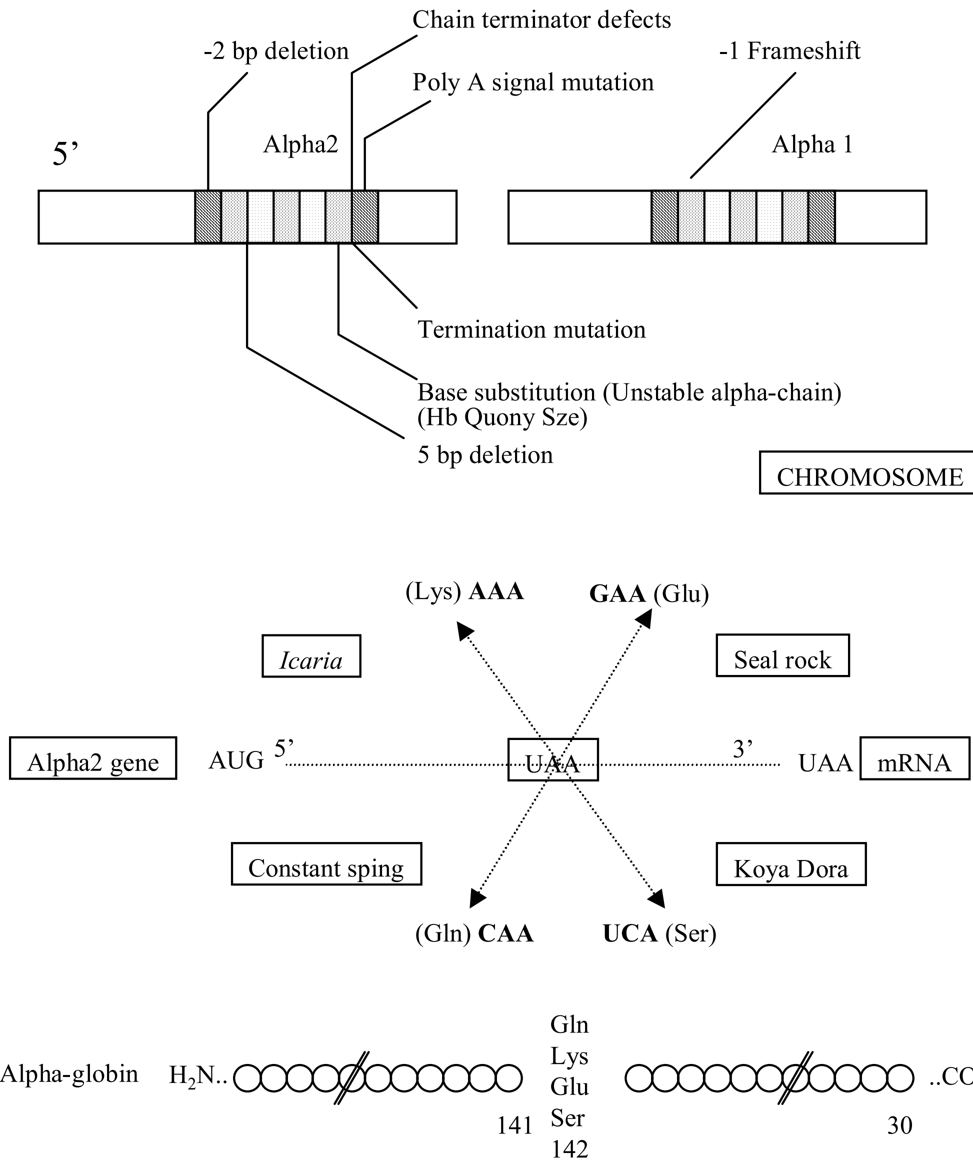
Figure 1.15: Non-Deletion Alpha-Thalassaemia



However, this extra length polypeptide is unstable, and is hence synthesized in smaller amount producing an α-thalassaemia phenotype. Other haemoglobins are produced as a result of mutation of the stop codon UAA to AAA (for Lys) or GAA (for Glu) or UCA (for Ser). These haemoglobins differ from each other in a single amino acid, have an extra 30 amino acid sequence at the carboxyl end and all are unstable.

(b) **The Molecular Genetics of the β-Thalassaemias**

Unlike α-thalassaemias, majority of the β-thalassaemias result from point mutations in the IVS, regulatory boxes, distant regulatory sequences, exons, and the polyadenylation sites. Some of these mutations result in the complete absence of β-globin gene expression and hence no β -globin chain synthesis and are referred to as the β°-thalassaemias, while others result in decrease synthesis of the β-globin chains and are referred to as the β^+-thalassaemias (Kazazian et al, 1990, 1983; Kazazian and Antonarakis, 1988; Kazazian & Boehm, 1988).

(I) Point mutations producing β-thalassaemias

(A) Mutations producing non-functional mRNA

Frameshift mutations, as a result of addition or deletion of one, two, four or five base pairs, have been reported in different populations. Majority of these mutations produce a β°-phenotype,

however, a few result in β^{+}-thalassaemias. Another group of mutations which produce non-functional mRNA are the nonsense mutations. A single base change in the exon, results in the conversion of a codon to a stop codon and hence a premature termination. All nonsense mutations produce a β°-thalassaemia phenotype. A number of nonsense mutations are known. These include the mutation in codons 39, 15, 21, 37, 43, 61, 35, 22 and others. Each of these is confined to a specific ethnic group i.e. codon 37 & 39 mutations are reported in Saudi Arabs, codon 17 in Chinese and so on.

(B) Transcriptional mutations

The transcriptional mutations occur upstream from the β-globin gene in the regions that are essential for control of β-globin gene expression and for accurate and efficient initiation of transcription at the promoter region. The mutations may occur in the conserved TATA box (28-31 bp upstream from the CAP), the CCAAT box (70 bp upstream from the CAP site) and in the CACACCC box (90 bp upstream from CAP site).

A large number of mutations, generally single base pair substitutions have been reported in the TATA and the CACACCC regulatory boxes, but none in the CCAAT box. Single point mutation in these boxes influences the rate of transcription and in

some cases the initiation of the transcription at the promoter region. The mutations do not abolish the transcription completely and hence cause a β^{+}-thalassaemia phenotypes. In vitro studies have confirmed that mutations in the promoter regions lead to hypofunction of the globin genes.

(C) RNA Processing Mutations

Studies at the nuclear level have confirmed that a number of β-thalassaemia patients have defective RNA processing. Sequence analysis and in-vitro functional characterization of β-thalassaemia mutations have confirmed these findings. A tremendous amount of knowledge has been acquired about the sequences involved in specific, selective and efficient RNA splicing. The RNA processing mutations include (a) the splice junction changes, (b) consensus changes, (c) internal IVS changes, and (d) coding region substitutions affecting RNA processing.

(a) **The Splice Junction Changes**

The exon-intron splice boundaries have sequences which are critical for RNA splicing. The first two nucleotides at both the 5' and the 3' end of the intron are essential for splicing. In almost all eukaryotic genes there is a GT sequence at the 5' end and an AG sequence at the 3' end of every intron. Mutations are known which result in substitution in the nucleotides at the splice junction and produce complete absence of mRNA

splicing and hence β°-thalassaemia phenotype. In addition, deletions in the 3' or 5' splicing junctions are reported which also result in β°-thalassaemia allele.

(b) **Consensus Changes**

The consensus regions are other key sequences at the exon-intron splice junctions which are necessary for splicing of the mRNA. For donor site (i.e. 5' end of introns) the consensus regions encompass the last 3 nucleotides of the exons and the first 6 nucleotides of the intron. For the acceptor sites (i.e. 3' end of introns) they are the last 10 nucleotides of the introns and the first nucleotides of the exons. At the donor site of intron 1 only over 7 mutations have been reported, occurring at -3, -1, +5 and +6 bp from the splice site. These mutations are single base substitutions and lead to a β^{+}-thalassaemia phenotype. However, the most diverse clinical consequences are observed in patients with +5 mutation, while the +6 mutation produces a mild clinical picture.

At the acceptor site of both intron 1 & 2, a single point mutation has been identified at position -3 bp from the splice junction. At the acceptor β+-position, a substitution of A for a C, 8 bp 5’ to the splice site in a mild β+-thalassaemia phenotype.

(c) **Internal IVS Changes**

Certain mutations which may occur within the introns or even the exons result in the creation of new consensus splice site sequence hence

producing donor or acceptor sites, where splicing occur. Three mutations have been reported in IVS2, approximately 100, 150 and 200 bp from the acceptor site. The first two decrease the production of normally spliced mRNA, while the last one results in complete absence of mRNA splicing and produces β°-thalassaemia phenotype.

Another interesting set of mutations are those in exons I which activate cryptic splice site in codons 24-27 leading to β^{+}-thalassaemia phenotype. A cryptic splice site has a sequence which resembles the consensus sequences for a splice site but in normal circumstances it is not used. However, mutation in a cryptic site can change it so that it resembles the normal splice site more closely and hence is used for splicing but at a lower efficiency, thus producing β^{+}-thalassaemia.

(d) **Coding Region Substitutions Affecting RNA Processing**

Several substitution mutations within the exons result in the substitution of amino acid in the β-globin gene to produce a variant haemoglobin and at the same time the rate or expression of the mRNA is decreased leading to thalassaemia phenotype. One such mutation is a G→A change in codon 26 of β-globin chain, which substitute a lysine for a glutamic acid and produces Hb E. The Hb E production is deficient and is referred to as Hb E β-thalassaemia phenotype.

(D) Polyadenylation Mutations

At the 3' end of the mRNA the polyadenylation signal i.e. AATAAA sequence

about, 10-20 bp 5' to the site of cleavage of mRNA transcript is essential for the addition of the 3'-poly A tail in the mRNA.

Several substitution mutations and five nucleotides deletion, in the polyadenylation signal have been reported in this site which result in inappropriate cleavage of the mRNA. The elongated mRNA so produced has lower stability and hence leads to β^+-thalassaemia.

(E) Cap Site Mutations

The β^+-thalassaemia phenotype is encountered in patients who have a mutation in the CAP site. This site is the first nucleotide in the transcription site and the purine base present is methylated (known as the CAP). Normally the A residue is seen at this site, but other nucleotides may be substituted which result in a reduced rate of transcription and hence mild β^+-thalassaemia.

(F) Mutations Producing Unstable Globins

Several of the haemoglobin variants produced as a result of substitution mutations in the exons of the globin genes are unstable and exhibit symptoms of mild β-thalassaemia. In this type of mutations the rate of transcription and translation is normal, however, the globin chain synthesized has lower stability.

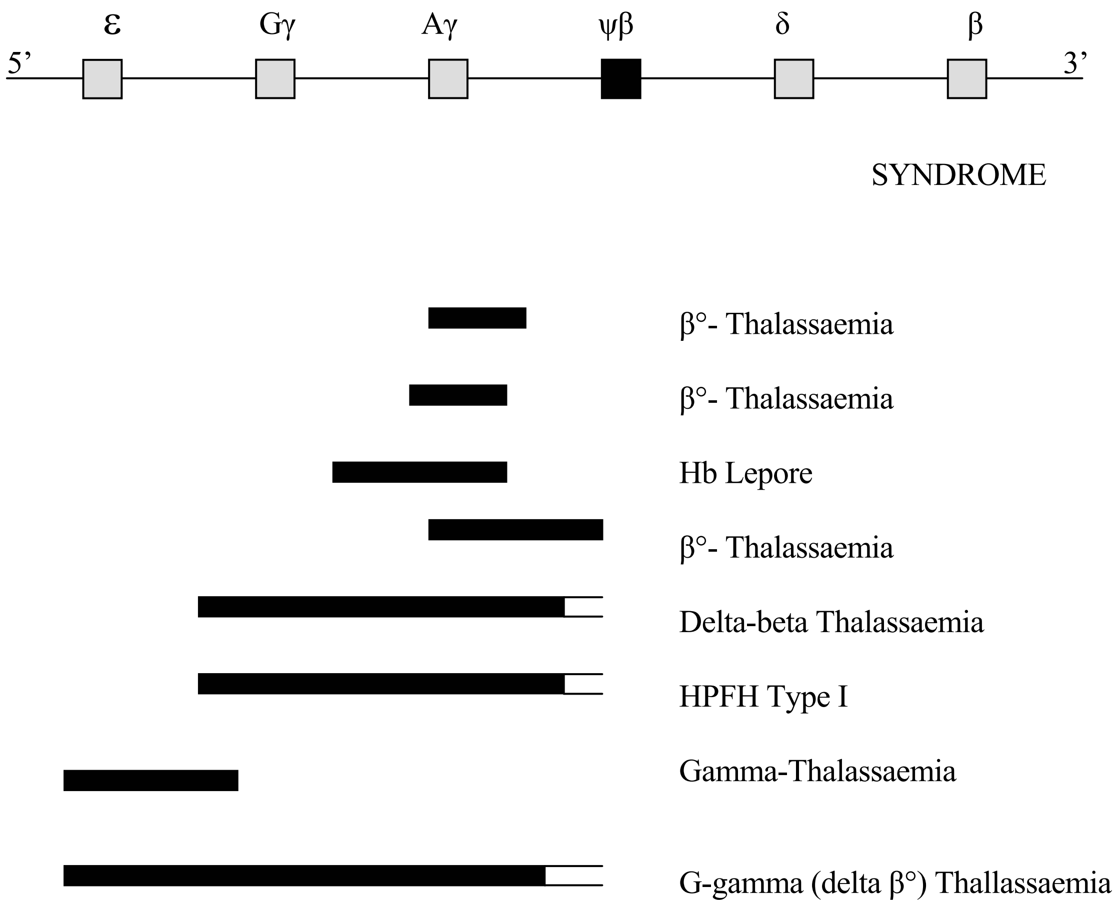
(G) Translation Mutations

Over one-third of the mutations discussed above affect the translation of the mRNA into globin chains and hence may be referred to as translation mutations. These include the nonsense mutations, the frameshift mutations and substitution in the initiation codon for translation. Generally these mutations have a more severe effect and result in β°-thalassaemia phenotypes.

