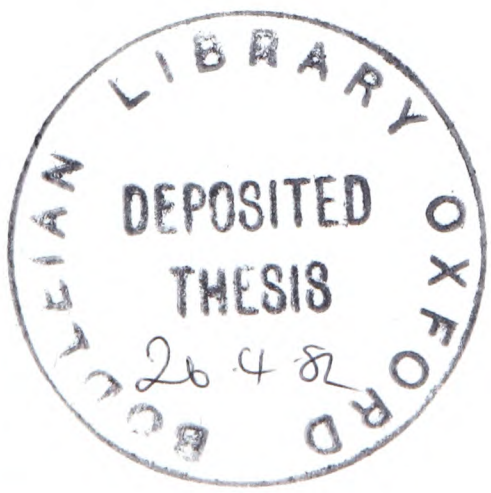
THE SYNTHESIS OF HAEMOGLOBIN E

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A thesis submitted for the degree of

Doctor of Philosophy

in the University of Oxford



Linacre College

Michaelmas Term, 1981

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ABSTRACT

Hb E ($\alpha_2\beta_2^{26\ glu\rightarrow lys}$) is one of the most common haemoglobin variants, found in an estimated 30 million people in South-East Asia. Homozygotes for Hb E are only mildly anaemic but compound heterozygotes with β thalassaemia have a severe clinical disorder which is the commonest form of symptomatic thalassaemia in S.E. Asia. The reasons for the high frequency of the β^E gene and the severity of its interaction with β thalassaemia have never been adequately explained. We have studied eight Hb E homozygotes and nine heterozygotes all of S.E. Asian origin.

In peripheral blood reticulocytes of homozygotes there was marked deficit of β^E chain synthesis relative to α chain synthesis (α/β^E ratio 2.0-3.3) and this was also observed to a lesser degree in the heterozygotes (1.23-2.19). There was no evidence that this was due to rapid destruction of the newly synthesised β^E chains, nor that Hb E was preferentially destroyed during the lifespan of the red cell.

Measurement of the ratios of α/β globin mRNA in the reticulocytes of these subjects showed a deficit of β^E mRNA consistent with the decreased β^E chain synthesis in these cells. Assessment of α/β mRNA ratios in bone marrow samples suggested normal transcription of β^E mRNA and transport out of the nucleus but that once in the cytoplasm the β^E mRNA was relatively unstable. The nature of the mRNA defect is unknown and could result either from the base substitution responsible for the amino acid change or from a second independent mutation in this gene.

Thus the β^E gene acts as a mild β thalassaemia gene, the defect acting at a pretranslational level. This explains why on interaction with β thalassaemia there is marked deficit of β chain production leading to a disorder of clinical importance.

ABBREVIATIONS

cDNA	complemetary DNA
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
TTP	Thymidine 5'-triphosphate
mRNA	messenger ribonucleic acid
EDTA	ethylenediaminetetraacetic acid
Hepes	N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid

CHAPTER 1

INTRODUCTION

The Structure and Function of Haemoglobin

The human haemoglobin molecule has been studied for over a century, and its structure and closely associated functions are now well understood. The detailed knowledge of its structure and the characterization of inherited disorders of haemoglobin synthesis make it an extremely useful model for defining the mechanisms of mammalian protein synthesis and their underlying genetic controls.

The haemoglobin molecule is composed of four polypeptide globin chains, to each of which a haem prosthetic group is attached. The iron containing haem was thoroughly investigated at the beginning of this century, but it was not until the middle of this century that the amino acid sequences of the globin moieties were determined. It was also at this time that the 3-dimensional structures of oxy- and deoxy-haemoglobin were elucidated and it was these studies that enabled the correlation of haemoglobin structure and function.

All the normal human haemoglobins have a molecular weight of 64,000-68,000 and a tetrameric structure consisting of two α-like and two β-like globin chains. The various haemoglobins that are formed during normal human development are summarised in Table 1.1. Despite extensive investigation (Stamatoyannopoulos and Nienhuis 1978; 1981) very little is known about the mechanisms

TABLE 1.1 HAEMOGLOBIN SYNTHESIS DURING NORMAL HUMAN DEVELOPMENT

DEVELOPMENTAL STAGE	HAEMOGLOBIN TYPE		PROPORTION PRESENT
Embryo	Hb Gower 1	$\zeta_2\varepsilon_2$	?
	Hb Gower 2	$\alpha_2\varepsilon_2$	?
	Hb Portland	$\zeta_2\gamma_2$	?
	? Hb F		
Foetal	Hb F	$\alpha_2{}^{G}\gamma_2$ / $\alpha_2{}^{A}\gamma_2$	90-95% ($3^{G}\gamma : 1^{A}\gamma$)
	Hb A	$\alpha_2\beta_2$	5-10%
Adult	Hb A	$\alpha_2\beta_2$	97%
	Hb A_2	$\alpha_2\delta_2$	2.5%
	Hb F	$\alpha_2{}^{G}\gamma_2$ / $\alpha_2{}^{A}\gamma_2$	0.5%

controlling the differential synthesis of globin chains during human development.

A tiny proportion of Hbs A and F undergo post-synthetic modification. The addition of glucose to Hb A forms Hb A_{1c} and Hb F_1 is formed by the addition of acetyl groups to Hb F.

The Structure of Globin

The primary amino acid sequences of all the human globins have been determined. The human α-chain has 141 amino acids whilst the β, γ and δ chains each have 146 amino acid residues. The β, δ and γ chains are highly analogous, β and δ globin differing in only 10 of 146 amino acids and the β and γ chains differing in 39 of 146 residues. There are, in fact, two distinct γ chains, one ($^{G}\gamma$) with glycine and the other ($^{A}\gamma$) with alanine at residue 136. The relative amounts of these two γ chains constituting Hb F change during development from foetal to adult life.

More recently, the amino acid sequences of the embryonic ζ and ε globins have been determined. The ε chain has 24 differences with β globin and 18 differences with γ globin. The ζ chain differs more from α globin than ε from β globin (Gale et al 1979).

In native haemoglobin about 75% of the globin is in the form of an α-helix. At the intermittent non-helical segments the polypeptide is more flexible and these allow the chain to take up its convoluted 3-dimensional structure. The 8 helical segments in β globin

(labelled A-H) have highly similar counterparts in α globin, except that the amino-acids composing the D helix are missing in the latter. All amino acid residues are numbered from the amino-terminal end.

The Structure of Haemoglobin

The tertiary and quaternary structure of Hb was worked out using the technique of X-ray crystallography (Perutz et al 1968). The spatial arrangements of the α- and β-chains are fairly similar and the complementary contours of the folded chains make a compact fit in the tetrameric Hb molecule. The ellipsoid Hb molecule measures approximately 64 x 55 x 50 Å (Angstrom units). The physiologically significant feature of the molecule is the difference in the conformation of the oxygenated and deoxygenated forms.

In both forms each subunit is in contact with each of the unlike subunits and they interact with one another by relatively weak, although extensive, non-polar bonds and some hydrogen bonds. Like subunits only have limited contact. The contacts between the subunits have been designated $\alpha_1\beta_1$ ($\alpha_2\beta_2$) and $\alpha_1\beta_2$ ($\alpha_2\beta_1$). In oxy-haemoglobin there are 31 Van der Waal's contacts and 3 hydrogen bonds at the $\alpha_1\beta_1$ interface. In contrast there are only 15 Van der Waal's bonds and 2 hydrogen bonds at the $\alpha_1\beta_2$ interface. Under physiological conditions oxyhaemoglobin is known to dissociate into a pair of identical dimers at the $\alpha_1\beta_2$ interface (Rosemeyer and Huehns 1967).

Following deoxygenation the β chains of the tetramer move apart by about 7Å. Little or no conformational change occurs at the $\alpha_1\beta_1$ interface but the residues at the $\alpha_1\beta_2$ contact become aligned differently.

Despite the difference in alignment of the $\alpha_1\beta_2$ contact in oxy- and deoxy-haemoglobin, there is little alteration in the number of Van der Waal's and hydrogen bonds. However, the greater stability of the tetramer in deoxyhaemoglobin is related to the presence of inter- and intra-subunit salt bonds. Some of these salt bonds play an important role in the Bohr effect (discussed below). In addition, the interaction of deoxyhaemoglobin with the inorganic phosphate 2,3-diphosphoglycerate (2,3-DPG) also enhances the stability of the deoxy conformation (see below).

The haem residue of each globin chain lies in a cleft between the E and F helices. It is held between 2 histidine residues. The iron atom at the centre of the porphyrin ring is covalently linked to the proximal histidine (residue 92) and the distal histidine (residue 63) lies opposite the oxygen binding site of the iron, but it is not directly linked to the haem group. The haem is stabilized by a large number of inter atomic contacts.

Perutz's studies have shown that it is the iron atom at the centre of each porphyrin ring that triggers the conformational changes which occur on the uptake and release of oxygen by haemoglobin (Perutz 1970; 1972).

These changes enable the cooperative binding of oxygen to haemoglobin (haem-haem interaction) and indirectly influence the Bohr effect.

Haem-haem Interaction As each haem binds with molecular oxygen the rate of oxygen uptake by the other haem groups is increased through the series of globin subunit conformational changes triggered by the movement of the haem iron. The sigmoidal oxygen dissociation curve of haemoglobin, reflecting the haem-haem interaction, enables haemoglobin to become almost completely saturated with oxygen in the lungs and yet allows it to deliver adequate quantities of oxygen to the tissues, despite the relatively small drop in partial pressure between the lungs and the tissues.

The uptake of oxygen by haemoglobin is influenced by several intracellular conditions. Directly, and more importantly indirectly through pH (the Bohr effect), carbon dioxide facilitates the release of oxygen by haemoglobin in the tissues. When the carbon dioxide enters the red cell it reacts with water, in the presence of carbonic anhydrase:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

The protons so formed are able to bind with, and stabilise, the deoxy conformation of haemoglobin. In addition, about 10% of the carbon dioxide is able to bind directly with the deoxyhaemoglobin conformation so stabilising it and promoting release of oxygen to the tissues.

The intracellular levels of 2,3 diphosphoglycerate (2,3-DPG), an intermediate in the glycolytic pathway, also influence the binding of oxygen to haemoglobin. The 2,3-DPG is able to bind with deoxyhaemoglobin, hence stabilizing this conformation and facilitating release of oxygen by haemoglobin.

All these oxygen linked reactions, so important in enabling haemoglobin to fulfill its physiological roles, demonstrate the elegant allosteric properties of haemoglobin.

The Genetics of Haemoglobin Synthesis

Formal genetic studies suggested that the α- and β-globin genes were situated on different chromosomes (see Weatherall and Clegg 1979) and it has since been established that the α-like globin genes are located on chromosome 16 and the β-like globin genes on chromosome 11 (Deisseroth et al 1977 ; 1978).

The α-chain locus is duplicated on each chromosome in most, if not all, normal individuals. This was initially established on the basis of genetic studies (see Weatherall and Clegg 1976) and has recently been verified by cDNA/DNA hybridization and restriction endonuclease mapping.

Genetic evidence suggested a single β and single δ gene. However, the existence of two non-allelic γ globins, $^{A}\gamma$ and $^{G}\gamma$, demonstrated at least two γ-gene loci.

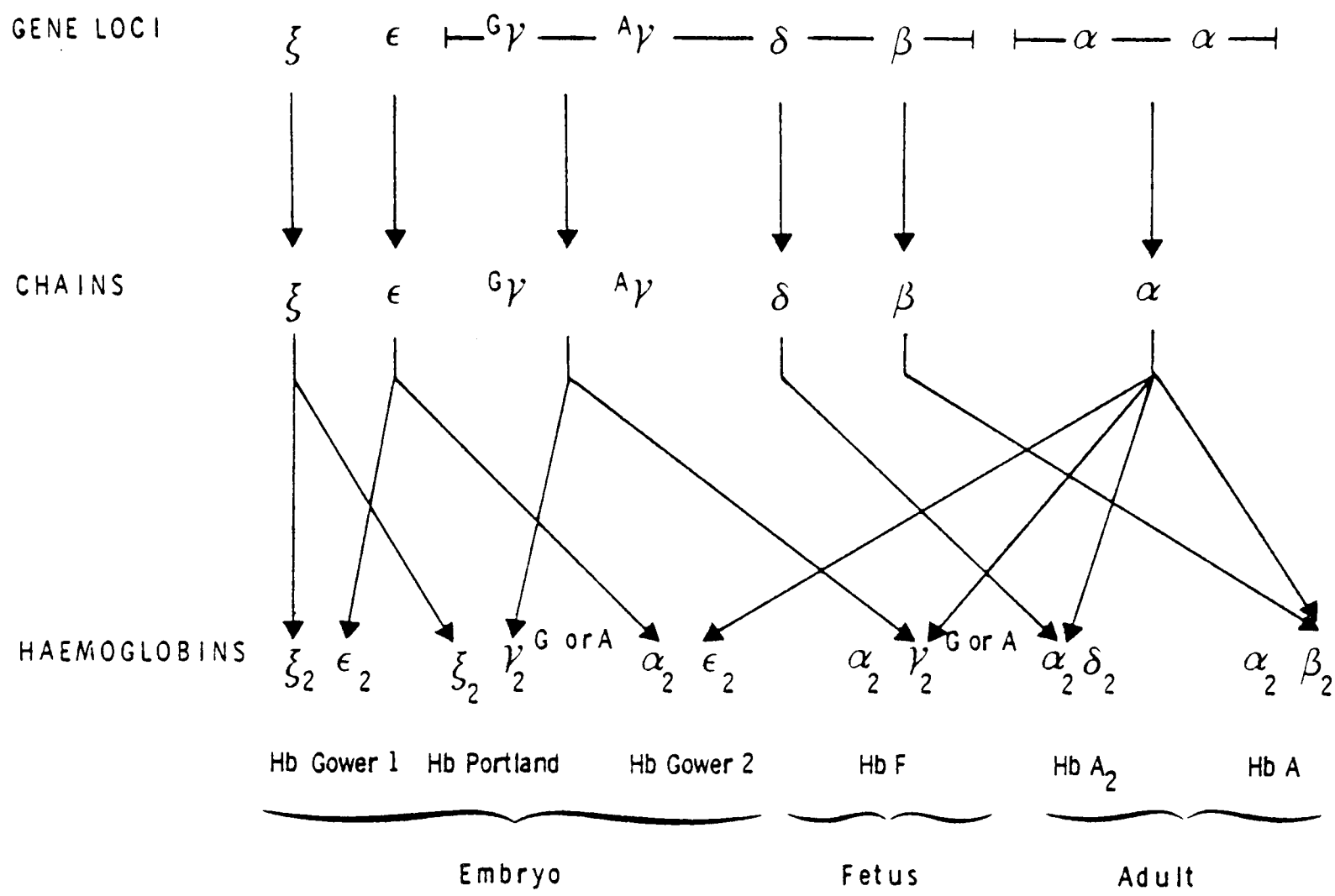


FIG. 1.1 The genetics of haemoglobin synthesis

The analysis of the fusion haemoglobins, Hb Lepore (α chains associated with $\delta\beta$ fusion globin chains - $\alpha_2\delta\beta_2$) and Hb Kenya (α chains with $\gamma\beta$ fusion globin chain $\alpha_2\gamma\beta_2$) indicated that the β-like genes were all linked on one chromosome, probably in the order $^{G}\gamma\text{-}^{A}\gamma\text{-}\delta\text{-}\beta$ (Weatherall and Clegg 1979). Gene mapping has now confirmed that the β-like globin genes (including the embryonic ε globin gene) are linked on chromosome 11 in the order $\varepsilon\text{-}^{A}\gamma\text{-}^{G}\gamma\text{-}\delta\text{-}\beta$ (Figure 1.2).

The genetic control of haemoglobin synthesis is illustrated in Figure 1.1.

The Chromosomal Arrangement of the Globin Genes

The arrangement of the ζ-α globin gene cluster is summarised in Figure 1.2. The genes are transcribed from the same DNA strand of the chromosome and are arranged in the order of their expression during development.

The two α genes, separated by about 3.7 kb DNA, are virtually identical (Lauer et al 1980) except in the 3' non-coding region. Each is interrupted by a small and large non-coding or intron sequence between the codons for amino acids 31/32 and 99/100 respectively.

The ζ genes are located 5' to the α genes with a pseudogene between the adult and embryonic α globin coding genes. It has been demonstrated that the ζ_2 gene is functional (Pressley et al 1980a) and recent evidence has shown that the ζ_1 gene is infact another pseudogene (Proudfoot, unpublished observation).

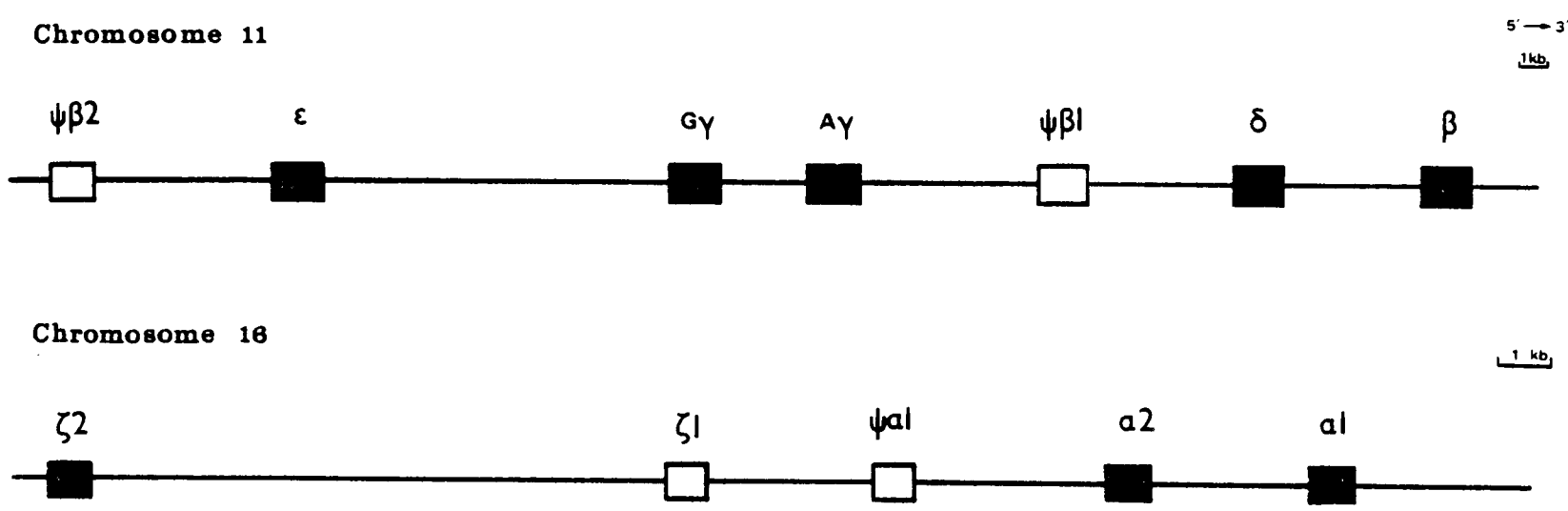


FIG. 1.2 Organisation of the α globin and β globin gene clusters.

The β-like genes are also transcribed from the same DNA strand and arranged in the order of their expression during development (see Figure 1.2).

The complete nucleotide sequence of the 5 β-like genes has been determined (Lawn et al 1980; Spritz et al 1980; Baralle et al 1980; Slightom et al 1980). Each gene has 2 introns, analogous to those in the α-like genes, the small one (122-130 base pairs long) between the codons for amino acids 30/31 and the large one (850-900 base pairs long) between the codons for residues 104/105.

The β and δ genes are closely homologous in their coding sequences but show great divergence in their non-coding flanking and intron sequences (Spritz et al 1980; Lawn et al 1980). The $^{G}\gamma$ and $^{A}\gamma$ gene coding sequences are virtually identical in every respect (Slightom et al 1980).

Alignment of the sequences 5' to each of the β-like genes reveals two areas of sequence homology (Efstradiadis et al 1980) which are analogous to those present in other eukaryotic genes. One sequence (the 'ATA' box) is found 30±1 b.p. 5' to the mRNA capping site and the second sequence (the 'CCAAT' box) is located 77±10 b.p. 5' to each gene. These sequences may have a role to play in the control of gene expression (Efstradiadis et al 1980; Proudfoot et al 1980).

Within the β gene cluster there are 2 pseudogenes, but as for the α-pseudogenes their function, if any,

is as yet unknown.

The Biosynthesis of Globin

The major steps in the synthesis of eukaryotic proteins, which includes globin, are summarized in Figure 1.3. The genetic information of the DNA is made into an RNA copy (transcription). After various modifications (processing) the mature mRNA is transported from the nucleus to the cytoplasm where it is decoded into protein by ribosomes (translation).

Before describing the steps outlined above, it is appropriate to describe the structure of DNA in general, for genes are more complex than a sequence of coding and non-coding nucleotide bases.

The General Structure of DNA

There are three types or classes of DNA sequences alternating within the chromosome (see Nienhuis and Benz 1977):

1. Highly repetitive DNA - usually small sequences represented many thousands of times.

2. Moderately repetitive DNA - sequences represented 10-1000 times and constituting about 10% of the DNA.

3. Unique sequences - present as only 1 or a few copies and accounting for more than 60% of the DNA. The globin genes are an example of unique sequence DNA.

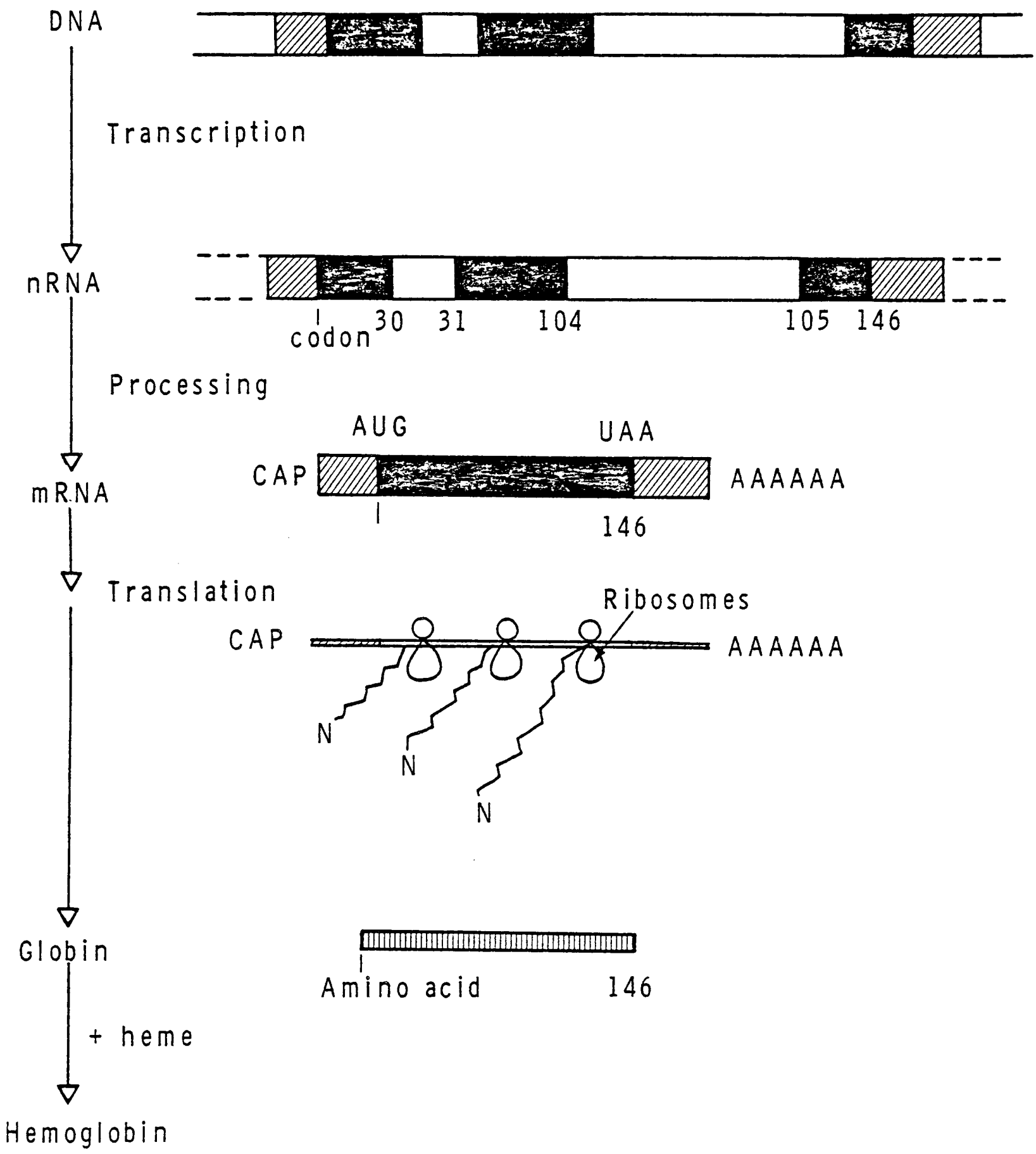


FIG. 1.3 Major steps in protein synthesis

The genes on the chromosomes of human DNA are not present as naked strands of nucleotide sequences but are complexed with proteins (histones and non-histones) forming chromatin. The transcription of DNA is greatly influenced by its incorporation into chromatin.

Chromatin consists of repeating structures called nucleosomes. Each nucleosome consists of 8 histone molecules associated with about 200 base pairs of DNA. Of the total DNA, about 90% is present in nucleosomes, and therefore interactions between the DNA and histones appear not to be DNA sequence specific.

There are 5 different histone molecules: H1, H2a and H2b, H3 and H4. They are basic proteins with a strongly conserved primary sequence. Some diversity in their structure is introduced by post-synthetic modification, for example acetylation or phosphorylation.

The core of the nucleosome consists of 2 symetrically paired histone tetramers (H2a, 2b, 3 and 4) around which is coiled (almost twice) about 140 base pairs of DNA. The spacer DNA of about 40-100 b.p. between the individual nucleosomes is associated with the histone H1 (Nienhuis and Benz 1977).

Chromatin may have 2 distinct configurations - the transcriptionally active or open fraction (euchromatin) and the condensed chromatin (heterochromatin) containing the genes not expressed in a specific cell. It is not known how the structure of chromatin is modified such that it may influence transcriptional activity of genes,

but somehow genes that are involved in protein synthesis in a specific cell are more 'accessible' as demonstrated by their selective destruction by nucleases (Nienhuis and Benz 1977).

Non-histone proteins probably also have an important regulatory role in gene activity. The heterogeneous group of non-histone proteins includes many enzymes, e.g. DNA and RNA polymerases, proteases and enzymatic modifiers of chromosomal protein.

Transcription

The sense-strand of unique sequence DNA is transcribed into RNA copies by type II RNA polymerase. (There are 3 types of RNA polymerase, types I and III transcribing the genes for ribosomal and transfer RNA's). The polymerase enzymes are multiple subunit complex molecules, requiring additional factors in order to function normally.

Processing of mRNA Precursors

The intial RNA transcript is an exact copy of the entire gene such that it includes the non-coding intron sequences as well as the coding sequences. There is evidence that the primary transcripts do not extend beyond the 5' and 3' extremes of the mature mRNA (Kinniburgh and Ross 1979; Grosveld et al 1981).

The heterogeneous nuclear RNA molecules (HnRNA) may be 2-3 times longer than the mature mRNA,and therefore undergo various modifications to yield mRNA.

In the mouse,the primary β globin RNA transcript

is 1860 nucleotides long and the largest α globin mRNA precursor is about 870 nucleotides long (Ross et al 1978). The globin mRNA coding sequences are separated into 3 regions by the 2 non-coding intervening sequences. In mouse and rabbit the β mRNA is processed in several steps. The small intervening sequence (IVS) appears to be excised first, followed by the removal of the large IVS, possibly in two steps (Ross et al 1978; Grosveld et al 1981).

Following each excision, the coding sequences are spliced together to form the mature mRNA. In addition to the excision of intervening sequences, the primary transcript undergoes a modification at the 5' end and 3' end of the molecule. One is the formation of a 5'-5' triphosphate linkage involving m^7G at the 5' end of the molecule, and the methylation of some nucleotide residues, to give the 'CAP' structure. The other is the addition of a series of adenylic residues (the poly A tail) at the 3' end of the molecule. The mature mRNA is then transported across the nuclear membrane into the cytoplasm where the globin chains are synthesised.

The Structure of Globin mRNA

Globin mRNA, like other eukaryotic mRNA's, is composed of 4 structural regions. (1) The 5' untranslated N-terminal sequence; (2) The coding sequence which is translated into globin; (3) The 3' untranslated C-terminal sequence; (4) The poly A sequence at the extrene 3' end of the molecule (Proudfoot and Longley 1976; Chang et al 1977; Baralle 1977; Kan et al 1978; Forget 1977).

The general structures of α- and β-globin mRNA are represented in Figure 1.4.

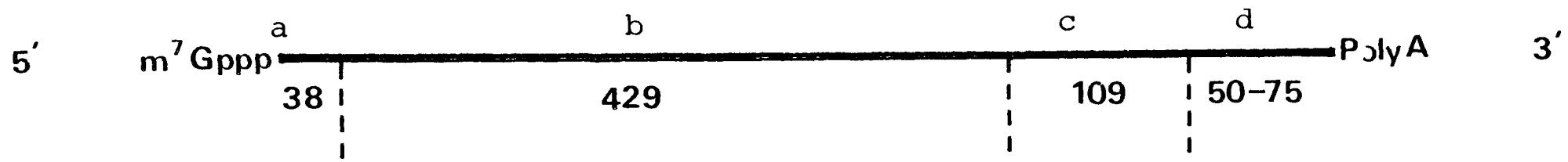
The CAP and Poly A Tail The modified 5'-end structure of human globin mRNA's consists of a 5'-5' Triphosphate linkage between 7-methyl guanosine and the penultimate nucleotide, leaving the 5' end without a free phosphate group. There are two distinct CAP structures, CAP 1 and CAP 2, which differ only in the number of methylated nucleotide residues. CAP 1 is found on both HnRNA and mRNA whereas CAP 2 is found only on cytoplasmic mRNA. CAP 2 is probably a derivative of CAP 1 and therefore all mature globin mRNAs have the CAP 2 structure (Kazazian et al 1978).

The CAP structure appears to play an important role in the formation of ribosome-mRNA complexes and in the initiation of protein synthesis (Kazazian et al 1978). Enzymic removal of the CAP reduces the capacity of mRNA to direct protein synthesis in cell free systems (Lodish 1976).

All the globin mRNAs have a poly A tail, the length of which appears to diminish as the RNA 'ages' (Brawerman 1976). The precise function of the poly A tail is still not known but it is not involved in the translational function of the mRNA (Sippel et al 1974). It is possible, however, that it is involved in maintaining mRNA stability (Brawerman 1976), although histone mRNA has no mRNA tail and appears to be quite stable.

Human α Globin mRNA

625-650 Nucleotides



Human β Globin mRNA

675-700 Nucleotides

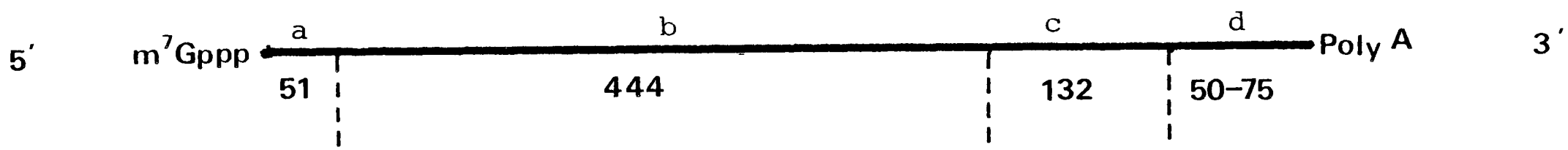


FIG. 1.4 a) 5' non-coding region; b) protein coding region; c) 3' non-coding region; d) Poly A tail

The Coding Sequence The complete nucleotide sequence of several globin mRNAs have been defined either directly (α, β and γ) or by extrapolation from the DNA sequence. All the mRNAs show a bias in the use of codons, some of which are common to all globin mRNAs and others which are selected only by certain globin mRNAs (Forget et al 1978).

The Non-coding Sequences The nucleotide sequences of the 5' untranslated regions of the human α, β and γ mRNAs differ considerably from one another. However, one invariant sequence is the hexanucleotide CUUCUG which occurs 5 nucleotides from the 5' end of the α-mRNA and 9 nucleotides from the 5' end of β- and γ-mRNA. The 5' untranslated region probably plays a role in ribosomal binding and it is suggested that this sequence is involved in ribosomal recognition. An alternative binding site may be nearer the AUG initiation codon (Kan et al 1978).

The 5' non-coding nucleotide sequences of globin mRNAs show the potential of forming hairpin loop structures. The structure of the 5' end of the mRNA molecule may influence the rate at which the mRNAs initiate globin synthesis. Alpha-globin chain synthesis is initiated more slowly than β-globin chain synthesis (Lodish and Jacobsen 1972) and it has been demonstrated that the initiator codon, AUG, of mouse and rabbit α globin mRNA may be less accessible than that of the mouse and rabbit β-globin mRNA (Pavlakis et al 1980).

The 3' untranslated regions of the globin mRNAs also show little homology and the role of this region is obscure besides the possibility that it may help to stabilise the mRNA molecule.

mRNA molecules appear to be associated with proteins to form ribonucleoprotein complexes. The proteins associated with the cytoplasmic mRNA are probably different to those associated with the nuclear RNA precursors of mRNA (Setyono and Greenberg 1981) and the RNA-associated proteins may play some role in the RNA stability and metabolism.

The Synthesis of Globin:- Translation

Once in the cytoplasm the mRNA is translated into globin polypeptide chain by the ribosomes in a series of steps involving also transfer RNAs and several cofactors.

The initiation of globin synthesis requires the formation of a tertiary complex between an initiation factor (eIF-2), GTP and the initiation transfer RNA (always $tRNA^{met}$). This tertiary complex binds to the small (40s) ribosomal unit, and then the globin mRNA and large ribosomal unit are added with the aid of further initiation factors. In this initiation complex the mRNA is bound to the ribosome with its initiator codon AUG aligned with the UAC anti-codon of the $tRNA^{met}$. Once translation commences the initiation factors dissociate from the initiation complex and are free to take part in another round of initiation.

Elongation of the amino-acid chain occurs in a stepwise process from the N-terminal end. With the participation of an elongation factor EF 1, an aminoacyl tRNA, with a complementary anti-codon, binds to the next free mRNA codon which is in the position of the acceptor site on the ribosome. Peptidyl transferase catalyses the formation of a peptide band between the previously added amino acid of the growing peptide chain and the amino acid of the latest aminoacyl tRNA. The tRNA that donated the growing peptide chain is released from the donor site of the ribosome and the newly elongated peptidyl tRNA and mRNA move from the acceptor to the donor site (translocation) involving GTP and a second elongation factor EF2 or translocase. The next mRNA codon in free acceptor site then binds with another complementary aminoacyl tRNA. The N-terminalmethionine incorporated during initiation is removed after the addition of about 20 amino acids. Each mRNA is long enough to support several ribosomes synthesising protein along its length so forming polysomes. Globin synthesis stops when the terminator codon (UAA, UGA or UAG) is reached. The protein chain is split from the tRNA and the mRNA releases the ribosome. Termination requires co-factors including releasing factor and GTP.

The Control of Globin Synthesis

The regulation of the synthesis of a protein could occur at one or more of many stages from the availability of the gene for transcription to the processing of the protein to its final form.

Under normal conditions the amounts of specific globins synthesised are proportional to the levels of the mRNA for that globin that are present. Therefore, it appears that the main regulation of globin synthesis takes place in the nucleus although effectively nothing is known of the precise mechanisms involved.

The translation of globin does not have a major role in determining the levels of globin synthesised. Initiation is the only rate limiting step (Lodish 1976) and although β mRNA initiates more efficienty than α mRNA, this difference is compensated by relatively greater levels of α mRNA resulting in almost equal α- and β-globin synthesis.

Haem appears to have an influence on the synthesis of globin (as in fact it does on the synthesis of all proteins). It can induce globin mRNA accumulation in mouse erythroleukaemia cells and it is apparently active in normal erythroblasts before globin mRNA begins to accumulate (Nienhuis and Benz 1977). In addition it controls initiation of translation by preventing the phosphorylation (and thus inactivation) of the initiation factor elF 2.

The Assembly of Haemoglobin

The α and β globin chains accumulate at more or less equal rates, with possibly a slight excess of α-chains (Weatherall et al 1965). The haem is probably incorporated into the globin chains as soon as they are released from the ribosomes. The α and β dimers then associate, followed

by formation of Hb tetramers.

Erythropoiesis and Haemoglobin Production Throughout Development

To complete the picture of normal haemoglobin production it is considered appropriate to summarize the process of normal red cell production (erythropoiesis).

Erythropoiesis in the Adult

The red blood cells are derived from pluripotent haemopoietic stem cells through a complicated series of cell divisions involving the transitory co-ordinated expression of different sets of genes. The production of red cells can be divided into two major stages. During the first stage, differentiation, the stem cells acquire the potential for the co-ordinated expression of the genes, which define the characteristics of the red blood cell. The second stage, maturation, is characterized by globin mRNA and haemoglobin accumulation such that in terminally differentiated erythroblasts more than 95% of the total protein synthesis is devoted to the accumulation of haemoglobin. In man, the process of erythropoiesis from an undifferentiated stem cell to a mature erythrocyte takes about 10 days and the main steps are illustrated in Figure 1.5.

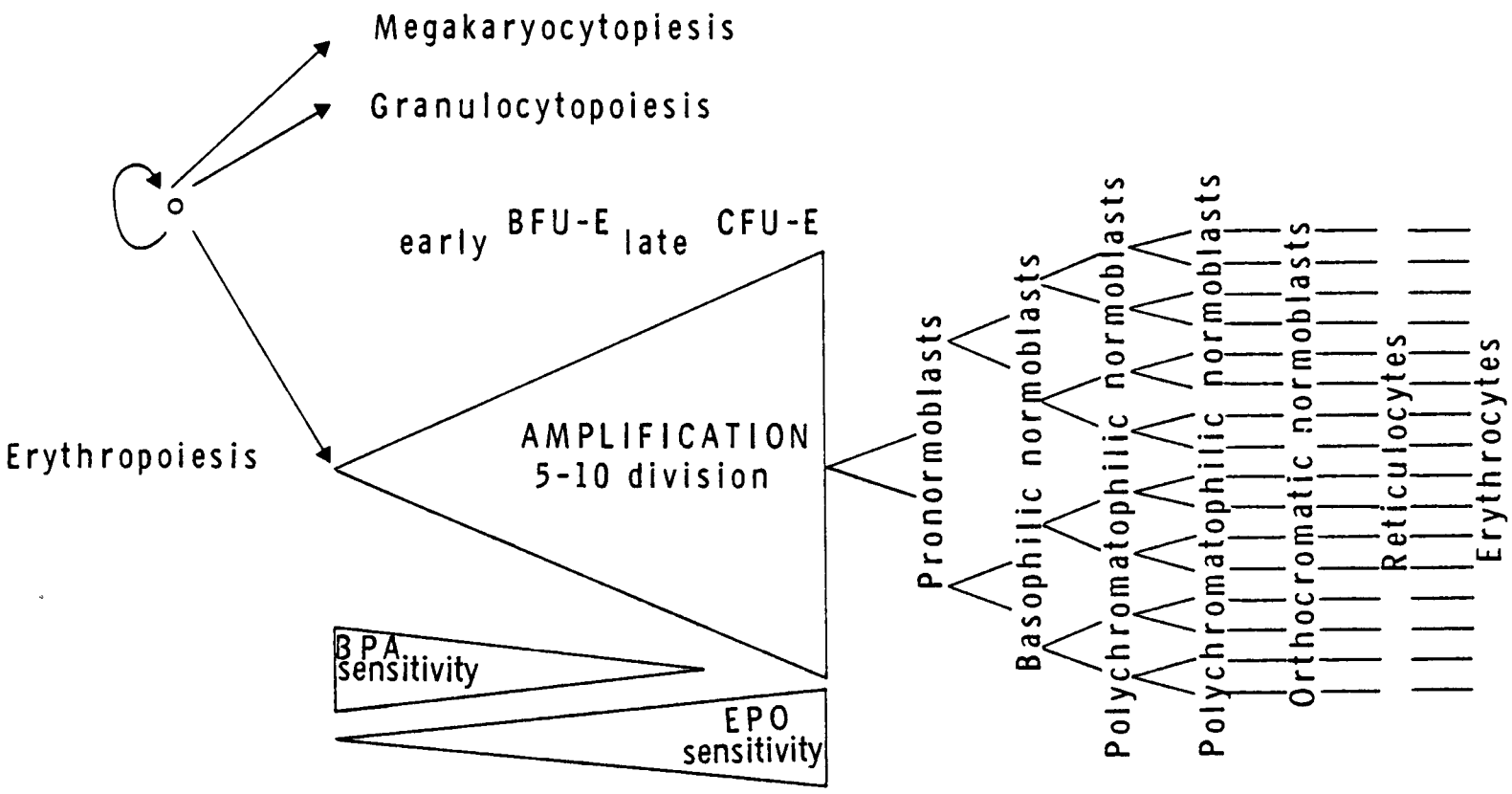


FIG. 1.5 Differentiation and maturation of red blood cell precursors.

Early cell differentiation All blood cells are the products of pluripotent haemopoietic stem cells which are capable of both self renewal (so maintaining their own numbers) and differentiation to the various precursors committed to form myelocytes, megakaryocytes or erythrocytes.

There is much evidence for the existence of pluripotent stem cell types in man, including that derived from certain clonal disorders and by analogy to other mammalian models, in particular the mouse.

Many of the characteristics of one pluripotent stem cell type have been defined. The CFU-S (colony forming unit - spleen, initially demonstrated by its ability to initiate colonies of erythroid, granulocytic, megakaryocytic and undifferentiated cells, either as mixed or pure colonies, in the spleen of an irradiated mouse which had been injected with syngenic mouse bone marrow) represent a slow-cycling cell population with a considerable proliferative potential (see Lajtha 1982) and under normal circumstances the renewal potential of the pluripotent stem cell does not seem to diminish with aging. However, the factors controlling the CFU-S proliferation rate and the mechanism by which cells derived from the CFU-S become programmed to enter the erythroid compartment are completely unknown.

Early Stages of Erythropoiesis

Preceding the first morphologically recognizable erythroid precursors there is a spectrum of committed but not fully differentiated erythroid progenitor cells

which ultimately give rise to the red cell series.

In vitro studies using culture techniques for adult marrow or fetal liver cells have demonstrated at least 2 separate classes of erythroid progenitor cells. They are the Burst Forming Units - Erythroid (BFU-E) and the Colony Forming Units - Erythroid (CFU-E). The BFU-E are more primitive than the CFU-E and in culture the red cell colonies derived from BFU-E are larger and appear later than those from CFU-E.

As the progenitor cells become more differentiated their proliferative capacity diminishes, but their cell size, density, proliferation rate and pool size increases (Cline and Golde 1979; Quesenberry and Levitt 1979).

The precise controls of progenitor cell differentiation are not clear. However, both BFU-E and CFU-E are responsive to the hormone erythropoietin or EPO (the kidney being the major site of production in the adult) and hence they are termed the 'erythropoietin responsive cell compartment' or ERC. EPO acts on the progenitor cells (in a manner as yet undefined) prompting them to enter the maturation phase. The more differentiated progenitor cells are more responsive to EPO and <u>in vivo</u> the CFU-E compartment is directly correlated to EPO activity. There are in addition other as yet undefined factors termed burst promoting activity (BPA) and they seem to have more influence on the BFU-E (Figure 1.5).

Red Cell Maturation

The CFU-E undergo a series of maturation steps and cell divisions to produce about 16 daughter cells from each precursor. These then undergo the final cellular maturation to form the red blood cell.

As the red cell mature there are well-defined morphological changes of the red cells associated with the alteration of gene expression and metabolic processes. The major recognizable cells are summarised in Figure 1.5. The basophilic cells have a large spherical nuclei, and although there is little haemoglobin accumulated as yet, the cells are actively synthesising nucleic acids and proteins. The large (less mature) polychromatophilic cells are similar to the basophilic cells, except for the greater levels of haemoglobin present. In the small polychromatophilic cells the nucleus is starting to condense. In these cells there is no DNA synthesis, little RNA synthesis, but still large amounts of protein synthesis (mainly haemoglobin) In the orthochromatophilic cells the nucleus is tightly condensed prior to extrusion and the cell is still synthesising protein. After the nucleus has been extruded, the reticulocyte remains in the marrow maturing for 24 hours, after which time it is released into the circulation (Denton and Arnstein 1973). Following a further 24 hours the red blood cell loses all mRNA and reticulum and the mature erythrocyte is unable to synthesise any more protein. Although the peripheral blood reticulocyte represents only the very final stages of globin synthesis, it provides a very useful system for investigating globin

synthesis in many situations.

The Inherited Disorders of Haemoglobin Structure and Synthesis

The preceding sections described the structure, function, genetics and synthesis of normal haemoglobins. However, there are many inherited disorders of haemoglobin synthesis, generally referred to as the haemoglobinopathies. Only those affecting globin synthesis (and not haem synthesis, about which little is known) will be discussed.

The genetic disorders of haemoglobin synthesis include the defective production of normal globins (the Thalassaemias), the genetic failure of the normal neonatal switch from foetal to adult haemoglobin synthesis (with features overlapping with those of the thalassaemias) and the synthesis of haemoglobins with abnormal globin chains.

The Thalassaemias

Thalassaemias are essentially characterized by a reduced rate of production of one or more globin chain. This definition includes the syndromes characterized by persistent γ (Hb F) production in adult life as a result of failure of the normal γ to β globin synthesis perinatal switch. In addition certain abnormal haemoglobins will be considered with the thalassaemias since the abnormal globin chain is synthesised at a reduced rate, so resulting in phenotypes similar to those of thalassaemia.

The thalassaemias can be divided into two main

categories - those affecting the synthesis of α globin and those affecting the synthesis of the β-like globins.

The Alpha Thalassaemias

The α-thalassaemias are a group of disorders resulting from deficient α-chain synthesis. The genetics of these syndromes have been difficult to characterise mainly because the carrier states are frequently unassociated with marked haematological abnormalities. However, with the more recent techniques of mRNA analysis and gene mapping greater progress has been made in defining the genetics and underlying molecular defects in many populations. In this brief summary of an extremely wide field the typical manifestations of the disorders and their underlying molecular lesions in the various populations will be outlined.

The Phenotypic Presentation of the α-thalassaemias

These will be described as they were originally characterized in the Oriental (Thai) population and only variation in other races will be outlined (see Weatherall and Clegg 1981).

Haemoglobin Barts Hydrops Fetalis Syndrome is incompatible with life and infants die either in utero or very shortly after birth. The severely anaemic clinical presentation and abnormal blood picture is a result of a complete deficit of α chain production. In such foetuses, the only physiologically functioning haemoglobin present is Hb Portland ($\zeta_2\gamma_2$) which constitutes 10-20% of the total Hb, the remainder being mainly Hb Bart's (γ_4) and some Hb H (β_4). In addition to its relatively wide recognition

in S.E. Asia, sporadic cases have been described in the Chinese, Cypriots (Sophocleous et al 1981), Greeks (Kattamis et al 1980) and Filipinos although the underlying molecular basis may vary (see later).

Haemoglobin H (Hb H) Disease The low production of α-globin chains (α/β globin synthesis 0.2-0.5) produces Hb H disease marked by anaemia, bone changes and splenomegaly. The level of haemoglobin is about half normal. The red cells are microcytic, hypochromic showing abnormal morphology and reticulocytosis. Hb H constitutes about 2-40% of the haemoglobin present and the levels of Hb A_2 are slightly below normal. At birth individuals with Hb H disease have 20-25% Hb Bart's (γ_4) which drop to lower levels in adult life.

Hb H and to a lesser extent Hb Bart's, are oxidatively unstable. In the presence of redox dyes, Hb H precipitates to form inclusions inside the red cell typical of Hb H disease.

The anaemia in Hb H disease results from gross under haemoglobinization of the red cells, shortened red cell survival (a result of globin chain imbalance and Hb H precipitation as cells age) and the high oxygen affinity of Hb H. However, the ability of the excess β chains to form relatively stable tetramers prevents the marked ineffective erythropoiesis which is the main cause of the more severe clinical course in homozygous β thalassaemia, despite the similar degree of globin chain imbalance (see later).

The preceeding description of Hb H disease also applies to sporadic cases reported in the Mediterranean and Saudi Arab populations. In Negroes, however, the rare cases reported seem generally to have a much milder anaemia with lower levels of Hb H.

<u>The α-thalassaemia Carrier States</u> In the Oriental populations there are commonly 2 such states:- the haplotype with no functioning α genes --/ (α thalassaemia 1) and the haplotype with one functioning α gene -α/ (α thalassaemia 2). Carrier states for α-thalassaemia in some other populations have different molecular lesions that result in the complete lack of output of α globin directed from one chromosome (α^{o} thalassaemia) or a reduced output of α globin (α^{+} thalassaemia). However, the phenotypic expression of the carrier states in all populations is similar.

The differentiation between the α^{o}- and α^{+}-thalassaemia carrier states is extremely difficult on the basis of haematological features and globin chain synthesis, since there is considerable overlap between the ranges observed in each group and between the normal and α^{+} thalassaemia ranges.

Usually the α thalassaemia carrier states are associated with little clinical or haematological abnormality. Individuals heterozygous for α^{+} thalassaemia may have minimal anaemia with slightly small under

haemoglobinized red cells and in α^{o} thalassaemia heterozygotes these changes may be more marked. In α^{o} heterozygotes there is generally greater chain imbalance than in α^{+} heterozygotes.

The Hb Bart's levels at birth approximately correspond to the α-thalassaemia phenotypes (1-2% α^{+} thal heterozygotes, 5% α^{o} thal heterozygotes) although the ranges still represent a continuum and it is not always possible to characterize the genotype from the levels of Hb Bart's with certainty.

The Molecular Bases of the α-thalassaemias

Original investigations to define the cause of the reduced α-chain production in α-thalassaemia did not demonstrate any defects at the protein synthesis level. Subsequent studies revealed that individuals with α-thalassaemia had reduced levels of α mRNA. The measurement of α/β mRNA ratios in normals, α^{+} and α^{o} heterozygotes and in Hb H disease (from Thai and Mediterranean populations) demonstrated that the α/β mRNA ratios fell into 4 non-overlapping groups (Hunt et al 1980) the greatest deficit of α-globin mRNA being observed in Hb H disease.

It soon became apparent that the α genes were completely absent in most Oriental Hb Barts hydropic infants (homozygosity for α-thal 1 or α^{o} thalassaemia) and that only one of the normal 4 α genes were present in Hb H disease in the same population.

Within the extension of gene mapping techniques,

various types of molecular defects underlying the α^{+} and α^{o} phenotypes have been characterized to date. They are summarized in Table 1.2 together with the population(s) in which they have been found. So far 4 α^{o}, 2 α^{+} deletion and 2 or 3 α^{+} non-deletion determinants have been identified. The deletion determinants are illustrated in Figure 1.6. The molecular and genetic basis of α-thalassaemia has been defined in the Oriental, Negro, Mediterranean and Saudi Arabian populations. Since Hb E is a haemoglobin variant commonly found in S.E. Asia, only the genetics of Oriental α-thalassaemia will be considered here.

The α^{o} thalassaemia lesion in S.E. Asia (Thailand) involves the total deletion of the α genes but leaves the ζ genes intact (Refer to Figure 1.6). The well recognised Bart's Hydrops Fetalis Syndrome results from homozygosity for this determinant (--/--).

There are 2 α^{+} determinants in this population which both result in the deletion of only one of the two α-genes from the α-ζ gene cluster (-α/). One results from a 3.7 kb deletion and the other in a 4.2 kb deletion. The former appears to be more common.

Compound heterozygotes with an α^{o} and an α^{+} thalassaemia determinant have Hb H disease (--/-α).

An α^{+} thalassaemia non-deletion determinant has also been described in an Oriental (Chinese) (Kan et al 1977) but is comparatively rare (P. Winichagoon, personal communication).

TABLE 1.2 SUMMARY OF THE MOLECULAR LESIONS UNDERLYING THE α-THALASSAEMIA PHENOTYPES

PHENOTYPE	MOLECULAR BASIS	POPULATION IN WHICH OBSERVED
<u>No α globin production</u>		
α^{o}	Complete deletion α genes; variation in 5' extent of deletion	Asian (Thai; Vietnamese, Chinese); Mediterranean (Greek, Cypriot, Sardinian).
α^{o}	'Dysfunctional' α-gene; part of α gene remaining with either:-	
	> 25 kb deleted	Medit.(Italian, Turkish, Cypriot/Sardinian)
	or 5.2 kb deleted	Greek
<u>Some α globin production</u>	One α gene remaining, probably arisen by unequal cross-over.	Mediterranean Middle East Asian
α^{+}	Various points of cross-over and extent deletion	Negro
α^{+}	Non-deletion lesion; both α genes present (Maybe more than one type)	Asian Mediterranean Middle East

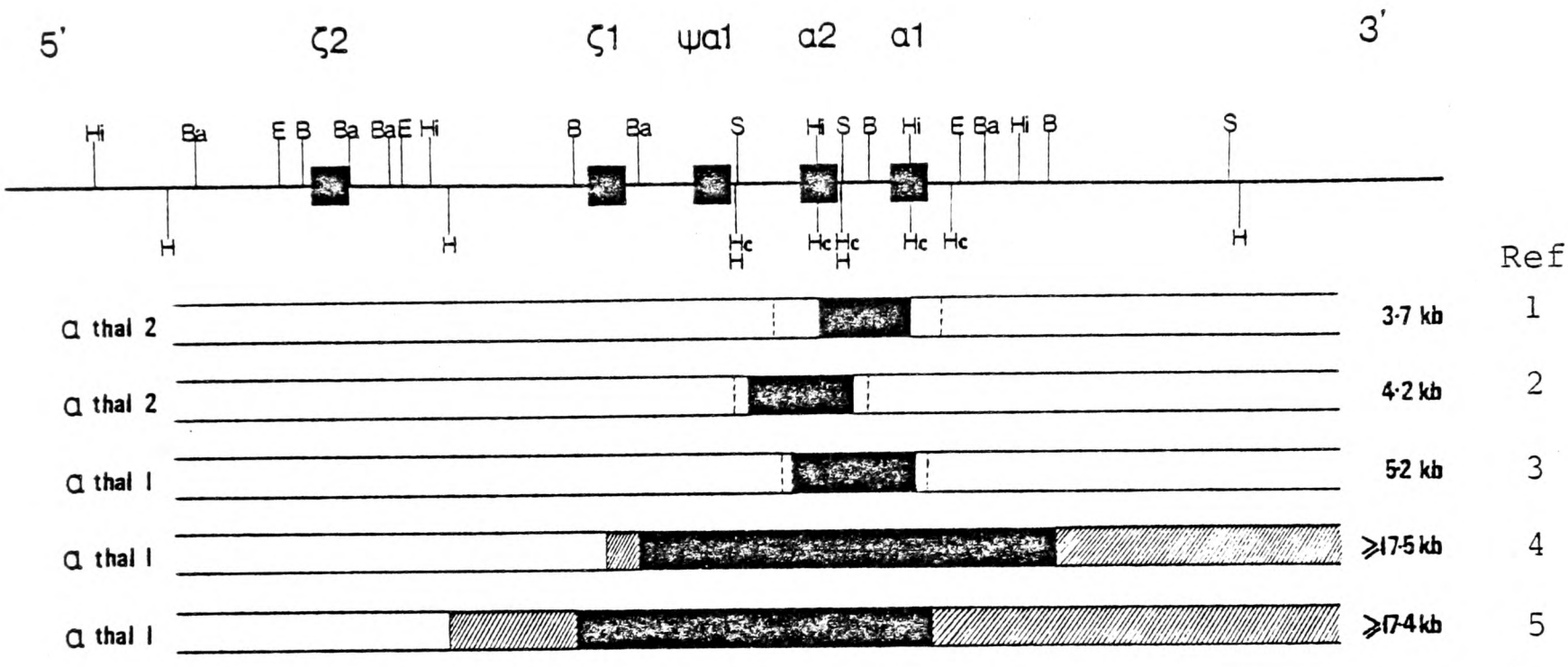


FIG. 1.6 Deletions in the various α thalassaemia haplotypes with the phenotype of α^{+} and α^{o} thalassaemia: 1) Orkin (1978); 2) Embury et al (1979); 3) Pressley et al (1980b); 4 and 5) Pressley et al (1980a); 6) Orkin and Michelson (1980). In the case of the 3.7, 4.2 and 5.2 kb deletions the black areas represent the amount of DNA that has been deleted from within the area defined by the vertical dotted lines. In the other three cases areas known to be deleted are shown in black; where limits of the deletion are not defined their maximum extremes are indicated by hatched areas. (Weatherall and Clegg 1981)

Closely associated with the phenotype of α-thalassaemia are various α chain variants. Some of these result from the reduced synthesis of the variant α chain. Others are found linked to an α^+ determinant which results in low or absent synthesis of normal α-globin directed by the α gene in cis to the abnormal α gene.

