CHAPTER II

MATERIALS AND METHODS

MATERIALS

A. Instruments

1. Autoclave : Hirayama Model HA-3d, Hirayama Manufacturing Corporation, Japan
2. Hot Air Oven : Heraeus Type T 5090 E, Germany
3. Hot Air Oven : Elektro Helios Type 282 08, Sweden
4. Centrifuge : Centra-4 International Centrifuge Company, U.S.A.
5. pH Meter : ϕ 43 pH meter Beckman, USA.
6. Spectrophotometer : Spectronic 710 Bausch & Lomb, U.S.A.
7. Analytical Balance : Sartorius Type 1602, Sartorius GMBH Göttingen, West Germany
8. Balance : Sartorius Type 1518, Sartorius GMBH Göttingen, West Germany
9. Shaker : G10 Gyrotory Shaker, Newbrunswick Scientific Co., Inc. Edison, NJ. U.S.A.
10. Incubator For Gyrotory Shaker : Boonchoo Engineering Thailand
11. Shaker Waterbath : Julabo sw 1, Julabo v, Julabo Labortechnik GMBH D-7633 Seelbach, West Germany

12. Incubator : Memmert, West Germany

13. Desiccator : Sanko C-2, Japan

14. Light Microscope : Nikon Japan

15. Light Microscope with Camera : Nikon Vanox Japan

16. Light Microscope with Camera : Olympus BH-2 PM-10ADS Japan with Olympus C-35 AD-2

17. Scanning Electron Microscope : Hitachi S-430, Japan

18. Transmission Electron Microscope : Hitachi H-300, Japan

19. Critical Point Dryer : HCP-2 Hitachi Critical Point Dryer

20. Gold Coating Unit : SEM Coating Unit E 5,000 Polarson Instruments Inc., U.S.A.

21. Ultramicrotome : Sorvall® Porter-Blum MT-2 Ultramicrotome Ivan Sorvall Inc., Norwalk Conn. U.S.A.

22. Refrigerated Centrifuge : International Refrigerated Centrifuge Model PR-6, International Equipment Co., Needham HTS., Mass., U.S.A.

23. Liquid Scintillation Counter : Beta Matic II, Kontron Analytical, U.S.A.(Scientific Promotion Co., Ltd.)

B. Chemicals

1. Chemicals used for tea seed cake extraction

Chloroform BP, n-Butanol, Isopropanol, Methanol and Diethyl Ether (from May & Baker Ltd. Dagenham, England)

Glacial acetic acid (from Riedel-DE Haën AG. Seelze-Hannover, Germany)

Kieselgel 60 (70-230 mesh ASTM) (from Merck, USA)

Ethanol 95% (from The Government Pharmaceutical Organization, Bangkok, Thailand)

2. Culture Media

Sabouraud dextrose agar (SDA), Sabouraud dextrose broth (SDB), Yeast nitrogen base (YNB) and Bacto-dextrose were obtained from Difco Laboratories, Detroit, Michigan, U.S.A.)

3. Chemicals used for the Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

Miconazole nitrate Batch no. 3/82 (from Instituto International Terapeutico SA, Italy)

Dimethyl sulfoxide (from Prosynth® Riedel-DE HAEN Ag. Seelze-Hannover, Germany)

4. Chemicals used for Microscopic Study

4.1 For Light Microscopy

Lactic acid and Glycerol (from BDH CHemicals Ltd. Poole, England)

Methylthionine chloride (Methylene blue) (from Dakin Brothers, Ltd., London, England)

Cotton blue (from Bayer, West Germany)

Phenol crystal (from May & Baker Ltd. Dagenham, England)

4.2 For Electron Microscopy

Araldite 502, Dodecanyl Succinic Anhydride (DDSA), 2, 4, 6-tri (dimethyl aminomethyl) phenyl (DMP-30), Uranyl acetate, Sodium cacodylatetrihydrate, Osmium tetroxide, Glutaraldehyde 25% solution (were obtained from Electron Microscopic Sciences : Box 251 Fortwashington, DA 19034, U.S.A.)

Propylene oxide EM Grade (from Polyscience, Inc., Warrington, PA 18976, U.S.A.)

Lead nitrate (Fisher Scientific Company, NJ, U.S.A.).

Sodium citrate $Na_3C_6H_5O_7.2H_2O$ (from JT Baker Chemical Co., Phillipsburg, N.J., U.S.A.)

Acetone BPC 1973 and Absolute alcohol (from The Government Pharmaceutical Organization, Bangkok, Thailand)

Alcian blue 8GX (Ingrain blue 1) (from Sigma Chemical Company, St. Louis, U.S.A.)

5. Chemicals used for the Determination of DNA and Carbohydrate Biosynthesis

(Methyl-^{3}H) Thymidine 5'-monophosphate ammonium salt (specific radioactivity = 50 Curie/millimole and D-(U-^{14}C) Glucose (specific radioactivity = 3m Curie/millimole were obtained from Amersham International plc, England)

PPO (2, 5-Diphenyl-oxazole, $C_{15}H_{11}NO$) and POPOP (1, 4-Bis-(5-phenyl-2-oxazolyl)-benzol) (from Nakarai Chemicals Ltd., Japan)

Triton X-100 (iso-octylphenoxy polyethoxyethanol polyethoxy chain contains approximately 10 ethoxy units) (from BDH Chemicals Ltd., Poole, England)

Toluene (from May & Baker Ltd. Dagenham, England)

Perchloric acid 70% (from E. Merck, Darmstadt, Germany)

6. Miscellaneous

Kieselgel F 254 precoated plate 0.25 mm. (from Merck, U.S.A.)

Sodium chloride (from BDH Chemicals Ltd. Poole, England)

Potassium dihydrogen phosphate (Potassium phosphate monobasic) and Monosodium hydrogen phosphate (Sodium phosphate dibasic) (from Mallinekredt, Inc. Paris Kentucky 40361, U.S.A.)

Tea Seed Cake (were obtained from Chinese drug store)

C. ORGANISMS

1. *Candida albicans* ATCC 10231 was obtained from Department of Microbiology, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

2. *Arthroderma benhamiae* Ajello et Cheng JCM 01886 was obtained from, Japan Collection of Microorganisms. The Institute of Physical and Chemical Research, Hirosawa, Wako-shi, Saitama 351, Japan)

METHODS

A. Tea Seed Cake Extraction and Purification

1. Excess volume of deionized water was added to tea seed cake fine powder and the mixture was boiled for 30 minutes.

2. It was filtered through two layers of gauze with cotton wool in the middle. Three volumes of 95% ethanol was added to the filtrate.

3. The mixture was filtered through whatman no.1 filter paper and the filtrate was evaporated in the vacuum evaporator to remove ethanol until the volume was reduced to 1/3.

4. One volume of chloroform was added to the ethanol-water fraction to extract impurities and repeated twice. The chloroform fraction was discarded.

5. The ethanol-water fraction was then extracted with 1 volume of butanol for 3 times and the butanol fraction was evaporated in the vacuum evaporator until it dried to brown powder which is called 'TK'.

6. TK was dissolved in deionized water (3gm : 3ml), then 5 ml of isopropanol was added to it to make clear brown solution.

7. Silica gel (Kieselgel 60) was immersed in excess volume of 2% H_2O in isopropanol overnight and it was packed in a 4 cm. x 60 cm. glass column with thin layers of cotton wool at the bottom, then it was eluted with 5% H_2O in isopropanol.

