CHAPTER III

MATERIALS AND METHODS

3.1 Animals

Male wistar rats weighing 220-270 g were used in this experiment. The rats were housed in groups of four to five rats and maintained on a standard light-dark cycle with a constant humidity and controlled temperature (28-32 ^{0}C). The animals were given free access to food and tab water ad libitum. To limit the effects of nonspecific stress, all animals were kept in animal center of faculty for at least 7 days before experimentation. Animal handlings and experiments has been approved by Ethical Committee, Faculty of Pharmaceutical Science, Chulalongkorn University. Wistar rats were obtained from the National Laboratory Animal Center, Mahidol University, Nakornpathom and Japan SLC Inco., Shizuoka, Japan.

3.2 Chemicals used in various methodology.

3.2.1 Anticonvulsant activity of VPU and VPA against pilocarpine-induced seizure

VPA, methyl-scopolamine nitrate,. carboxymethyl cellulose sodium salt (CMC) and pentobarbital sodium were purchased from Sigma chemical company (U.S.A.). Pilocarpine hydrochloride was purchased from Nacalai Tesque, Inc. (Japan). Moreover VPU was synthesized by Associate Professor Chamnan Patarapanich and coworkers at the Faculty of Pharmaceutical Science, Chulalongkorn University.

VPA and VPU were dissolved in 0.5% CMC solution. Pilocarpine and methyl-scopolamine nitrate were dissolved in normal saline solution (NSS) and distilled water, respectively.

3.2.2 Neuronal Morphological determinations by a Cresyl violet staining technique

Cresyl violet was purchased from Sigma chemical company (U.S.A.). 95% ethanol and xylene were purchased from Merck (Germany) and J.T. Baker Solusorb (Canada), respectively.

3.2.3 Effects of VPU and VPA on the level of hippocampal amino acid neurotransmitters in freely moving rats

GABA, glycine, glutamate, aspartate, homoserine, and o-phtalaldehyde (OPA) were purchased from Sigma chemical company (U.S.A.). Methanol was purchased from Merck (Germany).

The artificial cerebrospinal fluid (aCSF) pH 7.3 contained 120 mM NaCl, 15 mM $NaHCO_3$, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, and 6 mM glucose.

The mobile phase consisted of two components, 0.05 M sodium phosphate buffer (pH 6) with 50 % methanol for analysis of GABA and 0.05 M sodium phosphate buffer (pH 6) with 22% methanol for analysis of glutamate, aspartate and glycine with flow rate 0.5 ml/min.

3.2.4 Measurement of lipid peroxidation

2-thiobarbituric acid, 1,1,3,3-tetraethoxypropane, and sodium dodecyl sulphate were purchased from Sigma chemical company (U.S.A.). 1,1,3,3-tetraethoxypropane was used as the standard sample.

