Chapter 3

RESULTS

1. Haematological and Globin Chain Synthetic Studies.

1.1 Normal controls.

Haematological data of four normal controls are shown in table 1. The α/β ratio of globin chain synthesis was determined by measuring the total radioactivity incorporation into globin chains. Haematological means appeared to be within normal limits. A mean of radioactivity α/β ratio of the normal is 1.08±0.02 indicating that the α- and β-globin chains are approximately equally synthesised in a normal adult.

1.2 HbH disease (α-thal$_1$/α-thal$_2$).

Five patients with clinical manifestation of chronic haemolytic anemia, hepatosplenomegaly and positive for intraerythrocytic inclusion bodies were diagnosed as haemoglobin H disease. Since haemoglobin types of the patients were HbA + A_2 + H, the genotype designations of the patients are a double heterozygote for the α-thal$_1$ and α-thal$_2$ genes. Haematological data of the patients, as shown in table 2, indicated marked anemia, severe hypochromic microcytic red cells. A mean of radioactivity α/β ratio was 0.57±0.15. This result indicates a marked decrease of α-chain synthesis and the excess β-chain was then polymerized to be tetramer β_4 or HbH.

Table 1

Haematological data and α/β ratio of globin chain synthesis in normal control

Subject	Age	Sex	Hb g/100ml	RBC mill/mm^3	Hct %	MCV μ^3	MCH pg.	MCHC %	Alk.resist. Hb %	HbA_2 %	Hb Types	Total-radioactivity α/β ratio
S.A.	26	M	15.15	4.75	44.5	93.7	31.9	34.0	0.54	2.68	A_2 + A	1.09
S.P.	30	F	12.90	4.20	39.2	93.3	30.7	32.9	0.66	3.43	A_2 + A	1.04
S.S.	26	M	16.00	5.96	47.5	79.7	26.9	33.7	1.08	2.63	A_2 + A	1.10
S.B.	24	F	15.80	4.94	48.3	97.8	31.9	32.7	0.39	3.43	A_2 + A	1.09
Means	-	-	14.96	4.96	44.9	91.1	30.4	33.3	0.67	3.04	-	1.08
S.D.	-	-	1.42	0.74	4.1	7.9	2.4	0.6	0.30	0.45	-	0.02

RBC = Red Blood Cells.

Hct. = Haematocrit or Packed Cell Volume (PCV)

MCV = Mean Corpuscular Volume. = $\frac{PCV}{RBC} \times 10$

MCH = Mean Corpuscular Haemoglobin = $\frac{Hb}{RBC} \times 10$

MCHC = Mean Corpuscular Haemoglobin Concentration. = $\frac{Hb}{PCV} \times 100$

Table 2

Haematological data and globin synthesis in HbH disease (α-thal$_1$/α-thal$_2$)

Subject	Age	Sex	Hb g/100ml	RBC mill/mm^3	Hct %	Incl. %	MCV μ^3	MCH pg.	MCHC %	Alk.resist. Hb %	HbA$_2$ %	HbH %	Hb types	Total-radioactivity α/β ratio
T.K.	39	F	7.7	3.75	28.0	75.6	74.7	20.5	27.5	0.90	0.97	8.6	A$_2$+A+H	0.07
S.K.	23	M	9.8	6.44	32.0	99.0	59.0	16.3	27.6	0.60	1.00	-	A$_2$+A+H	0.64
P.S.	54	F	7.6	3.37	25.8	52.0	76.6	22.6	29.5	1.17	1.22	8.7	A$_2$+A+H	0.69
S.P.R.	15	F	10.4	5.67	36.3	73.2	64.0	18.3	28.7	1.03	2.30	7.4	A$_2$+A+H	0.46
S.N.R.	16	F	8.6	4.60	31.9	57.0	69.4	18.7	26.9	0.63	1.90	5.2	A$_2$+A+H	0.36
Means	-	-	8.8	4.77	30.8	71.4	68.7	19.3	28.1	0.87	1.48	7.5	-	0.57
S.D.	-	-	1.2	1.29	4.1	18.5	7.3	2.4	1.0	0.24	0.59	1.6	-	0.15

1.3 HbH disease with Hb Thai (α-thal$_1$/Hb Thai).

Haematological data of seven patients of HbH disease with Hb Thai are shown in table 3. The clinical features of the patients were similar to the previous group, HbH disease (α-thal$_1$/α-thal$_2$), except haemoglobin electrophoresis revealed HbA + H + Thai. A haemoglobin band corresponding to HbA_2 was not observed on starch-gel electrophoresis at pH 8.6. The genotype of the HbH disease with Hb Thai is evidently a double heterozygosity for α-thal$_1$ and Hb Thai trait. The haematological means of the patients were similar to the previous group, but a mean of radioactivity α/β ratio was 0.70±0.14, which was slightly higher than that of the previous group.

1.4 Haemoglobin Thai trait.

Haematological data and globin chain synthesis of 12 heterozygous Hb Thai are shown in table 4. Haematological means of eleven adults revealed within normal ranges. A mean of radioactivity α/β ratio is 1.34±0.09, which is higher than that of the normal. Normally, the α/nonα ($\alpha/\beta+\gamma$) ratio of globin chain synthesis in a newborn is approximately equal to one. It is of interest that the mean of $\alpha/\beta+\gamma$ ratio of the neonate with Hb Thai was 1.39 which is the same as observed in adult Hb Thai trait.

1.4.1 α/β ratio of haemolysate in Hb Thai trait after Sephadex G-100 filtration. Three ml of fresh haemolysate from incorporative studies of Hb Thai trait was passed through a 3 X 120 cm column of Sephadex G-100 in Tris-KCN buffer, in order to separate the tetrameric

Table 3

Haematological data and globin chain synthesis in HbH disease with Hb Thai (α-thal$_1$/Hb Thai)

Subject	Age	Sex	Hb g/100ml	RBC mill/mm^3	Hct. %	Incl. %	MCV μ^3	MCH %	MCHC %	Alk.resist. Hb %	HbXY + A_2 %	HbH %	Hb Types	Total-radioactivity α/β ratio
S.V.	15	F	8.9	4.35	35.0	90.2	80.5	20.6	25.6	3.35	2.94	11.2	XY+A+H+Bt.	0.64
M.J.	13	F	10.1	5.71	38.0	95.8	66.5	17.7	26.6	0.87	2.50	17.7	XY+A+H+Bt.	0.59
S.K.	16	M	8.5	4.26	33.8	98.8	79.3	19.9	25.0	0.42	2.56	16.8	XY+A+H	0.66
V.C.	27	F	10.9	4.28	39.0	81.6	91.1	25.5	27.9	4.68	2.72	9.1	XY+A+H	0.70
B.L.	28	F	4.7	2.51	18.7	45.2	74.5	18.7	25.1	4.93	3.20	10.7	XY+A+H+Bt.	0.88
D.L.	17	F	7.9	4.35	32.0	95.2	73.5	18.2	24.7	1.38	2.10	5.5	XY+A+H+Bt.	0.93
C.R.	47	M	10.0	4.31	37.2	91.2	86.3	23.2	26.9	1.15	2.90	4.5	XY+A+H	0.55
Means	-	-	8.7	4.25	33.4	85.4	78.8	20.6	25.9	2.40	2.70	10.8	-	0.70
S.D.	-	-	2.1	0.93	6.9	18.6	8.3	2.9	1.2	1.90	0.36	5.1	-	0.14

Table 4

Haematological data and globin chain synthesis in Hb Thai trait

Subject	Age	Sex	Hb g/100ml	RBC mill/mm^3	Hct. %	MCV μ^3	MCH pg.	MCHC %	Alk. resist. Hb %	HbXY+ A_2 %	Hb Types	Total-radioactivity α/β ratio
N.A.	29	F	11.4	4.16	35.0	84.0	27.4	32.5	0.57	2.97	XY+A_2+A	1.32
V.S.	27	F	11.2	4.48	36.0	80.0	25.0	31.1	0.55	2.88	XY+A_2+A	1.36
L.N.	20	F	11.3	3.90	36.0	92.3	28.9	31.4	0.61	2.85	X +A_2+A	1.38
K.N.	58	M	15.6	5.04	47.5	94.2	30.9	32.8	0.83	2.85	X +A_2+A	1.40
R.N.	53	F	12.0	3.80	37.0	97.4	31.6	32.4	0.41	2.56	XY+A_2+A	1.40
P.N.	12	F	12.1	4.28	37.0	86.4	28.2	35.4	0.69	2.76	XY+A_2+A	1.36
J.A.	37	F	10.9	4.12	33.1	80.0	26.5	32.9	0.56	3.13	Y+A_2+A	1.40
J.J.	42	M	15.0	5.96	46.0	77.2	25.2	32.6	0.67	2.66	A_2+A	1.22
A.J.	11	M	10.7	4.72	34.0	72.0	22.7	31.5	0.78	2.85	X +A_2+A	1.38
Y.K.	46	M	10.4	3.60	32.6	90.5	28.9	31.9	0.64	2.42	X +A_2+A	1.13
S.S.R.	10	F	12.3	5.22	39.6	74.9	23.3	31.1	0.28	1.65	XY+A_2+A	1.40
Means	-	-	12.1	4.49	37.6	84.4	27.2	32.3	0.60	2.69	-	1.34
S.D.	-	-	1.7	0.71	4.9	8.4	2.9	1.2	0.16	0.40	-	0.09
Cord-blood	-	-	15,8	5.22	48.3	92.0	30.7	32.8	68.48	0.89	XY+F+A+Bt.	2.96 ($\alpha/\beta+\gamma$ =1.39)

haemoglobin from free globin chains if they were present. The chromatography as shown in figure 2, revealed two peaks; A and B. Peak A was positive for radioactivity incorporation and for optical density at 540 nm indicating its corresponding to tetrameric haemoglobin. Peak B was evidently a high radioactivity incorporation, but negative for the optical density at 540 nm indicating the absence of haem in the peak B.

Fractions corresponding to peak A were pooled, and concentrated. The globin was then precipitated by acid-acetone method. Finally the α/β chain ratio was determined in two cases (table 5). It can be seen that the α/β ratio of the whole haemolysate and haemolysate after Sephadex G-100 were the same. This indicates that the imbalance of α/β ratio in Hb Thai trait is not due to a pool of free globin chain in the reticulocyte.

Table 5

α/β ratio of Hb Thai trait observed before and after gel-filtration on Sephadex G-100 column.

Subject	α/β ratio (total radioactivity)	
	Whole haemolysate	Haemolysate after passing G-100 column
V.S.	1.357	1.350
L.N.	1.380	1.420

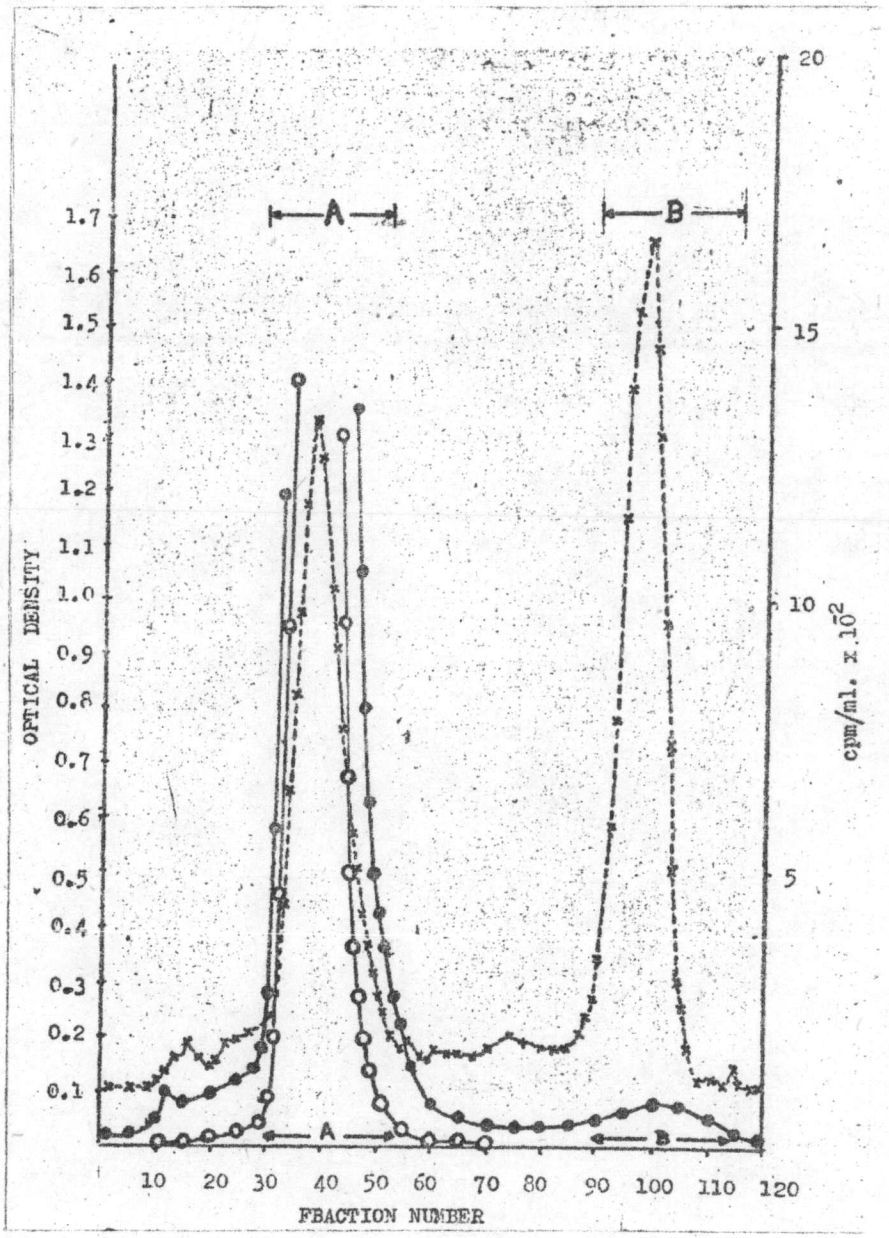


Figure 2 Chromatography of gel-filtration on Sephadex G-100 of the labelled fresh haemolysate from reticulocytes of Hb Thai trait. ●——● O.D. 280 nm. o——o O.D. 540 nm. x-----x radioactivity.

