CHAPTER 3

EXPERIMENT

Description of Experiment

An experiment was designed using two types of HA in disc - shape ; 14 mm. in diameter x 1 mm. in thickness (Hyakuna et al., 1990). The experiment could be classified into 5 steps : starting material preparation, specimen preparation, specimen characterization, dissolution test on *in vitro* study, and characterization of tested specimens and the solution as shown in the following flowchart. Further information in each step was described in Fig. 3.2.

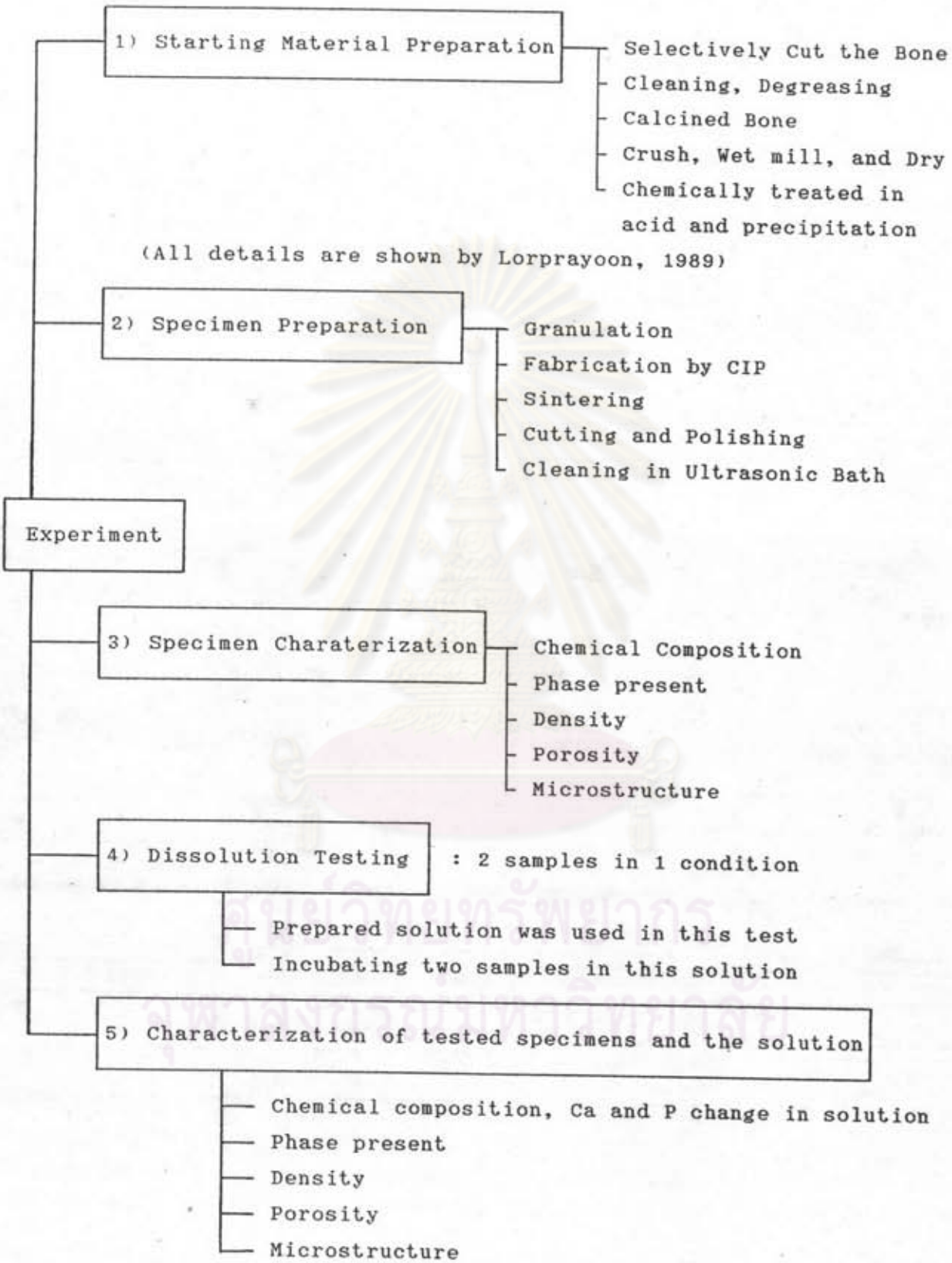

Fig. 3.1 Experimental description flow chart.

