DIAGNOSIS OF ALPHA THALASSAEMIA TRAIT IN DIFFERENT ETHNIC GROUPS

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Blood count measurements, haemoglobin electrophoresis and estimation of HbA_2 and F were performed by haematology laboratory staff as part of routine investigation of the patients. Serum ferritin, HbH inclusion tests and globin chain synthesis studies were performed by myself.

SUMMARY

The tests used in the diagnosis of α thalassaemia trait include measurement of red cell indices, measurement of HbA_2, measurement of serum ferritin, demonstration of HbH inclusions and globin chain synthesis ratios. The efficacy of these tests to substantiate this diagnosis may be impaired unless certain variables are carefully controlled and, in the mild form of the disorder, diagnosis may not be possible without resort to gene mapping techniques. In addition, the heterogeneity of the disorder becomes apparent when studying patients from different ethnic groups. Complicating disorders e.g. co-existent iron deficiency compound the diagnostic problem.

The main aims of this study are:-

1. Assessment of red cell volume distribution as a means of differentiating α thalassaemia trait from iron deficiency.
2. Assessment of the effect of different variables on the performance of HbH inclusion testing:
   (a) Variation in incubation temperature.
   (b) Variation in incubation time.
   (c) Use of potassium iodide as a stimulant.
   (d) Use of centrifuged blood methods as an alternative to the traditional whole blood method.
3. Assessment of the reliability of HbH inclusion testing in the diagnosis of all forms of α thalassaemia trait in different ethnic groups.
4. Assessment of globin chain synthesis ratios to diagnose the existence of α thalassaemia trait.

5. Study of the heterogeneity of α thalassaemia trait in different races.

The following conclusions were reached:-

1. Red cell volume distribution analysis using the red distribution width (RDW) parameter produced by the Coulter Counter Model S Plus IV is a useful measurement for distinguishing uncomplicated α thalassaemia trait from iron deficiency.
2. Standardisation of a number of variables is of importance in achieving the best results in HbH inclusion testing:
   (a) HbH inclusions develop more rapidly when incubation is conducted at 37°C rather than 25°C.
   (b) In general, the rate of development of HbH inclusions increases in direct proportion to the time of incubation. However, incubation of control blood for 2 hours at 37°C results in the emergence of false positive results.
   (c) The use of potassium iodide as a stimulant does not show any advantage.
   (d) The use of centrifugation techniques increases the number of inclusions seen particularly in the severe form of the disorder, but did not increase the diagnostic yield in the mild form.
3. The HbH inclusion test proved unreliable in the diagnosis of mild forms of α thalassaemia trait.
4. Globin chain synthesis studies showed statistically

significant differences between controls and mild forms of the disorder and between mild and severe forms of the disorder. However, some overlap of results occurred between control subjects and patients with mild forms, therefore this test must be considered unsuitable in a proportion of patients with mild α thalassaemia trait.

5. Examples of α thalassaemia trait were found in patients of Indian, Chinese and Negro origin. In spite of reports to the contrary, the severe form of the trait was identified in a significant proportion of Indian patients. A comparison of the severe form in Indian and Chinese subjects revealed differences in the number of HbH inclusions demonstrable and in the degree of suppression of globin chain synthesis.

INTRODUCTION

1. GENERAL INTRODUCTION

The thalassaemias are a group of inherited disorders of haemoglobin synthesis which result from a deficiency of either the α or β chains of haemoglobin. The α thalassaemia syndromes are a common worldwide group of disorders which result from inherited defects in the rate of synthesis of the α globin chain. Since α chains are present in both foetal (HbF,$\alpha_2\gamma_2$) and adult haemoglobins (HbA,$\alpha_2\beta_2$), a deficiency of α chain production will affect haemoglobin synthesis in foetal as well as in adult life. A reduced rate of α chain synthesis in foetal life results in an excess of γ chains, which form γ_4 tetramers or haemoglobin Bart's. In adult life a deficiency of α chains results in an excess of β chains which form β_4 tetramers or haemoglobin H.

The extent of the α chain deficit is very variable and is usually associated with four major clinical manifestations in most ethnic groups:-

(a) α thalassaemia 2, the milder form of the heterozygous state which is either silent or causes only mild red cell changes.

(b) α thalassaemia 1 which is a more severe variety of the heterozygous state resulting in the appearance of microcytic red cells, but little or no anaemia.

(c) Haemoglobin H disease, usually a combination of α thalassaemia 2 and α thalassaemia 1, resulting in mild to moderate anaemia with marked morphological changes in the red cells.

(d) Haemoglobin Bart's hydrops foetalis syndrome (HBHFS) an invariably fatal condition which represents the homozygous state of α thalassaemia 1.

The first form of α thalassaemia to be recognised was HbH disease, which was described independently in the United States and in Greece in 1955 (Rigas et al, 1955: Gouttas et al, 1955). Jones et al (1959) suggested that HbH results from a deficiency in α-globin chains since it was shown to consist solely of β chains.

In 1958 haemoglobin Bart's was described by Ager and Lehmann. Fessas (1960) assumed that this haemoglobin also resulted from a deficiency of α chains and was the foetal counterpart of HbH.

In 1960 Lie-Injo and Jo described a stillborn Indonesian infant whose haemoglobin consisted almost entirely of Hb Bart's. This was the first report of the entity now recognised as haemoglobin Bart's hydrops foetalis syndrome (HBHFS).

Subsequently an extensive series of genetic studies on the various forms of α thalassaemia in Thailand (Wasi et al, 1964; Pootrakul et al, 1967; Na-Nakorn et al, 1969) suggested that the disease resulted from the interaction of two main α thalassaemia determinants, α thalassaemia 2 and α thalassaemia 1.

2. GENETICS OF α THALASSAEMIA

Although α thalassaemia clearly has a wide geographical distribution, initially there appeared to be no straightforward genetic patterns which explained

the inheritance of α thalassaemia in all populations (Higgs and Weatherall, 1983).

There were two hypotheses proposed in the 1970's to explain the genetic basis of α thalassaemia. One hypothesis based on family studies in Thailand where α thalassaemia is common, proposed the existence of a single α chain locus and two thalassaemia alleles; the α thalassaemia 1 allele caused complete inhibition of α-chain synthesis. The observed phenotype of HbH disease was the result of interaction of these two genes. This hypothesis is no longer tenable (Na-Nakorn and Wasi, 1970). The second hypothesis remains partially tenable and was based on evidence that the α globin structural gene is duplicated (Lehmann and Carrell, 1968; Lehmann, 1970). A normal individual would, on this evidence, possess four α globin genes i.e. (αα/αα). α thalassaemia would then result from gene deletion, the severity depending on the number of α chain genes deleted up to a maximum of four (Kan et al, 1975). This hypothesis explains the inheritance of α thalassaemia in most human populations.

This gene deletion, thought to be the basic abnormality in α thalassaemia, results in a deficiency or absence of functional α globin messenger ribonucleic acid (mRNA). The nature of the underlying defect responsible for the decrease in α mRNA production has been studied. Two groups (Ottolenghi et al, 1974: Taylor et al, 1974) were able to show by hybridisation of deoxyribonucleic acid (DNA) and radioactive

synthesised DNA copy (DNA_c), DNA/DNA_c, that the α chain genes are substantially, if not completely, deleted in homozygous α thalassaemia 1 (Hb Bart's hydrops foetalis). These studies were the first demonstration of gene deletion as a cause of α thalassaemia. The same technique has been used (Kan et al, 1975) to demonstrate deletion of three-quarters of the normal complement of α globin genes (HbH disease). These results indicated that α thalassaemia 2 patients have three functional α globin genes (αα/-α); α thalassaemia 1 trait subjects have two (--/αα); individuals with haemoglobin H disease have one (--/-α), and infants with haemoglobin Bart's hydrops foetalis syndrome have none.

More extensive genetic studies followed which described the structure of α-globin genes. In 1977, Diesseroth et al reported that α-globin genes were located on chromosome 16. Using a Southern blotting technique (cleavage of DNA molecules into fragments by enzymes) it was shown that the α-globin genes on each chromosome are duplicated and separated by approximately 3.0 kb (kilobase pairs) of non-coding DNA (Orkin, 1978). Lauer et al (1980) using a radioactive labelled α-globin DNA_c probe, studied the fine structure of α genes. They isolated a cluster of five α-like globin genes. In addition to the α genes (α1 and α2) they also identified a pseudo α gene (ψα1, a non-functional α globin-like DNA sequence) and two embryonic α-like globin genes(ζ1 and ζ2) within a 26 Kb stretch of DNA. These genes

are arranged along the chromosome in the order in which they are expressed during development 5′-ζ2-ζ1-ψα1-α2-α1-3′ (Figure 1). Each gene is located in a region of homology approximately 4Kb long, interrupted by two small non-homologous regions (Orkin, 1978; Lauer et al, 1980: Liebhaber et al, 1981) (Figure 2).

It is thought that the homologous regions have resulted from an ancient duplication event and that the non-homologous segments may have subsequently arisen by insertion of DNA into the non-coding region around one of the two genes (Fritsch et al, 1980).

The structure of each α gene conforms to the general pattern observed for all globin genes. It consists of three coding regions (exons) and two intervening sequences (introns) as illustrated in Figure3. Comparison of the α1 and α2 coding sequences has revealed no differences in exons 1, 2 or 3 (Liebhaber et al, 1981). The first intron in each gene is identical but the second intron of α1 is nine bases longer and differs by three bases from that in the α2 gene (Liebhaber et al, 1980). In each gene the start codon is Thymine, Adenine, Guanine (TAG) and the terminator is Thymine, Adenine, Adenine (TAA). The exon/intron junctions all conform to the Chambon rule (5 -GT/AG-3)which appears to be a requisite for normal splicing of nuclear mRNA (Breathnach et al, 1981). The ζ1 and ζ2 genes are also highly homologous. The introns are much larger than those found in the α-globin genes (Higgs and Weatherall, 1983). There are three base changes in the coding sequence of the first exon of ζ1, one of which

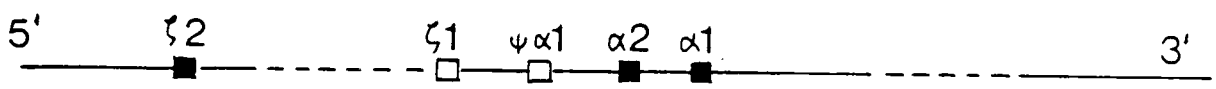

FIGURE 1: The α-gene complex.

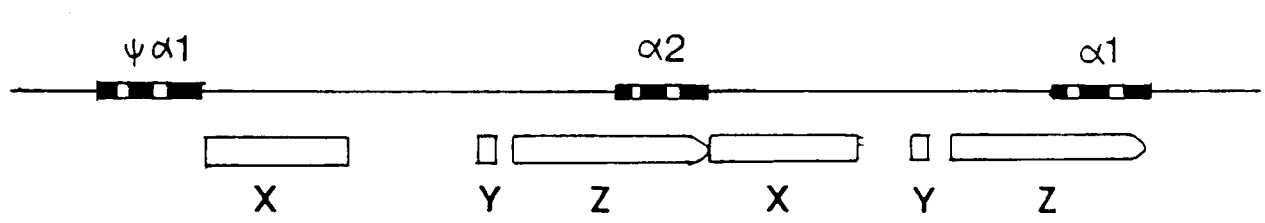

FIGURE 2: Details of homologous regions x, y and z surrounding the α1- and α2-genes.

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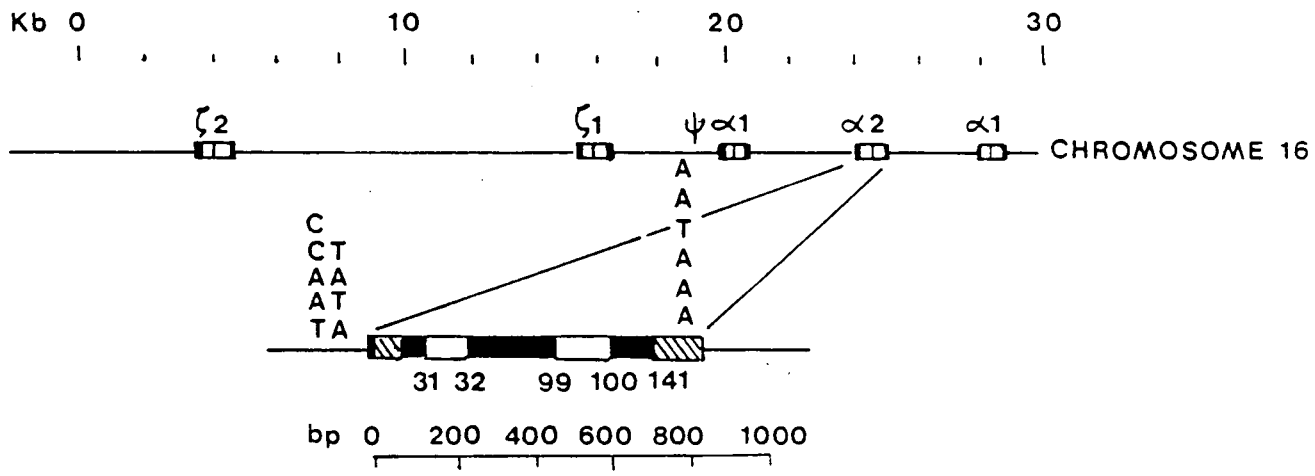

FIGURE 3: The α globin gene cluster. The α2 gene has been enlarged to show the non-coding regions (hatched), the exons (dark shading), and introns (unshaded).

Reproduced from Molecular Pathology of Thalassaemia by Weatherall and Wainscoat in Recent Advances in Haematology (1985), with kind permission of Churchill Livingstone, publishers.

gives rise to a premature stop codon. Thus it appears that ζ1 is an inactive pseudo-gene (Proudfoot, 1982).

Recent studies showed that variation in the clinical syndromes and patterns of inheritance of α thalassaemia are due to diversity in the underlying molecular defects rather than variation in the number of α-genes present in non-thalassaemic individuals (Higgs and Weatherall, 1983). Alpha thalassaemia 2 and α thalassaemia 1 could therefore be further classified into those which are due to deletion from the α-gene complex, and "non-deletion" or mutations in which the complex appears to be intact.

Last year, Weatherall and Wainscoat (1985) described the simplest way in which this disorder might arise (Figure 4). In this model they showed that α-thalassaemia 2 results from the deletion of only one of the pair of α-globin genes whereas α thalassaemia 1 results from the deletion (loss) of both the linked α-globin genes (α1 and α2). It turns out that α thalassaemia 1 trait is due to deletions which remove both α-globin genes. However, α thalassaemia 2 trait can be due to either gene deletion or alternatively to mutations which inactivate them partly or completely.

2.1 Deletion Types of α Thalassaemia

2.1.1 α thalassaemia 2

In the deletion forms, different size deletions (3.7 and 4.2 kb) remove one α globin gene and leave one functional α gene per haploid genome (-α) (Figure 5). These lesions are thought to have arisen by unequal crossover between homologous pairs on

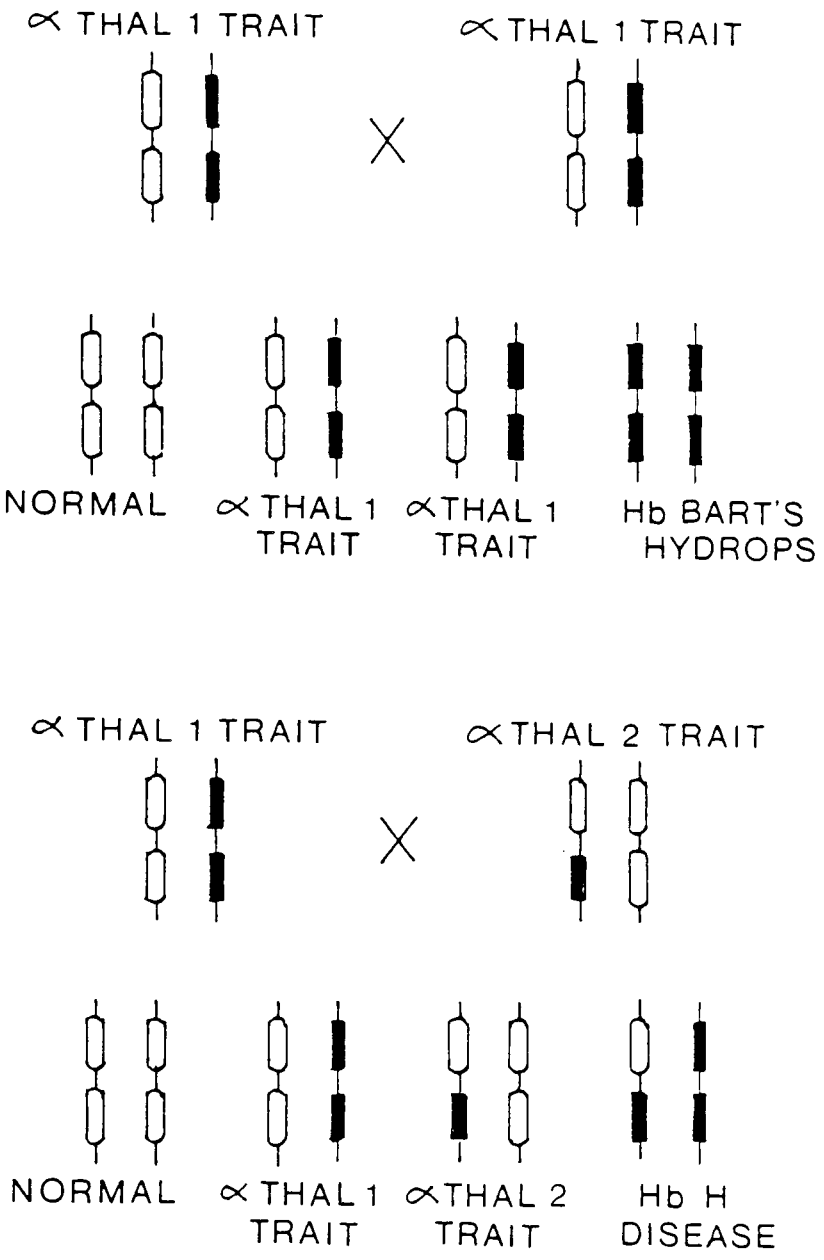

FIGURE 4: Models for the transmission of α thalassaemia 1 and α thalassaemia 2 and the production of Hb Bart's Hydrops and HbH disease. Normal α loci are shown by the open boxes and deleted or non-deleted α thalassaemia genes by the shaded boxes.

Reproduced from Molecular Pathology of Thalassaemia by Weatherall and Wainscoat in Recent Advances in Haematology (1985), with kind permission of Churchill Livingstone, publishers.

chromosome 16, leaving one globin gene on one of the pair and three on the other (Figure 6). A 3.7 kb deletion may involve both α genes and leave a single composite α gene while the other 4.2 kb deletion removes the α2 gene.

2.1.2 α thalassaemia 1

The common Southeast Asian or Mediterranean α thalassaemia 1 is caused by different deletions of the α-globin gene cluster which start downstream from the α1 globin genes (Figure 5) and extend upstream through the α-globin chain gene cluster, removing the α2 gene, the ψα gene and in one case, one of the ζ globin genes (Pressley et al, 1980). It is not clear how these deletions have arisen. They may result from unequal crossing over but the reciprocal arrangements have not been observed. It is possible that the major rearrangements of this type are lethal (Weatherall and Wainscoat, 1985).

2.2 Non-deletion Type

Several different "non-deletion" α thalassaemia 2 syndromes have been characterised (Figure 7) (Weatherall and Wainscoat, 1985).

