CHAPTER I

REVIEW OF LITERATURE

Hemoglobin performs the significant physiological function of supplying molecular oxygen to the tissues of animal body. This globular protein in most vertebrates is a tetramer consisting of two kinds of polypeptide chains. Each polypeptide chain has a prosthetic group, heme, which is combination of one iron atom with a molecule of protoporphyrin IX attached to it. Vertebrate hemoglobin vary in the globin part while heme moiety is of same composition. The genetic mechanisms that bring about such a variation in the protein part of different hemoglobins can be elucidated through structural studies of the molecule. The new found interest in the molecular genetics and evolution has brought about an intensive research activity in the field of hemoglobins. The data accumulating is both impressive and staggering.

I. Hemoglobin: its structure and function

1. X-Ray Crystallographic studies

The biological activity of protein molecule depends on its native conformation. X-ray crystallography and computer methods are now available for charting the biomolecular structure. The successful application of X-ray diffraction analysis to the conformation of fibrous proteins by William Astbury, Linus Pauling and Corey and some time later globular proteins by Kendrew (75)
and Perutz (103) yielded newer insights into the structure function relationship. Perutz and Lehmann (106) and also other workers (51,52,58,85,95) have investigated the disruption of chemical structure and function due to mutations by comparisons of the three-dimensional structures of normal and abnormal hemoglobins studied by X-ray crystallography. The three dimensional electron density maps of deoxyhemoglobin of horse and man reveal no significant differences in the conformations (103,107). X-ray crystallographic data has indicated that approximately eighty percent of the amino acid residues are in helical structure (35,36,106,110). In the β-chain there are eight α-helical segments designated by letters A to H in order from the N-terminus. The α-chain contains seven α-helical segments; the segment D of β-chain is absent in the α-chain. At some points the segments are separated by nonhelical portions, whereas at others, the helix comes to an abrupt end and another helix begins in a new direction without intervening residues. The non-helical segments are denoted by the helices at either end. According to the nomenclature introduced by Kendrew et al (74) residues within each helical or nonhelical segment are numbered from one at the N-terminal residues of each section. In the β-chain, 24th and 64th glycine residues are in close spatial contact, with each other. Histidine residue 92 β (87 α) is the so-called 'proximal' histidine and
Histidine residue 63 β (and 58 α) in helix E is the 'distal' histidine.

The three-dimensional structure of the subunits of hemoglobin is held together by weak noncovalent bonds such as salt bridges, hydrogen bonds and nonpolar interactions. The polar amino acid side chains are in contact with the aqueous environment while nonpolar side chains generally lie in the interior in contact with each other to form nonpolar hydrophobic surroundings. Some seryl or threonyl hydroxyl groups are hydrogen bonded to a carbonyl group within the same α-helix (104). Occasionally nonpolar side chains protrude into the surrounding water, for example, cysteine F9 (93) β or leucine E17 (68) β (108).

The heme group is embedded in an hydrophobic pocket which is present in each chain. It is located between the helices E (residues 57-76 β and 52-71 α) and F (residues 85-93 β and 80-88 α). Four coordinate positions of the ferrous iron are taken up by the four pyrroles of the porphyrin. The fifth position is occupied by a nitrogen atom from the imidazole ring of the 'proximal' histidine. The sixth position is empty in deoxyhemoglobin or has oxygen attached in oxyhemoglobin. In the general vicinity of the sixth coordinate position of the iron is the imidazole ring of the 'distal' histidyl residue. Access to the distal side at which oxygen attaches in
Oxyhemoglobin is not restricted. The heme is held by about sixty noncovalent interactions (104). All these interactions except one in the \( \alpha \)-chain and two in the \( \beta \)-chain are nonpolar. The polar contact in the \( \alpha \)-chain extends from one of the two propionic acid carboxyls to histidine CD 3 (45). In the \( \beta \)-chain one propionic acid carboxyl makes contact with serine CD 3 (44) and the other probably with lysine E 10 (66). In different mammalian species with the exception of leucine H 12 (129) all the residues involved in contact between \( \alpha \)-chain and heme are common to all normal \( \alpha \)-chains. Similarly, with the exception of two \( \beta \)-chain residues namely phenylalanine E 15 (71) \( \beta \) and serine CD 3 (44) \( \beta \) all the other residues involved in contact between \( \beta \)-chains and heme are common to all \( \beta \)-chains. In this connection it is interesting to note that the serine which is present in non human primates and man, is replaced by histidine in Ungulates (37). The invariance of the residues surrounding the heme group implies that they are nearly all essential for the function of the hemoglobin molecule.

