CHAPTER III

MATERIALS AND METHODS

Chemicals

Aminopyrene (Aldrich, St. Louis, U.S.A.) and sodium nitrite (BDH Chemicals Ltd. Poole, England) were used as precursors of standard mutagen in this assay. Sodium thiocynate (NaSCN), ammonium sulfamate ($NH_2SO_3NH_4$), bovine serum albumin (BSA), d-biotin, and sodium dihydrogen phosphate (NaH_2PO_4) were perchased from Sigma Chemical Co, (St. Louis, Missouri, U.S.A.). Hydrogen bromide (HBr), and N-(1-napthyl) ethylenediamine dihydrochloride ($C_{10}H_7$ NH CH_2 CH_2 NH_2 . 2HCl) were obtained from BDH Chemicals Ltd. (Poole, England). Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$), citric acid monohydrate GR ($C_6H_8O_7.H_2O$), L-histidine monohydrochloride, hydrochloric acid, gracial acetic acid, potassium chloride (KCI), sodium chloride (NaCl), crystal violet indicator, sulfanilic acid ($C_6H_7NO_3S$), and acetone (CH_3COCH_3) were supplied by E. Merck (Darmstadt, Germany). Sodium ammonium hydrogen phosphate tetrahydrate GR ($NaNH_4HPO_4.4H_2O$), di-potassium hydrogen phosphate anhydrous (K_2HPO_4), and D(+)-glucose monohydrate were furnished by Fluka, (Biochemica, Switzerland). Ampicillin sodium salt, was purchased from Vesco Pharmaceutical Ltd. (Bangkok, Thailand). Bacto agar was a product of Difco Laboratories, (Michigan, U.S.A.). Oxoid nutrient broth No. 2 was supplied by Oxoid Ltd, (Basingstoke, Hants., England). Other chemicals were of laboratory grade.

The bacterial tester strains

Salmonella typhimurium strains TA 98 and TA 100 were provided by Dr. Wannee Kusamran of National Cancer Institute (Ministry of Public Health). Overnight cultures of strain TA 98 and TA 100 were prepared in Oxoid nutrient broth No. 2 at 37°C with aeration. Manipulation of the cultures (Appendix 3) was done as suggested by Maron and Ames (1983).

Nutrient agar

Minimal agar plate. Each plate contained 30 ml of minimal glucose agar medium consisting of 1.5% Bacto-Difco agar and 2% glucose in Vogel-Bonner medium E (recipe in Appendix 1.2). A sterile petri dish was used.

Top agar. It contained 0.6% Difco agar and 0.5% sodium chloride (Appendix 1.7). It was autoclaved and kept warm in water bath (45°C). Before use, 10 ml of a sterile solution of 0.5 mM L-histidine and 0.5 mM biotin was added to each 100 ml of the molten agar and mixed throughly by swirling.

Sample selection and preparation

All of drug samples listed in Table 4 were purchased from drug store in Bangkok. Selection was based on the fact that they are usually taken with or after meal and used for a long period of time. Chemical structures of these drugs are shown in Appendix 4.

Each pulverized pharmaceutical preparation was dissolved in a suitable volume of DMSO and then it was centrifuged at 3000 rpm. for 20 min (IEC model) and the supernatant was kept in sterilized vial at 4°C before use.

Preparation of plant fiber

Plant fiber was isolated from stem and leaf of ivygourd (*Coccinia indica*). The vegetable was perchased from local markets in Bangkok. It was disrupted and blended with water and the homogenate was frozen overnight. Then it was washed repeatedly with water to remove water-soluble materials including protein and water soluble vitamins as suggested by Eastwood and Mitchell (1976). The fiber was extracted with a mixture of hexane and acetone (3:2) to remove lipid soluble compounds (Deutsh , 1984). Finally the resultant fiber was ground and passed through a 20 mesh screen. Steps in preparation of plant fiber was shown in Figure 3.