1.4.2 Study of the peak B on CM-cellulose chromatography.

Peak B, the labelled non-haem protein from whole haemolysate after gel-filtration on Sephadex G-100 of haemoglobin Thai trait (figure 2), was pooled and lyophilized. About 40 mg of cold globin prepared from HbA was added into the protein obtained from peak B and the sample was fractionated on a CM-cellulose column (figure 3). No radioactivity was observed in the peaks corresponding to α- and β-chains. Most of the radioactivity appeared to be unbound to the CM-cellulose. This indicates that the non-haem protein peak B is not a complete free globin chain, but consists of small molecular weight material.

1.5 α-thal$_2$/Hb Thai disease.

Pedigree of family R.P. and haematological data are shown in figure 4 and table 6 respectively. The father I-1, two daughters II-1 and II-2 presented clinical manifestation of HbH disease. The father I-1 had haemoglobin types of HbA + H + Thai, while the two daughters II-1 and II-2 had no Hb Thai in addition to HbA + H on starch-gel electrophoresis. Therefore the genotype designation of the father I-1 was α-thal$_1$/Hb Thai, but those of the two daughters II-1 and II-2 were α-thal$_1$/α-thal$_2$. The α/β radioactivity ratio of the three were 0.55, 0.36 and 0.46 respectively. The genetic evidence of the pedigree indicated that the α-thal$_2$ gene of the two daughters had to be transfered from her mother I-2. Haematological data of the I-2 showed low MCV, MCH and the α/β ratio of 0.98 which were consistent with the designation of α-thal$_2$ trait. Haematological data and α/β ratio of II-4 were compatible with a genotype of Hb Thai trait. It is of interest

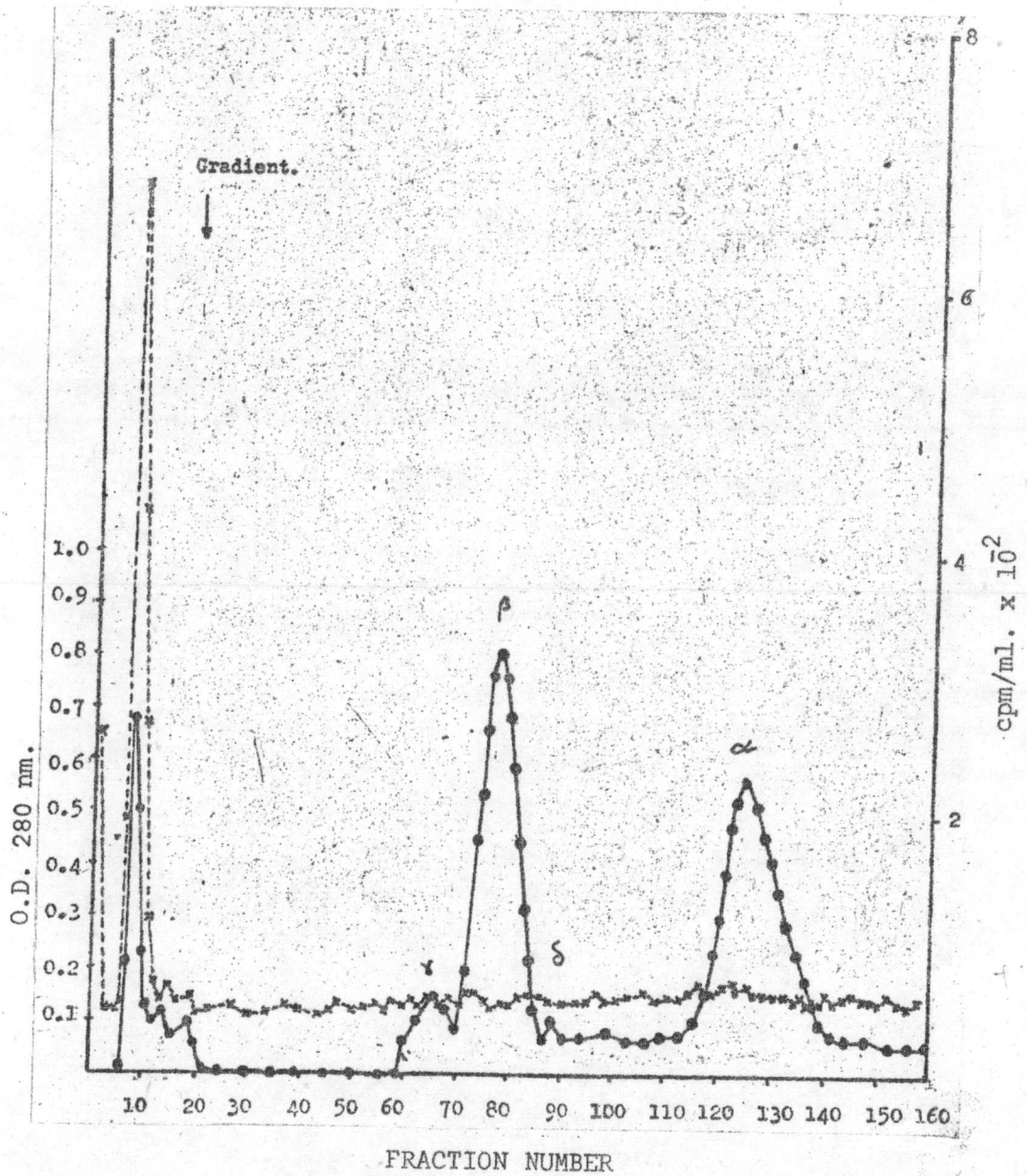


Figure 3 Chain separation by CM-cellulose chromatography of radioactivity peak B from figure 2 with non radioactive Hb A as carrier. ●——● O.D. 280 nm., x----x radioactivity.

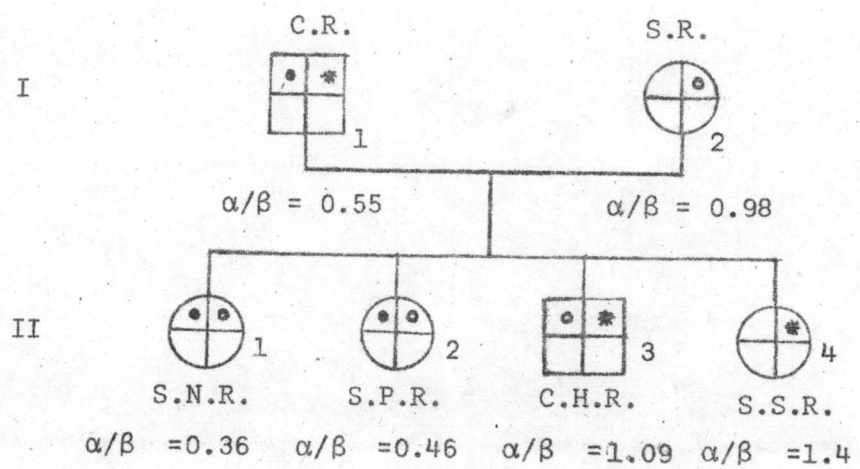
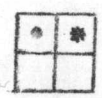
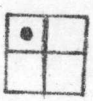


Figure 4 Pedigree of family R.P.

 = Male, α-thal$_1$/Hb Thai.

 = Female, α-thal$_2$ trait.

 = Female, Hb Thai trait.

 = Male, α-thal$_1$ trait.

The Roman and Arabic number represent generation and subject number in the pedigree respectively.

Table 6

Haematological data and α/β ratio of globin chain synthesis of family R.P.

Subject	Age	Sex	Hb g/100ml	RBC mill/mm^3	Hct %	Incl. %	MCV μ^3	MCH pg.	MCHC %	Alk.resist. Hb %	HbXY %	HbA_2 %	HbH %	Hb Types	Total-radioactivity α/β ratio
I-1	47	M	10.0	4.31	37.2	91.2	86.3	23.2	26.9	1.15	2.9	-	4.5	Xy+A+H	0.55
I-2	30	F	14.2	6.66	52.3	-	78.5	21.2	27.2	0.63	-	2.6	-	A_2+A	0.98
II-1	16	F	8.6	4.60	31.9	57.0	69.4	18.7	26.9	0.63	-	1.9	5.2	A_2+A+H	0.36
II-2	15	F	10.4	5.67	36.3	73.2	64.0	18.3	28.7	1.03	-	2.3	7.4	A_2+A+H	0.46
II-3	13	M	10.2	5.11	33.7	1	65.9	20.0	30.3	1.25	2.9	-	-	XY+A+F+Bt	1.09
II-4	10	F	12.3	5.29	39.6	-	74.9	23.3	31.1	0.28	1.7	-	-	XY+A_2+A	1.40

that II-3 was slightly anemia and occasionally jaundiced with palpable spleen. The red cells morphology revealed thalassaemic changes. Only few red cells were positive for inclusion body test. Haemoglobin types were HbA and Hb Thai and trace Hb Bart's. The α/β ratio was 1.09. The inclusion test, haemoglobin types and the α/β ratio of the II-3 were differed from HbH disease (either $\alpha\text{-thal}_1$/Hb Thai or $\alpha\text{-thal}_1/\alpha\text{-thal}_2$). It is believed that the II-3 is a double heterozygous state of $\alpha\text{-thal}_2$ and Hb Thai.

1.6 Homozygous Hb Thai.

1.6.1 Family N.K. Pedigree of family N,K. is shown in figure 5. The patient II-2 presented the clinical disorders very similar to the $\alpha\text{-thal}_2$/Hb Thai disease as already mentioned. The starch-gel electrophoresis also showed HbA + Hb Thai and trace Hb Bart's. The α/β radioactivity ratio of the patient was 1.66 which was significantly higher than that of the $\alpha\text{-thal}_2$/Hb Thai or a heterozygous Hb Thai. Haematological data and the α/β ratio of the parents were compatible with the diagnosis of Hb Thai trait (table 4). One of her sister II-4 also had the globin chain synthetic study suggesting the inheritance of Hb Thai trait.

1.6.2 Family P.V. The pedigree of family P.V. is summarized in figure 6. The propositus, II-1 had mild anemia and other haematological manifestations including the α/β ratio of 1.7, resembling to the patient with homozygote for Hb Thai (II-2 of N.K. family). Haemoglobin starch-gel electrophoresis of the parents with repeated examinations had undetectable of Hb Thai. However, the α/β radioactivity ratio of the

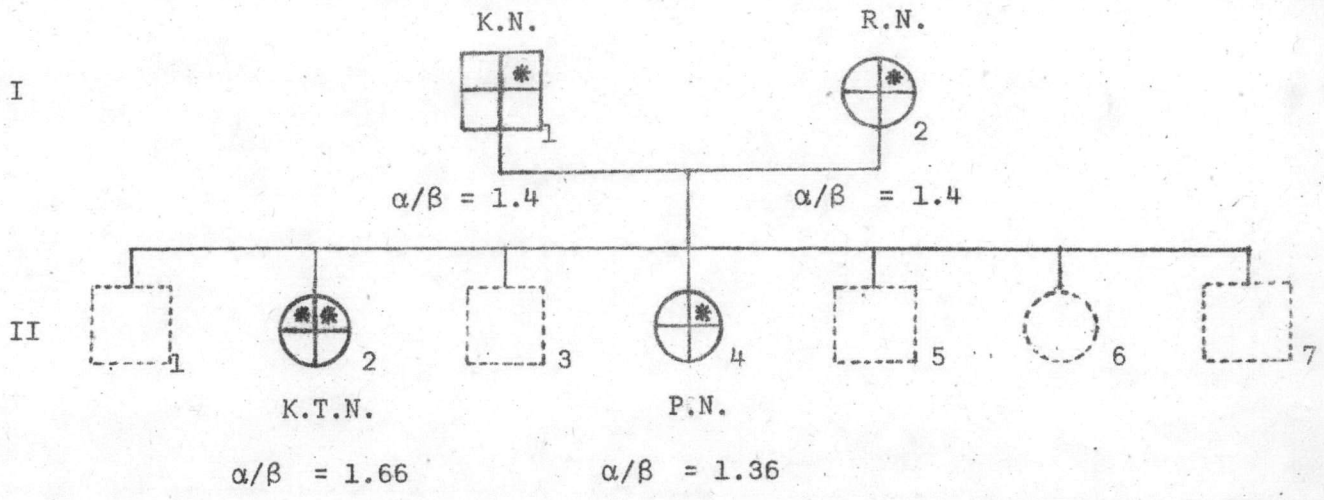


Figure 5 Pedigree of family N.K.

