Chapter 2

MATERIALS AND METHODS

1 Chemicals.

L-leucine-^{3}H(G) from the Radiochemical Centre, Amersham, Buckinghamshire, England.

L-amino acid sigma grade from Sigma Chemical Company, St. Louis, U.S.A.

POPOP (1,4-bis [2-(5-phenyloxazole)] -Benzene, Phenyl-oxazolyl-phenyl-oxazolylphenyl); 2,5-Diphenyloxazole (PPO) scintillation grade from Sigma Chemical Company, St. Louis, U.S.A.

2-Mercaptoethanol, Trizma base reagent grade from Sigma Chemical Company, St. Louise, U.S.A.

Naphthalin scintillation grade from E. Merck, Darmstadt, West Germany.

Carboxy methyl cellulose (CM-cellulose) CM 23 fines reduced from Whatman Column Chromedia Balston Ltd, England.

Sephadex G-25, Sephadex G-100, CM-Sephadex C 50, DEAE-Sephadex A 50 from Pharmacia, Uppsala, Sweden.

Starch-hydrolysed from Connaught Medical Research Laboratories, University of Toronto, Toronto, Canada.

Other chemicals were of analytical grade from E.Merck, Darmstadt, West Germany, BDH Laboratory Chemical Division England, Mallinckrodt Chemical Works, St. Louis, U.S.A.

2 Instruments.

Starch-gel electrophoresis (Home made).

Sorvall RC2-B refrigerated centrifuge, Ivan Sorvall Inc. Newtown Connecticut, U.S.A.

Beckman DB-G Grating Spectrophotometer, Beckman Instruments Inc. Fullerton, California,U.S.A.

LKB Ultrolac Fraction Collector with UVICORD II for the continuous monitoring the UV absorption at 280 nm of the effluent, LKB-Productor AB Bromma 1 Sweden.

High Voltage Electrophoresis, Peschel Instrument Inc., Patterson N.Y., U.S.A.

Virtis Lyophillizer, The Virtis Co., Inc., Gardiner, N.Y., U.S.A.

Packard Tri-Carb Liquid Scintillation Spectrometer, Packard Instrument Company Inc., Illinois, U.S.A.

3 Subjects.

Subjects for incorporation studies are Thai and Chinese. Four non-thalassaemic subjects were healthy with normal haematological data and normal HbA_2. There were no history of HbH disease or Hb Bart's hydrops in their families. They were used in the study of the rate of globin chain production as normal controls.

Twelve cases of patients with a clinical disorder of thalassaemia syndrome were positive for intra erythrocytic inclusion bodies. This is compatible with a diagnosis of HbH disease. Five of them had haemoglobin types of HbA + HbH + HbA_2, but the remainders had HbA + HbH

and Hb Thai. The former is known to be a case of double heterozygote for α -thal$_1$ and α -thal$_2$ while the latter is a double heterozygote for α -thal$_1$ and Hb Thai.

Twelve asymptomatic subjects, including a neonate, who were diagnosed as a heterozygous Hb Thai were studied for the globin chain synthesis. The designation for a heterozygote was made on the basis of having Hb Thai (either X or X and Y bands) in addition to the HbA and HbA$_2$ on starch-gel electrophoresis. The neonate had Hb Thai and 5.6% Hb Bart's besides the normal HbF and HbA.

Three others interesting cases included two cases of homozygous Hb Thai, and one case of double heterozygosity for α -thal$_2$ and Hb Thai presented with mild chronic haemolytic anemia and splenomegaly. Red blood cells revealed moderate thalassaemic changes. Their haemoglobin types were HbA + Hb Thai and trace Hb Bart's. The clinical and haematological findings of the three were similar to each other. The definite genotype designations were made by family studies. The three patients with members of their families were also examined.

All patients and members of the families investigated here were kindly supplied from Division of Haematology, Department of Medicine, Siriraj Hospital.

4 Reagents.

4.1 Reticulocyte saline solution

The solution was composed of 0.13 M sodium chloride (NaCl), 0.005 M potassium chloride (KCl), 0.0074 M magnesium chloride

hexahydrate ($MgCl_2.6H_2O$).

4.2 Amino acid mixture.

The L-amino acid were dissolved in saline solution with the following concentration:

2×10^{-3}M alanine	0.5×10^{-3}M arginine
2.85×10^{-3}M aspartic acid	0.4×10^{-3}M cysteine
8×10^{-3}M glutamine	5.3×10^{-3}M glycine
2.4×10^{-3}M histidine	1.1×10^{-3}M hydroxyproline
0.3×10^{-3}M isoleucine	1.8×10^{-3}M lysine
0.3×10^{-3}M methionine	1.6×10^{-3}M phenylalanine
1.4×10^{-3}M proline	1.65×10^{-3}M serine
1.7×10^{-3}M threonine	0.3×10^{-3}M tryptophan
0.8×10^{-3}M tyrosine	3.2×10^{-3}M valine

the suspension was heated to 100°C. After cooling the pH was adjusted to 7.75 with NaOH. The solution was not autoclaved because of the destruction of phenylalanine.

