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IDENTIFICATION AND SOME PROPERTIES OF HEMOGLOBIN QUEENS [α 34 (B15) LEU → ARG] IN A THAI SUBJECT

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(B.Sc. in Medical Technology)

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จาก
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entitled

IDENTIFICATION AND SOME PROPERTIES OF HEMOGLOBIN QUEENS [α 34 (B15) LEU → ARG] IN A THAI SUBJECT

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BAWP	n-butanol/acetic acid/water/pyridine
CM-cellulose	carboxymethyl-cellulose
DEAE-cellulose	diethylaminoethyl-cellulose
1,3 DPG	1,3-diphosphoglycerate
2,3 DPG	2,3-diphosphoglycerate
EDTA	ethylenediamine tetraacetic acid
Ga-3P	glyceraldehyde-3-phosphate
Ga-3PD	glyceraldehyde-3-phosphate dehydrogenase
Hb	hemoglobin
Hct	hematocrit
HVPE	high voltage paper electrophoresis
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
3PG	3-phosphoglycerate
Pi	inorganic phosphorus
PGM	phosphoglycerate mutase
TEMED	N, N, N', N'-tetramethylene ethylene-diamine
T_p	tryptic peptide
tris	tris-(hydroxymethyl) aminomethane

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ABBREVIATIONS FOR AMINO ACIDS

Amino acid	Three-letter abbreviation
Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic acid	Asp
Asparagine or aspartic acid	Asx
Cysteine	Cys
Glutamine	Gln
Glutamic acid	Glu
Glutamine or glutamic acid	Glx
Glycine	Gly
Histidine	His
Isoleucine	Ile
Leucine	Leu
Lysine	Lys
Methionine	Met
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophan	Trp
Tyrosine	Tyr
Valine	Val

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ABSTRACT

A twenty-four year old healthy Thai male of Chinese ancestry with normal hematologic profile was found to have an abnormal hemoglobin (about 12% of total hemoglobin) moving slower to the anode than Hb A on electrophoresis in starch gels and on cellulose acetate membranes in Tris-EDTA borate buffer, pH 8.6 and pH 9.1 respectively. Another faint abnormal band moving slower to the anode than Hb A_2 was also detected on starch gel electrophoresis so that the abnormality in the propositus was believed to be an α chain variant. Electrophoresis of globin chains on cellulose acetate in acid-urea-mercaptoethanol buffer, pH 6.5 and on Triton-X 100-acid-urea polyacrylamide gels indicated that the abnormal globin chain moved faster to the cathode than normal α chain. Preparative isolation of the abnormal hemoglobin was performed by DEAE-cellulose column chromatography and the abnormal α chain was purified by CM-cellulose column chromatography. A fingerprint of the tryptic peptides of the abnormal α chain from the propositus showed that $\alpha T_p V$ was lost but two new peptides were present, which had amino acid compositions indicating the replacement of a leucyl residue in position 34 by an arginyl residue. This amino acid interchange in the α_1 β_1 contact was first found in 1979 by Tatsis and named Hb Queens [α 34 (B15) Leu $\rightarrow$ Arg]. The whole hemolysate of the propositus showed normal heat stability and isopropanol stability, but the isolated abnormal

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hemoglobin was somewhat less heat stable than Hb A. Red blood cells showed normal morphology and osmotic fragility, and were negative for the inclusion body test.

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INTRODUCTION

Hemoglobin is a very important and abundant protein found in vertebrate erythrocytes or red blood cells, comprising about 97.5% of the red cell protein (1). Since the total blood volume in normal adult human is 5 liters, every milliliter of blood has approximately $5x10^{12}$ red blood cells and each cell is packed with $2.8x10^{8}$ molecules of hemoglobin, so that the adult human probably contains about $7.0x10^{21}$ molecules of hemoglobin (2). Hemoglobin plays an important role in blood circulation as an oxygen transporter permitting the body to indulge high oxygen consumption and also acts as a buffer to protect the body against acid metabolites (3). No other mammalian protein is as well understood from both the genetic and biochemical points of view as is hemoglobin (3).

1. Structure of hemoglobin

Hemoglobin is an allosteric protein with a molecular weight of approximately 65,000, consisting of a tetramer of two α subunits and two non-α subunits (4) : each subunit contains one heme group and one polypeptide chain. The heme group is composed of a porphyrin ring with an iron atom at its centre (Fig. 1). In humans, six different polypeptide chains may be synthesized at various stages : two α- like globin chains (α and ζ) containing 141 amino acid residues and 4 non α - like globin chains (β, δ, γ, and ε) containing 146 amino acid residues (5-13). The amino acid compositions of each polypeptide chain are summarized in Table 1. In addition, there are

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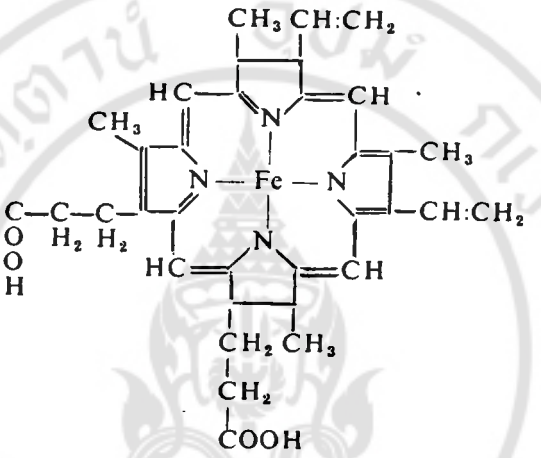


Fig. 1 Structure of heme, a porphyrin ring with an iron atom at its centre.

(taken from Lehman and Huntsman (1974) Ref. 3)

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Table 1 Primary Structure of Human Globin Subunits (taken from Bunn et al, 1977 Ref.43 and Clegg et al, 1981 Ref. 8)

Helix*	α	ζ	Helix*	β	δ	γ
NA1	1 Val	Ser	NA1	1 Val	Val	Gly
			NA2	2 His	His	His
NA2	2 Leu	Leu	NA3	3 Leu	Leu	Phe
A1	3 Ser	Thr	A1	4 Thr	Thr	Thr
A2	4 Pro	Lys	A2	5 Pro	Pro	Glu
A3	5 Ala	Thr	A3	6 Glu	Glu	Glu
A4	6 Asp	Glu	A4	7 Glu	Glu	Asp
A5	7 Lys	Arg	A5	8 Lys	Lys	Lys
A6	8 Thr	Thr	A6	9 Ser	Thr	Ala
A7	9 Asn	Ile	A7	10 Ala	Ala	Thr
A8	10 Val	Ile	A8	11 Val	Val	Ile
A9	11 Lys	Val	A9	12 Thr	Asn	Thr
A10	12 Ala	Ser	A10	13 Ala	Ala	Ser
A11	13 Ala	Met	A11	14 Leu	Leu	Leu
A12	14 Trp	Trp	A12	15 Trp	Trp	Trp
A13	15 Gly	Ala	A13	16 Gly	Gly	Gly
A14	16 Lys	Lys	A14	17 Lys	Lys	Lys
A15	17 Val	Ile	A15	18 Val	Val	Val
A16	18 Gly	Ser				
AB1	19 Ala	Thr				
B1	20 His	Gln	B1	19 Asn	Asn	Asn
B2	21 Ala	Ala	B2	20 Val	Val	Val
B3	22 Gly	Asp	B3	21 Asp	Asp	Glu
B4	23 Glu	Thr	B4	22 Glu	Ala	Asp
B5	24 Tyr	Ile	B5	23 Val	Val	Ala
B6	25 Gly	Gly	B6	24 Gly	Gly	Gly
B7	26 Ala	Thr	B7	25 Gly	Gly	Gly
B8	27 Glu	Glu	B8	26 Glu	Glu	Glu
B9	28 Ala	Thr	B9	27 Ala	Ala	Thr
B10	29 Leu	Leu	B10	28 Leu	Leu	Leu
B11	30 Glu	Glu	B11	29 Gly	Gly	Gly
B12	31 Arg	Arg	B12	30 Arg	Arg	Arg
B13	32 Met	Leu	B13	31 Leu	Leu	Leu
B14	33 Phe	Phe	B14	32 Leu	Leu	Leu
B15	34 Leu	Leu	B15	33 Val	Val	Val
B16	35 Ser	Ser	B16	34 Val	Val	Val
C1	36 Phe	His	C1	35 Tyr	Tyr	Tyr

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* The residues have been aligned to demonstrate homology between subunits.

Table 1 Primary Structure of Human Globin Subunits (continued)

Helix*	α	ζ	Helix*	β	δ	γ
C2	37 Pro	Pro	C2	36 Pro	Pro	Pro
C3	38 Thr	Gln	C3	37 Trp	Trp	Trp
C4	39 Thr	Thr	C4	38 Thr	Thr	Thr
C5	40 Lys	Lys	C5	39 Gln	Gln	Gln
C6	41 Thr	Thr	C6	40 Arg	Arg	Arg
C7	42 Tyr	Tyr	C7	41 Phe	Phe	Phe
CE1	43 Phe	Phe	CD1	42 Phe	Phe	Phe
CE2	44 Pro	Pro	CD2	43 Glu	Glu	Asp
CE3	45 His	His	CD3	44 Ser	Ser	Ser
CE4	46 Phe	Phe	CD4	45 Phe	Phe	Phe
			CD5	46 Gly	Gly	Gly
CE5	47 Asp	Asp	CD6	47 Asp	Asp	Asn
CE6	48 Leu	Leu	CD7	48 Leu	Leu	Leu
CE7	49 Ser	His	CD8	49 Ser	Ser	Ser
CE8	50 His	Pro	D1	50 Thr	Ser	Ser
			D2	51 Pro	Pro	Ala
			D3	52 Asp	Asp	Ser
			D4	53 Ala	Ala	Ala
			D5	54 Val	Val	Ile
			D6	55 Met	Met	Met
CE9	51 Gly	Gly	D7	56 Gly	Gly	Gly
E1	52 Ser	Ser	E1	57 Asn	Asn	Asn
E2	53 Ala	Ala	E2	58 Pro	Pro	Pro
E3	54 Gln	Gln	E3	59 Lys	Lys	Lys
E4	55 Val	Leu	E4	60 Val	Val	Val
E5	56 Lys	Arg	E5	61 Lys	Lys	Lys
E6	57 Gly	Ala	E6	62 Ala	Ala	Ala
E7	58 His	His	E7	63 His	His	His
E8	59 Gly	Gly	E8	64 Gly	Gly	Gly
E9	60 Lys	Ser	E9	65 Lys	Lys	Lys
E10	61 Lys	Lys	E10	66 Lys	Lys	Lys
E11	62 Val	Val	E11	67 Val	Val	Val
E12	63 Ala	Val	E12	68 Leu	Leu	Leu
E13	64 Asp	Ala	E13	69 Gly	Gly	Thr
E14	65 Ala	Ala	E14	70 Ala	Ala	Ser
E15	66 Leu	Val	E15	71 Phe	Phe	Leu
E16	67 Thr	Gly	E16	72 Ser	Ser	Gly
E17	68 Asn	Asp	E17	73 Asp	Asp	Asp
E18	69 Ala	Ala	E18	74 Gly	Gly	Ala

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*The residues have been aligned to demonstrate homology between subunits.

Table 1 Primary Structure of Human Globin Subunits (continued)

Helix*	α	ζ	Helix*	β	δ	γ
E19	70 Val	Val	E19	75 Leu	Leu	Ile
E20	71 Ala	Lys	E20	76 Ala	Ala	Lys
EF1	72 His	Ser	EF1	77 His	His	His
EF2	73 Val	Ile	EF2	78 Leu	Leu	Leu
EF3	74 Asp	Asp	EF3	79 Asp	Asp	Asp
EF4	75 Asp	Asp	EF4	80 Asn	Asn	Asp
EF5	76 Met	Ile	EF5	81 Leu	Leu	Leu
EF6	77 Pro	Gly	EF6	82 Lys	Lys	Lys
EF7	78 Asn	Gly	EF7	83 Gly	Gly	Gly
EF8	79 Ala	Ala	EF8	84 Thr	Thr	Thr
F1	80 Leu	Leu	F1	85 Phe	Phe	Phe
F2	81 Ser	Ser	F2	86 Ala	Ser	Ala
F3	82 Ala	Lys	F3	87 Thr	Gln	Gln
F4	83 Leu	Leu	F4	88 Leu	Leu	Leu
F5	84 Ser	Ser	F5	89 Ser	Ser	Ser
F6	85 Asp	Glu	F6	90 Glu	Glu	Glu
F7	86 Leu	Leu	F7	91 Leu	Leu	Leu
F8	87 His	His	F8	92 His	His	His
F9	88 Ala	Ala	F9	93 Cys	Cys	Cys
FG1	89 His	Tyr	FG1	94 Asp	Asp	Asp
FG2	90 Lys	Ile	FG2	95 Lys	Lys	Lys
FG3	91 Leu	Leu	FG3	96 Leu	Leu	Leu
FG4	92 Arg	Arg	FG4	97 His	His	His
FG5	93 Val	Val	FG5	98 Val	Val	Val
G1	94 Asp	Asp	G1	99 Asp	Asp	Asp
G2	95 Pro	Pro	G2	100 Pro	Pro	Pro
G3	96 Val	Val	G3	101 Glu	Glu	Glu
G4	97 Asn	Asn	G4	102 Asn	Asn	Asn
G5	98 Phe	Phe	G5	103 Phe	Phe	Phe
G6	99 Lys	Lys	G6	104 Arg	Arg	Lys
G7	100 Leu	Leu	G7	105 Leu	Leu	Leu
G8	101 Leu	Leu	G8	106 Leu	Leu	Leu
G9	102 Ser	Ser	G9	107 Gly	Gly	Gly
G10	103 His	His	G10	108 Asn	Asn	Asn
G11	104 Cys	Cys	G11	109 Val	Val	Val
G12	105 Leu	Leu	G12	110 Leu	Leu	Leu
G13	106 Leu	Leu	G13	111 Val	Val	Val
G14	107 Val	Val	G14	112 Cys	Cys	Thr

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*The residues have been aligned to demonstrate homology between subunits.

Table 1 Primary Structure of Human Globin Subunits (continued)

Helix*	α	ζ	Helix*	β	δ	γ
G15	108 Thr	Thr	G15	113 Val	Val	Val
G16	109 Leu	Leu	G16	114 Leu	Leu	Leu
G17	110 Ala	Ala	G17	115 Ala	Ala	Ala
G18	111 Ala	Ala	G18	116 His	Arg	Ile
G19	112 His	Arg	G19	117 His	Asn	His
GH1	113 Leu	Phe	GH1	118 Phe	Phe	Phe
GH2	114 Pro	Pro	GH2	119 Gly	Gly	Gly
GH3	115 Ala	Ala	GH3	120 Lys	Lys	Lys
GH4	116 Glu	Asp	GH4	121 Glu	Glu	Glu
GH5	117 Phe	Phe	GH5	122 Phe	Phe	Phe
H1	118 Thr	Thr	H1	123 Thr	Thr	Thr
H2	119 Pro	Ala	H2	124 Pro	Pro	Pro
H3	120 Ala	Glu	H3	125 Pro	Gln	Glu
H4	121 Val	Ala	H4	126 Val	Met	Val
H5	122 His	His	H5	127 Gln	Gln	Gln
H6	123 Ala	Ala	H6	128 Ala	Ala	Ala
H7	124 Ser	Ala	H7	129 Ala	Ala	Ser
H8	125 Leu	Trp	H8	130 Tyr	Tyr	Tyr
H9	126 Asp	Asp	H9	131 Gln	Gln	Gln
H10	127 Lys	Lys	H10	132 Lys	Lys	Lys
H11	128 Phe	Phe	H11	133 Val	Val	Met
H12	129 Leu	Leu	H12	134 Val	Val	Val
H13	130 Ala	Ser	H13	135 Ala	Ala	Thr
H14	131 Ser	Val	H14	136 Gly	Gly	Gly,Ala
H15	132 Val	Val	H15	137 Val	Val	Val
H16	133 Ser	Ser	H16	138 Ala	Ala	Ala
H17	134 Thr	Ser	H17	139 Asn	Asn	Ser
H18	135 Val	Val	H18	140 Ala	Ala	Ala
H19	136 Leu	Leu	H19	141 Leu	Leu	Leu
H20	137 Thr	Thr	H20	142 Ala	Ala	Ser
H21	138 Ser	Glu	H21	143 His	His	Ser
HC1	139 Lys	Lys	HC1	144 Lys	Lys	Arg
HC2	140 Tyr	Tyr	HC2	145 Tyr	Tyr	Tyr
HC3	141 Arg	Arg	HC3	146 His	His	His

*The residues have been aligned to demonstrate homology between subunits.

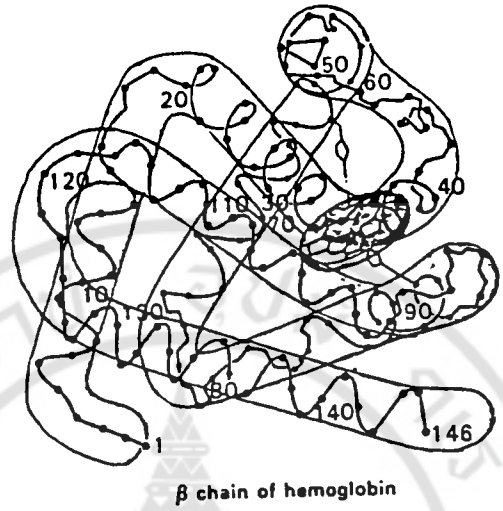
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two types of γ chain; $^{G}\gamma$ and $^{A}\gamma$ which differ in amino acid sequence at the position 136 in which an alanine is present in the $^{A}\gamma$ chain and glycine is present in the $^{G}\gamma$ chain. Normal adult human hemoglobin is composed of three major components : Hb A (α_2 β_2), Hb A_2 (α_2,δ_2), and Hb F (α_2 γ_2) in relative amounts of approximately 97%, 2.5% and 0.5% respectively.

Hemoglobin A, the predominant hemoglobin in normal adult human, consists of 2 α subunits and 2 β subunits. In both the α and the β subunit, about 80% of the peptide chain is α-helical (Fig. 2 a) (14) and the tertiary structures are strikingly similar to that of myoglobin. Accordingly, following the convention adopted for myoglobin (15-17), the α helices in hemoglobin are named by the letters A through to H starting from the amino terminus, and the non-helical segments between the helical regions are given the letters of the preceeding and the following helices (AB, CD, etc). The β globin chain contains eight helical segments (A-H) and the α globin chain has seven helical segments (the D helix is absent). In each chain, the heme group is buried in a pocket and is held by about 60 non covalent interactions with atoms of adjacent side chains (18). The iron atom of the heme is directly bonded to 2 nitrogen atom of proximal histidine (F8) which occupies the fifth coordination position (Fig. 2b). The sixth coordination position on the other side of the heme plane is the binding site for oxygen. A second histidine, termed the distal histidine (E7) lies next to this site. The distal and proximal histidines are residue

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a.



b.

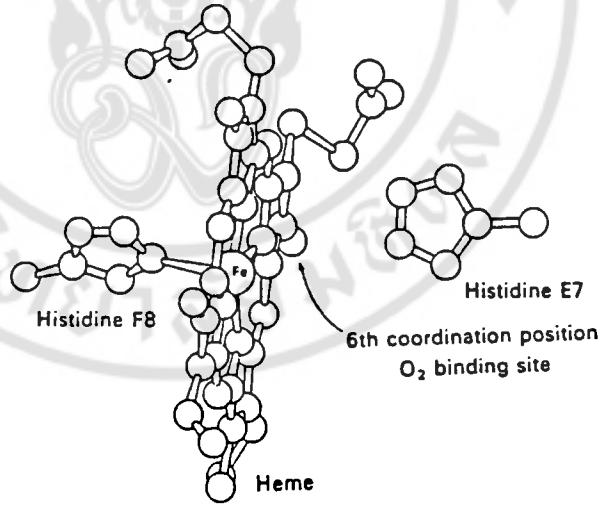


Fig. 2 The three dimensional structure of the β chain of hemoglobin showing the α helical structure (a), the proximal (His F8) and distal (His E7) histidine and the oxygen binding site (b)
(taken from Stryer, L. (1981) Ref. 14)

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numbers 58 and 87 respectively in the α chain and residues 63 and 92 respectively in the β chain. The quaternary structure arises from the association of four heme/globin units by means of hydrogen bonding and non-polar interactions. The charged residues are situated on the surface of the subunit where they can form contacts with water, and most of the non polar residues are directed to the interior of the subunit to contribute to the formation of the heme pocket and to stabilize the overall conformation (19).

The quaternary structure of oxy and deoxyhemoglobin show some differences in that the oxygenated molecule is more compact. For instance, the distance between the iron atoms of the β chains decreases from 39.9 to 33.4 A^{o} on oxygenation. The changes in the contacts between the α and β chain are of special interest, and as demonstrated by Perutz and his coworkers, most of the contacts between subunits are between side chains of unlike subunits (20-24). Two types of α β contacts can be defined namely $\alpha_1 \beta_1$ and $\alpha_1 \beta_2$ (Fig. 3). From considerations of symmetry the $\alpha_1 \beta_1$ contact is identical to $\alpha_2 \beta_2$ and the $\alpha_1 \beta_2$ contact is identical to $\alpha_2 \beta_1$. Thirty four amino acid residues are involved in the $\alpha_1 \beta_1$ contact and nineteen residues in $\alpha_1 \beta_2$ contact : These involve mainly Van der Waals forces and some hydrogen bonds. In the transition from oxy to deoxyhemoglobin, there is little change in the $\alpha_1 \beta_1$ contact. In contrast, there are large structural changes at the $\alpha_1 \beta_2$ contact; one pair of α β subunits rotates relative to the other α β pair by 15 degrees on oxygenation. Therefore the $\alpha_1 \beta_1$ contact

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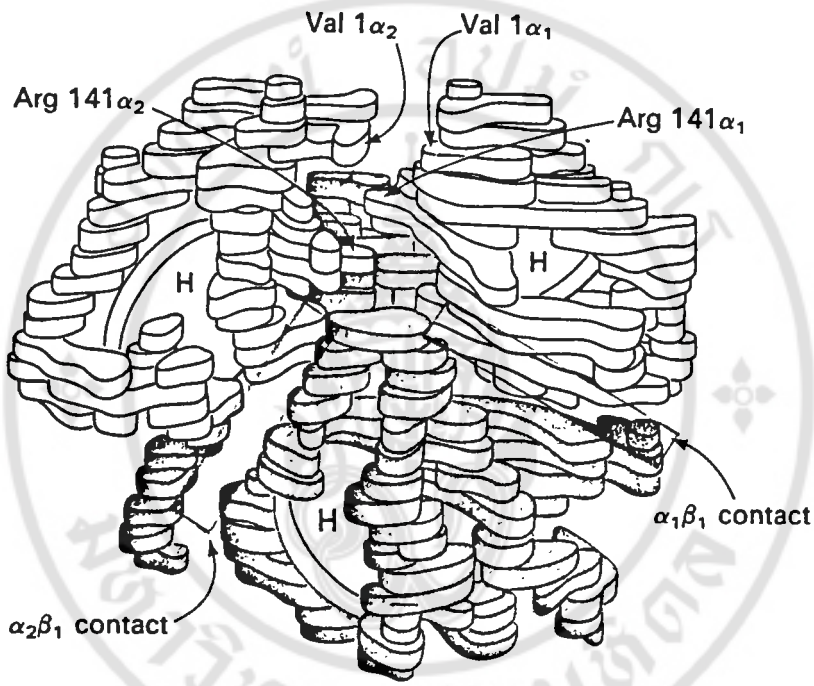


Fig. 3 Model of quaternary structure of hemoglobin showing areas of α_1 β_1 contact and α_2 β_1 contact. (taken from Stryer, L. (1981) Ref. 14)

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seems to be relatively fixed while the α_1 β_2 contact allows a sliding movement to take place (20). In addition, deoxyhemoglobin is stabilized by both inter-and intra-subunit salt bridges (ionic interactions) involving the carboxyl-terminal residues of the peptide chains. In contrast to oxyhemoglobin, the carboxyl-terminal residues of all four chains have almost complete freedom of rotation.

2. Normal human hemoglobin and development

The synthesis of each polypeptide chain is under the principal control of a specific structural gene which produces only one type of sequence : for α-like globin chains, the structural genes are located on the short arm of the 16^{th} chromosome and for β-like globin chains the genes are located on the short arm of the 11^{th} chromosome (Fig. 4) (25-28). Six polypeptide chains are synthesized at various stages during development from fetus to adult (Fig. 5). The ζ chain which is homologous to the α chain becomes supplanted by the α chain early in the prenatal period, and ε chain is replaced by γ chain at about the same time then finally the γ chain is replaced by β (or δ) chain shortly after birth (29, 30). Therefore, in the prenatal development, four hemoglobins are present : the original Hb Gower I (ζ_2 ε_2) is replaced first by Hb Gower II (α_2 ε_2) and Hb Portland (ζ_2 γ_2), and finally by Hb F (α_2 γ_2). After birth, the synthesis of Hb F is drastically reduced and a corresponding increase in HbA (α_2 β_2) occurs together with HbA_2 (α_2 δ_2).