3.2.5 Determination of neuronal mitochondrial activity

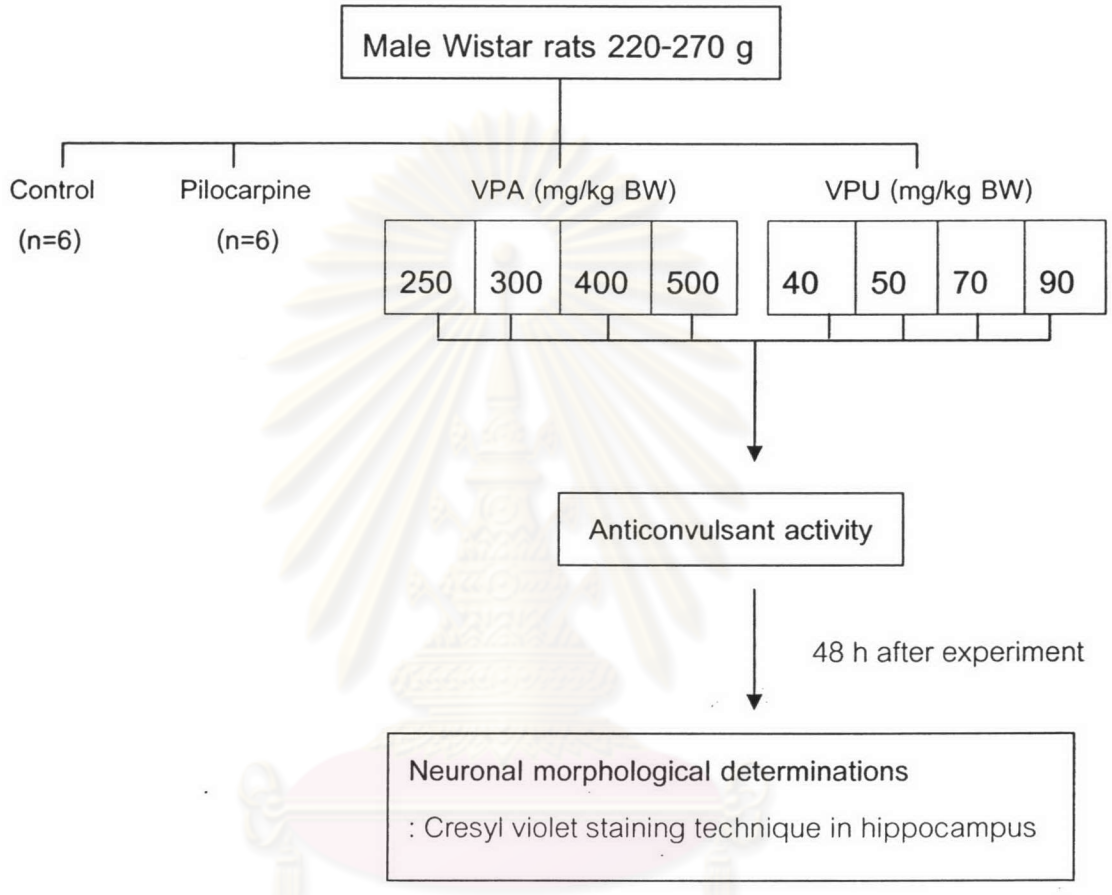
Adenosine diphosphate (ADP), N-[2 Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), bovine serum albumin, and digitonin were purchased from Sigma chemical company (U.S.A.). Mannitol and sucrose were purchased from Ajax Finechem (Australia).

Mitochondrial isolation medium (pH 7.4) contained 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1mg/ml bovine serum albumin. The standard respiration medium (pH7.4) contained 300 mM mannitol, 75 mM sucrose, 5 mM KCl, 10 mM TRIS, and 5 mM KH_2PO_4.