= Male, Hb Thai trait.

= Female, Homozygous Hb Thai

= Female, Hb Thai trait.

= Not examined.

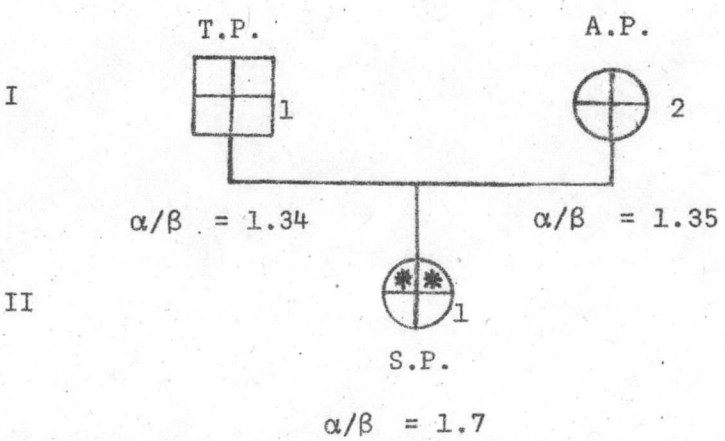
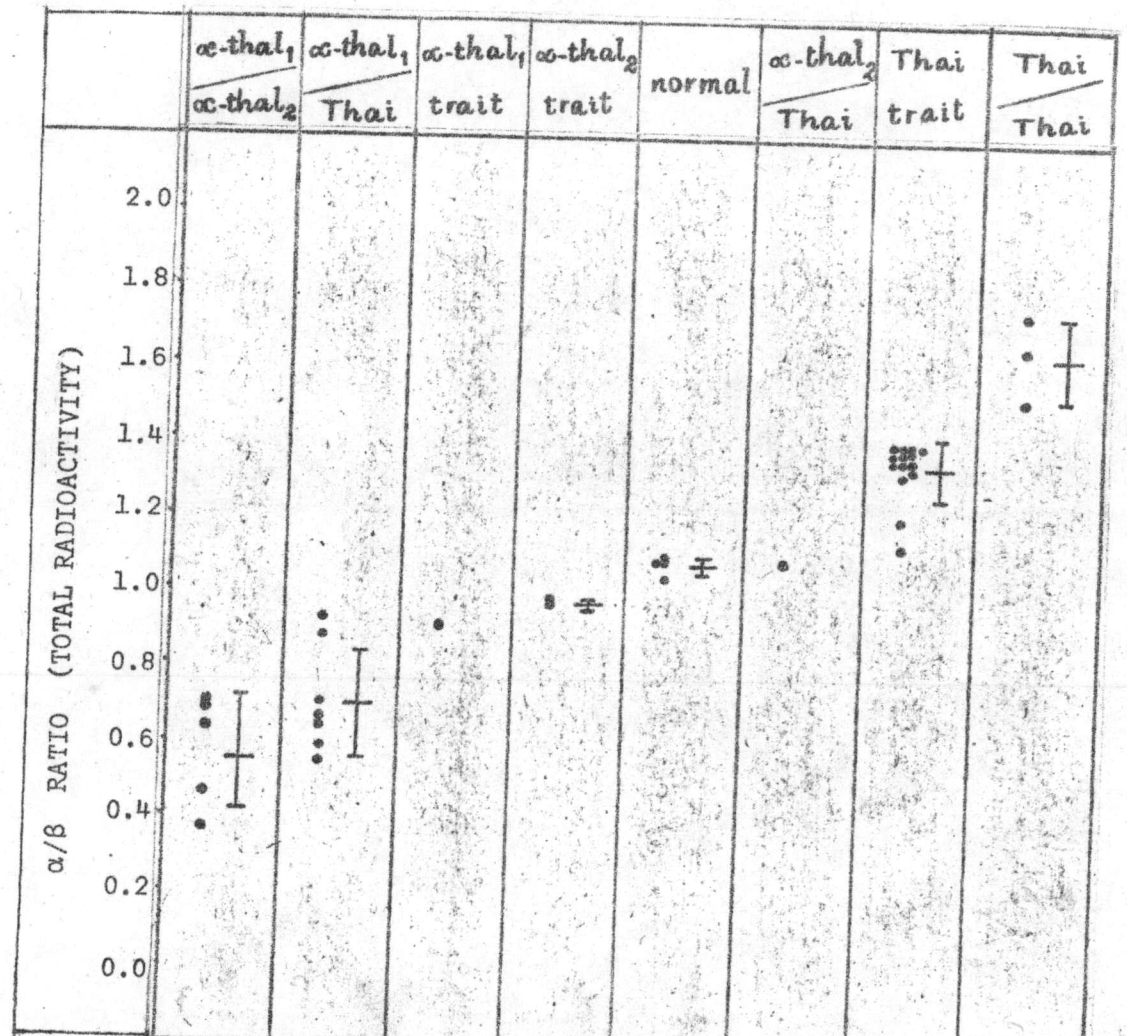


Figure 6 Pedigree of family P.V.

parents were 1.34 and 1.35 which were evidently compatible with designation of Hb Thai trait.

1.6.3 Patient S.P. The patient presented a history of mild anemia, and jaundice. Her haemoglobin was 8-9 g%. The red cell indices indicated hypochromic microcytic anemia. The haemoglobin types were Hb Thai + HbA + trace Hb Bart's. Her haematological data were consistent with the designation of either α-thal$_2$/Hb Thai or homozygous Hb Thai. Since the α/β ratio of the patient was 1.52 the genotype assignment of the patient should be homozygous Hb Thai. Fortunately incorporative study of the cord blood sample of her baby was also performed (table 4). The $\alpha/\beta+\gamma$ radioactivity ratio was 1.39 suggesting the genotype of Hb Thai heterozygote.

A summary of the means of radioactivity α/β ratio of various genotype designations in α-thalassaemia syndromes are shown in figure 7. It was shown that the means of the α/β ratio of Hb Thai trait was 1.34± 0.09 which was significantly higher than that of the control of 1.08± 0.02. However, the homozygous Hb Thai appeared significantly increased in the α/β ratio which was 1.64±0.11. Two α-thal$_2$ trait and a heterozygote of α-thal$_1$ were also studied for comparision, with the α/β ratio of 0.975 and 0.90 respectively. The α/β ratio of the other diseased forms; α-thal$_1$/α-thal$_2$, α-thal$_1$/Hb Thai and α-thal$_2$/Hb Thai were 0.57, 0.70 and 1.09 respectively.



	α-thal$_1$/α-thal$_2$	α-thal$_1$/Thai	α-thal$_1$ trait	α-thal$_2$ trait	normal	α-thal$_2$/Thai	Thai trait	Thai/Thai
MEANS	0.57	0.70	0.9	0.975	1.08	1.09	1.34	1.64
S.D.	0.15	0.14	0.0	0.005	0.02	0.00	0.09	0.11
CASES	5	7	1	2	4	1	14	3

Figure 7 A summary of the means of total radioactivity of α/β chain ratio in alpha-thalassaemia syndromes.

2 Studies on Haemoglobin Components in Haemolysate of HbH Disease with Hb Thai.

2.1 HbA_2 ($\alpha_2\delta_2$) in HbH disease.

Haemoglobin electrophoreses at pH 8.9 of HbH with or without Hb Thai are shown in figure 8. It can be seen that Hb types of HbH disease (α-$thal_1$/α-$thal_2$) were HbA + H + A_2, while that of HbH disease with Hb Thai (α-$thal_1$/Hb Thai) were HbA + H + Thai (X and Y bands). No haemoglobin corresponding to HbA_2 was observed in whole haemolysate of HbH disease with Hb Thai. Preparative isolation of the slow haemoglobin components from the whole haemolysate was also made on the DEAE-Sephadex chromatography as described by Wasi et al. (1968). The pooled fraction of the slow components were concentrated on CM-Sephadex column. After dialysed in water, the isolated slow haemoglobins were studied on starch-gel electrophoresis (figure 8.4). Only the X and Y bands of of Hb Thai were seen. Therefore, on starch-gel electrophoresis in our hands, no haemoglobin band corresponding to HbA_2 ($\alpha_2\delta_2$) was observed in the haemolysate of HbH disease with Hb Thai (α-$thal_1$/Hb Thai).

2.2 δ-chain is synthesized in a small amount in reticulocytes and erythroid cells in bone marrow of HbH disease with Hb Thai (α-$thal_1$/Hb Thai).

Since the HbA_2 is absent in α-$thal_1$/Hb Thai disease, it is of interest to know whether the δ-chain is synthesized or not. The radioactive incorporation in reticulocytes and in bone marrow cells of the patient were carried out.

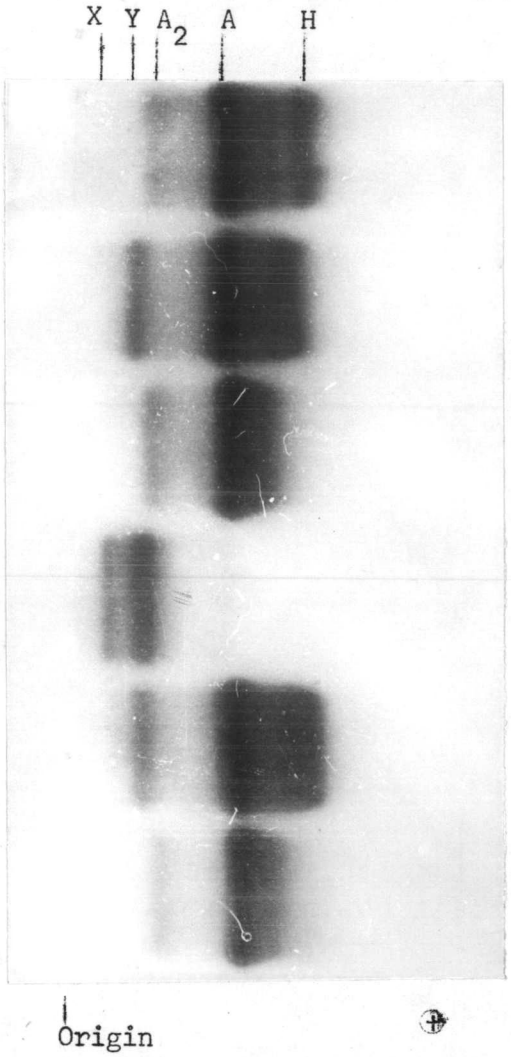


Figure 8 Starch-gel electrophoresis in Tris-borate-EDTA buffer, pH 8.6, of fresh haemolysate, stained with orthodianisidine.

1. Haemolysate of HbH disease (α-thal$_1$/α-thal$_2$).
2. Haemolysate of HbH disease with Hb Thai (α-thal$_1$/Hb Thai).
3. Haemolysate of normal control.
4. Isolated slow haemoglobin fractions-Hb Thai which were separated from the whole haemolysate of HbH disease with Hb Thai by DEAE-Sephadex chromatography.

CM-cellulose chromatography studies of the globin from reticulocytes and bone marrow cells are shown in figures 9 and 10 respectively. It is shown that a small peak of radioactivity corresponding to the δ-chain was observed in both reticuloeyte and bone marrow. This indicates the presence of δ-chain in the patient. Since the HbA_2 was undetectable on starch-gel, the δ-chain presumably did not join to the normal α-chain forming the tetramer of $\alpha_2\delta_2$.