8. The TK solution in 6. was applied to the silica gel column and was eluted with 5% H_2O in isopropanol 700 ml (elution rate = 3 ml/minute). The clear pale yellow fluid was discarded. The colorless fluid was collected thereafter.

9. The column was eluted again with 10% H_2O in isopropanol. All of the colorless fluid was collected.

10. All of the elutant fluids were combined in a round bottom flask and it was dried in the vacuum evaporator until brown powder was obtained

11. This brown powder was dissolved in a small volume of methanol then excess volume of cold diethyl ether was added to it to get the salting out effect and the light pale yellow precipitate (P) was obtained.

12. The precipitate (P) was seperated by centrifugation. at 1500 rpm x 30 minutes. The yield at this stage was 6.67% (0.6gm. from 9 gm. of crude tea seed cake)

Thin Layer Chromatography

Plate : Commercial plate Kieselgel F 254 precoated 0.25 mm. incubated in a desiccator for 5 days

Samples :TK

Light precipitate (P)

Samples were dissolved in deionized water before spotting.

Solvent systems

1. 50% ethanol in chloroform

or

2. Butanol : Acetic : Water 4:4:1

Detector : Short wave UV-light (254 nm)

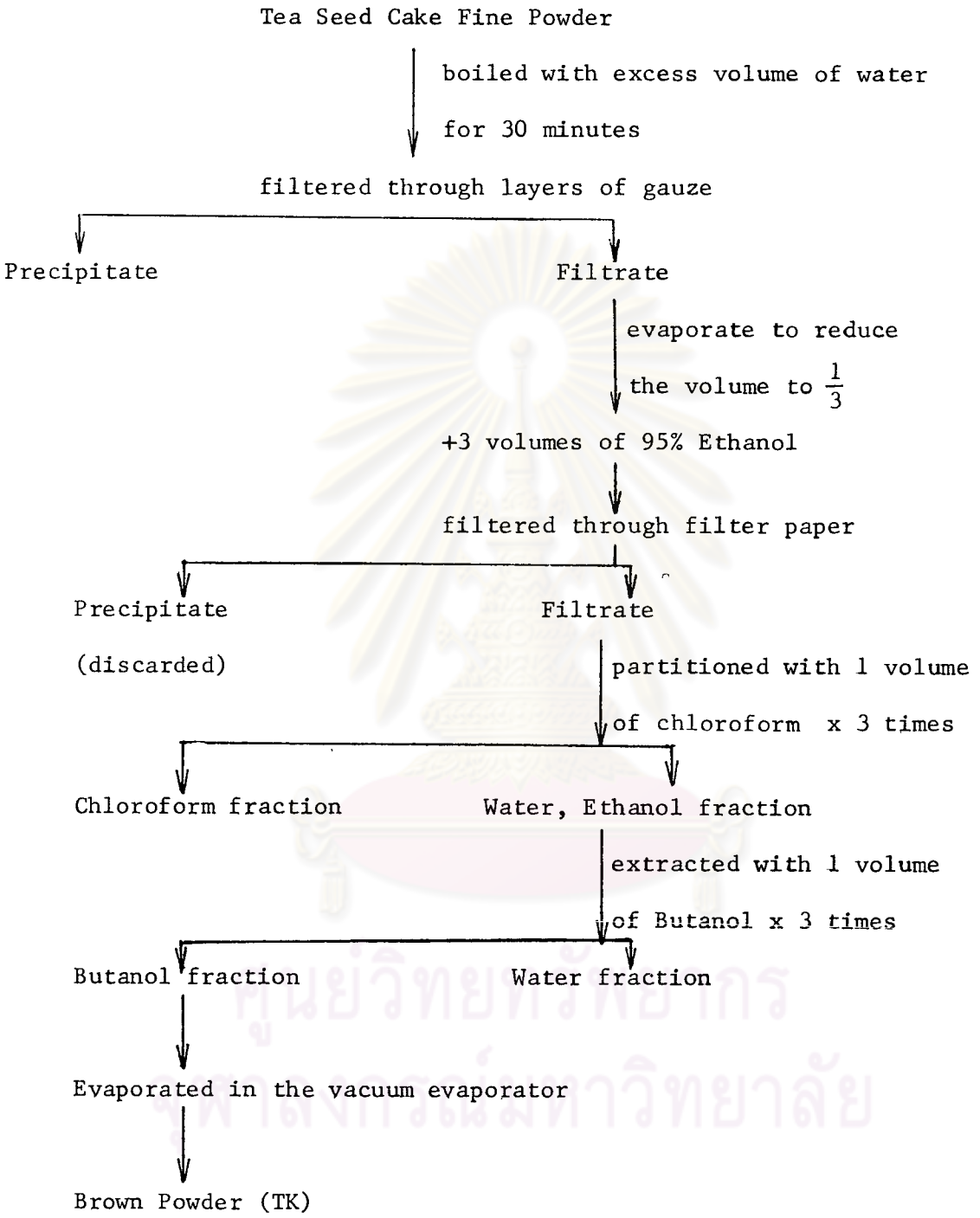

Figure 2.1 Tea seed cake extraction (Laohapaibul and Tosukhovong, 1981)