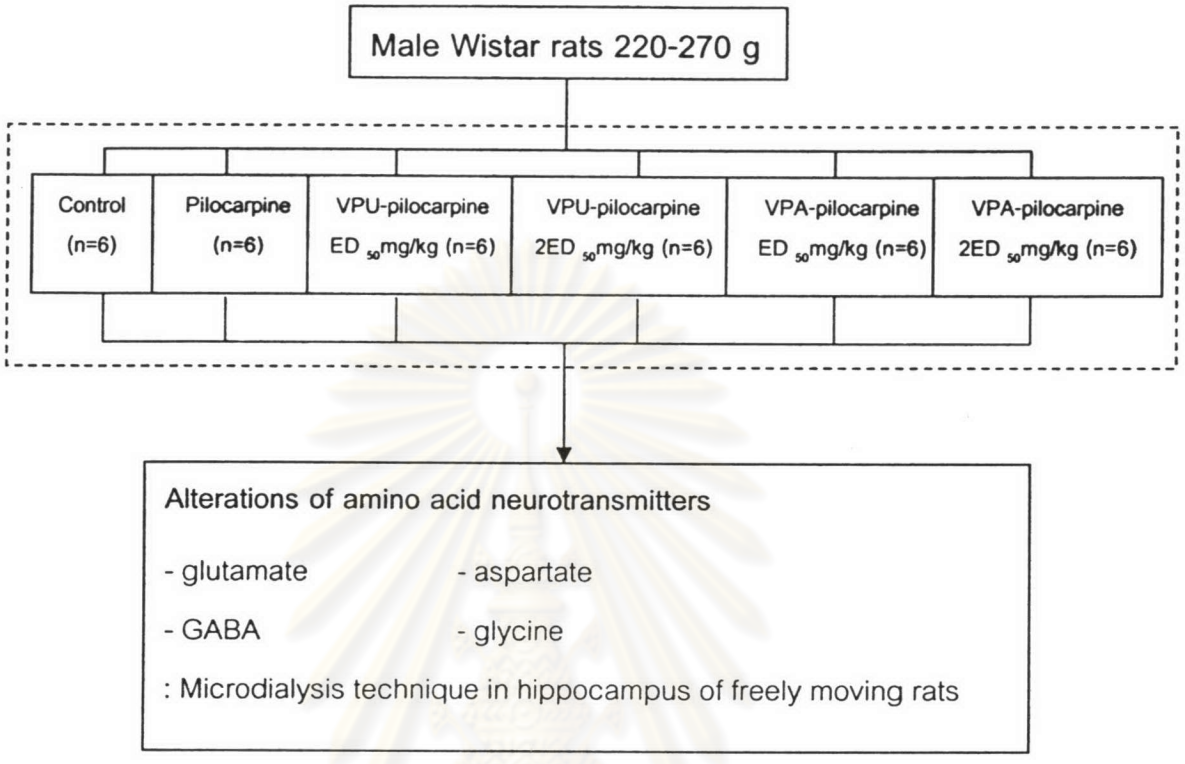
3.3 Study design

The experiment in this study was divided into three main parts as follows:

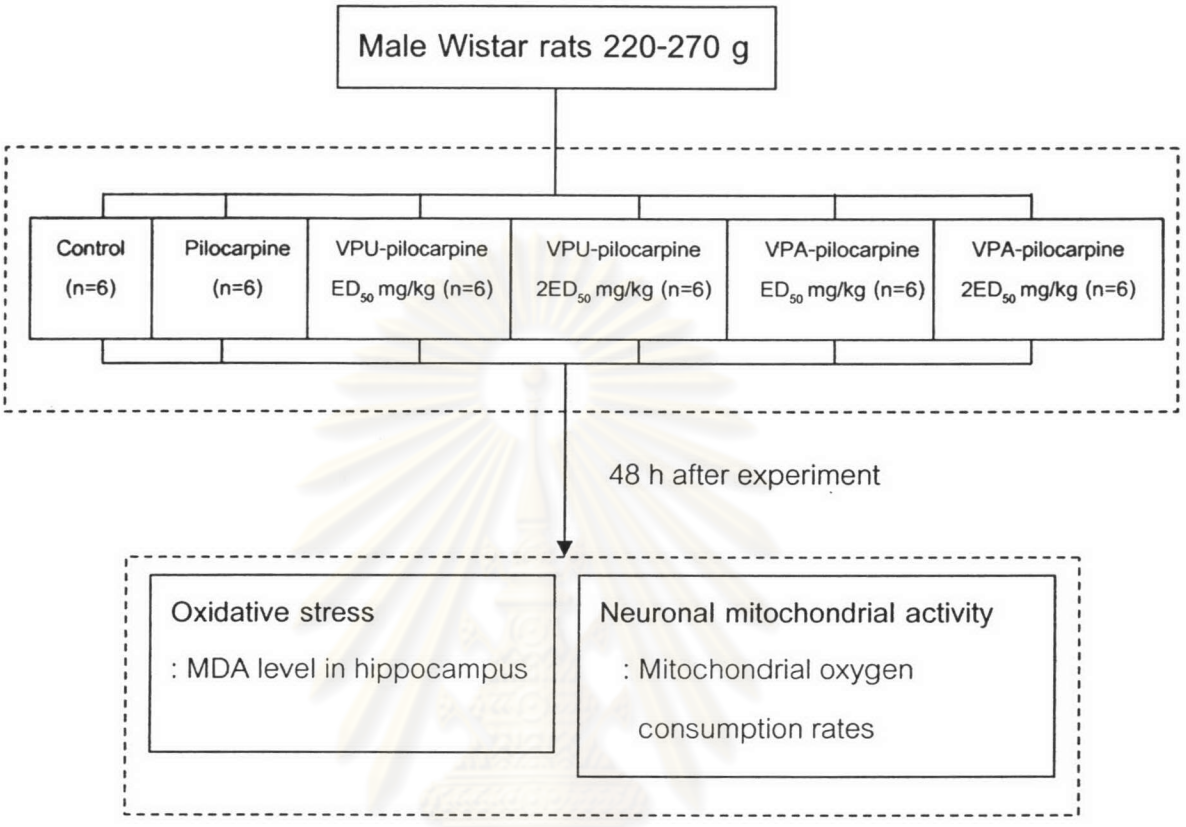
Part I Anticonvulsant activity and Neuroprotective effect of VPU and VPA on pilocarpine-induced seizure



