Chapter 2

Material and methods

The experiments were carried out in 2 parts

Part A: Electrophysiological method

Part B: Biochemical method

Part A: Electrophysiological method

Experimental Aminals and Anesthesia

Experimentals were performed on adult albino rats (Wistar strain) of both sexes weighing 190 - 320 g. The animals were anesthethized with urethane (1.5 gm/Kg body weight) by receiving single intraperitoneal injection. After complete anesthesia, the animals were mounted on a Narishige's stereotaxic apparatus. The animal's head was fixed firmly in the head holder. A proportionally controlled DC heating pad was used to maintain body temperature between 36.5° - 37.5° C. A midline incision was made in the scalp from the level of frontonasal suture to the neck. Craniotomy was performed to expose the cerebellar surface and the dura - matter removed under a dissecting microscope. Microelectrode was slowly inserted into the folium vermis lobe of the cerebellar cortex.

Before insertion of the microelectrodes, a small patch was made in the pia-matter to facilitate the insertion of glass microelectrodes. This step was considered necessary since advancing a microelectrodes through and intact pia-matter tended to cause blockade. After insertion of the electrodes, to reduce movement artifacts, and prevent the cerebellar surface from drying, the exposed cerebellar cortex was covered with layer of 3% agar in saline solution.

Recording and Microiontophoresis

Microiontophoresis is a method of providing a controlled application of chemical substance from fine microelectrode or micropipette by passing of electric current. The term is sometime interchangable with microelectrophoresis, but in a more strict sense the microiontophoresis should be applied only when describing the movement of ions by current flow. Thus, in microelectrophoresis, microiontophoresis occurs, which usually is associated with electro-osmosis.

Microiontophoretic application in this study was performed by using seven-barrel microelectrodes with 6 to 9 μm tip diameter for simultaneous recording of extracellular spontaneous discharge of Purkinje cells

via the center barrel, and microiontophoertic injection of the drugs via the peripheral barrels. The microelectrode was fabricated in the laboratory from borosilicated glass tubing (external diameter 1.55 mm± 0.05 mm., internal diameter 1.15 ± 0.05 mm., Modulohm I/S - Denmark). The electrode was pulled on a vertical type electrode puller and the tip was broken back to 6 - 9 μm. under microscopic control. Each barrel of the electrode contained a fine glass fiber along its length to facilitate the filling of the drug solutions to the tip by capillaric attraction.

The outer barrels of electrode contained drug solutions, usually kept refrigerated until need for use. One side barrel was filled with 4 M NaCl for automatic current balancing. The central barrel was filled with 4 M NaCl for recording action potentials (spikes) which were amplified, monitored on an oscilloscope, and converted to a uniform voltage pulses by a window discriminator. The pulses were integrated by a rate meter and displayed on a strip chart recorder.

After filling with drug solutions, the electrode was then subjected to electrical tests. The most convenient and useful assessment was to measure the DC resistance of each barrel in the electrode assembly.

In practice, the usual resistance range for 4 M NaCl filled barrel of a seven - barrel microelectrode was 4 - 8 Mohm (Megaohm), 8 -12 Mohm for 2 M.NaCl and 15 - 100 Mohm for barrel containing drug solutions.

Ejection of substances into the vinicity of neurones was achieved by passing current with the same polarity as that of the active ions through the electrode barrels. For a strongly ionised ions the ejection depended mostly on iontophoretic migration, while in case of poorly ionised substance, the release has to be dependent on electro - osmosis (Curtis, 1964; Kelly, Simmonds and Straughan, 1975). This process could be enhanced by dissolving the particular substance in 165 mM NaCl. When being released the expelled sodium ions were believed to be accompanied with water and solute (i.e.drug) molecules. Substances released by this process are shown in table I.