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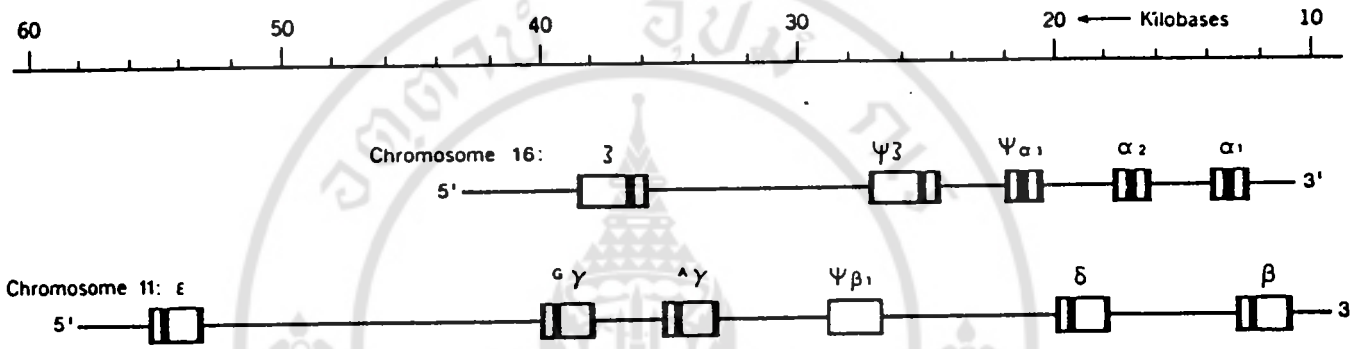


Fig. 4 Arrangement of hemoglobin genes along two human chromasomes. The α family of genes lies on chromosome 16, and the β family is on chromosome 11. Rectangular boxes are the transcribed genes, and thin horizontal lines are intervening sequences representing untranscribed spacer DNA. Exon are represented by vertical black bands and introns are shown in white. $\psi\alpha_1$ and $\psi\beta_1$ are pseudogenes, which are not translated into protein chains.
(taken from Dickerson and Gies (1983) Ref. 2)

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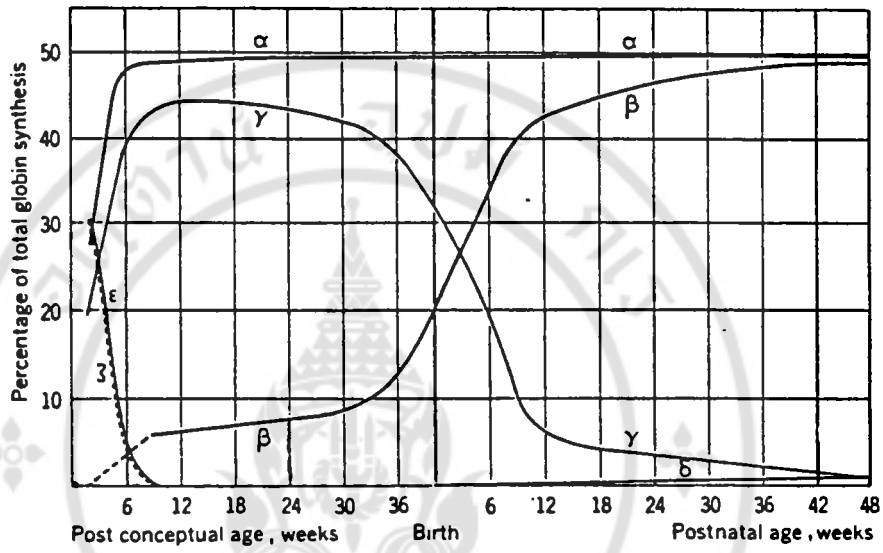


Fig. 5 Relative amounts of α, β, γ, δ, ε and ζ chains at different times in the development of the human embryo, fetus, infant and after birth.
(taken from Dickerson and Geis (1983) Ref. 2)

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In addition, there are several minor components found in normal adult hemoglobin designated as "glycosylated hemoglobin" : Hb A_{1a}, Hb A_{1b} and Hb A_{1c} (31), comprising approximately 1.6, 0.8 and 4% of the total hemoglobin. These minor components are produced by nonenzymatic post-translational modification of Hb A and formed slowly and continuously throughout the life span of the red cell (32). Hb A_{1c} is the most abundant minor component and structural analysis of Hb A_{1c} has shown that the NH_2 terminus of the β chains forms a Schiff base linkage with glucose and then undergoes an Amadori rearrangement to a stable ketoamine linkage (Fig. 6) (33-35). This reaction occurs slowly within the erythrocyte at a rate dependent on the blood glucose concentration (36) so that the level of Hb A_{1c} is increased about two fold in the patients with diabetes mellitus (37-39). Accordingly, a number of clinical investigators have suggested the usefulness of measuring Hb A_{1C} in the assessment of diabetic control (40-41).

3. Functional properties of human hemoglobin

In its function as the oxygen carrier of the blood, hemoglobin is capable of specifically binding large quantities of oxygen (1.34 ml O_2 per gram hemoglobin) (42). The oxygen binding site is at the sixth coordination position between the heme plane and the distal histidine as shown in Fig. 2b. To be able to fulfit its function as oxygen carrier, hemoglobin must combine with oxygen in the lungs and release it to the tissues. The oxygen binding affinity of Hb A is such that at the oxygen tension found in the air sacs of the lung, hemoglobin

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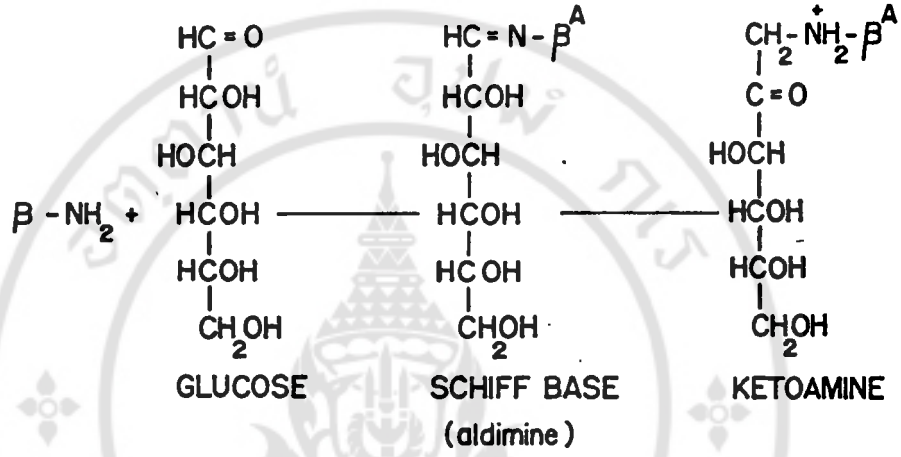


Fig. 6 Hb A_{1c} synthesis during the life span of red cell (taken from Bunn (1981) Ref. 34)

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becomes 97% saturated with oxygen. Thus, normal blood would carry about 1.34 ml O_2 per gram hemoglobin while at the capillary where the venous oxygen tension is normally about 40 mm Hg, hemoglobin is about 75% saturated with oxygen. Thus, blood normally give up an average of 0.03 ml O_2 per gram hemoglobin during its circulation through the tissues.

The oxygen binding capacity of hemoglobin can be expressed in terms of P_{50} : the oxygen tension at which hemoglobin is half-saturated with oxygen. Since hemoglobin is an allosteric protein, its oxygen dissociation curve is sigmoidal in shape compared to the hyperbolic curve of myoglobin (43, 44). This functional property is influenced by the intraerythrocytic concentration of three modifiers, protons, CO_2 and 2, 3-diphosphoglycerate (2, 3-DPG) (45-47). These modifiers modulate the binding of O_2 and act to facilitate its release at the tissues. The binding of oxygen to hemoglobin may be expressed as.

$$Hb + nO_2 \rightleftharpoons Hb\ (O_2)_n$$

Under acidic conditions with an excess of the protons, the equilibrium between oxy and deoxyhemoglobin is shifted towards the deoxyhemoglobin.

$$HbO_2 + H^+ \longrightarrow HbH^+ + O_2$$

As a result, the oxygen dissociation curve is shifted to the right; this is known as Bohr effect. Carbon dioxide also exerts its effect by directly binding to hemoglobin, forming carbamino compounds (48).

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$$Hb(O_2)_4 + nCO_2 \rightleftharpoons Hb(CO_2)_n + 4O_2$$

and by indirectly producing protons when it dissolves in blood plasma to form bicarbonate ions.

$$CO_2 + H_2O \longrightarrow HCO_3^- + H^+$$

In addition to protons and CO_2, 2, 3-DPG is another factor which binds to hemoglobin reciprocally with oxygen.

$$Hb(O_2)_4 + DPG \rightleftharpoons HbDPG + 4O_2$$

2,3 DPG is an important regulator of hemoglobin oxygen affinity. Its concentration within the red cell is normally about 5 mmoles per liter of packed red cells (49). The negatively charged 2,3-DPG can bind to deoxyhemoglobin but not to oxyhemoglobin, and stabilizes the deoxyhemoglobin structure by interacting with three positively charged groups : lysine (82), histidine (143) and val (the N-terminal amino group) of both β chains (50). On the other hand, in oxyhemoglobin, this complementarity with 2,3-DPG was lost because the central cavity becomes too small to accomodate 2,3-DPG molecule.

From X-ray diffraction studies on human and on horse hemoglobin, Perutz and his coworkers (43, 50) have suggested that hemoglobin can exist in two conformations, the R (for relaxed) state corresponding to that of fully oxygenated hemoglobin, and the T (for tense) state, corresponding to that of unoxygenated hemoglobin. The change from the T to the R state is produced by a shift, on oxygenation, in the position of the heme iron atom and the histidyl residue to which it is linked.

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However, among hemoglobins, differences between the two chains of the non-α globin chain cause the differences in the

functional properties of each hemoglobin. The structural characteristics of HbF, fetal hemoglobin result in a decreased binding of 2,3-DPG by this protein, causing an increase in oxygen affinity compared to that of HbA. Therefore the fetal hemoglobin (HbF) has a higher oxygen affinity than adult human hemoglobin (HbA) (51), promoting the transport of oxygen from the maternal circulation to the fetus through the placenta.

4. The hemoglobinopathies

The hemoglobinopathies or genetic disorders of hemoglobin involve two main groups : in the first group : the "thalassemias", where synthesis of the globin chain is reduced or absent, and the "abnormal hemoglobins" where an abnormal polypeptide chain is produced.

4.1 The thalassemias

The thalassemias refer to a defect in the synthesis of one or more of the normal globin chains of hemoglobin. This defect causes a decrease or absence of the affected chain. There are two major types of thalassemia, α-thalassemias and β-thalassemias (52).

4.1.1 α-thalassemia

The α thalassemias (α thal) are inherited disorders characterised by a decrease or lack of synthesis of the α globin chain (53). Fig. 4 shows arrangement of the structural genes of the α-like chains, indicating two α globin genes on each chromosome

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and representing four functional α genes per diploid genome.

In α thalassemia, the deletions may involve one, two, three or all four of the α genes. There are two main genetic determinants. The first is α thalassemia 1 (α thal 1) which is caused by deletion of two α globin genes in a cis manner (54, 55), so that the heterozygous genotype is written as --/αα and the haploid type as --. The size of the deletion in both α genes in the α thal 1 defect varies from one group of individuals to another (57), and the deletion may start to the 5' side of the ζ_2 gene and extents into the α_1 gene, leaving a non-functional 3' α_1 gene fragment (57). The other major defect is α thalassemia 2 (α thal 2) which is caused by deletion or dysfunction of one of the four α genes (58, 59) so that the heterozygous genotype is written as -α/αα and the haplotype as -α. The deletion in the α thal 2 may also vary (60) and two different deletions have been characterized : in the leftward deletion α thal 2 genotype, there is a 4.2 kb deletion involving the α_2 globin gene, and in the rightward deletion α thal 2 genotype, α 3.7 kb segment involving the region bridging the α_2 and α_1 globin genes is deleted (59, 61, 62).

The more severe forms of α thalassemias are quite widespread. Hb H disease which was first described by Rigas et al. in a Chinese family (63) and involves deletion or dysfunction of three of the four α genes due to compound heterozygosity for the α-thal 1 and α-thal 2 determinants (-α/--) (64-66). The ratio of α to β chain synthesis is therefore greatly reduced, and the excess β chains accumulate and form

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β4 tetramers (Hb H), which are characterized by the presence of inclusion or Heinz bodies in the erythrocytes. Patients with Hb H disease have a mild to moderate hemolytic anemia. The most severe form of α thalassemia is Hb Bart's hydrops fetalis which was first reported by Lie-Injo and Jo (67), where there is complete absence of α chain synthesis due to deletion or dysfunction of all four α genes (homozygous α thal 1) (68-70). This leads to the formation of Hb Bart's (γ_4) as the major hemoglobin in affected infants. Hb Bart's has a very high oxygen affinity, and therefore releases relatively little oxygen to the tissues. The resultant severe tissue hypoxia leads to hydrops fetalis, with fetal or neonatal death.

4.1.2 β-thalassemia

The β thalassemias are inherited disorders of human β globin synthesis characterized by decreased or lack of expression of the adult β globin gene.

In contrast to α thalassemias, gene deletion does not appear to be the underlying abnormality in β thalassemia. This disorder is very heterogeneous and may be caused by many different defects in the β globin gene, namely point mutation and small deletions or insertions in the nucleotide seguence (71). The classical β thalassemias are of two types, β^+ and β^0 thalassemia. β^+ thalassemia is characterized by a reduced level of β globin chain production which appears to be due to a reduced level of transcription or inefficient processing of the mRNA precursors

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(72, 73). In homozygous β^+ thalassemia, β globin chains are present at 5%-30% of normal levels and there is a proportional deficiency of β globin mRNA in the erythroid cells of affected individuals (74). In β^o thalassemia, β globin chains are completely absent, although small quantities of β globin mRNA may be either present or absent (75, 76). The molecular defect is quite heterogeneous in nature and may result from lack of transcription of the β gene itself (75, 76), nucleotide deletion or duplication (77, 78) or nonsense mutation (102) etc. For example a single nucleotide deletion at coden 44, resulting in a frameshift with termination at codon 60, transcription and mRNA processing appears normal but β-globin mRNA is deficient (79-81).

The heterozygous state of β thalassemia is essentially asymptomatic, although such individuals can be identified by typical abnormalities of red cell morphology and other indices. However, in homozygous β thalassemia, there is greater imbalance of α to β globin chain synthesis than in the heterozyous state. The excess α globin chains tend to precipitate, damaging the cell membrane of the β thalassemic red cell, and causing severe hemolytic anemia and hepatosplenomegaly.

4.2 Abnormal hemoglobins

Since sickle cell hemoglobin (HbS $\alpha_2 \beta_2^{6Glu \rightarrow Val}$) was first described (82), many other structural hemoglobin variants have now been discovered. Variation in structure of the globin chains can be divided into various groups :-

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4.2.1 Hemoglobins with single amino acid substitutions

Over 90% of known hemoglobin variants have arisen by substitution of a single amino acid residue in one chain type, and of these over 60% involve the β chain (19, 83). Most variants involve single base changes in the coding DNA of the structural gene (84). The amino acid substitution can occur in various parts of the molecule.

4.2.1.1 Substitutions on the outside of the subunit: e.g.

Hb S ($\alpha_2 \beta_2^{6Glu \rightarrow Val}$)

4.2.1.2 Substitution on the inside of the subunit : e.g.

Hb Sogn ($\alpha_2 \beta_2^{14\ Leu \rightarrow Arg}$) (85)

4.2.1.3 Substitution at the heme pocket : e.g.

Hb M Boston ($\alpha_2^{58\ His \rightarrow Tyr} \beta_2$) (86)

4.2.1.4 Substitution at the $\alpha_1 \beta_1$ contact : e.g.

Hb Philly ($\alpha_2 \beta_2^{33\ Tyr \rightarrow Phe}$) (87)

4.2.1.5 Substitution at the $\alpha_1 \beta_2$ contact : e.g.

Hb Chesapeak ($\alpha_2^{92\ Arg \rightarrow Leu}\ \beta_2$) (88)

4.2.1.6 Substitution in the internal cavity between like chains : e.g.

Hb Hiroshima ($\alpha_2 \beta_2^{145\ His \rightarrow Asp}$) (89)

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4.2.2 Hemoglobin with two amino acid substitutions in one chain

The structures of five variants containing two amino acid substitutions in one chain are shown in Table 2 (19).

Table 2 Hemoglobins with two amino acid substitution in a chain

Hemoglobin	Structure
J Singapore	α_2 78 Asn → Asp, 79 Ala → Gly β_2
C Harlem	$\alpha_2 \beta_2$ 6 Glu → Val, 73 Asp → Asn
C Ziguinchor	$\alpha_2 \beta_2$ 6 Glu → Val, 58 Pro → Arg
S Travis	$\alpha_2 \beta_2$ 6 Glu → Val, 142 Ala → Val
Arlington Park	$\alpha_2 \beta_2$ 6 Glu → Lys, 95 Lys → Glu

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4.2.3 Hemoglobin with deleted and inserted residues

Several β chain variants have been described (Table 3) in which up to five amino acids are deleted internally from the normal β chain. Althouth no α chain variants with deletions are known, one variant with a three residue insertion has been described; Hemoglobin Grady where the sequence of residues 116-118 (Glu-Phe-Thr) is duplicated in the variant (90).

4.2.4 Hemoglobins which contain extended chain

The hemoglobins which contain α or β globin chains longer than normal and the likely mechanism of their production are listed in Table 4 (19).

4.2.5 Hemoglobins which contain hybrid chains

There are variants which contain a type of polypeptide chain with an amino acid sequence identical in part to the β chain and identical in part to the δ or the γ chain. The structure of the various hybrid chains are summarized in Table 5 (91-93).

In Thailand, α and β thalassemia, Hb E and Hb Constant Spring are prevalent (94-97) with the frequencies being 20%-30% for α thalassemia, 3%-9% for β thalassemia, and up to 52% for Hb E in the North East (Surin province) and at least 4% for Hb Constant Spring.

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5. Diseases due to abnormalities of hemoglobin structure

Hemoglobin structural variants may lead to disease states.

Table 3 Hemoglobins with deleted residues

Hemoglobin	Residues deleted	
Leiden	β 6 or 7	Glu
Lyon	β 17-18	Lys, Val
Fréiburg	β 23	Val
Niteroi	β 42-44 or 43-45	Phe, Gly, Ser
Tochigi	β 56-59	Gly, Asn, Pro, Lys
St. Antonine	β 74-75	Gly, Leu
Tours	β 87	Thr
Gun Hill	β 91-95 or 92-96 or 93-97	Leu, His, Cys, Asp, Lys
Leslie	β 131	Glu

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Table 4 Hemoglobins with extended chain

Hemoglobin	Structure	mechanism of production
Constant Spring-1	α1-141-Gln-thirty residues	UAA ⟶ CAA
Icaria	α1-141-Lys-thirty residues	UAA ⟶ AAA
Seal Rock	α1-141-Glu-thirty residues	UAA ⟶ GAA
Koya Dora	α1-141-Ser-thirty residues	UAA ⟶ UCA
Wayne 1	α1-138-eight residues	frameshift
Tak	β1-146-eleven residues	frameshift
Cranston	β1-144-eleven residues	frameshift
Grady	β1-118-Glu-Phe-Thr-β119-146	in phase crossover

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Table 5 Hemoglobins which contain hybrid chain

Hemoglobin	Hybrid chain
Lepore Hollandia	δ1-22---β50-146
Lepore Baltimore	δ1-50---β86-146
Lepore Boston	δ1-87---β116-146
Miyada	β1-12---δ22-146
P Niloic	β1-22---δ50-146
P Congo	β1-22---δ87-146
Conventry	β1-140---δ142-146
Lincoln Park	β1-22---δ50-146 (δ 137 deleted)
Kenya	$^{A}\gamma$1-81---β86-146

--- region of crossover

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The disease changes caused will depend on the position and type of amino acid involved in the structural change, and whether that change affects an atomic interaction necessary for the stability or function of the molecule. The list of the abnormal hemoglobin with alterations in the function and stability of the molecule are shown in Table 6 (2).

5.1 Sickle cell disease

The sickling of red cells arises from the structural variant, Hemoglobin S, which, upon deoxygenation, undergoes a marked reduction in solubility (98, 99), and forms semisolid gels consisting of linearly aggregated molecules with the characteristics of long nematic fluid crystals, known as tactoids (100, 101). Since the basic defect is the production of a rigid deformed sickled red cell, these cells have an increased mechanical fragility which leads to sickle-cell anemia (102). Furthermore, there is a tendency for rigid sickle cells to aggregate in the small blood vessel resulting in the local hypoxia, and damage of multiple organs. There are several factors which modify the rate of sickling and hence the clinical severity of any sickling disorder. These include the absolute amount of hemoglobin S per red cell, the presence of other hemoglobins in the cell and their degree of interaction with hemoglobin S in fibre formation, and factors which may alter the oxygen affinity of hemoglobin S.

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5.2 Hemoglobin M diseases

Hemoglobins M are mutants either of the α or β chains where the amino acid substitution causes an alteration in the environment of the heme group, so that the iron atom is in the ferric (Fe^{3+}) state. In many cases they involve substitutions of tyrosine for either the proximal or distal histidines, such as hemoglobin M Iwate where histidine F8 is replaced by tyrosine in the α chain causing the heme to become liganded to both tyr F8 and His E7 (103, 104). These variants are associated with permanent cyanosis, and are also less stable than normal hemoglobin leading to mild hemolysis (105). The blood of these patients has a chocolate brown appearance but otherwise the hematological findings are normal. These conditions are diagnosed by the electrophoresis after conversion of the hemolysate to methemoglobin form (106), as well as by spectral studies of the acid methemoglobin derivative (107).

5.3 Hemoglobin with a raised oxygen affinity

Several of the high oxygen affinity variants such as hemoglobin Chesapeake ($\alpha_2^{92\ Arg \longrightarrow Leu}\beta_2$) (88, 108) result from amino acid substitutions which involve the $\alpha_1\ \beta_2$ contacts, while others involve the C-terminal end of the β chain which is involved in maintaining the overall stability of the deoxy or T conformation. Yet others involve the binding sites for 2,3-DPG. With higher oxygen affinity, the oxygen dissociation curve is shifted to the left, causing a defect in unloading oxygen to the tissue. This results in relative hypoxia, increased erythropoietin, and a variable degree of polycythaemia (109).

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5.4 Hemoglobin with decreased oxygen affinity

Fewer mutants are known that decrease oxygen affinity and these are frequently due damage to the oxy or R conformation. Forexample Hb kansas (110) which has a substitution of threonine for asparagine at β 102 in the $\alpha_1 \beta_2$ contact. When normal Hb A is oxygenated β 102 Asn forms a hydrogen bond with α 94 Asp, but in Hb kansas, this bond is lost, so that the low oxygen affinity appears to be due to a relatively unstable R structure. The affected patient suffers from cyanosis, but there are no clinical or hematological abnormalities. In addition, some Hb M variants have been found to have decreased oxygen affinity (104, 111).

5.5 The unstable hemoglobins

The unstable hemoglobins are one of the causes of congenital nonspherocytic hemolytic anemia, since the basis for the hemolysis in these conditions is intracellular precipitation of the unstable variant with subsequent damage to the red cell membrane. The clinical changes vary from patient to patient, some having only slight shortening of red cell life span, while others having a more severe degree of hemolysis. Several types of substitution can lead to hemoglobin instability :

5.5.1 Amino acid substitutions near the heme pocket : e.g. Hemoglobin Biras ($\alpha_2 \beta_2^{88\ Leu \longrightarrow Arg}$) (112), where the substitution of a polar for a non polar residue at the heme pocket allows

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the entrance of water into hydrophobic heme pocket and thereby weekens heme-globin linkage.

5.5.2 Disruption of secondary structure : e.g.

Hemoglobin Duarte ($\alpha_2 \beta_2^{62\ Ala \longrightarrow Pro}$), where the proline residue can not participate in an α helix, having a marked effect on the tertiary structure and the overall stability.

5.5.3 Substitution in the interior of the subunit : e.g.

Hemoglobin Sogn ($\alpha_2 \beta_2^{14\ Leu \longrightarrow Arg}$) (85), where the substitution of a polar for a non-polar residue disrupts the stability of interior hydrophobic interactions of molecule.

5.5.4 Amino acid deletion : e.g.

Hemoglobin Gun Hill ($\alpha_2 \beta_2^{91\text{-}95 \longrightarrow 0}$) (113), which lakcs heme binding to the β chain because of the deletion of five residues in the F-FG region.

5.5.5 Elongated subunits : e.g.

Hemoglobin Cranston ($\alpha_2 \beta_2^{1\text{-}146+\text{eleven residues}}$) (114), where instability is due to the attachment of a hydrophobic segment to the C-terminal end of the β chain.

