Chapter 4

DISCUSSION

Globin chain synthesis had been reported by several authors (Heywood et al., 1965; Weatherall, 1965; Bank and Marks, 1966; Clegg and Weatherall, 1967; Kan et al., 1968; Modell et al., 1969; Nathan, 1972). They found that the value of α/β ratio of globin chain synthesis in normal controls is nearly equel to 1. In this observation it was found that α/β ratio in normal control is 1.08±0.02. The results indicate an equally synthesis of α- and β-chains in normal adult. In normal cord blood inwhich HbA and HbF are the major haemoglobins, the $\alpha/\beta+\gamma$ ratio was the same as in normal adults.

1 Globin Chain Synthesis in HbH Disease.

It has been previously shown that the range of α/β ratio in globin chain synthesis in HbH disease (α-thal$_1$/α-thal$_2$) was 0.3-0.6 (Clegg and Weatherall, 1967; Kan et al., 1968; Nathan, 1972). In this investigation, the means of α/β ratio of HbH disease (α-thal$_1$/α-thal$_2$) and HbH disease with Hb Thai (α-thal$_1$/Hb Thai) were 0.57±0.15 and 0.70±0.14 respectively. Although the α/β ratio of the latter was higher than that of the former, the haematological means of the two groups were very similar (table 2, p 21 and table 3, p 23). The marked decrease of α-chain leads to an excess of β-chain which would polymerized to be β_4-HbH. Since the interaction of either α-thal$_2$ or Hb Thai with α-thal$_1$ cause a clinical disorder of HbH disease, it suggests that expression

of Hb Thai gene is equivalent to that of α-thal$_2$ gene.

2 α-thal$_2$/Hb Thai and Homozygous Hb Thai Syndromes.

The described genetic evidence shown in the pedigree of R.P. family (figure 4, p 29) indicated that the mother I-2 inherited α-thal$_2$ gene and her haematological data (table 6, p 30) showed mild hypochromic microcytic red cells. Furthermore, the radioactivity α/β ratio was 0.98, which was compatible with the designation of α-thal$_2$ trait (the α/β ratio of obligatory case of α-thal$_2$ trait studied by Pootrakul et al., 1974 ranged 0.86-0.98). The II-3 was slightly anemia with moderate hypochromic microcytic red cells. Spleen was just palpable. Inclusion body of red cells was negative. The haemoglobin electrophoresis showed Hb Thai and trace Hb Bart's besides HbA. Since the haematological data of the II-3 was distinguished from HbH disease or Hb Constant Spring trait, it was most likely that the genotype assignment of the II-3 was α-thal$_2$/Hb Thai which the abnormal genes were transfered from the mother and father respectively. The radioactivity α/β ratio of the patient II-3 was 1.09 which was higher and was different from the HbH disease either with or without Hb Thai.

The patient II-2 of the family N.K. (figure 5, p 32) presented the clinical and haematological data including haemoglobin starch-gel electrophoresis similar to that of the α-thal$_2$/Hb Thai syndrome. The haematological data and radioactivity α/β ratio of both parents are 1.4 (table 4, p 24) which indicating heterozygous Hb Thai. Therefore, the II-2 was presumably a homozygous Hb Thai. Although the clinical and haematological data including Hb typing of the α-thal$_2$/Hb Thai and

homozygous Hb Thai were very similar while the radioactivity α/β ratio were 1.09 and 1.66 respectively. It is no doubt that the incorporative study would enable one to distinguish the genotype assingnments between the two phenotypes.

The patient II-1 of the family P.V. (figure 6, p 32) was suffered from mild jaundice for years. Her haematological data and haemoglobin types of Hb Thai + A + trace Hb Bart's suggested the genotype designation either α-thal$_2$/Hb Thai or homozygous Hb Thai. Hb Thai of both parents was undetectable on starch-gel. It was cleared, after determining the globin chain production, that the α/β ratio of the patient was 1.70 which was compatible with homozygous Hb Thai, while that of the parents were 1.34 and 1.35 respectively, the results indicated that both parents were Hb Thai trait which the slow pigment escaped detection on starch-gel electrophoresis (Wasi et al., 1972).

Another patient S.P. also presented a phenotype resembling the α-thal$_2$/Hb Thai syndrome, but the α/β ratio was 1.52 suggesting a genotype designation of homozygous Hb Thai.

