PART II

EXPERIMENTAL
Chemicals

Acetylcholine chloride, acetylcholinesterase, acetyl-CoA, acetylthiocholine iodide, adenosine 5’-triphosphate, ammonium reineckate, bovine serum albumin, 1,5-bis(4-allyldimethyl-ammoniumphenyl)pentan-3-one dibromide (BW 284 C51), 1,4-bis(5-phenyl oxazole)benzene (POPOP), choline chloride, 2,5-diphenyloxazole (PPO), 5,5’-dithiobis-(2-nitrobenzoic acid) (DTNB), eserine sulfate, hyamine hydroxide, nicotine, polyethylenimine and tetraphenylboron were purchased from Sigma Chemical Co., USA. Ammonium acetate, and tetraethylammonium chloride were purchased from Merck, Germany. Ficoll-400 was purchased from Pharmacia Fine Chemicals, Sweden. Sucrose was purchased from BDH, England. Pirenzepine hydrochloride was purchased from Research Biochemicals Inc., Natick, USA. [14C]Acetyl CoA (200 mCi/m mole) and [3H]L-nicotine (82 Ci/m mole) were purchased from Radiolabelled Chemicals Inc., USA. [14C]Choline (45.5 mCi/m mole) and [U-14C]glucose (53 mCi/m mole) were purchased from Bhabha Atomic Research Center, India. [3H]Quinuclidinyl benzilate (43.5 Ci/m mole) was purchased from Dupont, NEN products, Boston, USA.

Animals

Adult albino Wistar rats weighing 200-240 g were used in the present study. Animals were maintained in cages with free access to food and water under natural light (12 hr.) and dark (12 hr.) cycles at 25°C in air conditioned rooms. Balanced pellet diet (Hindustan Lever Ltd., India) and water was provided ad libitum.

Induction of Hyperammononemia

Hyperammononemia was induced by intraperitoneal administration of either AA or MSI. AA is a direct source of ammonia, and MSI increases blood and brain ammonia levels by inhibiting glutamine synthetase.
Induction of Hyperammonemia with AA

Hyperammonemia was induced as per the method described by Ratnakumari and Murthy (1989).

Acute (convulsive) Group: Animals were rendered hyperammonemic by intraperitoneal administration of single dose (25 mmoles/Kg body wt.) of AA dissolved in saline and pH adjusted to 7.4. Animals were decapitated either during convulsive phase or at different time intervals after administration of the drug.

Subacute (non convulsive) Group: Hyperammonemia was induced in this group of animals by intraperitoneal administration of 2.5 mmoles of AA/Kg body weight. Animals were decapitated 20 min. after administration of the drug to keep parity with the acute group animals.

Induction of Hyperammonemia with MSI

Hyperammonemia was induced with MSI, following the method described by Subbalakshmi and Murthy (1983). A single dose of MSI, dissolved in saline and pH adjusted to 7.4 (300 mg/kg body wt.), was administered intraperitoneally to the animals. Rats in this group were killed 3.0 hr. after administration of the drug (just before entering into the convulsive phase).

In vitro Experiments

To test the direct effects of AA, all the experiments were performed with the preparations of normal rats in presence of 1, 2, 5, or 10 mM AA.

Preparation of Brain/Serum Extracts for Ammonia Estimation

Animals were decapitated and the head was allowed to fall directly into liquid nitrogen and was allowed to freeze for 15 min. at this temperature. Brains were chiseled out of the cranial vault and powdered in stainless steel mortar under liquid nitrogen. Powdered tissue was
immediately transferred to **pre-weighed** tubes containing 3 ml of 5% ice-cold PCA, the tubes were re-weighed and was homogenized at 4°C in **Potter-Elvehjem** homogenizer with serrated teflon pestle. Homogenate was centrifuged at 5000 **rpm** at 4°C and the supernatant was collected. The supernatant was neutralized with solid potassium carbonate in an ice bath and the precipitated potassium perchlorate was removed by **centrifugation** at 10,000 rpm for **15 min** at 4°C. The neutralized extract was used for ammonia estimation.

Blood from neck wound of the animal was collected into centrifuge tubes, allowed to clot at room temperature. This was centrifuged at 4°C and 5000 rpm and serum was collected. To 0.5 ml of serum, 1 ml ice-cold PCA was added and kept in ice for **15 min**. Supernatant was collected by centrifugation and was neutralized with solid potassium carbonate. The precipitated perchlorate was removed by centrifugation and the neutralized supernatant was used for ammonia estimation.