2.2.1 5 bp Deletion

This involves the first intron of the α2 gene and is characterised by abnormal splicing of the α2 globin mRNA. It results from the loss of five bases (TGAGG) following the G of the invariant G-T contained within the donor splice site. Since, almost without exception, intervening sequences of

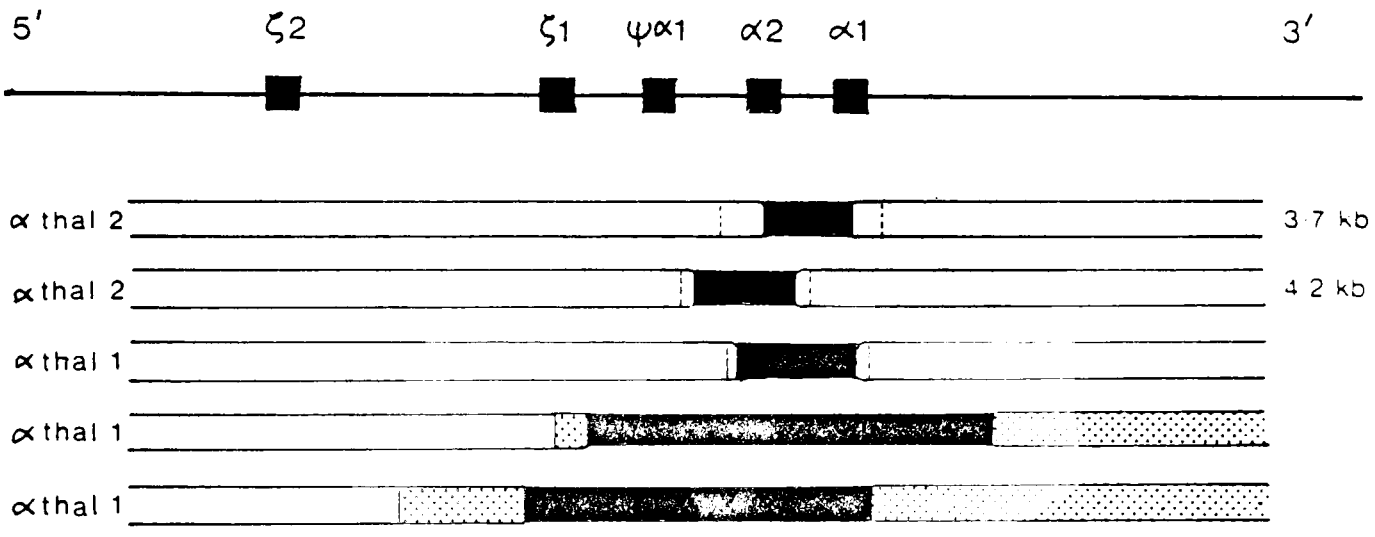

FIGURE 5: The different deletions responsible for various forms of α thalassaemia 1 and α thalassaemia 2

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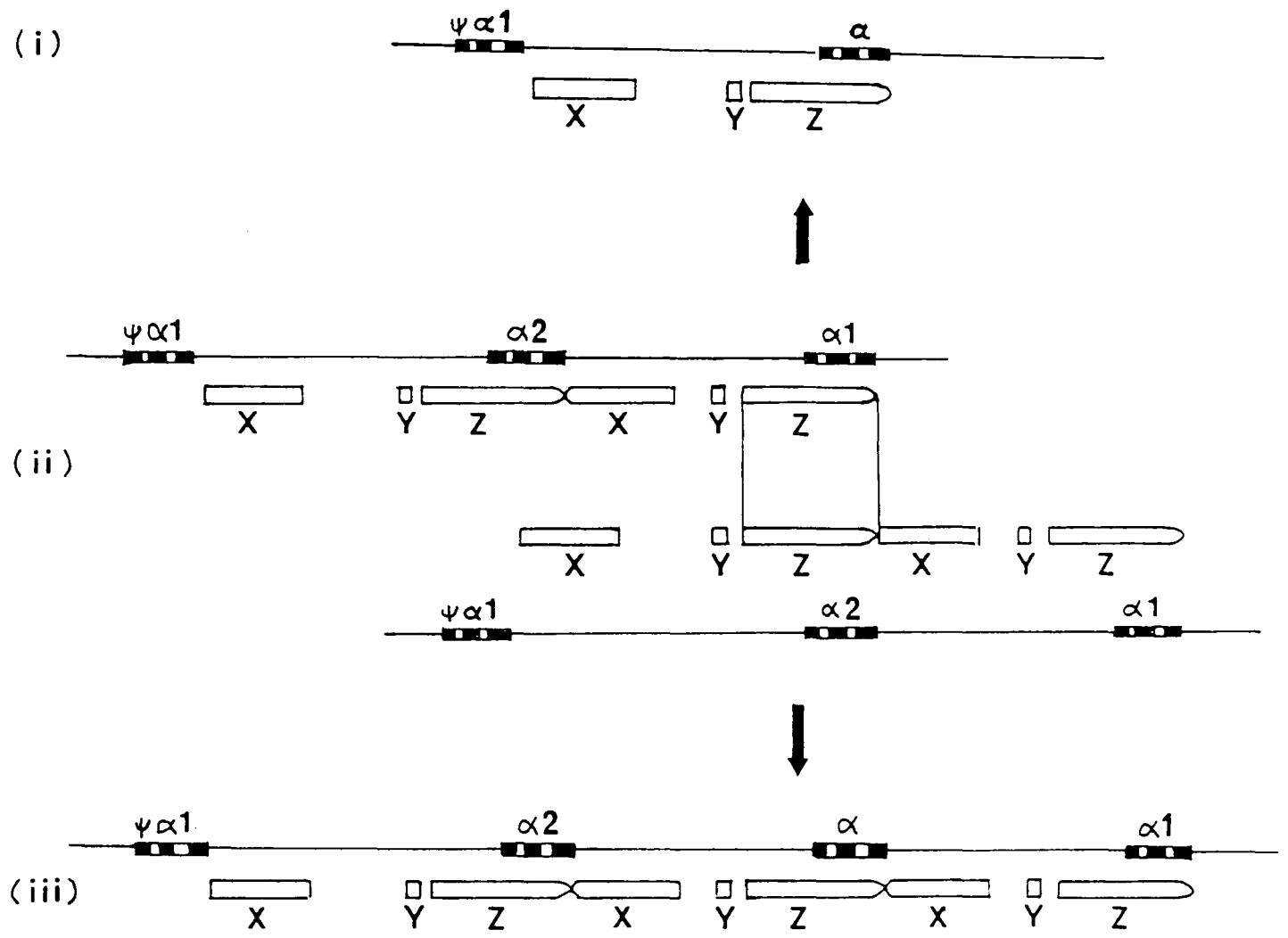

FIGURE 6: Example of crossover between misaligned chromosomes (ii), giving rise to (i) the -α haplotype and (iii) the ααα- haplotype.

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functional genes have the oligonucleotide GT at their 5′ end, it has been suggested that this small deletion, in a critical region of the gene, results in failure of stable α2 mRNA formation (Figure 8) (Felber et al, 1982). The 5 base deletion removes a particular restriction site (HpH 1) from the DNA sequence of the α2 gene and so it can be identified by restriction enzyme analysis (Orkin et al, 1981).

2.2.2 Base Substitution

The second syndrome results from mutation which produces a highly unstable α chain variant, Hb Quong Sze, which is rapidly destroyed and hence results in an α thalassaemia phenotype. In this case a leucine codon [Cytosine, Thymine, Guanine, (CTG)]in the α2-gene is changed to CCG, which codes for proline (Goossens et al, 1982) (Figure 8).

2.2.3 Chain termination defects

Furthermore non-deletion α thalassaemia can result from single base mutations in the α2 chain termination codon (Weatherall and Clegg, 1975; Hunt et al, 1982). Instead of reading 'stop', the point mutation allows an amino acid to be inserted at what is normally the position of the stop codon, and then sequences at the 3′ end of the α-globin mRNA which are not normally utilised, are translated. This results in the production of an α globin chain variant with 31 additional amino acid residues at the C terminal end; the prototype is Hb

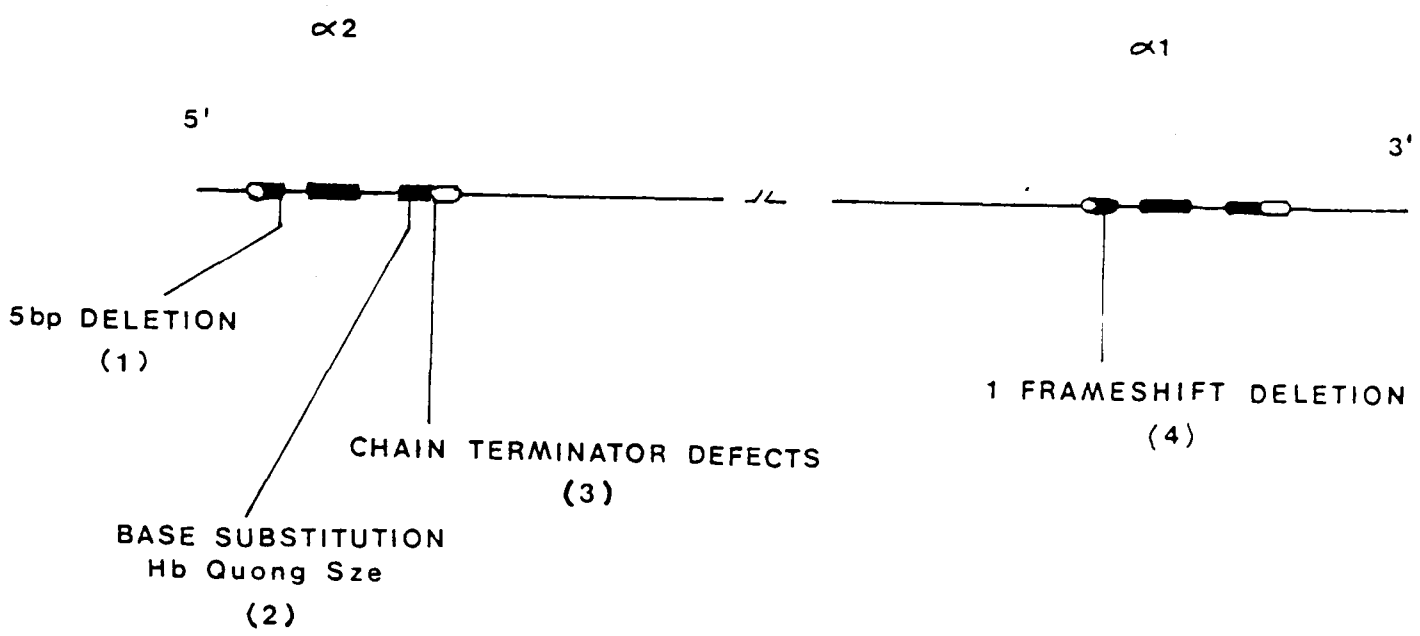

FIGURE 7: A summary of the different non-deletion forms of α thalassaemia. (1) Orkin et al (1981)
(2) Goosens et al (1982)
(3) Weatherall and Clegg (1975)
and (4) Higgs et al (1983)

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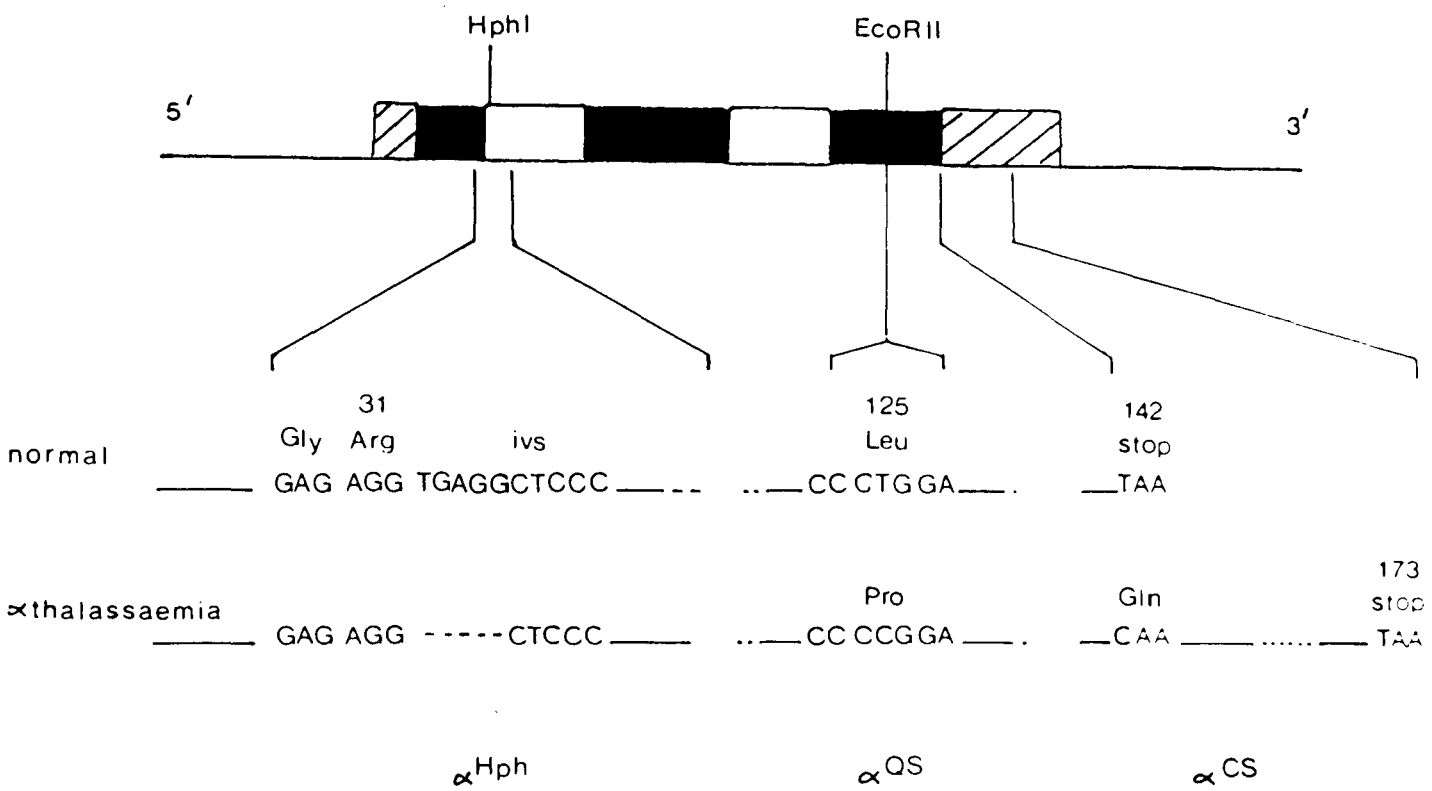

FIGURE 8: Summary of the molecular basis of "non deletion" α thalassaemia defects. Non coding regions are shown hatched exons by dark shading and introns are unshaded.

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Constant Spring (Figure 8). Hence Hb Constant Spring is produced in very low quantities and is associated with the phenotype of α thalassaemia 2.

2.2.4 Frameshift Deletion

Recently a form of α thalassaemia 2 has been found in Eastern Saudi Arabian populations (Pressley et al, 1980: Higgs et al, 1983). They found that one of the linked pair of α globin genes (α1) has a frame-shift mutation which totally inactivates it, while the other (α2) has a mutation in the highly conserved AATAAA sequence in the 3 non-coding region of the α-globin mRNA (AATAAA→ AATAAG). This lesion reduces the output from the gene.

3. DIAGNOSIS OF α THALASSAEMIA TRAIT

It is very difficult to diagnose α thalassaemia trait in adults, because the disorder is asymptomatic with minimal or no blood changes (Pornpatkul et al, 1969; Weatherall and Clegg, 1972; de Gruchy et al, 1978).

Alpha thalassaemia 2 trait patients have virtually normal blood pictures; as a group they have only slightly reduced mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH), with normal haemoglobin A_2 and serum ferritin (Weatherall and Wainscoat, 1985). Unlike α thalassaemia 1, HbH inclusions are not demonstrable (Pornpatkul et al, 1976; Chanarin et al, 1980). On the other hand α thalassaemia 1 patients show a significant reduction in MCV and MCH, the peripheral

blood film shows clear-cut microctyosis and hypochromia; HbA_2 and serum ferritin levels are normal, but HbH inclusions are demonstrable in most instances.

3.1 Screening of α thalassaemia trait by red cell measurements

A major diagnostic difficulty is the distinction of α thalassaemia trait from iron deficiency using blood counts only (Weatherall and Clegg, 1981). There have been several attempts to differentiate between thalassaemia trait and iron deficiency on the basis of red blood count measurements. England and Fraser (1973), using a simple "discriminant function" (DF) derived from the mean cell volume (MCV in fl), the red blood count (RBC x 10^6 per µl), and the haemoglobin concentration (Hb in g per 100 ml).

DF = MCV - RBC - (5 X Hb) - 3.4, claimed that a positive value indicated iron deficiency anaemia and a negative value thalassaemia trait. However, Menzer (1973) suggested that an even simpler index, the ratio MCV/RBC was equally capable of distinguishing the two conditions; values below 13 indicating thalassaemia trait and values greater than 13 indicating iron deficiency anaemia. Walford and Deacon (1976) found these formulae unreliable when applied to patients with iron deficiency or thalassaemia trait. England (1979) in an assessment study using both formulae found that the discriminant function was more satisfactory in distinguishing the two conditions, although it was not able to distinguish heterozygous α thalassaemia 2 from

iron deficiency.

There is also a tendency for measuring the heterogeneity of the red cell volumes to differentiate iron deficiency from thalassaemia trait i.e. the degree of anisocytosis. This measurement is called the Red Distribution Width (RDW) on modern automated blood cell counters.

Most reported work on red cell size distribution has been carried out using the Coulter Counter Model ZB1 with Channelyzer C1000 attachment. Heterogeneity of red cell volumes has been variously expressed as standard deviation of the natural logarithms of the red cell volumes, or as a volume ratio for 95% of the cells in the sample (volume of largest cell/volume of smallest cell) or as the percentage of microcytes (England and Down, 1974). Alternatively, Bessman and Johnson (1975) have used the co-efficient of variation (CV) which is the ratio of the standard deviation to the mean, based on a normal distribution (Gaussian). Both groups have used red cell volume analysis in the differentiation of iron deficiency from heterozygous thalassaemia (England et al, 1976; Bessman, 1980). In 1979 Bessman and Feinstein reported that the co-efficient of variation (CV) of the red cell volume distribution was less than 14% in normal subjects and in individuals with α thalassaemia trait, but greater than 14% in patients with iron deficiency.

3.2 Serum ferritin level

Patients with iron deficiency anaemia have low serum iron levels while those with thalassaemia trait have normal or slightly elevated levels. The diagnostic value of the serum ferritin level as a function of the amount of storage iron in normal subjects and in those with iron deficiency anaemia was described by Jacobs et al (1972). They found that a ferritin level below 10 ng/ml indicated iron deficiency. Hussein et al (1976) showed that serum ferritin was normal in patients with β thalassaemia trait, while Loria et al (1978) claimed that all patients with ferritin above 20 ng/ml had thalassaemia trait and those below 20 ng/ml had iron deficiency.

3.3 Demonstration of HbH inclusion bodies

At birth the two types of α thalassaemia trait are easily distinguished. In α thalassaemia 2 trait the levels of Hb Bart's found range from 1-3% (Wasi et al, 1964; Pootrakul et al, 1970). Whereas in α thalassaemia 1, levels of 5-10% are detected (Na-Nakorn and Wasi, 1970: Weatherall and Clegg, 1981). However, it is very difficult to diagnose α thalassaemia trait in the adult because low levels of HbH are not detectable by starch gel electrophoresis (Pornpatkul et al, 1969; Weatherall and Clegg, 1972).

Most red cells in patients with haemoglobin H disease produce multiple rounded inclusions which are

evenly distributed throughout the cell when incubated with redox dyes **in vitro** (Gouttas et al, 1955; Rigas et al, 1956; Hedenberge et al, 1958). In their studies Gouttas et al (1955) observed that the inclusions became progressively more visible with increase in incubation time and increase in temperature.

In α thalassaemia trait, unlike haemoglobin H disease, it is very difficult to find the occasional inclusion bodies (Pornpatkul et al, 1969; Weatherall and Clegg, 1972; Dacie and Lewis, 1984).

There is no agreement as to what constitutes the best method for demonstration of inclusions in trait subjects. The variables include redox dye used, incubation time and temperature. Various workers have recommended the use of 1% brilliant cresyl blue (BCB) to demonstrate inclusion bodies in trait patients using an incubation temperature of 37°C for periods varying from 10 minutes to 4 hours (Dacie and Lewis, 1968; Schwartz and Atwater, 1972; Walford and Deacon, 1976; Winichagoon et al, 1980). On the other hand Weatherall and Clegg (1981) used BCB at 25°C for two hours.

More recently an attempt has been made to define the type of trait in which these inclusion bodies may be found. Pornpatkul et al (1976) examined 34 children (aged 3-5 years) for inclusion bodies. These children were part of a cord blood study (Pootrakul 1970) and hence their haematological data and level of haemoglobin Bart's at birth were known. In addition the same workers

studied 11 parents of infants with the haemoglobin Bart's hydrops foetalis syndrome and 86 individuals who were parents of offspring with haemoglobin H disease. They found a very clear-cut relationship between the α thalassaemia genotype and the presence or absence of inclusion bodies. For example, nine of the 34 children had red cell inclusions and all were designated as α thalassaemia 1 trait at birth. The 25 who did not have inclusions, were previously diagnosed as α thalassaemia 2 trait from their level of Hb Bart's at birth. All the parents of babies with the HBHFS, who were thus obligatory carriers for α thalassaemia 1, had red cell inclusion bodies. These studies suggested that HbH inclusion bodies were found mainly in α thalassaemia 1, but that the method was not sensitive enough to detect the slight excess of β chains occurring in α thalassaemia 2.

3.3.1 Problems encountered in testing for HbH inclusion bodies

A positive test is essential for the diagnosis of α thalassaemia trait although with existing methods a negative result does not exclude the condition. As currently performed, identification of HbH inclusions cannot be used as a screening test particularly so in multi-racial populations as in the United Kingdom since the test is usually negative in Negroes and Indians with α thalassaemia trait (Walford and Deacon, 1976).

Technical difficulties also occur which are not uncommon depending on technique used. Thomas (1966) demonstrated the formation of extensive spicules resembling HbH inclusions in red cells from normal individuals. He concluded that rough handling induced the false inclusion bodies. In addition, false positive results may be induced by prolonged incubation. Wickramasinghe et al in 1981 reported that normal red cells show little or no ultra-structural change in their cell membranes after supravital staining for up to two hours at 37°C. However after 24 hours incubation red cells produced inclusions indistinguishable from those of HbH. Also Dacie and Lewis (1984) reported that false positive results could be induced by incubation at 37°C for more than one hour.

3.3.2 Improved techniques for the demonstration of HbH inclusions

In 1973 Raven and Tooze suggested that improved demonstration of HbH inclusions in α thalassaemia trait could be achieved by using a reticulocyte enriched sample obtained by centrifugation (Wintrobe method). This is so because younger cells contain more HbH inclusions than older red cells from the same individual. Wickramasinghe et al (1981) confirmed this observation in patients with HbH disease. However, Walford et al (1976) failed to confirm the superiority of this centrifugation method over the whole blood method for α thalassaemia trait.

Jones et al (1981) experienced similar difficulties using the Wintrobe method, however, improved reticulocyte enrichment occurred when a microhaematocrit technique was used. More recently Maungsapaya et al (1985) confirmed this observation.

