Chapter 2

Material and methods

1. Materials

The superfusion cannula was made from stainless steel tubes, and composed of two parts (1) an outer cannula with external diameter 0.9 mm., and (2) an inner concentric cannula with diameter 0.2 mm. The extent of the protusion of the inner cannula was 1.3 mm. below the outer cannula (Fig. 3). At this protusion range, best recovery of perfusate sample has been reported (Yask and Yamamura, 1974).

Two channels of the peristaltic infusion pumps (Gilson, minipuls 2, France) were used with silicone tubing (internal diameter 0.8 mm.) for continuous delivery of freshly prepared artificial cerebrospinal fluid (CSF). The composition of the artificial CSF was:-

- NaCl	120	mM
- $NaHCO_3$	15	mM
- KCl	5	mM
- $CaCl_2$	1.5	mM
- $MgSO_4$	1	mM
- Glucose	6	mM

The pH was adjusted to 7.2 and equilibration with O_2-CO_2 (95-5, v/v mixture). In some experiments,

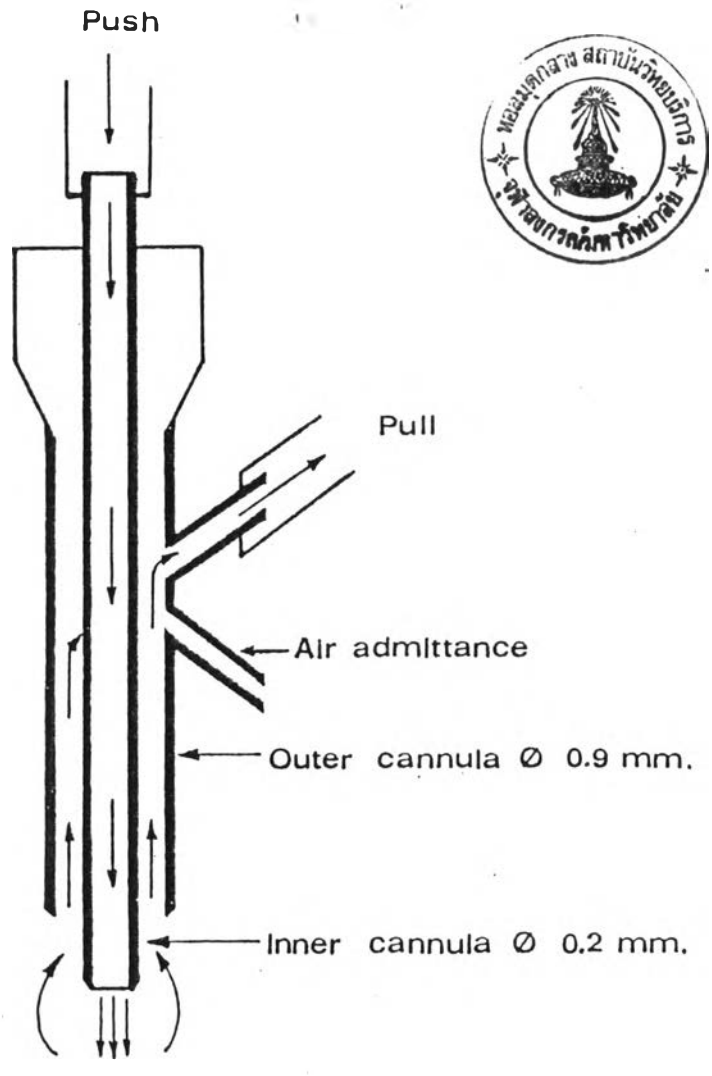


Figure 3. Schematic description of the superfusion push-pull cannula.

the Ca^{+2} content of the solution was omitted (i.e. Ca^{2+}-free solution) and replaced by an equimolar concentration of Mg^{2+} with the addition of 0.5 mM disodium EDTA. In other experiments, the Na^{+} content was reduced by either 50 or 100 mM and replaced by the same molar equivalent of K^{+} (i.e. high K^{+} solution; K^{+} conc 50 mM or 100 mM).

2. Superfusion Methods

Male albino rat (Wistar strain) weighing 280-300 g, were used in the present study. The animals was anesthethized with sodium pentobarbitone (40 mg/kg bodyweight) by receiving single intraperitoneal injection. After complete anesthesia the animal was mounted in a Takahashi stereotaxic instrument in a supine position, with head firmly fixed in the head holder. Tissues overlying the skull area was dissected and retrated away. The dorso-caudal surface of the cerebellum was exposed by craniotomy and the C_1 vertebra was removed by laminectomy. The dura-matter and pia-matter were dissected under the dissecting microscope. The exposed-surface of the cerebellum was covered by a mixture of vaseline and liquid paraffin to prevent dehydration. The animal's head was aligned downward in a 30° plane to facilitrate further surgery on the hind brain. The cerebellum was lifted away from the floor of the fourth ventricle by using a small

spatula and micromanipulator. The fourth ventricle with the vestibular nuclei lined bilaterally along its floor could be visible clearly under the microscope.

