2. EXPERIMENTAL
2.1. CHEMICALS AND REAGENTS:

All the chemicals were obtained commercially and used as described. The lithium chloride used in this study was supplied by Thomas Baker and Company, London. Aspartic, glutamic, pyruvic, alpha-ketoglutaric and oxaloacetic acids along with glycine, acetylcholine hydrochloride and ATP, were purchased from Patel Chest Institute, New Delhi, India. Hydrochlorides of DA, NE, 5-HT along with GABA, Coenzyme-A, Choline chloride and taurine were obtained from Sigma Chemical Company, U.S.A. Most of the solvents were used after fresh distillation. For each set of experiments a separate list of reagents is given below:

"BIOGENIC AMINES"

2.1.1. n-Heptane and n-Butanol: Reagent grade butanol and n-heptane were purified by shaking with an equal volume of 0.1 N NaOH followed by a wash with the same volume of 0.1 N HCl. They were finally washed twice with distilled water to remove the traces of alkali or acid.

2.1.2. Peroxide free ether: Diethyl ether was washed with saturated solution of ferrous sulphate to remove accumulated peroxide. Thereafter, washed with distilled water to remove the excess of sulphate.
2.1.3. **Ethylenediamine**: This was freshly distilled and stored in a dark bottle in cold prior to use.

2.1.4. **Stock solutions of biogenic amines**: The solutions of dopamine, norepinephrine and 5-HT were prepared in 0.01 N HCl at a concentration of 100 µg/ml and stored in a refrigerator. These solutions were not used beyond one month of their preparation.

2.1.5. **Standard amine solutions**: On the day of experiment, the stock solutions of the amines were diluted with 0.01 N HCl to get the working standards of each amine in the range of 1 µg/ml. Thereafter, 5 ml of NE and 10 ml of each of 5-HT and DA working standards were diluted to 50 ml to obtain standard amine solutions corresponding to 100 ng of NE and 200 ng of DA and 5-HT/ml.

2.1.6. **Acetate buffer (2 M, pH 6.8)**: 2 M acetic acid was adjusted to a pH 6.8 with 2 N NaOH.

2.1.7. **Phosphate buffer (0.5 M, pH 7.3)**: 77 ml of 0.5 M Na₂HPO₄ was mixed with 23 ml of 0.5 M KH₂PO₄.

2.1.8. **Sodium thiosulphate (0.1 M)**: 6.2 g of Na₂S₂O₃·5H₂O was mixed with 23 ml of 0.5 M KH₂PO₄.

2.1.9. **EDTA solution (10 per cent)**: 10 g of ethylenediaminetetra-acetic acid was dissolved in 100 ml distilled water by heating. The final volume was again made up to 100 ml.
2.1.10. **Alkaline sulphite - EDTA solution**: 12.6 g of sodium sulphite was dissolved in 10 per cent EDTA solution and diluted to 250 ml with 5 N NaOH.

2.1.11. **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 of previously redistilled ethylenediamine and 22.5 ml 10 N NaOH. This was continuously shaken to have a thorough mixing. The reagent was freshly prepared immediately before use.

2.1.12. **Iodine solution**: 3.175 g of iodine together with 12.5 g of sodium iodide were dissolved in 250 ml of water. The solution was stored in a dark bottle in cold.

"**MONOAMINE OXIDASE**"

The preparation of phosphate buffer, and the purification of n-heptane and n-butanol was done as described for monamines.

2.1.13. **1-Nitroso-2-naphthol reagent (0.1 per cent)**: 100 mg of 1-nitroso-2-naphthol was dissolved in 100 ml of 95 per cent ethanol.

2.1.14. **Nitrous acid reagent**: To 5 ml of 2N H₂SO₄ was added to 0.2 ml of 2.5 per cent sodium nitrite. The reagent was prepared fresh just before use.
2.1.15. **Standard solution of serotonin (0.03 m mole/ml):** This was prepared by dissolving 63 mg of serotonin hydrochloride in 10 ml of 0.01 N HCl.

