CHAPTER III

MATERIALS AND METHODS

MATERIALS

EQUIPMENT

- Portable air sampler for agar plates (Burkard, England)
- Data loggers (StowAway, USA)
- Light microscope (Bausch&Lomb, USA)
- Laminar air flow (ISSCO, Thailand)
- Balance (Sartorius, Germany)
- Autoclave (Tomy, Japan)
- Hot air oven (Memmert, Germany)
- Magnetic stirer hot plate (Chiltern, USA)
- Vortex mixer (Scientific Industries, USA)
- Refrigerator

SUPPLIES

- Macro pipette controller (Brand, Germany)
- Sterile disposable polystyrene Petri dishes (100×15 mm: Falcon 1029, USA)
- Glass Petri dishes (100×15 mm)
- Screw-capped test tubes (16×130 mm)
- Disposable plastic syringes with needle
- Glass slides and cover slips
- Pipettes
- Graduated cylinder
- Cork borer 3 mm internal diameter
- Erlenmeyer flasks
- Test tubes (16×160 mm)
- Inoculating needle
- Beaker
- Reagent bottle
- Forceps
- Cotton swabs
- Aluminium foil
- Parafilm
- Burner
- Vernier calipers

AGAR MEDIA

Potato Dextrose Agar (PDA)

Potato (peeled, diced)	200 g
Dextrose (Glucose)	20 g
Agar	20 g
Distilled water	1 litre

The potato was boiled for 15 min, then filtered through muslin. Dextrose and agar were added to the extract, made up the volume to 1 litre and sterilised by autoclaving at 121°C for 20 min.

To prepare the isolating medium, 0.03 g of Rose Bengal was added for restrict growth of spreading fungi and 0.03 g of Streptomycin was added to inhibit growth of bacteria. (Martin, 1950)

Carboxymethyl Cellulose Agar (Hankin and Anagnostakis, 1977)

Ammonium sulphate [$(NH_4)_2SO_4$]	1.0 g
Carboxymethyl Cellulose (CMC)	5.0 g
Yeast extract	1.0 g
Agar	10.0 g
Distilled water	1 litre

The CMC was gradually dissolved in boiling water with continuous mixing. The rests were added, made up the volume to 1 litre and sterilised by autoclaving at 121°C for 20 min. This medium was used for determination of the cellulolytic fungi.

SOLUTIONS

0.1 % Congo Red Solution

Congo red (CR)	1.0 g
Distilled water to made	1 litre

Dissolve Congo red in distilled water, then bring the volume to 1 litre. Store and use for the detection of cellulolytic fungi.

1 M Sodium Chloride Solution

Sodium chloride (NaCl)	58.44 g
Distilled water to made	1 litre

Dissolve sodium chloride in distilled water and bring the volume up to 1 litre.

Buffered Mineral Salt Solution (BMSS) (Upsher, 1977)

Ammonium nitrate (NH_4NO_3)	2.7 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	0.9 g
Dipotassium hydrogen orthophosphate (K_2HPO_4)	0.7 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.5 g
Potassium nitrate (KNO_3)	0.5 g
Distilled water	1 litre

Dissolve salts in distilled water then bring the volume up to 1 litre and sterilised by autoclaving at 121°C for 20 min.

Lactophenol

Phenol (pure crystals)	10 g
Lactic acid (specific gravity = 1.21)	10 g
Glycerol	20 g
Distilled water	10 g

The phenol and water were warmed until dissolved, subsequently lactic acid and glycerol were added and mixed to homogeneity.

For observing hyaline fungi, 0.03% Cotton Blue in Lactophenol was used.

METHODS

SAMPLING SITE

The present study was carried out in the Western Hall (*Mukdej Dan Tawantok*) located in the National Museum, Bangkok (Figure 3.1). This hall was 10 m wide, 14 m long and 8.5 m high, measuring 140 m^2 in area and 1,190 m^3 in volume. The schematic diagram of sampling points is shown in Figure 3.2. Description of each ancient woodcarving is depicted in Appendix A. Samplings of fungi were performed biweekly, between 9.00 a.m. to 12.00 a.m. Schedule of samplings is shown in Table 3.1.