Hb Constant Spring and Related Variants

These haemoglobins have elongated α globin chains. A point mutation appears to have arisen in the normal chain termination codon, resulting in the translation of 31 additional amino acids from the normally untranslated 3' region of the α-globin mRNA. In Hb CS the codon at position 142 (the normal chain termination codon) codes for glutamine.

Hb CS is found almost as commonly as the α-thal 2 determinant in the Thai population. It is found at very low levels in the red cells (0.5-1.0% in the heterozygous state; 5% in the homozygous state) and its reduced synthesis appears to result from the accumulation of low levels of the Constant spring mRNA due to its instability (Hunt et al 1982). As a result Hb CS has the phenotypic expression similar to the Oriental α-thal 2 determinant and it interacts with α-thalassaemia 1 to produce a condition similar to Hb H disease.

Although it results in reduced α globin chain output as does the α thal 2 determinant, homozygous CS is associated with a significant degree of haemolysis and relatively normal red cell indices and in this way

it is dissimilar to the Oriental α-thal 1 (or homozygous α-thal 2) phenotype.

The unexpected finding that Hb CS homozygotes and heterozygotes have α/β globin synthesis ratios greater than unity indicates that there may be an effect of mRNA degradation products on protein synthesis.

The other α chain elongation Hb variants vary only in the amino acid at position 142. Hb Icaria (142 lysine), reported in sporadic Greek cases; Hb Koya Dora (142 Serine) reported in the inhabitants of Andhra Pradesh in India; Hb Seal Rock (142 Glu) reported in a single Negro family. All these variants are thought to have arisen by a point mutation and they have the same phenotypic expression as Hb CS.

Hb variants with single amino-acid substitutions have been described associated with the phenotype of α thalassaemia. Their underlying molecular lesions and phenotypic expressions are summarized in Table 1.3.

The Beta Thalassaemias

The β thalassaemias are also an extremely diverse group of disorders resulting from the reduced synthesis of β globin.

There are basically two main groups of β thalassaemia, β^{o} thalassaemia (no output of β globin directed by the affected gene) and β^{+} thalassaemia (reduced output of β globin directed by the affected gene). There are

TABLE 1.3 SUMMARY OF SOME α-CHAIN VARIANTS FOUND ASSOCIATED WITH PHENOTYPE OF α-THALASSAEMIA

HAEMOGLOBIN VARIANT	MOLECULAR LESION	POPULATION
Hb Q α74 Asp → His	Always associated with α thal deletion lesion $-\alpha^{Q}/$. In addition poss. defect in α^{Q} globin synthesis (Higgs et al 1980).	Chinese
Hb G-Phil α68 Asp → Lys	When associated with α-thal phenotype, associated with α-thal deletion lesion $-\alpha^{G}/$. Sometimes not ($\alpha\alpha^{G}/$.)	Anerican Negro
Hb J Tongariki α115 Ala → Asp	Associated with α-thal deletion lesion $-\alpha^{JT}/$	Islands of the Pacific (Vanuatu).
Hb Hasharon α47 Asp → His	Associated with α-thal deletion lesion $-\alpha^{H}/$.	Po Valley, Italy
Hb I α16 Lys → Glu	Undefined	Negro
Hb J Mexico α54 Gln → Glu	Undefined (possibly associated with non-deletion α-thal lesion)	Algerian

various subgroups of β^{o} and β^{+} thalassaemia and these are summarized in Table 1.4.

To discuss this vast group of disorders in detail is impossible in such a small review. Therefore only the phenotypic manifestations and molecular lesions so far defined will be summarized. The phenotypic expression of β^{o} thalassaemia and Type 1 β^{+} thalassaemia are indistinguishable in the homozygous and heterozygous state and the following phenotypic descriptions apply to both.

Homozygous β thalassaemia (or β-thalassaemia 'major') is associated with severe anaemia, poor physical development, hepatosplenomegaly, skeletal changes and an abnormal blood picture. The levels of Hb F are significantly raised and in homozygous β^{+} thalassaemia a small amount of Hb A is present.

Despite the similar degree of chain imbalance to that seen in Hb H disease, the clinical course of homozygous β thalassaemia is far more severe. The excess α chains, unable to form stable tetramers, precipitate in the red blood cells. The α chain precipitates in the red blood cell precursors cause a large degree of ineffective erythropoiesis and consequently the anaemia cannot be fully compensated, despite the bone marrow expansion. In addition, the α chain precipitates in the peripheral blood red cells results in some haemolysis and hypersplenism. Homozygous β thalassaemia is also characterised by iron overload which causes endocrine dysfunction and often cardiac death. Life

TABLE 1.4 SUMMARY OF THE β THALASSAEMIA PHENOTYPES

PHENOTYPE	HOMOZYGOUS PRESENTATION	HETEROZYGOUS PRESENTATION	POPULATION
No β globin production			
β^{O}	Severe presentation (see text). Often transfusion dependent	Mild presentation. Raised Hb A_2	Most populations in which β thalassaemia described.
β^{O} (Ferrara)	As above. In addition evidence for induced endogenous β globin synthesis following transfusion with normal cells.	As above	Limited to Ferrara region, N.E. Italy
Some β globin production			
Type 1 'Mediterranean'	Severe presentation 5-15% Hb A	Mild presentation Raised Hb A_2	Mediterranean, M. East, SE Asian, Indian (occasionally Negro)
Type 2 'Negro'	Less severe presentation 'Thalassaemia intermedia' Not transfusion dependent.	Poss. more mild than other β-thal het states. (30% Hb A in compound het state with β chain variant) Less chain imbalance.	Negro
Type 3 'Silent'	Thalassaemia Intermedia Not transfusion dependent	No anaemia. Almost normal red cell indices. Normal Hb A_2 and F. α/β globin synthesis 1.4-2.1	Mediterranean

expectancy in homozygous β thalassaemia is infrequently beyond the second decade.

Homozygosity for the Negro Type 2 β^+ thalassaemia has a much milder clinical course with moderate anaemia, splenomegaly and bone changes and may be generally referred to as β-thalassaemia intermedia.

Heterozygous β thalassaemia (or β thalassaemia minor) is mainly asymptomatic. The haematological picture shows a small degree of anaemia with small, poorly haemoglobinised cells and there is some evidence of ineffective erythropoiesis in the bone marrow. The levels of Hb F are normal, but Hb A_2 is raised to levels of about 5% partly due to an absolute increase in δ globin output (apparently directed by the δ genes in both cis and trans to the β thalassaemia lesion). The globin chain synthesis is imbalanced in both reticulocytes (α/β synthesis 1.5-2.5) and bone marrow. In the latter absolute α/β synthesis ratios are harder to determine due to technical reasons (refer to Chapter 4) but after very short incubation periods the imbalance in the bone marrow is comparable to that in the reticulocytes. The mild anaemia in β thalassaemia heterozygotes is the result of some ineffective erythropoiesis which in turn appears to reflect the degree of β chain deficiency.

The Molecular Basis of the β Thalassaemias

Initial investigations into the possible defect in β globin synthesis during translation or post-translation provided no evidence of abnormal β globin

synthesis at these levels, suggesting that the defect was either deficient or abnormal β globin mRNA.

To date several mechanisms have been defined underlying the β thalassaemia syndromes and they are summarised in Table 1.5.

Unlike many of the α thalassaemia lesions, the defects underlying most of the common forms of β-thalassaemia so far characterised do not involve deletions of the β-gene.

In β^{o} thalassaemia where there is no β chain production, there is heterogeneity at the mRNA level, some cases having no detectable β mRNA (Old et al 1978; Benz et al 1978), while in other cases β mRNA-like sequences are readily detectable by cDNA hybridization, although they are apparently non-functional (Old et al 1978; Benz et al 1978). Several β^{o} thalassaemia genes have now been cloned and sequenced and the underlying defect identified. A partial deletion removing the 3' end of the β gene has been observed in several cases, all of Indian origin, but is apparently absent or very rare in other racial groups (Orkin et al 1979; Flavell et al 1979). Base substitutions leading to premature translation termination codons at positions 17 and 39 have been identified, the latter apparently being very common in Mediterranean cases (Chang and Kan 1979; Trecartin et al 1981). In other cases of Mediterranean origin, an abnormality has been detected at the 5' end of the large intervening sequence, suggesting that abnormal processing of the initial

TABLE 1.5 MOLECULAR LESIONS UNDERLYING THE β-THALASSAEMIAS

PHENOTYPE	MOLECULAR LESION	REFERENCE
β^+ thalassaemia	Processing defect - single base change near 3' end small IVS results in abnormal splice site	Maquat et al 1980 Kantor et al 1980 Spritz et al 1981 Westaway and Williamson 1981
β° thalassaemia	Abnormal β mRNA - defective 3' end?	Old et al 1978
	Full length mRNA - defective initiation site.	Old et al 1978
	Full length β mRNA - chain terminator mutation at: codon 17 AAG → UAG codon 39 CAG → UAG	Chang and Kan 1979 Trecartin et al 1981
	Full length β mRNA - soluble inhibitor of initiation?	Conconi et al 1972
	No β mRNA but HnRNA present - processing defect?	Comi et al 1977
	No β mRNA or HnRNA - transcriptional defect?	Comi et al 1977
	Single amino acid substitution at 5' end IVS 2 - processing defect?	Baird et al 1981
	Dinucleotide deletion early in β gene sequence - frame shift mutation to produce premature termination codon.	Orkin and Goff 1981
	Partial β gene deletion 600 bp at 3' end gene	Orkin et al 1979 Flavell et al 1979

transcript may be responsible for the absence of β globin production in those cases (Baird et al 1981; Maniatis et al cited by Orkin et al 1982). Functional studies are required to demonstrate that this is the primary lesion and not a secondary change occurring in an already defective gene. Another lesion has been identified in a Turkish β^{o} thalassaemic which results from a dinucleotide deletion early in the β-globin sequence that results in a frameshift and consequent production of a premature translation stop codon (Orkin and Goff 1981). Thus these lesions that have been identified to date demonstrate the extensive heterogeneity of β^{o} thalassaemia at the molecular level.

In the β^{+} thalassaemias, reduced levels of normal β chains are produced and in general the β mRNA levels correlate well with β globin synthesis (Benz et al 1981a). Studies on several β^{+} thalassaemia homozygotes suggested that the β mRNA precursors were being abnormally processed (Nienhuis et al 1977; Maquat et al 1980; Kantor et al 1980). More recently, sequencing of β genes in one individual clinically homozygous for β^{+} thalassaemia (Spritz et al 1981) and in one $\beta^{+}/\delta\beta^{o}$ thalassaemia double heterozygote (Westaway and Williamson 1981) has revealed a single nucleotide substitution 21 nucleotides upstream from the 3' end of the small intervening sequence (IVS) that results in a sequence which is almost the same as the normal splicing signal except for one base (see Figure 5.7). It appears that most of the β mRNA precursor is spliced at this abnormal

splice site, whereas only a small amount is spliced normally and presumably enters the cytoplasm where it directs the low levels of β globin synthesis characteristic of β^+ thalassaemia. To date, this is the only β^+ thalassaemia gene which has been characterised, so it remains to be seen what proportion of β^+ genes will turn out to be processing defects.

Hb Lepore, δβ Thalassaemias and Pancellular Hereditary Persistence of Foetal Haemoglobin (HPFH)

This group of disorders represents a continuum closely related to the β thalassaemias in which defective β and δ chain production is compensated by a variable persistence of γ chain production in adult life. In the Hb Lepore and δβ thalassaemia syndromes there is only slight persistence of γ chain synthesis and the still significant chain imbalance results in a clinical state similar to β thalassaemia although generally less severe. In the pancellular HPFH syndromes the defective synthesis of β and δ chains is almost totally compensated by γ chain production and consequently the clinical presentation is very mild.

Within each group of these disorders there is great heterogeneity at all levels from the phenotypic expression to the underlying molecular lesion.

Hb Lepore Syndromes The Lepore haemoglobins are abnormal haemoglobins which have a δβ fusion globin

chain associated with α-chains. The δβ fusion globin is the product of a δ and β fusion gene, thought to have arisen by a non-homologous cross-over event between β and δ genes of two chromosomes (see Figure 1.7). As a result the δ and β genes in cis to the δβ fusion gene have been deleted and since relatively low levels of δβ globin are produced (the result of an apparently unstable δβ mRNA), the Hb Lepores have a similar phenotypic expression to the β thalassaemias, ameliorated slightly by some increase in γ globin production directed by the γ gene in cis to the δβ fusion gene.

The types of Lepore haemoglobins so far described and their phenotypic expression are summarised in Table 1.6.

The opposite chromosome in the cross-over event that produces a Lepore haemoglobin has a βδ fusion gene (see Figure 1.8). Three βδ fusion genes have been described: Hb Miyada (β12 δ22); Hb P-Congo (β30,δ116); Hb P-Nilotic (β22 δ50). The βδ globin is also produced at reduced levels (βδ mRNA is also apparently unstable) and although there are intact β and δ genes in cis to the βδ fusion gene, heterozygotes have a very mild β-thalassaemia condition and in addition there is a marginal elevation of γ globin output by the cis-γ gene.

It is possible that some fusion genes have not yet been identified if their globin products have identical electrophoretic mobilities to either β or δ globins.

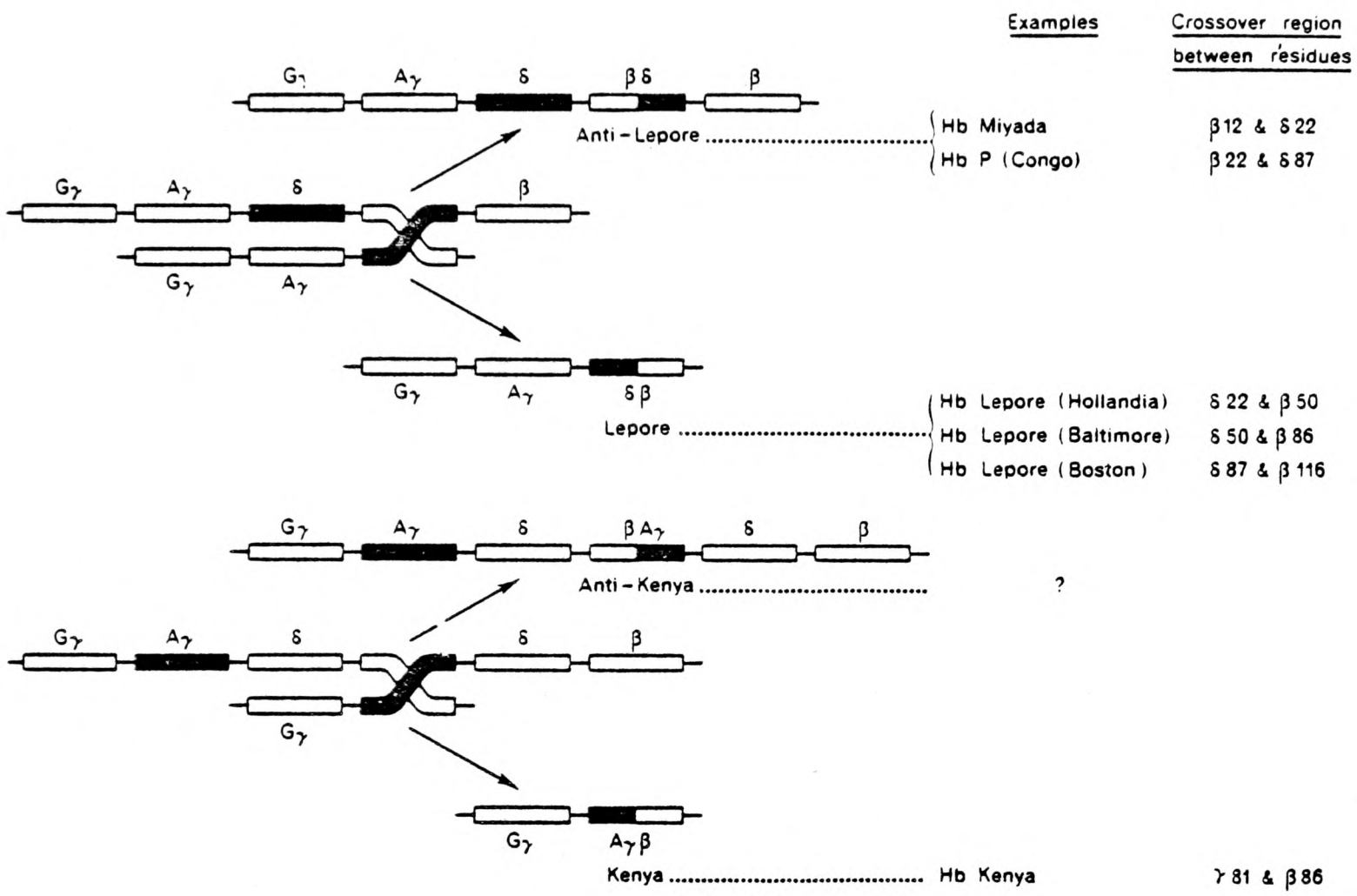


FIG. 1.7 Mechanism of production of the crossover variants Hb Lepore and Hb Kenya (from Weatherall and Clegg 1979)

TABLE 1.6 SUMMARY OF Hb LEPORES, δβ THALASSAEMIA AND HPFH SYNDROMES

		H O M O Z Y G O T E S				H E T E R O Z Y G O T E S			
DISORDER	RACIAL DISTRIBUTION	CLINICAL MANIFESTATIONS	% Hb F	$G_\gamma:A_\gamma$	GLOBIN SYNTHESIS	CLINICAL MANIFESTATIONS	% Hb F	$G_\gamma:A_\gamma$	GLOBIN SYNTHESIS
LEPORE (Boston, Hollandia and Baltimore)	Many populations. Boston most common	Thalassaemia Intermedia	75 (15% Lepore)	Between 3:1 and 2:3	Similar to β thalassaemia homozygotes	Asymptomatic	1-14% (generally greater than β-thal heterozygotes	1:2	1.2 - 2.5
$G_\gamma A_\gamma(\delta\beta)^o$ thal	Mediterranean, M. East, India, SE Asia, Negro	Thalassaemia Intermedia	100	1:1	1.4 - 1.8 (Cell selection)	Asymptomatic	5-20	2:3	Mild imbalance to balanced
$G_\gamma(\delta\beta)^o$ thalassaemia	Negro, India, China	Thalassaemia Intermedia	100	No A_γ	3.9	Asymptomatic	5-15	No A_γ	1.4
Sardinian δβ thalassaemia							10-20	G_γ << A_γ	
$G_\gamma A_\gamma$ HPFH	Negro		100	1:1	1.4 - 3.0 (no cell selection)		20-30	2:3	Essentially balanced no β output in cis
$G_{\gamma\beta}^+$ HPFH							15-20	No A_γ	Balanced
$G_\gamma(\gamma\beta)^+$ HPFH (Hb Kenya Form)							5-9 (7-12 Hb Kenya)	No A_γ	---
Greek $G_\gamma A_\gamma$ HPFH						Completely normal haematology	14.5	A_γ << G_γ	Mild imbalance to balanced. Some β production in cis.

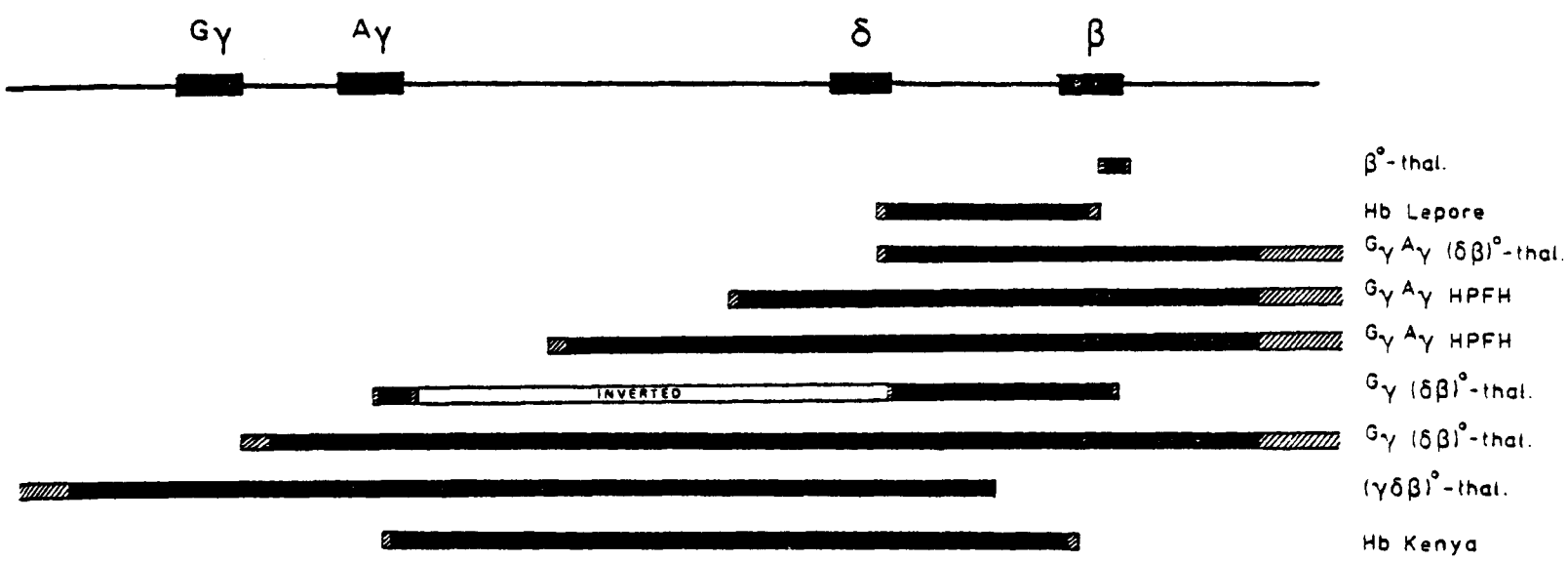


FIG. 1.8 Deletions in the γ-δ-β region of the genome on chromosome 11 which result in β^{o} thalassaemia, Hb Lepore, δβ thalassaemia, HPFH. (From Jones et al 1981).

Such Lepore-like lesions would result in β thalassaemia like conditions with low (β-like globin product) or high (δ-like globin product) Hb A_2 levels.

The δβ Thalassaemias These disorders have total absence of β and δ chain synthesis in homozygotes and are characterized by relatively raised levels of Hb F in heterozygotes. They are classified into two groups according to the type of the γ chain synthesis - $^{G}\gamma\delta\beta$ thalassaemia and $^{G}\gamma^{A}\gamma\delta\beta$ thalassaemia. Their essential features are summarised in Table 1.6.

Pancellular HPFH This group is characterised by the persistent synthesis of substantial levels of Hb F in adult life in the absence of significant clinical features. These conditions are also diverse, differing between the type of γ chains synthesised and whether there is any β globin output directed from the β gene in cis to the HPFH determinant (see Table 1.6). Pancellular HPFH may still be considered under the general term 'thalassaemia' despite the absence of clinical manifestations because there is still a significant degree of chain imbalance associated with these disorders. Pancellular HPFH may be considered a well compensated thalassaemia condition.

The Molecular Basis of Hb Lepore, δβ thalassaemia and Pancellular HPFH

Unlike the molecular defects underlying most of the β-thalassaemias, the molecular basis of many of these syndromes involves major deletions or rearrangements

of the β-like globin gene cluster. Those deletion lesions that have been characterized to date are illustrated in Figure 1.8. In addition to these determinants, $^{G}\gamma\beta^{+}$ thalassaemia and Greek HPFH appear to be non-deletion lesions (Jones et al 1982).

The preceding summary of the β, δβ thalassaemias and HPFH syndromes includes most of the conditions that have been characterised to date at least at the descriptive level. However, there are numerous interactions amongst these disorders and with other haemoglobinopathies (namely α thalassaemia and abnormal haemoglobins) that frequently modify the clinical and haematological features. It is not possible to include descriptive accounts of all these conditions in such a small review of the thalassaemia field.

Heterocellular HPFH

There is a group of conditions in which the level of Hb F is raised above the normal level in adult life, but the distribution of Hb F is limited to only a small proportion of the red blood cells.

Heterocellular HPFH differs from pancellular HPFH (and the thalassaemias) since, in addition to the cellular distribution of Hb F, the heterocellular HPFH conditions have no associated haematological abnormalities or globin chain synthesis imbalance. The observation that the F-cell number in normal individuals has some genetic control (Zago et al 1979) suggests that hetero-cellular HPFH results from an abnormality in the

regulatory factors which control the number of F-cells and the Hb F per cell.

There have been several types of heterocellular HPFH described, and they vary in the level of Hb F (and F cells) present and in the proportion of $^{G}\gamma$ and $^{A}\gamma$ chains present in the Hb F. The features of those types so far characterised at the descriptive level are summarised in Table 1.7.

The Abnormal Haemoglobins

There are more than 300 haemoglobin variants that have been described with abnormal α, β, δ or γ globin chains, approximately 200 of which affect the β chain.

The Molecular Basis of the Globin Chain Variants

Most of the abnormal globin chains have arisen by single-base substitutions in the globin gene, although other molecular defects underlie a few of the globin variants.

Single-base Substitutions The majority of Hb variants arose by single-base substitutions. Since the genetic code is degenerate, only about ¼ of the base mutations result in an amino acid alteration and of these only about 1/3 will alter the electrophoretic mobility of the haemoglobin so enabling the haemoglobin variant to be easily detected.

A few variants have been described with 2 amino acid substitutions at separate points in the globin chain and these appear to have arisen by homologous cross-over events between 2 allelic globin genes each carrying a

TABLE 1.7 THE TYPES OF HETEROCELLULAR HPFH DEFINED TO DATE

TYPE	% Hb F	pgF/CELL	$G_\gamma A_\gamma$
Swiss+	Up to 6%	3	Variable
British	5 - 13 (19 - 21)*		1/9
Georgia	2.6 - 6	3	$G_\gamma >> A_\gamma$
Atlanta	2 - 4		G_γ
Seattle+	3 - 8		

* Only British heterocellular HPFH has been described in the homozygous state and these values represent the level of Hb F observed.

+ These categories could be the same type but they are not yet well enough characterised.

single point mutation.

Single-base substitutions also account for some elongated abnormal globin chains and nonsense mutations resulting in premature termination of translation.

The chain termination variants include Hb Constant Spring and related variants (see before) which are α-chain variants. Three β chain variants have resulted from nonsense mutations. Hb McKees Rocks has 144 amino acids instead of the normal 146 in its β globin chain, the result of a mutation at codon 145 to a termination codon. The other two result in the phenotype of β^{o} thalassaemia since the mutations have occurred much earlier in the β mRNA, at codons 17 and 39 respectively (see before).

Deletions and Insertions of Bases Certain globin variants have been described in which bases are added or lost in groups of 3 such that the amino-acid sequence beyond the mutation is normal. There are 5 β chain variants in which up to 5 amino-acids have been deleted. They include Hb Gun Hill (β92-96 or 93-97 deleted) Hb Leslie (β131 deleted) and Hb Vicksburg (β75 deleted). One α-chain variant has arisen by the insertion of 3 amino acids (Hb Grady). Both such lesions probably result from the non-homologous cross-over of allelic genes.

The deletion or insertion of bases in numbers other than in groups of 3 results in a frameshift mutation. Three such frameshift variants have been described (β

chain variants Hb Cranston and Hb Tak; α chain variant Hb Wayne) and all have an extended C-terminal sequence as the result of an out-of-phase normal termination codon, as well as a completely altered amino acid sequence following the position of mutation in the coding sequence.

Finally, there are the fusion globin chains that have already been mentioned - Hb Lepore ($\alpha_2\delta\beta_2$) and Hb Kenya ($\alpha_2\gamma\beta_2$).

The Synthesis of Abnormal Haemoglobins

The genetics of haemoglobin synthesis were discussed previously. Normal individuals have two α genes per haploid chromosome and one β gene. The globin gene numbers therefore suggest that individuals heterozygous for α chain variants should have about 25% of the abnormal haemoglobin and homozygous individuals about 50% of the abnormal haemoglobin. Individuals heterozygous for β chain variants should have about 50% of the abnormal haemoglobin. However, particularly in the case of the β chain variants, the level of the abnormal haemoglobin is usually below that expected.

Itano (1957) proposed that the amino acid alteration of a protein may not only alter its structure but also decrease the amount of the protein synthesised. Although this is often observed to be the case with the haemoglobin variants, the level of protein synthesis at which the defect occurs is still obscure in many cases. It is possible that a defect may arise in one or more of the many steps of protein synthesis. They include:-

(a) Defective transcription or processing of the mRNA for the abnormal globin.

(b) Instability of the abnormal globin mRNA.

(c) Defective translation including initiation, assembly or termination.

(d) Instability of the globin chain.

(e) Defective assembly of the abnormal and normal globin subunits to form haemoglobin.

(f) Instability of the abnormal Hb tetramer.

The last three mechanisms have been demonstrated in various haemoglobin variants: Hb Bushwick (β74 Glu→Val) is an example of a β chain variant in which the abnormal β globin chain is unstable (Rieder et al 1975); there is evidence that the abnormal β^S chains of Hb S and β^E chains of Hb E do not associate readily with α globin chains as do β^A chains (Shaeffer et al 1978; Tuchinda et al 1967; Pagnier et al 1974); many Hb variants are unstable in their tetrameric form including Hb Shepherds Bush, Hb Leslie and Hb Saki.

Molecular Pathology of Abnormal Haemoglobins

The ma.jority of Hb variants do not have any clinical manifestations and therefore cause little alteration in the overall structure or function of the haemoglobin molecule. However, substitutions which do occur at a critical point of the molecule may drastically alter the stability or function of the haemoglobin tetramer (Perutz and Lehmann 1968).

Generally, alterations of amino acid residues on the surface of the Hb molecule rarely give rise to altered

function or haemolytic anaemia. On the contrary, substitutions of internal residues often result in abnormal properties of the molecule.

Substitutions at or near the haem pockets may cause haemoglobin instability (resulting in haemolytic anaemia) or alter the oxygen carrying properties of the haemoglobin by the formation of methaemoglobin (the M-haemoglobins).

Substitutions at or near the $\alpha_1\beta_2$ contact, which plays an important role in the haem-haem interaction, or in the region of the 2,3-DPG binding site may result in the altered oxygen affinity of the haemoglobin.

An amino acid replacement at or near the $\alpha_1\beta_1$ contact may result in haemoglobin instability by increasing the tendency of the haemoglobin to dissociate into monomers.

Deletions involving more than one amino acid, and even single amino substitutions at a critical point, have been shown to drastically alter the stability of the globin and therefore the haemoglobin.

The clinical expression of most unstable haemoglobins however, differ from that of the thalassaemias. In the case of most unstable haemoglobins, the precipitation of the haemoglobin appears to occur in the peripheral blood cells rather than the red cell precursors and consequently the anaemia tends to be haemolytic rather than diserythropoietic. In addition, even with the most unstable haemoglobins present at very low levels, the MCH is often normal. This raises the possibility that there

are differential effects on protein (globin) synthesis depending upon whether the precipitation of globins takes place early or late in erythroid maturation.

The Polymorphic Abnormal Haemoglobins

There are a few haemoglobin variants which are found at polymorphic frequencies in some populations, the most commonly encountered of which are haemoglobins S, C and E.

Haemoglobin S

Hb S is a β chain variant (β6 Glu→Val) found in very high frequencies in Negro populations.

The homozygous state results in the often severe disorder of sickle cell anaemia. The clinical manifestations include haemolytic anaemia, growth impairment, susceptibility to infection, acute 'crises' (for example vaso-occlusive, haemolytic) and organ damage due to the vaso-occlusive episodes.

In such individuals the levels of haemoglobin are reduced (6-10 g/dl) and the reticulocyte count is raised (15-25%).

The single amino-acid substitution appears to result in profound alteration of the properties of the haemoglobin molecule resulting in the intracellular polymerisation of Hb S molecules particularly under conditions of low oxygen tension. The polymerisation of the Hb S causes the red cells to assume a rigid sickle shape, with increased mechanical fragility, and hence under conditions when many cells become sickled, vaso-occlusive crises and

haemolysis often results.

The presence of certain other haemoglobins in the red blood cell prevents the polymerization to some extent. One such haemoglobin is Hb F.

The heterozygous state for Hb S (Hb S trait) is not associated with any clinical abnormality and there is no sickling under normal conditions. (The presence of Hb A seems to prevent significant polymerization of Hb S). The haematology is completely normal and Hb S constitutes about 40% of the haemoglobin present.

Haemoglobin C

The amino acid substitution in Hb C also occurs at residue no. 6 in the β globin chain. In the case of Hb C, lysine replaces glutamate. Hb C occurs very frequently amongst the West African Negro population.

Homozgyous Hb C is characterised by a variable haemolytic anaemia, associated with some target cells, moderate reticulocytosis and slightly raised levels of Hb F. The red cells contain mainly Hb C and they are abnormally rigid with intracellular crystals of Hb C (not always morphologically detectable) which are removed from the cells by the spleen. Despite the increased viscosity of homozygous Hb C red cells, the passage of the cells through the microcirculation is almost normal because of their smaller size and diameter (Fabry et al 1981).

Hb C trait is symptomless and the only abnormal feature is the presence of some target cells. Heterozygotes

for Hb C have 30-40% Hb C.

Besides the relative frequency of the homozygous and heterozygous states for these haemoglobins, their interaction with β thalassaemia is also commonly observed, in particular Hb S-β thalassaemia. Both S-β-thalassaemia and C-β-thalassaemia have wide clinical presentations which are essentially dependent upon the nature of the interacting β thalassaemia. The interaction with the mild Negro or Type 2 β^+ thalassaemia tends to produce a much milder condition than the interaction with β^o thalassaemia or severe β^+ thalassaemia (Type 1 or Mediterranean). Interaction with the latter usually results in a condition similar to the homozygous state for the haemoglobin concerned.

Haemoglobin E

In 1954, two groups independently reported the first electrophoretic identification of Hb E. In a child of part Indian origin with a clinical picture of mild or 'intermediate' Cooley's anaemia, paper electrophoresis at alkaline pH (9.0-9.2) of her haemoglobin revealed the presence of two components, one with the mobility of Hb F (subsequently shown to be so) and the other with a mobility very similar to that of Hb C. Moving boundary electrophoresis at acidic pH (pH 6.5) however, showed that the abnormal component had a mobility greater than that of Hb A but slightly less than Hb S. Thus this abnormal haemoglobin component was not Hb S, C or A and it was named Hb E (Itano et al 1954; Sturgeon et al 1955). Similarly, in some Thai patients,

described by Minnich et al 1954 to have a condition comparable to Cooley's or 'Mediterranean' anaemia, an abnormal component was identified with an electrophoretic pattern unlike any of the previously identified haemoglobins and which on paper electrophoresis at pH 8.8 had a mobility intermediate between that of Hbs S and C (Chernoff et al 1954). Exchange of blood samples between the groups of Itano and Chernoff suggested that this abnormal component was the same in both cases. Further analysis of the physical behaviour of Hb E demonstrated that its absorption spectrum, solubility and lability to alkali denaturation were similar to those of Hb A (Itano et al 1954).

The Structural and Functional Properties of Hb E

The Structure of Hb E Examination of the tryptic peptides of Hb E compared with those of Hb A showed that the difference in Hb E was within peptide, numbered at that time, as 26. Following the dissociation of the α and β globin subunits of various haemoglobins in the presence of sodium dodecylsulphate, separation of the globin tryptic peptides showed that peptide 26 was from the β chain. Thus it was concluded that the change in Hb E had occurred within the β chain (Ingram 1959). Subsequent sequencing of the abnormal peptide of Hb E generated by chymotrypsin and trypsin digestion demonstrated that the amino acid substitution in Hb E was glutamate to lysine at position 26 in the β chain (Hunt and Ingram 1959; 1961).

On the observation that Hb E does not manifest its charge as fully as does Hb C, despite the same amino acid change (Hb C β6 Glu→Lys), Hunt and Ingram (1961) suggested that the altered amino acid in Hb E may be buried deeper within the molecule, resulting in a different charge environment of the lysine residue. The construction of an atomic model of haemoglobin based upon high resolution X-ray diffraction (Perutz et al 1968) confirmed that in fact β6 is an external residue, whereas β26 is an internal residue. In Hb A β26 glutamate lies in a strongly basic region and probably forms hydrogen bonds with some of the surrounding residues which are involved in the $\alpha_1\beta_1$ contact, hence helping to stabilize the contact. In Hb E the substitution of a positively charged lysyl residue at β26 probably disrupts the system of hydrogen bonds which contribute to the stability of the contact. The destabilization of the contact may result in an increased dissociation of $\alpha\beta^E$ dimers into monomers (Perutz and Lehmann 1968).

Haemoglobin Stability The precipitation of Hb E has been observed *in vitro* in the presence of strong oxidising agents (Frischer and Bowman 1975; Ali et al 1980; Carrell and Lehmann 1981). The oxidative instability of Hb E is a property of this haemoglobin variant which is useful in distinguishing it from other haemoglobin variants which have a similar electrophoretic mobility under some conditions (eg. starch gel electrophoresis at pH 8.6), such as Hb O_{Arabia}, and particularly Hb C, which is also an extremely common structural variant (see Chapter 2). It has been postulated that the

tendency of Hb E to dissociate into monomer globin chains enables sulphydryl (SH) groups normally buried in the $\alpha_1\beta_1$ contact to react with oxidizing agents. If these sulphydryl groups become irreversibly oxidized the globin chains become less soluble and precipitate (Frischer and Bowman 1975).

The Oxygen Affinity of Hb E The replacement of amino acid residues at α and β subunit contact sites may affect the respiratory functions of a haemoglobin (Perutz and Lehmann 1968). In the case of Hb E it has been suggested that the positive lysyl side chain introduced into the vicinity of the $\alpha_1\beta_1$ contact is much closer to the basic residues of this region when the haemoglobin molecule is in the oxy conformation, but much further away when the haemoglobin is in the deoxy conformation. Hence, the amino acid substitution may tend to destabilize the oxy but not the deoxy structure and hence the oxygen affinity of Hb E may be decreased (Morimoto et al 1971).

Studies on the oxygen affinity of Hb E in whole cells and purified in solution have yielded conflicting results. Initial reports showed that homozygous Hb E red cells (which contain more than 90% Hb E) had a reduced oxygen affinity with a P_{50} of around 40 mmHg compared to a range of 30-32 for Hb A (Kolatat 1964; Bellingham and Huehns 1968). Bellingham and Huehns went on to suggest that the absence of erythropoietic response to compensate the mild degree of anaemia often seen in Hb E homozygotes was, in fact, because the slightly

reduced oxygen affinity of Hb E prevented oxygen deprivation of the tissues, despite the often mild anaemic state. However, these first studies were completed before the importance of red cell 2,3-DPG on oxygen binding was appreciated. Subsequent studies measured both the oxygen affinity and 2,3-DPG content of red cells with Hb E. Red cells from individuals with Hb E trait had a normal oxygen affinity (p_{50} 30.0-31.5) (Bunn et al 1972; May and Huehns 1975) and the measurement of red cell 2,3-DPG in one Hb E trait was well within the normal range (Bunn et al 1972). However, the greater proportions of Hb A in these cells may mask any decrease in the oxygen affinity of Hb E. In red blood cells of a Hb E homozygote studied by Gacon et al 1974, the p50 and intracellular 2,3-DPG levels were both raised above the respective normal range (p50 (mmHg): EE 36.0 and AA 29.5 $\pm$ 0.5; 2,3-DPG (μM/g Hb): EE 22.1 and AA 14.7 $\pm$ 1.0). Very similar results were observed in 2 Hb E homozygotes by May and Huehns (1975), but in a third Hb E homozygote the p50 was found to be just above the normal range (p50 (mmHg): EE 32.4 and AA 29.0 - 32.0) and the red cell 2,3-DPG was normal. In yet another Hb E homozygote studied by Fairbanks et al 1980a, the whole blood p50 was 30.5 mmHg compared with a p50 of 27.2 mmHg obtained in a concurrent normal specimen, suggesting a slight decrease in the oxygen affinity of Hb E.

The oxygen affinity, Bohr shift and haem-haem interaction of purified Hb E were found to be exactly the same as those of pure Hb A (Kolatat 1964; Bunn et al

1972; May and Huehns 1975).

There does not appear to be any consistent trend in the measurement of red cell p50 and 2,3-DPG in Hb E homozygotes. May and Huehns (1975) attempted to correlate their observations with the presence or absence of anaemia in the patients studied, claiming that their results demonstrated a reduced oxygen affinity of Hb E only when the individual was anaemic as a direct result of increased intracellular 2,3-DPG levels. This is exactly the same situation as occurs in individuals with Hb A who are anaemic.

These observations suggest that there is no major disturbance of oxygen affinity of either Hb E or EE red cells. However, they leave unexplained the observation of Bellingham and Huehns (1968) that the anaemia in Hb E does not seem to produce a normal reticulocyte response. This raises the question as to whether there is a significant degree of ineffective erythropoiesis in this condition.

Geographical Distribution and Frequency of Hb E

Hb E is one of the most common haemoglobin variants estimated to be carried by more than 20-30 million people in SE Asia and the Indian subcontinent (Flatz 1967; Fairbanks et al 1980b). Although accurate figures are not available, it has been estimated that the frequency of Hb E is similar to that of Hb S and exceeds that of all the other variants put together (Fairbanks et al 1980b). As illustrated in Figure 1.9 the highest incidence of Hb E is focused in a large area which

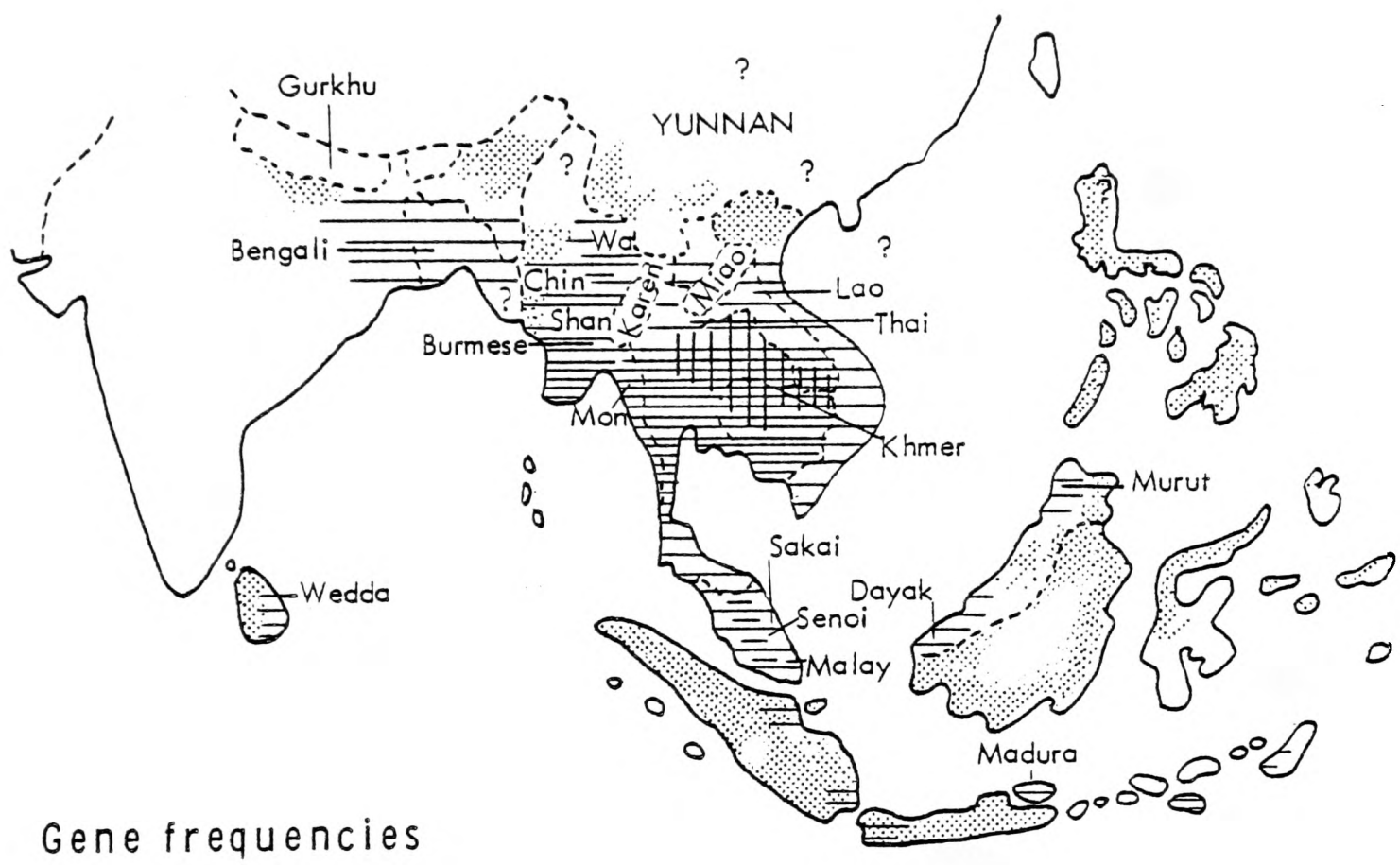


FIG. 1.9 The distribution of Hb E in SE Asia and the Indian subcontinent (from Flatz 1967)

includes NE Thailand, N. Kampuchea and S. Laos (and their mutual borders) where the incidence may be as high as 60% among the Khmer population (Na-Nakorn and Wasi 1978; Sicard et al 1979; Wasi et al 1980).

Moving away from this area, the incidence in Central and S. Thailand (Na-Nakorn et al 1956; Flatz et al 1965) and in Burma is between 10-20%, although in N. Thailand a surprisingly low incidence of less than 10% is observed (Na-Nakorn et al 1956; Flatz et al 1965). In Malaysia (Lehmann and Singh 1956), Vietnam (Blackwell et al 1965) and NE India (Chakravarti et al 1979) the β^E gene is carried by between 2-10% of the population and in 2 tribal groups in Assam, NE India (with origins in SE Asia), the incidence of Hb E is more than 20% (Das et al 1971). In the Veddahs of Ceylon there is also a high incidence of Hb E (17%) (Saha and Banerjee 1973) although the frequency in the native Singalese is much lower. In S. China (Blackwell et al 1965; Shio 1979), Indonesia (Lie-Injo 1955), Philipines (Flatz 1967) and Nepal (Gupta et al 1979) the incidence is below 2% and a single case of Hb E-thalassaemia has been reported from the Punjab (Dash et al 1980). In fact occasional reports of Hb E are not uncommon in other areas of India (Flatz 1967), but Hb E is rarely observed in populations outside those of SE Asia and the Indian subcontinent. Sporadic cases of Hb E have been reported in Turkey, Greece (see Weatherall and Clegg 1981), Middle East (see Flatz 1967), in a German family (Keller and Kohne 1976) and in occasional black Americans (Schroeder et al 1976/7; Altay et al 1976/7; El-Shirbiny

et al 1980). Whether these cases outside SE Asia and the Indian subcontinent are the result of racial admixture or independent mutations has not been determined.

The wide distribution and high frequency of the β^E gene in SE Asia and parts of India imply that there is intense selection for the β^E, since such polymorphic frequencies cannct be achieved by spontaneous mutation. If there is a selective environmental factor which carries a high risk of mortality or reduced fertility in normal individuals, but from which heterozygotes are relatively protected, then such carriers will increase in numbers in successive generations for as long as the particular environmental conditions which favour them are present. A balanced polymorphism is when a situation of equilibrium is reached, whereby the increase in the number of heterozygotes is balanced by the loss of increasing resultant homozygotes and in this way the mutant haemoglobin does not ever totally replace Hb A.

Flatz (1967) attempted to define the population dynamics of Hb E in SE Asia. In SE Asia there are 3 common β alleles: β^A, β^E and $\beta^{Thalassaemia}$ and they are presumed to interact with one another.

The genotype fitness of the 6 genotypes of this tri-allelic system were assessed using the scanty clinical data available. Clinical observations demonstrated the certain disadvantage of the β thalassaemia homozygote and the Hb E-β-thalassaemia double heterozygote. In populations with high Hb E frequency Flatz observed a

deficit of Hb E homozygotes from the expected number in healthy adults, but nearer the expected number in hospital patients. Despite the benign clinical manifestations of homozygous Hb E reported (see below) this suggested an increased morbidity (and possibly mortality) of Hb E homozygotes. In addition he found evidence for a decreased reproductive performance in Hb E homozygotes. He concluded that there was a slight selective disadvantage of Hb E homozygotes, but a slight selective advantage of Hb E and β thalassaemia heterozygotes in the light of the high gene frequencies of these alleles. Flatz suggested that the tri-allelic system is unstable and that the β^E gene is replacing the β thalassaemia gene in most SE Asia populations.

Although the limited clinical data suggests no obvious fitness advantage of individuals with Hb E trait, it has been suggested that the distribution of Hb E appears to be limited to areas with a past or present high incidence of P. falciparum malaria (Flatz 1967). With respect to the β^S gene, protection against malaria-induced mortality, mediated through the Hb S heterozygote, is well established (Allison 1961). Several studies have been carried out in an attempt to demonstrate a similar mechanism for Hb E.

Artificial infection of individuals with Hb E with malarial plasmodia, usually Plasmodium vivax (a milder strain than P. falciparum) yielded conflicting results. One study found the individuals with Hb E-thalassaemia were resistant, but those homozygous or heterozygous for Hb E as susceptible to infection as normals. The

other study found the Hb E-thalassaemia patients were most severely affected by malaria and in Hb E heterozygotes parasite counts were similar to normals (see Flatz 1967). However, besides the small numbers in each study, no consideration was made of the possibility that smaller children may have different susceptibility compared to adults.

Results from studies which attempted to compare the incidence of malaria infection in normal individuals and those with Hb E trait were rather negative. In a random survey of 211 village children in Thailand, there were no significant differences in any age group (1-3 years, 4-6 years, 7-9 years), in the incidence of malaria (irrespective of the type of Plasmodium) between AA and AE individuals (Kruatrachue et al 1961). Analysis of hospital records, for a 12 month period, of patients of all ages (adults and children) with malaria infection showed no significant difference of parasite densities, in Hb E homozygotes, heterozygotes and normals. Moreover, the incidence of Hb E trait in the area in which the study was carried out and the incidence of Hb E trait in the malarial patients admitted to a hospital in the same area were identical (18.9%) further suggesting no selective advantage against malaria infection carried out by the Hb E heterozygote (Kruatrachue et al 1961).

A further study (Kruatrachue et al 1969) that included

600 infants under 3 years of age showed no difference in the incidence of P. falciparum infection between Hb E heterozygotes and other individuals in age groups of under 1 year or 1-3 years. In 82 children under 3 years of age admitted to hospital with malaria infection, there was no evidence that the parasite counts differed significantly between Hb E heterozygotes and normals.

In a group of 204 children suffering from P. falciparum malaria, mortality and morbidity rates (including cerebral, gastrointestinal and renal complications) were compared. No significant differences in morbidity rates were observed between Hb E heterozygotes and normals, and although children with Hb E trait showed a lower mortality than normal children, the difference was not statistically significant (Kruatrachue et al 1970).

An alternative way to examine a possible protection of Hb E heterozygotes against malaria involves the investigation of the ability of red blood cells to support growth of P. falciparum in short term cultures in vitro. In red blood cells from 10 AA controls, eight Hb E heterozygotes and 15 Hb E homozygotes, Santiyanont and Wilairat (1980) were unable to demonstrate any significant difference in the multiplication of P. falciparum after 96 hours between the three groups under conditions of 17% oxygen. The red cells were also cultured under a higher oxygen tension of 30% to investigate a differential effect of oxidant damage on malaria parasite growth between the three groups, since it has been suggested that oxidant damage mediates red cell resistance to

malaria (Friedman 1979). However, cells with Hb E were no more resistant to parasite growth than normal cells. A similar lack of difference in parasite growth between normal and homozygous Hb E red cells was reported by Pasvol and Wilson (1982). On the other hand, Nagel et al (1981), using similar culture conditions and 17% oxygen observed a marked impairment of parasite growth in homozygous Hb E red cells compared to those of Hb E heterozygotes and normals, as judged by the percentage of parasitized cells in the culture.

Taken together, the epidemiological data and the *in vitro* culture data provide little or no direct support for the malaria hypothesis operating to maintain the β^E gene levels. However, given the difficulties, inherent to both these approaches (epidemiological studies are notoriously difficult to operate and control properly, while the sensitivity of the *in vitro* techniques has not been established) the lack of supportive data cannot be taken as evidence against the hypothesis. Certainly no better candidate has been proposed as the selective agent.

Clinical and Haematological Studies of Hb E

Homozygous Hb E Hb E homozygotes have been found to be essentially asymptomatic (Chernoff et al 1956; Lehmann et al 1956; Fairbanks et al 1980a). Haematological studies have shown normal or slightly reduced levels of haemoglobin, with microcytic, hypochromic red blood cells and normal reticulocyte counts. Red cell morphology shows

a significant increase in the number of target cells. Red cell osmotic fragility was shown to be greatly reduced (Chernoff et al 1956; Lehmann et al 1956).

Haemoglobin analysis demonstrated the presence of mainly Hb E with a slight, but significant, increase in Hb F levels, comprising up to 5 or 6% of the total haemoglobin.