2.1.16. **Borate buffer (pH 10):** 9.42 g of boric acid was dissolved in 300 ml of distilled water and the solution was added with 16.5 ml of 10 N NaOH. The buffer solution was then saturated with purified n-butanol and sodium chloride by adding these substances in excess. Excess of salt was permitted to settle down while the excess butanol was removed by aspiration. The final pH was adjusted about 10, on a pH meter by adding NaOH.

"AMINO ACIDS"

2.1.17. **Ninhydrin spraying solution (1 per cent):** 1 g of ninhydrin was dissolved in 100 ml acetone.

2.1.18. **2,4,6-Collidine:** Collidine obtained commercially was used as a second spraying reagent on paper chromatograms for better development of spots.

2.1.19. **Standard solutions of amino acids (1 mg/ml):** Solutions of each of GABA, taurine, glutamic acid, aspartic acid and glycine were prepared by dissolving 5 mg of amino acid in 5 ml of 1 per cent isopropanol. The standard solutions were placed in a refrigerator at 0°C.
2.1.20. Trichloroacetic acid (TCA) (10 per cent): Every month the fresh solution was prepared and stored in a refrigerator.

2.1.21. Hydrazine reagent (0.1 per cent): 2,4-Dinitrophenylhydrazine (50 mg) was dissolved in 50 ml of 2 N HCl. The reagent was placed in a brown bottle in cold.

2.1.22. Sodium carbonate (10 per cent): This was prepared by dissolving 10 g of anhydrous salt in 100 ml of distilled water, filtered and kept in a pyrex container.

2.1.23. Stock solutions of alpha-ketocarboxylic acids: The solution of sodium pyruvate (0.2 M) was prepared by dissolving 2.2 g of sodium pyruvate in 100 ml of distilled water. Whereas, alpha-ketoglutaric acid (192 mg) and oxalosuccinate (178 mg) were dissolved in 100 ml of 5 per cent acetic acid solution to obtain 0.01 M solutions. All the stock solutions were placed in a refrigerator at 0°C.

2.1.24. Working standards: The working solutions of all the dilutions, of sodium pyruvate, alpha-ketoglutaric acid and oxalosuccinate, were prepared by diluting their stock solutions with distilled water and acetic acid respectively.

2.1.25. Aniline citrate: This was prepared by mixing equal parts of aniline and 5 per cent citric acid.
2.1.26. **Glutamic-oxaloacetate transaminase (GOT) substrate (200 ml aspartic acid and 2 ml of alpha-ketoglutaric acid):** 2.66 g of DL aspartic acid was dissolved in a minimum amount of 1 N sodium hydroxide. This was added with 29.2 mg of alpha-ketoglutaric acid. If needed 1 N NaOH was further added to dissolve alpha-ketoglutaric acid. The pH of solution was adjusted to 7.4 and the total volume was made up to 100 ml with phosphate buffer (pH 7.4). The substrate was stored in small fractions (5 or 10 ml) at -15°C.

2.1.27. **Glutamic-pyruvate transaminase (GPT) substrate (200 ml alanine and 2 ml alpha-ketoglutaric acid):** 4.5 g of alanine was dissolved in 45 ml distilled water and the pH was adjusted to 7.4 with 1 N NaOH. The final volume was made up to 250 ml with phosphate buffer. 5-10 ml fractions of the substrate were stored frozen at -15°C.

2.1.28. **Stock pyruvate standard (20 ml):** 220 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer and stored at -15°C.

2.1.29. **Working pyruvate standard (5 ml):** The stock standard solution of pyruvate (1 ml) was diluted to 5 ml with phosphate buffer and stored frozen at -15°C.
2.1.30. **Dinitrophenylhydrazine reagent (1 mL):** 19.3 mg of 2,4-dinitrophenylhydrazine was dissolved in 10 mL of concentrated hydrochloric acid and the volume was made up to 100 mL with water. The reagent was placed in a brown bottle.