The two \( \alpha \)- and \( \beta \)-chains of hemoglobins are arranged in a tetrahedral manner. Because the molecule has a two-fold axis of symmetry, any one type of chain has two different areas of contact with its opposite type and two of each type of contact are present. The few points
of contact between like chains (α to α or β to β) probably consists of salt bridges involving the terminal residues. The α₁β₁ contact involves 34 residues with about 110 atoms in contact. The majority of these interactions are nonpolar. These contacts are contributed mainly by amino acid side chains belonging to the helices B, G and H.

The α₁β₂ contact has 19 residues and about 80 atoms. These residues belong chiefly to the helices C and G and the nonhelical segment FG. All but two of the interactions are nonpolar (104). There is a hydrogen bond between the side chains of aspartic acid Gl (94) α and Asx G4 (102) β, and another one between the side chains of threonine C6 (41) α and histidine FG4 (97) β. The α₁β₂ contact represents the shortest route between two hemes in the molecule. This has led Perutz et al (104) to suggest that the α₁β₂ contact is of primary importance as a pathway for functional interactions between the hemes.

In solution, hemoglobin tetramer is in dynamic equilibrium with dimers and monomers (53,54). In suitable conditions hemoglobin tetramer dissociates into dimers and also into free α and β chains. This takes place at the contacts α₁β₁ and α₂β₂, further splitting into free α and β chains at the contacts α₁β₁ and α₂β₂ (125). Dissociation is favoured by high concentration of neutral electrolyte which are known to weaken polar interactions and to strengthen nonpolar ones. Therefore high concentration
of neutral electrolytes weakens the polar contacts between like chains and strengthens the predominantly nonpolar ones between unlike chains. Out of the two types of nonpolar contacts $\alpha_1\beta_2$ has the smaller area and would therefore be the first to break.

(ii) Oxygenation and deoxygenation

The configurational changes occurring during the reversible reaction of oxygen binding and oxygen release has been studied by many workers (21,94,105,108,109). During oxygenation the hemes of the two $\beta$-chains are brought closer together, while on deoxygenation they separate. This phenomenon is illustrated by considering the distance between the iron atoms of the heme groups of the four polypeptide chains. The $\beta_1 - \beta_2$ distance decreases by 6.5 Å on oxygenation. There is also a corresponding increase by about 1Å in the distance between the two $\alpha$ chains. The $\alpha_1 \beta_1$ distance decreases by 1.9 Å while $\alpha_1 \beta_2$ distance remains almost same. During oxygenation the molecule expands, resulting in further separation of the $\alpha$-terminal parts of the $\beta$ polypeptide chains. It has been suggested that during this process of expansion two histidinyl residues - His-97 $\beta$ and His-146 $\beta$, will be uncovered. The increased buffering capacity of deoxyhemoglobin may be the result of such an exposure of the imidazole rings of these histidine amino acid residues. Experimental results
suggests that there is a rotation of chain about an appropriate axis. Such a rotation will change the distance between $\alpha_{12}^\alpha$ and the $\beta_{12}^\beta$ contact will be broken. The $\alpha_{12}^\alpha$ contact is closely connected with the heme groups and as a result a shift in the $\alpha_{12}^\beta$ contact will influence their environment. Perutz et al (109), using Difference Fourier analysis between normal oxy and deoxyhemoglobin and their N-ethylsuccinimide derivative, have identified changes in chain conformation upon ligand binding at the terminal regions of both chains.

In deoxyhemoglobin the $\alpha$-terminal histidine, $146^\beta$ forms a loop around helix H; its imidazole makes a salt bridge with aspartate, $94^\beta$, of the same $\beta$-chain, and its $\alpha$-carboxyl makes a salt bridge with lysine $40^\alpha$, in the $\alpha_{12}^\beta$ contact. In the electron density map of oxyhemoglobin at $2.8\AA$ resolution only the penultimate tyrosine, $145^\beta$ is visible but not the $\alpha$-terminal histidine. Model building shows that at physiological salt concentration the $\alpha$-terminal carboxyl groups of one $\beta$-chain may be linked to lysine $132^\alpha$, of the other $\beta$-chain, but that the imidazole group is either free or weakly linked to the $\alpha$-amino group of valine $1\beta$.

The electron density map of horse oxyhemoglobin at $2.8\AA$ resolution shows the $\alpha$-amino group of valine, $1\alpha$ to be free. In the electron density maps of human and horse
deoxyhemoglobin at 5.5Å resolution, on the other hand, a bridge extends from the amino terminus of each α-chain to the carboxyl terminus of its partner chain, suggesting a link between the α-amino and α-carboxyl groups.