(II) **β-Thalassaemia due to gene deletions** (Figure 1.16)

Beta-thalassaemia due to partial or complete deletion of the β-globin gene cluster are reported in different populations though at a lower frequency compared to the β-thalassaemias resulting from point mutations. One deletion affects only the β-globin gene

Figure 1.16: Deletions in the β-Globin Gene Cluster



and produces β-thalassaemia. Among the Asians almost 30% of the β-thalassaemias are due to deletion of 619 bp from the IVS-2, exon 3 and sequences 3' of the β-globin gene. Other types of deletions 5' to the β-globin gene are known which leave the β-globin gene intact yet silence its expression. It is shown that these mutations eliminate the DNA required for activating transcription of all genes in the β-globin gene cluster i.e. the locus control region.

Depending upon the extent of the deletion, the thalassaemia type may be β°, (δβ) (γδβ)° and (εγδβ) thalassaemias. A condition in which γ-globin chain persists is known as Hereditary Persistent of Fetal Haemoglobin (HPFH). In this state, the δ, β and some times ψβ-globin genes are deleted and the γ-globin gene remains fully functional. The (δβ°) thalassaemia are also accompanied excessive γ-globin chain production. However, the amount of γ chains are insufficient to make up for the lack of δ and β-globin production.

These conditions are rare and are always associated with β°-thalassaemia phenotype. However, the HPFH are associated with a mild clinical picture due to compensation of Hb A by Hb F.

Unknown Mutations

A few cases of β-thalassaemia have been reported which do not appear to have any mutation in the β-globin cluster. It is suggested that the mutations in these cases occur in another gene located elsewhere in the genome but which plays a role in the β-globin gene expression. So far the exact mutation in these β-thalassaemia patients have not been identified and so these are referred to as β-thalassaemia due to unknown mutations. Further

studies are continuing to identify the molecular basis of these mutations.

1.4. Molecular Pathophysiology of the Haemoglobin Disorders

In every normal healthy individual a steady state is maintained within the body system in the relationship of the red blood cells (erythrocytes) to the lean body mass and to the haemoglobin level. Under normal circumstances an adult synthesizes almost 80 mg haemoglobin per Kg lean body weight per day and on average almost 1% of the lean body mass consists of haemoglobin. In growing infants the haemoglobin produced is more than that in adults but is never more than 120 mg/kg/day.

The body maintains the amount of haemoglobin necessary for sufficient oxygen supply to the tissues, mean corpuscular volume (MCV) and the haemoglobin concentration in the red cells more or less constant. Haematological and acquired states resulting in reduced haemoglobin level are associated with a compensatory mechanism whereby the body increases the synthesis of haemoglobin in the bone marrow, provided all requirements are sufficiently fulfilled.

Several of the haemoglobin structural variants are not associated with any significant haematological or biochemical abnormality and hence are of no clinical significance. Others, on the other hand, are associated with changes in the structure, solubility and functions of the haemoglobin and hence produce a clinical disorder of varying degree of severity. The unstable haemoglobin variants precipitate or crystalize in the red cells leading to cell membrane damage and shortened life span, others have altered oxygen affinity and may lead to cyanosis and polycythaemia, still others may be associated with decreased production of the haemoglobin variant and hence a hypochromic state.

The pathophysiology of the thalassaemias is significantly variable depending on the extent of globin chain produced. In the homozygous α°- and β°-thalassaemias the α- or β-globin chain production is completely inhibited and hence severe, life threatening states result, while in the α+ and β+-thalassaemias, since some α- or β-globin chains are produced the associated pathophysiology ranges from a very mild to moderate clinical presentation (Weatherall & Clegg, 1981).

The molecular pathophysiology of the haemoglobin structural and biosynthetic disorders is presented in the following section.

1.4.1 Pathophysiology of haemoglobin structural variants

The four major clinical abnormalities associated with haemoglobin variants are (a) haemolysis, (b) cyanosis, (c) polycythaemia, and (d) hypochromic anaemia (Lehmann & Huntsman, 1966; Livingstone, 1967; Weatherall et al, 1982; Hardisty & Weatherall, 1982; Edwards et al, 1991; Williams et al, 1983).

The precipitation or crystallization of the unstable or insoluble haemoglobin variant in the red cells may lead to secondary damage to the red cell membrane and hence shortened life span of the red cells, leading to haemolytic anaemia. The most commonly encountered haemoglobin variants which result in excessive haemolysis are Hb S and Hb C. Several unstable haemoglobin variants also produce haemolysis, however, they are rare in most populations of the world.

Cyanosis results from decreased oxygen transport to the tissues. This state is associated with haemoglobin M (Methaemoglobins) in which the haem iron is oxidized from ferrous to ferric state and is unable to bind oxygen thus these haemoglobins have a

significantly reduced affinity for oxygen. In these states the oxygen in the lungs is unable to oxygenate completely the haemoglobin variants and thus oxygen transport to tissues is severely reduced.

Similar effect is also seen in patient who have haemoglobin variants with increased oxygen affinity. In these cases, since the haemoglobin bind oxygen with a significantly higher affinity than normal, it does not release the oxygen to the tissues thus leading to a state of hypoxia and cyanosis. The body compensates for this by excessive production of the red blood cells and hence results in polycythaemia.

Finally, some haemoglobin variants are produced in lower amounts (i.e. Hb E), or the globin chains are prematurely terminated (i.e. the chain termination mutants and abnormal crossing-over mutants). This leads to a hypochromic state since the amount of haemoglobin in the red cell is low.

The molecular pathophysiology of haemoglobin S, the most frequent haemoglobin variant is discussed in the following section in detail.

1.4.1.1 Molecular Pathophysiology of Sickle Cell Disease (SCD)

The sickle cell haemoglobin (Hb S) results from a single point mutation which converts the codon 6 in β-globin gene from GAG to GTG and hence a signle amino acid change in the β-globin chain (Figure 1.17). The homozygous Hb S state referred to as sickle cell disease (SCD) is associated with a chronic state of haemolytic anaemia with a varying degree of other complications. The pathophysiological mechanisms involved in the haemolytic episodes and other complications are presented in Figure 1.18.

The Hb S has a lower solubility compared to the Hb A in the deoxygenated

state. In the lungs where the haemoglobin is oxygenated the Hb S is soluble and the red cells exist in the normal biconcave shape. When the red cells deliver their oxygen to the

Figure 1.17: The Sickle Cell (Hb S) Mutation

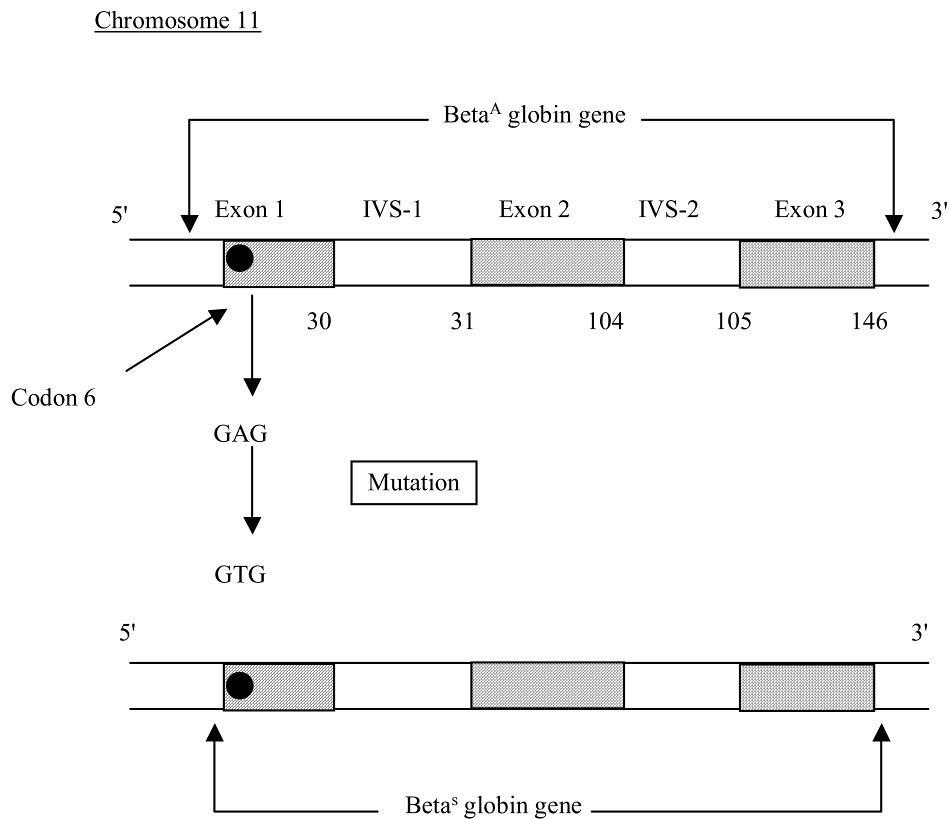
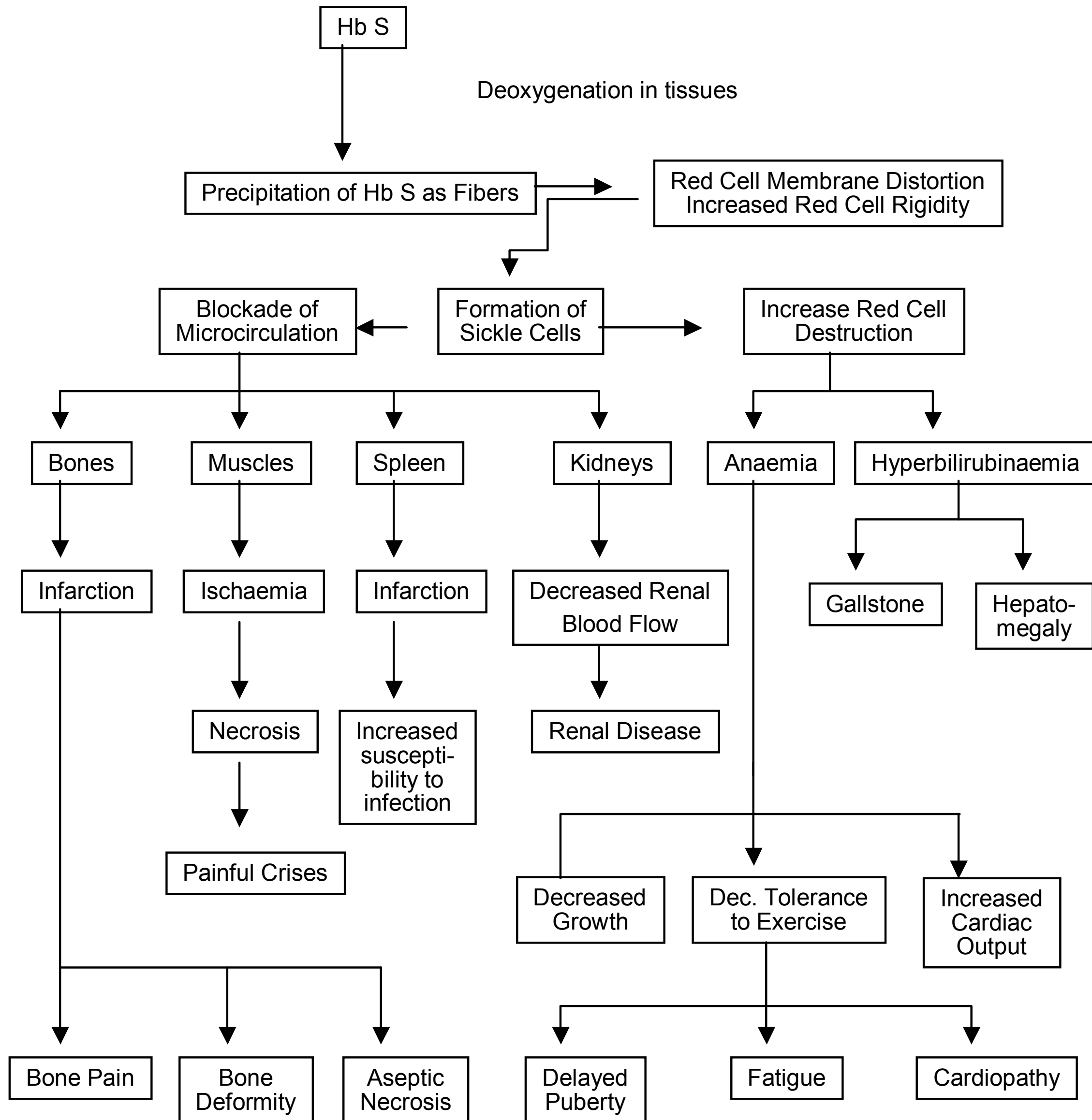


Figure 1.18: Pathophysiology of Hb S



tissues, the Hb S becomes insoluble and precipitates forming a gel-like substance which eventually becomes crystalline forming long fibers and distorts the red cell membrane. This decreased solubility results from the substitution of a hydrophobic amino acid (i.e. valine) on the haemoglobin surface in place of the acidic amino acid (i.e. glutamic acid). The valinyl residues in the different Hb S molecules attract each other due to hydrophobic interactions and form bundles of long fibers. Electron microscopy has shown that these bundles are of long straight fibers whose axis is parallel to the long axis of the sickled red cells. The approximate diameter of the fibers is 200°A with spacing between them ranging from 180 to 240°A. The valine at position 6 is believed to enhance and stabilize a mechanism which leads to lower the solubility of the deoxygenated haemoglobin (Harris, 1950; Stetson, 1966; White, 1968; Finch et al, 1973; Edelstein et al, 1973).

Initially the reoxygenation of the Hb S results in solubilization of the fibers in the lungs where the red cells return to their original shape. However, with continuous reversion between the normal and sickled shape the red cell become fragile and changes also occur in the red cell membrane cytoskeleton, thus leading to membrane damage and excessive haemolysis. Some of the cells become irreversibly sickled and there are irreversible changes in the red cell membrane, which are often responsible for infarcts and sickle cell crises. The irreversibly sickled cells are remarkably rigid, with extremely short life span and contribute to the haemolytic anaemia of sickle cell disease. Thus the basic deficit in sickle cell anaemia and other sickling disorders is the production of rigid deformed sickled red cells, known as irreversibly sickled cells (ISC).

