MATERIALS AND METHODS
CHEMICALS

The following chemicals used in the present study were obtained from Sigma Chemical Co., St. Louis, USA.

Acrylamide, Brilliant blue G-250 (Coomassie brilliant blue), bovine serum albumin (Type V), β-nicotinamide adenine dinucleotide phosphate reduced form tetrasodium salt (NADPH), β-nicotinamide adenine dinucleotide phosphate monosodium salt (NADP), β-nicotinamide adenine dinucleotide reduced form disodium salt (NADH), β-nicotinamide adenine dinucleotide (NAD), pyruvic acid (sodium salt, Type II), cytochrome c (Type III, from horse heart), DL-isocitric acid trisodium salt, adenosine 5'-triphosphoric acid disodium salt, ouabain octahydrate, p-nitrophenyl-N-acetyl-β-D-glucosaminide, glutathione reduced form (GSH), oxidized glutathione, 2-thiobarbituric acid, 2-phosphoglyceric acid sodium salt, DL-glyceraldehyde 3-phosphate diethylacetal monobarium salt, Dowex-50W (hydrogen form), pargyline hydrochloride, 4-dimethyl-amino antipyrine (aminopyrine), kynuramine dihydrobromide, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride, 4-hydroxyquinoline, glutamate diethylester, N-methyl-D-aspartate (NMDA), quisqualic acid, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), phenolthalein glucuronic acid (from rabbit urine), Triton X-100, 1,4-dithio-DL-threitol (DTT) and glutathione reductase (Type III from baker's yeast).
SKF-525 A hydrochloride; 1-methyl-4-phenyl-pyridinium (MPP⁺) iodide; 5-methyl-10, 11-dehydro-5H-benzo- (α,β) cyclo-
hepten-5,10-imine maleate (MK-801); β-oxalylamino-L-alanine
(L-BOAA); β-methylamino-L-alanine (L-BMAA); 2-amino-7-phos-
phono heptanoic acid (AP7); 6,7-dinitroquinoxaline-2,3-dione
(DNQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX); α-amino-
3-hydroxy-5-methylisoxazole-4-propionate hydrobromide (AMPA)
and kainic acid were procured from Research Biochemicals
Inc., USA.

Piperonylbutoxide, acetonyl acetone (2,5-hexanedione)
were obtained from Aldrich Chemical Company, USA.

N-Acetylaspartylglutamate was a gift from Vittal Mallya
Scientific Research Foundation, Bangalore, India.

2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo (F) quinoxaline
(NBQX) sodium salt was a gift from Novo Nordisk, Denmark.

All the other chemicals and reagents were of analytical
grade and were procured locally.

INSTRUMENTS

Spectrophotometric assays were carried out using UVIKON
810 spectrophotometer (Kontron Instruments, Switzerland) or
DU-64 Spectrophotometer (Beckman, USA). Flame photometer
(Systronics, Medi Flame 127) was used for the estimation of
potassium. Microsomes and crude mitochondria were prepared
using high speed refrigerated centrifuge (J2-21 Beckman,
USA). Fluorescence estimations were monitored using Aminco-Bowman spectrophotofluorometer (American Instrument Company, USA).

STATISTICAL ANALYSIS

Statistical analysis was carried out using students 't' test or One Way Analysis of Variance (ANOVA) with Duncan's Multiple range test, where appropriate.

ANIMALS

Swiss Albino mice (3-4 months old) or Wistar rats obtained from Institute's Central Animal Research Facility (CARF) were used for all experiments. Animals had free access to pelleted diet (Lipton India Ltd., Calcutta) and water ad libitum.

PREPARATION OF BRAIN SLICES

Sagittal slices of rodent brain were prepared using an indigenously fabricated slicer. The slicer consisted of two metal screws (2 inch long) with four bolts fitted on them. One set of bolts was placed at the end of each of the screws. Locally available razor blades were placed equidistant from each other, separated by polyethylene or perspex sheets (0.5 mm thick and 1x1 cm square). The sheets had a hole in the center through which they were threaded onto screws and were placed between the blades. The other set of bolts was placed at the end of the screws and tightened to prevent movement of the blades (Figs. 1 and 2).
Fig. 1: Frontal view of the slicer. Blades are placed equidistant from each other with the help of polyethylene sheets (1 x 1 cm) of uniform thickness (0.5 mm) and mounted on 2 screws. The blades are positioned by placing bolts on either side of the blades and tightened them.

Fig. 2: Lateral view of the slicer.
Animals were decapitated and the brain were quickly removed. The brain was placed on a plastic lid, the edges of which were cut on opposite sides to facilitate movement of the slicer (Fig. 3). The slicer and the plastic lid were pre-cooled at 4°C. The slicer was placed above the brain with equal number of blades on either side of the median plane and gently pressed. The slicer was lifted and the slices present between the blades (Fig.4) were carefully transferred using a fine needle to a preweighed beaker (25 ml capacity) containing artificial cerebrospinal fluid (ACSF). Six to eight slices could be prepared from each mouse or rat brain. Slices of uniform thickness (500 mm) were obtained. The time taken for preparing 6-8 slices from each mouse or rat brain was 1-2 min.