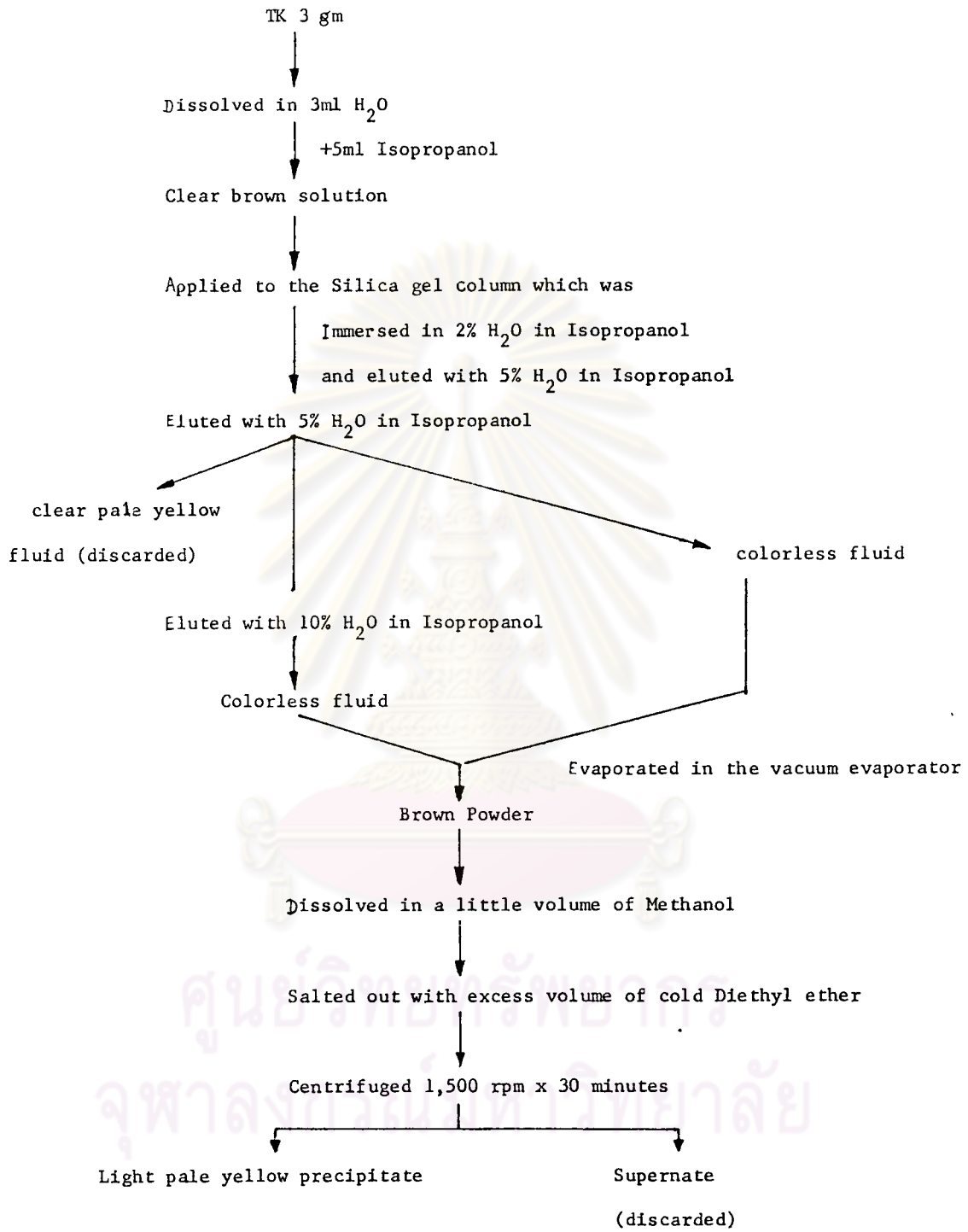

Figure 2.2 Purification of TK by column chromatography

B. Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

1. Broth Dilution Method

Organisms : a) *Candida albicans* ATCC 10231

b) *Arthroderma benhamiae* Ajello et Cheng JCM 01886

These two organisms were cultivated on Sabouraud dextrose agar slant at 30°C for 2 days (for *C. albicans*) and 15 days (for *A. benhamiae*). Cell suspensions were prepared in sterile normal saline solution and adjusted to a transmission of 88%-90% as measured at 530 nm. using normal saline solution as blank ($\simeq 1 \times 10^6$ colony forming units (CFU)/ml) (McGinnis, 1980).

Media : Sabouraud dextrose broth (SDB), Yeast nitrogen base (YNB). Buffered Yeast nitrogen base (buffered YNB)

Drug Solutions :

A 200,000 µg/ml Stock solution of TK powder was prepared in deionized water and sterilized by autoclaving. This solution was stored at 4°C and was used through the study without activity loss.

A 100,000 µg/ml stock solution of miconazole nitrate powder was prepared by solubilizing in dimethyl sulfoxide (DMSO). This solution must be protected from light and was allowed to stand for 30 minutes before use to permit autosterilization. (Shadomy et al., 1980). This stock solution was kept at -70°C and used within 1 year.

Each serial drug solution was prepared in 12 sterile tubes (16x125 mm). A 5.0 ml of the YNB or buffered YNB or SDB was added to tubes 2 through 12.

A 5.0 ml of the 10,000 µg/ml TK solution or 5.0 ml of the 100 µg/ml Miconazole nitrate solution was added to tubes 1 and 2 of each series. The contents of tube 2 were mixed and then the drug were serially diluted, using fresh 5.0 ml pipette for each dilution through the remaining tubes. The 5.0 ml from the last tube was discarded. This gave a serial dilution of TK solution ranging in concentration from 10,000 µg/ml to 5 µg/ml and that of miconazole nitrate ranging in concentration from 100 µg/ml to 0.05 µg/ml. (Table 2.1)

Table 2.1 Serial drug dilution in broth dilution method

Tube number / Content (ml)	1	2	3	4	5	6	7	8	9	10	11	12
Media SDB or YNB or buffered YNB	-	5	5	5	5	5	5	5	5	5	5	5
Drug: Miconazole 100 µg/ml or TK 10,000 µg/ml	5	5	5	5	5	5	5	5	5	5	5	5
	5	10	10	10	10	10	10	10	10	10	10	10
		5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml	5 ml discarded
Total (ml)	5	5	5	5	5	5	5	5	5	5	5	5

A 1.0 ml from each dilution was transfered to each of four sterile tubes. The remaining 1.0 ml volumes were kept as controls for contamination of the serial dilution.

A 1.0 ml of drug free broth was added to each of four additional sterile tubes for growth controls of the organisms.

Two tubes each of each concentration of drug were inoculated with 0.05 ml of the standardized suspensions of *C. albicans* or *A. benhamiae* (Fig. 2.3a).

Two tubes of drug-free media were also inoculated with each suspension for the growth controls. (Fig 2.3b)

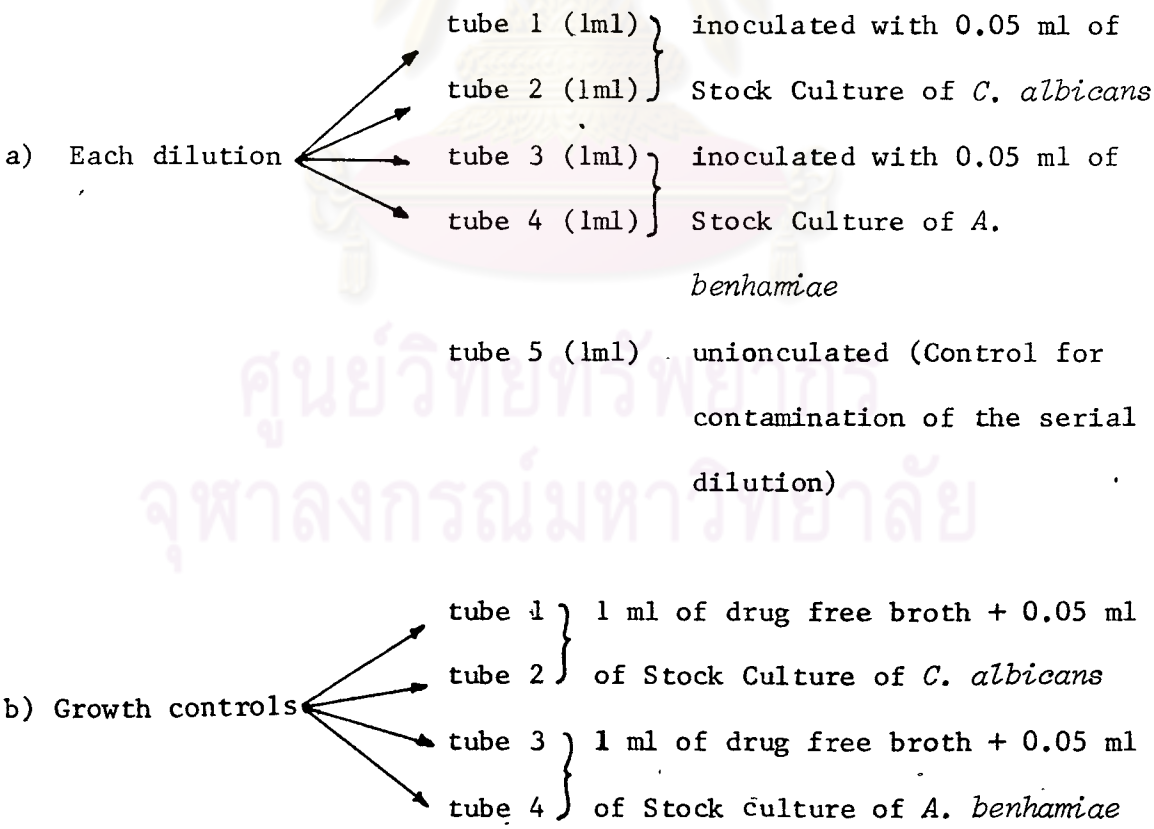

Fig. 2.3 Broth dilution method :inoculation

They were all incubated at 30°C for 48 hours (for *C. albicans)* and 15 days (for *A. benhamiae).* After incubation, the tubes were examined and the MIC was recorded. The MIC was defined as the lowest concentration of drug which inhibited clearly visible growth, ignoring a faint haze or slight turbidity.

