CHAPTER II

MATERIALS AND METHODS

Animals

All experiments used male albino Wistar rats weighing 180-200 grams each and fed ad libitum. The animals were purchased from Nation Experimental Animals Center, Mahidol University, Salaya, Nakornprathom Province.

Preparation of Intact Rat Liver Mitochondria.

Rat liver mitochondria were prepared by the method of Hogeboom [33] as described by Myers and Slater [34]. The isolation media, homogenizer, centrifuge tubes and mitochondrial suspension were kept in an ice-bath thoughout the preparation period. The temperature of the refrigerated centrifuge was set at 4 ^{0}C.

1. Preparation of liver homogenate.

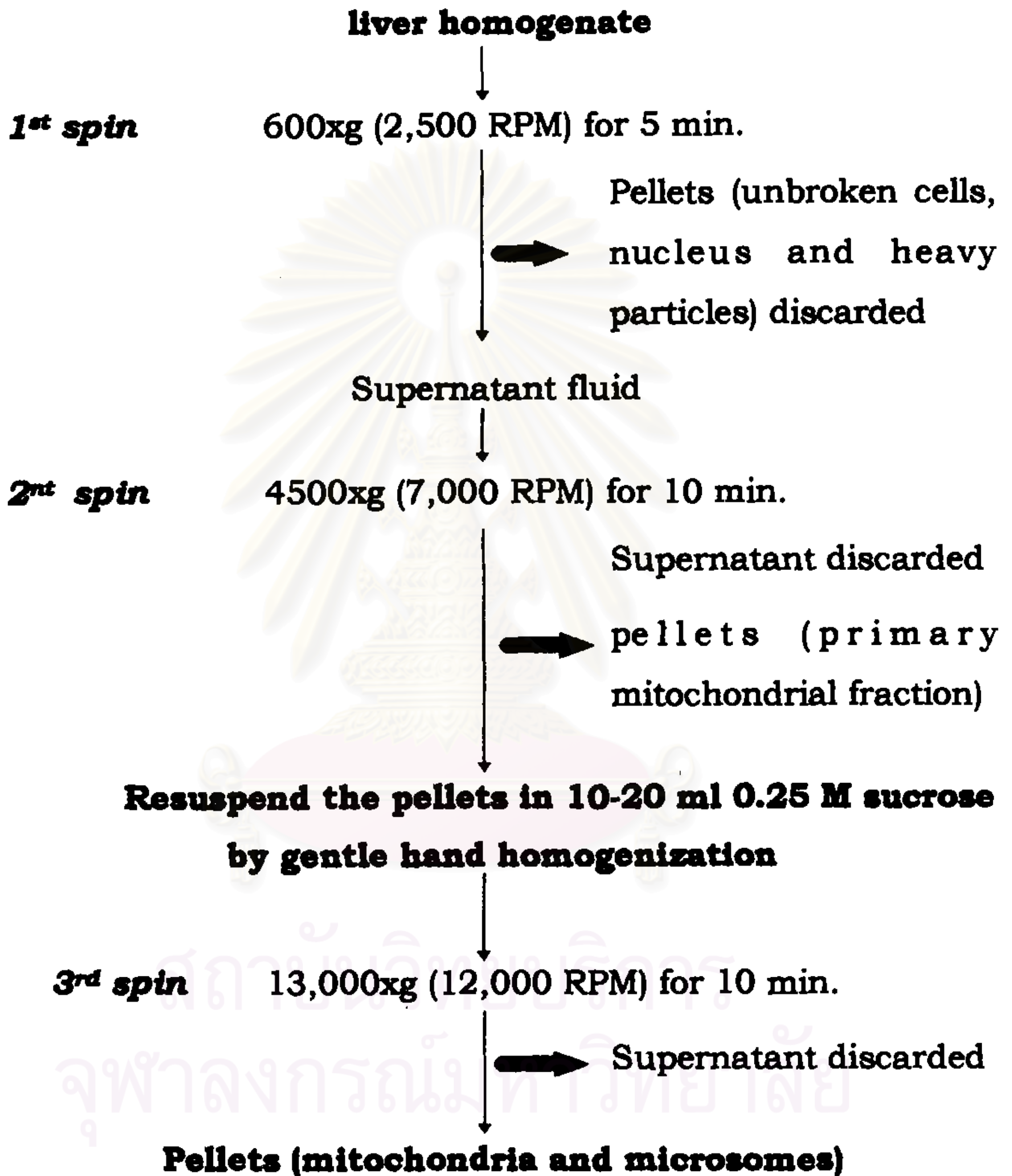
The rat was sacrificed by cervical dislocation. The abdomen was opened and the liver was quickly removed and placed in a ice-cold small beaker. The liver was cut into small pieces and washed 2-3 times with homogenizing medium (0.25 M sucrose + 1 mMEGTA, pH 7.2) to remove blood as much as possible. The chopped liver was suspended in 30 ml homogenizing medium and