**ARTIFICIAL CEREBROSPINAL FLUID**

Artificial cerebrospinal fluid (ACSF) used for incubation of brain slices, was prepared as described by Elliott (1969) and contained (in mM)-NaCl(122), KCl (3.1), CaCl₂ (1.3), MgSO₄ (1.2), glucose (10), NaHCO₃ (25) and KH₂PO₄ (0.4). The ACSF was well oxygenated and the pH was adjusted to 7.4 by bubbling carbon-dioxide just before use.

In addition, ACSF described by McIlwain (1975) was also used for some experiments. In this preparation the bicarbonate was replaced by glycyl glycine (30 mM) and contained (in mM)- NaCl (124), KCl (5), KH₂PO₄ (1.2), MgSO₄
Fig. 3: Freshly isolated rat brain mounted on a plastic lid suitably modified by cutting on both edges. This prevents movement of the brain during the slicing procedure. The slicer was placed above the brain and gently pressed.

Fig. 4: A sagittal slice of the rat brain prepared using the above slicer.
(1.3), CaCl₂ (1.3) and glucose (10). The pH of the ACSF was adjusted to 7.4 by adding 0.1N NaOH. The ACSF was well oxygenated before use.

INCUBATION CONDITIONS

Brain slices bathed in ACSF were incubated in a shaking water bath. Incubations were carried out at 37°C in an atmosphere of oxygen.

Estimation of lactate dehydrogenase and potassium in the medium -

Following incubation of brain slices in ACSF, the medium was filtered through Whatman No. 1 filter paper. Leakage of lactate dehydrogenase and potassium from the brain slices into the ACSF was measured in the filtered ACSF.

ASSAY OF LACTATE DEHYDROGENASE (LDH)

The leakage of LDH from the slices into the medium was measured using the method of Yoshida et al (1975).

Principle:

\[
\text{LDH} \\
\text{Pyruvic acid } \xrightarrow{\text{LDH}} \text{ Lactic acid} \\
\text{NADH} \quad \text{NAD}
\]

Lactate dehydrogenase reduces pyruvic acid to lactic acid in the presence of NADH. NADH gets oxidized in the
process to NAD. Rate of conversion of NADH to NAD is directly proportional to the LDH activity and was monitored by measuring change in absorbance at 340 nm.

Reagents:
Phosphate buffer - Equimolar concentrations (50 mM) of potassium dihydrogen phosphate (KH$_2$PO$_4$) and dipotassium hydrogen phosphate (K$_2$HPO$_4$) were prepared in water and the solutions were mixed such that the final pH was 7.4.
Pyruvate solution - Pyruvic acid (0.63 mM) was dissolved in phosphate buffer.
NADH solution - NADH (11.3 mM) was prepared fresh in phosphate buffer.

Method: Pyruvate solution (100 µl), ACSF (20 µl) and phosphate buffer (860 µl) were added to a 1 ml spectrophotometric cuvette and mixed. The reaction was initiated by the addition of NADH (20 µl). The rate of decrease in absorbance at 340 nm was monitored at 25°C. Blanks containing no ACSF were also run simultaneously. LDH activity was calculated from the molar extinction coefficient of NADH (6220 M$^{-1}$ cm$^{-1}$).

**ESTIMATION OF POTASSIUM**

Potassium concentration in the medium (ACSF) was measured using flame photometer.

**Principle:**
The solution evaporates in the flame and the solute is
converted to atomic state. Electrons in the outer orbit of the atom absorb energy and move to higher energy orbits. In the higher energy orbits, electrons are in metastatic state and return to lower energy orbits or ground state. Absorbed energy is released as quanta of light, which is characteristic of the solute.

Reagents:
Standard Solution-Combined standards consisting of both sodium chloride and potassium chloride were used. NaCl (1.169 gm) and KCl (0.075 gm) were dissolved in 100 ml distilled water separately in standard flasks. Both the salts were dried at 60-70°C for 2 hr before the preparation of standard solution. Aliquots of both the salt solutions were mixed and the volume was made up to 100 ml with distilled water so as to give varying range of standard solutions. Standard solutions containing 1-9 mEq/L of potassium were used.

Method: The flame photometer was calibrated using standard solutions. Hundred microliter of ACSF was mixed with 900 µl of distilled water and aspirated into the flame photometer. Values are expressed in mEq/L of ACSF.

ENZYME ASSAYS CARRIED OUT IN BRAIN SLICES

Following incubation, the brain slices were rinsed, blotted and homogenized (5%, w/v) in 0.32 M sucrose. The homogenate was centrifuged at 1,000 g for 10 min and the
post-nuclear supernatant was used for the estimation of following enzymes:

(i) Na\(^+\)-K\(^+\) ATPase,
(ii) glyceraldehyde 3-phosphate dehydrogenase (GAPDH),
(iii) lactate dehydrogenase (LDH),
(iv) enolase,
(v) NADH - dehydrogenase (NADH-DH),
(vi) isocitrate dehydrogenase (ICDH),
(vii) cytochrome c oxidase,
(viii) acid phosphatase (ACP),
(ix) N-acetylglucosaminidase (NAG),
(x) β-glucuronidase (β-GLU),
(xi) monoamine oxidase (MAO) and
(xii) aminopyrine N-demethylase (APD)

The concentration of GSH in the slices was also estimated.

ASSAY OF Na\(^+\)-K\(^+\) ATPase

Ouabain sensitive Na\(^+\)-K\(^+\) ATPase activity was estimated according to Delicostantinos et al (1987).

