Chapter 2

MATERIALS AND METHODS

Chemicals

List of chemicals in this experiment

- Amino acids from Sigma Laboratory grade, U.S.A.
- 2, 5 diphenyloxazole and p-bis [2, (5-phenyloxazole)] - benzene scintillation grade from Sigma Laboratory, U.S.A.
- ^{3}H leucine from the Radiochemical Centre Amersham, England
- Naphthalene, dioxane ethylene glycol, methanol, pyridine, n-butanol were reagent grade from E. Merck, Darmstadt, West Germany.
- Other chemicals were reagent grade from BDH Laboratory Chemical Division, England, E. Merck, Damstadt, West Germany; and Sigma Laboratory, U.S.A.

Instruments

1) Sorvall, Super speed RC 2-B automatic refrigerated centrifuge.
2) LKB Fraction collector with Uvicord I for the continuous monitoring the U V absorption at 280 nm of the effluent.
3) Spectrophotometer (Beckman DBG spectrophotometer).
4) High voltage electrophoresis.
5) Starch gel electrophoresis.

6) Lyophilizer.

7) Packard Tricarb Liquid Scintillation spectrometer Model-3390.

Reagents

Saline solution (NKM isotonic solution)

The solution contains 0.13 M NaCl

0.005 M KCl

and 0.0074 M $MgCl_2 . 6H_2O$

Amino acid mixture

The amino acid mixture was made up in NKM solution by using L-form amino acids with the following concentrations.

Amino acids	Concentration (M x 10^{-3})
Alanine	2.0
Arginine	0.5
Aspartic acid	2.85
Glycine	5.3
Histidine	2.4
Isoleucine	0.3
Methionine	0.3
Lysine	1.8
Phenylalanine	1.6
Proline	1.4
Serine	1.65

Amino acids	Concentration ($M \times 10^{-3}$)
Threonine	1.7
Tryptophan	0.3
Tyrosine	0.8
Valine	3.2
Hydroxyproline	1.1
Cysteine	0.4
Glutamine	8.0

The amino acids were dissolved in water at 100°C. After cooling the pH was adjusted to 7.75 with NaOH. The amino acid solution was divided into small portions and kept in freezer until used.

Dialyzed plasma

The human plasma was dialyzed at 4°C against ten volumes of NKM solution for four hours or more before using in the incorporative study.

Reagent mixture

The composition of reagent mixture was made up as follow.

Amino acid mixture	54	ml.
0.25 M. $MgCl_2$ and 10 % glucose in saline solution	2.7	ml.
0.164 M. tris HCl pH 7.75	27	ml.
10^{-2}M trisodium citrate in dialyzed plasma	21.6	ml.
10^{-2}M sodium bicarbonate in dialyzed plasma	32.4	ml.

Bray's solution

Naphthalene	60	gm.
2,5 diphenyloxazole (PPO)	4	gm.
p-bis[2(5-phenyloxazole)]-benzene (POPOP)	200	mg.
Methanol	100	ml.
Ethylene glycol	20	ml.
Dioxane to make	1000	ml.

Subjects

Subjects were Thai and Chinese. Seven healthy persons with normal hematologic data and normal Hb A_2, which were believed to represent non-thalassemic subjects, were studied for globin chain synthesis as controls.

Thalassemic patients and members of their families which had been investigated in this study were kindly supplied from the Division of Hematology, Department of Medicine, Siriraj Hospital.

Six thalassemic patients with clinical features of chronic hemolytic anemia, hepatosplenomegaly and hemoglobin types of E+F (Figure 5) were diagnosed as classical β-thalassemia/Hb E disease.

Other five thalassemic patients were clinically similar to the previous group but the hemoglobin types showed E+F+A (Figure 5) without a history of blood transfusion. The latter were compatible with a designation of mild β-thalassemia/Hb E disease.

Nine subjects with an elevation of Hb A_2 were either parents or offspring of the classical β-thalassemia/Hb E disease families. They were considered as a case of obligatory β-thalassemia$_1$ trait.

In the same manner, another nine obligatory β-thalassemia$_2$ traits from the mild β-thalassemia/Hb E families were also examined.

Six heterozygous Hb E (Hemoglobin type A+E) from the members of thalassemia families were also incorporatively studied.

Reticulocyte preparation

Fifty ml. of heparinized venous blood obtained from a patient was spun in a refrigerated centrifuge in a polyethylene tube for 5 minutes at 5000 rpm to remove plasma and then washed three times with cold isotonic NKM solution (Borsook et al. 1957). The packed red cells were suspended in one to two volumes of cold NKM solution and spun at 15,000 rpm for one hour. The supernatant was removed by suction. The 5 ml upper layer of the packed cells which enriched with reticulocytes was carefully removed by pasteur pipette and then the reticulocyte sample was readily used for incorporative study. The left packed red cells were used in other studies.

