal on the platform can go to NREM sleep but not REM sleep. Controls are placed on platforms which are large enough for them to procure both NREM as well as REM sleep but the rest of the conditions are the same as the experimental animal on the smaller platform.

PROCEDURAL DETAILS:

Along with the experimental, suitable controls other than the large platform control experiments were also done to eliminate the possibility of stress whatsoever. The procedural details are broadly divided as follows:

EXPERIMENTAL ANIMALS FOR REM SLEEP DEPRIVATION:

Experimental (E) rats were maintained on a 6.5 cm diameter
island projecting above a pool of water in a polypropelene cages. Food and water were supplied ad lib. The size of the platform was selected based on the weight of the animals as reported earlier (Mendelson et al. 1974, Yanick and Radulovacki, 1989). REM sleep deprivation was continued for one, two, four and eight days.

FREE MOVING CONTROLS (FMC):

These were normal free moving rats were taken randomly from a common pool of rats and were placed in the same room where the experimental rats undergoing REM sleep deprivation were placed. Food and water were supplied ad lib.

LARGE PLATFORM CONTROLS:

To rule out the possibility of non-specific effects, a group of animals was maintained on a large circular island of 12.5-13.5 cm diameter projecting above a pool of water for identical period as that of the E rats. These large platform control animals (LPC) were maintained in the condition similar to the E rats except that the platform size was a little larger so that the animal can have both the REM sleep in addition the NREM sleep. The rats were also maintained in the same room where the experimental animals undergoing REM sleep deprivation and the free moving control rats were placed.

MOVEMENT RESTRICTION CONTROLS:

One of the non-specific factors might be the movement restriction of the experimental animals during REM sleep deprivation. To overcome the effect of movement restriction, rats were maintained individually on normal litter for 8 - 15 days in cages of 12.5 cm diameter so that the movement was restricted (RM) but the rats did not undergo possible stress induced by raised platform surrounded by water used in the E situation. These were also maintained in the same room where the experimental animals were
undergoing REM sleep deprivation as well as other control rats were maintained.

SWIMMING CONTROLS:

To eliminate the possibility of the effect of muscular hyperactivity on smaller platform, Swimming (SW) control was done in two groups of rats, by allowing the rats to swim continuously for 2 and 5 h in a pool of water 35 cm deep and 32 cm dia at room temperature. Swimming controls were done with an idea that since it is difficult to equate the stress, if at all, induced by deprivation and due to swimming, if the effect on the enzyme activity would be due to stress the activity would be significantly different in SW5 as compared to that of SW2.

RECOVERY CONTROLS:

In a separate set of experiment, a group of REM sleep deprived animals were allowed to recover for at least equivalent period, by maintaining them individually, in normal rat cages. These recovered (R) animals were provided with food and water ad lib and were placed in the same room where the experimental and other control animals were kept.

METHODOLOGY USED FOR BIOCHEMICAL ESTIMATIONS:

Biochemicals estimation were performed in controls and the experimental animals in whole brain, as well as in different areas of the brain viz. the cerebrum, the cerebellum, the brain stem, the medulla, the pons and the midbrain. The details of the methodology used are classified as follows:

BIOCHEMICAL ESTIMATIONS:

Biochemical estimation conducted in this study can be described, for convenience into three broad sub-headings as follows:

Enzyme Activity Estimations
Phospholipid Estimation and
Membrane Fluidity Estimation  
ENZYME ACTIVITY ESTIMATIONS:

The methodology used for enzyme activity estimation is broadly divided as follows:

I] PREPARATION OF TISSUES FOR HOMOGENIZATION
II] HOMOGENIZATION OF THE TISSUE
III] ESTIMATION OF THE PROTEIN CONCENTRATION
IV] ESTIMATION OF THE SPECIFIC ACTIVITY OF ENZYMES

PREPARATION OF THE TISSUES FOR HOMOGENIZATION:

Control or experimental rats were sacrificed by decapitation after cervical dislocation. The skull was cut open and the brain was removed in a chilled petri dish containing the homogenizing buffer which was kept on ice, so as to maintain the tissue at 4°C. The whole procedure took about 2 or 3 min. The tissue was then washed with ice cold homogenizing buffer to remove the blood. After washing, either the whole brain was homogenized or different regions viz. cerebrum, cerebellum, brain stem, midbrain, pons and medulla were dissected out within two-three min. After removing the brain, the cerebellum was first dissected out. The brain stem was separated from the cerebrum by giving a dorsoventral cut by a plane passing through the anterior border of the superior coliculli dorsally and posterior end of the corpus mammillary ventrally. The brain stem was further dissected into the midbrain, the pons and the medulla. The part of the brain stem from anterior border to the first deep notch ventrally (approx. 3 mm) anterior to that of N. trigeminus corresponding approximately to the level of N. trochlearis was taken as midbrain. Thereafter, up to the anterior margin of the corpus trapezoideum was taken as the pons. The anterior border of the pons would pass anterior to that of locus coeruleus. After dissecting out
the pons the remaining area of the brain stem up to the level corresponding to the posterior border of the N. hypoglossus was taken as medulla. Thus, as per the transections shown in Zeman and Innes (1963), the medulla in this study would approximately correspond to the transection levels between A3 to A8, pons between A8 to A11 and the midbrain A11 to A14. After dissecting out the different areas the tissue was then homogenized. All the procedures was done on ice and the temperature was maintained around 4°C.

HOMOGENIZATION OF THE TISSUE:

Tissue was homogenized in the Potter-Elvjham homogenizer using different homogenization buffers, depending on the biochemical parameter to be studied.

III] ESTIMATION OF THE PROTEIN CONCENTRATION:

Protein concentration of different regions of the brain was estimated by the method of Lowry et al. (1951).

Assay System:

The reaction mixture contained a suitable aliquot of the homogenate and double distilled water was added to make up the volume to 0.5 ml. 5 ml of freshly prepared Lowry’s reagent (Lowry’s reagent contained 48 ml of 2% sodium carbonate dissolved in 0.1 N NaOH, to this 1 ml of 0.5% of copper sulphate and 1 ml of 1% Na-K tartrate solutions were added) was added and the mixture was vortexed and incubated at room temperature for 30 min. After incubation 0.5 ml of Folin’s reagent was added. The reaction mixture was again vortexed and incubated at room temperature for 10 min. The color developed was read, within 10 min at 700 nm in Shimadzu UV 260 or Hitachi 2000 spectrophotometer. Bovine serum albumin was used as a standard.

ESTIMATION OF THE ACTIVITIES OF ENZYMES:

The following enzymes activities were estimated in control
animals and in REM deprived animals of different time durations:

1] Acetylcholinesterase (AChE) and its Molecular forms
2] Monoamine Oxidase (MAO) and Monoamine Oxidase-A (MAO-A) and Monoamine Oxidase-B (MAO-B)
3] Hexokinase
4] Lactate Dehydrogenase (LDH)
5] Glucose-6-Phosphatase (G-6-Pase)
6] Glucose-6-Phosphate Dehydrogenase (G6PDH)
7] 5'-Nucleotidase

In detail the estimation of

**THE ACTIVITY OF ACETYLCHOLINESTERASE** :

[Systemic Name: Acetylcholine acetyl hydrolase, EC: 3.1.1.7]

AChE is a specific cholinesterase which hydrolysis the neurotransmitter acetylcholine (ACh).

\[
\text{(AChE)} \\
\text{Acetylcholine + H}_2\text{O} \rightarrow \text{Choline + Acetate}
\]

In this study AChE was estimated in the whole brain, the cerebrum, the cerebellum the brain stem, the medulla, the pons and the midbrain. The methodology in detail is as follows:

Whole brain and its different regions viz. cerebrum, cerebellum and brain stem were homogenized in 1 M Saline phosphate buffer (pH 7.4) containing 1% Triton X-100(v/v). The Triton X-100 was used to solubilize the membrane bound enzyme. The activity of AChE was assayed by the method of Ellman et al. (1961).

**Assay System** :

For AChE estimation the reaction mixture contained 0.168 M phosphate buffer (pH 8.0), 0.01 mM DTNB and 0.01mM acetylthiocholine and increase in the absorbance was observed spectrophotometrically at 412 nm for 5 min, at room temperature
using Shimadzu UV 260 recording spectrophotometer. The activity of acetylcholinesterase was expressed as μmoles/min/mg protein.