2.1.31. **Sodium hydroxide (0.1 M):** 8 g of sodium hydroxide was dissolved in 500 mL of distilled water.

"ACHE & Chac"

2.1.32. **2 M Hydroxylamine solution:** 34.75 g of hydroxylamine hydrochloride was dissolved in 250 mL distilled water and stored in cold.

2.1.33. **Acid reagent (50 per cent):** 50 mL of concentrated HCl (sp. gr. 1.8) was diluted with equal volume of distilled water.

2.1.34. **Ferric chloride reagent:** 0.37 M solution was prepared by dissolving 15 g of ferric chloride (anhydrous) in 250 mL of 0.1 M HCl.

2.1.35. **Stock solution of ACh (0.1 M):** The stock solution of acetylcholine was prepared by dissolving 95 mg of acetylcholine chloride in 50 mL of 0.001 M sodium acetate.

2.1.36. **Alkaline hydroxylamine:** This was freshly prepared by mixing equal volumes of 2 M hydroxylamine and 3.5 M NaOH.
2.1.37. **ATP solution (0.02 M):** 50.7 mg of adenosine triphosphate solution was dissolved in 5 ml of distilled water.

2.1.38. **Histidine/HCl buffer (0.2 M, pH 7.4):** Histidine (3.1 g) was dissolved in 100 ml of 0.15 N HCl and the pH was adjusted to 7.4 on a pH meter by gradually adding dilute hydrochloric acid from a burette.

2.1.39. **Magnesium chloride (0.1 M):** 1.906 g of salt was dissolved in 200 ml of water.

2.1.40. **Ammonium molybdate reagent (2.5 per cent):** 2.5 g of ammonium molybdate was dissolved in 100 ml water.

2.1.41. **AINA reagent:** Sodium bisulphite (30 g), sodium sulphite (6 g) and 0.5 g of recrystallised 1,2,4-aminonaphthal sulphonic acid were mixed thoroughly and dissolved in 250 ml distilled water. The solution was filtered after placing it in dark for three hours and finally stored in cold.

2.1.42. **Standard KH₂PO₄ (1 ml):** The stock solution of potassium dihydrogen phosphate (10 ml) was prepared by dissolving 0.6805 mg of the salt per litre of distilled water. 1 ml of this solution was diluted ten fold with water to get the working standard (1 ml). One ml of working standard contains 1 μ mole phosphate/ml. A few drops of chloroform were added to the above solution and refrigerated at 0°C.
2.2. MAINTENANCE OF ANIMALS:

Male albino rats weighing 150-250 g were used during all the experiments. They were maintained on a pallet diet (Hindustan Lever Ltd., India) and had free access to tap water. The control rats were simultaneously maintained under similar conditions except that the injections of lithium chloride were replaced by physiological saline.

2.3. DRUG ADMINISTRATION:

For assay of monoamines, amino acids and enzymes (*in vivo* studies), the rats were administered lithium chloride intraperitoneally under the following dose schedules:

2.3.1. Chronic schedule: Four groups each comprising of at least six rats were used. Three groups were separately injected lithium at a rate of 1, 2 and 3 m eq/kg body weight for two weeks. The rats of the control group were concurrently sham injected with the equal volume of normal saline.

2.3.2. Acute schedule: Two injections of 5 m eq/kg body weight of lithium were given at four hourly intervals to a group of six albino rats. The animals were sacrificed after two hours of the second injection. A control group comprising of identical number of rats, was simultaneously injected with an equal volume of normal saline, or distilled water.
2.4. PREPARATION OF BRAIN HOMOGENATES FOR ENZYME ASSAYS:

After completing the injections, the rats of experimental as well as control groups (as described under "Drug administration"), were sacrificed by decapitation. Their cerebrum, cerebellum and brain stem were dissected, weighed and homogenized in ice cold distilled water to make the 5 per cent concentration of the wet weight of the tissue in water. The samples were centrifuged at a slow speed and supernatant fractions were stored in a refrigerator. For in vitro experiments the cerebrum, cerebellum, brain stem and whole brain homogenates were prepared similarly as described above. Normal healthy male rats, not previously treated with lithium were used.