The superfusion push-pull cannula was stereotaxically inserted into the vestibular nucleus to a depth of 1.3-1.8 mm below the surface by another micromanipulator. The constant flow rate was adjusted within 10-30 μl/min. The amount of flow collected (efflux) was always equal to the amount of flow perfused (influx). Superfusate fractions (10 min) were collected at ten min intervals, and the samples were stored at -20 °C until analysis (within 1 week).

3. Electrical Stimulation Method

A bipolar platinum electrode (diameter 0.1 mm), insulated except for the tip and having a tip separation of 1.0 mm was stereotaxically implanted to the vestibular nerve. Localization of the vestibular nerve was based on stereotaxic coordinates adapted from the stereotaxic atlas of the rat brain by Pellegrino, Pellegrino and Cushman (1979). Precision of stimulating electrode placement was controlled by observing typical field potentials recorded at the ipsilateral vestibular nucleus through a single barrel microelectrode filled with 4 M NaCl solution (tip diameter 3 μm, resistance 2-7 mΩ). Survey stimulating pulses were square wave with 2 Hz frequency, and pulses

adjusted within 50-60 μA by measuring voltage drop across a 100 Ohm-resister on an oscilloscope. The position of the stimulating electrode was adjusted so that the field potential was at a maximum. The recording electrode was then replaced with the push-pull cannula. The perfusion was started. After controlled sample No. 3 (i.e. 30 min) the vestibular nerve was stimulated using current 1 mA, frequency 100 Hz, duration 0.1 msec, for 10 min with stimulus current being monitored continuously on an oscilloscope (Fig.4).

4. Amino Acid Assays

4.1 Chromatograph

Quantitative analysis was performed on a high performance liquid chromatography (HPLC) which was constituted of two Shimadzu HPLC pumps (LC-3A) for delivery of mixture of two solvents (ethanol : acetate buffer) whose compositions was change in gradient. The gradient was controlled by a solvent programmer (GRE-3A). An autoinjector (LC injector SIL-1A) with 200 μl loop was used for sample injector. Amino acids in the sample were separated on a Zorbex-ODS (C_{18}) column (25 cm X 4.6 mm I.D.) packed with $-C_{18}H_{37}$, particles diameter 5 μm (Shimadzu, Kyoto, Japan).

The column effluent was monitored by a Shimadzu filter-type fluorometric detector FLD-1 with a 14 μl flow-through cell operated at an excitation wavelength

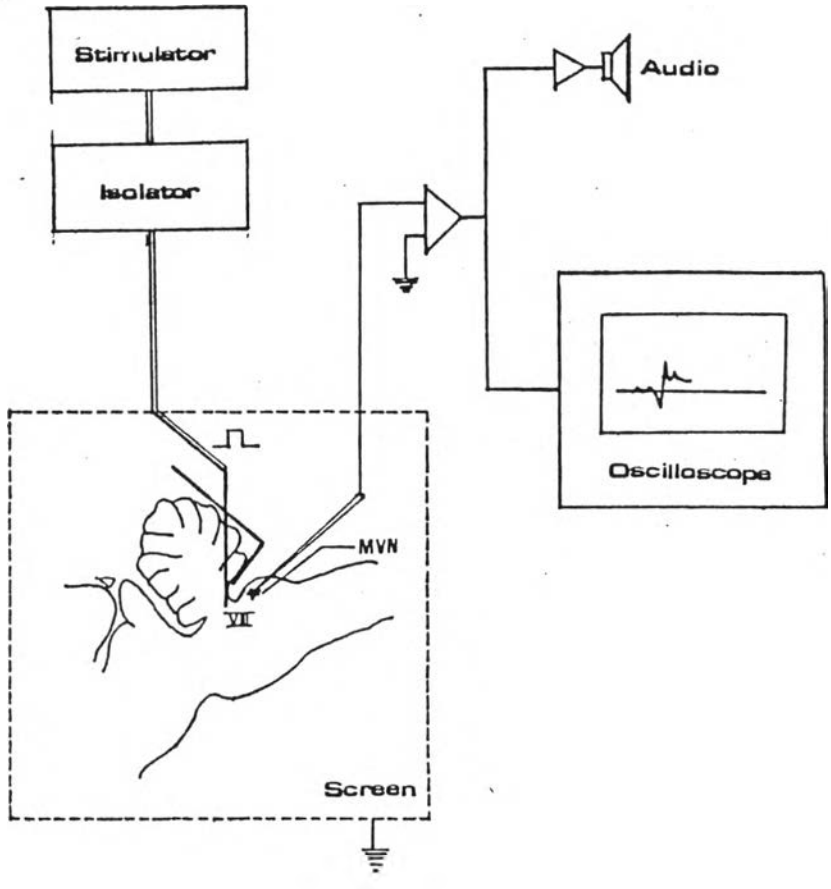


Figure 4. Schematic illustration of the stimulation, recording and localized perfusion arrangements used in the present studies.