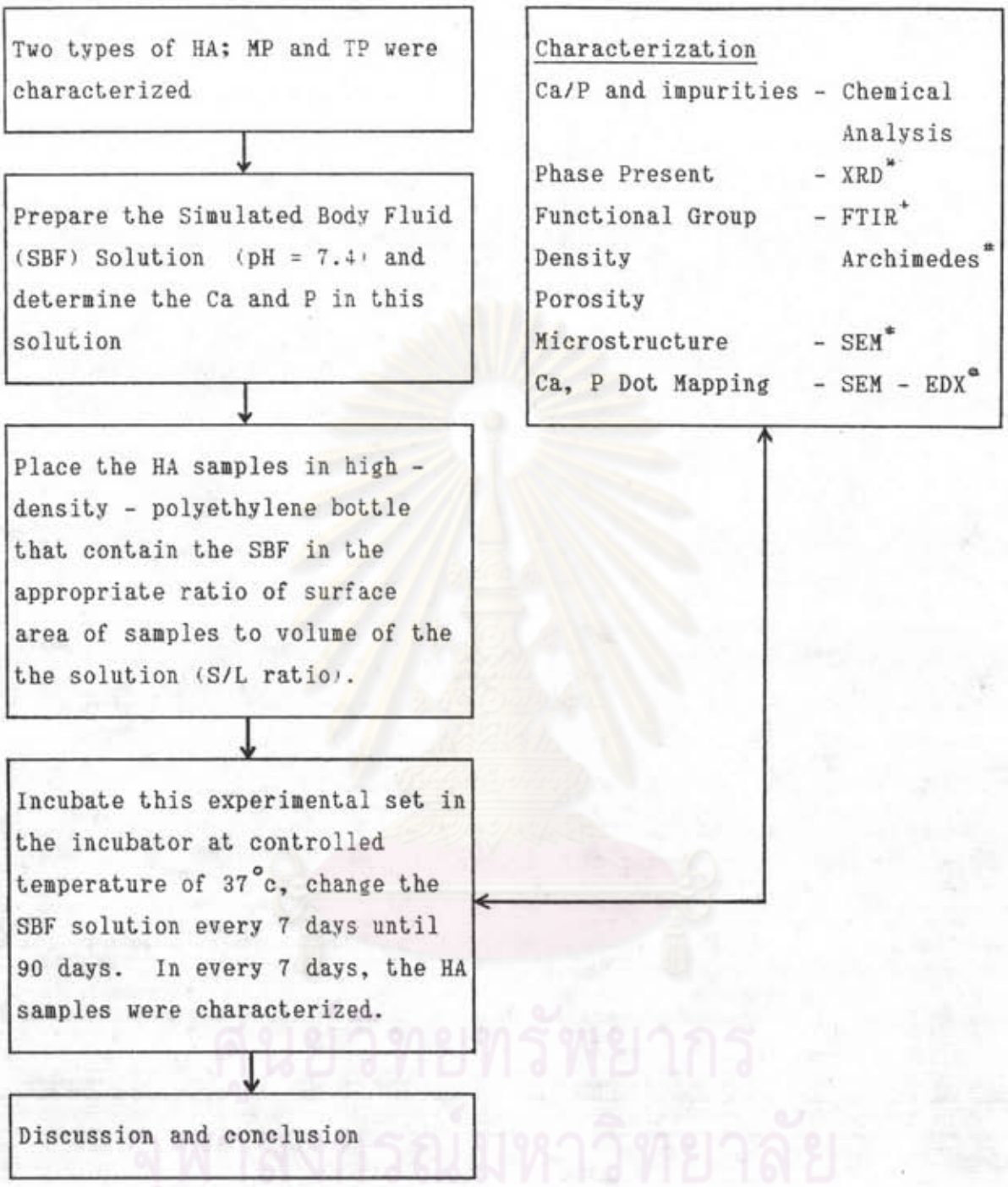

Fig 3.2 Information in each step of experiment

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* X-ray Diffraction

\+ Fourier Transform Infrared Spectroscopy

\# Archimedes - ASTM Designation B212-82

* Scanning Electron Microscopy

@ Scanning Electron Microscopy - Energy Dispersive Analysis

Experimental Procedure and Equipment

3.1 Starting Material Preparation

MP and TP used in this experiment were hydroxyapatites derived from cattle bone ash. MP is cattle bone ash and TP is chemically treated of cattle bone prepared by dissolution the bone ash in acid and precipitation in ammonia under controlled condition. The details for MP and TP preparation were reported by Lorprayoon, 1989.

3.2 Specimen Preparation

MP and TP powders obtained in 3.2.1 were fabricated by Cold Isostatic Pressing (CIP) into disc-shape (15 mm. in diameter x 12 mm. in thickness). Green disc-shape of MP and TP samples were heated to remove PVA binder, and sintered. The methods were as described by Lorprayoon et al., 1991. The sintered samples were cut by low-speed refined saw machine (Fig. 3.3) into a specimen of 15 mm. in diameter x 1.2 mm. in thickness, polished and cleaned using acetone and ethyl alcohol in ultrasonic bath. The first step was rough polishing using various sequences of abrasive papers number : 360, 400, 600, 1000 and the second step was fine polishing using alumina powder (BUEHLER Micropolish) with various diameters : 1.0, 0.3 and 0.5 μm by the Metaserv Universal Polisher in Fig 3.4. These specimens were further

cleaned with acetone and ethyl alcohol (respectively) in ultrasonic bath. A flow chart of the specimen preparation was as shown in Fig. 3.5.