homogenized in glass homogenizer equipped with a motor-driven Teflon pestle to break the cell. The liver homogenate obtained was approximately 70 ml per rat.

2. Preparation of the liver mitochondria.

The liver homogenate was transferred to centrifuge tubes and centrifuged by Hitachi high speed refrigerated centrifuge (model CR. 20B3, rotor no. 9) to separate the mitochondria according to the following flow-chart.

The pellets obtained from the third spin consisted of two distinct layers, the lower brown layer of tightly-packed mitochondria and the upper pink one of loosely packed microsomes. The microsomes were washed out with 1-2 ml 0.25 M sucrose for 2-3 times. The mitochondrial precipitates were finally resuspended in 2-3 ml of 0.25 M sucrose by gentle manual homogenization. The final yield was approximately 30-60 mg mitochondrial protein per ml and the RCI value fell between 5 and 8 at 37^{0} C with glutamate plus malate as substrates. The mitochondrial suspension was kept in an ice-bath throughout the experiments (fig 13).



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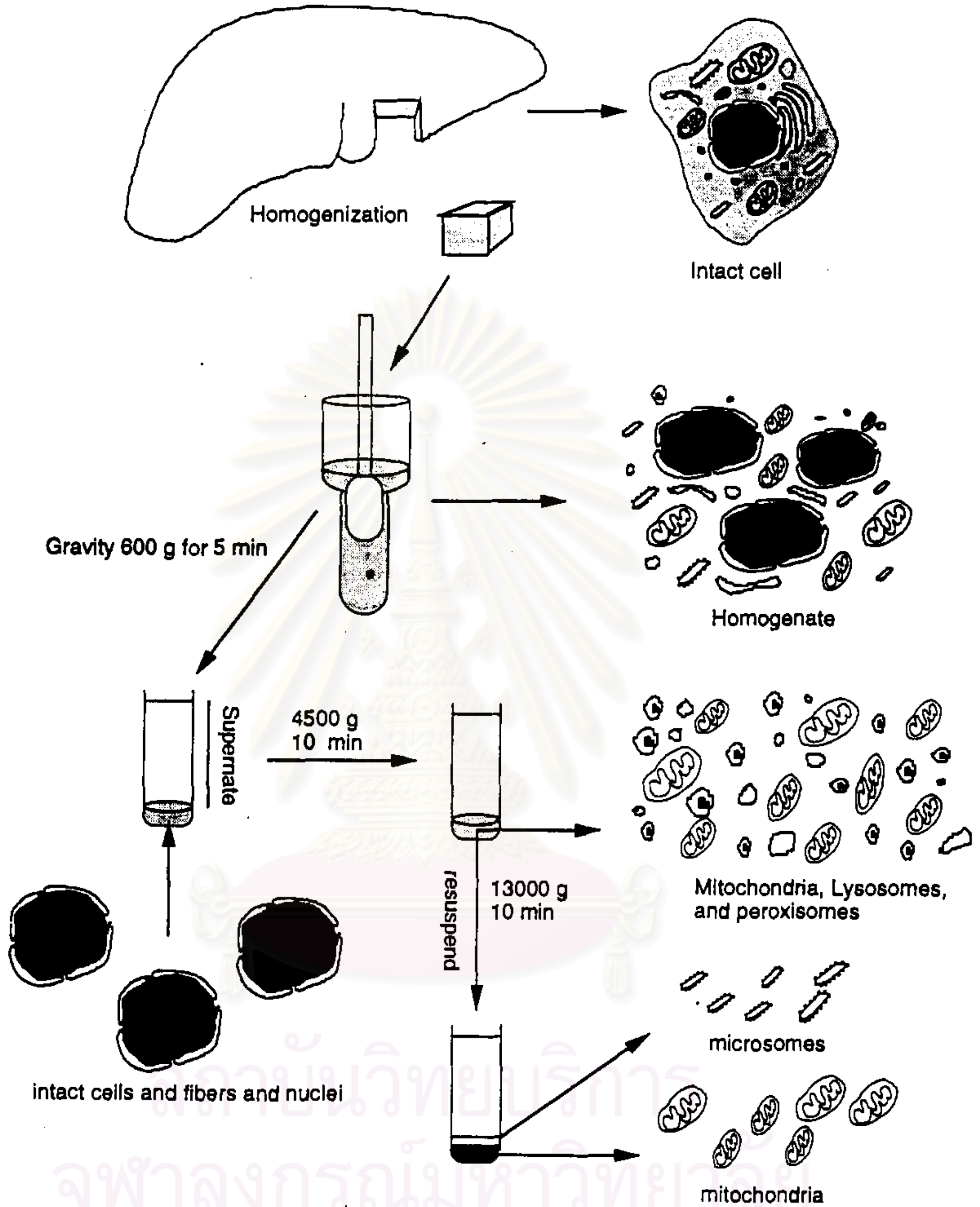