Part II Effects of VPU and VPA on the level of hippocampal amino acid neurotransmitters in freely moving rats



Part III *Ex vivo* determination of Lipid peroxidation and Neuronal mitochondrial activity



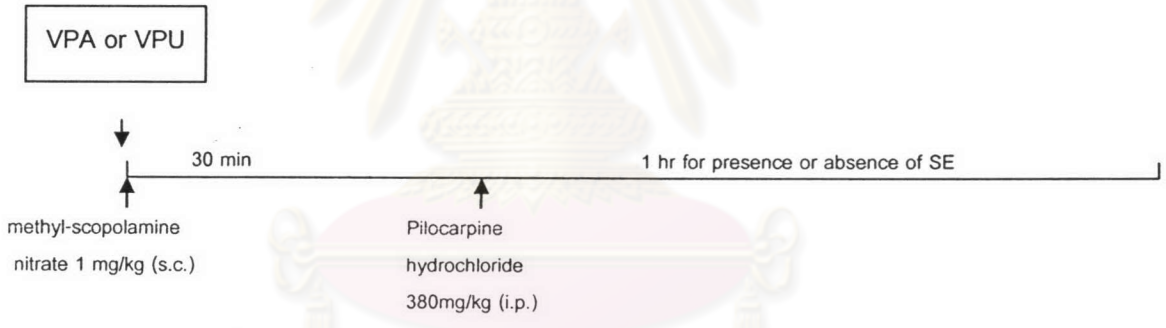
Pilocarpine-induced seizure model

Pilocarpine hydrochloride (380 mg/kg BW) was injected intraperitoneally (i.p.). Methyl-scopolamine nitrate (1 mg/kg BW) was injected subcutaneously (s.c.) 30 min before administration of pilocarpine in order to minimize the peripheral cholinergic effects, thus reducing the severity of symptoms not related to seizures such as secretion or a fine involuntary movement of muscles.

Part I Anticonvulsant activity and Neuroprotective effect of VPU and VPA on pilocarpine-induced seizure

Anticonvulsant activity

To determine the median effective dose (ED_{50}) of VPU and VPA in pilocarpine-induced seizure rats



VPU and VPA were dissolved in 0.5% CMC. Anticonvulsant activity of test substance was performed on 10 groups of six animals each. The first group (0.5 % CMC-NSS) was used as control group and the second group was injected with 0.5%CMC and pilocarpine. The other 8 groups were used for the determination of the ED_{50} of VPU (4 groups) and VPA (4 groups). The test substances, VPA (in varying doses) or VPU (in varying doses) were administered intraperitoneally 30 min prior to the injection of pilocarpine.

After pilocarpine injection the rats were place in a large cage and observed for the presence or absence of motor limbic seizure (≥ stage IV according to Racine 1972).

The behavioural changes were observed for 60 min for presence or absence of seizure.

Stages of pilocarpine-induced seizure are classified as follows;

0 : no response

1 : stereotype mouthing, eye blinking and/or mild facial clonus

2 : head nodding and / or severe facial clonus

3 : myoclonic jerks in the forelimbs

4 : clonic convulsions in the forelimbs with rearing

5 : generalized clonic convulsions associated with loss of balance

Fourty eight h after experiment, the rats were killed by injection with over dosing of pentobarbital sodim (60-80 mg/kg BW, i.p.) to determine morphological changes.

Neuronal Morphological Determinations

Detection of neuronal damage was performed with a Cresyl violet staining technique. Cresyl violet is a synthetic dye that is widely utilized to stain neuronal tissues. Because it is a basic stain, it readily binds to the acidic components of the neuronal cytoplasm such as RNA-rich ribosomes, as well as the nuclei and nucleoli of the nerve cells.

