Chapter 2

Material and methods

1. Animals and materials

Albino rats (Wistar strain) of male sex weighing 280-320 g , were used in the present study. The drugs used in producing olivary lesion were:

- 3-Acetylpyridine (Sigma)
- Harmaline HCl (Sigma)
- Niacenamide (Sigma)

The push-pull canula consisted of two concentric stainless steel tubes with external diameters of 0.9 mm. and 0.2 mm. respectively. The extent of protusion of the inner canula was 1.3 mm. below the outer (pull) canula (Fig.4). At this position, a maximum efficiency of collection has been reported (Yaksh and Yamamura,1974).

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

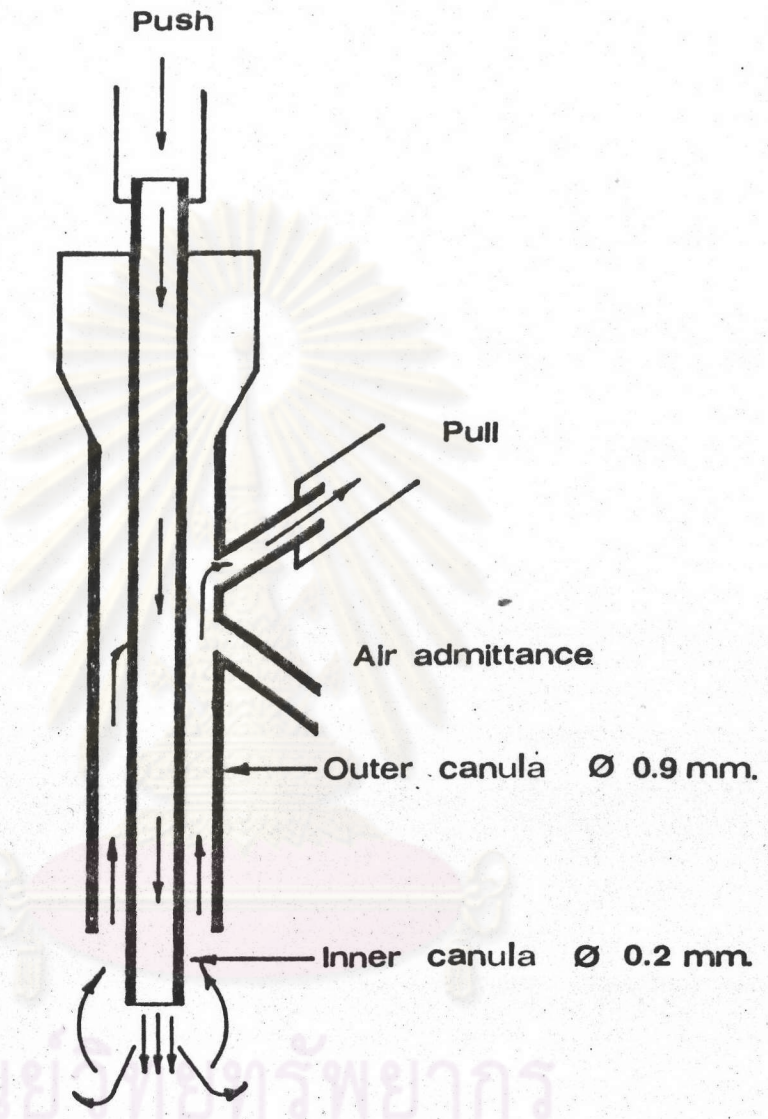


Figure 4. SUPERFUSION PUSH-PULL CANULA

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 pH 7.2 by aerating with O_2 - CO_2 (95-5 v/v) mixture. In some experiments, the Ca^{2+} content of the solution was omitted and replaced by an equimolar concentration of Mg^{2+} with the addition of 0.5 mM disodium EDTA (Ca^{2+} - free solution). In other experiments, the Na^+ content was reduced by either 50 or 100 mM and replaced by either K^+ (high K^+ solution).

2. Preperation of olivary lesioned rats

The rats were given one intraperitoneal injection of 3-acetylpyridine (3-AP) diluted in physiological saline at a dosage of 60-65 mg/kg bodyweight, which is known to destroy the inferior olivary nucleus and its climbing fiber projection into the cerebellum (Desclin and Escubi,1974). Restriction of the central lesion to the inferior olive was made by a subsequent injection of harmaline (15 mg/kg bodyweight) 3 hours later. This 3-AP dose would

ordinary be lethal to most animal. The injection of niacenamide (300 mg/kg bodyweight) 4.5 hours after the 3-AP injection prevented death and protected the rest of the nervous system from otherwise extensive lesion, especially in the brain stem nuclei. Symptoms of the effects of 3-AP became apparent within a few hours after injection in the form of tremors and difficulty in swallowing and breathing. After this initial period, the animal survived well and gained weight, although their final weight was less than that of control animals. Symptomatology often assosiated with cerebellar dysfunction such as intention tremors, a general lack of co-ordination and impaired locomotion. Control rats were injected with normal saline.