2.3 Peptide mapping of a peak corresponding to δ-chain in HbH disease with Hb Thai.

Haemolysate from a patient (α-$thal_1$/Hb Thai) was fractionated on Sephadex G-100 chromatography in order to separate the tetramer haemoglobin molecules from a free δ-chain, if it was present. A preparative CM-cellulose chromatography of the globin from the haemolysate after Sephadex G-100 chromatography is shown in figure 11. Besides the two major chains, α- and β-, and one minor γ-chain, two small peaks corresponding to δ-chain and presumably α^{Thai} chain, were observed. The peak corresponding to δ-chain was tryptic digested and its peptide map compared with the peptide map of normal β-chain (figure 12). Generally, peptide maps of β- and δ-chains are different for a few peptides. One is the Tp III (tryptic peptide No. 3) which is easily recognised by a positive arginine staining. δ Tp III is known to migrate slightly ahead of δ Tp V in H.V.E. (high voltage electrophoresis), while β Tp III moves behind the β Tp V (the δ Tp V and β Tp V are identical in amino acid sequences). After arginine staining, it was concluded that the material eluted off the CM-cellulose column behind

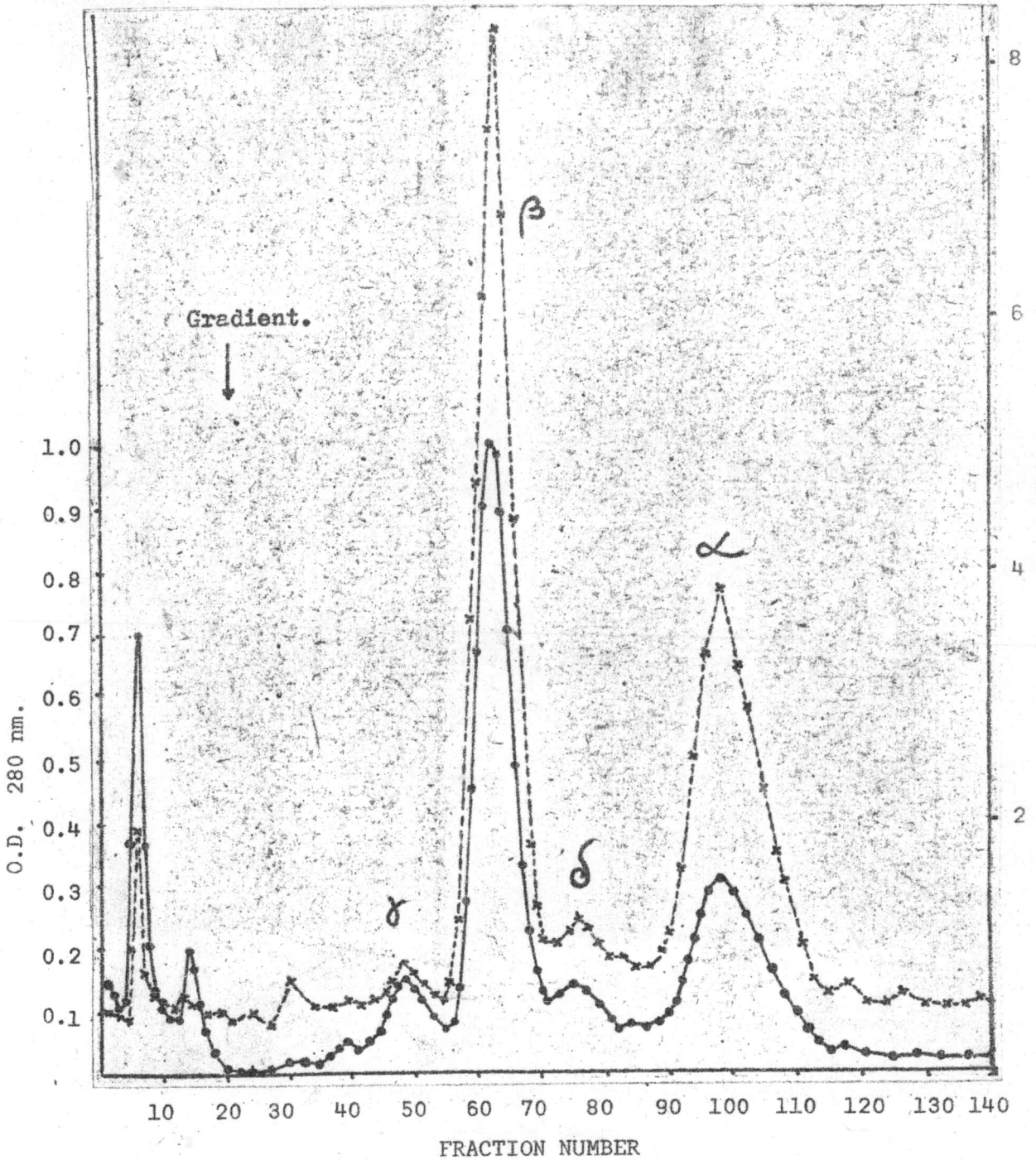


Figure 9 CM-cellulose chromatography of labelled globin which was prepared from reticulocytes of Hb H disease with Hb Thai. ●——● O.D. 280 nm., x-----x radioactivity.

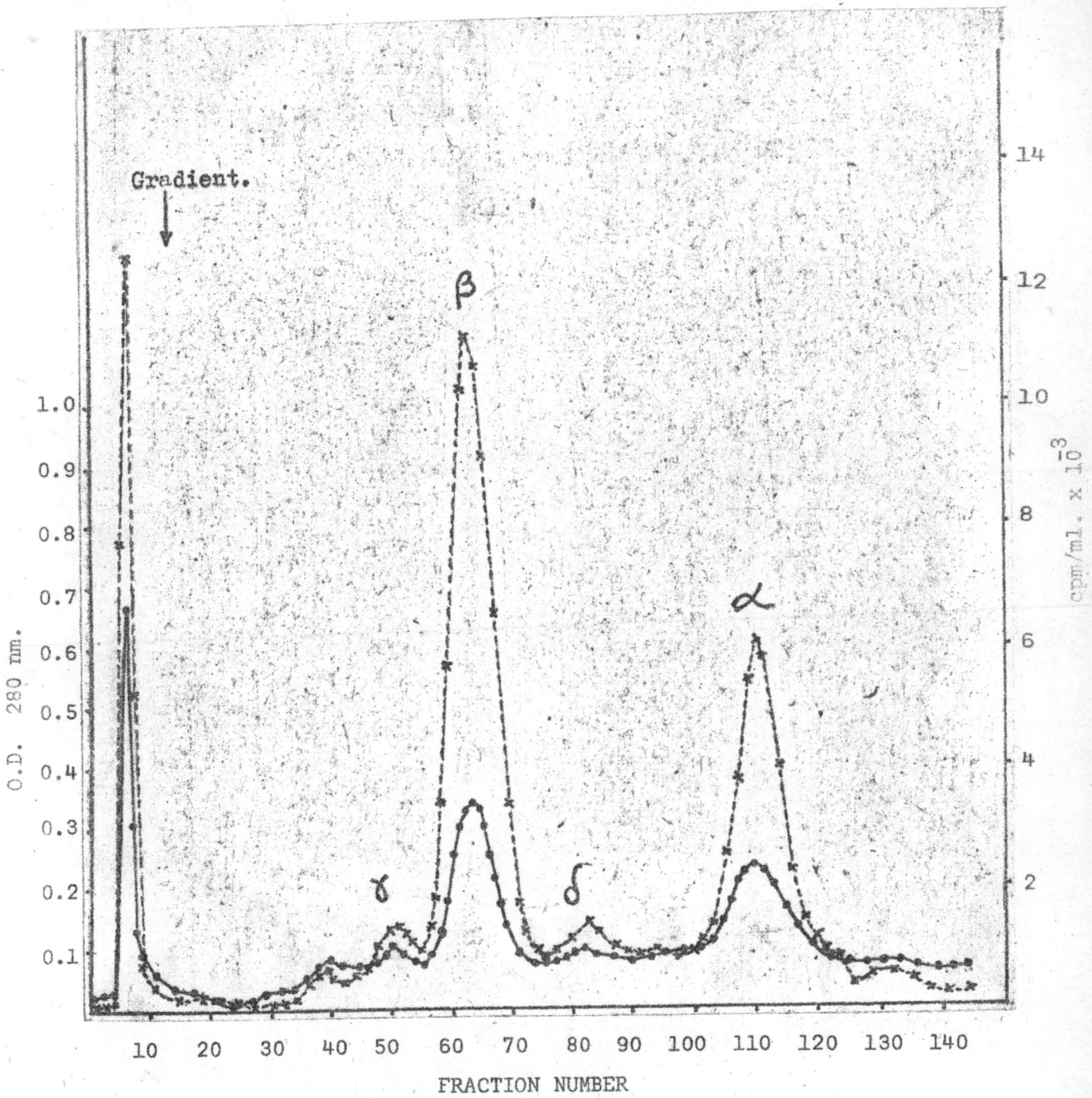


Figure 10 Chain separation by CM-cellulose chromatography of labelled globin from bone marrow of Hb H disease with Hb Thai.
●——● O.D. 280 nm., x-----x radioactivity.

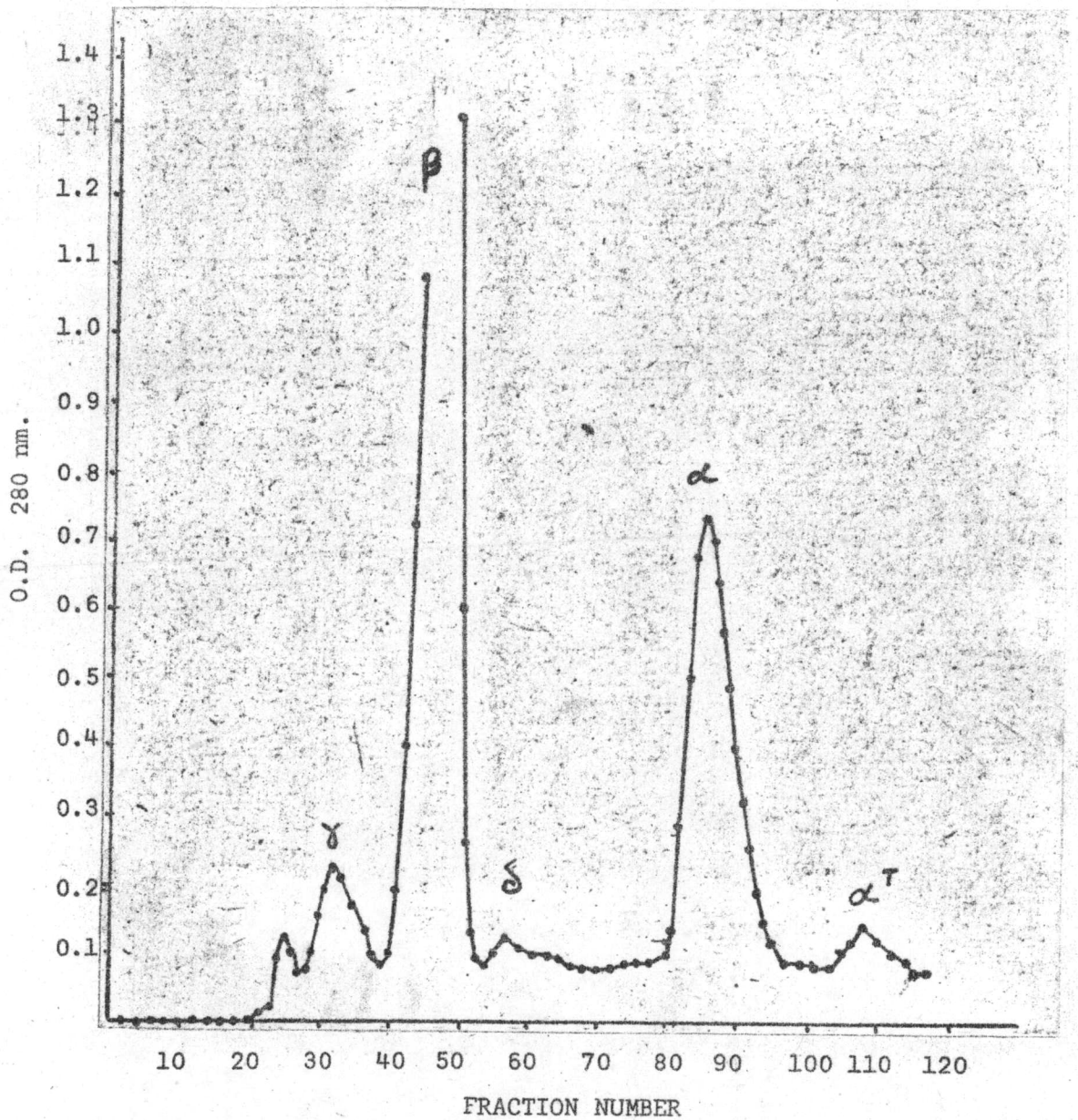
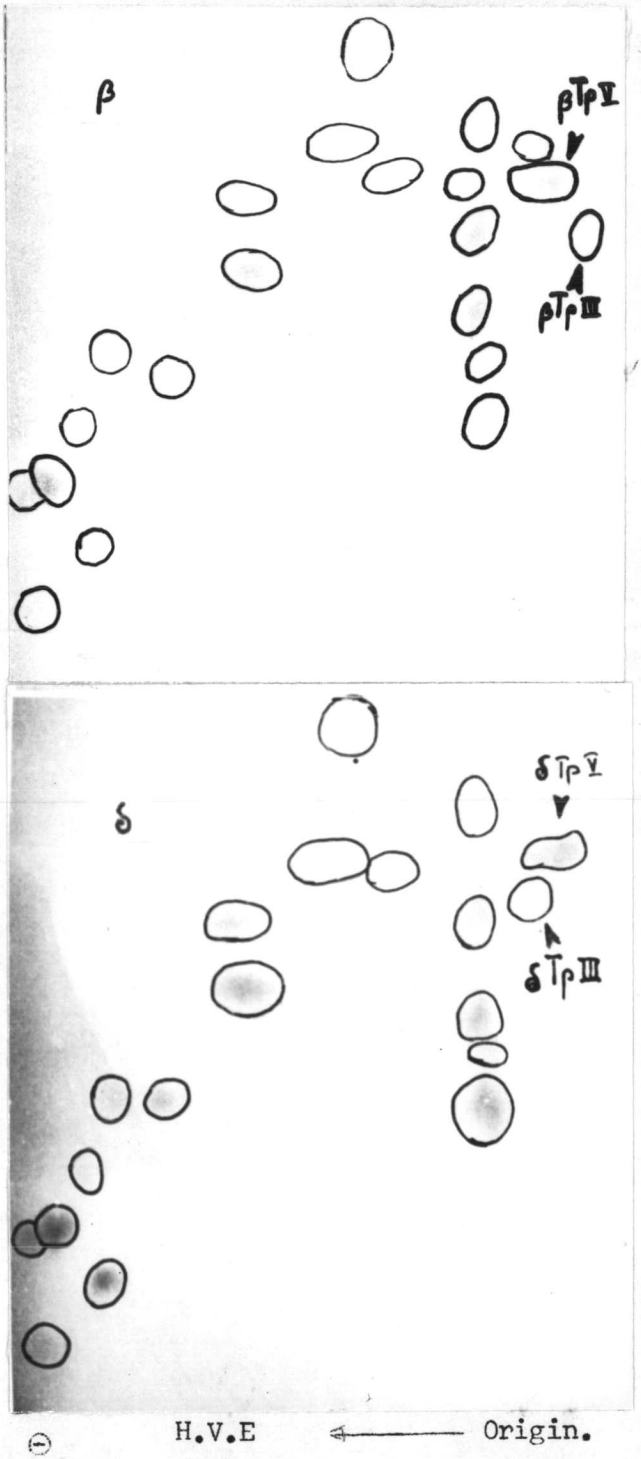


Figure 11 Preparative chain separation by CM-cellulose chromatography of whole haemolysate from peripheral blood of Hb H disease with Hb Thai, previously fractionated on a Sephadex G-100 column.