Principle:

Na\(^+\)-K\(^+\) ATPase converts ATP to ADP and inorganic phosphate. Molybdic acid reacts with inorganic phosphate to form molybdiphosphate which is reduced by ferrous ions to produce molybdenum blue having an absorption maxima at 660 nm.
Reagents:

Tris-HCl buffer - Tris (40 mM, pH 7.4) containing MgCl₂ (5mM), NaCl (80mM) and KCl (20 mM) was prepared.

Adenosine triphosphate (ATP) solution - ATP (3 mM) was dissolved in water. This solution was prepared fresh.

Ouabain solution - Ouabain (1mM) was prepared in distilled water.

Ammonium molybdate solution - 1.6 gm of ammonium molybdate was dissolved in 100 ml of 1 N H₂SO₄.

Ferrous sulphate solution - Ferrous sulphate (1%, w/v) solution was prepared in 0.15 N H₂SO₄. This reagent was prepared fresh.

Trichloroacetic acid (TCA) solution - Trichloroacetic acid (10 gm) was dissolved in 100 ml of water (10%, w/v).

Standard solution - Potassium dihydrogen phosphate (100 µmoles/ml) was dissolved in water.

Method: Incubation mixture (125 µl) contained the enzyme (20-40 µgs of the homogenate protein), tris buffer (10-25 µl) and ouabain (12.5 µl). The reaction was initiated by the addition of ATP (12.5 µl) and the mixture was incubated at 37°C for 30 min. TCA (100 µl) was added to stop the reaction at the end of incubation period. Color was developed by adding ammonium molybdate (1 ml) and ferrous sulphate (150 µl) and the absorbance at 660 nm was recorded against reagent blank. Blanks did not contain ATP, ouabain or brain homogenate. Assays were carried out with and without ouabain. Incubations
carried out without ouabain measure total ATPase activity. Ouabain sensitive Na\(^+\)-K\(^+\) ATPase activity was obtained by subtracting enzyme activity in assays containing ouabain from total ATPase activity. Various amounts of standards were added to test tubes and the color was developed as mentioned above. The amount of inorganic phosphorus liberated was calculated from the standard curve. Enzyme activity was expressed as μmoles of inorganic phosphorus liberated (Pi)/hr/mg protein.

ASSAY OF GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (GAPDH)

GAPDH activity was measured using the method of Howland et al (1980a).

Principle:

GAPDH converts glyceraldehyde 3-phosphate to dihydroxyacetone phosphate and 1,3-diphosphoglycerate. The reaction requires NAD as a co-factor. Reduction of NAD to NADH was monitored by measuring change in absorbance at 340 nm.

Reagents:

Sodium pyrophosphate buffer - Sodium pyrophosphate (25 mM) was dissolved in water and pH was adjusted to 8.5 using phosphoric
acid. The buffer also contained DTT (0.3 mM) and sodium arsenate (17 mM).

Substrate solution - Glyceraldehyde 3-phosphate was prepared from glyceraldehyde 3-phosphate diethyl acetal. To 150 mg of Dowex-50W suspended in 600 µl of water, 10 mg of glyceraldehyde 3-phosphate diethyl acetal was added and mixed. The mixture was heated in a boiling water bath for 3-5 min, cooled, centrifuged and the supernatant was collected. The process was repeated thrice by adding 100 µl of water each time. The supernatants were pooled, mixed and stored at 20°C. The glyceraldehyde 3-phosphate solution, thus prepared was stable for 2 days.

NAD solution - NAD (1 mM) was dissolved in buffer.

Method: In a spectrophotometric cuvette (1ml), the slice homogenate containing 10-30 µg protein and glyceraldehyde 3-phosphate (20µl) were added. The volume was made upto 950µl by the addition of buffer. The reaction was initiated by the addition of NAD (50 µl). The increase in absorbance at 340 nm was monitored. The enzyme activity was calculated from the molar extinction coefficient of NAD (6220 M⁻¹ cm⁻¹) and was expressed as µmoles of NAD reduced/min/mg protein.

ASSAY OF ENOLASE

Enolase activity was estimated as described by Howland et al (1980b).
Principle:

Enolase converts 2-phosphoglycerate to phosphoenolpyruvate. The increase in absorbance due to the formation of phosphoenolpyruvate, which has an absorption maxima at 240 nm was monitored.

Reagents:
Imidazole buffer - Imidazole buffer (50 mM, pH 6.8) containing MgSO$_4$ (1.5 mM) and KCl (0.5 M) was prepared.
2-Phosphoglycerate solution - 2-Phosphoglycerate (10 mM) was prepared in water.

Method: To 880 μl of buffer, the slice homogenate containing 20-40 μg protein was added. The reaction was initiated by the addition of 100 μl of substrate. The increase in absorbance was monitored at 240 nm. Blanks containing no substrate were also run simultaneously. The enzyme activity was expressed as μmoles of phosphoenolpyruvate formed/min/mg protein using a linear absorbance increase of 0.100 units as being equivalent to the formation of 0.226 μmoles of product (phosphoenolpyruvate).

ASSAY OF LACTATE DEHYDROGENASE (LDH)

LDH activity in slice homogenate was assayed as mentioned for ACSF except that brain slice homogenate containing 5'-20 μg protein was added instead of ACSF.
ASSAY OF NADH-DEHYDROGENASE (NADH-DH)

The activity of NADH-DH was measured using the method of Ramsay et al (1986).