3 Delta-Chain in HbH Disease with Hb Thai.

The absence of a haemoglobin component-corresponding to HbA$_2$ on starch-gel electrophoresis at alkali pH in a patient with HbH disease with Hb Thai (figure 8.2, p 36) has been observed in Division of Haematology, Siriraj Hospital for years. The isolated slow haemoglobin fractions from the whole haemolysate of the patient by DEAE-Sephadex chromatography also revealed no observable HbA$_2$ on starch-gel electrophoresis (figure 8.4, p 36). The globin chain synthetic studies in

reticulocytes and in bone marrow of the patient (figures 9 and 10, p 38, 39) showed a small radioactive peak corresponding to δ-chain which was present in both studies. Since HbA_2 consists of two α-chains and two δ-chains as the formular of $\alpha_2\delta_2$, it was believed that the δ-chain in the patient did not join to α-chain forming $\alpha_2\delta_2$-HbA_2. This was indicated by the absence of HbA_2 band on starch-gel. The δ-chain in the patient was possibly present as a tetramer δ_4, migrating at the same position as HbA (Dance et al., 1963). The explanation of the δ_4 formular was that the α-chain, which was decreased in synthesis due to α-thalassaemia, had a greater affinity to β-chain than that to δ-chain (Weatherall, 1969) and the free δ-chain polymerized to be δ_4. The presence of δ-chain was unlikely as monomer or dimer since the preparative CM-cellulose chromatography of globin prepared from haemolysate after Sephadex G-100 gel-filtration showed a small peak corresponding to δ-chain (figure 11, p 40). Furthermore the peptide mapping corresponding to δ-chain of the patient was also studied in order to identify the globin chain being δ-chain by comparing with the normal β-chain (figure 12, p 41). From the diagram of amino acid sequences of β- and δ-chain is shown in figure 23, p 64, it can be seen that the tryptic peptide of both chains are very similar. However, the key peptide to distinguish between the β- and δ-tryptic peptide maps is the tryptic peptide No. III (Tp III) (Dance et al., 1963) which is positive for arginine staining. δTp III has more positive net charge than βTp III, since amino acid residue 22 of β-chain is glutamic acid while that of the δ-chain is alanine. The tryptic peptide map of the corresponding δ-chain from a patient of HbH disease with Hb Thai, as compared with