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Fig 13. Illustration of mitochondrial preparation.

Measurement of Mitochondrial Oxygen Consumption Rates.

Rates of mitochondrial oxygen consumption were measured by polarographic oxygen electrode technique [35,36] in a Gilson reaction chamber connected with YSI oxygen monitor Model 53 and recorder (fig 14).

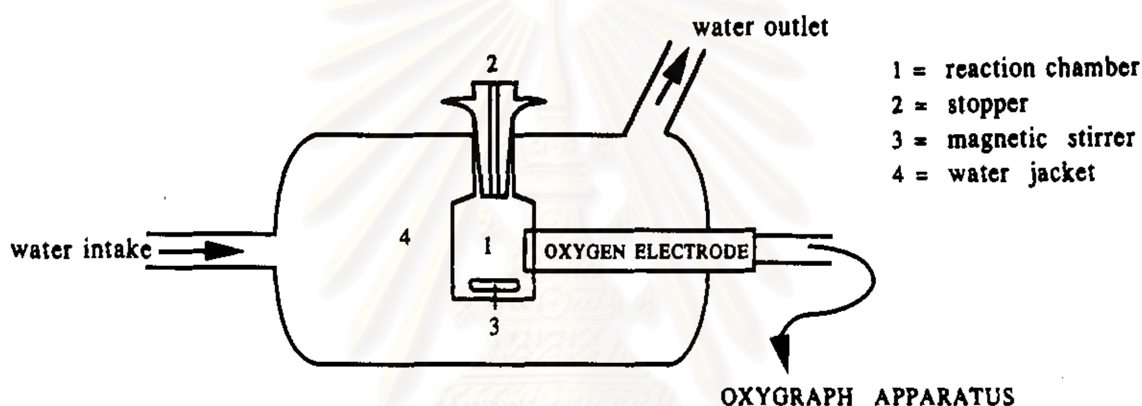

Fig. 14 Gilson reaction chamber

The chamber is about 2 ml in volume and consisted of the water jacket encompassed the chamber and a hollow glass stopper through which the substrates and reagents were added into the chamber. The temperature of the chamber was kept at 37^0 C by circulating water bath. The reaction chamber was equipped with a Clark oxygen electrode (Fig 15.) which connected to an amplifier (YSI model 53). The signal from the amplifier was recorded on a stripchart recorder (Gilson model N2). The tracings thus obtained were called "oxygraph" or "polarographic" tracings. The standard incubation medium ued for measuring