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Table 6 Human hemoglobin variants leading to alteration in functions and stability of molecule

I. SICKLE CELL AND ASSOCIATED FORMS—β CHAIN			
6(A3)	Glu→Val	S	Sickling (XRD).
6(A3)	Glu→Lys	C	Enhances sickling in S/C heterozygote (XRD).
6(A3)	Glu→Ala	G Makassar	
121(GH4)	Glu→Gln	D Los Angeles, D Punjab, D North America, D Portugal, Oak Ridge, D Chicago	Enhances sickling in S/D heterozygote.
121(GH4)	Glu→Lys	O Arab, Egypt	Enhances sickling in S/O heterozygote.
II. HEMOGLOBIN M (FERRIC IRON)			
A. α CHAIN			
58(E7)	His→Tyr	M Boston, M Osaka, Gothenburg, M Kiskunhalas	DEC; heme liganded to Tyr E7, not His F8 (XRD).
87(F8)	His→Tyr	M Iwate, M Kankakee, M Oldenburg	FERRI; DEC; heme liganded to both Tyr F8 and E7 (XRD).
B. β CHAIN			
28(B10)	Leu→Gln	St. Louis	FERRI; INC; UN; polar substitution tends to open heme pocket (XRD).
63(E7)	His→Tyr	M Saskatoon, M Emory, M Kurume, M Hida, M Radom, M Arhus, M Chicago, Leipzig, Hörlein-Weber, Novi Sad, M Erlangen	FERRI; INC; Tyr ligand stabilizes Fe(III).
63(E7)	His→Pro	Bicetre	UN; auto-oxidizing; Pro disrupts center of helix E.
66(E10)	Lys→Glu	I Toulouse	FERRI; UN; replacement of positive charge by negative stabilizes Fe(III).
67(E11)	Val→Glu	M Milwaukee-I	FERRI, DEC; negative charge stabilizes Fe(III) (XRD).
70(E14)	Ala→Asp	Seattle	Charge repels O_2 and stabilizes Fe(III). Side chain opposes heme tilt of T state.
92(F8)	His→Tyr	M Hyde Park, M Akita	FERRI; His–heme bond disrupted, 25% heme loss (XRD).
III. INCREASED O_2 AFFINITY (INC)			
A. α CHAIN			
6(A4)	Asp→Ala	Sawara	
6(A4)	Asp→Asn	Dunn	
45(CE3)	His→Arg	Fort de France	Salt bridge to heme propionate favors R.
84(F5)	Ser→Arg	Etobicoke	Positive charge on inner side of helix F.
85(F6)	Asp→Asn	G Norfolk	
92(FG4)	Arg→Gln	J Capetown	Slight stabilization of R state (XRD).
92(FG4)	Arg→Leu	Chesapeake	Substitution stabilizes R state (XRD)
95(G2)	Pro→Leu	G Georgia	Dissociation } Alters chain geometry at subunit contact.
95(G2)	Pro→Ala	Denmark Hill	
95(G2)	Pro→Ser	Rampa	Dissociation }
112(G19)	His→Asp	Hopkins-II	UN
126(H9)	Asp→Asn	Tarrant	Eliminates bond to Arg 141 in deoxy.
141(HC3)	Arg→His	Suresnes	Eliminates bond to Asn 126 in deoxy (XRD)
141(HC3)	Arg→Leu	Legnano	Eliminates bond to Asn 126 in deoxy

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B. β CHAIN

9(A6)	Ser→Cys	Porto Alegre	Polymerization; diminished heme–heme cooperativity.
15(A12)	Trp→Arg	Belfast	UN, bulk replaced by positive charge at contact with helix E.
17–18	(Lys-Val)→O	Lyon	
20(B2)	Val→Met	Olympia	Possibly alters AB bend, disturbing N-terminal bonds in deoxy?
23(B5)	Val→O	Freiburg	Shifts A, B helices, breaks terminal bonds, favors oxy or met.
28(B10)	Leu→Gln	St. Louis	FERRI, UN; polar substitution tends to open heme pocket and oppose T state heme tilt. (XRD).
28(B10)	Leu→Pro	Genova	UN, substitution alters packing in heme pocket between helices B and E.
34(B16)	Val→Phe	Pitie-Salpetriere	
35(C1)	Tyr→Phe	Philly	UN, loss of H-bond favors R state and high O_2 affinity even in deoxy.
37(C3)	Trp→Ser	Hirose	Helps free Tyr 145 in deoxy, favoring oxy.
40(C6)	Arg→Lys	Athens-Ga, Waco	Disturbs subunit packing in flexible joint.
40(C6)	Arg→Ser	Austin	Dissociation; disturbs subunit packing in flexible joint.
51(D2)	Pro→Arg	Willamette	Positive charge on inner side of helix D.
62(E6)	Ala→Pro	Duarte	UN; Pro interrupts helix E.
63(E7)	His→Tyr	M Saskatoon, etc.	See hemoglobin M.
63(E7)	His→Arg	Zürich	UN, Arg swings out, opens O_2 pocket (XRD).
74(E18)	Gly→Asp	Shepherds Bush	UN; anion repels DPG, favoring oxy (XRD).
75(E19)	Leu→Arg	Pasadena	UN; introduces charge into hydrophobic corner.
79(EF3)	Asp→Gly	G-Hsi-Tsou	
81(EF5)	Leu→Arg	Baylor	UN; introduces charge into hydrophobic corner.
82(EF6)	Lys→Thr	Rahere	Eliminates positive charge for DPG binding.
82(EF6)	Lys→Met	Helsinki	
85(F1)	Phe→Ser	Bryn Mawr, Buenos Aires	UN; polar group in heme pocket.
87(F3)	Thr→O	Tours	UN, shortens chain and disturbs F helix.
89(F5)	Ser→Asn	Creteil	Probably disturbs packing of H helix, weakens C-terminal bonds in deoxy (XRD).
91–95	(Leu-His-Cys-Asp-Lys)→O	Gun Hill	UN, radically shortens F helix near FG corner.
92(F8)	His→Gln	St. Etienne, Istanbul	UN; dissociation; His–heme bond disrupted.
97(FG4)	His→Gln	Malmö	Slight stabilization of R state.
97(FG4)	His→Leu	Wood	
98(FG5)	Val→Met	Koln, San Francisco (Pacific), Ube-I	UN
98(FG5)	Val→Gly	Nottingham	UN — Substitution alters critical heme contact.
98(FG5)	Val→Ala	Djelfa	UN
99(G1)	Asp→Asn	Kempsey	
99(G1)	Asp→His	Yakima	(XRD) — Breaks deoxy intersubunit bonds to αC7 and αG4.
99(G1)	Asp→Ala	Radcliffe	
99(G1)	Asp→Tyr	Ypsilanti	
99(G1)	Asp→Gly	Hotel-Dieu	
100(G2)	Pro→Leu	Brigham	Changes chain geometry at subunit contact.
101(G3)	Glu→Lys	British Columbia	Alters side-chain size in switch region
101(G3)	Glu→Gly	Alberta	
101(G3)	Glu→Asp	Potomac	
103(G5)	Phe→Leu	Heathrow	Smaller chain at bottom of heme pocket
106(G8)	Leu→Pro	Southampton, Casper	UN, interrupts helix G, alters bottom of heme pocket
106(G8)	Leu→Gln	Tubingen	UN, introduces polar group in bottom of heme pocket
109(G11)	Val→Met	San Diego	Larger chain disturbs $\alpha_1\beta_1$ packing contact, more serious in deoxy (XRD)

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121(GH4)	Glu→Gln	D Los Angeles, D Punjab, D North Carolina, D Portugal, Oak Ridge, D Chicago	
124(H2)	Pro→Gln	Ty Gard	
135(H13)	Ala→Pro	Altdorf	UN, interrupts helix H, breaks up C-terminal interactions in T state.
136(H14)	Gly→Asp	Hope	Asp binds N terminus, decreases DPG affinity.
142(H20)	Ala→Pro	Toyoake	UN; interrupts helix H, breaks up C-terminal interactions in T state.
142(H20)	Ala→Asp	Ohio	Anion repels DPG, favoring R state.
143(H21)	His→Arg	Abruzzo	Bulky side chain at DPG binding site.
143(H21)	His→Gln	Little Rock	Eliminates positive charge for DPG binding.
143(H21)	His→Pro	Syracuse	Probably disturbs C-terminal bonds in deoxy.
144(HC1)	Lys→Asn	Andrew-Minneapolis	Upsets C-terminal bonds, favoring R state.
145(HC2)	Tyr→His	Bethesda	Alkali resistant; forms disulfide bridge; (XRD).
145(HC2)	Tyr→Cys	Rainier	Upsets C-terminal bonds (XRD)
145(HC2)	Tyr→Asp	Fort Gordon, Osler, Nancy	Eliminates C-terminal bonds.
145(HC2)	Tyr→Term	McKees Rocks	Disrupts T state salt bridge and eliminates Bohr proton source (XRD).
146(HC3)	His→Asp	Hiroshima	Disrupts T state salt bridge and eliminates Bohr proton source.
146(HC3)	His→Pro	York	
146(HC3)	His→Leu	Cowtown	
IV. DECREASED O_2 AFFINITY (DEC)			
A. α CHAIN			
43(CD1)	Phe→Val	Torino	UN, Phe assists oxy heme tilt; removal favors T state.
58(E7)	His→Tyr	M Boston, etc	See hemoglobin M.
86(F7)	Leu→Arg	Moabit	UN; introduces charge into invariantly hydrophobic corner of heme pocket.
87(F8)	His→Tyr	M-Iwate, etc.	See hemoglobin M.
94(G1)	Asp→Asn	Titusville	Dissociation; alters intersubunit bond to αG4 in R state.
B. β CHAIN			
1(NA1)	Val→Ac-Ala	Raleigh	Dissociation, deletes positive charge and introduces bulky acetyl group at N terminus.
24(B6)	Gly→Asp	Moscva	UN; upsets close contact with Gly E8.
42(CD1)	Phe→Ser	Hammersmith, Chiba	UN, UN: Phe assists oxy heme tilt; removal favors deoxy.
42(CD1)	Phe→Leu	Louisville, Bucuresti	
42–44 or 43–45	(Phe-Glu-Ser)→O or (Glu-Ser-Phe)→O	Niteroi	UN; drastically alters subunit contacts at flexible joint.
48(CD7)	Leu→Arg	Okaloosa	UN, replaces invariant hydrophobic group by positive charges
67(E11)	Val→Glu	M Milwaukee	See hemoglobin M.
70(E14)	Ala→Asp	Seattle	Charged group at heme contact opens pocket (XRD).
73(E17)	Asp→Tyr	Vancouver	Replaces surface negative charge by bulk or hydrophobicity.
73(E17)	Asp→Val	Mobile	
82(EF6)	Lys→Asn→Asp	Providence	Bond to C terminus traps Tyr 145, favoring deoxy
90(F6)	Glu→Lys	Agenogi	UN, introduces charge into invariantly hydrophobic corner of heme pocket
91(F7)	Leu→Arg	Caribbean	

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102(G4)	Asn→Thr	Kansas	Dissociation, breaks oxy intersubunit bond to αG1, favors deoxy (XRD).
102(G4)	Asn→Ser	Beth Israel	
107(G9)	Gly→Arg	Burke	
108(G10)	Asn→Asp	Yoshizuka	Introduces change in $\alpha_1\beta_1$ contact.
108(G10)	Asn→Lys	Presbyterian	
111(G13)	Val→Phe	Peterborough	UN, upsets $\alpha_1\beta_1$ contact, worse in oxy.
V. UNSTABLE HEMOGLOBINS (UN)[c]			
A α CHAIN			
43(CD1)	Phe→Val	Torino	DEC, loss of heme contact. Milder anemia than Hammersmith.
47(CD5)	Asp→His	Hasharon, Sinai, Sealy, L Ferrara	
53(E2)	Ala→Asp	J Rovigo	
80(F1)	Leu→Arg	Ann Arbor	Introduces positive charge into hydrophobic EF corner.
86(F7)	Leu→Arg	Moabit	DEC; introduces charge into invariantly hydrophobic corner of heme pocket.
91(FG3)	Leu→Pro	Port Phillip	Rigidifies geometry of chain at FG corner.
94(G1)	Asp→Tyr	Setif	Alters subunit contacts at flexible joint.
95(G2)	Pro→Arg	St. Lukes	Dissociation; alters chain geometry at subunit contact.
102(G9)	Ser→Arg	Manitoba	Positive charge at bottom of heme pocket.
109(G16)	Leu→Arg	Suan-Dok	Positive charge at contact with helix B.
112(G19)	His→Asp	Hopkins-II	INC
136(H19)	Leu→Pro	Bibba	Dissociation; Pro disrupts helix H.
B. β CHAIN			
6 or 7	Glu→O	Leiden	
14(A11)	Leu→Pro	Saki	Pro interrupts helix A.
15(A12)	Trp→Arg	Belfast	INC; bulk replaced by positive charge at contact with helix E.
24(B6)	Gly→Arg	Riverdale-Bronx	Upsets close contact with E helix at E8.
24(B6)	Gly→Val	Savannah	
24(B6)	Gly→Asp	Moscva	DEC; upsets close contact with E helix at E8.
27(B9)	Ala→Asp	Volga, Drenthe	Negative charge on inside surface of helix.
28(B10)	Leu→Gln	St Louis	FERRI; INC; very polar group in heme pocket (XRD).
28(B10)	Leu→Pro	Genova	INC, Pro interrupts helix B, alters contact with helix E.
30(B12)	Arg→Ser	Tacoma	Decreased Bohr and heme–heme; normal O_2 affinity.
32(B14)	Leu→Pro	Perth, Abraham Lincoln	Pro interrupts helix B.
32(B14)	Leu→Arg	Castilla	Positive charge in molecular interior.
35(C1)	Tyr→Phe	Philly	INC, loss of $\alpha_1\beta_1$ H bond favors monomers and precipitation (XRD).
42(CD1)	Phe→Ser	Hammersmith, Chiba	DEC; loss of heme Water attracted into pocket
42(CD1)	Phe→Leu	Louisville, Bucuresti	DEC, heme misorientation, but no loss or H_2O. Milder anemia.
42–44 or 43–45	(Phe-Glu-Ser)→O or (Glu-Ser-Phe)→O	Niteroi	DEC; drastically alters subunit contacts at flexible joint.
48(CD7)	Leu→Arg	Okaloosa	DEC, charged group inside subunit
56→59	(Gly-Asn-Pro-Lys)→O	Tochigi	Drastic shortening of helix E beginning.

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57(E1)	Asn→Lys	G Ferrara	INC; Pro interrupts helix E.
62(E6)	Ala→Pro	Duarte	INC; larger group swings out, opening pocket. Favors FE(III) and heme loss (XRD).
63(E7)	His→Arg	Zürich	Auto-oxidizing; Pro disrupts center of helix E.
63(E7)	His→Pro	Bicetre	FERRI; replacement of positive charge by negative stabilizes Fe(III).
64(E8)	Gly→Asp	J Calabria, J Bari, J Cosenza	Charged group at heme contact opens pocket.
66(E10)	Lys→Glu	I Toulouse	Smaller group at heme contact. Water enters, weakening heme pocket (XRD).
67(E11)	Val→Asp	Bristol	Pro interrupts helix E.
67(E11)	Val→Ala	Sydney	Smaller, polar replacement. Less sensitive than Hammersmith.
68(E12)	Leu→Pro	Mizuho	INC; charged group in EF corner (XRD).
71(E15)	Phe→Ser	Christchurch	Shortens end of helix E.
74(E18)	Gly→Asp	Shepherds Bush	Pro interrupts helix E.
74–75	(Gly-Leu)→O	St. Antoine	INC; introduces charge into hydrophobic corner.
75(E19)	Leu→Pro	Atlanta	INC; introduces charge into hydrophobic corner.
75(E19)	Leu→Arg	Pasadena	INC; polar group in heme pocket.
81(EF5)	Leu→Arg	Baylor	INC; shortens chain and disturbs F helix.
85(F1)	Phe→Ser	Bryn Mawr, Buenos Aires	Charged group at heme contact opens pocket.
87(F3)	Thr→O	Tours	Helix disruption by Pro.
88(F4)	Leu→Arg	Boras	DEC; introduces charge into invariantly hydrophobic corner of heme pocket.
88(F4)	Leu→Pro	Santa Ana	INC; radically shortens F helix near FG corner.
91(F7)	Leu→Pro	Sabine	INC; dissociation; ruptures heme attachment. Very polar Gln opens pocket.
91(F7)	Leu→Arg	Caribbean	INC; Substitution alters critical heme contact.
91–95	(Leu-His-Cys-Asp-Lys)→O	Gun Hill	INC; Pro interrupts helix G.
92(F8)	His→Gln	St. Etienne, Istanbul	INC; introduces polar group in bottom of heme pocket.
98(FG5)	Val→Met	Köln, San Francisco (Pacific), Ube-I	DEC; upsets $\alpha_1\beta_1$ contact at β112.
98(FG5)	Val→Gly	Nottingham	Positive charge in molecular interior.
98(FG5)	Val→Ala	Djelfa	Pro interrupts helix G.
101(G3)	Glu→Gln	Rush	Positive charge at $\alpha_1\beta_1$ subunit contact.
106(G8)	Leu→Pro	Southampton, Casper	Negative charge at $\alpha_1\beta_1$ subunit contact.
106(G8)	Leu→Gln	Tübingen	Charged group in subunit interior.
111(G13)	Val→Phe	Peterborough	Shortens helix H, disturbs C-terminal contacts.
112(G14)	Cys→Arg	Indianapolis	Negative charge at bottom of heme pocket.
115(G17)	Ala→Pro	Madrid	INC, interrupts helix H, breaks up C-terminal interactions in T state.
124(H2)	Pro→Arg	Khartoum	Pro interrupts helix H.
128(H6)	Ala→Asp	J Guantanamo	INC; interrupts helix H, breaks up C-terminal interactions in T state.
130(H8)	Tyr→Asp	Wien	
131(H9)	Gln→O	Leslie, Deaconess	
134(H12)	Val→Glu	North Shore	
135(H13)	Ala→Pro	Altdorf	
138(H16)	Ala→Pro	Brockton	
142(H20)	Ala→Pro	Toyoake	

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6. Case study

The propositus "S.B." was a medical student; a twenty-four year old healthy Thai male of Chinese ancestry. Hemoglobin typing on starch gel electrophoresis in tris-EDTA-borate buffer, pH 8.6 (115) showed the presence of an abnormal hemoglobin moving more slowly than hemoglobin A, designated as hemoglobin "S.B." and another faint band moving slower than hemoglobin A_2 (Fig. 7). The relative amounts of the abnormal component Hb S.B., Hb A_2, slow HbA_2 as determined by elution of cellulose acetate membranes after electrophoresis (116) was 11.8, 2.18 and 0.64 % respectively. The presence of a slow HbA_2 band as well as the more intense Hb S.B. band suggested that mutation has occurred in the α globin chain, so that the abnormal α chain would combine with both the β and the δ chains to give two hemoglobins of abnormally slow mobility.

Based on this information, further studies were planned in order to characterize this abnormal hemoglobin, with the following objectives :

1. Study of the hematological features

2. Identification of the amino acid interchange in the abnormal polypeptide chain.

3. Study of some properties of the abnormal hemoglobin molecule including inclusion body formation and stability.

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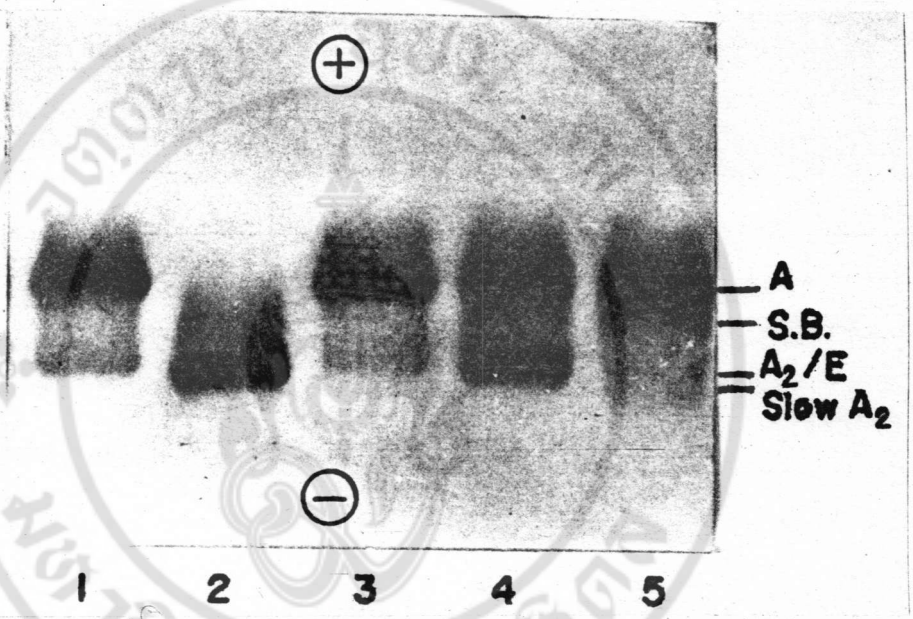


Fig. 7 Hemoglobin typing by starch gel electrophoresis of hemolysates in tris-EDTA-borate buffer, pH 8.6.

1,3 : normal subject

2 : homozygous HbE

4 : heterozygous HbE

5 : propositus "S.B."

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(Results taken from Thalassemia Center, Mahidol University)

MATERIALS AND METHODS

MATERIALS

Cellulose acetate strip (Sepraphore® III) 5.7x14.4 cm. was obtained from Gelman Instrument Company, Ann Arbor, Michigan, U.S.A. Pre-swollen anion exchanger diethylaminoethyl cellulose (DE 52), pre-swollen cation exchanger carboxymethyl cellulose (CM 52), and No. 3 chromatography papers (46x57 cm.) were from Whatman Ltd., England. Urea (ultrapure) was from Schwarz/Mann, U.S.A. Trypsin (302 U/mg) and Soybean trypsin inhibitor were from Millipore Corp., Freehold, N.J., U.S.A. Phosphoglycerate mutase (PGM 2400 U/ml) and Glyceraldehyde phosphate dehydrogenase (GAPD 800 U/ml)/Phosphoglycerate kinase (3PGK 450 U/ml) mixture were obtained from Sigma Chemical Company , St. Louis, Mo., U.S.A. HCl for hydrolysis was obtained as a 37% solution (Analytical®, Carlo Evba.) and diluted with an equal volume of water, phenol (1 mg/ml) was added to prevent destruction of tyrosine (117).

Other chemicals such as acetic acid, acetone and formic acid etc. were of analytical grade.

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METHODS

1. Hematological studies

A venous blood sample was collected in heparin (15-20 I.U./ml). Hemoglobin concentration, packed red cell volume and red blood cell count were determined by an electronic Coulter-counter Model ZF6. Alkali resistant hemoglobin was measured according to Singer et al (118).

2. 2,3-Diphosphoglycerate determination (119)

2,3-Diphosphoglycerate (2,3-DPG) is hydrolysed to 3-phosphoglycerate (3PG) by the phosphatase activity present in PGM stimulated by glycolate-2-phosphate. This reaction is linked to the conversion of NADH to NAD^+ by glyceraldehyde-3-phosphate dehydrogenase (Ga-3-PD) and phosphoglycerate kinase (PGK). The fall in absorbance at 340 nm, as NADH is oxidized, is measured:

1. 2,3 DPG $\xrightarrow[\text{(glycolate-2-phosphate)}]{\text{2,3 DPG phosphatase}}$ 3PG + Pi

2. 3PG + ATP $\xrightarrow{\text{PGK}}$ 1,3 DPG + ADP

3. 1,3 DPG + NADH $\xrightarrow{\text{Ga-3-PD}}$ Ga-3-P + Pi + NAD^+

Method

1 ml of fresh heparinized blood is deproteinized by 3 ml of ice-cold trichloroacetic acid (80 g/l) in a 10 ml conical centrifuge tube and shaken vigorously on a vortex mixer. The tube is allowed to

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stand for 5-10 min at 4°C to obtain a clear supernatant.

The reaction was carried out in a glass cuvette, with a 1 cm light path. Reagents were added as follow.

	Test (ml)	Blank (ml)
*triethanolamine buffer, pH 7.6	1.25	1.25
ATP (20 mg/ml in buffer)	0.05	0.05
NADH (10 mg/ml)	0.05	0.05
acid extract	0.125	-
water	-	0.125
Ga-3-PD + PGK	0.01	0.01
PGM	0.005	0.005

*0.2 M triethanolamine HCl, 0.05 M disodium EDTA, 0.04 M $MgSO_4 \cdot 7H_2O$

The mixture were warmed at 25°C for 5 min, and the absorbance of both test and blank mixture were recorded at 340 nm. The reaction is then started by the addition of 0.05 ml of glycolate-2-phosphate. After completion of the reaction, the absorbance of test and blank mixtures were remeasured at 340 nm.

Calculation :

$$\text{2,3-DPG } (\mu\text{mole/g Hb}) = (\Delta A \text{ 340 test} - \Delta A \text{ 340 blank}) \times \frac{49.28 \times 100}{6.22 \text{ Hb(gm)}}$$

where 49.28 = dilution of original blood sample

6.22 = μM extinction coefficient of NADH at 340 nm.

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3. Structural analysis

3.1 Preparation of blood sample

A venous blood sample was collected with heparin as an anticoagulant. The hemolysate was prepared according to Clegg et al (120). About 40 ml of blood was obtained and centrifuged at 3,000 rpm, 4°C for 10 min, the plasma and the buffy coat were removed out. Red blood cells were washed three times with 0.9% NaCl and other substances adhering to the cell membrane were removed. The packed red cells were lysed with an equal volume of distilled water and 0.4 volume of toluene by frequent shaking, and then centrifuged at 5,000 rpm for 30 min at 4°C. The red cell stroma at the upper layer were removed out, so that the clear hemolysate at the bottom was obtained. Hemoglobin solution was stored at -20°C until used.

3.2 Identification of the abnormal hemoglobin

The abnormal hemoglobins were identified by cellulose acetate electrophoresis in tris-EDTA borate buffer, pH 9.1 (132.9 mM tris, 4.6 mM EDTA (disodium), 14.9 mM boric acid) according to Miale (121). The cellulose acetate strip was soaked in this buffer for 1 hr, and finally drained and blotted before applying samples. Electrophoresis was performed at a constant voltage of 350 V at room temperature for 1.5 hr. Then the strip was stained with Ponceau S (0.5% Ponceau S in 5% (w/v) trichloroacetic acid solution) for a few minutes and washed then with 5% (v/v) acetic acid.