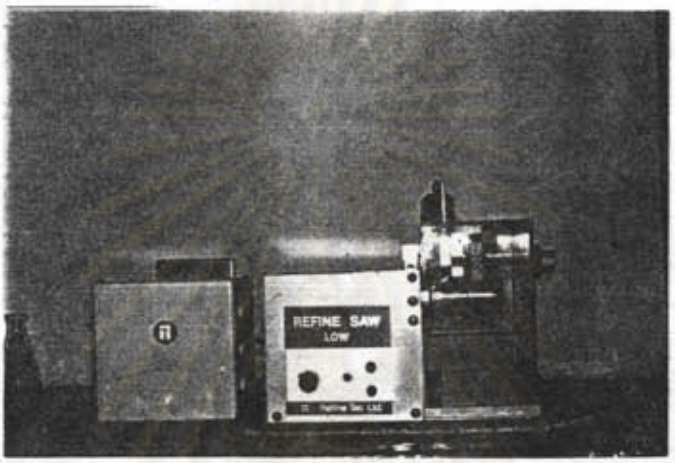


Fig. 3.3 Low - Speed Refined Saw Machine

Fig 3.4 Metaserv Universal Polisher

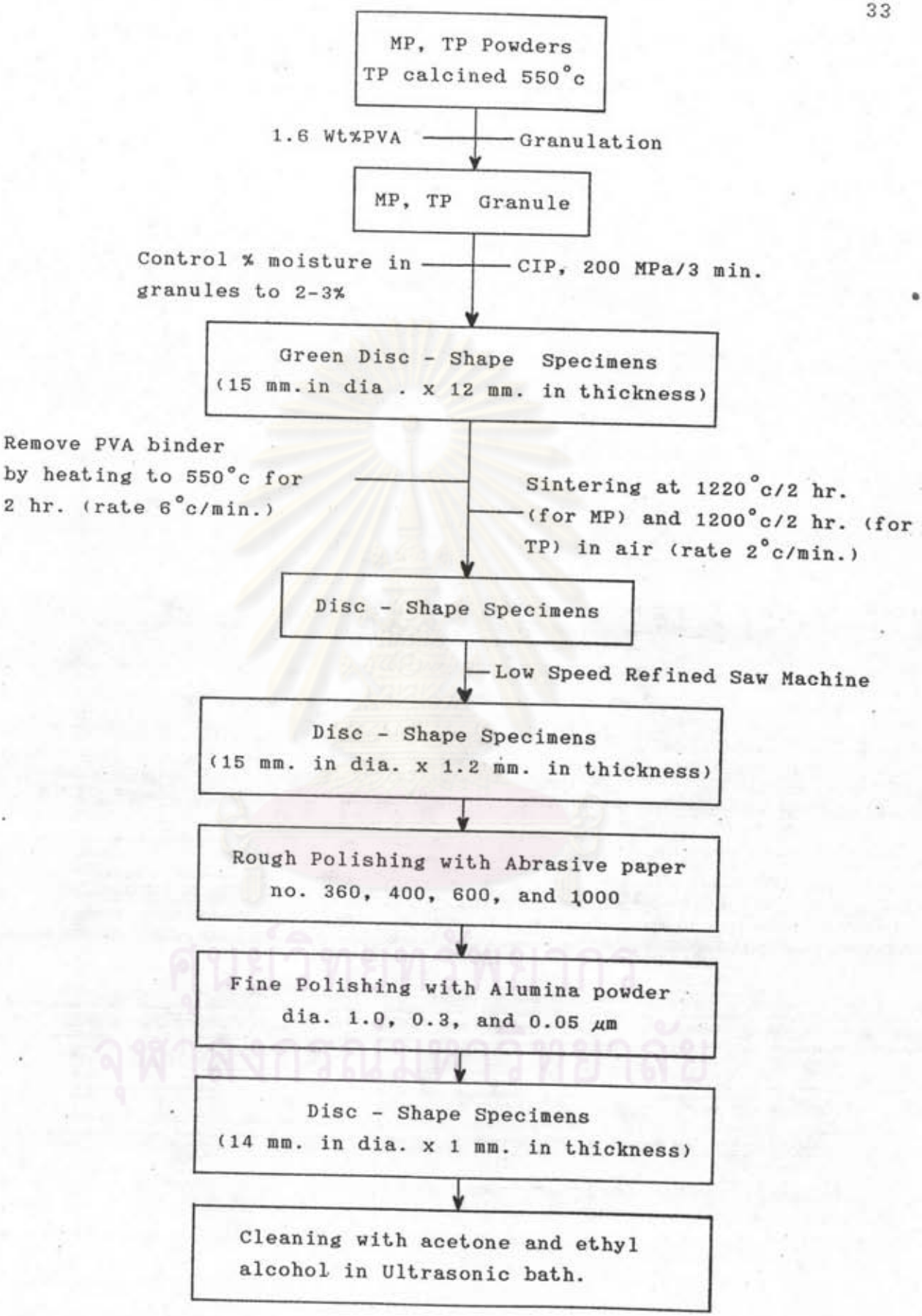


Fig 3.5 Flow chart of Specimen Preparation

3.3 Specimen Characterization

Specimens were characterized before incubation in Simulated Body Fluid (SBF) in terms of the following characteristics.

3.3.1 Chemical Composition and Impurities

The concentration of Ca and P was determined by EDTA chelate and molybdenum blue methods respectively. All impurities were determined by inductively coupled plasma and instrumental neutron activation analysis (ICP/INAA) method. All analyses were done by Mineral Assays and Service Co., Ltd.

3.3.2 Phase Present

The phase of the specimens were characterized by X-ray Diffraction (XRD). The sintered specimens were crushed to fine powders and examined crystal phases using Philips diffractometer (PW 1730/10) in Fig. 3.6 with copper $K\alpha$ radiation at 30 mA and 40 kV, and Nickel filter. A time constant of 1 s. and a scanning rate of 2 min^{-1} were used. The XRD patterns were recorded with a chart drive speed of 2 cm min^{-1} and the 2θ values were varied from 16° to 60°.