Bone marrow examination has only been reported in two cases, one of which had a normal cellular pattern and the other showed minimal erythroid hyperplasia (Chernoff et al 1956). No significant haemolytic component has been demonstrated in Hb E homozygotes (Chernoff et al 1956; Lehmann et al 1956) and red cell survival was found to be normal in one case (Fairbanks et al 1980 a).

Heterozygous Hb E Hb E heterozygotes were reported to be totally asymptomatic and initial studies concluded that other than a possibly slightly decreased red cell osmotic fragility, all other haematological features were normal (Chernoff et al 1956). More recently, however, it has become clear that Hb E trait is associated with moderately reduced MCV and MCH (Pootrakul et al 1978; Fairbanks et al 1979).

In Hb E heterozygotes, Hb E constitutes about 30% of the haemoglobin present, although this includes a small percentage of Hb A_2 which is not separated from Hb E on electrophoresis. The levels of Hb F are within the normal range (Pootrakul et al 1978; Fairbanks et al 1979).

Interaction of Hb E and α-thalassaemia

The α-thalassaemias are also prevalent in SE Asia and their interaction with Hb E is frequently observed.

Considering only the deletion types of α-thalassaemia as they occur in the SE Asian population, Hb E and α-thalassaemia may occur in eight different combinations, many of which have been identified and are summarised in Table 1.8. In Hb E homozygotes, the interaction of α-thalassaemia 1 or 2 does not reportedly alter the haematological presentation of the condition (Wasi et al 1969) although no details have been presented. However, the apparent interaction of homozygous Hb E with Hb H disease (--/-α) produces a condition which is distinguishable from both Hb E and Hb H disease. In a patient described by Wasi et al (1969), the haemoglobin level was 5 g/dl, lower than in either Hb E or Hb H disease. Hb E + F + Bart's were present in proportions of 84, 13.3 and 2.7 per cent respectively. Hb H-homozygous Hb E appears to be more severe than Hb H disease alone. No haemoglobin corresponding to a β^E tetramer was observed.

In Hb E heterozygotes the only reported effect of interaction with an α-thalassaemia carrier state is to reduce the levels of Hb E present. In a study by Tuchinda et al 1964 it was suggested that modal values for Hb E were observed at about 21% and 27% representing interaction of Hb E trait with α-thalassaemia 1 and 2 respectively. However, Wasi et al (1969) observed no sharp divide in the levels of Hb E produced by interaction

TABLE 1.8 THE INTERACTION OF Hb E AND α-THALASSAEMIA

GENOTYPE		HAEMOGLOBIN ANALYSIS	REFERENCE
αα/αα	AE	25-30% Hb E; remainder Hb A	
αα/α-	AE	17-25% Hb E; remainder Hb A	Wasi et al 1969; Tuchinda et al 1964
αα/--	AE	17-25% Hb E; remainder Hb A	Wasi et al 1969; Tuchinda et al 1964
α-/α-	AE	17-25% Hb E; remainder Hb A	Wasi et al 1969; Tuchinda et al 1964
α-/--	AE	14% Hb E; 8% Hb Barts remainder Hb A	Tuchinda et al 1964; Wasi et al 1967, 1969
αα/αα	EE	> 90% Hb E; slightly raised Hb F	Wasi et al 1969
αα/α-	EE	> 90% Hb E; slightly raised Hb F	Wasi et al 1969
α-/α-	EE	> 90% Hb E; slightly raised Hb F	Wasi et al 1969
αα/--	EE	> 90% Hb E; slightly raised Hb F	Wasi et al 1969
α-/--	EE	84% Hb E; 13% Hb F; 27% Hb Barts	Wasi et al 1969

with either of the α-thalassaemia carrier states. Instead, the Hb E levels ranged from 17-25% compared with 25-30% in Hb E heterozygotes without α thalassaemia. Infants whose cord blood contained 5-6% and 1-2% Hb Bart's in addition to Hb F + A + E were believed to represent α thalassaemia 1 Hb E and α thalassaemia 2 Hb E respectively (Wasi et al 1969).

Hb H and Hb E trait interact to produce a condition with the clinical manifestations of Hb H disease. However, the intraerythrocytic inclusions so characteristic of Hb H disease were only induced in a few red cells and Hb H was observed only as a faint band. In addition, the mean level of Hb Bart's in 32 such patients was about 7%. Hb E was reduced to a mean of about 14% and Hb F was slightly raised to about 3% (Wasi et al 1967; 1969). Newborns with this condition are found to have 25% Hb Barts in addition to Hb A + F + E (Wasi et al 1969).

In all the interaction of α thalassaemia with Hb E trait, the levels of Hb E present are found to be reduced from the levels of about 30% Hb E observed in the absence of α-thalassaemia to 14% (--/-α) or 17-25% (-α/-α), (--/αα), (-α/αα) Hb E respectively. In an experiment in which equal amounts of β^A and β^E chains were allowed to reassociate with limiting quantities of α-chains, it was demonstrated that β^A chains associate with α chains at a faster rate than β^E chains (Tuchinda et al 1967). This mechanism underlying reduced levels of Hb E trait in the presence of an α thalassaemia would be in accordance with the

postulated weakened $\alpha_1\beta_1$ contact (Perutz and Lehmann 1968).

Although both Hb E and α thalassaemia are extremely common in SE Asia, detailed characterization of the effects of their interaction is still required. With the advent of gene mapping techniques it should now be possible to accurately identify α thalassaemia carriers among Hb E heterozygotes and homozygotes and thus provide a more precise picture of their interaction.

Interaction of Hb E and β Thalassaemia - Hb E-β Thalassaemia

Both Hb E and β thalassaemia are commonly encountered in SE Asia. They interact to produce a disease with a variable clinical presentation but which may often be as severe as homozygous β thalassaemia Since β^o thalassaemia is the predominant form of β thalassaemia in SE Asia, the most common form of symptomatic thalassaemia in this part of the world is Hb E-β^o thalassaemia. It has been estimated that in Thailand, with a population of ∿ 30 million, there are 48,750 Hb E/β thalassaemia patients, almost three times as many as β thalassaemia homozygotes (Wasi et al 1969). Out of 1,352 cases involving β thalassaemia seen in the Department of Medicine, in the Siriraj Hospital in Bangkok, 1,249 had Hb E/β^o thalassaemia. (Wasi et al 1981).

Clinical and Haematological Features of Hb E β^o thalassaemia

The first clinical and haematological descriptions of Hb E thalassaemia appeared in a paper by Minnich et al (1954), and some of these cases were re-examined by

Chernoff et al (1956), in great clinical detail. Hb E β^{o} thalassaemia has a wide clinical spectrum but in most of the 32 patients described by Chernoff et al (1956) and the 408 patients described by Wasi et al (1969) the condition was marked by severe anaemia, retarded physical development, gross hepatosplenomegaly and bone changes as observed in homozygous β thalassaemia. In addition, other clinical aspects such as susceptibility to infection, and iron loading were very similar to those seen in homozygous β thalassaemia.

The haematological findings in Hb E β^{o} thalassaemia are indistinguishable from those found in homozygous β thalassaemia. The levels of haemoglobin observed by Chernoff et al (1956) ranged from about 3.0-7.0 g/dl and in the 408 patients reported by Wasi et al (1969) the mean level of haemoglobin was 6.37 g/dl. The red cells were severely microcytic and hypochromic and reticulocyte counts were between 4 and 6% (Chernoff et al 1956). Haemoglobin analysis demonstrated only Hbs E and F. The levels of Hb F were seen to vary between a wide range of about 5-85% (Chernoff et al 1956; Wasi et al 1969), but were mostly in the 40-60% range.

The anaemia in Hb E thalassaemia is partly haemolytic and partly diserythropoeitic. There appears to be a shortened red cell survival (Chernoff et al 1956; Swarup et al 1961) and increased autohaemolysis in vitro (Swarup et al 1960a). There is evidence for splenic sequestration (Ruymann et al 1978) and bone marrow examination shows marked erythroid hyperplasia (Chernoff et al 1956) with significant ineffective erythropoiesis

and diserythropoiesis (Swarup et al 1961a; Ruymann et al 1978; Wickramasinghe et al 1981). Electron microscopy studies suggest that the characteristics of intracellular inclusions and the ultrastructural abnormalities encountered in Hb E thalassaemia bone marrow cells are very similar to those seen in homozygous β thalassaemia implying the mechanisms underlying the anaemia in the two conditions are similar (Wickramasinghe et al 1981).

The red blood cells in Hb E thalassaemia also have abnormal metabolic properties. The glutathione instability test demonstrates red cell sensitivity to oxidant stress, usually associated with G6PD deficiency. In Hb E-β thalassaemia the red cells are sensitive to oxidant stress (Swarup et al 1960b), but not as a result of G6PD deficiency (Swarup et al 1961b). Instead there are reduced intracellular levels of reduced glutathione (GSH) and NADP (Swarup et al 1966) suggestive of a disturbance in the oxidising-reduction mechanism of the red cells.

Clinical and Haematological Features of Hb E β^+ thalassaemia

Hb E β^+ thalassaemia is much less commonly encountered. It appears to be a slightly milder condition than Hb E β^o thalassaemia. The degree of anaemia, splenomegaly and bone changes were less marked in 10 cases reported from Thailand (Wasi et al 1969). In these individuals, the levels of haemoglobin were generally slightly higher than in cases with Hb E β^o thalassaemia, having an average of 8.2 g/dl. Haemoglobin analysis demonstrated Hb's A + E + F, with mean levels of 29%, 55% and 16% respectively.

In 14 cases of Hb E β^+ thalassaemia reported from India (Mehta et al 1980), the clinical presentation was not as mild as in those reported from Thailand. The mean level of haemoglobin was 6.2 g/dl and 6 cases were on regular transfusion.

However, the only criteria for diagnosing the interaction of a β^+ thalassaemia, in these cases was the presence of Hb A and it is noted that half of the 14 cases had Hb E levels below 50%. This raises the question of whether some of these cases represented transfused Hb E-β^o thalassaemia patients.

Aims of the Thesis

Quite clearly, Hb E-β thalassaemia does represent an interaction of considerable clinical significance. In the first case of Hb E β thalassaemia described by Minnich et al (1954) it was noted that despite its similar clinical presentation to Cooley's anaemia, its mode of inheritance was unlike that of classical Cooley's anaemia (homozygous β thalassaemia): although one parent had haematological findings compatible with heterozygous β thalassaemia, similar abnormalities were often absent in the other parent (Chernoff et al 1956). It is relevant to quote the question raised by Sturgeon et al as long ago as 1955: "Why does this rather typical Cooley's anaemia exist in an individual who is only a carrier of the trait?". In this same paper, Sturgeon et al went on to note that there appeared to be "... relative retardation or inhibition of haemoglobin production" of Hb E.

This observation has been made on several occasions since this time, but to date no one has offered a satisfactory explanation for the apparently reduced synthesis of Hb E, or why, despite its asymptomatic homozygous and heterozygous state, its interaction with β thalassaemia produces a state of clinical significance.

The interaction of Hb E with β thalassaemia is unique amongst the polymorphic β chain variants: Hb D thalassaemia is comparable to heterozygous β thalassaemia; Hb C thalassaemia and Hb S thalassaemia both represent heterogeneous groups but their most severe clinical presentations are more comparable to the homozygous state for the respective haemoglobin variant rather than to homozygous β thalassaemia.

This thesis set out to investigate the synthesis of Hb E, with the possibility of defining the mechanism underlying its interaction with β thalassaemia, so answering the question raised by Sturgeon et al 27 years ago.

CHAPTER 2

MATERIALS AND METHODS

Blood Samples and Chemicals

The blood and bone marrow samples were freshly obtained either in England, or in Bangkok, and all blood analyses and experiments were begun within 2 hours of collection. Three blood and bone marrow samples from homozygous Hb E cases, used in RNA studies were shipped from Bangkok to Oxford on dry ice. In these 3 cases the haematological analyses and washing of samples were carried out in Bangkok using exactly the same procedures as those used at Oxford.

Samples from individuals homozygous or heterozygous for Hb E were identified either incidentally during haematological examination for conditions unrelated to the presence of Hb E, or as relatives of patients with Hb E-β thalassaemia.

The individuals who were used as controls were either laboratory staff known not to have any haematological disorders or hospital patients being treated for conditions thought not to affect normal erythropoiesis and haemoglobin synthesis.

Most of the chemicals used in the experiments described in this thesis were from Sigma London

Chemical Company Limited or British Drug Houses Chemicals Limited (BDH) 'AnalarR' grade. Any other sources are stated appropriately.

Haematological Parameters and Haemoglobin Analysis

In all cases approximately 20-30 ml of blood were taken by venepuncture, most of which was anti-coagulated with heparin and retained for the experimental studies. The remaining 3-5 ml were anticoagulated with EDTA and used for haematological analyses. Bone marrow samples were aspirated from the iliac crest and collected in heparin.

The haemoglobin (Hb), red cell count (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were determined using a Coulter Counter Model S.

Cell morphology was examined on Romanovsky-stained blood and bone marrow films (Dacie and Lewis 1975). For reticulocyte counts, blood was incubated with an equal volume of brilliant cresyl blue in citrate saline at room temperature for 15-20 minutes.

Haemoglobin analysis was carried out on haemo-lysates prepared from packed red cells. After washing three times with 0.9% saline, the cells were lysed by the addition of two volumes of distilled water. The lysate was shaken vigorously with half a volume of carbon tetrachloride and cleared of stroma by

centrifugation at 3000x g for 20 minutes. The clear supernatant haemoglobin solution was aspirated and one drop of 1% potassium cyanide solution added for every 2 ml of lysate.

To identify the haemoglobins present, the lysates were subjected to starch gel electrophoresis using a Tris-EDTA-borate buffer system pH 8.6 (a modification of the method of Boyer et al 1963) or by cellulose acetate electrophoresis using a Tris-EDTA-borate buffer at pH 7.9.

The levels of Hb E and Hb A_2 were quantitated by elution after cellulose acetate electrophoresis in a Tris-EDTA-borate buffer at pH 8.9 (Marengo-Rowe 1965).

The levels of Hb F were determined by a modification (Pembrey et al 1972) of the two minute alkali denaturation technique of Betke et al (1959).

All the above methods for the characterization and quantitation of haemoglobin present are described fully by Weatherall and Clegg (1981).

Further Identification of Hb E

With the starch gel and cellulose acetate electrophoresis methods just described, other haemoglobins such as Hb C and Hb O_{Arabia} have the same electrophoretic mobility as Hb E. Since Hb C is also a very common variant (although normally found

in people of African decent) it is necessary to differentiate between Hb E and Hb C.

There are several relatively simple methods by which Hb E may be distinguished from Hb C and all use the property that Hb E is unstable and rapidly precipitates in the presence of strong oxidizing agents.

The dichlorophenolindophenol (DCIP) precipitation test for Hb E (Frischer and Bowman 1975) was the method used for the samples in this study. 0.2 ml haemolysate were mixed with 5 ml of working reagent (72 mM Trizma base (Sigma); 14.4 mM Na_2EDTA; 0.19 M DCIP (Sigma). After standing at room temperature for 10 minutes the mixture was incubated for one hour at $37^{o}C$ without further mixing. The absorbance at 800 nm was read against a buffer or water blank and compared to that of a sample known not to contain Hb E. Samples with Hb E (homozygous or heterozygous) have at least twice the absorbancy of that observed in normal controls, or those with Hb C.

The other methods described for the identification of Hb E use isopropanol (Ali et al 1980) or zinc acetate (Carrell and Lehmann 1981) as the oxidizing agent.

In 2 Hb E homozygotes the present of β^{E} was

also demonstrated by digestion of the purified abnormal β chain with trypsin and fingerprinting as described by Clegg et al (1966).

Biosynthesis Studies

The synthesis of β^E globin relative to α globin (and β^A globin when present) was investigated in the peripheral blood reticulocytes and the bone marrow red cell precursors by incubating the cells in vitro and assessing the radioactivity incorporated into each globin chain. Individuals with no known haematological defect were used as controls to demonstrate the normal synthesis of β relative to α chains in reticulocytes.

About 10-15 ml heparinized whole blood were required from each individual as starting material and 0.2 ml packed bone marrow cells.

The Preparation of Reticulocyte Enriched Blood Fractions for Globin Biosynthesis

Most of the blood samples from the individuals of this study had low reticulocyte counts and for satisfactory biosynthesis results incubations had to be carried out on reticulocyte-enriched samples.

The method commonly used to achieve this is to spin washed red cells suspended in a small volume of reticulocyte saline for about 30 min at 3,000 x g. However, this method does not always yield sufficient enrichment if the sample is to be incubated for very

short times.

Consequently, before beginning the biosynthesis studies described in this thesis, other methods for obtaining highly enriched reticulocyte samples were investigated.

The first method tried was modified from that described by Vettore et al 1980. 0.5 ml of packed red cells (previously freed from white blood cells - see below) were mixed with 10 ml of 35% Percoll (Pharmacia) and 9-12% Urografin (Renografin ER Squibb and Sons) and centrifuged in 10x100 mm polypropylene centrifuge tubes at 35,000 x g for 10 minutes (4°C) in a Beckman L5-65 with a fixed angle 65 rotor. The lighter 2-4 fractions contained the majority of reticulocytes.

The results obtained by various modifications of the method are summarized in Table 2.1 and demonstrate that the enrichment of reticulocytes (in terms of percent of total red cells) was very high in all cases. However, less than 25% of the total reticulocytes were recovered in the top fraction and as a consequence the volume of the enriched fraction was too small to carry out time course incubations. The biosynthetic capability of the reticulocyte enriched fraction was not investigated.

TABLE 2.1 RETICULOCYTE ENRICHMENT BY THE METHOD OF VETTORE ET AL 1980

EXPT	ALTERATION TO GRADIENT	VOLUME PACKED RED CELLS LOADED ml	% RETICULOCYTES IN WHOLE BLOOD	% RETICULOCYTES IN ENRICHED FRACTION	% YIELD OF RETICULOCYTES	VOLUME OF ENRICHED FRACTION ml	COMMENTS
1	10% Urografin	0.5	0.7	12.0	--	<0.1	Good Separation
	11% Urografin	0.5	0.7	15.0	--	<0.1	of fractions
2	11% Urografin	0.5	0.7	5.0	25	0.1	Good Separation
		1.0	0.7	6.0	15	0.1	of fractions
		1.5	0.7	---	--	---	Separation of
		2.0	0.7	---	--	---	fractions not good

The second method tried was that described by Desimone et al (1974) using Urografin (Renografin) and Ficoll gradients. The trial spins were carried out using 20 ml of whole blood instead of the recommended 100 ml.

The first trial produced high enrichment but a low yield of total reticulocytes, so the procedure was modified using higher concentrations of Ficoll in the lower fraction of the discontinuous gradient.

Table 2.2 summarizes the results. Although the enrichment was very good, and in the third trial the yield of total reticulocytes was quite high, there were certain drawbacks with the method.

The most important of these was the white blood cell contamination of the reticulocyte enriched fraction as observed on the slides of the fraction. The aspiration of the zone of white cells from the gradient was not effective enough to prevent a high amount of non-globin radioactive contamination on incubation (Figure 2.1). If the white blood cells were removed by the cellulose column (see later) very few red cells were recovered for incubation and to remove all white blood cells from at least 10 mls of packed red blood cells before enrichment would be very time consuming. Furthermore, the method required at least 20 ml whole blood as starting material to give adequate quantities of red cells for

TABLE 2.2 RETICULOCYTE ENRICHMENT BY THE METHOD OF De SIMONE ET AL 1974

EXPT	ALTERATION TO GRADIENT	% RETICULOCYTES IN WHOLE BLOOD	% RETICULOCYTES IN ENRICHED FRACTION	% YIELD OF RETICULOCYTES	VOLUME OF CELLS (BEFORE WHITE CELLS REMOVED) ml
1	NONE	1.3	29	20	0.1
2	13% Ficoll	1.2	22	20	0.1
	14% Ficoll	1.2	14	40	0.2

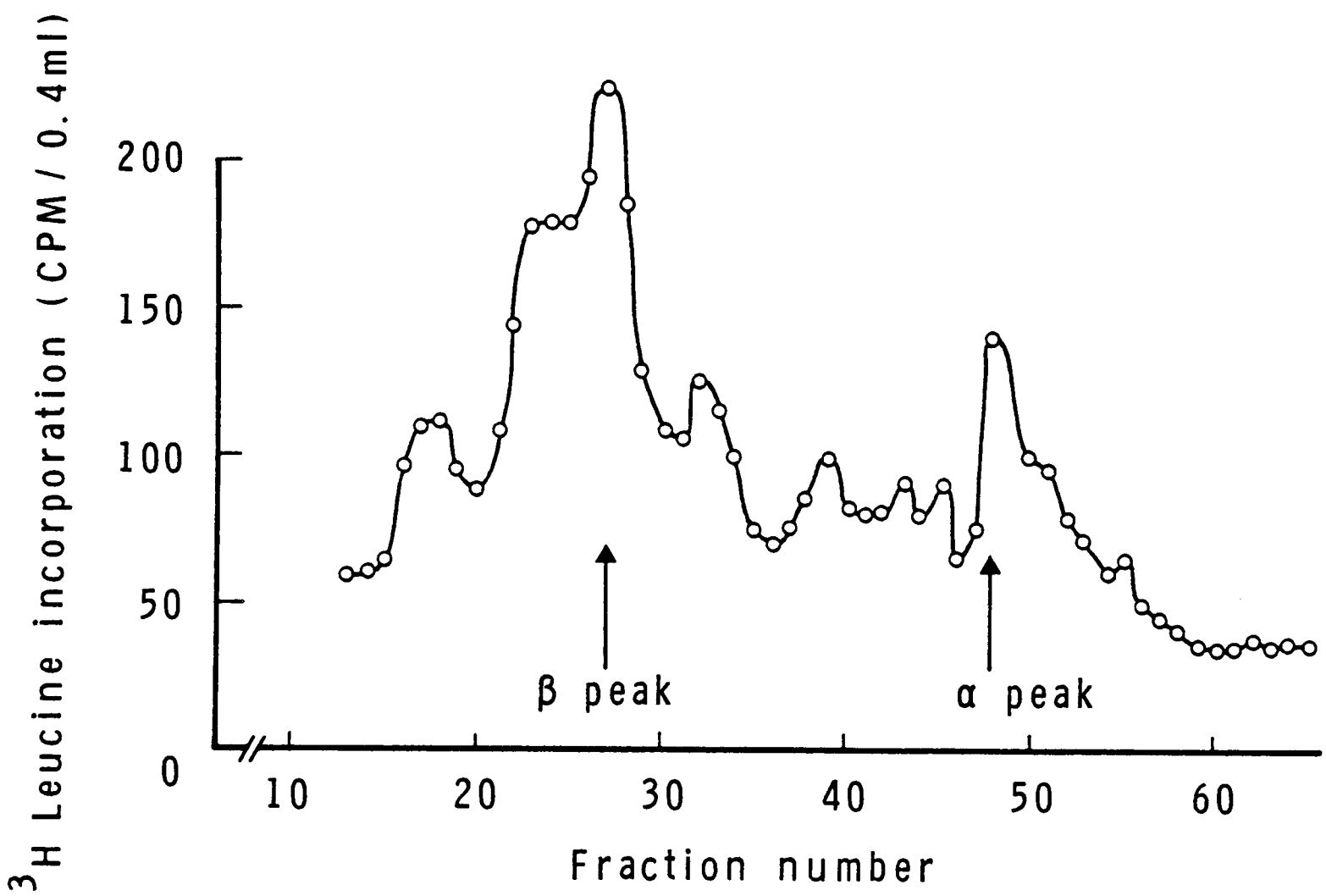


FIG. 2.1 CM-23 Separation of globins from reticulocyte-enriched fraction obtained by the method of DeSimone et al 1974.

incubation and the method took 2 hours to complete.

The third method was to spin about 5-10 ml of washed packed red cells suspended in a small volume of reticulocyte saline (see below) at 111,700 x g for 45 mins at 4°C. As table 2.3 demonstrates the enrichment of the top fraction was not as good in terms of percentage of reticulocytes, as the other methods, but the recovery of total reticulocytes was greater and moreover the volume of the reticulocyte-enriched fraction was more appropriate (even after white cell removal) particularly when time-course incubations were required. Furthermore, the method was simple and relatively quick.

The drawbacks of the first two methods outlined above outweighed the great enrichment of reticulocytes achieved. Therefore, the third method was chosen for obtaining a reticulocyte-enriched fraction for all biosynthesis incubations since it was the simplest, quickest and the enrichment of reticulocytes was more than adequate for the incubations.

Globin Chain Synthesis in Peripheral Blood Reticulocytes

Roughly 10 mls of heparinised whole blood were used as starting material for biosynthesis experiments. The cells were maintained at 4°C for most steps prior to incubation.

The whole blood was spun at 2,500 x g for 20 minutes and the plasma (retained for iron measurements) and the

TABLE 2.3 RETICULOCYTE ENRICHMENT BY HIGH-SPEED CENTRIFUGATION

EXPERIMENT	% RETICULOCYTES IN WHOLE BLOOD	% RETICULOCYTES IN ENRICHED FRACTION	% YIELD OF RETICULOCYTES	VOLUME OF ENRICHED FRACTION ml
1	0.8	11	30	0.5
2	0.5	9	30	0.6
3	1.4	9	50	0.8
4	1.0	7	65	0.8

buffy coat (retained for DNA mapping) were removed. The packed red cells were washed three times with reticulocyte saline (130 mM NaCl; 5 mM KCl; 7.4 mM $MgCl_2.6H_2O$ - Borsook et al 1957), and then centrifuged at 111,700 xg for 45 minutes at 4°C as just described. After centrifugation the top 0.5-0.8 ml of cells (representing the reticulocyte-enriched fraction) was passed through a small column of microcrystalline cellulose (1 part) and α-methyl cellulose (2 parts) (Beutler et al 1976) to remove the white blood cells present.

The packed red cells (about 0.5 ml) recovered from the cellulose column were pre-incubated in 1-1.3 ml of incubation medium as described by Lingrel and Borsook (1963) and modified by Weatherall et al (1969) (see Appendix II) containing 0.075 ml of ferrous ammonium sulphate (1 mg/ml of reticulocyte saline), for 10 minutes in a shaking water-bath at 37°C. Fifty-100 μCi of ^{3}H-Leucine (L 4,5 (^{3}H) Leucine Radiochemical Centre, Amersham 50 Ci/mM) were added and samples were removed after appropriate incubation times. The incubation was stopped by putting the cells in cold reticulocyte saline (4°C) and washing them three times with reticulocyte saline in the cold. Washed packed cells were lysed by freezing/thawing or by the addition of more than 1 volume of distilled water. Globin was prepared from the whole cell lysate by the addition of 20 volumes of acid-acetone (2% concentrated HCl in acetone) at -20°C and the globin allowed to precipitate by leaving at -20°C for 15-20 minutes. The globin was washed three times

with acetone at -20°C and finally in ether, after which it was air-dried. The globin was stable in this state when stored at -20°C.

The procedure for the incubation of reticulocytes described above gives reproducible results as long as all conditions are well controlled.

The degree of radioactive incorporation into globin can vary from batch to batch of incubation medium and new batches of medium were checked by incubating known normal samples to verify equal incorporation into α and β chains.

Experiments have also shown that the temperature of the incubation can influence the relative α/β chain synthesis in normal reticulocytes (Higgs, DR, cited by Clegg 1982). Temperatures above 37°C result in low α/β ratios (e.g. 0.76 to 0.88 at 40°C) while lower temperatures result in higher ratios (e.g. 1.3 to 1.4 at 34°C). At 37°C the ratios are nearer unity (e.g. 1.06-1.18).

In order to maximise the incorporation of ^{3}H leucine in globin biosynthesis studies of short incubation time periods, the effect of adding increasing amounts of ^{3}H leucine on the radioactivity incorporated into globin was examined. As shown in Figure 2.2, incorporation increases linearly with the addition of up to 100 μCi of ^{3}H leucine, demonstrating that the incorporation is concentration dependent and is not saturated at these levels. Thus, in short incubations (less than 5 minutes

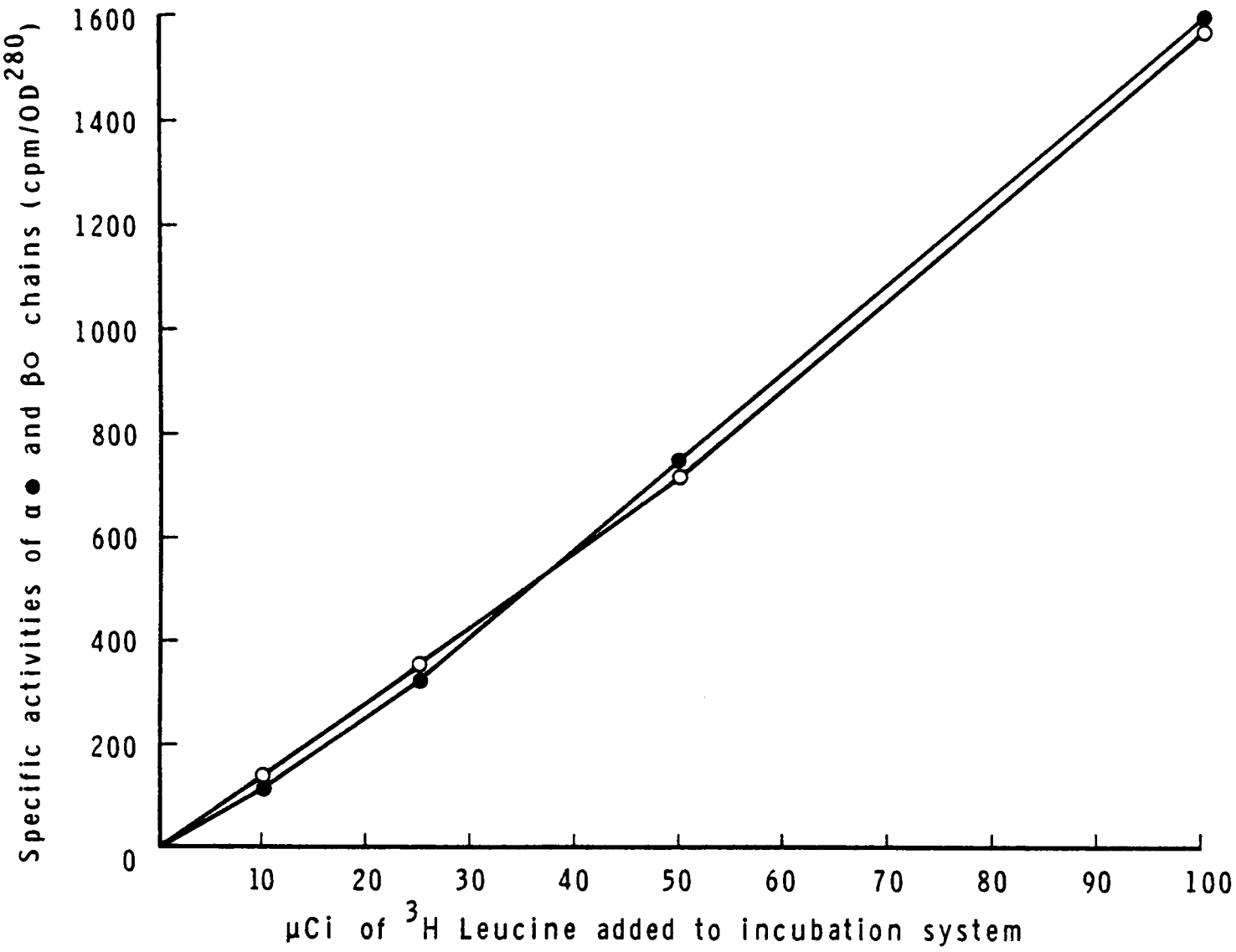


FIG. 2.2 Specific activities of α and β globin chains in presences of increasing ^{3}H Leucine.

duration) up to 250 μCi ^{3}H leucine were added.

Globin Chain Synthesis in Bone Marrow Cells

In most cases about 0.2 ml packed bone marrow cells were used as starting material. The bone marrow samples were washed three times with reticulocyte saline without any prior separation of cell fractions or the removal of contaminating peripheral blood cells. The samples were incubated and made into whole cell globin as described for the peripheral blood reticulocytes.

The Separation of Globin Chains

The globin chains were separated using the method described by Clegg et al 1966, with a slightly modified buffer gradient to improve the separation of the β^{E} and pre-α globin peaks.

Urea (Merck), freshly dissolved in distilled water to a concentration of 8 M, was filtered through Whatman No. 3 paper to remove undissolved impurities. Following filtration, mercaptoethanol (Kodak Eastman Ltd.) was added to a final concentration of 0.05 M. The buffer was made to the appropriate salt concentration (see below) by the addition of disodium hydrogen phosphate and the buffer was adjusted to pH 6.4-6.5 using 20% orthophosphoric acid.

The globin sample (15-40 mg) to be separated was dissolved in 2 ml starting buffer (see below) and dialysed for 1 hour against 50 ml starting buffer.

While the globin sample was dialysing, 2.5 g of CM-23 cellulose (Whatman) was allowed to swell for 20

minutes in 50 ml starting buffer and the slurry was then poured into a 1.5 x 25 cm glass column (with sintered-glass base) to give a bed height of about 20 cm. The CM-cellulose column was equilibrated by washing with starting buffer for about 1 hour at a flow rate of 0.5 ml/ /min (used throughout the chain separation), after which time the dialysed globin sample was gently applied to the column and washed in with 2-3 ml of starting buffer. The column was washed for a further 30-40 minutes with the buffer to elute the unbound material, after which the salt gradient was started. Five ml fractions were collected and the optical density (OD) of the effluent was monitored at 280 nm.

For the separation of α and β^A globin only, a linear gradient was used with 0.008 M Na_2HPO_4 in the starting buffer and 0.035 M Na_2HPO_4 in the finishing buffer. When separating samples containing β^E globin, a 3-chambered concave gradient was used to produce better separation at the β^E and pre-α peaks (see Fig 2.3). With respect to Na_2HPO_4 the starting buffer was 0.009 M, the middle buffer 0.024 M and the finishing buffer 0.031 M. All buffers were used at pH 6.4-6.5.

The radioactivity in 0.4 ml aliquots of each fraction was determined in 5 ml of xylene based scintillation fluid (ES 299 Packard Instruments Ltd.) with the addition of 0.1 ml water to prevent precipitation of the urea. The total counts incorporated into each globin peak were calculated by summing the counts of the appropriate fractions (judged by plotting the radioactive profile) and substracting the background counts.

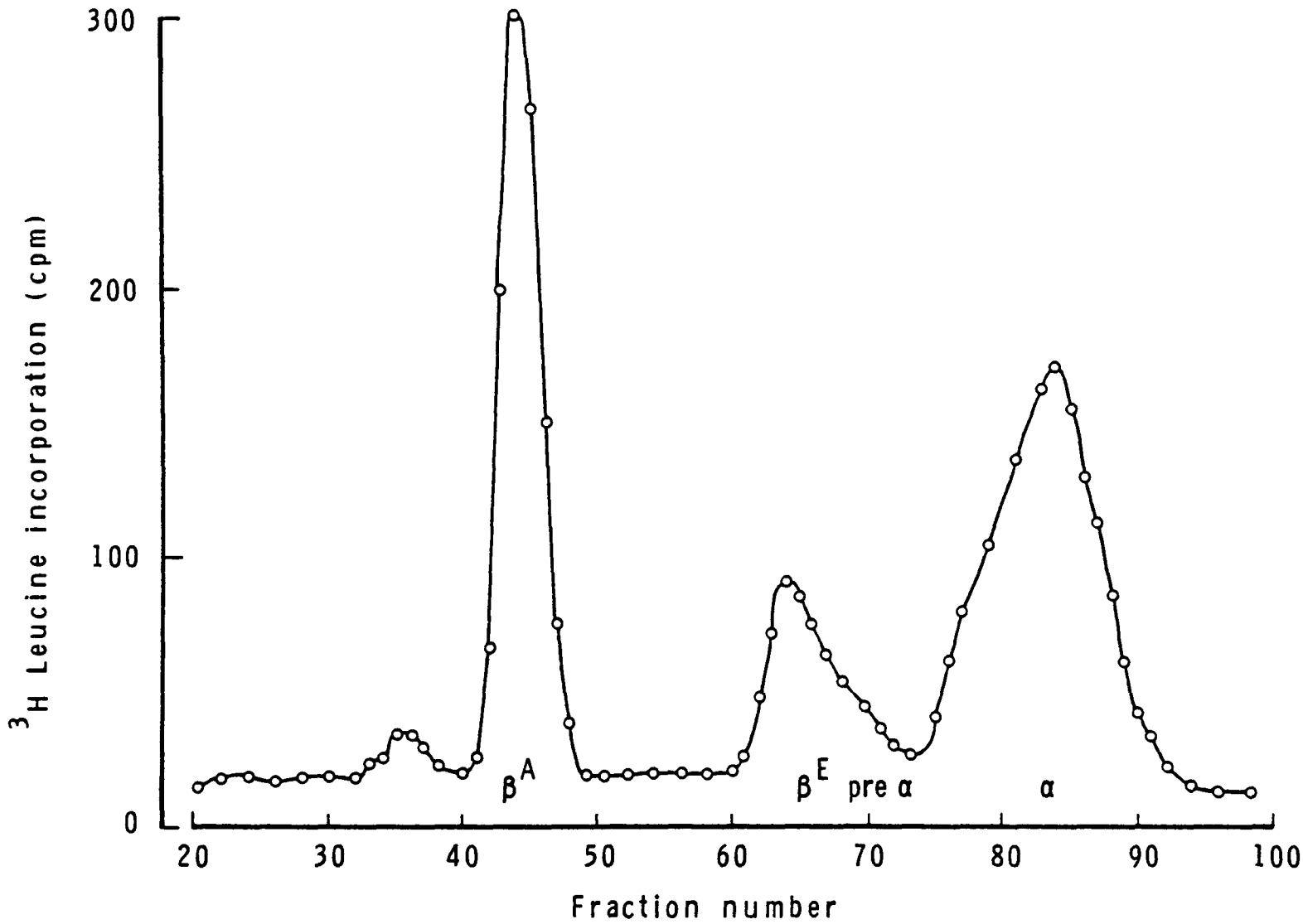


FIG. 2.3 CM-23 separation of Hb E heterozygote globin with unmodified 2-chambered gradient.

The specific activity of each chain was determined by measuring the absorbance at 280 nm and the radioactivity in 0.5 ml duplicate samples of each globin peak tube previously dialysed against 3 changes of 0.5% formic acid for 36 hours. The difference in absorbance of the α and β chains was corrected by using the values of α 0.56 and β 0.84 (Schwartz 1969).

Removal of non-globin proteins from marrow samples

The marrow globin samples also contained significant quantities of labelled non-globin protein which had to be removed from the globin in order to obtain accurate α/β globin synthesis ratios. Purification of the globin samples was achieved by gel filtration on G-100 Sephadex columns as described by Chalevelakis et al (1976).

G-100 Sephadex (20 g) was allowed to swell in 2L of 5% formic acid for 2-3 days at room temperature, degassed under vacuum and poured into a 2.5 x 100 cm glass column. Globin from incubated marrow (30-50 mg) was loaded in 5% formic acid together with blue dextran and ε-2,4-dinitrophenyl-1-lysine as high and low molecular weight markers respectively. The column was run at 4°C at a rate of 0.25 ml/minute and each 3 ml fraction collected was monitored for absorbance at 280 nm and counted (Fig. 2.4). The fractions containing the globin were pooled and freeze-dried ready for chain separation.

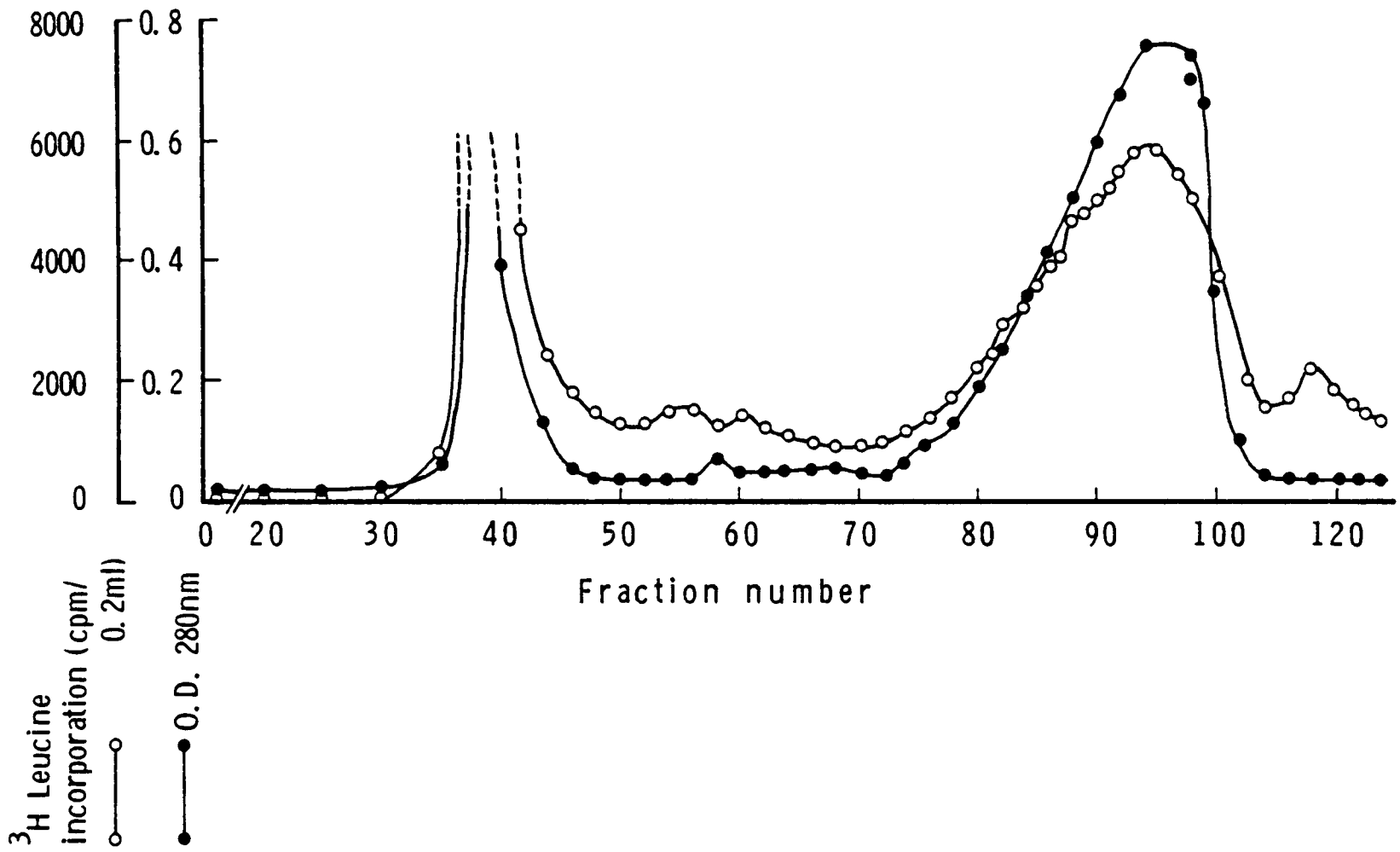


FIG. 2.4 Purification of marrow globin sample by gel-filtration.

Isolation of free α-chains using G-100 Sephadex gel filtration

Freshly incubated and washed red blood cells were lysed by the addition of 1-2 volumes of distilled water. Stroma was removed by centrifugation at 102,000 x g for 60 minutes at 4°C. The stroma-free lysate was carefully applied to the G-100 Sephadex column (made as before but in 0.05 M Tris HCl, 0.1% KCN pH 7.4), together with the blue dextran and ε-DNP lysine markers, and washed in 2 or 3 times with the Tris buffer. The column was run at 4°C at a rate of 0.15 ml/minute and 2-3 ml fractions were collected.

The radioactive content of each fraction was monitored by counting 0.3 ml aliquots diluted with 0.2 ml distilled water in 5 ml ES 299 using a Packard Instruments Ltd. 2650 liquid scintillation counter with a programmed quench correction (external standard method) for haemoglobin. The absorbance of each fraction at 415 nm was also measured.

The haemoglobin fractions and those possibly representing free α-chains were independently pooled. 'Carrier' haemoglobin was added to the free α-chain peak and both fractions were made into globin. The pooled fractions usually amounted to large volumes and the procedure for making globin was slightly modified by adding the fractions to a large beaker containing acid-acetone (2% concentrated HCl in acetone) at -20°C. The globin was washed and dried as for the preparation of whole cell globin.

The separation of haemoglobins by DEAE-cellulose chromatography

The method used was that described by Abraham et al 1976/7 using DEAE-cellulose as the ion-exchange resin and glycine-KCN-NaCl solutions as developers.

The stock developer (Developer A) consisted of 0.2 M glycine/0.1% KCN. The pH was not adjusted. For each 1.5 x 25 cm column, 15 g DEAE cellulose (DE 52 Whatman) was suspended in 100 ml of developer A and stirred for 10 minutes. The supernatant was then decanted from the settled cellulose and the procedure repeated twice with fresh developer to remove the 'fines'. After pouring, the column was equilibrated for 1 hour before loading.

Stroma free lysates, diluted in the starting buffer were loaded onto the column and eluted at a rate of 3 ml/minute to give fractions of 6-7 ml. The optical density (OD) was monitored continuously at 415 nm. The haemoglobins were eluted using an initial linear gradient of 0.005 M and 0.025 M NaCl in developer A, followed by 0.04 M NaCl in developer A to elute the final haemoglobins bound to the column.

Messenger RNA Studies

The relative amounts of α and β globin specific messenger RNA sequences (α/β mRNA) were determined in the total cell RNA extracted from the peripheral blood or bone marrow cells of individuals with Hb E by comparing hybridization of the total RNA with α- and β- specific cDNA probes.

In most cases the globin RNA was extracted from 10 ml of heparinized whole blood, without prior enrichment of reticulocytes, or from 0.2 ml packed bone marrow cells.

All RNA samples were extracted with the utmost care in order to minimise the loss of globin RNA sequences by nuclease degradation. Unless otherwise stated, all procedures were carried out at $4^{o}C$.

Preparation of RNA from Peripheral Blood

The plasma and buffy coat were removed from the whole blood samples after centrifuging at 2500 x g for 20 minutes. The packed red cells were then washed three times in reticulocyte saline.

The washed red cells were lysed with 2 volumes of cold 1 mM $MgCl_2$/0.1% diethylpyrocarbonate (Sigma) and the membranes were precipitated by spinning at 28,000 x g for 15 minutes ($4^{o}C$). Sodium dodecyl sulphate (SDS) was added to the supernatant to a final concentration of 0.75% and the lysate warmed to room temperature for all extraction steps. The SDS lysate was extracted with an equal volume of saturated phenol:chloroform: 8 hydroxyquinoline (made by saturating phenol (BDH special-for-chromatography grade) with NETS buffer (0.1 M NaCl; 1 mM Na_2 EDTA; 10 mM Tris-HCl ; 0.2% SDS pH 7.4), mixing with an equal volume of chloroform and adding 0.1% w/v 8-hydroxyquinoline (Sigma)) for 1 hour, after which the mixture was centrifuged at 21,000 x g for 10 minutes. The upper aqueous layer was re-extracted with an equal volume of phenol, and the lower phase with

an equal volume of NETS buffer, each for 15 minutes. Both mixtures were spun as above and the aqueous layers were pooled and extracted once more with an equal volume of phenol:chloroform for 10 minutes. The lower phenol phases were discarded. Following the final extraction the mixture was again centrifuged. The RNA was precipitated from the aqueous layer by standing at -20°C for 12-16 hours after the addition of 1/10th volume 4 M NaCl and more than two volumes ice cold absolute ethanol. The RNA was pelleted by centrifuging at 28,000 xg for 1 hour at 4°C. The ethanol was decanted and following drying in air the pellet was dissolved on ice in a small volume (1.0-1.5 ml) of distilled water and used as 'total RNA' without further purification. The concentration of RNA was calcuated by assuming that a 35 µg/ml RNA solution had an absorbance of 1.0 at 260 nm (Rosenfeld et al 1972).

In some cases the RNA was not immediately extracted from the fresh whole blood samples, but extracted from washed, frozen packed red cells or from the frozen SDS lysate, prepared after washing and lysing the blood cells, stored at -90°C in all cases. Experiments have shown that when RNA was extracted from frozen packed red cells or from SDS lysate, there was no significant alteration in the α/β mRNA ratios measured (Lynne Pressley D. Phil Thesis 1980).

Preparation of Nuclear and Cytoplasmic RNA from Bone Marrow Cells

The method used in this study was similar to that

described by Knowler et al (1973) as modified by Nienhuis et al (1977).

Immediately after collection, bone marrow aspirates were washed 3 times with reticulocyte saline and then frozen at -90°C in entirety until required.

The frozen packed bone marrow cells (about 0.2 ml) were thawed in the presence of 2-3 ml 1 mM $MgCl_2$/0.1% diethylpyrocarbonate, homogenized (10 strokes) in a Teflon-glass homogenizer (Thomsas Co. USA), diluted with an equal volume of 0.1 M citrate/1 mM $MgCl_2$ and homogenized again. The suspension was centrifuged at 2,500 x g for 5 minutes, the pellet resuspended in 3 ml citric acid/$MgCl_2$, homogenized and spun again. The supernatants from both centrifugations were retained on ice for the extraction of cytoplasmic RNA.

The pellet containing the nuclei was then resuspended in 3 ml of 0.5 M citrate/1 mM $MgCl_2$/1% Triton X-100, diluted with an equal volume of 0.5 M sucrose and homogenized (8 strokes). This solution was layered over a 6 ml cushion of 0.32 sucrose/1 mM $MgCl_2$/0.05 M citrate/1% Triton X 100 and centrifuged at 800 x g for 5 minutes. The resuspension of nuclei and the centrifugation through the sucrose cushion was repeated and the final nuclear pellet was suspended in 10 ml of 0.1 M NaCl; 10 mM NaOAC pH 5; 1 mM EDTA; 0.5% SDS. Examination of the nuclei under light microscopy revealed minimal contamination by cytoplasmic fragments.

Before extraction of the cytoplasmic RNA, the

cytoplasmic fraction was adjusted to pH 5.0 with 1 M NaOAc and 200 mM EDTA and 5% SDS were added to concentrations of 5 mM and 0.2% respectively. After this the extraction of the nuclear and cytoplasmic fractions was identical. To reduce the loss of globin RNA sequences by nuclease degradation, a 20 mg/ml of E. coli RNA (B.D.H.) was added to a final concentration of 10 µg/ml as carrier to each fraction. The fractions were then extracted at room temperature with an equal volume of phenol:chloroform:β hydroxyquinoline (as for the peripheral blood RNA extraction) for 15 minutes and centrifuged at 15,000 x g. The phenol extraction was repeated with the aqueous and interphase layers twice and finally with the aqueous layer alone. The RNA was precipitated from the final aqueous fraction for 12-16 hours at -20°C in the presence of 4 M NaCl, to a final concentration of 0.3 M, and 3 volumes of cold ethanol. In the nuclear extractions much of the DNA remained in the interphase and any DNA strands that appeared after the addition of ethanol were spooled out. The RNA was recovered by centrifuging at 22,000 x g for 1 hour at 4°C, the ethanol decanted and the RNA air dried before being taken up in distilled water (1.5 ml). The solution was then spun through a bed of G-25 Sephadex in a 10 ml plastic syringe at 4°C to remove the salts, lyophilized and redissolved in 0.5-1.0 ml of distilled water. The total RNA was not purified and the concentration present was calculated as for the peripheral blood total RNA.

All glassware used in the extraction was siliconized,

rinsed with 0.1% diethylpyrocarbonate and baked prior to use.

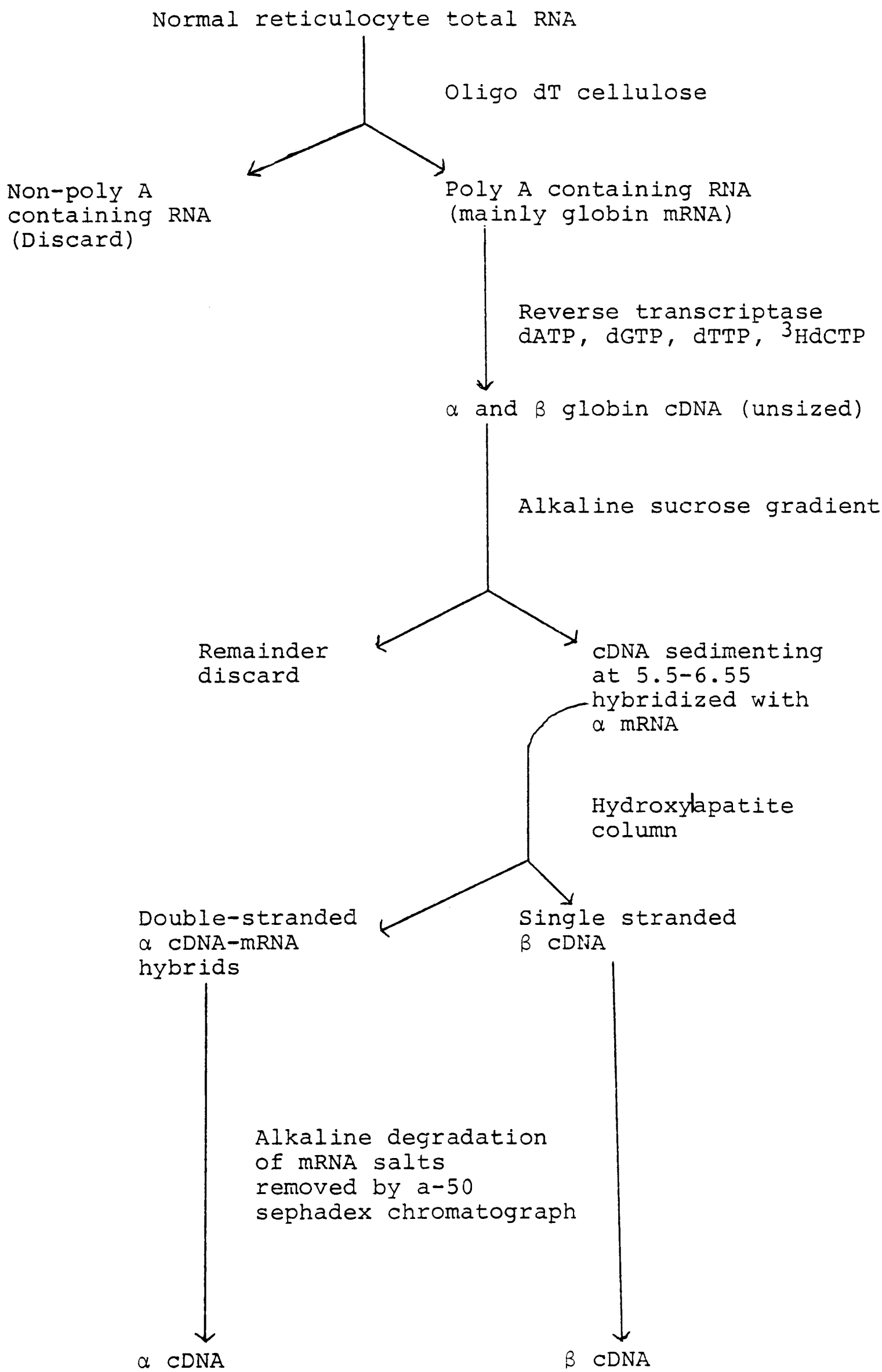
The Preparation of cDNA

Labelled complementary DNA (cDNA) copies of α- and β- globin messenger RNA sequences were synthesised from tritiated nucleotides by reverse transcriptase, followed by the separation and purification of the $cDNA_{\alpha}$ and $cDNA_{\beta}$ probes. The procedure is summarized in Figure 2.5.

