CHAPTER II

MATERIALS AND METHODS

1. Experimental Animals.

Male albino rats of Wistar strain weighing about 200-250 g, supplied by the National Experimental Animal Center, were used throughout the study. All animals were allowed free access to food (Laboratory chow) and tap water.

2. Drugs and Chemicals.

The following agents were employed in the highest purity available without further purification and most reagents were dissolved in tri-distilled water: sucrose, HEPES, manitol, EDTA, EGTA, potassium hydroxide, sodium carbonate, copper sulfate, potassium tartrate, Folin-Ciocalteu reagent, bovine serum albumin, benzylamine, beta-phenylethylamine, norepinephrine, dopamine, tryptamine, pargyline, dibasic sodium phosphate, sodium bihydrogen phosphate. Piperine was purchased from Sigma Co. and dissolved in absolute ethanol.

3. Preparation of Rat Liver Mitochondria.

Rat liver mitochondria were prepared by the method of Hogeboom (1955) as described by Mayers and Slater (1957). Ice-cold 0.25 M sucrose was used as an isolation medium throughout the preparation of mitochondria and ice-cold 0.25 M sucrose, mixed with 1 mM EGTA (pH 7.4) and 5 mM HEPES buffer (pH 7.4), was

used as a homogenizing medium. All centrifugations were done in Hitachi automatic refrigerated centrifuge with temperature setting at 2°C. Homogenizers and centrifuge tubes were kept cold in an ice bath.

Livers were removed from sacrificed rats and rinsed two or three times with 20-30 ml of homogenizing medium. The livers were cut into small pieces and homogenized in a 30-ml glass homogenizer by mean of a motor-driven Teflon pestle, and the homogenate was transferred to cellulose nitrate tubes. The first spin was carried out at 500 x g for 6 minutes to remove unbroken cells, cell debris and heavy materials. The supernatant was saved and centrifuged again at 4,500 x g for 10 minutes. The supernatant was discarded and the remaining pellet was resuspended in about 10 ml of 0.25 M sucrose, gently homogenized by hand and finally centrifuged at 12,000 x g for 10 minutes. The mitochondria then precipitated as a tightly packed brown pellet underneath a loosely packed, pink layer of microsomes which was floated off with 0.25 M sucrose solution. The mitochondria was finally suspended in the ice-cold 0.25 M sucrose by hand homogenization. The final volume was approximately 2-3 ml and the preparation was kept in an ice bath throughout the experiment. In some experiments, however, aliquots of the mitochondrial preparation was stored in a freezer (-20 °C) for further use. It was found that the enzymatic activity of monoamine oxidases was not significantly changed at least for 3 days after the storage.

4. Preparation of Rat Brain Mitochondria.

Rat brain mitochondria are isolated by a modification of the method of Student and Edwards (1977). The isolation medium (ME medium) consists of 0.3 M manitol and 0.1 mM ethylenediamine tetraacetic acid (EDTA) adjusted to pH 7.4 with KOH. The rat was decapitated, the brain was rapidly removed and placed in chilled ME medium. Cerebellum and brainstem were removed while keeping in the chilled medium. Two brains were homogenized in 10 volumes of ME medium with Potter-Elvejhem homogenizer and a motor-driven Teflon pestle. The homogenate was centrifuged at 600 x g for 8 minutes and the supernatant was aspirated and centrifuged at 10,000 x g for 10 minutes. The resulting pellet was resuspended in 40 ml of ME medium and centrifuged at 5,000 x g for 10 minutes. The supernatant is decanted and saved. A loosely packing, white sediment surrounding a brown pellet (mitochondria) should result and was removed by gentle swirling with ME medium and combined with decanted supernate from previous steps. The remaining brown mitochondrial pellet was suspended in ME medium (F1 fraction). The combined supernate was centrifuged at 5,000 x g for 10 minutes whereby a second mitochondrial pellet (brown) was obtained and combined with the F1 fraction. The combined fraction was finally suspended in a small volume (approximately 0.8 ml per two rat brains) of ME medium, yielding a suspension of 10 to 20 mg mitochondrial protein per ml.

5. Determination of Mitochondrial Protein.

Mitochondrial protein was determined by the method of Lowry et al (1951) as modified by Miller (1959). The blue color was developed when the complex of Folin phenol reagent, protein and alkaline copper was formed. In each assay tube, 1 ml alkaline copper reagent (which composed of 10 parts of sodium carbonate and 1 part of 0.5% copper sulfate in 1% potassium tartrate) was added to 1 ml aliquots of protein sample solution (tha same volume of distilled water was used in blank assay), mixed thoroughly and left standing for 10 minutes at room temperature, then 3 ml of diluted Folin phenol reagent (concentrated Folin & Ciocalteu's phenol reagent was diluted with distilled water in a ratio of 1:10 v/v) was added. The reaction mixture was mixed immediately and heated at 50 ^{o} C in a water bath for 10 minutes. After the mixtures were cooled to room temperature, optical densities of the blue color were read at 540 mu in an Ultrospec II spectrophotometer. The concentration of mitochondrial protein in each sample was calculated from a bovine serum albumin standard curve.