3.3.3 Functional Groups

The functional groups of the samples were characterized by Fourier Transform Infrared Spectrophotometer (FT-IR 1760X Perkin - Elmer) in Fig. 3.7. The sample preparation used KBr disc method. The IR spectra were recorded with wave number varied from 4000 to 400 cm^{-1}.

3.3.4 Specimen Density and Porosity

The density of the specimens was determined by Archimedes method (ASTM C373-72) using distilled water as the immersion medium. The procedure was as follows :

(1). Test specimens were dried to constant weight by heating in an oven to 80 °c and the dry weight, D was determined.

(2). Test specimens were placed in distilled water and boiled for 2 hr., taking care that the specimens were covered with water at all times. Then the specimens were immersed in water for 24 hr. before weighing and the weight, S was determined. This weight was accomplished by suspending the specimen in a loop of copper wire hung from the balance and immersed in water.

(3). After determine the suspended weight, each specimen was blotted lightly with moistened cotton cloth to remove all excess water from the surface and the saturated weight, M was determined by weighing in air.

(4). The calculated data from D, S, and M parameters were shown below :

a) Exterior volume, V (cm^3) = M-S

b) Volume of open pores, cm^3 = M-D

Volume of impervious portions, cm^3 = D-S

c) Apparent porosity, %P = [(M-D)/V] x 100

d) The water absorption, %A = [(M-D)/D] x 100

e) The apparent specific gravity, T = D/(D-S)

f) The bulk density, B = D/V

3.3.5 Specimen Microstructure

Specimen surfaces and fresh fractured specimens were coated with a thin layer of gold. Microscopic examinations were made by reflected - light microscopy and electron microscopy (SEM JEOL JAPAN, JSM - T 220A) in Fig.3.8. If the microstructure of HA specimens was not revealed, 0.25% H_3PO_4 etching for 15 sec. at room temperature was used before coating with gold.

3.3.6 Ca, P Dot Mapping

Specimen surfaces were coated with a thin layer of carbon. The SEM-EDX (Link-Systems) analyzed the Ca, P in the surface of specimens in the form of dot mapping. The time used for this analysis was 100 sec.

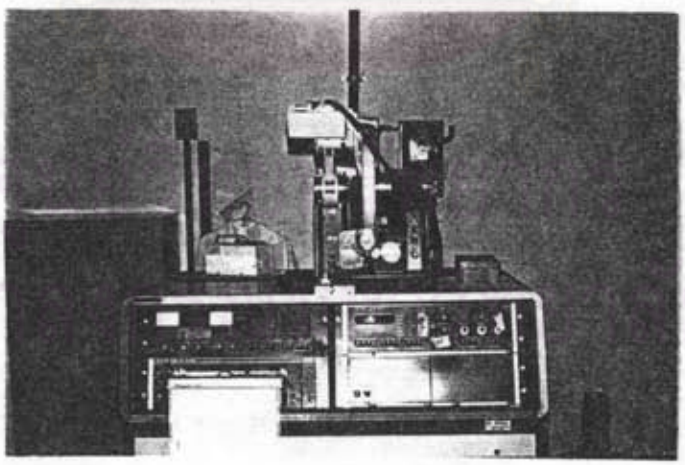


Fig. 3.6 Philips diffractometer (PW 1730/10)

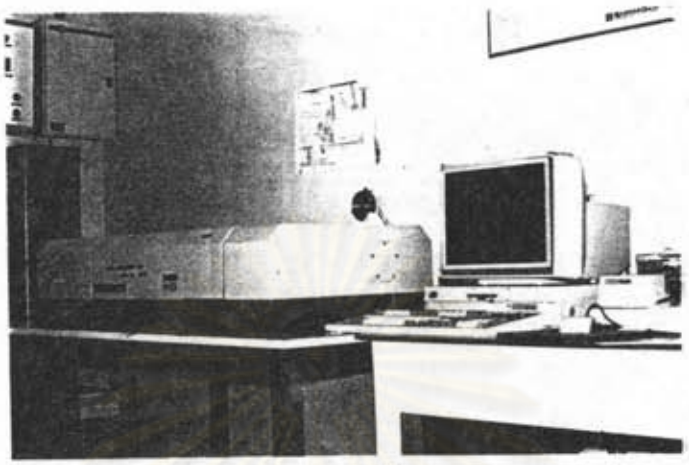
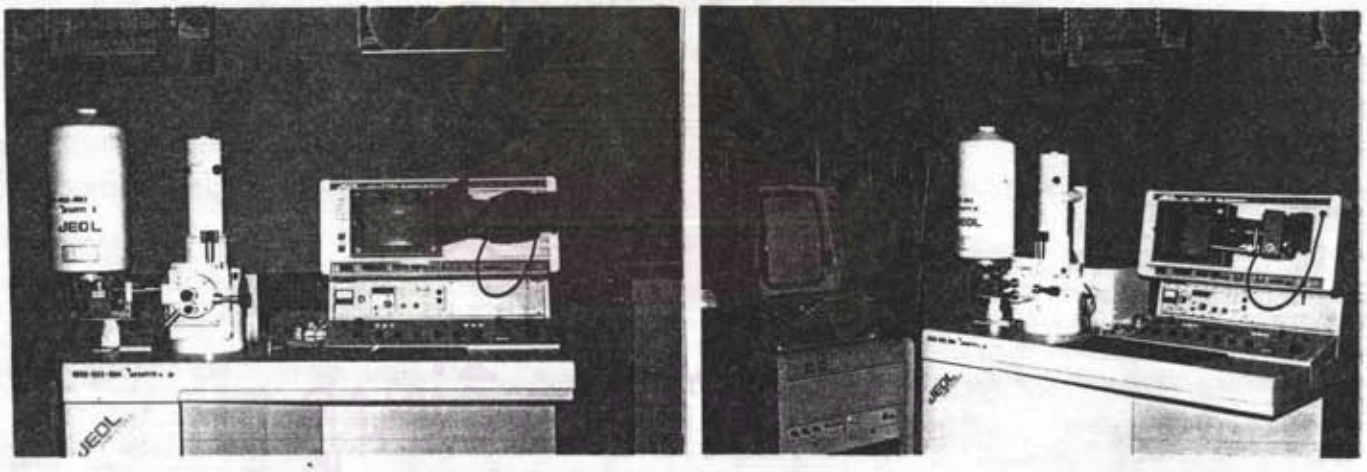