The increased red cell destruction leads to anaemia, hyperbilirubinaemia and the heart has to function at a significantly increased rate. The patients have decreased tolerance to exercise and thus get fatigued easily. The growth is retarded due to the decreased supply of the nutrients to the tissues and excessive utilization of the essential nutrients and this is followed by delayed puberty. The hyperbilirubinaemia leads to jaundice and increased activity of the liver causing hepatomegaly. Gall stones are more frequent in these patients due to the excessive serum bilirubin content.

The rigid sickled cells have a tendency to aggregate, particularly in the micro-circulation. This causes stasis and local hypoxia and further deoxygenation and sickling of the red cell. An area of vascular occlusion is formed and is amplified by a local vicious circle of occlusion, stasis, sickling and further occlusion (Diggs, 1965; Serjeant, 1988; Konotey-Ahulu, 1974).

Tissue damage is a frequent finding in the patients with sickle cell disease. This is due to the blockage of the micro-circulation which leads to different forms of crises. Painful bone crises occurs due to decrease blood flow to the bones leading to infarction followed by aseptic necrosis. Since the bones do not obtain the normal content of the nutrients, deformity of the weight bearing bones are frequent. Gradual changes in the bones (e.g. facial) occur due to malformation caused by hypertrophic bone marrow.

The spleen becomes blocked and as a result of continuous uptake of the sickled red cells, splenomegaly results initially. Soon the spleen is reduced to a fibrotic tissue which is unable to perform its functions. This is a major cause of increase susceptibility to infections in patients suffering from sickle cell disease.

In the muscles, blockage in microcirculation produces ischaemia, and necrosis. This causes the painful crises of sickle cell disease. Depending on the tissues involved the location of the painful crises varies. Abdominal crises are a frequent finding.

Blockage of the microcirculation in the kidneys results in reduced blood flow to the kidney, hypoxia, hence renal damage. In the initial stages the renal capacity to concentrate urine is reduced and is accompanied by polyuria. Spells of dehydration may occur leading to a increased risk of infarction. In young age these renal defects are reversible, however, in the adults the damage is irreversible due to loss of nephrons. Hydration is thus necessary since prolonged periods of fluid abstinence may produce dehydration and hence crises.

Aplastic crises is frequent in children and is caused by viral infections. Any factor which causes even a temporary dysfunction of the bone marrow leads to aplastic crises. Regular supply of vitamin B_{12} and folate are necessary to provide for the excessive rate of blood production.

Another abnormality in patients with sickling disorder is a lower oxygen affinity of Hb S. The oxygen dissociation curve of Hb S shifts to the right and hence the oxygen delivery to the tissues is rapid and so is the sickling phenomenon following deoxygenation.

Several factors determine the formation of vascular blocks and hence influence the clinical severity of the sickling disorders. The factors which precipitate crises include infections, dehydration, exercise, fevers, cold and decrease in the oxygen saturation of blood. Other coexisting genetic and environmental factors may also influence the frequency of development of the crises in the sickle cell disease patients.

In older patients retinal detachment, cardiac complications, chronic leg ulcers,

pulmonary infarction and aseptic necrosis of the head of the femur are frequently encountered, particularly in patients with a severe disease.

In patients with sickle cell trait (Hb AS) generally, no major abnormality is noticed. Renal concentration capacity is often impaired, but haematuria is not frequent. Some symptoms only occur in conditions which result in decrease oxygen concentration of air e.g. at high altitude, or during anaesthesia (Figure 1.19).

Other sickling disorders i.e. Hb SC, Hb SD, Hb SO-Arab and Hb Sβ°-thalassaemia are also associated with the same molecular pathophysiology, however, the conditions are generally less severe compared to sickle cell anaemia (Hb SS), though often Hb Sβ°-thalassaemia patients suffer from a severe disease, similar to Hb SS.

1.4.2 Molecular Pathophysiology of the Thalassaemia

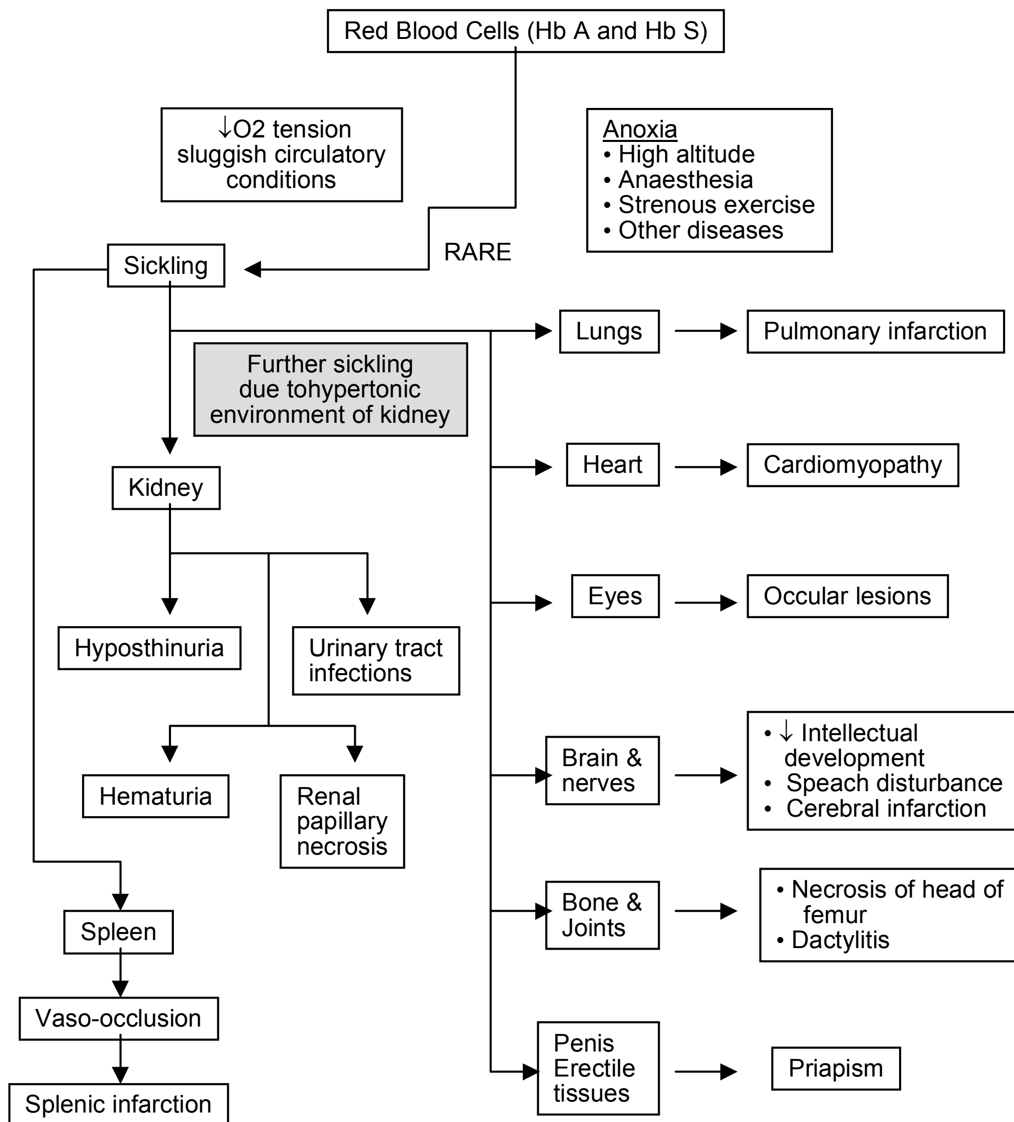
The pathophysiological mechanisms involved in the morbidity associated with the thalassaemias are different from those associated with structural haemoglobin abnormalities. The defect in the thalassaemia is a decreased production of one or more of the globin chains of haemoglobin, thus resulting in a decrease in the amount of haemoglobin formed in the red cell. In addition, the un-utilized excessive chains precipitate and hence lead to damage of the red cells. The following section presents the pathophysiology of the α- and β-Thalassaemias.

1.4.2.1 The β-thalassaemias

Patients homozygous to β+ or β°-thalassaemia suffer from severe anaemia which is hypochromic and microcytic. The basic defect in the β-thalassaemia is a decreased production of β-globin chains, thus altering the α/β globin chain ratio. The severity of the

disease depends on the extent of β-globin chains synthesized. In β + or β°-thalassaemia (homozygous) the β-globin chain synthesis is completely absent (in β°) or significantly

Figure 1.19: Pathophysiology of complications in Hb S heterozygotes



reduced (in β+) and the disease is significantly severe. The deficiency of the β-chains are partially compensated for by γ and δ globin chain synthesis and both Hb F and Hb A_2 may be elevated to a varying degree. However, the excess α-chains form tetramers and precipitate in the red cells forming inclusion bodies. The red cells become vulnerable and life span is reduced leading to excessive haemolysis. The abnormal red cells are sequestrated in the spleen and splenomegaly results. Elevated level of bilirubin due to increased destruction of red cells results in hyperbilirubinaemia and jaundice (Weatherall & Clegg, 1981).

The state of chronic anaemia produces several effects. There is decreased tolerance to exercise and the patient feels fatigued. The susceptibility to infections in increased and the children suffer frequently from severe infections and fever. Growth is delayed and puberty is affected both due to the chronic anaemia and due to endocrine abnormalities. To compensate for the anaemic state the bone marrow becomes hypertrophic and marrow hyperplasia leads to skeletal defects. The hypertrophy of the facial bones, leads to mongoloid features. The thinning of the cortex followed by compensatory subperiosteal thickening occurs and may cause fractures. Malformation e.g. of the vertebral spine, are frequent. Characteristic changes occur in the distal portion of the skeleton (hands) but these usually disappear during the retarded puberty.

As a compensatory mechanism chronic anaemia leads to an increase in the amount of iron absorption from the intestine, which is stored in the reticulo-endothelial system. The iron accumulation becomes more marked when repeated blood transfusions are given. The iron overload is a major cause of damage to several organs. The heart, liver and

endocrine glands are damaged due to deposition of the iron and lead to cardiopathy, liver abnormalities and decrease hormone synthesis. The most frequent of these are hypothyroidism and decrease insulin production due to destruction of β-cells of islets of Langerham leading to diabetes mellitus in some patients. Growth hormone abnormalities have been reported in some studies and result in delayed growth, while hypogonadism may play a role in delayed puberty.

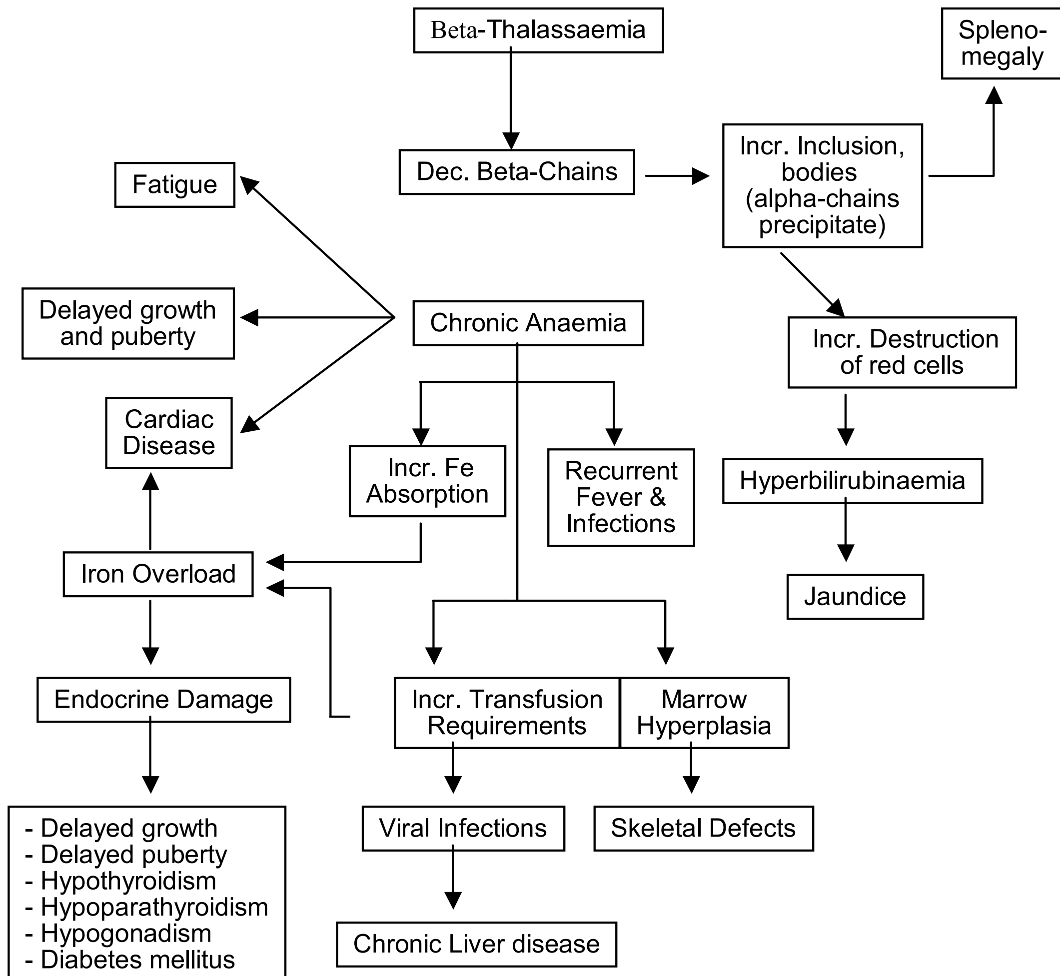
Blood transfusions are regularly required by the patients with homozygous form of β-thalassaemia associated with severe anaemias. Complications of blood transfusion regimes i.e. viral infections and iron overload may lead to tissue damage. Iron overload must be treated as early as possible to avoid tissue damage. The iron chelators such as desferroxamine, if used regularlydecreases the complications associated with the iron overload. On the other hand, if the patients are not transfused the typical clinical picture of homozygous β-thalassaemia develops. The molecular pathophysiology of β-thalassaemia is presented in Figure 1.20.