(1) Beta-chain.

(2) Delta-chain.

Figure 12 Fingerprint of tryptic peptide globin chains, stained with ninhydrin, from HbH disease with Hb Thai. δ TpIII peptide migrated slightly ahead δ TpV in H.V.E., while the β TpIII moved behind β TpV. The δ TpV and β TpV are known to be identical in amino acid sequence.

the β-peak corresponds to δ-chain. Since the globin prepared from haemolysate after Sephadex G-100 chromatography, which a free globin chain was excluded, the δ-chain was presumably present in a tetrameric molecule. As already indicated in 2.1, HbA_2 was absent in the patient, thus the δ-chain presumably did not join to the α-chain forming a tetrameric haemoglobin molecule of $\alpha_2\delta_2$ -HbA_2. It was possible that the δ-chain was present in polymerized form of δ_4, like HbH (β_4), or in the unusual structure.

2.4 Sephadex G-100 chromatography of whole haemolysate prepared from bone marrow incorporative study of a HbH disease with Hb Thai.

About 2 ml of haemolysate which was prepared from a 3 hour incorporative study of bone marrow cells from a patient was fractionated on a 3 X 120 cm column of Sephadex G-100 in Tris-KCN buffer. The chromatogram is shown in figure 13. Based on the optical density at 540 and 280 nm, and radioactivity profile, two major haem-proteins, peaks A and C were noted. B was the haem-protein fractions between peak A and C. Peak D presumably represented non-haem protein as peak B in figure 2.

The fractions; A, B and C were separately pooled, concentrated by CM-Sephadex and carbowax method. The haemoglobin fractions were then studied on starch-gel electrophoresis (figure 14). Fraction A revealed a major component of HbA and trace Y and Z bands of Hb Thai. Fraction C showed almost of HbH. Fraction B revealed components of overlapping fraction A and C.

Haemoglobin fractions A, B and C were concentrated and treated

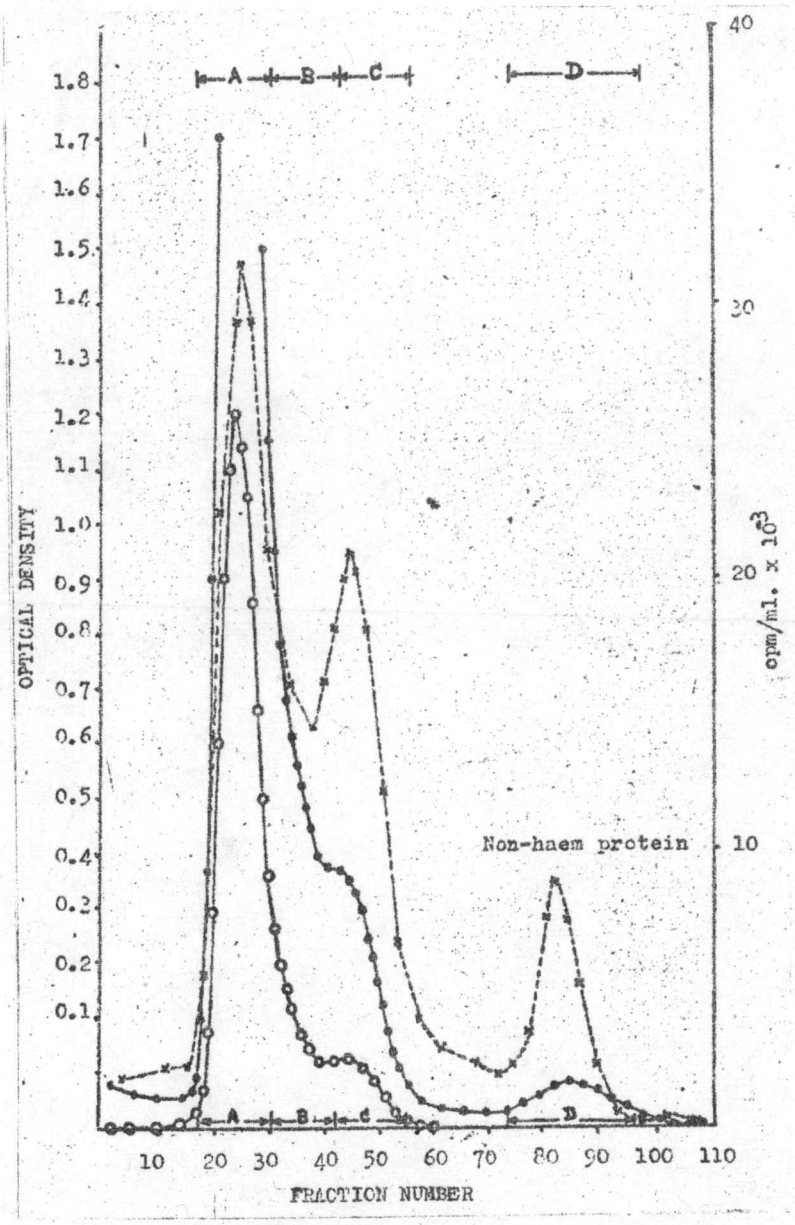
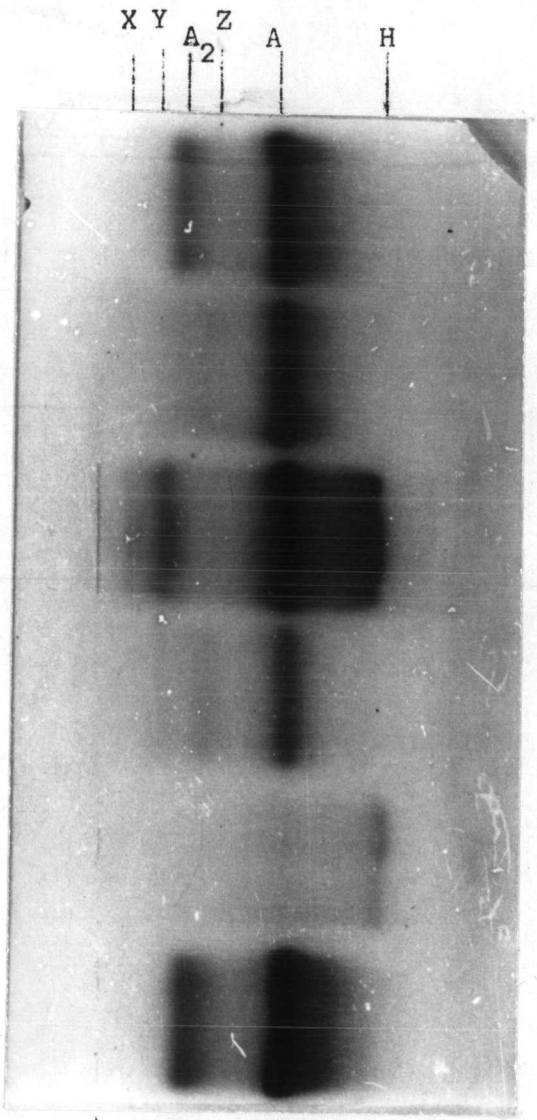


Figure 13 Chromatography of gel-filtration on Sephadex G-100 of the fresh lysate of labelled nucleated bone marrow cells. ●——● O.D. 280 nm., o——o O.D. 540 nm., x-----x radioactivity.



(1) A_2+ A

(2) FRACTION A (Y + Z + A)

(3) XY + A + H

(4) FRACTION B (Y + Z + A +trace H)

(5) FRACTION C (H + ?δ–chain)

(6) A_2+ A

Figure 14 Starch-gel electrophoresis in Tris-borate-EDTA buffer, pH 8.6, of haemoglobin fraction from figure 13, stained with orthodianisidine.

with acid acetone to obtain globin. Cold HbA as carrier was added into fraction B and C except in fraction A. The CM-cellulose chromatography of each globin chain is shown in figures 15, 16 and 17 respectively. The CM-cellulose chromatography of radioactive globin of fraction A which contained mostly HbA (figures 14 and 15) shows that the radioactivity of α-chain was markedly increased approximately three folds than that of β-chain in spite of 3 hours incorporative study. This indicated a large pool of β-chain in reticulocytes and nucleated red cells. Only a radioactive peak corresponding to β-chain, not to α-chain (figure 17), was observed on the CM-cellulose chromatography of labelled globin from fraction C-HbH from figure 14. This substantiates the concept of the existence of a pool of β-chain in reticulocytes and nucleated red cells. Fraction B contained Y and Z band of Hb Thai + HbA + trace HbH (figure 14). Radioactivity peak of β-chain was definitely higher than that of α-chain on CM-cellulose chromatography of fraction B globin (figure 16). It was evident that two peaks of radioactivity eluted after α^A-chain presumably represented the α-slow globin chain which were α^Y and α^X of Hb Thai. Small peaks of radioactivity corresponding to γ- and δ-chain were also noted. It can be seen that the radioactive unbound protein on CM-cellulose chromatography of figure 16 was remarkably high when compared with those of figures 15 and 17, in spite of the fractions A, B and C already passing through Sephadex G-100 column.

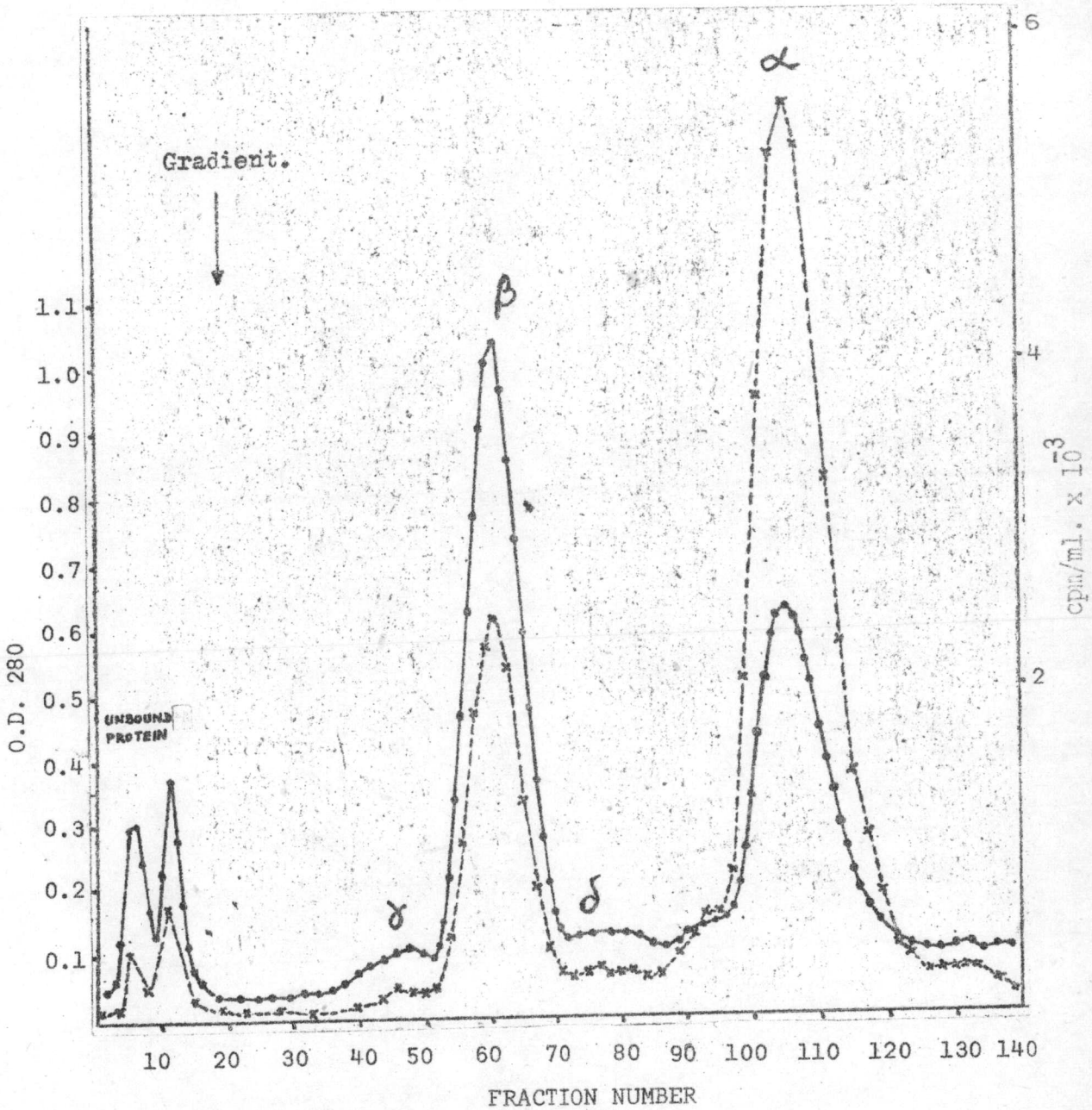


Figure 15 Chain separation by CM-cellulose chromatography of labelled globin from fraction A in figure 13 without Hb A carrier.