Fig. 3.7 FT-IR 1760X (Perkin-Elmer)



(a) (b)

Fig. 3.8 (a) SEM JEOL JAPAN
JSM - T220A
(b) SEM - EDX

3.4 Dissolution Testing

Dissolution testing of HA derived from cattle bone ash was designed by incubating of HA specimens (MP, TP) in simulated body fluid (SBF) of which composition was shown in Table 3.1. The fluid was prepared by dissolving reagent or analytical grade chemicals of NaCl, $NaHCO_3$, KCl, K_2HPO_4, $MgCl_2\ 6H_2O$, and $CaCl_2$ into deionized-distilled water. It was buffered at physiological pH 7.4 at 37°c with 50 mM trishydroxymethyl aminomethane $[(CH_2OH)_3\ CNH_2]$ and 45 mM hydrochloric acid (HCl). The fluid was reported to be capable of reproducing the process of bone-like apatite formation on the surfaces of various kind of bioactive glasses and glass-ceramics occurring *in vivo* (Li et al., 1994). After being soaked in SBF at 37°c for every 7 days, specimens were taken out of the fluid and cleaned in deionized-distilled water, and finally dried at ambient temperature for analysis.

3.4.1 Preparation of the Simulated Body Fluid (SBF)

SBF and Tris-buffer solutions were prepared from reagent-grade chemicals and deionized-distilled water. SBF contained the following ingredients, as shown in Table 3.1.

Table 3.1 Composition of Simulated Body Fluid (Kangasniemi et al., 1993).

Reagent-grade chemicals	Ingredients (mmol/l)
NaCl	137.8
$NaHCO_3$	4.2
KCl	3.0
K_2HPO_4	1.0
$MgCl_2 \cdot 6H_2O$	1.5
$CaCl_2$	2.5

One millilitre of formaldehyde per 1 litre of solution was added in order to prevent the growth of algae or bacteria (Scholze and Conradt, 1987). Solution was buffered with 50 mM trishydroxymethyl aminomethane and 45 mM hydrochloric acid (5:5 ml) and adjusted to pH 7.40 $\pm$ 0.05 with 95:5 by volume of N_2 : CO_2 (from TIG (Thai Industrial Gas Co., Ltd.), as shown in Fig. 3.9.

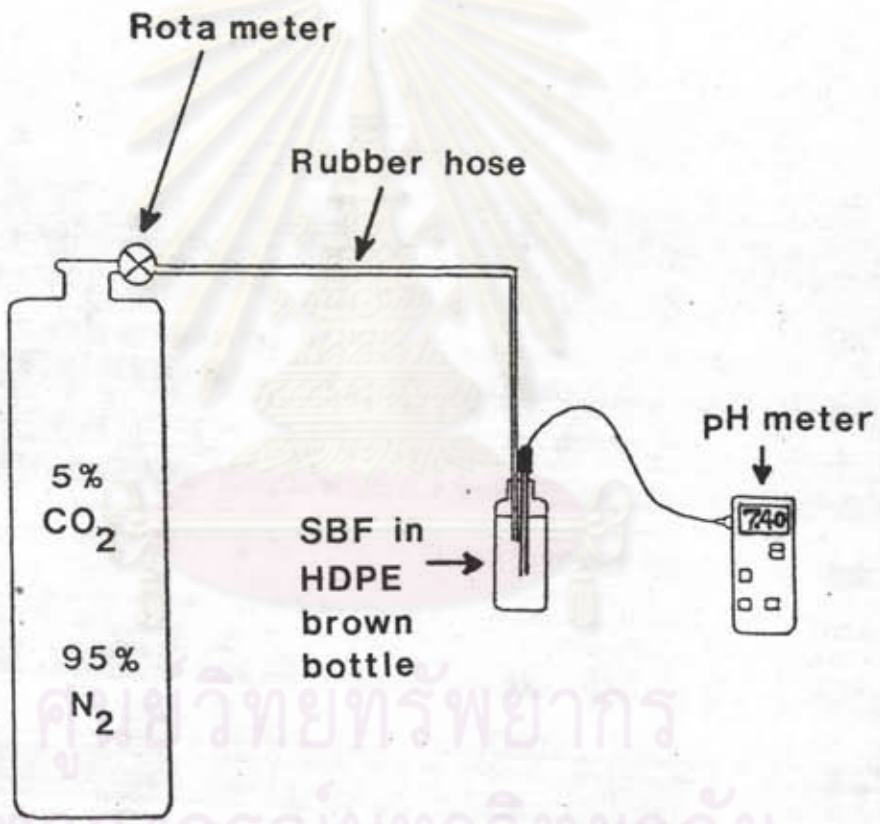


Fig. 3.9 N_2 - CO_2 flow system.