4.3 Dialysed plasma.

The plasma was dialysed at 4°C against three changes of 10 volumes in saline solution.

4.4 Reagent mixture.

The reagent mixture was composed of : 54.0 ml. of amino acid mixture; 2.7 ml. of a solution of 0.25 M magnesium chloried plus 10% glucose in saline ; 27.0 ml. of 0.164 M Tris-HCl, pH 7.75; 21.6 ml. of

1 X 10^{-2}M trisodium citrate in dialysed plasma ; and 32.4 ml. of 1 X 10^{-2}M sodium bicarbonate in dialysed plasma. The plasma was used for facilitated amino acid incorporation in reticulocyte (Borsook et al., 1957) and in marrow cells (Morell et al., 1958) and reduced lysis during incubation and washing of the cells.

4.5 Bray's solution.

Naphthalin (recrystalized)	60 g.
PPO	4 g.
POPOP	200 mg.
Methanol (AR)	100 ml.
Ethylene glycol (AR)	20 ml.

Make volume to 1 L. with dioxane.

5 Experimental Procedures.

5.1 Reticulocyte preparation.

About 50 ml of venous blood was drawn into a 50 ml. syringe which had been previously moistened with heparin. The blood was centrifuged at 5,000 rpm for 5 min, at 4°C in a Sorvall RC2-B refrigerated centrifuge. The plasma was then discarded. After washing three times with reticulocyte saline solution, the washed red cells were centrifuged at 15,000 rpm for 1 hour, at 4°C, and 5 ml of the upper layer of the packed cells inwhich the reticulocyte is enriched was collected for the incorporation study.

5.2 Marrow cell preparation.

About 1-2 ml of bone marrow aspiration was transfered into 10 ml of normal saline-heparin solution at 4° C. The sample was well shaken. After washing three times with normal saline, the marrow cells were ready for incorporation.

5.3 Haematological studies.

Routine haematological examinations were made by standard method (Dacie and Lewis, 1968). Haemoglobin concentration was determined by cyamethemoglobin method. The red cells were counted in electronic cell counter. Haematocrit was measured by a microhaematocrit technique. Intra-erythrocytic inclusion bodies were detected by mixing blood with brilliant cresyl blue or methylene blue solution (Na-Nakorn et al., 1965). The alkali resistant haemoglobin was quantitated by the one minute method of Singer et al. (Singer et al., 1951).

5.4 Preparation of haemolysate.

The packed red cells were haemolysed with equal volume of distilled water. Stroma was removed by centrifugation at 15,000 rpm for 30 min.

5.5 Biochemical analysis of haemoglobin.

5.5.1 Starch-gel electrophoresis. Using 15% hydrolysed starch in Tris-borate-EDTA buffer (0.09 M Tris,0.5M boric acid, 0.02 M EDTA) at pH 8.6 (Smithies, 1955, 1959). Electrophoresis was carried out at 220-300 V. in a cold room for 3-4 hours. The sliced starch-gel was stained with orthodianisidine.

5.5.2 DEAE-Sephadex chromatography. The separation of slow haemoglobin component such as HbA_2, HbE, Hb Thai were carried out by DEAE-Sephadex chromatography (Wasi et al., 1968). DEAE-Sephadex was well equilibrated in phosphate buffer (0.01 M $Na_2HPO_4.2H_2O$, 0.01% KCN) at pH 8.6. After applying the haemolysate, the slow haemoglobin was eluted with phosphate buffer (0.013 M $Na_2HPO_4.2\ H_2O$, 0.01% KCN) at pH 9.5. Finally the haemoglobin A was eluted with the same buffer.

5.5.3 Haemoglobin concentration. A large volume of haemoglobin solution was concentrated by loading on a small column of CM-Sephadex with 0.05 M Tris-maleic acid buffer at pH 6.5 (Schroeder et al., 1970). The haemoglobin solution obtained from DEAE-Sephadex was diluted with distilled water and adjusted to pH 6.5 with a molar maleic acid. The conductivity of the diluted haemoglobin solution must be equal to or less than equilibrated buffer before it can be applied to the CM-Sephadex column. The bounded haemoglobin on the column was eluted with a small volume of 0.05 M Tris-maleic acid buffer at pH 8.6.

5.5.4 Fractionation of haemolysate by Sephadex G-100 gel-filtration. Two to five ml of whole haemolysate was applied to a 3.5 X 120 cm Sephadex G-100 column previously equilibrated with a Tris-KCN buffer (0.1 M NaCl, 0.01 M Tris, 0.001 M KCN) at pH9.8, and eluted with the same buffer at a flow rate of 30 ml/hr. Each fraction contained about 6-7 ml. The optical density of the effluent was measured at 540 nm and 280 nm.

5.5.5 Preparation of globin. The globin was prepared by acid-acetone (2% HCl in acetone) precipitation of the haemolysate. The haem is soluble in the supernatant was discarded. The globin was

washed with cold acetone until the supernatant was clear and then dried under nitrogen gas or lyophilized.