Pereira in 1984 reported that potassium iodide is capable of "sensitising" certain blue dyes,e.g. methylene blue enabling them to precipitate and stain the ribonucleic acid of reticulocytes supravitally on incubation. With more prolonged incubation the haemoglobin H inclusion bodies in α thalassaemia can also be demonstrated.

3.4 Globin Chain Synthesis Studies

In 1965 Clegg et al succeeded in separating the α and non-α chains of human haemoglobin. This technique, combined with **in vitro** radioactive labelling (Weatherall et al, 1965) has now been widely used to study haemoglobin synthesis in the cells of patients with α thalassaemia syndrome. The first studies of this kind were reported by Kan et al (1968) and Schwartz et al (1969) who examined relatives of infants with Hb Bart's hydrops foetalis syndrome and haemoglobin H disease. They measured the α/β globin chain synthesis ratios in an attempt to classify them into four different groups, i.e. normal, α thalassaemia 2, α thalassaemia 1 and haemoglobin H disease. They reported that, while there is a significant

reduction in α chain synthesis in certain forms of α thalassaemia, overlap occurred between α thalassaemia 2 and α thalassaemia 1, and between α thalassaemia 2 and normal individuals. Other workers seem to have been slightly more successful in their attempts to separate the different forms of α thalassaemia by globin chain synthesis studies. For example, Pootrakul et al (1975a) examined haemoglobin synthesis in 28 obligatory carriers of α thalassaemia. These parents of babies with the haemoglobin Bart's hydrops foetalis syndrome, were found to have α/β globin chain synthesis ratios of 0.76± 0.04. The parents of patients with haemoglobin H disease were found to fall into two clear groups: one with a mean α/β ratio of 0.78 ± 0.03; the other with a ratio of 0.92 ± 0.03 (control range 1.0 - 1.2).

Walford and Deacon (1976) used globin chain synthesis studies to confirm the disorder in random patients suspected to have α thalassaemia trait because of abnormalities in their red cell indices. While, in 64 subjects studied (44 patients, 20 controls), no numerical overlap of results between the groups was detected i.e. control and α thalassaemia trait, the results were so close that in a larger series some degree of overlap might reasonably be anticipated. It was not possible to subdivide trait patients from this data.

Weatherall and Clegg (1981) concluded that many heterozygous α thalassaemia subjects have a significant reduction in α/β globin chain synthesis, although in some cases of α thalassaemia 2 the diagnosis can only be

suspected by this technique and requires confirmation by gene mapping.

4. ALPHA THALASSAEMIA IN DIFFERENT RACIAL GROUPS

Alpha thalassaemia syndromes constitute an heterogenous group of disorders with wide geographical distribution being particularly prevalent in Southeast Asia, the Mediterranean, the Middle East, parts of the African continent and in India. Recently it has been described in persons of North European origin. Clearly, it is also found in Asian, Negro, Mediterranean and Indian subcontinent emigrant populations worldwide.

4.1 Alpha Thalassaemia in Oriental Races

The disorder is most common in Southeast Asia (Na-Nakorn et al, 1956: Lie-Injo et al, 1957; Flatz et al, 1965; Wasi et al, 1967; Pootrakul et al, 1967; Na-Nakorn et al, 1969; Pootrakul et al, 1975b) with a very high incidence in Northern Thailand (Thumasathit et al, 1968; Na-Nakorn and Wasi, 1970). Twenty seven cases of Hb Bart's hydrops foetalis were reported in a period of two years at Chiengma (Thumasathit et al, 1968) where an incidence of 30.7% at birth have elevated levels of Hb Bart's (Na-Nakorn and Wasi, 1970). Based on this incidence of Hb Bart's at birth, these workers estimated a frequency of α thalassaemia 2 of 17.4%, α thalassaemia 1 of 12.2%, and an incidence of HbH disease in approximately 1% of the population. Based on these frequencies the haemoglobin Bart's hydrops foetalis syndrome is expected to occur with a frequency of about 0.4%.

A high incidence of α thalassaemia has also been reported in other parts of Southeast Asia. Cord blood surveys have shown that 3-4% of Chinese infants living in other parts of Southeast Asia, have increased levels of Hb Bart's (Vella, 1959: Lie-Injo, 1959: Todd et al, 1969: Wong, 1970). Alpha thalassaemia is well documented in Chinese populations who have moved from Southeast Asia. Gray and Marion (1971) found a 6.7% incidence of α thalassaemia trait in Chinese persons living in Canada. Haemoglobin H disease is well recognised in Chinese immigrants in the USA, UK and Caribbean (Weatherall and Clegg, 1981). In 1976 Walford and Deacon studied adult Chinese immigrants in the UK with α thalassaemia trait, but they could not differentiate them into α thalassaemia 2 and α thalassaemia 1. The same observation was reported by Todd and Chan (1978) who examined the levels of haemoglobin Bart's in infants born to mothers with haemoglobin H disease.

Generally the four types of α thalassaemia i.e. α thalassaemia 2, α thalassaemia 1, HbH disease and Hb Bart's hydrops foetalis syndrome occur frequently in oriental populations with deletion genotypes (-α/αα), (--/αα), (--/-α) and (--/--) respectively. However, non-deletion forms of α thalassaemia are very rare in this population (Weatherall and Clegg, 1981).

4.2 Alpha Thalassaemia in Mediterranean Races

Although previously thought to be rare, more recent evidence suggests that α thalassaemia does occur not

infrequently in certain Mediterranean areas, notably Sardinia and Cyprus (Weatherall and Clegg, 1981). Elevated levels of Hb Bart's were found in 4.3% of new born infants in Northern Sardinia (Bianco et al, 1972). In 1979, Hadjaminas et al found that of 1200 Greek Cypriot and 132 Turkish Cypriot newborns 12.4% and 6.8% respectively had elevated levels of haemoglobin Bart's.

The genetic and the haematological features of α thalassaemia trait are similar to those observed in oriental races (Weatherall and Clegg, 1981). Although the surveys thus far are limited, it would appear that the non deletion form of the disease occurs more commonly in these Mediterranean popualtions than in those of South East Asia (Weatherall and Wainscoat, 1985).

4.3 Alpha Thalassaemia in Negroes

Recent studies in African Negro populations suggest that approximately 3% of African infants have elevated levels of Hb Bart's ranging from 3 - 10% (Weatherall and Clegg, 1981). A similar incidence was observed in Negro infants in Jamaica and the USA. However, there have been very few attempts to estimate the frequency of α thalassaemia in the adult Negro populations. Pierce et al (1977) assessed the frequency of α thalassaemia in the American Negro using globin synthesis studies. They suggested that about 5% of adult Negroes possessed haematological and globin chain synthesis ratios comparable with those in α thalassaemia 1 in oriental races. 2.5% of this population was classified as α thalassaemia 2. Using globin gene mapping

Dozy et al (1979) and Higgs et al (1980) showed that about 3% of the Negro population were homozygous for α thalassaemia 2 and about 25% heterozygous for this determinant.

It had been suggested that the genetic and possibly the molecular basis of Negro α thalassaemia may differ from those of the oriental or the Mediterranean races, because the disorder is relatively milder in Negroes (Weatherall and Clegg, 1972). This was confirmed by Schwartz and Atwater(1972a) who reported that the genetic basis of α thalassaemia trait in the American Negro differs from other racial groups. They thought that there may be only one gene deletion (-α/αα),i.e. only α thalassaemia 2,in this group. That idea was also supported by Wasi et al (1974). They claimed that α thalassaemia in the Negro and non-Asian groups may not be identical with α thalassaemia 2 and α thalassaemia 1. However, Higgs et al (1979) and Dozy et al (1979) unlike the previous studies found the two forms of α thalassaemia trait in some Negro populations. Using gene mapping studies, they discovered that the mild forms,i.e. α thalassaemia 2,resulted from the deletion of one α gene (-α/αα) while the severe form, which resembled α thalassaemia 1, is in fact the homozygous form of α thalassaemia 2 with genotype (-α/-α). The genotype (--/αα) is very rare in Negroes (Figure 9). These studies explain the rare occurrence of HbH disease and the complete absence of HbBHF syndrome in Negroes.

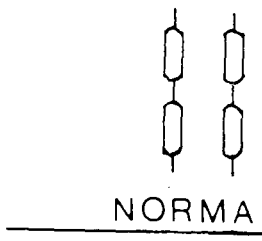

FIGURE 9: Comparison of homozygous α thalassaemia 2 in Negroes with heterozygous α thalassaemia in Orientals. (open boxes indicate normal genes and shaded boxes deleted genes).

A more detailed analysis of Negro α thalassaemia 2 haplotype has been carried out by Higgs et al (1980). These experiments have shown that it has probably arisen by gene deletion (-α) as is found in oriental races.

4.4 Alpha Thalassaemia in India

There is very limited information on the incidence of and molecular defects in α thalassaemia in Indians (Weatherall and Clegg, 1981).

Early studies on the incidence of the disorder in infants suggested that about 1% of newborn Indian infants in Singapore or Malaysia have elevated levels of haemoglobin Bart's (Vella, 1959; Lie-Injo and Ti, 1961). Chauhan et al (1970) found elevated levels in 4% of Bengali infants and in 1% of Maharashtrians. There are no reports of HbBHF syndrome in the Indian population.

There have been sporadic reports of α thalassaemia in the adult. Chatterjea (1959) and Swarup et al (1963) described cases of HbH disease in single Indian families and further reports of HbH disease are reviewed by Saha and Banerjee (1973). However, the genetic basis of HbH disease in India is unknown (Weatherall and Clegg, 1981). The heterozygous state for α thalassaemia in adult Indians has only been reported once by Walford and Deacon (1976). They found that α thalassaemia trait is a common disorder in Indian immigrant populations in the UK with less striking changes in the red cell indices when compared to that of Oriental trait subjects (Chinese).

They were unable to distinguish two clear cut varieties of α thalassaemia trait in this population. They proposed that the genetic defect in Indian α thalassaemia trait is the same as that in Oriental α thalassaemia 2 i.e. -α/αα. On this genetic model, they thought that the homozygous form (-α/-α) would result in a 50% deficit in α chain production and would thus resemble in severity oriental α thalassaemia 1 trait (as found recently in Negroes). So far no such Indian homozygote has been identified.

4.5 Alpha Thalassaemia in the Middle East

Alpha thalassaemia has been reported in many Middle Eastern populations although there is a limited amount of information about true gene frequencies (Weatherall and Clegg, 1981).

One of the first reports of α thalassaemia in Israel was that of Ramat and her colleagues (1959) who described a form of haemoglobin H disease with persistence of high levels of haemoglobin Bart's in an oriental Jewess. In a survey of newborn infants in Israel, Goldschmidt et al (1968) reported an incidence of 1.3% for increased amounts of haemoglobin Bart's. Affected infants had morphological changes suggestive of α thalassaemia.

The incidence and distribution of α thalassaemia in Israel have been studied more extensively over the last few years. The disorder appears to be confined mainly to Yemenite and Iraqi Jewish communities.

Increased levels of haemoglobin Bart's have been found in cord bloods in 17% of Yemenite and 11% of Iraqi newborn infants (Zaizov and Natoth, 1972). Furthermore, haemoglobin H disease is not uncommon in these Jewish communities. The genetics of α thalassaemia in these populations was studied by Zaizov et al, 1972; 1973. Based on haematological and globin synthesis studies, these authors were not able to demonstrate the presence of two clear α thalassaemia phenotypes in these families. They favoured an inheritance of α thalassaemia through a single mutant gene with variable expressivity in these populations. Recently both deletion and non-deletion forms of α thalassaemia have been found in patients with haemoglobin H disease from Israel (Weatherall and Clegg, 1981).

Surveys of newborn infants in the eastern oases populations of Saudi Arabia have shown one of the highest incidences for increased levels of haemoglobin Bart's found anywhere in the world (Pembury et al, 1975). They found elevated levels of haemoglobin Bart's ranging from 1 to 16% in 50% of newborn infants.

Although haemoglobin H disease has been reported in the oases populations, it is relatively rare and haemoglobin Bart's hydrops foetalis has not been observed (Weatherall and Clegg, 1981). McNiel (1971) reported family studies on haemoglobin H disease in this population and came to the conclusion that the disorder, unlike that of Oriental or Mediterranean types, results from the interaction of two α thalassaemia genes which were not

clinically or haematologically distinguishable. More recently in 1980, Pressley et al supported McNiel's conclusion of the presence of two identical α thalassaemia genes in HbH disease. From a combination of haematological, globin chain synthesis, mRNA and gene mapping studies, they showed that both deletion (-α) and non-deletion ($\alpha\alpha^T$) haplotypes are present in the Saudi population. However, individuals with haemoglobin H disease had normal gene maps and family studies proved that they had a normal complement of four α chain genes and they were,in fact,homozygous for a non-deletion defect ($\alpha\alpha^T/\alpha\alpha^T$). They suggested that the expressed phenotype of non-deletion α thalassaemia gene present in this population was intermediate between those of the Oriental α thalassaemia 1 and α thalassaemia 2 deletion genes. Also from those studies they suggested that the genotype (--/αα),i.e. α thalassaemia 1,may be extremely rare in Saudi Arabia which could explain the absence of HbBHFS there.

4.6 Alpha Thalassaemia in North Europeans

Alpha thalassaemia seems to be relatively uncommon in North European races. In small cord blood surveys elevated levels of haemoglobin Bart's have not been observed (Vella, 1959; Weatherall, 1963) and there have only been sporadic reports of the occurrence of HbH disease (Hedenburg et al, 1958; Woodrow et al, 1964). During their studies on single Swedish and English families, they noted that the haematological findings

were similar to those in Oriental families.

There is no doubt that forms of α thalassaemia trait do occur occasionally in North European populations (Weatherall and Clegg, 1981). Such patients have had thalassaemia-like blood pictures with normal levels of HbA_2 and HbF; α/β globin chain synthesis ratios however showed unequivocal evidence of α thalassaemia although precisely which category these cases fall into is not known.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. SAMPLE POPULATION

1.1 Controls

Twenty healthy adult volunteers aged 18-72 years (10 males and 10 females) were chosen as normal controls.

1.2 Patients

After informed consent 32 patients were selected because of abnormality in their red cell measurements.

Patients were suspected to possess α-thalassemia trait when all the following abnormalities were present (Weatherall and Clegg, 1972; Pearson et al, 1973; Carrell and Lehmann, 1983);-

(a) Mean corpuscular volume (MCV) less than 80 fl.

(b) Mean corpuscular haemoglobin (MCH) less than 27 pg.

(c) Red cell count (RBC) greater than $4 \times 10^{12}/l$.

(d) Haemoglobin concentration 10 g/dl or greater

(e) Normal haemoglobin electrophoresis

(f) Normal HbF percentage (HbF $<$ 1%)

(g) Normal HbA_2 percentage (HbA_2 $<$ 3.3%)

(h) Normal serum ferritin.

In addition the red cell volume distribution (red distribution width [RDW]) was assessed to see if it was suitable for use as a screening test to differentiate between iron deficiency and α-thalassaemia trait.

2. SAMPLING

30 ml of venous blood was withdrawn from each patient and control subject following venous occlusion using a syringe and needle of bore size 19-SWG. All sampling was carried out between 9.00 a.m.-11 a.m. A 10 ml aliquot of blood was anticoagulated in dipotassium ethylene-diaminetetra-acetic acid (K_2EDTA) to give a final concentration of 1.5 mg/ml for routine blood counts, HbH inclusion demonstration and serum ferritin estimation. The remainder of the original sample (20 ml) was anticoagulated with Lithium heparin (LH) at a final concentration of 15± 2.5 I.U./ml for globin chain synthesis studies.

All investigations were commenced within one hour of sample collection with the exception of ferritin where the plasma was separated immediately and kept frozen at -20°C for batch analysis.

3. RED CELL MEASUREMENTS

3.1 Principle

Blood counts were performed on an aperture impedance counter (Coulter Counter Model S Plus IV). The haemoglobin was measured by the cyanmethaemglobin method.

3.2 Method

The following parameters were measured: red blood cell count (RBC x 10^{12}/l), haemoglobin concentration (HGBg/dl), haematocrit (HCT l/l) mean corpuscular volume (MCV fl), mean corpuscular

haemoglobin (MCH pg), mean corpuscular haemoglobin concentration (MCHC g/dl) and the new parameter,red distribution width (RDW%).

Red Distribution Width

The RDW is a sensitive electronic measurement of red cell anisocytosis i.e. an expression of the volume distribution of the erythrocyte population. It is computed from the RBC volume histogram.

Calculation of RDW

The red cells are counted and then channelysed using three pulse height analysers each of 256 channels. A total of 75,000 cells may be accumulated in up to five count periods to generate a representative volume distribution histogram. A particle of volume range 36-360 fl is classed as a red cell. The mean cell volume is obtained from this distribution by Simpson's integration, a new method for MCV determination. The method of RDW calculation is also new and the way in which this measurement is generated is shown in Figure 10. From any volume distribution histogram the volume data below 20% of scale is excluded and the co-efficient of variation (CV) of the remainder of the distribution calculation using the 16th and 84th percentile volumes which are obtained from a continuously variable threshold circuit. The threshold which commences at a level equivalent to 360 fl is moved progressively lower until 16% of all cells present have passed the threshold. The cell

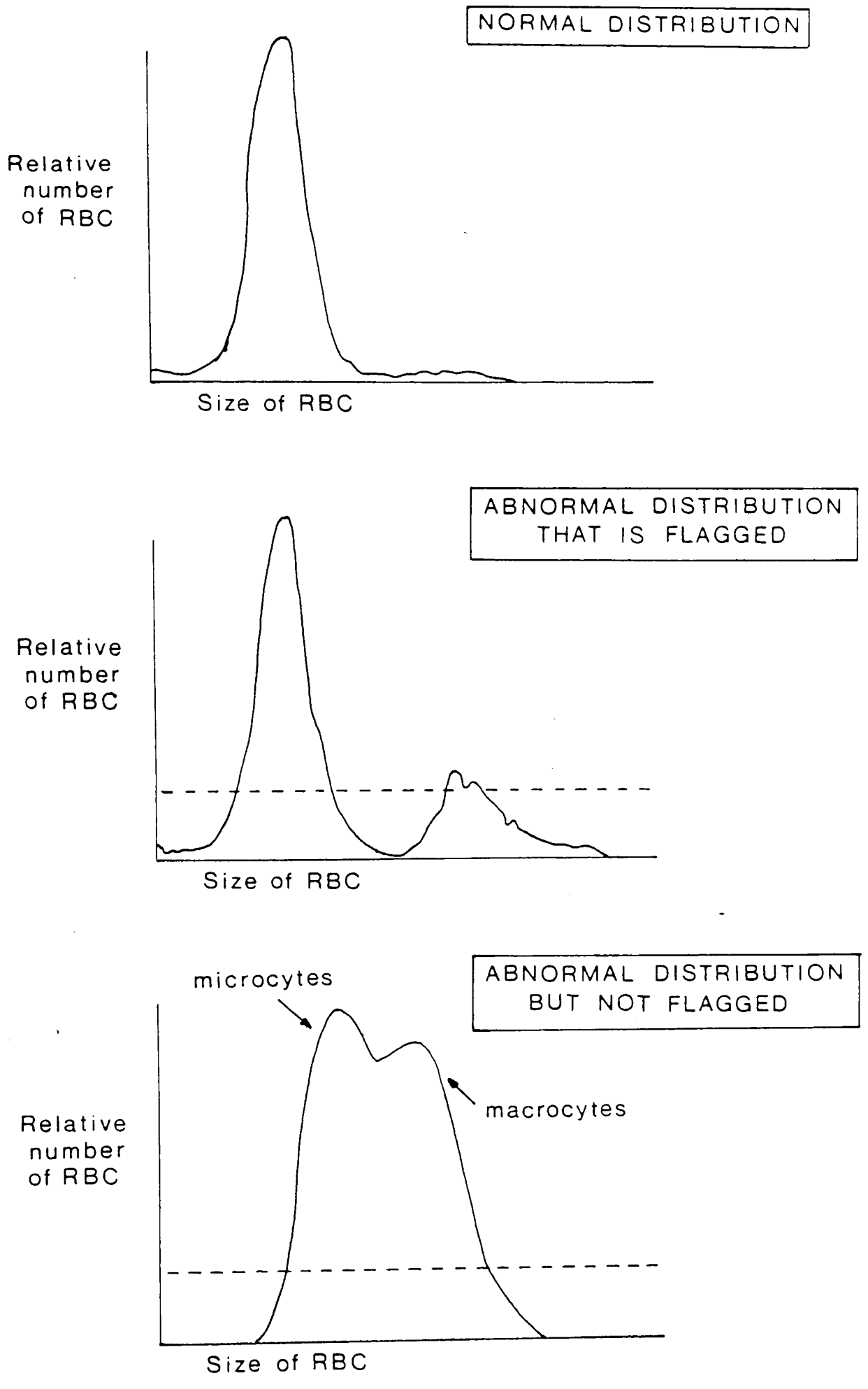

FIGURE 10: Describes the principles used to calculate the RDW on the Coulter Model S Plus II and all subsequent models.

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size at which this event occurs is recorded as the 16th percentile volume. The threshold continues to move downwards until 84% of the cells present have passed. The cell size at which this event occurs is recorded as the 84th percentile volume. The data truncation before the calculation of CV is to exclude possible interference due to electrical noise and debris at the lower end of the red cell size distribution. The CV of the truncated distribution is then scaled up by a factor of 1.15 to compensate for the excluded data and this modified CV is called the RDW (Rowan, 1983).