(iii) Hemoglobin: an allosteric protein

The binding of the first oxygen molecule to one heme group enhances the binding of succeeding oxygen molecules to the three remaining heme groups. Hills equation expresses the heme-heme interaction.

\[ Y = \frac{KP^n}{1 + KP^n} \]

Where \( Y \) = fraction of hemoglobin saturated with oxygen.

\( P \) = Partial oxygen pressure.

\( K \) and \( n \) = constants, 'n' being the interaction coefficient. Greater the value of 'n' greater is the interaction. Value of 'n' for mammalian hemoglobins is around 2.8. Since the heme groups are spatially distant from each other, heme-heme interaction must be transmitted through the globin. It has been suggested that interactions of the hemes are due to entropy effects associated with similar or identical conformational changes accompanying the reactions (146). Wyman and Allen (145) have observed that combination of hemoglobin with ligand is accompanied by profound conformational changes and have suggested that the homotropic and heterotropic interactions are both basically allosteric. The sigmoid saturation curve
of hemoglobin indicates a heme-heme interaction. It has been postulated that there is a conformational change in the α chain when it combines with oxygen molecule which is mechanically transmitted to the tightly bound β chain. This β chain has a higher oxygen affinity, since it is in a new conformation. In this way the binding of one molecule of oxygen enhances the binding of the second. The new internal stress developed in the hemoglobin molecule results in the oxygenation of the other functional subunit, (αβ).

(iv) Bohr Effect

The dependence of oxygen affinity on the pH of the environment is known as Bohr effect (24). In human and other mammalian hemoglobins the pH dependence of the affinity consists of alkaline Bohr effect (above pH 6) where an increase in pH increases ligand affinity, and an acid Bohr effect (below pH 6) where the affinity for the ligand changes in an opposite for changes in pH. Bohr effect is very similar in various mammalian hemoglobins (4,5,132) and it is also very similar for the reaction with various ligands (3,6).

The isolated chains as well as Hemoglobin H which is a tetramer (β4) have no Bohr effect (18). This observation and a study of ligand equilibria of artificial intermediates led to the conclusion that the α- and β-chains contribute equally to the alkaline Bohr effect (15,40).
The Bohr effect in the acid range presents a different picture. A modified hemoglobin, carrying heme, only on \( \alpha \)-chains, which are bound to heme-free \( \beta \)-chains, has a substantial Bohr effect (130). This observation and kinetic study results on the uptake of ligand and release of Bohr protons, indicate that the Bohr effect results from intra-chain conformation changes, not directly correlated with changes involving the molecule as a whole (7). In the light of the allosteric model proposed by Monod et al (90) Bohr effect can be considered to result from heterotropic interactions.

The \( \alpha \)-amino groups have been considered as the source of the Bohr protons. But blocking of the terminal amino groups of all the four chains reduces Bohr effect only by 15 to 25 percent (79). The terminal amino groups involved in this effect have been shown to belong to the \( \alpha \)-chains (79). About 75 percent of the Bohr effect has, therefore, to be determined by other groups, presumably imidazoles. Removal of the \( \alpha \)-terminal ends of both \( \alpha \)-and \( \beta \)-chains as well as reaction of the \( \beta \) 93 SH groups with appropriate reagents results in marked reduction of Bohr effect (8). Perutz et al (112) have suggested that about one-half of the alkaline Bohr effect may be due to a change in \( pK \) of the imidazole group of the \( \alpha \)-terminal histidine of the \( \beta \)-chain which has a normal \( pK \) in oxyhemoglobin and a higher one in deoxyhemoglobin; one quarter of the Bohr
effect would arise from a change in pK of the α-amino group of the α-chain. From X-ray studies Perutz et al have shown that during oxygenation-deoxygenation cycle, the molecule changes its shape and in this process imidazole groups of the last histidine residues in the two β-chains are involved. In oxyhemoglobins these residues are free for ionization; in deoxyhemoglobins they interact with carboxyl groups of aspartic acid residues in the β-chain. Likewise during deoxygenation the free amino group at the end of one-chain interacts with the free carboxy group at the opposite end of its twin, so that the two α-chains are linked head to tail. Both these interactions which take place on deoxygenation, will increase the affinity of basic groups in the molecule for hydrogen ion and decrease the affinity for oxygen.