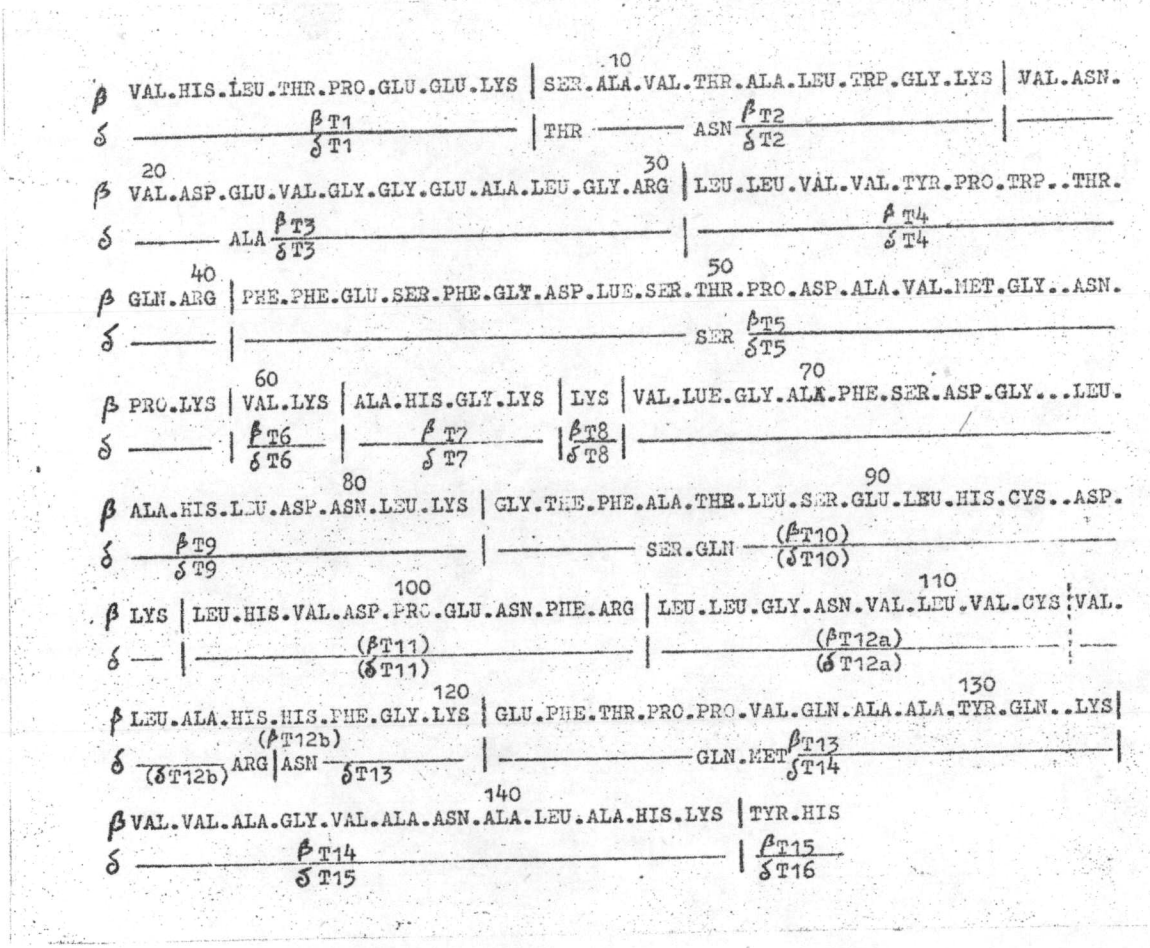

Figure 23 The amino acid sequence of β- and δ-chain. The part of the δ-chain which were identical to those of the β-chain, are indicated by drawn lines. Trypsin-sensitive bonds are indicated by vertical bars; the dotted bars represent the bonds which can be split only after aminoethylation. The peptide numbers in parentheses indicate the "core" peptides (De Jong, 1969).

normal β-chain (figure 12, p 41), was compatible with δ-chain.

Based on the present available evidence, the synthesized δ-chain in Hb H disease with Hb Thai is presumably not either combined with α-chain to form $\alpha_2\delta_2$ - Hb A_2 or was present in a monomer or dimer. It may be found as a tetramer δ_4 or in an unusual haemoglobin molecule.

4 Studies on Sephadex G-100 Chromatography of Bone Marrow Lysate in Hb H Disease with Hb Thai.

The chromatogram of bone marrow lysate from Hb H disease with Hb Thai fractionated by Sephadex G-100 filtration showed two haem protein peaks corresponding to pooled fractions of A and C (figure 13, p 43). Fraction B was the overlap of peaks A and C. CM-cellulose chromatography of globin from fraction A which essentially contained Hb A (figures 14 and 15, p 44, 46) shows that radioactivity incorporated into α-chain was greater than that into β-chain and the α/β ratio was over 3. Since the means of α/β radioactivity ratio of the whole haemolysate in the patient was 0.70 ± 0.14, as already shown in figure 7, p 34, the discrepancy of the α/β ratio between the purified Hb A and whole haemolysate indicated a presence of β-chain pool. The newly synthesized α-chain which was labelled with ^{3}H-leucine would readily join to the non radioactive β-chain in the pool to form Hb A. This study also confirmed the previous report of the existence of β-chain pool in Hb H disease as described by Clegg and Weatherall, (1967).

Starch-gel electrophoresis at pH 8.6, of haemolysate from fraction C (figure 14, p 44) showed a major fast component corresponding to Hb H and minute amounts of haemoglobin component corresponding to Hb A. The CM-cellulose chromatography of globin prepared from fraction C with Hb A as carriers revealed only a radioactive peak at β-chain (figure 17, p 48). No radioactivity was observed at α-chain. This implied that the fast haemoglobin component-Hb H fraction consisted of only β-chain. Since the fraction C which contained Hb H was eluted after fraction A-essentially represented Hb A on Sephadex G-100 chromatography (figure 13, p 43). It suggested that the fast component had a smaller molecule than Hb A and was believed to be a monomer of β-chain. This was consistent with the fact that the unstable tetramer of β-chain (Hb H) dissociated into dimer and finally into monomer during the process of chromatography.