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3.3 Identification of the abnormal globin chain

3.3.1 Acid-urea-mercaptoethanol cellulose acetate electrophoresis

The abnormal globin chains were identified by cellulose acetate electrophoresis in acid-urea-mercaptoethanol buffer, pH 6.5 (80 mM tris-EDTA borate buffer containing 6 M urea, 71.5 mM 2-mercaptoethanol and adjustment of the pH with concentrated citric acid as described by Schneider (122). The cellulose acetate strip was first soaked in acid-urea buffer without 2-mercaptoethanol for 1 hr, drained and soaked again for 1 hr in the acid-urea buffer containing 71.5 mM 2-mercaptoethanol, finally drained and blotted before sample application. Samples were prepared by adding 20 µl each of hemolysate and acid-urea buffer without 2-mercaptoethanol, and 10-20 µl of 2-mercaptoethanol. The prepared samples were place into the sample wells after standing at room temperature for about 0.5 hr. (but not longer than 4 hr). Electrophoresis was performed at room temperature for 1.5 hr at a constant voltage of 350 Volts. The cellulose acetate strip was stained in Ponceau S and destained as described above.

3.3.2 Triton X-100-acid-urea polyacrylamide gel electrophoresis

The abnormal globin chains were identified using the method of Alter et al (123), by electrophoresis on 12% polyacrylamide gels, containing 6 M urea and 2% Triton X-100, in 5% acetic acid. The gel solution consisted of 5 ml of 60:0.4% acrylamide : bis-acrylamide,

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1.25 ml of glacial acetic acid, 18.75 ml of 8 M urea which has been freshly deionized with AG 501x8(D), 0.125 ml of TEMED, 0.5 ml of Triton-X 100, and 15 mg of ammonium persulphate. The slab gel was 12 cm long, 16 cm wide and 1.5 mm thick. Gelling occured within 30 min, but the gels were left for 2 hr prior to use. The electrophoresis buffer was 5% acetic acid. The gel were pre-electrophoresed for 60 min at 200 V with the anode at the top. Then the anode buffer was removed, the gels were overlayed with 30 µl of 2-mercaptoethanol and fresh electrophoresis buffer was added and a second pre-electrophoresis performed for 45-60 min at 150 V. The 2-mercaptoethanol was then removed entirely. 20-30 µg of hemoglobin was mixed with 35 µl of sample buffer and placed in the wells of the slab gel. The sample buffer was made up of 5 ml of deionized 8 M urea, 0.5 ml of glacial acetic acid, 0.5 ml of 2-mercaptoethanol and 2 mg of pyronin Y. the buffer was made up just before use. All procedures were performed at room temperature. Electrophoresis was carried out for 17 hrs at a constant current of 8.5 mA. The gel was stained with 0.5% Coomassie brilliant blue in 7% acetic acid, 30% methanol and destained in 10% acetic acid and 50% methanol for 1-2 hr, followed by 7% acetic acid and 5% methanol.

3.4 Preparative isolation of the abnormal hemoglobin

The abnormal hemoglobins were isolated by DEAE-cellulose column chromatography. A 3.5x40 cm column was prepared, using the developer 0.2 M glycine, 0.1 g/L KCN containing varying amounts of NaCl (124). The hemolysate containing 600-700 mg hemoglobin was

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dialysed against distilled water overnight at 4°C and applied to the column equilibrated with the initial developer (0.2 M glycine, 0.1 g/L KCN and 10 mM NaCl). Chromatography was performed at room temperature at a flow rate of 60 ml/hr, initially eluting with starting buffer, and then with starting buffer containing linear NaCl gradients (500 ml + 500 ml) of 10-20 mM NaCl, 20-30 mM NaCl and 30-60 mM. Effluent fractions of 6 ml were collected and optical densities of the fractions were determined at 415 nm, Since the molecular extinction of oxyhemoglobin at 415 nm was much higher than at 580 nm (125). The conductivity of every 10th tube was measured. The effluent fractions corresponding to each peak were pooled, and concentrated by adsorption on DEAE-cellulose. The hemogolbin fraction was diluted until the sodium chloride concentration was less than 10 mM, applied to a 2 x 10 cm DEAE cellulose column and eluted out with a small volume of 0.2 M NaCl in 0.2 M glycine buffer. The concentrated hemoglobin was finally dialysed against distilled water at 4°C, and then lyophilized. The purity of each peak was checked by cellulose acetate electrophoresis under normal (section 3.2) and denaturing (section 3.3) conditions.

3.5 Preparative isolation of the abnormal globin chain

Firstly, globin was prepared from hemoglobin by acid-acetone precipitation according to Clegg et al (120). The hemoglobin was dissolved in a small volume of distilled water, the globin was precipitated by slowly adding the solution drop by drop to a 20-fold volume of acid acetone (2% (v/v) conc HCl in acetone) that had be cooled to -20°C, under vigorous stirring. The mixture was stirred

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for an additional 0.5 hr. After the globin had settled, the supernatant solution was decanted as completely as possible, and then the remaining suspension was centrifuged at 4°C, and the precipitate was washed several times with cooled acetone at 4°C and lyophilized to give a white powder.

The abnormal globin chain was isolated on CM cellulose column chromatography in 8 M urea, 0.05 M 2-mercaptoethanol in phosphate buffer, pH 6.7 (126). 8 M urea was prepared by dissolving the solid in distilled water at room temperature and deionized by mixing with AG-501x 8 (D) mixed bed resin, stirred for 5 min and filtered through two sheets of Whatman filter paper No. 1. The urea solution, developer A (0.005 M Na_2HPO_4, 0.05 M 2-mercaptoethanol in 8 M urea, adjusted to pH 6.7 with 85% phosphoric acid) and developer B (0.04 M Na_2HPO_4, 0.05 M 2-mercaptoethanol in 8 M urea, adjusted to pH 6.7 with 85% phosphoric acid) were immediately prepared before use. The 3x17 cm CM-cellulose column was prepared and equilibrated with developer A. About 300-400 mg globin was dissolved in a few milliliters of developer A and dialysed against a 50-fold excess of developer A for 2-3 hr at room temperature before loading into the column. Chromatography was carried out at room temperature at a flow rate of 60 ml/hr. A linear sodium ion gradient containing 500 ml of developer A and 500 ml of developer B was started after 100 ml of developer A had passed through the column. Effluent fractions of 5 ml were collected, and the conductivity and the optical density at 280 nm of these fractions were measured. The isolated globin was dialysed against 0.5% formic acid solution to remove urea and then lyophilized.

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3.6 Tryptic digestion of globin chain

The globin chain was digested with trypsin which cleaves the polypeptide chain specifically on the carbonyl side of lysine and arginine residues to give smaller peptides. Digestions were carried out in 1% (w/v) NH_4HCO_3 with an enzyme : substrate ratio of 1:100 (by wt.) for 4½ hr. at 37°C. The globin was suspended in distilled water and 1% NH_4HCO_3 was added in an equal volume, so that the final protein concentration was 10 mg/ml. The reaction was stopped by adding Soybean trypsin inhibitor. Finally, the tryptic digest was concentrated by lyophylization.

3.7 Separation of peptides by fingerprinting

Fingerprinting : Electrophoresis

High voltage paper electrophoresis was carried as described by Ambler (127-128). About 7 mg of digested globin was redissolved in a small amount of distilled water (1 mg/10 µl) for application a sheet of Whatman No. 3 chromatographic paper (46x57 cm). Samples were applied (1 mg/cm paper) side by side 18 cm from the end of the paper (Fig. 8). The paper was buffered up lightly with 0.5% acetic acid, 10% pyridine buffer, pH 6.5 from both ends until wet to help concentrate the sample at the origin. The paper was then lightly blotted with another sheet of filter paper to remove surplus buffer, avoiding contact with the spot itself. Electrophoresis was carried out at 3,000 volt at 10-15°C for 45 min in a tank cooled with white spirit (129). DNP lysine and Xylene cyanol FF were used as the external markers for following the course of the run.

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Chromatography

After electrophoresis, the paper was hung up to dry without heating. Small sample strips of electrophoretogram were cut out and stained with cadmium ninhydrin reagent (130). These strips were used as a guide in determining the section of paper to be cut for the chromatography. The remaining unstained section of the paper containing the peptides was then stitched with a sewing machine to a second sheet (46x57 cm) of Whatman No. 3 paper (Fig. 9) and then subjected to descending chromatography using the solvent BAWP (n-butanol/acetic acid/water/pyridine : 15/3/12/10 by vol.) (131) at right angles to the first dimension for 16-18 hr using the same markers. The chromatogram was dried by hanging it in a fume cupboard for 2-3 hr.

3.8 Detection of peptide on paper

(a) Cadmium ninhydrin (mixture of 15 ml solution B with 100 ml of solution A where solution B is 1 gm cadmium acetate $\cdot 2H_2O$, 50 ml glacial acetic acid, 100 ml water; solution A is 1% (w/v) ninhydrin in acetone). The papers were sprayed with this mixture and heated to $100^{o}C$. Most peptides give a reddish-pink colour. However, glycine and threonine as N-terminal residues impart a permanent yellow colour to the stained peptide whereas N-terminal asparagine and cysteic acid result in an orange colour that slowly turns red. Isoleucine and valine at the N-terminus give a low colour yield.

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This staining method was used to render the peptide visible for photography, but does not allow determination of the amino acid composition of the peptides.

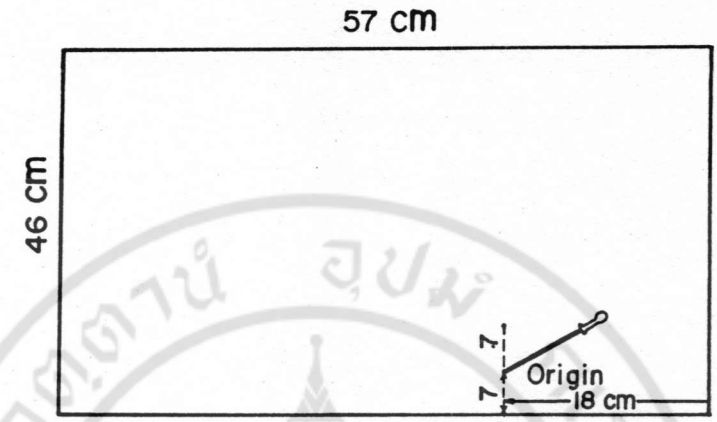


Fig. 8 Figure to show point of application of sample in first dimension, high voltage paper electrophoresis.

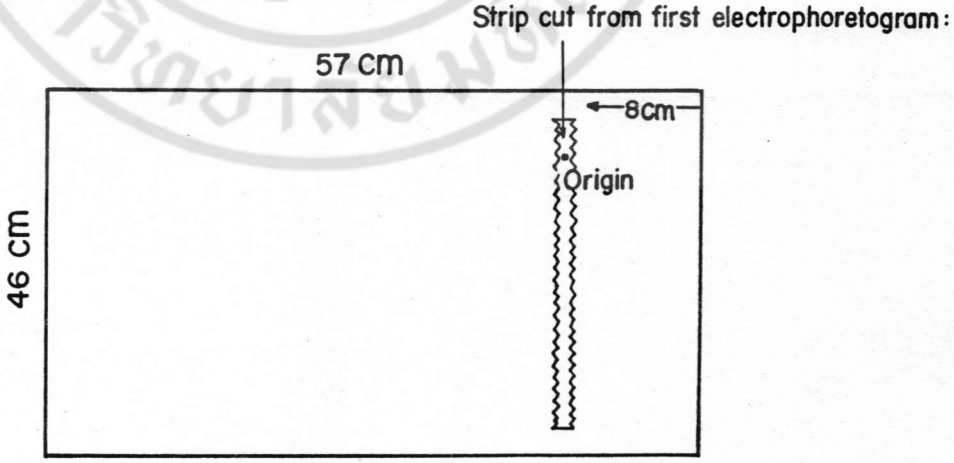


Fig. 9 The strip from the first electrophoretogram was stitched with a second sheet of Whatman No. 3 paper; for the second dimension, paper chromatography.

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(b) Dilute ninhydrin (0.03% ninhydrin in acetone) (132)

After staining, the colour was allowed to develop overnight at room temperature. All peptide spots give a purple colour, however, valine or isoleucine at the N-terminus give very poor colour yields and are sometimes not stained at all.

This staining mathod was used when peptides were to be eluted for subsequent amino acid analysis.

3.9 Amino acid analysis

The peptide spots to be analysed were cut out in the shape of a rectangle with a pointed end from the dilute ninhydrin stained paper, and were eluted with double distilled water (Fig. 10). The eluted peptides were dried in a vacuum desiccator and then hydrolysed with 6 N HCl containing 1mg/ml phenol in vaccuum sealed tubes for 22 hr at 105-110^{o}C. The hydrolysed sample was evaporated to dryness again in a vaccuum desiccator and the amino acid compositions of the peptides were determined on LKB 4101 amino acid analyzer.

4. Properties and stability

4.1 The isopropanol stability test (133)

Hemolysate preparation

Venous blood was collected with heparin as an anticoagulant, and washed three times with isotonic saline. A hemolysate freshly prepared by adding 2 vol. of distilled water to the packed cells and centrifuging at 15,000 rpm for 20 min at 4^{o}C to remove the cell

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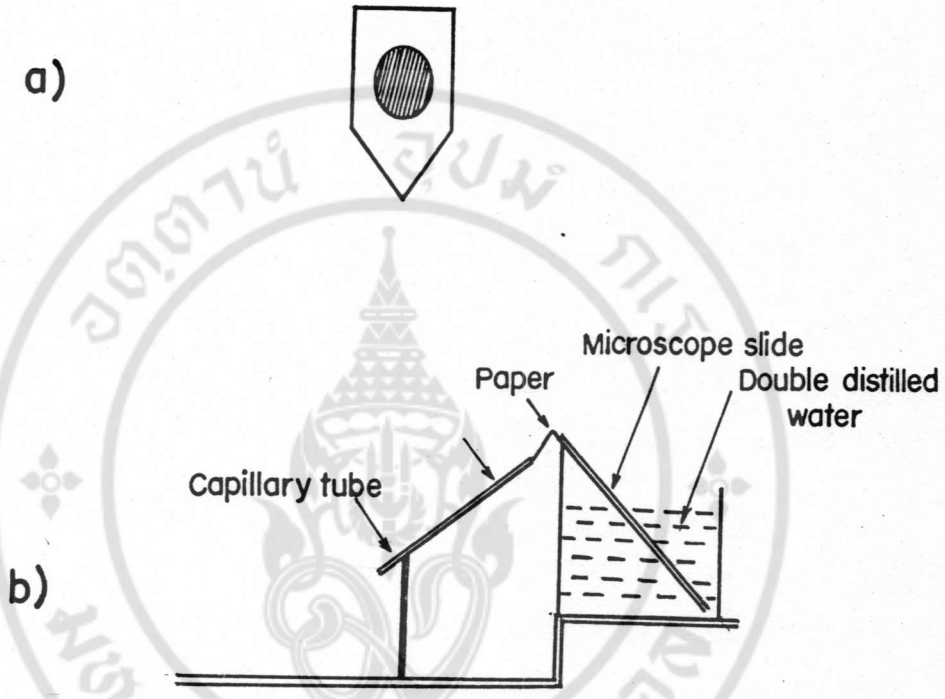


Fig. 10 The elution of a peptide from a fingerprint.

a) peptide cut from fingerprint.

b) elution of peptide from the paper.

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membrane. Hemoglobin concentration was measured by the cyanmethemoglobin method (134), and the hemoglobin solution was made 5 gm % in concentration.

Procedure

4 ml of 5 gm % freshly prepared hemolysate was added to 40 ml of 17% isopropanol buffer (isopropanol 17% by volume in 0.1 M Tris/HCl buffer, pH 7.4). 2 ml fractions of this diluted hemoglobin solution was pipetted into 10 test tubes in duplicate. These tubes were placed in a water bath at 37°C for periods of 0, 5, 10, 15, 20, 25, 30, 35 and 40 min. After incubation, each tube was cooled in an ice bath for about 5 min and centrifuged at 3000 rpm. for 10 min to remove the precipitate. 1 ml of the supernatant was added to 4 ml of Drabkin solution (134) and the optical density was measured at 540 nm. The percentage of the precipitated hemoglobin was calculated as follows

$$\% \text{ precipitate Hb} = \frac{\text{OD of tube(o)} - \text{OD of tube(t)}}{\text{OD of tube(o)}} \times 100$$

4.2 The heat stability test (124, 135)

4.2.1 The heat stability of whole hemolysate

A 5 gm % hemolysate was prepared as previously described. 2 ml of the hemolysate was added into 100 ml of 0.1 M tris/HCl, pH 7.4 and 5 ml fractions of the diluted hemolysate were pipetted into 10 test tubes in duplicate, tubes were then placed in a water bath at a constant temperature of 60°C for periods of 0, 5, 10, 15, 20, 25, 30 and 35 min. Each tube was cooled in an ice bath after incubation, and the precipitate was removed by centrifugation at 3000 rpm for

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10 min. The optical density of the clear supernatant was measured at 523 nm. Then the percent heat precipitable hemoglobin was calculated from the original amount present (tube o) and that remaining in solution as follows :

$$\% \text{ precipitate Hb} = \frac{\text{OD of tube(o)} - \text{OD of tube(t)}}{\text{OD of tube(o)}} \times 100$$

4.2.2 The heat stability of the purified abnormal hemoglobin (Hb SB)

Blood was freshly drawn from the propositus, the hemolysate was prepared as described in section 4.3 and was fractionated by DEAE-cellulose column chromatography as described in section 3.4 but the buffer used contained 0.2 M glycine without 0.1 g/l KCN. Pools of the abnormal hemoglibin (Hb SB) and of normal HbA were made from selected tubes, and the hemoglobin and salt concentrations of the two pools were adjusted to be approximately equal by dilution. Then the heat stability of the normal and abnormal hemoglobins was studied as described in section 4.4.1.

4.3 The osmotic fragility of red blood cells

The red cell osmotic fragility was automatically recorded using a Fragiligraph Model D-2 (Elron), which is based on the technique developed by Danon (136). Heparinized blood sample is suspended in isotonic salt buffer, pH 7.4. During the test, the initial salt concentration of the blood suspension is automatically decreased by dialysis across a membrane which is part of the test cell. With decreasing

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ionic strength, osmotic hemolysis of red blood cell occurs and this is accompained by changes in the light transmitting properties of the suspension. By measuring and recording the light transmission during the hemolysis process, the cumulative fragility curve and the derivative fragility curve are obtained, where the cumulative fragility curve represents the percentage hemolysis with time and the derivative fragility curve shows the rate of hemolysis at any given time.

4.4 The inclusion body test

According to the method described by Fessas (137), 10 µl of sodium nitrite-glucose solution (0.18 M $NaNO_2$, 0.28 M glucose in distilled water) (138) was added to 0.2 ml of fresh heparinized blood in a small glass test tube and incubated at 37°C for 30 min. After removal from the incubator, 0.2 ml of methyl violet solution (2% w/v of 0.85% NaCl) was added. The tube was left for 15-20 min at room temperature before preparing the blood films, which were then directly examined under the microscope.

Inclusion or Heinz bodies in the red blood cell represent an end product of the degradation of hemoglobin and appear as irregular inclusions, approximately 1-3 µ in size, at or near the periphery of the red blood cells. The smaller the number of inclusions per cell, the larger is their size.

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RESULT

1. Hematological studies

The hematological data for the propositus "S.B." are shown in Table 7. The hematological values when compared to that of normal males indicated that the propositus "S.B." had slightly low levels of hemoglobin, red blood cell count, hematocrit (PCV) and MCV, which result in normal MCH and higher MCHC. Red blood cell morphology was as follows : few microcytes (red blood cells smaller than 6 μ in diameter), few poikilocytes (red blood cells that are variable in shape) and rare target cells (red blood cells that appear as a central rounded area of pigment material surrounded by a clear ring without pigment). The level of Hb F as determined by alkali resistance method (117) was 0.69% which was in the normal range (less than 1%).

2. 2,3 DPG determination

The 2,3 DPG level of propositus "S.B." was 8.9 μmole/g Hb which was in the low range of normal value (8.6-11.5 μmole/g Hb, SD = 1.04, $\bar{x}$ = 11.05, n = 8).

3. Electrophoretic studies

3.1 Identification of abnormal hemoglobin

Hemoglobin typing of the hemolysate of the propositus "S.B." on cellulose acetate electrophoresis in tris-EDTA borate buffer, pH 9.1 showed two bands; a major one moving identical to Hb A and a faint bond moving slower to the anode than Hb F and assigned as Hb S.B.

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Table 7 The hematological data* of the propositus "S.B." and normal Thai male.

Subject	Hb (g/dl)	RBC (cells/L)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RBC morphology microcytosis	poikilocytosis	target cell	%HbF
Normal Thai male	15.9±0.9	5.3±0.3 $x10^{12}$	49.0±3.1	92.5±3.7	31.1±1.1	33.0±0.9	normocyte	normocyte	-	0.48±0.24
propositus "S.B."	13.8	$4.4x10^{12}$	38.2	86.8	31.3	36.0	few	few	rare	0.69

*obtained from Mrs. Pranee Fucharoen, Thalassemia Center, Mahidol University.

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(Fig. 11). Hb A_2 was present as a very faint band, while the other abnormal band migrating slower than Hb A_2 (Fig. 7), which was believed to be a slow Hb A_2, could not be detected in this system.

Family studies were carried out by cellulose acetate electrophoresis in this system and are shown in Fig. 12. The results indicate that the propositus' mother (lanes 1,5) and brother (lane 4) are also carriers. The pedigree of propositus' family deduced from these studies is shown in Fig. 13.

3.2 Identification of abnormal globin chain

3.2.1 Acid-urea-mercaptoethanol cellulose acetate electrophoresis

Globin chains were analysed on cellulose acetate electrophoresis using a denaturing system as described by Schneider as shown in Fig. 14. The hemolysate of the propositus "S.B." (lanes 1,3) was separated into three bands: two bands had mobilities identical to β^A and α^A, and a faint band moving slightly faster to the cathode than α^A was believed to be the α chain variant designated as $\alpha^{S.B.}$.

3.2.2 Triton x-100-acid-urea polyacrylamide gel electrophoresis

The hemolysate of propositus "S.B." was also separated into three bands in this system (Fig. 15). Comparisons of the propositus' bands (lanes 1,4) with those of normal hemolysate (lanes 2,3) and with cord blood (lane 5) indicate that in addition to the α and β globin chains, the propositus "S.B." contained a faint abnormal band of the $\alpha^{S.B.}$ chain moving faster to the cathode than α^A.

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Fig. 11 Hemoglobin typing by cellulose acetate electrophoresis in tris-EDTA-borate buffer, pH 9.1.

1,6 : hemolysate of normal subject

2 : purified HbF

3 : hemolysate of the propositus "S.B."

4 : purified Hb A_2/E

5 : hemolysate of un-identified abnormal hemoglobin

Fig. 12 Hemoglobin typing by cellulose acetate electrophoresis of hemolysates in tris-EDTA-borate buffer, pH 9.1.

1,5 : S.B.'s mother

2,6 : S.B.'s father

3 : normal subject

4 : S.B.'s brother

7 : propositus "S.B."