In the earlier days with poor nutritional status, poor hygiene and insufficient management protocols, the life span of patients with homozygous form of β-thalassaemia were significantly low and very few patients reached adulthood. However, with the recent improvement in the management protocols the patients do not suffer from severe complications and are able to lead a more or less normal life.

The heterozygous states of β-thalassaemias are generally associated with mild to moderate hypochromic-microcytic anaemia, with normal or elevated levels of Hb A_2 and often slightly elevated or normal Hb F. Other complications may or may not be present

depending on the severity of the anaemic state.

Figure 1.20: Pathophysiology of Beta-Thalassaemia



1.4.2.2 The α-Thalassaemias

The α-thalassaemic state particularly in the heterozygotes is significantly different in severity and symptoms. However, the symptoms depend on the extent of α-chain synthesized.

In complete absence of α-chain synthesis (--/--), hydrops fetalis results. This is a state in which the child is born dead or dies soon after birth as there are no α chains for the production of normal haemoglobins. The birth weight is significantly low, Hb Barts (γ4), H (β4) and Hb-Portland, may be seen on electrophoresis. Haematological abnormalities are common. The anaemia is severe with reticulocytosis and hypochromasia are present. Strong extramedullary haematopoiesis occurs accompanied by hepato- and splenomegaly and edema. Hyperplasia of the bone marrow is observed. The in-utero survival is normal due to the high oxygen affinity of Hb-Barts and Hb H and, therefore, sufficient availability and supply of oxygen to the fetus.

In patients with Hb H disease, three α-genes are non-functional (--/-α) and electrophoresis shows the presence of Hb H. There is always a severe degree of anaemia which increases as the child grows. Bone changes typical of thalassaemia (e.g. thalassaemic faces) have been reported. Splenomegaly is the rule and hepatomegaly occurs in some patients. The patients are more prone to infections. They occasionally have growth retardation, skeletal changes, splenomegaly with associated hypersplenism and gallstone.

In the α-thalassaemias due to one or two α-gene deletion (i.e. the carrier states:- α/αα; -α/-α; --/αα no serious clinical abnormalities have been reported. Haemoglobin synthesis is reduced due to deficiency of the α-chains and in the α/β-globin chain ratio is

decreased. Haemoglobin Barts may be detected in some patients during the neonatal cells are hypochromic and microcytic with or without a varying degree of anaemia. Occasional red cells containing Hb H inclusions may be found in those with abnormal red cell morphology. The pathophysiological mechanisms involved in the α-thalassaemia states are presented in Figure 1.21.

Since the heterozygous state of α-thalassaemia are not associated with any specific clinical or haematological abnormalities, they are more difficult to diagnosis and the patients are often identified during routine screening or diagnosis of other disease states. The patients with α-thalassaemia state survive normally into adult life and lead a normal life.

1.5 The Enzymopathies

The enzymopathies affecting the red cells are a heterogenous group of metabolic disorders, resulting from mutation in the gene of an enzyme. Depending on the function of the enzyme in the red cells, the enzyme defect may produce a variety of clinical consequences ranging from severe, often life threatening abnormalities to mild or asymptomatic states. The red cells depend mainly on glucose metabolism for obtaining energy. Glucose is utilised by the process of glycolysis (> 90%) (Figure 1.22) and the ATP generated during this process is utilised for all energy requiring processes in the red cells.

period, but disappears after the first few months of life, and is not replaced by Hb H. The red

Figure 1.21: Pathophysiology of α-thalassaemia

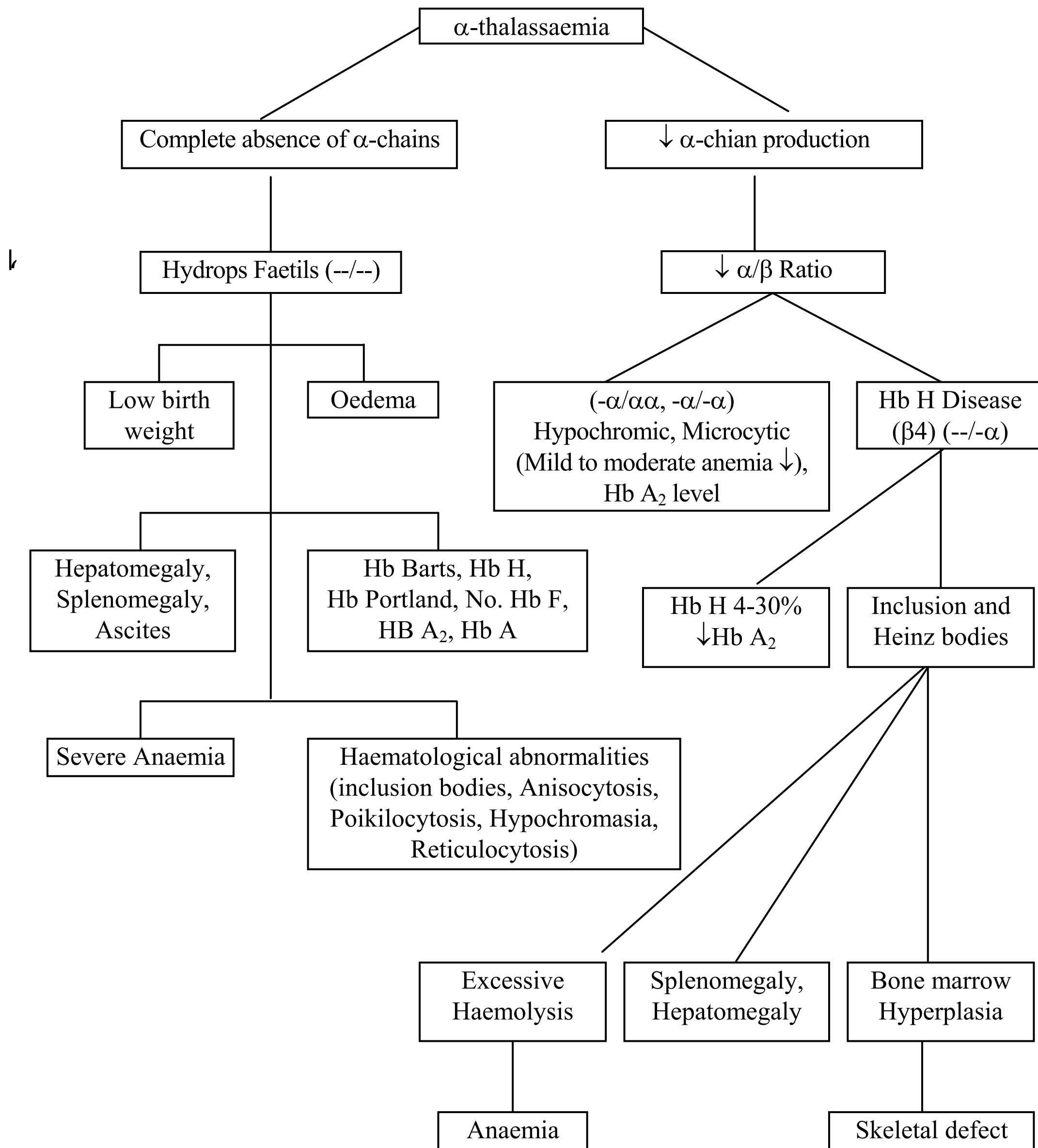
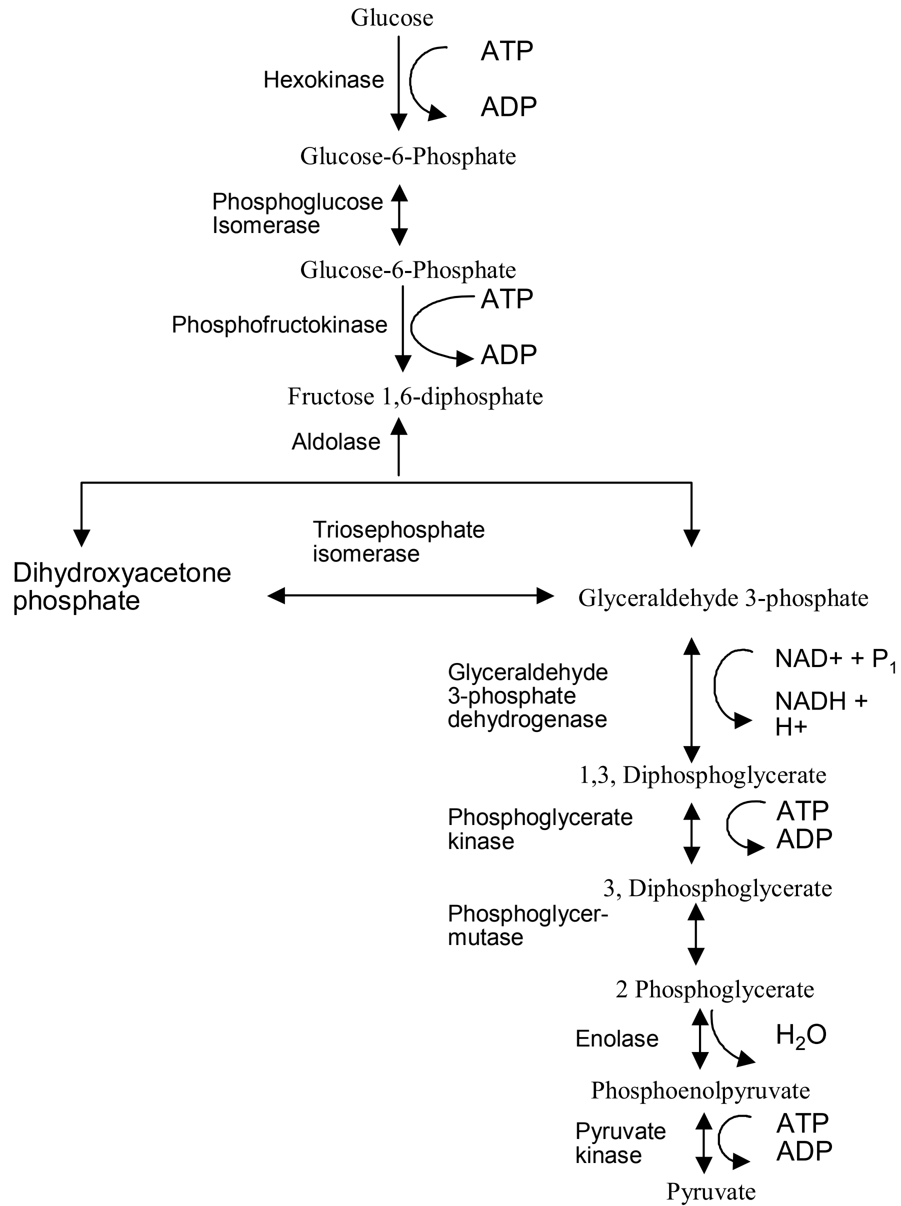


Figure 1.22: The Glycolytic Pathway



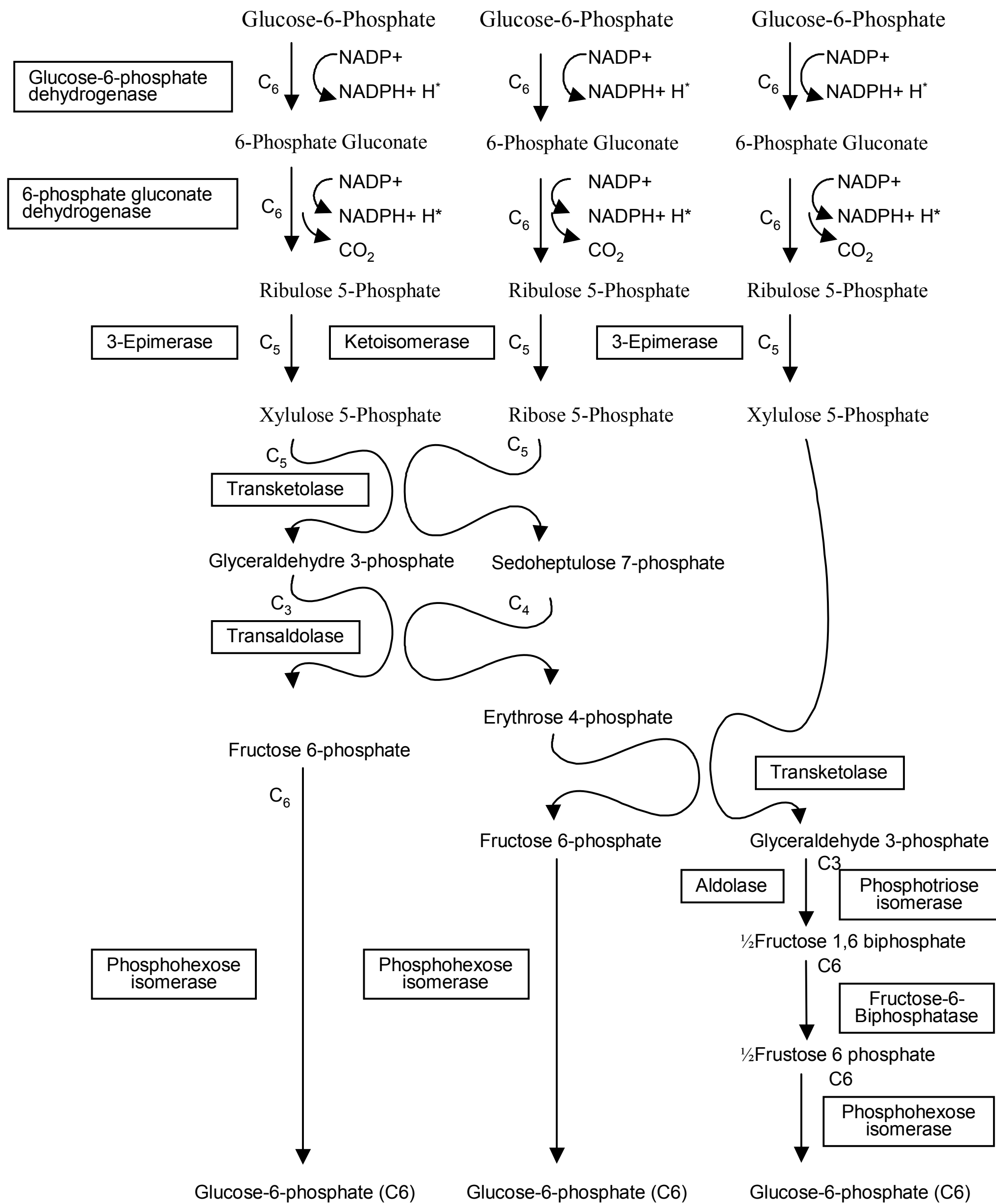
A small percentage of glucose is utilised by the hexose monophosphate shunt (Figure 1.23). The main purpose of which is to produce the reduced coenzyme NADPH and pentose sugars. The NADPH is essential in red cells as it helps provide a reduced environment by removal of oxidising radicals generated during the red cell life cycle and keeps the red cell membrane and other proteins in a reduced state.