**Principle:**

The thio analogue of ACh, acetylthiocholine iodide is used as a substrate for AChE assay. Acetyl-thiocholine iodide is hydrolysed by AChE to form a reactive sulfhydryl (-SH) group on thiocholine.

\[
(AChE) \quad \text{Acetylthiocholine iodide} + H_2O \quad \rightarrow \quad \text{-SH-choline iodide} + \text{Acetate}
\]

This product reacts with dithionitrobenzene (DTNB) to form a yellow anion, 5-thio-2-nitrobenzoate.

\[
\text{-SH-choline iodide} + \text{DTNB} \quad \rightarrow \quad \text{5-thio-2-nitrobenzoate}
\]

(yellow color)

This change in the absorbance (absorbance increases with an increase in yellow coloration) is followed at 412 nm in a recording spectrophotometer.

**THE ACTIVITY OF MOLECULAR FORMS OF ACETYLCHOLINESTERASE:**

The enzyme AChE is known to exist in at least two different forms; the membrane bound form and the free or the soluble form, though their functions are not very clear. It has been proposed that the membrane bound form (10S) of AChE is located mainly in the nerve terminals and primarily responsible for the degradation of ACh (Sirvio et al. 1989). Crude soluble and membrane-bound forms were prepared by the method of Rakonczay et al. (1981). The dissected regions were homogenized in 12.5 mM sodium phosphate buffer (10%) (pH 7.2) containing 0.4 M NaCl. This was then centrifuged at 100,000g for 1 hr (Beckman Ultracentrifuge L-5-50B using 80Ti rotor). Supernatant (S1) was used as the source of crude soluble form of AChE. Pellet obtained was further homogenized in the same
volume of glass distill water and 9 volumes of 13.75 mM (pH 7.2) containing 0.44 M NaCl and 0.55% Triton X-100 (w/v). The suspension was stirred for 30 min and centrifuged at 100,000g for 1 hr (Beckman Ultracentrifuge L-5-50B using 80Ti rotor). The supernatant (S2) was used as the source of crude membrane-bound form of AChE. The AChE activity was estimated in both S1 and S2 by the method of Ellman et al. (1961), the details of which are as mentioned above. The activity of different molecular forms of acetylcholinesterase was expressed as μmoles/min/mg protein.

THE ACTIVITY OF MONOAMINE OXIDASE:
[Systemic Name: Amine: Oxygen oxidoreductase [deaminating], EC: 1.4.3.4]

MAO catalyses the oxidative deamination of many biogenic amines.

\[
\text{(MAO)} \\
\text{R-NH}_2 + \text{O}_2 \rightleftharpoons \text{R-CHO} + \text{H}_2\text{O}_2 + \text{NH}_3
\]

MAO is localized in the mitochondria (Finberg and Youdim, 1983).

Isolation of crude mitochondrial pellet:

The activity of MAO was estimated in the crude mitochondrial pellet in whole brain homogenate, the cerebrum, the cerebellum, the brain stem, the medulla, the pons and the midbrain. Each regions from two different rats were pooled for each assay and homogenized in 0.32 M Sucrose buffer containing 12.5 mM Tris- HCl (pH 7.4). The homogenate was centrifuged at 3,000 g (5,000 rpm) for 10 min. The pellet was discarded and the supernatant was again centrifuged at 27,000 g (15,000 rpm) for 30 min to pellet out the crude mitochondria. In case of whole brain, cerebrum, cerebellum and brain stem, the pellet was further homogenized in appropriate quantity of 0.32 M sucrose buffer so that the final protein concentration became 4-10 mg/ml of the crude mitochondrial homogenate. In case of
medulla, pons and midbrain, the protein concentration was made up to 1 mg/ml. This crude mitochondrial preparation was used for MAO assay.

