MATERIALS AND METHODS
I  EXPERIMENTAL ANIMALS

Adult Albino rats of Holtzman strain, maintained at temperature 24 ± 3°C in the School of Life Sciences, were used in all experiments. The rats were fed with the standard laboratory diet, obtained as pellets from Hindustan Lever, Bombay, and given water ad libitum.

II  PREPARATION OF HOMOGENATES AND SUBCELLULAR FRACTIONATION

1. Preparation of Homogenates

Rats were sacrificed by cervical dislocation and the whole brain or regions, namely, cerebral hemispheres, cerebellum and brain stem were excised, washed in 0.9 per cent saline and weighed immediately.

A 10 per cent homogenate (w/v) of the tissue was prepared using a Potter-Elvehjem type of homogeniser fitted with a teflon plunger in a homogenising medium containing 0.25 M sucrose, 20 mM triethanolamine and 0.1 mM dithiothreitol, pH 7.4. All procedures were carried out at 4°C, unless otherwise stated.

2. Subcellular Fractionation

Subcellular fractionation was performed by a combination of the methods of Hess and Brand (1974) and Student and Edwards (1977).
a) Differential Centrifugation:

A crude nuclear fraction (P₁) was obtained by centrifugation at 1,000 × g for 10 min in K-24 (MLW) refrigerated centrifuge. The resulting supernatant (S₁) was centrifuged at 12,000 × g for 40 min to yield the crude mitochondrial pellet (P₂) and the supernatant (S₂). Centrifugation of S₂ at 70,000 × g for 1 hour gave a microsomal fraction (P₃) and the cell soluble fraction (S₃).

b) Preparation of Synaptosomes by Discontinuous-Density Gradient Centrifugation

The crude mitochondrial pellet (P₂) was washed twice in the homogenising medium and after centrifugation (12,000 × g for 40 min), the pellet (P₂) was suspended in the iso-osmotic (0.32 M) sucrose (3 ml/g of original tissue). The suspension (5 ml) was then layered on a Ficoll-Sucrose discontinuous gradient. The gradient was previously prepared by layering 7 ml each of solutions containing 5, 7.5, 10 and 13 per cent Ficoll in 0.32 M sucrose in 10 mM potassium phosphate buffer, pH 7.4, and then allowing them to equilibrate for 1-2 hours at room temperature, and finally at 4°C for a further 1-2 hours.

The gradient with the crude mitochondrial pellet on top was then centrifuged in a MSE superspeed centrifu-
Whole Homogenate (10/ W/V)

1,000 x g | 10 min, 4°C

↓

Crude Nuclear Pellet (P₁)  |  Supernatant (S₁)

12,000 x g | 40 min, 4°C

↓

Crude Mitochondrial Pellet (P₂)  |  Supernatant (S₂)

70,000 x g | 1 hr, 4°C

↓

Microsomal Pellet (P₃)  |  Soluble Fraction (S₃)

↓

P₂

↓

5%  7.5%  10%  13%

105,000 x g | 30 min, 4°C

FLOW SHEET OF THE SUBCELLULAR FRACTIONATION

Myelin (My)  Synaptosomes (Sym)  Mitochondria (M)
fuge at 105,000 x g for 30 min using 6 x 38 ml swingout rotor. The two separated layers containing myelin (My) (on top of the 5 per cent layer and at the 5 - 7.5 per cent interface) were collected and pooled by pipetting off all the material from the top of the gradient through half of the 7.5 per cent Ficoll sucrose layer. The synaptosomal (Syn) fractions were collected and pooled by pipetting off all material from the middle of the 7.5 per cent layer to the middle of the 13 per cent layer. The mitochondrial pellet (M) was suspended in the remaining 13 per cent Ficoll-Sucrose solution.

Since Ficoll was found to interfere with the Lowry protein estimations, the myelin, the synaptosomal and the mitochondrial fractions were diluted with 0.32 M sucrose buffered with 10 mM potassium phosphate buffer, pH 7.4, and centrifuged at 29,000 rpm for 45 min in Sorvall RC-5 refrigerated centrifuge in SS 34 rotor. The resulting pellets were then suspended in 0.32 M sucrose buffered with 10 mM potassium phosphate buffer, pH 7.4.