Nitrosation

All drugs were nitrosated using the method described by Takeda and Kanaya (1981). Breifly explain, the reaction mixture was composed of 0.1 ml of sample solution and 0.25 ml of 2 M sodium nitrite. The pH of the reaction mixture was adjusted to 3.0-3.5 with diluted hydrochloric acid, the tube was stoppered and shaken in a 37°C water bath for 4 h. Then the tube was placed in an ice bath with the addition of 0.25 ml of 2 M ammonium sulfamate to the reaction mixture in order to decompose the residual nitrite for 10 min.

Table 3 List of Drug samples.

Generic name	Pharmaceutical preparation used	
Tranquilizer		
Bromazepam	Lexotan tablets	(1.5 mg)
Chlordiazepoxide	Benpine capsules	(10 mg)
Dipotassium Clorazepate	Tranxene capsules	(5 mg)
Diazepam	Valium tablets	(2 mg)
Clobazam	Frisium tablets	(5 mg)
Prazepam	Prazepine capsules	(5 mg)
Antidepressant		
Amitriptyline	Tryptanol tablets	(10 mg)
Nortriptyline	Nortilen tablets	(10 mg)
Thioridazine	Melleril tablets	(10 mg)
Anticonvulsant		
Chlorpromazine	Matcine tablets	(100 mg)
Phenytoin	Dilantin capsules	(100 mg)
Trihexyphenidyl HCl	ACA tablets	(5 mg)
Carbamazepine	Tegretol tablets	(200 mg)
Cardiovascular drug		
Atenolol	Oraday tablets	(100 mg)
Nifedipine	Adalat capsules	(10 mg)
Isosorbide dinitrate	Isordil tablets	(10 mg)
Enalapril	Enaril tablets	(5 mg)
Mexilitine	Mexitil capsules	(100 mg)
Antituberculosis		
Pyrazinamide	Pyrazinamide tablets	(500 mg)
Rifampicin	Rimactan capsules	(300 mg)
Isoniazid	Isoniazid tablets	(100 mg)

Table 3 (cont) List of Drug samples.

Generic name	Pharmaceutical preparation used	
Antiulcerative drug		
Cimetidine	Cimulcer tablets	(400 mg)
Ranitidine	Ranidine tablets	(150 mg)
Antifungal drug		
Griseofulvin	Fulcin tablets	(500 mg)
Ketoconazole	Ketocox tablets	(200 mg)
Hypoglycemic drug		
Metformin	Glucophage tablets	(500 mg)