SAMPLING OF FUNGI

The Sampling of Atmospheric Fungi

The portable air sampler for agar plates was used for the collection of airborne fungi (Figure 3.3). The sampler was manufactured by Burkard Manufacturing, Hertfordshire, England. This sampler was designed for isokinetic sampling and shielded from wind speed and direction. The apparatus was used for collecting bioaerosols direct onto culture medium in a Petri dish through a sieve plate with 100 holes (1 mm diameter).

Thirty five mL of the appropriate medium, Rose Bengal Potato Dextrose Agar with Streptomycin, was poured into a sterile plastic Petri dish and allowed the material to solidified. The distance between the grid plate and medium was critical, as the final gap should be maintained at between 0.50 to 0.65 mm. In this study, the top of agar to bottom of jet inlets distant range was 0.49-0.61 mm (average = 0.55; n = 163). Duplicate samples were also collected at each point by using the sampler operating at 20 Litre/min for 2 min.

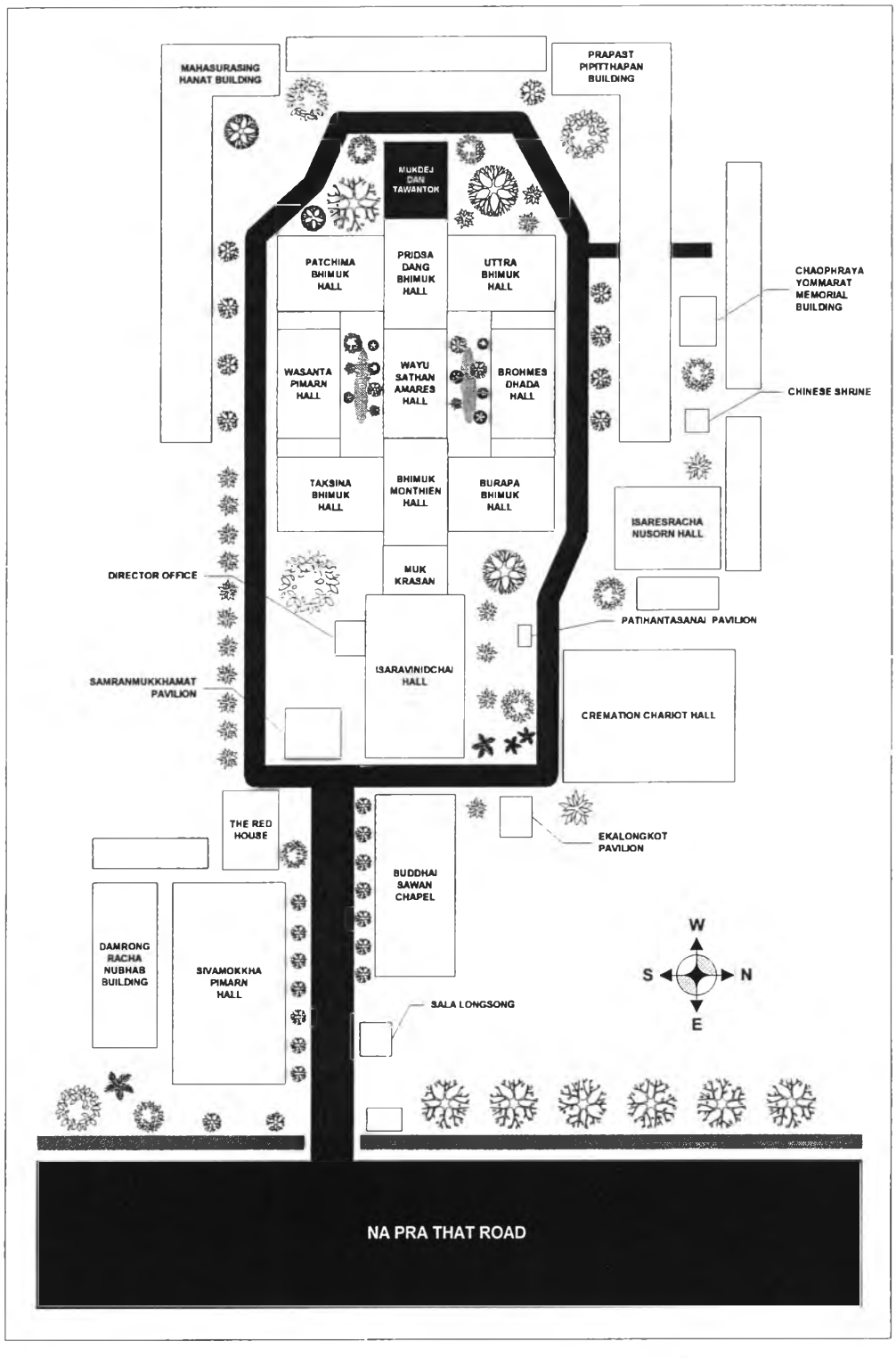

Figure 3.1: The location of the Western Hall.

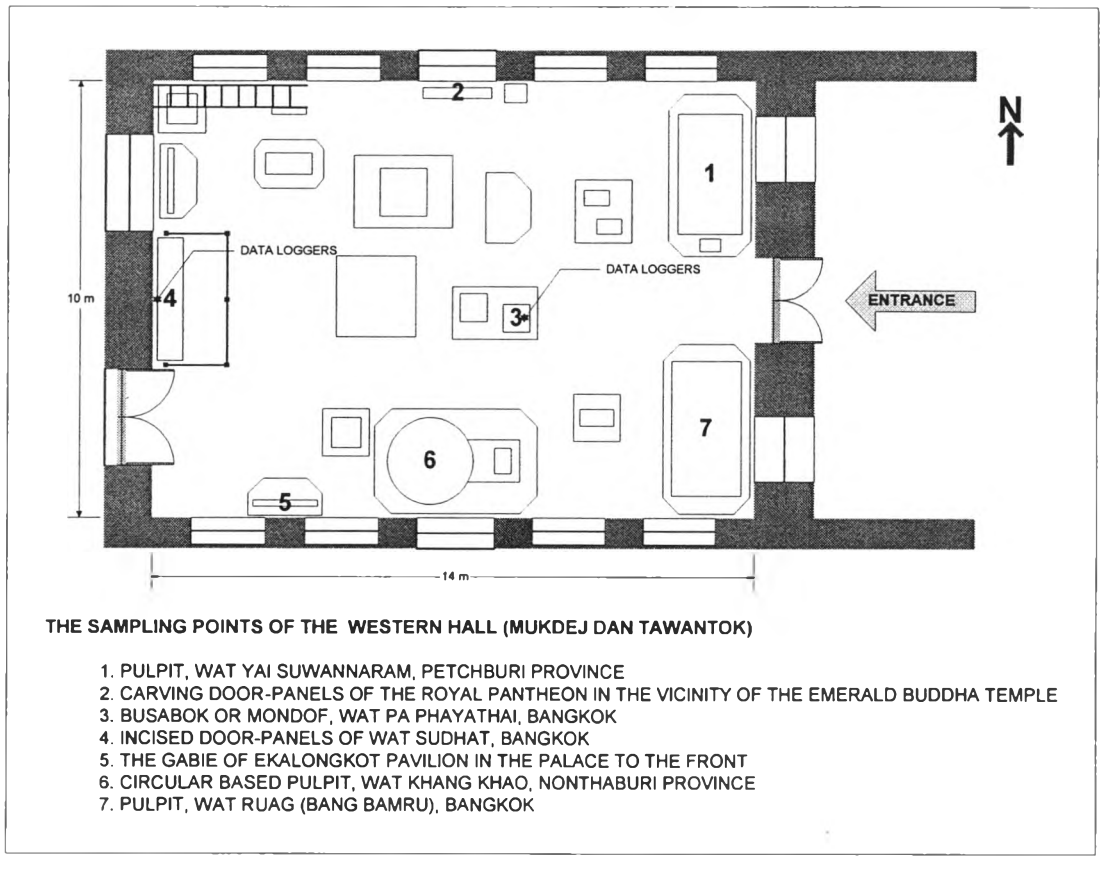

Figure 3.2: The schematic diagram of sampling points.