Principle:
NAD-dehydrogenase oxidizes NADH to NAD. Rate of conversion of NADH to NAD was monitored by measuring the decrease in absorbance at 340 nm.

Reagents:
Phosphate buffer - Equimolar amounts (0.05 M) of KH₂PO₄ and K₂HPO₄ were dissolved in water and mixed in proportions such that the final pH was 7.6. The buffer contained 0.25 M sucrose.
NADH solution - NADH (0.28 mM) was dissolved in phosphate buffer.

Method: To 450 μl of phosphate buffer, slice homogenate containing 90-120 μg of protein was added. The reaction was initiated by the addition of NADH (20 μl). The decrease in absorbance was monitored at 340 nm. Blanks without NADH were run simultaneously. The activity was expressed as nmoles of NADH oxidized/min/mg protein and was calculated using molar extinction coefficient of NADH (6220 M⁻¹ cm⁻¹).

ASSAY OF ISOCITRATE DEHYDROGENASE (ICDH)

The activity of the mitochondrial enzyme, ICDH was measured using the method of Cleland et al (1969).
Principle:

Isocitric acid is oxidised to α-ketoglutarate by isocitrate dehydrogenase in the presence of NADP as a cofactor. The rate of reduction of NADP to NADPH is monitored by measuring the rate of increase in absorbance at 340 nm.

Reagents:
Tris-HCl buffer - Tris-HCl (100 mM, pH 7.4) was prepared in distilled water. The buffer contained DTT (0.3 mM), MnSO₄ (20 mM) and 0.1% Triton X-100.
NADP solution - NADP (1.5 mM) was prepared in water.
Substrate solution - Isocitric acid (80 mM) was dissolved in water.

Method: NADP (50 µl) and slice homogenate containing 20-40 µg of protein were added to a spectrophotometric cuvette and the volume was made up to 980 µl with tris buffer. The reaction was initiated by the addition of isocitric acid (20 µl). The linear increase in absorbance was monitored at 340 nm. Blanks without the substrates were also run simultaneously. The enzyme activity is expressed as µmoles of NADP reduced/min/mg protein using molar extinction coefficient.

ASSAY OF CYTOCHROME c OXIDASE

Cytochrome c oxidase activity was measured by the method of Gibson and Hilf (1983).
Principle:

Cytochrome c is reduced by the addition of dithionate. The reduced cytochrome c is oxidized by cytochrome c oxidase to the oxidized form of cytochrome c. The disappearance of reduced cytochrome c is monitored by measuring the decrease in absorbance at 550 nm.

Reagents:

Phosphate buffer - Phosphate buffer (0.5 M, pH 7.5) was prepared in distilled water.

Cytochrome c solution - Cytochrome c (1.2 mM) was dissolved in phosphate buffer. This solution was reduced by adding a small quantity of sodium dithionite (Na₂S₂O₄). Excess dithionate was oxidized by bubbling air through the solution.

Method: The brain slice homogenate containing 10-30 μg protein was placed in 1 ml spectrophotometric cuvette and the volume was made up to 950 μl by the addition of phosphate buffer. The reaction was initiated by the addition of cytochrome c solution (50 μl). The disappearance of cytochrome c was monitored by measuring the decrease in absorbance at 550 nm. The rates of oxidation were linear up to 2 min. The specific activity was calculated from molar extinction coefficient of cytochrome c and was expressed as μmoles of cytochrome c oxidized/ min/ mg protein.
ASSAY OF ACID PHOSPHATASE (ACP)

Acid phosphatase activity was measured as described by Cotman and Mathews (1971).

Principle:

Acid phosphatase hydrolyses p-nitrophenyl phosphate to p-nitrophenol. The product, p-nitrophenol gives yellow colored complex under alkaline conditions. The absorbance of the yellow colored complex is measured at 412 nm.

Reagents:

Sodium acetate buffer - Sodium acetate buffer (0.05 M, pH 5.0) containing MgCl₂ (2 mM) and Triton X-100 (0.1%, v/v) was prepared.

Sodium hydroxide solution - NaOH (0.2N) was prepared in water.

Standard solution - p-Nitrophenol (0.1%, w/v) was dissolved in water.

Method: To the sodium acetate buffer (450 µl), substrate solution (50 µl) and slice homogenate containing 20-40 µg of protein were added, mixed and incubated at 37°C for 30 min. The reaction was stopped by the addition of NaOH (500 µl) and the contents were centrifuged. The absorbance of the supernatant was read against a blank containing no substrate at 412 nm. Various concentrations of the standard solution were added to test tubes and the color was developed as mentioned above. Acid phosphatase activity was calculated from the standard curve of p-nitrophenol.
ASSAY OF β-GLUCURONIDASE

β-Glucuronidase activity was measured by the method of Levvy and Conchie (1966).

Principle:

β-Glucuronidase hydrolyses phenolphthalein glucuronic acid to phenolphthalein under acidic conditions. Phenolphthalein gives pink colored product in the alkaline medium which has an absorption maximum at 545 nm.

Reagents:
Sodium acetate buffer - Sodium acetate buffer (0.2M, pH 4.5) was prepared in distilled water.
Glycine-sodium hydroxide buffer - Glycine (0.4 M) was dissolved in water and the pH was adjusted to 10.7 with NaOH.
Bovine serum albumin (BSA) solution - BSA (0.08 %, w/v) was prepared in distilled water.
Substrate solution - Phenolphthalein glucuronic acid (5 μM) was dissolved in water.
Standard solution - Phenolphthalein (1mM) was dissolved in ethanol (100 μl) and the volume was made up with water.