To avoid spontaneous efflux of the contents inside the electrode (Kelly et al, 1975) a current with opposite polarity to that of the active ion species was continuously passed through each barrel. The currents of 10 -30 nA. were usually used in this study. Theoretically, the amount of this retaining current was dependent upon the resistance of each particular barrel. Retention and ejection periods were automatically

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Table I Substances used in Microiontophoretic study

Substance	Abbreviation	Conc. (M)	pH	Supplier
Angiotensin II	AII	0.002	4.5	Calbiochem
Saralasin		0.002	4.5	Calbiochem
gamma-aminobutyric acid	GABA	0.2	3.5-4	Sigma
GLycine HCl	GLy	0.2	3.5	Sigma
GLutamate	GLu	0.2	7	Sigma
Aspartate	Asp	0.2	7.5	Sigma
Amino-oxyacetic acid	AOAA	0.2	3.5-4	Sigma

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sequenced and maintained constant throughout an experiment by the use of digital electronic circuitry. This circuit also incorporated an automatic current balancing channel. At all time this channel automatically provided the current which was equivalent to algebraic sum of the current flow at the electrode tip but of opposite polarity through one barrel which contained 4 M NaCl. By so doing, tip potentials that could directly influence cell discharges were minimized. Cells that displayed nonspecific responses to drug applications were eliminated from the study. To minimize electromagnetic and electrostatic interferences successful recording was performed on the animal housed in a screened cage Fig.6 shows detail of experimental arrangement used in all electrophysiological investigation in this study.

Processing of spike data

In order to obtain signals which were compatible with the input stage of the instruments, the amplified spike potentials were converted into corresponding pulses of standard amplitude and duration. This was achieved firstly by feeding the amplified spike into a pulse height selector window- discriminator which by adjusting a variable gate, would reject undesirable

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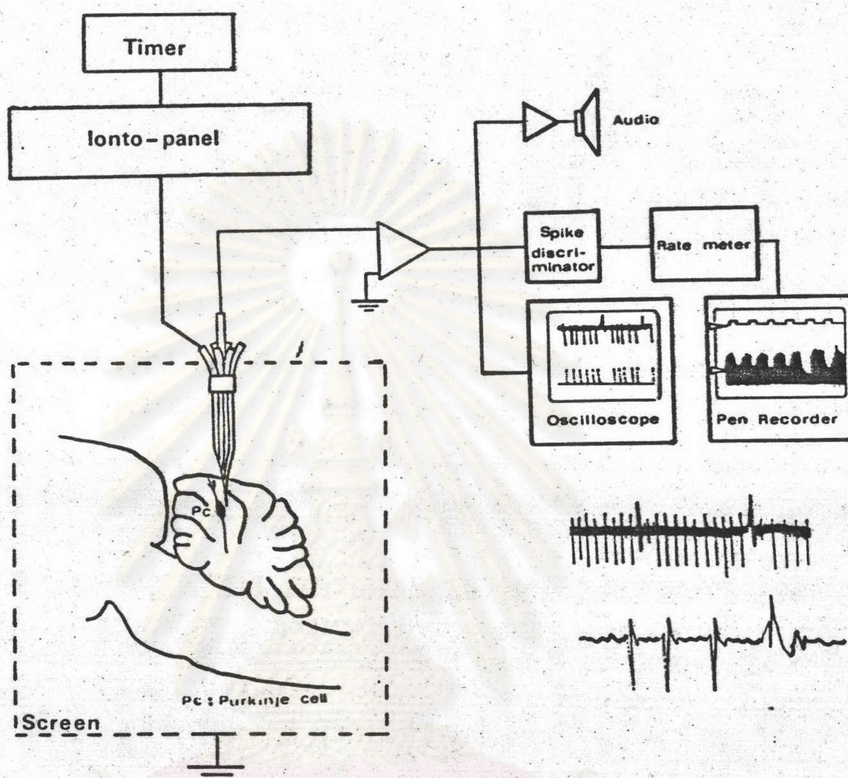

Figure 6 Diagramatic representation of experimental arrangements routinely employed in experiments involving electrophysiclogical investigation and microiontophoresis. For detail, see text. (from Saguanrungsirikul, S., 1983)

signals (i.e.noise) which had amplitudes below or above a set level. This method together with maneuvering the electrode position in relation to a particular neurone enabled a single unit spike activity to be isolated. The selected amplified spikes were then used for tiggering standard pulses (one pulse per spike). The standard pulses were fed into a digital rate meter and epoch counter, the output from epoch counter was recorded on the curvilinear pen recorder to obtain the neuronal firing rates and the counts displayed on a strip chart recorder as histogram of number of pulses over each consecutive epoch of 0.5 sec. The records were subsequently converted to neuronal firing rates of spikes per second for data presentation. The neuronal activities before, during, and after iontophoretic applications of various substances were compared and analysed.