The top cover and alloy grid plate were removed together. At this stage, the sliding 'SHUT-OFF' valve could be seen to operate above the turbine chamber. It was ensured that the slide was in the closed position. A Petri dish with medium was inserted onto the 3 brass mounting standards within the unit. The grid plate was refitted and ensured to be resting accurately onto the rim of the Petri dish. The top cover was replaced onto the grid plate. It was checked that the lower rim of the cover is completely level with the plastic housing. The timer switch was selected to 2 min. The power switch was moved to the 'ON' position and the slide valve was then opened. A red LED indicator fitted in the front of the base flashed when the sampler was running and indicated that air flow was correct. At the end of the timed cycle, the flashing LED stopped but audible bleep would sound every 4 seconds. The slide valve was closed and the power switch was turned OFF. The top cover and grid plate were removed again. The exposed Petri dish was closed with the lid and taken to the laboratory.

Table 3.1: Date of the samplings.

Sampling No.	Date
I	Wednesday 5th February 1997
II	Wednesday 19th February 1997
III	Wednesday 5th March 1997
IV	Wednesday 19th March 1997
V	Wednesday 2nd April 1997
VI	Friday 18th April 1997
VII	Wednesday 30th April 1997
VIII	Wednesday 14th May 1997
IX	Wednesday 28th May 1997
X	Wednesday 11th June 1997
XI	Wednesday 25th June 1997
XII	Wednesday 9th July 1997
XIII	Wednesday 23rd July 1997
XIV	Wednesday 6th August 1997
XV	Wednesday 20th August 1997
XVI	Wednesday 3rd September 1997
XVII	Wednesday 17th September 1997
XVIII	Wednesday 1st October 1997
XIX	Wednesday 15th October 1997
XX	Wednesday 29th October 1997
XXI	Wednesday 12th November 1997
XXII	Wednesday 26th November 1997
XXIII	Thursday 11th December 1997
XXIV	Wednesday 24th December 1997
XXV	Wednesday 7th January 1998
XXVI	Wednesday 21st January 1998

Figure 3.3: The portable air sampler for agar plates.

The Sampling of Surface Fungi

The nondestructive method can be used for investigation of the valuable art objects. The swabbing technique was the method which was used for collecting of surface fungi on the ancient woodcarving.

The cotton swabs were placed in a test tube which is then closed with a cotton plug and sterilised by autoclaving at 121°C for 20 min.

The sterile swab was removed from the tube, dipped in BMS solution with a wetting agent, then swab was performed on selected surface (about 25 cm^2). The terminal portion (~4 cm) of the swab stick was then snapped off into a screw-capped tube with 10 mL of BMS solution. The tube was capped and taken to the laboratory for microbiological examination.

ISOLATION AND IDENTIFICATION OF FUNGI

For surface fungi, the sample in BMS solution was shaken vigorously, preferably with a vortex mixer, until the swab was dispersed. The suspension was removed by using a sterile pipette and placed into a sterile Petri dish. Molten sterile agar medium, Rose Bengal Potato Dextrose Agar with Streptomycin, was then poured on to the suspension in the Petri dish and mixed thoroughly, allowing the medium to gel.

All Petri dishes of atmospheric sampling and surface sampling were incubated at room temperature for 5 days. The appearing fungal colonies were counted. For atmospheric fungi, colony counts were converted for possible multiple impactions by using the conversion factor, and Colony Forming Units per cubic metre (CFU/m^3) of air were calculated. The colonies of surface fungi were calculated as Colony Forming Units per square decimetre (CFU/dm^2).

Fungi were subcultured several times until the pure cultures were obtained. Fungal isolates were classified and identified based on cultural and morphological characteristics by observing culture growth, and performing wet mount and/or slide culture technique. Fungal structures were examined under the light compound microscope. Mounting fluids used for identification processes were either lactophenal or lactophenol cottonblue. Fungal isolates were identified when possible according to references and literature cited for fungal Taxonomy, Mycology, etc. (Raper and Fennell, 1965; Barron, 1968; Koneman, Allen, Dowell Jr., and Sommers, 1979; Barnett and Hunter, 1987; Samson and Van Roenm-Hoekstra, 1988; Koon, 1990; Hawksworth, Kirk, Sutton, and Pegler, 1995). Fungal isolates were transferred and maintained on PDA slant for determination of cellulolytic fungi.

