CHAPTER IV

DISCUSSION

1. Heterogeneity of Haemoglobins

It is well established that alterations in the globin portion of the haemoglobin molecule are responsible for interspecies and intraspecies differences in vertebrate haemoglobins. Certain structural alterations in the haemoglobin molecule can directly be correlated with alterations of genetic loci that apparently control the globin synthesis and are incorporated into the structure at the time of synthesis. This resulted in the various haemoglobin types in human and infra-human species. Although, a vast number of animals are available for the study of their haemoglobins, we have undertaken a detailed comparative study of a few groups of animals representing both closely and distantly related species to human. This is done with a view to evaluate the range of variation in haemoglobin structure throughout the evolution. These groups involve two mammalian classes and one of avians. The two mammalian classes are primates and ruminants. Human haemoglobins, of course, serve as a model with which the structure of haemoglobins of the rest of the animals is compared.

Primates

From the various monkey species langur (Presbytes entellus)
and bonnet (Macaca radiata) which are common to this part of India were selected. In India, there are different types of langurs or Presbytes locally called as Hanuman, Himalayan, Madras, Malbar, Nilgiri, Phayres or Silvery, Purple-faced or Wanderoo, Red-bellied, Capped, Banded, Barbe's, Dusky, Rutledge's and Bear langur or Great Wanderoo. Out of these we have selected the common langur or Hanuman (Black faced monkey). The second type of monkey, the bonnet (Red faced monkey) is also common to Western and Southern part of India. Our survey of haemoglobin types from 21 langurs showed the variation in the number of haemoglobin components. Out of twentyone langurs about fourteen langurs showed the presence of two component haemoglobins, while the remaining animals contained only one haemoglobin component. All the seventeen bonnets surveyed, on the contrary, revealed the presence of two component haemoglobins in each animal. It may be noted that the preliminary survey work carried out by Barnabas and Goodman (personal communication) showed single band in twelve animals and double band in two animals. Several species of monkeys are known to have more than one haemoglobin type (22, 62, 96, 135, 141). The presence of several haemoglobins within an individual monkey or among monkeys of the same species has been reported. Similar finding has been reported in other primate species also (22, 96). M. speciosa has two haemoglobin components
in each and every animal examined (135). The haemoglobins of *M. nemestrina* and orangutans exhibit polymorphism (22, 62). Haemoglobin heterogeneity in the primates has been also reported for chimpanzee, *gibbon*, Java monkey and black ape (96).

**Ruminants**

A considerable amount of work has been done on ruminant haemoglobins. The results from these studies however are in conformity with our work (11, 17, 37, 102, 202, 201, 224, 225). In the present thesis we have compared the results of ruminant haemoglobins with those of primate haemoglobins.

Of the water-buffalo breeds, one pedigree herd namely 'Murrah' was selected. This is bred in large numbers in the Western part of Uttar Pradesh and Patiala State. This is also bred in considerable numbers at the Military Farms in Ahmednagar and Poona from where the blood samples were obtained. Our survey of haemoglobin types from twelve buffaloes showed the presence of two components in each animal. The presence of two component haemoglobins has also been observed in American buffaloes (*Bison bison*) (41).

Among cow breeds one pedigree herd namely 'Gir' was selected. As the name suggests, this breed probably originated in the Gir forest of India. The blood samples of cow were also obtained from Military Farm at Ahmednagar.
This breed shows polymorphism involving two haemoglobin types. Such a phenomenon has also been reported in many of the European (53, 54), British (19) and American breeds (81, 215, 227).

The 'Deccany' breed of sheep, as the name suggests, is from this part of Maharashtra. Blood samples of sheep were obtained from small farms and slaughter house. Our analysis showed that the Deccany breed shows polymorphism involving two component haemoglobins.

All the blood samples from goat surveyed were from three mixed breeds locally known as 'Lambkani', 'Kuri' and 'Mudi'. From the samples surveyed most of them were of the fast type, while some were of the mixed type. Thus, in general it appears that the goat haemoglobins occur either as two components together or one component singly, corresponding to the faster type. This is apparently true of the other Indian mixed breeds (87, 132). However, in two cases we have observed a third fast component having a higher anodic mobility (at alkaline pH) than the normal types (Fig. 6).

Avians

From avians we have selected three locally available species which included chick, duck and pigeon. Each of these species represents a separate natural order. Thus, chick represents Galliformes, domestic duck Anseriformes
and pigeon Columbiformes. The blood samples were obtained by bleeding the birds. Chick and duck showed the presence of one major and one minor haemoglobin in each and every bird examined. Pigeon, on the contrary revealed the presence of only one component. The presence of two haemoglobin components in chick has also been reported by other workers (177, 213, 91, 147). Matsuda and Takei (169) reported the presence of three haemoglobin components. White leghorn chick also showed the presence of three haemoglobin components (6). Hashimoto and Wilt (88) have observed five different haemoglobin types in chick. Two haemoglobin types in adult duck have also been observed in White Peking duck (35).

Thus in primates and ruminants, the haemoglobins exhibit a distinctive genetic heterogeneity. In genetic heterogeneity a distinction is made between situations in which members of a given species contain the same number of multiple haemoglobins as in case of pigeon and those in which there is a variation in the number of haemoglobin components in the members of the same species. The latter types where haemoglobins are polymorphic and allelomorphs are segregating in the population is found in various species such as langurs, bovine and sheep. In view of our limited survey it is difficult to ascertain whether or not the goat species exhibit polymorphism in
their haemoglobins, since the slow haemoglobin type referred to as Hb-1 did not occur singly. Avians in general exhibit a minor component heterogeneity. Chick and duck for example contain a minor haemoglobin component along with a major one.