The minimal fungicidal concentration (MFC) was determined by subculturing approximately 0.01 ml from each negative tube and from the positive growth control tubes onto drug-free Sabourand dextrose agar (SDA), with subsequent incubation at 30°C for 48 hours (for *C. albicans)* and 15 days (for *A. benhamiae).* The minimal fungicidal concentration (MFC) was defined as the lowest concentration of drug from which subcultures were negative or which yielded fewer than three colonies.

In this experiment, miconazole nitrate was used as the standard drug for testing susceptibility of the organisms to antifungal agent.

All of the experiments were repeated 3 times.

2. Agar Dilution Method

Drug Solutions and Media :

Stock drug solutions were prepared as above. Different concentrations of drugs were prepared in appropriate broth each at ten times the final desired concentrations, therefore, various concentrations from 100,000 µg/ml to 50 µg/ml for TK and 1,000 µg/ml to 0.5 µg/ml for miconazole nitrate (Table 2.2). Each of these

Table 2.2 Serial drug dilution in agar dilution method

Content (ml) \ Tube number	1	2	3	4	5	6	7	8	9	10	11	12
SDB	-	5	5	5	5	5	5	5	5	5	5	5
Drug Solutions : Miconazole 1,000 µg/ml or TK 100,000 µg/ml	5	5	-	-	-	-	-	-	-	-	-	-
			5 ml	10 5 ml	10 5 ml	10 5 ml	10 5 ml	10 5 ml	10 5 ml	10 5 ml	10 5 ml	10 5 ml discarded
Total (ml)	5	5	5	5	5	5	5	5	5	5	5	5

dilutions were then added inratio of 1:10 to sterile molten SDA (using 2 ml drug solution for 18 ml SDA).

Inocula and performance of test

Inocula for agar dilution tests were prepared as above for broth dilution test. The inoculum size, was 0.01 ml of standard stock cultures (approximately $1x10^6$ CFU/ml). (Fig. 2.4 a)

There was a pair of drug-free plates, one was inoculated before and one after inoculation of the drug dilution plates. (Fig. 2.4 b)

Positive growth response must be obtained on both control plates for results to be valid.

The incubation was at 30°C for 3 days (for *C. albicans*) and 30 days (for *A. benhamiae*).

The MIC was defined as the lowest concentration of drug preventing macroscopic growth of colonies. Hazy responses were regarded as negative.

C. Kinetics of Inhibition of Growth and Killing of Organisms

1. *Candida albicans*

C. albicans cultivated on SDA slant at 30°C for 2 days was used to inoculate 50 ml of buffered YNB in a 250 ml Erlenmeyer flask. The culture was incubated at 30°C and 150 rpm

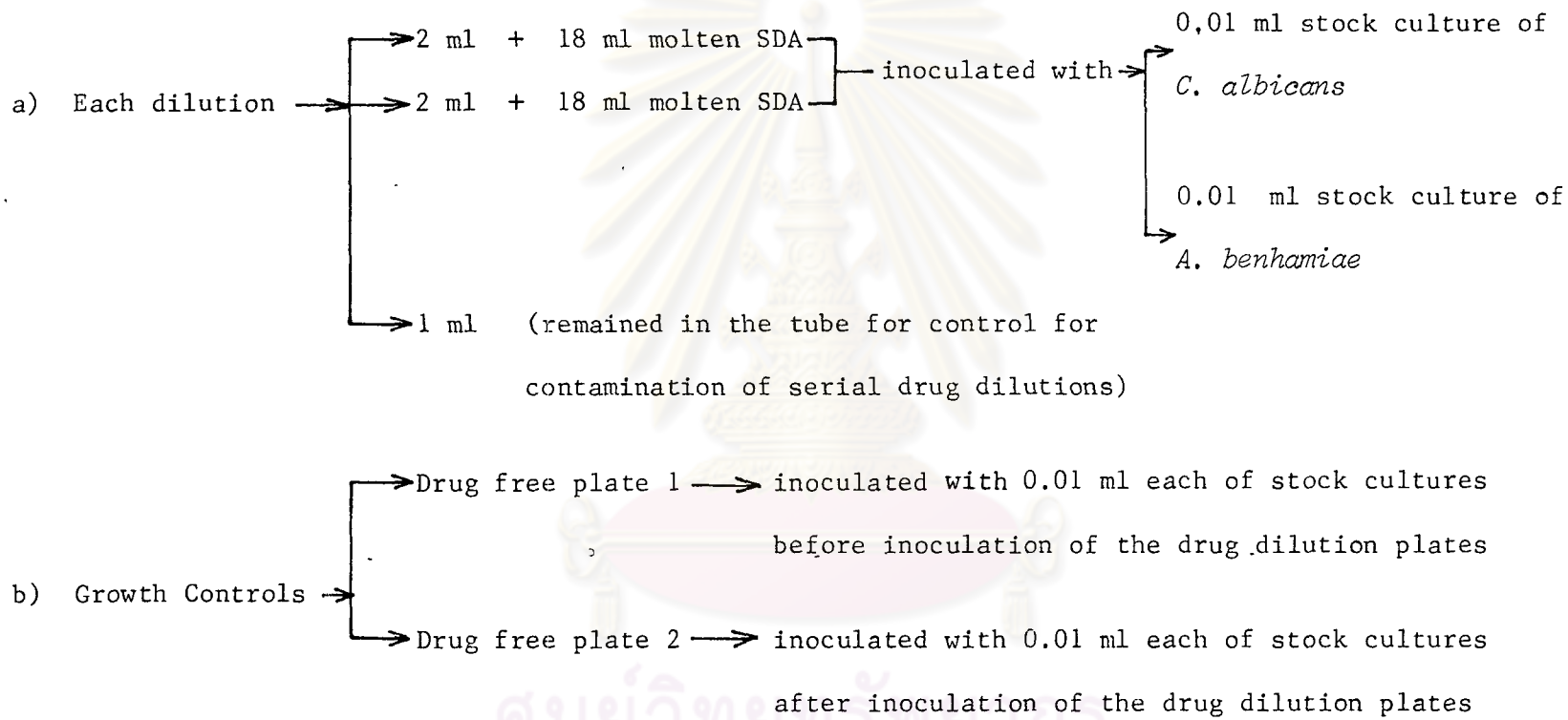

Figure 2.4 Agar dilution method : inoculation

on a gyrotory shaker for 24 hours then it was diluted with fresh buffered YNB and adjusted to the concentration of $2x10^6$ cells/ml (Count in a hemacytometer).