Slide Culture Technique

This method resulted in preparations in which the sporulation characteristics remain undisturbed and the spores remain attached to the sporophores thus facilitating their identification.

A bent glass rod was placed into a Petri dish, then a glass slide and cover slip were placed on the top of it. The preparing Petri dishes were sterilised by autoclaving at 121°C for 20 min. Fifteen mL of a suitable agar medium was poured in a sterile Petri dish. After solidification, the medium was cut into 0.5× 0.5 cm blocks by using a sharp sterile scalpel. An agar block was removed and placed on the sterile slide. The fungus was inoculated on the edges of an agar block between a slide and cover slip (Figure 3.4). A clump of cotton wool soaking with sterile water was placed into a Petri dish. The Petri dish was incubated under conditions favourable for sporulation of the fungus. The growth of the fungus on the agar block was observed periodically. After sporulation occurred, the cover slip was carefully lifted, then the agar block was discarded. Both slide and cover slip could then be mounted with lactophenol. The permanent slides might be made by sealing the edges of the cover slip with clear nail varnish.

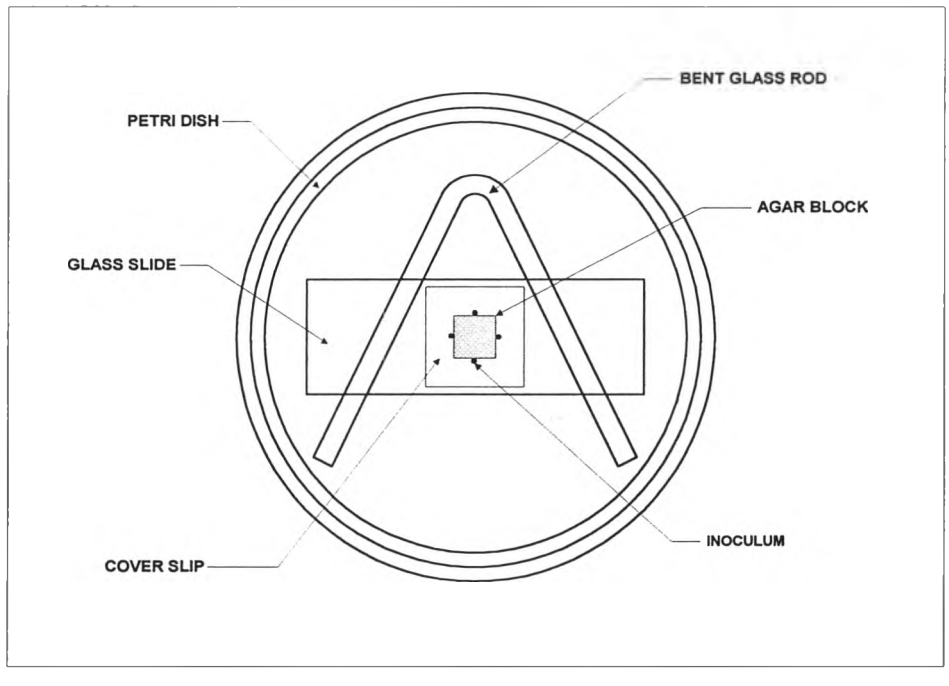

Figure 3.4: The slide culture technique.

DETERMINATION OF CELLULOLYTIC FUNGI

A solid medium containing Carboxymethyl cellulose (CMC) was used to detect C_x cellulase-producing fungi. Hydrolysis of CMC was seen as a clear zone around colony when used Congo red as a staining indicator. A qualitative measure of cellulase production may be obtained by calculating the ratio of zone size to colony diameter. A CMC agar medium providing rapid assay would be useful for the direct enumeration and isolation of cellulase-producing microorganisms because the incubation times required may be much shorter than using native cellulose (Hankin and Anagnostakis, 1977). This method was modified from Hankin & Anagnostakis (1977) and Teather & Wood (1982).