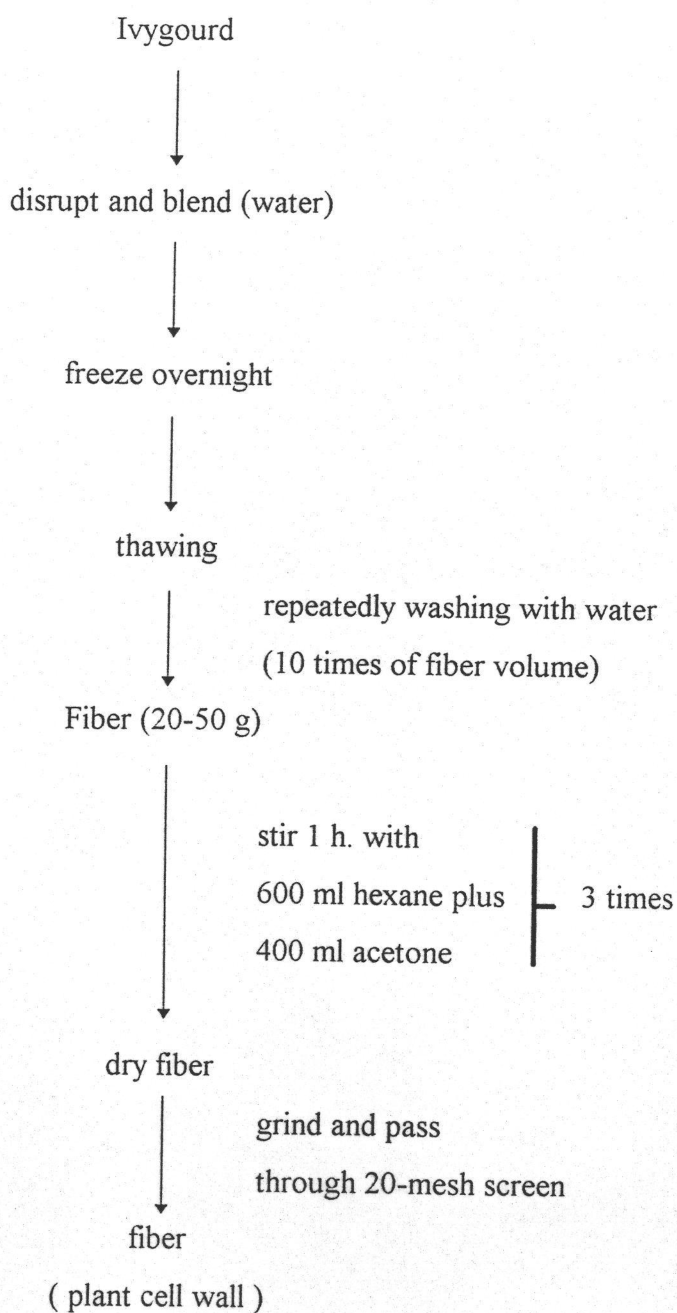

Figure 3 Steps in preparation of plant fiber.

Mutagenesis assay

The pre-incubation method of Yahagi et al.,1977 was used throughout this study (Figure 4). For the determination of direct mutagenicity, an aliquot of 0.1 ml of each nitrite treated drug was mixed with 0.5 ml of Na_3PO_4-KCl buffer (pH 7.4) and 0.1 ml of fresh overnight culture of tester strain (*S. typhimurium* TA 98 or TA 100). The contents were mixed and incubated at 37°C for 20 min in a shaking water bath. After incubation, 2.0 ml of molten top agar (45°C) was added, mixed well and poured on to a minimal glucose agar plate. His^+ revertant colonies were counted after incubation at 37°C for 48 h. Duplicate tests were carried out for each sample. The specific mutagenicity was calculated by number of His+revertants per plate per mmol of sample.

Positive and negative control plates were included in each assay. Negative control containing the bacteria and solvent was required to establish spontaneous mutation of each tester strain. Aminopyrene (0.06, 0.12 or 0.24 µg/plate) was used as positive control with and without the treatment of excess of nitrite in a gastric liked condition. The mutagenicity of each drug was presented as number of histidine (His^+) revertants per plate. The sample expressed its mutagenicity higher than 2 times of spontaneous revertants with a dose-response relationship was evaluated mutagenic (Brusich, 1982).