4. HAEMOGLOBINOPATHY INVESTIGATIONS

The blood sample was washed three times in normal saline. After each wash the sample was centrifuged at 1170 g for 5 minutes. An equal volume of distilled water was added to the cells together with 6 drops of toluene [BDH]. The haemolysate thus produced was mixed on a mechanical shaker for 10 minutes and then centrifuged for a further 10 minutes. The haemolysate was then filtered using a Whatman filter paper No. 1 and the haemoglobin concentration adjusted to 5 mg/dl.

4.1 Haemoglobin electrophoresis

4.1 Principle

Haemoglobin, like all proteins, has a buffering potential. The iso-electric point of a protein is

the pH at which the total charges carried cancel out resulting in neutrality. At a pH alkaline to the iso-electric point, the protein or haemoglobin is negatively charged and moves to the positive electrode (anode) on electrophoresis. The opposite occurs at acid pH where positively charged material moves to the negative electrode (cathode) [Lehmann and Huntsman, 1974].

4.1.2 Material

The following instruments and reagents were provided by Helena Laboratories (Beaumont, Texas, U.S.A.):-

Instruments

Titan electrophoresis chamber

Titan power supply

Micro dispenser

Applicator

Staining rack

Aligning base

Reagents

14.6 g Tris Borate EDTA buffer

(Supre Heme dissolved in 980 ml H_2O)

Titan 111-H Cellulose acetate plate (76 x 60 mm)

0.5% (W/V) Ponceau S stain.

4.1.3 Method

100 ml of Tris buffer (pH 8.6) was added to each of the outer compartments of the electrophoresis

chamber. The cellulose acetate plates were soaked in buffer, gently blotted and placed on an aligning base, cellulose acetate side up and application point 25 mm from the cathode. Using the microdispenser, each well in the sample plate was filled with 5 µl of the haemolysate. An applicator was then loaded by depressing the tips into the sample well several times. The loaded applicator was then applied to the wet plates, the latter being very quickly placed in the electrophoresis chamber, cellulose acetate side down, and point of application nearest to the cathode. Electrophoresis was carried out for 25 minutes at a potential of 350 volts across the strip. The strips were then stained by Ponceau S for 5 minutes and washed three times in 5% acetic acid (BDH) each for 2 minutes, to remove excess stain, and finally left to dry.

4.2 HbF Estimation by Alkali Denaturation Method

4.2.1 Principle

Measurement of the total foetal haemoglobin is dependent on the γ chain lacking an internal cysteine residue which is present in the β globin chain. This cysteine residue in the β globin chain is ionised causing instability and precipitation. The γ chain on the other hand, is stable at alkaline pH and is therefore described as alkali-resistant. Sodium hydroxide (NaOH) is used to denature adult haemoglobin (HbA) which is then readily precipitated by ammonium sulphate $[(NH_4)_2\ SO_4]$.

4.2.2 Method

HbF was measured by the method described by Singer, Chernoff and Singer (1951) modified by Went and MacIver (1961).

The haemolysate (0.2 ml) was added to 3.2 ml of N/12 NaoH (33.3 ml 1N NaoH made up to 400 ml with distilled water).

At precisely 60 seconds, 6.8 ml of acidified ammonium sulphate (152 gm ammonium sulphate dissolved in 400 ml distilled water and 1 ml of 10N HCl) was added, mixed, allowed to stand for one minute and then filtered using Whatman No.50 filter paper.

The optical density (OD) of the filtrate (HbF) was then measured spectrophotometrically at 540 nm. To determine the optical density of 100% haemoglobin, 0.1 ml of the haemolysate was added to 10 ml of ammoniated water (0.2 ml ammonia solution [specific gravity 0.85] made up to 500ml with distilled water) mixed and measured exactly as the filtrate.

The percentage of HbF was then calculated using the following equation:-

$$\% \text{ HbF} = \frac{\text{OD of HbF}}{\text{OD of 100\% Hb} \times 2} \times 100$$

4.3 Estimation of HbA_2 by micro-column method

4.3.1 Principle

HbA_2 is selectively eluted from a chromatographic column under specified conditions of pH and chloride ion concentration.

4.3.2 Method

HbA_2 was measured by the micro-column method described by Huisman et al (1975) and Abraham et al (1976 - 1977) using HbA_2 test kits (Isolab Inc.) which include all reagents and columns.

50 µl of the haemolysate (prepared as above) was loaded on to the surface of a preconditioned DEAE-cellulose column bed. Any residual sample adhering to the walls of the column was carefully washed on with 200 µl of the HbA_2 elution agent and the eluate discarded. The HbA_2 fraction was eluted by a further 4 ml of elution agent and the eluate retained for analysis. To elute the other haemoglobin fractions (HbA and HbF), 4 ml of a second elution agent was added and the eluate diluted with 16 ml of distilled water. The absorbance values of the two fractions were measured spectrophotometrically at 540 nm. Estimation of HbA_2 percentage was carried out as follows:-

$$\% \ HbA_2 = \frac{100X}{X + 5Y}$$

where X = Absorbance of HbA_2 fraction

Y = Absorbance of "other haemoglobin" fraction

5. SERUM FERRITIN BY RADIOIMMUNOASSAY (RIA)

5.1 Principle

The assay employs constant amounts of two pre-mixed antibodies, one (goat anti-human ferritin) covalently bonded to glass particles and the second (rabbit ferritin antibody) radio-iodinated and free in solution. A variable amount of antigen is provided by the patient samples. The ferritin antibody bonded to the glass particles will bind to any ferritin present and forms a solid phase complex to which the second tracer antibody binds. The solid phase complex is separated by centrifugation and counted for radio-activity. The amount of labelled antibody is proportional to the serum ferritin concentration and calculated from a standard curve (Figure 11).

5.2 Method

The method of Addison et al (1972) was used (Corning, Immunophase Ferritin [^{125}I] RIA kits).

Seven standards (included in kits) of known ferritin concentrations are arranged in ascending order of concentration (0-1000 ng/ml) and used to plot a standard curve. Also two controls with ferritin concentration in the normal and the low range are provided for internal quality control purposes.

50 µl aliquots from each of the standards, controls and patients were placed into appropriately labelled test tubes (12 x 75 mm) in duplicate. 500µl of antibody slurry was added to each tube and mixed for 4 seconds. All tubes were then incubated for

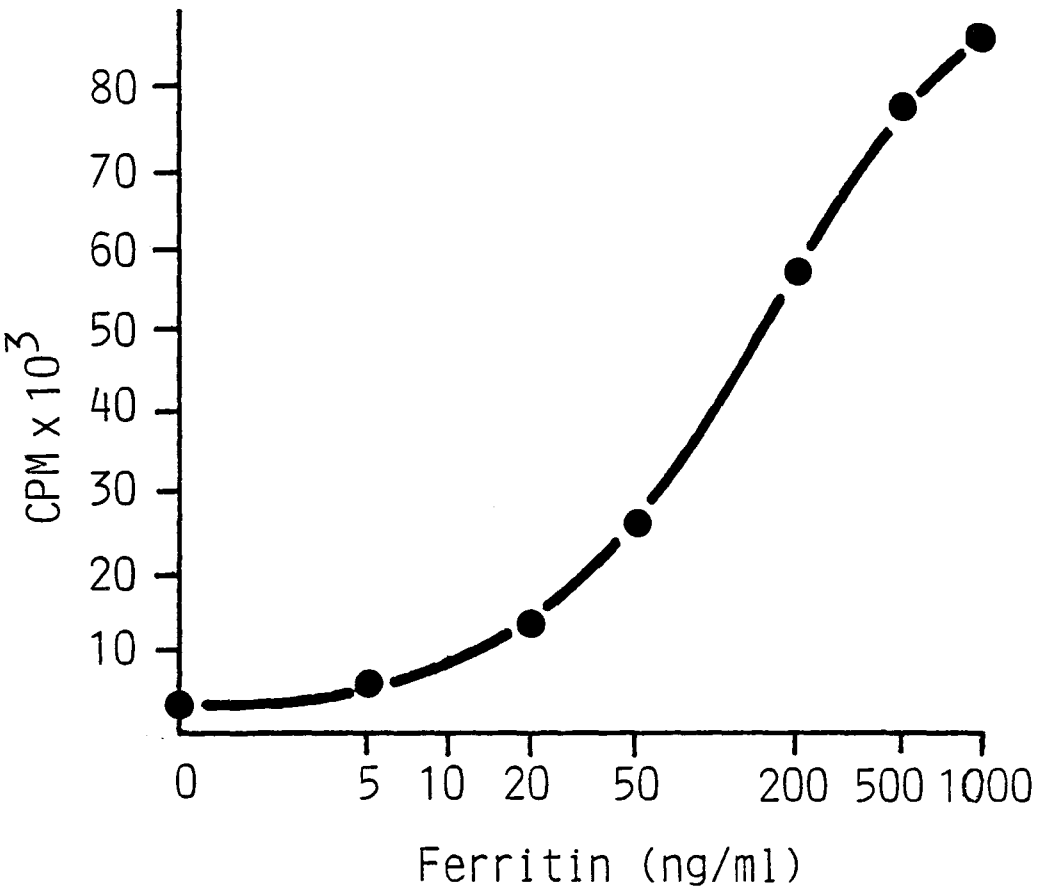

Fig. 11 Standard curve of serum ferritin.

30 minutes at room temperature. To each tube 500 µl of [^{125}I] ferritin antibody was added, mixed for a further 4 seconds and finally incubated for two hours at room temperature. 2 ml of distilled water was then added to each tube followed by centrifugation at 1170 g for 10 minutes. The tubes were decanted and counted in an L.K.B. Gamma Counter.

6. DEMONSTRATION OF HbH INCLUSIONS

6.1 Principle

Incubation of red blood cells with a redox dye such as brilliant cresyl blue (BCB) causes oxidative denaturation and precipitation of unstable haemoglobin H(β_4) resulting in diffuse stippling of the red cell (Figure 12).[Schwartz and Atwater, 1972b].

6.2 Method

6.2.1 Routine Method

Equal volumes of 1% BCB (Gurr) and blood were incubated at 25°C for 24 hours, after which blood smears were prepared.

6.2.2 Experimental Methods

6.2.2.1 whole blood method:

To see if the HbH test could be improved in the laboratory, changes were made in incubation temperature; variation in incubation time and finally, the effect of the addition of potassium iodide as a stimulant.

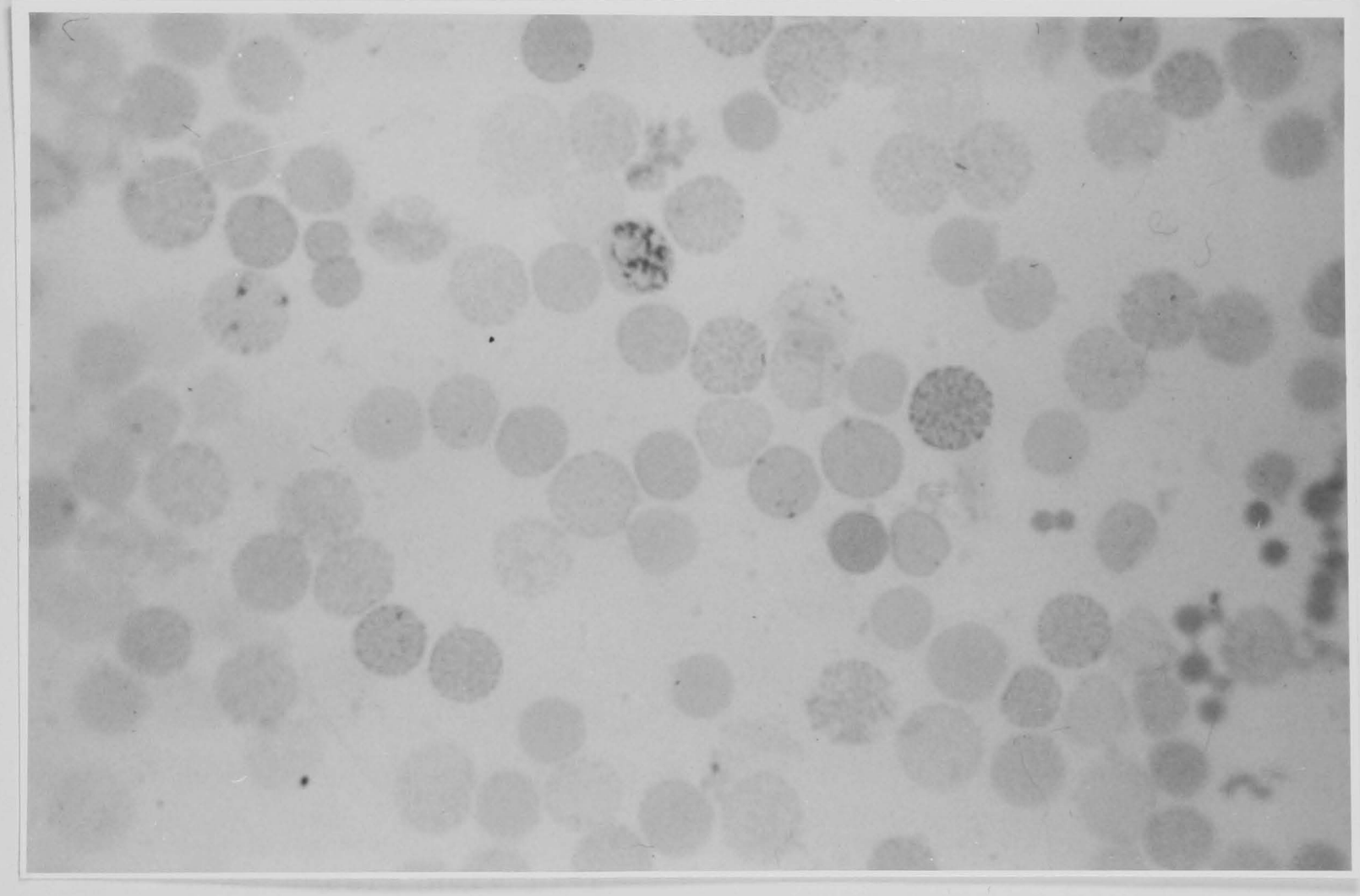

FIGURE 12: HbH inclusion bodies in blood of patient with HbH disease.

For each patient and each control four tubes (75 x 10 mm) were labelled as in Table I.

	1st	2nd	3rd	4th
Blood volume (drop)	3	3	3	3
1% BCB volume (drop)	3	3	-	-
Incubation temperature (°C)	25	37	25	37
*1% BCB-KI volume (drop)	-	-	3	3

TABLE I: Whole blood method varying temperature with and without potassium iodide.

*1 gm BCB/100 ml KI.

Potassium iodide (BDH) is dissolved in the proportion of 1 mg/ml in citrate-saline solution (one part 3 gm/100 ml sodium citrate [BDH] to four parts normal saline).

The tubes were incubated at 25°C and 37°C for 10, 20, 30 and 60 minutes, 2, 3, 4 and 5 hours and thereafter dry blood smears were prepared.

6.2.2.2 Centrifuged blood method

This technique was performed to determine any advantage over the whole blood method.

The Wintrobe tubes (2.5 mm x 100 mm) were filled with K_2 EDTA blood to provide sufficient packed red cells for subsequent experimentation (Raven and Tooze, 1973). These were centrifuged at 4500 g for 10 minutes. One drop from each Wintrobe tube, at approximately 3 mm above and 5 mm below the plasma-cell interface (Jones et al, 1981) was mixed with

three drops of 1% BCB in 75 x 10 mm test tube. The tubes were then incubated at 37°C for 10, 20, 30 and 60 minutes, 2, 3, 4 and 5 hours and then dry blood smears were prepared. Because of the high incidence of false positive results in control samples beyond one hour at 37°C, patient samples were studied only to one hour i.e. 10, 20, 30 and 60 minutes.

6.2.3 counting of inclusion bodies

Smears were examined using an oil immersion objective (magnification x 1000). To ensure that sufficient red cells were assessed, 300 fields (approximately 30,000 red cells) were examined using the technique illustrated in Figure 13. If only one cell was found, the test was considered as positive (Walford and Deacon, 1976).

7. GLOBIN CHAIN SYNTHESIS STUDIES

7.1 Principle

The **in vitro** measurement of globin chain synthesis can be a useful method of characterising the α-thalassaemias, since the disease arises from an inherited defect in the rate of synthesis of the alpha globin chain. Reticulocyte-enriched blood is incubated in a buffer containing all amino acids necessary to sustain globin synthesis and a radio-active tracer amino acid, usually ^{3}H-leucine. Leucine is chosen because there are equal numbers of leucine residues in both the α and β chains and, therefore, there is no need for correction of radio-activity. The entrance of leucine into the red

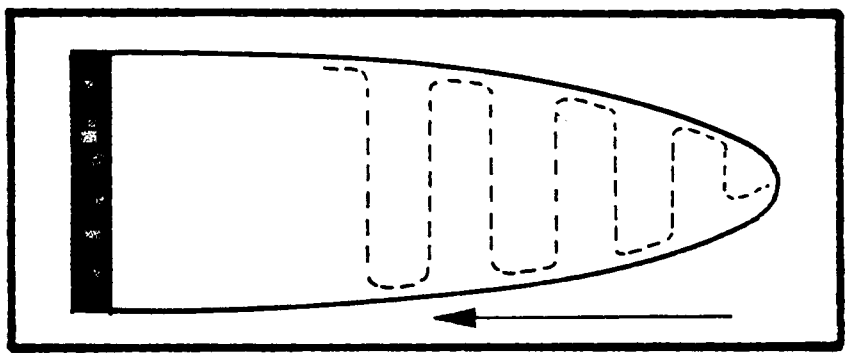

Fig. 13 Schematic drawing illustrating method of counting the inclusion bodies.

cells is rapid and its passage through the membrane is not the rate-limiting step (Schwartz,1974). The cells are then washed, haemolysed and the globin extracted with acid-acetone. The α and non-α chains are dissociated and separated by ion exchange chromatography on carboxy methyl cellulose (CM cellulose) in 8M urea. The chains are eluted separately from the column by a linear gradient of sodium ions at acidic pH e.g. the more positively charged α chains are eluted at a higher Na^+ concentration. Addition of mercaptoethanol to the buffer keeps the sulphydryl groups of the globin chains in the reduced state and prevents aggregation of the α and β chains. The total number of counts incorporated into each peak are calculated and the

$$\alpha/\beta \text{ ratio is expressed as } \frac{\text{total counts } \alpha \text{ peak}}{\text{total counts } \beta \text{ peak}}$$

The α/β ratio approximates at 1.0 in normal individuals whereas it is increased in β-thalassaemia and decreased in α-thalassaemia in proportion to the severity of the defect.

7.2 Method

7.2.1 Globin preparation

7.2.1.1 Preparation of a reticulocyte rich suspension:

20 ml of heparinised blood was centrifuged at 1,100 g for 5 minutes and the packed cells washed three times with normal saline. The washed red cells

were subjected to ultracentrifugation (Beckman Model L5-65) at 9200 g for 10 minutes at 4°C.

7.2.1.2 Globin incubation

1 ml from the upper layer of the packed cells was added to 3 ml of leucine-free amino acid mixture, prepared as described by Lingrel and Borsook (1963) and stored in batches at -20°C until used. Glutamine (0.15 ml of a 0.1461 g/100 ml saline solution) was added to the incubation medium to replace that of the amino acid mixture which denatures during storage. Ferrous ammonium sulphate (0.18 ml of 0.1 g/100 ml H_2O solution) was added as a soluble source of iron.

The incubation mixture was completed by the addition of 100 µl H^3 leucine (radioactive concentration 1.0 m Ci/ml; specific activity 45 Ci/mmol, [Amersham]).

This mixture was then incubated at 37°C for one hour and washed three times with 10 ml cold phosphate buffered saline (Sigma Chemicals) to remove any free isotope. Finally the red cells were lysed in 5 ml distilled water.

7.2.1.3 Globin separation

To remove the haem group, the haemolysate was added dropwise to 100 ml of a 1.5% (W/V) solution of concentrated hydrochloric acid(HCl) in acetone with continuous stirring. The precipitated globin was centrifuged and the brown supernatant containing

the haem discarded. The globin was washed three times in acetone (BDH, ANALAR) to remove any trace of acid, dried in a nitrogen stream and stored at -20°C until used.

7.2.2 Globin chain separation

The globin chains derived from the haemoglobin were separated by the method of Clegg et al (1966).

7.2.2.1 Reagents/Equipment

Solutions

The following solutions were freshly prepared before use:-

8M Urea

This was prepared by dissolving 720 gm Urea (BDH Analar grade) in 900 ml of distilled water. The solution was filtered to remove any impurities and made up to 1.5 l with distilled water.

Buffers

Buffer	8M urea	$Na_2HPO_4.2H_2O$ Sörensen's salt	2-mercapto-ethanol
Inital(0.005M)	1200 ml	1.068 gm	4 ml
Final (.03M)	300 ml	1.602 gm	1 ml

The solutions were adjusted to pH 6.7 with concentrated phosphoric acid (H_3PO_4) [BDH].

Carboxymethyl Cellulose (CM)

5 gm of fines reduced CM 23 cellulose (Whatman) was made into a slurry with 100 ml initial buffer and left to settle for one hour.

Chromatography column

A column of dimension 1.5 cm x 30 cm (Pharmacia) was used.

Dialysis membrane

Dialysis membrane of size 8/32 (Scientific Instrument Limited).

7.2.2.2 Method

100 mg of H^3-labelled globin were dissolved in 5 ml initial buffer, and dialysed against 100 ml of initial buffer for 2 hours to reduce the sulphydryl groups of globin. Meanwhile the cellulose slurry was poured into the column and packed very carefully at atmospheric pressure to form a bed 20 cm in height. The column was then equilibrated with initial buffer at a flow rate of 1 ml/min. for 30 minutes, the rate of flow being adjusted by a peristaltic pump (Pharmacia). The sample was then loaded onto the column under gravity using a Pasteur Pipette and the residual proteins were washed on with a further 2 ml of the initial buffer. The globin remains firmly bound to the CM cellulose at the top of the column.