The 5.0 ml of 2,000 or 10,000 µg/ml TK solution was added to 45.0 ml of the culture above in each flask to make the final concentrations of 200 and 1,000 µg/ml of TK respectivety. The cultures were incubated at 30°C and 150 rpm on a gyrotory shaker. Control was made by replacing the TK solution with 5.0 ml of sterile distilled water (Table 2.3).

Table 2.3 Kinetics of inhibition of growth and killing of organisms (*Candida albicans*)

Treatment / Content (ml)	Control	200 µg/ml TK	1,000 µg/ml TK
Culture ($2x10^6$ cells/ml)	45.0	45.0	45.0
Sterile H_2O	5.0	-	-
2,000 µg/ml TK	-	5.0	-
10,000 µg/ml TK	-	-	5.0

Samples of 3.0 ml each were taken at the 0-, 2-, 4-, 6-, 8- and 24-hour incubations to determine the optical density (OD) and the number of viable cells. The OD was measured at 530 nm (Spectronic 710) using unionculated media containing matching concentrations of the TK solution as blanks. The viable counts were made by staining with Loeffler's methylene blue which is a vital dye (Loeffler's methylene blue : culture = 1:9) by which only dead cells were stained and the number of viable cells and dead cells were counted in a hemacytometer. The experiment was duplicated and repeated twice.

2. *Arthroderma benhamiae*

The organism was rubbed from 15 days old culture on SDA slant which had been incubated at 30°C and was diluted in Sabourand dextrose broth (SDB). The culture was incubated at 30°C, 150 rpm on a gyrotory shaker for 2 days then they were diluted with SDB to the concentration of 3 mg dry weight/ml (100°C 1 hour) (Nose, 1971).

Add 3.0 ml of 2,000 µg/ml or 10,000 µg/ml of TK solution to 27.0 ml of the culture in each flask to make the final concentration of 200 and 1,000 µg/ml of TK solution. Control was made by replacing 3.0 ml of TK solution by 3.0 ml of sterile distilled water (Table 2.4).

Table 2.4 Kinetics of inhibition of growth and killing of organisms *(Arthroderma benhamiae)*

Treatment / Contents (ml)	Control	200 µg/ml TK	1,000 µg/ml TK
Culture (3 mg dry weight/ml)	27.0	27.0	27.0
sterile H_2O	3.0	-	-
2,000 µg/ml TK	-	3.0	-
10,000 µg/ml TK	-	-	3.0

One flask of each concentration was sampling at the 0-, 1-, 3-, 5- and 7-day incubations and the mycelia were harvested by filtration through preweighed filter paper (Whatman no.1), washed twice with sterile normal saline solution (NSS), then the harvested mycelia on the filter paper were dried in a hot air oven (Elektro Helios Type 282 08, Sweden) at 100°C for 1 hour.

The dry mycelia and filter paper were weighed on an analytical balance and the weight of mycelia in each flask was the difference between the weight of the dry mycelia plus filter paper and the corrected weight of filter paper (approximately 3% loss at 100°C and 1 hour). The experiment was duplicated and repeated twice.

D. Light Microscopic Study

Candida albicans and *Arthroderma benhamiae* cultivated on SDA slants at 30°C for 2 days and 15 days consequently, were washed with sterile NSS then diluted with buffered YNB (for *C. albicans*) or SDB (for *A. benhamiae)* to the final concentration of $1x10^6$ colony forming units/ml.

8.0 ml of 2,000 µg/ml or 10,000 µg/ml of TK solution was added to 72.0 ml of the culture above to make the final concentration of 200 or 1,000 µg/ml of TK solution controls were made by replacing 8.0 ml of TK solution by 8.0 ml of sterile distilled water. They were all incubated at 30°C, 150 rpm on a gyrotory shaker. The experiments were duplicated (Table 2.5).

Table 2.5 Light microscopic study, *Candida albicans* and *Arthroderma benhamiae*

Treatment / Content (ml)	Control	200 µg/ml TK	1,000 µg/ml TK
Cultures - *C. albicans*, in buffered YNB - *A. benhamiae*, in SDB	72.0	72.0	72.0
Sterile H_2O	8.0	-	-
2,000 µg/ml TK	-	8.0	-
10,000 µg/ml TK	-	-	8.0

Samples of 10.0 ml each were taken at the 0-, 4-, 8- and 24-hour incubations (for *C. albicans)* and the 0-, 1-, 3-, 5- and 7-day incubations (for *A. benhamiae).*

C. albicans were stained with Loeffler's methylene blue (in the ratio of 9:1) while *A. benhamiae* were stained with lactophenol cotton blue. All of them were examined under a light microscope and photographed (phase contrast).

E. Electron Microscopic Study

1. The microbiological method was the same as that of the light microscopic study described above but the samples were treated differently.

2. Samples of 10.0 ml each were centrifuged 3,000 rpm for 15 minutes to remove media, washed twice with sterile NSS and once with 0.1 M cacodylate buffer pH 7.4. The specimens were primary fixed with 2.5% glutaraldehyde in cacodylate buffer pH 7.4 at 4°C for 2 hours, washed three times with the same buffer, post-fixed in 1% osmium tetroxide in the same buffer for 2 hours, and then washed three times in cold distilled water. The specimens at this stage were then suitable for further treatment for scanning or transmission electron microscopic studies (Fig. 2.5).