**Preparation of Synaptosomes using Sucrose Density Gradients**

Synaptosomes were prepared followed by the method of Whittaker and Barker (1972). **Immediately** after decapitation, brains were removed from cranial vaults of normal and hyperammonemic rats and washed with ice-cold 0.32 M sucrose. Brains were pressed between two sheets of Whatman No. 1 filter paper to remove excess fluid, surface capillaries and **meninges**. CC, CE and BS were separated from the brains and homogenates were prepared (5% **W/V**) using motor driven Potter-Elvehjem homogenizer with a serrated teflon pestle at 800 rpm with 8-10 up and down strokes. Homogenates were centrifuged at **1000g** for 5 min. and the resultant pellet (**P**₁) was discarded. The supernatant (**S**₁) was centrifuged at 15,000g for 15 min. to obtain crude mitochondrial pellet (**P**₂) and **post-mitochondrial** supernatant (**S**₂). The **P**₂ pellet was suspended in
0.32 M sucrose and was layered on to a pre-formed discontinuous sucrose density gradient (15 ml each of 0.8 M and 1.2 M sucrose). Tubes were centrifuged at 53,000g for 2 hrs. Synaptosomes, present at the interface of 0.8 M and 1.2 M sucrose layers were aspirated, diluted with 0.32 M sucrose and centrifuged at 15,000g for 20 min. The synaptosomal pellet, thus obtained, was suspended in 0.32 M sucrose and used for enzyme assays. All the centrifugations were done at 4°C.

**Preparation of Synaptosomes using Ficoll-400 Gradients**

Synaptosomes were prepared following the method of Cotman (1974). The P₂ pellet obtained as described above was suspended in 0.32 M sucrose. This was layered on pre-formed discontinuous Ficoll-400 density gradient (10 ml each of 4%, 6% and 13% Ficoll-400 in 0.32 M sucrose). The tubes were centrifuged at 63,000g for 45 min. Synaptosomes present at the interface of 6% and 13% Ficoll layers, were aspirated, diluted with 0.32 M sucrose and centrifuged at 15,000g for 20 min. Final pellet was suspended in appropriate medium and used for further studies.

**Preparation of Membranes**

Membranes were prepared from CC, CE, and BS of normal and hyperammonemic rats according to the method of Rao et al., (1991). Homogenate (5%) was prepared in 0.32 M sucrose containing 5 mM Tris-HCl buffer pH 7.4. The homogenate was centrifuged at 1000g for 5 min. at 4°C. The supernatant was diluted with 3 volumes of ice-cold double distilled water and centrifuged at 36,400g for 15 min. at 4°C. The pellet was homogenized in 5 mM Tris buffer and centrifuged at 36,400g for 15 min. at 4°C. The pellet was homogenized in 40 mM Tris-HCl buffer pH 7.4 and centrifuged at 36,400g for 15 min. This step was repeated twice. The final pellet containing membranes was suspended in 40 mM Tris-HCl buffer and used for binding studies.
Estimation of Serum and Brain Ammonia

Ammonia content of brain and serum was determined by using phenol-hypochlorite reagent as described by Rao (1991). To 1 ml of supernatant (obtained after neutralization with potassium carbonate), 1.5 ml of solution A (50g of phenol and 250 mg sodium nitroprusside in 3.75 l water) and 2 ml of solution B (8.4g NaOH, 89.2g disodium hydrogen phosphate and 10 ml of 5% sodium hypochlorite/1) were added. After 20 min at room temperature, absorbance of the sample was read at 630 nm. Ammonium chloride (0.1-1.0 μmoles) was used as standard.

Protein Estimation

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

Cholinesterases

Both acetyl (AChE) and pseudocholinesterases (PChE) were assayed by the method of Ellman et al., (1961) as described by Whittaker and Barker (1972). Thiocholine formed by the action of AChE on acetylthiocholine, reacts with DTNB to give a yellow anion 5-thio-2-nitrobenzoate and 2-nitrobenzoate-5-mercaptothiocholine. The formation of yellow anion was continuously monitored colorimetrically for five minutes at 420 nm. The absorption coefficient of yellow anion 1.36 x m mol⁻¹ x mm⁻¹ was used for the calculation of enzyme activity.

Reaction mixture for total cholinesterase consisted of 100 mM phosphate buffer pH (7.9), 0.5 mM acetylthiocholine iodide, 0.3 mM DTNB and 10 μg synaptosomal protein in a final volume of 1 ml. The reaction was initiated with the addition of substrate and change in absorbance was measured at 420 nm for 5 min.

For the assay of PChE, protein was preincubated for 10 min with 0.2 mM 1,5 bis(4-allyldimethylammonium-phenyl)pentan-3-one dibromide
(BW 284 C51) (AChE inhibitor) and reaction was initiated with the addition of acetylthiocholine iodide. Difference in the activities of cholinesterases measured in absence (total cholinesterase) and in presence (PChE) of inhibitor was taken as AChE activity. For the assay of cholinesterases, synaptosomes were kept frozen at -20°C, and the assay was done, a day after the isolation.