of 360 nM (7-60 corning filter) and an emission wavelength of 450 nM (cut-off filter). The area of each chromatograph peak was autoมatically determined by a computing integrator C-R1A chromatopac (Shimadzu).

4.2 Reagent and chemicals :

- Potassium acetate (J.,T Baker Chemical co.)
- Methanol HPLC grade (J.T Baker Chemical co.)
- Standard amino acids (Sigma)
- O-Phthalaldehyde (OPA) (Sigma)
- Absolute ethanol (J.T Baker Chemical co.)
- 2-mercaptoethanol (BDH Chemical Ltd.)
- Boric acid (Mallinckrodt.)

The mobile phase was composed of 0.1 M potassium acetate, pH 5.50 and methanol, HPLC grade. A standard stock amino acid solution was prepared by dissolving known amount of each amino acid in 10 mM HCL to provide a concentration of 100 µmol/ml. The solution was stored at 4 °C until use. Working standard solution was a mixture of all amino acid solutions prepared from the stock solution by diluting with triple distilled water.

The derivatization reagent was prepared as follow : 10 mg O-phthalaldehyde were dissolved in 500 ul absolute ethanol. To this solution 500 µl of 2-mercaptoethanol were added and then diluted up to 10 ml with 0.4 M boric acid adjusted to pH 10.4. The

solution was freshly prepared everyday and kept in the dark.

4.3 Chromatography :

The mobile phase was degassed by ultrasonic agitation and vaccuum suction. The mobile phase gradient was increased from 20 % to 65 % methanol in one linear steps at a flow rate of 2 %/min. The solvent was pumped through the column at a rate 1 ml/min at a pressure 300 Psi. The gradient elution program was followed by a 10-min washing step (100 % methanol) and, finally, the column was equilibrated with 20 % methanol. The column temperature was maintained at 40 °C. The samples were applied to the column with an autoinjection valve (Fig. 5).

4.4 Precolumn Derivatization

One volume (100 µl) of amino acid standard or perfusate sample was mixed with two volume (200 µl) of the derivatization reagent solution by a vortex-mixer at room temperature. After exactly 90 sec the reaction mixture were injected into the chromatograph.

5. Histological Study :

At the end of each experiment, stimulation site was marked electrolytically by passing positive currents 1 mA for 1 min through the electrodes. This current denatured tissues surrounding electrode tip.