The activity of MAO in the cerebrum, cerebellum, and the brain stem was assayed by the method of spectrophotometric method of Catravas, et al. (1977). In case of medulla, pons and the midbrain, as the protein content was low, MAO was estimated by the spectrofluorimetric method of Karml, (1965)

**Assay system for the spectrophotometric method:**

The reaction mixture contained 0.44 mM Tris-HCl buffer (pH 7.4) containing 0.08 mM MgCl₂, 0.22 mM Kynuramine dihydrobromide (final concentration) and the crude mitochondrial extract (0.4-1 mg protein) as the source of enzyme. The reaction was carried out for 90 min at 37°C and then terminated by 0.2 ml of 0.5 N NaOH and 0.4 ml of 10% ZnSO₄. The mixture was vortexed and heated in the water bath for 10 min, cooled and centrifuged at 4,300 g (6,000 rpm) for 10 min to remove the precipitate. The clear supernatant was then read at 330 nm at room temperature using Shimadzu UV 260 recording spectrophotometer. 4-hydroxy quinoline was used as the standard. The activity of monoamine oxidase was expressed as μmoles/90 min/mg protein.

**Assay system for the spectrofluorimetric method:**

In case of medulla, pons and the midbrain, as the protein content was low, MAO was estimated by the spectrofluorimetric method. The reaction mixture contained 20 μM kynuramine dihydrobromide (final concentration), 100 mM phosphate buffer and 100-200 μg protein (crude mitochondrial pellet as source of enzyme). The mixture was incubated at 37°C for 10 min and the reaction was terminated with 400 μl 10% TCA. That was then centrifuged at 10,000 g (10,000 rpm) for 10 min. There after 800 μl of the supernatant was added to 2 ml
of 1N NaOH and the fluorescence was read at 315 nm (excitation) 380nm (emission) at room temperature using Shimadzu RF 540 spectrofluorimeter. In case of blank, 40μl of TCA was added before the addition of Kynuramine. 4-hydroxyquinoline was used as standard. The activity of monoamine oxidase was expressed as μmoles/90 min/mg protein.

**Principle:**

Both the methods estimate 5-hydroxyquinoline, hence the principle of both the methods is the same. MAO oxidatively deaminates kynuramine dihydrobromide to generate an intermediate amine aldehyde product, which undergoes non-enzymatic intramolecular condensation to give 4-hydroxyquinoline which can be estimated spectrophotometrically at 330 nm or spectrofluorometrically with excitation at 315 nm and emission at 380nm.

**THE ACTIVITY OF MAO-A AND MAO-B:**

MAO is known to exhibit two different forms viz. MAO-A and MAO-B (Johnston, 1968) and the former is primarily responsible for the breakdown of NE whereas the latter in rat brain, is a non-specific enzyme for the break down of amines in general. The activity of MAO-A and MAO-B were estimated in the crude mitochondrial preparation of the cerebrum, the cerebellum, the brain stem, the medulla, the pons and the midbrain. Each region from two different animals of the same group were pooled together washed and homogenized and the crude mitochondrial pellet isolated in the same way, as in the case of MAO, as stated above. The activity of MAO-A and MAO-B in the cerebrum, cerebellum and the brain stem were estimated by the spectrophotometric method of Catravas, et al. (1977) with slight modification. The assay system included specific inhibitors of MAO-A and MAO-B. In case of MAO-A estimation 1 umol (final concentration) of (-) deprenyl (MAO-B
inhibitor) was added and in case of MAO-B 1 μmol (final concentration) of clorgyline (MAO-A inhibitor) was added to the reaction mixture. The rest of the procedure used was the same as in case of MAO. 4-hydroxyquinoline was used as the standard.

In case of medulla, pons and the midbrain, as the protein content was low, MAO-A and MAO-B were estimated by the spectrofluorimetric method of Karml, (1965) with the same modifications. In case of MAO-A estimation 1 μmol (final concentration) of (-) deprenyl (MAO-B inhibitor) and in case of MAO-B 1 umol (final concentration) of clorgyline (MAO-A inhibitor) was included in the reaction mixture. The rest of the procedure used was the same as in case of MAO. 4-hydroxyquinoline was used as the standard. The activity of MAO-A and MAO-B was expressed as μmoles/90 min/mg protein.

ESTIMATION OF ACTIVITY OF HEXOKINASE :
[Systemic Name: ATP:D-Hexose-6-phosphotransferase EC: 2.7.1.1]

Hexokinase catalyses the following reaction.