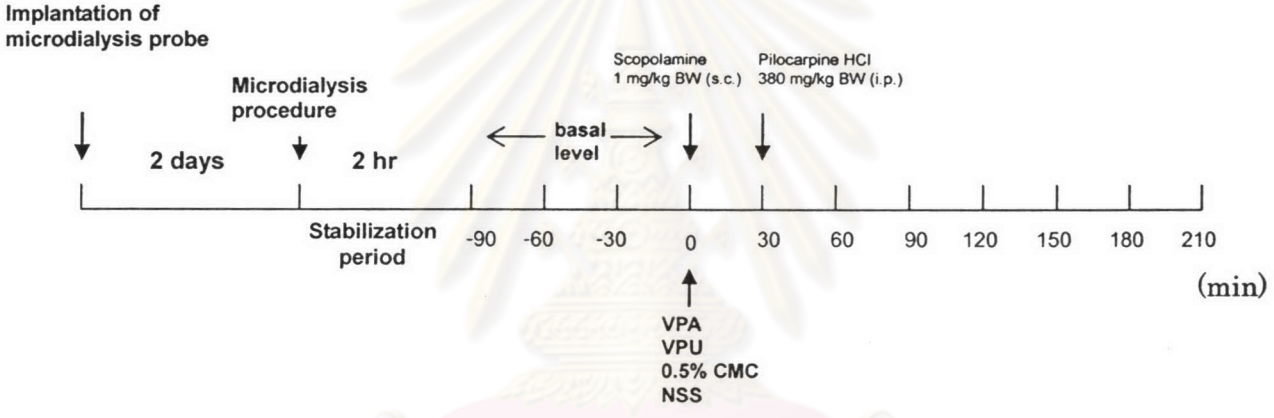
To investigate neuronal damage in hippocampal formation (CA1 and CA3) by using light microscopy, the rats were anesthetized with pentobarbital sodium and killed by decapitation at 48 h after administration of drugs. The brains were then removed and quickly frozen in dry ice. Subsequently, coronal sections (10μm thick) were cut at the level of the hippocampus (1.5-mm. caudal to the bregma) by using a cryostat and stained with 1% Cresyl violet for the microscopic observation. The CA1 and CA3 subfields of hippocampus were photographed (X20), and then the number of pyramidal cell per 0.05 mm^2 in CA1 and CA3 subfields was counted. Only the neuron with a distinct nucleus was counted as an undamaged cell. Average surviving cell numbers were counted over consistent fields, over both hemisphere, and over three sections in

each brain. The degree of neuronal cell damage at the hippocampal CA1 and CA3 area was expressed as the density of surviving CA1 and CA3 pyramidal cells according to the following equation: Density = the number of surviving CA1 or CA3 pyramidal cells / the area of CA1 or CA3 region ($0.05mm^2$) (Ni et al., 1995; Nanri et al, 1998).

Part II Effects of VPU and VPA on the level of hippocampal amino acid neurotransmitters in freely moving rats

Alterations of amino acid neurotransmitters

To determine the effect of VPU with reference to VPA on the alterations of amino acid contents in hippocampus of pilocarpine-induced seizure rats



The rats were anesthetized with pentobarbital sodium (50 mg/kg BW, i.p.) and placed in a stereotaxic frame. Microdialysis tubes (molecular weight cut-off 50,000, polyacrylonitrile capillary hollow fiber AHF-UN, Asahi medical Co., Tokyo, Japan) were inserted transversely into the hippocampus. The microdialysis tube was covered with epoxy resin glue along its entire length except for region corresponding to the brain areas to be studied. The stereotaxic coordinate used for the transverse implantation of the microdialysis tubing was followed Paxinos and Watson, 1986; AP -3.6 mm and H -3.3 mm for hippocampus. All coordinates were referred to bregma. The probe was fixed by polycarboxylate cement. After microdialysis probe implantation, the rats were allowed at 24-48 h for recovery before starting the experiments.

Microdialysis Procedure

Two days after surgery, rat was placed in the collecting sample instrument, which allowed freely moving. One side of probe was connected to a constant flow perfusion pump by polyethylene tube, and the other was placed into a collecting tube. The artificial cerebrospinal fluid (aCSF) pH 7.3 was perfused into the probe by perfusion pump at the constant flow rate of 2 μl/min. After 2 hours of stabilization period, 3 samples were collected for the basal level every 30 min. Then, substances (VPA or VPU) which were dissolved in 0.5% CMC solution were intraperitoneally injected and followed immediately by subcutaneous injection of methyl scopolamine nitrate. Pilocarpine was administered at 30 min after the methyl scopolamine nitrate application. After drug administrations seven dialysate samples were collected every 30 min from freely moving animals. The animals are divided into 6 groups with 6 rats in each group.