2.5. EXPERIMENTS PERFORMED ON MONOAMINE NEUROTRANSMITTERS:

2.5.1. Estimation of monoamines: The lithium treated and control rats were killed by decapitation, their cerebrum, cerebellum and brain stem were dissected, weighed and treated according to the solvent extraction procedure of Welch and Welch.\(^{44}\)

2.5.1.1. Extraction procedure: The brain parts were homogenized in 1.5 ml ice cold 0.01 N HCl to which 0.1 ml of 10 per cent EDTA was added. These homogenates followed by a rinse of 1.5 ml 0.01 N HCl, were transferred to glass stoppered bottles containing 2.5 ml of n-butanol and 4 g of NaCl. The bottles were shaken for ten minutes and centrifuged at 3000 x g for about 8 min in cold. The butanol layer
was separated and transferred to another set of glass stoppered bottles each containing 40 ml of n-heptane and then added 1.5 ml of phosphate buffer. The mixture was shaken for 10 min and centrifuged in cold. Care was taken to keep the pH of buffer not below 7. The phosphate buffer (1.5 ml) was drawn from the butanol phase and transferred to a clean 30 ml bottle and the pH was adjusted to 3.5 - 4 using 3 N HCl. Thereafter, 20 ml of peroxide free ether was added and the bottles were shaken and centrifuged as described above. The acidic aqueous layer was taken from the bottom with a 0.5 ml pipette and three 0.5 ml aliquots were collected separately for the determination of NE, DA and 5-HT respectively.

2.5.1.2. Preparation of standard curves: The standard curves for DA, NE and 5-HT were prepared by analysing fluorometrically (as described under "Analysis of monoamines"), the standard amine solutions in the range of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 ml corresponding to 20, 40, 60, 80, 100, 120 and 140 mg of NE and 40, 80, 120, 160, 200, 240 and 280 mg of each of DA and 5-HT.

2.5.1.3. Analysis of monoamines: The samples of monoamines obtained after the extraction, were analysed using Turner Model 430-Spectrofluorophotometer (C. K. Turner Association, U.S.A.) as given below:
2.5.1.3.1. **5-Hydroxytryptamine:** The 5-HT samples were mixed with an equal volume of 6 N HCl and the native fluorescence was immediately read at 295/535 nm using a 5 mm slit. Observations in each tube were made one by one. After taking the readings, these tubes were used as blanks for dopamine analysis.

2.5.1.3.2. **Noradrenaline:** To the each 0.5 ml extracted sample were added 0.5 ml of 2 N acetic acid buffer, 0.1 ml iodine solution, 0.15 ml of 0.1 N sodium thiosulphate and 0.2 ml of alkaline ascorbic acid; Ethylenediamine mixture in respective order thereby making a total volume of 1.45 ml. Each reagent was added after an interval of 5 minutes and the viscous mixture was thoroughly mixed by shaking. The test tubes were placed under fluorescent desk lamp at a distance of 5-10 cm from reflector, for about 35 minutes. Thereafter, the fluorescence was read at 400/510 nm using 2 or 3 mm slit.

2.5.1.3.3. **Dopamine:** The samples (0.5 ml) were added at 5 minutes intervals, with 0.5 ml 2 N acetic acid buffer, 0.1 ml of 0.1 iodine solution, 0.2 ml alkaline sodium sulphite - EDTA solution and 0.25 ml 1:1 glacial acetic acid and HCl reagent respectively to make a final volume of 1.5 ml. The tubes were placed in a boiling water bath for 45 minutes, after which they were allowed to cool to room temperature. Fluorescence was read within 1-2 h at 335/380 nm using 2 mm slit.
2.5.1. **Per cent recovery**: This was evaluated to test the reliability of results. The method described by Welch and Welch was followed and the recoveries for individual monoamines were estimated.