[hexokinase]
D-Glucose + ATP -------------> D-Glucose-6-phosphate + ADP

In this study hexokinase was estimated in the non synaptic mitochondrial preparations of the cerebrum, the cerebellum, the brain stem, the medulla, the pons, and the midbrain. The methodology in detail is as follows:

In the brain, majority of hexokinase activity is found in the mitochondrial fraction (Chou and Wilson, 1972). Hence, in this study mitochondrial fraction was used to assay the activity of hexokinase. Non-synaptic mitochondrial fraction was prepared by the method of Lai and Clarke (1979) as follows:

Each region from two different animals of the same group were pooled together washed and homogenized in the isolation medium
containing 0.25 M Sucrose, 0.05 mM potassium salt of EDTA and 10 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 2,000 g (5,000 rpm) for 3 min and the supernatant obtained was recentrifuged at 2,000 g (5,000 rpm) for 3 min. The supernatant of the recentrifugation was centrifuge at 12,500 g (10,000 rpm) for 8 min to obtain the crude mitochondrial pellet. Crude mitochondrial pellet was suspended in 3% Ficoll containing 0.24 M mannitol, 60 mM sucrose 50 μM EDTA and 10 mM tris-HCl (pH 7.4). This was layered over 6% Ficoll and spun at 12,500 g (10,000 rpm) for 30 min. The resultant pellet was then suspended in the isolation medium and centrifuged at 12,500 g (10,000 rpm) for 10 min. The pellet obtained was used for the estimation of the hexokinase activity. The activity of hexokinase was assayed by the method of Chou and Wilson (1972).

**Assay System:**

For estimation of hexokinase activity the reaction mixture contained 40 mM HEPES buffer (pH 8.5), containing 6.7 mM MgCl₂, 3.3 mM Glucose, 1 unit of glucose-6-phosphate dehydrogenase, 0.32 mM NADP, 100-200 μg of protein (non-synaptic mitochondrial pellet as source of enzyme) and 6.7 mM ATP. The increase in the absorbance was observed, in a recording spectrophotometer (Shimadzu UV 260), at 340 nm for 5 min against the substrate blank (ATP replaced by distil water). The activity of hexokinase was expressed as μmoles/min/mg protein.

**Principle:**

This method is based on the coupled assay. Hexokinase catalyses the following reaction

\[
\text{[hexokinase]} \\
\text{D-Glucose + ATP} \rightarrow \text{D-Glucose-6-phosphate + ADP}
\]

Glucose-6-phosphate formed is used as one of a substrate by the
enzyme G6PDH.

\[
\text{[G6PDH]} \\
\text{D-Glucose-6-phosphate + NADP'} \rightarrow \text{D-Glucono-\(\gamma\)-lactone-6-phosphate + NADPH}
\]

The product of G6PDH action NADPH can be monitored at 340 nm in a recording spectrophotometer.

**THE ACTIVITY OF GLUCOSE-6-PHOSPHATASE**

[Systemic Name: D-Glucose-6-phosphohydrolase. EC: 3.1.3.5]

G-6-Pase catalyses the following reaction.

\[
\text{[G-6-Pase]} \\
\text{D-Glucose-6-phosphate} \rightarrow \text{D-Glucose + Pi}
\]

**Preparation of the microsomal fraction**

In this study G-6-Pase was estimated in the microsomal fraction of the cerebrum, the cerebellum, the brain stem, the medulla, the pons, and the midbrain. Each region from two animals of the same group was pooled, and homogenized in the isolation medium containing 0.25 M Sucrose, 0.05 mM potassium salt of EDTA and 10 mM Tris-HCl (pH 7.4). The homogenate was centrifuged at 10,000 rpm (12,500 g) for 30 min and the supernatant recentrifuged again at 40,000 rpm (1,00,000 g) for 1 h (Beckman Ultracentrifuge). The pellet obtained was used as the microsomal fraction for the estimation of G-6-Pase.

**Assay System**

The reaction mixture for the assay of G-6-Pase contained 0.1 M Tris-HCl buffer (pH 7.0), 100 mM Glucose-6-phosphate and 400-800 \(\mu\)g of protein (microsomal pellet as the source of enzyme). The reaction mixture was incubated at 37°C for 30 min, then 1 ml of 10\% TCA was added to terminate the reaction. This was then centrifuged at 3000 rpm (1,000 g) for 10 min and 1 ml of the clear supernatant
was taken for the estimation of free phosphorous (Simpson et al. 1987). The activity of G-6-Pase was expressed as μmoles/min/mg protein.