The Criteria Used in Identifying Purkinje Cells

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On passing the microelectrode into the cerebellar cortex, the microelectrode encountered an increased level of maintained multiunit activity. Of the units that could be isolated in or near the Purkinje layer, most generated two distinctly different kinds of spike (Fig 7) A Simple spike - so-called because of its relatively simple wave - form, was first

recorded as an initially negative potential that commonly became positive - negative on further advance of the microelectrode and fired (without indications of injury) at maintained frequencies about 20 - 150 spikes/sec. Discharge of this simple spike was frequently interrupted by another waveform that was more complex in shape (Figure 7, black dot, Tongroach et al, 1984). The "complex" spike occured in a sporadic pattern at a frequency about 1 - 4 spikes/sec, and was followed by a brief silent period. Units whose activity was recorded extracerebellarly in the cerebellar cortex and which generated these two different spike shapes are subsequently refered to as "Purkinje cells" (Eccles, 1973; Szentagothai and Arbib, 1974; Tebecis, 1974).

DATA ANALYSIS

Drug effects qualitatively were assayed by tabulating the total number of cells tested versus the number of cells evidencing a prescribed response. For all integrated histograms, drug effects were quantitated by comparing the discharge rate during the iontophoresis of the drug with the rate before the drug and expressing the value as a per cent change.

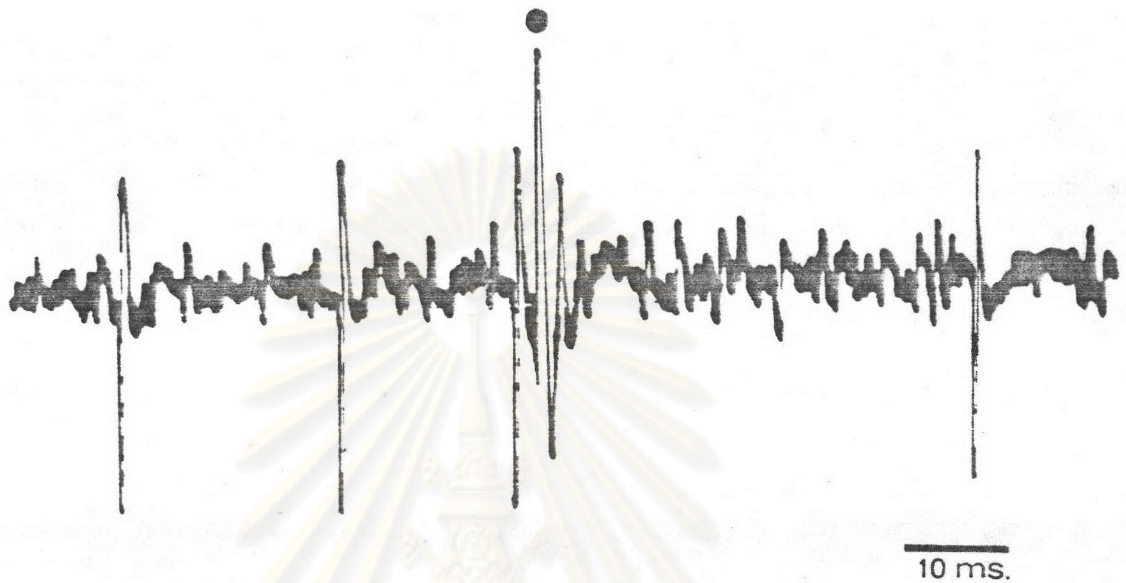

Figure 7 Maintained discharge of a Purkinje cell, recorded extracellularly, showing its two different spike potentials the "simple" and the "complex" (black dot). The traces shows their different pattern of discharge and shape. Positive is up. (From Saguanrungsirikul, S., 1983)

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Part B: Biochemicalphysiology method

Superfusion methods

Male albino rat (Wistar strain) weighing 280-320 gm., were used in the present study. The animal was anesthetized with nembutal (40 mg/Kg body weight) by receiving single intraperitoneal injection three to four hours before the begining the perfusion, amino-oxyacetic acid (AOAA) was injected subcutaneously (30 mg/Kg bodyweight) in an attempt to prevent the enzymic distruction of the released GABA (Van Gelder, 1965). The animal was mounted on a stereotaxic instrument (Takahashi co.) in a supine position, with head firmly fixed in the head holder. Tissues overlying the skull area was dissected and retracted away. The dorso - caudal surface of the cerebellum was exposed by craniotomy and the dura - matter was dissected under the dissecting microscope. The animal's head was aligned downward in a 30° plane to facilitate further superfusion procedure. Then a small patch was made in the pia - matter to facilitate the insertion of push - pull canula (PPC).