2. Structural Basis for Genetic Heterogeneity

(i) Electrophoresis and Column Chromatography

A comparative study of the relative mobilities of haemoglobins of primates, ruminants and avians by paper electrophoresis using veronal buffer pH 8.8 is presented in Fig. 4. From this figure it is evident that the haemoglobins of langur, bonnet and buffalo do not separate into two components. The haemoglobins of these three species have identical anodic mobility and migrate as a single homogeneous component. The haemoglobins of these species, in addition, have comparable paper electrophoretic mobility with that of human. The haemoglobins of bovine, sheep, goat and avians, on the contrary, undergo a clear separation into two components. In case of bovine, there is a maximum net charge difference between Hb-B and Hb-A. Bovine Hb-A, Goat Hb-1 and Sheep Hb-A exhibit more or less same anodic mobility. Among ruminants, Goat Hb-2 has the least anodic mobility. Avian haemoglobins, in general, have less anodic mobility as compared to that of primates and ruminants. The major and minor haemoglobin components
of chick and duck have comparable mobility while pigeon haemoglobin has intermediate mobility.

Thus, it is apparent that fast moving haemoglobin component constitutes relatively a major fraction in the ruminants while a reverse is true in case of avians.

After a preliminary characterization by electrophoresis the component haemoglobins were isolated through CM-cellulose column chromatography. The typical elution patterns of primate, ruminant and avian haemoglobins are presented in Fig. 8. From this figure it is clearly seen that the haemoglobins of langur and bonnet are separated into two components, namely, Hb-1 and Hb-2. This separation into two components was not shown by electrophoretic procedures. Thus, the column chromatography is the only method to demonstrate the presence of two haemoglobin components in case of langurs and bonnets. An interesting correspondence is observed between the electrophoretic mobilities and elution pattern of different haemoglobin components on CM-cellulose columns. Usually the haemoglobins with higher anodic mobility at alkaline pH are eluted with buffer of low pH value. However, in ruminants, bovine Hb-A which has a higher anodic mobility than goat Hb-1 (slow moving component) requires buffer of higher pH value, (Table 4). This inconsistency could probably be explained on the basis of differences in solubility (257).
Thus, the results presented in Fig. 8 and Table 4 indicate that human Hb-A, langur Hb-1 and bonnet Hb-1 are the major haemoglobins and are eluted at the same pH. Likewise, langur Hb-2 and bonnet Hb-2 have the same pH of elution. In bonnets the relative percentages of Hb-1 and Hb-2 remain the same within the experimental errors in all the animals examined. In langurs, on the other hand, the relative percentages of Hb-1 and Hb-2 vary in different animals. The percentage of Hb-1 varies between 60 to 40 percent while that of Hb-2 varies between 40 to 60 percent. A similar observation is made in case of M. speciosa where the proportion of two haemoglobins is not the same in different individuals (32).

The presence of shoulder in the elution pattern of bonnet haemoglobin is clearly seen in Fig. 8. This shoulder is observed in case of all the bonnet haemoglobins surveyed. It constitutes approximately 10 to 15 percent of the total haemoglobin. It was difficult to collect this small fraction in pure form i.e. free from Hb-1. We have collected the Hb-1 fraction along with this small fraction.

In primates and ruminants the major haemoglobin component has the faster rate of elution on CM-cellulose column. In avians, on the other hand, the minor haemoglobin component has the faster rate of elution.
(ii) Subunit Evaluation

After isolation of component haemoglobins, globins were prepared in the usual manner for further evaluation. The separation of polypeptide chains of globins was done by paper electrophoresis in veronal buffer pH 8.1 using 6 M urea.

Fig. 9 represents the comparative mobilities of polypeptide chains of human, monkey and ruminant haemoglobins. Under these conditions, the polypeptide chains migrate towards the cathode, $\alpha$-chains having the greater cathodic mobility than the $\beta$-chains.

Primates

As in case of human haemoglobins, the globins of langur and bonnet also undergo dissociation into $\alpha$ and $\beta$-chains. The $\alpha$ and $\beta$-chains of langur Hb-1 have comparable mobilities with those of langur Hb-2. Likewise, the mobility of $\alpha$ and $\beta$ subunits of bonnet Hb-1 corresponds to those of bonnet Hb-2. Thus, no mobility differences in the subunits of langur and bonnet haemoglobins are apparent with this electrophoretic procedure. The inter-species comparison of the electrophoretic mobility of the $\alpha$ and $\beta$ subunits of human, langur and bonnet haemoglobins do not reveal any characteristic differences.

The $\alpha$ subunits of human, langur and bonnet exhibit the identical electrophoretic mobility. A similar pattern
is also shown by the β subunits of the haemoglobins of all these three species. From these results it is apparent that haemoglobins of human, langur and bonnet may be structurally similar.

Ruminants

In case of water-buffalo the α subunit of Hb-1 is referred to as α\textsuperscript{I} and that of Hb-2 as α\textsuperscript{II}. The mobility differences between the α\textsuperscript{I} and α\textsuperscript{II} subunits are clearly shown in Fig. 9. It appears that the β chains of Hb-1 and Hb-2 have identical mobility. The α-chains of the two bovine haemoglobins Hb-A and Hb-B have similar mobilities while the non-α-chains have differing mobilities. In case of sheep a similar situation as in bovine is seen. The polymorphic haemoglobins of sheep have α-chains of comparable mobility while the non-α-chains differ.