In addition, other enzymes exist in the red cells which play a role in the metabolism of glutathione and nucleotides.

It has been known for almost three decades that the mature red cells do not produce any proteins due to the lack of a nucleus and the ribosomes, the site of protein synthesis. The enzyme synthesis occurs upto the stage of reticulocytes and during the life cycle of the red cells these enzymes continue to function. There are certain "age dependent" enzymes which decline during the circulatory life-span of the red cells. Red cell death is believed to result from one or more critical enzymes decaying to a threshold level that was too low to sustain an adequate metabolic rate. Deficiency of an enzyme due to mutations in the genes also influences metabolic rates in the red cells and in some cases the red cell is unable to survive due to the decrease rate of a metabolic reaction. Each enzyme has its own gene which is under specific control mechanisms by which the gene expression are regulated. Mutation within the genes or within these control regions may have a profound effect on the structure or the rate of synthesis of the enzyme. Enzymes are highly sensitive molecules and changes in their structure, even of a single amino acid, particularly those close to the active site of the enzyme may result in complete loss of the activity of the enzyme. This demonstrates an exceptionally close correlation between enzyme structure

and function.

Figure 1.23: The Hexose monophosphate shunt



The mutation in the genes of these enzymes, is transcribed in the mRNA transcript which is then translated in the amino acid sequence of the protein. The protein structure and conformation is a consequence of a specific amino acid sequence and determines the protein functions. Alterations in the protein structure are often accompanied by alterations in its function. Enzymes may lose activity completely or partially, resulting in a "deficiency state" or in some cases the enzyme may even become more active.

A large number of enzyme defects affecting the red cell metabolic pathway are known (Beutler, 1978; 1990; Williams, 1983; Weatherall et al, 1982). Some of the enzyme defects have no effect on the red cells, others affect both structure and function of the red cells, while still others produce a harmful effect in tissues other than the red cells. A few examples of enzyme deficiencies are listed in Table 1.7.

Deficiency of the enzymes of glycolysis, result in an overall deficiency of ATP and hence metabolic activities of the red cells decline. Often the clinical consequences of this deficiency are severe resulting in shortened life span and haemolytic anaemia, which may be cute or chronic or may be precipitated by drugs, chemicals, infections and other environmental factors.

The most well known of the red cell enzyme defects which produces a clinical abnormality include glucose-6-phosphate dehydrogenase, pyruvate kinase, adenosine deaminase, hexokinase and glutathione reductase deficiencies.

Since the advent of the techniques of molecular biology extensive studies have been directed towards the study of the molecular pathology of the red cell enzymopathies. Considerable progress has been made in the study of the glucose-6-phosphate

dehydrogenase deficiency genes and these studies provide an accurate and indepth insight

Table 1.7: Mode of inheritance and clinical manifestations of red cell enzymopathies

Pathway	Red Cell Enzymopathy*	Genetics	Clinical Manifestations
- Glycolysis	Aldolase	AR	Mental retardation, NSHA
	Enolase	AR	NSHA
	GPI	AR	NSHA
	HK	AR	NSHA
	PFK	AR	NSHA, Myopathy
	PGK	XL	NSHA, Mental retardation
	PK	AR	NSHA
	PK (elevation)	AD	Polycythemia
	TPI	AR	NSHA, Neuromuscular impairment
	DPGM/DPGP	AR	Polycythemia
- PPP	G-6-PD	XL	NSHA, favism
- Nucleotide metabolism	ADA	AD	DIHA
- Glutathione Metabolism	P-5'-N	AR	DIHA
	GSI	?	NSHA
	γ-Glutamyl cysteine synthetase	AR	NSHA, NM
	GSH synthetase	AR	NSHA, favism, DIHA
	GR	AR	Favism

PGR: = Phosphyglyceride kinase
PK = Pyruvate kinase
TPI = Triose Phosphate isomerase
DPGM/ = Diphosphoglyceromutase
DPGP = Phosphatase
G6PD = Glucose-6-Phosphase Dehydrogenase
ADA = Adenosine deaminase

AR = Autosomal recessive
AD = Autosomal dominant
XL = Sex linked
NSHA = Non-spherocytic hemolytic anaemia
NM = Neuromuscular impairment
P-5'-N = Pyrimidine-5-nucleotidase
GST = Glutathione-S-transferase

HK = Hexokinase
PFK = Phosphofructokinase
GSH = Glutathione
GR = Gutathione reductase.

into the molecular pathology of the enzymopathies.

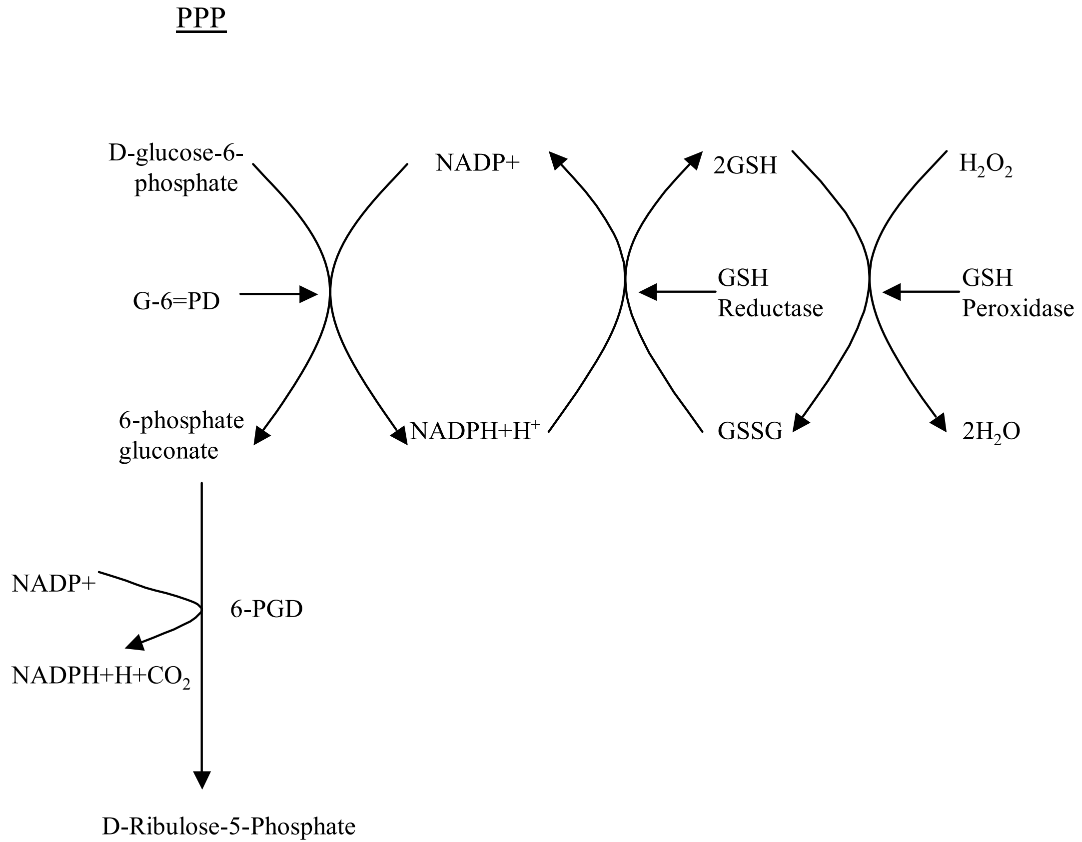
1.5.1 Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G-6-PD) is the first enzyme of the hexose monophosphate shunt and catalyses the conversion of glucose-6-phosphate to 6-phospho-glucono lactone with the concomitant conversion of NADP to NADPH (Figure 1.24). The NADPH is an essential cofactor for several reactions in the red cells. It is necessary for the reduction of oxidised glutathione to reduced glutathione by glutathione reductase. It is also utilised during the reduction of hydrogen peroxide (H_2O_2), generated during several reactions taking place in the red cells. Any deficiency in the level of NADPH, reduces the concentration of reducing equivalents in the red cells, and this is reflected in the shortened life span of the red cells, particularly under oxidative stress, such as exposure to antimalarial drugs, infections, fava beans and certain chemicals. Under normal circumstances the red cells are normal and G-6-PD deficient individuals are more or less asymptomatic. However, under the oxidative stress caused by one or more agents, an acute deficiency of NADPH results in severe haemolytic anaemia with haemoglobinuria and acute abdominal pain.

Glucose-6-phosphate dehydrogenase (G-6-PD) deficiency is the most common enzyme deficiency affecting the red cells in several populations of the world. The frequency shows a close correlation with malaria endemicity, and regions with a past or present history of malaria endemicity have a high frequency of the G-6-PD deficiency gene. The G-6-PD deficiency provides an inborn resistance to the growth of malarial parasite (*Plasmodiun falciparum*), which is one of the most frequent killers in certain

populations of the world. The deficient individuals (carriers) do not develop malaria since

Figure 1.24: Reaction catalysed by G-6-PD and its significance



the parasite is unable to grow in the red cells in the absence or deficiency of NADPH. In addition, the parasitised red cells are either haemolysed at a higher rate or destroyed by the spleen and thus the parasite does not have enough chance to multiply and infest in other cells. This provides a natural resistance against malaria.

1.5.1.1 Glucose-6-phosphate dehydrogenase gene

The G-6-PD is considered as a typical and essential house keeping enzyme as it is found in every cell of the body and plays a major role in metabolism particularly at the interface between carbohydrate and lipid metabolism (NADPH is essential for fatty acid synthesis). The G-6-PD gene is located on the long arm of X-chromosome and is subject to the phenomenon of X-chromosome inactivation. The G-6-PD gene has been isolated, cloned and investigated in considerable detail.

The G-6-PD gene has 13 exons and 12 introns and is located over an area of 20114 base pairs on the long arm of X-chromosome (Figure 1.25). The coding area of the gene consists of 1548 base pairs. The gene is extremely sensitive to environmental factors and over 400 mutants of G-6-PD have been reported in the world populations. Of these 86 variants have been classified as polymorphic.

1.5.1.2 The G-6-PD Variants

The G-6-PD variants result from point mutations in the G-6-PD gene. The normal G-6-PD in all populations investigated so far is G-6-PD-B^+. The other variants differ from the normal enzyme in their electrophoretic mobility, stability, enzyme activity and enzyme kinetics. On the basis of the activity and associated complications, G-6-PD variants have been classified into 5 major classes by World Health Organisation. These are listed in

Figure 1.26. The most frequent of the G-6-PD variants is G-6-PD Mediterranean which