3. Perfusion Methods

At 7-21 days after injection, a group of control and 3-AP injected rat were perfused by the push-pull canula. All the animals were anesthethized with nembutal (40 mg/kg bodyweight) by a single intraperitoneal injection three to four hours before beginning the perfusion, amino-oxyacetic acid (AOAA) was injected subcutaneous (30 mg/kg bodyweight) in an attempt to prevent the enzymic destruction of the released GABA (Van Gelder,1965). The animals was mounted on a sterotaxic instrument (Takahashi Co.).The

dorsocaudal surface including part of the os parietale bone covering the cerebellum was removed by craniotomy. The exposed cerebellar mass was then lifted 3-5 mm away from the floor of the fourth ventricle, using a small spatula manipulated by a micromanipulator. The fourth ventricle with the vestibular nuclei lined bilaterally along its floor was visible.

The push-pull canula was slowly inserted at the vestibular nucleus ridge until the canula submerged 1.3-1.8 mm below the surface . The perfusion was then commenced. The flow rate was adjusted within 10-30 ul/min. The amount of flow collected (efflux) always equal to the amount of flow perfused (influx). The pooled perfusate sample collected 10 min were weighed and stored at -20 c until analysis (within 1 week). At the end of the experiments the brain was removed and the position of the canula was examined histologically.

4. Amino acid assays

4.1 Materials :

High performance liquid chromatography (HPLC) was carried out on a Shimadzu HPLC-3A chromatograph equipped with a gradient accessory (GRE-3A). An autoinjector (LC injector SIL-1A) with 200 ul loop was

used. Amino acid was seperated on a Zorbax-ODS (C 18) column (25 cm x 4.6 mm I.D.), packed with $-C_{18}H_{37}$, particle diameter 5 um (Shimadzu, Kyoto, Japan).

The column effuent was monitored by fluorescent detector FLD-1 (Shimadzu) equipped with a 14-ul flow through cell operated at an exitation wavelength of 360 nM (7-60 corning filter) and an emission wavelength of 450 nM (cut-off filter). The areas of each chromatographic peak was automatically 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 acid (Sigma)
- O-Phthaladehyde (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 solution was prepared by dissolving each amino acids in 10 mM HCl to provide a concentration of

100 umol/ ml. Working standard solution was a mixture of all amino acids containing 200 nmol/ml of each, prepared from the stock solutions by dilution with distill water.

The derivatization reagent was prepared by dissolving 10 mg OPA in 500 ul absolute ethanol. To this solution 500 ul of 2-mercaptoethanol were added and then diluted up to 10 ml with 0.4 M boric acid adjusted to pH 10.4.

4.3 Chromatography :

The mobile phase was degassed by ultrasonic agitation in combination with vaccuum suction. The mobile phase gradient was run from 20% to 65% methanol in one linear steps at a rate of 2%/min. The mobile phase 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 sample were applied to the column with a autoinjection valve. (Fig. 5).