Goat haemoglobins present a different picture. The two haemoglobins Hb-1 and Hb-2 from the same animal have α-chains of different mobilities while the β-chains seem to be similar. Furthermore, the α-chain of Hb-3 has a comparable mobility with that of Hb-2 but its β-chain is different from that of either Hb-1 or Hb-2.

Avians

In case of avian haemoglobins, the globins could not be resolved into subunits by the above paper electrophoretic procedure. Hence the globins were subjected to starch gel
electrophoresis in Na-formate buffer pH 1.8. Under these conditions the polypeptide chains migrate towards the cathode with α-chains having the highest cathodic mobility. In case of chick the electrophoretic mobilities of the α- and β-subunits of haemoglobin major and haemoglobin minor are different (Fig. 10a). The electrophoretic mobilities of the α and β subunits of the major haemoglobins of chick, duck and pigeon have comparable mobilities (Fig. 10b).

(iii) Structural comparison of Globins and their Subunits

The intra-species and inter-species comparison was further carried out by evaluation of fingerprints of S-aminomethyl derivatives of globins and their subunits.

Primates

Langur

Examination of the globins of langur Hb-1 and langur Hb-2 for their colour tested fingerprints and their peptide pattern make-up reveals no differences indicating that these two haemoglobins are mostly similar. Differences, if any, must be confined to the neutral peptides only. Alternatively, the differences may involve the tryptic insoluble core peptides or substitution of similarly charged groups which do not show net charge differences.

The peptide patterns of the α and β subunits of the langur haemoglobins Hb-1 and Hb-2 show that these two haemoglobins have similar α-chains while the β-chains differ (Fig. 15).
Fig. 13  Fingerprints of aminoethylated globins of Human Hb-A, Langur Hb-1 and Bonnet Hb-1.
Electrophoretic buffer: Michl's buffer pH 6.4.
Fig. 14 TRACING OF FINGERPRINTS OF AMINOETHYLATED GLOBINS OF HUMAN Hb-A, LANGUR Hb-1 & BONNET Hb-1.

* ELECTROPHORETIC BUFFER - MICHL'S BUFFER (pH 6.4), CHROMATOGRAPHIC SOLVENT - PYRIDINE: Α-BUTANOL: ACETIC ACID: WATER [80:120:36:144 %].
* FINGERPRINTS WERE STAINED FOR SULPHUR (S), TRYPTOPHANE (TR), TYROSINE (TY), ARGinine (A) & HISTIDINE (H).
Fig. 15  Fingerprint of Langur Hb-1β and Langur Hb-2 β.

Electrophoretic buffer: Michl's buffer pH 6.4
Fig. 16 TRACING OF FINGERPRINTS OF BONNET Hb-1/3 & BONNET Hb-2/3.

- ELECTROPHORETIC BUFFER: MICHL'S BUFFER (pH 6.4), CHROMATOGRAPHIC SOLVENT: PYRIDINE,
- n-BUTANOL, ACETIC ACID, WATER (180:20:14A V/V/V).
- FINGERPRINTS WERE STAINED FOR SULPHUR (S), TRYPTOPHANE (TR), TYROSINE (TY), ARGinine (A) & HISTIDINE (H).
Fig. 17 TRACING OF FINGERPRINTS OF HUMAN Hb-A\textsubscript{c}, LANGUR Hb-1\textsubscript{c} & BONNET Hb-1\textsubscript{c}.

- ELECTROPHORETIC BUFFER—MICHL'S BUFFER (pH 6.4), CHROMATOGRAPHIC SOLVENT—PYRIDINE:A-BUTANOL:ACETIC ACID:WATER (180:120:36:144 \%).
- FINGERPRINTS WERE STAINED FOR SULPHUR (S), TRYPTOPHANE (TR), TYROSINE (TY), ARGinine (A) & HISTIDINE (H).
Fig. 18 Tracing of fingerprints of human Hb-A/β, langur Hb-1/β & bonnet Hb-1/β.

Electrophoretic buffer: Michl's buffer (pH 6.4), Chromatographic solvent: pyridine: butanol: acetic acid: water (80:120:36:144 vol%).

Fingerprints were stained for sulphur (S), tryptophane (Tr), tyrosine (Ty), arginine (A) & histidine (H).
Differences in the \( \beta \)-chains are attributable to the position of histidine-positive peptide spot (marked by arrow in the figure). The second difference involves a histidine and tyrosine containing peptide which is strong in langur \( \text{Hb-2} \) \( \beta \) and faint in langur \( \text{Hb-1} \) \( \beta \). On the basis of these results it appears that the two haemoglobins in langur share a common \( \alpha \) chain and differ in \( \beta \) chains.

**Bonnet**

The fingerprints of bonnet \( \text{Hb-1} \) and \( \text{Hb-2} \) are completely indistinguishable. The peptide patterns of the \( \alpha \) subunits of these two haemoglobins are also similar while there are differences in their \( \beta \) chains (Fig. 16). The two \( \beta \) chains probably differ in one histidine-containing peptide (marked by arrow in figure). The second additional difference may involve the presence of a faint peptide in the far basic region in the bonnet \( \text{Hb-1} \) \( \beta \) subunit only. Thus, the bonnet haemoglobins, namely, \( \text{Hb-1} \) and \( \text{Hb-2} \) have similar \( \alpha \) chains and different \( \beta \) chains.