Figure 1.25:The Glucose-6-Phosphate Dehydrogenase

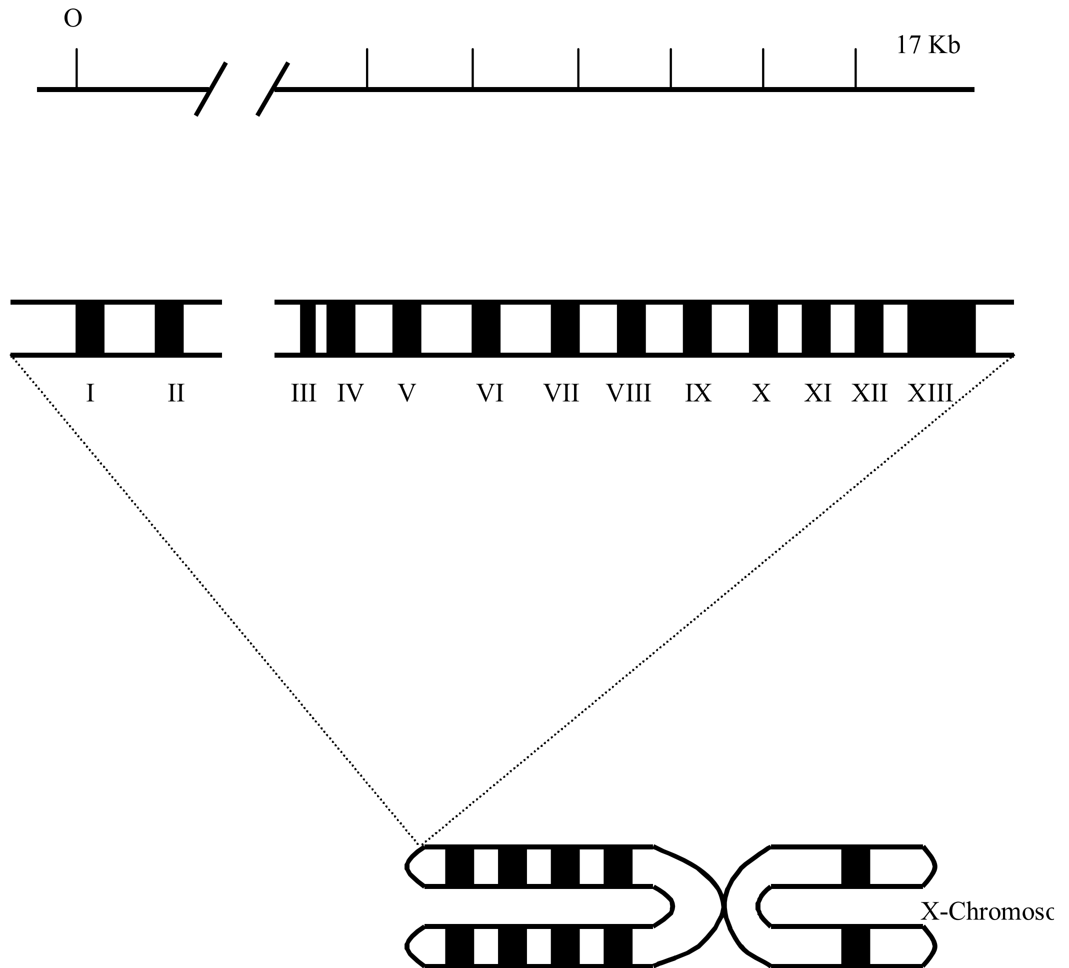
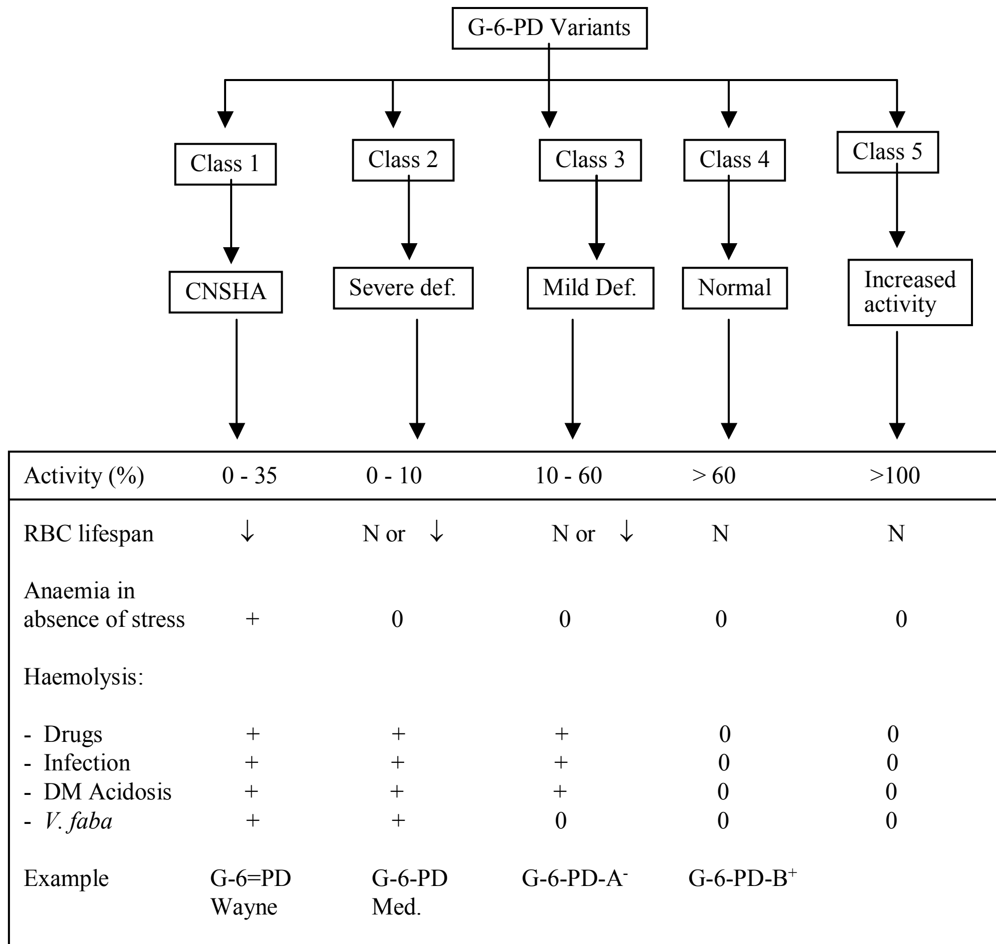


Figure 1.26: Classification of G-6-PD variants



Activity (%)	0 - 35	0 - 10	10 - 60	> 60	>100
RBC lifespan	↓	N or ↓	N or ↓	N	N
Anaemia in absence of stress	+	0	0	0	0
Haemolysis:					
- Drugs	+	+	+	0	0
- Infection	+	+	+	0	0
- DM Acidosis	+	+	+	0	0
- *V. faba*	+	+	0	0	0
Example	G-6=PD Wayne	G-6-PD Med.	G-6-PD-A^-	G-6-PD-B^+	

has the same mobility as G-6-PD-B^+ but has less than 10% of the normal activity. G-6-PD-A^+ is the most frequent normal G-6-PD which differs from the wild-type enzyme i.e. G-6-PD-B^+ in its electrophoretic mobility and occurs at a frequency of 20% in the people of African ancestry. A deficient variant G-6-PD-A^- is the most frequent deficient variant encountered in these populations and occurs at a frequency of 20% in some African population.

The G-6-PD deficiency occurs at a high frequency in several populations of the world and is closely correlated with the past or present history of malaria endemicity.

The molecular basis for enzyme deficiency in the red cells might be (a) decreased synthesis, (2) accelerated breakdown (c) decreased catalytic activity, or any combination of these. Among the 400 or more G-6-PD deficient variants that have been so far identified, there are majority which are unstable and due to the accelerated breakdown result in a G-6-PD deficient state. A large number of variants have a decreased activity, several are broken down rapidly and have a reduced activity at the same time. No G-6-PD variant has so far been recognized which is due to a decreased rate of synthesis. This would suggest that the mechanisms contributing to the expression of G-6-PD deficiency gene are very stable and not influenced by the environmental or other factors which result in mutations in the G-6-PD gene.

The G-6-PD-B^+, the deficient variant and some non-deficient variants have been investigated in detail to identify the molecular basis of the deficient state.

1.5.1.3. Molecular pathology of G-6-PD variants

Sequence analysis of Italians, Iraqis, Iranians, Jordanians, Lebanese and Saudis

subjects have revealed that the molecular basis of G-6-PD Mediterranean is a single C → T transition at nucleotide position 563 causing the replacement of serine by phenylalanine at the position 188 in the G-6-PD molecule. Most G-6-PD Mediterranean subjects also have silent C - T transition, but this does not replace any amino acid at nucleotide position 1311.

Another mutation at the amino acid position 437 has also been reported in an individual from South Italy who had G-6-PD deficiency due to G-6-PD Mediterranean.

The G-6-PD-A^+ has arisen from an A → G transition in nucleotide 376. In G-6-PD A^- this mutation is also identified, and in addition a second mutation i.e. a G → A mutation at nucleotide 202, which replaces a valine by methionine has been identified in some G-6-PD A^- individuals. A few black G-6-PD A^- individuals have the nucleotide 376 mutation and a T → C mutation at nucleotide 968 (not at nt 202). Thus G-6-PD A^- is a result of two single point mutations in the same gene.

Several other G-6-PD variants have been identified by DNA analysis. The molecular basis of these variants is a single point mutation (transversion or transition) and a few examples are shown in Table 1.8.

1.5.1.4. Clinical Problems in G-6-PD deficiency

The most frequent clinical abnormalities associated with G-6-PD deficient variants are acute haemolytic anaemia under oxidative stress, (i.e. drug induced, triggered by infection and favism) and neonatal jaundice. However, the behaviour of the G-6-PD deficient variants is extremely erratic, particularly with respect to favism. Some G-6-PD deficient individuals eat fava beans all their lives and never have a

clinical problem, others suffer from a severe haemolytic anaemia upon its ingestion (~ 25% of G-6-PD deficient individual), still others have no problem with fava beans for years but

Table 1.8: Examples of a few G-6-PD variants and the mutations producing these variants

Variant	Nucleotide substitution	WHO Class*	Amino Acid substitution
Metaponto	172 G-A	3	58 Asp-Asn
A- Distrito Federal	202 G-A 376 A-G	3	68 Val-Met 126 Asn-Asp
A	376 A-G	4	126 Asn-Asp
Mahidol	487 G-A	3	163 Gly-Ser
Santamaria	542 A-T 376 A-G	2	181-Asp-Val 126 Asn-Asp
Mediterranean	563 C-T	2	188 Ser-Phe
Minnesota	637 G-T	1	213 Val-Leu
Hanilaou	648 T-G	1	216 Phe-Leu
A-	680 G-T 376 A-G	3	227 Arg-Leu 126 Asn-Asp
Wayne	769 G-C	1	257 Arg-Gly
Seattle	844 G-C	2	282 Asp-His
Montalbano	854 G-A	3	285 Arg-His
A-	968 T-C	3	323 Leu-Pro
Iowa	1156 A-G	1	386 Lys-Glu
Beverly Hills	1160 G-A	1	387 Arg-His
Nashville	1178 G-A	1	393 Arg-His
Alhambra	1180 G-C	1	394 Val-Leu
Puerto Limon	1192 G-A	1	398 Glu-Lys

Riverside	1228 G-A	1	410 Gly-Cys

Table 1.8 ……..contd

Santiago de Cubs	1339 G-A	1	447 Gly-Arg
Andalus	1361 G-A	1	454 Arg-His
Taiwan-Hakka	1376 G-T	2	459 Arg-Leu
Kaiping	1388 G-A	2	463 Arg-His

may develop the acute haemolytic state upon ingesting fava beans on a later occasion. Several studies have been directed towards the identification of genetic factors required

Population differences are seen in the frequency of both favism and neonatal jaundice. Some differences may be explained on the basis of different variants in the different populations i.e. the Africans have a very low frequency of favism compared to the Mediterranean populations. This is due to the presence of G-6-PD-A^- in the former group which is not sensitive to fava beans while G-6-PD Mediterranean is very sensitive.

All in all it can be summarised that the study of clinical expression of the common G-6-PD deficient variants has not yet fully answered why and when the haemolytic anemia will be severe. Further studies are in progress to clarify this aspects of G-6-PD deficiency.

1.5.2. Other enzymopathies

Extensive studies are directed towards the study of the molecular basis of other enzymopathies. Some of these produce haemolytic anaemia (e.g. pyruvate kinase deficiency). Others produce non-haematologic disease (i.e. galactosaemia, caused by galactose-1-phosphate uridyl transferase deficiency), while still others are considered as "nondiseases" (i.e. NADP diaphorase deficiency). Extensive population studies have not been conducted on these enzyme defects. The molecular basis of a few sporadic cases of some has been investigated. Mutations have been identified in a few of these variants. The molecular biology studies conducted so far on red cell enzyme are presented in Table 1.9.

Extensive studies are still required to identify the molecular pathology of a large number of red cell enzymopathies.

Table 1.9: The Molecular biology of red cell enzymes

Enzyme (abbrev)	Effect of deficiency	Inheritence *	Molecular biology
Hexokinase (HK)	NSHA*	A	Unique reticulocyte enzyme number of loci controversial
Glucose-6-phosphase dehydrogenase (G-6-PD)	Drug-induced hemolytic anemia & NSHA*	x	Many variants sequenced on DNA level cloned.
Glucose phosphate isomerase (GPI)	NSHA	A	Cloned and partly sequenced.
Glutathione peroxidase (GSGPx)	NSHA	A	Cloned
Glutathione reducttase (GR)	Favism	A	cDNA
Phospho-fructokinase (PFK)	Compensated hemolysis ± erythrocytosis & myopathy	A	Both subunits M and L have been cloned and sequenced. An M subunit mutation has been identified.
Fructose-6-phosphate-2-kinase (PF 2-K)	Deficiency unknown	A	Rat nmucle enzyme cloned.
Aldolase	NSHA: ? mental retardation and glycogen storage	A	Cloned. Mutation identified in one case.
Triosephosphate isomerase (TPI)	NSHA & severe neuromuscular disease	A	Cloned. Mutation identified in several patients..
Diphosphoglycerate mutase/ diphosphoglycerate phos-phatase (DPGM/DPGP)	Erythrocytosis	A	Cloned. A mutation detected.
Phosphoglycerate kinase (PGK)	NSHA, mental retardation	x	Cloned. One mutation detected by protein sequencing.
Catalase	Oral ulcers	A	Cloned. Mutation identified in one case
Enolase	?NSHA	A	
Pyruvate kinase (PK)	NSHA	A	Cloned. One mutation detected.
NADH-diphorase (ND)	Methemoglobinemia with or without mental retardation	A	Mutation identified in one case.

*A = Autosome X = Linked
*Selected emphasize recent advances in molecular biology.
*Hereditary nonpherocytic hemolytic anaemia.

1.6 Methods used for diagnosis of haemoglobin disorders and enzymopathies

The diagnosis of the homozygous states of haemoglobinopathies, thalassaemias and enzymopathies can generally be made accurately from a complete clinical picture, for the haemolytic attack in G-6-PD deficient individuals, but these attempts have so far failed to identify any specific factor. It is now suggested that the decisive factors may be

acquired and the effects are erratic as the factors are variable and difficult to quantitate family history and routine haematological examination of the patient and members of the family. Differential diagnosis and confirmation of the condition requires further studies using more specific tests for evaluation of the haemoglobin components or the enzyme activity. However, the heterozygous states (carriers) may not be identified during routine investigations as several of these states are asymptomatic and haematological parameters are more or less normal. Thus, diagnosis of the carrier state requires more detailed investigations. Majority of the carriers are identified during investigations of their relations with more severe disease, during screening programs or during investigation of chronic state of mild anaemia.