Method: To sodium acetate buffer (125 μl), slice homogenate containing 80-100 μg of protein, BSA (242.5 μl) and substrate solution (62.5 μl) were added. The mixture was incubated at 37°C for 1 hr. The reaction was stopped by the addition of glycine-sodium hydroxide buffer. The mixture was centrifuged to pellet down the proteins and absorbance of the pink
colored supernatant was measured at 545 nm. Blanks containing no substrate were also run simultaneously. Various concentrations of the standard solution were added to the test tubes and the color was developed by the addition of glycine-sodium hydroxide buffer. The quantity of phenolphthalein formed in the test sample was calculated from the standard curve.

ASSAY OF N-ACETYLGUCOSAMINIDASE (NAG)

NAG activity was estimated as described by Froehwein and Gatt (1969).

Principle:

N-Acetylglucosaminidase hydrolyzes p-nitrophenyl-N-acetylglucosaminide to p-nitrophenol. In alkaline conditions, p-nitrophenol forms yellow colored complex with an absorption maxima at 412 nm.

Reagents:
Sodium phosphate-citrate buffer - Equimolar (0.5 M) concentrations of tri-sodium orthophosphate (Na₃PO₄ 2H₂O) and citric acid were dissolved in water and mixed so that the final pH was 4.2.

di-Sodium tetraborate (borax) solution - Borax (0.125 M) was prepared in water.

Substrate solution - p-Nitrophenyl-N-acetyl-ß-D-glucosaminide (4 mM) was dissolved in water.
Trichloroacetic acid (TCA) solution - TCA (2%, w/v) was prepared in distilled water.

Sodium hydroxide solution - Sodium hydroxide (2%, w/v) was dissolved in water.

Standard solution - p-Nitrophenol (0.1%, w/v) was prepared in water.

Method: The incubation mixture contained substrate (50 μl), buffer (25 μl), water (40 μl) and slice homogenate containing 10-40 μg of protein. Incubations were carried out at 37°C for 1 hr. The reaction was stopped by the addition of TCA (250 μl). Color was developed by the addition of NaOH (50 μl) and borax (325 μl), centrifuged and the absorbance of the supernatant was read at 420 nm. Various concentrations of the standard solution were pipetted into the test tubes and the color was developed as mentioned above. NAG activity was calculated using p-nitrophenol standard curve and was expressed as μmoles of product formed/hr/mg protein.

ASSAY OF MONOAMINE OXIDASE (MAO)

Monoamine oxidase activity was measured as described by Krajl (1965).

Principle:

Monoamine oxidase deaminates kynuramine to give an aldehyde which undergoes spontaneous cyclization to form 4-hydroxyquinoline. Under alkaline conditions, the fluorescence of 4-hydroxyquinoline is measured at an excitation wavelength of 315 nm and emission wavelength of 380 nm.
Reagents:

Potassium phosphate buffer - Potassium phosphate buffer, (0.5 M, pH 7.4) was prepared using distilled water.

Kynuramine solution - Kynuramine dihydrobromide solution (1mg/ml) was prepared in distilled water.

TCA solution - TCA (10%, w/v) was dissolved in water.

Sodium hydroxide solution - Sodium hydroxide (1N) was prepared in water.

Standard solution - 4-Hydroxyquinoline (10 mM) was prepared in water.

Method: The reaction mixture containing phosphate buffer (0.5 ml), kynuramine (0.1 ml) and slice homogenate containing 40-80 µg of protein was made up to 3 ml with distilled water and incubated at 37°C for 30 min. TCA (2 ml) was added to stop the reaction and centrifuged. One ml of the supernatant was added to two ml of sodium hydroxide and the fluorescence was measured at an excitation wavelength of 315 nm and emission wavelength of 380 nm. The standard curve was plotted using different concentrations of 4-hydroxyquinoline in alkaline medium. Blanks containing no substrate were run and the blank values were subtracted from test values. The enzyme activity was expressed as nmoles of 4-hydroxyquinoline formed/min/mg protein.

ASSAY OF AMINOPYRINE N-DEMETHYLASE (APD)

Aminopyrine N-demethylase activity was determined according to Werringloer (1978).
Principle:

The N-methyl group of aminopyrine is oxidized by cytochrome P-450 dependent monooxygenase to formaldehyde and 4- aminoantipyrine. Formaldehyde reacts with acetylacetone and ammonia present in NASH reagent to give a yellow colored complex called 3,5, diacetyl-1, 4 - dihydrolutidine which exhibits an absorption maxima at 412 nm.

Reagents:

Tris-HCl buffer - The assay buffer consisted of tris (50 mM) and MgCl₂ (20 mM) dissolved in water. The pH was adjusted to 7.5 with HCl.

NASH reagent - Ammonium acetate (15 gm), glacial acetic acid (0.3 ml) and acetylacetone (0.2 ml) were mixed and the volume was made upto 100 ml with water.

NADPH solution - NADPH solution (3.1 mM) was prepared in the assay buffer. This solution was prepared fresh.

Aminopyrine solution - Aminopyrine (1.80 mM) was dissolved in assay buffer.

Trichloroacetic acid solution - Trichloroacetic acid (10%, w/v) was prepared in distilled water.