4.4 Derivatization :

One volume (100 ul) of amino acid standard

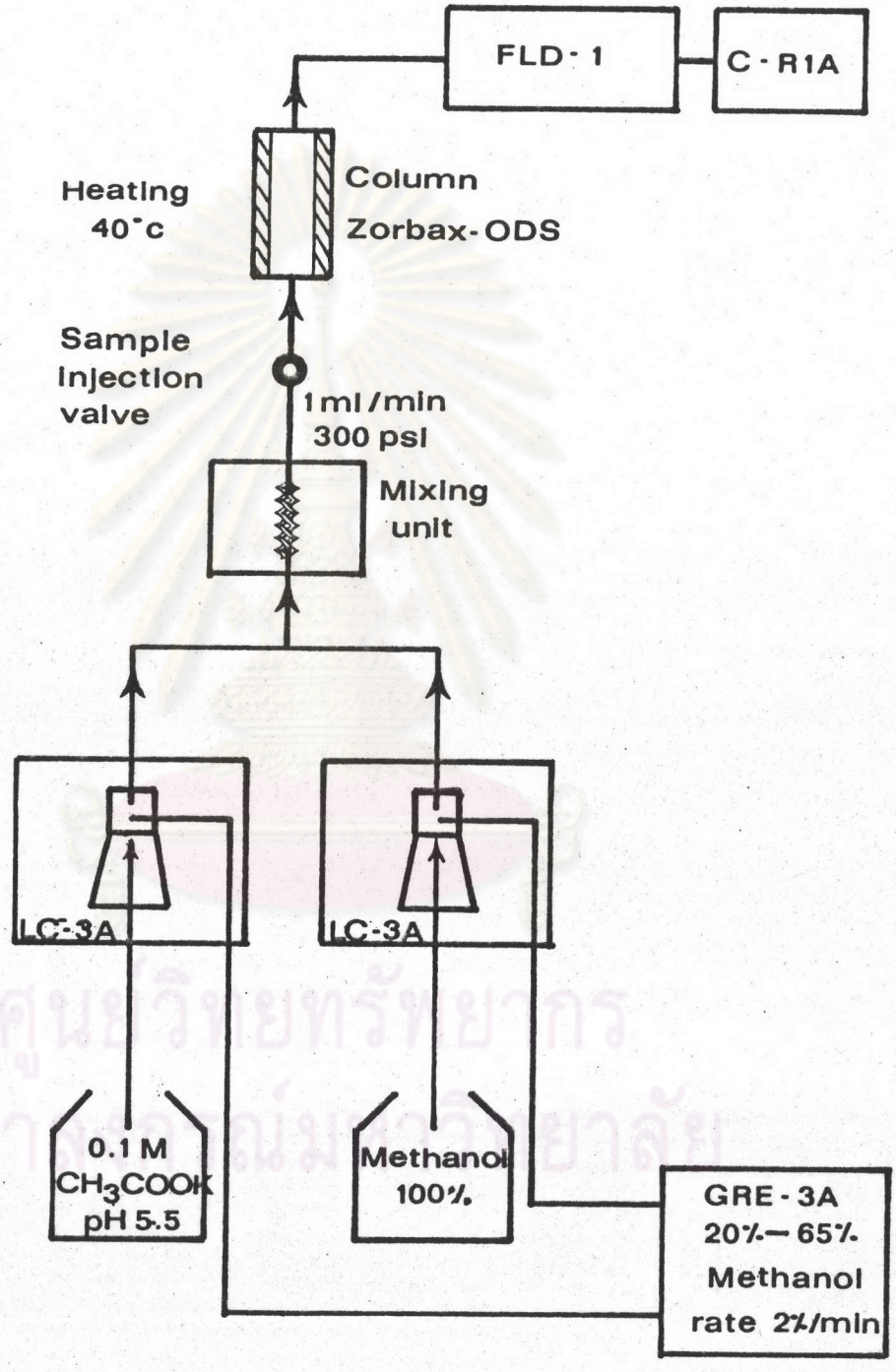
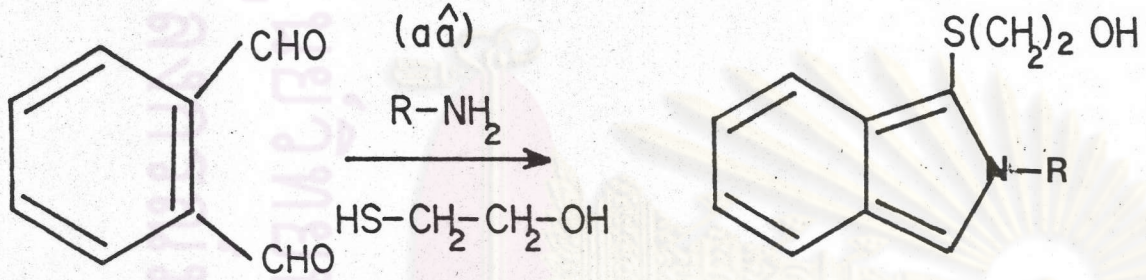


Figure 5. The flow diagram of high performance liquid chromatography (HPLC).

solution or perfusate sample was mixed with two volume (200 ul) of the derivatization reagent solution. The content were mixed by a vortex- mixer and injected after 90 sec standing at room temperature. (Fig. 6).

The values obtained in individual experiments are everaged and graphs of the mean release of each amino acid plotted against sample number. In order to ascertain whether any observed changes in the release were statistically significant, the mean of the three resting release values was calculated to give a mean resting release and each succeeding value compared with this mean resting value using an unpaired Student's t test. In these experiments in which a change in amino acid release has occured and in which release was allowed to return to the prestimulation stage, result are expressed in terms of the total number of p-mol of each amino acid released.



OPA 2 - Mercaptoethanol Fluorescent derivatives

Ex = 340 nm

Em = 460 nm

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