2.5.2. **Assay of monoamine oxidase (MAO)**: The enzyme was assayed according to the procedure described by Udenfriend et al., and the serotonin was used as a substrate.

2.5.2.1. **Preparation of calibration curve**: The standard curve was prepared by analyzing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 ml fractions of 0.01 M serotonin hydrochloride solution corresponding to 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 μ mole of serotonin respectively. The colorimetric measurements were done as described under the "Procedure".

2.5.2.2. **Procedure**: The incubation mixture contained 0.5 ml of 0.5 M phosphate buffer, 1 ml of 0.01 M serotonin hydrochloride and 0.5 ml of enzyme. The total volume in all the tubes was made up to 2 ml by distilled water. The incubation was carried on a water bath at 37°C. After 20 min, the 0.5 ml fractions of assay mixture were taken in 60 ml glass stoppered bottles. The pH of the samples was made approximately 10, by adding 0.1 N sodium carbonate solution. The final volume of each bottle was made up to 15 ml by the addition of 5 ml of borate buffer and distilled water. Thereafter, 5 g of sodium chloride and 15 ml of purified butanol were added.
and the bottles were shaken for 10 m, centrifuged at 3000 x g for 5 m and decanted the solution from solid material into other precleaned bottles. The aqueous layer was removed by aspiration. 10 ml of the butanol phase was transferred to another bottle containing 20 ml of n-heptane, 1.5 ml 0.1 N HCl and centrifuged to separate the acid layer containing serotonin.

The in vitro experiment was carried out in cerebrum, cerebellum and brain stem by adding different concentrations of lithium to the incubation media before the addition of the substrate. Lithium chloride solution (0.1 m eq/ml) was taken in the range of 0.5, 1 ml and 1.5 ml corresponding to 0.05, 0.10 and 0.15 m eq lithium to the experimental test tubes. In the control tubes lithium was replaced by an equal volume of distilled water.

2.6. EXPERIMENTS PERFORMED ON AMINO ACID NEUROTRANSMITTERS;

2.6.1. Estimation of amino acids: The cerebrum, cerebellum and brain stem of lithium treated and control rats, were dissected out, weighed and homogenized in ice cold 0.01 N HCl (500 mg of wet tissue/ml of HCl) and collected in precooled test tubes.

2.6.2. Extraction: The extraction of different amino acids was carried out according to the procedure as described by Avapara. To each sample was added 8 ml of ice cold absolute alcohol. After keeping the test tubes at 0°C for
one hour, the centrifugation was done at 16000 x g for 10 min under refrigeration in a preparatory ultracentrifuge. The supernatants along with the washes of the sediment with 3-5 ml of 70 per cent alcohol were collected and dried under reduced pressure at 60-90°C. The residue was added with 1 ml distilled water, 2 ml methanol and 2 ml chloroform in the respective order, centrifuged again at 1000 x g for 10 min on a clinical centrifuge. The upper layer was carefully separated and used for the estimation of GABA, glycine, glutamic acid, taurine and aspartic acid by two dimensional descending paper chromatography.

2.6.1.2. Preparation of calibration curves: Three different volumes (10, 20 and 30 mm) of each of the amino acid standard solutions, were gently spotted by a lambda micropipette on the separate filter paper sheets (Whatman No. 1). After drying the spots the sheets were run using Butanol : Acetic acid : Water (4:1:1.6) as solvent and developed using ninhydrin spray. The spots were cut out and eluted with 5 ml of distilled water by boiling at 60°C for 10 min. Aliquots thus obtained were read at 750 nm, on a Bosch and Lomb Spectrophotometer.