**Principle:**

G-6-Pase catalyses the following reaction.

\[
\text{[G-6-Pase]} \\
\text{D-Glucose-6-phosphate} \quad \longrightarrow \quad \text{D-Glucose + Pi}
\]

The liberated inorganic phosphorus is estimated.

**THE ACTIVITY OF LACTATE DEHYDROGENASE:**

[Systemic Name: L-Lactate:NAD oxidoreductase. EC: 1.1.1.27]

LDH catalyses the following reaction.

\[
\text{[LDH]} \\
\text{Pyruvate + NADH+H+} \quad \longrightarrow \quad \text{Lactate + NAD+}
\]

In this study LDH was estimated in the cytosolic fraction of the cerebrum, the cerebellum and the brain stem. The methodology in detail is as follows:

**Preparation of the cytosolic fraction:**

Cerebrum, cerebellum and the brain stem were separately dissected out, and homogenized in 10 mM Tris-Cl (pH 7.4) buffer containing 0.25 M Sucrose. The homogenate was centrifuged at 10,000 rpm (12,500 g) for 30 min, the supernatant recentrifuged at 40,000 rpm (1,00,000 g) for 1 h (Beckman Ultracentrifuge). The supernatant obtained was used for the estimation of LDH.

**Assay System:**

The reaction mixture consisted of 50 mM phosphate buffer (pH 7.4), 0.6 mM Sodium pyruvate, 0.2 mM NADH and 20-80 μg of protein (cytosolic fraction as the source of the enzyme). The decrease in absorbance at 340 nm was observed for 3 min (Hitachi 2000). The activity of LDH was expressed as μmoles/min/mg protein.
Principle:
LDH catalyses the following reaction.

\[ \text{LDH} \]
\[ \text{Pyruvate + NAD}^+ + \text{H}^+ \rightarrow \text{Lactate} + \text{NAD}^+ \]

The released NAD+ is monitored in a recording spectrophotometer at 340 nm.

THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE:
[Systemic Name: D-Glucose-6-phosphate: NADP oxidoreductase.
EC:1.1.1.49]

G6PDH (G6PDH) catalyses the following reaction.

\[ \text{G6PDH} \]
\[ \text{D-Glucose-6-phosphate} + \text{NADP}^+ \rightarrow \text{D-Glucono-Y-lactone-6-phosphate} + \text{NADPH} \]

In this study G6PDH was estimated in the microsomal preparation of the cerebrum, the cerebellum and the brain stem. The methodology in detail is as follows:

Preparation of the microsomal fraction:
Microsomal pellet was isolated in the same way as mentioned in the case of G-6-Pase. The pellet obtained was used as the microsomal fraction for the estimation of G-6-PDH. The activity of G6PDH was assayed by the method of Baquer and McLean (1972).

Assay System:
The reaction mixture contained 0.1 M glycyl-glycine buffer (pH 8.0), containing 0.1 M MgCl₂ and change in the absorbance was observed spectrophotometrically at 340 nm for 5 min (Hitachi 2000). For G6PDH estimation the reaction mixture contained 0.1 M glycylglycine buffer (pH 8.0), containing 0.1 M MgCl₂ and change in the absorbance was observed spectrophotometrically at 340 nm for 5
min. The activity of G-6-PDH was expressed as μmoles/min/mg protein.

**Principle**:

The product of G6PDH action NADPH can be monitored at 340 nm in a recording spectrophotometer.

**THE ACTIVITY OF 5'-NUCLEOTIDASE**:

[Systemic Name: 5'-Ribonucleotide phosphohydrolase. EC: 3.1.3.5]

5'-Nucleotidase catalyses the following reaction.

\[
[5'\text{-Nucleotidase}] \\
\text{AMP} \rightarrow \text{Adenosine} + \text{Pi}
\]

In this study 5'-Nucleotidase was estimated in the microsomal fraction of the cerebrum, the cerebellum and the brain stem. The methodology in detail is as follows:

Microsomal pellet was isolated in the same way as mentioned in the case of G-6-Pase. The pellet obtained was used to estimate the activity of 5'-Nucleotidase. The activity of 5'-Nucleotidase was assayed by the method of Ipata (1969) as modified by McIntosh and Plummer (1976).