III INDUCTION OF HORMONAL CONDITIONS

1. Alloxan Diabetes

Rats weighing between 200 - 250 g were starved for 24 hours. Each rat was injected with alloxan monohydrate sub-cutaneously, prepared fresh in 0.154 M sodium acetate
buffer, pH 4.5, at the dose of 15 mg per 100 g body weight. Control rats were injected similar volumes of the vehicle. From the next day, the alloxan treated rats were injected two units of LENTE zinc insulin suspension, intraperitoneally for 6 days. The control rats were injected similar volumes of saline. This treatment greatly reduced the mortality rate and the toxic effects of alloxan.

The alloxan-treated rats were then divided into two groups. The first group received one unit of saline everyday and was termed the 'diabetic group' and the second was continued with the injections of 1 unit of insulin daily and formed the 'diabetic + insulin'.

Blood and urine glucose was monitored daily by means of glucose detection strips (Boehringer Corp., London; and Miles Laboratories, England). The rats were sacrificed at a fixed hour after three, eight, fifteen and twenty-two days of insulin withdrawal.

2. Insulin-Induced Hypoglycemia

Rats weighing between 200 - 250 g were starved for 24 hours, and then were injected intraperitoneally with 5' units per 100 g body weight per rat of LENTE zinc insulin diluted in 0.9 per cent saline. Blood glucose
was monitored by means of glucose detection strips, and the rats were sacrificed after one, two and three hours of insulin administration. Another group of control rats were injected similar volumes of the saline, and were sacrificed together with the hypoglycemic group.

3. **Thyroidectomy**

Thyroidectomy was performed according to the method of Zarrow et al. (1964). The rats were starved for 24 hours and placed under ether anaesthesia prior to operation. The two lobes of the thyroid glands, lying on either side of the trachea, were then surgically removed. Thyroidectomised and the sham operated control rats were given food and water *ad lib* after the operation. One per cent (w/v) calcium lactate was given in their drinking water to compensate for the loss of calcium ions due to the removal of parathyroid glands.

Three days before sacrifice, the thyroidectomised rats were divided into two groups. One group received intraperitoneal injections of 3,3',5'-triiodothyronine ($T_3$; 100 μg per ml in 0.001 N NaOH) daily at a dose of 15 μg/100 g body weight. This group was designated the $T_3$ treated thyroidectomised group ($T_x + T_3$). The other group which received equal volumes of the vehicle for
the same period, was termed the thyroidectomised group ($T_x$ group). The sham operated controls were injected similar volumes of the solvent. The rats were then sacrificed at 7, 14, 21, 30 and 60 days after thyroidectomy.

4. Hyperthyroidism

Control rats were injected intraperitoneally with $3,3'\text{-}5$-triodothyronine ($T_3$) dissolved in 0.02 N NaOH at the dose of 50 µg per 100 g body weight per rat, for four alternate days to make them hyperthyroid. The last injection was given 24 hours before sacrifice. The control rats were injected similar volumes of the vehicle.

5. 6-Aminonicotinamide Administration

6-Aminonicotinamide dissolved in 0.9 per cent saline was injected intraperitoneally (3.5 mg/100 g body weight) to each rat. Control rats were injected with similar volumes of saline. Rats were killed at 2, 4, 8 and 16 hours after the injection.

6. In vivo Monoamine Oxidase Inhibitor Studies

Rats were given intramuscular injection of clorgyline and deprenyl (1.2 mg/100 g body weight) dissolved in 0.9 per cent saline, 2 hours before sacrifi-
fice to achieve maximum inhibition of MAO-A and MAO-B. The control rats were injected with the same volume of saline. Subcellular fractionation of the whole brains was then carried out as described earlier.

IV DETERMINATION OF ENZYME ACTIVITY

1. Monoamine Oxidase

Monoamine oxidase (amine: oxygen oxidoreductase [deaminating] EC 1.4.3.4) (MAO) is a mitochondrial enzyme, which catalyses the oxidative deamination of a variety of biogenic amines.