●——● O.D. 280 nm., x-----x radioactivity.

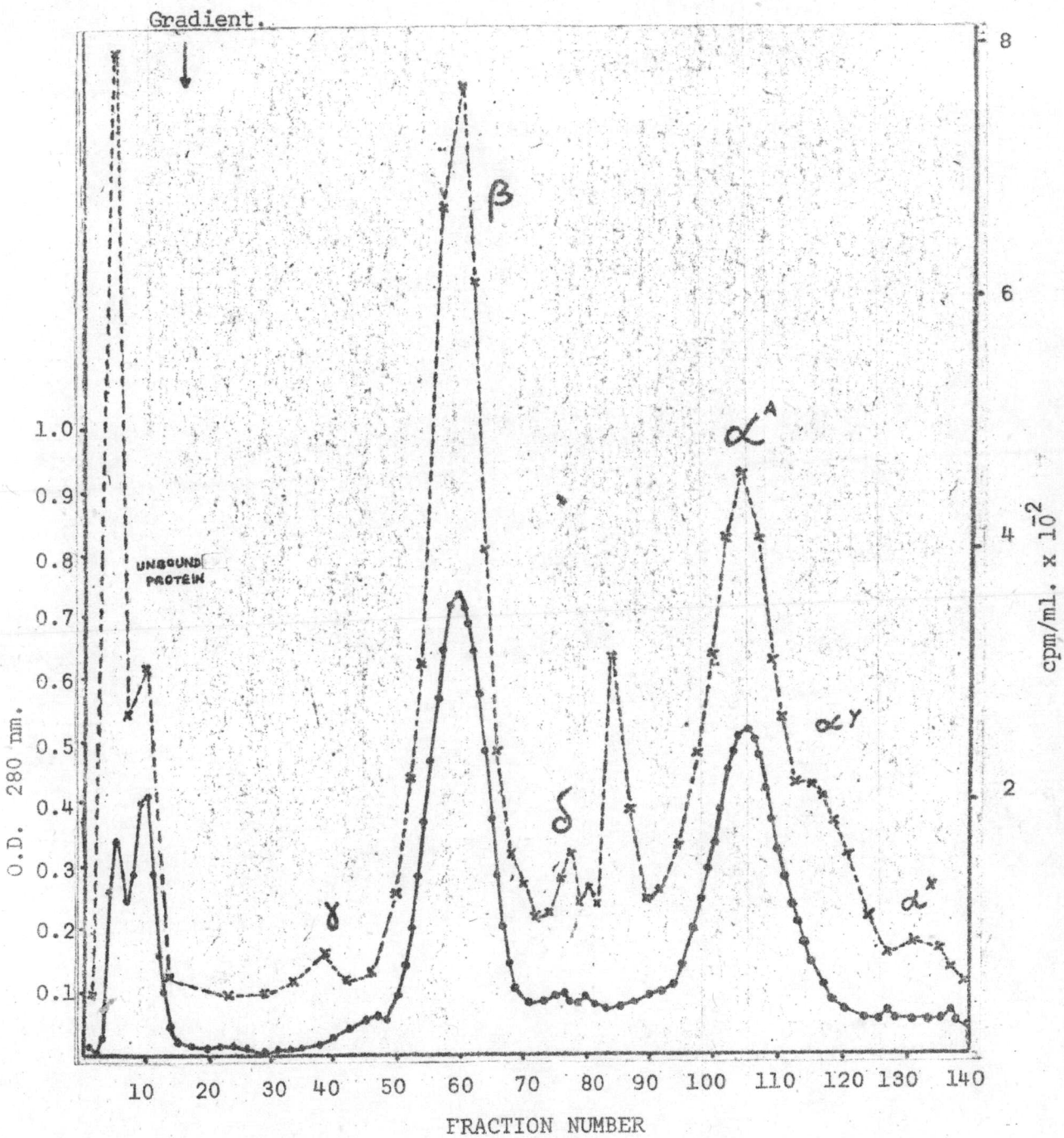


Figure 16 Chain separation by CM-cellulose chromatography of labelled globin from fraction B in figure 13 with cold Hb A carrier. ●——● O.D. 280 nm., x----x radioactivity.

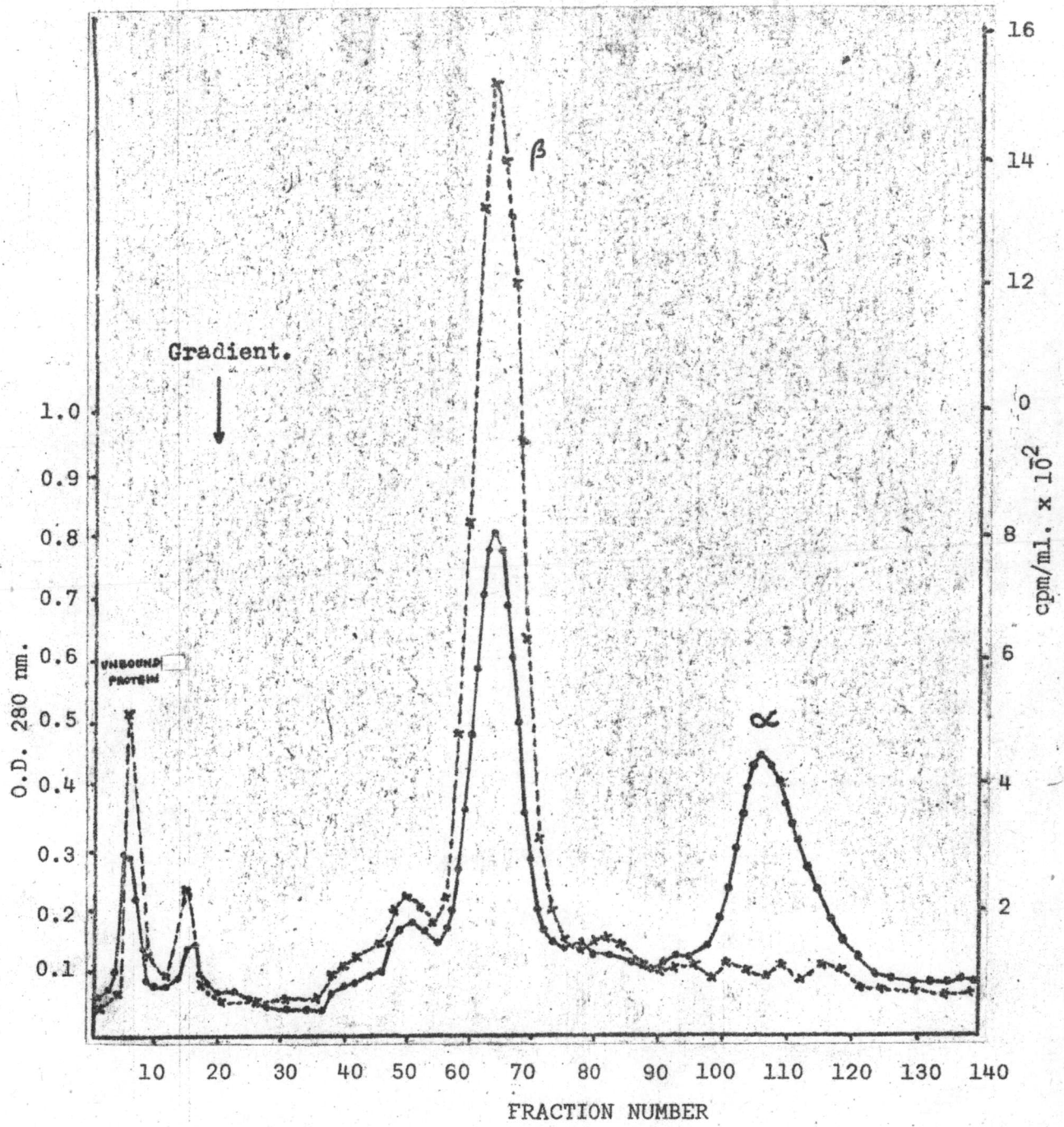


Figure 17 Chain separation by CM-cellulose chromatography of labelled globin fraction C in figure 13 with cold Hb A carrier.
●——● O.D. 280 nm., x----x radioactivity.

3 Studies on Biosynthesis of Hb Thai.

3.1 CM-cellulose chromatography of isolated slow haemoglobin-Hb Thai.

Radioactive haemolysate from HbH disease with Hb Thai was fractionated on DEAE-Sephadex. The slow haemoglobin component corresponding to Hb Thai was concentrated and mixed with cold haemolysate of HbA as carrier. Globin prepared from the mixed haemolysate was separated by CM-cellulose chromatography as shown in figure 18. It can be seen that radioactivity appeared in two major and two minor peaks. The two major peaks correspond to the β- and α^A globin chains. The two minor peaks which were eluted after the α^A, one was a small peak presumably α^X which was well separated from the α^A, the other appeared as a "hump" (α^Y) at the shoulder of the α^A. It should be pointed out that a large incorporation of radioactivity was expected to be at α^Y and α^X chain, instead of the α^A. The radioactivity of the α^A was supposed to be absent since the labelled globin was prepared from the isolated Hb Thai, which has been known to contain no α^A chain in the haemoglobin molecule.

3.2 Hb Thai derivatives demonstrated on starch-gel electrophoresis.

On starch-gel electrophoresis at pH 8.6 of a fresh haemolysate, Hb Thai consists of two slow components namely X and Y (figure 8.2). At least within 15 hours during the isolation process on DEAE-Sephadex chromatography, the X and Y components of Hb Thai were not changed (figure 8.4, p 36). After 20 hours however, another component-Z band besides X and Y of Hb Thai was observed (figure 19.2). The increased

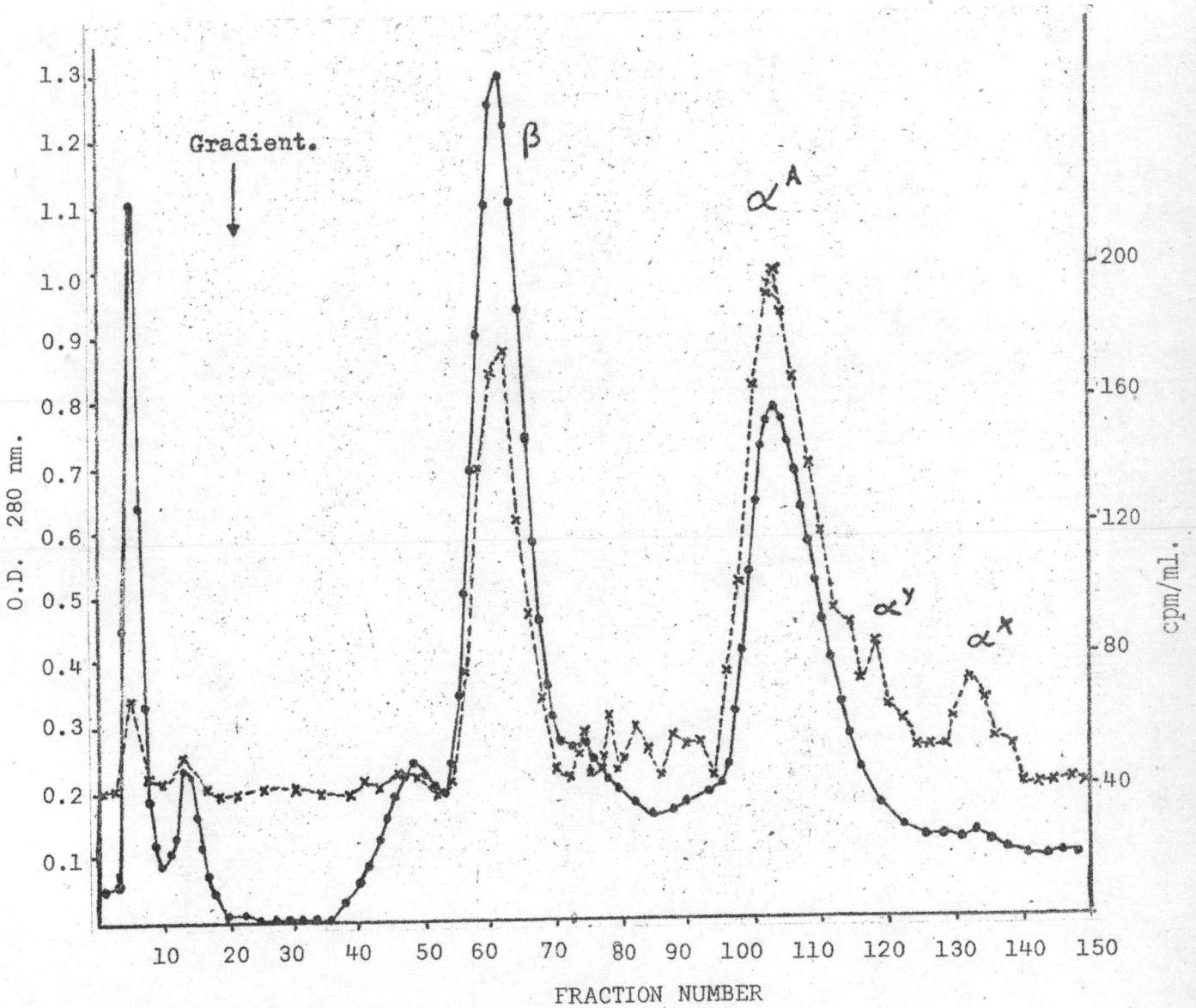
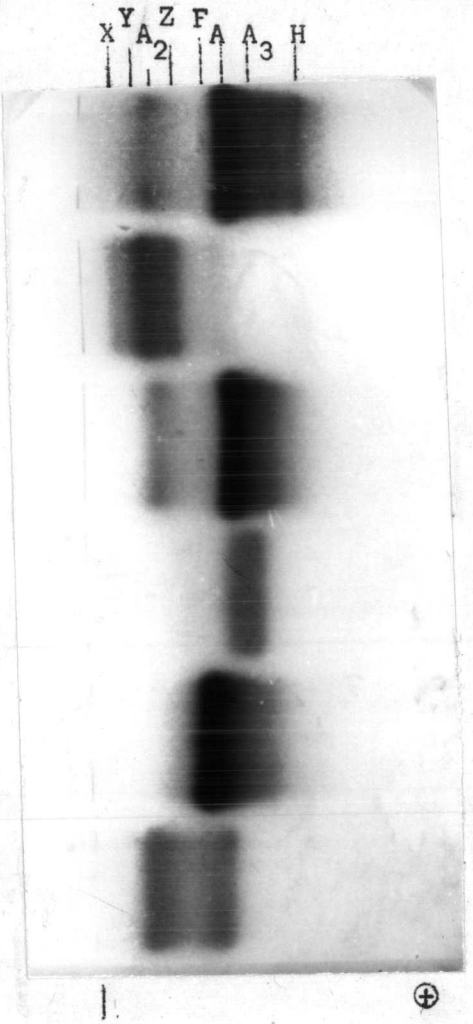