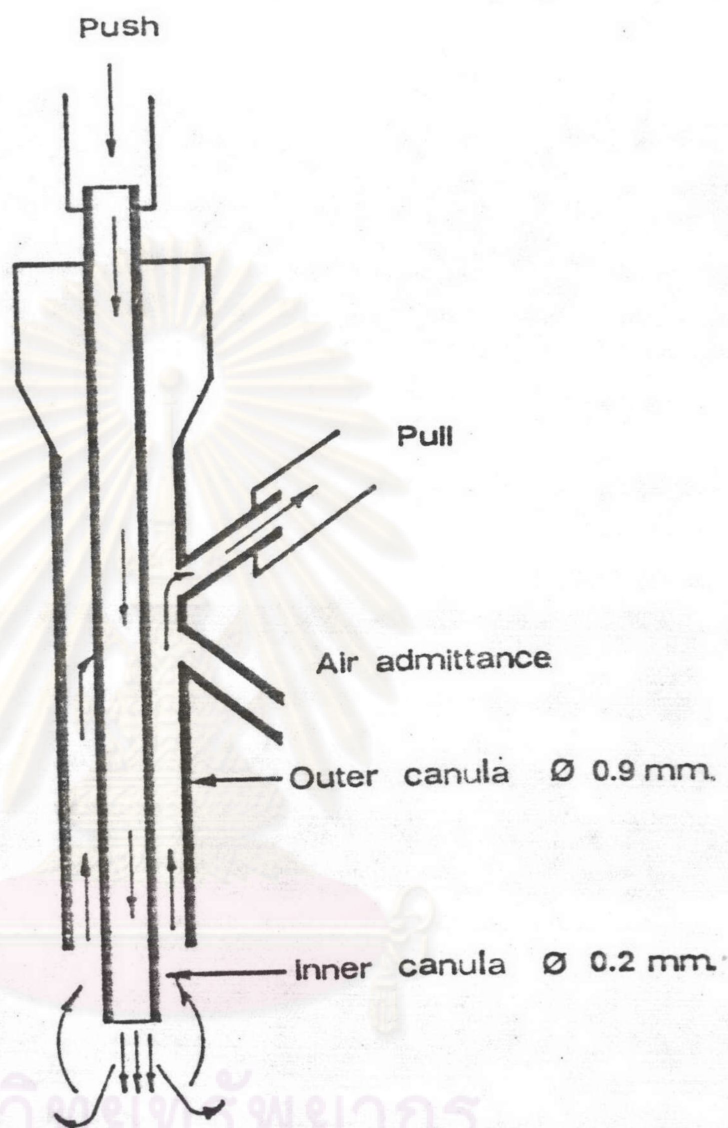
The push - pull canula was slowly inserted into the folium vermis lobe of the cerebellar - cortex until the canula submerged 0.3 - 0.5 mm. below the surface.

The exposed surface of the cerebellum was covered by a mixture of vaseline and liquid paraffin to prevent dehydration. The perfusion was then commenced. 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 were collected at 10 min. intervals, and the samples were 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.

The superfusion push-pull canula consisted of two concentric stainless steel tubes, and composed of two parts (1) an outer canula with external diameter 0.9 mm., and (2) an inner concentric canula with diameter 0.2 mm. and protruding 0.3 mm. beyond the outer tube. (Fig.8)

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

- NaCl 120 mM
- $NaHCO_3$ 15 mM



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SUPERFUSION PUSH-PULL CANULA

Figure 8

- KCl 5 mM
- $CaCl_2$ 1.5 mM
- Mg SO_4 1 mM
- Glucose 6 mM

The pH was adjusted to 7.2 by equilibration with O_2-CO_2 (95 - 5,v/v) mixture. In some experiments, the Na^+ content was reduced by 100 mM and replaced by the same molar equivalent of K^+ (high K^+solution ; K^+ 100 mM). In other experiment, artificial CSF containing 40 mM Angiotensin II was used.