The column was washed for 30-40 minutes using the initial buffer to elute unbound protein. The globin was then eluted from the column by a linearly increasing Na^+ ion concentration generated in a two chamber mixer containing equal volumes (300 ml) of initial and final buffers. At acidic pH, α and non α-chains elute separately from the column at

characteristic Na^+ ion concentrations. 10 ml fractions were collected (ISCO Model 328 fraction collector) and the optical density of the eluate was monitored continuously at 280 nm (ISCO Model UA-5 absorbance/fluorescence monitor).

7.2.3 Counting

A 1 ml aliquot from each fraction was pipetted into a polyethylene vial (Packard) containing 10 ml of a scintillation fluid (Scintillator 299^{TM} [Packard]) and mixed thoroughly. The radioactivity of each aliquot was counted for one minute in a liquid scintillation counter (Packard Model 3300).

The radioactivity incorporated in α and β chains was counted and the α/β ratio expressed as follows:-

$$\alpha/\beta = \frac{\text{Total counts under } \alpha \text{ peak}}{\text{Total counts under } \beta \text{ peak}}$$

RESULTS

1. RED CELL MEASUREMENTS

Table II contrasts red cell measurements in normal controls and in subjects with suspected α thalassaemia trait. Males and females were analysed separately. Significant differences in data sets between the groups are shown using the 'p' value (Table III). It is clear that the mean values of MCV, MCH and MCHC of patients of both sexes were significantly lower than controls ($p < 0.001$). While the RBC was increased in both sexes, the increase only attains significance in females ($p < 0.001$). The HGB was slightly reduced in patients of both sexes when compared with controls ($p < .01$). Male and female patients did not differ with the exception of the RBC ($p < 0.001$).

Red cell measurements	MALES		FEMALES	
	Controls n = 10	Suspected α thalassaemia trait n = 14	Controls n = 10	Suspected α thalassaemia trait n = 18
RBC (10^{12}/l)	5.03±0.29	7.3±2.4	4.38±0.2	5.11±0.3
HGB (g/dl)	15.4±0.9	13.4±1.7	13.3±0.7	12.2±1.0
HCT (l/l)	0.44±0.02	0.40±0.02	0.38±0.02	0.36±0.02
MCV (fl)	89.5±4.2	68.0±4.9	89.0±4.0	72.0±5.6
MCH (pg)	30.7±1.5	22.3±2.1	30.8±1.8	23.8±2.0
MCHC (g/dl)	33.9±0.9	32.5±0.7	34.4±0.6	32.6±1.0
RDW (%)	13.1±0.8	14.3±0.9	13.2±0.8	14.1±1.0

TABLE II: Red cell measurements in controls and in suspected α-thalassaemia trait subjects (32) expressed as mean ± standard deviation.

Red cell parameters	Control males vs control females	Control males vs patients (male)	Control females vs patients (female)	Patients (male) vs patients (female)
RBC (10^{12}/l)	$p < .001$**	$p < .01$*	$p < .001$**	$p < .001$**
HGB (g/dl)	$p < .001$**	$p < .01$*	$p < .01$ *	$p < .03$
HCT (l/l)	$p < .001$**	$p < .01$*	$p < .01$ *	$p < .02$
MCV (fl)	$p < .7$	$p < .001$**	$p < .001$**	$p < .04$
MCH (pg)	$p < .8$	$p < .001$**	$p < .001$**	$p < .04$
MCHC (g/dl)	$p < .1$	$p < .001$**	$p < .001$**	$p < .7$
RDW (%)	$p < .7$	$p < .01$ *	$p < .02$	$p < .5$

TABLE III: Statistical analysis of red cell measurements for normal controls and suspected α thalassaemia trait subjects

$p < .001$** = highly significant

$p < .01$ * = significant

2. HAEMOGLOBINOPATHY INVESTIGATIONS

All patients had normal Hb electrophoresis, normal levels of HbF and HbA_2 (Table IV).

The normal ranges in the laboratory for HbF and HbA_2 are $<$ 1% and $<$ 3.2% respectively.

	Hb electrophoresis	HbF %	HbA_2 % (mean±S.D.)
controls	normal	HbF < 1%	2.5±0.3
patients	normal	HbF < 1%	2.3±0.4

TABLE IV: Hb electrophoresis, HbF percentage and HbA_2 percentage in the controls (20) and suspected α thalassaemia trait patients (32)

3. SERUM FERRITIN LEVEL

Six out of the thirty-two suspected α thalassaemia trait subjects were found to have iron deficiency (ferritin < 12 ng/ml), the remainder having normal levels (normal range 12 - 400 ng/ml). Results are shown in Table V. As shown in Table VI males, both controls and patients whose serum ferritin levels fall within the normal range, had significantly more ferritin than was detected in females ($p<.01$). No significant difference was noted between controls and patients of the same sex (Table VI).

Sex	control	patients with normal ferritin	patients with iron deficiency
Males	n = 10	n = 12	n = 2
	133±105	186±125	4±.7
Females	n = 10	n = 14	n = 4
	43± 34	66± 42	7±3

TABLE V: Serum ferritin levels (ng/ml) in normal controls and suspected α thalassaemia trait subjects expressed as mean ± standard deviation

control males vs control females	control males vs patients(male)	control females vs patients(female)	patients (male) vs patients(female)
$p < .01$*	$p < .3$	$p < .1$	$p < .01$*

TABLE VI: Statistical analysis of serum ferritin levels (ng/ml) in controls and suspected α thalassaemia trait subjects with normal ferritin levels.

patient: suspected α thalassaemia trait subjects with normal ferritin

$p < .01$* significant

4. DEMONSTRATION OF HbH INCLUSIONS

Routine Method

As shown in Table VII approximately half the patients gave positive results with the HbH inclusion test indicating the presence of α thalassaemia trait. However, globin chain synthesis studies were performed on all patients irrespective of whether HbH inclusions were detected or not.

	Number of patients	Number of patients with positive results
patients with normal ferritin	26	14
patients with iron deficiency	6	2*

TABLE VII: ROUTINE METHOD

HbH inclusion test in suspected α thalassaemia trait at 25°C for 24 hours using 1% BCB.

* These 2 patients were diagnosed as having α thalassaemia trait/ iron deficiency by globin chain synthesis studies.

5. GLOBIN CHAIN SYNTHESIS STUDIES

The results are expressed as α/β-radioactivity ratios. Precision studies were performed by measuring five replicated analysis on one control subject and on one patient. The results of these and their respective coefficients of variation are shown in Table VIII.

Patients and control results

The results of globin chain synthesis studies are shown in Table IX and are categorised as follows (Figure 14):-

A - The control group was found to have a mean α/β ratio of 1.16±0.10.

B - The four suspected α thalassaemia trait patients with iron deficiency and negative HbH inclusion tests, had normal α/β ratios (1.15±0.14) and were, therefore, diagnosed as iron deficient. The other two patients suspected to have iron deficiency on the basis of low serum ferritin levels and who showed positive HbH inclusion tests had low α/β globin chain ratios (0.63 and 0.71) indicating that they had combined α thalassaemia trait/ iron deficiency, and are, therefore, included with α thalassaemia trait in category D.

C - This patient had a normal serum ferritin and a negative HbH inclusion test but was found to have a high α/β ratio (2.14) and was, therefore, diagnosed as β thalassaemia trait with a normal HbA_2 level.

D - The remaining patients with normal ferritin levels

who either gave positive or negative results in the HbH inclusion test were found to have reduced α/β ratios (0.85 ± 0.12) when compared with controls ($p < .001$). These patients were diagnosed as α thalassaemia trait.

	Replicate of α/β ratios	Mean±SD	Co-efficient of variation
Control	1.00; 1.01; 0.99; 1.00; 1.01	1.0±0.01	1%
Patient	0.70; 0.69; 0.70; 0.69; 0.71	0.70±0.01	1.4%

TABLE VIII: Precision of α/β globin chain ratio method

controls		patients with iron deficiency	patients with normal serum ferritin	
1.00	1.19	1.04	0.63	0.91
1.01	1.19	1.05	0.66	0.91
1.02	1.26	1.16	0.70	0.93
1.04	1.26	1.36	0.72	0.94
1.10	1.30	0.63	0.73	0.96
1.10	1.36	0.71	0.77	0.97
1.10	1.36		0.77	0.97
1.10			0.84	0.98
1.14			0.85	0.98
1.15			0.86	0.99
1.17			0.87	0.99
1.17			0.88	1.00
1.18			0.90	2.14

TABLE IX: α/β ratios in controls and suspected α thalassaemia trait subjects

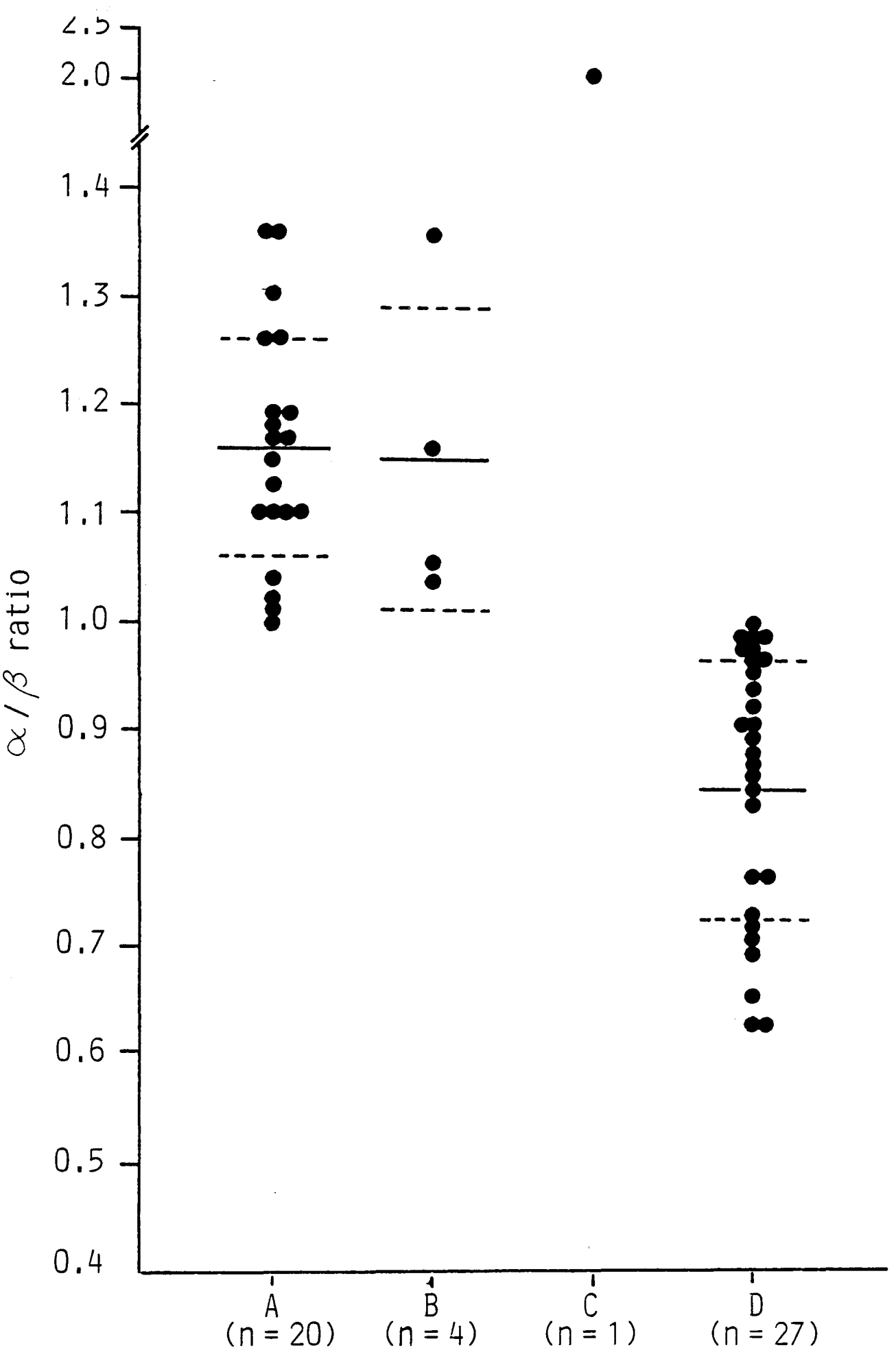

Fig. 14 Globin chain synthesis in controls and suspected ∝ thalassaemia trait.

A = controls B = iron deficiency
C = β thalassaemia trait D = ∝ thalassaemia trait
——— mean ----- standard deviation .

6. RDW IN α THALASSAEMIA TRAIT AND IRON DEFICIENCY

The normal range of RDW is from 11.8 - 14.8%. All controls lay within this range (Figure 15). Of the 27 patients with α thalassaemia trait, 6 had marginally elevated RDW levels in the range 14.8 - 15.0%. In the α thalassaemia group were two patients with iron deficiency in addition (RDW of 13.6 and 14.5). Four of the original patient group did not have α thalassaemia but simply iron deficiency. Of these, one patient possessed an RDW in the normal range, the other three having elevated levels. See Appendice I.

7. DEMONSTRATION OF HbH INCLUSIONS IN α THALASSAEMIA TRAIT

7.1 Routine Method

Results of HbH inclusion testing in the group of α thalassaemia trait patients performed on whole blood incubated for 24 hours at 25°C are shown in Table X. The mean value for HbH inclusions in this group is 2.6 per 30,000 RBC with a range of 0 - 8 per 30,000 RBC. However, it is important to note that of the 27 patients with α thalassaemia trait, 11 patients did not show HbH inclusions.

7.2 Experimental Method

7.2.1 Whole blood method varying incubation time and temperature with and without potassium iodide (KI) stimulation

Table XI shows results obtained at 25°C incubation. Table XII shows results obtained at 37°C.

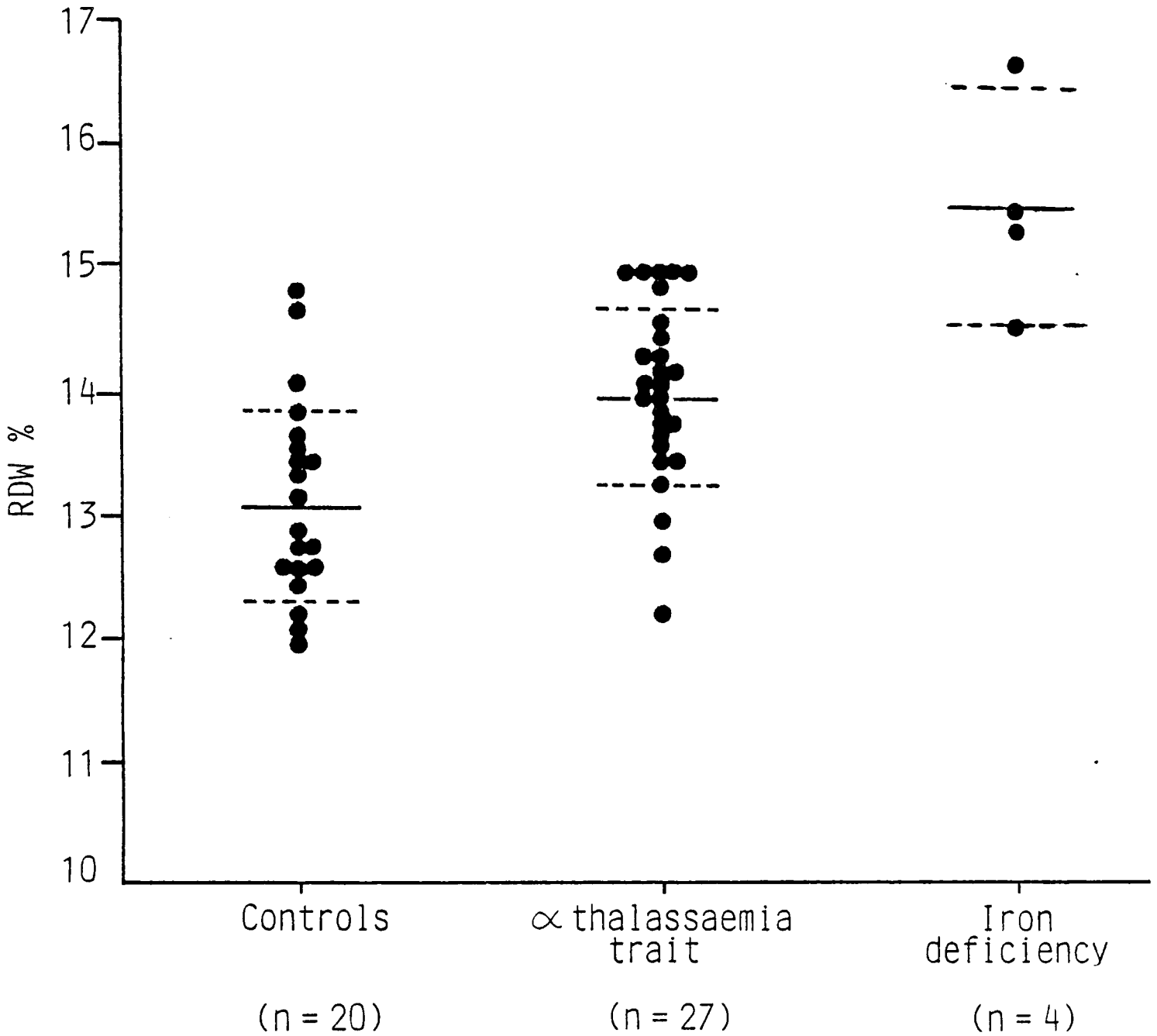

Fig. 15 Comparison of RDW (%) in controls, ∝ thalassaemia trait and iron deficiency.

—— mean ---- standard deviation

HbH inclusion bodies start to appear after two hoursincubation at 25°C. No HbH inclusion bodies were found in control subjects. The addition of KI had no effect. However, at 37°C the HbH inclusions started to appear in patients after 10 minutes incubation and increased progressively. At 37°C following 2 hours incubation controls started to show HbH inclusion positivity but not of the magnitude observed in patients. Again the addition of KI does not produce any particular advantage.

From these experiments:-

(a) The number of HbH inclusions apparently reaches a maximum at 5 hours (Figure 16), at 25°C.

(b) Incubation at 37°C for 1 hour results in the formation of similar numbers of HbH inclusions (Figure 16).

(c) False positive results emerge in the control group after 2 hours incubation at 37°C.

7.2.2 Centrifuged blood method varying incubation time (temperature 37°C only;no KI addition)

Using this method controls showed false positivity commencing after 2 hours incubation and increasing progressively. Patients, on the other hand, showed a major progressive increase in HbH inclusions from 10 minutes incubation to 1 hour incubation (Table XIII).

Case number	Routine examination	Case number	Routine examination
1	1	17	5
2	1	18	0
3	8	19	0
4	8	20	8
5	3	21	0
6	0	22	0
7	1	23	0
8	0	24	4
9	0	25	1
10	5	26	0
11	0	27	5
12	4		
13	0		
14	5		
15	1		
16	6		

TABLE X: The number of HbH inclusion bodies counted in 30,000 RBC in α thalassaemia trait patients by routine method.

Incubation time	controls n=20		α thalassaemia trait . n=27	
	1% BCB	1% BCB-KI	1% BCB	1% BCB-KI
10 minutes	nil	nil	nil	nil
20 minutes	nil	nil	nil	nil
30 minutes	nil	nil	nil	nil
60 minutes	nil	nil	nil	nil
2 hours	nil	nil	* 0.4 ≠ 0-3	* 0.3 ≠ 0-2
3 hours	nil	nil	* 1.1 ≠ 0-6	* 1.1 ≠ 0-5
4 hours	nil	nil	* 1.8 ≠ 0-8	* 1.6 ≠ 0-8
5 hours	nil	nil	* 2.7 ≠ 0-8	* 2.8 ≠ 0-8

TABLE XI: Whole blood method varying incubation time at 25°C with and without potassium iodide

KI: potassium iodide

* mean

≠ observed range

	controls n=20		α thalassaemia trait n=27	
Incubation time	1% BCB	1% BCB-KI	1% BCB	1% BCB-KI
10 minutes	nil	nil	* 0.1 ≠ 0-1	nil
20 minutes	nil	nil	* 1.1± ≠ 0-4	* 1.1 ≠ 0-3
30 minutes	nil	nil	* 1.9 ≠ 0-6	* 1.7 ≠ 0-5
60 minutes	nil	nil	* 2.7 ≠ 0-10	* 2.8 ≠ 0-10
2 hours	* 0.2 ≠ 0-2	* 0.3 ≠ 0-1	* 3.8 ≠ 0-12	* 3.7 ≠ 0-11
3 hours	* 0.8 ≠ 0-3	* 0.8 ≠ 0-2	* 4.0 ≠ 0-12	* 4.1 ≠ 1-10
4 hours	* 1.1 ≠ 0-3	* 1.1 ≠ 0-2	* 4.7 ≠ 1-13	* 4.6 ≠ 2-12
5 hours	* 1.5 ≠ 1-4	* 1.6 ≠ 1-3	* 5.0 ≠ 2-13	* 5.3 ≠ 2-14

TABLE XII: Whole blood method varying incubation time at 37°C with and without potassium iodide.