Figure 18 CM-cellulose chromatography of slow haemoglobins, previously separated on DEAE-Sephadex chromatography, with cold Hb A carrier. α^Y and α^X presumably represented two minor radioactive peaks. ●——● O.D. 280 nm., x----x radioactivity.



(1) A_2+ A + H

(2) XYZ

(3) A_2+ A

(4) A_3

(5) F + trace A

(6) Z + A?

Figure 19 Starch-gel electrophoresis in Tris-borate-EDTA buffer pH 8.6, of slow haemoglobins after separation on DEAE-Sephadex, stained with orthodianisidine.

1. Haemolysate of HbH disease (α-thal$_1$/α-thal$_2$).
2. The slow haemoglobins isolated by DEAE-Sephadex from lysate of HbH disease with Hb Thai.
3. Haemolysate of normal control.
4. Haemolysate of HbA_3.
5. Haemolysate of cord blood.
6. The same as (2), after storage for 24 hours.

amounts of the Z component was correlated to the diminished amounts of the X and Y components. Furthermore, when the isolated Hb Thai haemolysate was stored over 24 hours,a derivative band-migrated at the same rate of HbA appeared on starch-gel electrophoresis (figure 19.6). This suggested that the Hb Thai molecule was unstable leading to the formation of Z and the band corresponding to HbA.

3.3 Characterization of globin chains in the slow haemoglobin components-Hb Thai.

The slow haemoglobin components from a large amounts of haemolysate of HbH disease were separated by a preparative DEAE-Sephadex chromatography. The isolated slow haemoglobin, whithout carrier, were then prepared for globin. The CM-cellulose chromatography, as shown in figure 20, revealed five peaks namely A, B, C, D and E respectively. Fractions of each peak, as indicated in figure 20, were separately pooled and carried out for tryptic peptide mapping studies. Peptide maps of the peaks A, B and C were identical to the peptide maps of β, δ (figure 12, p 41) and α-chain (figure 21.2) respectively. Peptide maps of the peaks D and E, as shown in figures 21.3 and 21.4 respectively, were similar to pattern of normal α-chain (figure 21.1), but three extra peptides were also noted. Based on the studies of Clegg et al. (1971), the peptide maps of the peaks D and E were compatible with the pattern of α^{Y} (α^{CS1}) and α^{X}(α^{CS2}) respectively. The peptide map of the slow α-chain of Hb Thai (figure 21.5) from figure 11, p 40 was also similar to pattern of α^{Y} (figure 21.3).

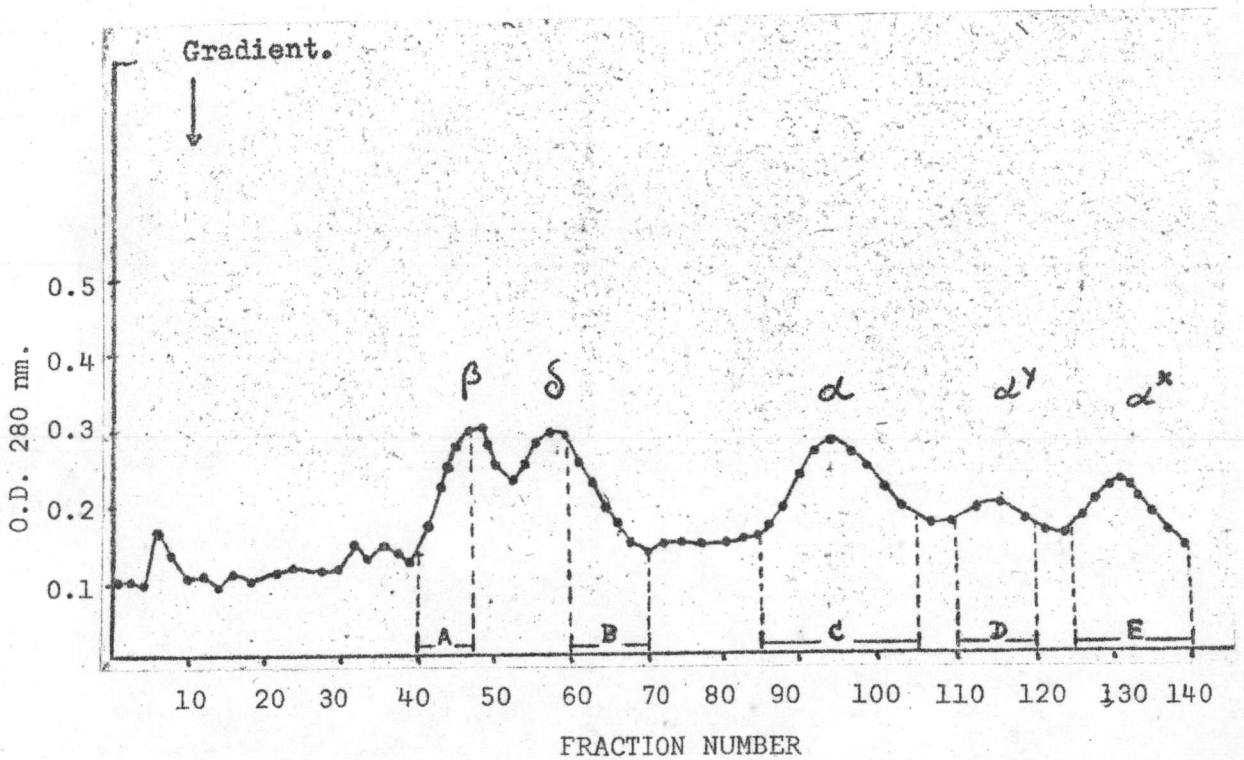


Figure 20 Preparative CM-cellulose chromatography of globin which was prepared from slow haemoglobin fractions of Hb H with Hb Thai separated on DEAE-Sephadex. A, B, C, D and E represented the peaks and also indicated fractions separately pooled for chain characterization.

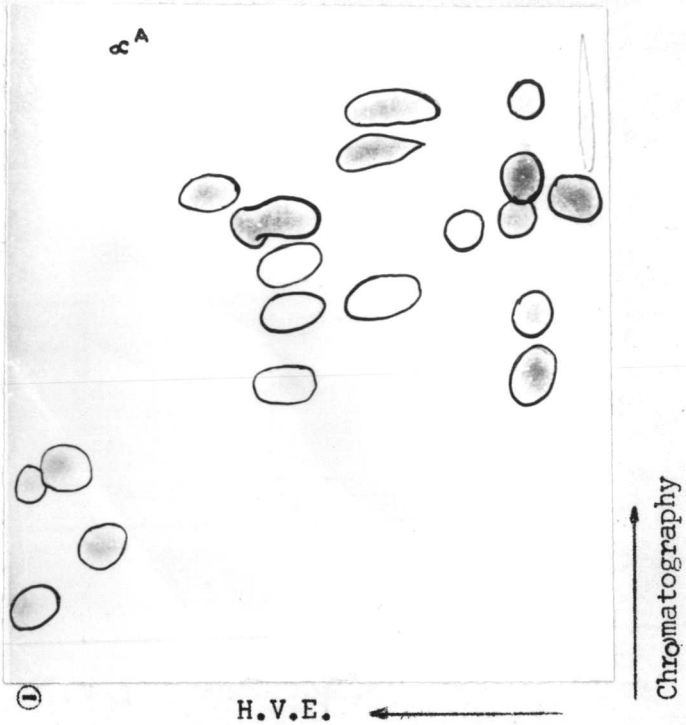


Figure 21.1 Fingerprint of tryptic peptide of α^A-chain control, stained with ninhydrin.

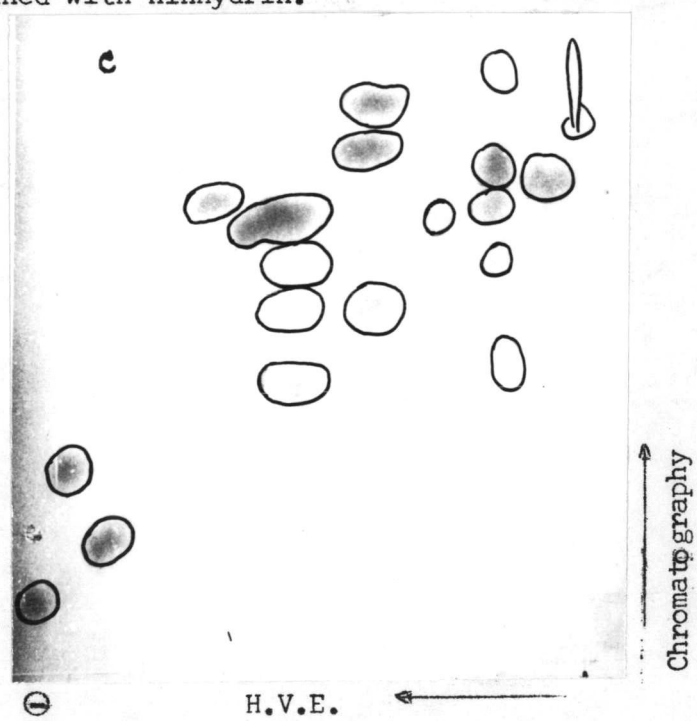


Figure 21.2 Fingerprint of tryptic peptide of peak C globin from figure 20. The peptide map was identical to the peptide map of α^A.

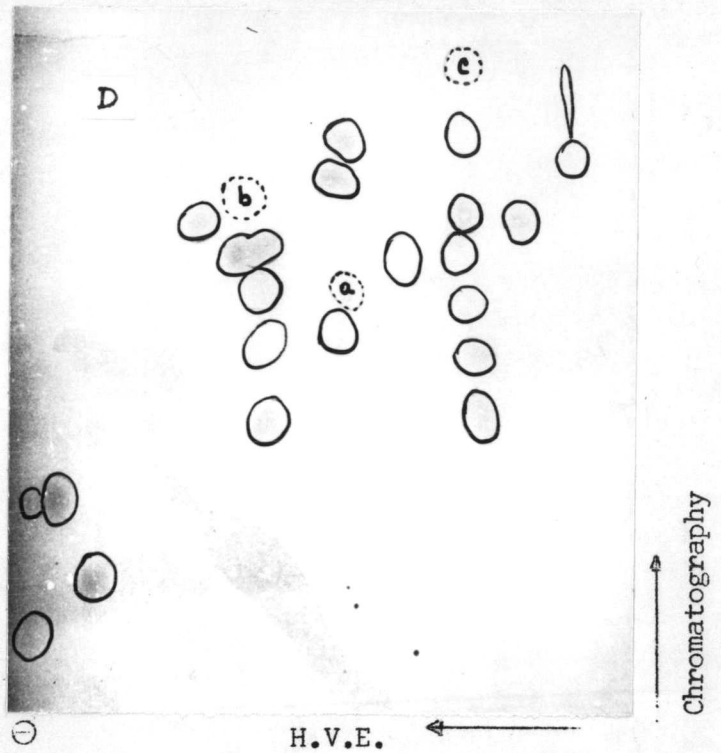


Figure 21.3 Fingerprint of tryptic peptide of peak D globin from figure 20.