8 : heterozygous HbE

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Fig.11

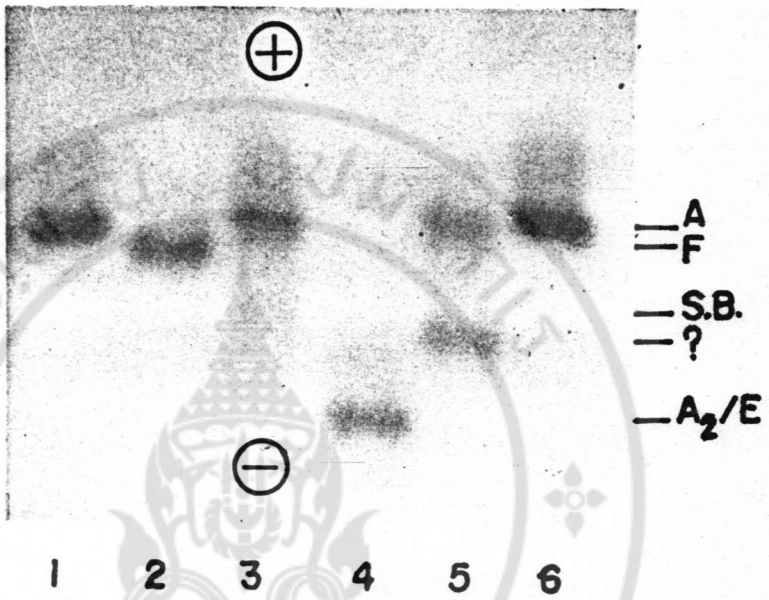
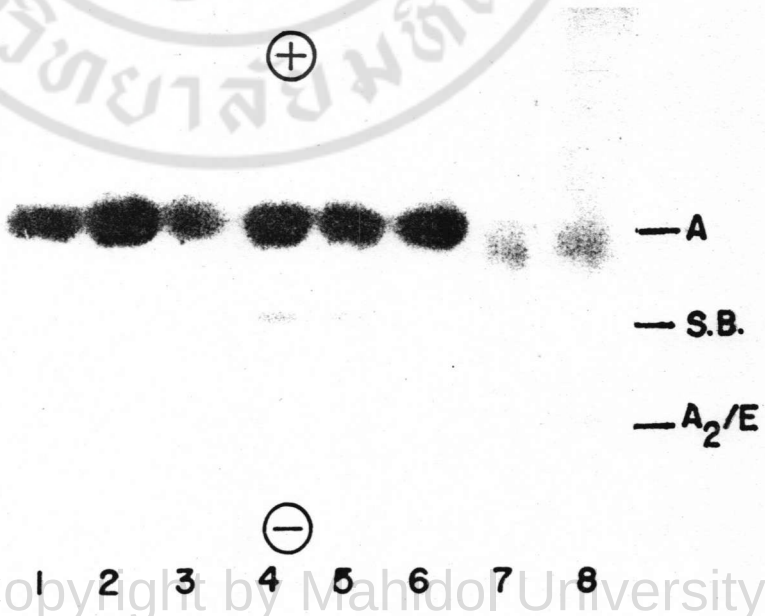


Fig.12



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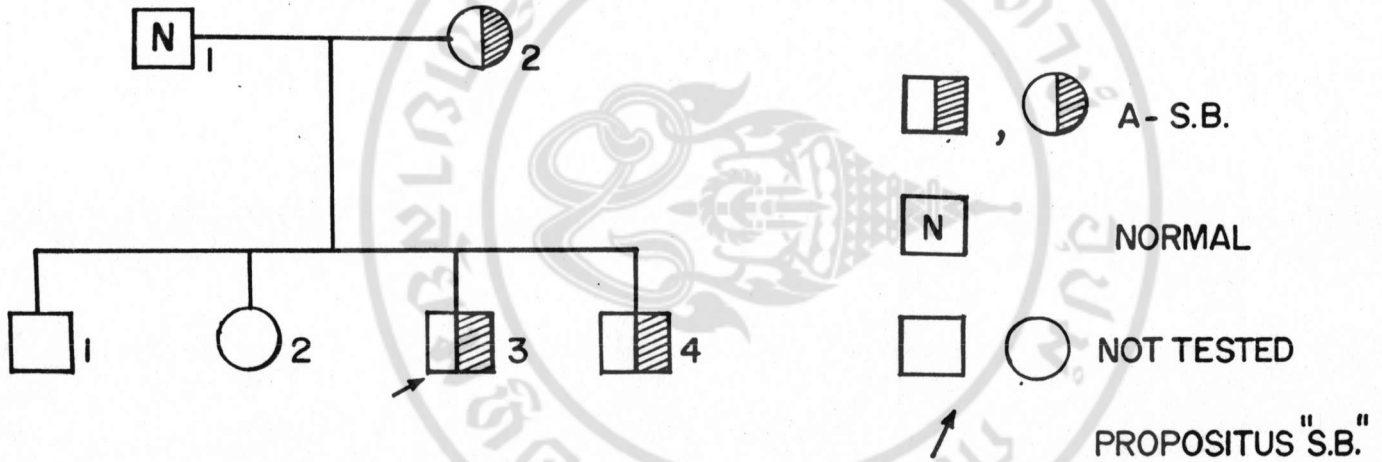


FIG.13 Pedigree diagram of propositus "S.B." Who has the abnormal hemoglobin . Squares indicate males and circles indicate females .

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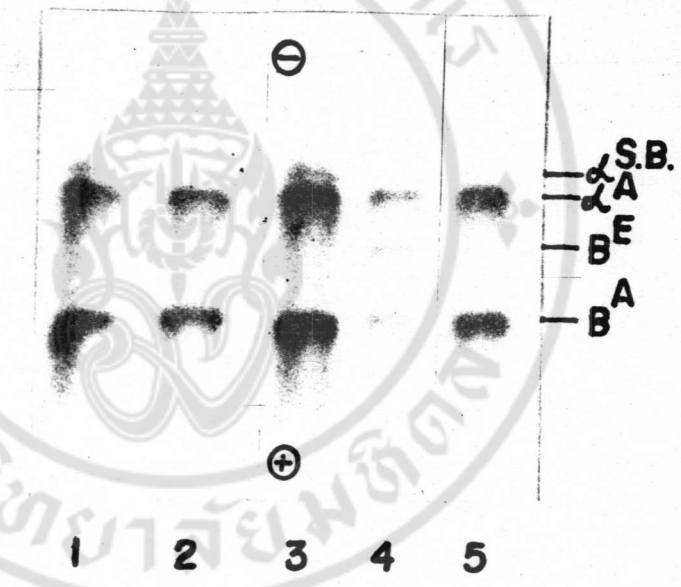


Fig. 14 Globin chain separation by cellulose acetate electrophoresis of hemolysates in acid-urea-mercaptoethanol buffer, pH 6.5.

1,3 : propositus "S.B."

2,5 : normal subject

4 : heterozygous HbE

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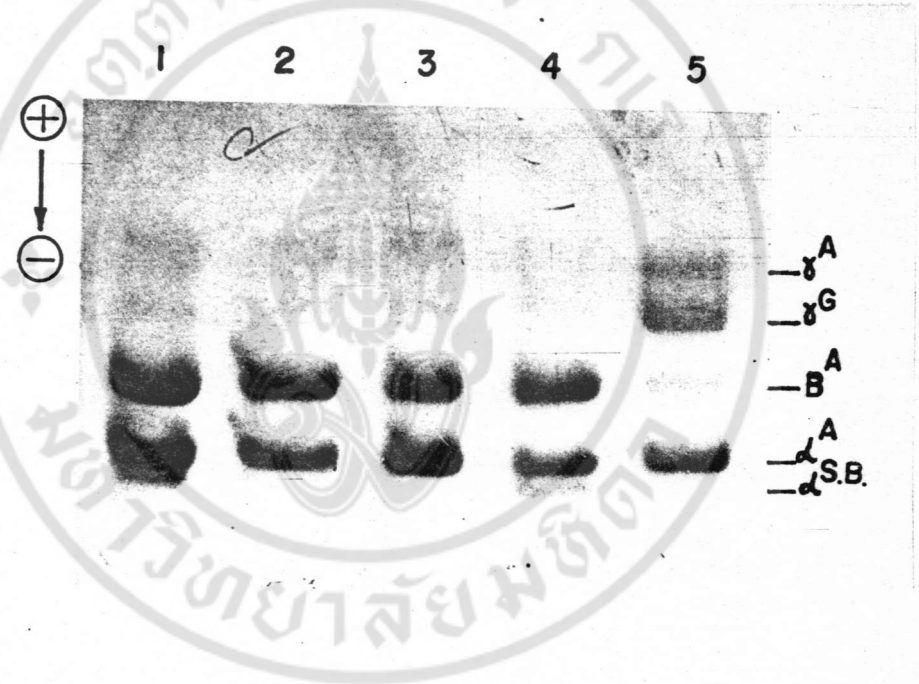


Fig. 15 Globin chain separation of hemolysates by Triton X-100 - acid-urea polyacrylamide gel electrophoresis.

1,4 : propositus "S.B."

2,3 : normal subject

5 : cord blood

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Both these methods cause the separation of globin chains and permit detection of the abnormal $\alpha^{S.B.}$ chain of the propositus "S.B.". Electrophoresis on cellulose acetate under denaturing conditions is simple, offering rapid results using very small volumes of sample (0.25 µl) but requires a high concentration of hemoglobin in the sample. On the other hand, Triton x-100-acid-urea polyacrylamide gel electrophoresis is more complex and takes a longer time, but samples contianing low concentrations of hemoglobin can be used because larger volumes (up to 40 µl) can be loaded.

4. Preparative isolation of abnormal α chain

4.1 Preparative isolation of abnormal hemoglobin

The hemolysate of the propositus "S.B." was fractionated on DEAE-cellulose column chromatography using a NaCl gradient from 10 mM to 60 mM as shown in Fig. 16. The chromatogram showed a clear separation of the abnormal hemoglobin from Hb A. Analysis of each peak on cellulose acetate electrophoresis in tris-EDTA borate buffer, pH 9.1 gave the results shown in Fig. 17 a. The pool of fractions 78-88 (lane 8) moved slower than other fractions and was believed to be slow Hb A_2, while tubes 160-174 (lane b) moved slightly slower than purified Hb A_2/E (lane 7) and were probably Hb A_2. Tubes 318-350 (lane 4) constituted the major peak in the profile (Fig. 16) and moved identically to Hb A, while tubes 406-428 (lane 2), which were eluted out behind Hb A at the position of Hb F, moved faster to the anode than Hb A and was suggested to be glycosylated hemoglobin.

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The pooled fractions of interest (nos. 260-278), eluted out before Hb A (Fig. 16), moved slower than Hb F and was believed to be Hb S.B. (lane 5). The identify of this pool was confirmed by running a mixture of this pool and the propositus' hemolysate on electrophoresis (Fig. 17 b) with the result that only two bands of Hb A and S.B. (lane 1) could be detected. Furthermore, globin separation of this peak on cellulose acetate electrophoresis under denaturing conditions showed two bands (Fig. 18); one is β^{A} and the other, moving faster to the cathode than α^{A}, is $\alpha^{S.B.}$.

The chromatogram also indicated that abnormal Hb S.B. was present at low levels (13.1% of total hemoglobin) corresponding to the results, from elution of cellulose acetate membranes after electrophoresis (11.8 % of total hemoglobin).

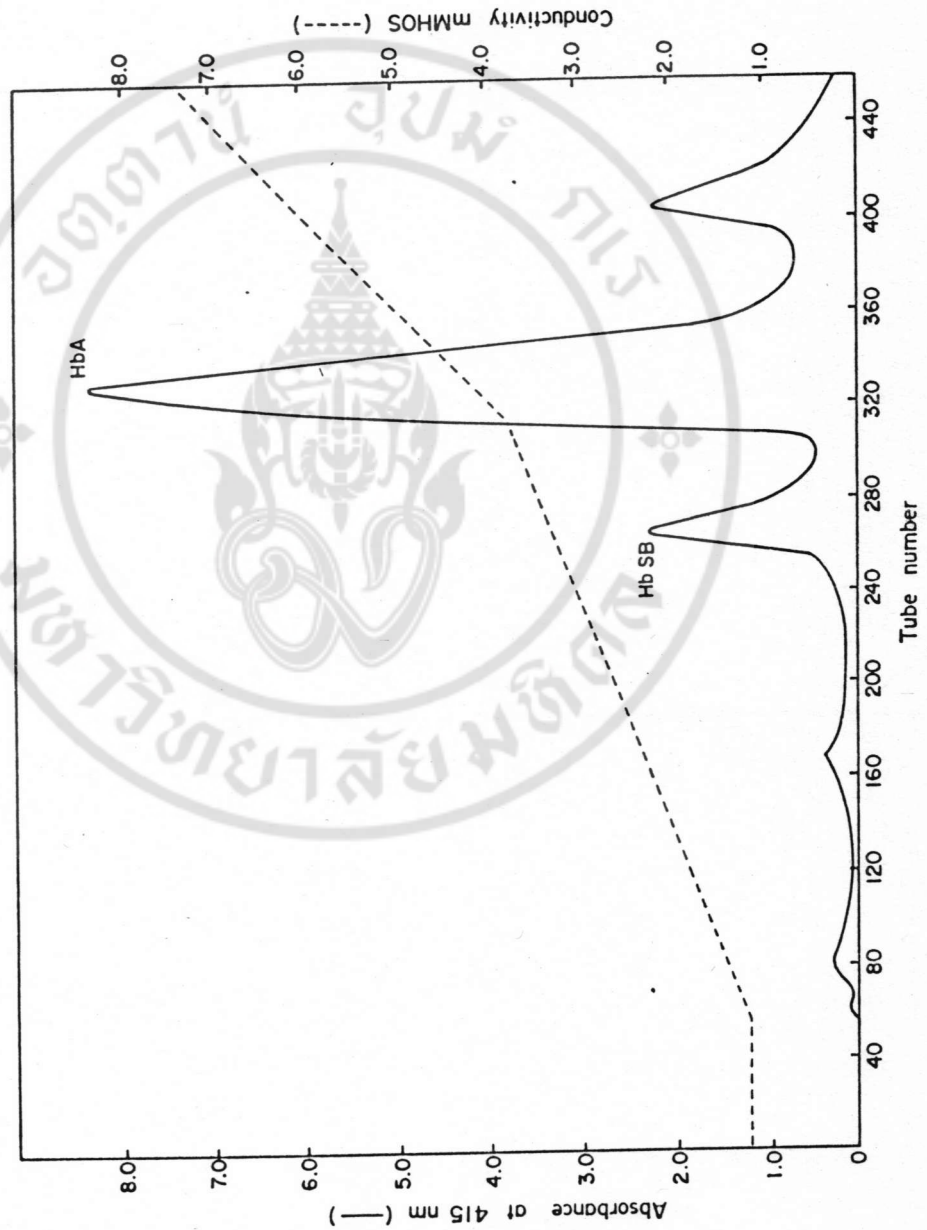
4.2 Preparative isolation of abnormal globin chain

Hemoglobin was converted to globin by acid-acetone precipitation. When globin prepared from purified Hb A was fractionated by CM-cellulose column chromatography in 8 M urea, the chromatogram (Fig. 19) showed two major peaks : β^{A} was eluted out first followed by α^{A}. A similar pattern was observed from the chromatogram of globin prepared from purified Hb S.B. where β^{A} was eluted first followed by $\alpha^{S.B.}$ (Fig. 20), but when these two chromatograms are compared, $\alpha^{S.B.}$ was eluted at higher salt concentration than α^{A} (conductivity 3.2 m MHO for $\alpha^{S.B.}$ compared to 2.9 mMHO for α^{A}) while their β^{A} chains were eluted at the same salt concentration (conductivity 2.0 mMHO).

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Fig. 16 Chromatographic separation of the abnormal hemoglobin (Hb S.B.) from the hemolysate of the propositus "S.B." by DEAE cellulose chromatography using NaCl-glycine-KCN.

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Fig. 17 Cellulose acetate electrophoresis in tris-EDTA-borate buffer, pH 9.1 of fractions from the DEAE-cellulose column of the propositus "S.B." hemolysate fractions from the DEAE-cellulose column of the propositus.

a) 1 : purified HbF

2 : tube nos 406-428

3 : purified HbA

4 : tube nos 318-350

5 : tube nos 260-278

6 : tube nos 160-174

7 : purified Hb A_2, E

8 : tube nos 78-88

b) 1 : mixture of propositus "S.B." hemolysate and tube nos 260-278

2 : purified Hb F

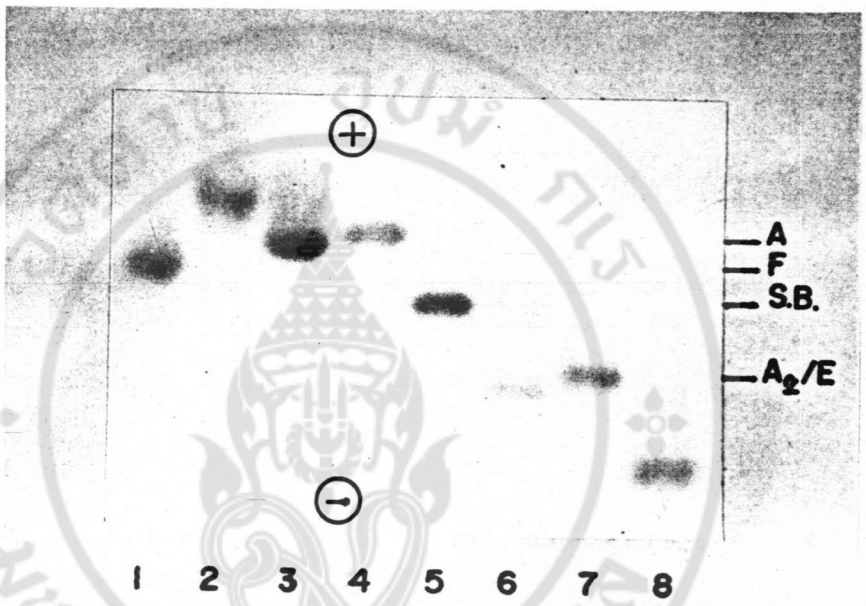
3 : tube nos 260-278

4 : purified Hb A

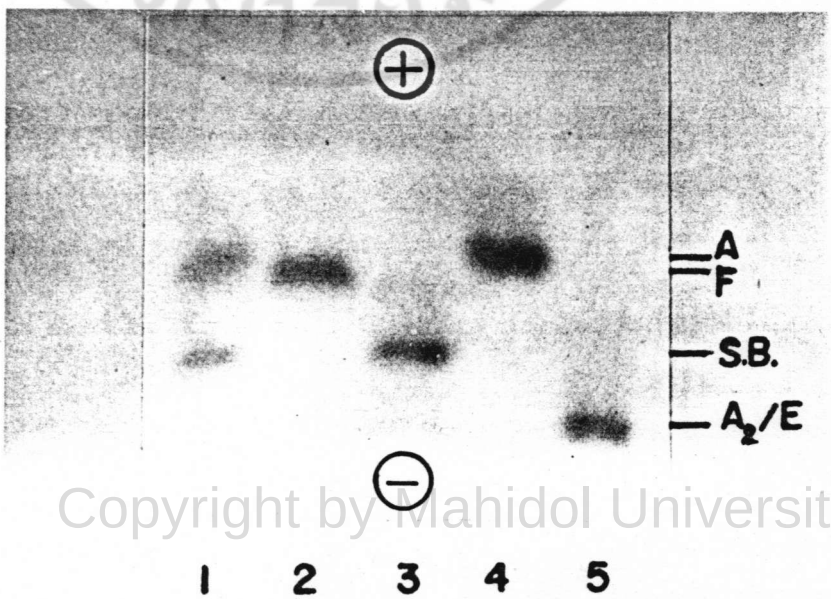
5 : purified Hb A_2/E

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a.



b.



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Fig. 18 Globin chain typing by cellulose acetate electrophoresis in acid-urea-mercaptoethanol buffer, pH 6.5.

1,5 : tube nos 260-270

2 : tube nos 406-428

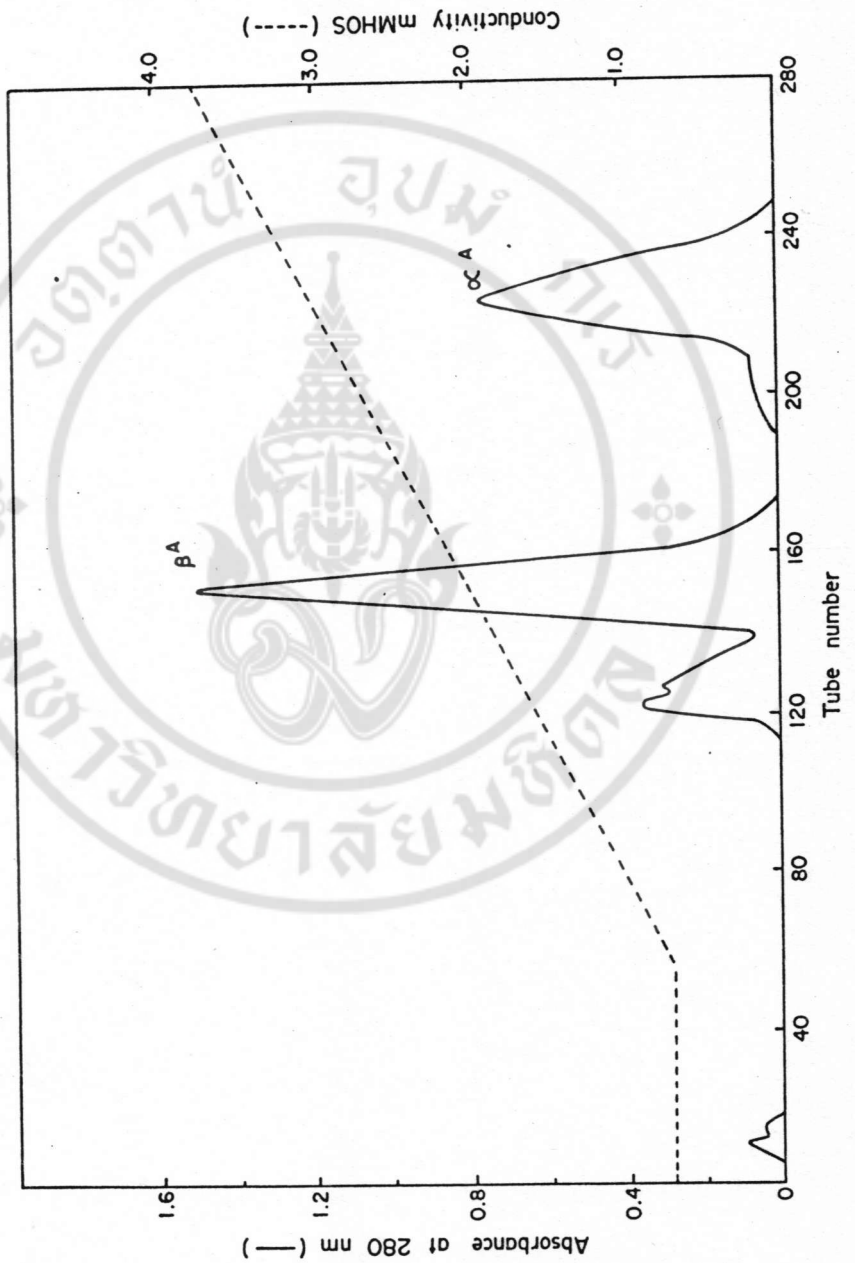
3 : normal hemolysate

4 : tube nos 318-350

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Fig. 19 The separation of β^A and α^A globin chain from purified hemoglobin A by CM-cellulose column chromatography in 8 M urea-2-mercaptoethanol-phosphate buffer, pH 6.7.

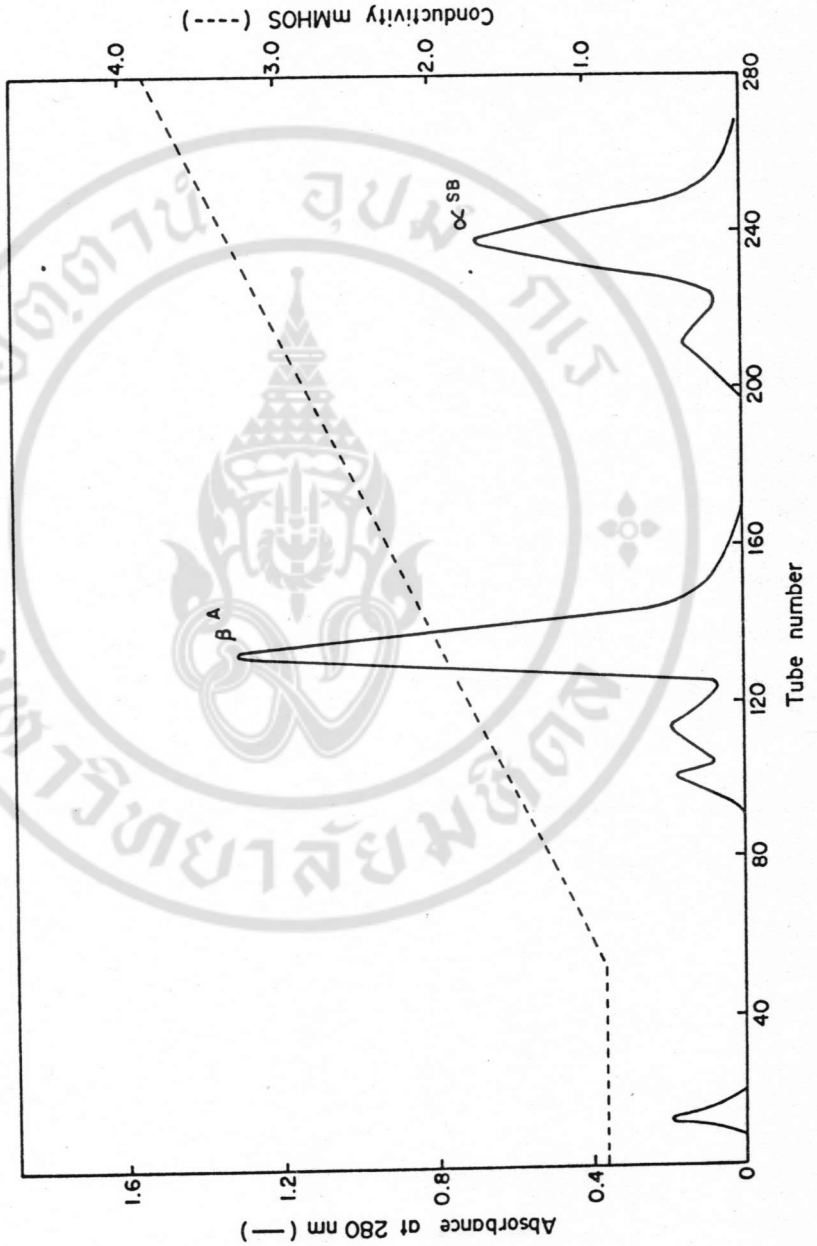
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Fig. 20 The separation of β^{A} and $\alpha^{S.B.}$ globin chains from purified hemoglobin S.B. by CM-cellulose column chromatography in 8 M urea-2-mercaptoethanol-phosphate buffer, pH 6.7.