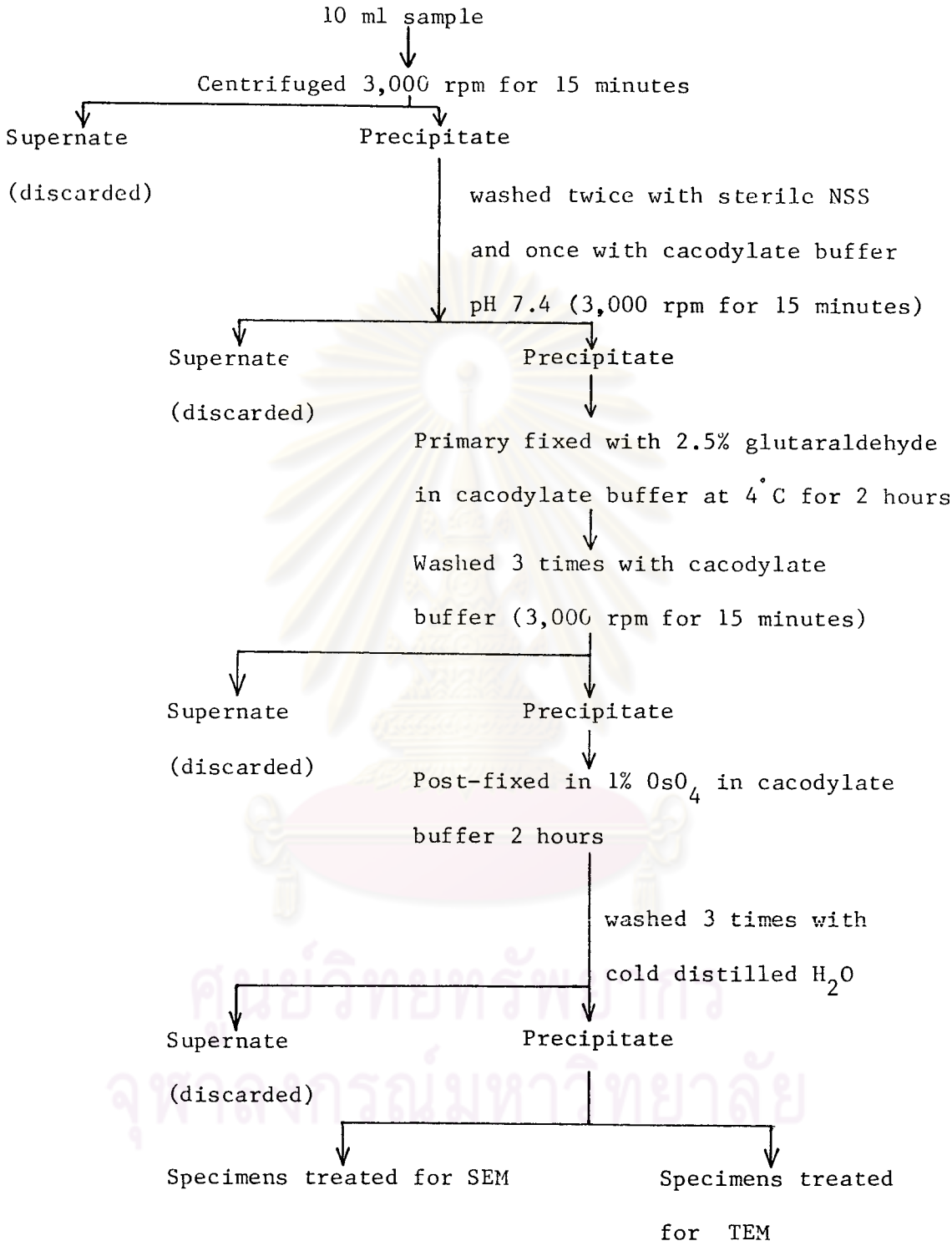

Figure 2.5 Specimens treated for electron microscopic studies

1. Scanning Electron Microscopic Study (SEM)

For scanning electron microscopy, the specimens treated above were settled on cover slips which were previously immersed in acetone, water and 1% alcian blue for 15 minutes. The specimens on cover slips were then dehydrated through series of ethanol by adding various percentage of aqueous solution of ethanol (50%, 70%, 80%, 90%, 95% and twice with absolute (100 %) ethanol). Each time, the ethanol solution was left 10 minutes before removal by using pasteur pipette.

When the dehydration process was completed, all of the specimens were dried in a Hitachi HCP-2 critical point dryer using liquid CO_2 as a transitional medium and they were mounted onto aluminium planchettes and coated with gold in an ion-sputtering apparatus Polarson E-505 with setting at 1.4 kv and 12 mA for 3 minutes.

The specimens were then examined by a Hitachi S-430 scanning electron microscope operating at 20 kv (Fig. 2.6).

2. Transmission Electron Microscopic Study (TEM)

For transmission electron microscopy, the specimens described before were subsequently fixed with 0.5% aqueous solution of uranyl acetate containing 45 mg/ml sucrose at 0°C for 30 minutes in a light-tight container and washed 3 times with cold distilled water. Subsequently, the fixed specimens were dehydrated through series of ethanol by adding 2-3 ml. of various percentage of cold ethanol solution in water : 50%, 70%, 80% and 90% ethanol for 5 minutes each, at 4° C

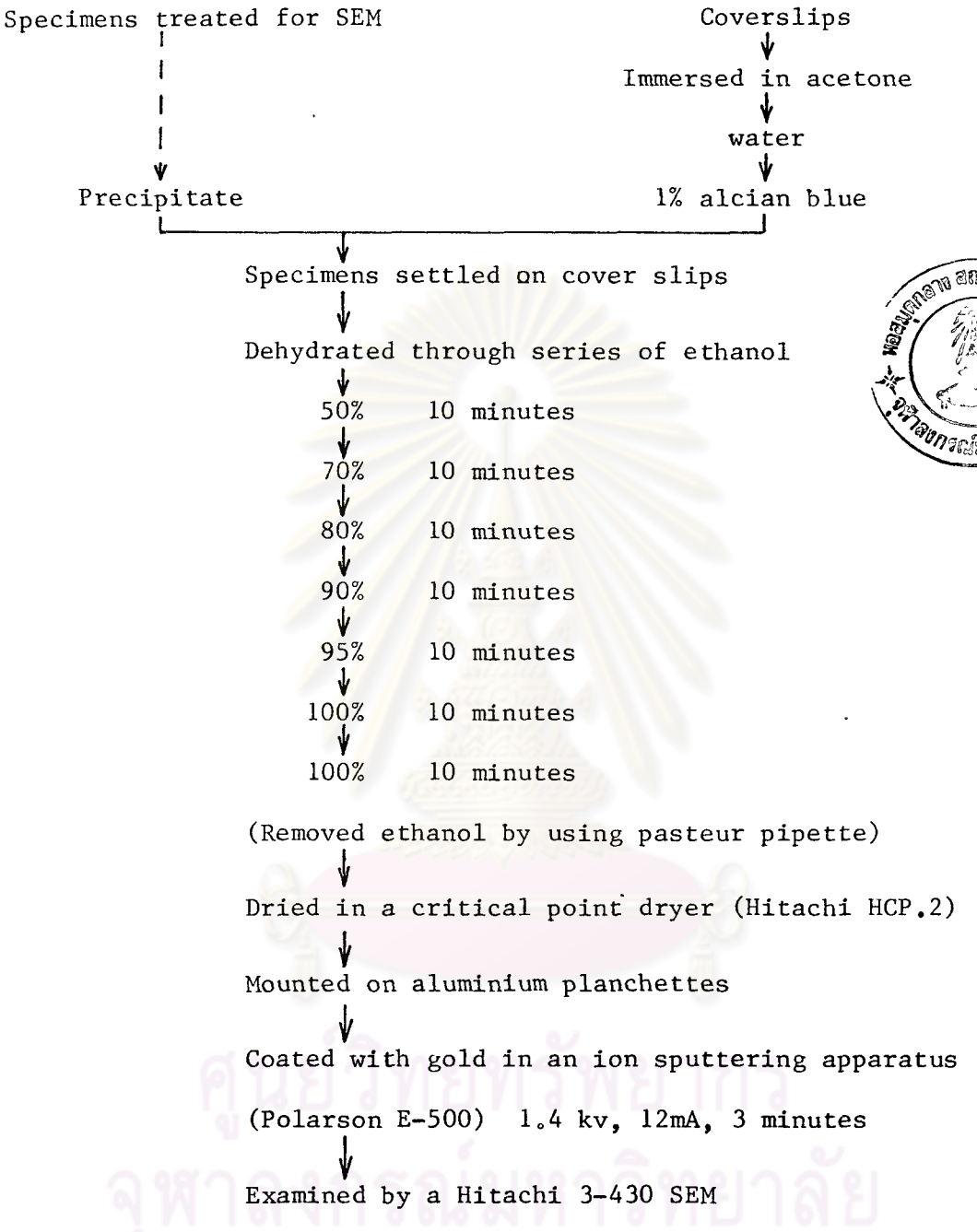

Figure 2.6 Specimens treated for scanning electron microscopy

- 95% ethanol for 5 minutes at room temperature
- 100% ethanol twice for 10 minutes each at room temperature (the last step of dehydration was made in plastic capsules).