**Interspecies comparison of tryptic peptide maps of human, langur and bonnet haemoglobins**

Figures 11 to 18 present the photographs of fingerprints of soluble tryptic peptides from the amino-ethyalted derivatives of the different globins of human, langur and bonnet and their corresponding polypeptide subunits. Examination of the fingerprints of human \( \text{Hb-A} \) and langur \( \text{Hb-1} \) reveals that these two haemoglobins differ only in the
position of one tryptic peptide containing tryptophane. This differing peptide is a $\beta$-Tyr one (Figs. 11 and 12). This is confirmed by studying the tryptic peptides of the isolated $\beta$ chains. This difference is obtained clearly only by using Baglioni's chromatographic solvent system involving pyridine - isoamyl alcohol and water (35:35:27). The interspecies comparison of the tryptic peptide maps of human, langur and bonnet is shown in Figs. 13 and 14. In this case pyridine:butanol:acetic acid : water (180:120:36:144) is employed as a chromatographic solvent.

In this system the tryptic peptide maps of human and langur haemoglobins are almost identical except for the slight difference in the position of TR-containing peptide. On the contrary, the fingerprint of bonnet haemoglobin can be clearly distinguished from those of human and langur in the position of the TR-containing peptide. In bonnet haemoglobin, the TR containing peptide is just above the His-Tyr-containing $\alpha$ chain peptide. The second difference involves the presence of a strong peptide in the neutral region present in bonnet Hb-1. The position of the two peptides, one containing His and other containing sulphur and His may be an additional point of difference in human, langur and bonnet haemoglobins.

Comparison of the homologous polypeptide chains of human, langur and bonnet shows that the respective
α and β chains of these three species are different (Figs. 17 and 18).

**Ruminants**

**Buffalo**

Examination of the fingerprints of soluble tryptic peptides of aminopropylated buffalo Hb-1 and Hb-2 reveals that these two haemoglobins may share a β chain in common. This is confirmed by studying tryptic peptide maps of the isolated chains. The β chain fingerprints of Hb-1 and Hb-2 were indistinguishable. The aminopropylated derivatives of α chains showed differences in two tryptic peptides (Fig. 19), one involving tryptophane. The total number of tryptophane-containing peptides in the fingerprints of the two α chains was, however, the same. The tryptophane positive peptide near the basic region of the fingerprint of α II subunit served as a marker to identify this polypeptide chain. On the basis of these results, it is probable that the two α chains may differ in two or more amino acid residues.

**Bovine**

Examination of the globins as well as the polypeptide chains of bovine Hb-A and bovine Hb-B for their colour tested fingerprints and peptide pattern make up reveals that these two haemoglobins may share the α chains while β chains are different (Fig. 20). Beta chains are differing by the presence of an extra peptide spot in the near basic
Fig. 19  Fingerprint of Buffalo Hb-1 α and Buffalo Hb-2 α.
Electrophoretic Buffer: Michl's buffer pH 6.4
Fig. 20 Tracing of fingerprints of Bovine Hb-A and Bovine Hb-B. 
Electrophoretic buffer: Michl's buffer pH 6.4. 
Fingerprints were stained for sulphur (S), tryptophane (TR), tyrosine (Ty), arginine (A) and histidine (H).
Tracing of fingerprints of Sheep Hb-A and Sheep Hb-B.

Electrophoretic Buffer: Michl's buffer pH 6.4

Fingerprints were stained for sulphur (S), tryptophane (TR), tyrosine (Ty), argine (A) and histidine (H).
Fig. 22 Fingerprint of Goat Hb-1 α and Goat Hb-2 α.
Electrophoretic Buffer: Michl’s buffer pH 6.4
region of bovine Hb-A pattern (marked by arrow), besides other probable minor differences. This peptide is absent in bovine Hb-B. Since the extra tryptic peptide spot in Hb-A did not show arginine on colour test, it is likely that lysine may be involved in this difference. On the basis of this it appears that the differences between the two β chains may be due to two or more amino acid residues involving lysine. These results are recently confirmed by Schroeder et al. (225) who have reported a comparison of amino acid sequence in the β chains of bovine Hb-A and Hb-B. According to these workers, there are at least three differences in the amino acid sequence of these two haemoglobins. They are:

<table>
<thead>
<tr>
<th></th>
<th>15</th>
<th>18</th>
<th>119</th>
</tr>
</thead>
<tbody>
<tr>
<td>β^B</td>
<td>Ser</td>
<td>His</td>
<td>Asn</td>
</tr>
<tr>
<td>β^A</td>
<td>Gly</td>
<td>Lys</td>
<td>Lys</td>
</tr>
</tbody>
</table>

Comparison of the homologous polypeptide chains of buffalo and bovine shows that the respective α and β chains of these two species are different. For example, the α chain of buffalo Hb-2 differs from those of bovine haemoglobins in four peptides, while the β chain of buffalo haemoglobins has at least two differing peptides from that of bovine Hb-A (Fig. 20).
Sheep and Goat

The peptide pattern of sheep Hb-A and Hb-B shows that these two haemoglobins have similar α chains (val chains), while there are multiple differences in their β chains (met chain) (Fig. 21). This is in agreement with the result reported by Muller (177). Huisman et al (106) have also reported that sheep haemoglobins Hb-A, Hb-B and Hb-C have identical α chains, while there are multiple differences in their β chains. Beal and Coworkers (25) have reported partial sequences of α and β chains of sheep Hb-A. Recently, Boyer et al (37) have proposed nearly complete sequence analysis of the β chains of Hb-A, Hb-B and Hb-C. The two β chains of Hb-A and Hb-B differ from each other in at least seven amino acid residues.

The inter-species comparison of the haemoglobins of sheep and goat indicates that the α chains of sheep haemoglobins may be identical with that of goat Hb-2. The goat Hb-1 differs from goat Hb-2 in the α chains while β chains may be similar (Fig. 22). On the other hand, goat Hb-3 has similar α chain as that of goat Hb-2, while β chains differ.