Dialysate samples were analysed for amino acid contents (glutamate, aspartate, glycine and GABA) by using high performance liquid chromatography (HPLC) with electrochemical detection (ECD). Samples were mixed with homoserine (internal standard) and pre-column derivatised with OPA before automatic injection by using HPLC autosampler 465 (ESA Inc., Bedford, MA, USA) into a HPLC-ECD (Eicom, Kyoto, Japan) with an analytic column (SC-5ODS 3.0 x 150 mm). After each experiment, the rats were anesthetized and then perfused intracardially with 10% formalin to examine the probe position.

Output data from detector was changed from analog to digital by PowerChrom (ADInstruments, Australia), recorded by Power Chrom v. 2.2.2 (ADInstruments, Australia) in Power Macintosh G3 computer (ADInstruments, Australia).

Part III *Ex vivo* determination of Lipid peroxidation and Neuronal mitochondrial activity

Measurement of Lipid Peroxidation

As an index of lipid peroxidation we used the formation of thiobarbituric acid reactions (TBARS), which is widely adopted as a sensitive method for measurement of lipid peroxidation, as previously described (Draper and Hadley., 1990). Malondialdehyde (MDA), oxidative stress parameter, was usually assayed by their reaction with 2-thiobarbituric acid, which formed chromogen adduct at acid pH, under heating (100°C). The chromogen was then measured spectrophotometrically at 532 nm by using 1,1,3,3-tetraethoxypropane for standard sample.

The rat was anesthetized with pentobarbital sodium (60mg/kg BW, i.p.) and killed by decapitation at 48 h after administration of drugs. The hippocampus was separated from rat brain. Tissue sample was thawed and homogenized with 10 times (w/v) ice-cold 0.1 M phosphate buffer (pH7.4). Aliquots of homogenates from rat brain was used to determine lipid peroxidation.

MDA was measured as described by Gupta et al. (2003). The reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulphate (8.1%) were added to 0.1 ml of processed tissue samples, then heated at 100°C for 60 min. The mixture was cooled with tap water and 5 ml of n-butanol/pyridine (15:1), 1 ml of distilled water was added. The mixture was vortexed vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was separated and absorbance was measured at 532 nm by using a spectrophotometer. The concentration of MDA was expressed as nmol/g tissue.

Neuronal Mitochondrial Activity

Isolation of mitochondria

Brain mitochondria was isolated from male rats by the method of Rosenthal et al. (1987). One rat was decapitated, and the whole brain excluding

cerebellum was rapidly removed, washed, minced, and homogenized in ice-cold mitochondrial isolation medium. Single brain homogenates was brought to 30 ml, divided equally into three tubes, and then centrifuged at 3,000 g for 3 min. The supernatants were pooled and centrifuged in two tubes at 10,000 g for 10 min. The pellets, including the fluffy synaptosomal layer, were resuspended in two tubes to 10 ml each in isolation medium containing 0.02% digitonin and centrifuged at 12,000 g for 10 min. The brown mitochondrial pellets without the synaptosomal layer were then resuspended again in 10 ml of medium and recentrifuged at 12,000 g for 10 min. The mitochondrial pellets were resuspended in 0.5 ml of medium/tube and combined.

Measurement of Mitochondrial Oxygen Consumption Rates

The mitochondria was incubated in an oxygraph apparatus in standard respiration medium containing substrate and phosphate and then ADP was added. It caused a sudden burst of oxygen uptake as the ADP was converted into ATP. The actively respiring state is sometimes referred to as stage 3 respiration, while the slower rate after all the ADP has been phosphorylated to form ATP is referred to as stage 4 respiration (Fig. 3.1).

Rates of mitochondrial oxygen consumption was measured with a thermostatically controlled (37°C) Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.) in water-jacketed chamber (Gilson, Middleton, WI, U.S.A.) according to the method of Sordahl et al. (1971). Oxygen consumption was expressed as nano-atoms of oxygen consumed per minute and per mg protein. From these values were calculates the respiratory control ratio (RCR) and the P/O ratio.