* mean ≠ observed range

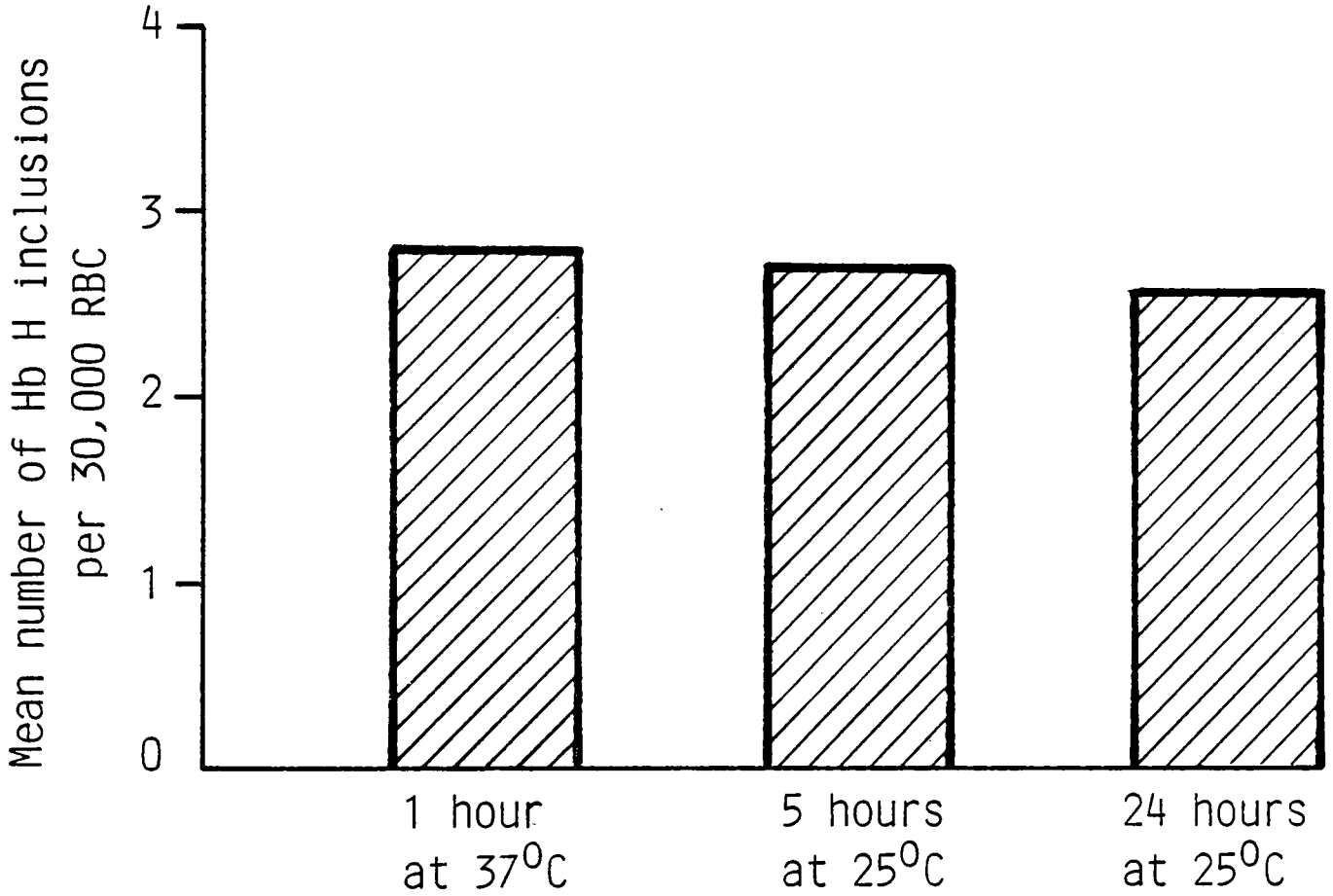

Fig. 16 Mean number of Hb H inclusions in α thalassaemia trait (27) using whole blood for 1 hour at 37°C, 5 and 24 hours at 25°C.

Incubation time	Controls n=20	α thalassaemia trait n=14
10 minutes	nil	* 0.6
		≠ 0-4
20 minutes	nil	* 4.8
		≠ 0-20
30 minutes	nil	* 7.0
		≠ 0-35
60 minutes	nil	* 15.0
		≠ 0-41
2 hours	*0.2	
	≠ 0-1	
3 hours	* 0.3	
	≠ 0-2	
4 hours	* 0.6	
	≠ 0-2	
5 hours	* 1.2	
	≠ 1-3	

TABLE XIII: Centrifuged blood method varying incubation time at 37°C

* mean ≠ observed range

8. CLASSIFICATION OF α THALASSAEMIA TRAIT

Based on blood count measurements globin chain synthesis studies and the demonstration of HbH inclusion bodies in routine and experimental methods, the 27 patients with α thalassaemia trait could be further classified into two types:-

8.1 α Thalassaemia 2 "mild form"

There were 9 patients in this group who were characterised by :-

(a) MCV values between 75-79 fl and MCH values between 25-27 pg with mean values significantly less than controls ($p < .001$) [Figures 17 and 18].

(b) Negative results with HbH inclusion test.

(c) Mean α/β globin chain ratio 0.96±0.04 [Figure 19] which is significantly less than control ($p < .001$).

8.2 α Thalassaemia 1 or homozygous α thalassaemia 2 "severe form"

There were 18 patients included in this group. These patients were characterised by:-

(a) MCV values between 60-72 fl, MCH values between 19-24 pg with mean values significantly less than the mild state ($p < .001$) [Figures 17, 18].

(b) Positive results with HbH inclusion test.

(c) Mean α/β globin chain ratio of 0.79±.11 which is also significantly less than the mild group ($p < .001$) [Figure 19].

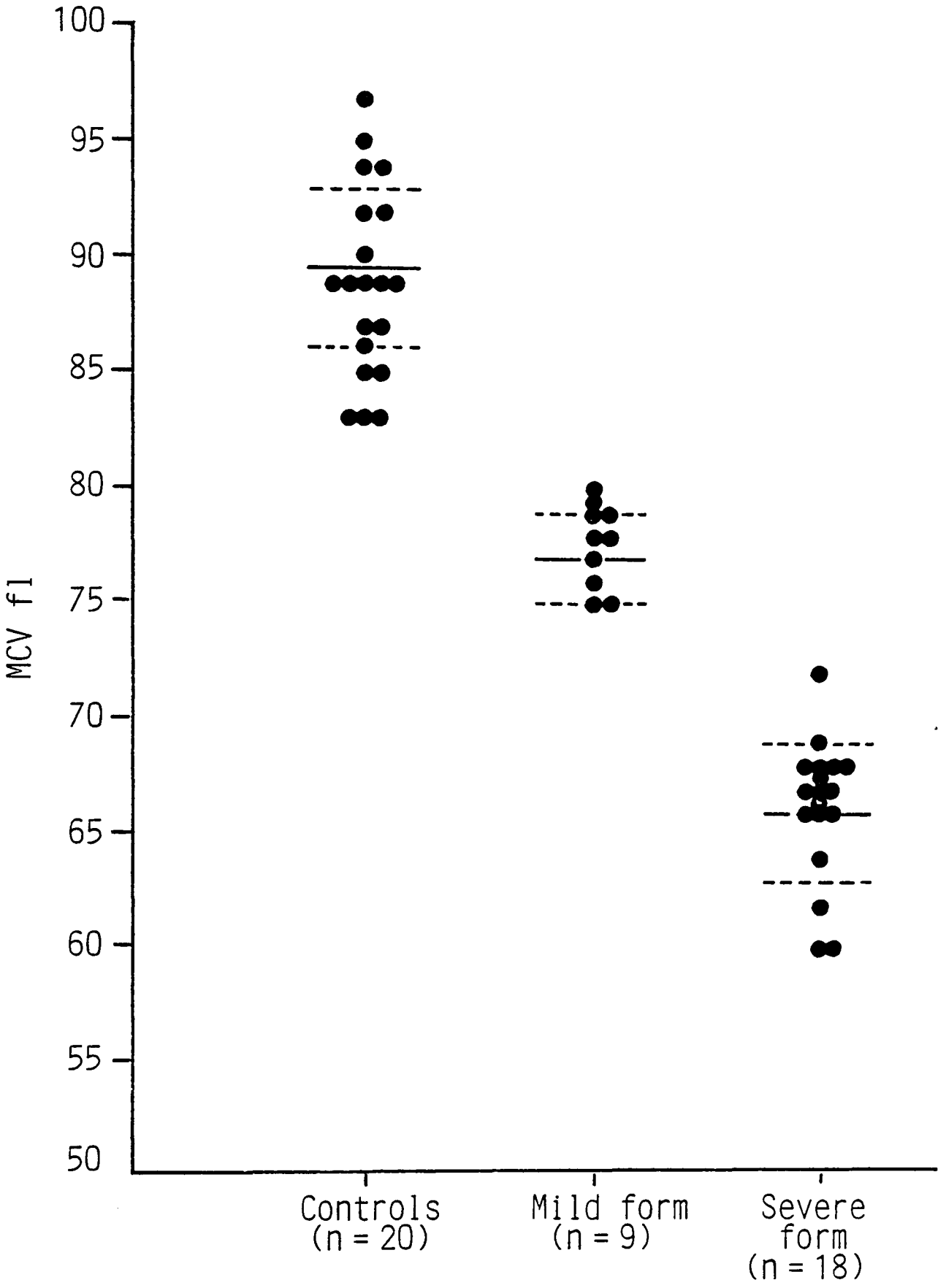

Fig. 17 Comparison of MCV in controls, mild and severe forms of α thalassaemia trait.

——— mean ----- standard deviation

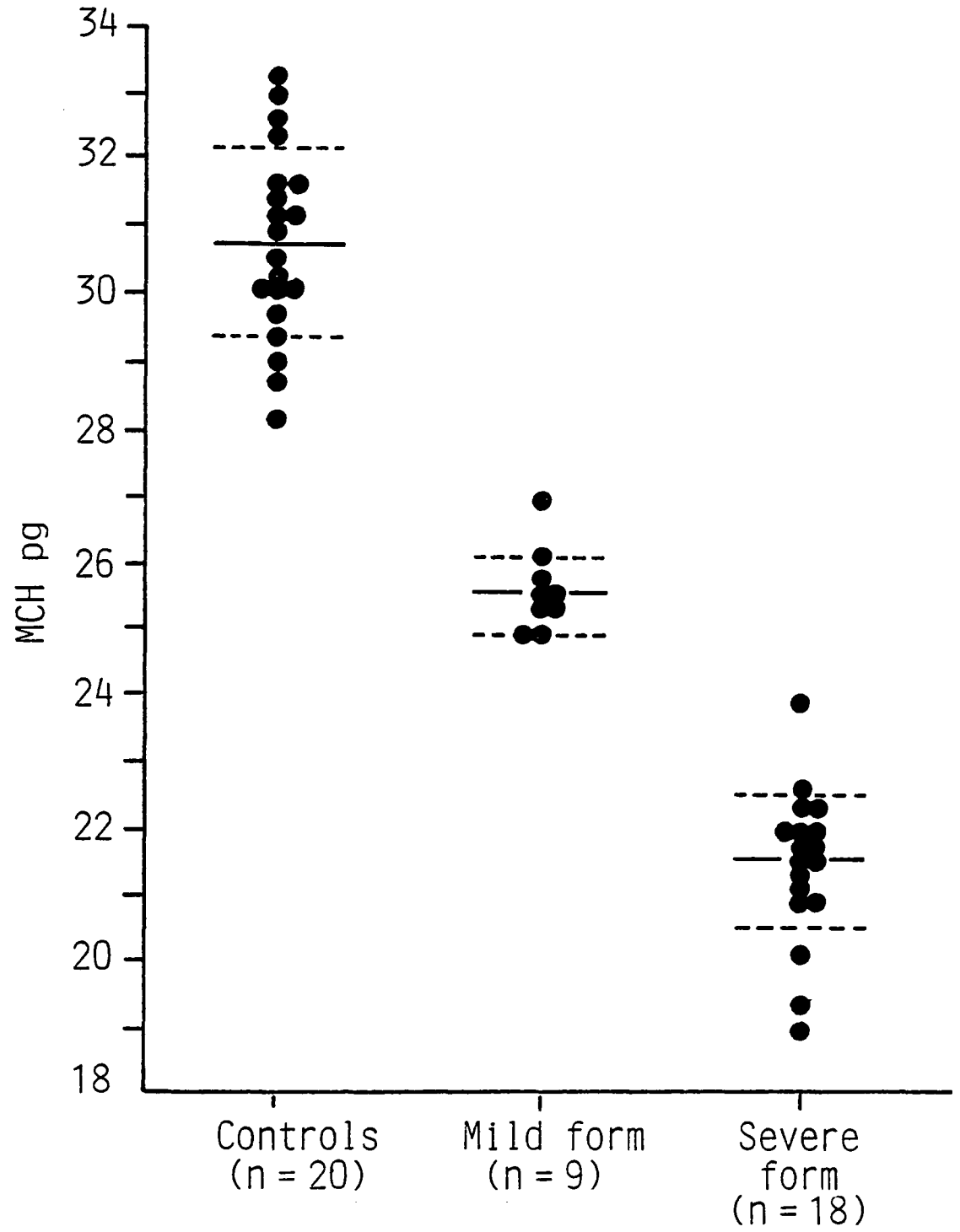

Fig. 18 Comparison of MCH in controls, mild and severe forms of ∝ thalassaemia trait.
—— mean ---- standard deviations.

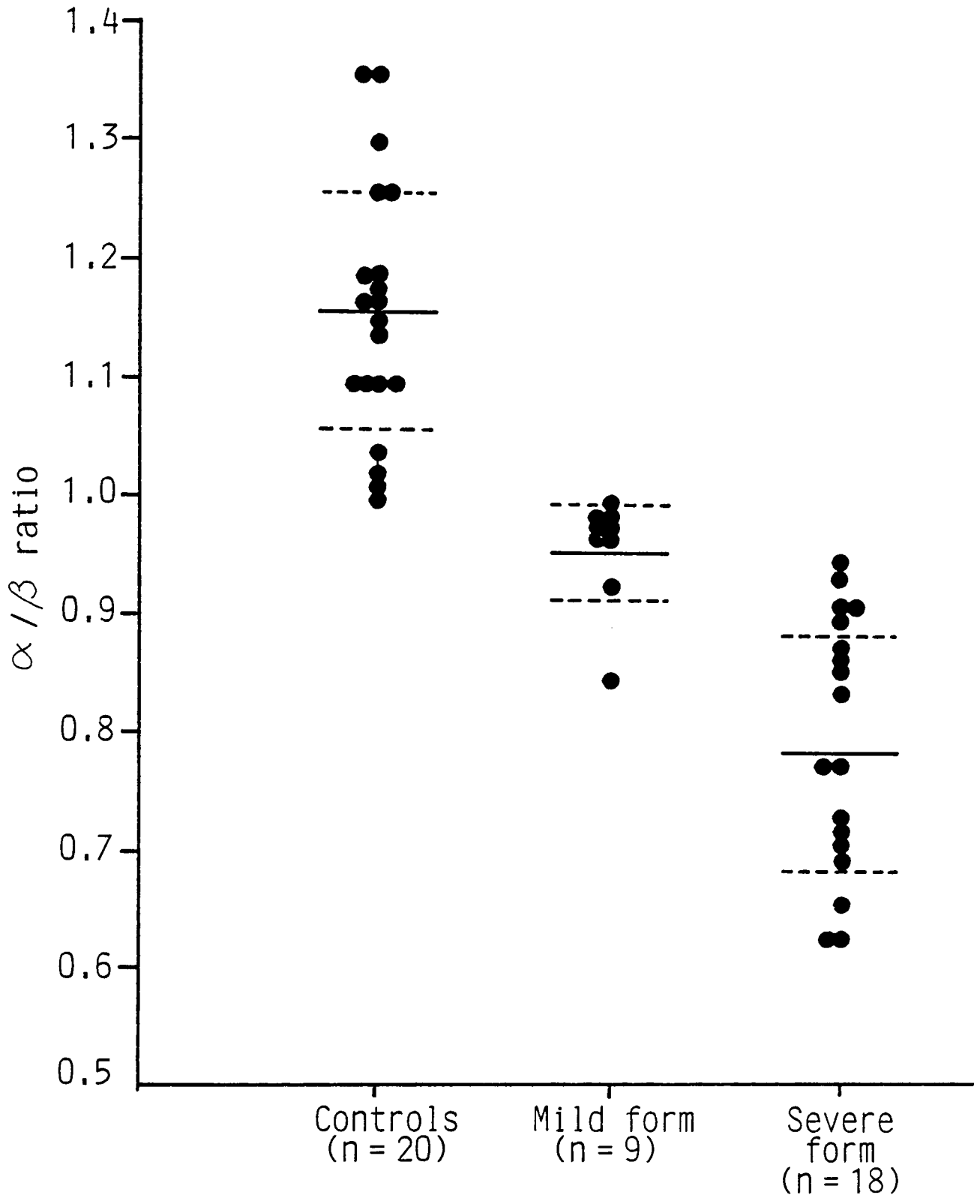

Fig. 19 Comparison of α/β ratios in controls, mild and severe forms of α thalassaemia trait.
—— mean ----- standard deviation

9. α THALASSAEMIA TRAIT IN DIFFERENT RACIAL GROUPS

The 27 α thalassaemia trait patients were further divided by race into:-

14 Indians (8 mild form and 6 severe form)

12 Chinese (1 mild form and 11 severe form)

1 Negro (severe form)

None of the patients were related to each other.

9.1 Red Cell Measurements

A comparison of red cell measurements in Indian and Chinese patients with the severe form of α thalassaemia trait shows no significant difference between the two ethnic groups (Table XIV). Comparison of mild and severe forms in Indians demonstrated highly significant differences in the MCV and MCH ($p < .001$) [Table XV]. There were insufficient Chinese mild form patients to permit any comparison with the mild Indian patients.

9.2 Serum Ferritin Level

Serum ferritin levels in Chinese males are significantly greater than those observed in control males ($p < .01$), Chinese females ($p < .001$) and Indian males ($p < .001$)[Tables XVI, XVII]. There is no significant difference between males and females in the Indian group ($p < .3$).

9.3 Demonstration of HbH Inclusion Bodies

As mentioned before inclusion bodies are observed only in the severe form of the disorders. Therefore the following experimental studies to assess racial

differences in occurrence of HbH inclusions were restricted to those patients with the severe form (α thalassaemia 1 or homozygous α thalassaemia 2).

The mean number of HbH inclusions observed in Indian, Chinese and Negro patients with the whole blood method at 25°C and 37°C are shown in Table XVIII. At 37°C tests were only performed with incubation periods ranging from 10-60 minutes, because of the occurrence of false positive results beyond that time in the control group. The number of inclusions in the Chinese group is six and eight times greater than Negro and Indian values respectively following incubation for 5 hours at 25°C. The number of inclusions present following incubation for 1 hour at 37°C is six and nine times greater in the Chinese group than in the Negro and Indian values respectively [Figure 20].

Although centrifugation increases the number of inclusions in the Chinese group approximately five times over that obtained in the whole blood method, the increase in the Indian group is only by a factor of three (Table XIX)[Figures 20,21].

9.4 Globin Chain Synthesis Studies

Results of globin chain synthesis studies are illustrated in Table XX. The α/β ratios in the different geographical groups are illustrated in Figure 22. The α/β ratios in Indians with the severe form of α thalassaemia trait are significantly less than in the Chinese group ($p < .001$).

Globin chain synthesis correlates positively with MCV in both forms of the trait in the Indian group [Figure 23].

10. TWO FORMS OF α THALASSAEMIA TRAIT IN INDIANS

Table XXI summarised variation in the haematological studies between mild and severe forms in Indians. These indicate clear separation of the two groups.

Red cell Measurement	Severe form in Indians n=6	Severe form in Chinese n=11	Indians vs Chinese
RBC X 10^{12}/l	5.43±0.66	6.16±0.73	$p < .06$
HGB g/dl	11.9±1.5	12.8±1.6	$p < .2$
HCT l/l	0.36±0.04	0.39±0.05	$p < .2$
MCV fl	67.9±0.9	65.2±3.1	$p < .05$
MCH pg	21.9±0.5	21.1±1.0	$p < .08$
MCHC g/dl	32.2±0.7	32.4±0.6	$p < .5$
RDW %	13.9±0.9	14.4±0.7	$p < .22$

TABLE XIV: Comparison of red cell measurements between Indian and Chinese severe form of α thalassaemia trait. Results expressed in mean ± standard deviation.

Red cell measurements		mild form n=8	severe form n=6	mild form vs severe form
RBC	X 10^{12}/l	5.25±0.60	5.43±0.66	$p < .6$
HGB	g/dl	13.5±1.4	11.9±1.5	$p < .06$
HCT	l/l	0.40±0.04	0.36±0.04	$p < .08$
MCV	fl	77.9±1.8	67.9±0.9	$p < .001$**
MCH	pg	25.9±0.6	21.9±0.5	$p < .001$**
MCHC	g/dl	32.8±1.5	32.2±0.7	$p < .38$
RDW	%	13.6±0.5	13.9±0.9	$p < .43$

TABLE XV: Comparison of red cell measurements between the mild and the severe form of α thalassaemia trait in Indians. Results expressed in mean ± standard deviation.

$p < .001$** highly significant

Sex	Controls	Indians	Chinese
Males	n=10	n=4	n=7
	133±105	55±36	272±90
Females	n=10	n=8	n=5
	43±34	70±52	46±30

TABLE XVI: Serum ferritin level (ng/ml) in controls, Indian and Chinese α thalassaemia trait. Results expressed in mean ± standard deviation.