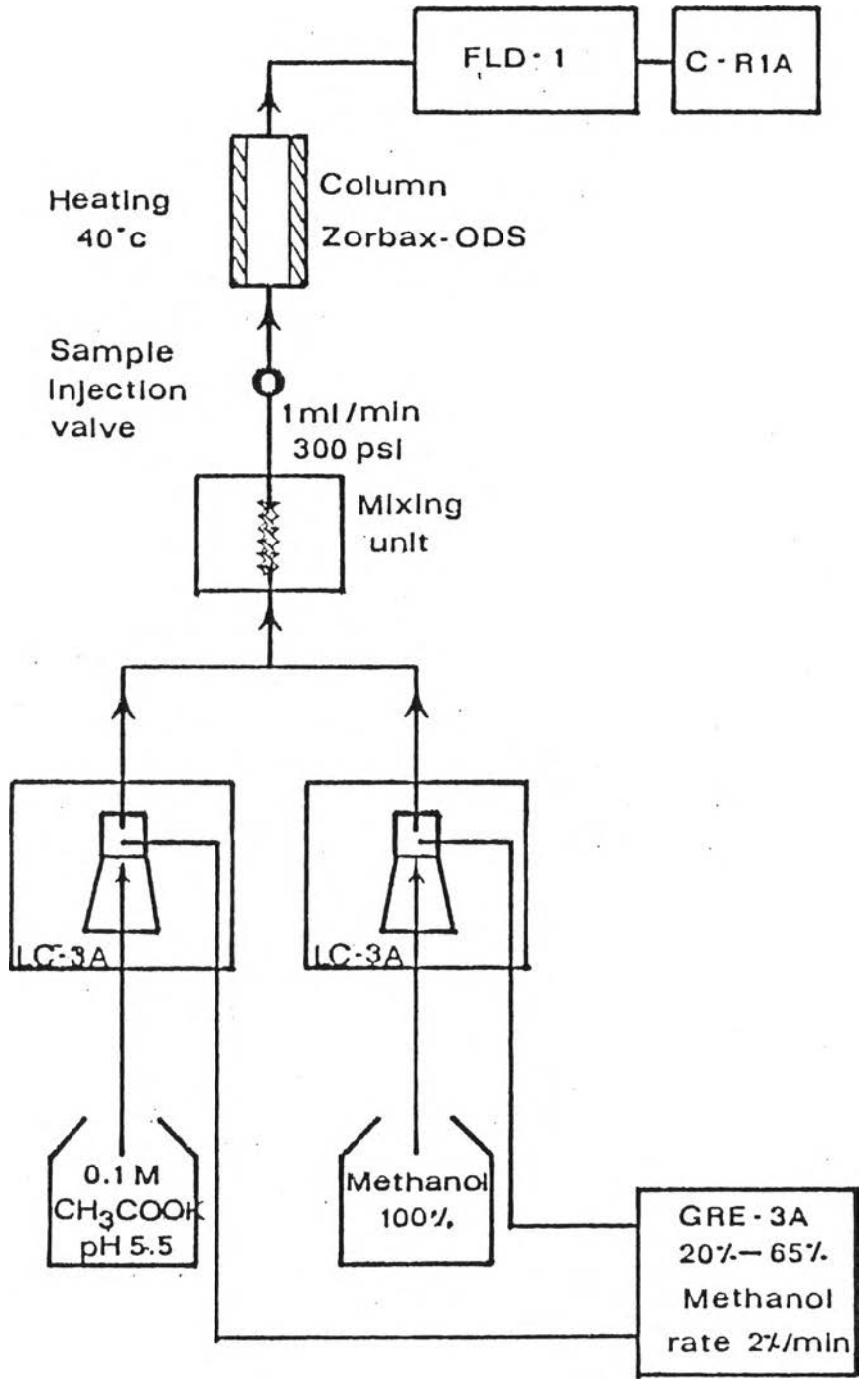
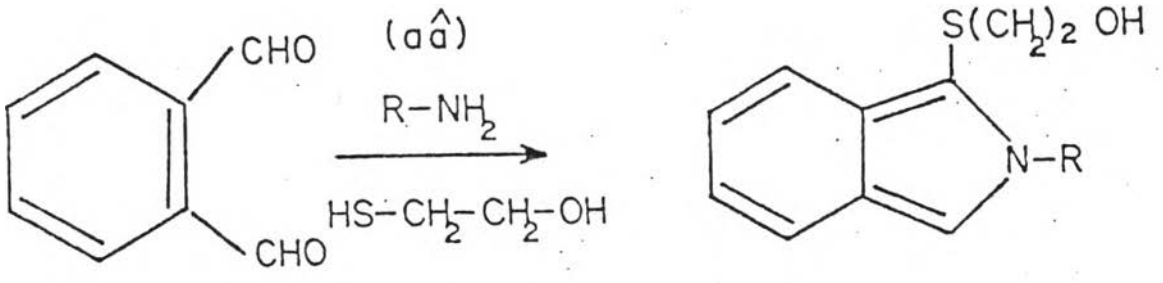


Figure 5. Diagrammatic representation of high performance liquid chromatography used in determination of amino acid contents in superfusate from the vestibular nuclei.

The animal was deeply anesthetized further, and prepared for brain extraction. After cutting the jugular veins to provide drainage, the animal was perfused through the left ventricle with 10 % formal saline containing 0.2 % potassium ferricyanide. The brain was then removed and stored in 10 % formal saline. After fixation the brain was cut in coronal sections (50-100 μm) on a freezing microtome. The sections were studied under light microscope for the tissue damage caused by a push-pull cannula and a blue pigment of iron cyanide at stimulation electrode tip location. This histological examination was performed in all experiments.

The values obtained in individual experiments are everaged and graphs of the mean release of each amino acid plotted against sample number. The mean of the three resting release values was calculated to give a mean resting release and each stimulating value compared with this mean resting value using an unpaired Student's t test.



OPA 2 - Mercaptoethanol Fluorescent derivatives

Ex = 340 nm

Em = 460 nm

Figure 6. o-Phthalaldehyde (OPA) forms fluorescent derivatives in aqueous solution when reacts with amino acids in the presence of the reducing agent 2 - mercaptoethanol.