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

Monoamine oxidase activity was assayed according to the method of Catravas et al. (1977). The assay mixture contained the following: Tris-HCl, 0.05 M (pH 7.4); Kynuramine dihydro bromide, 0.22 mM; MgCl₂, 0.08 mM; and the appropriate enzyme preparation containing 0.5 to 1 mg protein in a final volume of 3 ml. The reaction was started by the addition of the substrate and was carried out for 90 min in a water bath maintained at 37°C. The reaction was stopped by the addition of 0.2 ml of 0.5 M NaOH and 0.4 ml of 10 per cent ZnSO₄. The mixture was heated in a boiling water bath for 5 min and centrifuged at 10,000 x g for 10 min.
The concentration of the reaction product, 4-hydroxyquinoline, was determined spectrophotometrically in the supernatant by measuring the absorbance at 330 nm (Catravas and McHale, 1974). A blank was prepared by replacing kynuramine with distilled water. A standard graph of 4-hydroxyquinoline (0.1 μmol/ml to 1 μmol/ml) was drawn by measuring the absorbance at 330 nm.

One unit of enzyme activity is defined at 1 μmol of 4-hydroxyquinoline produced per 90 min per g of tissue at 37°C.

2. Na⁺K⁺ATPase

Na⁺K⁺ATPase (Na⁺, K⁺ ATPase, Mg²⁺ dependent ATP phosphohydrolase, EC 3.6.1.3) catalyses the hydrolysis of ATP to form ADP and inorganic phosphate.

\[
\text{ATP} \xrightarrow{\text{ATPase}} \text{ADP} + P_i
\]

Na⁺ K⁺ ATPase activity was assayed according to the method of Zaheer et al. (1968). For total ATPase assay, the assay mixture in a final volume of 3 ml contained: 40 mM Tris-HCl buffer, pH 7.4; 1 mM EDTA, pH 7.0; 6 mM MgCl₂, 100 mM NaCl; 20 mM KCl and 5 mM Tris-ATP.
For Mg\textsuperscript{2+} ATPase assay, the assay mixture in a final volume of 3 ml contained: 40 mM Tris-HCl buffer, pH 7.4; 1 mM EDTA, pH 7.0; 6 mM MgCl\textsubscript{2}; 100 mM NaCl; 20 mM KCl. The enzyme was preincubated for 10 min at 37°C in the presence of 1 mM ouabain and the reaction was started by addition of 5 mM Tris-ATP. The reaction was stopped by addition of 0.6 ml 50 per cent cold TCA.

The tubes were centrifuged at 600 rpm for 10 min and one ml of the supernatant was taken for inorganic phosphate estimation, according to the method of Fiske and Subbrow (1925).

Preparation of Tris-ATP:

Dowex-50 was suspended in 0.1 N HCl and stirred slowly for about half an hour. The slurry was then filtered on a sintered glass funnel, to form a solid cake. It was then washed with distilled water, till the pH of the eluent was about 5.5. The slurry was filtered and suspended in 0.1 M Tris-HCl, pH 7.6. The suspension was degassed by the use of a suction pump, lest any air bubble remain occluded inside the bead. The fines were decanted, and the slurry poured slowly inside a column (0.5 x 4 cm), having some glass wool at its bottom. The Dowex-50 was allowed to pack in the
column at 5°C. The flow rate was adjusted to 5-6 ml per min. The column was then equilibrated with 0.1 M Tris-HCl pH 7.6. Solution of the disodium salt of ATP, 0.3 M, 5 ml was poured in the column, and ATP as Tris salt was collected. Five ml of 0.1 M Tris-HCl, pH 7.6, was then added to wash the column, so that the final concentration of ATP became 0.15 M. The pH of the Tris-ATP solution was then adjusted to 7.4 by solid Tris. The resin was then regenerated by suspending it in 0.1 N HCl, and left overnight, washed with distilled water and reused again.

One unit of enzyme activity is expressed as one μmol inorganic phosphate liberated per g of tissue per min.

V OTHER ESTIMATIONS

1. Protein

Protein was estimated according to the method of Lowry et al. (1951), using bovine serum albumin as standard.