Control males	Indian males	Chinese males	Indian males	Indian females
vs	vs	vs	vs	vs
Control females	Indian females	Chinese females	Chinese males	Chinese females
$p < .01$*	$p < .3$	$p < .001$**	$p < .001$**	$p < .3$

TABLES XVII : Statistical analysis of serum ferritin levels (ng/ml) in controls, Indian and Chinese α thalassaemia trait

$p < .01$* significant

$p < .001$** highly significant

Incubation time	Incubation temperature					
	25°C			37°C		
	Negro (n=1)	Indians (n=6)	Chinese (n-11)	Negro (n=1)	Indians (n=6)	Chinese (n=11)
10 minutes	nil	nil	nil	nil	nil	* 0.1
						≠ 0-1
20 minutes	nil	nil	nil	nil	nil	* 2.7
						≠ 1-4
30 minutes	nil	nil	nil	nil	nil	* 4.6
						≠ 2-6
60 minutes	nil	nil	nil	1	* 0.7	* 6.2
					≠ 0-1	≠ 3-10
2 hours	nil	nil	* 1.0			
			≠ 0-3			
3 hours	nil	* 0.16	* 2.5			
		≠ 0-1	≠ 1-6			
4 hours	nil	* 0.3	* 4.2			
		≠ 0-1	≠ 2-8			
5 hours	1	* 0.7	* 5.8			
		≠ 0-1	≠ 3-9			
24 hours	1	* 0.7	* 5.5			
		≠ 0-1	≠ 3-8			

TABLE XVIII: Comparison of number of HbH inclusions (per 30,000 RBC) in Indian, Chinese and Negro α thalassaemia trait patients using 1% BCB at 25°C and 37°C with whole blood.

* mean

≠ range

Incubation time (minutes)	Indians (n=4)	Chinese (n=8)
10	nil	* 1.1
		≠ 0-4
20	nil	* 9.7
		≠ 1-30
30	* 0.75	* 13.4
	≠ 0-1	≠ 4-37
60	* 2.0	* 29.7
		≠ 18-41

TABLE XIX: Comparison of number of HbH inclusions per 30,000 RBC in Indian and Chinese α thalassaemia trait patients using centrifuged blood with 1% BCB at 37°C up to hour.

* mean

≠ observed range

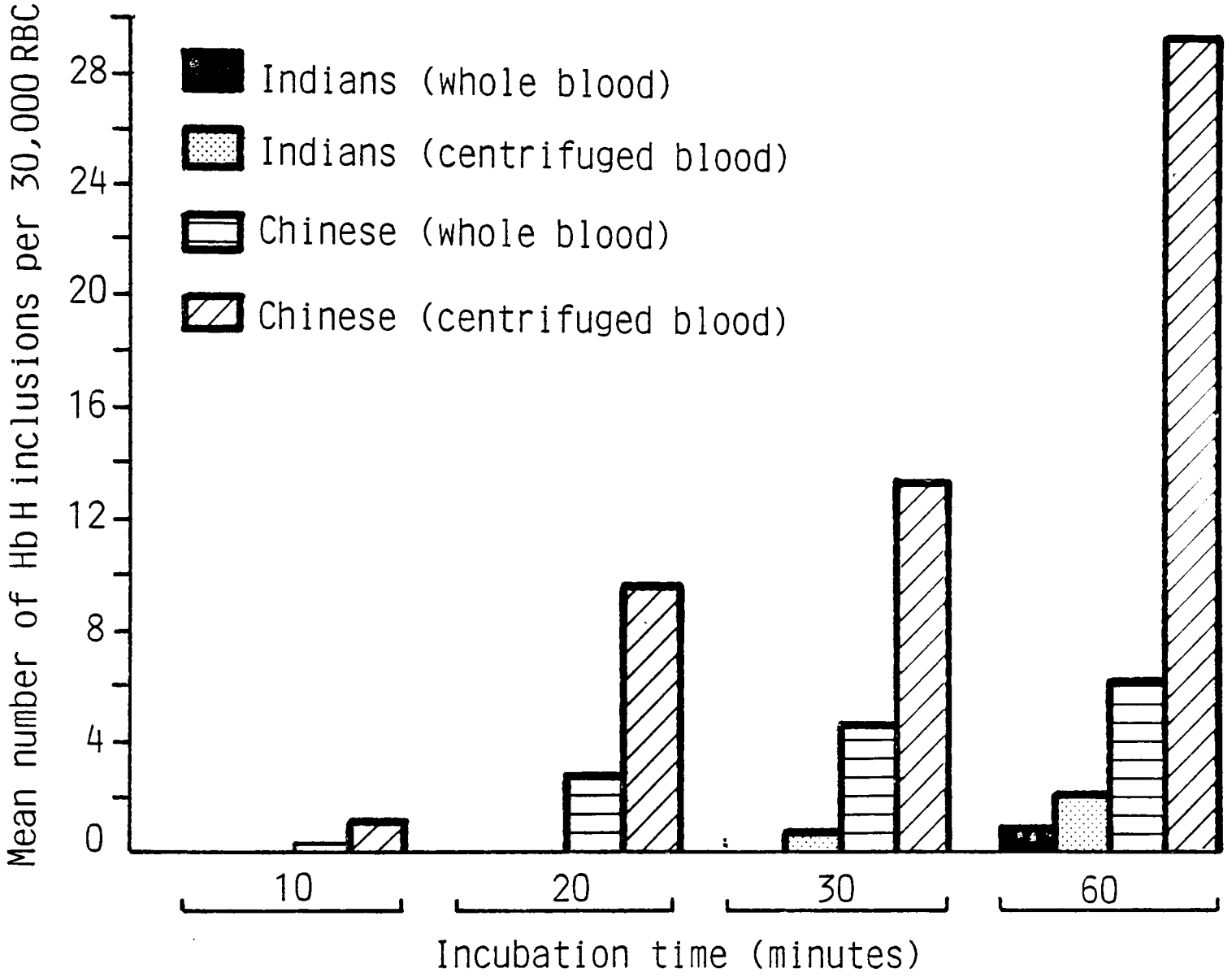

Fig. 20 Comparison of number of Hb H inclusions of α thalassaemia in Indian and Chinese patients using (1) whole blood (2) centrifuged blood with 1% BCB at $37^{o}C$.

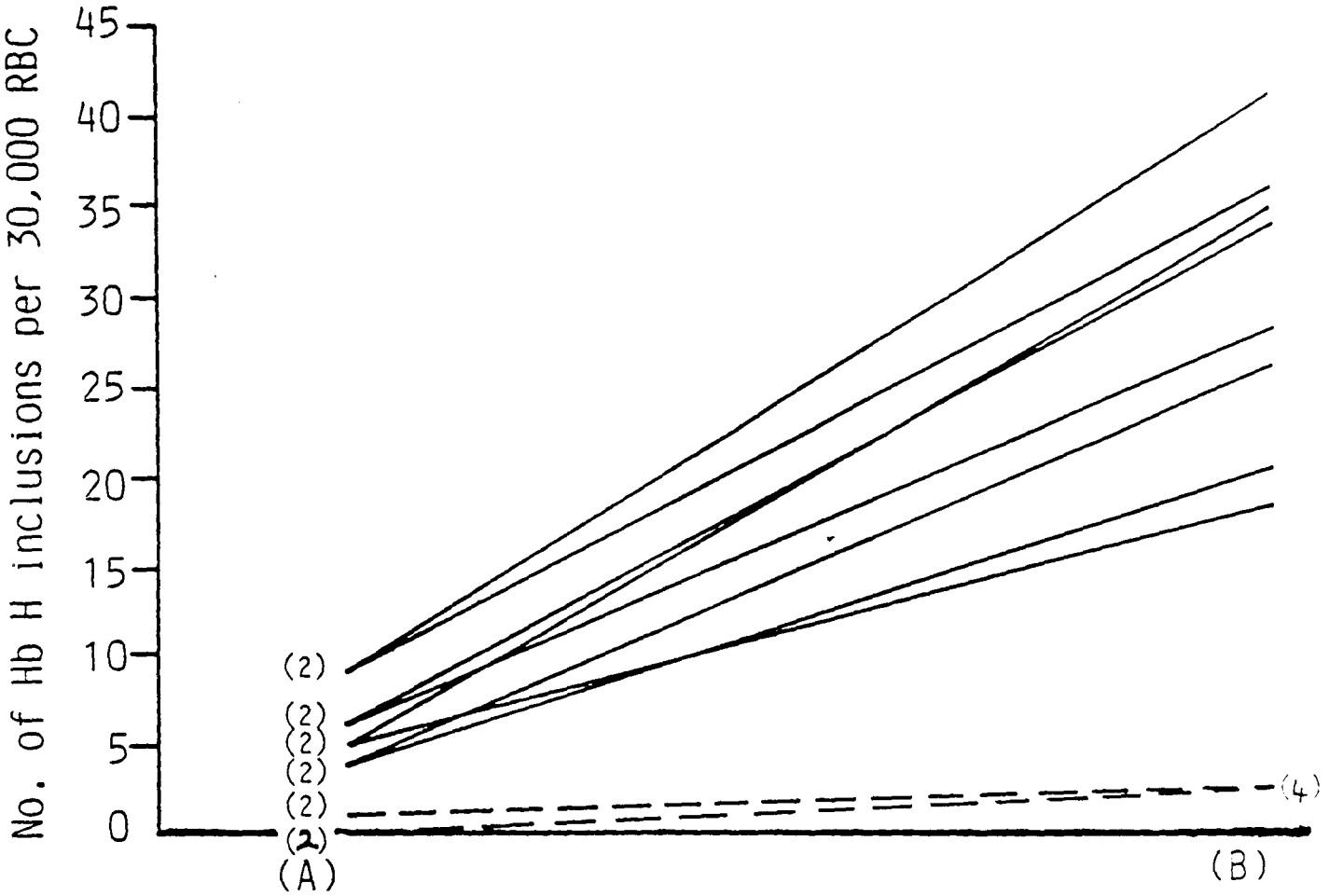

FIGURE 21: Comparison of HbH inclusions found in Indian (4) and Chinese (8) α thalassaemia trait paitents using 1% BCB at 37°C for one hour with (A) whole blood, and (B) centrifuged blood.

——— Chinese

— — — Indians

SEVERE FORM			MILD FORM	
Indians (n=6)	Chinese (n=11)	Negro (n=1)	Indians (n=8)	Chinese (n=1)
0.63	0.77	0.72	0.85	0.99
0.63	0.77		0.93	
0.66	0.84		0.97	
0.70	0.86		0.97	
0.71	0.87		0.98	
0.73	0.88		0.98	
	0.90		0.99	
	0.91		1.00	
	0.91			
	0.94			
	0.95			

TABLE XX: α/β globin chain ratios in Indian, Chinese and Negro α thalassaemia trait.

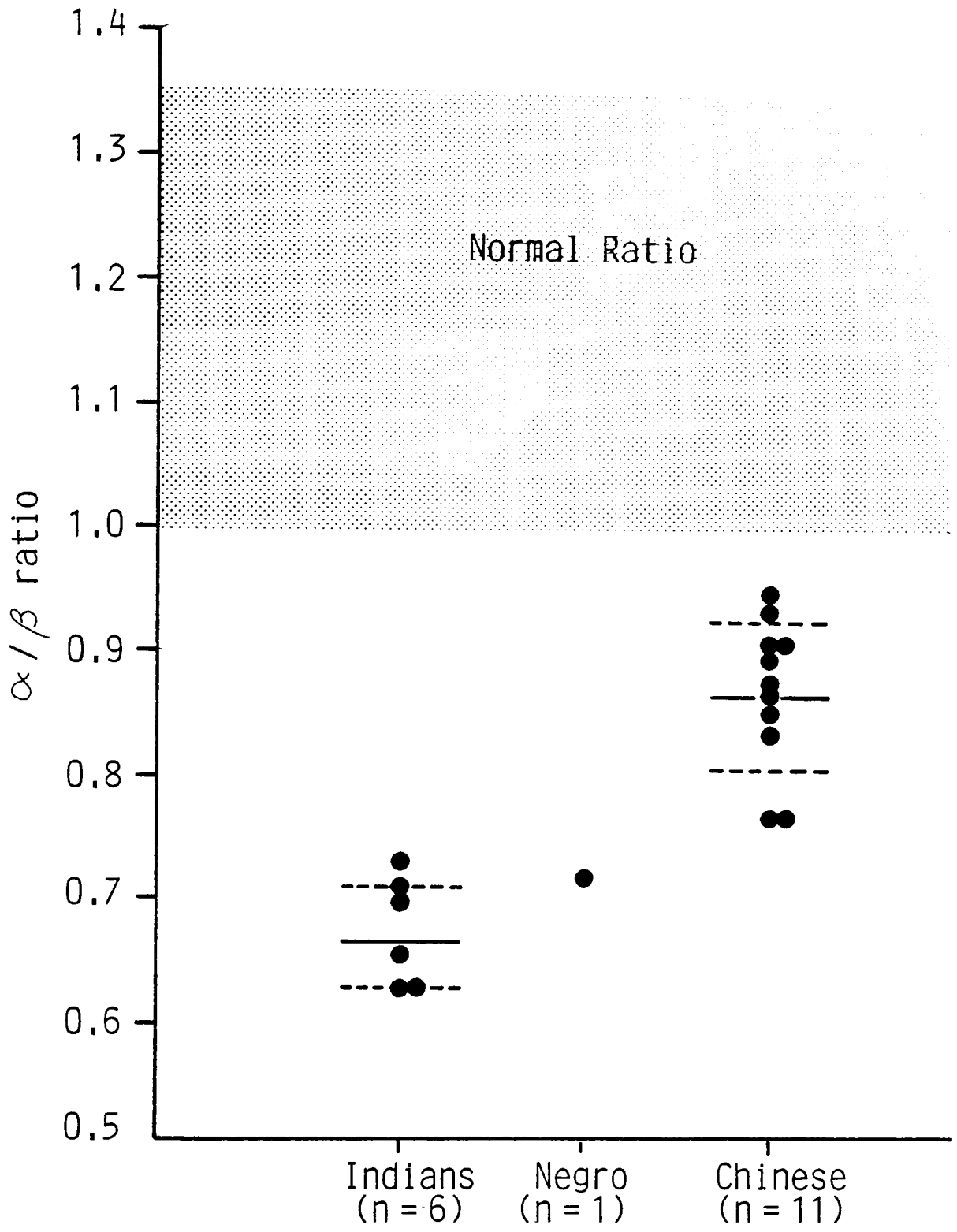

Fig. 22 α/β ratios in Indian, Negro and Chinese severe form of α thalassaemia trait.

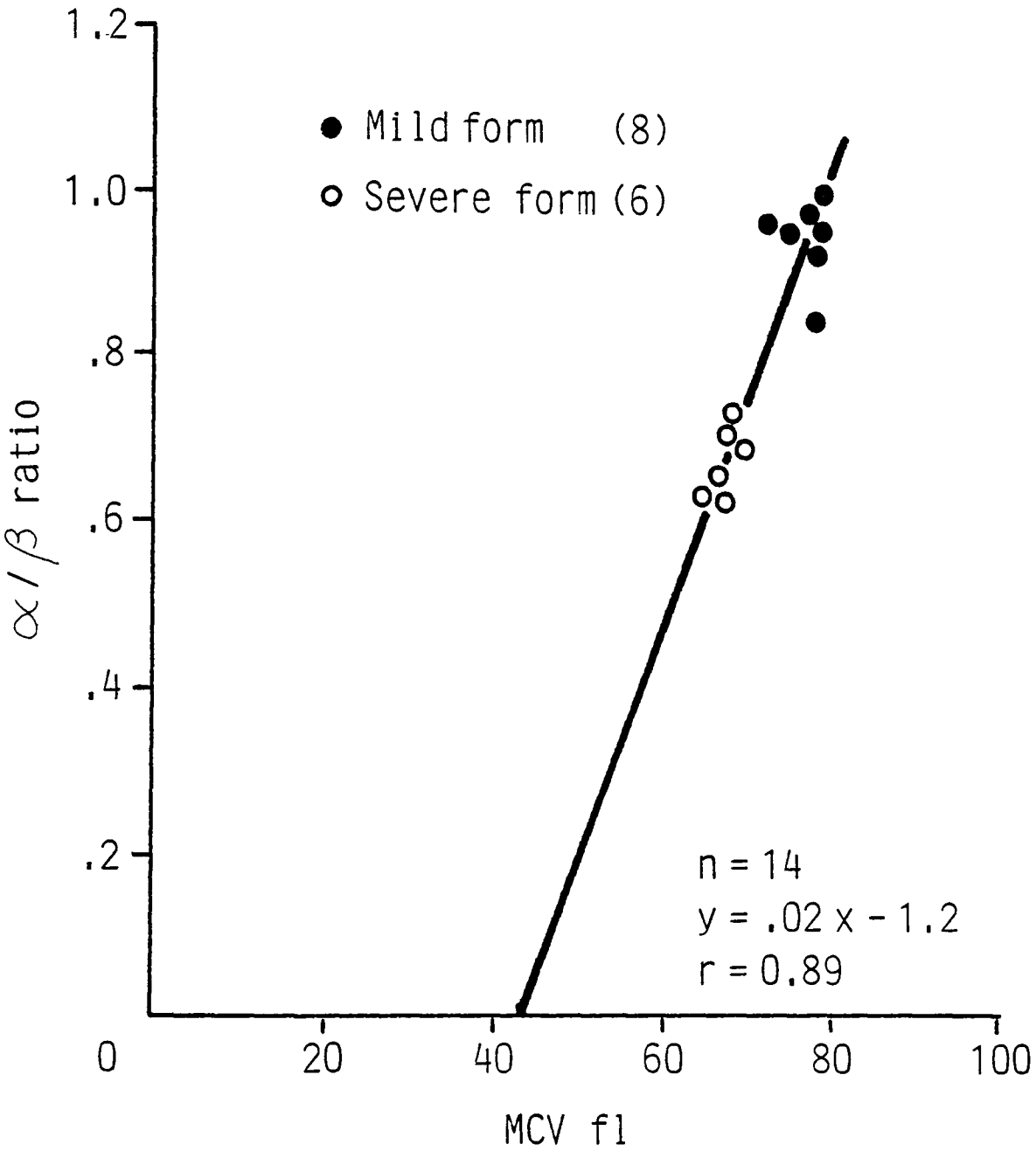

Fig. 23 Correlation between α/β ratios and MCV fl in Indian α thalassaemia traits.

	mild form (n=8)	severe form (n=6)	mild form vs severe form
MCV fl	≠ 77.9±1.8	≠ 67.9±0.9	$p < .001$**
MCH pg	≠ 25.9±0.6	≠ 21.9±0.5	$p < .001$**
HbH inclusion test	All negative	All positive	
α/β ratio	≠ 0.96±.04	≠ 0.67±0.04	$p < .001$**

TABLE XXI: Comparison of haematological studies between the mild and the severe forms of α thalassaemia trait in Indians.

≠ mean ± standard deviation

$p < .001$** highly significant

DISCUSSION

DISCUSSION

The routine laboratory tests used to diagnose α thalassaemia trait include (1) measurement of red cell indices i.e. MCV, MCH and MCHC, (2) examination of peripheral blood film and (3) demonstration of HbH inclusions.

Difficulties can arise in differential diagnosis:-

a. Discrimination of thalassaemia trait from the other causes of red cell microcytosis e.g. iron deficiency. This can be achieved by measuring the serum ferritin level (ferritin levels below 12 ng/ml indicating iron deficiency).

b. Differentiation of α thalassaemia trait from β thalassaemia trait by measurement of the HbA_2 level (HbA_2 levels greater than 3.3% indicating β thalassaemia trait).

1. RED CELL VOLUME DISTRIBUTION

In this study the red cell distribution width (RDW) has been examined to assess its ability to distinguish between α thalassaemia trait and iron deficiency. The RDW is a very rapidly performed test available on second generation fully automated blood cell counters such as the Coulter Counter Model S Plus IV. The other second generation fully automated blood cell counters such as the Technicon H1, the Ortho ELT8,and the Sysmex E5000 produce similar measurements of red cell anisocytosis but use different methods of obtaining the red cell volume distribution data and none is strictly comparable with

any of the others.

The red cells on the peripheral blood smear in iron deficiency are well known to show hypochromia and microcytosis (Price, 1933; Daland et al, 1946) and those two features have formed the corner stone of the morphological diagnosis. England et al (1976) in a study of subjects with latent iron deficiency showed that red cell anisocytosis was an early and sensitive measurement of this disorder. This observation received further support from the work of Bessman and Feinstein (1979). On the other hand Bessman and Feinstein (1979) showed that patients with α and β thalassaemia trait possessed red cells with normal or only slightly increased variation in size.

In this work the RDW values obtained in α thalassaemia trait and in iron deficiency patients agreed with those obtained by England et al (1976) and Bessman and Feinstein (1979). Nineteen out of twenty five patients with uncomplicated α thalassaemia trait had no anisocytosis (normal RDW) while the remainder had very slight anisocytosis (Figure 15). Two patients with combined α thalassaemia trait/iron deficiency showed no anisocytosis (normal RDW). However, three out of four patients with iron deficiency showed anisocytosis (elevated RDW). Overall, therefore, of 28 patients who either had very slight or no anisocytosis (RDW $\leq$ 15%) but with significant reduction in MCV and MCH, 96% were classified as α thalassaemia trait and 4% as iron deficiency.

According to Bessman and Feinstein (1979),RDW values are increased in iron deficiency and normal in α thalassaemia trait, since red cell abnormalities in thalassaemia trait are not progressive, while in iron deficiency, without iron replacement, progressive change in red cell morphology occurs. In evolving iron deficiency the red cells most recently produced are likely to be the smallest cells in the patient population resulting in an increased percentage of microcytes and an increased degree of anisocytosis (England et al, 1976). Such does not exist in α thalassaemia trait. Although the morphological abnormalities described in thalassaemia trait include variation in red blood cell size (Wintrobe, 1974) this anisocytosis really reflects the existence of leptocytosis or poikilocytosis (Bessman and Feinstein, 1979).