Total RNA was extracted from the peripheral blood of patients with high reticulocyte counts by the method previously described. The poly-A containing RNA (mostly globin mRNA) was purified from the total RNA using oligo (dT) cellulose chromatography by a slight modification of the method described by Aviv and Leder (1972). A small column of oligo (dT) cellulose was prepared in eluting buffer (10 mM Tris; 0.5% SDS pH 7.4). Before use the column was washed throughly with high salt binding buffer (0.4 M NaCl; 10 mM Tris; 0.5% SDS pH 7.4) in which the sample was loaded. The unbound material was eluted from the column with binding buffer, after which the mRNA was eluted with the low salt eluting buffer. The OD_{260} of each fraction was read and the mRNA was precipitated from the appropriate pooled fractions by the addition of salt and cold ethanol as described previously. The mRNA was recovered by centrifugation and dissolved in a small volume of water.

This messenger RNA, containing both α- and β-globin mRNA sequences, was used as a template in the preparation

FIG. 2.5 THE PREPARATION OF cDNA



of ^{3}H cDNA, using reverse transcriptase from avian myeloblastosis virus (a gift from Dr. J. Beard, Life Sciences Research Laboratory, Florida, USA) with oligo (dT) as a primer. The mRNA (17 µg/ml) was heated to 70°C for 5 minutes, rapidly cooled on ice and added to the following mixture: 0.5 mM each dATP, dGTP and dTTP; 0.1 mM ^{3}HdCTP (14.8 Ci/mmol, Radiochemical Centre, Amersham, U.K.); 3.5 µg Oligo dT_{12-18} (PL Biochemicals); 100 µg actinomycin D/ml; 50 mM Tris-HCl pH 8.2; 10 mM magnesium acetate; 10 mM dithiothreitol followed by the addition of 290 units reverse transcriptase/ml. The reaction was carried out at 45°C for 12 minutes and stopped by cooling on ice and by the addition of 100 mM Na_2EDTA to a final concentration of 30 mM. The RNA was denatured by treatment with 0.3 M sodium hydroxide for 2 hours at 37°C. The solution was neutralized with hydrochloric acid using 40 mM HEPES as a buffer and phenol red as indicator. This was then desalted on a G-50 Sephadex column. The cDNA was sized by alkaline sucrose gradient sedimentation (Harrison et al 1974) and the fractions sedimenting at 5.5-6.5 S taken. The peak tube was 6.3 S and therefore the average molecular weight of the cDNA was 160,000. Following neutralization and passage through G-50 Sephadex the $cDNA_{\alpha}$ and $cDNA_{\beta}$ were separated by hybridizing the $cDNA_{\alpha\beta}$ (525 ng) to an excess of mRNA (105 µg) extracted from the blood of an individual with $\delta\beta/\beta^{o}$ thalassaemia previously shown not to have β-specific mRNA sequences (Ottolenghi et al 1975). The hybridization reaction was carried out in 1 ml buffer containing

25 mM HEPES; 0.5 M NaCl; 10 mM EDTA pH 6.8 and 50% formamide (Fluka Chemicals) at 43°C for 24 hours by which time the reaction had reached completion. The double stranded fraction ($cDNA_\alpha$ enriched) was separated from the single-stranded fraction ($cDNA_\beta$ enriched) on a hydroxylapatite column (Bio Rad Laboratories DNA Grade Bio Gel HTP) at 60°C, by eluting with 0.15 M and 0.4 M sodium phosphate buffers (pH 6.8) respectively. After alkali digestion to remove the RNA, the reaction was neutralized, the $cDNA_\alpha$ and $cDNA_\beta$ desalted on G-50 Sephadex (as described before), decalcified with Dowex 50X8 (Na^+ form) and freeze-dried. The $cDNA_\alpha$ and $cDNA_\beta$ were each taken up in a small volume of distilled water and stored at -90°C after counting aliquots to determine the yields and concentrations present.

All glassware used in the procedure was siliconized, rinsed with 0.1% diethylpyrocarbonate and baked. The Sephadex G-50 and Dowex columns were pre-run with E. coli DNA (1 mg/ml) to prevent non-specific losses and the Sephadex columns were run with 0.05 M ammonium bicarbonate.

RNA/cDNA Hybridization and Assay

In order to calculate the α/β mRNA ratio for any RNA sample, increasing amounts of RNA were titrated against a fixed amount of $cDNA_\alpha$ or $cDNA_\beta$ until all hybridization had gone to completion (Young et al, 1974). The hybridized material was assayed with S_1 nuclease by a slight modification of the method described by Harrison et al (1974), and the relative amounts of RNA required to saturate 50% of each cDNA probe were compared.

The RNA and cDNA to be hybridized were mixed with 10 µg E. coli rRNA (BDH) as carrier, lyophilized and suspended in a fixed volume (usually 2 µl) of hybridization buffer (25 mM HEPES; 0.5 M NaCl; 10 mM Na_2 EDTA pH 6.8; 50% formamide). The reaction was carried out in small sealed glass tubes at 43°C for not less than 96 hours, after which the samples were flushed out with 350 µl nuclease assay buffer (0.1 m NaCl; 2.88 mM $ZnSO_4$; 0.03 M sodium acetate, pH 4.5 containing 20 µg/ml calf thymus DNA, which was denatured by boiling before addition to the buffer) and incubated for 2.5 hours at 37°C with about 45 units of S_1 single-stranded specific nuclease (Aspergillus oryzae, Sigma). The amount of S_1 reuqired varied slightly from batch to batch. Following nuclease digestion of the single-stranded nucleotides, the total amount of ^3H cDNA present was determined in a 100 µl aliquot from each sample. To precipitate the hybridized, undigested ^3H cDNA, 50 µl of a 5 mg/ml catalase solution (as carrier) and 50 µl of 6 M perchloric acid were added to a second aliquot of 200 µl. The mixture was kept at 4°C for 30 minutes to precipitate the S_1 resistant DNA, after which the aliquot was centrifuged and the radioactive content of 200 µl of the supernatant was determined.

Radioactivity was measured by liquid scintillation counting in the presence of xylene based ES 299 (Packard) after adjusting the total and supernatant aliquots to the same acidity to reduce the amount of quench correction required. The samples were counted in a Packard Instruments Ltd. 2650 liquid scintillation

spectrometer with a programmed quench correction (external standard method).

The percentage of cDNA hybridized for each point was calculated as:

$$100 - \frac{\text{quench corrected dpm in supernatant fraction}}{\text{quench corrected dpm in total fraction}} \times 0.75$$

Purity of $cDNA_\alpha$ and $cDNA_\beta$ probes

This was assessed by hybridizing the probes with normal mRNA, mRNA from an infant with Hb Bart's hydrops fetalis syndrome (i.e. no α mRNA sequences) and mRNA from a patient with $\delta\beta/\beta^o$ thalassaemia (who had no β mRNA sequences).

The results showed that approximately 5% of $cDNA_\alpha$ and $cDNA_\beta$ were resistant to S_1 digestion in the absence of any mRNA sequences and were therefore double-stranded and unable to hybridize with mRNA. The plateau values of hybridization with the normal mRNA (Fig. 2.6) were approximately 80% for $cDNA_\alpha$ and $cDNA_\beta$ indicating that a further 20% of each probe was non-hybridizable.

Hybridization of $cDNA_\alpha$ with mRNA from the hydropic infant showed a plateau value of 15% showing the $cDNA_\alpha$ contained about 10% of $cDNA_\beta$ sequences (see Fig. 2.6). Similarly, when $cDNA_\beta$ was hybridized to mRNA from the patient with $\delta\beta/\beta^o$ thalassaemia the plateau value was 9% indicating that $cDNA_\beta$ contained about 4% $cDNA_\alpha$ sequences (see Fig. 2.6).

Therefore the α and β cDNA probes are relatively pure

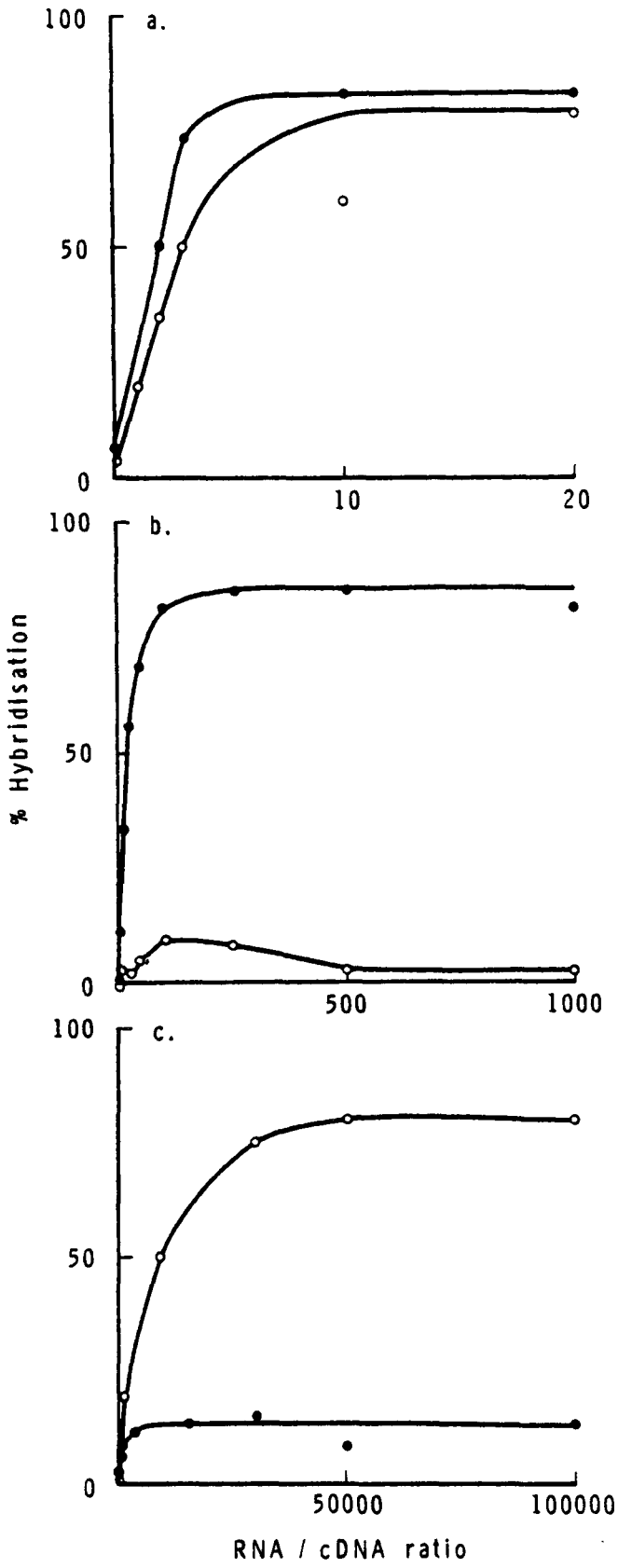


FIG. 2.6 Characterization of $cDNA_{\alpha}$ and $cDNA_{\beta}$ probes by hybridization with:

a) Normal mRNA
b) $\beta^{o}/\delta\beta$ thalassaemia mRNA
c) Hydrops mRNA

Closed circles - $cDNA_{\alpha}$; Open circles - $cDNA_{\beta}$

(10% and 4% contamination respectively) and that about 75% of each cDNA probe is hybridizable.

Measurement of relative α/β mRNA ratios

Hybridization curves were set up with each of the $cDNA_\alpha$ and $cDNA_\beta$ probes and increasing quantities of the RNA sample to be assessed. The quantity of hybridized cDNA was assayed and calculated for each point as described in the previous section. The amount of hybridized cDNA was plotted against the RNA/cDNA concentration ratio and from the curve of best fit the amounts of RNA required for 50% hybridization of $cDNA_\alpha$ and $cDNA_\beta$ respectively were compared.

With most RNA samples there was sufficient RNA such that 100 pg cDNA could be used for each point of hybridization. However, the yield of RNA extracted from most bone marrow samples was much lower and consequently there was only sufficient RNA to hybridize with 50 pg of each cDNA probe per point. When lower quantities of cDNA (50 pg) were used, the hybridization time was doubled to allow the hybridization reactions to go to completion.

Since total RNA samples were used it was not possible to assess the quantity of mRNA present and consequently a trial hybridisation curve was carried out using $cDNA_\alpha$ with each RNA sample (Figure 2.7) and this was used to estimate the most appropriate RNA/cDNA ratios with which to set up the $cDNA_\alpha$ and $cDNA_\beta$ curves for the calculation of the α/β mRNA ratio.

A typical pair of $cDNA_\alpha$ and $cDNA_\beta$ curves with a

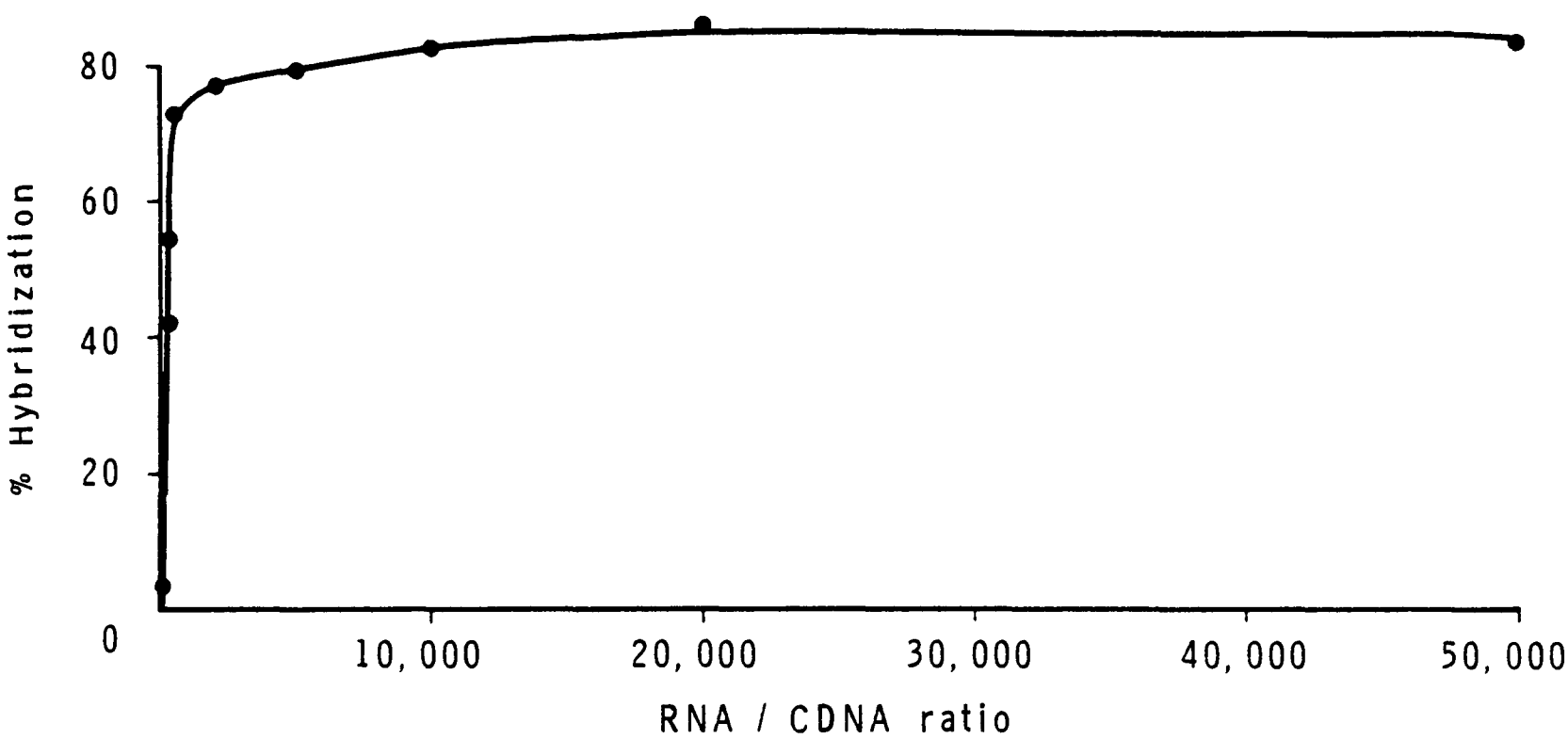


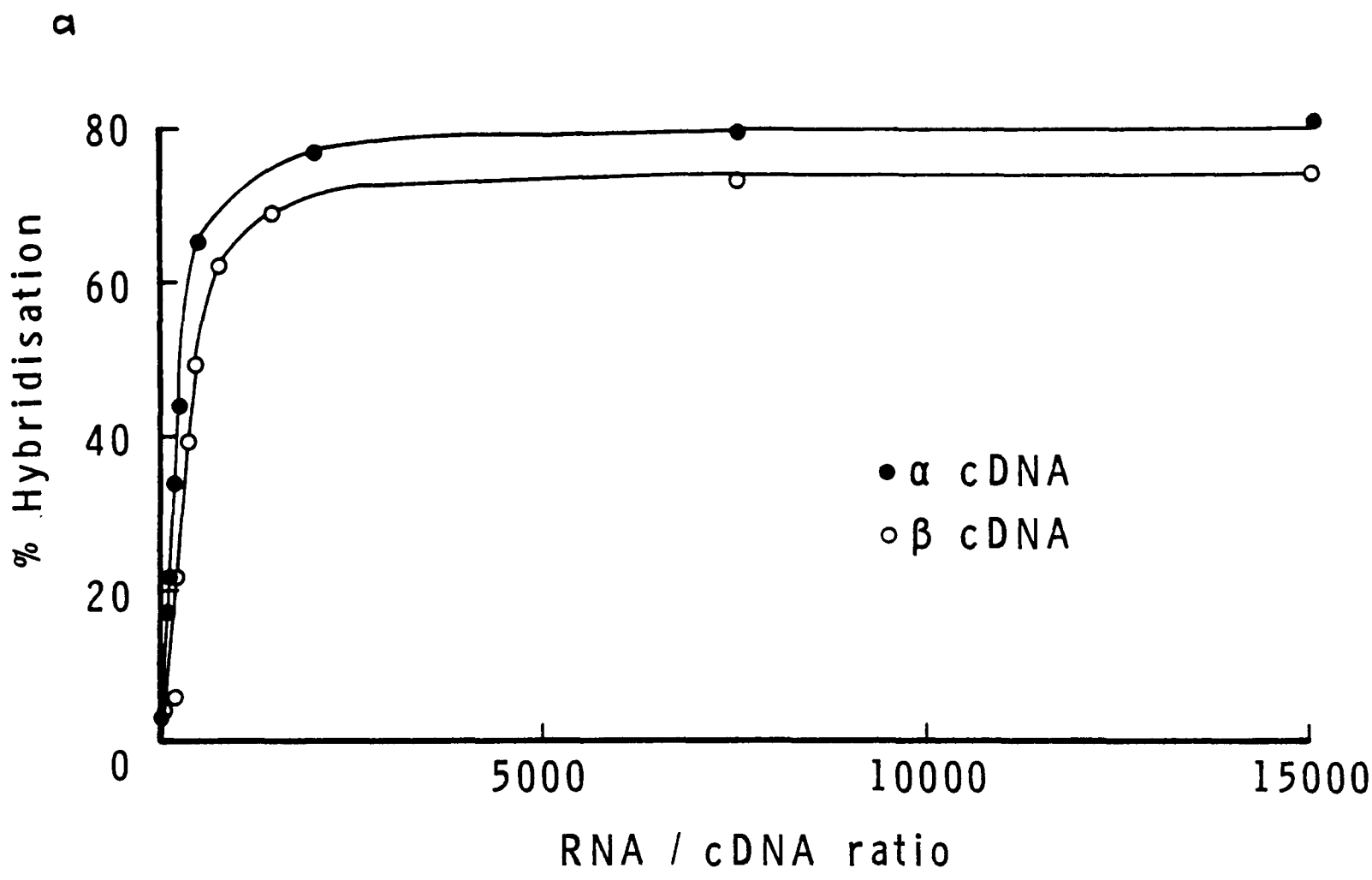
FIG. 2.7 Trial α mRNA curve

normal total RNA sample is shown in Figure 2.8. To assess the general form and maximum plateau of each curve, they were plotted on a small scale (Figure 2.8a) and the scatter of the points of each curve was assessed by plotting on a larger scale (Figure 2.8b). This pair of normal RNA/cDNA hybridization curves was considered acceptable (see below) and the relative α/β mRNA ratio was calculated from the RNA/cDNA ratio required for 50% hybridisation of the respective cDNA. This ratio is inversely proportional to the concentration of mRNA complementary to that cDNA and that the α/β mRNA ratio is:

$$\frac{\text{RNA/cDNA ratio required to saturate 50\% cDNA}_{\beta}}{\text{RNA/cDNA ratio required to saturate 50\% cDNA}_{\alpha}}$$

In the calculation corrections were made for the percentage of radioactivity that was non-hybridizable for each probe, and also for any difference in the average amounts of $cDNA_{\alpha}$ and $cDNA_{\beta}$ used for each curve.

For the curve depicted in Figure 2.8, the $cDNA_{\alpha}$ curve had a zerovalue of 3.5% and a plateau value of 80% and the $cDNA_{\beta}$ curve had a zero value of 4.0% and a plateau value of 74%. Therefore, the percentage of the probes that were hybridizable were 80-3.5 i.e. 76.5% for $cDNA_{\alpha}$ and 74-4.0 i.e. 70.0% for $cDNA_{\beta}$ and the normalised 50% ratios were 76.5/2 + 3.5 i.e. 41.75% ($cDNA_{\alpha}$) and 70.0/2 + 4.0 i.e. 39.0% ($cDNA_{\beta}$). From the curves in Figure 2.7b the RNA/cDNA ratios at which each probe was 50% saturated was 200 for $cDNA_{\alpha}$ and 340 for $cDNA_{\beta}$. Finally, the average amount of $cDNA_{\alpha}$



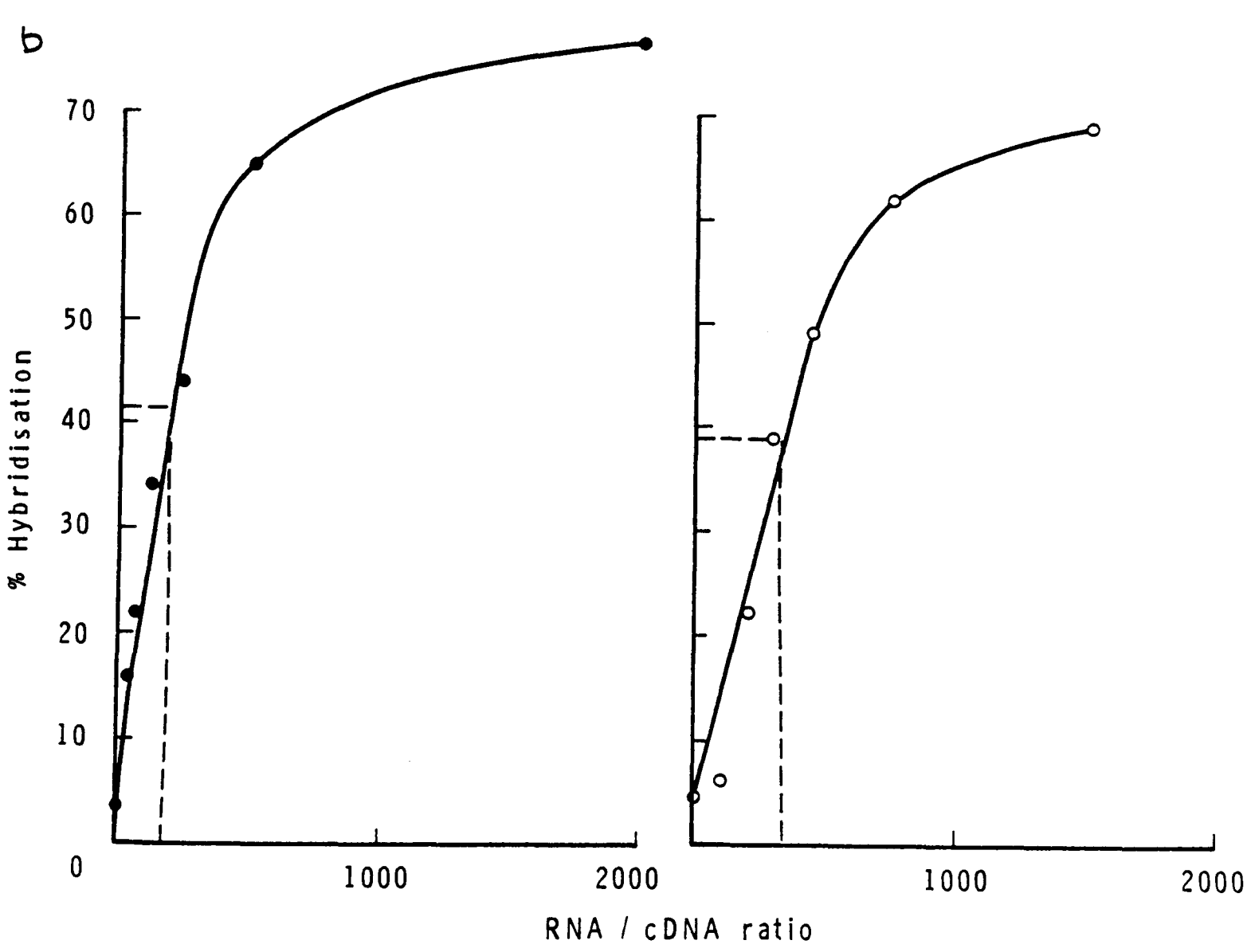


FIG. 2.8 Normal α/β mRNA curve

a) Plotted on small scale
b) Plotted on large scale

was 90 pg and for $cDNA_\beta$ was 89 pg, as calculated from the dpm in the 'total' aliquot. Therefore, the α/β mRNA ratio in this normal individual was:

$$\frac{340}{200} \times \frac{76.5}{70.0} \times \frac{90}{89} = 1.87$$

The Acceptability of α/β mRNA Curves

Before calculating the α/β mRNA ratio for any RNA sample, the curve had to fulfill certain criteria before being considered acceptable. The general form, zero and plateau values were judged by plotting the curve on a small scale (2.7a) and the scatter of points by plotting the curve on a larger scale (2.7b) as already described.

Zero values, representing the amount of S_1 resistent non-hybridizable DNA, were accepted if they were between 1-10%. Occasionally, zero points of -5 to 0 were accepted since such values probably arose through bad calibration of the automatic pipette which resulted in soluble fraction aliquots of slightly greater than 200 µl. The zero values were very important in the calculation of a curve and since pipetting errors have greater effect at lower values for the percentage hybridization, quadruplicate assays were set up for the zero points, and the acceptable values were averaged.

The plateau values of each curve were also important in the calculation of α/β mRNA ratios. Acceptable curves should have a plateau defined by 2 or 3 points, and a curve was not accepted if insufficient RNA had been added

to produce a plateau of hybridization. The peripheral blood RNA curves were accepted if the plateau values were within the ranged defined when the probe was first tested for purity i.e. 80%. Values below this usually indicated nuclease activity in either the cDNA or the RNA. However, plateau values for most hybridization curves of bone marrow nuclear and cytoplasmic RNA's were frequently below 75% with both $cDNA_{\alpha}$ and $cDNA_{\beta}$. This was probably the result of some degradation of the mRNA sequences during extraction, producing mRNA fragments smaller than the cDNA probes. Hybridization of these fragments with the probe would result in the susceptibility of cDNA 'tails' to S_1 digestion, thereby reducing the apparent quantity of hybridizable cDNA. If such curves fulfilled the other criteria defined for acceptability the α/β mRNA ratios were calculated.

Finally, the scatter of points, as observed in the larger scale plot of the curves, had to be minimal. particularly in the region of 50% cDNA hybridization. If there was excessive scatter in this region the curve was not calculated. However, if there was scatter in other regions of the curve, judged not to affect the calculation of the 50% hybridization RNA/cDNA ratio, the curve was calculated.

For many RNA samples the α/β mRNA ratio was determined from two acceptable sets of $cDNA_{\alpha}$ and $cDNA_{\beta}$ curves (In particular this applied to the bone marrow nuclear and cytoplasmic RNA samples) and close

comparability of the α/β mRNA ratios of a sample calculated from two sets of good curves demonstrates the reproducibility of the technique.

Restriction Endonuclease Mapping

The DNA of most individuals in this study was mapped with restriction enzymes in order to assess their α-gene status using the method of restriction enzyme mapping as described by Old and Higgs (1982).

CHAPTER 3

HAEMATOLOGY, HAEMOGLOBIN ANALYSIS AND HAEMOGLOBIN STABILITY

The haematological indices and haemoglobin analysis of the Hb E cases studied are summarised in Tables 3.1-3.3.

Homozygous Hb E

Eight cases of homozygous Hb E were investigated, seven of Thai origin and one of Indian origin. None of the individuals had any clinical symptoms other than mild anaemia in some cases.

The blood films from all the Hb E homozygotes showed microcytosis, hypochromia and many target cells (Fig. 3.1).

The haematological indices of each homozygous individual are summarised in Table 3.1. Since relatively few individuals are included in this group, the data have not been separated on the basis of sex or age.

The haemoglobin levels (range 8.3 - 14.2 g/dl; mean 11.7 $\pm$ 1.9) were generally towards the lower end of the normal range* and two cases (Nos. 3 and 4) showed

* The normal haematological indices quoted in this thesis refer to Caucasian population. Equivalent figures for the SE Asian population are not available.

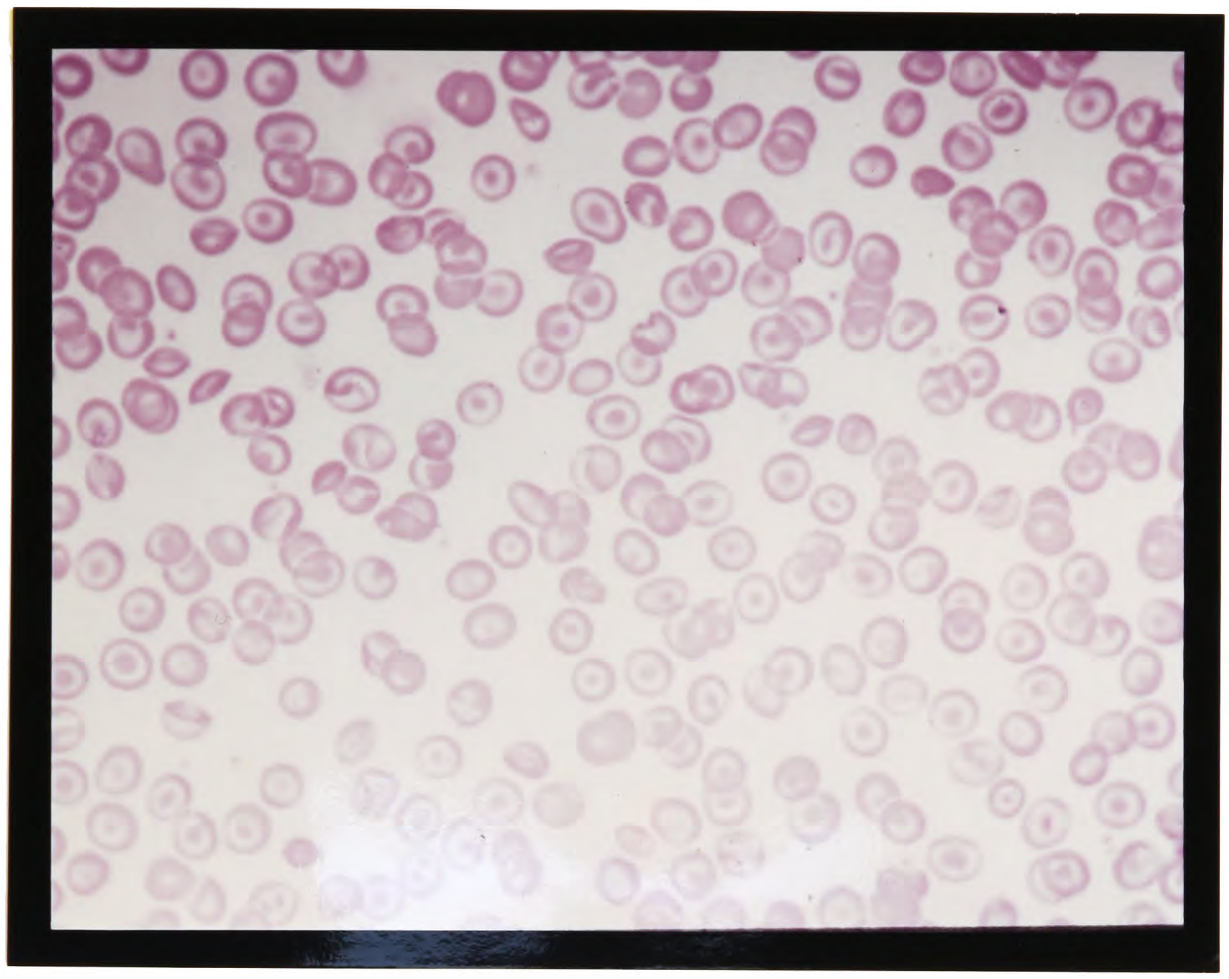


FIG. 3.1 Blood film of Hb E homozygote (Case 1)

TABLE 3.1 HAEMATOLOGICAL INDICES AND HAEMOGLOBIN ANALYSIS IN Hb E HOMOZYGOTES

INDIVIDUAL	RACIAL ORIGIN	SEX	AGE YRS	Hb g/dl	RBC x $10^{12}/\ell$	Hct	MCV fl	MCH pg/RBC	MCHC g/dl	RETICULOCYTES %	SERUM IRON µm/ℓ	TOTAL IRON BINDING CAPACITY µm/ℓ	% IRON SATURATION	% Hb F	α GENE STATUS
1	Thai	F	~25	12.2	5.81	38.3	64	20.9	32.3	1.5	15	70.5	21	4.0	αα/αα
2	Indian	F	~35	12.3	6.52	38.3	57	19.1	32.4	0.8	3.0	24.0	12.5	1.1	αα/αα
3	Thai	M	50	8.3	3.41	24.9	74	24.0	33.0	0.8			81	4.5	αα/αα
4	Thai	F	20	9.7	4.48	27.6	64	22.0	35.0	0.4			45	2.3	αα/αα
5	Thai	M	20	12.5	5.97	37.6	65	21.0	33.0	0.6			45	0.8	αα/αα
6	Thai	F		14.2	6.19	43.9	72	22.9	32.3	---			34	---	-α/αα
7	Thai			11.5	5.01	33.5	68	23	34.3	---			37	---	αα/αα
8	Thai			12.8	5.91	39.5	68	21.7	32.4	---			33	---	αα/αα
Mean ± SD				11.7±1.9	5.4±1.0	35.5±6.4	66.5±5.3	21.8±1.5	33.1±1.02	0.82±0.41				2.5±1.7	
Normal Range				15.5±2.5(M)	5.5±1.0(M)	47±7(M)									
Male + Female				14.0±2.5(F)	4.8±1.0(F)	42±5(F)	85±8	29.5±2.5	33±2		13-31	45-72	>15		

a significant degree of anaemia with haemoglobin levels well below the normal range (see Fig. 3.2).

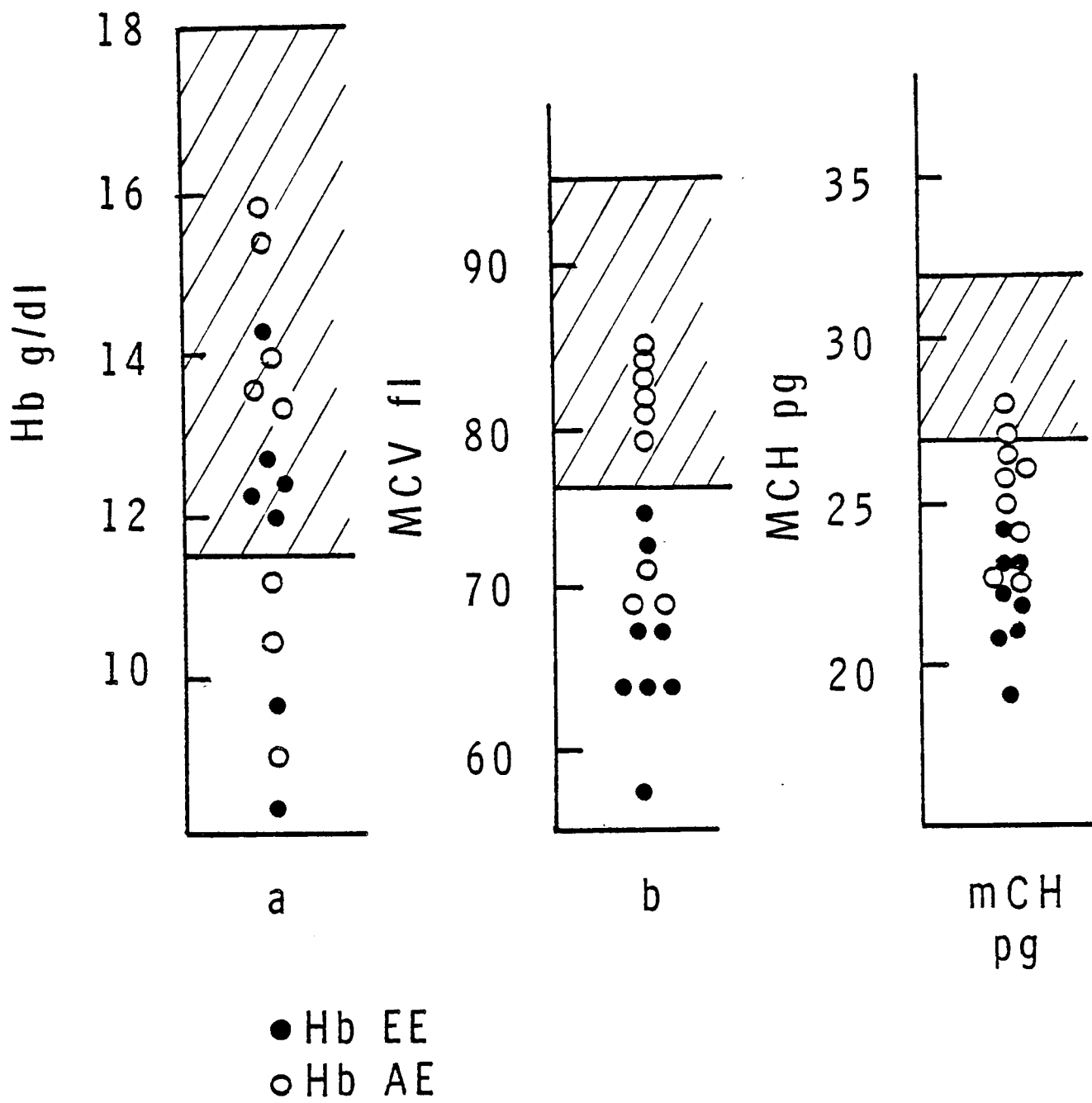
The values for MCV ranged from 57-74 (mean 66.5 ± 5.3) and those for MCH ranged from 19.1-24.0 pg (mean 21.8 ± 1.5) Both of these ranges were well below normal (see Fig. 3.2) and are consistent with the small, poorly haemoglobinised red blood cells seen on the blood film. On the other hand the MCHC in each case was within the normal range or only slightly below.

Reticulocyte counts were available for only 5 cases and all had normal values.

Iron deficiency was excluded in all cases except No. 2 whose serum iron and total iron binding capacity were well below the normal range. The resulting low iron saturation of 12.5% is indicative of iron deficiency and this appears to have further reduced her red cell indices.

Haemoglobin Analysis In all the Hb E homozygotes, Hb E constituted more than 90% of the haemoglobin present. The level of Hb F in the 5 individuals from whom data was available ranged from 0.82-4.54% which generally represented a marginal elevation above the normal range (Table 3.1). The similarity of electrophoretic migration of Hb E with Hb A_2 precluded measurement of the latter.

α-gene Status of Hb E Homozygotes Although this is not strictly a 'haematological parameter' it is



Shaded area represents normal range (From Dacie and Lewis 1975)

FIG. 3.2 Haematological indices in Hb E homozygotes and heterozygotes

considered important to establish the α-gene status of the individuals as far as is possible. As was mentioned in the introduction, the incidence of α-thalassaemia is quite high in the populations of the Indian sub-continent and SE Asia, with some of the highest gene frequencies being found in Thailand. It is virtually impossible to detect an interacting α-thal 1 or α-thal 2 in an individual with homozygous Hb E from consideration of haematological data alone. However, the presence of a deletion α-thalassaemia 2 gene can be demonstrated by the restriction endonuclease mapping of the DNA of the individuals. The principle of the technique is demonstrated in Fig. 3.3a and the results obtained in some of the 8 Hb E homozygotes are depicted in Fig. 3.3b. Only case 6 has an interacting α-thal 2 gene which did not appear to have affected the haematological picture in this patient.

The Oxygen Affinity of a Fresh Sample of Homozygous Hb E Whole Blood

The oxygen affinity was measured, using the Aminco oxygen dissociation analyser, on a fresh whole blood sample from individual number 1 who had 96% Hb E.

As a control, the oxygen affinity was measured in an equally fresh sample of normal whole blood and as the oxygen dissociation curves demonstrate (Fig. 3.4) there was no significant difference in the oxygen affinity of the two samples, which is in agreement with most previous studies (see Chapter 1).

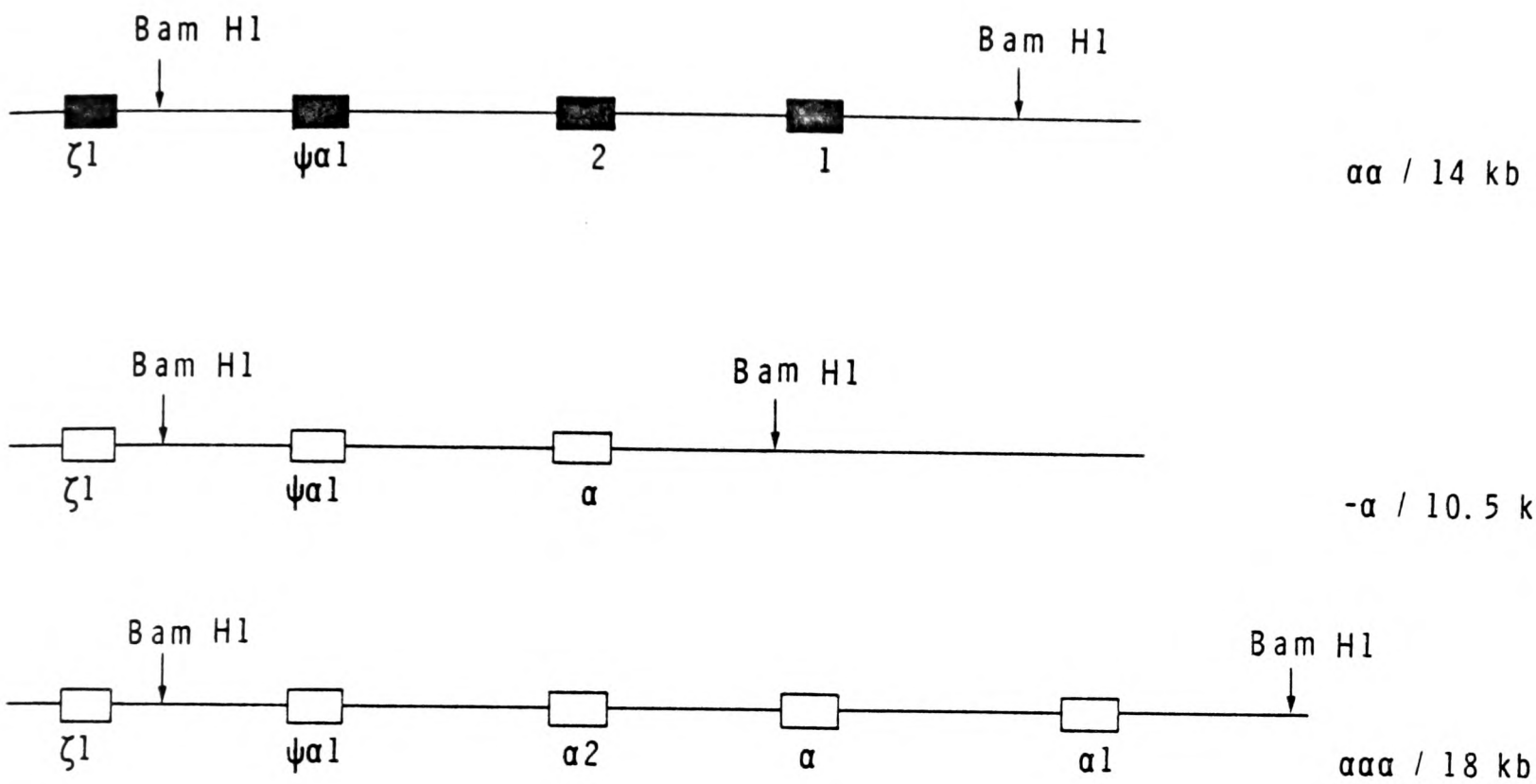


FIG. 3.3a Restriction enzyme Bam Hl - the fragment sizes generated by normal and abnormal α gene arrangements.

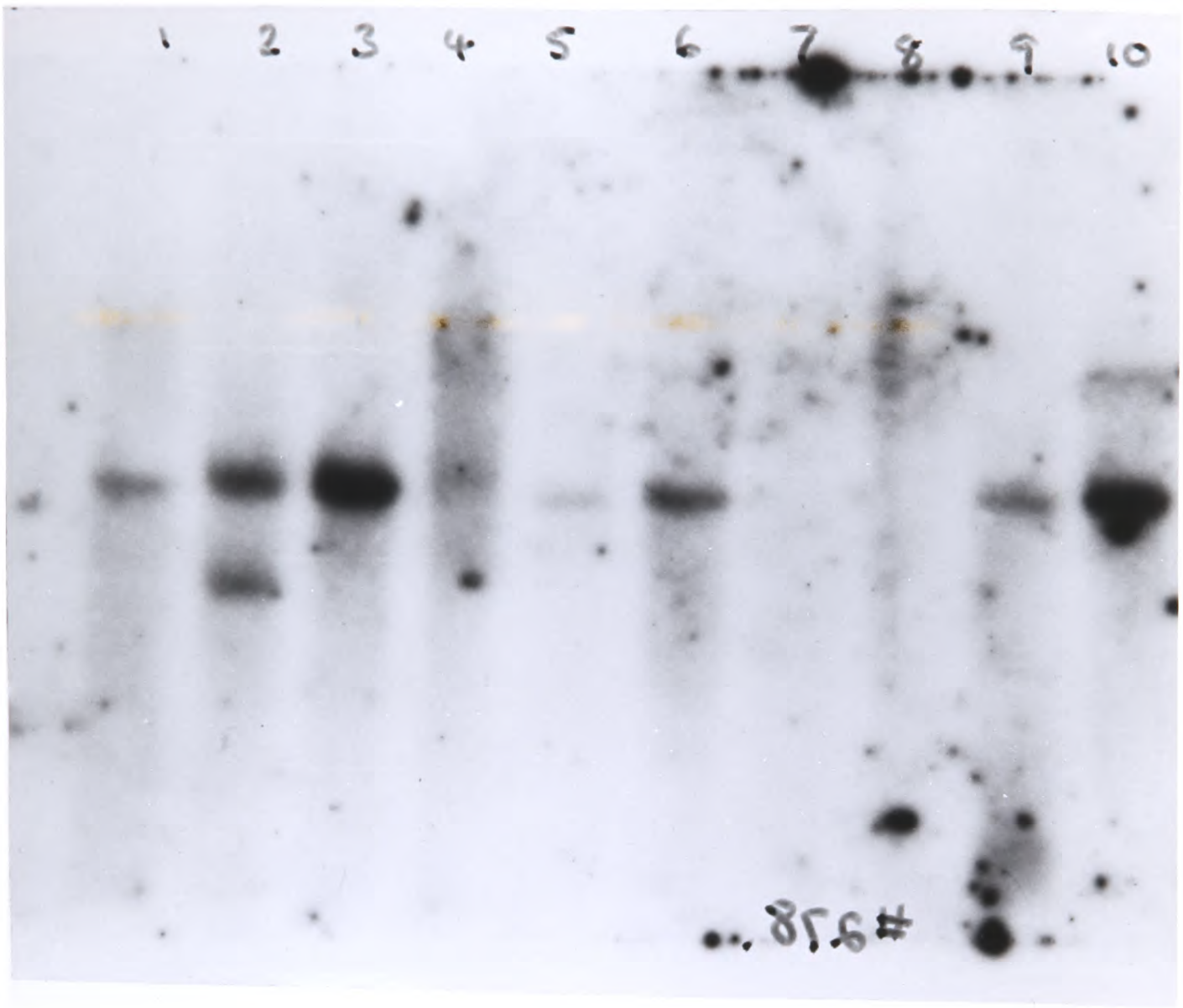


FIG. 3.3b Example of restriction endonuclease analysis of α-gene status in some individuals included in this study:

Track no.	Case no.
1	33
2	6
3	8
4	7
5	20
6	14
7	--
8	--
9	2
10	11

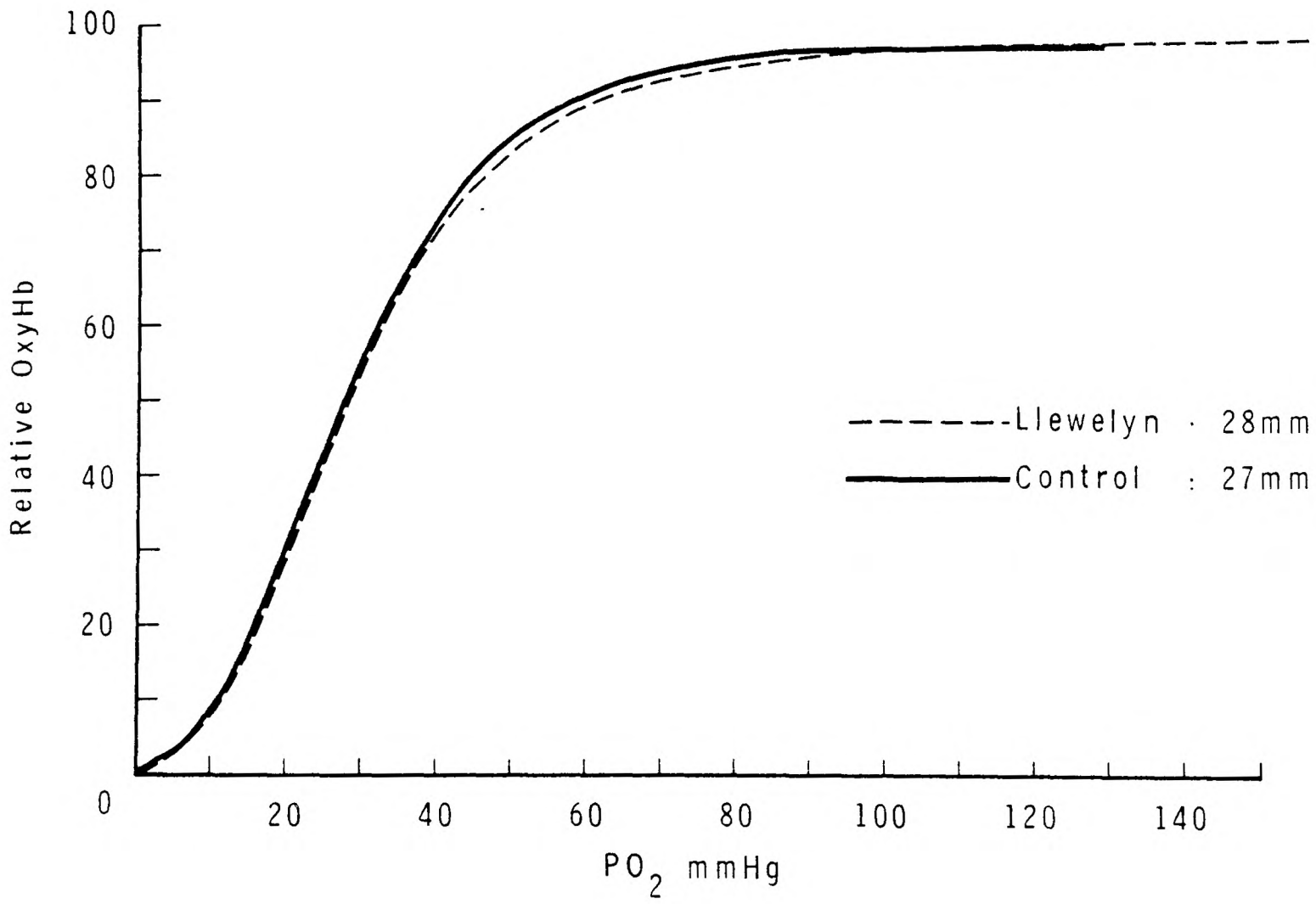


FIG. 3.4 Whole blood oxygen dissociation curve in an AA control and a Hb E homozygote

Solid line - control (Case 35)

Broken line - Hb E homozygote (Case 1)

Heterozygous Hb E

The red cell indices of 10 Hb E heterozygotes are summarised in Table 3.2. The data is not separated according to the sex or age of the individuals. Three of the individuals were from Thailand and the other seven of Indian origin. None of the cases had any clinical symptoms.

In one case (No. 13) a clotted blood specimen prevented the measurement of the red cell indices. Three of the Hb E heterozygotes had reduced haemoglobin levels but those of the four remaining individuals were within the normal range (mean 12.9 $\pm$ 2.3 g/dl) (See Fig. 3.2).

The values for MCV were generally within the normal range (mean 77.6 $\pm$ 5.8 fl) but most of those for MCH were below normal with a mean of 25.4 $\pm$ 1.8 pg (see Fig. 3.2). The MCHC values were within the normal range or very slightly below.

Only one Hb E heterozygote (No. 15) showed reticulocytosis of about twice the normal figure. The remaining individuals had normal reticulocyte counts.

None of the Hb E heterozygotes were iron deficient except case 13 from whom no other haematological parameters were available. The possible significance of iron deficiency on the level of Hb E in her peripheral blood is considered later.