2. Blood Glucose

Blood glucose was assayed enzymatically by the method of Bergmeyer et al. (1974), using a coupled assay system linked to hexokinase and glucose-6-phosphate dehydrogenase. The assay system contained the following:
0.25 ml of glycyglycine/Mg\textsuperscript{++} (0.25 M/0.1 M, pH 7.6); 0.1 ml of NADP (2 mg/ml); 0.5 ml of ATP/Mg\textsuperscript{++} (0.2 M/0.05 M, pH 7.3); 0.01 ml of extract (blood) (1:5); 0.01 ml of glucose-6-phosphate dehydrogenase (1:5) and one unit of hexokinase in a final volume of 1.25 ml. The increase in optical density (OD) was measured after addition of hexokinase and the change in OD was taken as a measure of the glucose in the extract. The blood glucose was expressed as m mol/litre.

VI  PURIFICATION OF MONOAMINE OXIDASE FROM RAT BRAIN

1. Mitochondrial Monoamine Oxidase

Mitochondrial monoamine oxidase was purified by the procedure of Tipton (1971) and Youdim (1975) with some modifications. All steps were performed at 4°C. Ammonium sulfate (AR grade) was recrystallised before use and the dialysis tubes were prepared by boiling twice in 5 per cent Na\textsubscript{2}CO\textsubscript{3} rinsed with distilled water, and boiled (100°C) again in 50 mM EDTA, pH 8.0. The tubes were stored at 4°C in 0.1 mM EDTA. Before use, they were boiled and rinsed in distilled water.

Step 1: Preparation of Mitochondria

Pure mitochondria were prepared according to the method of Student and Edwards (1977), as described earlier in 'Subcellular fractionation'.
Step 2: **Extraction of MAO from Mitochondria**

This step was essentially that of Tipton (1971). Pure mitochondria obtained from Step 1, were suspended in one tenth of the original homogenate volume in ice-cold distilled water. The suspension was homogenised by hand, using a Potter-Elvehjem homogeniser and centrifuged at 18,000 x g for 45 min in a Sorvall RC-5 centrifuge in SS 34 rotor. The residue was taken up in the same volume of 10 mM sodium phosphate buffer, pH 7.6, and stored frozen for a period of not less than 3 days. The preparation was then thawed at room temperature (25°C) and diluted with 10 mM sodium phosphate buffer, pH 7.6 to give a final protein concentration of about 15 mg/ml.

The suspension was then sonicated in a MSE ultrasonic disintegrator fitted with half inch probe at an output ampearage of between 6 and 8 for 45 min, the suspension being cooled in ice-brine solution during sonication. The sonicate was centrifuged at 105,000 x g maximum (76,000 x g average) for 120 min in a MSE superspeed centrifuge in 6 x 38 ml swing out rotor.

The supernatant from the sonicate was carefully decanted. This cream colored and opalescent supernatant
was stored at $4^\circ\text{C}$ and the residue resuspended in $10\ \text{mM}$ sodium phosphate buffer (pH 7.6) and homogenised and sonicated again as before. The sonicated suspension was stored frozen overnight, allowed to thaw at room temperature, and centrifuged as before.

The procedure of sonication, freezing, thawing and centrifugation was carried out at least 6 times, so as to release 60 - 75 per cent of the enzyme activity into the supernatant. The efficiency of this procedure solubilizing the enzyme depends on the output efficiency of the sonication system used, and the amount of enzyme liberated in each step (and hence the number of sonication steps required). The active supernatant was then pooled and stored at $4^\circ\text{C}$.

**Step 3:** *Ammonium Sulfate Precipitation*

Recrystallised solid ammonium sulfate was added slowly to the active supernatant containing the MAO activity obtained from Step 2 to achieve 30 per cent saturation. After 2-4 hours at $4^\circ\text{C}$, the precipitated protein was removed by centrifugation at $14,000 \times g$ for 20 min. The precipitate was discarded, and the solution carefully poured off. More solid ammonium sulfate was added to yield 60 per cent saturation. The preparation was allowed to stand overnight at $4^\circ\text{C}$ and the precipitate was collected by centrifugation at $14,000 \times g$ for
20 min. It contained 60 - 70 per cent of the monoamine oxidase activity. The precipitate was then dissolved in a small volume of 10 mM sodium phosphate buffer, pH 7.6 to give a concentration of 12-15 mg protein per ml.