Standard solution - Formaldehyde (15 µl of 37.5% solution, v/v) was diluted to 250 ml with distilled water in a standard flask.

Method: The reaction mixture containing aminopyrine (10 µl) and slice homogenate containing 10-30 µg of protein was made
up to 750 μl with assay buffer and preincubated for 3 min at 37°C. The reaction was initiated by the addition of NADPH (250 μl). The reaction mixture was mixed and incubated for 10 min at 37°C. The reaction was terminated by the addition of trichloroacetic acid (0.5 ml) and centrifuged at 1,000 g for 5 min. The supernatant (1 ml) was transferred to a test tube containing NASH reagent (0.5 ml). The tubes were vortexed and warmed at 70°C for 20 min, cooled and the absorbance measured at 412 nm against a reagent blank. The reagent blank contained equal amounts (0.5 ml) of assay buffer, trichloroacetic acid and NASH reagent. Blanks containing no NADPH or homogenate protein were also run simultaneously. Blank values were subtracted from test values. Various concentrations of standard formaldehyde solution were pipetted, the colour developed as described above and the standard curve was drawn. The amount of formaldehyde formed in the test samples was calculated from the standard curve. The specific activity of the enzyme was expressed as nmoles of formaldehyde formed/min/mg protein.

ASSAY OF GLUTATHIONE (GSH)

Glutathione levels were estimated as described by Akerboom and Sies (1981).

Principle:

Glutathione (GSH) reduces 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to 5-thio-2-nitrobenzoic acid. GSH concomitantly
gets oxidized to GSSG. Glutathione reductase reduces GSSG back to GSH in the presence of NADPH. The GSH again reacts with DTNB, thus setting up a cyclic process. The rate of formation of 5-thio-2-nitrobenzoic acid is monitored at 412 nm by measuring the increase in absorbance.

Reagents:
Phosphate buffer - Potassium phosphate buffer (0.1M, pH 7.4) containing EDTA (1mM) was used.
Assay buffer - Phosphate buffer containing DTNB (0.8 mM) and glutathione reductase (1 unit/ml) was used as assay buffer.
Standard solution - Oxidized glutathione (10 μg/ml) was prepared in water.
NADPH solution - NADPH (1.2mM) was prepared in phosphate buffer. This solution was prepared fresh.
Method: Brain slices were homogenized (10%, w/v) in perchloric acid (1%, v/v). The homogenate was centrifuged at 8000 g for 10 min and the perchloric acid supernatant was used for the assay. The assay buffer (0.5 ml) was mixed with perchloric acid supernatant (10-20 μl). The reaction was initiated by the addition of NADPH (50 μl). Blanks containing no GSSG or perchloric acid supernatants were also run simultaneously. GSH levels were calculated from the standard curve of oxidized glutathione. GSH levels were expressed as μmoles of GSH/gm tissue.
ESTIMATION OF THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS)

The concentration of thiobarbituric acid reactive substances was estimated as described by Ohkawa et al (1979).

Principle:

The thiobarbituric acid reactive substances are indicative of lipid peroxidation products. Malondialdehyde is one of the end products of lipid peroxidation. Under acidic conditions, malondialdehyde reacts with thiobarbituric acid at 100°C to give malondialdehyde-thiobarbituric acid complex, which has absorption maxima at 535 nm. The absorbance at 535 nm is directly proportional to the amount of malondialdehyde-thiobarbituric acid complex.

Reagent:

Thiobarbituric acid reagent - Thiobarbituric acid (0.375% w/v), 75 ml of trichloroacetic acid (100%) and 10.4 ml of HCl (0.2 N) were mixed and the volume made upto 500 ml with distilled water. Butylated hydroxytoluene (0.1 mM, v/v) was added to the reagent.

Method: Slices were homogenized in phosphate buffer (0.1 M) since sucrose interferes with the assay. Slice homogenate (50-200 µl) was mixed with thiobarbituric acid reagent so that the final volume was 750 µl. The reaction mixture was heated in a boiling water bath for 20 min. After heating, the reaction mixture was cooled under tap water and
centrifuged for 10 min at 8000 g. The absorbance of the supernatant was read at 535 nm against reagent blank. The amount of TBARS formed was estimated using molar extinction coefficient of malondialdehyde-thiobarbituric acid complex, $1.56 \times 10^{-5}$ M$^{-1}$ cm$^{-1}$.

**ESTIMATION OF PROTEIN**

Protein was estimated using the dye binding method (Bradford, 1976).

**Principle:**

In acidic pH, Coomassie brilliant blue G-250 binds to protein to form a blue colored complex. The protein-dye complex exhibits absorbance maxima at 595 nm and the intensity of the absorbance is directly proportional to the concentration of the protein.

**Reagents:**

Coomassie brilliant blue dye reagent - Coomassie brilliant blue G-250 (150 mg) was dissolved in methanol (50 ml) and orthophosphoric acid (100 ml). This stock dye reagent is stable at 4°C for more than two months. Fifteen ml of the stock reagent was diluted to 100 ml with distilled water and kept for 5-6 hr. The diluted dye was filtered through Whatman No. 1 filter paper and used.