The perfusion push-pull canula was held in a suitable manipulator for insertion into the brain. The inflowing perfusion fluid must be warmed to 37° C. The outflow polyethylene tube, after priming, passed directly to a collection tube. Before inserting the PPC into the brain, perfusion through the system should be started and the flow rate adjusted and any air bubbles in the system removed. The canula can then be lowered into the brain, taking care to avoid damage to surface blood vessels.

After insertion into the brain, the perfusion flow should continue at the same rate as when the canula was suspended in the air. The tissue at the tip of the canula may cause some blockage of the out flow system.

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Clearly this situation must be corrected quickly to prevent perfusion fluid building up within the brain tissue. If blockage of the outflow occured, it was usually sufficient to withdraw the canula a small distance, when flow was normally re-start. If flow did no recommence after this manoeuvre it was necessary to remove the canula from the brain and clear it.

Once a steady flow has been established, the inflow and outflow rates must be carefully monitored to ensure equality and any necessary drugs can then be added to the perfusion fluid.

Amino acid assays

High performance iuquid chromatography (HPLC) was carried out on a Waters Model 440 chromatograph. The system employed a gradient elution method which started with 20% methanol in acetate buffer and ended with 65% in a linear increment at the rate of 2% per min The gradient was controlled by a solvent programmer (Model 680, Waters). An injector (Model U 6 K, Waters Associates) with 250 µl. loop was used for sample injector. Amino acids in the sample were separated on a (C_{18}) column (25 cm x 4.6 mm. I.D.) packed with $C_{18}H_{37}$, particles diameter 5 µm (Waters Associates).

The column effluent was monitored by Waters fluorescent detector Model 420 C or 420 E (Waters Associates), operated at an excitation wavelength of 340 nm and an emission wavelength of 455 nm (cut - off filter) The detector was fitted with a computing integrator (Waters 740 Data Module), by which the areas of each chromatographic peak was automatically determined.

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.)

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The mobile phase composed of 0.1 M Potassium acetate, pH 5.50, and methanol HPLC grade. A standards stock solution was prepared by dissolving each amino acids in 10 mM HCl to provide a concentration of 100 µmol/ml. The solution was stored at 4° C until used. Working standard solution was a mixture of all amino acids containing 200 nmol/ml of each, prepared from the

stock solutions by dilution with distrilled water.

The derivatization reagent was prepared by dissolving 10 mg OPA in 500 µl 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. The solution was freshly prepared everyday and kept in the dark.

Chromatography

The mobile phase was degassed by ultrasonic agitation in combination with vaccum suction. The mobile phase gradient was run from 20% to 65% methanol in one linear steps at a rate of 2% /min. It 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 $40^{o}C$. The samples were applied to the column with an injection volume 250 µl. (Fig.9)

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Derivatization

One volume (100 µl) of amino acid standard

solution or perfusate sample was mixed with two volume (200 μl) of the derivatization reagent solution (Fig.10). The content were mixed by a vortex-mixer and injected after 90 sec standing at room temperature.

The result from such experiment have been expressed. In the first, the values obtained in individual experiments was averaged and graphs of the mean release of each amino acid plotted against sample number. In order to ascertain whether any observed changes in release were statistically significant, the mean of the two 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 this experiments in which a change in amino acid release has occured and in which release was allowed to return to the prestimulation stage, results have been expressed in terms of the total number of p-mol of each amino acid released.