To find a time requirement for rebubbling the mixture of gas, the pre-test was set up by bubbling N_2 : CO_2 (pressure of gas was measured by rota meter : metal ball was on scale no. 2). The starting pH of SBF was 8.20 $\pm$ 0.05. To adjust pH by bubbling the mixture of gas for 10 min., pH changed to 7.40 $\pm$ 0.05. Changing of pH in SBF at 37°c was shown in the following figure.

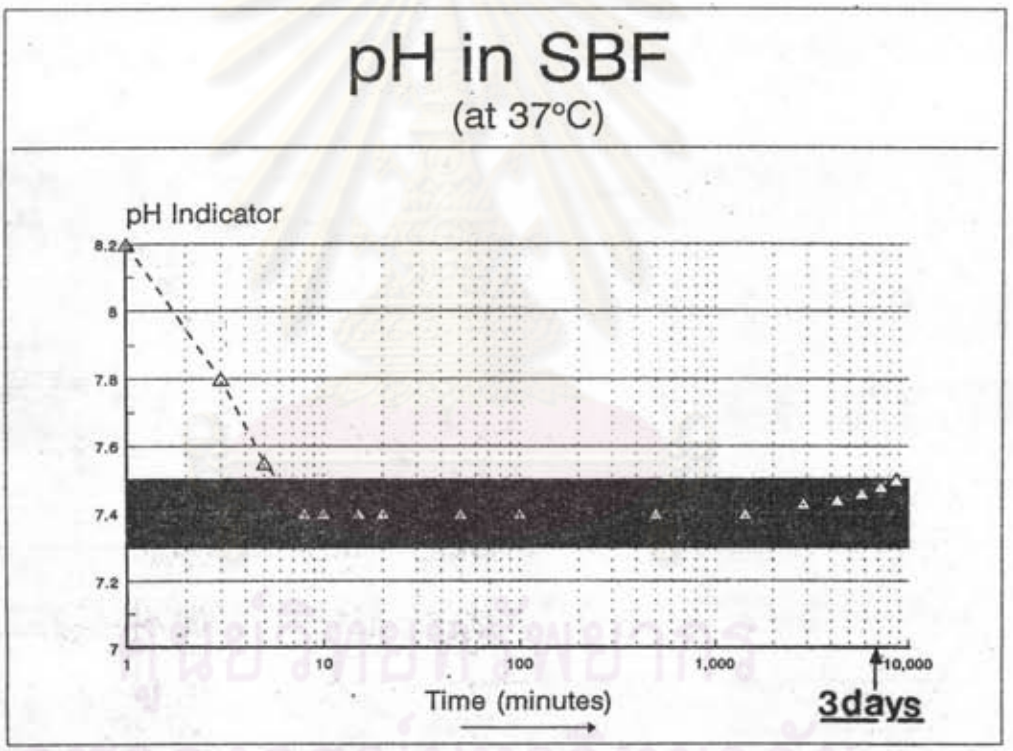
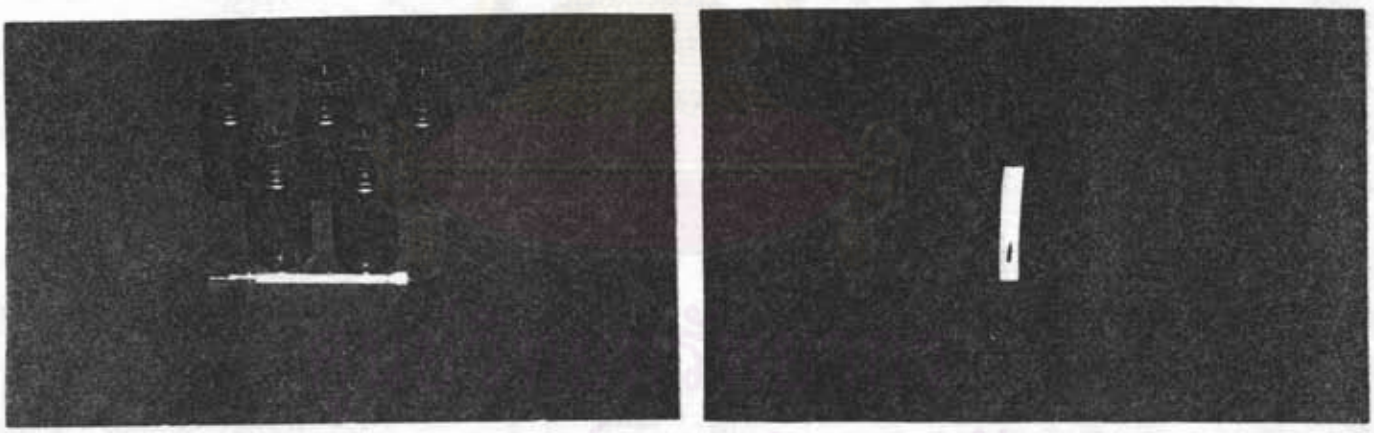


Fig. 3.10. Changing of pH in SBF at 37°c when bubbling with N_2 : CO_2

From Fig. 3.10, it could be summarized that the proper time for rebubbling the mixture of gas to maintain pH of SBF at 7.40 $\pm$ 0.05 was 3 days.