2.6.1.3. Chromatography of unknown samples: In order to achieve a better separation of various amino acids of the unknown samples two phase run was carried using Butanol : Acetic acid : Water (4:1:1.6) as solvent in first phase and Phenol : Water (3:1) as solvent in the second phase. Authentic samples were simultaneously run for the location
of the amino acids of unknown samples. Elution of spots and their colorimetric measurements were done essentially according to the procedure mentioned earlier for standard curves.

2.6.2. 

**Estimation of alpha-ketocids:** The cerebrum, cerebellum and brain stem of lithium treated and control rats, were dissected, weighed and homogenised in a known volume of ice cold 10 per cent TCA. The samples were then centrifuged at a slow speed on a clinical centrifuge and finally the sediments were discarded.

2.6.2.1. 

**Determination of pyruvic acid and alpha-ketoglutaric acid:**

The assay method of Friedman et al.\(^{148}\) as described elsewhere was followed. Test tubes each containing 3 ml of tissue extract, along with reagent blanks containing 3 ml of TCA were incubated for 10 min in a water bath at 25°C. After addition of 1 ml DNP reagent the incubation was continued at the same temperature for specified time and then the hydrozones formed were extracted by a definite volume of appropriate solvent as described below:

**Method 1: Determination of pyruvic acid:** The incubation was carried out for 5 min and then 3 ml of ethyl benzene was used as a solvent for extraction.
Method 2: Determination of alpha-keto glutaric acid: Incubation was carried out for 25 m and the hydrazones was extracted by the addition of 3 ml of benzyl alcohol.

Immediately after the addition of solvents the contents in the test tubes were mixed thoroughly by vigorous shaking for 10 m, centrifuged at a slow speed (if required) and the lower phase of water was removed. The remaining organic phase was once again washed with 1 ml water, added with 6 ml of sodium carbonate and the contents were mixed by rapid shaking. The lower carbonate extract (0.5 ml) was transferred by gentle pipetting to the other test tubes, placed in a water bath at 25°C. To each of these tubes was added 5 ml of 0.4 M NaOH. After 5 m the colour density was measured on a photoelectric colorimeter at 420 nm. The calculations were done as described by Friedman et al.143

2.6.2.2. Determination of oxaloacetate acid: The fresh tissue extracts (3 ml) as obtained above, were immediately mixed with 3 ml of TCA and 0.5 ml of aniline citrate. After completing the decarboxylation of oxaloacetate which took about 30 m, the pyruvate was estimated according to the procedure already mentioned. The pyruvate content thus obtained, was corrected for preformed pyruvate (as determined above), to evaluate oxaloacetate concentration in each brain region.
2.6.3. Assay of GOT: The enzyme activity was determined by the colorimetric method of Reitman and Frankel\(^{150}\) as described elsewhere\(^{151}\).

2.6.3.1. Procedure: The homogenates of different brain regions obtained from lithium treated and control rats, were used as the enzyme source for in vivo study. For each brain region a separate experiment was carried out as described below:

A. Test: A number of test tubes were added in respective order, 0.5 ml of GOT substrate, 0.7 ml of phosphate buffer (pH 7.3), warmed at 37\(^{\circ}\)C in a water bath and then mixed with 0.2 ml of enzyme. After incubation for 30 min the tubes were removed from water bath and immediately treated with 0.5 ml of DNP\(^{2}\) solution.

B. Comparative control: For the correction due to background colour produced by alpha-ketoglutaric acid the duplicate test tubes were run as a check. Each tube was added with 0.5 ml of GOT substrate, 0.7 ml distilled water, 0.5 ml DNP\(^{2}\) solution and 0.2 ml of the homogenate of the given brain regions respectively.

C. Standard: Duplicate tubes containing working pyruvate standard (0.1 ml) were added with 0.4 ml of GOT substrate and the total volume was made up to 1.4 ml by distilled water. Thereafter, 0.5 ml of DNP\(^{2}\) reagent was added to get a coloured hydrazone of pyruvate.
D. **Blank**: Each blank tube was containing 0.5 ml of G6P substrate 0.9 ml distilled water and 0.5 ml of DNP reagent.