(v) Hemoglobin affinity for organic phosphates

Organic phosphates like 2,3 diphosphoglyceric acid (2,3 DPG), adenosine triphosphate (ATP) and inositol hexaphosphate (IHP) bind to hemoglobin bringing about a shift in the oxygen dissociation curve. Benesch and Benesch (19,20) have reported that 2,3 DPG which is present in a relatively large amount in human adult red cells acts as a regulator of hemoglobin oxygen affinity. The 2,3 DPG greatly facilitates the release of oxygen from hemoglobin because of the greater affinity of the
compound for oxygenated form. The 2,3 DPG content of human fetal red cells may be lower than that of adult erythrocytes (124). It seems that an increase in the formation of 2,3 DPG after birth is a possible regulator in decreasing oxygen affinity of the red blood cells during this period. Erythrocytes of sheep and goats have virtually no 2,3 DPG (117). It has been proved that 2,3 DPG, ATP and GTP decrease oxygen affinity of hemoglobin (20, 50).

Arnone (9) has shown from X-ray crystallography studies that 2,3-diphosphoglycerate pulls the A-helices further apart, inducing subtle changes in the tertiary deoxy structure of the β-chains. He has also shown that one molecule of DPG binds to one molecule of deoxyhemoglobin and takes up a stereochemically complementary position on the two-fold symmetry axis, plugging the entrance to the central cavity. Its anionic group forms salt bridges with catonic groups of the β-chains. This complementary stereochemistry is specific for the quarternary deoxy structure and is lost on transition to the oxy form. Thus DPG stabilizes the former at the expense of the latter.

II. Biosynthesis of hemoglobins

Mammalian erythrocytes though devoid of a nucleus is well equipped for the synthesis of hemoglobin, which in fact is its primary product. The immature erythroid
cell undergoes various developmental stages, namely stem cell - proerythroblast - basophilic erythroblast - polychromatophilic erythroblast - orthochromic erythroblast - reticulocyte and erythrocyte, to become the mature non-nucleated cell (88). Aronstein, Cox and Hunt (10) obtained direct evidence for the presence of m-RNA in rabbit reticulocytes. Messenger RNA synthesised during basophilic erythroblast stage remains in a stable functional form through the reticulocyte stage (87). Globin biosynthesis takes place in four major steps, each of these steps requires specific enzymes and cofactors. Hemoglobin polypeptide chain synthesis takes place in ribosomes and polysomes which are free and not attached to endoplasmic reticulum. On the average the polysome unit contains some five ribosomes per mRNA (68).

Even though protein synthesis initiation by N-formylmethionyl-tRNA (fMet-tRNA\textsubscript{F}) received experimental evidence in bacterial systems its role in higher organisms was doubtful. Later on experimental evidence (57,133,140) have indicated that the initiating tRNA involved in hemoglobin synthesis in rabbit reticulocytes is methionyl-tRNA\textsubscript{F} but that the amino group of the methionyl-tRNA\textsubscript{F} was free and was not formylated. Since rabbit hemoglobin begins with valine it is assumed that the intact cell contains an enzyme which cleaves the terminal methionine. The various steps in peptide chain initiation include
formation of a complex between the 30s subunit, mRNA and fMet-tRNA. GTP is also required. Three distinct initiation factors, F₁, F₂, and F₃ also take part in this process (139). It is found that temperature above 40°C inhibit the rate of rabbit hemoglobin synthesis in intact reticulocytes by as much as 30 - 90 percent at 44°C, but the chain assembly time is immeasurably fast. The chains at this temperature are made on polyribosomes with few number of ribosomes. This data indicate that the temperature sensitive step is chain initiation (135).

Chain elongation has been studied in bacterial systems (73,101,131) and most of the evidence point that the same mechanisms operate in eucaryotes. During elongation the ribosome move along the mrNA in the 5' to 3' direction and the message is read in groups of 3 nucleotides at a time (39,120). The elongation factors recognized in bacterial systems are Ts, Tu and G factors (73,101). In mammalian system corresponding factors are aminoacyl transferase I and rat liver aminoacyl transferase II. Factors Tu and Ts combine to form a complex which then interacts with GTP to form Tu-GTP complex with liberation of Ts. This complex then interacts with amino acyl tRNA to form the amino acyl-tRNA-Tu-GTP complex. In the next step Tu is released and GTP is hydrolysed. At this point the aminoacyl tRNA of the next amino acid is in position on the ribosome and
peptide bond formation takes place catalysed by peptidyl transferase. Translocation of peptidyl tRNA from amino site to peptidyl site is the last step in the elongation process. Factor G and GTP are required for this step (55,96,101). The overall conclusion that the peptide chain grows linearly from the N-terminus in hemoglobin synthesis has been reported (22). Mintzi's (39) elegant experiments with radioactive amino acids confirmed this view. Although the assembly of a polypeptide chain is apparently linear it is not necessarily at a uniform rate along the length of the chain. Various workers (66,80) who redetermined the assembly time for hemoglobin peptide chains at different temperatures concluded that α chain messages are translated more rapidly. Experimental evidence are also available to indicate that the α and β chains are synthesized independently of one another (97).