**Assay System**:

The assay system contained 33 mM Tris-HCl buffer (pH 7.4) containing 100 mM MgCl, 150 mM Na-glycerophosphate, 0.5 units of adenosine deaminase, and 100-400 μg of protein (microsomal pellet). 100 mM AMP was used as the substrate and the change in the absorbance was observed spectrophotometrically at 265 nm for 3 min. The activity of 5'-Nucleotidase was expressed as μmoles/min/mg protein.

**Principle**:

The method to assay 5'-Nucleotidase is a coupled assay.

5'-Nucleotidase catalyses the following reaction
Adenosine formed, is the substrate for the enzyme adenosine deaminase. The reaction it catalyzed is as follows:

\[
[\text{Adenosine deaminase}] \\
\text{Adenosine} \rightarrow \text{inosine} 
\]

The product of adenosine deaminase action inosine can be monitored at 265 nm in a recording spectrophotometer (Hitachi 2000).

**MEMBRANE FLUIDITY ESTIMATION:**

In this study membrane fluidity was estimated in the microsomal preparation of the cerebrum, the cerebellum and the brain stem. The methodology in detail is as follows:

**Preparation of the microsomal fraction:**

Microsomal pellet was isolated in the same way as mentioned in the case of G-6-Pase. The pellet obtained was used as the microsomal fraction for the estimation of membrane fluidity (Lebel and Schatz, 1990). Microsomal membrane were diluted in 50 mM Tris-HCl, pH 7.4, to a final protein concentration of 50 µg/ml. 1 ml volume of microsomal membranes was mixed with 1 ml of 2 uM DPH in 50 mM Tris-HCl, pH 7.4. The DPH solution was diluted from stock solution of 2 mM in tetrahydrofuran. The mixture was incubated at 37°C for 45 min. The fluorescence intensity and polarization were monitored at excitation wavelength 365 nm and an emission wavelength of 430 nm.

**PHOSPHOLIPID ESTIMATION:**

The methodology used for phospholipid estimations is broadly divided under following sub-headings:

I] **ISOLATION OF PHOSPHOLIPIDS**

II] **SEPARATION OF PHOSPHOLIPIDS**
III] QUANTIFICATION OF DIFFERENT PHOSPHOLIPIDS

ISOLATION OF PHOSPHOLIPIDS

Total phospholipids were isolated from the cerebrum the cerebellum, the brain stem by the method of Horrock and Sun (1972). The detailed procedure is as follows:

Different areas were dissected out and homogenized in 10 volumes of a mixture of chloroform methanol (2:1 v/v) which contained 0.005% butylated-hydroxy toluene as antioxidant. The homogenate was filtered with Whatman Filter paper No.1. The residue was washed three times with the same mixture. The filtrate collected and washed with 0.66 volumes of a mixture of chloroform methanol water (2:1:2 v/v) containing 0.005% butylated-hydroxy toluene as antioxidant. This was shaken vigorously. It was allowed to stand so as the two phases could separate. The upper phase was removed and discarded and the lower phase was again washed again with 0.66 volumes of a mixture of chloroform methanol and water (1:15:16 v/v) containing 0.005% butylated-hydroxy toluene as antioxidant. After the separation of the two phases, the upper phase was removed and discarded. The lower phase was evaporated to dryness under N2 gas. 1 ml of the mixture of chloroform methanol (1:1 v/v) containing 0.005% butylated-hydroxy toluene as antioxidant and stored at -20°C.