Standard solution - Bovine serum albumin (0.1%, w/v) was dissolved in normal saline.
Method: The brain homogenate (10 µl) was mixed with sodium hydroxide (20 µl of 0.1N) and heated at 60°C for 10 min in a water bath. The diluted dye (5 ml) was added after cooling the samples. Various amounts (10-100 µl) of standard bovine serum albumin solution and sodium hydroxide (20 µl, 0.1N) were pipetted and 5 ml of the dye was added and mixed. The absorbance of both standard and samples were read at 595 nm against reagent blank, 10 min after the addition of the dye. The protein concentration in the sample was estimated from the standard curve.

STUDIES WITH ACRYLAMIDE

Female wistar rats (3-4 months old) weighing 200 gm were used for all experiments.

Brain slices (400-500 µm thickness) were prepared using an indigenous slicer as described earlier. Brain slices were incubated with various concentrations of acrylamide in ACSF for 1 hr at 37°C for studying the effect of varying doses of acrylamide on rat brain slices. Incubations were carried out in an atmosphere of oxygen. Incubations carried out without acrylamide served as controls. Following incubation, the activities of lysosomal enzymes-acid phosphatase, β-glucuronidase and N-acetylglucosaminidase; glycolytic enzymes-glyceraldehyde 3-phosphate dehydrogenase and enolase and membrane marker enzyme - Na⁺-K⁺ ATPase were measured in the slice homogenate as described earlier.
Studies with structural analogs of acrylamide were carried by incubation of rat brain slices with acrylamide, acétylamide or methylene bis-acrylamide. Incubations were carried out for 1 hr at 37°C in an atmosphere of oxygen. At the end of the incubation period, the activity of the subcellular marker enzymes were estimated in the slice homogenate.

In vivo studies:

Acrylamide (150 mg/kg body weight) was injected to rats intraperitoneally (i.p) and sacrificed 2 and 4 hr later. Control rats received vehicle alone. Brain and sciatic nerve were dissected and homogenized in 0.32 M sucrose (5%, w/v). Homogenate was centrifuged at 1,000 g for 10 min and post-nuclear supernatant was separated. Glyceraldehyde 3-phosphate dehydrogenase, β-glucuronidase and N-acetylgucosaminidase activities were assayed in brain homogenate, while β-glucuronidase and acid phosphatase activities were measured in the sciatic nerve.

STUDIES WITH 2,5-HEXANEDIONE

Wistar rats, 3-4 months old, weighing about 200 gm were used for all experiments.

Studies with rat brain homogenate:

Rat brain homogenate (5%, w/v) was prepared in 0.32 M sucrose and centrifuged at 1,000 g for 10 min. The supernatant was incubated with 2,5-hexanedione, acetone and
methyl ethyl ketone at various concentrations for 20 min at 37°C. The activities of glycolytic enzymes, namely, glyceraldehyde 3-phosphate dehydrogenase and enolase; and lysosomal enzymes, acid phosphatase and N-acetylglucosaminidase were estimated in the homogenate at the end of the incubation period. Control incubations containing no ketones were carried out simultaneously. All ketones were freshly distilled prior to use.

Studies with rat brain slices:

Sagittal slices of rat brain were incubated in ACSF in an atmosphere of oxygen at 37°C. 2,5-Hexanedione (100 mM) and acetone (100 mM) were dissolved in ACSF and added to the slices at the beginning of the experiment. At the end of incubation period of 1 hr, slices were homogenized in 0.32 M sucrose as described earlier. Slices incubated in ACSF simultaneously without the ketones served as controls. Subcellular marker enzymes were estimated in the slice homogenate as mentioned earlier.

STUDIES WITH MPTP AND MPP+

Male Swiss Albino mice (3 months old) were used for all experiments.

Mouse brain slices (400-500 μm thick) were incubated in ACSF containing various concentrations of MPTP or MPP+ at 37°C for one hr. Incubations were carried out in an
atmosphere of oxygen. NADH-dehydrogenase activity was monitored in the slices following incubation as mentioned earlier. In addition, leakage of LDH from the slice into the medium was also determined as a measure of cell damage.

Mouse brain slices were preincubated with MAO inhibitors (pargyline and deprenyl) or cytochrome P-450 inhibitors (piperonyl butoxide, SKF-525 A and metyrapone) or dopamine uptake blocker, namely, GBR-12935 for 30 min at 37°C. Following preincubation, MPTP (10 nM) or MPP⁺ (100 μM) was added to one set of slices and the incubation was continued further for 1 hr. The other set of slices served as controls. Simultaneously, slices were also incubated with and without inhibitor, MPTP (10 nM) or MPP⁺ (100 μM). Leakage of LDH from the slice into the medium and the activities of MAO, aminopyrine N-demethylase and NADH-dehydrogenase were measured in the slice following incubation.

Pargyline treatment:

Mice were injected pargyline (15 mg/kg/body weight) intraperitoneally (i.p.), and sacrificed 4 hr later. Control animals received vehicle alone. Brain microsomes and mitochondria were prepared from control and pargyline treated animals. Total cytochrome P-450 concentration was measured in the microsomes and mitochondria. The activity of MAO was estimated in the mitochondria.
Preparation of brain microsomes

The brain microsomes were prepared from both control and pargyline treated animals using the calcium aggregation method (Ravindranath and Anandatheerthavarada, 1990).

Buffers:
Homogenization buffer - Tris-HCl (0.1 M, pH 7.4) buffer containing KCl (1.15%, w/v), EDTA (0.2 mM), DTT (0.1 mM), phenylmethyl sulfonyl fluoride (PMSF, 0.1 mM), butylated hydroxytoluene (20 µM) and glycerol (10%, v/v) was prepared.
Storage buffer - Homogenization buffer containing glycerol (20%, v/v) was used as storage buffer.