When the polypeptide chain is complete the last polypeptide tRNA ester linkage is cleaved, releasing the chain and allowing the ribosome-mRNA complex to dissociate. The nonsense codons act as termination and releasing signals. Two protein releasing factors $R_1$ and $R_2$ mediate termination and hydrolysis process. A third factor $S$ or $\langle $ factor may also be involved in the rate of release of the chain by one of the factors (55,96).

Heme and globin are synthesized separately and later on coupled to form the hemoglobin molecule. It is
proposed that heme regulates its own synthesis by feedback inhibition, stimulates the synthesis of globin and promotes the coordinate synthesis of α and β-chains (137). Levere and Granick (86) content that heme is required for the appropriate three dimensional folding of the globin chain as it is synthesized on the ribosomes. Globin folds around heme to form a compact unit and then it is released from ribosome.

Heme and globin synthesis take place more over at the same rate (81,92). The steps of heme synthesis are well elucidated (30,72,138). The first step is the condensation of glycine with succinyl-coA in presence of pyridoxal phosphate to form α-amino-β-ketoacidic acid which is decarboxylated to α-aminolevulinic acid. This reaction is catalysed by α-aminolevulinic acid synthetase. This is followed by the condensation of two molecules of α-aminolevulinic acid, catalysed by α-aminolevulinic acid dehydrase to form porphobilinogen. This reaction apparently involves the formation of a schiff's base between the keto group of one molecule of α-aminolevulinic acid and an e-amino group of a lysine residue of the enzyme. Uroporphirinogen is then formed by the combination of four molecules of porphobilinogen catalyzed by porphobilinogen deaminase. The four acetic acid side groups of uroporphirinogen are next enzymatically decarboxylated to form methyl groups of coproporphyrinogen III
which on dehydrogenation and decarboxylation yields protoporphyrin IX. Iron is incorporated enzymatically into protoporphyrin to form heme.

III. Molecular genetics and Evolution of Hemoglobins

1. Molecular Genetics

Chemical investigation of abnormal hemoglobins has brought about a great deal of understanding of inheritance of characters. In man, genes with specific locus are placed along 23 pairs of homologous chromosomes. Chromosomes which determine sex (female XX, male XY) also carry other genes which are not determinant of sex and are called sex linked genes. Chemical heterogeneity of hemoglobins has been explained by several hypotheses. Allelism of a single structural gene, ambiguous translation of genetic material and duplication of the structural gene followed by mutations are few of the hypotheses advanced.

Allelic genes on the paired chromosomes may be of equal dominance as with the abnormal hemoglobins or one may be dominant or recessive to its partner. The dominant gene can be recognised in the heterozygous state while the recessive gene can only be recognised in the homogygous state. When the dominant gene appears to mask completely the presence of the recessive allele, it is complete dominance; while incomplete dominance will be the expression
of the phenotype which is intermediate between two homozygotes. Codominance is the condition in heterozygotes where both members of an allelic pair contribute to the phenotype which will be the mixture of phenotypic traits produced in either homozygous condition. In animal population the frequencies of alleles vary considerably. However an ultimate equilibrium frequency is attained by alleles, governed by breakdown of isolating mechanisms, frequency of mutation, selection and random genetic drift. This genetic equilibrium is described by Hardy-Weinberg theorem, which is used to determine the frequency of each allele of a pair or a series and of homozygotes and heterozygotes in the population. The gene frequencies of two alleles is calculated using the expression $p^2 + 2pq + q^2$ which is the expansion of $(p+q)^2$ where $P$ is the frequency of one of the allele while $q$ is that of the other. When multiple alleles are present more terms are added to the expression (44). Vertebrate hemoglobin show a wide variety of allelic variations. Apart from a host of mutant hemoglobins known to be present in man (37), incidences of allelism of two abnormal hemoglobins, Hb-C and Hb-S has also been reported. Ranney (116) attributed the inheritance of two abnormal hemoglobins to allelism of genes in case of family with Hb-C and Hb-S which was later confirmed by the finding that the 6th residue of the $\beta$ chain is differently substituted in Hb-C
and Hb-S (64). Another incidence of allelism of hemoglobin genes is found in horse hemoglobin, which consists of two electrophoretically distinct components whose structural difference lies in the \( \alpha \) chains (32, 78, 134). Position 60 of the more acidic \( \alpha \) chain is occupied by glutamine while the more basic \( \alpha \) chain has lysine in this position. Position 24 of the \( \alpha \) chain can be occupied by either a tyrosine residue or by a phenylalanine residue or both. This heterogeneity occurs simultaneously in the \( \alpha \) chains of both fast and slow hemoglobin components. A mutation in the transfer RNA cistron has been offered to explain this heterogeneity in horse hemoglobins. Ohno (99) advanced an attractive alternative explanation for the horse hemoglobin genetics highlighting an unequal exchange between two chromatids of the same chromosome. According to this report an ancestral equus possessed a single alpha structural locus - \( \alpha^{60} \) Lys. At this stage horses might have already maintained two alleles, \( \alpha^{24} \) Tyr and \( \alpha^{24} \) Phe (78, 99).