3.4.2 Container, Support

In this experiment, brown HDPE bottles and white HDPE plates were selected for the container and the support respectively. The reason for using two kinds of HDPE were reported by Geasee (1993) that they had high resistivity to chemical attack when tested in 1M NaCl at 37 °c and 90 °c for 7 days, furthermore the brown HDPE bottle could prevent daylight. A shape of support was designed which allowed good access of the solution from all sides of the sample. The support was put in a bottle as shown in the following figure.

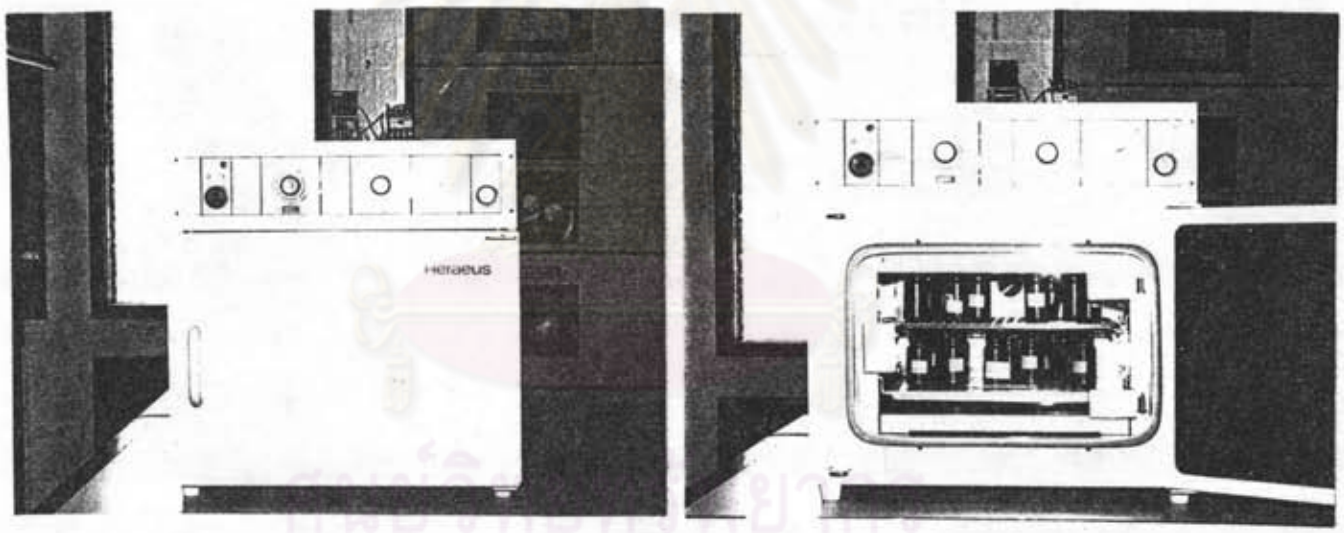


(a) (b)

Fig. 3.11 (a) Container (Nalgene, brown HDPE bottle
(b) Support (white HDPE plate)

3.4.3 Incubator

Heraeus Incubator (type B6030) was chosen for this experiment. The chamber was equipped with 2 levels, as shown in the following figure. For the experiments, a highly accurate long-term temperature control was required. Temperature controller and thermostat were installed in order to control temperature at 37 $\pm$ 1 c. To make double sure that this was the correct temperature, a medical thermometer was used (Geasee, 1993).



(a) (b)

Fig. 3.12 Heraeus Incubator (type B6030)

(a) Outside

(b) Inside

3.4.4 Dissolution Model

After two kinds of HA specimens derived from cattle bone were already cut and cleaned, these samples were fixed in HDPE white support (Fig. 3.13). The SBF solution was put into the HDPE brown bottle by volumetric pipettes. The volume of solution in each bottle was limited by the factor of sample surface to leachant volume (S/L) = 0.1 cm^{-1} (Geasee, 1993). The surface area of disc-shape samples could be calculated by the following equation.

surface area of disc-shape = $2\pi r^2 + 2\pi rh$

In the equation, r and h indicated the radius and the thickness of disk-shape sample respectively (r and h were measured by vernier - caliper).

The mixed gas N_2 : CO_2 = 95:5 was bubbled into the bottle until the pH meter (Fig. 3.14) showed 7.40 (7.40 $\pm$ 0.05) for SBF solution. The bottles which contained the specimens in SBF, pH = 7.4 were kept at 37 $\pm$ 1 c in the Heraeus incubator usually for 90 days. The temperature was checked by a medical thermometer everyday. The mixture of gas (N_2 : CO_2) was bubbled every 3 days, and the solution was renewed every 7 days. After various exposure periods, the samples were removed to examine and the solution changes were evaluated by measurement of Ca, and P.