Methods

Hematologic studies

A heparinized peripheral blood sample was used in the hematologic studies. Standard hematologic techniques were employed

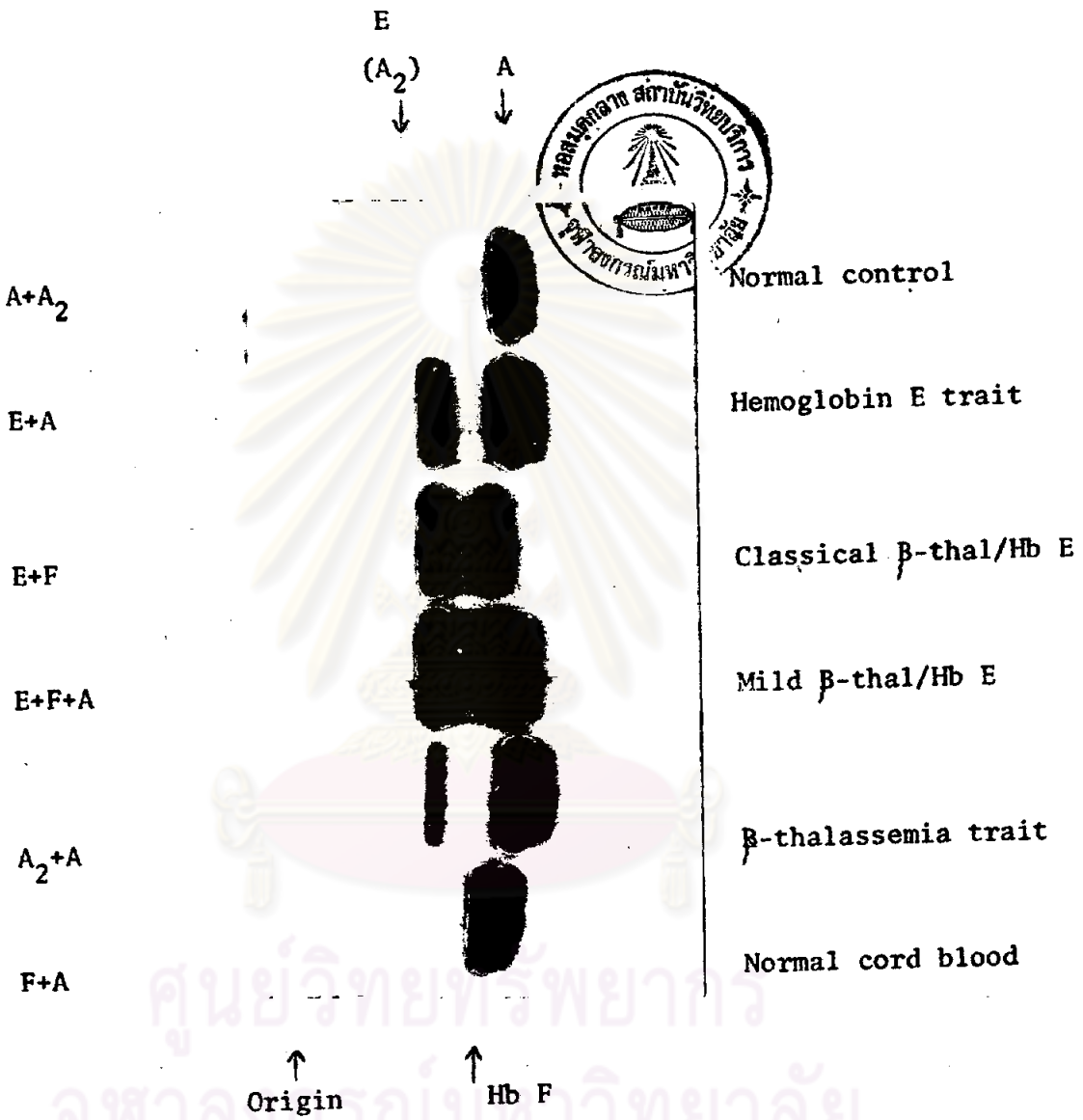

Figure 5. Starch-gel electrophoresis in tris-EDTA-borate buffer, pH 8.6, with orthodianisidine stain, demonstrating hemoglobin types in various genotype designations.

(Dacie & Lewis, 1968). Hemoglobin concentration was measured by cyamethemoglobin method (Dacie & Lewis, 1968). The red cell count was carried out in Coulter electronic cell counter. Packed cell volume (PCV) was obtained by microhematocrit method (Dacie & Lewis, 1968). The red cell osmotic fragility was determined on Danon's fragiligraph (Danon 1963). Hb A_2 and Hb E was quantitated by DEAE Sephadex-chromatography. The normal Hb A_2 by this method in our laboratory is 2.65 ± SD 0.28 % (Pootrakul et al. 1973). Hb F was quantitated by 1 minute alkali denaturation technique (Singer et al. 1951). The alkali resistant hemoglobin in a normal adult is less than 1 %.

Hemoglobin starch gel electrophoresis

The packed red cells were washed three times with isotonic saline. Hemolysate was prepared by lysing packed red cells with two volumes of distilled water and one volume of toluene. After vigorous shaking the mixture was centrifuged and filtered. The clear hemolysate was applied to horizontal starch gel electrophoresis in tris-EDTA borate pH 8.6 according to Smithies (1959). The gel was sliced and stained with orthodianizidine.