If duplication of ancestral equus gene locus was accomplished by two separate events involving unequal exchange within the same chromosome, an original and duplicate loci would have received same codon for the 24th position (99). These events would lead to the existence of two different types of homologous chromosomes within horse population; an original and a duplicate on
one chromosome that specify tyrosine at 24th position and both on the other chromosome specifying phenylalanine at 24th position (78,99). But only tyrosine was observed at the 24th residue position of α-chain sequences of 12 Indian horses (83).

Multiple site alleles are found in the hemoglobins of bovid population. Bovine \( \beta^A \) and \( \beta^B \)-polypeptide chains of hemoglobins which are products of allelic genes differ by three amino acid substitutions (128). Recent report of Lalthantluanga and Barnabas (84) presents evidence of alpha chain allelic variants in gayal (Bos gaurus frontalis) which showed the presence of one hemoglobin component. The allelic α chains, \( \alpha^A \) and \( \alpha^B \) were identified by peptide mapping and the differing peptides located. In sheep also polymorphism of hemoglobins is observed. Sheep \( \beta^A \) and \( \beta^B \)-chains which are also known to be products of allelic genes differ by 7 amino acid substitutions (141). Polymorphism of goat hemoglobin was reported by many workers from many laboratories (1,2,14,42,43,56,59,60,61,62,77,144). Phenotype AB has three α chains \( I_\alpha, II_\alpha \) and \( L_\beta \). The two alpha chains \( I_\alpha \) and \( L_\beta \) are the products of allelic genes, while \( II_\alpha \) and \( II_\beta \) are products of duplicated genes. Adams et al (2) reported a β-chain variant hemoglobin, Hb-D in domestic goats. Homozygous individuals for both Hb-D and Hb-B have been reported (2,59). Another hemoglobin variant, Hb-E has also been reported (28).
The β-chain of this hemoglobin - β^E, differs from β^A in three positions. In none of the animals were these three types of β chains observed (63). On the basis of these observations and other data, it has been suggested that the occurrence of β^D and β^E variants result from the existence of alleles at a single structural locus. Probably β^D arose from point mutation while β^E chain is the product of a multisite allele (2,144).

Hemoglobin polymorphism in Rodents is complicated with evidence for several alleles at both α and β chain loci, for gene duplication, for codons ambiguity and for SH dependent polymerisation (34). The hemoglobin electrophoretic pattern often indicated by the terms 'diffuse' and 'single' is controlled by allelic genes which have been identified as β chain structural genes (113,114). A third type of allele 'Hbb P' determines another type of 'diffuse' pattern (93). The solubility properties of specific hemoglobin types are also possibly controlled by allelic α chain structural genes (114). A gene duplication followed by a single mutation also can explain the production of α chain variants associated with high and low solubility phenotypes (113). Rabbit hemoglobins with one major component has two α chains which differ in positions 29, 48 and 49. Studies on these chains led to the conclusion that two linked sets of amino acid variants in the rabbit α chains are the products
of two alleles at one Hb α-structural locus (Set I: 29-Val, 48-Phe, 49-Thr; Set II 29-Leu, 48-Leu, 49-Ser) (66). Evidence confirming this has been reported (126, 127).