**Choline Acetyltransferase**

Choline acetyltransferase was assayed according to the method of Fonnum (1975). Reaction mixture consists of 0.2 mM $[^3]$Hacetyl CoA (4800 dpm/nmole), 50 mM phosphate buffer, 300 mM sodium chloride, 8 mM choline chloride, 20 mM EDTA, 0.2 mM BW 284 C51, 0.02% Triton X-100 and 30 μg of synaptosomal protein in a final volume of 50 μl. In blanks, BW 284 C51 was excluded. The protein was preincubated with BW 284 C51 for 20 min. except for blanks. Reaction was initiated with the addition of $[^3]$Hacetyl CoA and incubated at 37°C for 15 min. The reaction was stopped by transferring the Eppendorf tubes into a scintillation vial containing 3 ml of 10 mM phosphate buffer. Acetylcholine was extracted into 10 ml toluene scintillation fluid (0.05% PPO and 0.02% POPOP in toluene) with 2 ml acetonitrile containing 10 mg tetraphenylboron. Radioactivity was determined with Beckman LS-1800 liquid scintillation spectrometer for 5 min. Prior to these experiments, optimum protein and optimum time of incubation were determined.

**Choline Uptake into Synaptosomes**

Incubation medium for choline uptake consists of well oxygenated Krebs Ringer-phosphate-glucose-HEPES medium pH 7.4 (HEPES 10 mM, NaHPO$_4$ 10 mM, NaCl 110 mM, KCl 5 mM, CaCl$_2$ 1 mM, Mg SO$_4$ 1.3 mM, glucose 5 mM), eserine sulphate 0.2 mM, and $[^14]$Ccholine (0.2 μCi, 0.5 μM to 100 μM) in a final volume of 500 μl. The reaction mixture was
incubated at 37°C for 5 min. and uptake was started by the addition of 300 μg of synaptosomal protein and incubation was continued at 37°C for 5 min. with constant shaking. Uptake was terminated by rapid centrifugation at 10,000 rpm for 1 min. and the supernatant was discarded. Pellet was washed twice with one ml of buffer containing the same concentration of non-radioactive choline. The protein pellet was dissolved in 100 μl of 0.1 N sodium hydroxide and transferred to a scintillation vial containing 5 ml Bray's mixture (PPO 4 g, POPOP 200 mg, naphthalene 60 g, methanol 100 ml, and ethylene glycol 20 ml made to 1 litre with 1,4-Dioxan). After twelve hours of storage at room temperature (to reduce the chemiluminiscence), radioactivity was determined in Beckman LS-1800 liquid scintillation spectrometer. Optimum amount of protein and optimum time was determined as a part of preliminary standardization. In the former, 100 to 1000 μg of synaptosomal protein was incubated for 15 min. in reaction mixture containing 100 μM choline. For the latter, 300 μg protein was incubated in reaction mixture containing 100 μM choline for 2 to 60 min. In each experiment, non-specific uptake was determined by carrying out incubations at 0°C and due corrections were made to obtain the specific uptake.

$^{14}$CO$_2$ Production from [U-$^{14}$C]Glucose

$^{14}$CO$_2$ produced from [U-$^{14}$C]glucose was measured by the method of Ratnakumari (1990). Reaction mixture contained NaCl 110 mM, KCl 5 mM, CaCl$_2$ 1 mM, Mg SO$_4$ 1.3 mM, glucose 2.5 mM, and 1 mg synaptosomal protein in a final volume of 1 ml. The reaction was performed in vials closed with air tight rubber stoppers from which center wells containing a strip of Whatman No.1 paper, were suspended. Reaction was started by the injection of 1 mg protein into the vial and incubated at 37°C for 45 min. in a shaker water bath. The reaction was
terminated by injecting 200 μl of hyamine hydroxide into a center well and 200 μl of 3 M PC A into a reaction vial. To controls, PC A was added before commencing the reaction. Incubations were continued for another 1 hr. at 37°C, then the center wells were removed and transferred to scintillation vials containing 10 ml Bray's scintillation fluid. Radioactivity was determined in LS-1800 liquid scintillation spectrometer for 5 min.

Synthesis of Acetylcholine from [U-14C]Glucose

Synthesis of acetylcholine from [U-14C]glucose was studied by the method of Gibson et al., (1975) with few modifications. Reaction mixture contains sodium phosphate 10 mM, CaCl2 1 mM, MgSO4 1.3 mM, KC1 5 mM, Choline chloride 2 mM, glucose 2.5 mM and eserine sulfate 0.5 mM adjusted to pH 7.4 in a final volume of 1 ml. Synaptosomes were pre-incubated with 0.5 mM eserine sulfate for 20 min. and the reaction was initiated by the addition of labeled glucose (2.5 mM) and incubated for 45 min. at 37°C with constant shaking. In blanks, eserine sulfate was omitted. The reaction was terminated with the addition of 0.5 ml of 1.4 M PCA containing 35 mM tetraethylammonium chloride (TEAC). Flasks were rinsed twice with 0.5 ml of 0.2 M PCA containing 5 mM TEAC and transferred to centrifuge tubes, homogenized, centrifuged and the supernatant was used for acetylcholine estimation.