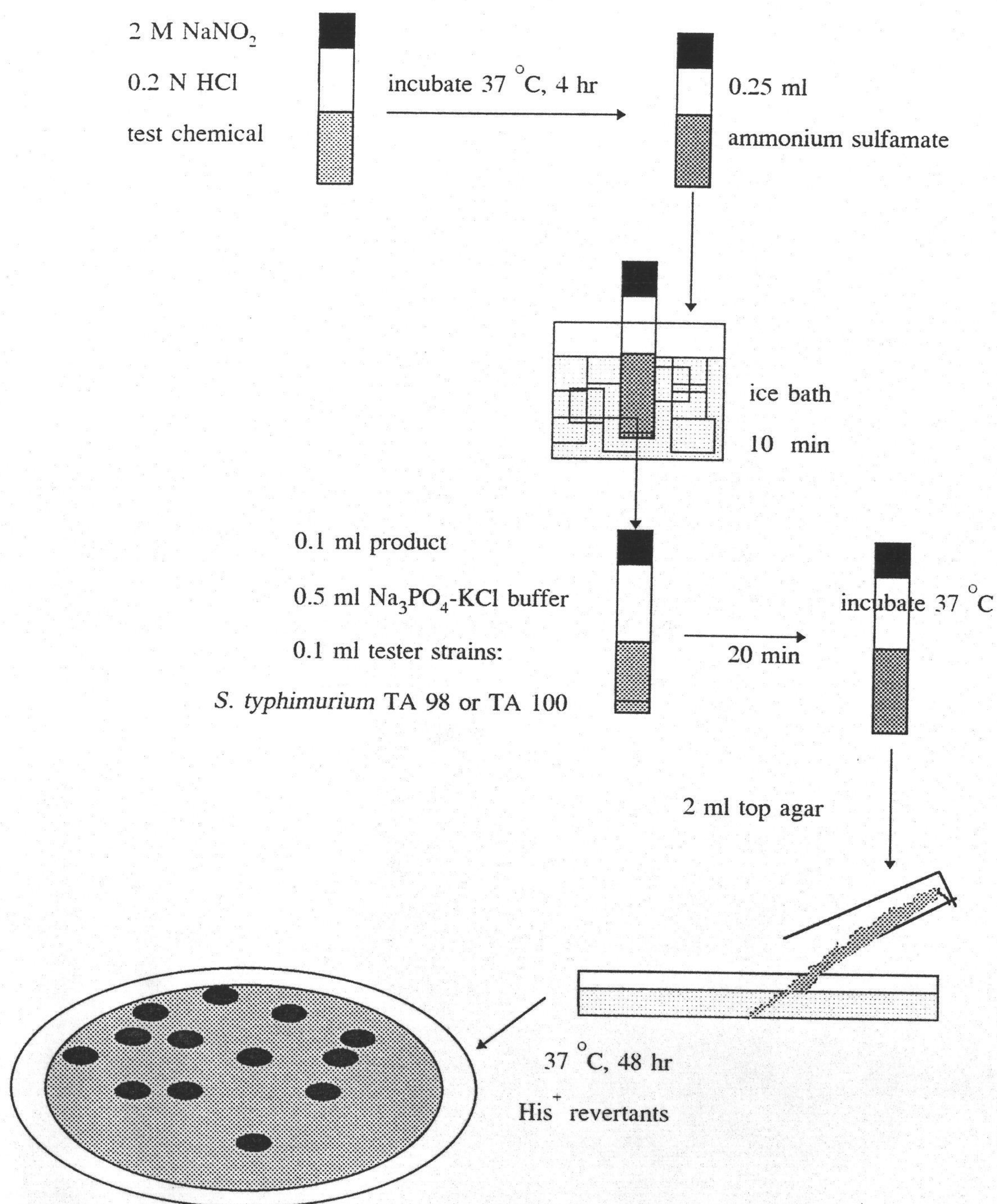

Figure 4. Steps in mutagenicity evaluation using the Ames test (pre-incubation method)

Antimutagen formation of ivygourd fiber

In a reaction tube containing drug sample (100mg/ml), ivygourd fiber 100 mg was added with 1.95 ml 0.2 N HCl (pH 3.0 - 4.0) and it was stored refrigerated overnight. Before starting the experiment, 0.75 ml of 2 M sodium nitrite was added and the reaction was incubated at 37°C with shaking for 4 h. After the tube was placed in an ice bath, 0.75 ml of 2 M ammonium sulfamate was added to decompose the residual nitrite. The reaction tube was, then, centrifuged (3000 rpm, 10 min) and the supernatant was collected and filtered through a steriled Milipore filter. The filtrate was subjected to the Ames test described earlier.

Antimutagen formation of proteins

Sample extracts (100 mg/ml) was added to 2 ml of simulated gastric condition mixture containing BSA 300 mg/lit, 0.3 mM sodium thiocyanate and 2 g/lit of sodium chloride. Added 0.75 ml of 2 M sodium nitrite and acidified with hydrochloric acid to pH 3.0-3.5. The reaction was conducted at 37°C with shaking for 4 h. then, it was stopped by placing the tube in an ice bath and was added with 0.75 ml of 2 M ammonium sulfamate to decompose the residual nitrite. The reaction products were subjected to the Ames test described earlier.