Avians

Figs (23-25) represent the peptide patterns of soluble tryptic peptides of the aminooethylated derivatives of chick, duck and pigeon haemoglobins. Examination of the colour tested peptide pattern make up of chick major and
chick minor haemoglobins reveals that these two
haemoglobins differ at least in six peptides. The
differing peptides are distributed in all the three
regions i.e., basic, acidic and neutral, of the fingerprint
indicating that all the four polypeptide chains may be
different. This result along with that of the subunit
evaluation by starch gel electrophoresis in Na-formate
buffer confirms the suggestion of Muller (177), that all
the four polypeptide chains in chick haemoglobin may be
different. These results are in contrast with those from
White leghorn chicken (147), where the $\beta$ chain is common
in both fractions while $\alpha$ chains are different, and
there is a clear difference in seven peptide spots while
three more spots are faint in component II and strong in
component I. In general, the fingerprints of the two
components showed that they possessed in common a neutral
backbone of an equal number of peptides (since they had
a common $\beta$ chain) and the majority of the peptides were
identical.

In case of duck haemoglobins the major and minor
components are varying in more than five peptide spots
(Fig. 24). The total number of neutral peptides in both
the haemoglobins are not similar. Also, the acidic
region of duck minor haemoglobin contains one extra
spot containing tryptophane.
Fig 23 TRACING OF FINGERPRINTS OF AMINOETHYLATED GLOBINS OF CHICK Hb-MAJOR & CHICK Hb-MINOR

- ELECTROPHORETIC BUFFER - MICHEL'S BUFFER (pH 6.4), CHROMATOGRAPHIC SOLVENT - PYRIDINE:
  9: BUTANOL, ACETIC ACID, WATER (60:100:30:46:4%)
- FINGERPRINTS WERE STAINED FOR SULPHUR (S), TRYPTOPHANE (TR), TYROSINE (TY), ARGinine (A) & HISTIDINE (H).
Fig 24 TRACING OF FINGERPRINTS OF AMINOETHYLATED GLOBINS OF DUCK Hb - MAJOR & DUCK Hb - MINOR.

- ELECTROPHORETIC BUFFER - MICHL'S BUFFER (pH 6.4), CHROMATOGRAPHIC SOLVENT - PYRIDINE:
  n-BUTANOL: ACETIC ACID: WATER (180:120:36:144) V/V.
- FINGERPRINTS WERE STAINED FOR SULPHUR (S), tryptophane (TR), tyrosine (TY), arginine (A) & histidine (H).
Fig. 25 Tracing of fingerprints of aminoethylated globins of CHICK Hb-Major, DUCK Hb-Major & PIGEON Hb.

- FINGERPRINTS WERE STAINED FOR SULPHUR (S), TRYPTOPHANE (TR), TYROSINE (TY), ARGININE (A) & HISTIDINE (H).
The interspecies comparison of the major haemoglobins of chick, duck and pigeon is given in Fig. 25. The differences are very clear from the figure. From these studies it appears that perhaps all the avian haemoglobins are quite different from each other, indicating how specific and complex the haemoglobins of different avian species are.

3. Genetic Basis for the Haemoglobins of Human and Infra-human Species

Primates

Molecular evolution stems from the alteration that takes place in the DNA molecule. These alterations, in general are of three types. The first of these is point mutations which often occur as replacement of one base-pair in a DNA molecule. This change could result in a codon that would code for a different amino acid resulting in a single amino acid substitutions. The mutational mechanism is referred to as either transition if the exchange involves like-bases or a transversion if it involves a purine and a pyrimidine. This type of change is mostly found in abnormal human haemoglobins. Allelism (199) and non-allelism (231) of genes controlling the synthesis of abnormal haemoglobins are also known in man. Multiple but random point mutations occur in the animal species. The second type of alteration in the DNA molecule is duplication of DNA double strands, either as entire
chromosomes, as genes or as segment of genes. Crossing over and recombination may be the principal mechanisms involved in the later two cases. Gene duplication is a common phenomenon in the evolution of haemoglobins. Ingram (117) has proposed an evolutionary scheme in which the gene for a primitive myoglobin-like protein, by a series of duplications, gave rise to the genes for $\alpha$, $\gamma^0$, $\beta$ and $\delta$-chain precursors. The most recently discovered chain, the $\epsilon$-chain (101) is characteristic of early human embryos and probably originated at a time near that of origin of the $\alpha$-chain. As a result of these repetitive duplications, there now appear to be at least six different loci in man derived from the original gene. Furthermore, the close linkage of genes responsible for the synthesis of $\beta$ and $\delta$ chains has been established (113). Crossing over within a pair of genes would produce hybrid genes ($\beta$-$\delta$ in one DNA strand and $\delta$-$\beta$ in the other) and also deletion of both genes from one of the strands. Such products have actually been observed in Lepore haemoglobins (12,20). This type of haemoglobin was found to have a $\delta$-like amino terminal portion and a $\beta$-like carboxy terminal portion. The third type of event is deletion of portion of DNA strands. All these types of alterations of the DNA molecule apparently have occurred during the evolution of primates and ruminants (Artiodactyla).
The genetic control of haemoglobins in man is fairly well established. The normal genes permit the synthesis of normal polypeptide chains and an individual is a homozygote for haemoglobins $A, F$ and $A_2$. The individual who synthesizes both haemoglobins $A$ and $S$ is a heterozygote and has inherited from both parents the ability to make normal $\alpha$, $\gamma$, and $\delta$ chains but from one parent the ability to make $\beta$ chains and from the other $\beta^S$ chains.