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When DEAE-cellulose column chromatography was used, this step had to be used four times to provide enough Hb S.B. for the CM-cellulose step. Therefore, fractionation of globin prepared from the whole hemolysate was attempted directly on the CM-cellulose column without the prior DEAE-cellulose step. The chromatographic separation of globin chains from globin prepared directly from whole hemolysate of a normal subject and of the propositus "S.B." are shown in Figs. 21 and 22. The chromatogram produces a clear separation of the abnormal $\alpha^{S.B.}$ chain which was eluted behind α^{A} chain (Fig. 22). Cellulose acetate electrophoresis as described in methods section 3.3.1 was used to check the purity of purified $\alpha^{S.B.}$ (Fig. 23). The results showed clear separation of $\alpha^{S.B.}$ (Fig. 23 lane 1 & 4), indicating that abnormal $\alpha^{S.B.}$ globin chain can be isolated directly from globin prepared from the whole hemolysate of propositus "S.B. by CM-cellulose column chromatography.

5. Structural analysis

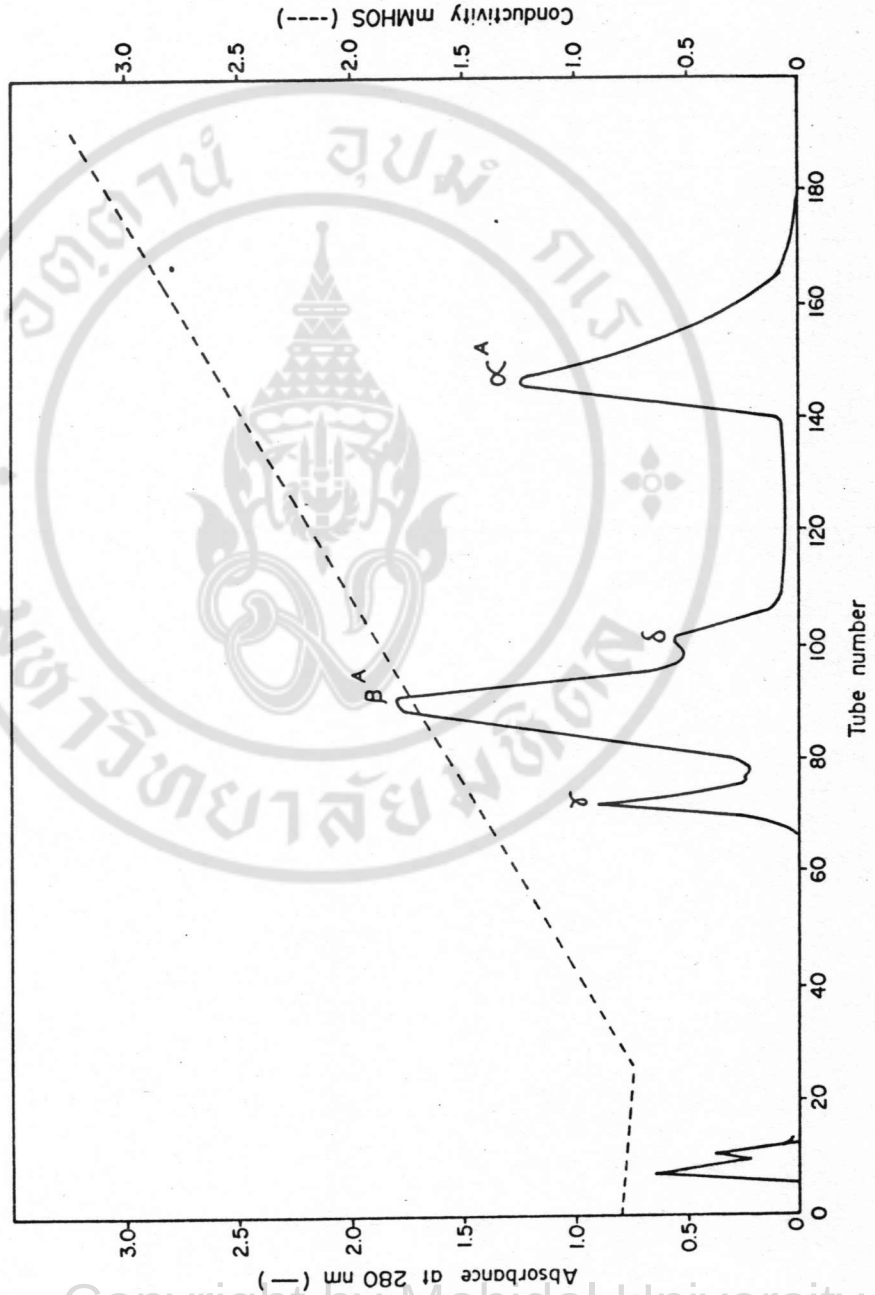
5.1 Fingerprinting

The purified globin chains were digested with trypsin which has a total of fourteen lysine or arginine cleavage sites in the normal α chain, producing free lysine and a number of peptides varying in size from two to twenty nine amino acid residues. The peptides obtained were fractionated by fingerprinting on high voltage paper electrophoresis (HVPE) and descending paper chromatography. In general, electrophoretic mobility depends on the size and charge of the peptide, the smaller

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Fig. 21 Chain separation of globin from whole normal hemolysate by CM-cellulose column chromatography in 8 M urea-mercaptoethanol-phosphate buffer, pH 6.7.

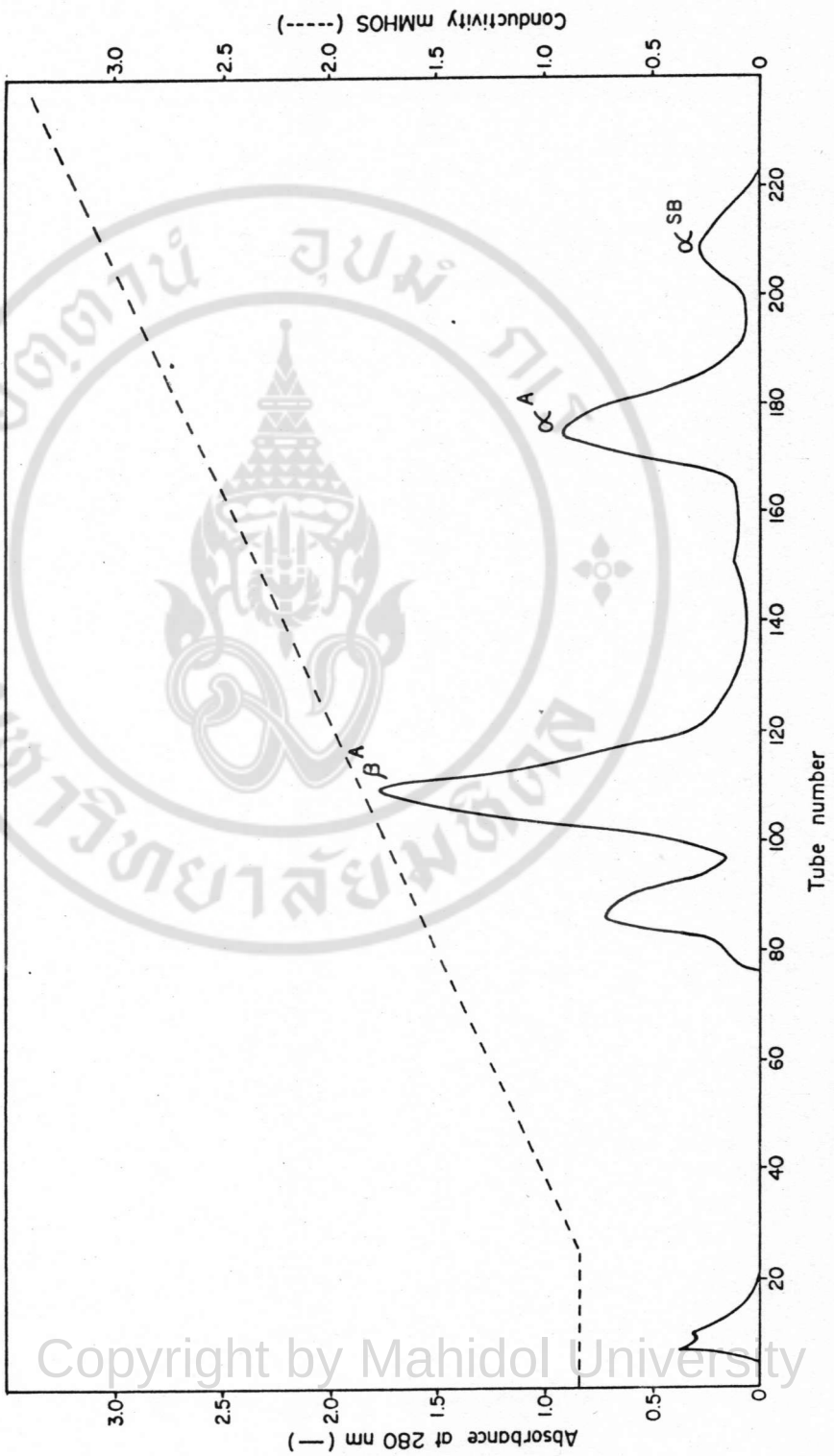
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Fig. 22 Chain separation of globin from whole hemolysate of propositus "S.B." by CM-cellulose column chromatography in 8 M urea-mercaptoethanol-phosphate buffer, pH 6.7.

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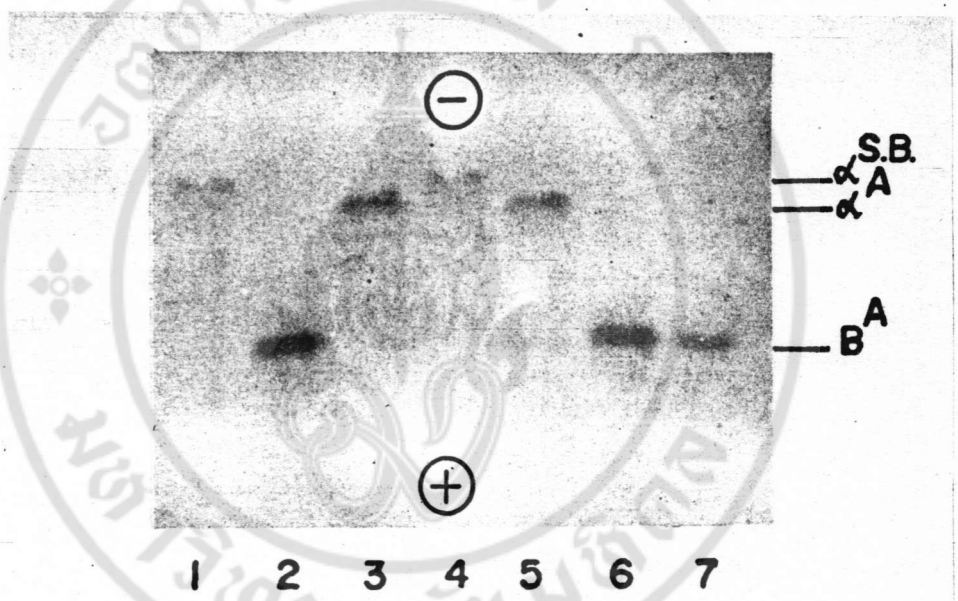


Fig. 23 Globin chain cellulose acetate electrophoresis of purified fractions from CM-cellulose column in acid-urea-mercaptoethanol buffer, pH 6.5.

1,4 : $\alpha^{S.B.}$ chain

2,6,7: β^{A} chain

3,5 : α^{A} chain

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and more highly charged a peptide is, the faster it will migrate. On the other hand, chromatographic separation depends largely in polarity, with hydrophobic peptides moving faster.

The fingerprint of the digested $\alpha^{S.B.}$ chain and digested α^{A} chain are shown in Fig. 24a & b. The results showed clear separation of small peptides both from the $\alpha^{S.B.}$ and from the α^{A} chain. Comparison of the fingerprint of the digested $\alpha^{S.B.}$ chain and of the digested α^{A} chain indicated that there are about thirteen spots of small peptides present in the α^{A} chain whereas the digested $\alpha^{S.B.}$ chain showed fourteen spots of small peptides. The spot corresponding to the normal T_pV (Fig. 24a) was missing in the $\alpha^{S.B.}$ map and new two spots, 'a' and 'b' were detected (Fig. 24b). The colour given by peptides vary from red to orange and to yellow. Both spots 'a' and 'b' gave reddish pink colour, and moved slightly faster to the cathode in electrophoresis but slower in chromatography compared to spot αT_pV of the α^{A} chain and are thought to be smaller and more polar peptides than αT_pV. Comparison of the two new spots of $\alpha^{S.B.}$ chain indicated that spot 'a' moved slightly faster to the cathode in electrophoresis and also faster in chromatography than spot 'b'.

5.2 Amino acid analysis

The spots of interest were cut out, eluted and hydrolysed with 6N HCl containing 1 mg/ml phenol. The amino acid analyses of the hydrolysates of peptides 'a', 'b' and αT_pV are shown in Table 8. For the most part, peptides 'a' and 'b' appeared to be composed of amino acid

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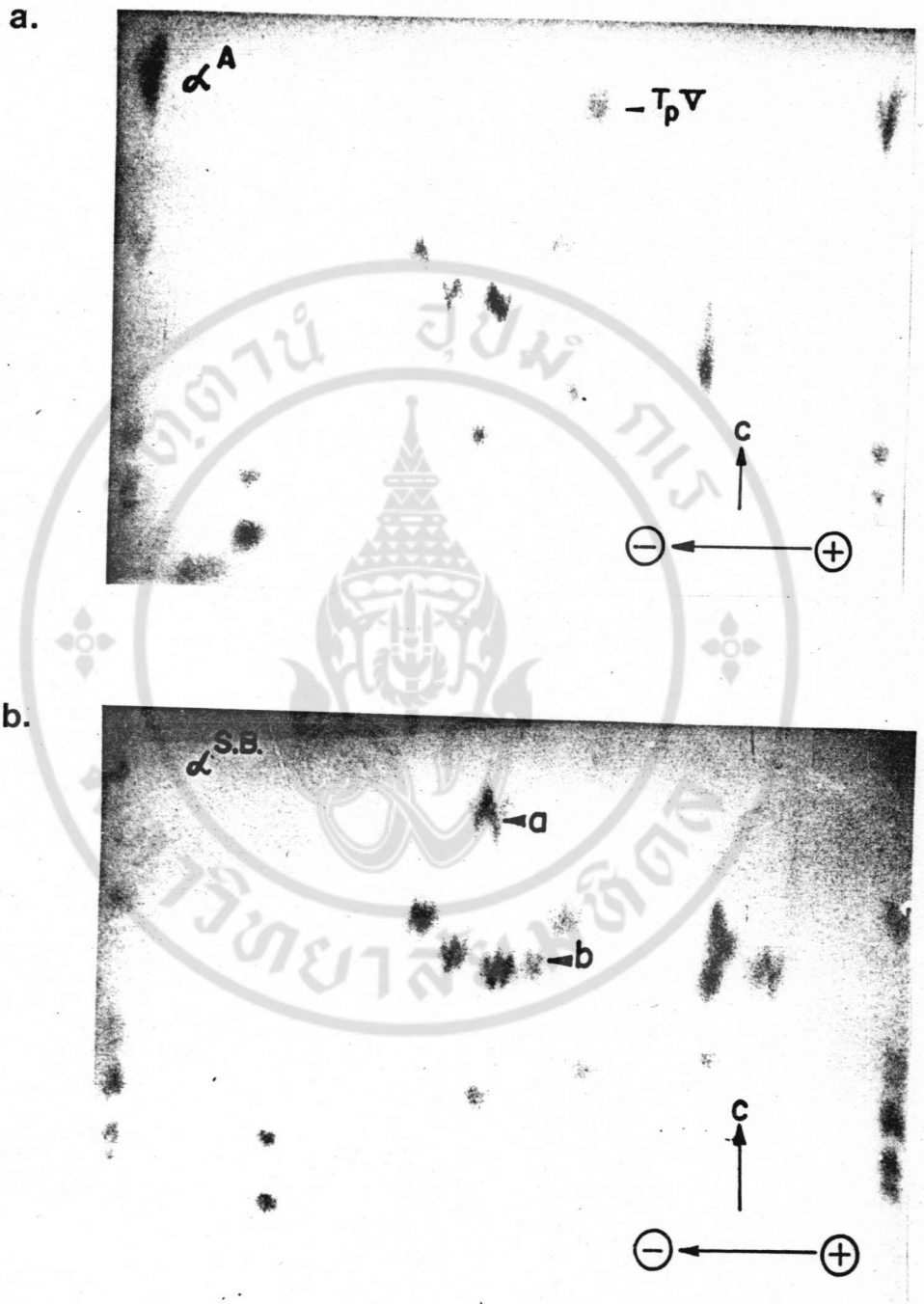


Fig. 24 Fingerprints of α^A chain (a) and $\alpha^{S.B.}$ chain (b) after tryptic digestion. First dimension, high voltage paper electrophoresis in acetic acid/pyridine buffer pH 6.5, Second dimension, chromatography in n-butanol/acetic acid/pyridine/water (BAPW) at right angles, stained with ninhydrin.

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residues present in the amino acid composition of $\alpha T_p V$. The amino acid composition of spot 'b' was identical to the C-terminal six residues of $\alpha T_p V$ (residues 35-40), while the amino acid composition of spot 'a' is compatible with the N-terminal of $\alpha T_p V$ (residues 32-34) except that the leucyl residue was absent but an extra arginyl residue was present. The evidence indicated that leucyl residue at the 34^{th} position of normal α chain was replaced by arginine producing the abnormal $\alpha^{S.B.}$ chain, and causing $\alpha^{S.B.}$ $T_p V$ to be digested into two spots, 'a' and 'b'. From the amino acid composition (Table 8), the methionine content in both peptides $T_p V$ and peptide 'a' and the serine content in peptide 'b' are rather lower (0.65, 0.63 and 0.63 residues/mole peptide, respectively) than the expected value (1 residue /mole peptide). This is due to destruction of these amino acids because they are at the N-termini of the peptides and consequently react with ninhydrin. The recovery of these three peptides; $T_p V$, 'a' and 'b' were 32.9%, 45.4% and 45.4% respectively and the purity of these three peptides were quite good.

In conclusion, the structural identification indicated the leucyl residue at position 34 of α globin chain of propositus "S.B." was replaced by an arginyl residue. The abnormal hemoglobin in the propositus was therefore $\alpha_2^{34\ Arg}\ \beta_2$, which has been reported as Hb Queens, first described in sixteen year old Korean girl in 1979 by Tatsis (139).

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Table 8 Amino acid composition (residues/mole peptide) of tryptic peptides T_pV from the α^A map, and peptides a and b from the $\alpha^{S.B.}$ map.

Amino acid	a		b		T_pV	
	found	expected	found	expected	found	expected
Thr	-	-	2.03	2	1.90	2
Ser	-	-	0.63	1	0.91	1
Pro	-	-	1.07	1	1.00	1
Met	0.63	1	-	-	0.65	1
Leu	-	-	-	-	1.00	1
Phe	1.07	1	1.10	1	2.12	2
Lys	-	-	0.92	1	1.04	1
Arg	0.99	1	-	-	-	-
% Yield	45.4	-	45.4	-	32.9	-

note : Amino acids present in less then 0.05 residues/mole peptide were omitted.

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6. Properties

6.1 The isopropanol stability test

The instability of hemoglobin was studied by using 17% isopropanol to make the solution more non-polar, so that the internal bonding forces of hemoglobin molecule would be weakened, there by decreasing stability of the molecule and causing precipitation of denatured hemoglobin. Normal hemoglobin will begin to precipitate by 40 minutes, whereas the unstable hemoglobins rapidly precipitate within 5 minutes and become flocculant by 20 minutes. For the purpose of comparison, the whole hemolysates of the propositus "S.B.", of normal subjects and of a patient with Hb H were tested in the isopropanol stability test. The percent precipitation was measured at various times up to 40 minutes as shown in Fig. 25. Propositus "S.B." showed very little precipitation during incubation from 5 to 40 minutes, similar to the results obtained with normal hemolysates. On the other hand, Hb H well known for its instability, showed rapid precipitation within 5 minutes and gave much more precipitation after prolonged incubation.

6.2 The heat stability test

Unstable hemoglobins are heat labile *in vitro*, and can be detected by the precipitation of denatured hemoglobin on heating at $60^{o}C$. Hemoglobin in 0.1 M Tris-HCl buffer, pH 7.4 from the whole hemolysate of the propositus "S.B." and of normal subjects were heated at $60^{o}C$ for various times in 0.1 M Tris-HCl buffer, pH 7.4. After centrifugation, a reddish brown precipitate was observed, and the

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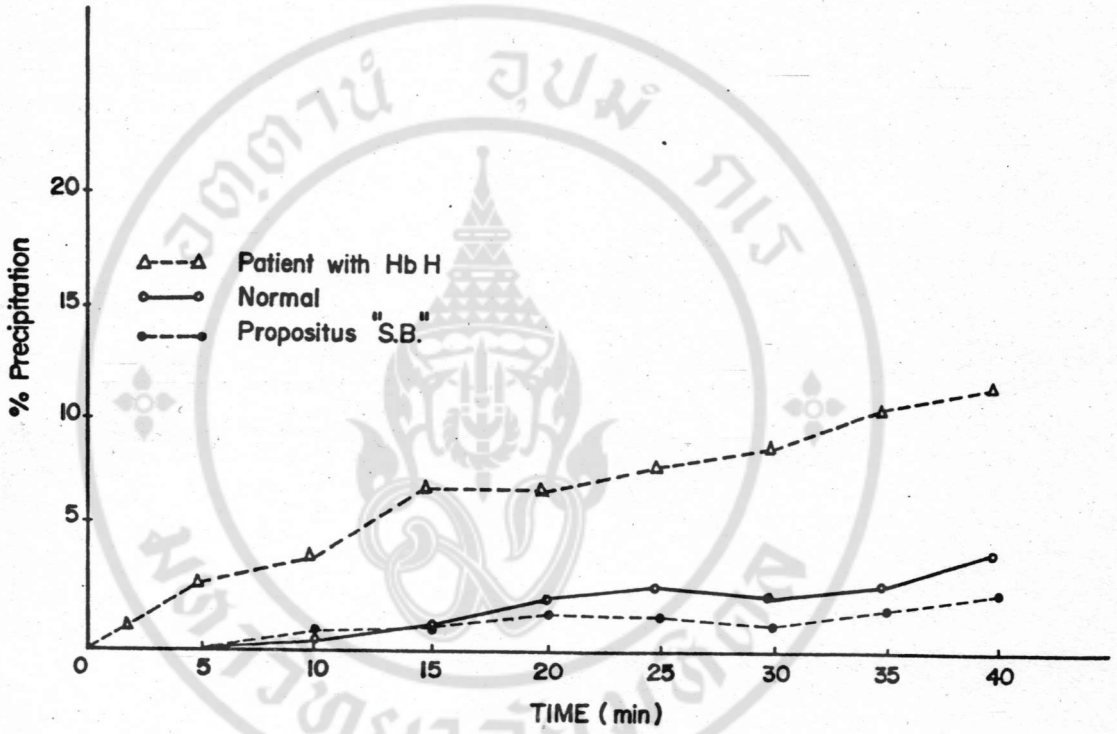


Fig. 25 Isopropanol stability test of whole hemolysate of normal (0 —— 0), propositus "S.B." (0 --- 0) and patient with Hb H (Δ --- Δ).

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percent precipitation was calculated from the absorbance at 523 nm of the supernatant at zero time and at various times up to 40 minutes. The results indicated that the percent precipitation from propositus was not significantly different from those of normal subjects as shown in Fig. 26. The ratio of the absorption at 523 nm and 280 nm in the supernatant was also measured and was found to be constant with time for both the propositus "S.B." and also for normal subjects. Furthermore, precipitates from both the propositus "S.B." and normal subjects were analysed for globin chain composition on Triton X-100-acid-urea polyacrylamide gel electrophoresis. The results (Fig. 27) indicate that in the precipitate from the normal hemolysate, β^A and α^A chains were present in similar amounts while in the propositus hemolysate, all three bands of β^A and α^A and also $\alpha^{S.B.}$ chains were present with no evidence that $\alpha^{S.B.}$ was enriched relative to α^A.