When the dehydration process was completed, the specimens were infiltrated by adding in the following sequence :

1. Propylene oxide for 20 minutes, twice
2. Propylene oxide : Plastic Araldite 502 epoxy resin 2:1 for 1 hour
3. Propylene oxide : Plastic Araldite 502 epoxy resin 1:2 over night

The specimens were centrifuged 3,000 rpm for 15 minutes in between each step to remove the remaining solvents.

The specimens were then embedded in pure plastic Araldite 502 epoxy resin, cured at 35°c for 1 day, 45°c for 2 days and at 60°c for 3 days, respectively.

Ultra-thin sections were cut with a Sorvall Mt-2 ultramicrotome using glass knives at 300-500 A°. Sections were picked up with 300-400 mesh copper grids coated with formvar and stained sequentially with uranyl acetate and Reynold's lead citrate. Specimens were then examined under a Hitachi H-300 transmission electron microscope operating at 75 kv (Fig. 2.7).

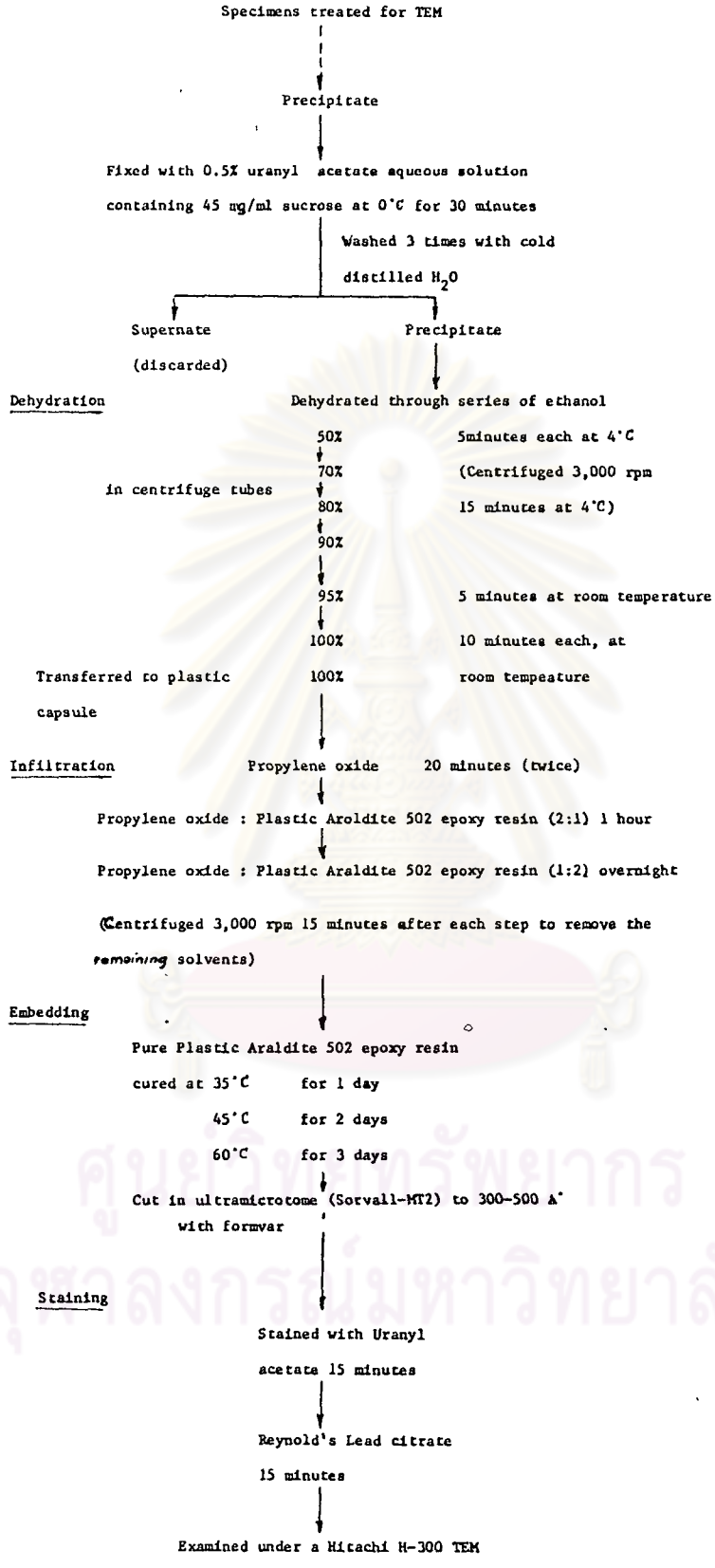

Figure 2.7 Specimens treated for transmission electron microscopy

F. Determinations of Macromolecular Biosynthesis

1. DNA Biosynthesis

1.1 *Candida albicans*

C. albicans cultivated on SDA slant at 30°C for 24 hour was used to inoculate 50 ml. buffered YNB in a 250 erlenmeyer flask. The culture was incubated at 30°C and 150 rpm on a gyrotory shaker for 24 hours, then it was diluted with fresh buffered YNB and adjusted to the concentration of $2x10^6$ CFU/ml (count in a hemacytometer).

0.5 ml. of (methyl-^{3}H) thymidine-5 monophosphate ammonium salt aqueous solution containing 100 µCi/ml (specific radioactivity = 50 Ci/m mole) (Amersham, England) and 5.0 ml of 2,000 or 10,000 µg/ml TK solution were added to 44.5 ml of the culture above in 250 ml erlenmeyer flask. The final concentration of ^{3}H-thymidine was 1 µCi/ml and that of TK solution was 200 or 1,000 µg/ml. Control was made by replacing TK solution by 5.0 ml distilled water (Table 2.6).

Table 2.6 The Experiment of DNA biosynthesis in *Candida albicans*

Treatment / Content (ml)	Control	200 µg/ml TK	1,000 µg/ml TK
C. albicans culture ($2x10^6$ CFU/ml)	44.5	44.5	44.5
^{3}H-Thymidine (100 µCi/ml)	0.5	0.5	0.5
H_2O	5.0	-	-
2,000 µg TK	-	5.0	-
1,000 µg TK	-	-	5.0

The cultures were incubated at 30°C 150 rpm on a gyrotory shaker.

Samples of 10 ml each were taken at the 0-, 1-, 2- and 4-hour incubations.

Each sample was centrifuged 3,000 rpm for 15 minutes to remove media, washed twice with 1% NSS, then 5% cold perchloric acid was added to the washed sample. It was centrifuged 3,000 rpm for 15 minutes to separate the supernatant fluid and the precipitate.

To determine the radioactivity, 1.0 ml each of the supernatant fluid was transferred to a vial and was added with

10.0 ml of scintillation fluid cocktail, then it was ready to be counted in a Betamatic II liquid scintillation counter.

The precipitate was solubilized with 10.0 ml of the same scintillation fluid cocktail and was counted in a Betamatic II liquid scintillation counter too (Fig. 2.8).