5.5.6 Globin chain separation. The globin was separated on a 1 X 25 cm column of CM-cellulose with 8 M urea/mercaptoethanol buffer at pH 6.7 (8 M urea, 0.05 M 2-mercaptoethanol and 0.005 M disodium hydrogen phosphate) as a starting buffer, according to the technique of Clegg et al. (Clegg et al., 1966, 1968).

Approximately 60 mg of globin was dissolved in 4 ml of starting buffer. The globin solution was dialysed for 3 hours against two changes of the buffer. The dialysed globin was then applied onto the CM-cellulose column, previously equilibrated with the starting buffer. After washing the CM-cellulose column until unbound material was eluted, the globin chains were then fractionated by means of a linear Na^+-ion gradient (200 ml of starting buffer and 200 ml of 8 M urea in 0.05 M 2-mercaptoethanol, 0.03 M sodium hydrogen phosphate at pH6.7). Each fraction of 2-3 ml of eluent was then collected and its UV absorption at 280 nm was continuously monitered by LKB Fraction Collector with UVICORD II Spectrophotometer.

5.5.7 Peptide maping. After separation by CM-cellulose chromatography, the fraction corresponding to a particular globin chain were pooled and desalted on Sephadex G25 in 0.2 M acetic acid and the globin solution was lyophilized. The purified globin was digested with trypsin at the enzyme substrate ratio of 1:200 in 1% ammonium bicarbonate (NH_4HCO_3) buffer pH 8.3. The digestion was carried out at 37 °C for 2 hours. The digested globin was then diluted with distilled water and lyophilized 2-3 times to remove the salts.

About 3-5 mg of tryptic digested globin was applied to a 9 X 57 cm sheet of Whatman No. 3 filter paper. The sample was loaded 1 cm wide at the origin and 15 cm from the anode margin. High voltage electrophoresis was carried out in pyridine : acetic acid : water buffer (25:1:224 by volume) at pH 6.4, at 1900 volts, for approximately 1½ hours or until a methyl-green marker moved 33 cm from the origin. The unstained sheet from high voltage electrophoresis was then sewn onto a 46 X 57 cm sheet of Whatman No. 3 filter paper 6 cm from the edge. Descending chromatography was then performed perpendicular to the direction of the electrophoresis for 16 hours in butanol:acetic acid:water:pyridine system (15:3:12:10 by volume) (Waley and Watson, 1953).

5.5.8 Peptide staining. The peptide map was stained by dipping the chromatogram in 0.5% ninhydrin in acetone. The purple spots of peptides were developed after paper was dried or heated.

Sakaguchi staining for arginine was conducted as follows. The paper was immersed in solution A (0.1% 8-hydroxyquinoline in acetone), then dried and sprayed with a freshly prepared solution B(0.3% bromine (v/v) in 0.5 N NaOH). A transient orange spot was positive for arginine.

5.6 Incorporation studies.

5.6.1 Reaction mixture. The reaction mixture composed of 5 ml reticulocytes or packed marrow cells, 13.2 ml reagent mixture (Lingrel and Borsook, 1963), 0.75 ml ammonium iron sulphate [$(NH_4)_2Fe(SO_4)_2.6H_2$)] (10.5mg/ml 10 ml saline) and 0.1 ml penicillin and streptomycin (each

60 mg/ml in saline). Each component was added sequently into 250 ml Erlenmeyer flask.

5.6.2 Incubation procedure. The flask containing the reaction mixture was incubated in a water bath at 37°C. After incubation of 15 min. 1 ml of ^{3}H-leucine (100-250 µCi/m mole) was added. The flask was gently shaken every 15 min. After 3 hours of incubation the reaction mixture was transfered into 2 volumes of ice cold normal saline and centrifuged at 5,000 rpm, for 5 min. at 4°C. The supernatant was discarded and the cells were washed three times with cold normal saline. The packed red cells were separated into portions and kept in a freezer.

5.7 Measurement of radioactivity.

The fractions containing globin from CM-cellulose chromatography were separately pooled and desalted by dialysing against four changes of 0.05% formic acid for 48 hours. The optical density of the dialysed globin was determined at 280 nm. in a Beckman DB-G Grating Spectrophotometer. One ml of dialysed globin solution was pipetted into a counting vial and 10 ml of Bray's solution was added (Bray, 1960). The radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer. The total count was obtained by multiplying the count per min./ml by its total volume. The specific activity was obtained by dividing the count per min./ml by its optical density.

Experimental Protocol

The investigation can be divided into three parts

Part 1: Comparative studies of haematology and globin chain synthesis in normal and various types of the patients.

1 Haematological studies and globin chain synthesis in normal controls.

2 Haematological studies and globin chain synthesis in Hb H disease (α-thal$_1$/α-thal$_2$)

3 Haematological studies and globin chain synthesis in Hb H disease with Hb Thai (α-thal$_1$/Hb Thai)

4 Haematological studies and globin chain synthesis in Hb Thai trait.

5 Haematological studies and globin chain synthesis in α-thal$_2$/Hb Thai disease.

6 Haematological studies and globin chain synthesis in homozygous Hb Thai.

Part 2: Studies on haemoglobin components in haemolysate of Hb H disease with Hb Thai.

Part 3: Studies on biosynthesis of haemoglobin Thai.