However, since the abnormal Hb S.B. was present in very low amounts compared to normal Hb A in the propositus' whole blood, it was considered interesting to study the heat stability of the purified abnormal hemoglobin. Accordingly, purified Hb S.B. and Hb A were prepared and studied as described in Methods section 4.4.2. The results indicated that the percent precipitation at various times of Hb S.B. was quite different from that of Hb A. With purified Hb A, precipitation was detectable at 15 minutes and increased after further incubation whereas in purified Hb S.B., the precipitation occurred within 10 minutes and increased with time to a higher level than with purified Hb A (Fig. 28). These results suggest that Hb S.B. was less stable

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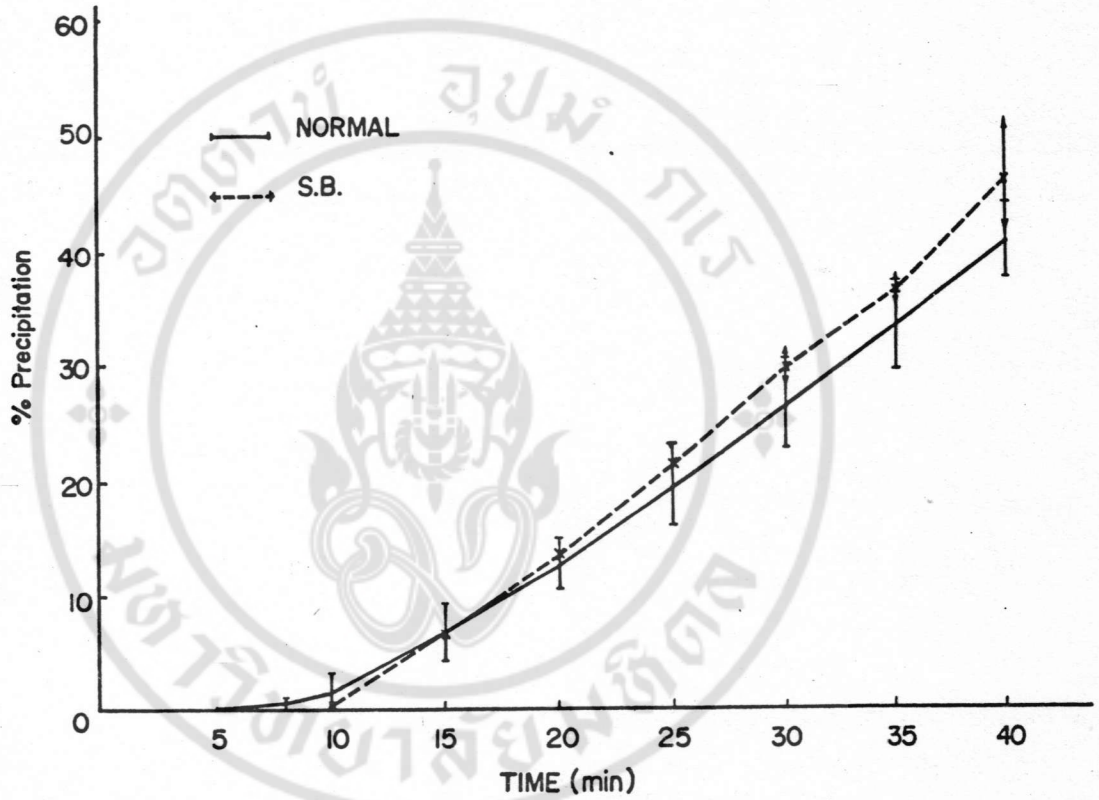


Fig. 26 Heat stability of whole hemolysate of normal (0 ⟷ 0) and propositus "S.B." (<--->) at 60°C using 0.1 M Tris-HCl buffer, pH 7.4 Data were the means ± S.D. of six values at each time point.

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Fig. 27 Analysis of precipitates from heat stability test by Triton x-100-acid-urea polyacrylamide gel electrophoresis;

1 : precipitate from propositus "S.B." at 40 minutes

2 : precipitate from normal subject at 40 minutes

3 : hemolysate of propositus "S.B."

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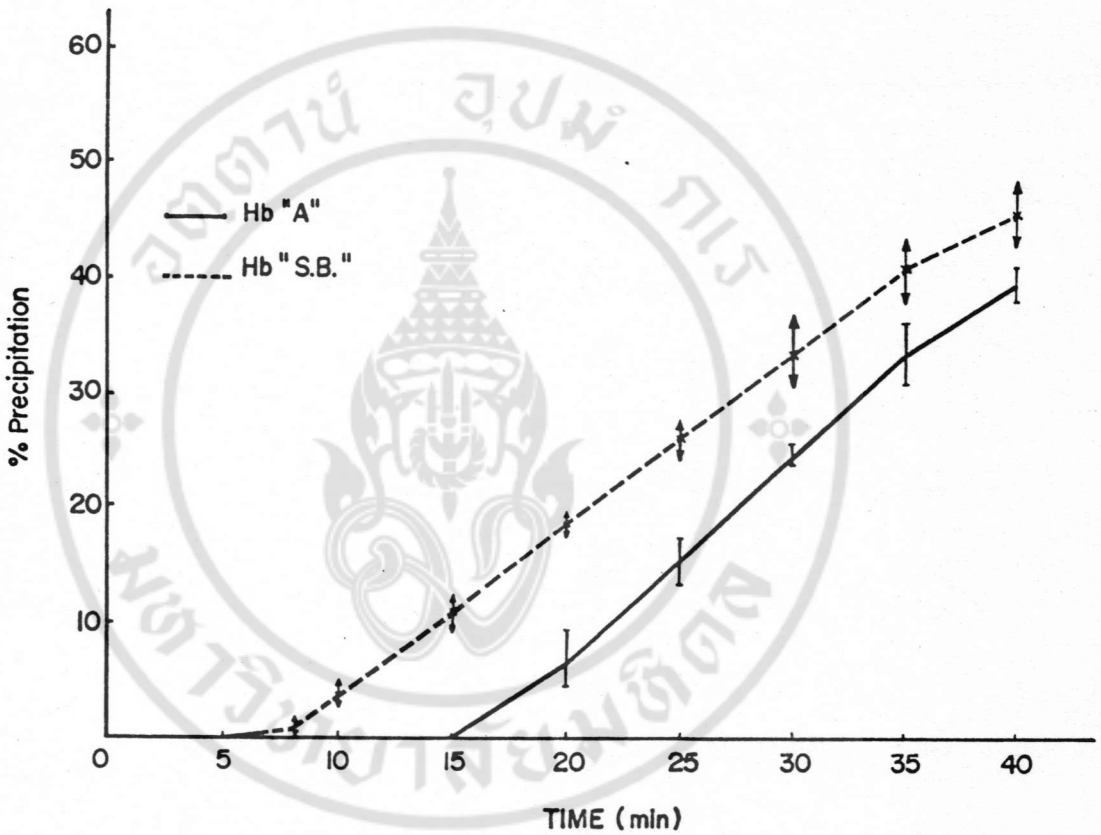


Fig. 28 Heat stability of purified Hb S.B. and purified Hb A at 60°C. Data represent mean ± S.D. of four values at each time point.

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than Hb A. However, a reddish brown precipitate was also observed, and absorption at 523 nm and 280 nm in the supernatant of Hb S.B. and Hb A was constant at different times. Triton X-100-acid-urea polyacrylamide gel electrophoresis of the precipitate from Hb A shows two bands of β^A and α^A chain, and the precipitate from Hb S.B. shows β^A and $\alpha^{S.B.}$ chain (Fig. 29), but it was not possible to detect enrichment of any particular chain.

6.3 The osmotic fragility of red blood cell

Fresh heparinized blood was prepared and the osmotic fragility of red blood cell containing abnormal Hb S.B. was automatically determined. Fig. 30 shows the osmotic fragility curve of propositus "S.B. red cell (b) compared to that of normal red blood cell (a). The results indicate that osmotic fragility of propositus' red cell is normal; hemolysis of red blood cell begins at 1 minutes and is complete within 3½ minutes.

6.4 Inclusion body test

Red blood cells containing unstable hemoglobin usually have a shortened life span and are particularly sensitive to further destruction by oxidizing agents, giving intraerythrocytic clumps, known as inclusion bodies. Fresh heparinized blood from normal subjects, the propositus "S.B." and a patient with Hb H were tested using sodium nitrite as an oxidizing agent. The results are shown in Fig. 31. Normal blood (a) used as a negative control showed red blood cell without inclusion bodies whereas Hb H (b) as a positive control showed irregular inclusions, at or near the periphery of the red blood cell, while the red blood cell of propositus "S.B. (c) had no inclusion body formation.

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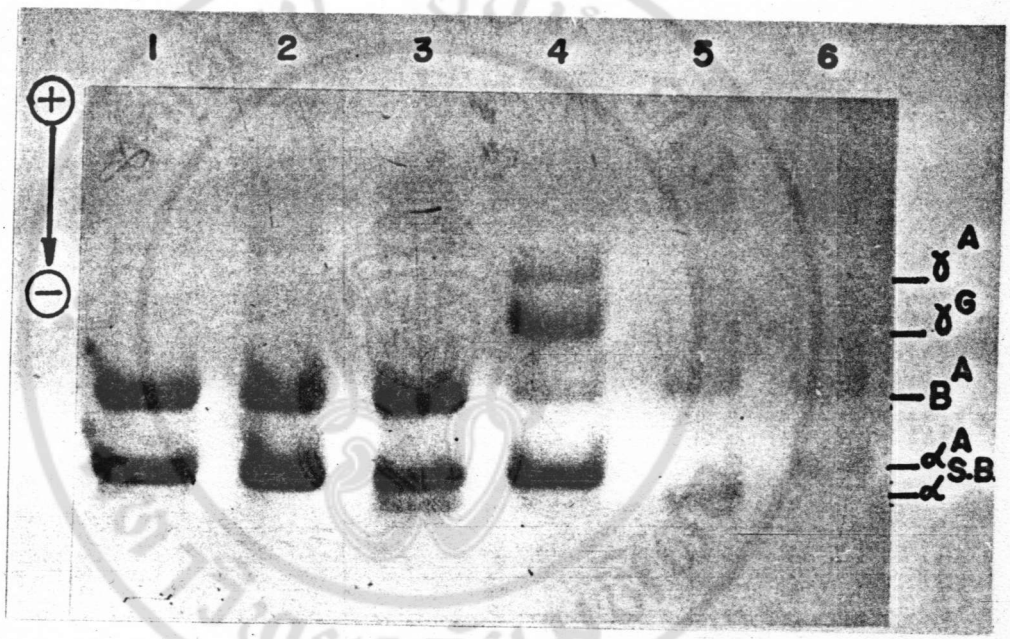


Fig. 29 Analysis of precipitates obtained on heating purified Hb A and Hb S.B. by Triton x-100-acid-urea polyacrylamide gel electrophoresis

1,2 : hemolysate of normal subject

3 : hemolysate of propositus "S.B."

4 : hemolysate of cord blood

5 : precipitate of purified Hb S.B.

6 : precipitate of purified Hb A

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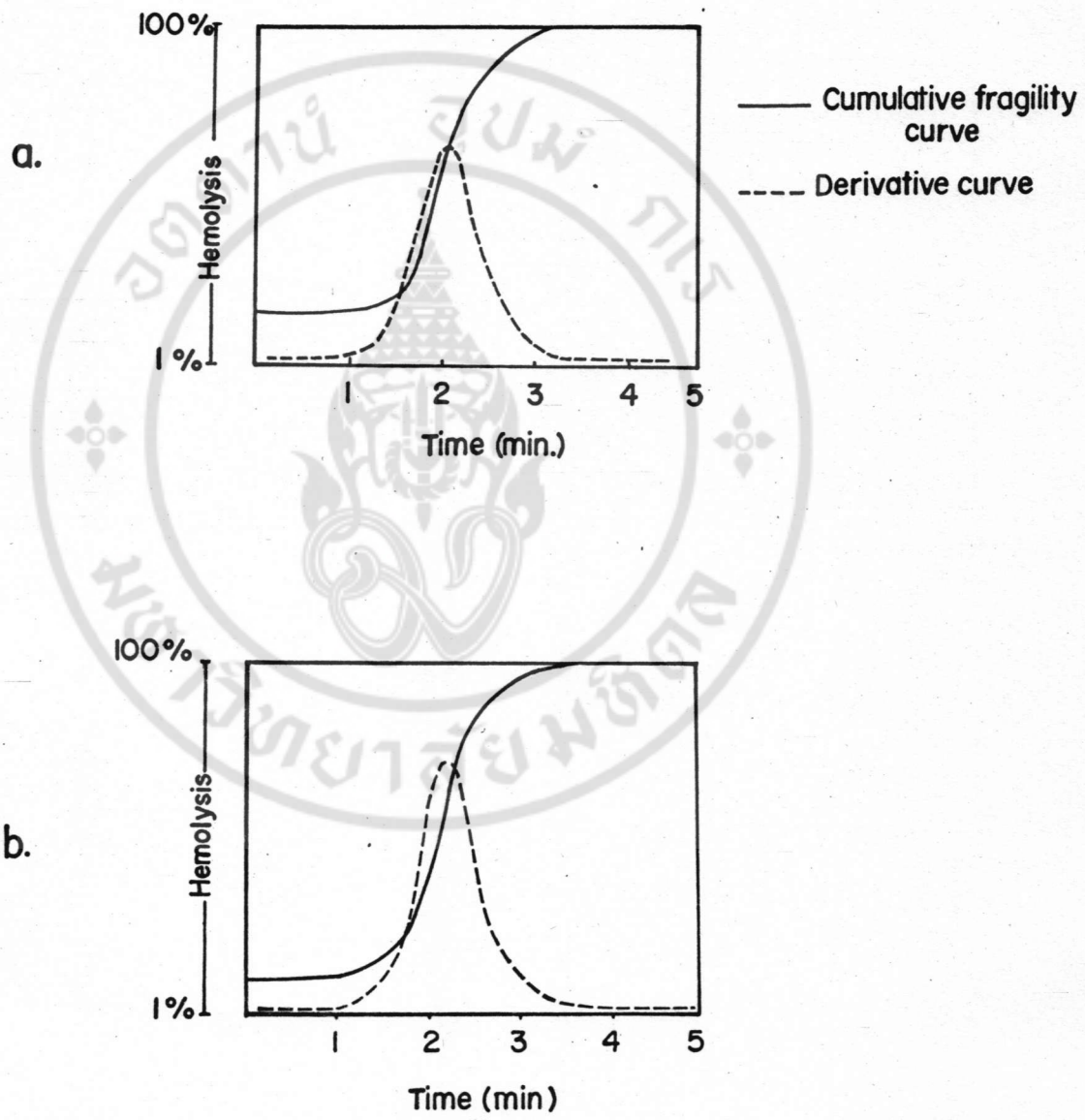


Fig. 30 The osmotic fragility curves of a) normal red blood cell b) propositus "S.B." red blood cell.

(Data from the thalassemia center, Mahidol University).

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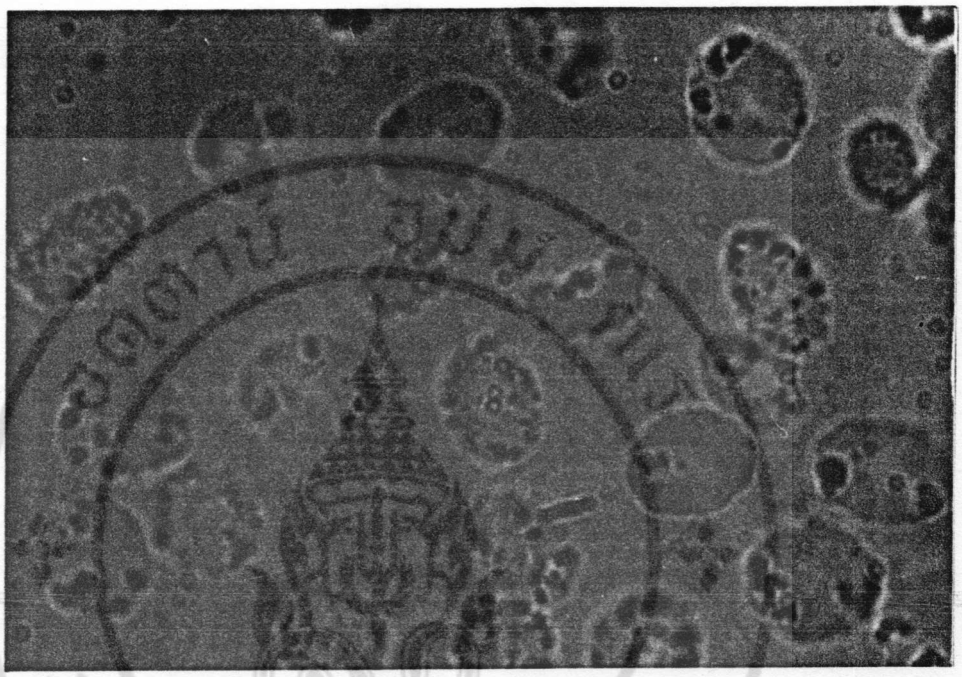
a.



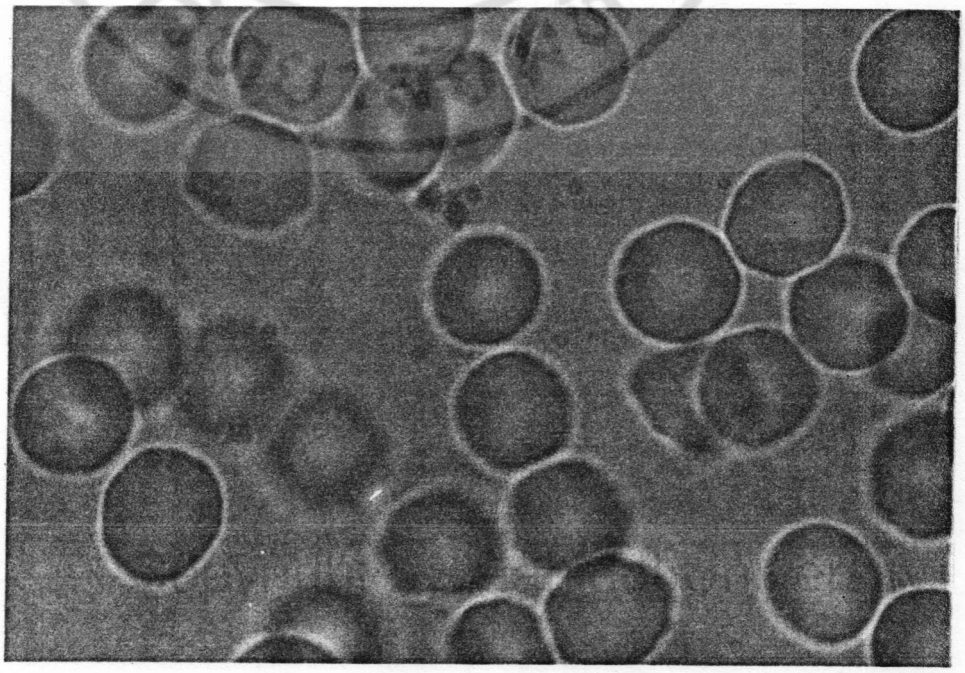
Fig. 31 Inclusion bodies test of red blood cell from normal subject (a), patient with HbH (b) and propositus "S.B."(c). Stained with methyl violet. (1,500 x).

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b.



c.



DISCUSSION

1. Identification of abnormal hemoglobin and abnormal globin chain by electrophoresis

Normal hemoglobins, Hb A, HbA_2 and Hb F and many hemoglobin variants can be identified by starch gel electrophoresis which is useful for routine screening of thalassemia and abnormal hemoglobins because of its low expense and ability to analyse many samples simultaneously. Starch gel electrophoresis of the propositus "S.B." hemolysate in tris-EDTA borate buffer, pH 8.6 revealed the presence of two abnormal bands, with the major band moving slower than Hb A and the faint minor band moving slower to the anode than Hb A_2 (Fig. 7). In general, α chain variants will produce abnormalities in three hemoglobins, Hb A, Hb A_2 and Hb F because these hemoglobins share a common α chain. Non-α chain variants, on the other hand, will result in only one abnormal hemoglobin. Therefore, the abnormality in the hemoglobin of the propositus "S.B." hemolysate was believed to be an α chain variant, with the major abnormal band being thought to be abnormal of Hb A (α variant$_2$ β_2) and the faint abnormal band believed to be abnormal Hb A_2 (α variant$_2$ δ_2). However, the abnormality in Hb F (α variant$_2$ γ_2) could not be detected because it is probably present in very low amounts.

Electrophoresis of the propositus "S.B." hemolysate on cellulose acetate membranes at pH 9.1 also showed an abnormal band moving slower to the anode than Hb A (Fig. 11). This method is simple, offering rapid results and may be used without any staining to quantitate Hb A_2, Hb E,

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Hb H and others (124). However, the band believed to be slow Hb A_2 in propositus' hemolysate cannot be detected by this method because very low amounts of hemolysate were loaded; so that components present at low concentration in hemolysate cannot be detected.

Since the electrophoresis was carried out at pH 9.1 and hemoglobins moved towards the anode, the rate of movement of hemoglobins will depend on their net negative charge. Thus the slower movement of the abnormal hemoglobin found in propositus "S.B." indicates that it probably carries less negative charge than normal hemoglobin, Hb A.

Identification of the abnormal globin chain can be achieved by electrophoresis on cellulose acetate, in the presence of urea and 2-mercaptoethanol in buffer, pH 6.5. The method can be used with the whole hemolysate to which 2-mercaptoethanol and urea have been added, causing the removal of heme from globin, and the dissociation of the α-and non α-globin chains, which will then migrate on cellulose acetate according to their electrical charge. The propositus' hemolysate showed the presence of an abnormal globin chain moving faster to the cathode than α^A chain (Fig. 14), suggesting that the abnormal α chain was more positively charged than the α^A chain, in agreement with the results obtained from electrophoresis of native hemoglobins on starch gel and cellulose acetate, where the abnormal hemoglobin moved slower to the anode than Hb A at alkali pH.

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Electrophoresis of human hemoglobins on Triton X-100-acid-urea gel leads to separation of all the major normal globin chains;

ζ,ε, $^{A}\gamma$, $^{G}\gamma$, β and α chain (140). The separation of the polypeptides is based on the hydrophobicity of molecule, since the detergent forms mixed micelles with the hydrophobic regions of polypeptide melecule (141). The abnormal globin chain of propositus' hemolysate can also be separated by Triton X-100-acid-urea polyacrylamide gel electrophoresis (Fig. 15). This technique provides analysis of very small amounts of hemoglobins, in determining $\gamma^{G}/^{A}\gamma$ ratio, and in detecting silent hemoglobin variants, e.g. Hb Knossos (β 27(B9) Ala $\longrightarrow$ Ser) (142).

2. Preparative isolation of abnormal hemoglobin and abnormal globin chain by column chromatography

Anion exchange chromatography on DEAE-cellulose columns has been one of the standard techniques for the separation of the different hemoglobins, and a sodium gradient is often used to elute the hemoglobin. Small amounts of potassium cyanide (100 mg/L) are usually added to buffers to prevent the formation of methemoglobin on the column, since ferri-hemoglobin cyanide and oxyhemoglobin show no difference in chromatographic behavior (143). DEAE-cellulose column chromatography of the propositus' hemolysate (Fig. 16 & 17) indicated the abnormal hemoglobin (about 13.1%) was eluted before Hb A at a conductivity about 3.0 mMHOs, while the main component Hb A (about 69.1%) was eluted at a higher salt concentration (conductivity 4.0 mMHOs). Since glycosylated hemoglobins are generally eluted with the Hb F fraction on DEAE cellulose column chromatography and have a distinctly higher mobility than Hb A on starch gel electrophoresis, pH 8.6 (143), the fraction (approx. 14.7%), which eluted after Hb A in the DEAE-cellulose column and moving faster to the anode than

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Hb A on cellulose acetate electrophoresis (Fig. 17) was believed to be glycosylated hemoglobin. This suggestion was supported by the fact that Hb F, which is also eluted out after Hb A, could only be detected in very small amounts (0.69%) by alkali resistance method. Two small hemoglobin peaks were eluted out before these three major fractions: the first was believed to be slow Hb A_2 (about 0.9%) and the second was probably Hb A_2 (2.0%). The purified Hb A_2 fraction was always moved slightly slower than Hb E to the anode on electrophoresis at pH 9.1 (Fig. 17) (143). The relative elution rates of hemoglobins on DEAE cellulose column chromatography as measured by their elution volumes were slow A_2 > A_2 > S.B. > A > A_1 = F.

Cation exchange chromatography on CM-cellulose columns in 8 M urea is a standard technique for the preparative isolation of the globin chain (126). Deionizing and filtering the 8 M urea are necessary to eliminate any additional contribution of urea to the neccessarily low initial strength and to prevent the accumulation of insoluble matter on the surface of the column. The 2-mercaptoethanol is also required in the buffers to prevent aggregation of the α and non-α chains through disulfide bonds formed by disulfide interchange reactions in 8 M urea at neutral pH (126).

In the present study, CM-cellulose column chromatography was used for the preparative isolation of the α and β globin chains from Hb A (Fig. 19) and of the abnormal α globin chain and the normal β chains from the abnormal hemoglobin of propositus "S.B." (Fig. 20). This method has also been successfully used to prepare globin from the total hemoglobin

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of the normal hemolysate (Fig. 21) and to prepare the abnormal globin from the total hemoglobin of propositus' hemolysate (Fig. 22) in a high degree of purity (Fig. 23) without preliminary purification of the particular hemoglobin.

3. Fingerprinting and amino acid analysis of the abnormal globin chains

The fingerprint of both $\alpha^{S.B.}$ and α^{A} globin chain provided a clear separation of the small tryptic peptides (Fig. 24a & b). The fingerprint does not represent the whole of the α globin chain because the tryptic resistant core (residues 93-139) is insoluble and was removed by centrifugation at the end of the tryptic digestion. The "core" region of the α and β chains may be studied by S-amino-ethylation of cysteine residues with either bromoethylamine (BEA) (144) or ethylene imine (145) to introduce additional sites for tryptic cleavage.

In our experiment, abnormal peptides can be detected without the aminoethylation of globin chain prior to the tryptic digestion. There are fourteen peptide spots found in $\alpha^{S.B.}$ map (Fig. 24b). Compared to the α^{A} map (Fig. 24a), one spot corresponding to the peptide $\alpha T_p V$ was absent in the $\alpha^{S.B.}$ map and two new spots assigned as peptides 'a' and 'b' were present. Amino acid analysis (Table 8) indicated that peptides 'a' and 'b' were derived from normal $\alpha T_p V$. Normal peptide $\alpha T_p V$ is composed of nine amino acid residues (residue 32-40) while peptide 'b' contains the six amino acids at the C-terminus of peptide $\alpha T_p V$, and peptide 'a' is composed of the amino acids Met, Phe and Arg, similar to the N-terminus of peptide $\alpha T_p V$ except that Leu was

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missing and an additional Arg was present (Table 8). This data suggests that the leucine residue at position 34 of α globin chain has probably been replaced by arginine, creating an additional tryptic cleavage site giving rise to two abnormal peptides 'a' and 'b' in fingerprinting (Fig. 32).