TABLE 3.2 HAEMATOLOGICAL INDICES AND HAEMOGLOBIN ANALYSIS IN Hb E HETEROZYGOTES

INDIVIDUAL	RACIAL ORIGIN	SEX	AGE YRS	Hb g/dl	RBC x $10^{12}/\ell$	Hct	MCV fl	MCH pg/RBC	MCHC g/dl	RETICULOCYTES %	SERUM IRON µm/ℓ	TOTAL IRON BINDING CAPACITY µm/ℓ	% IRON SATURATION	% Hb E	% Hb F	α GENE STATUS	
9	Thai	F	8	13.5	4.98	40.4	81	27.1	33.3	0.83	16.5	60	27.5	28.6	0.8	-α/αα	
10	Indian	M		----	----	----	71	24.0	33.4	----	----	--	----	29.7	1.9	-----	
11	Indian	F	10	13.4	5.86	40.4	69	22.8	33.1	<1.0	19.5	87	22.4	24.2	---	αα/αα	
12	Indian	F	8	13.9	6.11	41.9	69	22.7	33.2	<1.0	31.0	84.0	37.0	24.5	---	αα/αα	
13	Indian	F	~30	----	----	C L O T T E D		S P E C I M E N		----	9.0	66	13.6	24.8	0.51	αα/αα	
14	Indian	M		15.9	5.96	49.1	82	26.6	32.3	<1.0	18.0	78	23	26.9	0.35	αα/αα	
15	Thai	F	52	10.4	3.98	32.5	83	26.0	32.0	2.4			84	28.5	0.37	αα/αα	
16	Thai	F	25	11.1	3.96	33.7	84	28.0	33.0	0.2			65	28.5	0.95	αα/αα	
17	Indian	M	45	15.6	5.96	47.4	80	26.2	32.9	1.0	12.5	64.5	19	28.0	0.38	αα/αα	Normal
18	Indian	F		9.0	3.68	28.3	79	24.9	31.5	----	-----	--	----	----	---	-----	
Mean ± SD				12.9±2.5	5.1±1.0	39.2±7.3	77.6±5.8	25.4±1.8	32.7±0.7					27.0±2.0	0.75±0.6		
Normal Range				15.5±2.5(M)	5.5±1.0(M)	47±7(M)											
Male + Female				14±2.5(F)	4.8±1.0(F)	42±5(F)	85±8	29.5±2.5	33±2		13-31	45-72	>15				

Haemoglobin Analysis The level of Hb F in the Hb E heterozygotes was within the normal range except in case 10, who had 1.9% Hb F. This slightly raised level of Hb F is probably unconnected with the carrier state for Hb E. No family studies were available to exclude the presence of a Swiss HPFH condition.

The level of Hb E in the heterozygotes ranged from 24.2-29.1%. This range is significantly below those values observed in heterozygotes for two other common β chain variants, haemoglobin S and C (Fig. 3.5). In 7 Hb S heterozygotes and 7 Hb C heterozygotes the mean values for the percent abnormal haemoglobin (as measured by the same technique in the same laboratory) were 34.4 $\pm$ 4.1 and 37.0 $\pm$ 3.0 respectively. This proportional difference underestimates the difference between the levels of Hb E and haemoglobins S and C since the MCH is also reduced in Hb E heterozygotes. Allowing for the reduced MCH in the Hb E heterozygotes, these individuals have about 7.0 pg Hb E per cell as opposed to approximately 10.5 pg Hb S and 11.5 pg Hb C in the carriers for these haemoglobins. (It is relevant to note that 7.0 pg Hb E estimated per cell includes about 0.7 pg Hb A_2).

Both iron deficiency (Wasi et al 1968) and α-thalassaemia (Wasi et al 1969; Tuchinda et al 1967, 1964) can reduce the proportions of Hb E in heterozygotes and have to be excluded before conclusions are finally drawn about the level of Hb E in these Hb E heterozygotes.

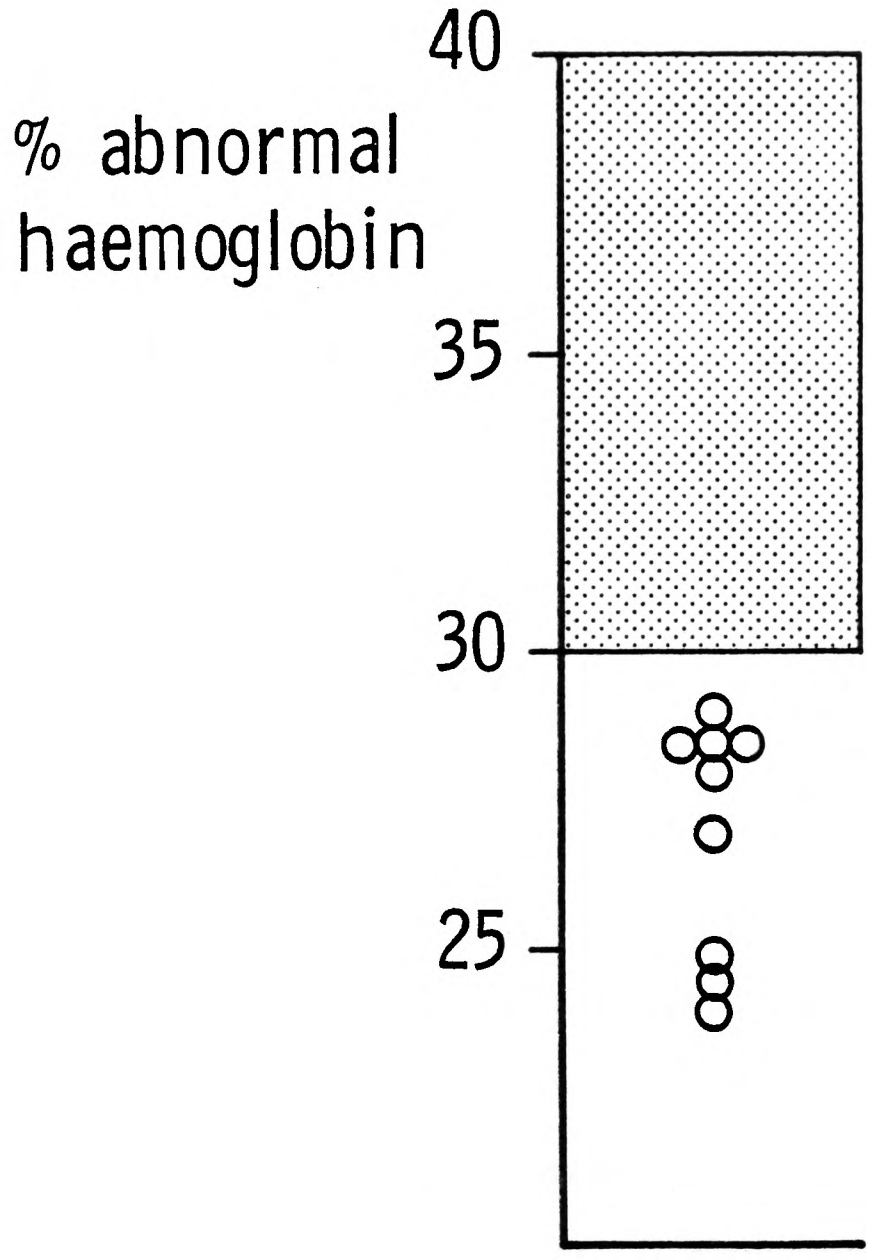


FIG. 3.5 The percentage Hb E in Hb E heterozygotes

Shaded area represents the % abnormal haemoglobin in a series of 14 Hb S and Hb C heterozygotes.

With the exception of case 13, the iron status of the Hb E carriers was normal. The iron deficient individual had 24.8% Hb E, which was not significantly lower than the other cases.

It is not possible to discern if there is interacting α-thal 1 or α-thal 2 with Hb E trait from the haematological indices and haemoglobin analysis. Although Wasi et al (1969) showed that α-thal 1 or 2 in combination with Hb E caused reduction in the level of Hb E from about 30% to approximately 17-25% there was no sharp divide in this range between the levels caused by an interacting α thal 1 or α thal 2 gene.

By restriction endonuclease mapping of the DNA (Fig. 3.3a) the presence of a deletion α-thalassaemia 2 gene was detected only in case No. 9
Even with an interacting α-thal 2 gene the level of Hb E in this individual was not reduced below the value of 25%, demonstrating the difficulty in assessing the presence of interacting α-thalassaemia from haematological data.

The individuals with the lowest Hb E levels (cases 11 and 12 - siblings) were both less than 10 years of age and Fairbanks et al (1979) have observed that children aged less than 10 years tend to have slightly lower levels of Hb E than adults or older children even in the absence of iron deficiency and α-thalassaemia.

Recalculating the levels of Hb E in uncomplicated Hb E trait, excluding cases 9, 11, 12 and 13, gives a mean level of Hb E of 28.2 $\pm$ 0.8 and allowing for the MCH in these individuals there are about 7.5 pg Hb E per cell in Hb E heterozygotes without associated iron deficiency or α-thalassaemia, which is still significantly below the quantities of Hb S and Hb C observed in such heterozygotes.

Haemoglobin E/β-thalassaemia (Hb E Thalassaemia)

Seven cases of Hb E thalassaemia were included in this study. Two were Thai (Mother and son) and the remaining 5 were Indian. The clinical presentation of the disease was seen to vary widely, partly dependent on the type of interacting β-thalassaemia.

Three cases (20, 23 and 26) were transfusion dependent. Globin chain synthesis demonstrated a complete absence of β chain production in case 23 (Hb E/β^{o} thalassaemia), while in the other two cases (20 and 26) a small radioactive peak was observed in the β^{A} chain region. It is not clear whether this represented a small amount of residual β^{A} globin synthesis directed by the β-thalassaemia gene or a contaminating non-globin protein which has been described in cases of β^{o} thalassaemia (J. Longley 1979; Cividalli et al 1979).

The remaining cases were not transfusion-dependent and were considered as cases of thalassaemia intermedia. Cases 28 and 29 were siblings from India in whom Hb E

TABLE 3.3 HAEMATOLOGICAL INDICES AND HAEMOGLOBIN ANALYSIS IN Hb E-β THALASSAEMIA

INDIVIDUAL and GENOTYPE	RACIAL ORIGIN	SEX	AGE	Hb g/dl	RBC $\times 10^{12}/\ell$	Hct	MCV fl	MCH pg/RBC	MCHC g/dl	% RETICULOCYTES	SERUM IRON µm/ℓ	TOTAL IRON BINDING CAPACITY µm/ℓ	% IRON SATURATION	% Hb F	% Hb E
Hb E/β^{+}_{19} thalassaemia	Indian	F	25	9.5	---	---	58	20.6	34.4	4.35	----	----	----	12.0	78.0
Hb E/β_{20} thalassaemia	Indian	M	∿10	----	T R A N S F U S E D					----	17.0	64.5	26.0	----	----
Hb E/β^{++}_{22} thalassaemia	Thai	F	∿30	12.5	5.7	37.9	64	21.6	33.1	3.27	----	----	----	0.8	43.5
Hb E/β^{o}_{23} thalassaemia	Thai	M	∿12	----	T R A N S F U S E D					----	----	----	----	----	----
Hb E/β_{26} thalassaemia	Indian	M		----	T R A N S F U S E D					----	42.5	61.5	69.0	5.0	12.0
Hb E/β^{+}_{28} thalassaemia	Indian	M		7.8	4.03	22.1	55	19.5	35.3	9.0	15.6	33.3	46.8	20.2	64.8
Hb E/β^{+}_{29} thalassaemia	Indian	F		7.0	3.42	20.0	58	20.5	35.0	8.3	15.6	35.4	44.1	20.5	63.0
Normal Range Male + Female				15.5±2.5(M) 14.0±2.5(F)	5.5±1.0(M) 4.8±1.0(F)	47±7(M) 42±5(F)	85±8	29.5±2.5	33±2		13-31	45-72	>15		

was interacting with a severe β^+ thalassaemia gene. Only trace amounts of Hb A (less than 10%) were observed on haemoglobin analysis. Both were mildly affected and had required only occasional transfusions. Their haemoglobin levels were 7.8 and 7.0 g/dl, with low MCVs (55 and 58 fl) and MCHs (19.5 and 20.5 pg) and reticulocyte counts of more than 8%.

The haemoglobin analysis demonstrated approximately 20% Hb F, trace amounts of Hb A and the remainder Hb E in both individuals.

Case 19, from India was pregnant at the time she was studied but was maintaining a haemoglobin level above 9.0 g/dl. She had a low MCV and MCH and a reticulocyte count of only 4.4%. Haemoglobin analysis showed 12% Hb F, 78% Hb E and 10% Hb A.

The remaining case with Hb E/β thalassaemia (no.22) showed only a mild degree of anaemia, (Hb 12.5 g/dl) with reduced MCV and MCH and a slightly raised reticulocyte count of 3.3%. Haemoglobin analysis showed an unusual pattern with a normal Hb F level, 43.5% Hb E, and the remainder Hb A. The unusual haemoglobin pattern and the mild clinical presentation of this individual is probably accounted for by the presence of an α thalassaemia 2 gene as revealed by restriction endonuclease mapping.

The Stability of Hb E

Previous studies have proposed that Hb E is unstable (Feldman and Rieder 1973; Finer et al 1979) and there have been several reports that Hb E is oxidatively unstable in vitro (Frischer and Bowman 1975; Ali et al 1980; Carrell and Lehmann 1981).

To investigate whether instability of Hb E might be responsible for the low levels of Hb E in the red cells, the proportions of Hb E were measured in red cells separated according to age. On centrifugation of a column of packed red cells the older cells, being more dense, are found towards the bottom (Borun et al 1957). Therefore, if Hb E is unstable the levels of Hb E should be lower in the older cells. Packed red blood cells from 2 Hb E heterozygotes (9 and 10) and 2 Hb E/β^+ thalassaemia compound heterozygotes (19 and 22) were spun at 111,700 x g for 30 minutes and the MCV, MCH, MCHC and % Hb E were measured in several fractions down each spin. The results obtained are summarised in Tables 3.4a and 3.4b. In all cases, the MCHC increases from the top to the bottom fractions, indicating that the cells were indeed separated according to their density.

As Table 3.4a and Figure 3.6a show, in Hb E heterozygotes, the amount of Hb E per cell decreased very slightly as the cells aged. In both cases the difference in the amount of Hb E from the youngest to the oldest cells is about 0.4-0.5 pg, representing a loss of about 4-5% of the Hb E initially present in

TABLE 3.4a HAEMOGLOBIN ANALYSIS OF FRACTIONATED Hb E HETEROZYGOTE WHOLE BLOOD

INDIVIDUAL	FRACTION	MCV μm^3/RBC	MCH pg/RBC	MCHC g/dl	% Hb E	pg Hb E
	Whole Blood	81	27.1	33.3	27.4	7.4
	1 (Top)	82	27.0	32.6	29.4	7.9
	2	81	27.1	33.4	29.0	7.9
9	3	80	26.9	33.4	26.8	7.2
	4	79	26.8	33.8	28.2	7.6
	5 (Bottom)	78	27.6	35.0	27.5	7.6
	Whole Blood	71	24.0	33.4	29.4	7.1
	1	74	23.9	32.0	34.4	8.2
	2	72	23.2	31.6	35.9	8.3
10	3	71	24.4	33.9	35.5	8.7
	4	70	24.0	34.0	30.9	7.4
	5	68	23.5	34.3	32.8	7.7

TABLE 3.4b HAEMOGLOBIN ANALYSIS OF FRACTIONATED Hb E/β THALASSAEMIA WHOLE BLOOD

INDIVIDUAL	FRACTION	MCV μm^3/RBC	MCH pg/RBC	MCHC g/dl	% Hb E	pg Hb E	% Hb F
	Whole Blood	58	20.6	34.4	78	16.1	12.0
	1	59	18.7	30.5	86	16.1	7.1
19	2	57	19.8	33.8	81	16.0	10.8
	3	57	20.0	33.9	74	14.8	12.7
	4	55	20.9	36.6	72	15.0	15.1
	Whole Blood	64	21.6	33.1	43.5	9.4	0.75
	1	74	23.2	31.0	44.3	10.4	0.25
22	2	68	22.4	32.6	43.4	9.7	0.34
	3	63	21.2	33.3	45.0	9.5	0.55
	4	61	20.9	33.6	45.1	9.4	0.59
	5	58	20.2	34.2	43.7	8.8	0.76

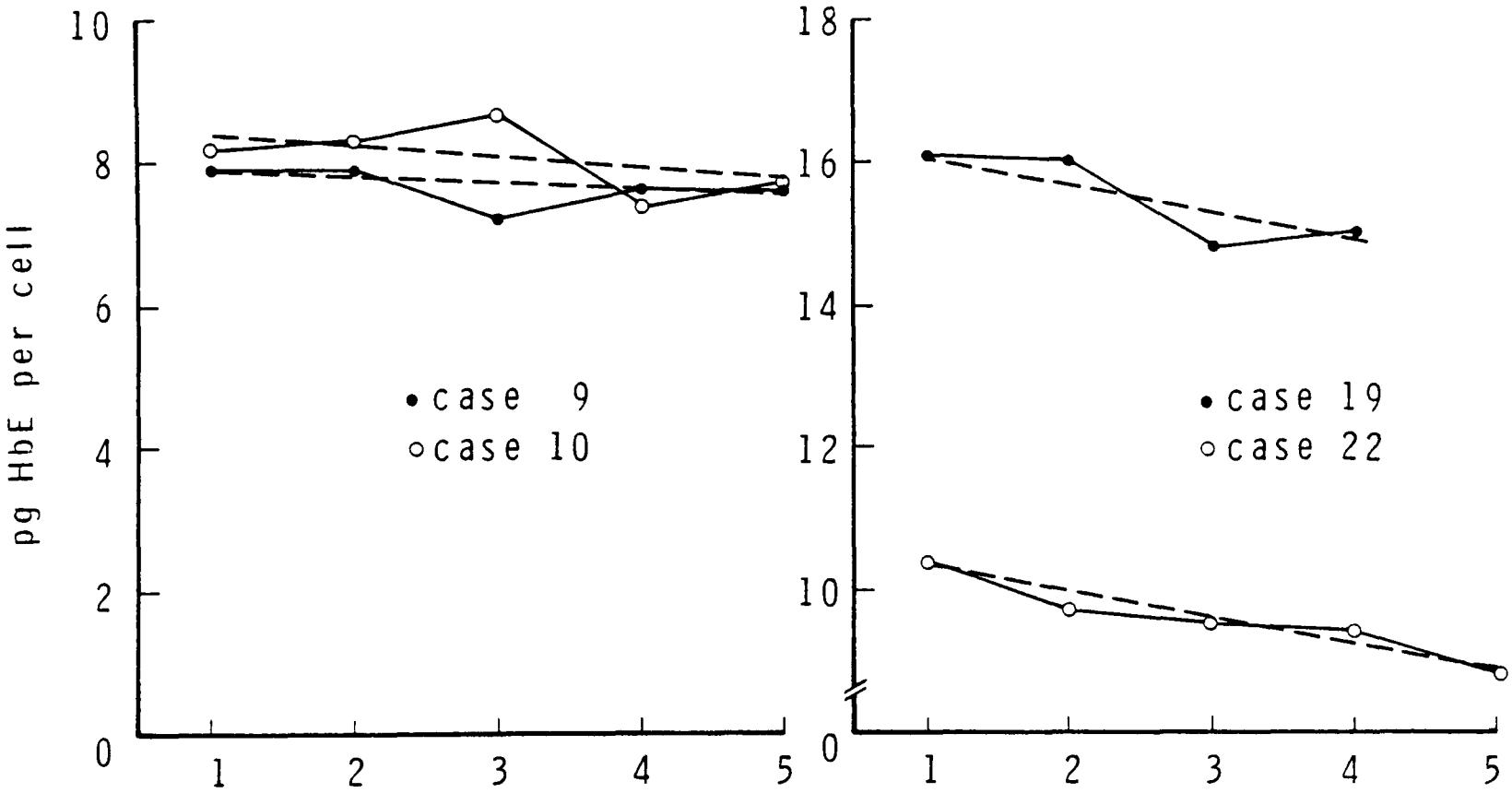


FIG. 3.6 pg Hb E per cell in fractionated peripheral blood cells from two Hb E heterozygotes (cases 9 and 10) and two Hb E-β thalassaemia compound heterozygotes (cases 19 and 22).

the youngest red cells. However, even the youngest cells contain only approximately 8 pg Hb E in these Hb E heterozygotes.

The results observed in the untransfused Hb E/ β^+ thalassaemia cases are summarised in Table 3.4b and illustrated in Fig 3.6b. These cases also demonstrated a decrease in the intracellular levels of Hb E as the red blood cells aged. In case 19 about 1.2 pg (or 7.5%) Hb E was lost between the youngest and oldest cells and in case 22 the loss was 1.5 pg (or 16%). The apparently larger loss in case 22 is infact accounted for by a better separation of the oldest and youngest cells as represented by a larger difference in the MCHC between the top and bottom fractions.

DISCUSSION

Clinical Presentation and Red Cell Indices

The clinical presentation and red cell indices of the 8 individuals with homozygous Hb E demonstrate that it is an extremely mild disorder associated with microcytic, hypochromic red blood cells and sometimes slight anaemia.

These observations are similar to those of previous studies (Chernoff et al 1956; Lehmann et al 1956; Fairbanks et al 1980a) and the red cell indices of the Hb E homozygotes closely resemble those found in β-thalassaemia heterozygotes (see Weatherall and Clegg 1981 and Appendix I).

It is unclear whether or not the anaemia sometimes found in Hb E homozygotes is a result of the homozygous state for Hb E. It has been suggested that anaemia observed in Hb E homozygotes should not be attributed to the haemoglobinopathy but result from iron deficiency or an interacting thalassaemia (Fairbanks et al 1980a). The two anaemic Hb E homozygotes (cases nos. 3 and 4) included in this study do not have iron deficiency and there is no evidence for an interacting α-thalassaemia by gene mapping or by globin biosynthesis (see Chapter 4). (The haematological presentation of Hb E homozygotes is not altered by the presence of α-thalassaemia (Wasi et al 1969)). The possible presence of a mild β-thalassaemia gene in these individuals is excluded by the absence of detectable Hb A or significantly raised levels of Hb F. Therefore the anaemia in these two individuals does not appear to result from interacting iron deficiency or thalassaemia. The data used by Fairbanks et al (1980a)was highly selected and drawn from very diverse sources without appropriate control data. There is too little information about the natural haematological status of Hb E homozygotes to conclude that it has little or no effect on haemoglobin levels.

The red cell indices in the 9 Hb E heterozygotes show that Hb E trait is associated with poorly haemoglobinized, occasionally small, red blood cells.

Previous studies reported similar values for MCV

and MCH in Hb E heterozygotes (Chernoff et al 1956; Pootrakul et al 1978; Fairbanks et al 1979) although in the much larger series of Pootrakul et al and of Fairbanks et al it was demonstrated that the red blood cells in iron replete Hb E heterozygotes are generally smaller than those of the small group of Hb E heterozygotes of this study.

The wide clinical and haematological presentation of the 7 Hb E β-thalassaemia compound heterozygotes included in this study are in accordance with other reported cases of Hb E β-thalassaemia (Chernoff et al 1956; Wasi et al 1969; Mehta et al 1980). Previous studies, in fact, indicate that the interaction of Hb E with the severe form of β^+ thalassaemia generally results in a slightly milder condition than found in Hb E β^o thalassaemia (Wasi et al 1969) although this is not apparent from this study since so few cases were investigated.

In conclusion, the low red cell indices observed in the Hb E homozygotes and heterozygotes suggest that the red blood cells are under-haemoglobinised, although the clinical consequence of this appears to be negligible.

Haemoglobin Analysis

The marginal elevation of Hb F levels observed in several of the Hb E homozgyotes is in agreement with the findings of previous studies (Chernoff et al 1956; Lehmann et al 1956) and once again it is noted that this is also observed in heterozygous

β-thalassaemia (Weatherall and Clegg, 1981).

The Hb E levels in the Hb E heterozygotes corresponded closely to those observed in previous studies (Pootrakul et al 1978; Fairbanks et al 1979; Ali et al 1980). As discussed previously in this chapter the levels of Hb E in Hb E trait are much lower than the levels of Hb S and Hb C in heterozygotes for these haemoglobins.

The relatively reduced levels of Hb E are further illustrated in reports of double heterozygotes for haemoglobins S and E or haemoglobins C and E.

In two compound heterozygotes for Hb S with Hb E, the levels of Hb S were 60% and 64% and those of Hb E were 32% and 35% respectively (Altay et al 1976/7; Schroeder et al 1976/7). In one individual with Hb C and E there was 50% Hb C, 37% Hb E and the remainder was a minor component which was not specified (Schroeder et al 1976/7).

The reduced intracellular levels of Hb E (∿7.5 pg/cell) to levels almost half those expected from gene dosage explains why the red blood cells in Hb E homozygotes and heterozygotes are under haemoglobinized.

The Stability of Hb E

In both the Hb E heterozygotes and the Hb E/β-thalassaemia compound heterozygotes studied there was a slight loss of Hb E as the red cells aged. In the Hb E/β^+ thalassaemia heterozygotes the data are complicated by the selection of red cells with less

chain imbalance, which generally means a higher Hb F content. The cell selection occurs mainly in the bone marrow, (Testa et al 1980) but it continues in the circulation as demonstrated by the slight increase in the Hb F levels in each fraction down the spin. Since the synthesis of γ and β globin chains are reciprocally related (Weatherall et al 1978) the cell selection for high Hb F content is at the expense of Hb E content. Thus the greater decrease in the intracellular Hb E level as the cells age in compound heterozygotes, compared to the loss observed in Hb E heterozygotes is not necessarily solely due to the direct loss of Hb E.

Other reports have suggested that Hb E is unstable and that the degree of instability accounts for the relatively reduced levels of Hb E observed in the red blood cells (Feldman and Rieder 1973; Finer et al 1979). Both groups presented biosynthesis data that demonstrated a greater synthesis of β^{E} globin relative to the level of Hb E in the blood of Hb E heterozygotes but the data presented were rather ambiguous in both cases and in conflict with the biosynthesis data to be presented in Chapter 4 of this study.

Hb E has been observed to be unstable under oxidant stress (Frischer and Bowman 1975; Ali et al 1980; Carrell and Lehmann 1981), the Hb E precipitating when oxidizing agents are added to solutions containing Hb E. This instability appears to be a direct result of the amino acid substitution in the

region of the $\alpha_1\beta_1$ contact (refer to Chapter 1). The postulated tendency for Hb E to dissociate into monomers more readily than Hb A as a result of this substitution (Perutz and Lehmann 1968) may cause sulphydryl groups, normally buried in the $\alpha_1\beta_1$ interface, to become available to react with oxidants. The oxidation of sulphydryl groups results in the decreased solubility of globin subunits, which precipitate.

Even if the Hb E molecule is inately unstable, the loss of Hb E in the circulation is insufficient to account for the large discrepancy between the expected quantities of haemoglobin output directed by one β gene (12-14 pg) and the 6-7 pg observed in the Hb E heterozygotes. It has already been noted that even the youngest red blood cells in the Hb E heterozygotes contain only 8 pg Hb E. Furthermore, the haematological data give no indication of a significant haemolytic component, even in Hb E homozygotes.

In conclusion, the slight instability of Hb E is not a major cause for the low levels of Hb E found in red blood cells.

SUMMARY

The low red cell indices observed in the Hb E homozygotes and heterozygotes suggest that the red blood cells of these individuals are poorly haemoglobinized.

In Hb E heterozygotes it was demonstrated that the level of Hb E in the red blood cells was approximately

half of that expected if the β^A and β^E genes directed equal synthesis of Hb A and Hb E respectively. The reduced levels of Hb E probably account for the low red cell indices and slight anaemia sometimes observed in Hb E disease and Hb E trait. Furthermore the β-thalassaemia-like phenotype of the β^E allele probably explains its often severe interaction with β-thalassaemia in Hb E-β thalassaemia.

The reduced levels of Hb E in red blood cells could be a result of instability of the Hb E molecule. However, although Hb E is slightly unstable in vivo, the loss of Hb E observed in peripheral blood cells as they age is much too small to account for the low levels of Hb E present.

Alternatively, the reduced levels of Hb E could result from reduced synthesis of the haemoglobin tetramer. Its reduced amount could be caused by premature destruction of the β^E globin prior to tetramer formation or by reduced synthesis of β^E globin itself.

The next chapter presents the results of experiments carried out to investigate these possibilities.

CHAPTER 4

THE BIOSYNTHESIS OF β^E GLOBIN

The synthesis of β^E globin and the question of its stability can be investigated by the measurement of globin chain synthesis during in vitro time course incubations of reticulocytes from individuals with Hb E. The experimental procedures used were described in Chapter 2.

The incubation of normal reticulocytes with tritiated leucine for periods ranging from a few minutes to several hours generally produces equal radioactive incorporation into α and β^A chains such that the α/β^A total counts ratio is unity at any time (see Fig 4.1a). Since the red cells contain equal amounts of α and β^A chains the specific activities (cpm/mg protein) of the α and β^A chains are equal.

If β^E globin is synthesised at reduced levels then the α/non α globin counts ratios in reticulocytes of Hb E homozygotes and heterozygotes should be greater than unity. If the β^E globin is unstable such that some of the newly synthesised β^E chains are degraded before they are incorporated into haemoglobin tetramers, then the synthesis of β^E globin will exceed the proportion present within the red cell and its specific activity should be greater than that of the α and/or β^A chains. This would be most marked after short incubation periods (when the rate of synthesis

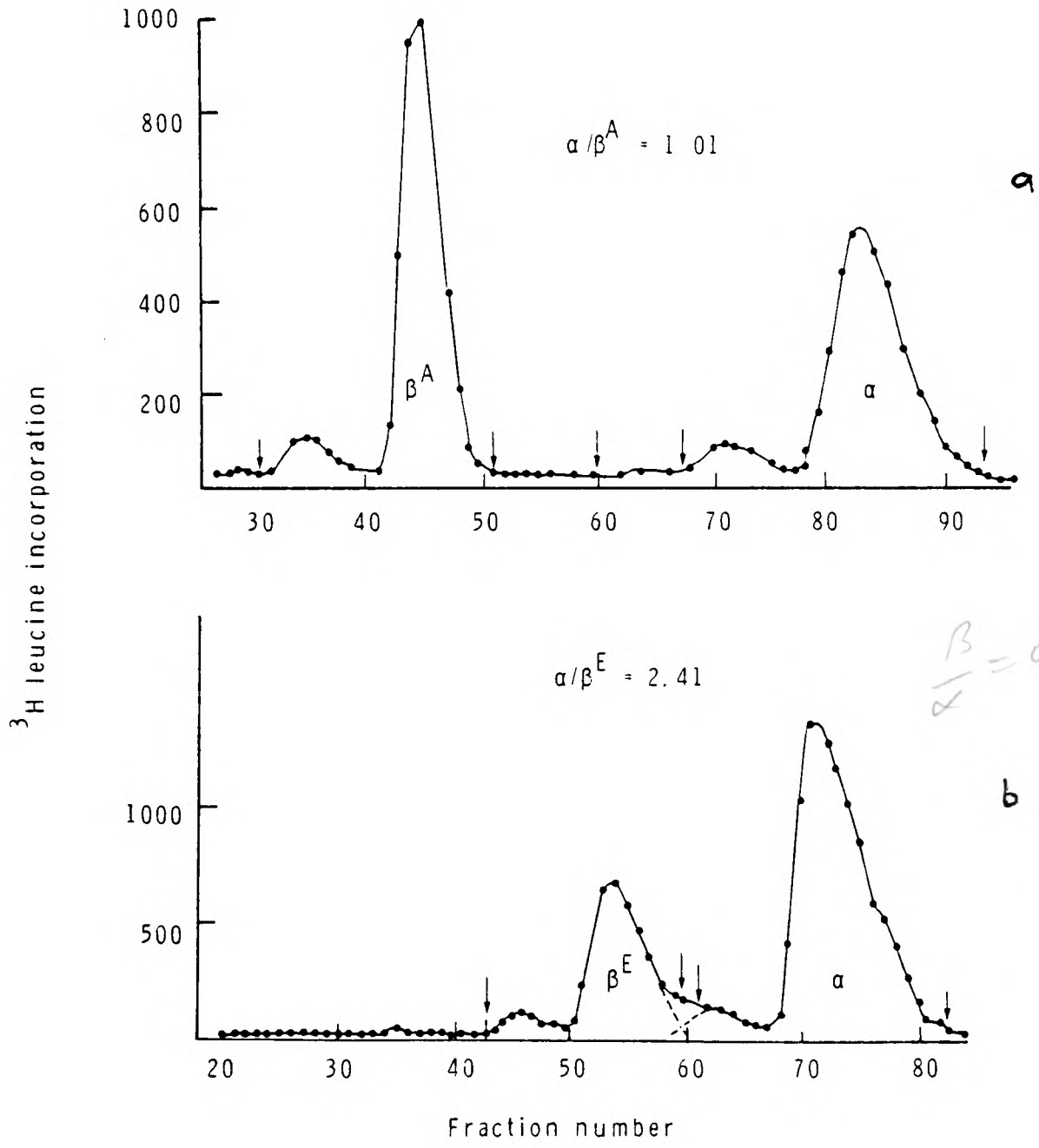


FIG. 4.1a and b

The incorporation of ^{3}H leucine into globin chains during a 60 minute incubation of reticulocytes from

a) A normal (case 35) and
b) A Hb E homozygote (Case 5)

may exceed degradation) but diminish with increasing time as a steady state is achieved.

As discussed in Chapter 2, the conditions of in vitro reticulocyte incubations have to be well defined, and in practice the α/β^A ratios observed in normal individuals range from about 0.80-1.20. Thus it may be difficult to detect small deviations from the expected ratio of 1.0 in any individual experiment. Therefore, control incubations of reticulocytes from normal individuals were carried out in parallel with each incubation (or group of incubations) of samples containing Hb E.

RESULTS

The Biosynthesis of β^E Globin in Peripheral Blood Reticulocytes.

Incubations of 1 hour Duration

The reticulocytes from 5 Hb E homozygotes, 9 Hb E heterozygotes and 7 Hb E-β-thalassaemia compound heterozygotes were incubated for 1 hour under the standard conditions described in Chapter 2. The results obtained are summarized in Tables 4.1a, b and c.

A globin chain separation from each of a typical Hb E homozygote, heterozygote and Hb E-β-thalassaemia compound heterozygote are shown in Figure 4.1b, c and d. As the radioactive counts profile illustrates, the radioactive incorporation into each globin peak was much greater than the background counts. Furthermore, each globin peak was well-defined, although there

BIOSYNTHESIS DATA IN Hb E RETICULOCYTES AFTER 1 HOUR INCUBATION

TABLE 4.1a Hb E HOMOZYGOTES

INDIVIDUAL	TOTAL COUNTS RATIO α/β^E	SPECIFIC ACTIVITY RATIO α/β^E
1	2.36	2.25
2	2.34	2.41
3	2.42	2.06
4	3.31	2.99
5	2.41	2.67
Mean $\pm$ S.D.	2.57 $\pm$ 0.42	2.48 $\pm$ 0.36

BIOSYNTHESIS DATA IN Hb E RETICULOCYTES AFTER 1 HOUR INCUBATION

TABLE 4.1b Hb E HETEROZYGOTES

INDIVIDUAL	TOTAL COUNTS RATIOS			SPECIFIC ACTIVITY RATIOS		
	$\alpha/\beta^A+\beta^E$	α/β^E	β^A/β^E	α/β^A	α/β^E	β^A/β^E
9	0.85	3.02	2.19	0.86	1.39	1.56
11	1.47	4.03	1.75	1.54	1.86	1.20
12	1.50	4.07	1.71	1.58	1.75	1.11
13	1.23	4.38	2.55	1.20	1.54	1.38
14	1.54	5.08	2.30	1.71	2.15	1.25
15	1.57	4.74	2.02	1.34	1.53	1.15
16	1.25	4.27	2.64	0.90	1.37	1.51
17	2.19	6.38	1.91	2.04	2.06	1.00
18	1.41	4.99	2.55	1.52	1.60	1.10
Mean$\pm$ SD*	1.52$\pm$0.30	4.74$\pm$0.77	2.18$\pm$0.38	1.48$\pm$0.34	1.73$\pm$0.27	1.21$\pm$0.16

* Omitting Case 9

BIOSYNTHESIS DATA IN Hb E RETICULOCYTES AFTER 1 HOUR INCUBATION

TABLE 4.1c Hb E-β THALASSAEMIA COMPOUND HETEROZYGOTES

INDIVIDUAL	TOTAL COUNTS RATIOS		SPECIFIC ACTIVITY RATIOS
	α/non α	α/β^E	α/β^E
19	1.92	2.23	1.43
20	2.76	3.21	Transfused
22	1.85	2.76	1.33
23	3.03	4.08	Transfused
26	2.63	3.68	Transfused
28	3.30	4.31	3.19
29	2.70	3.17	3.37
Mean ± SD	2.60 ± 0.54	3.35 ± 0.73	2.33 ± 1.10

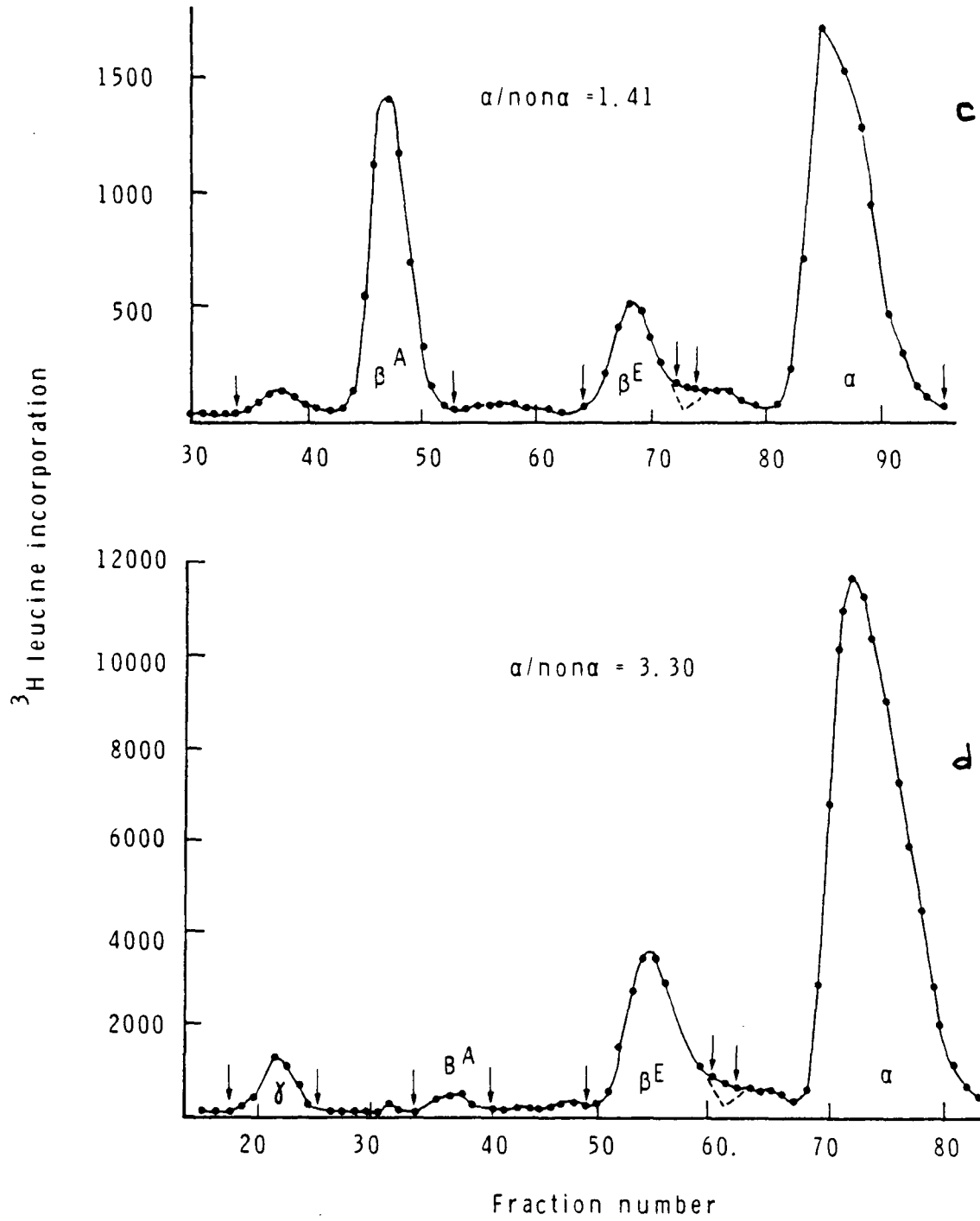


FIG. 4.1c and d

The incorporation of ^{3}H leucine into globin chains during a 60 minute incubation of reticulocytes from

c) A Hb E heterozygote (Case 13) and
d) A Hb E β thalassaemia compound heterozygote (Case 28)

was slight overlap between the end of the β^E peak and the beginning of the pre-α peak and some judgement was required in allocating the counts in this region to one or other of the peaks (Figure 4.1). The degree of error introduced into the calculation of the α/non α globin counts ratios by this is estimated to be very minor since the number of counts in the fractions which are allocated in this way are never more than 10% of the total radioactivity in either peak.

In the normal control individuals the total α/β^A total counts ratios ranged from 0.92-1.14 (mean 1.01 $\pm$ 0.06) (see Appendix III and Figure 4.2).

However the α/β^E counts ratios in the 5 Hb E homozygotes (cases 1-5) ranged from 2.34-3.31 (mean 2.57 $\pm$ 0.42) (Figure 4.2). These ratios represent marked chain imbalance and are in fact comparable to the α/non α counts ratios of 1.91, 2.57 and 2.22 observed in 3 β-thalassaemia heterozygotes, (cases 21, 25 and 27) that were incubated as part of family studies (Appendix I).

In the 9 Hb E heterozygotes the $\alpha/\beta^A+\beta^E$ total counts ratios ranged from 0.85-2.19 (Figure 4.2). The ratio of 0.85 (Case 9) was the only ratio found to be within the normal range and as already discussed in Chapter 3, this individual had an interacting α-thal 2 gene. If the ratio from this individual is excluded, the $\alpha/\beta^A+\beta^E$ counts ratios in the other 8 Hb E heterozygotes ranged from 1.23-2.19

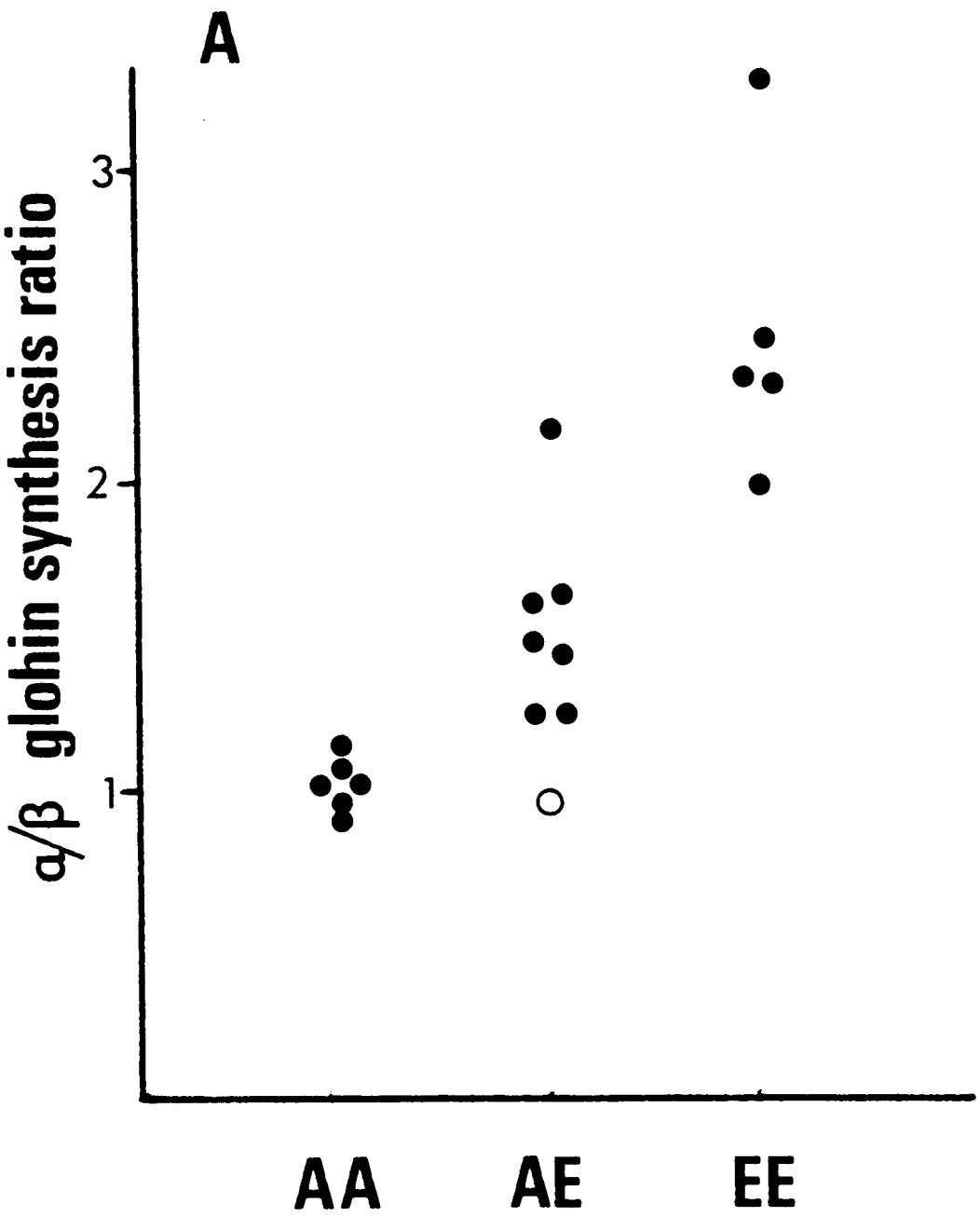


FIG. 4.2 Globin chain synthesis ratios in peripheral blood reticulocytes in normal (AA), heterozygous Hb E (AE) and homozygous Hb E (EE) individuals measured after 60 minute incubation with ^{3}H leucine.

The open symbol represents the result from a Hb E heterozygote (Case 9) who had one out of four α genes deleted.

(mean 1.52 ± 0.28). In every case the α/β total counts ratio of the respective normal sample incubated in parallel was lower. The α/β^A counts ratios (mean 2.14 ± 0.54) are greater than the α/β^E counts ratios (mean 4.74 ± 0.77) indicating that it is the β^E chain that is synthesised in deficient quantities, and so causing the chain imbalance observed. These data corroborate those observed in the Hb E homozygotes.

All of the 7 Hb E/β thalassaemia compound heterozygotes demonstrated chain imbalance after a 1 hour incubation with the α/non α total counts ratios ranging from 1.90-3.30 (Table 4.1c). The value of 1.90 was observed in case 22 who had an α-thal 2 in addition to a mild β-thalassaemia and Hb E. Excluding this value the range in the remaining 6 individuals was 2.18-3.30. As a result of the variable expression of the interacting β-thalassaemia in these individuals, the increased γ chain synthesis and the cell selection that occurs in the bone marrow, it is more meaningful to assess the α/β^E total counts ratios which ranged from 2.71-4.31 (mean 3.35 ± 0.73) (Table 4.1c) and were comparable to those observed in the Hb E heterozygotes.

Time Course Incubations

The 60 minute incubations of reticulocytes described above indicate that there is reduced synthesis of the β^E chain. However, the above experiments do not exclude the possibility that the β^E chains are synthesised at normal levels but that some of the β^E chains are rapidly destroyed before they are able to associate

with α chains to form stable Hb E tetramers. Previous studies have proposed that α chains and β^E globin chains do not associate as readily as α and β^A chains (Tuchinda et al 1964; Pagnier et al 1974) and it is possible that any β^E chains that are not immediately incorporated into Hb E are degraded.

Peripheral blood reticulocytes were incubated as for the 1 hour incubations but samples of cells were removed after much shorter times as the incubation proceeded (Tables 4.2a, b and C).

Table 4.2a summarizes the results obtained in 3 Hb E homozygotes (cases 1, 2 and 3). The α/β^E counts ratios and specific activity ratios did not alter significantly throughout the incubations of all three homozygotes. Even at very early times of incubation the specific activity of the β^E chain was always less than that of the α chain in each case (Figure 4.3).

Similarly in 2 of 3 Hb E heterozygote time course incubations (individuals 9 and 13) the α/β^E and β^A/β^E total counts ratios did not alter significantly between each time point (Fig. 4.4). In addition the α/β^E and β^A/β^E specific activity ratios were relatively constant throughout and they were never less than unity (Table 4.2b).

In Hb E heterozygote no. 15 the α/β^A and α/β^E specific activity ratios were less than unity at 2 minutes (Table 4.2b), but, there was so little

TABLE 4.2a TIME COURSE INCUBATIONS OF Hb E HOMOZYGOTE RETICULOCYTES

INDIVIDUAL	INCUBATION TIME (MINS)	TOTAL COUNTS RATIO α/β^E	SPECIFIC ACTIVITY RATIO α/β^E
1	10	2.36	2.49
	20	2.40	2.41
	60	2.36 .42	2.25
2	2	2.26	2.37
	10	2.83	2.81
	60	2.34 .42	2.41
3	2	----	2.46
	10	2.65	2.34
	60	2.42 .41	2.06

.42 ± .004

TABLE 4.2b TIME COURSE INCUBATIONS OF Hb E HETEROZYGOTE RETICULOCYTES

INDIVIDUAL	INCUBATION TIME (MINS)	TOTAL COUNTS RATIO $\alpha/\beta^A+\beta^E$	β^A/β^E	SPECIFIC ACTIVITY RATIO α/β^E		β^A/β^E
9	5	UNSATISFACTORY CHAIN SEPARATION				
	10	1.01	2.22	1.39		1.28
	20	0.99	2.14	2.05		1.93
	60	0.85	2.19	1.34	.74	1.56
13	5	1.26	2.56	1.54		1.10
	20	1.31	2.39	1.52		1.37
	60	1.23	2.55	1.54	.65	1.38
15	2	COUNTS TOO LOW		0.58		0.43
	10	COUNTS TOO LOW		1.30		1.00
	60	1.57	2.02	1.53	.65	1.15

.68 ± .05

TABLE 4.2c TIME COURSE INCUBATIONS OF Hb E-β THALASSAEMIA RETICULOCYTES

INDIVIDUAL	INCUBATION TIME (MINS)	TOTAL COUNTS RATIO α/non α	TOTAL COUNTS RATIO α/β^E	SPECIFIC ACTIVITY RATIO α/β^E
19	5	2.32	3.09	1.99
	10	2.57	3.18	1.69
	20	1.90	2.35	1.53
	60	1.92 .52	2.23 .45	1.43
22	5	1.93	Not good β^E/ pre α separation	1.43
	10	1.95	2.92	1.75
	20	1.88	2.84	1.44
	60	1.85 .54	2.76 .36	1.33
23	5	3.06	4.14	Transfused
	10	3.30	4.53	Transfused
	20	3.16	4.21	Transfused
	60	3.03 .33	4.08 .25	Transfused
		.46 ± .12	.35 ± .10	

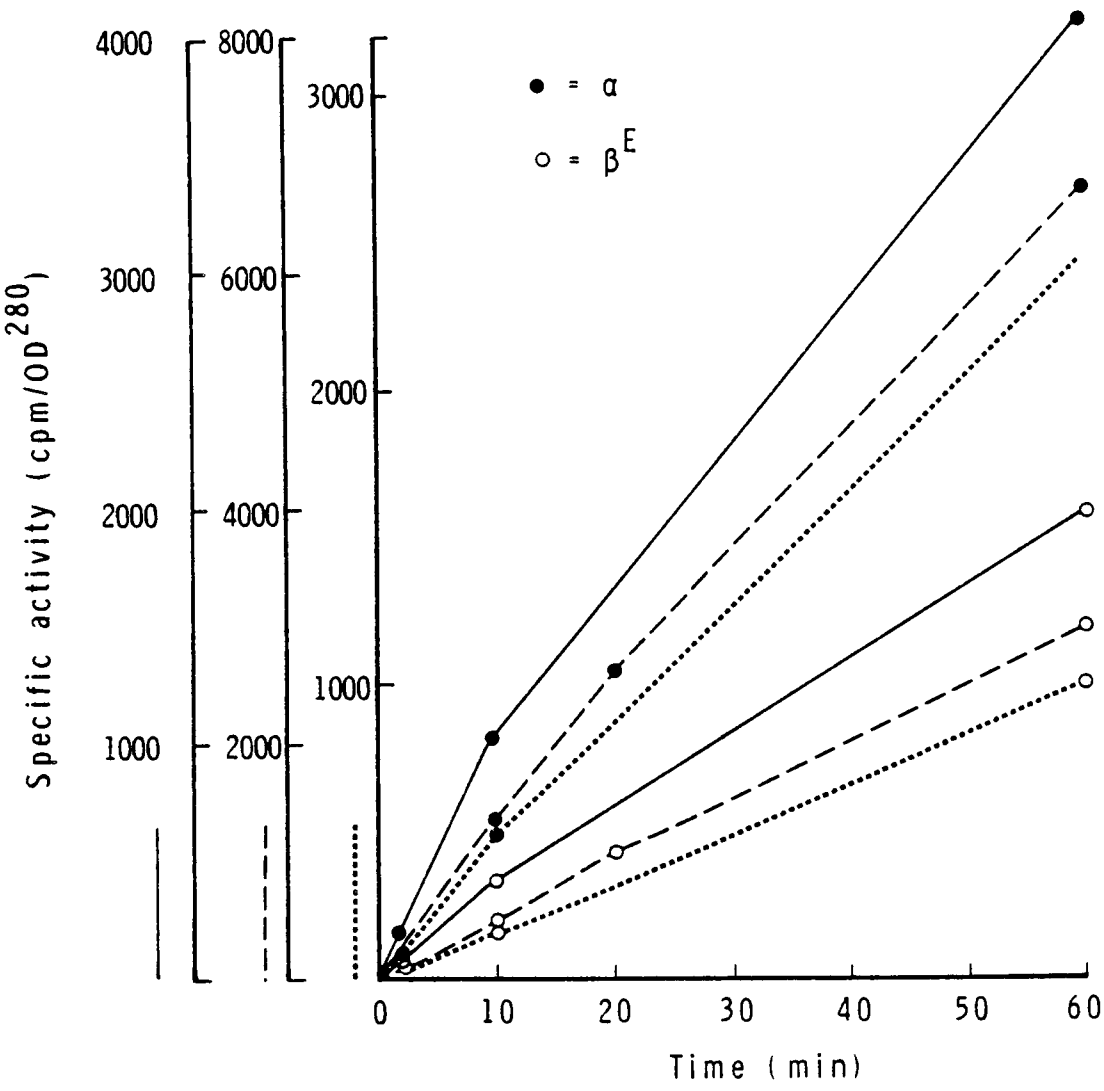


FIG 4.3 The specific activities of globin chains synthesised during time course incubations of homozygous Hb E reticulocytes

---------- Case 1
.... Case 2
_____ Case 3

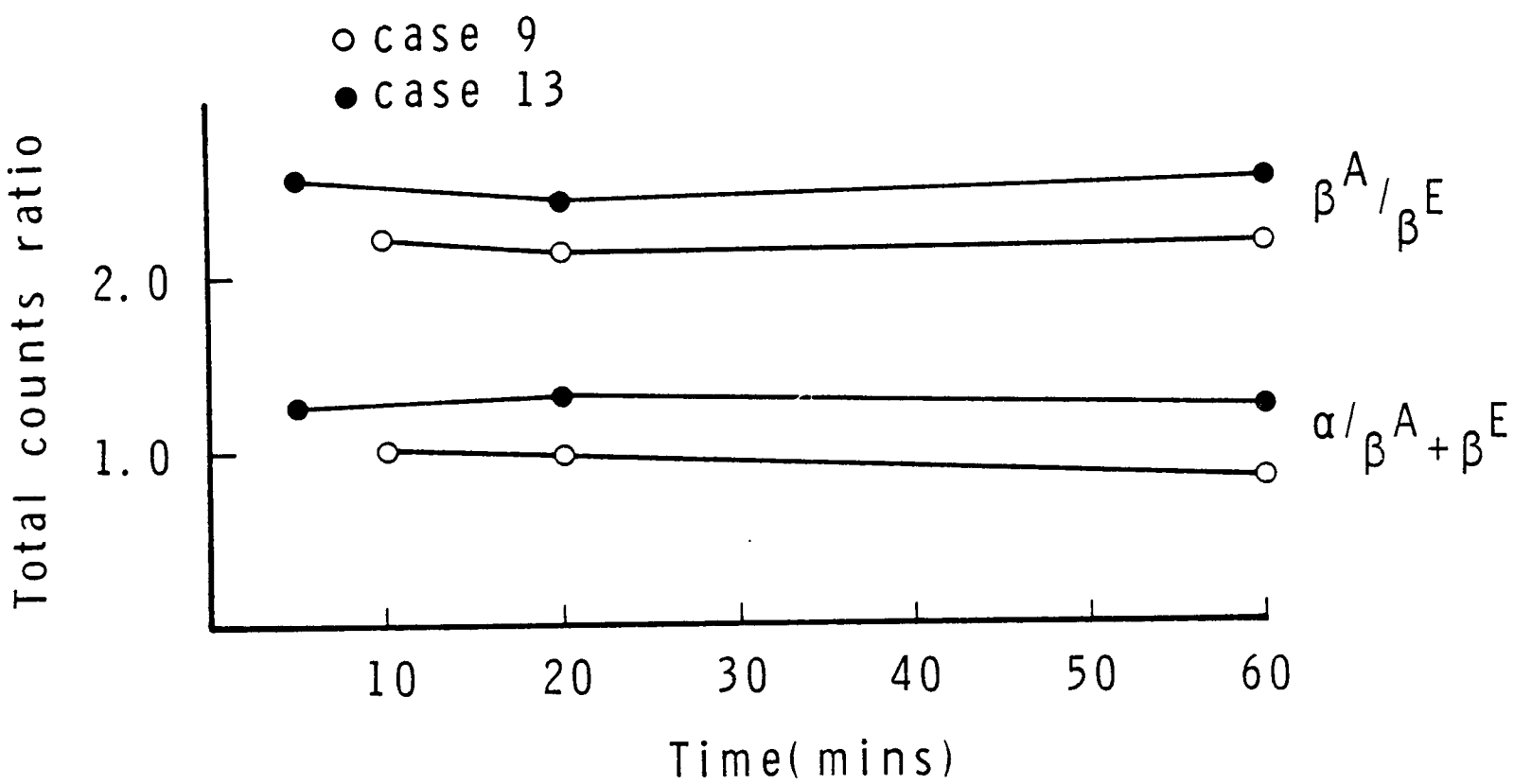


FIG. 4.4 Globin chain total counts ratios during time course incubations of two Hb E heterozygotes.

radioactive incorporation into the chains after 2 (and 10) minutes, that the specific activities were probably unreliable.