SEPARATION OF PHOSPHOLIPIDS:

Separation of phospholipid mixture was done with thin layer chromatography (TLC) by the method of Horrock and Sun (1972). Principle:

Separation of different phospholipids from a mixture of phospholipids is achieved by utilizing the different degrees to which the lipids are adsorbed on a solid support, and then eluted by the use of suitable solvent systems.
**METHODOLOGY:**

**Preparation of the plates:**

Silica Gel (type G), a highly porous amorphous silicic acid coated on a glass plate, was used as an absorbent. Glass plates (20x20 cm., 3 mm thick) were washed in double distilled water, dried and again cleaned by a mixture of chloroform and methanol (1:1 v/v) so as to make the plates free of any grease. 5 plates were placed on a platform. Using the feeler gauge the clearance between the plates and leading edge of the gel spreader was adjusted to 0.5 mm. 35 g of silica gel (type G) was mixed with 85 ml of Sodium Carbonate solution (1.06 g in 1 lit of glass distilled water). The mixture was stirred vigorously. This slurry was poured into the spreader and 5 plates were coated. The plates were left as such till the slurry dried. Once the slurry had dried the plates were activated in a oven at 100°C for at least 1 h. The plates were stored at 100°C in the oven, if not used.

**Development of the plates:**

Separation of phospholipids was performed using two dimensional solvent system. Two separate development jars containing 2 different solvent systems were allowed to equilibrate/saturate for at least one hour before putting the plate for development. The two solvent systems used were

1) chloroform:methanol:ammonia solution (sp.gra. 0.88) in the ratio 75:25:5 (v/v/v) and


After activation the plate was removed from the oven allowed to cool and then the a known volume extracted phospholipid mixture (containing 30-70 μg of bound phosphorous) was applied as a discrete spot approximately 2 cm away from the left hand and the bottom
edge of the plate. The spot was allowed to dry and then developed in the developing jar contain a mixture of chloroform, methanol and ammonia solution in the ratio of 75:25:5 (v/v/v) containing the 0.005% antioxidant butylated hydroxytoluene. The plate was removed thoroughly dried and then turned through 90°C in anti-clockwise direction and put to develop in the second solvent mixture. After the development of the plate, it was dried at room temperature for at least 1 h. The plate was then transferred to the iodine chamber and the phospholipids were visualized by iodine vapors. The spots were marked and then scrapped and transferred to glass test tubes.

Quantification of the phospholipids:

Quantification of different phospholipids was done by estimating the amount of bound phosphorous.

Assay system:

1 ml of 70% perchloric acid was added to the glass test tubes containing different phospholipids adsorbed on silica gel and the mixture was heated in the sand bath at the boiling point of perchloric acid for at least 2 h. The test tubes were then allowed to cool at room temperature. 8 ml of glass distilled water was added and the volume was adjusted to 9 ml by the addition of perchloric acid. 0.5 ml of 5% ammonium molybdate and 0.4 ml of ANSA solution was added. The blue color was developed by heating the mixture in a boiling water bath for 10 min. The mixture was centrifuged at 3,000 rpm for 10 min and read at 820 nm in a recording spectrophotometer and the concentration of phosphorous calculated from the standard curve.

[C] STATISTICAL ANALYSIS OF THE DATA:

I) STATISTICAL METHODS:

The data obtained in this study showed a normal distribution which is the essential precondition for many of the parametric tests.
like t test, F test etc. used for testing the validity of difference between two conditions (e.g. enzyme activity in normal free moving control and enzyme activity after REM sleep deprivation and various controls). Hence, in this study, parametric statistical methods were applied for testing the statistical significance.

The tests applied for analyzing the data included:

1) Student’s t-test.

1) **STUDENT'S T-TEST**:

This test was applied for comparing the means of enzyme activities in controls and REM sleep deprived animals as well as FMC and other controls groups. In this method, following steps were followed to determine the level of significance:

(i) Mean and standard deviation of individual groups were calculated separately.

(ii) The value of test statistic (t) was calculated using the following formula:

\[
\frac{\text{MEAN}_1 - \text{MEAN}_2}{\sqrt{\frac{s^2_1}{N_1} + \frac{s^2_2}{N_2}}}
\]

(iii) t calculated was finally compared with the tabulated value of t given at different level of significance, according to the following rules:

- H₀ (Null hypothesis) rejected if \( t \leq t_\alpha \) for observed value of 'N'.
- H₀ accepted if \( t > t_\alpha \) for observed value of N.

(Here, \( t_\alpha = \) tabulated value of t at \( \alpha \) level of significance).

Therefore, the difference in the enzyme activity between the controls and the experimental groups was accepted to be significant when the calculated value of t was < the tabulated value of \( t_\alpha \) at a given level of significance.