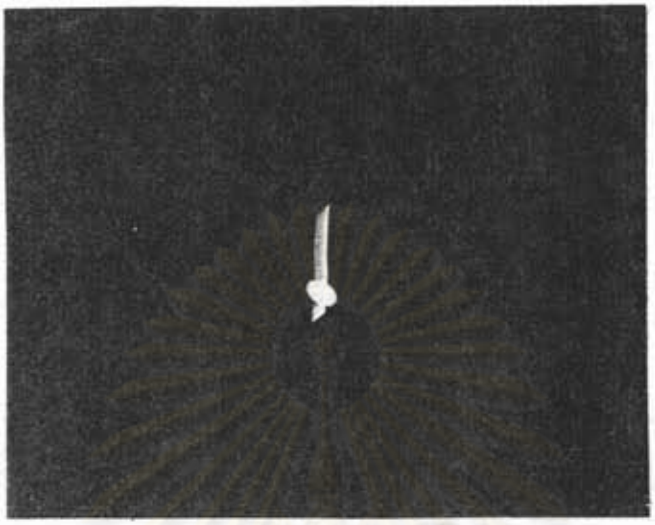


Fig. 3.13 HA sample was fixed in HDPE white support

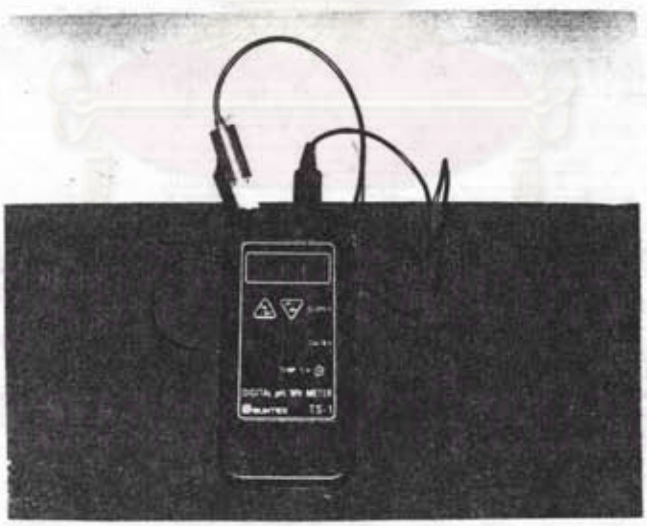


Fig. 3.14 pH meter (Suntex TS-1)

NB : The supports, bottles, pipettes were sterilized in hot water to kill all microorganisms and later on they were cleaned with ethyl alcohol before using.

3.5 Characterization of Tested Specimens and the Solution

3.5.1 Evaluation of the Ca, P Changes in SBF.

Every 7 days, the specimens were removed, and solution changes were evaluated by measurement of Ca and P. The concentration of Ca and P was determined by inductively coupled plasma (ICP) emission spectroscopy (Plasma 1000, Perkin Elmer in Fig. 3.15) before and every 7 days after immersion of the specimens. Fluid (5 ml) was pipetted from the bottle and diluted to 25 ml by deionized-distilled water in volumetric flask for ICP measurement.

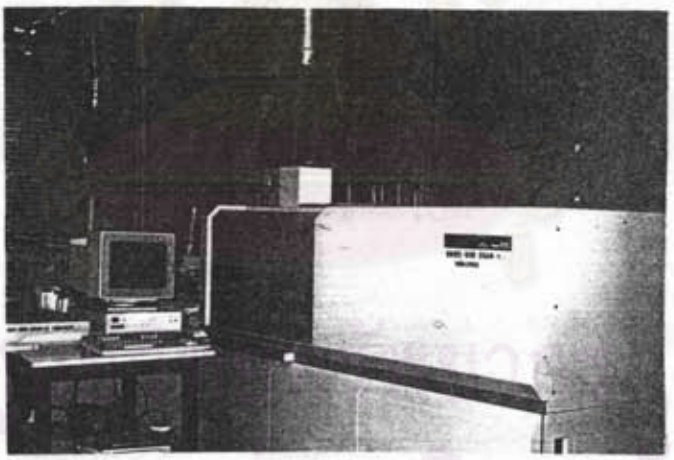


Fig. 3.15 ICP (Plasma 1000, Perkin Elmer)

3.5.2 Analysis of the Surface Changes

After the specimens were removed and dried in desiccator, the specimen surface was coated with a thin layer of gold and observed by SEM (JEOL JSM-T220A). Some of these specimens were dipped in deionized water for

1 hr. before drying and coating with a thin layer of carbon in order to remove the interfere ion species from SBF. Carbon - coated specimens were analyzed the elemental composition of the formed solid phase on the specimen surfaces by SEM-EDX.

3.5.3 Analysis of the Formed Solid Phase

Surfaces evidencing changes were subjected to XRD. The specimen and new formed solid phase were ground together in mortar in order to analyze by XRD (Philips PW 1730/10). The FTIR (Perkin - Elmer 1760X) was used for the reflection spectroscopy, the reflection angle being taken at 45. This technique enables to detect effectively about 0.5 - μm thick layer at the surface. The bulk specimens that covered with the formed solid can be used directly for the reflection spectroscopy.

3.5.4 Analysis of Ca, P and Impurities in Bulk Specimen

The quantities of Ca, P in bulk specimens were determined by EDTA chelate and molybdenum blue respecitvely. All impurities were determined by inductively coupled plasma - instrumental neutron activation analysis (ICP/INAA) method. (All analyses were done by Mineral Assays and Service Co., Ltd.)

3.5.5 Analysis of the BulK Density and Porosity of the Specimen

Bulk density of the specimen was performed by Archimedes method ASTM C373-72 (Reapproved 1982).