In 3 Hb E/β thalassaemia compound heterozygotes, the α/non α and α/β^E specific activity ratios were always greater than unity even at the earliest times of incubation (Table 4.2c).

Therefore, these times course experiments provide no evidence for the destruction of β^E globin, even very shortly after its synthesis, and the imbalanced α/non α globin chain ratios observed appear to be the result of reduced synthesis of β^E globin in the reticulocytes.

<u>The Synthesis of β^E Globin during Erythroid Cell Maturation</u>

In the Hb E heterozygotes the β^A/β^E chain specific activity ratio was greater than 1.0 in almost every case (Refer to Table 4.2b). As stated at the beginning of this chapter, the synthesis of normal α and β^A chains in reticulocytes is synchronous and proportional to the level of Hb A accumulated in the red blood cells. Consequently the α/β^A specific activity ratio in normal reticulocytes is 1.0. However, the β^A/β^E specific activity ratios were consistently greater than 1.0 in the Hb E heterozygotes and yet there was no evidence of β^E chain degradation. This raises the possibility that the synthesis of β^E chains may decrease as the red cells age and that the proportion of β^E synthesis may be greater in bone marrow normoblasts

than in reticulocytes.

To investigate this possibility, bone marrow samples from 3 Hb E homozygotes and 2 Hb E heterozygotes were incubated for 1 hour together with peripheral blood samples from the same individuals. Figures 4.5a and b depict typical bone marrow globin chain separations in a homozygous and heterozygous individual, demonstrating good radioactive incorporation into the globin chains, clear separation of the globin peaks and negligible radioactive background (the globin was partially purified by gel filtration, prior to separation of CM-23 cellulose, as described in Chapter 2).

The α/β^{E} total counts ratios observed in the 3 Hb E homozygotes (cases 3, 4 and 5) are summarised in Table 4.3a. After 1 hour incubation the α/β^{E} total counts ratios were lower in the bone marrow than in the peripheral blood (Fig. 4.6) in every case, suggesting that either the β^{E} chain was synthesised in greater quantities in the bone marrow red cell precursors or the excess α chains synthesised were more effectively degraded in the marrow cells. A time course experiment in Case 3, however, showed a constant α/β^{E} ratio in incubations ranging from 2 to 60 minutes (Table 4.3a), providing no evidence for substantial α chain proteolysis in the red cell precursors in this case.

The results obtained in 2 Hb E heterozygotes (15 and 16) are summarised in Table 4.3b and again

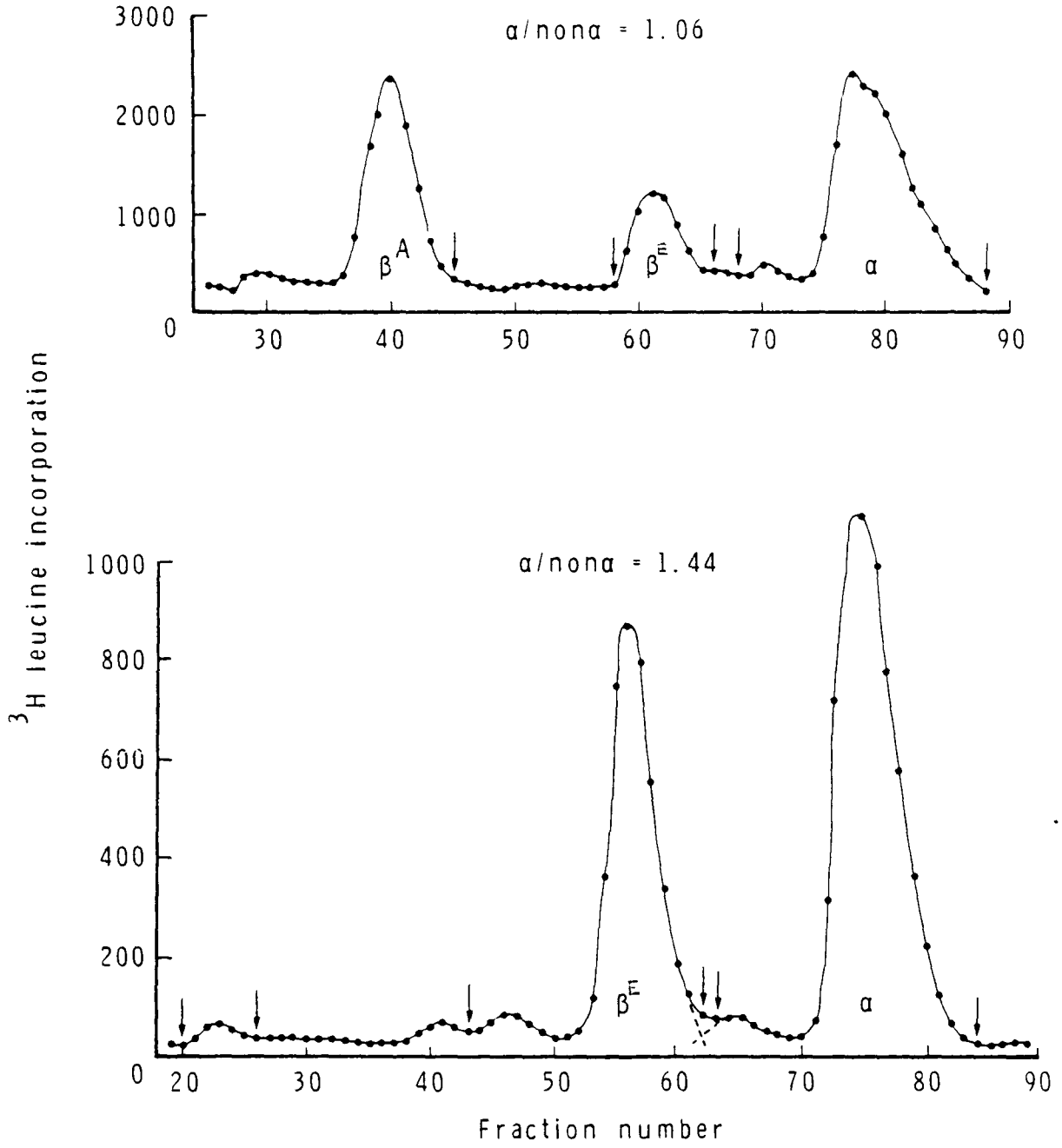


FIG. 4.5 The incorporation of ^{3}H leucine into globin chains during a 60 minute incubation of bone marrow from

a) A Hb E heterozygote (Case 15) and
b) A Hb E homozygote (Case 3)

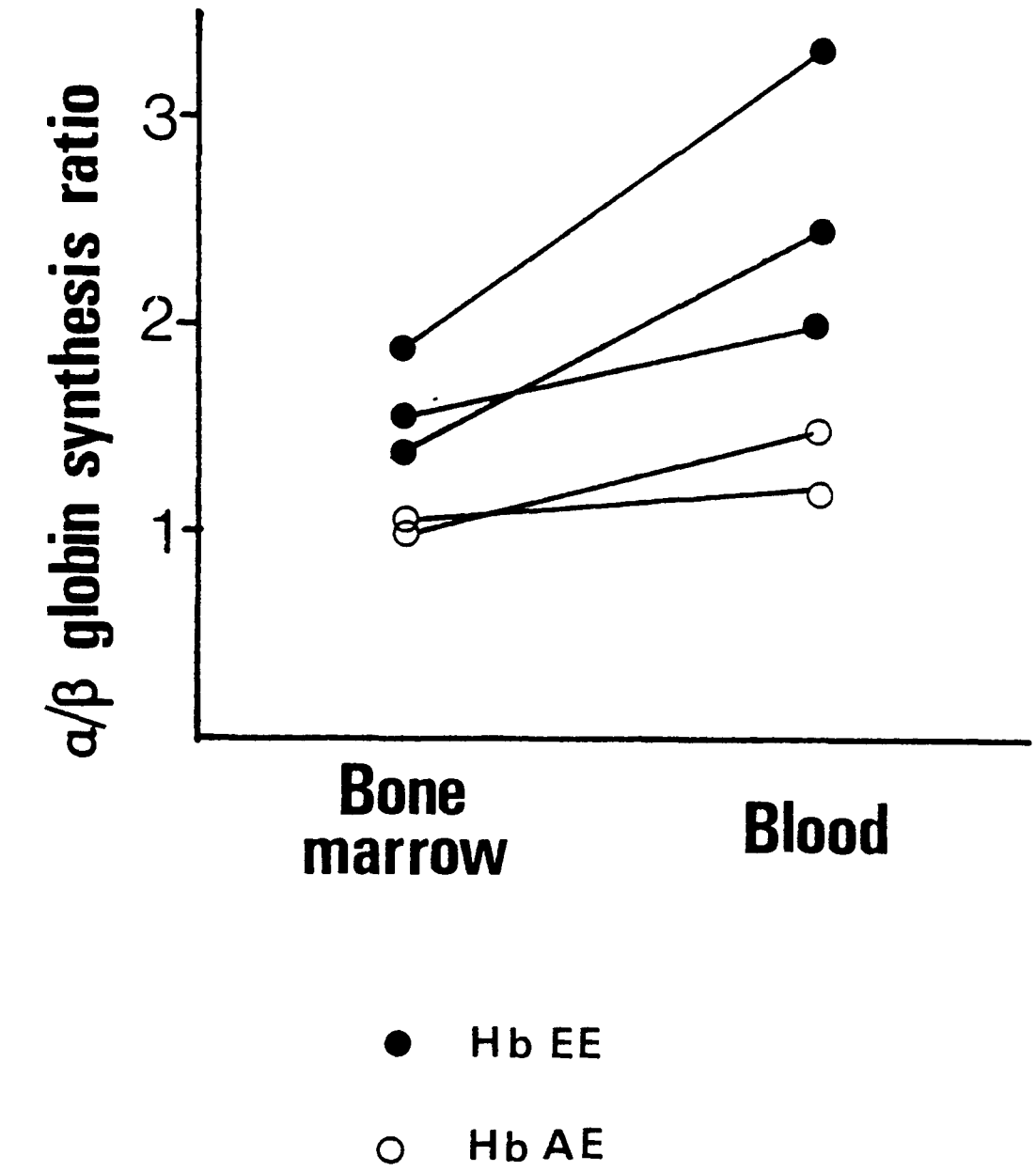


FIG. 4.6 Comparison of the α/β globin chain synthesis ratios in peripheral blood reticulocytes and bone marrow in 3 Hb E homozygotes (●) and two Hb E heterozygotes (O).

TABLE 4.3a BONE MARROW INCUBATIONS IN Hb E HOMOZYGOTES

INDIVIDUAL	INCUBATION TIME (MINS)	TOTAL COUNTS RATIO α/β^E		SPECIFIC ACTIVITY RATIO α/β^E
4	60	1.74	.57	1.55
5	60	1.40	.71	1.61
3	2	1.56		1.14
	10	1.60		1.11
	60	1.49	.67	1.19
			.65±.07	

TABLE 4.3b BONE MARROW INCUBATIONS IN Hb E HETEROZYGOTES

INDIVIDUAL	INCUBATION TIME (MINS)	TOTAL COUNTS RATIO $\alpha/\beta^A + \beta^E$	β^A/β^E	SPECIFIC ACTIVITY RATIO β^A/β^E
16	60	0.94	2.12	1.1
15	2	0.94	2.76	0.85
	10	1.10	2.27	0.88
	60	1.06	2.28	0.91

show that the α/non α globin chain synthesis was more balanced in the bone marrow than in the peripheral blood after 60 minutes of incubation.

The time course incubation of 1 Hb E heterozygote bone marrow (number 15) demonstrated constant α/non α counts ratios throughout, corroborating the results observed in the Hb E homozygote. More importantly, the β^A/β^E specific activity ratios in the 2 Hb E heterozygotes were greater in the peripheral blood than in the respective bone marrows.

In other words the proportion of β^E globin synthesis was greater in the marrow than in the peripheral blood when measured in a situation where α-chain proteolysis was irrelevant.

In conclusion, all these results are consistent with a decreasing rate of β^E globin synthesis as the erythroid cells mature.

To return to the question of the stability of the β^E chain, it is noted that the β^A/β^E specific activity ratios in the bone marrow time course incubation of case 15 did not change significantly between 2 and 60 minutes of incubation, providing further evidence against rapid degradation of the β^E chain, even in the red blood cell precursors.

Characterisation of Free α Chain Pools in Reticulocytes

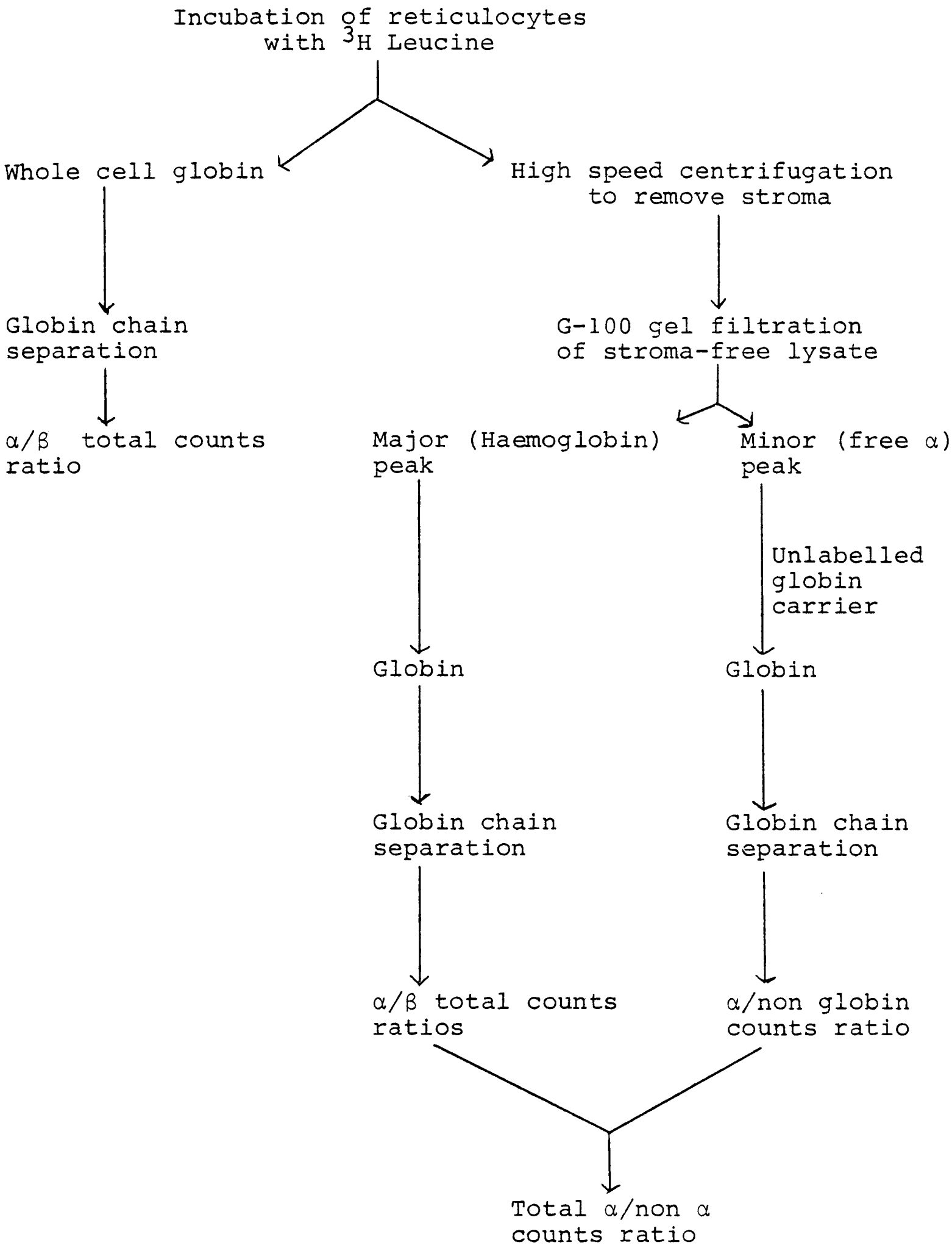
The chain imbalance observed in the peripheral blood reticulocytes of Hb E homozygotes and heterozygotes suggests that there should be a greater free α chain

pool than that found in the reticulocytes of normal individuals in which α and β chain synthesis is essentially balanced. Substantial free α chain pools have been observed in the peripheral blood reticulocytes of individuals with various forms of β-thalassaemia (Huehns and Modell 1967; Bank 1968; Weatherall et al 1969; Clegg and Weatherall 1972) and although some of the excess α chains are rapidly removed from the free α-pool (Weatherall et al 1969) there should be a detectable pool of free α chains in homozygous and heterozygous Hb E reticulocytes that have been incubated for a short period of time.

The method commonly used to investigate the possible presence of a pool of free α-chains in red cells is outlined in Figure 4.7.

Gel filtration (which separates on the basis of molecular weight differences) of stroma-free lysate from normal blood previously incubated for 1 hour demonstrates a major peak of radioactivity which coincides with the haemoglobin optical density (OD) peak, followed by a small shoulder of radioactive counts representing the small pool of free α-chains present in normal reticulocytes (Weatherall et al 1969). This pattern was observed in two normal individuals in this study and in 4 Hb E heterozygotes (9, 11, 12 and 14) (Figures 4.8a and b). In both normal cases and in one Hb E heterozygote (no. 14) the small shoulder was converted to globin (after the addition of carrier haemoglobin) and the globin

FIG. 4.7 THE INVESTIGATION OF α CHAIN POOL SIZE IN RETICULOCYTES



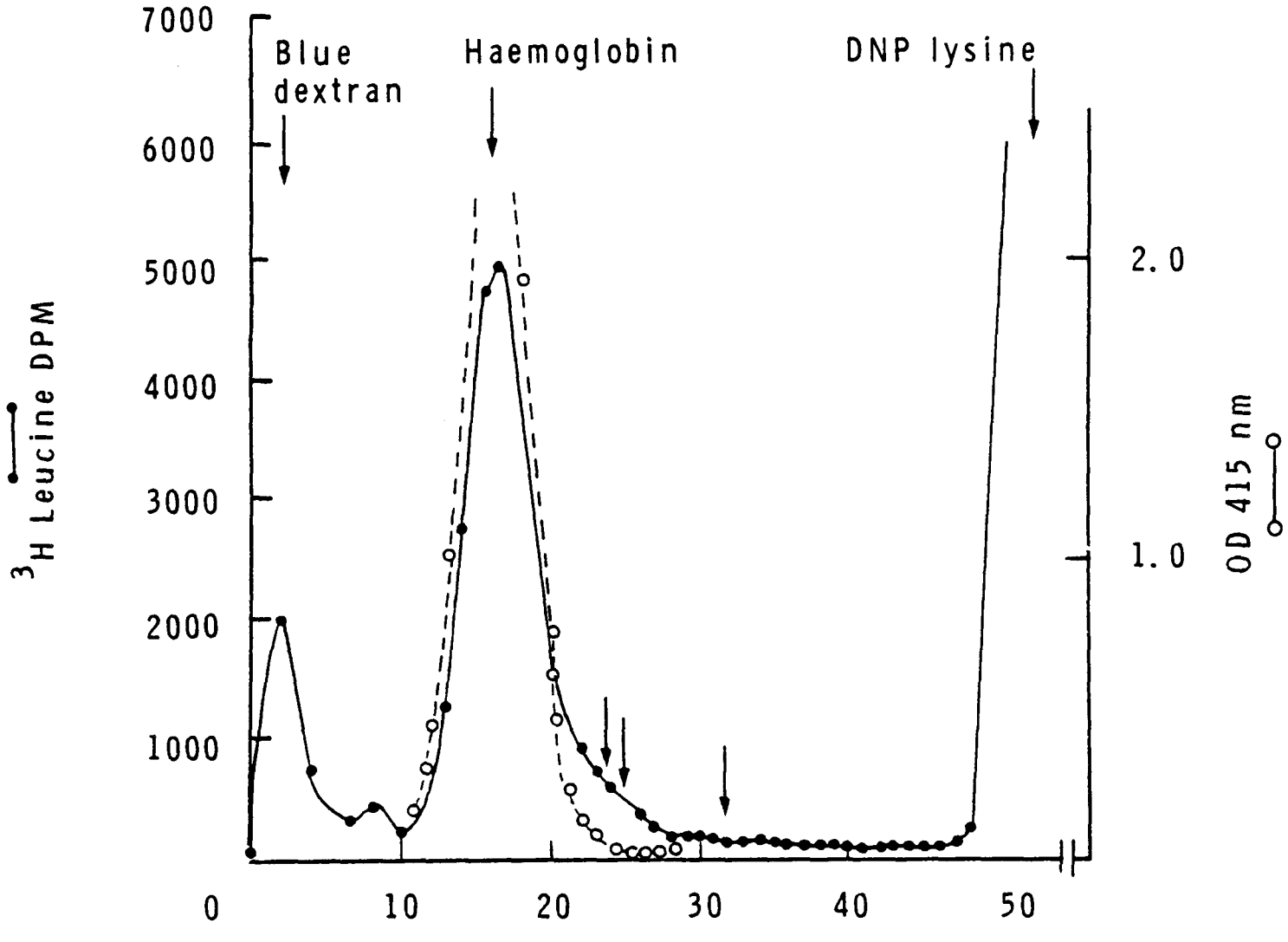


FIG. 4.8a Gel filtration of stroma-free haemolysate following the 60 minute incubation of reticulocytes from a normal (Case 37)

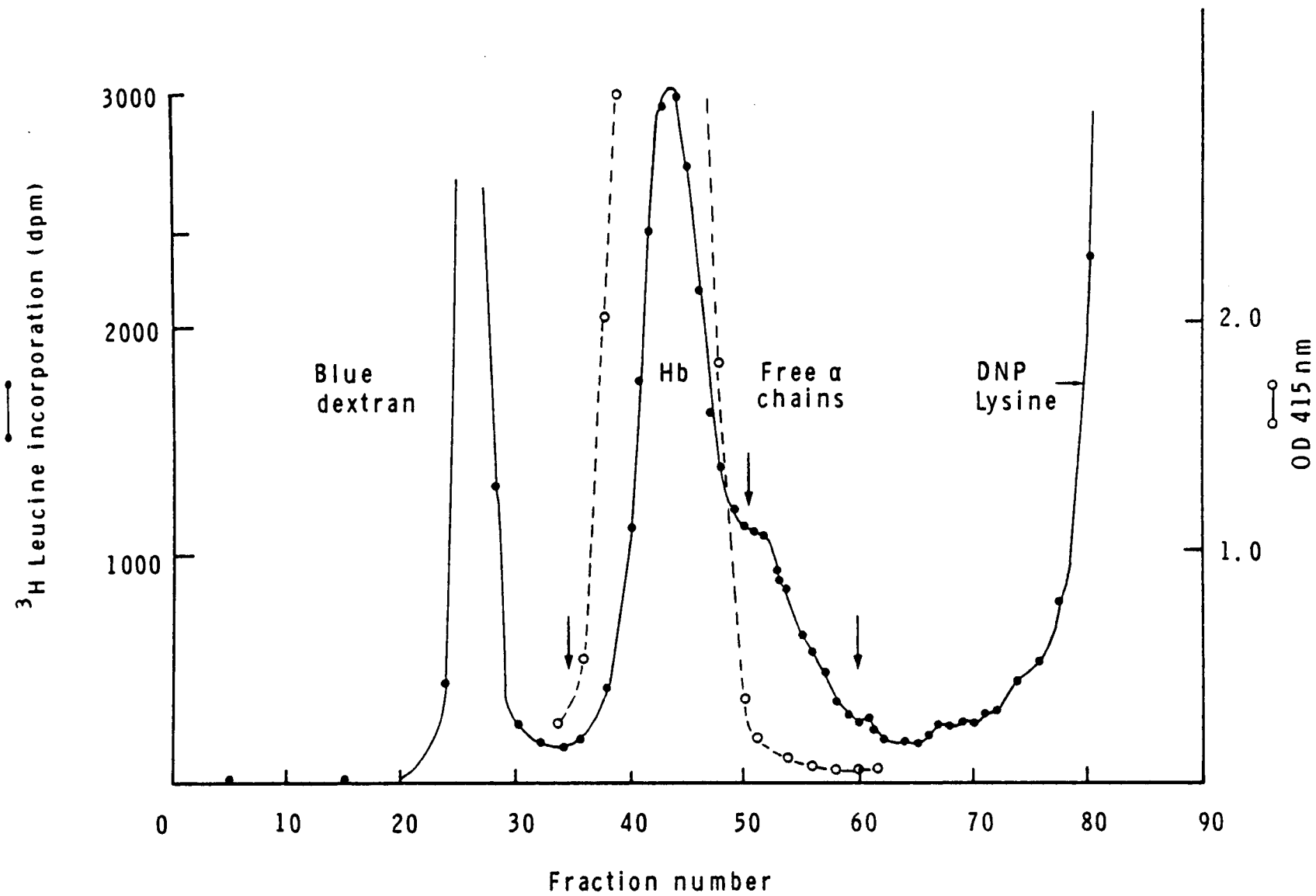


FIG. 4.8b Gel filtration of stroma-fre haemolysate following the 60 minute incubation of reticulocytes from a Hb E heterozygote (Case 14).

chains were separated on CM-cellulose. Most of the radioactivity of this small shoulder was associated with the α-chain peak, suggesting that the reticulocytes of Hb E heterozygotes contain a small pool of free α-chains although it does not appear to be larger than that observed in normal reticulocytes despite the α/non α chain synthesis imbalance in the former.

The gel filtration of stroma free lysate from 2 Hb E homozygotes (1 and 2) demonstrated a major radioactive haemoglobin peak which was followed by a more pronounced peak of radioactivity in the free globin chain position (Fig. 4.9a). To confirm that the minor peak represented free α chains and to quantitate the size of the probable α-pool, the fractions of the haemoglobin peak and the minor peak were independently pooled and made into globin as described in Chapter 2. The globin chains were separated on CM-23 cellulose and as Figure 4.9b illustrates the globin from the haemoglobin peak contained both labelled α and β^E chains, whereas the minor peak contained only labelled α chains. This confirms the presence of a free α chain pool in Hb E homozygotes.

The size of the free α chain pool in each individual is summarised in Table 4.4. In Case 1, 18% of the α chains synthesised during a 60 minute incubation were in the free α pool. In case 2, 11% of the total α chains were free after 10 minutes of incubation and 6% were free after 60 minutes. The

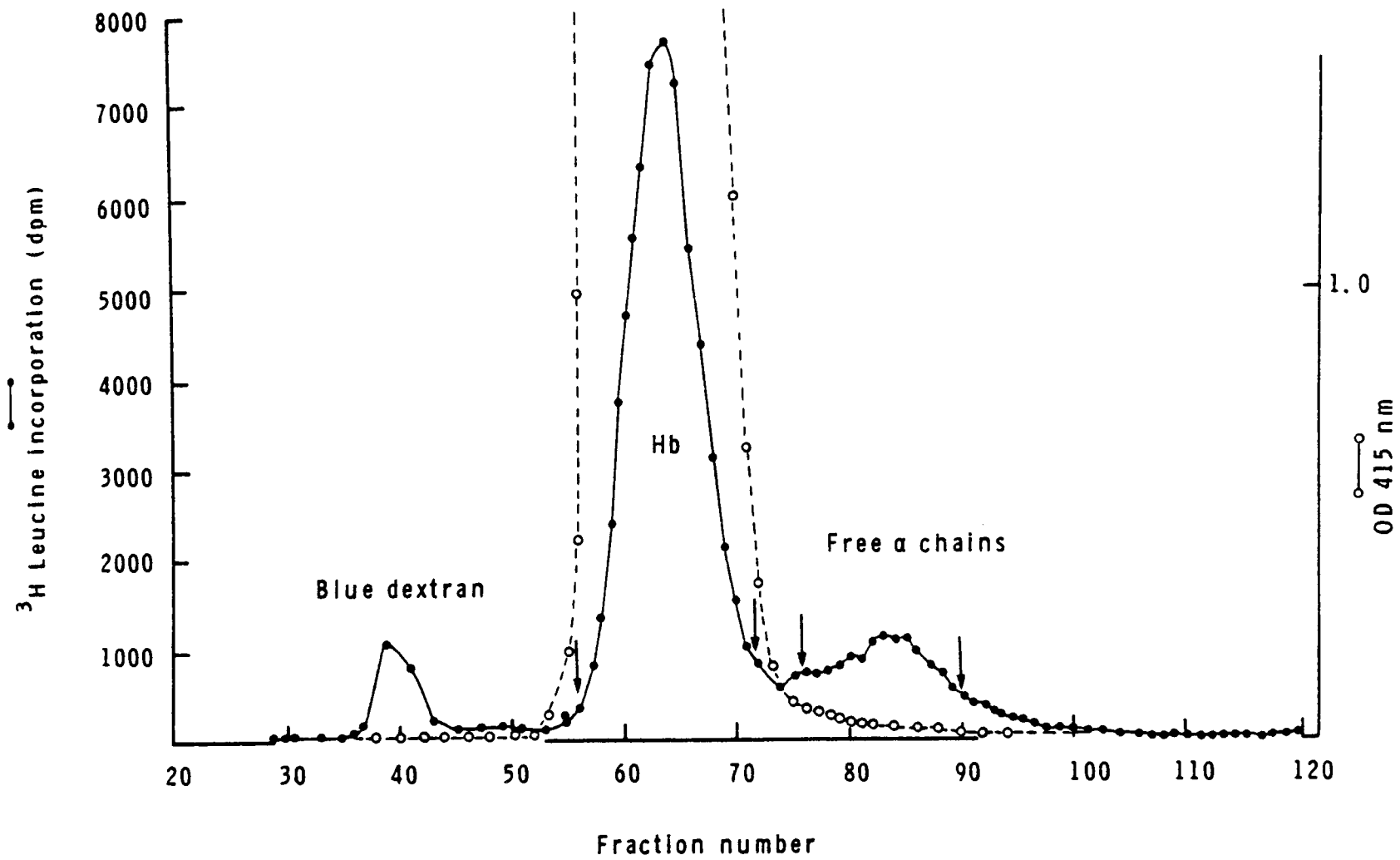


FIG. 4.9a Gel filtration of stroma-free haemolysate following the 60 minute incubation of reticulocytes from a Hb E homozygote (Case 1).

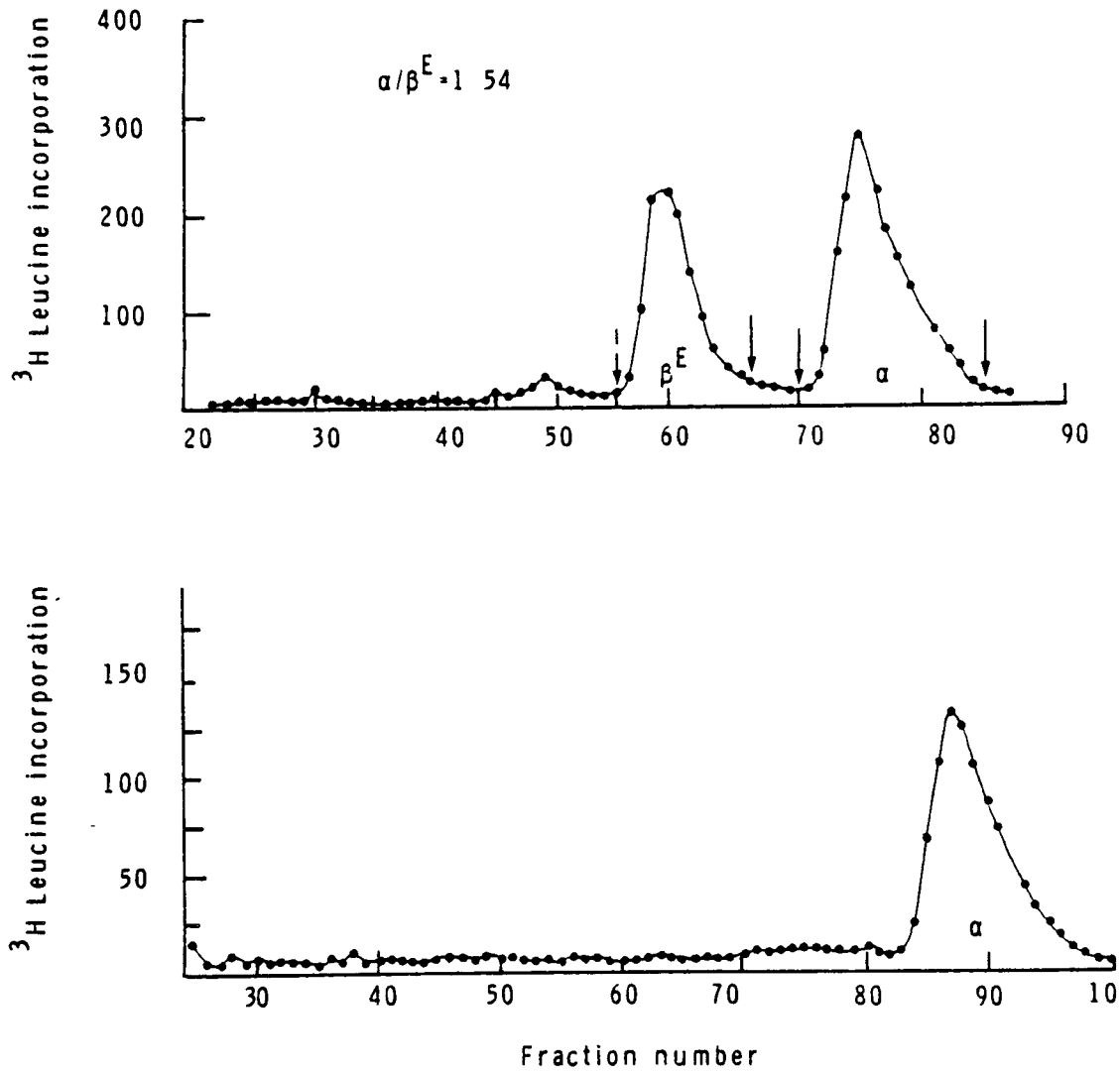


FIG. 4.9b ^{3}H leucine incorporation into globin made from

i) The major (haemoglobin) peak and

ii) The minor peak from the gel filtration of homozygous Hb E haemolysate (Case 1).

TABLE 4.4 THE MEASUREMENT OF THE FREE-α CHAIN POOL SIZE IN 2 Hb E HOMOZYGOTES

INDIVIDUAL	TIME OF INCUBATION (MINS)	G-100 Hb PEAK (TOTAL COUNTS)	G-100 Free α CHAIN PEAKS (TOTAL COUNTS)	% FREE α CHAINS	Hb PEAK α/β^E RATIO	α/β RATIO * CALCULATED FROM G-100 Hb AND FREE α PEAKS	α/β WHOLE GLOBIN RATIO (Table 4.2a)
1	60	60,793	13,703	18	1.54	2.11	2.25
2	10	93,133	11,400	11	2.05	2.42	2.83
	60	574,273	38,150	6	1.84	2.03	2.34

* This ratio was calculated as follows:

The number counts of the G-100 Hb peak were allocated to α of β^E globin chains using the α/β^E globin counts ratio obtained from the CM-23 chain separation.

Any non globin counts, as judged from the CM-23 separation of the free-α G-100 peak were subtracted from the G-100 free α peak counts.

The total α counts from the G-100 column were summated and divided by the β^E counts.

decrease in the size of the free α-pool in case 2 from 10 to 60 minutes of incubation suggests that during the incubation period some of the free α chains were removed from the pool, possibly due, at least in part, to proteolysis, since there was a significant drop in the α/non α ratio between these time points.

The α/β^{E} counts ratio calculated from the counts in the haemglobin and free α peaks was 2.11 in Case 1. In Case 2 the ratios were 2.42 after 10 minutes and 2.03 after 60 minutes. In both cases the ratios are similar to the α/β^{E} counts ratio, in case 1 of 2.36 and in case 2 of 2.83 (10 minutes) and 2.34 (60 minutes) observed in the whole cell globin sample separated directly on CM-23 cellulose (Refer to Table 4.2a).

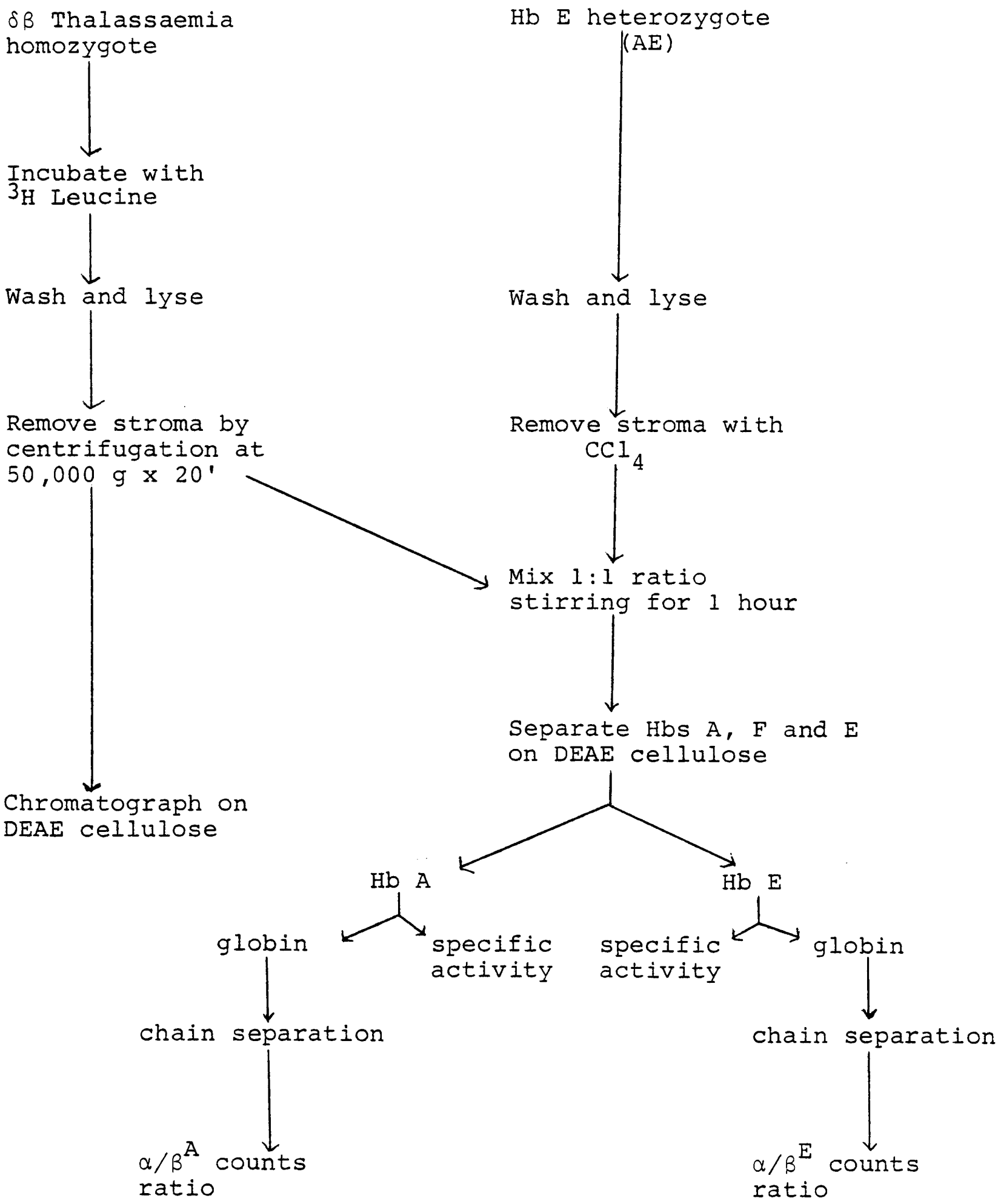
The free α-chain pools in the red blood cells of Hb E homozygotes were smaller than have been observed in β thalassaemia heterozygotes (Clegg and Weatherall 1972; Wood and Stamatoyannopoulos 1975) and yet they both have a similar overall degree of α/non α globin chain imbalance. The comparable degree of chain imbalance in the two conditions makes it unlikely that excess α-chain proteolysis accounts for the relatively smaller α-chain pool in the Hb E homozygotes. It is believed that during the synthesis of haemoglobin, nascent α-chains first enter the free α-chain pool and then combine with nascent β chains to form haemoglobin tetramers. Thus, after short incubation times the α/β ratio in the haemoglobin tetramers should be less than unity. However in the Hb E homozygotes this

α/β counts ratios of the haemoglobin peak were considerably greater than 1.0. One possible explanation for this is that the newly synthesised α-chains in the free α-pool are rapidly exchanging with non-radioactive α-chains already present in the haemoglobin molecules. In some individuals with β-thalassaemia exchange of α-chains between the free α pool and Hb A has been demonstrated (Huehns and Modell 1967; Bank 1968; Weatherall et al 1969). If free α chains exchange more readily with Hb E than with Hb A, it would explain the differences in the α chain pool size observed in the Hb E homozygotes and β-thalassaemia heterozygotes.

To investigate this possibility, unlabelled whole blood lysate from a Hb E heterozygote was mixed with lysate from a δβ thalassaemia homozygote which had just been incubated with ^{3}H leucine, and therefore contained a substantial number of radioactive free α chains (Figure 4.10 illustrates the experimental design).

The radioactive profiles from the chromatography of the δβ thal homozygote lysate, alone and after mixing with AE lysate are shown in Fig. 4.11. The mixed lysates showed radioactivity not only in the peak corresponding to Hb F, but also in the positions of Hb E and Hb A, with considerably more counts in the Hb E peak. The δβ thal lysate showed a major radioactive peak corresponding to Hb F but in addition there were two minor peaks of radioactivity, one of which (peak X) ran in the position of Hb E, and one (peak Y) immediately following the Hb E position.

FIG. 4.10 THE INVESTIGATION OF FREE α-CHAIN EXCHANGE WITH Hb's E AND A



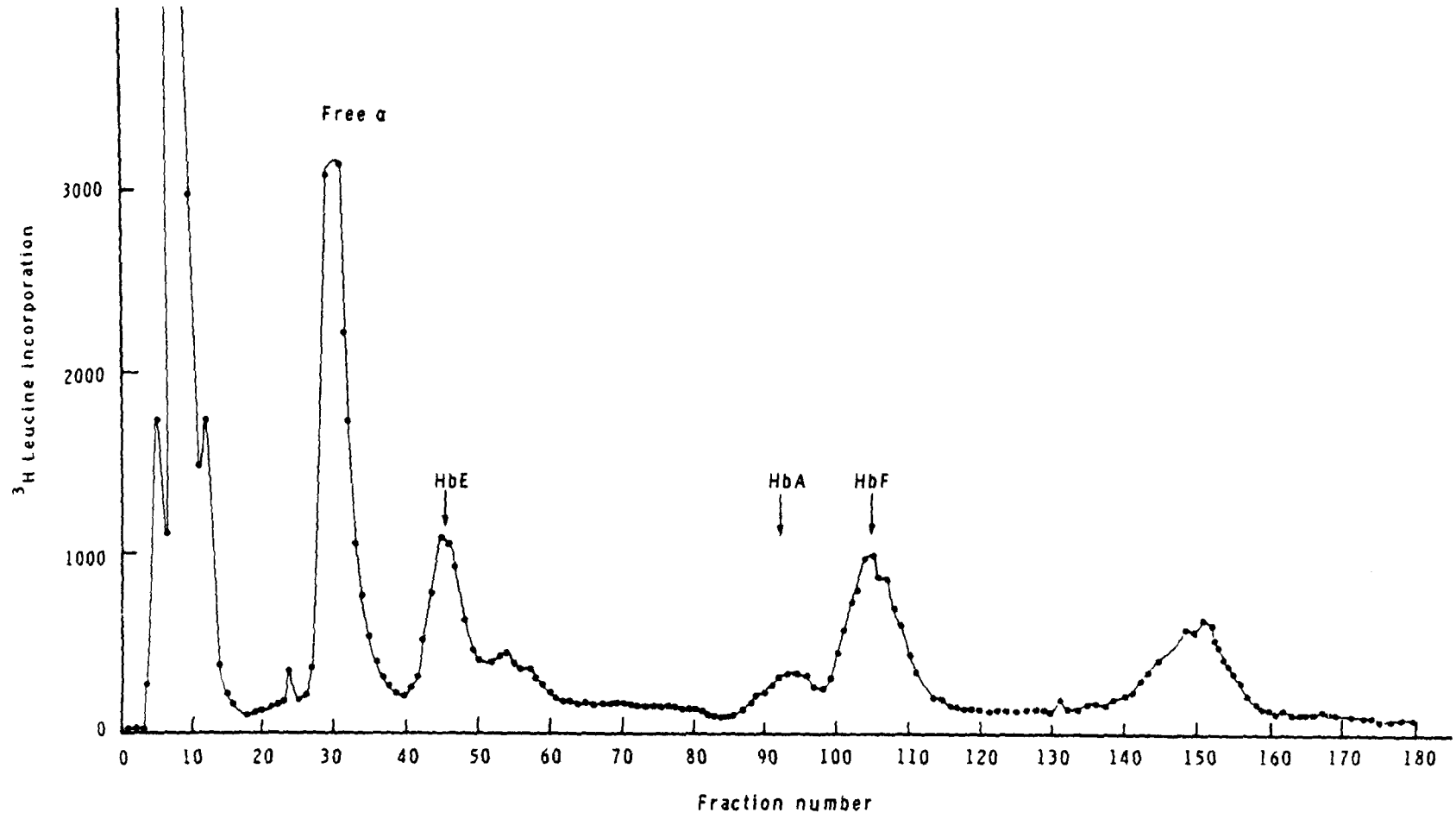


FIG. 4.11 ^{3}H leucine incorporation into fractions of

a) Unlabelled heterozygous Hb E and labelled homozygous δβ thalassaemia haemolysate and

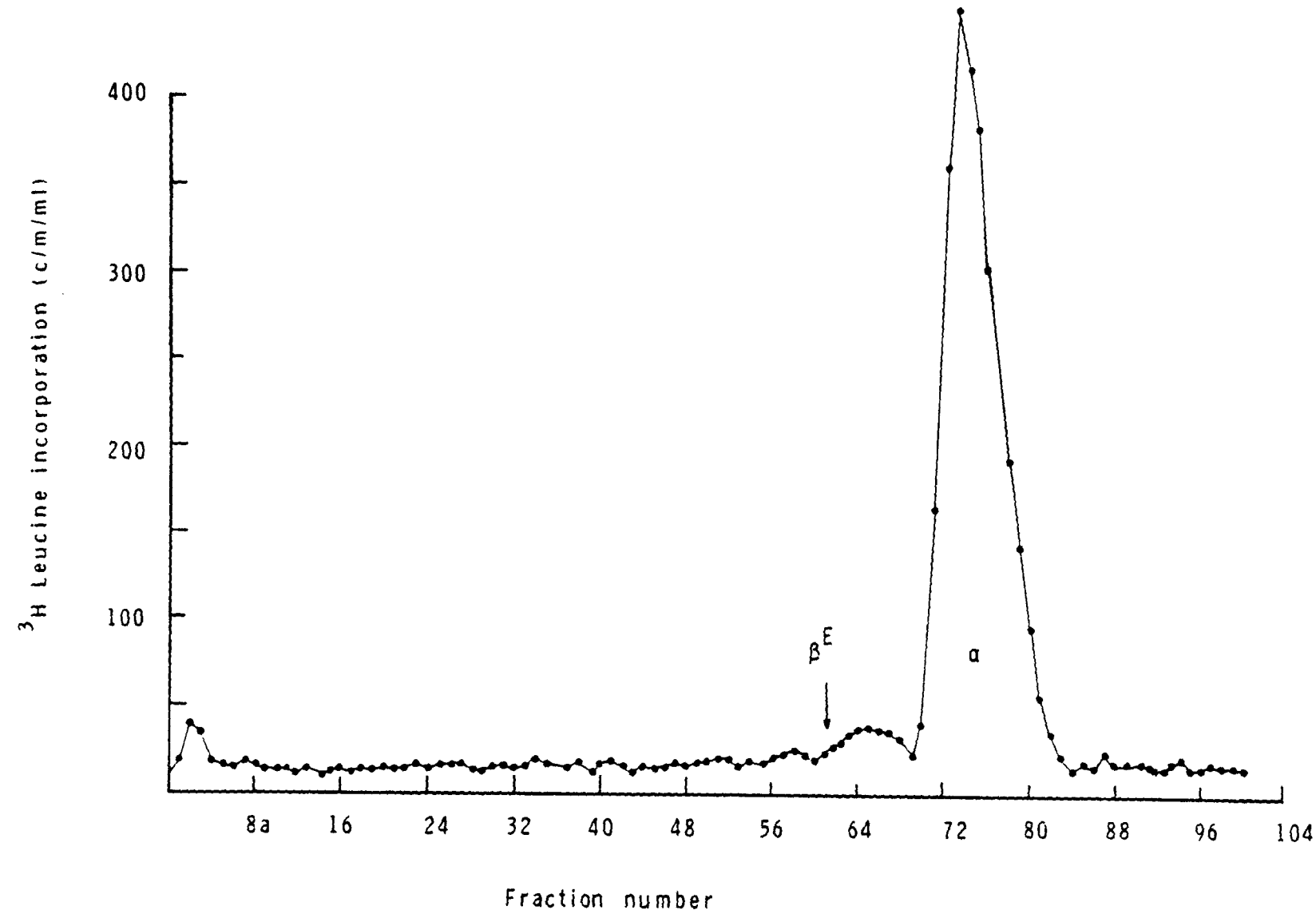
b) Labelled homozygous δβ thalassaemia separated on DEAE-cellulose

(This peak is also evident in the chromatography of the mixed lysate).

When the globin chains of the Hb A and Hb E peaks were separated on CM-cellulose both demonstrated radioactivity under the α globin peak (Figure 4.12a and b) showing that both haemoglobins had exchanged α chains with the radioactive α-chains of the δβ thal lysate.

To assess the relative exchange of α chains with Hb E and Hb A the specific activity of each peak fraction was calculated (Table 4.5a). The specific activity of the Hb E peak fraction was approximately 5 times greater than that of the Hb A peak fraction. However, since the DEAE separation of the δβ thal lysate alone demonstrated radioactivity in the region of the Hb E peak (mainly peak X), this presumably also contaminates the Hb E peak of the mixed lysates. When carrier haemoglobin lysate (containing Hbs A, E and F) was added to peak X and the globin chains separated on CM-23 cellulose, no radioactivity was associated with any of the globin chains from the X fraction, implying that the contaminant was a non-globin protein.

The total counts in peak X amounted to about 20% of the counts in the Hb F peak in the δβ thalassaemia lysate and presumably a similar proportion in the mixture with the AE lysate. Thus it can be calculated that about 25% of the counts in the Hb E peak are due to contamination with X. Clearly, this is insufficient to account for the five fold difference in the specific activities of the Hb E and Hb A fractions.