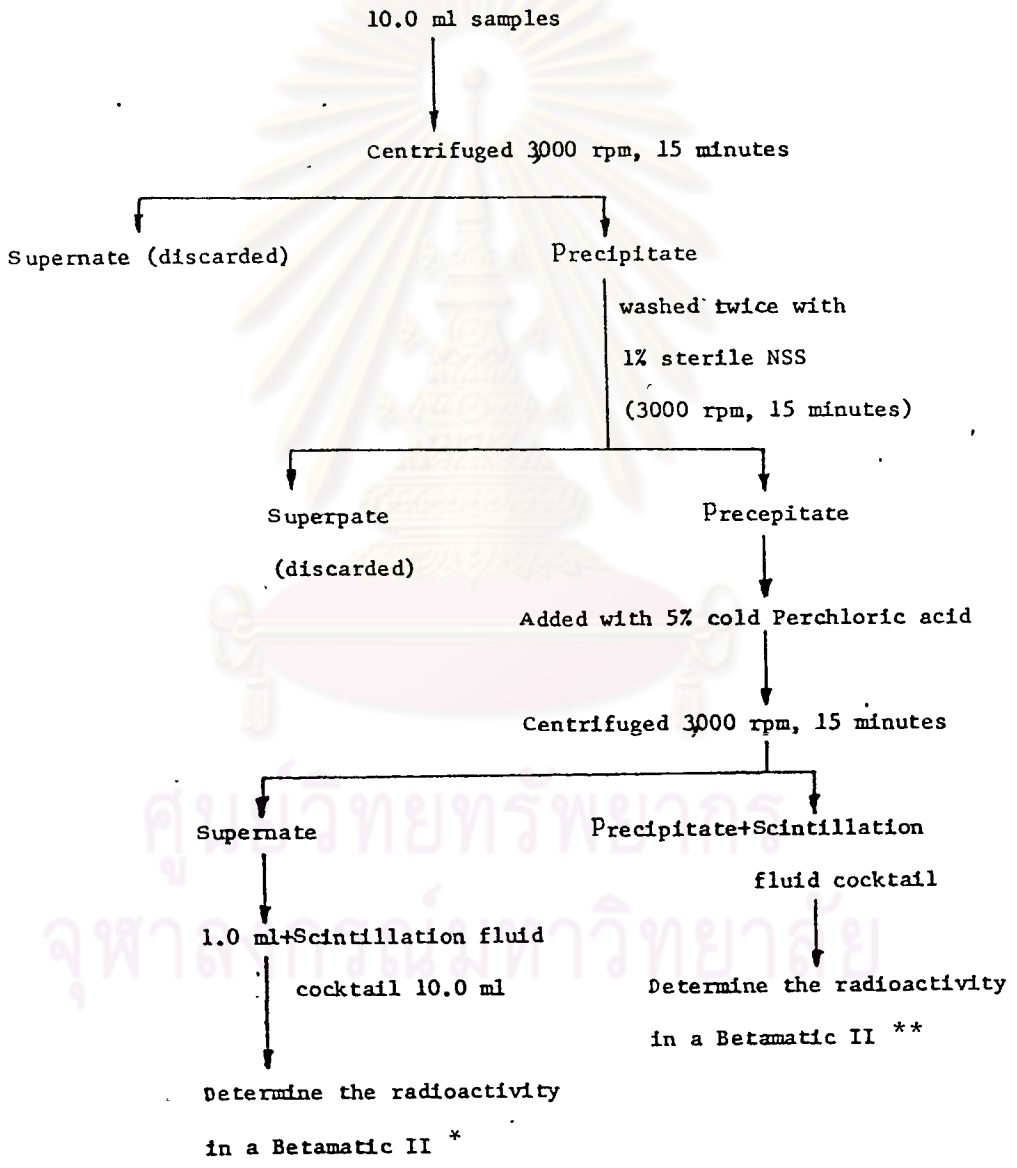

Figure 2.8 The treatment of samples for radioactivity

Note : * The dpm obtained must be multiplied by the exact volume of supernate for corrected total dpm.

** Total dpm

1.2 *Arthroderma benhamiae*

A. benhamiae cultivated on SDA slant at 30°C for 15 days was used to inoculate 50ml of SDB. The culture was incubated at 30°C and 150 rpm on a gyrotory shaker for 2 days then they were diluted with SDB to the concentration of 3 mg dry weight/ml (Nose, 1971).

The 0.4 ml of (methyl-^{3}H) thymidine-5'-monophosphate ammonium salt aqueous solution containing 100 µCi/ml (specific radioactivity = 50 Ci/m mole) (Amersham, England) and 5.0 ml of 5,000 µg/ml of TK solution were added to 44.6 ml of the culture above. The final concentration of ^{3}H-Thymidine was 0.8 µCi/ml and that of TK solution was 500 µg/ml. Control was made by replacing the TK solution by 5.0 ml of sterile distilled water (Table 2.7)

Table 2.7 The experiment of DNA biosynthesis in *Arthroderma benhamiae*

Treatment / content (ml)	Control	500 µg/ml TK
A. benhamiae culture (3mg dry weight/ml)	44.6	44.6
^{3}H-Thymidine (100 µCi/ml)	0.4	0.4
H_2O	5.0	-
5000 µg/ml TK	-	5.0

The cultures were incubated at 30°C and 150 rpm on a gyrotory shaker.

Samples of 10.0 ml each were taken at the 0-, 1-, 3- and 6-day incubations.

The samples were then treated as same as those of *C. albicans* described above and were counted for radioactivity too.

2. Carbohydrate Biosynthesis

C. albicans and *A. benhamiae* cultivated on SDA slants at 30°C for 2 days (for *C. albicans)* and 15 days (for *A. benhamiae)* were used to inoculate 50 ml of buffered YNB (for *C. albicans*) or 50 ml of SDA (for *A. benhamiae).* The cultures were incubated at 30°C and 150 rpm on a gyrotory shaker for 24 hours (for *C. albicans)* and 2 days (for *A. benhamiae).* They were diluted with buffered YNB or SDB to the concentration of $2x10^6$ cells/ml *(C. albicans)* and 3mg dry weight/ml (for *A. benhamiae).*

0.5 ml of D- U-^{14}C Glucose aqueous solution (100 µCi/ml) (specific radioactivity = 3mCi/m mole) (Amersham, England) and 5.0 ml of 2,000 or 10,000 µg/ml TK solution were added to 44.5 ml of the cultures above. The final concentration of ^{14}C-Glucose was 1µCi/ml and that of TK solution was 200 or 1,000 µg/ml. Controls were made by replacing the TK solution by 5.0 ml of sterile distilled water. The cultures were incubated at 30°C, 150 rpm on a gyrotory shaker (Table 2.8).

Table 2.8 The experiments of carbohydrate biosynthesis in *Candida albicans* and *Arthroderma benhamiae*

Contents (ml) \ Treatment	Control	200 µg/ml TK	1,000 µg/ml TK
Cultures - *C. albicans* ($2x10^6$ cells/ml) - *A. benhamiae* (3mg dry weight/ml)	44.5	44.5	44.5
^{14}C-Glucose (100 µCi/ml)	0.5	0.5	0.5
H_2O	5.0	-	-
2,000 µg/ml TK	-	5.0	-
10,000 µg/ml TK	-	-	5.0

Samples of 10.0 ml each were taken at the 0-, 1-, 2- and 4-hour incubations (for *C. albicans*) and the 0-, 1-, 3- and 7-day incubations (for *A. benhamiae*). The samples were treated for radioactivity counting the same as those of DNA biosynthesis experiment.