The nature of genetic control of monkey haemoglobins appears to be on similar lines as that of human. The general survey of monkey haemoglobins shows a wide diversity of genetic control as in man. Like in man, langurs and bonnets exhibit heterogeneity in their haemoglobins.

The presence of more than one haemoglobin component within an individual monkey or among monkeys of the same species has been reported. Similar findings have been observed in other primate species also (22, 62, 96, 135, 141). Several explanations have been proposed to account for the haemoglobin heterogeneity in primates. The haemoglobin heterogeneity may be due to the occurrence of either multialleles, resulting perhaps from duplication or mutation of alleles (183).

In most primate haemoglobins, the structural differences in the $\beta$ chains have been responsible for the haemoglobin polymorphism. This appears to be the case in langur and bonnet haemoglobins where the polymorphism
is observed. The two component haemoglobins of langurs and bonnets appear to be the product of similar $\alpha$ genes and allelic $\beta$ genes.

In some M. irus (245) the heterogeneity of haemoglobins seems to result from both nonallelic and allelic mutations. The three haemoglobins out of which two are major and one is minor - in M. irus appear to share a common $\beta$ chain but have different $\alpha$ chains. The $\alpha$ chains of haemoglobin - fast and slow appear to be the products of alleles, but the $\alpha$ chain of haemoglobin minor component is not a product of alleles for either of the other two $\alpha$ chains. The $\alpha$ chain of haemoglobin minor component has been proposed to be the product of duplication of $\alpha$ cistron.

In M. speciosa, the two haemoglobins which are present in all the animals, differ in $\alpha$ chains, $\beta$ chains being similar (135, 133). The two structural loci have been proposed for the two $\alpha$ chains and a single locus for the $\beta$ chain. A possible explanation for the two $\alpha$ chains could be duplication of the single $\alpha$ cistron followed by a mutation in one of the resulting $\alpha$ cistrons.

It was interesting to note that both langur and bonnet monkeys did not show a minor component comparable to $\text{Hb-A}_2$ of human. However $\text{Hb-A}_2$ has been found in Apes and New-World monkeys (141). In man, $\text{Hb-A}_2$ usually is about 3 percent of the total haemoglobin while in New-World primates it ranges between 0.6 to 6 percent (36).
Boyer et al. (36) have further suggested from comparisons of peptide compositions that the \( \delta \) gene of Hb-\( A_2 \) arose in a common ancestor to man and New-World monkeys.

Based on the above, the distribution of \( \beta \) genes and \( \delta \) genes can schematically be given as in Fig. 26. Since the New-World monkeys split from the common ancestor Anthropoidea, much earlier than Old-World monkeys, it appears that either (i) \( \delta \)-gene is present in all the primates descending from Anthropoidea and that it is silent in New-World monkeys, or (ii) \( \delta \)-gene arose by duplication followed by mutation, from the \( \beta \)-gene in Hominoids and Ceboids. In other words, \( \delta \)-gene of New-World monkeys arose by the duplication of the ancestral Ceboid \( \beta \)-chain. Likewise, the \( \delta \)-gene of man, chimpanzee and gorilla arose by duplication of the ancestral \( \beta \)-gene of Hominoids.

The interpretation of the available sequence data throws some light on the problem, even though conclusive answers cannot be given at this stage.

This is summarised in Table 5a which gives the number of differences between sequences of \( \beta \) and \( \delta \)-chains of Ceboids and Catarrhins (67). From this Table it is evident that:

1. The \( \beta \) chains of man and chimpanzee are identical while gorilla differs by one amino-acid substitution from man.
ANTHROPOIDEA

β & δ LIKE LINKED GENES

PLATYRRHINI

CEBOIDEA
(New World monkeys)
include Spider monkey,
Tamarin, Squirrel monkey.

BOTH β LIKE AND δ LIKE CHAINS PRESENT

CERCOPITHECOIDEA

CERCOPITHECOIDEA
(Old World monkeys)
includes Rhesus
monkey, Langur and
Bonnet.

ONLY β LIKE CHAIN PRESENT

HOMINOIDEA

BOTH β LIKE AND δ LIKE CHAINS PRESENT

CATARRHINI

HOMINOIDEA
(Includes Man, Chimpanzee and Gorilla)

Fig. 26 β and δ like genes in Higher Primates.
**Table 5a.**

**BETA-TYPE HAEMOGLOBIN CHAINS**

**NUMBER OF AMINO ACID DIFFERENCES BETWEEN SEQUENCES**

<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Gorilla</th>
<th>Spider Monkey</th>
<th>Human</th>
<th>Spider Monkey δ</th>
<th>Rhesus Monkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Human</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>10</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>β-Gorilla</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>11</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>β-Spider Monkey</td>
<td>6</td>
<td>7</td>
<td>0</td>
<td>10</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>δ-Human</td>
<td>10</td>
<td>11</td>
<td>10</td>
<td>0</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>δ-Spider Monkey</td>
<td>9</td>
<td>10</td>
<td>7</td>
<td>9</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>β-Rhesus Monkey</td>
<td>8</td>
<td>7</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

* Taken from 'Atlas of Protein Sequence and Structure' by Dayhoff, M.O., 4, 22 (1969).
(ii) The human $\delta$-chains, rhesus $\beta$-chains and the Ceboid $\beta$- and $\delta$-chains have about 6 to 10 amino-acid differences from that of human $\beta$. This suggests that $\delta$-genes of human and Ceboids might have originated at the time of their common ancestor, Anthropoida.

(iii) The $\delta$- and $\beta$-chains of Ceboids are closer to one another than either of them to human $\beta$ or $\delta$-chains. This is also evident from the recent sequence determination of $\beta$ and $\delta$-chains of Spider monkey, Squirrel monkey and Tamarin (36).