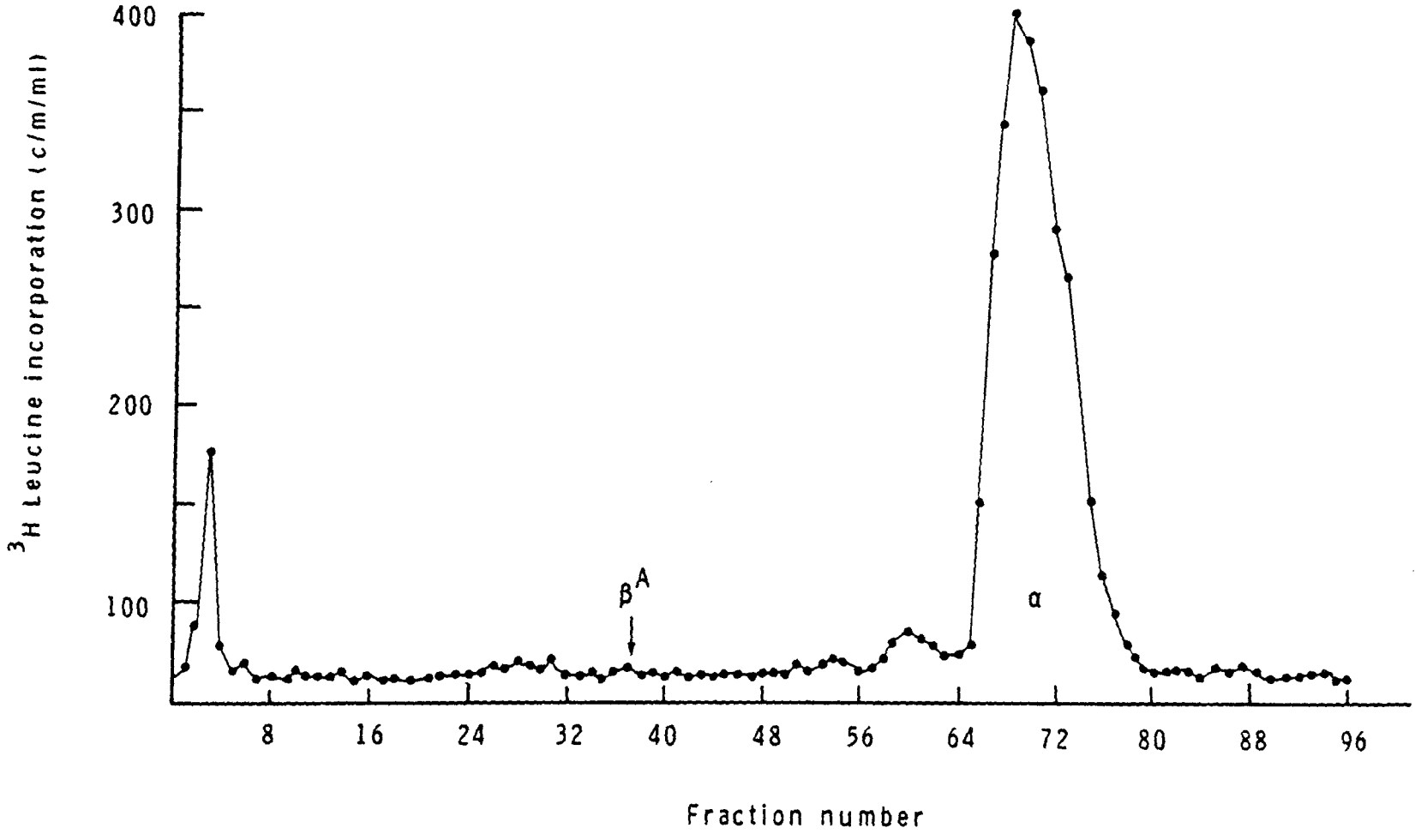


FIG. 4.12 ^{3}H leucine incorporation into globin made from

a) The Hb E peak and

b) The Hb A peak of heterozygous Hb E haemolysate separated on DEAE-cellulose

TABLE 4.5 THE SPECIFIC ACTIVITY OF HAEMOGLOBIN PEAKS FROM THE SEPARATION OF AE + δβ/β THAL LYSATES ON DEAE CELLULOSE

LYSATE MIXTURE	Hb PEAK	OD_{415}	DPM	SPECIFIC ACTIVITY
AE + δβ Thal	Hb E	0.896	1375	1535
AE + δβ Thal	Hb A	1.587	557	351
AE + δβ Thal	Hb F	2.066	1342	649
δβ Thal	Hb F	0.853	675	791

Thus, although a precise figure cannot be obtained it is apparent from this experiment that there is a far greater exchange of free α-chains with Hb E (or $\alpha\beta^E$ dimers) than with Hb A ($\alpha\beta^A$ dimers) probably by a factor of about three fold. This may well explain the relatively low proportions of free α chains seen on the gel filtration of homozygous Hb E lysates and the high α/β^E ratio in the haemoglobin fraction.

In conclusion, Hb E homozygote reticulocytes have a pool of free α chains which is greater than that observed in normal reticulocytes. However, the free α-chain pool in the Hb E homozygotes is smaller than that expected from the degree of chain imbalance observed. This is probably due to exchange between the labelled free α chains of the pool and the unlabelled α chains of the Hb E tetramers, whereby the labelled free α-chain pool is diluted with cold α chains.

DISCUSSION

The results of biosynthesis experiments on peripheral blood reticulocytes of Hb E homozygotes, heterozygotes and E-β thalassaemia compound heterozygotes incubated for 1 hour indicated that β^E globin was synthesised at a lower rate than normal globin chains.

The α/β^E counts ratios in 5 Hb E homozygotes showed a marked chain imbalance. The range of ratios observed was slightly greater than the range of α/non α counts ratios observed in 3 β-thalassaemia heterozygotes of this study and those reported in β-thalassaemia

heterozygotes (Weatherall and Clegg 1981; Chalevelakis et al 1975).

As already discussed,the α thal 2 genotype is quite prevelant in the populations from which these 5 Hb E homozygotes originate. It is possible that the gene deletion giving rise to this genotype results from an unequal cross over between the α gene pairs on homologous chromosomes and by analogy the opposite chromosome in this event will have 3 α genes, as has been observed in some individuals (Higgs et al 1980). The possibility that the high α/β^{E} globin counts ratios observed in the Hb E homozygotes results from the presence of extra α-genes in these individuals, (which direct α-globin synthesis) was excluded by restriction endonuclease mapping of their DNA (see Chapter 3). Therefore it appears that the chain imbalance observed is the result of reduced β^{E} globin synthesis.

The production of a relative excess of α chains in the homozygous Hb E reticulocytes was confirmed by the increased α chain pool demonstrated in 2 Hb E homozygotes. However, the free α chain pool was observed to be smaller than predicted by the degree of chain imbalance.

This could be due to loss of free α chains, either by proteolysis or because they are unstable and become rapidly associated with the cell membrane (Weatherall et al 1969). Alternatively, the labelled free α chains may exchange with the unlabelled α chains

of the haemoglobin molecules already present in the cells, so reducing the apparent size of the pool. Results from individuals with β thalassaemia suggest that exchange of α chains between Hb A and the free α pool is probably dependent upon the size and stability of the pool and the manner in which the lysates are treated immediately following haemolysis of the red cells (Weatherall et al 1969).

There is no reason to believe that in Hb E homozygotes proteolysis or membrane binding of the free α chains should be any more active than in β thalassaemia heterozygotes with a similar degree of chain imbalance. Indeed, although a small decrease in the α/β^{E} specific activity ratios was observed in the time course incubations of 3 Hb E homozygotes (Table 4.3a) indicative of α chain proteolysis, the loss of α chains appeared to be insufficient to explain the small size of the free α chain pools found in the Hb E homozygotes. The similarity of the α/non α chain synthesis ratios measured directly from 'whole cell globin' and after gel filtration argues against appreciable loss of free α chains during removal of the stroma prior to gel filtration. Furthermore, the mixing of δβ thalassaemia lysate (containing labelled free α globin chains) and Hb E heterozygote lysate (containing Hb E and Hb A) demonstrated directly that free α chains exchange more readily with Hb E than with Hb A. It has been postulated that Hb E may have a tendency to dissociate into monomers more readily than Hb A as a result of the lysine substitution for glutamic acid at the $\alpha_1\beta_1$ contact (Perutz and Lehmann 1968).

This would readily explain why free α chains may exchange with the α chains of the Hb E tetramers more rapidly than with Hb A.

In the Hb E heterozygotes the degree of chain imbalance was rather less than that observed in the Hb E homozygotes and nearer to the normal range than expected from the β^E gene dosage. It has been observed in β^O thalassaemia heterozygotes that there is some compensation for the deficient output of β globin by the abnormal gene such that red blood cells of β^O thalassaemia heterozygotes contain approximately 20 pg Hb A (reviewed by Weatherall and Clegg 1981) compared with two normally functioning β genes. In the Hb E heterozygotes the level of Hb A is also slightly raised, to approximately 19 pg per cell, suggesting that the β^A gene in trans to the β^E gene does direct slightly more β^A globin synthesis than normal. This could account for the slightly lower α/non α counts ratios observed in the Hb E heterozygotes relative to those predicted from gene dosage.

In the 7 individuals with Hb E β thalassaemia, the imbalanced α/non α counts ratios varied widely. This is partly due to the heterogeneity of the interacting β thalassaemia gene. The picture is further complicated by the selection of cells with least chain imbalance that probably occurs in Hb E β thalassaemia compound heterozygotes as a result of ineffective erythropoiesis (Ruymann et al 1978; Wickramasinghe et al 1981), and in addition proteolysis of the excess α chains, as demonstrated by the slight decrease in

α/β^{E} counts ratios noted in the time course incubations of 3 cases of Hb E β thalassaemia (Refer to Table 4.2c), and the fact that the α/β^{E} ratios were generally lower in the compound heterozygotes than in the Hb E heterozygotes.

Clearly, the results of reticulocyte incubations from Hb E homozygotes, heterozygotes and compound heterozygotes, provide strong evidence for the decreased synthesis of β^{E} globin, such that effectively, the β^{E} gene acts as a mild β thalassaemia gene. In the light of this result it can be understood why Hb E/β thalassaemia is often such a severe clinical disorder, the low β^{E} chain synthesis enhancing the chain imbalance that results from the β thalassaemia gene. Although the first cases of Hb E β thalassaemia were described more than 25 years ago it has only recently been observed that the mechanism underlying the unusual interaction of Hb E and β thalassaemia is the defective synthesis of β^{E} globin. In retrospect, there were several indications that this was the mechanism but in general globin synthesis studies involving Hb E have been sporadic and usually concerned with isolated cases.

In 1969 Weatherall et al reported $\alpha/\gamma+\beta^{E}$ ratios in 2 individuals with Hb E/β^{o} thalassaemia of 2.1 and 2.4 and α/β^{E} counts ratios of 3.6 and 3.7. These latter ratios were considerably higher than expected of a normal β globin gene in trans to a β^{o} thalassaemia gene. Subsequently, other groups have published similar results in individuals with Hb E/β thalassaemia (Pagnier et al 1974; Ruymann et al 1978; Finer et al 1979; Testa

et al 1980). The only notable exception was in a study by Feldman and Rieder (1973) on a family with both β^{O} thalassaemia and Hb E. The single family member with Hb E/β^{O} thalassaemia gave an almost balanced $\alpha/\gamma+\beta^{E}$ ratio of 1.1 and an α/β^{E} ratio of 1.4. (Two other family members with Hb E trait had $\alpha/\beta^{A}+\beta^{E}$ counts ratios of 0.91 and 0.83). The α/β counts ratio in one other member of the family without Hb E or β thal was 0.75, which the authors considered to be within the range for normal individuals. In fact it is more typical of ratios observed in individuals with α-thalassaemia 1 or 2 (reviewed by Weatherall and Clegg 1981) and re-examination of the whole family is entirely consistent with the presence of an α thalassaemia gene in the Hb E heterozygotes and the Hb E/β^{O} thalassaemia compound heterozygote.

Balanced $\alpha/\beta^{A}+\beta^{E}$ counts ratios were reported in Hb E heterozygotes by Pagnier et al (1974) and Finer et al (1979) but in neither study was α thalassaemia excluded. In the case of Pagnier et al a defect of β^{E} globin synthesis was considered, based on the high β^{A}/β^{E} ratio, but these findings were complicated by studies on an unrelated Hb E homozygote who reportedly had a balanced α/β^{E} synthesis ratio. Inconsistent with this balance ratio, however, was a large free α pool in the reticulocytes, similar in size to that observed in the 2 Hb E homozygotes reported in this thesis. They concluded that the balanced synthesis ratio together with the free α chain pool indicated a decreased association of α and β^{E} chains although

they could not satisfactorily explain the fate of the unassociated β^E chains.

During the course of the studies described here, Fairbanks et al (1980a) reported an α/β^E ratio of 2.1 in a Hb E homozygote compared to a ratio of 1.1 in a normal control. The presence of α thalassaemia in this individual was essentially excluded by a family study.

Even more recently imbalanced $\alpha/\beta^A+\beta^E$ synthesis ratio of 1.45 was also observed by Benz et al (1981) in a Hb E heterozygote with no evidence of an interacting α thalassaemia gene.

The synthesis ratios observed by both of these latter groups in one Hb E homozygote and one hetero-zygote are in agreement with the respective ranges observed in the much larger series in this study and they both conclude that β^E globin is synthesised at reduced levels.

The possibility that the decrease in β^E globin synthesis in reticulocytes was the result of rapid post-synthetic destruction of the β^E chain was investigated by the incubation of reticulocytes from very short time periods up to 1 hour. In Hb E homozygotes, heterozygotes and Hb E β thalassaemia compound heterozygotes there was no evidence for any increased proportion of β^E chain synthesis even at very early incubation times as should be the case if some of the β^E chains are rapidly destroyed soon after

their synthesis. Furthermore the specific activity of the β^E chain was always constant relative to the normal α and/or β^A globin chains present at all times of incubation. Therefore, the low synthesis of β^E globin does not appear to be the result of premature destruction of the β^E chain confirming the preliminary observations of Weatherall et al 1969, Pagnier et al 1974 and Benz et al 1981.

The above results do not exclude delayed association of α and β^E chains, which was proposed as the mechanism for the relatively low levels of Hb E observed in reticulocytes before comprehensive biosynthesis studies were carried out (Pagnier et al 1974). The data in that study were difficult to interpret, but, in a more conclusive study (Tuchinda et al 1967) it was demonstrated that β^A chains compete more efficiently than β^E chains for α chains to form haemoglobin tetramers. This probably explains why the levels of Hb E in individuals with Hb E trait and an interacting α-thal gene are lower than those found in uncomplicated Hb E trait. However this mechanism is probably only relevant when the levels of α-chains are limiting and is unlikely to play a major role in defining the levels of Hb E found in individuals with Hb E unassociated with α thalassaemia.

In addition to the lower levels of β^E synthesis relative to β^A synthesis observed in reticulocytes, the β^A/β^E specific activity ratios in Hb E heterozygotes suggest that the synthesis of β^E and β^A globin is asynchronous. The β^A/β^E specific activity ratios exceeded one in eight out of the nine cases (range 1.00 -

1.56 mean 1.25±0.19). Since the specific activity reflects the amounts of a globin chain synthesised during the incubation period relative to the proportion already present in the peripheral blood, these results suggest that β^E chain synthesis declines more rapidly than that of β^A chains, such that earlier in erythroid maturation, β^E chain synthesis constituted a higher proportion of the total β chain production. With respect to this observation in the Hb E heterozygotes, it was noted that in Hb S heterozygotes the β^A/β^S specific activity ratios were often similarly greater than 1.0. In 4 Hb S heterozygotes studied in this laboratory, the β^A/β^S specific activity ratios were found to range from 1.02-1.23 (mean 1.14±0.03) after incubations lasting for 60 minutes (D.R. Higgs, personal communication). Furthermore, in 5 out of 6 Hb E heterozygotes studied by Shaeffer et al (1976) the β^A/β^S specific activity ratios ranged from approximately 1.05-1.20 after a 1 hour incubation and were even greater at shorter incubation times.

In order to ensure that these observations are not a characteristic of β globin chain variants in general (for some obscure reason) the relative synthesis of globin chains were examined in a Hb D heterozygote. At all times of incubation from 2-60 minutes, the $\alpha/\beta^A+\beta^D$ total counts ratios were balanced and the β^A/β^D specific activity ratios were very close to unity (Figure 4.13). The finding of β^A/β^D specific activity ratios of 1.0 in the Hb D heterozygote suggest that the $\beta^A/\beta^{Variant}$ specific activity ratios

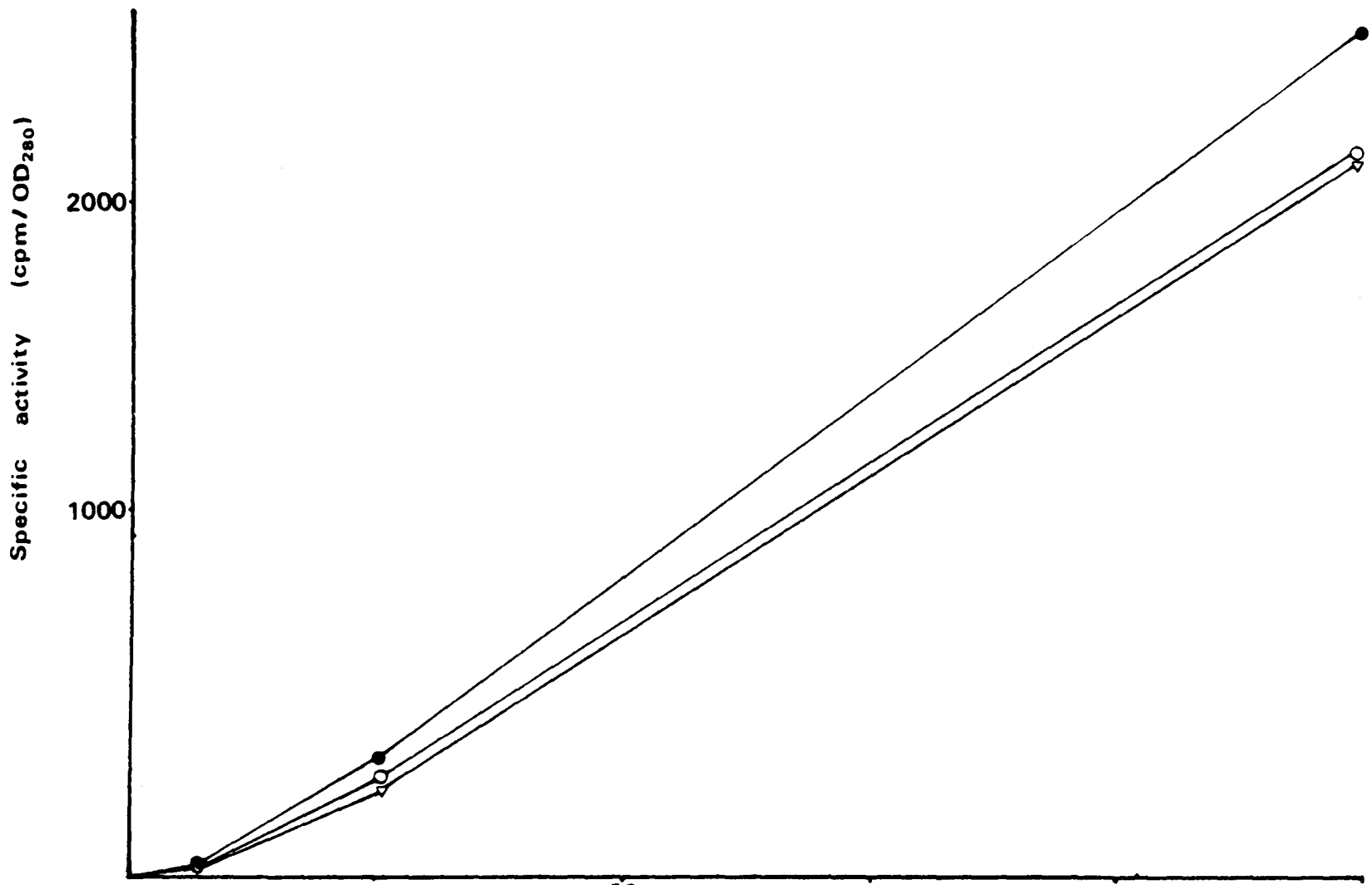


FIG. 4.13 The specific acitivties of globin chains synthesised during a time course incubation of reticulocytes from a_AHb D heterozygote$_D$ (●) α globin; (○) β^A globin; (▽) β^D globin.

of greater than 1.0 frequently observed in Hb E and Hb S heterozygotes probably does reflect some asynchrony in β chain production in these individuals, and furthermore that the synthesis of β^S globin in Hb S heterozygotes be further investigated.

The α/non α chain synthesis ratios in the bone marrow red cell precursors of 3 Hb E homozygotes and 2 Hb E heterozygotes were nearer unity that those observed in whole blood reticulocytes of the same individuals, tending to confirm the suggestion of a higher proportion of β^E chain synthesis earlier in erythroid maturation. However, results from bone marrow incubations must be interpreted with caution. Bone marrow globin biosynthesis studies in β thalassaemia heterozygotes were initially reported to show balanced α/β counts ratios (Schwartz 1970). In later studies, the α/β synthesis ratios in heterozygous β thalassaemia bone marrows were shown to be imbalanced and closer to those observed in peripheral blood, particularly at short incubation times (Chalevelakis et al 1975). There are several probable reasons for the apparently balanced synthesis ratios originally observed in these bone marrow incubations (Chalevelakis et al 1976). Firstly, there are non-globin proteins that co-chromatograph with β^A globin which must be removed prior to globin chain separation if accurate α/β globin synthesis ratios are to be observed. Secondly, and more importantly, excess α chains are rapidly removed by proteolysis which occurs much more efficiently in red blood cell

precursors than in reticulocytes (Clegg and Weatherall 1972; Wood and Stamatayannopoulos 1975; Hanash and Rucknagel 1978). Thus, after very short periods of incubation the α/non α counts ratios in β thalassaemia bone marrows were observed to be significantly greater than after long incubation periods.

The globins from marrow incubations of Hb E cases in this study were purified by gel-filtration prior to separation of CM-23 cellulose, thereby removing much, if not all, of the non-globin proteins that have been shown to contaminate the β^A globin peak, and possibly the region of the β^E globin peak. The problem of α chain proteolysis could not be avoided but it was observed that the time course incubations in one Hb E homozygote and one Hb E heterozygote showed constant α/non α total counts ratios from 2 to 60 minutes implying that no significant degradation of excess α chains occured in these particular samples. Furthermore in the Hb E heterozygotes the β^A/β^E specific activity ratios were greater in the reticulocytes than in the earlier red cell precursors of the bone marrow, a factor which should not be affected by α-chain proteolysis, and this point is also indicative of asynchronous β^A and β^E chain synthesis during erythroid maturation.

CHAPTER 5

STUDIES ON β^E mRNA

The Levels of β^E mRNA in Peripheral Blood Reticulocytes

The results presented in the two preceding chapters imply that there are low levels of Hb E in peripheral blood and this is a direct result of a reduced rate of β^E globin synthesis. The defect in the production of β^E globin does not appear to be due to inherent instability of the mutant protein and the possibility arises that the defect is pre-translational, at the level of the messenger RNA.

The initial approach was to quantitate the relative levels of α and β mRNA in peripheral blood reticulocytes of homozygotes and heterozygotes for Hb E using specific tritiated α and β globin cDNA probes.

As a result of slight contamination of α cDNA with β cDNA sequences and vice versa, and the variation in probe length, (see Chapter 2) the α/β mRNA ratios calculated are not a measurement of the absolute ratio of α to β mRNA present in the sample. Therefore the probe is characterised using the reticulocyte RNA from normal individuals and the mRNA ratios in the individuals with Hb E are compared to the normal α/β mRNA range. If the levels of β^E mRNA are lower than those of normal β^A mRNA, the RNA ratios in Hb E homozygotes and heterozygotes should be greater than

the normal range.

Typical curves obtained for total RNA from normal reticulocytes hybridized to α and β specific cDNA probes are illustrated in Figure 2.8. The range of values in 7 normal individuals was 1.62-2.18 (mean 2.06± 0.23) (See Table 5.1).

Homozygous Hb E

The α/β mRNA ratios were measured in 5 individuals with Hb E disease. Three of the α/β mRNA curves are depicted in Fig. 5.1. All the curves met the criteria defined in Chapter 2. The α/β^{E} mRNA ratios in the Hb E homozygotes ranged from 3.27-5.63 (mean 4.63±0.78) and the values are summarized in Table 5.2. These RNA ratios are approximately twice the values of the normal range (Figure 5.3) indicating that the amount of β^{E} mRNA present in the reticulocytes of Hb E homozygotes (relative to α mRNA) is only half that of β^{A} mRNA in normal reticulocytes.

Heterozygous Hb E

The α/β mRNA ratios were measured in 8 Hb E heterozygotes. Three representative α/β mRNA curves are presented in Fig. 5.2. All the curves essentially fulfilled the standards defined in Chapter 2. The α/β mRNA ratios are summarized in Table 5.3 and ranged from 2.05-2.78 (mean 2.32 ± 0.23), excluding the ratio of 1.29 observed in individual 9 who had α thalassaemia 2 in addition to Hb E trait (see Chapter 3). In this individual, the α thal 2 gene appears to have reduced the α/β mRNA ratio below the normal range as is usually

TABLE 5.1 α/β mRNA IN PERIPHERAL BLOOD CELLS FROM NORMAL (AA) INDIVIDUALS (using Probe I)

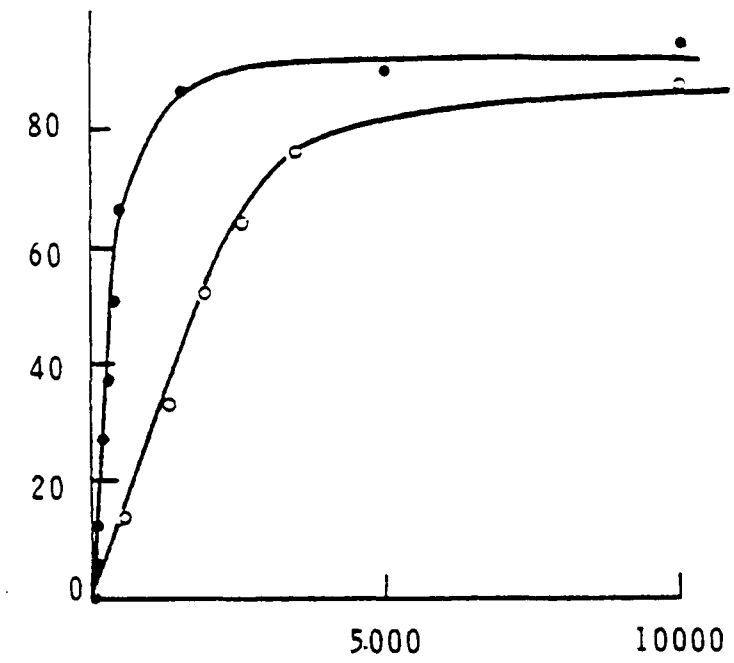
INDIVIDUAL	RETICULOCYTE α/β mRNA
30	1.6
31	2.18
36	2.12
38	1.87
40	2.11
41	2.06
43	2.06
Mean + SD	2.06 ± 0.23

TABLE 5.2 α/β mRNA IN PERIPHERAL BLOOD CELLS FROM Hb E HOMOZYGOTES (using Probe I)

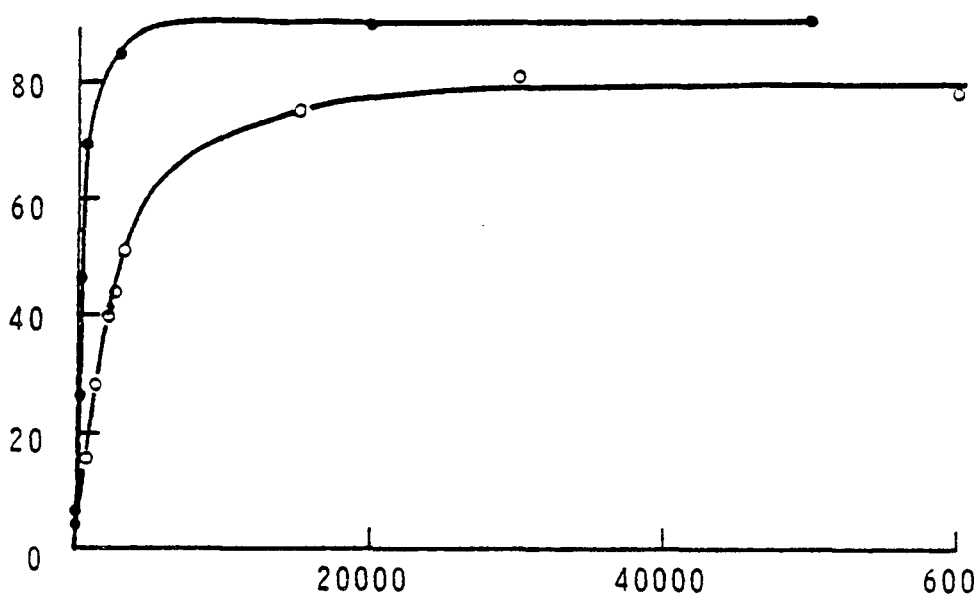
INDIVIDUAL	RETICULOCYTE α/β mRNA	STANDARDIZED α/β mRNA $\left(\frac{\text{EE Ratio}}{\text{mean AA ratio}}\right)$	α/β GLOBIN SYNTHESIS RATIO (TOTAL COUNTS)
1	4.47	2.17	2.36
2	4.82	2.34	2.34
3	5.00	2.43	2.42
4	5.81, 5.46	2.73	3.31
5	3.29, 3.25	1.59	2.41
mean ± SD	4.63 ± 0.78	2.25 ± 0.38	2.57 ± 0.37

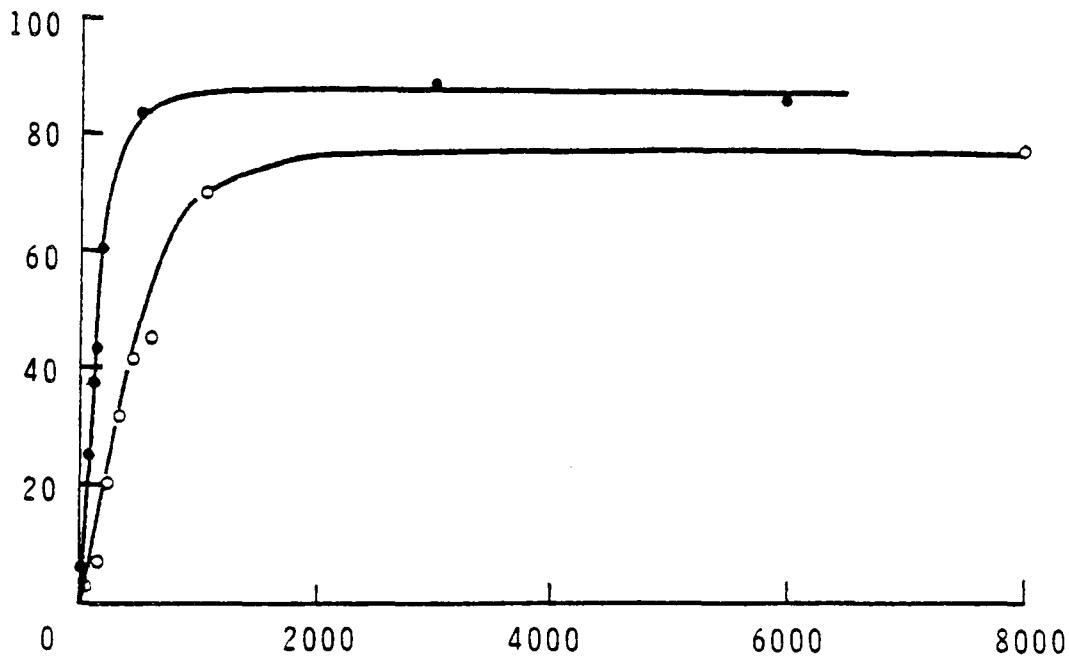
TABLE 5.3 α/β mRNA IN PERIPHERAL BLOOD CELLS FROM Hb E HETEROZYGOTES (using Probe I)

INDIVIDUAL	RETICULOCYTE α/β mRNA	STANDARDIZED α/β mRNA $\left(\frac{\text{AE Ratio}}{\text{mean AA ratio}}\right)$	α/β GLOBIN SYNTHESIS RATIO (TOTAL COUNTS)
9	1.29	0.62	0.85
11	2.48	1.20	1.47
12	2.15	1.04	1.50
13	2.34	1.14	1.23
14	2.05	1.00	1.54
15	2.78	1.35	1.57
16	2.12	1.03	1.25
17	2.34	1.14	2.19
mean ± SD	2.32 ± 0.23	1.07 ± 0.20	1.45 ± 0.36



Case 4



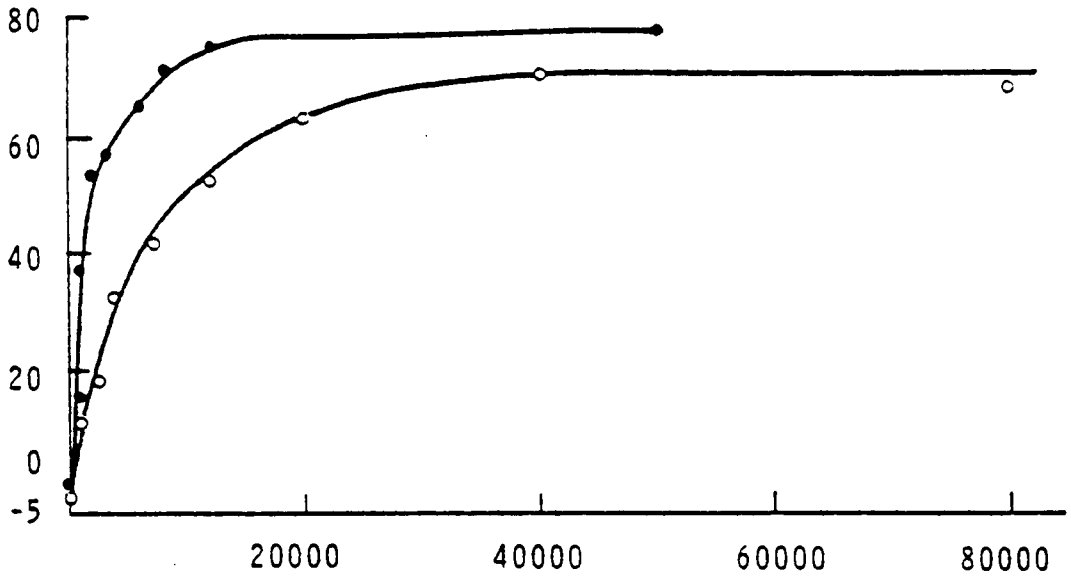


Case 1

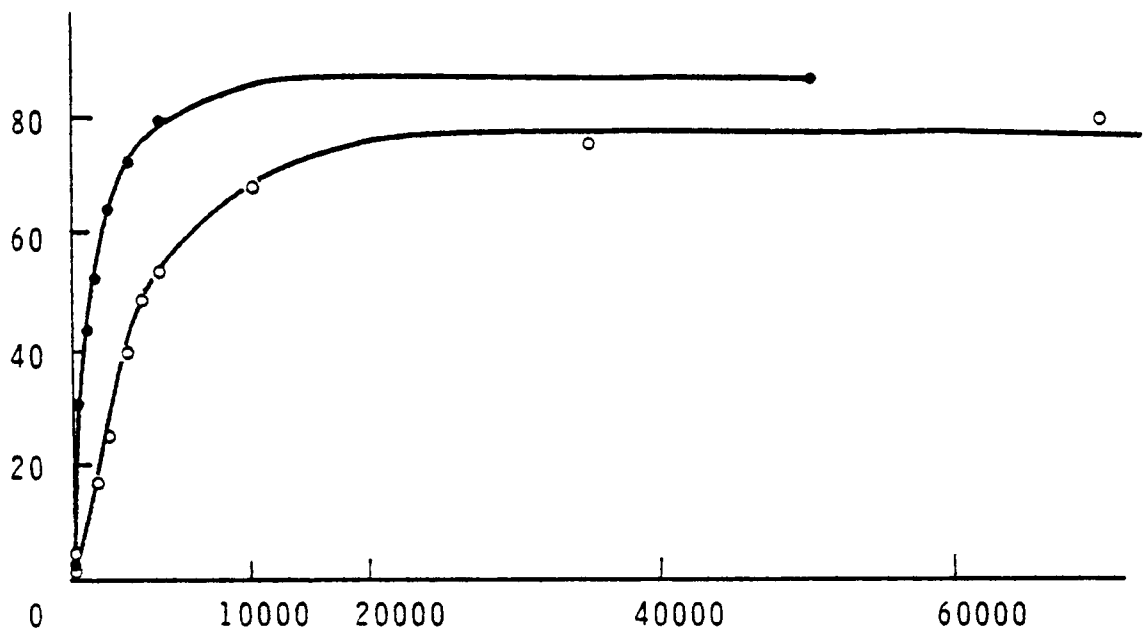
Case 5

FIG. 5.1 Hb E homozygote reticulocyte α/β mRNA curves

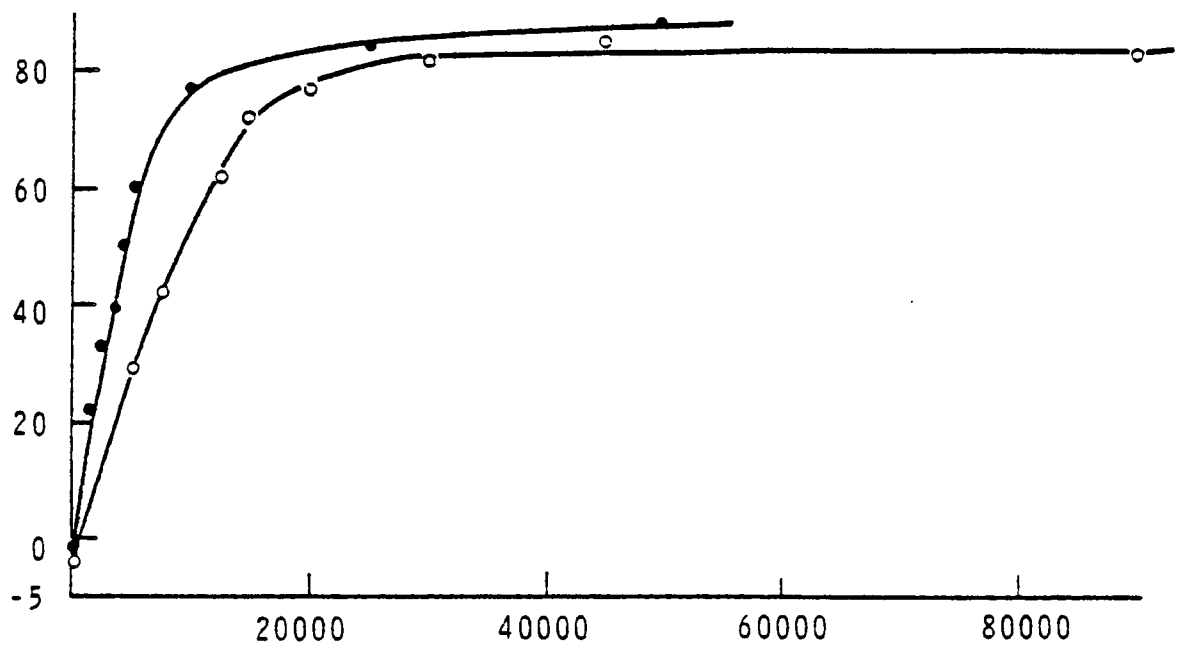
Closed circles - with cDNA
Open circles - with cDNA $^{\alpha}_{\beta}$



Case 13



Case 15



Case 12

FIG. 5.2 Hb E heterozygote reticulocyte α/β mRNA

Closed circles - with cDNA$^{\alpha}_{\beta}$
Open circles - with cDNA$^{\alpha}_{\beta}$

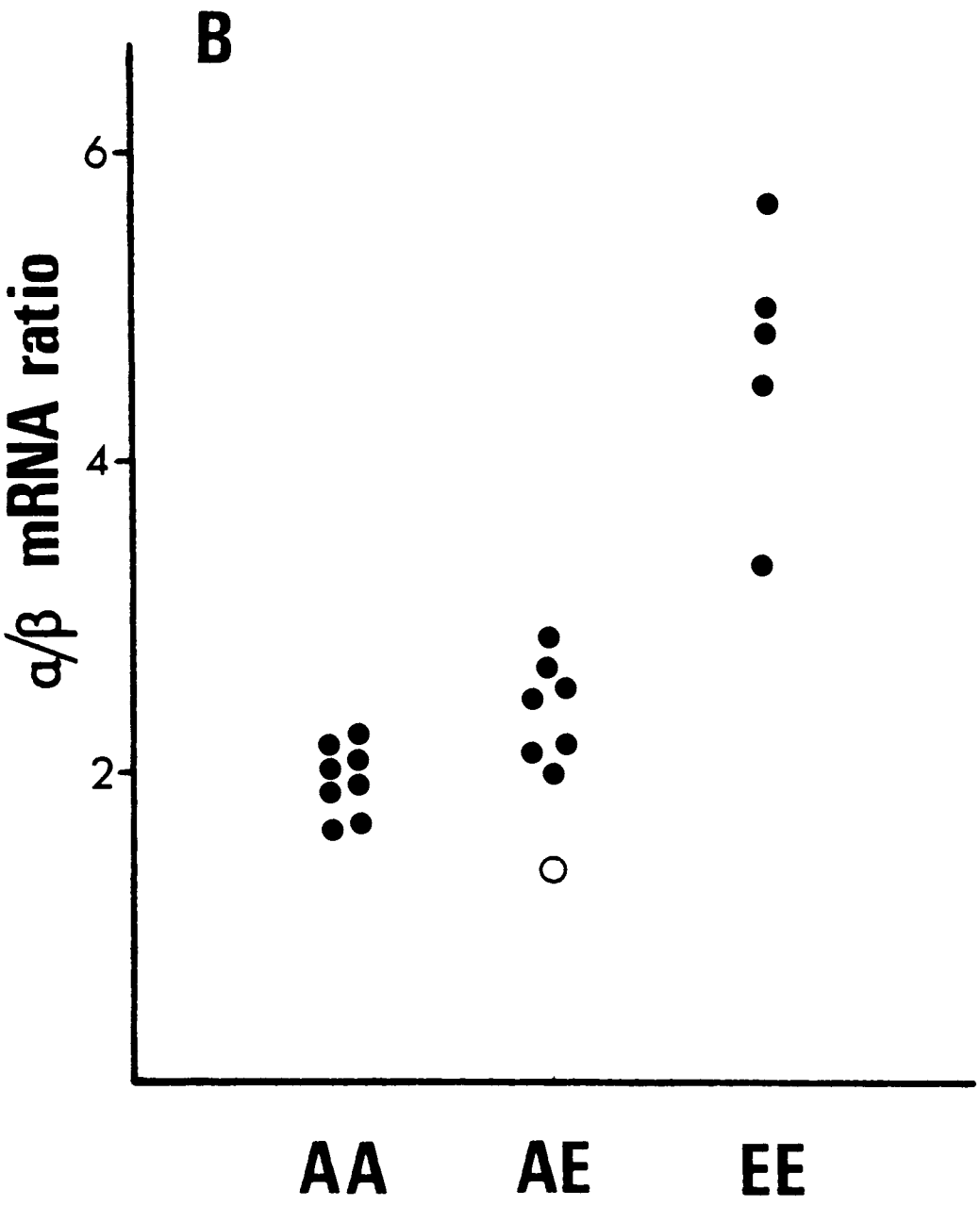


FIG. 5.3 α/β globin mRNA in peripheral blood reticulocytes in normal (AA), heterozygous Hb E (AE) and homozygous Hb E (EE) individuals.

observed in patients with α thalassaemia (Hunt et al 1980).

The range of values in the Hb E heterozygotes is slightly higher than the range observed in the normal group (Figure 5.3). It is not possible to differentiate between β^A and β^E mRNA with this method of quantitating mRNA levels, but presumably the raised α/β mRNA ratios in the heterozygotes is a result of reduced levels of β^E mRNA. These results corroborate those observed in the Hb E homozygotes.

The Levels of β^E mRNA in Nucleated Red Blood Cells

Several mechanisms may account for the failure of β^E mRNA to accumulate at normal levels in peripheral blood reticulocytes. The defect could be in transcription, in the processing of the initial RNA transcripts to mature mRNA or with decreased stability of the abnormal mRNA.

Globin mRNA is synthesised in the nucleated red blood cells (up to the polychromatophilic stage, Denton and Arnstein 1975) and in order to define the level at which β^E globin mRNA production is defective, the relative concentrations of α and β mRNA were measured in the total RNA extracted from both the nuclei and the cytoplasm of homozygous Hb E bone marrow cells, and in the total reticulocyte RNA from the same individuals.

A control series of bone marrow and peripheral blood RNA samples from patients with a variety of disorders (see Table 5.4), but whose marrow showed normal

TABLE 5.4 HAEMATOLOGICAL INDICES IN AA CONTROLS

	Case No	Diagnosis	Hb g/dl	MCV fl	MCH pg	MCHC %
	1	Treated iron deficiency	13.9	87	28	33
	2	AML in remission	13.9	101	32	32
AA	3	Chronic idiopathic thrombocytopaenia	12.6	85	28	33
	4	ALL in remission	15.6	98	33	34
	5	Treated pernicious anaemia	14.9	92	31	33

cell morphology at the time of study, were treated similarly.

Note: In order to complete this series of experiments a new set of α and β cDNA probes were made. Since different batches of cDNA probes have slightly different properties (e.g. length and purity) the new set of probes (numbered II for convenience) was standardized with normal reticulocyte RNA samples (see Table 5.5) as was the first probe (now numbered I). For any individual, the α/β globin mRNA ratios in the bone marrow nuclei, cytoplasm and reticulocyte RNA fractions were measured using the same set of probes.

RESULTS

The α/β mRNA ratios in the bone marrow nuclei and cytoplasm and in the peripheral blood reticulocytes of the AA controls and the Hb E homozygotes are summarized in Tables 5 a and b. Representative RNA curves from a Hb E homozygote and an AA control are illustrated in Figure 5.4. All of the curves that were calculated were acceptable, as defined in Chapter 2. The α and β mRNA curves were set up at least twice for many samples and the close comparability of α/β mRNA ratios, as calculated from acceptable RNA curves, demonstrates the reproducibility of the method. The α/β mRNA curves of two samples, however, (cytoplasmic RNA curves of case 6 and the nuclear RNA curves of case 34) were not calculable and there was insufficient RNA sample in both cases to repeat them.

TABLE 5.5 α/β mRNA in peripheral blood cells from normal (AA) individuals (using Probe II)

	Individual	Reticulocyte α/β mRNA
	32	1.42
	33	1.64
	34	2.07
	35	1.32
	36	2.03
	38	1.45
Probe II	Mean + S.D.	1.66 ± 0.30
Probe I	Mean + S.D.	2.06 ± 0.2

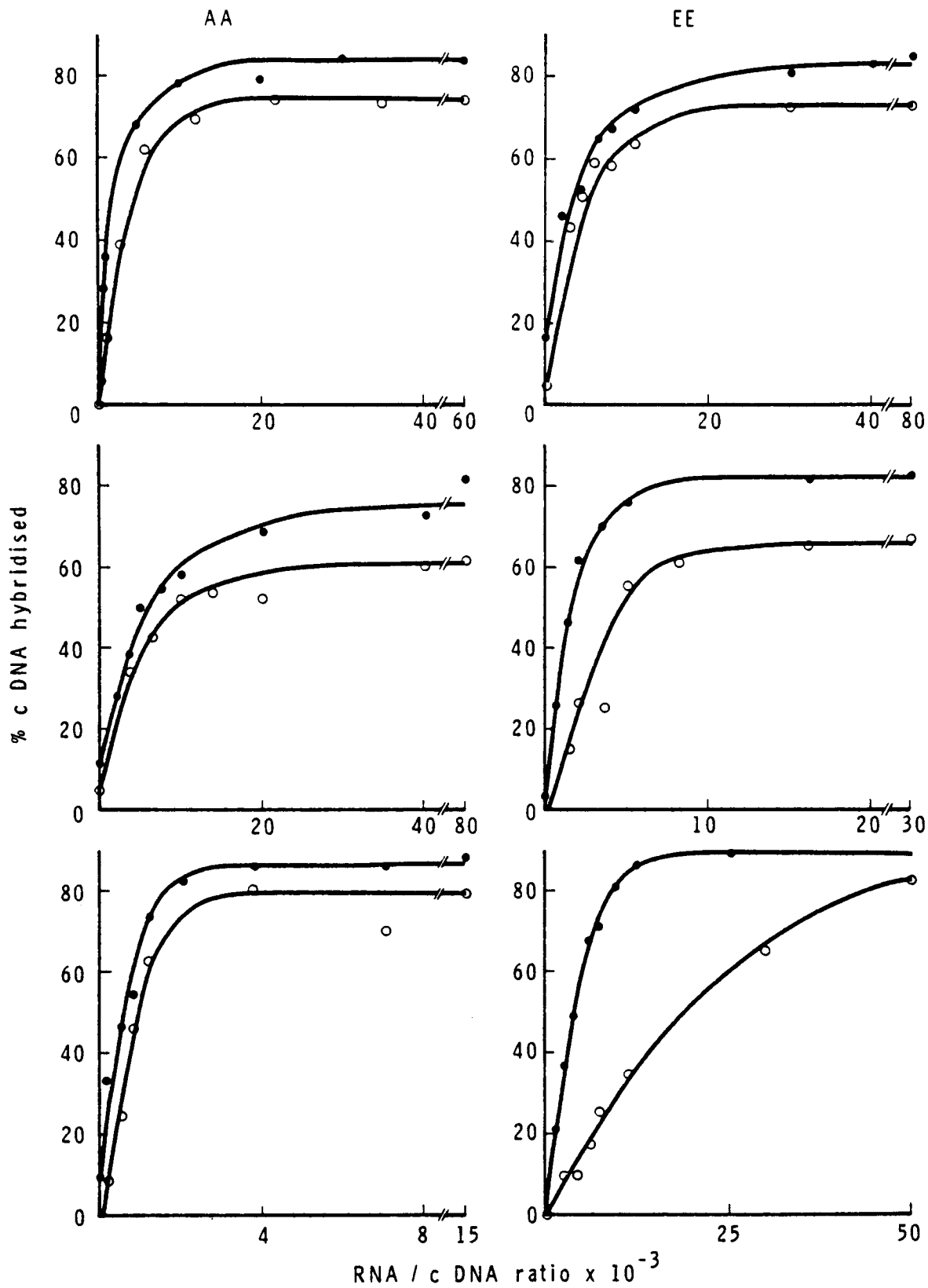


FIG. 5.4 The hybridization of cDNAα (●) and cDNAβ (○) to total RNA prepared from bone marrow nuclei (top), bone marrow cytoplasm (middle) and peripheral blood (bottom) of AA control case 32 (left) and, homozygous Hb E case 7 (right).

As Table 5.6 shows, there was little difference in the α/β mRNA ratios across the three samples for each AA control (Figure 5.5), although in three out of the five cases the α/β mRNA ratio in the bone marrow nuclear fraction was somewhat lower than in the other two fractions. However, among the Hb E homozygotes there was a consistent increase in the α/β mRNA ratio in going from the marrow nuclei to marrow cytoplasm to reticulocytes. (Table 5.6 and Figure 5.5).

Since the two different sets of probes gave slightly different mean ratios when used to measure α/β mRNA ratios in normal reticulocyte RNA samples (I 2.06±0.23; II 1.66±0.30; see Table 5.5), comparisons of the RNA ratios of the three fractions in both the AA controls and Hb E homozygotes were made after the results were corrected for this difference by dividing the observed α/β mRNA ratios by the mean normal reticulocyte RNA ratio for whichever probe was used. The normalised α/β mRNA ratios are summarised in Table 5.7.

In the AA individuals the α/β mRNA ratios in the marrow nuclei, marrow cytoplasmic fraction and the reticulocytes were 0.70 ± 0.19, 1.02±0.26 and 0.99±0.18 respectively (Table 5.7). In paired t-tests the differences between the ratios in the marrow nuclei and cytoplasm, marrow nuclei and reticulocytes and marrow cytoplasm and reticulocytes were not statistically significant. In the Hb E homozygotes (excluding case 6 with α thalassaemia 2) the corresponding mean α/β

TABLE 5.6 α/β mRNA ratios in reticuloyctes and bone marrow fractions

INDIVIDUAL		BONE MARROW NUCLEI α/β mRNA	BONE MARROW CYTOPLASM α/β mRNA	RETICULOCYTE α/β mRNA	PROBE USED
AA Controls	30	1.04	1.41	1.62	I
	31	1.45	2.24	2.18	I
	32	1.59	1.45, 1.59	1.42	II
	33	1.02, 1.11	1.77, 1.70	1.88, 1.40	II
	34	----	2.30	2.07	II
EE Cases	4	1.65	2.60	5.81, 5.46	I
	5	1.59	2.67, 2.44	3.29, 3.25	I
	6	1.14, 1.30	----	2.10, 2.00, 1.81	II
	7	1.30, 1.40	2.97	3.40, 3.92	II
	8	1.87, 1.64	2.30	5.18	II

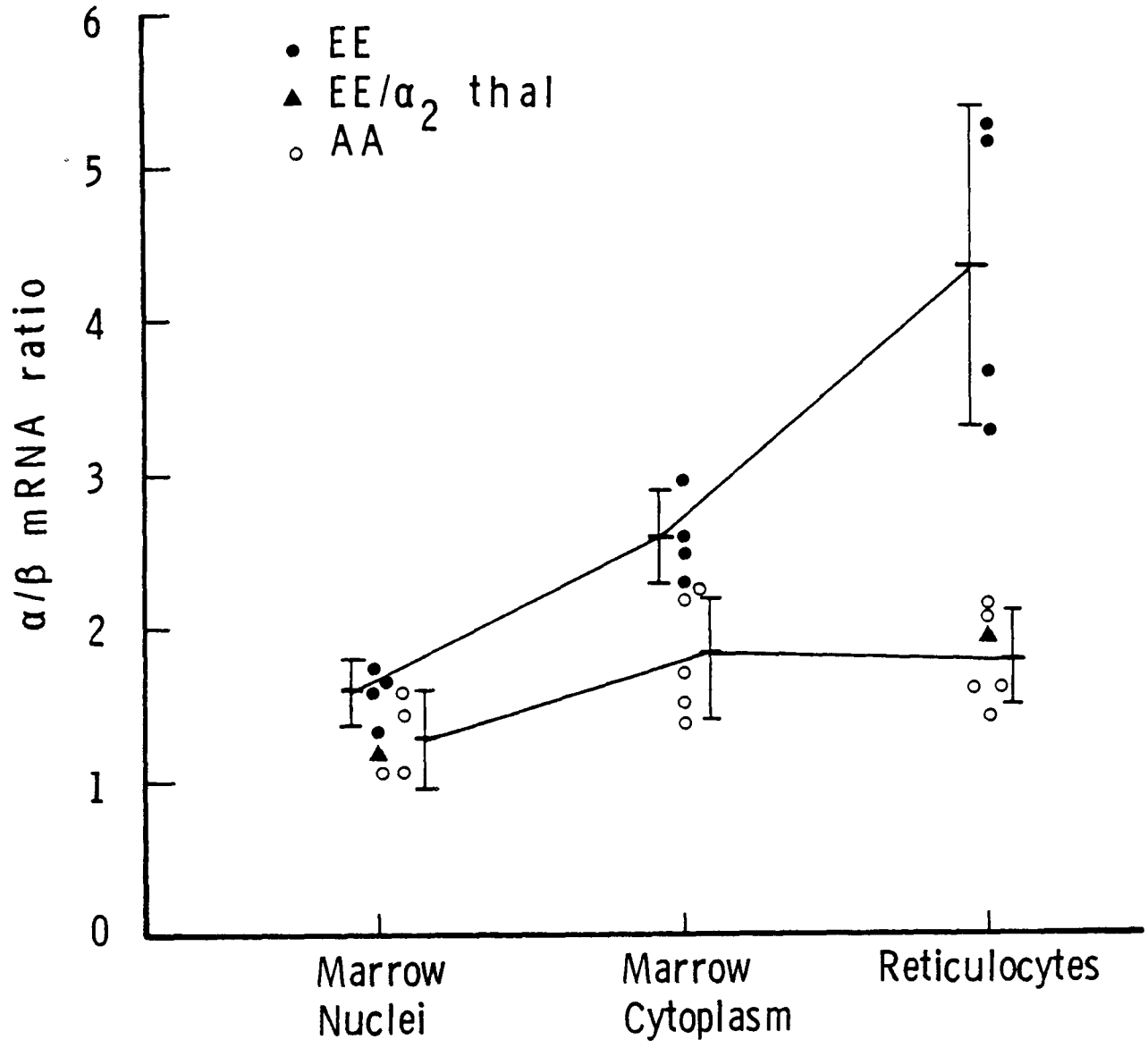


FIG. 5.5 α/β mRNA ratios, determined by cDNA solution hybridization in bone marrow nuclei, bone marrow cytoplasm and peripheral blood of AA controls and Hb E homozygotes.