The cork borer (3 mm inner diameter) was used to cut the mycelium end of fungal isolate that grown on PDA at room temperature for 2 days and was then inoculated on CMC agar medium. All Petri dishes were incubated at room temperature for 3 days. After incubation, the size of the colony was measured and the surface of the medium was flood with an aqueous solution of Congo red (1 mg/mL) for 15 min. The Congo red solution was then poured off, and further treated by flooding with 1 M sodium chloride solution for 15 min. The clear zone was seen around fungal colony. The visualised zone of hydrolysis could be stabilised for at least 2 weeks by flooding the agar with 1 M hydrochloric acid, which changed the dye colour to blue and inhibited further enzyme activity. The size of the clear zone was measured and the ratio of zone size to colony diameter was calculated.

THE MEASUREMENT OF ATMOSPHERIC TEMPERATURE AND RELATIVE HUMIDITY

In this research, the Data Loggers were used for monitoring of atmospheric temperature and relative humidity in everyday of the year. The StowAway® XTI was a temperature logger and the StowAway® RH was a relative humidity logger (Figure 3.5). The loggers were operated to start, readout and plot the data with LogBook™ software for Windows. They were programmed for multiple sampling with averaging. The data was exported to spreadsheet programme (MS–Excel) for analysis. Two series of Data Loggers which were located in the Western Hall i.e. Busabok (sampling point no.3: P3) and Incised Door-Panels (sampling point no.4: P4). The location is shown in Figure 3.2.

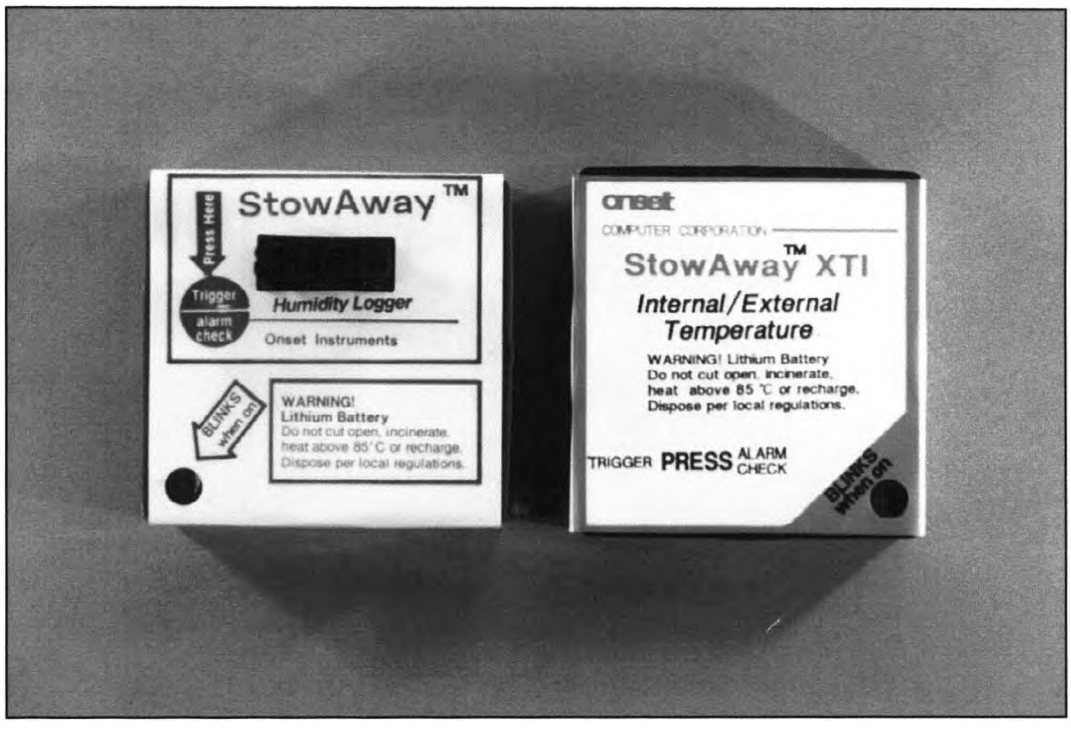

Figure 3.5: The Data Loggers.