mitochondrial respiratory rate composed of 40 mM HEPES buffer pH 7.2, 2 mM $MgCl_2$ and 92 mM KCl which is isotonic with the mitochondria (i.e., 250 mOsm). About 1.8 ml incubation medium and 100 μl mitochondrial suspension were incubated in the chamber with the substrates and other reagents. The reaction mixtures were agitated by a small rotating magnet. The electrode was calibrated with distilled water saturated with air at 37^{0} C .

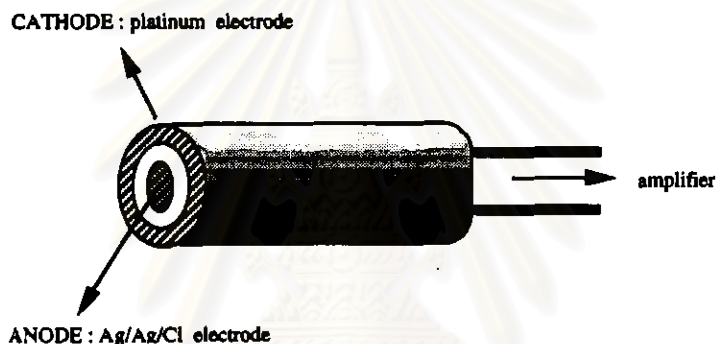

Figure 15. Clark oxygen electrode

1. Determination of the respiratory control index (RCI).

Chance and Williams [37] have classified the mitochondrial metabolic states as follows.

State	Condition
1	Oxygen only
2	Oxygen and ADP
3	Oxygen, ADP and substrates
3u	Uncouple
4	Oxygen and substrates
5	Substrates only
6	The respiration is inhibited by excess calcium

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The method used for calculation the RCI value was also described by Chance and Williams.

$$RCI = \frac{\text{Rate of state 3 respiration}}{\text{Rate of state 4 respiration (after state 3)}}$$

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

Thus from figure 16:

$$RCI = \frac{Y1/X}{Y2/X} = \frac{Y1}{Y2}$$

Y1 and Y2 are the length of line Y1 and Y2 respectively.

The RCI value indicates the tightness of the coupling mechanism, that is, whether the substrate oxidation is tightly coupled to ATP synthesis. The RCI value of good intact mitochondria should be at least 5 at 37° C with glutamate plus malate as substrates.

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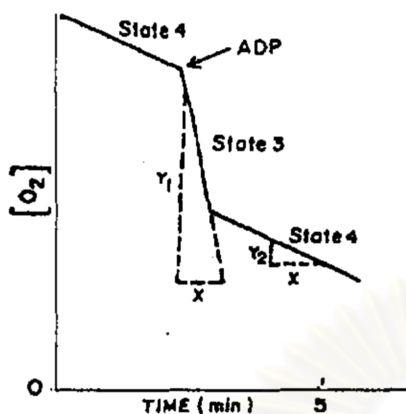

Fig 16. An oxygraph tracing illustrating the measurement of RCI value

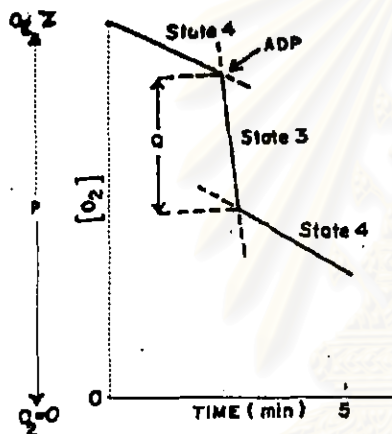

Fig 17. An oxygraph tracing illustrating the measurement of P/O ratio.