RCR is a ratio of oxygen consumption in stage 3 to stage 4 that indicates the functional integrity of the mitochondria. The method used for calculation the RCR value was also described by Chance and Williams (1956).

$$RCR = \frac{\text{Rate of state 3 respiration}}{\text{Rate of state 4 respiration}}$$

$$\text{Or} = \frac{\text{slope of the tracing in state 3 respiration}}{\text{Slope of the tracing in state 4 respiration}}$$

Thus from Figure 3.2 : $$RCR = \frac{Y_1/X}{Y_2/X} = \frac{Y_1}{Y_2}$$

The P/O ratio is the relationship between ATP synthesis and oxygen consumption. This value is an index of oxidative phosphorylation which indicates the efficiency of mitochondrial ATP synthesis. The P/O ratio can be calculated according to Estabrook (1967) as follows (Fig. 3.3).

$$\text{P/O ratio} = \frac{\text{nmoles of ATP synthesized}}{\text{nano-atoms of oxygen consumed in state 3 respiration}}$$

The nmoles of ATP synthesized is equal to nmoles of ADP added to the reaction mixture and can be calculated from the concentration and volume of ADP added. The nano-atoms of oxygen consumed in state 3 respiration, calculated from oxygraph tracing (Fig. 3.4), are [Q/P]X Z where P and Q are the length of line P and Q respectively. Z is nano-atoms oxygen initially dissolved in the reaction mixture. Z value depends on volume of reaction mixture and temperature. It is calculated by multiplying the amount of oxygen dissolved in 1 ml water (A) with total volume of the reaction mixture. The value A can be calculated from the following formula.

$$A = [S/V][P/100]xNx10^9 \text{ nano-atoms oxygen/ml}$$

where A = nano-atoms oxygen dissolved in 1 ml of water

S = absorption coefficient at 37 °C (volume of oxygen reduced to O °C and 760 mm, absorbed by one volume of water when the pressure of the gas itself amount to 760 mm) = 0.02373

P = percentage of oxygen in atmospheric air = 21%

N = number of atoms in a molecule of oxygen = 2

V = volume of gas (at 0 ° C and 760 mm) corresponding to 1 gm-mole = 22,400 ml

Substituting these values in the above equation, the amount of oxygen dissolved in 1 ml of water at 37 ° C = 444.9 nano-atoms oxygen/ml.

Calculation of rates of oxygen consumption.

Rates of oxygen consumption in various metabolic states can be calculated form oxygraph tracing as shown in figure 3.4.

Method 1; rate of oxygen consumption in state 3= [R/P]xZ nano-atoms oxygen/min

Method 2; rate of oxygen consumption in state 3= [R/P]xA nano-atoms oxygen/min/ml where, R and P = the length of line R and P respectively

Z = nano-atoms oxygen initially dissolved in the reaction mixture

A = nano-atoms oxygen dissolved in 1ml of water

Oxygen comsumtion rates, calculated from method 1, can be divided by divided by mitochondrial protein used in the reaction, and the unit becomes ng-atoms oxygen/min/mg protein.

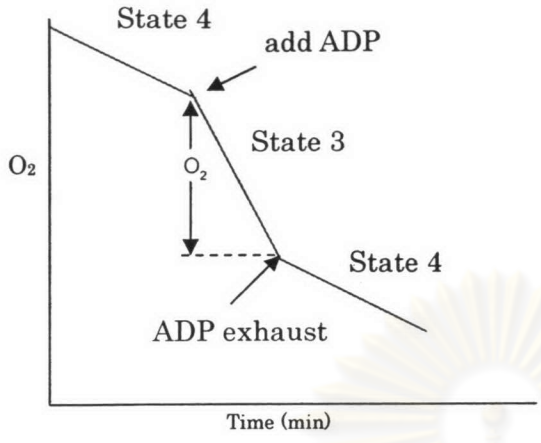


Figure 3.1 Representative trace of mitochondrial respiration. Oxygen consumption was first measure in isolated mitochondria with glutamate plus malate or succinate as substrate, prior to any experiment. Additon of ADP caused respiratory stimulation which was followed by a cut-off (transition from state 3 to state 4 respiration) when the added ADP had been phosphorylated to ATP. State 3 respiration was measured after addition of ADP, and state 4 respiration was measured after cut-off.