Method: The brain was homogenized in homogenization buffer (20%, w/v) with ten up and down strokes. The homogenate was centrifuged at 17,000g for 20 min. The pellet was resuspended in 4 volumes of homogenization buffer and centrifuged at 17,000g for 20 min. The post-mitochondrial supernatants from both the centrifugations were pooled. Calcium chloride (8 mM) was added to the pooled supernatant, mixed and allowed to stand for 5 min at 4°C and centrifuged for 30 min at 30,000g. The pellet was gently homogenized in homogenization buffer and centrifuged at 30,000 g for 30 min. The pellet was resuspended in a small volume of storage buffer and stored in liquid nitrogen till use.

Isolation of crude mitochondria

Crude mitochondria were isolated from mouse brain as described by Lai and Clark (1979).
Reagent:
Buffer - Tris-HCl buffer (0.1 M, 7.4) containing EDTA (0.2 mM), DTT (0.1 mM), PMSF (0.1 mM), butylated hydroxytoluene (20 μM) and glycerol (10%, v/v) was used.

Method: The brain was homogenized in the buffer (20%, w/v) with ten up and down strokes. The homogenate was centrifuged at 2,000g for 5 min and the supernatant was carefully decanted. This supernatant was centrifuged at 12,500g for 10 min to obtain crude mitochondrial pellet. The crude mitochondrial pellet was resuspended in the buffer and stored until use.

Estimation of cytochrome P-450

Cytochrome P-450 levels were estimated in the microsomes and mitochondria by the method of Matsubara et al (1976).

Principle:

The heme protein, cytochrome P-450 is complexed with carbon monoxide and reduced with dithionate. The cytochrome P-450 content is calculated from the reduced carbon monoxide spectra.

Reagents:
Assay buffer - Potassium phosphate buffer (0.1M, pH 7.4) containing EDTA (1 mM), glycerol (20%, v/v), sodium cholate (0.5%, w/v) and Triton N-101 (0.4%, w/v) was prepared.
Anhydrous sodium dithionate.

Carbon monoxide gas - Formic acid was added through a dropping funnel to concentrated sulphuric acid contained in a side arm flask to generate carbon monoxide gas. The gas was bubbled through sodium hydroxide solution to remove carbon dioxide prior to use.

Method: Microsomal or mitochondrial protein (0.6-2 mg) was made up to 2 ml with assay buffer. Carbon monoxide was bubbled through the mixture at a rate of one bubble per second for 1 min. The reaction mixture was then divided into two spectrophotometric cuvettes of 1 ml capacity (test and reference) and the base line was recorded between 400-500 nm. A few granules of sodium dithionate were added to the test cuvette, mixed gently and the spectrum was recorded between 400-500 nm. Cytochrome P-450 content was calculated as follows:

\[
(A_{450} - A_{490})_{\text{Test}} - (A_{450} - A_{490})_{\text{Baseline}} = \text{nmoles of P-450/ml}
\]

0.106

The specific content of cytochrome P-450 was expressed as nmoles of P-450/mg protein.

Phenobarbital treatment:

Phenobarbital was administered to mice in drinking water (0.1%) for 10 days, followed by intraperitoneal injections (80 mg/kg body weight) every day for ten days. Control
animals received vehicle alone. Control and phenobarbital treated animals were sacrificed 24 hr after the last injection.

Brain slices were prepared from both the groups of animals and incubated with various concentrations of MPTP or MPP⁺⁺ for 1 hr at 37°C. NADH-dehydrogenase activity was estimated in the slice homogenate and LDH activity was monitored in the ACSF after incubation.

Studies with rat brain slices:

Rat (Wistar, 3 months old) brain slices were incubated in ACSF containing varying amounts of MPTP for 1 hr at 37°C. NADH-dehydrogenase activity and LDH activity were monitored in slice homogenate and ACSF respectively, after incubation.

STUDIES WITH EXCITATORY AMINO ACIDS

Swiss Albino mice (3-4 months old) were used for the experiments with excitatory amino acids.

Mouse brain slices were incubated in ACSF containing varying concentrations of excitatory amino acids, namely, N-acetylaspartylglutamic acid (NAAG), L-glutamic acid, quisqualic acid, β-methylamino-L-alanine (L-BMAA), β-oxalylamino-L-alanine (L-BOAA), kainic acid, NMDA or AMPA. Incubations were carried out for 1 hr at 37°C in an atmosphere of oxygen. Slices incubated in ACSF alone served as controls. Following incubation, LDH and potassium concentrations were measured in the medium.
CNQX (30 mg/kg body weight), NBQX (30 mg/kg body weight) or MK-801 (5 mg/kg body weight) were administered to mice intraperitoneally and the animals were sacrificed 4 hr later. Control animals received vehicle alone. Brain slices prepared from treated and untreated animals were incubated with and without excitatory amino acids for 1 hr at 37°C. LDH and potassium levels were measured in the medium after incubation.

Studies with rat brain slices:

Rat (Wistar, 3-4 months old) brain slices were incubated in ACSF containing varying concentrations of L-BOAA for 1 hr at 37°C. NADH-dehydrogenase and LDH activity were monitored in slice homogenate and ACSF respectively following incubation.