In this section the laboratory approach to the diagnosis of haemoglobinopathies, the thalassaemias and enzymopathies is described with a major emphasis on the tests which may be performed in any routine hospital laboratory. However, since, during the last decade the DNA technology has been applied successfully for the diagnosis of carriers and patients suffering from haemoglobinopathies, thalassaemias, and enzymopathies, the main methodologies of this technology are presented (Weatherall & Clegg, 1981; Dacie &

Lewis, 1991).

1.6.1. Diagnosis of Haemoglobinopathies

Haemoglobinopathies result from mutation in the haemoglobin structural gene which produces one or more amino acid alterations in the globin chains and thus alters structure and/or functions. These include alteration in the charge, the isoelectric pH, the solubility, the stability and the oxygen affinity of haemoglobin. These alterations often result in increased haemolysis of the red cells and thus affect the haematological picture.

The laboratory investigations applicable for the diagnosis of haemoglobinopathies are briefly outlined in Table 1.10.

1.6.1.1. Estimation of haematological parameters

Complete blood count (CBC), red cell indices, white blood cell differential and reticulocyte count, provide useful information regarding the haematological status of a patient. The CBC measurements include the determination of total haemoglobin (Hb), red blood cell count (RBC), haematocrit (PCV), and white blood cell count, and are obtained in most haematology laboratories using automated instruments such as Coulter Counter. The values are extremely useful as they give a clear assessment of the degree and severity of anaemia and indicate the presence of infection (i.e. elevated WBC) or neoplastic disorders (e.g. elevated WBC). Reticulocyte count is a fairly accurate and useful indicator of erythropoietic activity. In patients suffering from homozygous haemoglobinopathies, there is mild to severe anaemia and reticulocytosis depending on the severity of anaemia. WBC may be raised if there is infection as is generally the case

in these patients.

In patients suffering from heterozygous states, there may or may not be anaemia and all parameters may be either slightly reduced or within the normal range.

Table 1.10: Diagnosis of Haemoglobinopathies

- Haematological parameters.
- Red cell indices.
- Red cell morphology.
- Reticulocyte count.
- Sickling test.
- Hb Electrophoresis
  - Alkaline pH (Cellulose acetate)
  - Acidic pH (Citrate Agar)
  - Isoelectric focussing
- Haemoglobin types
  - Hb F
  - Hb A_2
- Structural Studies
- Biosynthetic Studies
- Gene Studies
  - Polymerase chain reaction (PCR)
  - RFLP (with or without PCR)
  - Dot blot analysis (with or without PCR)
  - Direct sequencing (with or without PCR)
  - Oligonucleotide hybridization (with or without PCR)
- Others

1.6.1.2. Red Cell Morphological Studies

A simple well prepared and well stained blood smear provides useful information about the red cell morphology when examined microscopically. In patients with homozygous haemoglobinopathies, several morphological abnormalities may be observed. These include

presence of sickled cells, inclusion bodies, Heinz bodies, Hb H precipitations, target cells, hypochromic-microcytic cells, reticulocytosis, polychromesia and basophilic stippling.

The morphological abnormalities are more pronounced in patients who have associated thalassaemia.

Sickling of the red cells is generally seen in patients with sickle cell disease (e.g. SCA, Hb Sك°-thalassaemia, Hb SC disease etc.) However, the absence of sickled cells does not rule out these disorders. Sickling can be brought about by addition of 2% sodium metabisulfite or 10% formalin. Target cells formation is a frequent finding in many haemoglobinopathies and are seen with Hb O-Arab, Hb E, Hb C, Hb S and or-ك thalassaemias. In homozygous Hb S, presence of numerous target cells and frequently Howell-Jolly bodies reflects 'autosplenectomy' due to recurrent infections.

Patients suffering from haemoglobinopathies who also have associated thalassaemias, generally have hypochromic, microcytic cells. In some unstable haemoglobins (e.g. Koln) hypochromic cells may occur in association with macrocytosis. This finding has a significant diagnostic value.

1.6.1.3 Red Cell Indices

The red cells indices i.e. mean cell volume (MCV), mean cell haemoglobin

(MCH)and mean cell heamoglobin concentration (MCHC) can be either calculated or can be obtained directly from the Coulter Counter. In patients with haemoglobinopathies, generally the red cell indices are normal. However, if associated α- or β-thalassaemia is present, then the value of MCV and MCH are reduced and the cells microcytic are hypochromic. This effect is also seen in patients with iron deficiency and, therefore, care should be taken to rule out iron deficiency anaemia for definitive diagnosis.

1.6.1.4 Sickling Test

Blood cells from sickle cell disease patients can be made to sickle under conditions of reduced oxygen tension as that obtained by addition of 2% sodium metabisulfite. The red cells change to sickled shape and upon oxygenation return to normal shape. There are, however, certain number of cells which become irreversibly sickled and even upon oxygenation they remain irreversibly sickled. SDC patients with a more severe disease have a higher number of Irreversibly sickled cells (IRS) compared to those with a mild disease presentation.

1.6.1.5. Stability Test

Stability test of haemoglobin molecule is carried out to investigate the stability of the mutant haemoglobin. Several haemoglobinopathies result from unstable haemoglobins. The presence of unstable haemoglobins may be detected by heat treatment or precipitation with isopropanol. If the haemoglobins are unstable, haemoglobin variant, precipitate at about 50°C while in isopropanol precipitation test the precipitation is brought about by addition of propanol buffer at 37°C.

Thus by applying suitable buffers, lysing and reducing agents it is possible to

differentiate between Hb AA, AS & SS.

1.6.1.6 Electrophoresis

Alterations in the amino acid composition of haemoglobins may be associated with a change in the charge on the haemoglobin molecule. Thus it is possible to separate some of the mutant haemoglobins from the normal haemoglobins by electrophoresis (Figures 1.27 and 1.28).

Haemoglobin electrophoresis both at alkaline and acid pH are recommended as part
of a protocol for the identification of mutant haemoglobins (International Committee for the Standardization in Hematology).

At alkaline pH (pH 8.4 - 8.9) electrophoresis is performed on cellulose acetate plates. This is generally followed by electrophoresis at acid pH with pH range from pH 6.0 - 6.2 on citrate agar plates. The results of the two methods together can be differentiated between several of the commonly encountered mutant haemoglobins e.g. Hb S, Hb C, Hb F, Hb O-Arab, Hb D etc.

In Hb S the mutation in the β-globin chain is β^6 glu → val i.e. a negatively charged amino acid has changed to an unchanged amino acid, thus the charge on Hb S is reduced and in alkaline pH it moves slower than Hb A. In β , β^E , βδ the amino acid substitution is Glu → Val at position β^{121}, β^{26}, β^6 , respectively. These three haemoglobins can be easily distinguished by their mobility in acid and alkaline pH (Figures 1.27 and 1.28).

Some mutant haemoglobins do not separate well on electrophoresis and have to be

identified by more sophisticated structural studies. However, majority of these mutant haemoglobins are rare and may not be associated with any serious clinical consequences.

Figure 1.27: Separation of Haemoglobin Variants on Electrophoresis at Alkaline pH

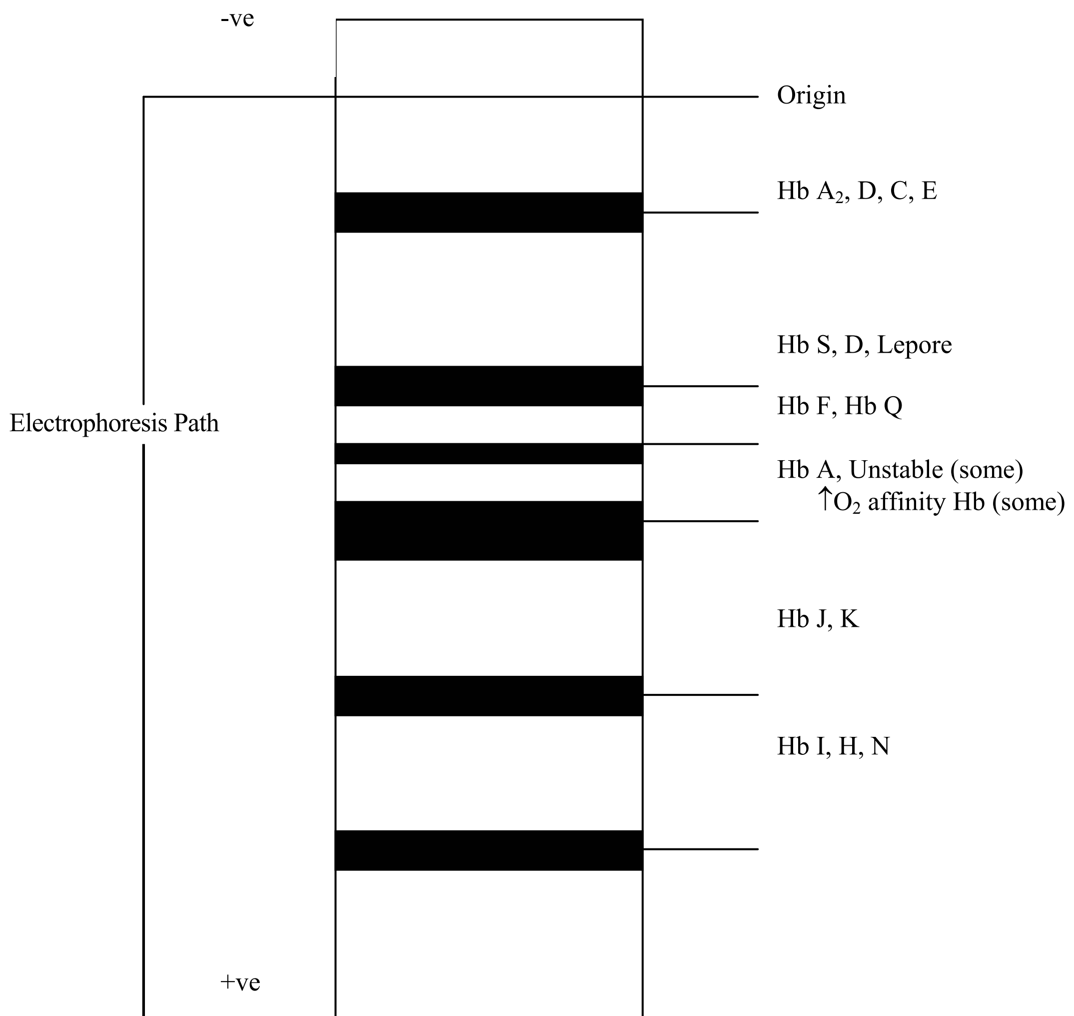
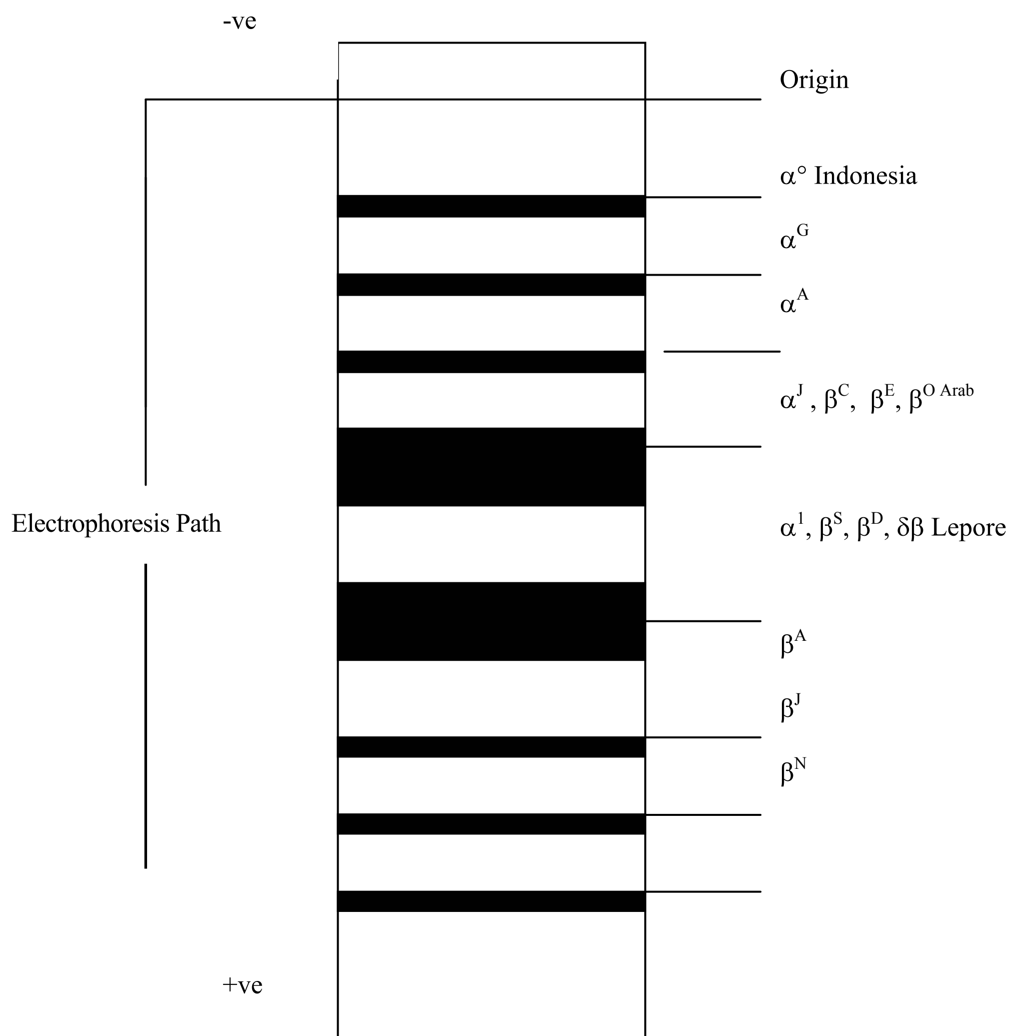


Figure 1.28: Separation of Haemoglobin Variants on Electrophoresis



1.6.1.7 Isoelectric Focussing

Isoelectric focusing has been used extensively for the identification of mutant haemoglobins. As the amino acid is altered in the mutant haemoglobin, its isoelectric pH may be altered. Thus alteration is made use of in the separation of abnormal haemoglobins in a pH gradient. The advantage of this procedure is twofold. A better resolution may be obtained between mutant haemoglobins which are not separated well on electrophoresis and minor component of haemoglobins present in small amounts may be seen as isoelectric focusing has the ability to concentrate the proteins. In addition, this is a more specific method and other methods may not be required. Either commercially available isoelectric focusing plates (pH range 6 - 9.5) or plates prepared in the laboratory from commercially available ampholytes may be used.