Estimation of Acetylcholine

Acetylcholine present in the supernatant was precipitated with 3.5 ml of ice-cold ammonium reineckate, adjusted to pH 2.0 with concentrated PCA. The precipitation was allowed to proceed for 1 hr in ice. Tubes were centrifuged at 25,000g for 20 min. and the precipitate was washed twice with 5 ml of 0.2 M PCA. The final pellet was suspended in 2 ml methanol adjusted to pH 4.5 with concentrated acetic acid. The tubes were centrifuged and 0.5 ml supernatant was added to scintillation vials
containing 10 ml Bray's scintillation fluid. Radioactivity was determined with LS-1800 liquid scintillation spectrometer.

**Binding Studies**

Nicotinic and muscarinic receptor binding was studied with specific ligands $[^3\mathrm{H}]\text{nicotine}$ and $[^3\mathrm{H}]\text{quinuclidinyl} \text{benzilate (QNB)}$ in synaptic membranes isolated from CC, CE and BS of normal and AA treated rats.

**$[^3\mathrm{H}]\text{Nicotine Binding}$**

Synaptic membrane protein (200 $\mu\text{g}$) was incubated with 10 nM $[^3\mathrm{H}]\text{nicotine}$ in 500 $\mu\text{l}$ of 40 mM Tris-HCl buffer pH 7.4 for 15 min. at 37°C. Binding was terminated by diluting the incubation mixture with 6 ml ice-cold Tris-HCl buffer and rapid filtration under negative pressure through GF/C glass microfiber filters followed by two washes with 6 ml ice-cold buffer. Non-specific binding was determined by parallel assays performed in presence of 100 $\mu\text{M}$ unlabelled nicotine. Filters presoaked in 0.1% polyethylenimine were used, to reduce the non-specific binding of ligand to filters. Filters were transferred to scintillation vial containing 5 ml of Bray's mixture and radioactivity was determined with liquid scintillation spectrometer. Specific binding was taken as the difference between total binding and non specific binding.

Reversibility of nicotine binding was studied by incubating 200 $\mu\text{g}$ of membrane protein with 20 nM $[^3\mathrm{H}]\text{nicotine}$ in 40 mM Tris-HCl buffer for 30 min. at 37°C, and various concentration of unlabelled nicotine was added. Binding was stopped 10 min. after the addition of unlabelled nicotine. Amount of nicotine bound was determined as described above.

**$[^3\mathrm{H}]\text{QNB Binding}$**

Synaptic membrane protein (200 $\mu\text{g}$) was incubated with 1 nM $[^3\mathrm{H}]\text{QNB}$ in 500 $\mu\text{l}$ of 40 mM Tris-HCl buffer pH 7.4 for 15 min. at 37°C. Non-specific binding was determined in parallel assays performed in
presence of 100 μM atropine. In order to study the subtypes of muscarinic receptors, 50 nM pirenzepine was used to block the M₁ receptors and [H]QNB bound under these conditions was considered as M₂ binding. M₁ binding was taken as the difference between the binding in absence and in presence of pirenzepine. The binding was stopped by diluting the assay mixture with 6 ml ice-cold Tris-HCl buffer and rapid filtration under vacuum through GF/C glass microfiber filters followed by two washes with 6 ml Tris-HCl buffer. To reduce the non specific binding of the ligand to filters, filters presoaked in 0.1% (W/V) polyethylenimine were used. Filters were transferred to scintillation vial containing 5 ml Bray's mixture and radioactivity was determined with Beckman LS-1800 liquid scintillation spectrometer at 55% efficiency. Specific binding was taken as difference between binding performed in absence and in presence of atropine. Prior to this optimum protein concentration and optimum time of incubation were standardized.

Reversibility of QNB binding was studied by incubating 200 μg of membrane protein with 1 nM [³H]QNB in 40 mM Tris-HCl buffer for 30 min at 37°C, and 100 μM atropine was added. Binding was stopped either immediately or at various time periods by dilution and filtration as described above. To determine the Kᵋ and B_max values, assay was performed with various QNB concentrations (0.005-5.0 nM)

**Calculation of Kinetic Constants**

Kinetic constants for enzyme assays, uptake studies and binding experiments were calculated by Scatchard analysis of the data using Sigma Plot program and plot statistics option.
Statistical Methods

Neuman-Keul’s multiple range test was used to calculate the statistical significance between more than two groups and Student's t-test was used to calculate the statistical significance between two groups.