Step 4: **Sephadex G-25 Column Chromatography**

The 30-60 per cent ammonium sulfate precipitate from Step 3 was passed through a column of Sephadex G-25 (10 x 2.5 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.6. A single peak of protein was eluted with the same buffer.

Step 5: **DEAE-Cellulose Column Chromatography**

The enzyme solution from Step 4 was placed on a column of DEAE cellulose (15 x 2.5 cm) which had been equilibrated with 10 mM sodium phosphate buffer, pH 7.6. The enzyme was eluted using the same buffer containing NaCl in increasing concentrations ranging from 0.1 M to 0.4 M. Monoamine oxidase was eluted with 0.1 M NaCl.

The elution pattern was followed by measuring the absorbance of the fractions at 280 nm to give the protein profile as well as the activity of monoamine oxidase. Fractions of 4 ml were collected at a rate of 15 ml/hour. The fractions containing monoamine
oxidase activity were pooled and precipitated at a concentration of 60 per cent solid (NH₄)₂SO₄. The precipitate was dissolved in a minimum volume of 10 mM sodium phosphate buffer, pH 7.6, to give a protein concentration of 5-10 mg/ml. This solution was then dialysed against the same buffer for 12 hours in the cold (4°C).

2. Cytosolic Monoamine Oxidase (MAO S)

The presence of the monoamine oxidase activity in the soluble fraction was reported earlier by Mayanil and Baquer (1982) from rat brain, and by Copeland et al. (1983) in rat liver. In the brain, the enzyme was found to be clorgyline and deprenyl insensitive when kynuramine was used as a substrate. To know the nature of this inhibitor insensitive monoamine oxidase, it was essential to purify the soluble monoamine oxidase and to compare its properties with the monoamine oxidase associated with the mitochondria.

Step 1: Extraction

The soluble fraction was obtained according to the method of Student and Edwards (1977) as described earlier in 'Subcellular Fractionation'.
Step 2: Ammonium Sulfate Precipitation

Recrystallised solid ammonium sulfate was added to the soluble fraction from Step 1 to achieve 30 per cent saturation. After 2-4 hours at 4°C, the precipitated protein was removed by centrifugation at 14,000 x g for 20 min. The precipitate was dissolved in a small volume of 10 mM sodium phosphate buffer, pH 7.6, to give a concentration of 10-12 mg protein/ml.

Step 3: Sephadex G-25 Column Chromatography

The 0-30 per cent saturated ammonium sulfate precipitate from Step 2 was passed through a column of Sephadex G-25 (10 x 2.5 cm) equilibrated with 10 mM sodium phosphate buffer, pH 7.6. A single peak of protein was eluted with the same buffer.

Step 4: DEAE-Cellulose Column Chromatography

The enzyme solution from Step 3 was placed on a column of DEAE cellulose (15 x 2.5 cm) which had been equilibrated with 10 mM sodium phosphate buffer, pH 7.6. The enzyme was eluted using the same buffer containing NaCl in increasing concentrations ranging from 0.1 M to 0.3 M. Monoamine oxidase was eluted with 0.2 M NaCl.
The elution pattern was followed by measuring the absorbance of each fraction at 280 nm, as well as following monoamine oxidase activity. Fractions of 2 ml were collected at a rate of 15 ml/hour. The fractions containing monoamine oxidase were pooled and precipitated as before at a concentration of 30 per cent solid \((\text{NH}_4)_2\text{SO}_4\). The precipitate was dissolved in a small volume of 10 mM sodium phosphate buffer, pH 7.6, to give a solution of 2-5 mg protein/ml. This solution was then dialysed against the same buffer for 12 hours in the cold (4°C).