1.6.1.8. Haemoglobin A_2 and F quantitation

The quatitation of Hb A_2 and F is routinely carried out when investigations are conducted for the suspected haemoglobinopathies. At birth almost 70% of the total haemoglobin is Hb F. However, by the age of one to two years, the synthesis of l-chains of Hb F is largely switched off and the level of Hb F reaches the adult level of 1-2%. The normal level of Hb A_2 is from 2.5 - 3.5% and no major alterations are seen with age. Hb F is generally raised in patients with sickle cell haemoglobin while Hb A_2 is normal, unless there is associated β-thalassaemia, in which the Hb A_2 level is elevated or α-thalassaemia in which the level is decreased.

The methods utilized for the estimation of Hb F and Hb A_2 include the elution method, following alkaline denaturation and electrophoresis. The commercially available

Beta-thalassaemia, Hb A_2 Quick Column Kit or Sickle-Thal Quick Column Kit (Helena Laboratory) provide an accurate estimate of the Hb A_2 level. A radioimmune diffusion method using antigen-antibody reaction provides a more accurate estimate of Hb F level.

1.6.1.9 Serum Iron, Total Iron Binding Capacity and/or Ferritin

To assess iron status in patients with haemoglobinopathies and thalassaemias, it is a frequent practice to determine serum iron and total iron binding capacity. More recently serum ferritin estimation has replaced these assays. The serum ferritin, a good indicator of iron status of the body is reduced in patients with iron deficiency and elevated in patients with iron overload. Elevated levels occur frequently in patients receiving frequent blood transfusions and must be controlled by chelation therapy in order to prevent tissue damage particularly cardiac, liver and endocrine damage. Excessive iron deposits in the liver parenchyma may result in liver fibrosis, cirrhosis and hepatoma while in the heart muscles haemosiderosis eventually leads to cardiac failure or arrhythmia.

1.6.1.10 Structural Studies

The introduction of peptide mapping or finger printing provides means of determining the amino acid substitution in abnormal haemoglobin chains. Ultimate characterization of all haemoglobins requires amino acid analysis followed by amino acid sequencing that is carried out following finger printing of an abnormal haemoglobin chain. In some rare cases the abnormal haemoglobins are separated from the associated normal haemoglobin fraction using affinity for oxygen before finger printing the suspected haemoglobin chain.

The globin chains are first separated by chromatography and under optimal

conditions, the chains are degraded by treatment with trypsin. The peptide so obtained are separated by a combination of electrophoresis and chromatography in opposite direction carried out on the same filter paper. The 'finger print' of the abnormal globin is compared with that of the normal and any altered peptides observed is extracted from the paper. It is subjected to hydrolysis for determining amino acid composition and the sequencing is carried out using automated amino acid sequences.

1.6.1.11 Recombinant DNA Technology

Recombinant DNA technology can be applied for the diagnosis of haemoglobin structural disorders by studies at the gene level. These methods are highly specific and have been applied for the prenatal diagnosis of several abnormal haemoglobins and α- and β-thalassaemias.

A. Diagnosis of Abnormal Haemoglobin

Restriction fragment length polymorphism (RFLP) studies have been utilized for the diagnosis of several abnormal haemoglobins. The most widely used method in the application of Mst II for the diagnosis of sickle cell mutation. Mst II recognizes a palin-dromic site CCTGAGG. In the β^A gene it produces 1.15 kb DNA fragment containing the β^A gene. In the β^S since a mutation occurs which converts GAG to GTG, the Mst II site is lost and a longer 1.35 kb fragment is produced carrying the β^S globin gene. In Hb S heterozygous (Hb AS) both 1.35 and 1.15 kb fragment are produced while Hb S homozygous (Hb SS) only one 1.35 Kb fragment is obtained. Other restriction endonucleases used for Hb S diagnosis include Dde I and Cvn I.0.

Amplification of the DNA by polymerase chain reaction results in the production

of several thousand copies of the β-globin gene from a few copies e.g. those obtained from a mouth wash, chorionvillus or amniotic cells. Once the amplification has been brought about the diagnosis can be made using specific allele specific oligonucleotide probes, or RFLP. The method is highly specific and the false positive results are few.

Other abnormal haemoglobins diagnosed by RFLP include Hb O-Arab using EcoRI and Hb Lepore.

B. Diagnosis of the Thalassaemias

In addition to the methods listed above for haemoglobinopathies diagnosis, the recent DNA techniques are widely used for the diagnosis of both deletion and non-deletion types of α- and β-thalassaemias (Table 1.11) and a flow chart of the steps involved in diagnosis of thalassaemic state are presented in Figure 1.29.

Majority of the α-thalassaemias are caused by α-gene deletion. This can be diagnosed by RFLP using the restriction endonucleases Bam HI or Bgl II. Bam HI restricts on both sides of the α-globin genes and produces a 14.5 kb fragment containing oth α-genes. However, deletion of an α-gene results in a 10.5 kb fragment. If two α-genes are deleted (-α/-α) a single 10.5 Kb fragment is obtained, while in cases with one α-gene deletion (-α/α α and 10.5 Kb fragments are obtained. Bgl II can differentiate between rightward and leftward deletion α-thalassaemia and produces different size fragments in the two types of deletions. The β-thalassaemias generally result from point mutations and are a much more heterozygous group due to the presence of over 90 mutations. There are several recombinant DNA techniques that have been applied for the diagnosis of β-thalassaemias and for differentiating between the β-thalasseamias depending on their

pathophysiology. These include restriction endonuclease mapping (RFLP) since a mutation either adds or removes a restriction site. Allele specific oligonucleotide probes are small oligonucleotides with a base sequence complementary to a portion of the globin gene where the mutation is suspected. If mutation has taken place the binding of the ASO

Table 1.11: Methods used for the diagnosis of the thalassaemias

A. Routine analysis
- Haematological parameters
- Red cell indices
- Red cell morphology
- Discriminant factors
- Biosynthetic studies

B. Gene Analysis

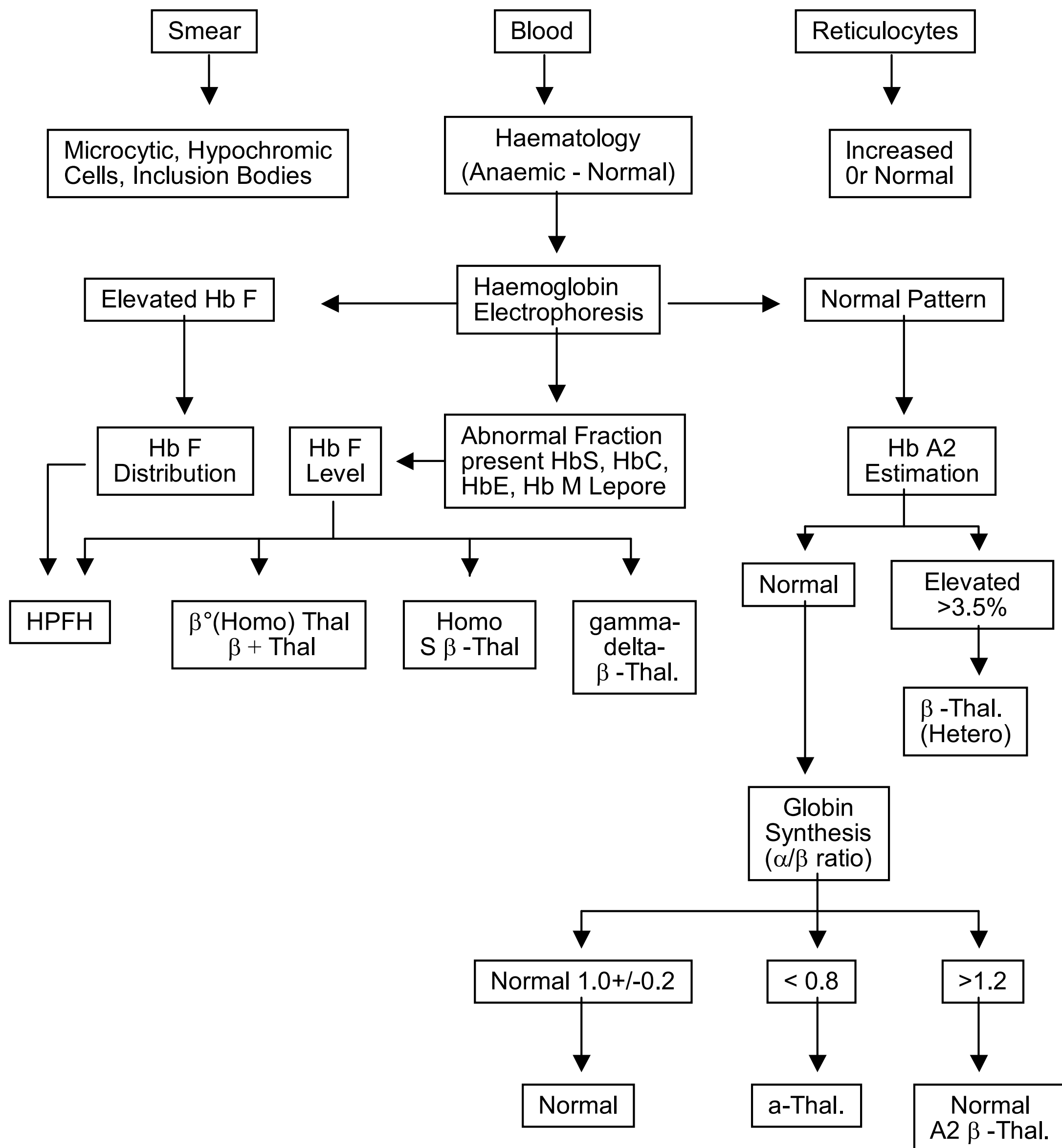
(i) Direct detection

(a) Point mutations:
- Restriction endonuclease analysis (with or without PCR)
- Southern blot analysis
- Oligonucleotide hybridization (with or without PCR)
- Dot blot hybridization (with or without PCR)
- Direct sequencing (after PCR or cloning)
- Denaturing gel electrophoresis (after PCR or cloning)
- Riboncule ase A analysis
- Solution melting method
- Other enzymatic and chemical methods

(b) Additions, deletions, rearrangements:
- Restriction fragment analysis
- Southern blot
- Polymerase chain reaction (PCR)

(ii) Indirect detection
- Restriction fragment length polymorphism (RFLPs)
- Restriction site polymorphism
- Hypervariable regions (HVRs)

Figure 1.29: Flow Chart for Thalassaemia Diagnosis



does not occur. On the other hand an ASO may have a base sequence which is complementary to the mutated gene and hence the ASO does not bind to the normal gene. The gene or portion of the gene is first amplified by PCR and the amplified gene is used for diagnostic purpose either on agarose-gel or by dot-blot analysis. More recently amplification refractory mutation system (ARMS) and denaturing gradient gel electrophoresis (DGGE) have been applied for the diagnosis of the ك-thalassaemia mutations. PCR plays an important role particularly in prenatal diagnosis since a minute quantity of the DNA from a few cells can be amplified considerably to provide enough DNA for successful diagnosis. The amplified DNA can be cloned or hybridized to an allele-specific oligonucleotide (or analyzed directly by electrophoresis without using radioactive probes) and analyzed by DGGE and ARMS.

Diagnosis of enzymopathies

The clinical and haematological manifestations of enzymopathies vary widely. Some of the enzymopathies in the homozygous states produce an asymptomatic state with normal haematological picture, while others are associated with haematological and clinical abnormalities. However, except for a few conditions i.e. glucose-6-phosphate dehydrogenase deficient individuals, in whom favism or drug induced haemolytic anaemia produces severe clinical and haematological abnormalities, other enzymopathies may not be diagnosed from the clinical manifestation. The diagnosis of these states requires the estimation of enzyme activity using specific substrate under specific conditions of pH, cofactors and temperatures. The enzyme converts the substrate to the product and this reaction may be followed either by measuring the amount of product formed or substrates used or cofactor modified. A simple means of estimating these changes is by following the reaction spectrophotometrically. In other

cases if the reaction is not accompanied by any spectrophotometric change, two reactions are coupled together and in one of these a significant spectrophotometric change occurs, thus allowing the monitoring of the reaction.

Glucose-6-phosphate dehydrogenase deficiency is the most frequently encountered enzymopathies and the estimation of G-6-PD activity requires washed red cells which are first haemolysed, and small quantity of the haemolysate is added to the substrate reaction mixture (glucose-6-phosphate, $NADP^+$). The reaction is followed for several minutes in the spectrophotometer at 340 nm. As $NADP^+$ is reduced to NADPH during this reaction, the OD_{340} increases and the amount of substrate consumed and the rate of reaction can be obtained from the change in OD_{340}/min. The activity of pyruvate kinase, hexokinase, glutathione reductase and several other red cell enzymes can be estimated spectrophotometrically, using suitable sub-strates and coenzymes, thus providing a simple procedure for diagnosis of the deficiency state.

For G-6-PD and some other enzymes it is a routine procedure to analyze for the type of G-6-PD phenotypes present. This phenotyping procedure involves separation of the different phenotypes on electrophoresis at alkaline pH and staining the gel specifically to determine the enzyme activity. Most frequently encountered G-6-PD deficient phenotype is G-6-PD-Mediterranean in most populations of the world. However, in the African population, $G\text{-}6\text{-}PD^-$ A^- is more frequent.

Phenotyping of the enzymes provides useful information regarding the microhetero-geneity of red cell enzymes.

More recently recombinant DNA techniques have been utilized for the determination of the mutation in the G-6-PD gene.