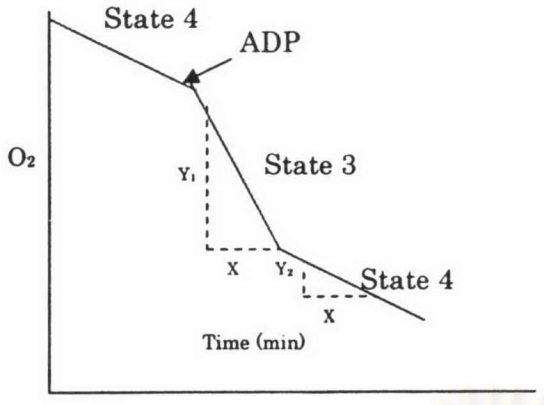


Figure 3.2 An Oxygraph tracing illustrating the measurement of RCR value

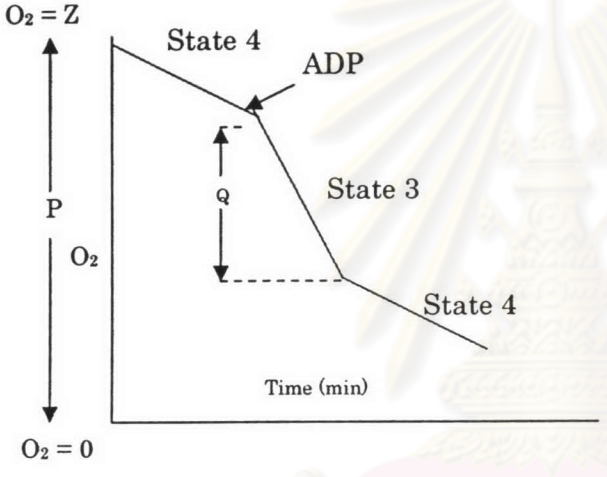


Figure 3.3 An Oxygraph tracing illustrating the measurement of P/O ratio

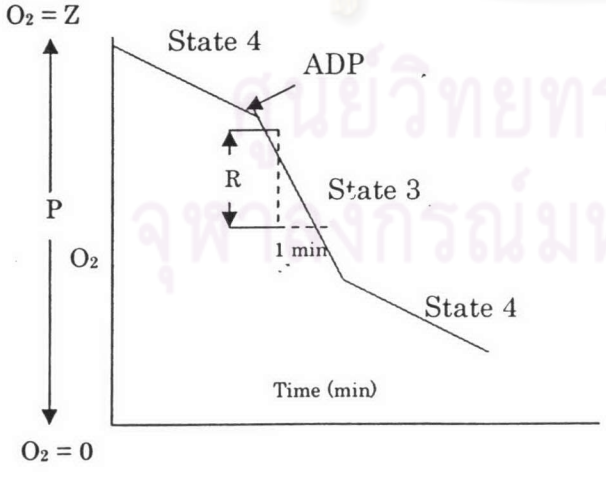


Figure 3.4 An Oxygraph tracing illustrating the measurement of oxygen consumption rates

Determination of Total Protein

Protein was determined by the method of Lowry et al. (1951) by using bovine serum albumin as standard. The assay procedures are as follows:

1. 10 μl of mitochondrial suspension or processed tissue sample from brain was diluted with 3 ml distilled water: solution A

2. In each assay tubes, 1 ml of alkaline copper reagent was added to 1 ml aliquot of solution A or to 1 ml of standard bovine serum albumin solution, mixed thoroughly and left for 10 min at room temperature.

3. 3 ml of 1:10 diluted Folin-phenol reagent was added, immediately mixed and incubated in 50 °C water bath for 10 min.

4. Then the mixtures were cooled to room temperature, the absorbances of the developed blue color were read at 540 mm wavelength in spectrophotometer. The amount of mitochondrial protein in each sample (mg/ml) was calculated from a standard curve of bovine serum albumin.

Annotation: - 1:10 diluted Folin-phenol reagent was freshly prepared by diluting 1 part of concentrated Folin-Ciocalteu's reagent with 10 parts of distilled water.

3.4 Data analysis

The ED_{50} was calculated by a simplified method of evaluating dose-effect experiments of Litchfield and Wilcoxon (1949).

The experimental data was presented as mean ± SEM. The differences between various groups were performed by analysis of variance (ANOVA) followed by Bonferroni's test. A difference with $P < 0.05$ was considered statistically significant.