All the tubes were then centrifuged at 3000 x g for 10 min and supernatants were transferred to other precleaned test tubes. After about 20 min of the addition of DNP reagent, 5 ml of 0.4 M NaOH was added to each tube and after another 10 min the colour density was recorded at 510 nm.

The mmoles of pyruvate formed per hour per g of wet tissue (0.2 ml of 5 per cent brain tissue contains 1 mg of wet tissue) were calculated from the undergiven equation:

\[ P = \frac{T - C}{S - B} \times 0.8 \]

where \( P \) represents the mmoles of pyruvic acid formed. The symbols \( T, C, S \) and \( B \) indicate the optical densities of test, comparative control, standard and blank respectively.

The *in vitro* experiments were also carried out in cerebrum, cerebellum and brain stem by adding 1 M lithium chloride solution in the range of 0.01, 0.02, 0.04, 0.08, 0.12, 0.16, 0.20, 0.24, 0.26, 0.28 ml corresponding to 5, 10, 20, 40, 60, 80, 100, 120, 140, 160 mM concentration of lithium in a final volume of 2 ml in the experimental tubes (Test). On the other hand, in control tubes the final volume was made up to 2 ml with distilled water alone.
2.6.4. **Assay of Glutamic pyruvate transaminase (GPT):** Both *in vivo* and *in vitro* studies were carried out by following essentially the same procedure as described for GOT. However, in this experiment GPT substrate was used instead of GOT substrate.

2.7. **EXPERIMENTS PERFORMED ON CHOLINERGIC NEUROTRANSMITTER:**

2.7.1. **Assay of acetylcholinesterase:** The enzyme activity was determined in the different brain regions by using tissue homogenates without prior centrifugation. Both *in vivo* and *in vitro* studies were performed as described below:

2.7.1.1. **Preparation of calibration curve:** The standard solution of ACh (0.01 M), was analysed colorimetrically\(^{153}\) in the range of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 ml by the method as described for unknown samples under "Procedure". The recorded O.D. of standard samples was plotted against their respective concentrations in \(\mu\) moles.

2.7.1.2. **Procedure:** The assay mixture was comprised of 0.2 ml homogenate of the specified brain region, 0.5 ml of phosphate buffer (pH 7.4) and 0.5 ml of 0.01 M ACh solution. Finally the total volume was made up to 2 ml by distilled water. The incubation was carried out at 37° C in a water bath for 30 m. The ACh remained unhydrolysed, was determined colorimetrically by the method of Bestrin\(^{153}\). To each sample was added 2.5 ml of freshly prepared alkaline hydroxylamine reagent and shaken for 2 m. After the addition of 0.5 ml of 10 per cent trichloroacetic
acid, the tubes were centrifuged at 5000 x g for 10 min. For the colorimetric measurements the supernatant fractions were mixed with 1.5 ml of 4 N HCl and 1.5 ml of 3.7 N FeCl₃ and the colour density was recorded on a spectrophotometer at 540 nm. For each set of experiments the enzyme and substrate blanks were simultaneously run throughout the process. The enzyme activity was expressed in terms of μ moles of ACh hydrolysed/h/g of wet tissue.

During the in vitro experiments the components of the assay mixture were same, except that, in experimental tubes different volumes of 1 M lithium chloride were added, to make the final concentration in the range from 5-160 mM, in a total volume of 2 ml (see 00T). However, in control tubes this volume of assay mixture was maintained by distilled water alone.