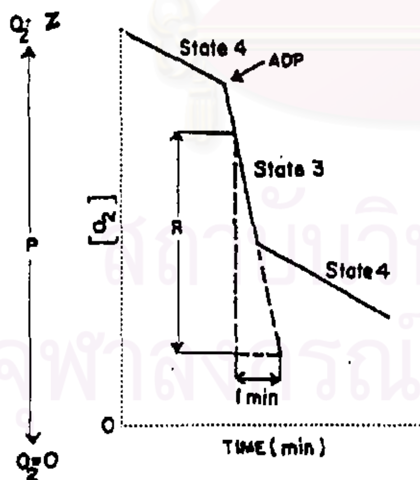

Fig 18. An oxygraph tracing illustration the measurement of oxygen consumption rates.

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2. Determination of P/O ratio.

The **P/O** ratio is the number of ATP molecules synthesized per one oxygen atom consumed during state 3 respiration. It indicates the efficiency of mitochondrial ATP synthesis. The **P/O** ratio can be calculated according to Estabrook [38] as follows.

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

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 ng-atoms of oxygen consumed in state 3 respiration, calculated from oxygraph tracing (fig 17), are **[Q/P]xZ** where P and Q are the length of line P and Q respectively. Z = ng-atoms oxygen initially dissolved in the reaction mixture.

Z value depends on volume of reaction mixture and temperature; the larger the volume, the lower the temperature, the more oxygen dissolved in the reaction mixture. Z value 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

$$A = [S/V][P/100] \times N \times 10^9 \text{ ng-atoms } 0/\text{ml}$$

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

S = absorption coefficient at 37° C (volume of oxygen reduced to $0^{\circ}C$ 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 O^{o} 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 ng-atoms O/ml.

3. Calculation of rates of oxygen consumption.

Rates of oxygen consumption in various metabolic states can be calculated from oxygraph tracing as shown in fig 18.

Method 1; rate of oxygen consumption in state 3 = [R/P]xZ ng-atoms 0/min.

Method 2; rate of oxygen consumption in state 3 = [R/P]xA ng-atoms 0/min./ml where, R and P = the length of line R and P respectively

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

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

Oxygen consumption rates, calculated from method 1, can be divided by mitochondrial protein used in the reaction, and the unit becomes ng-atoms 0/min./mg protein.

The Study of Mitochondrial Monoamine Oxidase Activity.

Monoamine oxidase (MAO). the marker enzyme of the mitochondrial outer membranes, oxidizes monoamine compound by oxidative deamination process as depicted by the following reaction

$$R\text{-}CH_2NH_2 + O_2 \longrightarrow R\text{-}CHO + NH_3 + H_2O_2$$

Since the reaction employs molecular oxygen, therefore the MAO activity can be studied by measuring the rate of mitochondrial oxygen consumption in medium supplemented with monoamine substrate.

The incubation medium in this experiment was 0.025 M phosphate buffer pH 7.2 and benzylamine was used as a substrate for MAO. The respiratory chain inhibitor, rotenone, was also added to the reaction mixture to inhibit the oxidation of endogenous substrates by mitochondrial respiratory chain. Thus the mitochondrial oxygen consumption under this condition was due to MAO activity.

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Measurement of Mitochondrial ATPase Activity.

The mitochondrial ATPase is synonymous to ATP synthase and located on the inner membrane. This enzyme complex catalyzes the following reactions.

ATP synthase

$$ADP + Pi + H^+ \rightleftharpoons ATP + H_2O$$

The ATP activity can be determined by measuring the amount of inorganic phosphate [39] or proton [40] liberated from ATP hydrolysis. In this investigation the former method was employed. The inorganic phosphate (Pi) liberated was quantitated by the calorimetric method of Fiske and Subbarow [41]. The mitochondrial ATPase activity was measured as described below.

1. 200 µl of mitochondrial suspension was incubated with 2.63 ml standard incubation medium in appropriate chambers surrounded by circulating water bath with temperature setting at 37° C . The reaction was stirred magnetically.

2. DMSO or ketoconazole was added and incubated for 1 min.

3. 150 µl of 0.1 M ATP was added to start the reaction and the reaction mixtures were further incubated for 10 min.