It is of interest to discuss the presence of small amounts of haemoglobin component corresponding to Hb A in fraction C (figure 14, p 44). It was unlikely to be a contaminant Hb A because the pooled fraction C was apparently separated from fraction A which contained Hb A. Since the radioactivity incorporated into α-chain of Hb A was remarkably high when compared with β-chain (figure 15, p 46), a small radioactive peak at α-chain should be observed if only small amount of Hb A was presented. Infact, no radioactivity at α-chain was observed (figure 17, p 48). Dance and his co-workers (1963) described excess δ-chain, like excess β-chain forming Hb H, in a patient with Hb H disease. Presumably the excess δ-chain was a monomeric state which was similar to the fast component of Hb H, it was no doubt that

the two components would come off in the same fraction on Sephadex G-100 chromatography. Thus the haemoglobin component migrating at Hb A in starch-gel analysis of fraction C (figure 14, p 44), as a matter of fact, was an monomer of δ-chains. This was consistant with the studies by Dance et al., (1963) reported that the excess δ-chain had electrophoretic mobility the same as Hb A in starch-gel at pH 8.6.

Sephadex G-100 chromatography of fraction B as shown in figure 14, p 44 contained almost of Hb A, slow haemoglobin-Hb Thai and a very small Hb H. The CM-cellulose chromatography of globin from this fraction with carrier-Hb A showed that two small radioactivity peaks following the normal α-chain peak. These were presumed to represent the abnormal chains of Hb Thai (figure 16, p 47). How can one explain the decreased radioactive incorporation in abnormal α -chain of Hb Thai ? This problem will be discussed later. Another finding would be mentioning here is that the labelled unbound material were markedly high (figure 16, p 47) inspite of the fraction B having already separated the labelled non-haem protein (figure 13, p 43) on Sephadex G-100 chromatography.

5 The Structure of Hb Thai.

Hb Thai has been known to comprise less than 1 % of the total haemoglobin in a heterozygote. It is evident that its molecule is unstable since the abnormal haemoglobin bands would disappear when old haemolysate are studied. This unstable property together with the presence in the very small quantity of Hb Thai lead to difficulty

in detecting the abnormal pigment in starch-gel electrophoresis. The fresh haemolysate of Hb Thai consists of two components designated as Y and X on starch-gel electrophoresis. The Y component migrates just behind Hb A_2 while the mobility of X component moves slower than the Y component (figure 8, p 36). Recently, it has been decribed that the primary structure of Hb Thai and Hb Constant Spring (Hb CS) were identical (Fessas et al., 1972). Therefore the haemoglobin components, Constant Spring 1 (CS1) and Constant Spring 2 (CS2) correspond to the haemoglobin component Y and X respectively. Based on the studies by Clegg et al., (1971), the α-chain of CS1 component (α^{CS1}) had 31 amino acid residues extending from the C-terminal of normal α-chain. The α-chain of CS2 component (α^{CS2}) were identical to α^{CS1} except for the disappearence of three amino acids; valine, phenylalanine and glutamic acid from the C-terminal of α^{CS2}. Similar to the previous studies by Efremov et al., (1971), another haemoglobin component-Z band which migrated ahead Hb A_2 was observed on starch-gel of old haemolysate of Hb Thai (figure 19, p 51). It was believed that the component Z was a derivative of the component X and Y since the increased amounts of the Z component correlated with the decreased amounts of the X and Y components. However, the primary structure of the Z component has not been determined.

The studies of biosynthesis of Hb Thai were carried out in reticulocytes of Hb H disease with Hb Thai. The labelled slow haemoglobin fractionated by DEAE-Sephadex chromatography from the whole haemolysate presumably contained mainly Hb Thai and probably none or has a very small amounts of Hb A_2 ($\alpha_2\delta_2$), since Hb A_2 was not observed

on starch-gel electrophoresis (figure 8, p 36). Therefore, the radioactivity was expected to be found in the α-chain of Hb Thai (α^Y and α^X corresponding to α^{CS1} and α^{CS2} respectively). The labelled slow haemoglobin was mixed with non radioactive haemolysate of Hb A carrier and globin prepared from the mixture. The CM-cellulose chromatography, as shown in figure 18, p 50, showed that two major radioactivity peaks were at β- and α-chain and two small radioactivity peaks-probably corresponding to α^Y and α^X chains of Hb Thai were also observed. As already mentioned, radioactive peaks at α-abnormal chain; α^Y and α^X chain of Hb Thai, were expected, but the major radioactive peak appeared at normal α-chain. This implied that the radioactive normal α-chain was probably a product of degradation of the α^Y and α^X chains. In other words, Hb Thai had been changed to Hb A during the cause of DEAE-Sephadex chromatography. The isolated Hb Thai, after storage over 24 hours, revealed another band with a mobility the same as Hb A (figure 19.6, p 51). It can be readily explained that the appendage of 31 amino acid residues in the α-abnormal chain in Hb Thai was unstable and gradually degraded by either chemical or enzymatic means (Clegg et al., 1971). This suggestion is supported by the observation that Hb A is present in an old haemolysate of the isolated Hb Thai (figure 19.6, p 51) and also to the presence of the two main radioactive peaks α - and β-chains, in the incorporative studies of the isolated Hb Thai (figure 18, p 50).