TABLE 5.7 NORMALISED α/β mRNA RATIOS IN RETICULOCYTES AND BONE MARROW FRACTIONS

INDIVIDUAL		MARROW NUCLEI α/β mRNA	MARROW CYTOPLASM α/β mRNA	RETICULOCYTE α/β mRNA	MEAN NORMAL RETICULOCYTE α/β mRNA FOR PROBE
AA controls	30	0.50	0.68	0.79	2.06
	31	0.70	1.09	1.06	2.06
	32	0.96	0.92	0.86	1.66
	33	0.64	1.04	0.99	1.66
	34	----	1.39	1.25	1.66
mean ± SD		0.70 ± 0.19	1.02 ± 0.26	0.99 ± 0.18	
EE cases	4	0.80	1.26	2.73	2.06
	5	0.77	1.24	1.59	2.06
	6	(0.73)*	----	(1.19)*	1.66
	7	0.81	1.80	2.20	1.66
	8	1.05	1.39	3.12	1.66
mean ± SD		0.86 ± 0.13	1.42 ± 0.26	2.41 ± 0.66	

( )* value observed in EE with α thal 2. Excluded from mean value calculation.

mRNA ratios were: 0.86±0.13, 1.42±0.26 and 2.41± 0.66, clearly demonstrating an increase across the fractions. The mean marrow nuclei RNA ratio was significantly different from the marrow cytoplasmic and reticulocyte ratios ($p = <0.01$), while the marrow cytoplasmic and reticulocyte RNA ratios differed only at the 5% significance level, although this was as a result of the large standard deviation in the reticulocyte samples (see Figure 5.5).

When the mean values for each fraction are compared between the AA controls and the Hb E homozygotes, there is no significant difference between the nuclear RNA ratios, the difference between the mean marrow cytoplasmic RNA ratios just fails to reach significance at the 5% level, and the difference between the reticulocyte RNA ratios is highly significant ($p = <0.01$).

As expected, the ratios obtained in the Hb E homozygote with an interacting α thalassaemia 2 gene (case 6) were lower than in the other Hb E homozygotes due to the deficiency of α mRNA (Hunt et al 1980).

Discussion of Results

The α/β mRNA ratios observed in the peripheral blood reticulocytes of Hb E homozygotes demonstrated a marked deficit of β^E mRNA (relative to α mRNA) when compared to the α/β mRNA ratios in the reticulocytes of normal individuals. A deficit of β mRNA of about half that found in the Hb E homozygotes, was observed in the Hb E heterozygotes.

In both the Hb E homozygotes and heterozygotes, the deficit in β^E mRNA reflected the deficit in β^E globin production found in biosynthesis experiments on peripheral blood reticulocytes of the same individuals (see Chapter 4).

It is not possible to correlate the α/β globin synthesis and the observed α/β mRNA ratios directly, since the α/β mRNA ratios are not an absolute measurement of the concentrations of α and β globin mRNA sequences present, for reasons already outlined. However, in normal reticulocytes the α and β mRNAs, although not present in equal quantities (there are generally greater levels of α mRNA relative to β mRNA-Lodish 1971) direct the synthesis of equal amounts of α and β globin (because β globin mRNA has a higher rate constant for ribosome attachment than α mRNA whereby each β mRNA initiates the synthesis of more globin than each α mRNA - Lodish and Jacobsen 1972). Thus, for the purpose of correlating the globin synthesis and mRNA ratios observed for each Hb E homozygote and heterozygote, it may be assumed that the mean α/β mRNA ratio of 2.06, observed in the reticulocytes of the normal group (with Probe I), reflects the proportions of α and β mRNA required to produce balanced globin chain synthesis. The normalized Hb E α/β mRNA ratios were obtained by dividing the observed reticulocyte α/β mRNA by the mean normal reticulocyte α/β mRNA ratio of 2.06.

The normalised α/β mRNA ratios and the α/β globin synthesis ratios in the Hb E individuals are summarized

in Tables 5.2 and 5.3. In most of the cases the RNA ratios and globin synthesis ratios match closely and the correlation (r=0.89) is highly significant at the 0.1% level (see Figure 5.6), demonstrating that the defect in the synthesis of β^E globin in reticulocytes results directly from deficient levels of β^E globin mRNA and is therefore pre-translational.

In the majority of cases the normalised α/β mRNA ratio is slightly lower than the α/β globin synthesis ratio (see Tables 5.2 and 5.3). Although it is a consistent trend, this observation could be an artefact since variability (up to 10%) is observed to occur with both α/β synthesis and mRNA ratios for any individual from one experiment to another. The normalizing calculation for the α/β mRNA ratio may introduce further inaccuracy. Alternatively, this observation may reflect the presence of β mRNA sequences in Hb E reticulocytes which are detectable by cDNA hybridization but not functional in protein synthesis.

In interpreting the above results it has been assumed that the β^A cDNA probe has an equal affinity for the β^A and β^E mRNA sequences. It has been demonstrated that β^A cDNA hybridizes as well with β^S globin mRNA as β^A mRNA, since the α/β mRNA ratio in an individual homozygous for Hb S was within the normal range (Nienhuis et al 1977). Presumably β^E globin mRNA, like β^S globin mRNA, differs from β^A globin mRNA by only a single nucleotide and thus would also hybridize as well to the β cDNA probe.

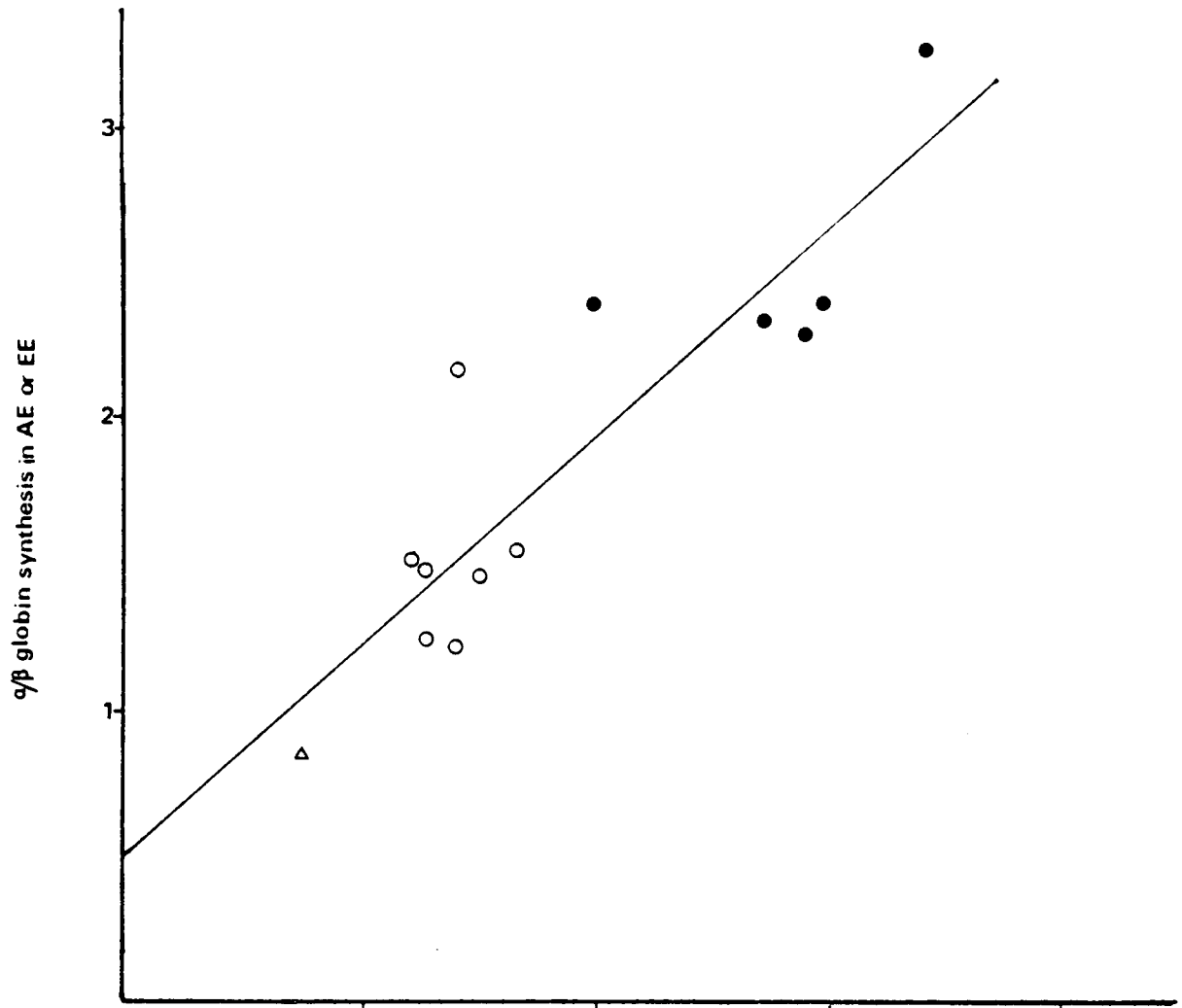


FIG. 5.6 α/β globin synthesis vs normalised α/β mRNA ratio in Hb E homozygotes (●), Hb E heterozygotes (○) and Hb E heterozygote with α-thalassaemia 2 (△).

The measurement of α/β mRNA ratios in marrow nuclei, cytoplasm and in peripheral blood reticulocytes showed consistent values in a group of AA controls. However, in Hb E homozygotes the α/β mRNA ratios increased in going from the marrow nuclei, to the marrow cytoplasm and then to the reticulocytes, presumably as a result of decreasing levels of β^E mRNA.

When assessing the α/β mRNA ratios observed in the two groups, various potential technical problems must be considered. Since both groups of samples were treated in the same manner, the trend of results observed in the Hb E homozygotes does not appear to be due to technical artefacts, such as differential loss or degradation of mRNA sequences. However, the marrow nuclei α/β mRNA ratios are somewhat lower than those in the marrow cytoplasm or in reticulocytes in most AA cases. In previous studies that used techniques almost identical to those used here, no such difference was observed by Nienhuis et al (1977), but Hunt et al (1982) obtained α/β mRNA ratios of 1.36 and 1.74 in marrow nuclei of 2 non-thalassaemic individuals compared to a range of 2.00-2.45 α/β mRNA observed in the reticulocytes of non-thalassaemic controls. It is possible that the much lower yields of total RNA often obtained from marrow nuclei, with a lower proportion of globin mRNA, contribute to this unexplained observation.

The close comparability of the marrow nuclear α/β mRNA ratios in the AA controls and the Hb E homozygotes suggests that the major defect in β^E mRNA production is not at the stage of transcription. While there may be

some loss of β^E mRNA between the marrow nuclei and cytoplasmic fractions the major loss of β^E mRNA sequences appears to occur once the message is within the cytoplasm. If there is any contamination of marrow cytoplasmic RNA by reticulocyte RNA in the homozygous Hb E samples, the cytoplasmic α/β mRNA ratios would tend to be falsely raised, exaggerating the difference between the marrow nuclear and marrow cytoplasmic RNA ratios. Certainly, the loss of β^E mRNA following the apparently normal transcription suggests that the β^E mRNA is in some way defective and that this results in its reduced stability within the cytoplasm.

The instability of β^E mRNA is consistent with the biosynthesis data presented in Chapter 4. In the Hb E heterozygotes the β^A/β^E specific activity ratios were consistently greater than unity, suggesting asynchrony of β^A globin and β^E globin synthesis during erythroid cell maturation. In addition the β^A/β^E specific activity ratios were lower in the marrow than in the blood of the Hb E heterozygotes, in accordance with a higher level of β^E mRNA in normoblasts than in reticulocytes.

Recently, the deficit of β^E mRNA observed in reticulocytes was confirmed by Benz et al (1981). In this study the relative concentrations of α and β mRNA sequences were measured using radioactive α or β cDNA specific probes in the reticulocytes of a Hb E heterozygote and in 2 Hb E-β thalassaemia compound heterozygotes. In the single Hb E heterozygote a ratio

of 2.11 was obtained, compared with a mean ratio of 1.15±0.09 observed in 10 normal individuals and just within the range of 2.44±0.27 found in 6 β thalassaemia heterozygotes. This deficit of β mRNA is greater than that seen in any of the 8 Hb E heterozygotes included in this study, although the globin synthesis ratio of 1.45 observed in the Hb E heterozygote studied by Benz et al was similar to those of the present series. The α/β mRNA ratios obtained in two Hb E/β^+ thalassaemia heterozygotes of 3.57 and 4.55, were similar to the range of about 3.0 and above observed in 31 β thalassaemia homozygotes.

A similar pattern of decreasing β mRNA during erythroid cell maturation was observed in the marrow and reticulocyte RNA fractions from β^+ thalassaemia homozygotes, obtained using similar techniques as those employed in this study (Nienhuis et al 1977). In each of 3 β^+ thalassaemia homozygotes the α/β mRNA ratios increased from the marrow nuclei (1.1, 1.6 and 4.4), to the marrow cytoplasm (2.5, 3.0 and 5.5), to reticulocyte RNA (3.2, 3.8 and 8.8 respectively) compared to non-thalassaemic controls in which the α/β mRNA ratios remained close to unity in all three fractions. Although these results are very similar to those found in this study, they are more difficult to interpret.

In β^+ thalassaemia homozygotes, cells synthesising more Hb F have a selective advantage, since in such cells the relative excess of α-globin is less marked.

There is, in fact, a decrease of α/γ mRNA ratios (presumably due to a relative increase in γ mRNA) from the marrow fractions to the reticulocytes observed in these β^+ thalassaemia homozygotes, compared to a constantly high α/γ mRNA ratio of 40 in the 3 RNA fractions of the controls. It has also been shown that the production of γ and β globin (and presumably, therefore, γ and β mRNA) within a cell is inversely related (Weatherall et al 1978). Thus, selection of cells with most γ chain synthesis may, as a result, select for cells with reduced β chain production, and this could be an explanation for the apparent loss of β mRNA sequences observed in the β^+ thalassaemia heterozygotes.

More recent analysis of the molecular defect in β^+ thalassaemia demonstrated a possible splicing (processing) defect of the mRNA precursors (Spritz et al 1981; Westaway and Williamson 1981). It is proposed that a single nucleotide mutation within the small intron, 21 nucleotides upstream from its 3' end (Figure 5.7) creates a new splicing site which most probably competes or interferes with RNA splicing at the authentic splice site. Any RNA molecules spliced at this abnormal site would have additional nucleotides within their sequence derived from the 3' end of the small intron. These extra nucleotides within the mRNA sequence would also cause a frame shift mutation generating a premature stop codon at the 3' border of the small intron sequence (Spritz et al 1981). If this abnormally spliced RNA enters the cytoplasm

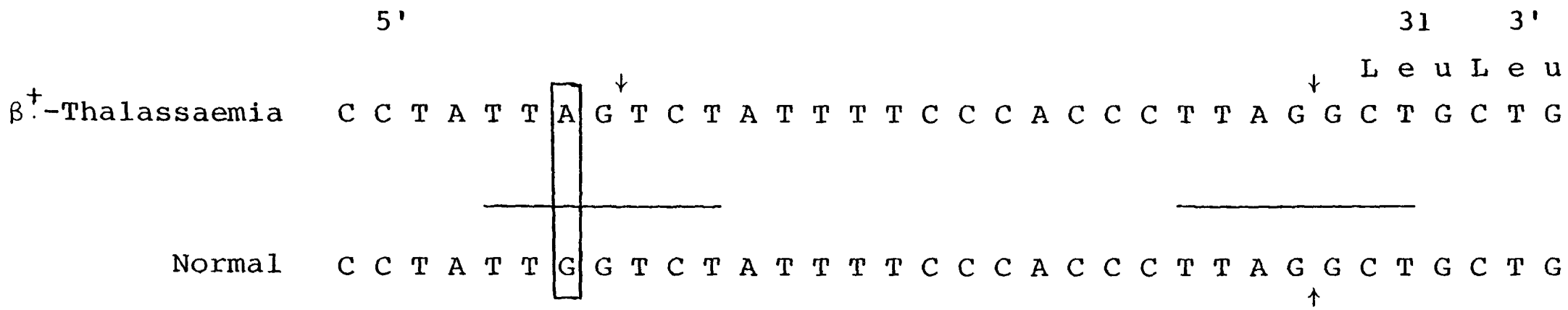


FIG. 5.7 Nucleotide sequence of the human β globin gene near the 3' junction between the small intervening sequence and the adjacent protein-encoding sequence. The homologous authentic and putative internal 3' splice junctions are indicated by the solid lines and the potential sites of intron excision are indicated by the arrows. The single nucleotide change in the β^+-thalassaemic DNA sequence is indicated by the box. Normal β globin mRNA codon position 31 is numbered. (From Spritz et al 1981).

it may be translated as far as codon 29 or 30 (depending on the precise site of splicing) and then into the additional sequences until the premature stop codon is reached. It has been proposed that if globin chain synthesis terminates prematurely, a large part of the mRNA will not be covered by ribosomes and so may be exposed to nuclease degradation (Chang and Kan 1979). This appears to be the case in the β^{o} thalassaemias that are the result of premature stop codons or nonsense mutations in the β mRNA. Two nonsense mutations have been identified, one in the Chinese population (β^{17UAG} - Chang and Kan 1979) and another in the Sardinian population (β^{39UAG} - Trecartin et al 1981), and in patients with thalassaemia major in whom at least one β thalassaemia gene contains a nonsense mutation, the otherwise apparently normal β mRNA is present in reticulocytes at markedly reduced levels (β/α mRNA 0.14 and 0.043-0.063 in Chinese and Sardinian cases respectively). Thus it is possible that the apparent instability of the β mRNA in β^{+} thalassaemia, suggested by Nienhuis et al (1977) as an interpretation of their results may, in fact, be correct. The observation that the α/β mRNA ratios (3.2, 3.8 and 8.8) were always lower than the corresponding α/β globin synthesis ratios (3.5, 6.4 and 12.5) in the 3 β^{+} thalassaemia homozygotes studied, is consistent with the presence of some hybridizable, but non-functional β mRNA surviving in these cells.

In the Hb E homozygotes there is little or no

ineffective erythropoiesis and hence no significant selection of cells with greater Hb F content. Therefore, the loss of β^E mRNA during red cell maturation is unlikely to be the result of cell selection and is probably real. The instability of β^E mRNA, as these results imply, could arise from abnormal nuclear RNA processing, abnormal secondary mRNA structure, abnormal binding of ribonuclear proteins important in maintaining mRNA stability or some other defect, either as a direct result of the nucleotide base substitution responsible for the amino acid change, or due to a second nucleotide mutation within the mRNA sequence. These possibilities will be considered in detail in Chapter 6.

CHAPTER 6

DISCUSSION

In Hb E homozygotes, and to a lesser degree in Hb E heterozygotes, the MCH values were below the range observed in normal individuals. This observation, plus that of the low proportion of Hb E relative to Hb A in Hb E heterozygotes, suggest that the levels of Hb E in peripheral red cells are reduced.

Theoretical consideration of the amino acid substitution in Hb E suggested that Hb E may have a tendency to dissociate into monomers (Perutz and Lehmann 1968). Although Hb E has been shown to be unstable *in vitro* under conditions of oxidant stress (Frischer and Bowman 1975; Ali et al 1980: Carrell and Lehmann 1981) experiments carried out in this study failed to demonstrate any substantial instability of Hb E *in vivo*. Thus the low levels of Hb E in peripheral blood are not apparently due to its instability.

Biosynthesis experiments, however, gave clear evidence of reduced synthesis of β^E globin in reticulocytes, with no suggestion of rapid β^E globin degradation. Instead analysis of the relative levels of α and β mRNA in reticulocytes demonstrated reduced amounts of β^E mRNA which correlated very well with the level of β^E globin synthesis.

A comparison of the α/β mRNA ratios in bone marrow and reticulocytes of AA controls and Hb E homozygotes

showed that the levels of β^E mRNA were equivalent to those of β^A mRNA in bone marrow nuclei, but that there were slightly reduced levels of β^E mRNA in the cytoplasm of bone marrow cells and significantly reduced levels in the reticulocytes. These results implied that there was decreased stability of β^E mRNA. They were supported by the observations in the biosynthetic studies: in the peripheral blood of Hb E heterozygotes that β^A/β^E globin specific activity ratios were most often greater than unity, suggesting comparatively more synthesis of β^E globin in red blood cell precursors; more directly incubation of bone marrow cells of individuals with Hb E suggested relatively greater synthesis of β^E globin in those cells than in reticulocytes.

The single amino acid substitution in β^E globin is presumably the result of a single base mutation in the DNA. Thus the fact that this appears to result in decreased stability of the mRNA, is a most unexpected finding.

Possible Mechanisms of β^E mRNA Instability

Normal globin mRNA appears to be very stable, as implied by the finding of functional globin mRNA sequences in peripheral blood reticulocytes despite the cessation of mRNA synthesis at about the polychromatophilic stage of erythroid cell maturation. This is supported by direct measurement of the half-life of globin mRNA in induced Friend cells. Initially values of about 17 hours were obtained (Aviv et al 1976; Lingrel

et al 1978) but more recent studies have shown that the half-life is probably greater than 60 hours (Volloch and Housman 1981).

Very little is known about the mechanisms involved in maintaining mRNA stability. Presumably the secondary structure of the mRNA, closely related to its association with specific proteins to form ribonucleoprotein (RNP), and its interaction with translation machinery of the cell all play a role. In addition, there is evidence that normal processing of the mRNA precursors is important in the formation of a stable mRNA (Breathnach and Chambon 1981).

Based on the above assumptions and what little is known about globin mRNA stability, several mechanisms may be proposed to account for the decreased stability of β^E mRNA:

(1) Abnormal secondary structure and/or abnormal formation of RNP and/or abnormal translation.

(2) Abnormal processing of mRNA precursors

(3) An independent nucleotide mutation from that which underlies the amino acid change in β^E globin.

Abnormal β^E mRNA Secondary Structure or Function

From the known sequence of the β globin gene (Forget 1977; Lawn et al 1980), the codon substitution thatgives rise to the glutamate to lysine amino acid change in β^E globin is GAG → AAG at codon 26. Since little or nothing is known about the secondary structure of globin mRNA, the possible functional effects of such

a substitution must be speculative.

Globin mRNA normally exists in the cytoplasm in association with proteins as ribonucleoprotein particles, the vast majority of which are found as polysomes undergoing active translation. It is not clear to what degree secondary structure of the RNA is determined by its sequence and how much is imposed by protein binding. If protein binding is itself sequence specific the two will clearly be interrelated. Furthermore, it may be that the correct RNP structure is necessary for translation of the mRNA by ribosomes which may itself be an essential requirement for mRNA stability.

Internal Base Pairing Internal base-pairing of nucleotides may be a basic requirement for maintaining mRNA secondary structure. Although attempts have been made to predict the secondary structure of globin mRNAs through computer simulations based on the most stable base pairing arrangements (Heindell et al 1978; Pavlakis et al 1980) there is no evidence that these are in any way comparable to the structures in vivo. However, it could be postulated that the guanidine to adenosine base change is at a critical position within the mRNA structure whereby the new adenosine base interferes with any base pairing in its vicinity. This may also have consequences on the base-pairing in other regions of the mRNA molecule,so further disrupting the secondary structure of the mRNA and leading to its instability.

Formation of RNP The binding of specific proteins to mRNA is suggested to be an important mechanism in the maintenance of mRNA stability. Presumably, the proteins protect the mRNA against nuclease degradation, possibly by acting as a physical barrier and/or by maintaining a secondary mRNA structure which is inaccessible to nucleases. Specific proteins have been observed associated with globin mRNA (Blobel 1972) and a specific protein associated with the poly (A) tail of many eukaryotic mRNAs has been partially characterized (Seytono and Greenburg 1981). The binding of this protein to the poly (A) tail of mRNA appears to be highly sequence specific possibly also involving sequences other than the poly (A) tail itself (Barrieux et al 1976). It has been postulated that the binding of proteins to the poly (A) tail prevents degradation of the mRNA by 3' exonucleases (Brawerman 1976). Evidence suggests that the poly (A) of mRNAs becomes shorter as the mRNA ages in the cytoplasm (Brawerman 1976) and results presented by Volloch and Housman (1981) suggest that there is a very close relationship between poly (A) metabolism and mRNA stability.

Other mRNA specific proteins may also occur. It could be postulated that the G→A base change that occurs in β^E mRNA may interfere with the binding of proteins to the mRNA in one of two ways. Either the nucleotide change is at a critical point in the sequence usually involved in specific binding with a protein, or that any disruption of the secondary structure of the mRNA caused by a disruption of normal base pairing

prevents the association of proteins at one or several regions of the mRNA molecule, for example with the poly (A) tail.

The substitution of the neighbouring nucleotide in codon 26 does not appear to lead to a low level of β chain production. Thus a heterozygote for Hb Henri Mondor (β26 Glu→Val, presumed substitution GAG→GUG) had 37.5% of the abnormal variant i.e. the level found in most β chain variants, despite the observation that this haemoglobin is slightly unstable (Blouquit et al 1976) Furthermore, in suggesting the possible disruption of base-pairing in β^E mRNA and/or binding of mRNA specific proteins to β^E mRNA as possible mechanisms which lead to β^E mRNA instability, it should be considered that such a deleterious effect of just a single base change would place considerable restraints on the nucleotide sequences of genes.

Translation of mRNA There is considerable evidence that abnormal translation of the mRNA can lead to its premature degradation. Thus homozygotes for β^o thalassaemia resulting from nonsense mutations (i.e. premature termination codons) at codons 17 and 39 have β mRNA levels which are markedly reduced (Chang and Kan 1979; Trecartin et al 1981). Presumably in these cases translation ceases when the stop codon is reached and the ribosomes dissociate from the mRNA at this point, leaving the remainder of the coding sequence untranslated. The low levels of β mRNA in the reticulocytes suggest that this leads to destruction

of the message. Conversely continued translation of the mRNA through the normal termination codon and into normally untranslated 3' end of the mRNA leads to its instability (Hunt et al 1982) and considerably reduced protein synthesis in the chain termination mutants such as Hb Constant Spring or the frame shift mutants such as Hb Tak (Lehmann et al 1975). The mechanism of the effects of premature or extended translation on mRNA stability is unclear. Possibly ribosomal attachment of the coding sequence is necessary for the protection of the mRNA from nucleases, while the unravelling of the 3' untranslated region by continued ribosomal passage also exposes the mRNA to nucleases.

It seems unlikely that abnormal translation of β^E mRNA is responsible for its instability. In normal globin mRNA, all the codons appear to be translated at an equal rate (Lodish et al 1978). However it has been postulated (Itano 1957) that if a tRNA for a specific codon is in limited supply, then the translation of this codon may proceed at a slower rate and result in the delayed translation of the remainder of the mRNA. In β^E mRNA the codon substitution is GAG→AAG. An analysis of codon usage in globin mRNAs (Forget et al 1978) shows that the AAG codon is used 8 times in normal β globin mRNA (out of a total of 11 lysine residues) and is the predominant lysine codon in the α and γ chains (Forget et al 1978). Therefore it is most unlikely that this tRNA is in limited supply. Furthermore, there are four other haemoglobin variants (Hb C (β6)

(Hunt and Ingram 1957), Hb Siriraj (β7) (Tuchinda et al 1965), Hb Agenogi (β90) (Miyaji et al 1966) and Hb British Columbia (β101) (Jones et al 1976/7)) in which the Glu→Lys substitution presumably results from a GAG→AAG nucleotide change. These are found at levels of at least 40% in the heterozygous state, implying that the GAG→AAG nucleotide change per se does not impair the rate of mRNA translation and hence decrease the stability of β mRNA.

Abnormal Processing of β^E mRNA Precursors

The presumed nucleotide change in codon 26 lies very close to the 5' end of the small IVS in the β globin gene, which is between codons for amino acids 30 and 31. Removal of this IVS from mRNA precursors requires 'splice-signals' which include a GT dinucleotide at the 5' splice junction and an AG dinucleotide at the 3' splice junction, each surrounded by a consensus sequence. Although the nucleotides of codon 26 do not appear to be involved in the consensus sequence of the 5' splice junction of the small IVS, it is possible that this region of the nucleotide sequence may be important in maintaining the correct secondary structure of the mRNA precursor in order that the small IVS is efficiently removed. There is some evidence to suggest that small nuclear RNAs (sn RNAs) may play some role in splicing by hybridising to the mRNA sequence near the splice points and guiding the splicing enzymes (see Breathnach and Chambon 1981). It could be speculated that the G→A nucleotide substitution occurs at a critical signal sequence involved in binding snRNA required for correct

splicing of the small IVS such that not all the β^E mRNA is efficiently spliced. If any β^E mRNA is incorrectly processed but reaches the cytoplasm, it is quite likely to be degraded for any of the reasons suggested previously for reduced mRNA stability: abnormal secondary structure, RNP formation or translation.

It is interesting to note that all the amino acid residues β24-30 (with the possible exception of β25) are invariant amongst the mammalian haemglobins of known sequence (Perutz and Lehmann 1968). Most of the haemoglobins with amino acid substitutions at these residues are unstable, suggesting that this sequence of the β chain plays an important role in maintaining haemoglobin stability. It is also possible that the nucleotide sequence is conserved along with the amino acid sequence in order to maintain the functional properties of the DNA and RNA.

It may be possible to determine whether processing of β^E mRNA is abnormal using the techniques applied to normal and β thalassaemia mRNA (Ross et al 1978; Maquat et al 1980). Until then, abnormal processing remains a formal possibility for the instability of β^E mRNA.

Independent Nucleotide Mutation

Although the mechanisms discussed above (abnormal mRNA secondary structure or abnormal processing) cannot be excluded as the basis of the β^E mRNA instability, there is no strong evidence in favour of either of them. Thus an alternative possibility must be considered in that the base substitution responsible for the amino acid change in β^E globin is not itself responsible for the mRNA instability

and that a further defect occurs in the β^E gene. In other words, it is quite possible that the G→A mutation is quite incidental and that the defect that underlies the decreased stability of β^E mRNA is a separate lesion present in the mRNA sequence. This putative β^+-thalassaemia like lesion present in cis to the β^E mutation could have arisen by several mechanisms. Homologous crossing-over between two chromosomes that each have single lesions is observed in the generation of abnormal haemoglobins which have amino acid changes at two different sites within the same globin chain, for example, Hb Arlington Park (β6 Glu→Lys; β95 Lys→Glu). Such a cross-over event could have occurred between a gene carrying the β^E determinant and another carrying a mild β^+ thalassaemia determinant giving rise to a gene with both lesions. Another mechanism could have involved a non-homologous cross-over between a gene with the β^E determinant and a normal β gene with one of the resultant genes having a β^E determinant along with either an insertion or deletion of a nucleotide sequence presumably within one of the non-coding sequences of the mRNA. Alternatively, independent sequential β^E and β^+ thalassaemia mutations may have occurred in either order within the same gene.

<u>The Synthesis of Globin Chains with Apparently Unstable mRNAs</u>

In addition to β^E globin, several other globin chains have been reported to be synthesised in reduced amounts due, at least in part, to instability of the mRNA which codes for them. An understanding of the mechanism behind such defects may shed light on the

mechanism which causes β^E mRNA to be unstable.

The δ chain of Hb A_2 and the δβ and βδ fusion chains of Hbs Lepore and Miyada respectively, are synthesised at reduced levels despite the similarity between their amino acid sequences and that of the β chain. In all of these cases synthesis appears to cease early in erythroid maturation since it is detectable in bone marrow, but not reticulocytes (Rieder and Weatherall 1965; Roberts et al 1972; 1973).

In bone marrow cells, fractionated according to their relative maturity, the β/δ chain synthesis ratios are constant in cells still synthesising RNA (up to the polychromatophilic stage) but in cells beyond this stage of erythroid maturation the δ chain synthesis decreases relative to β chain synthesis until little or no δ chain synthesis is detected in late normoblasts or reticulocytes. Evidence suggests that the decrease of δ globin synthesis is concomittant with the decrease in δ mRNA levels, the δ mRNA having a shorter half-life than β mRNA. (Wood et al 1978). More recent studies have supported the observation of a reduced δ mRNA half-life. In pulse incubations on both normal and β thalassaemic erythroblasts, the low levels of δ mRNA synthesised decline further during a chase incubation (Benz et al 1981a). Although the low levels of δ globin synthesis do not seem to be primarily caused by reduced δ mRNA stability (evidence now confirms the suggestion that δ globin mRNA is transcribed or processed at a slower rate than β globin mRNA - Wood et al 1978; Benz et al 1981a) there may be some comparisons to be made

between the reduced stabilities of δ mRNA and β^E mRNA. However, although the observation that the fusion globin variants also have unstable mRNAs suggesting that both the 3' and 5' sequences of δmRNA may be involved in its shorter half-life, the complete nucleotide sequence of the δ-globin gene has not revealed any obvious structural basis for the low expression of this gene either through transcription or, more significantly to Hb E, mRNA instability (Spritz et al 1980).

Some haemoglobin variants with elongated globin chains, Hbs Constant Spring (CS) and Tak, also appear to be synthesised at reduced levels due to instability of the variant globin mRNA. Hb Constant Spring is an α chain variant which results from a premature termination codon and is produced in such low amounts that it gives rise to the phenotype of α-thalassaemia. In two individuals with Hb CS-H disease, α/β mRNA ratios in peripheral blood were comparable to individuals with classical Hb H disease (α/β mRNA 0.38 and 0.25; α/β mRNA 0.23-0.40 respectively). In two individuals with Hb H disease, the α/β mRNA ratios in the blood and bone marrow nuclei were comparable (reticulocyte α/β mRNA 0.46 and 0.39, nuclei α/β mRNA 0.44 and 0.40). However, in an individual with Hb CS-H disease the α/β mRNA ratio in the bone marrow nuclei was greater (0.55) than in the peripheral blood (0.38) (Hunt et al 1982) clearly suggesting that the mRNA gets rapidly degraded within the bone marrow cells.

In a Hb Tak heterozygote, the incubation of peripheral

blood reticulocytes for 60 minutes demonstrated 27% β^{Tak} synthesis of total non-α synthesis, yet Hb Tak constituted 36% of the haemoglobin present in the blood. This implied that the production of β^{Tak} was decreasing as the red cells matured and was consistent with the proposal of an unstable β^{Tak} mRNA (Lehmann et al 1975).

The possible mechanism underlying reduced stability of α^{CS} mRNA and β^{Tak} mRNA could be that the translation of the message beyond the normal stop codon in some way renders the mRNA susceptible to nuclease attack. However, it should be noted that in the frame shift mutants Hb Wayne and Hb Cranston, in which there is also translation into the 3' untranslated region of the mRNA, there is no evidence of mRNA instability (Bunn 1978).

Other Haemoglobin Variants Synthesised in Reduced Amounts

In heterozygotes for β chain variants, the β gene dosage suggests that the haemoglobin variant and Hb A should be present in equal proportions. Similarly, the α gene dosage suggests that α chain variants should constitute about 25% of the total haemoglobin (Nute 1974). However, many variants are found at lower levels than expected. In many cases the reduced levels can be ascribed to instability of the haemoglobin molecule. However, several variants, stable and unstable, have been reported to be synthesised in reduced amounts (Table 6.1) and, in the light of the results reported here with Hb E, are worthy of further consideration.

TABLE 6.1 β CHAIN VARIANTS WITH SINGLE AMINO ACID CHANGES

HAEMOGLOBIN VARIANT	SUBSTITUTION	% VARIANT IN HETEROZYGOTES	% VARIANT SYNTHESISED	REFERENCE
Hb S	β6 Glu→Val	35 - 40	35	See text
Leiden	β6 or 7 Glu→O	25	43	Rieder and James 1974
G-San Jose	β7 Glu→Gly	32	30	Marinucci et al 1981
Riverdale Bronx	β24 Gly→Arg	27		Bank et al 1970
Abraham Lincoln	β32 Leu→Pro	18 - 20		Honig et al 1974
Bushwick	β74 Gly→Val	5 - 15		Rieder et al 1975
Vicksburg	β75 Leu→O	7.6*		Adams et al 1981
Sabine	β91 Leu→Pro	12		Shaeffer 1973
Koln	β98 Val→Met	12	20	White and Brain 1970
Indiannapolis	β112 Cys→Arg	None detectable		Adams et al 1979, 1981a
K-Woolwich	β132 Lys→Gln	30 - 40	30	Lang et al 1974

* Hb Vicksburg was described in a compound heterozygote for Hb Vicksburg and β^{O} thalassaemia

In order to distinguish whether the globin (or haemoglobin) is unstable or in fact synthesised in reduced amounts, ideally short pulse incubations and time course incubations should be carried out. Some of the unstable variants reported to be synthesised in reduced amounts are summarised in Table 6.2. Of those variants (Hb Bushwick, Hb Sabine, Hb Indiannopolis) on which shorter pulse and time course incubations have been carried out, rapid post-synthetic destruction of the variant is demonstrated, and extrapolation of the $\beta^{variant}/\beta^{A}$ chain synthesis back to zero time suggests approximately equal synthesis of the β variant and β^{A} chains. In the unstable variants which have not been studied at short times of incubation (Hb Riverdale Bronx, Hb Abraham Lincoln, Hb Koln), the EJSan data suggested no reason other than post-translational precipitation of the abnormal chain (Table 6.2).

The remaining 5 variants listed in Table 6.1 - haemoglobins S, Leiden, G-San Jose, Vicksburg and K-Woolwich - all appear to be synthesised at reduced levels, but without evidence of any rapid post synthetic degradation.

The results of biosynthetic experiments on haemoglobins Leiden, G-San Jose, and K-Woolwich are summarised in Table 6.3. In each case there were internal inconsistencies in the data which make some of the interpretations questionable.

In the reticulocytes of two Hb Leiden heterozygotes, although there was a deficit of β chain synthesis, this

TABLE 6.2 SYNTHESIS OF HAEMOGLOBIN VARIANTS WITH UNSTABLE ABNORMAL β CHAINS

Hb VARIANT	INCUBATION TIME (minutes)	$\beta^{Variant}/\beta^{A}$ SPECIFIC ACTIVITY	$\beta^{Variant}/\beta^{A}$ TOTAL COUNTS	COMMENT
Riverdale Bronx	60	1.3 - 1.5	--	If instability Hb RB sol reason for reduced, level then would expect β^{RB}/β^{A} ratio to be nearer 2.5 assuming 28% Hb RB in peripheral blood. Thus results imply destruction newly synthesised β^{RB}
Abraham Lincoln	30, 60 and 120	Approximately 1.25, 1.9 and 3.4		β^{AL}/β^{A} specific activity ratios >1.0 which suggest rapid post-synthetic destruction β^{AL}. Although trend of ratios with time inconsistent with this, the same electophoretic mobility of β^{AL} and β^{A} required indirect measurement of the specific activities, thus possibly inaccurate
Bushwick	5-15; 50-120		0.83 - 0.33; 0.14 - 0.05	Rapid post-synthetic destruction $\beta^{Bushwick}$ chain
Sabine	2.5, 5, 10 and 20	5.0, 4.0, 3.0 and 3.0		Rapid post-synthetic destruction β^{Sabine} chain
Koln	60, 120		0.3, 0.25	Post-synthetic destruction of β^{Koln}
Indiannapolis	1.25, 3, 5, 10 and 20		β^{I}/β^{Total} 0.38, 0.32 0.27, 0.2, 0.13	Very rapid post-synthetic destruction β^{I} chain

TABLE 6.3 SYNTHESIS OF SOME ABNORMAL HAEMOGLOBINS WITH STABLE ABNORMAL β GLOBIN CHAINS

HAEMOGLOBIN VARIANT	INCUBATION TIME	TOTAL COUNTS RATIOS			SPECIFIC ACTIVITY	COMMENTS
		α/total β	α/β^{A}	$\beta^{A}/\beta^{Variant}$	$\beta^{A}/\beta^{Variant}$	
+ Leiden	3 - 60	1.5 - 2.0	2.2 - 3.7	2.0 - 1.22	1.1 - 0.62	Mild haemolytic state in heterozygotes
G-San Jose	5 - 120	mean 1.0	---	mean 0.3*		
Woolwich	3 - 60	mean 0.79	mean 1.2	mean 0.3*		

* Ratios of $\beta^{Variant}$/total β total counts

\+ Counts ratios and specific activity ratios for Hb Leiden are those observed at 3 and 60 minutes respectively. Ratios at times between 3 and 60 minutes were intermediate and showed an increasing/decreasing trend.

appeared to be partly due to a lack of β^A chains, since the α/β^A ratio was greater than 2.0, even at short incubation times, and increased until by 60 minutes it was 3.7. This together with the decreasing β^A/β^{Leiden} counts and specific activity ratios suggest post-synthetic destruction of the β^A chain, so making interpretation of the results with respect to β^{Leiden} synthesis rather uncertain.

In reticulocytes of individuals heterozygous for Hb G-San Jose the β^G chain synthesis remained constant at about 30% of the total $\beta^A + \beta^G$ counts over a 5-120 minute time course, but despite this, the α/total β synthesis ratios were balanced. However, α thalassaemia was not conclusively excluded from these individuals.

A situation resembling Hb E synthesis was observed in biosynthesis studies on reticulocytes of a heterozygote for Hb K-Woolwich in that the specific activity of the β^{KW} chain was found to be much lower than that of the β^A chain throughout a time course incubation from 3-60 minutes (see Lang et al 1974). However, inconsistent with this was the observation that the proportion of β^{KW} synthesised of total β chains was directly comparable to the levels of Hb K-Woolwich in the peripheral blood of the individuals studied.

Hb Vicksburg is an abnormal haemoglobin variant which results from a single amino acid deletion in the β chain (Table 6.1). It was discovered in a Black American child who also carried a β^o thalassaemia gene and comprised 7.6% of the total haemoglobin present (with 87.5% Hb F and

3.4% Hb A_2). Its electrophoretic properties were identical to those of Hb A but structural analysis of Hb Vicksburg demonstrated the deletion at β75 and the variant was not found to be unstable.

In a time course incubation of reticulocytes from the propositus there was evidence for reduced synthesis of the $\beta^{Vicksburg}$ chain but none for its post-translational degradation. Since the measurement of the α/β mRNA levels in the reticulocytes of the propositus gave a ratio far below the level expected in a β thalassaemia heterozygote, the authors concluded that the reduced synthesis of Hb Vicksburg was the result of decreasing levels of $\beta^{Vicksburg}$ mRNA, in turn due to a β^{+} thalassaemia determinant in cis to the $\beta^{Vicksburg}$ lesion (Adams et al 1981). Whether the defect resulting in the reduced synthesis of this variant is analogous to that underlying the reduced synthesis of Hb E is not clear, since the levels of Hb Vicksburg are far lower. The mother of the propositus was presumed to be a Hb Vicksburg heterozygote but it is of course, extremely difficult to characterise the synthesis of a haemoglobin variant which has exactly the same electrophoretic mobility as that of Hb A. However, the similarity of the levels of $\beta^{Vicksburg}$ globin synthesis and $\beta^{Vicksburg}$ globin mRNA to those of β globin and mRNA synthesis directed by a β^{+} thalassaemia lesion raises the question of whether the amino acid deletion characterised in Hb Vicksburg was correct. Loss of an internal leucine residue from globin peptides is not an uncommon artefact (J.B. Clegg, personal communication) and instead Hb Vicksburg may simply be a β^{+} thalassaemia lesion without an abnormal β globin chain. Re-examination of the structure of Hb Vicksburg would clarify the situation

Finally, the synthesis of β^S chains must also be considered. Although these chains are found at levels of 34-40% and have been shown to have a reduced affinity for α-chains compared to the affinity of β^A and α chains (Shaeffer et al 1978) there is also evidence that they are synthesised in reduced amounts, proportional to the level of Hb S present in the peripheral blood (Shaeffer et al 1976). Closer inspection of this data in fact shows that the β^A/β^S specific activity ratios are generally greater than 1.0, implying a reduction in β^S synthesis during erythroid maturation. There is no evidence for the rapid destruction of the β^S chain, although when α-chains are in limited amounts (as in the presence of α-thalassaemia) some β^S chains are rapidly destroyed (Shaeffer et al 1976). In addition there is evidence that newly synthesised β^S chains become rapidly associated with cell stroma (Bank et al 1974). The synthesis of Hb S differs from that of Hb E in that there is no evidence of chain imbalance in Hb S heterozygotes (D.R. Higgs, personal communication), or even in Hb S homozygotes (Kim et al 1977; Wood et al 1980) and furthermore the β^S mRNA levels appear to be normal throughout red cell maturation with an α/β^S mRNA near to 1.0 (Nienhuis et al 1977). Thus, further investigation into the defect underlying reduced Hb S synthesis is required.

It is apparent that, with the possible exception of Hb K-Woolwich pending further investigation of its synthesis, none of the defects underlying the reduced synthesis of any of the haemoglobin variants considered

are comparable to that which results in reduced synthesis on Hb E and therefore no analogies can be drawn to suggest possible reasons for the reduced stability of β^E mRNA.

Whatever the mechanism for the decreased stability of β^E mRNA, the result is the reduced synthesis of β^E globin. From this point the β^E gene may be considered analogous to a mild β^+ thalassaemia gene. The consequences of this on the interaction of Hb E with β thalassaemia and on the maintenance of the β^E gene in the SE Asian population will be considered.

Reasons for the Maintenance of High β^E Gene Frequencies

Hb E is found in very high frequencies in the SE Asian population. For such high frequencies to occur there is presumably a selective advantage of the Hb E heterozygotes over normal individuals. The geographical distribution of Hb E and *P. falciparum* imply that, as for some other haemoglobinopathies, the β^E gene is selected, through the Hb E heterozygote, by its protection against malaria-mediated morbidity or mortality. However, as presented in Chapter 1, there is, as yet, no direct evidence to confirm this.

Suggestions of potential mechanisms by which Hb E may protect the red cell against *P. falciparum* have tended to concentrate on any abnormal features of the Hb E molecule itself, such as its oxidative instability. However, the results of the studies described in this thesis raise the possibility that the β^E gene may be selected for its mild β thalassaemia-like characteristics.

In the case of β thalassaemia, it has been demonstrated that the oxidative sensitivity of β thalassaemia red cells inhibits the growth of malaria parasites in vitro (Friedman 1979). The exact cellular mechanisms which mediate this inhibition are complex but it appears that the presence of the malaria parasite, together with the free α globin chains, overloads the redox systems of the red cells whereby they are unable to prevent oxidative damage to the red cell proteins and particularly the cell membranes. It appears that oxidative damage to the red cell membrane results in increased cation (K^+) permeability and hence parasite growth is inhibited since the parasites require a high potassium environment (Friedman 1979). It is possible, therefore, that an analogous mechanism for mediating red cell resistance to malaria growth is present in cells with Hb E, since they also contain free α chains. Of course, the tendency of Hb E to dissociate into monomers may still be considered as an additional mechanism and yet another way in which Hb E could protect against malaria is through its tendency to bind reversibly to the red cell membrane and enhance membrane oxidation (Friedman 1980). Thus, assuming the selective environmental factor for the β^E gene is P. falciparum, the β^E gene may be selected not only for its β thalassaemia like phenotype but also for the role that Hb E itself may play in increasing the oxidative sensitivity of the red blood cell.

Although the selective agent for Hb E has not yet been unequivocally defined, the selection for the β^E

gene appears to be very strong. This is indicated by the observed frequencies of more than 50% in some areas (Na-Nakorn and Wasi 1978; Sicard et al 1979; Wasi et al 1980). Since the fitness of the homozygotes does not appear to be substantially reduced, it may be that the β^E gene is going towards fixation, provided that the selective agent (malaria?) remains prominent in the environment. Of course there is some β^E gene loss when combined with β thalassaemia, which also is quite prevelant in these populations and the effect of interacting α thalassaemia on the fitness of an individual who also has Hb E is completely unknown. Certainly, considerably more investigation is required into the frequency and distribution of the various interacting haemoglobinopathies in these populations, as well as studies on the natural history of the different genotypes that exist in order that the population dynamics of Hb E can be defined.

The Interaction of Hb E with β-thalassaemia

The often severe clinical presentation of Hb E/β thalassaemia seems at last explicable with the finding that the β^E gene directs reduced synthesis of β^E globin and hence acts as a mild β thalassaemia. Thus, in the presence of another β thalassaemia gene an often severe condition results. The pathophysiological mechanisms underlying homozygous β thalassaemia and Hb E thalassaemia appear to be similar and stem from the excess α chain production which leads to intra-medullary destruction of red cell precursors and in turn to ineffective erythropoiesis. The presence of

excess α chains, which cause some oxidant stress in the red cells, possibly results in some precipitation of Hb E, but whether this has any role to play in the pathophysiology of Hb E β thalassaemia is unclear.

Further Studies on Hb E

One question that remains unanswered in this thesis is the defect which results in the reduced stability of β^E mRNA. There are several possible routes of investigation which may yield an answer.

In order to investigate whether a second mutation is responsible for the reduced stability of the β^E mRNA, the β^E gene could be cloned and the mRNA coding portion sequenced.

Sequencing of the entire β^E gene could possibly demonstrate a splicing defect. In addition the characterization of the mRNA precursors synthesised in pulse-labelled Hb E bone marrow cells, would also indicate a splicing defect.

The absence of a second abnormality within the β^E mRNA coding sequence would suggest that the single nucleotide change is in fact responsible for the mRNA instability and experiments could then be directed towards investigating the structure of the β^E mRNA: the presence or absence of a poly (A) tail; any differences in the secondary structure of β^E mRNA compared to β^A mRNA as possibly revealed by the sizes of nucleotide fragments generated by limited S_1 nuclease digestion (Pavlakis et al 1980).

The presence of a second lesion in the β^E gene could be indicated by examining the synthesis of Hb E in individuals with Hb E from other racial groups. Presumably, if the β^E gene in these individuals arose by a separate mutation (rather than racial admixture) the presence of a β^+ thalassaemia-like phenotype associated with their β^E gene would suggest that the β^E lesion itself is responsible for the reduced synthesis of β^E globin; the absence of a β^+ thalassaemia phenotype would suggest that a second mutation had arisen on the SE Asian β^E gene.

Recently analysis of the restriction enzyme polymorphisms within the β gene cluster have shown that, in the Mediterranean population at least, there are several different haplotypes defined by the pattern of polymorphic sites, and that specific mutations within the β gene itself, are in linkage disequilibrium with these haplotypes (Antonarakis et al 1982; Orkin et al 1982). If the haplotype(s) associated with the β^E genes and other β genes were defined in the SE Asian population,patterns may be observed which could indicate that age of the β^E gene or even a possible origin of the putative second mutation.

This thesis set out to investigate the reasons for the reduced synthesis of Hb E with the possibility of defining the unique interaction of a polymorphic haemoglobin variant with β thalassaemia. In fulfilling the aims of the thesis, a molecular lesion has been revealed which on characterization may possibly provide

some insight into mechanisms involved in controlling the half-life of mRNA's, so important for the normal process of protein synthesis.

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PUBLICATIONS

Some of the data presented in this thesis have also been published.

Traeger, J., Wood, W.G., Clegg, J.B., Weatherall, D.J. Wasi, P. (1980) Defective synthesis of Hb E is due to reduced levels of β^E mRNA. Nature 288, 497.

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APPENDIX I HAEMATOLOGICAL PARAMETERS AND GLOBIN BIOSYNTHESIS OF β-THALASSAEMIA HETEROZYGOTES RELATED TO INDIVIDUALS WITH Hb E INCLUDED IN THIS STUDY.

CASE NUMBER	SEX	Hb g/dl	RBCx $10^{12}/\ell$	Hct	MCV fl	MCH pg	MCHC g/dl	% RETICULOCYTES	SERUM IRON	TIBC	Hb TYPE	% F	α/β^A TOTAL COUNTS	α/β^A SPECIFIC ACTIVITY
21	M	13.7	6.6	43.1	65	20.8	31.7	2.58	----	----	A + F + A_2	1.6	1.91	1.85
26	M	13.8	6.6	42.5	64.6	21.0	32.5	1.6	12.5	88.5	A + F + A_2	---	2.57	2.26
28	F	11.3	5.37	35.6	66	21.2	31.9	2.0	----	----	-----	---	2.32	2.7

APPENDIX II

Incubation mixture (IM)

The mixture is made up as follows:

1 54 ml amino-acid mixture, made up in RS
2 2·7 ml 0·25M $MgCl_2$ with 10% glucose, made up in RS
3 27 ml 0·164M Tris–HCl pH 7·75 *made up in* H_2O
4 21·6 ml 0·01M trisodium citrate, made up in dialysed AB plasma
5 32 ml 0·01M $NaHCO_3$, made up in dialysed AB plasma.

Dialysed plasma

AB plasma dialysed exhaustively at 4 °C against multiple changes of RS.

Amino-acid mixture

Amino acids (10 × concentration): weight in mg dissolved in final 100ml H_2O.

Alanine	178	
Arginine	87	
Aspartic acid	379	
Asparagine	264	(To be diluted 1 in 10 before use.
Glycine	398	Check pH and adjust if necessary
Glutamine	1169	to 7·75 with NaOH). If a labelled
Histidine	372	amino acid other than leucine is
Isoleucine	39	used, omit the unlabelled amino
Lysine	263	acid from this mixture and replace
Methionine	45	with leucine (1312 mg).
Phenylalanine	264	
Proline	161	
Serine	173	
Threonine	202	
Tryptophan	61	
Tyrosine	145	
Cysteine	48	

The final incubation mixture can be sterilised by filtration, first through a glass-fibre filter and then through a 0·22 μm pore-size Millipore filter and stored frozen at −20 °C.

APPENDIX III α/β TOTAL COUNTS RATIOS IN RETICULOCYTES OF NORMAL INDIVIDUALS USED AS CONTROLS FOR BIOSYNTHESIS EXPERIMENTS

CASE NUMBER	α/β^{A} TOTAL COUNTS RATIO
35	1.01; 0.94; 1.01; 1.08
36	1.14; 1.00; 1.01
37	0.94
38	0.99