From the amino acid compositions (Table 8), the recovery of these three peptides $\alpha T_p V$, 'a' and 'b' were very good i.e. 32%, 45.4% and 45.4% respectively. The purity of these three peptides was also good, since contamination with other amino acids was very low (less than 0.05 residue/mole peptide). Peptide $\alpha T_p V$ and peptide 'a' showed a rather low methionine content (0.65 and 0.63 residues/mole peptide respectively) and peptide 'b' also showed a low serine content of 0.63 residues/mole. This is not surprising because these three amino acids are the N-terminal residues of the peptides and were therefore partly destroyed by reaction with 0.03% ninhydrin, which was used to stain the paper.

In conclusion, the abnormality found in the hemoglobin variant of propositus "S.B." is a replacement of leucine by arginine at position 34 of the α chain. This replacement introduces an extra positive charge in the α globin chain of hemoglobin variant resulting in its faster movement to the cathode than normal α globin chain on cellulose acetate electrophoresis at pH 6.5 and on Triton X-100-acid-urea polyacrylamide gel electrophoresis. This variant was first discovered in a Korean girl in 1979 and was named Hb Queens by Tatsis (139). It has also been called Hb Ogi in Japan (146), and has also been found in China (147), and reported in association with Hb E in a Vietnamese boy (148).

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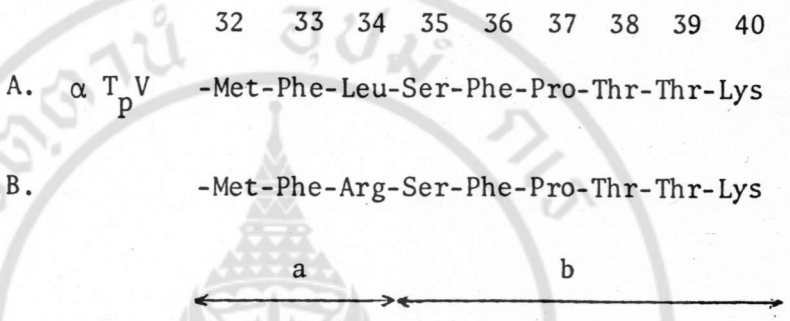


Fig. 32 A diagram of amino acid sequence from residue 32 to 40 of the α chain. A is the normal T_pV, B in Hb S.B. arginine substitutes leucine at position 34th. Tryptic cleavage after the arginine at this position leads to the abnormal spot a and b.

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It can be deduced from the recently determined nucleotide sequence of the α chain mRNA (149) that the substitution of leucine by arginine at position α 34 must be due to a single base mutation CUG → CGG.

4. Properties and stability

The stability of hemoglobin depends on bonds between amino acid residues and the heme groups, bonds between the chains, and bonds within the chains. Thus, when the replacement of an amino acid residue affects any of these many bonds, the stability of the molecule may be decreased. The "unstable" hemoglobin may then precipitate more easily in the isopropanol stability test and heat stability test, or may form inclusion bodies when subjected to suitable test conditions.

With unstable hemoglobins, oxidizing agents such as sodium nitrite may cause the formation of inclusion bodies in red blood cell (150). For example, Hb H which has a molecular instability due to an abnormal quaternary structure (β4) will rapidly precipitate in the red cell after oxidation (151). Fresh heparinized blood of propositus "S.B.", normal and patient with Hb H were collected and subjected to the sodium nitrite inclusion body test. Inclusion bodies could not be detected in either the propositus "S.B." or in the normal red blood cell but were present in treated red blood cell of a patient with Hb H (Fig. 31). Methylene blue staining (152) was also used to test for inclusion body formation, and inclusion bodies could be detected in the red blood cells of a patient with Hb H but not in the red blood cells of the propositus or of a normal subject (data not shown).

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With the isopropanol and heat stability tests, fresh hemolysates were used and cell membrane extraction was required. Also 0.1 M Tris HCl buffer was employed instead of phosphate buffer since both normal and unstable hemoglobins precipitated more rapidly in tris buffer (153). The results indicated that the hemolysate of the propositus "S.B." containing the abnormal hemoglobin showed no significant differences from the normal hemolysate in both the isopropanol and the heat stability tests (Fig. 25 & 26).

Although studies with whole hemolysate did not suggest any instability associated with the propositus' hemoglobin, the fact that an abnormal hemoglobin is present in low amounts often increases the difficulty of detecting slight instability (154). Accordingly studies were carried out to compare the heat stability of purified Hb A and abnormal hemoglobin isolated from the propositus. The results (Fig. 18) showed that the purified abnormal hemoglobin showed significantly greater precipitation than Hb A purified from the same subject. This is not surprising because in the structure of hemoglobin, the residue leucine 34 occupies the 15^{th} position of the helical B segment of the α chain and lies in the $\alpha_1 \beta_1$ contact (155), being in contact with the side chains of the residues Ala 128 (H 6), Pro 125 (H 3), Pro 124 (H 2) of the β chain. The replacement of leucine by arginine introduces a positive charge which could cause a loss of some or all of these contacts. The consequent weakening of the $\alpha_1 \beta_1$ contact would account for the slightly lower stability compared to Hb A under heating at $60^{o}C$. This is similar to Hb Khartoum which is a β chain variant

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where Pro 124 (H 2) in the $\alpha_1 \beta_1$ contact is replaced by positively charged arginine. Hb Khartoum comprises about 30% of the total hemoglobin, and red blood cells showed no intracellular inclusion bodies but the hemolysate was slightly less heat stable than that of a normal control sample (156).

The lack of clinical symptoms, the normal osmotic fragility of the red blood cell, and the normal hematologic profile of the propositus "S.B." indicate no gross abnormalities in the function of this hemoglobin variant. This agrees well with the previous observations that variants with mutations at the $\alpha_1 \beta_1$ contacts exhibit little or no departure in function from normal hemoglobin (3). The $\alpha_1 \beta_1$ contact, though involving a large area, undergoes only slight movement or alteration in the reversible transition between the oxy and deoxy tetrameric conformation. Thus, structural alterations involving the $\alpha_1 \beta_1$ contact are expected to cause only instability, of variable extent, of the specifically involved $\alpha\beta$ dimer (157).

With regard to the relative content of abnormal hemoglobins in heterozygotes, genetic variants of the β chain of human hemoglobin are usually found in a concentration approaching half of the total hemoglobin. Some variants occur in lower proportions, probably as a result of a diminished rate of synthesis of the abnormal chain or as a result of selective precipitation within the erythrocyte (154). In contrast, the α chain variants is more complex due to the presence of multiple alleles for the α chain, so that most variants are present at

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a level of 25% of the total hemoglobin. The abnormal hemoglobin of the propositus "S.B., which is an α chain variant, comprises only about 12% of the total hemoglobin. Several other α chain variants; Hb Chad α 23 Glu → lys (158) and Hb Audhali α23 Glu → Val (159) which lie in the B helix occur in concentration of less than 20% in heterozygotes; all without apparent alteration of red cell morphology or hemoglobin concentration. Another example is Hb Manitoba α102 (G9) Ser → Arg (160) which lies in the vicinity of $\alpha_1 \beta_1$ contact and is also found in similarly low concentrations.

Other reports of Hb Queens

There have been a number of reports of Hb Queens in Oriental races (139, 146-148). Heterozygous Hb Queens does not exhibit any clinical symptoms (139, 146-147), in agreement with the present study. Hyperbilirubinemia was observed in a Vietnamese newborn, homozygous for Hb E and heterozygous for Hb Queens (148), but it was believed that this condition was unrelated to the presence of the variant hemoglobin.

An interesting feature of all Hb Queens cases so far reported is the low percentage of Hb Queens in the hemolysate of heterozygous (12-18% total hemoglobin). The reasons for this are still unclear. However, globin chain synthesis studies suggested balanced synthesis (139), and whole hemolysates containing Hb Queens appear to show normal heat and isopropanol stability (139, 146-148), as was the case with the propositus' hemolysate (Fig. 25 & 26). However, when heat stability

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was studied using purified hemoglobins, Hb Queens appeared to be somewhat less heat stable than Hb A (Fig. 28). This discrepancy could be due to the low content of Hb Queens in the whole hemolysate, so that the properties of the abnormal hemoglobin tend to be masked by the properties of Hb A. Thus with low content variants, it may be more informative to study the properties of the purified variant rather than of the whole hemolysate. Indeed, when the oxygen affinity of isolated Hb Queens was studied, it was found to be slightly increased relative to Hb A (146).

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SUMMARY

1. An abnormal hemoglobin was found in a healthy twenty-four year old Thai man with a normal hematologic profile.

2. Electrophoresis on starch gel and on cellulose acetate showed an abnormal hemoglobin moving slower to the anode than Hb A, while another faint abnormal band was detected only on starch gel and moved slower to the anode than Hb A_2. This suggested that the abnormal hemoglobin was of the α chain type.

3. Electrophoresis on cellulose acetate in the presence of urea and mercaptoethanol, and on Triton-x 100-acid-urea-polyacrylamide gels revealed that the abnormal α chain moved faster to the cathode than normal α chain.

4. The abnormal hemoglobin was first purified by DEAE-cellulose column chromatography. The isolated globin were then fractionated on a CM-cellulose column to obtain the abnormal α chain.

5. The fingerprints of tryptic peptides from the abnormal α chain were compared to those of normal α chain obtained from a normal subject. The $\alpha T_p V$ spot was lost from the map of the abnormal chain, but two new spots were present.

6. Amino acid analysis of the two abnormal peptides revealed that the substitution in the abnormal hemoglobin was a replacement of a leucine residue at position 34 of the α chain by arginine. The α 34 residue lies on the $\alpha_1 \beta_1$ contact, and This identified

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the variant as Hb Queens [α 34 (B 15) Leu → Arg].

7. The whole hemolysate of the propositus showed normal heat stability and isopropanol stability, but the isolated abnormal hemoglobin appeared to be less heat stable than Hb A. Red blood cells showed normal osmotic fragility and were negative for the inclusion body test.

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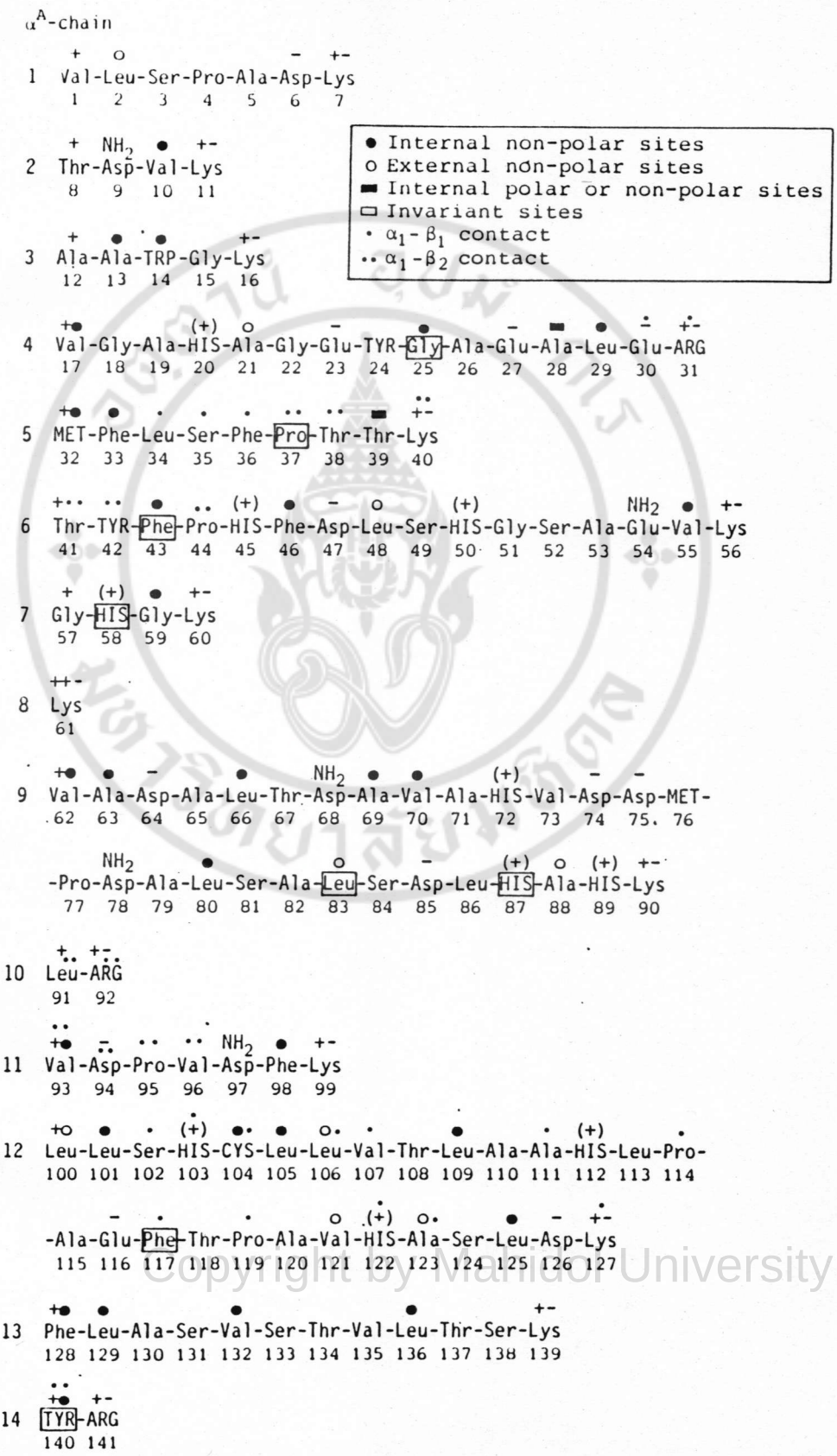
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APPENDIX I

The tryptic peptides of the α chain

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Tryptic peptides



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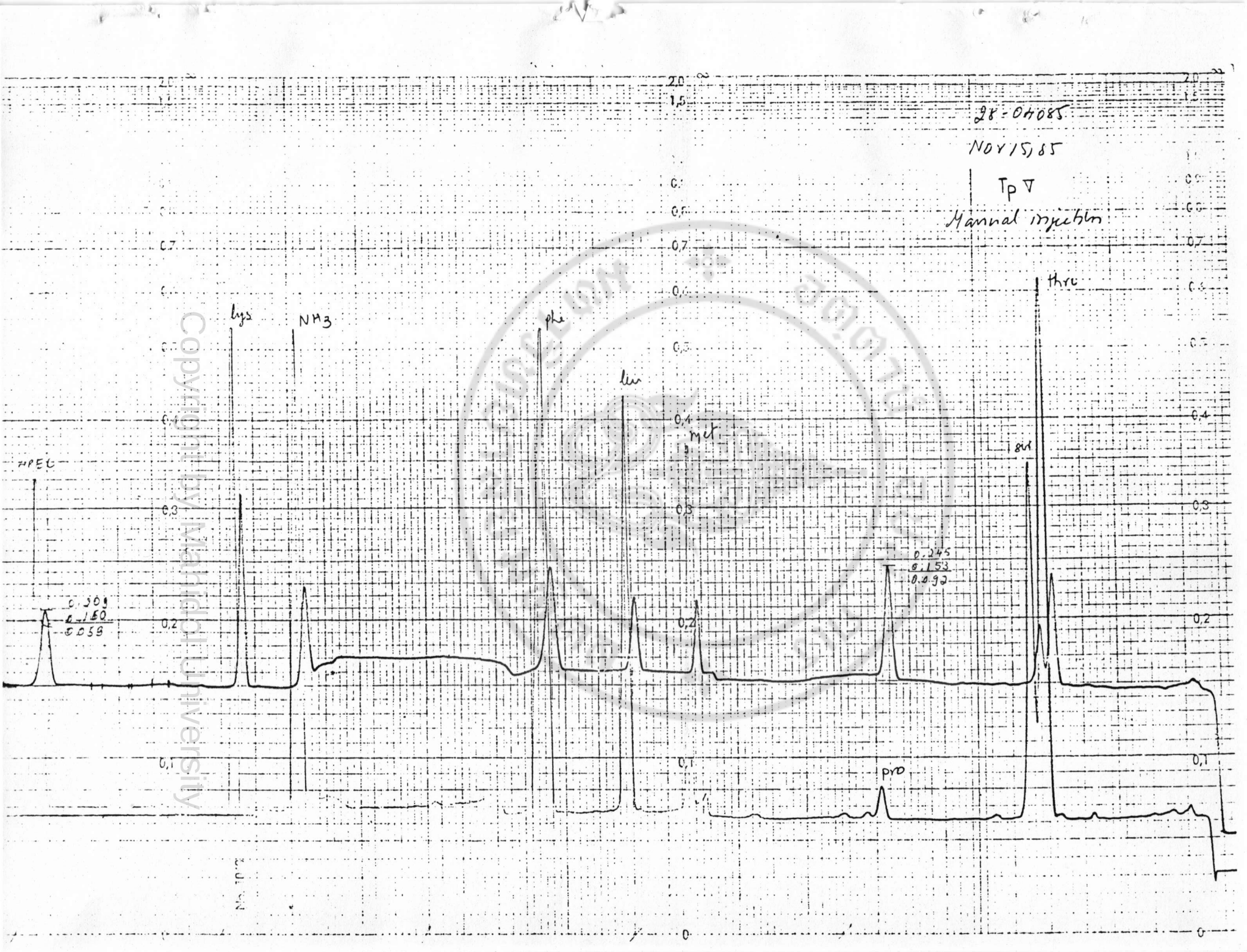
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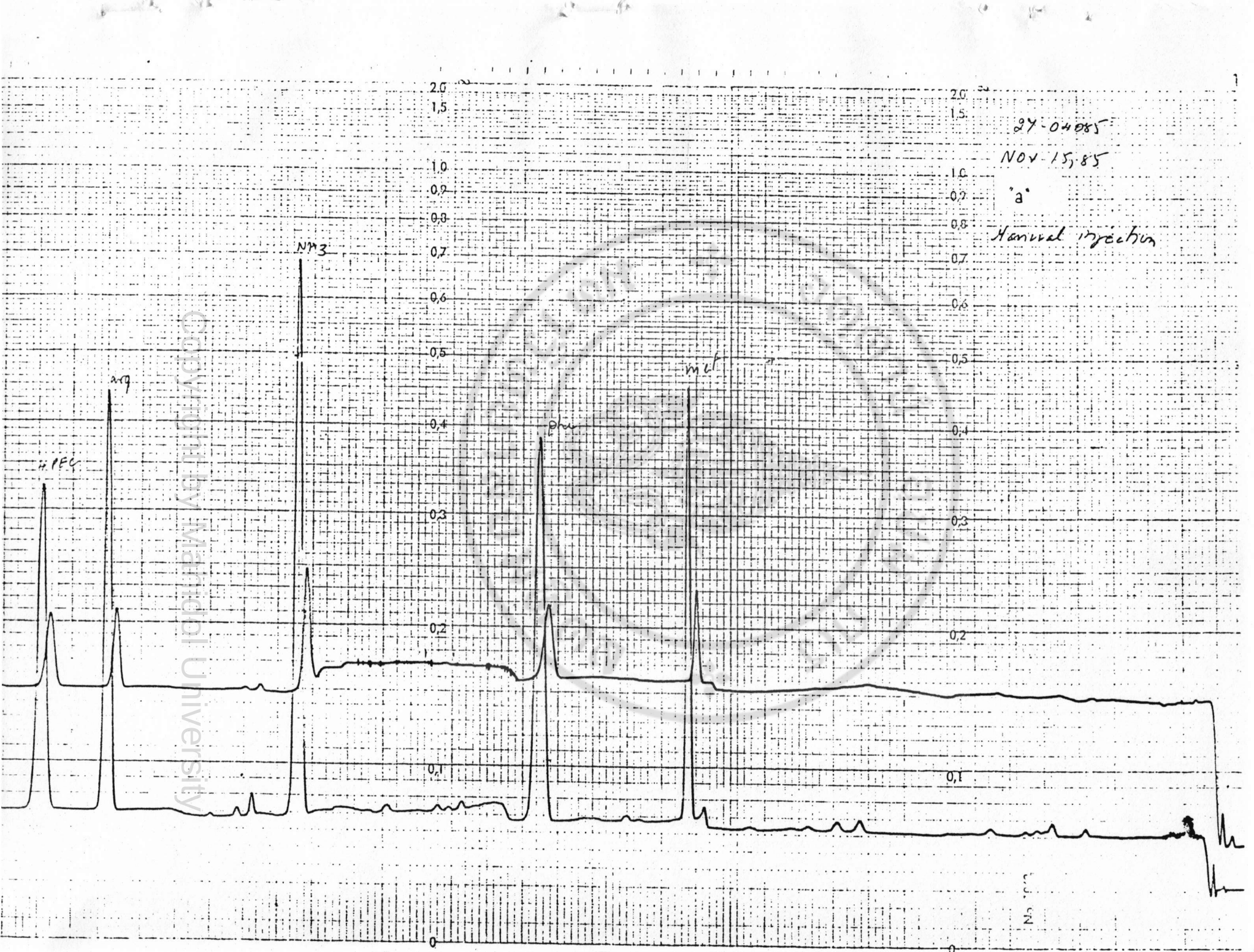
APPENDIX II

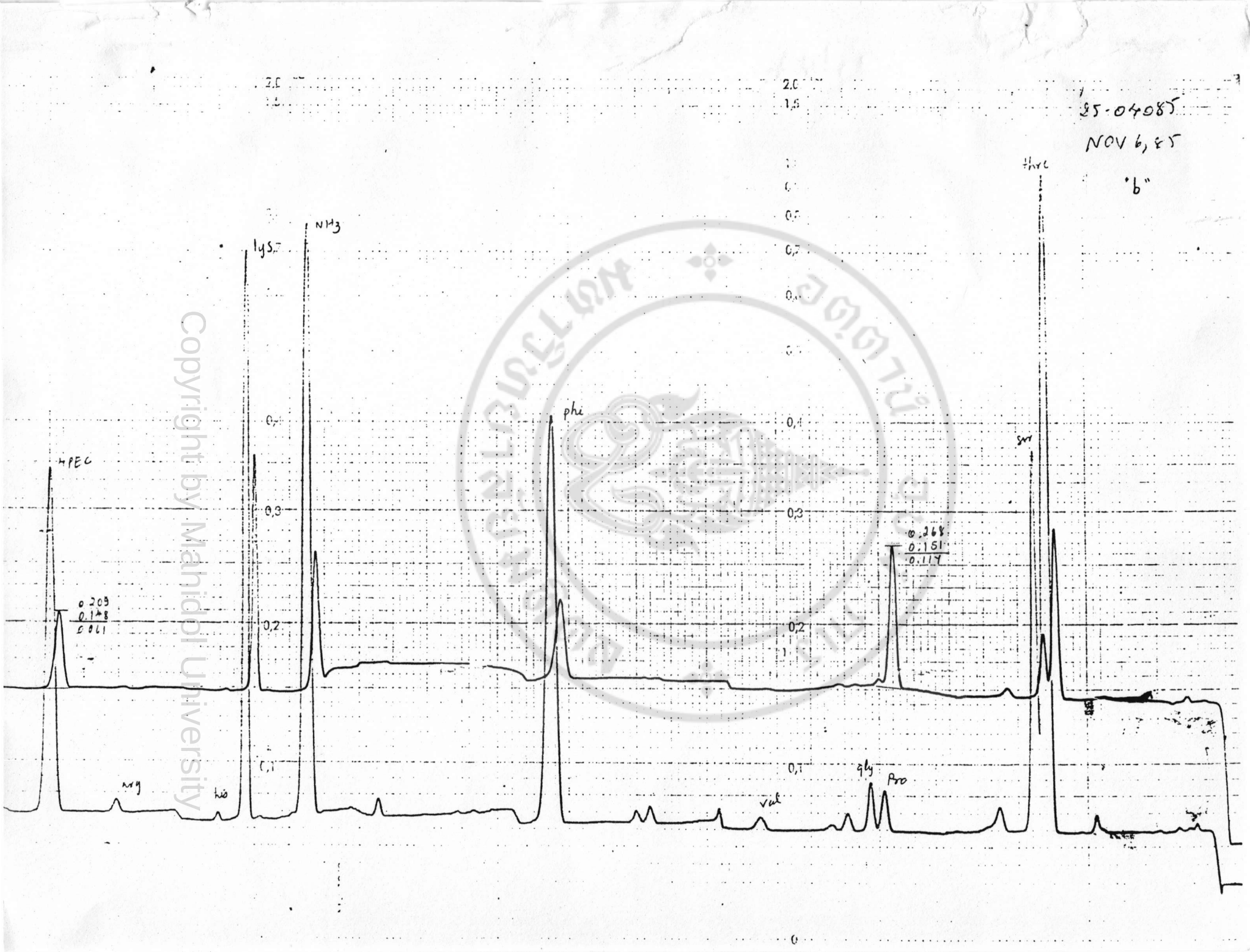
Amino acid elution profile of tryptic peptide T_pV of α^A chain and of tryptic peptide a and b of $\alpha^{S.B.}$ chain

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2.0
1.5
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
NH3
arg
phe
met
27-04085
NOV 15, 85
"a"
Manual injection



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