A preparative isolation of non-radioactive Hb Thai in Hb H disease with Hb Thai by DEAE-Sephadex and then globin chain separation by CM-cellulose chromatography were under taken as shown in figure 20,

p 53. Similar to the previous study by Efremov et al., (1971), five peaks designated as A, B, C, D and E were demonstrated. The tryptic peptide maps of the peaks A, B and C were identical to the peptide maps of the normal β-, δ- and α-chains respectively (figure 12 and and 21, p 41, 54). Based on their electrophoretic mobility, the globin chains of peak D and E were likely to correspond to α^{Y} (α^{CS1}) and α^{X} (α^{CS2}) respectively. The peptide mapping of α^{Y} and α^{X} were similar to the normal α-chain but had three extra peptides (figure 21.3 and 21.4, p 55). Based on the study by Clegg et al., (1971), the α^{Y} and α^{X} chain of Hb Thai, as expected, corresponded to the α^{CS1} and α^{CS2} chains in Hb CS respectively.

According to the study by Efremov and his colleagues (1971), the isolated slow haemoglobin from a Chinese patient with Hb H disease by DEAE-Sephadex chromatography contained Hb CS and Hb A_2. The CM-cellulose chromatography of the isolated slow haemoglobin revealed peaks representing β-, δ, normal α- and two slow α-chains, which were similar to the figure 20, p 53. The author believe that the δ-chain joined to the normal α-chain as Hb A_2, while the slow α-chains joined to β-chain forming Hb CS.

In this study, as already shown in figure 8, page 36, the isolated slow haemoglobin by DEAE-Sephadex contained no Hb A_2 ($\alpha_2\delta_2$) or had a very small amount of Hb A_2 which was undetectable on starch-gel. The CM-cellulose chromatography of the isolated haemoglobin (figure 20, p 53) indicated the presence of β, δ, normal α and two abnormal α-chains. This finding can be explained by the suggestion that δ-chain probably joined to the α-abnormal chain instead of

normal α-chain, forming $\alpha_2^{Thai}\delta_2$. Since δ-chain possess more positive net charge than that of β-chain, the component $\alpha_2^{Thai}\delta_2$ must migrate very slow, probably behind the X component of Hb Thai. However, no slow haemoglobin fraction behind the X component of Hb Thai was observed in haemolysate of Hb H disease with Hb Thai on starch-gel electrophoresis at pH 8.6. Another point should be mentioned here that the optical density of the peak corresponding to β-chain was approximately equal to the peak corresponding to δ-chain (figure 20, p 53). This implied that the same amount of the β- and δ-chains are present in the isolated slow haemoglobin-presumably containing mainly Hb Thai. Since Hb A_2 was undetectable on starch-gel it is possible that the Hb Thai molecule consists of $\alpha_2^{Thai}\beta\delta$, instead of $\alpha_2^{Thai}\beta_2$. However, this hypothesis needs further investigations.

6 Haemoglobin Synthesis in Hb Thai.

It is evident as previously described that Hb Thai is unstable and is present in less than 2-3 % in haemolysate of Hb H disease. The study described in chapter 3 indicated that the isolated slow haemoglobin from whole haemolysate of Hb H disease by DEAE-Sephadex contained entirely Hb Thai, Hb A_2 was undetectable on starch-gel electrophoresis. The CM-cellulose chromatography of the preparative isolated slow haemoglobin(figure 20, p 53) revealed the presence of δ-chain besides the β, α and abnormal α-chains of Hb Thai. Although δ-chain is present in the isolated slow haemoglobin, the CM-cellulose chromatography of the isolated slow globin from incorporative study

in reticulocytes revealed two major radioactivity peaks at β- and α-chain and two small radioactive peaks at two abnormal α-chains of Hb Thai, and none or a very small radioactivity at δ-chain peak was observed. This implied that the radioactivity of the isolated haemoglobin probably represents the labelled Hb Thai, presumably α^{Thai} and β-chains.