Thus, on one hand, it is possible that the $\delta$-gene of Hominoids and Ceboids arose in a common ancestor of man and New-World monkeys. On the other hand, it is also possible that $\delta$-gene of man, chimpanzee and gorilla arose at the time of separation of Catarrhins and that the $\delta$-gene of Ceboids arose in the Ceboid line.

Another aspect of Hb-$A_2$ which is becoming important in recent years is that the $\delta$-gene is neutral in the face of Natural Selection. This is supported by the fact that it is present in small quantities and also that no function has yet been assigned to Hb-$A_2$. The idea that many mutations could be selectively neutral becomes evident when we look at the genetic code. It appears that a large proportion of all base changes in the codon do not result in amino acid substitutions because of the degeneracy of the genetic code.
Likewise, a study of the nature of base-pairing between codon and anticodon suggests that pairing at the third position will display a certain amount of flexibility or wobble, resulting in certain non-standard base pairings. Thus most changes in the IIIrd position might result in synonymous codons. In addition a large percentage of total possible nucleotide changes apparently has no immediate harmful effect on the organism. To verify this, if one looks at the structure of abnormal haemoglobins, one notices that most changes in residues occurring on the exterior of the molecule appear to be harmless at least in the heterozygous state. In fact, among the 34 mutants examined by Perutz and Lehman (191) 48 had substitution which were selectively neutral.

Thus, Boyer et al. (36) suggest that Hb-A₂ may be neutral in the face of Natural Selection. This however may not be true. It is known that in Thalassemia minima, Hb-A₂ percentage is apparently doubled from 2.5 percent to 5.0 percent of the total. Hb-A₂ apparently has some Regulatory function to play.

Interpretation of the sequence data of a wide variety of polypeptide chains from primates alone could answer this.

Ruminants

The adult water-buffalo has two haemoglobins which differ in α chains while β chains appear to be identical.
Situations similar to this observation have recently been made in other animal species such as mice (207), rabbit (72), deer (136), horse (134), monkey (245) and goat (102) where two types of \( \alpha \) chains are present. Several explanations have been put forward to account for such type of variability in the amino acid sequence. One such explanation is that the two types of \( \alpha \) chains may be formed as a result of ambiguity in the translation of the genetic code. In this case, one codon codes for more than one amino acid. In other words, two different t-RNA's carrying different amino acids recognize the same codon. The \( \alpha \) chain variations in mice (207), rabbit (72), and horse (134) are particularly explained in the light of the above explanation. For example, in an inbred strain of mice (SEC) two types of \( \alpha \) chains are present. One type has a serine at position 68, while the other has a threonine residue at this location. This is apparently because the nucleotide triplet coding for the residue at 68 can be recognized both as a serine or a threonine codon by the t-RNA's responsible for the incorporation of these amino acids. That a particular triplet can code for more than one amino acid has also been shown in the case of suppressor of a nonsense triplet in alkaline phosphatase from E. coli (246, 247).

Another explanation that has been given to account
for the \( \alpha \) chain variations is that the two \( \alpha \) chains could have arisen as a result of gene duplication of the \( \alpha \) chain gene followed by mutations in one of the two genes. The scheme of gene duplication followed by mutation and translocation has been suggested earlier by Ingram (117) to account for the evolution of the four different types of polypeptide chains of human haemoglobins from a common precursor. This reasoning has been particularly extended to M.irus monkey haemoglobin in which a possible duplication of the \( \alpha \) chain gene is assumed to account for the presence of a polymorphic minor component with variant \( \alpha \) chains (245).

We propose that the two variant \( \alpha \) chains, \( \alpha^I \) and \( \alpha^II \) in water buffalo have arisen due to a duplication of \( \alpha \) chain gene. This appears to be the most satisfactory explanation. It is supported by the fact that the two variant \( \alpha \) chain genes are expressed together in each water buffalo from birth throughout the life span. It is this fact that made us to rule out the possibility that translational errors could probably be responsible for variant \( \alpha \) chains. The sequential studies of the differing peptides in buffalo alone will give insight to understand the exact nature of amino acid replacements. This has not been possible so far in our laboratory because of lack of equipment particularly an amino acid analyser.
The two bovine haemoglobins apparently are the product of identical α genes and allelic β genes because the latter substitute for each other in homozygous animals. A similar situation is observed in sheep where the two polymorphic haemoglobins are under the control of identical α genes and allelic β genes. But unlike that in man, where, in general, single amino acid substitutions are seen in normal and mutant haemoglobins, the two bovine β chains differ from each other by more than one amino acid substitution. This was earlier deduced by us based on the tryptic peptide pattern of the two β chains. This has recently been confirmed by Schroeder et al. (225) by sequence studies of these two polypeptide chains. Apparently in bovines, 3 amino acids glycine, lysine and lysine in β₁ are replaced by serine, histidine and aspargine respectively in β₂. Earlier, we had also shown that polymorphic haemoglobins of Indian sheep differ in multiple substitutions in β chain. This was in conformity with that of Muller (177) in Texel breeds. Recently, these observations have been substantiated by sequence studies and it is now known that β₁ chain and β₂ chains of sheep differ in as many as 7 amino acid residues. This again is a case of multiple substitutions in haemoglobin. Thus it becomes necessary to reconcile these apparent large differences in amino acid substitution with the current
picture of effects of mutations upon the primary structure of protein. The two alleles in each species would have to reflect a series of successive single nucleotide changes accumulated over a relatively large evolutionary time with selection favouring only the present two haemoglobin types present in these animals. Now that variant haemoglobins Hb-C and Hb-D have been known in bovines, it would be interesting to find out the extent to which they differ from normal bovine haemoglobins. A possibility that translational errors could be responsible at least in part, in different haemoglobin types in bovines or in sheep cannot be ruled out.