The in vitro effects of lithium calcium and magnesium ions on the enzyme were evaluated by adding to the assay mixture, 0.5 ml of 0.1 M LiCl, 0.5 ml of 0.1 M CaCl₂ and 0.5 ml of 0.1 M MgCl₂ respectively. To investigate the interactions of Ca²⁺ and Mg²⁺ with lithium, the experimental tubes were respectively, mixed with 0.5 ml of 0.1 M CaCl₂ + 0.5 ml of 0.1 M LiCl and 0.5 ml of 0.1 M MgCl₂ + 0.5 ml of 0.1 M LiCl. These metal salt solutions were added to the reaction media before the addition of the substrate, in addition to other components of incubation. This experiment was, however, carried out using whole brain homogenates.
2.7.2. **Assay of cholinesterase:** The enzyme activity was determined by the method of Smallman, B.N.\textsuperscript{154} as described elsewhere\textsuperscript{155}. The enzyme extract from whole brain homogenate (0.2 ml) was mixed with 0.3 ml of 0.01 M phosphate buffer, 0.01 ml of 1 M KCl, 0.01 ml of 1 M MgCl\textsubscript{2}, 0.01 ml of 0.1 M sodium salt of cysteine, 0.3 ml of 0.1 M eserine sulphate, 0.01 ml of 0.1 M sodium acetate, 0.1 ml of 0.01 M ATP and 0.2 ml of CoA solution (1 μM/ml). The mixture was incubated in a water bath for 2 h to obtain a sufficient amount acetyl-CoA, formed, in the reaction mixture. The reaction was started by adding 0.5 ml of 0.01 M of choline chloride and the final volume was made up to 2 ml by distilled water in control tubes or by lithium chloride and water in experimental tubes. The tubes were incubated in a water bath for 20 min and the acetylcholine formed was estimated by Horserin's method as given under acetylcholinesterase. In experimental test tubes the lithium chloride solution (1 M) was introduced in such a manner that the concentration of lithium ions was in the range from 5-160 mM (see GOT).

2.8. **ASSAYS OF ADENOSINETERIPHOSPHATASE:**

The activities of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase and Mg\textsuperscript{2+}-ATPase were determined in cerebrum, cerebellum and brain stem of rat. Both in vivo and in vitro experiments were performed. The aforementioned brain regions of each of the control and treated animals were removed, weighed and homogenized at 0°C in 9 volumes of 0.32 M sucrose solution. For total ATPase the media contained 0.2 ml of enzyme sample, 0.5 ml
2 M of histidine/HE1 buffer (pH 7.4), 0.3 ml of 1 M sodium chloride, 0.02 ml of 1 M potassium chloride and 0.01 ml of 1 M magnesium chloride corresponding to 50, 150, 10 and 5 mM concentrations of histidine buffer, sodium chloride, potassium chloride and magnesium chloride in a final volume of 2 ml respectively. Similarly, for Mg\(^{++}\)-ATPase, the histidine buffer and magnesium chloride were added in a final concentrations of 50 and 5 mM respectively\(^{145}\). In the end 0.1 ml of 0.02 M ATP was added and the total volume in all the tubes was made upto 2 ml. The tubes were incubated for 10 min at 37°C. The reaction was stopped by the addition of 5 ml of cold 10 per cent perchloric acid. Reaction blanks were prepared by adding perchloric acid without incubation. Thereafter, the mixture was centrifuged and the inorganic phosphate liberated during the reaction, was measured by the method of Fiske and Subbarow\(^{157}\). To each supernatant fraction was added 1 ml of ammonium molybdate solution (2.5 per cent) and enough volume of distilled water to make the final volume 9.9 ml. In the end 0.1 ml of ANSA reagent was added and the colour density was measured at 660 nm using Bosch and Lomb Spectrophotometer. Na\(^{+}\), K\(^{+}\)-ATPase activity was calculated by subtracting the Mg\(^{++}\)-ATPase activity from the total activity\(^{145}\).

During in vitro experiments lithium chloride solution was added prior to the addition of ATP, in a final concentration from 5-150 mM (see GOI). Finally, the volumes of incubation media were maintained upto 2 ml by distilled water, in control as well as in experimental tubes.