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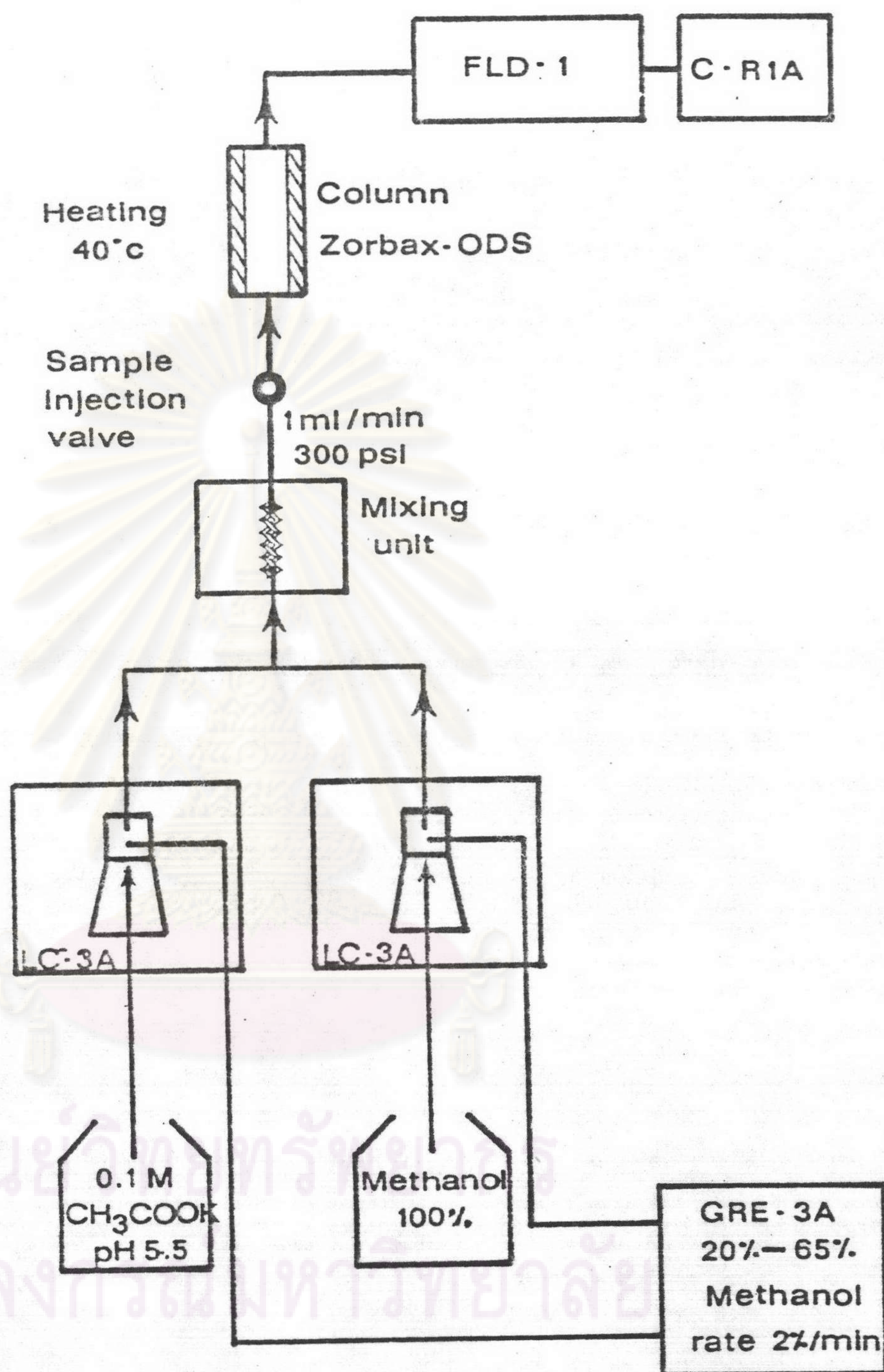

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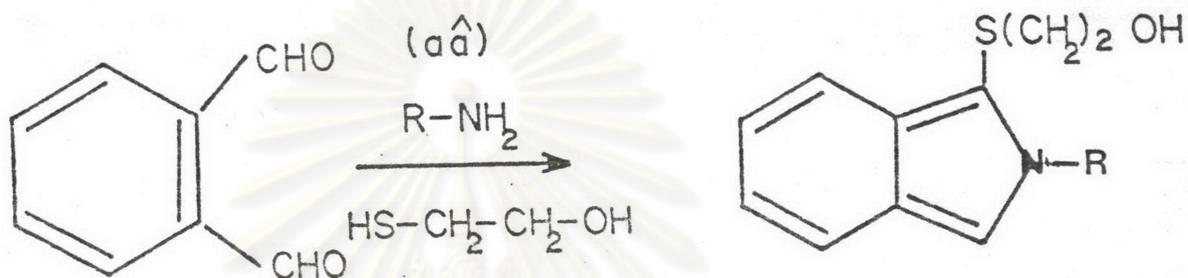
Figure 9 The flow diagram of high performance liquid chromatography (HPLC).



Ex = 340 nm

Em = 460 nm

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Figure 10 o - Phthaldehyde (OPA) forms fluorescent derivatives in aqueous solution when reacts with amino acids in the presence of the reducing agent 2 - mercaptoethanol.