The results obtained in the present study indicate that the RDW may be used as a rapid and reliable test to distinguish between iron deficiency and α thalassaemia trait. Patients with an MCV $\leq$ 79 fl, and MCH $\leq$ 27 pg and a normal HbA_2 level are most likely to have α thalassaemia trait if in addition, they have an RDW value $\leq$ 15% and iron deficiency if the RDW value is greater.

2. HbA_2 LEVEL

The level of HbA_2 can be used to differentiate between α thalassaemia trait and β thalassaemia trait on most occasions being elevated in the latter. However,patients with heterozygous "silent" β thalassaemia trait

have normal HbA_2 levels (Weatherall and Clegg, 1981). Of the twenty-eight patients studied, one patient fell into this category. The diagnosis of β thalassaemia trait being confirmed on the basis of the α/β globin chain synthesis ratio. The remaining twenty-seven patients had normal HbA_2 levels $< 3.3\%$ and were diagnosed as α thalassaemia trait.

3. SERUM FERRITIN LEVEL

Patients with uncomplicated α thalassaemia trait had normal serum ferritin levels. This finding is consistent with reports that serum ferritin is normal in both α and β thalassaemia trait (Weatherall and Clegg, 1972; Hussein et al, 1976; Loria et al, 1978; Carrell and Lehmann, 1983). On the other hand, two patients with the severe form of α thalassaemia trait were found to have low serum ferritin levels indicating combined α thalassaemia trait/iron deficiency. This last finding supports the observation that while iron status in patients with thalassaemia trait is normal, these patients can become iron deficient for any reason (Hussein et al, 1976).

4. DEMONSTRATION OF HbH INCLUSION BODIES

4.1 Whole Blood Method

The effect of temperature and incubation time on the number of inclusion bodies (Gouttas et al, 1955) was examined in this work. From the results obtained [Table XII], it is obvious that at 37°C inclusion bodies start to appear in a relatively short time (after 10 minutes).

In contrast, at 25°C, inclusion bodies only start to appear after 2 hours incubation [Table XI]. The number of inclusion bodies increases progressively with increasing incubation time reaching a peak after 5 hours at 25°C. However at 37°C, "false positive" results were obtained at two hours' incubation in control subjects. This agreed with the observation of Dacie and Lewis (1984). Wickramasinghe et al (1981), however, reported that normal red cells showed little or no ultrastructural change at 2 hours incubation.

Other previous work on the HbH inclusion test did not report false positives as only patients seemed to be used with no control groups (Schwartz and Atwater, 1972; Walford and Deacon, 1976; Winichagoon et al, 1980).

In the whole blood method used in this study, the number of inclusions obtained at 37°C for one hours incubation was the same as that obtained by the routine method at 25°C for 24 hours. As a result the inclusion body preparations could be examined after one hour instead of 24 hours provided an incubation temperature of 37°C was used (Figure 16). At both temperatures (25°C and 37°C) with varying incubation times, the use of potassium iodide (Pereira, 1984), as a stimulant to increase the number of HbH inclusions formed, does not show an advantage [Tables XI, XII].

4.2 Centrifuged Blood Method

As reported previously (Jones et al, 1981; Maungsapaya et al, 1985), the number of HbH inclusion

bodies produced using a centrifugation method was greatly enhanced in patients with α thalassaemia trait. Unlike the findings of some other workers, (Walford and Deacon, 1976; Jones et al, 1981), centrifugation by the Wintrobe technique as described by Raven and Tooze (1973), proved to be a suitable technique to obtain sufficient reticulocyte-enrichment. The percentage of reticulocytes obtained by the Wintrobe technique in this study (about 10%) is nearly the same as that obtained by the microhaematocrit technique (13%) (Jones et al, 1981). An increase in the speed of centrifugation is known to augment the percentage of reticulocytes obtained. However the centrifugation speed of 6,000 rpm used in this study with the Wintrobe technique is actually half that used by the microhaematocrit method (12,000 rpm) which might indicate that, with equivalent speed, a higher percentage of reticulocytes could be obtained by the Wintrobe method. With the Wintrobe technique, it is quite easy to obtain a drop of centrifuged blood from the tube by a Pasteur pipette; with the microhaematocrit technique it is very difficult to expel the centrifuged blood from the area below and above the plasma/cell interface.

Raven and Tooze (1973) using centrifuged blood, recommended 3 hours incubation at 37°C. In the present study, control subjects started to show "false

positive" results for incubation of more than one hour, at 37°C. It can, therefore, be concluded that at 37°C incubation time must not exceed one hour.

5. GLOBIN CHAIN SYNTHESIS STUDIES

5.1 Normal individuals

The normal range of globin chain synthesis ratios in 20 control subjects in this study was found to be 1.06 - 1.26 in agreement with that obtained by Higgs et al (1979, 1980b). In early studies by Kan et al (1968) and Schwartz et al (1969), the normal range in 10 control subjects was 0.95 - 1.10. In 1975 Pootrakul et al demonstrated a range of 1.0 - 1.2 in 8 controls while Walford and Deacon (1976) observed a range of 0.9 - 1.26 in 20 controls. This shows the variation in normal range from one study to another.

5.2 α Thalassaemia Trait Patients

The mean α/β ratio in the severe form of α thalassaemia trait in this study (0.79±.10) was found to be comparable to that observed in the obligatory carriers of α thalassaemia 1 trait reported by Kan et al (1968), Schwartz et al (1969) [0.77±.05] and Pootrakul et al (1975) [0.78±0.03]. While in the mild form of α thalassaemia trait, the mean α/β ratio (0.96±0.04) encountered in the present study, is comparable with that reported for obligatory carriers

of α thalassaemia 2 trait (0.92±0.03) (Pootrakul et al, 1975a).

Although there are significant differences observed in this study between controls and mild forms, and mild and severe forms of α thalassaemia trait ($p < .001$), overlap between the two broad groupings does occur (Figure 19). Because of this overlap between the groups, it is very difficult to deal with individual cases solely on the basis of globin chain synthesis studies (Weatherall and Clegg, 1981). For example, the mild form patients with ratios of 0.99 and 1.0 are very comparable with several in the control group (Figure 19). These patients are, however, categorised as α thalassaemia trait because of a significant reduction in MCV and MCH and the presence of normal ferritin levels. Globin chain synthesis studies, therefore, can be used to separate the severe form of the trait from normals, but is unsuitable for use as a diagnostic test for all mild forms, since minor reduction in α globin chain synthesis is not always detected by this technique (Higgs et al, 1980b; Clegg and Weatherall, 1981; Higgs and Weatherall, 1983; Weatherall and Wainscoat, 1985). If real doubt exists regarding diagnosis of α thalassaemia trait, gene mapping techniques must be undertaken.

6. MCV AND MCH α THALASSAEMIA TRAIT PATIENTS

MCV and MCH ranges were used to separate mild and severe forms of α thalassaemia trait in this study (Figures 17 and 18). These results are consistent with those reported by Chanarin et al (1980) indicating their values as discriminators for the mild and the severe forms of this disorder.

7. HbH INCLUSIONS IN α THALASSAEMIA TRAIT PATIENTS

All patients in the present study with the mild form of α thalassaemia trait showed negative results on HbH inclusion testing, consistent with that reported in obligatory carriers of α thalassaemia 2 (Pornpatkul et al, 1976). The present study showed that the HbH inclusion test, even with centrifugation could not detect red cell inclusions in the mild form of the disorder, supporting the suggestion that HbH production is too low in those patients to be detected by this test (Pornpatkul et al, 1976). Therefore, the HbH inclusion test has no value in the diagnosis of the mild form of α thalassaemia trait. However, in the severe form most of the patients showed positive results by the routine and experimental whole blood methods (Pornpatkul et al, 1976; Chanarin et al, 1980). However by the

centrifugation method, all patients with severe forms give a positive result. This indicates that the demonstration of HbH inclusions by the centrifugation method can be used as a reliable test to detect the severe form of the disease.

According to McNeil (1971), the co-existence of iron deficiency and α thalassaemia trait may lead to "false negative" results in HbH testing. This report is inconsistent with the results obtained in the present study, where two patients with combined severe α thalassaemia trait/iron deficiency, demonstrated a positive HbH inclusion test.

8. HETEROGENEITY OF α THALASSAEMIA TRAIT IN DIFFERENT ETHNIC GROUPS

8.1 Comparison of α Thalassaemia Trait in Indians and Chinese

8.1.1. Indian patients

As suggested in the literature, the severe form of α thalasssaemia trait does not exist in the Indian community (Walford and Deacon, 1976). In this study both forms of the disorder have been identified, the severe form being demonstrated in six Indian patients. The two forms can be clearly separated by measurement of MCV, MCH values, HbH inclusions and α/β chain synthesis ratios [Table XXI]. Because of the small number of patients examined in this work, it was not easy to reach a definite conclusion about

the major form in the Indian community, but the ratio of mild to severe forms of 4:3 observed in this group may indicate the co-existence of the two forms with the same frequency.

8.1.2 Chinese

In the Chinese group, a ratio of mild to severe of 1:11 may indicate that the severe form is the most common form in Chinese.

8.1.3 Red cell measurement

A comparison of the severe form observed in the Indian and Chinese populations fails to show any significant difference in red cell indices [Table XIV].

8.1.4 Serum ferritin level

8.1.4.1 Controls

Although regardless of sex all serum ferritin levels fell within the normal range, values in males were significantly higher than in females.

8.1.4.2 Patients

Although there is no significant difference in serum ferritin levels between all male patients and male controls, comparison of Chinese male patients differs significantly from male controls ($p < .01$) and male Indians ($p < .001$)[Figure 24]. This may be due to the fact that all Chinese males have serum ferritin levels in the upper normal range, as an ethnic characteristic, while Indians have ferritin levels in the lower range. These differences do not exist in

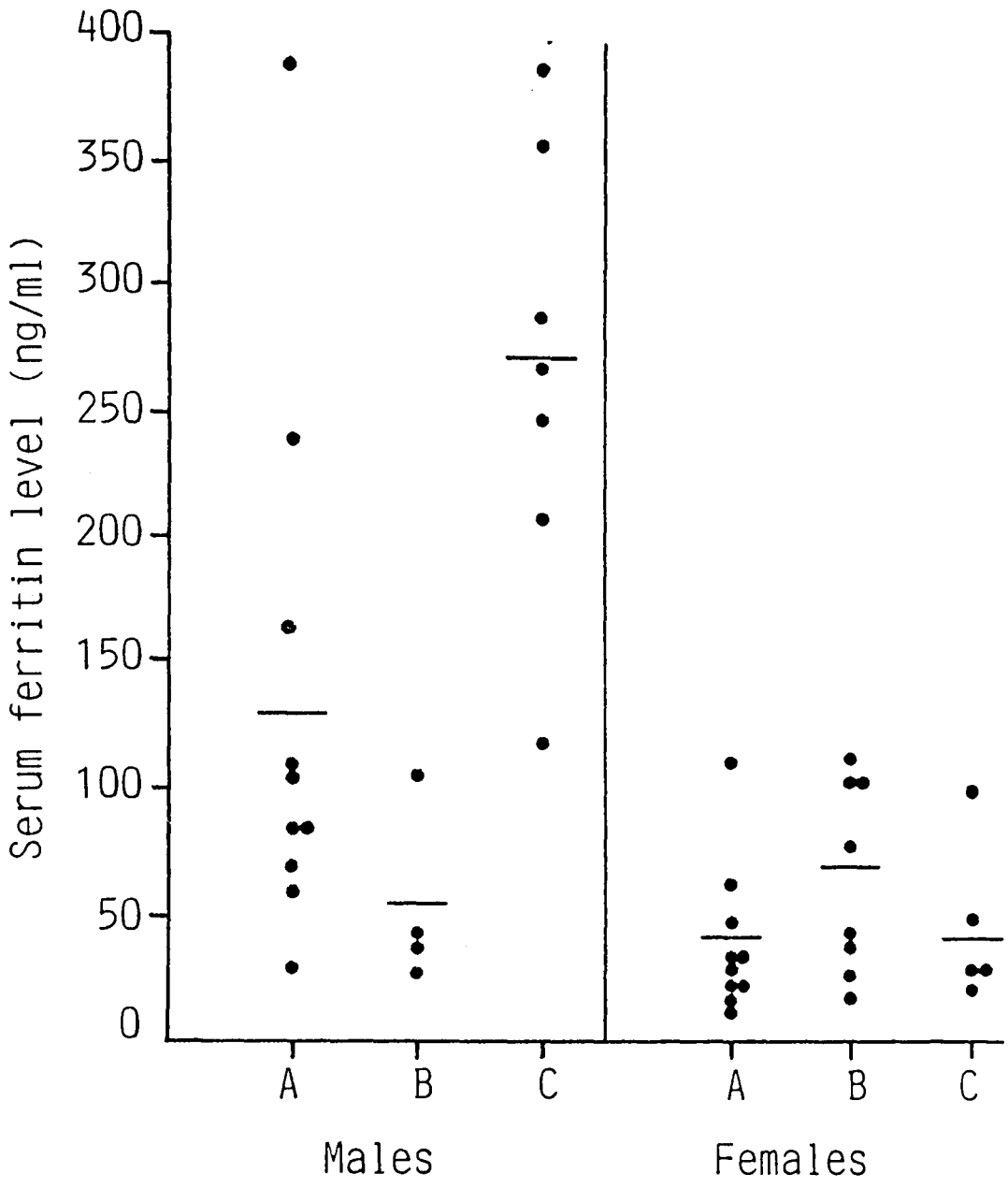

Fig. 24 Serum ferritin (ng/ml) levels in controls, Indian and Chinese α thalassaemia trait patients.

A Controls B Indians C Chinese

——— MEAN

females of the different groups. It should be noted that the sex difference in serum ferritin levels found in most ethnic groups did not occur in Indians.

8.1.5 HbH Inclusions

Although there is no significant difference in red cell measurements between Indian and Chinese with the severe form of the disorder, the numbers of inclusion bodies differ greatly between the two races regardless of method of HbH inclusion testing (Figure 20). When ranked in order of severity, HbH inclusions are more easily seen and more numerous in the Chinese group (mean 29/30,000 RBC) than in the Indian group (mean 2/30,000 RBC) using the centrifugation method. With centrifugation the Chinese group showed an increase of inclusion numbers about five times that obtained with whole blood, while the Indians showed only an increase of a factor of three. This indicates that with any improvement in the HbH inclusion test the ethnic origin of the patients examined should be taken into consideration.

8.1.6 Globin Chain Synthesis Studies

Despite the fact that the number of HbH inclusions in Indians is about 1/5th that observed in Chinese using the whole blood method and 1/15th using the centrifugation method, α/β ratios in the Indian severe form were more significantly reduced than that in the Chinese severe form. These differences in α/β ratios between the two racial groups are not consistent with

the observations of Walford and Deacon (1976).

The above comparisons between the severe forms of α thalassaemia trait in Indians and Chinese showed that there is no correlation between the number of HbH inclusions and the degree of reduction in α/β ratio. This might indicate that the precipitation of HbH(β_4) could be affected by other factors, related to a genetic variation between different populations, rather than the amount of HbH alone.

In this study the Chinese severe form showed a higher α/β ratio (0.87±0.06) than that reported previously by Kan et al (1968), Schwartz et al (1969) for mixed Chinese and Italian populations with α thalassaemia 1 (0.77±.05).

8.2 COMPARISON OF α THALASSAEMIA TRAIT IN INDIANS AND NEGROES

The only Negro patient studied had the severe form of α thalassaemia trait with red cell parameters, HbH inclusion bodies and globin chain synthesis ratio exactly as those of Indian origin. However, both Negro and Indians with the severe form ef α thalassaemia trait showed red cell indices and α/β ratios comparable with those observed previously for obligatory carriers of α thalassaemia 1 trait in Oriental and Mediterranean races (Kan et al, 1968; Schwartz et al, 1969). However, the number of inclusion bodies was very low or negative, unlike those of Oriental races (Walford and Deacon, 1976). This observation has been demonstrated previously in

Negroes with the severe form of the disorder (Higgs et al. 1979).

The absence of Hb Barts hydrops foetalis syndrome and the rare occurrence of HbH disease in both Indian (Weatherall and Clegg, 1981) and Negro populations (Higgs et al, 1979; Dozy et al, 1979) together with the similarities noted in the trait form suggests that the genetic disturbance in α thalassaemia in Indians is similar to that of Negroes. Based on these similarities the genetic studies on α thalassaemia trait in Negroes (Higgs et al, 1979; Dozy et al, 1979) can be used to propose the inheritance of α thalassaemia trait in Indians. Accordingly, the mild form "α thalassaemia 2" in Indians could be due to a deleted "or inactivated" α chain gene (-α/αα) as in Oriental α thalassaemia 2 (Walford and Deacon, 1976). While the severe form may be due to homozygous α thalassaemia 2 trait, characterised by a deletion of 2 α chain genes in trans (-α/-α), unlike that of Oriental (Chinese) heterozygous α thalassaemia 1, which is caused by a deletion of 2 α chain genes in Cis (--/αα) i.e. from the same chromosome. Therefore, the genotype --/αα is most likely to be rare or absent in Indians due to the complete absence of Hb Bart's hydrops foetalis syndrome (homozygous α thalassaemia 1) in this community.

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Appendice I

20 successive patients will a low ferritin (13 ng/ml), MCV $\leq$ 79 and Hb between 10 and 15 g/dl constitute the iron deficiency group in Figure 25. These are compared with the 25 uncomplicated α thalassaemia trait patients examined in this study.

The iron deficiency patients have a higher RDW (17.6 $\pm$ 2.1) compared with 13.9 $\pm$ 0.7 for those with α thalassaemia trait ($p < 0.001$).

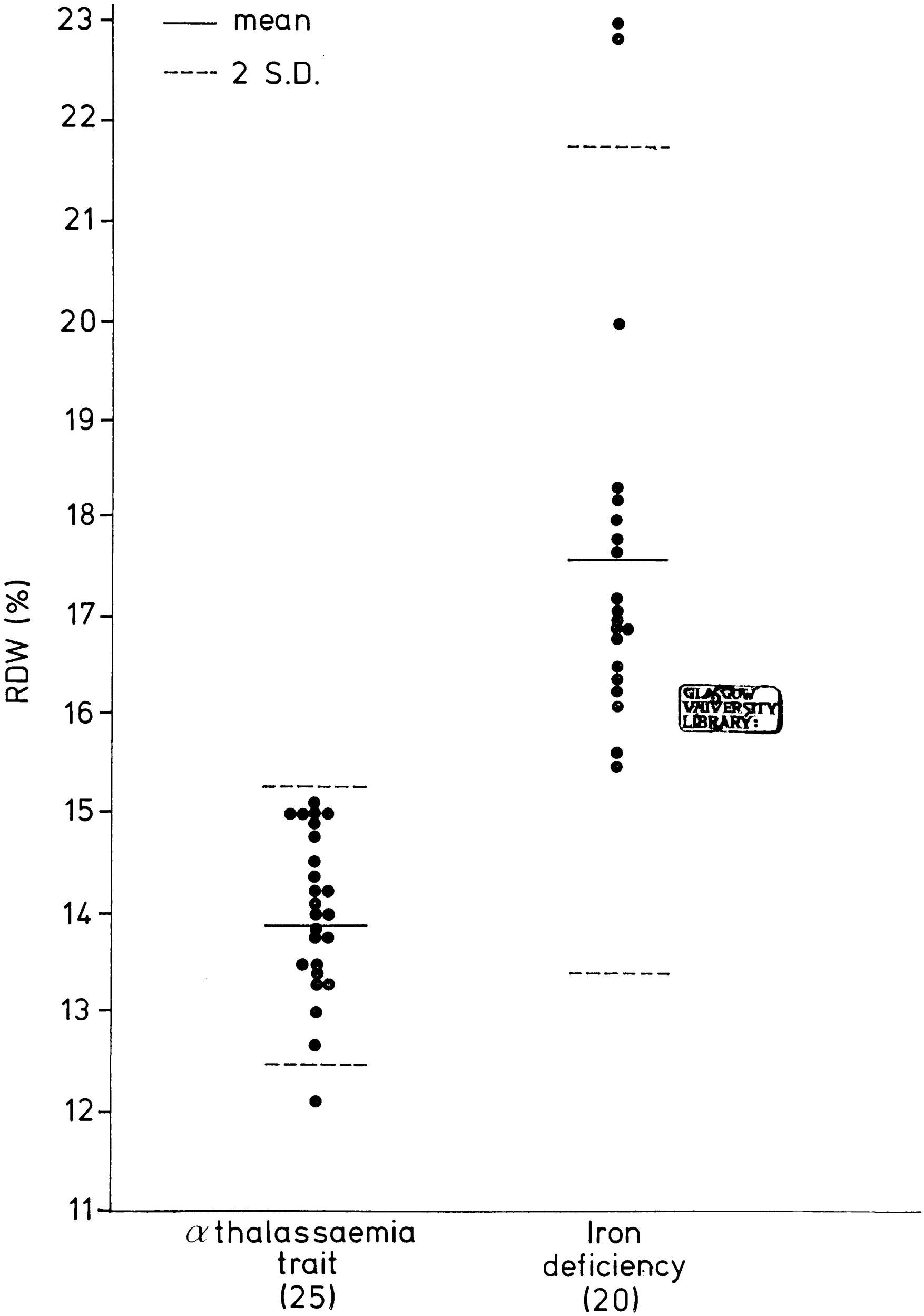

Fig.25 Comparison of RDW(%) in α thalassaemia trait and iron deficiency