Gene duplication resulting in the production of identical genes has been accepted as an attractive mechanism for an increase in the amount of genetic material which is significant in the process of evolution (99). Mutations in the duplicated section of DNA in the chromosome is readily accepted because one of the protein copies still can perform the original function. Schroeder et al (129) reported the presence of two different γ-chains in Hb-F from human cord blood, Gy (Gly=136) and a second, Ay (Ala=136). The ratio of Gy : Ay- is about 3 : 1 in normal cord blood. Abnormal Hb-F contain either Gy or Ay-, but not both. Also other data indicate the presence of at least two γ chain loci on each chromosome and possibly more. It is suggested that probably three γ-chain structural loci on each chromosome direct the synthesis of γ-chain chains and one directs that of an Ay- chain, but these structural genes are not necessarily expressed equally in individuals with a heterozygosity for an allele of one of these genes. Following the report of Penwick (118) on the occurrence of wild type allele in man Steadman, Yates and Huenhs (136) suggested that the human β chain may be of two types; one representing a 'wild type' from which the mutant hemoglobin Bristol (β 67 Val → Asp)
arose and the second giving rise to a mutant Hb-M, Milwaukee (β 67 Val → Glu). Kattamis and Lehmann (71) reported that the two α-chain loci model best explains their observations of Hb-H and α thalassemia. Also the genetic pattern of inheritance of Hb Constant Spring (89) indicates two α chain loci. Evidence for the two structural α chain loci also comes from the study of a Hungarian family (31, 33). Boyer et al (27) described a normally silent hemoglobin 3α locus in gorillas and chimpanzees. The gorilla and chimpanzee 3α chains differ at only two residue positions. The 3α locus originated through gene duplication. It is formulated that in some common ancestor of apes, a regulatory nonsense mutation must have silenced the locus and in the present ape populations in few animals this mutation has been recurred. A complementary explanation has been offered by Goodman, Barnabas and Moore (49) to account for the wide distribution of the 3α locus. They content that a duplication producing the first 3α gene must have occurred just after catarrhine population split into basal cercopithecoids and hominoids. A transcription blocking mutation must have silenced the new gene which in time accumulated mutations. A crossing over between alpha and 3α genes in misaligned chromatids during synapsis can restore the activity of 3α locus in some animals, resulting in the production of alpha - 3α
hybrid chains.

Lepore hemoglobins offer evidence for yet another genetical mechanism which brings about a chemical heterogeneity in human hemoglobins. The three types of Lepore hemoglobins - hemoglobin Lepore - Hollondia (16,98), hemoglobin Lepore - Washington (45) hemoglobin Lepore - Baltimore (100) are composed of two normal \( \alpha \)-chains and two chains that are part \( \alpha \) and part \( \beta \)-chains (11,12,82). A nonhemologous crossing over between corresponding points of the \( \alpha \) and \( \beta \)-structural genes have been put forward to explain such a formation of \( \alpha \beta \)-chains (12,82). An unequal crossing over between homologous genes resulting in the deletion of one or more than one of the corresponding nucleotide base triplets may be the genetical cause of hemoglobin variants like hemoglobin Freiburg (\( \beta \); 23-Val deleted), (70), hemoglobin Leiden (\( \beta \); 6 or 7-Glu deleted) (38) and hemoglobin Gun Hill in which five sequential amino acid residues including the \( \beta \); 33-Cys are deleted (28,121). Hemoglobin C-George Town (46) and hemoglobin C-Harlem (25,26) are hemoglobin variants with two substitutions in a single polypeptide chain. The replacement of glutamyl residue by valyl residue in position 6 and that of an aspartyl residue by an asparaginyl residue in position 73 mark the structural abnormalities of Hb-C-Harlem. Probably a second mutation in the gene for hemoglobin S produced a gene resulting in
the hemoglobin-C-Harlem. A homologous crossing over within the \( \beta \)-chain loci between determinants \( \beta-6 \) and \( \beta-76 \) has also been postulated as the possible mechanism (25,26). Gene duplication could explain the intra species heterogeneity of the \( \alpha \)-chains of buffalo (13,115) goat and barbary sheep (59,142) and \( \alpha \) and \( \beta \)-chains of some strains of mice (67,113,122). In 1965 Balani and Barnabas (13) reported for the first time that the \( \alpha \)-chains present in the hemoglobins of water buffalo may be the products of a duplicated gene locus. Since then a number of animal species are known to possess duplicated \( \alpha \) chains in their hemoglobins. Among caprines goat and barbary sheep possess duplicated \( \alpha \)-hemoglobin genes. Duplicated \( IX \) and \( IIX \)-chains of goat hemoglobins differ at 4 residue positions (59). Barbary sheep hemoglobin has two \( \alpha \)-chains, \( IX \) and \( IIX \)-with close structural similarity with \( IX \) and \( IIX \)-of goat hemoglobins (142). These two \( \alpha \) chains are also the products of duplicated genes.

Structural studies on the \( \alpha \)-chain of hemoglobins of C3H strains of mice indicated a possible variation in position 62 and 68. \( \alpha-62\text{-Ile, } \alpha-68\text{-Ser; } \alpha-62\text{-Val; } \alpha-68\text{-Asn} \). These data along with other structural studies suggest that these \( \alpha \)-chains may be products of a closely linked nonallelic genes (113,123). The 'diffuse' and 'single' phenotypes of mice differ among different strains (119). A \( \beta \)-gene duplication of the \( \beta \)-structural
locus has been suggested to explain the 'diffuse' phenotype; one locus directs the synthesis of 80 percent Hb while the other directs the synthesis of 20 percent Hb (67).