Synthetic rate of the isolated slow haemoglobin and isolated Hb A by DEAE-Sephadex was carried out in both reticulocytes and bone marrow of a patient of Hb H disease with Hb Thai. The specific activity of the slow haemoglobin comparing with Hb A at one and four hours incorporation in reticulocytes was shown in figure 22.1, p 58. The specific activity of Hb Thai at one hour incubation exceeded that of Hb A 1.6 times. The specific activity of Hb Thai at four hour incubation was only slightly higher than that of the one hour incubation unlike the continuous increase for Hb A. The explanation was that due to the instability of the abnormal haemoglobin molecule, Hb Thai may be degraded to Hb A as shown in figure 19.6, p 51. This was consistent with the constant specific activity of Hb Thai at four hour incubation and with the radioactivity incorporating into the normal α-chain instead of the abnormal α-chains. The synthesis in bone marrow at one and four hour incubation, as shown in figure 22.2, p 59, revealed that the specific activity of both Hb Thai and Hb A increased linearly. The radioactivity of Hb Thai was about 8 fold that of Hb A. This implied that the synthetic rate of Hb Thai was high, presumably resulting from an increase in synthesis of mRNA of Hb Thai from nucleated erythroid cells in bone marrow to compensate

for the instability of Hb Thai molecules. Based on the assumption that the same incorporative rate of the β-chain into both isolated Hb A and Hb Thai, the increased specific activity of Hb Thai had to come from the instability of the α abnormal chains of Hb Thai.

The Hb Thai heterozygote has normal haematological data with haemoglobin types of Hb Thai + A_2 + A (table 4, p 24). A very minute amounts of Hb Thai, normally less than 1 % in heterozygote, was measurable. The mean of radioactivity α/β ratio of a heterozygote was 1.34 ± 0.09, which was apparently a higher in comparison with the normal of 1.08 ± 0.02. The significant increase of radioactivity of the heterozygous Hb Thai was unlikely due to the increase incorporating in additional leucine amino acids contained in the 31 amino acid residues of the appendage of the α^{Thai}, since the quantity of Hb Thai was less than 1 % of the total haemoglobin and the normal α-chain was also separated from the α abnormal chains of Hb Thai. It was evident from this study that α abnormal chain of Hb Thai revealed a rapid turnover rate (figure 22). Furthermore, the appendage peptide of the α abnormal chain of Hb Thai was degraded to yield normal α-chain. The explanation of the increased radioactivity α/β ratio presumably was due to newly synthesized labelled normal α-chain including the yield from the rapid degradation of α abnormal chain of Hb Thai that joined to the non radioactive β-chain in the pool leading to the discrepancy of radioactivity of α- and β-chains. However, the radioactivity α/β ratio of the whole haemolysate and of haemolysate after fractionated by Sephadex G-100 chromatography were determined in two cases of heterozygous Hb Thai in order to exclude

a presence of a free chain in red cell pool. The result (table 5, p 25) showed no difference in α/β ratio in both cases. This was not compatible with the hypothesis of the β-chain pool in the heterozygote. Therefore the precise mechanism is still obscure.

The α/β ratio of at least two proven cases of a heterozygous Hb Thai (figure 5 and 6, p 32) are 1.66 and 1.7 respectively which were remarkably increased. This would support the fact that Hb Thai trait had the increased radioactivity α/β ratio.

Since either the Hb Thai trait or α-thal$_2$ trait would give rise to a Hb H disease upon the interaction with α-thal$_1$ trait, the Hb Thai trait presumably is equivalent to the α-thal$_2$ trait. It was evident that a mean of α/β ratio in obligatory α-thal$_2$ trait was 0.92 ± 0.03 (Pootrakul et al., 1974). This also agreed with the result of two cases of α-thal$_2$ trait in this study (figure 7, p 34). As already described, the mean of radioactivity of α/β ratio in Hb Thai trait was 1.34 ± 0.09, which was significantly different from that of the α-thal$_2$ trait. Therefore the actual pathogenesis of Hb H disease by interaction of either the Hb Thai or the α-thal$_2$ trait with the α-thal$_1$ trait is probably due to different mechanisms.