4. The reaction was terminated by adding 1 ml aliquot of reaction mixtures to 1 ml of 20% ice-cold trichloroacetic acid.

5. The resulting mixture was then centrifuged at 4000 RPM for 10 min.

6. 1 ml of the supernatant or 1 ml of phosphate standards (concentrations 0.1, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM) was added into the test tube containing 5 ml of 0.2 M H_2SO_4 and mixed.

7. 0.8 ml of 2.5% W/V of ammonium molybdate and 0.4 ml of Fiske-subbarow reducing agent (composed of 97.5 ml of 15% sodium bisulfite, 0.25 gm of 1-amino-2-naphthol-4-sulfonic acid and 2.5 ml of 20% sodium sulfite) were added. The reaction mixtures were mixed and then left standing at room temperature for 10 min.

8. The absorbances of the developed blue color were read at 650 nm wavelength in Ultrospec II Spectrophotometer. The amount of Pi in each sample was determined from a standard phosphate curve.

Annotation: In the preparation of Fiske Subbarow reducing agent, some of the undissolved 1-amino-2napthol-4-sulfonic acid was discarded by filtration and the filtrate was kept, no longer than one month, in the bottle protected form light.

Determination of Mitochondrial Protein.

The mitochondrial protein was determined by the method of Lowry et al [42] as modified by Miller [43] using bovine serum albumin as standard. The assay procedures are as follows:

1. 10 µl of mitochondrial suspension 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

(concentrations 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mg/ml), 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 50^0C water bath for 10 min. .

4. After the mixtures were cooled to room temperature, the absobances of the developed blue color were read at 540 nm wavelength in Ulthrospec II Spectrophotometer. The amount of mitochondrial protein in each sample (mg/ml) was calculated from a standard curve of bovine serum albumin.

Annotation:- Alkaline copper reagent composed of 1 part of 0.5% $CuSO_4$ in 1% W/V of potassium tartrate and 10 parts of 10% Na_2CO_3 in 0.5 M NaOH.

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

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Reagent, and drugs

Most reagents were dissolved in triple-distilled water and some were dissolve in absolute ethanol or dimethylsulfoxide (DMSO). If the pH of some solutions have to be adjusted , KOH and HCl were used.

Reagent dissolved in triple-distilled water: 10 µl of 1 M glutamate + 1 M malate (pH 7.2), 10 µl of 1 M succinate (pH7.2), 1M HEPES buffer (pH 6.8, 7.2, and 7.6), 2.3 M KCl, 1mM EGTA (pH 7.2), 2 µl of 0.3 M ADP + 0.6 M Pi, 2-6 µl of 0.05 M DNP, 90-150 µl of 0.1 M ATP (pH 7.2), 0.25 M sucrose, 2µl of 1 M DTT, 20-80 µl of 250 mg/ml BSA, 2µl of 0.1 M benzylamine, 0.025M KH_2PO_4 (pH7.2), 10 µl of 0.05 M KH_2PO_4 , 1µl of 0.1 M pargyline, 10 µl of 0.4 M $CaCl_2$.

Reagent dissolved in absolute ethanol : 2 µl of 5 mg/ml oligomycin.

Reagent dissolved in DMSO was ketoconazole.

L-Glutamic acid, malic acid, succinic acid, ADP, ATP, DNP, potassium phosphate (KH_2PO_4), sucrose, EGTA, HEPES, KCl, $CaCl_2$, benzylamine, pargyline, DTT, BSA, oligomycin, rotenone, DMSO, Folin-Ciocalteu Phenol reagent, sodium sulfite, sodium bisulfite, ammonium molybdate, cupric sulfate, sodium hydroxide, potassium tartrate, sodium carbonate were obtain from Sigma Chemical Company.

Absolute ethanol, sulfuric acid, hydrochloric acid, potassium hydroxide were obtained from E. Merck, Darmstadt Company.

Statistics

The differences in rate of oxygen consumption between control and tested groups were evaluated for statistical significance by two-tailed paired Student's t-test. A p value of 0.05 or less was considered statistically significant.

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