VII POLYACRYLAMIDE GEL ELECTROPHORESIS

1. Preparation of 5 Per cent Gels

The separating gels of 5 per cent acrylamide were prepared by mixing solutions (i), (ii) and (iii) and distilled water in the ratio of 1: 2: 1: 4. The solution (i) contained 24 ml of 1 N HCl, 18.1 g Tris, and 0.12 ml TEMED in a total volume of 50 ml distilled water. The solution (ii) contained 10 g acrylamide and 0.5 g bisacrylamide in 50 ml distilled water. The solution (iii) contained 0.07 g ammonium persulfate in 50 ml distilled water.

The solution (mixture of [i], [ii], [iii] and distilled water) was stirred and poured immediately in the
gel tubes (0.5 x 11 cm). A drop of water was layered on the top of the gels, and the gels were then allowed to polymerise for 4 hours.

2. Sample Loading and Electrophoresis

Twentyfive microlitres of the purified enzyme (0.5 mg/ml treated with Triton X-100, [0.02 per cent final concentration]) for 10 min at 4°C was introduced at the cathodal end of the polyacrylamide gel column. Disc electrophoresis was performed using a continuous Tris-HCl buffer system (0.05 M, pH 8.3) for 6 hours at 4°C with a constant current of 3 mA/tube.

3. Gel Staining

Gel staining for monoamine oxidase detection was performed as described by Youdim et al. (1970) by the method of Glenner et al. (1957), using nitroblue tetrazolium. Gels were incubated with a freshly prepared solution containing tryptamine hydrochloride (15 mg), sodium sulfate (9 mg), and nitroblue tetrazolium (5 mg) dissolved in 10 ml potassium phosphate (0.05 M, pH 7.4). Incubation was carried out at 37°C for 12 hours. Monoamine oxidase appeared as blue mauve bands. Gel chromoscopy was performed in Joyce Loebl Chromoscan 200 at 465 nm.
VIII  PURIFICATION OF Na\textsuperscript{+}K\textsuperscript{+}ATPase FROM RAT BRAIN

Na\textsuperscript{+}K\textsuperscript{+}ATPase was purified essentially according to the method of Lingham and Sen (1982).

Step 1: Preparation of Synaptosomal Membrane

Rat brain synaptosomes were lysed in a hypotonic medium containing 10 mM Tris-HCl, pH 7.4, followed by centrifugation at 39,000 \( x \) g for 20 min. The supernatant thus obtained was centrifuged at 105,000 \( x \) g for 3 hours to get the synaptosomal membrane pellet.

Step 2: Purification of Na\textsuperscript{+}K\textsuperscript{+}ATPase

Microsomes/synaptosomal membranes were treated with 0.055 per cent sodium dodecyl sulfate (SDS) in the presence of 3 mM Na\textsubscript{2}ATP, according to the procedure of Jorgensen (1974). Following slow stirring at room temperature (25\textdegree{}C) for 30 min, the SDS-treated preparations were layered on a discontinuous sucrose density gradient consisting of 10, 15, 20 and 50 per cent sucrose in 5.0 mM imidazole-HCl, 1.0 mM Na\textsubscript{2}EDTA, pH 7.6. After centrifugation at 105,000 \( x \) g for 3 hours, the gradient was fractionated at each interface. The Na\textsuperscript{+}K\textsuperscript{+}ATPase activity was found at the 35 per cent sucrose interface (between 20 and 50 per cent) and at the 17.5 per cent sucrose interface (between 15 and 20 per cent).
IX  CHEMICALS

Clorgyline (N-methyl-N-propargyl-1-3-(2,4-dichlorophenoxy) propylamine hydrochloride M and B 9302 was kindly provided by Dr R.A. Robinson of May and Baker Ltd., Dagenham, England. (-)-Deprenyl, (phenylisopropyl methyl propinoylamine hydrochloride, E-250) was a gift from Dr K. Magyar, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary. 3-Methoxy-4-hydroxy benzaldehyde and 3-methoxy benzaldehyde were gifts from the Organic Chemistry Laboratory, Indian Institute of Technology, New Delhi, India.

LENTE zinc insulin were obtained from Boots Co. (India). All biochemicals including substrates, cofactors and chromatographic materials were obtained from Sigma Chemical Co., St. Louis, USA. All other chemicals were from BDH and were of analytical grade.