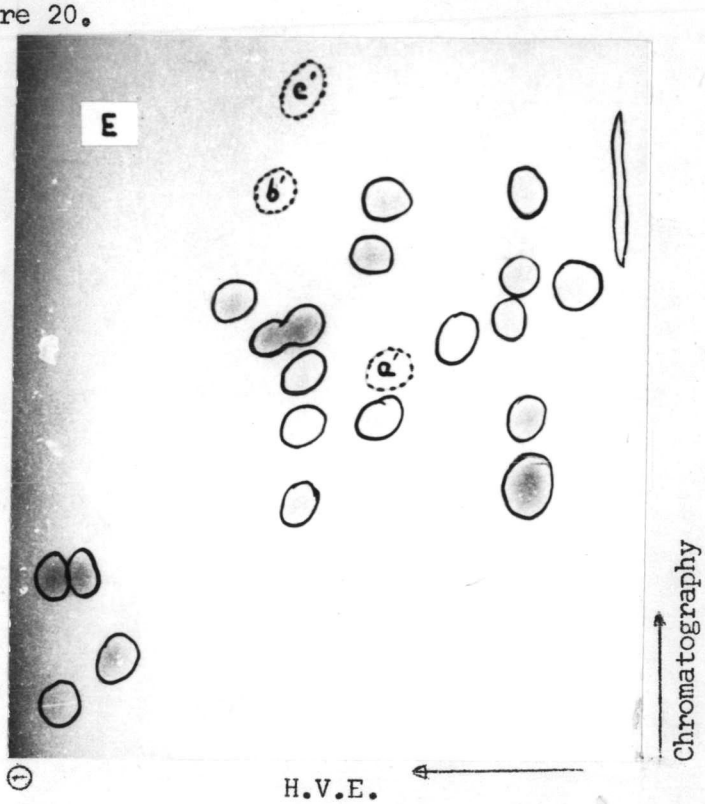


Figure 21.4 Fingerprint of tryptic peptide of peak E globin from figure 20.

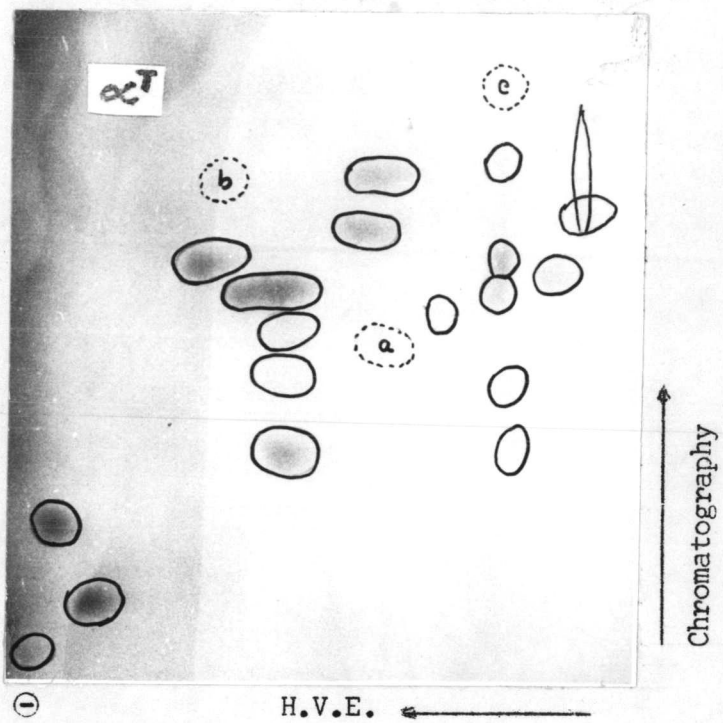


Figure 21.5 Fingerprint of tryptic peptide of minor peak globin followed α^{A}-chain from figure 11.

3.4 Studies on synthetic rate of Hb Thai and HbA.

Haemoglobin synthesis was studied in reticulocyte and bone marrow samples, obtained from a patient of HbH disease with Hb Thai, for one and four hours. The labelled haemolysates were fractionated by DEAE-Sephadex chromatography. Fractions corresponding to the slow haemoglobin component-Hb Thai and corresponding to HbA were pooled. The optical density and radioactivity were determined. The specific activity (cpm/O.D.) after one and four hours of incorporation in both Hb Thai and HbA from reticulocytes are shown in figure 22.1. Similar studies in bone marrow are shown in figure 22.2. In reticulocyte incorporation, the specific activity of the HbA at one and four hours incorporation revealed a continuous increase, while that of Hb Thai was about 1.6 times higher at one hour incubation than that of HbA. However, at the four hour incorporation the specific activity of Hb Thai was less than that of HbA.

In bone marrow studies, the specific activity of Hb Thai revealed a sharper linear increase. The specific activity of Hb Thai was about 8 fold greather than that of HbA throughout the incorporative study. However, this experiment was not repeated because of the limitation of getting the specimen, the results are very encouraging and further studies are highly suggested.

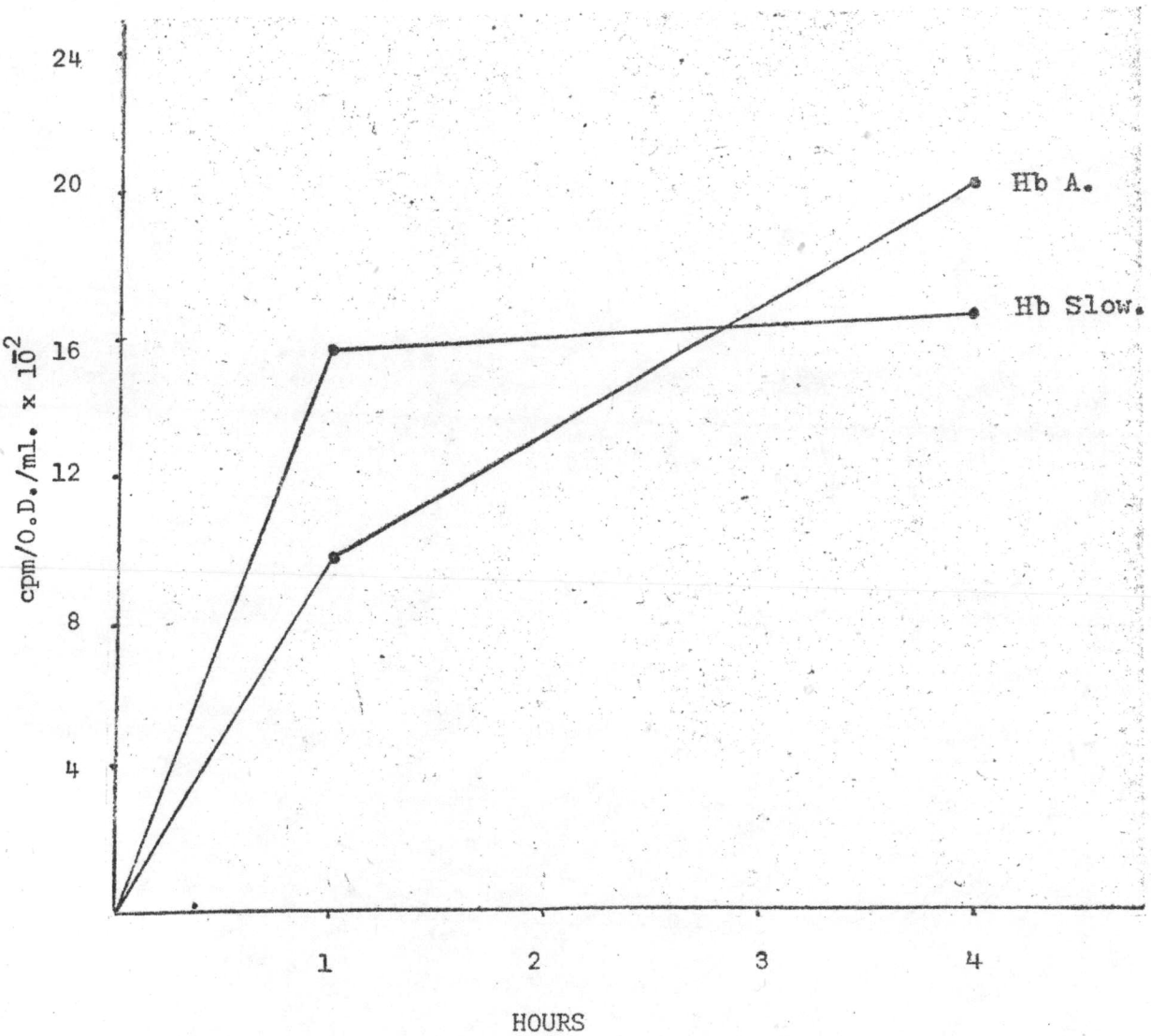


Figure 22.1 Specific activity of the isolated Hb Thai and Hb A synthesis at 1 and 4 hours from reticulocytes of Hb H with Hb Thai.

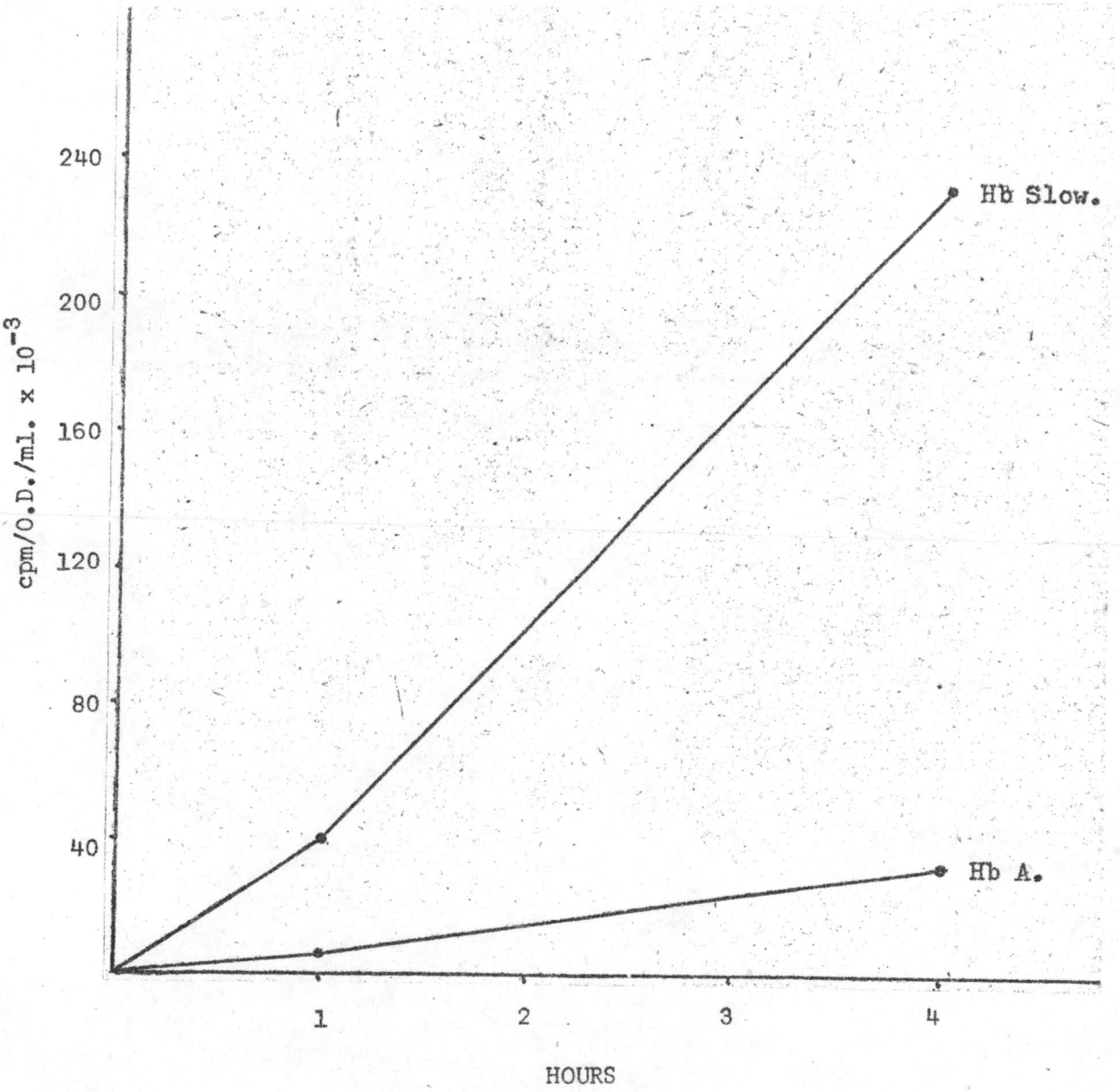


Figure 22.2 Specific activity of the isolated Hb Thai and HbA synthesis at 1 and 4 hours from bone marrow of the same patient HbH disease with Hb Thai.