Zuckerkandl and Pauling (148) suggested that up to an optimum number of duplications, the duplicated gene will be selectively retained with a structure identical to and a position near to the original gene. Beyond this point, duplicated gene will be progressively more strongly selected against. Such genes may be reduced to 'dormant genes', their activity being zero. These dormant genes may be reactivated through a change of the intracellular environment inducing the reactivation of the chromosomal region where the dormant gene is located.

A dormant gene - Hb- \( C_\beta \) gene - exists in caprinae species, which becomes activated when subjected to severe anemia. This activation of dormant \( C_\beta \)-gene also can be achieved, when these animals are injected with human urinary protein extracts with high erythropoietic activity (41,91). The occurrence of Hb- \( C \) is restricted to animals carrying an Hb- \( A \) gene. No such variant was detectable in sheep homozygous for a second adult hemoglobin type (Hb- \( B \)) after similar stress (23,91). Hemoglobin \( C_\beta \)-chain has also been detected in anemic moufflon and anemic barbary sheep. Results of limited structural studies suggest that these \( C_\beta \)-chains are closely related to the
$^\alpha$ chains, which are produced in the domestic sheep and goat during experimental anemia and rather different from the $^\alpha$ chain of certain non anemic barbary sheep (72). This non anemic $^\alpha$ of barbary sheep is not replaced by anemic $^\beta$ chain under anemic stress.

(ii) Hemoglobin Evolution

Studies of amino acid sequence of hemoglobins lead to the understanding of biological mechanisms of its genetics and evolution. Eukaryotes must have possessed genetic material to produce globin like proteins. Many invertebrates, protozoa, yeast and higher green plants also have oxygen binding globins. Based on the extensive structural and genetical studies of human hemoglobins Ingram (69) formulated an evolutionary scheme for hemoglobin genes. According to this widely accepted hypothesis duplication followed by mutations and translocations of a common ancestral gene played a vital role in the evolutionary history of present day hemoglobin genes. The primitive hemoglobin was a single polypeptide chain with heme group. Lamprey, a lower vertebrate still possess such a single chain hemoglobin. During the course of evolution the ancestral hemoglobin chain genes duplicated and slowly underwent mutations. These modified genes became distributed in chromosomal systems through translocations. Such events gave rise to separate $^\alpha$ and $^\beta$ chain genes. The tetramic form, normal to all
mammalian hemoglobins resulted from a mutal adaption of these chains. The β gene duplicated yielding genes for β-and γ-chain genes. The β gene again duplicated presumably recently in primate ancestry giving rise to a β gene and a δ gene. Both β and δ chains are found in normal adults. There appears to have been a γ-gene duplication just before the human - chimpanzee species divergence; man and chimpanzee possess the same two non allelic γ-chains.

Zuckerkandl and Pauling (102,147) trace back the hemoglobin-myoglobin relationship through evolution based on the number of differences in the various polypeptide chains. Evolutionary pattern and rates of molecular evolution in vertebrate globins have also been studied in detail by Barnabas, et al (17,47,48). According to these workers the average rate of evolution of α-chain genes is 13.8 per $10^3$ years per 100 codons as against that of β-chain genes which is 22.2 per $10^3$ years per 100 codons. These data indicate that following gene duplication, the duplicate evolve at a faster rate than the original.

IV. Nomenclature of Caprinae Hemoglobins

The following system of nomenclature based on the differences in the electrophoretic mobilities of hemoglobins when run at alkaline pH is being followed in the thesis.
(1) **Domestic goat (Capra species)**

The one type hemoglobin is referred to as Hb-A while the two type is referred to as Hb-AB, the slow moving component being Hb-B. Two type hemoglobin in which faster component having same mobility as Hb-A and the slow component having faster mobility than Hb-B is referred to as Hb-AX, since complete characterisation of this β variant hemoglobin is not yet done. Hemoglobin types in which the β-chain is of Cβ-type is referred to as Hb-C. Hb-C has faster anodic mobility than Hb-A.

(ii) **Mountain goat (Capra ibex sibirica)**

The major component with similar electrophoretic mobility as goat Hb-A is referred to as Hb-A and the minor component with faster mobility than Hb-A is, Hb-i.

(iii) **Nilgiri tahr (Hemitragus jemlahicus hylocrius)**

The major hemoglobin component is referred to as Hb-A while the faster minor component is Hb-T.

(iv) **Cashmere goral (Nemorhaedus goral goral)**

It has only one type hemoglobin.

(v) **Domestic sheep (Ovis aries)**

The one type hemoglobin with faster anodic mobility is referred to as Hb-A while the one type with slow mobility is Hb-B. The two type hemoglobin is referred to as Hb-AB.
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