Incubation procedure

The reaction mixture was made by sequently adding the following

1)	packed reticulocytes	5 ml.
2)	reagent mixture	13.2 ml.
3)	0.105 % $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.75 ml.

4) 6 % penicillin and streptomycin in NSS 0.1 ml.

5) ^{3}H-leucine 200 uci/ml in NSS 1 ml.

This reaction mixture was transfered to an erlenmayer flask of ten times in volume and incubated in a waterbath at 37°C with frequent shaking for three hours. At the end of the incubation, the incubation mixture was centrifuged. The packed red cells were washed three time with NKM solution.

Preparation of globin

The wash-packed cells from incubation study were lysed by four volumes of distilled water. The hemolysate was centrifuged at 15,000 rpm for 20 minutes to remove cell-stroma. Globin was prepared from the clear hemolysate by acid acetone precipitation (Fanelli et al. 1958). The precipitated globin was washed with 4 times of cold acetone. The washed globin was then lyophilized and stored at 4°C.

Separation of globin chains

The globin chains were separated by a column chromatography on carboxymethyl cellulose (CMC) at pH 6.7 according to the method of Clegg et al. (Clegg et al. 1965) with slight modifications. Sixty mg. of lyophilized globin was dissolved in 6 ml of starting buffer (8 M. urea, 0.05 M 2-mercaptoethanol, and 0.005 M. Na_2HPO_4, which was adjusted to pH 6.7 with phosphoric acid). The globin solution was dialysed at room temperature against the same buffer

for three hours.

The CMC (CM 23-Whatman) was suspended in starting buffer and adjusted to pH 6.7 with NaOH. The suspension was allowed to precipitated and the supernatant was decanted. The precipitated CMC was resuspended in starting buffer and packed in a 1 x 20 cm column. The column was equilibrated with starting buffer. After applying the dialized globin, the column was washed with starting buffer in order to remove unbound materials until the absorbance and conductivity was returned to that of the starting buffer. The effluent was monitored continuously at 280 nm and collected with the LKB Fraction collector with Uvicord I. The globin chains were eluted by a linear Na^+ ion gradient made by mixing 200 ml of starting buffer and 200 ml of the same buffer with 0.5 M. Na_2HPO_4. Two ml per fraction was collected. The actual optical density of each tube was finally determined at 280 nm with a Beckman DBG spectrophotometer.

Measurement of globin chain synthesis

The fractions corresponding to each globin chain were pooled and dialyzed against four changes of 100 fold excess of 0.5 % formic acid at $4^{o}C$ for four hours. The optical density and total volumes of each globin chain was measured and then one ml aliquot of each globin chain was added to 10 ml of Bray's solution in a vial. The sample was counted in Packard Tricarb Liquid scintillation spectrometer counter with 50 % efficiency for ^{3}H counting.

The specific activity was computed as counts per minute per absorbance unit (cpm/min/OD)

The radioactivity of each peptide chain was determined by multiplying the 1 ml aliquot radioactivity with total volumes of that pooled globin chain.

The absorbance of β-chain exceeds that of α chain by the factor of 1.52 (Kan et al. 1968). An appropriate correction for this absorbtion difference at 280 nm was made in calculating the specific activity.

Peptide mapping

The corresponding fractions of normal β chain (β^A) and abnormal β chain (β^E) were pooled and desalted by passing the solution through a column of sephadex G-25 which was previously equilibrated with 0.2 M. acetic acid. The effluent was monitored at 280 nm, with the LKB Fraction collector and Uvicord II, and the desalted globin chain was lyophilized, and stored at 4°C.

The lyophilized globin chain was digested with trypsin in 1 % NH_4HCO_3 buffer, pH 8.3 by dissolving the globin chain in water (20 mg/ml). An equal volume of 2 % NH_4HCO_3 was then added, this leads to some precipitation. The suspension was digested by adding trypsin (Worthington Biochemical Corporation) in concentration of 1/200 of enzyme-substrate ratio. The digestion was carried out at 37°C for 2 hours.

Two dimensional chromatography was performed by high voltage electrophoresis and descending chromatography. Four milligrams of tryptic peptides were applied on a strip of Whatman No 3 paper, of

which the applied origin was 16 cm from the anode margin. The first dimension of high voltage electrophoresis at pH 6.5 in pyridine-acetic acid-water system (25:1:225) by volume was run until the dye marker, methyl green, reached 33 cm from the origin. The unstained strip paper from high voltage electrophoresis was then sewn into another fresh sheet and separated at right angle by descending chromatography in n-butanol-acetic acid-water-pyridine buffer with the proportion of 15:3:12:10 by volume respectively for 15-18 hours. Finally the chromatogram was stained with ninhydrin for the visualization of the tryptic peptides. The peptide maps of β^E which were obtained either from classical β-thalassemia/Hb E or from mild β-thalassemia/Hb E disease patients were studied for comparison with normal tryptic peptides of β-chain.

Peptide maps staining with ninhydrin

A dry chromatogram was stained with 0.5 % ninhydrin in acetone for peptides visualization. The paper was dipped in the ninhydrin solution and allowed to dry at room temperature or may be accelerated by heating in the oven at 60°C for 15 minutes.