On the basis of Huisman's work (102) it appears that goat haemoglobins A and B, more or less, follow the same pattern of inheritance as sheep haemoglobins except that the differences exist in the \( \alpha \) chains rather than in the \( \beta \) chains. Moreover, the goat Hb-C like sheep Hb-C differs from the normal goat haemoglobins in \( \beta \) chain by multiple amino acid substitutions. Furthermore, goat Hb-C differs from sheep Hb-C by perhaps 1 amino acid residue. During our survey of over 15 Indian goats we have found that most of the goats occur as Hb-2 type (faster type) or as two components together with very low gene frequency. In our survey, we did not come across goat Hb-1 singly. Hence it is difficult to establish whether or not the Indian goats
exhibit polymorphism. However, it is probable that goat Hb-1 and goat Hb-2 correspond to goat Hb-A and goat Hb-B reported by Huisman (102). This is supported by the fact that the two haemoglobins of the Indian goats differ in \( \alpha \) chains and have similar \( \beta \) chains. Likewise, goat Hb-3 obtained by us in our random survey may be the same as goat Hb-C since the former differs from goat Hb-2 in the \( \beta \) chains, the \( \alpha \) chains being similar. There is yet another possibility that like in deer (136) which shows two \( \alpha \) chain variants and five \( \beta \) chain variants, goat also may exhibit different haemoglobin types.

Based on the number of amino acid differences between different animal species of ruminants (Order-Artiodactyla) (Table 5b). Dayhoff (67) has given gene phylogeny of \( \alpha \) and \( \beta \) chains (Fig. 27). From the figure it is evident that in \( \alpha \) gene phylogeny, after \( \alpha \) gene of Carp separated, the different \( \alpha \) genes separate from the ancestral gene in the following order: rabbit, mouse, primates, horse, bovine and sheep. From our work, we can further say that in the evolution of \( \alpha \) genes of Artiodactyla, in the line leading to water buffalo, \( \alpha \) genes have again duplicated giving rise to two \( \alpha \) genes. The duplication of \( \alpha \) gene in water-buffalo is surmised by the following reasons, in short.

Each and every water buffalo examined contained two haemoglobins differing in the \( \alpha \) chain. Likewise, these two chains are also present in the two fetal haemoglobins
Table No. 5b

BETA-TYPE HAEMOGLOBIN CHAINS

NUMBER OF AMINO ACID DIFFERENCES BETWEEN SEQUENCES*

<table>
<thead>
<tr>
<th></th>
<th>Fetal Bovine</th>
<th>A Sheep</th>
<th>A Goat</th>
<th>B Sheep</th>
<th>B Bovine</th>
<th>C Sheep</th>
<th>C Barbary Sheep</th>
<th>C Goat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal Bovine</td>
<td>0</td>
<td>24</td>
<td>24</td>
<td>25</td>
<td>23</td>
<td>30</td>
<td>27</td>
<td>29</td>
</tr>
<tr>
<td>A Sheep</td>
<td>24</td>
<td>0</td>
<td>4</td>
<td>7</td>
<td>16</td>
<td>16</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>A Goat</td>
<td>24</td>
<td>4</td>
<td>0</td>
<td>7</td>
<td>13</td>
<td>19</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>B Sheep</td>
<td>25</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>12</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>B Bovine</td>
<td>23</td>
<td>16</td>
<td>13</td>
<td>12</td>
<td>0</td>
<td>24</td>
<td>24</td>
<td>25</td>
</tr>
<tr>
<td>C Sheep</td>
<td>30</td>
<td>16</td>
<td>19</td>
<td>21</td>
<td>24</td>
<td>0</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>C Barbary Sheep</td>
<td>27</td>
<td>18</td>
<td>18</td>
<td>21</td>
<td>24</td>
<td>8</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>C Goat</td>
<td>29</td>
<td>15</td>
<td>18</td>
<td>21</td>
<td>25</td>
<td>1</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

* Taken from Atlas of Protein Sequence and Structure, by Dayhoff, M.O., 4, 22 (1969).
Fig. 27 Gene Phylogeny of α- and β-Genes in Vertibrates.

of water buffalo. Evidently the structural genes for two \( \alpha \) chains of water buffalo are present in each and every animal from birth through maturity.

In the line leading to goat there has been a duplication of the \( \alpha \) genes giving rise to two \( \alpha \) chains present in goat Hb-1 and goat Hb-2 respectively. Huisman et al (103) have reported the results of structural evaluation of polymorphic goat haemoglobins. The \( \alpha^A \) and \( \alpha^I \) which are products of duplicated genes differ in at least 4 amino acids. An allelic haemoglobin of goat with one difference in the \( \alpha \)-chain, has also been reported. This is referred to as goat Hb-B. Thus, so far in goat, three different types of \( \alpha \) chain genes have been observed. Two of them are non-allelic and one allelic.

We have, however, found only two types of \( \alpha \) chains, \( \alpha^I \) and \( \alpha^{II} \) which are probably products of duplicated genes. Thus in the \( \alpha \) gene phylogeny of Artiodactyla, there are a number of duplicated genes within species. Also, a case of an allelic \( \alpha \) gene in goat has been reported by Huisman et al (103).

When we look at the \( \beta \) gene phylogeny (Fig. 27), we find that, the \( \beta \) genes separate from the common ancestor in the following order: mouse, rabbit, primates, horse, foetal haemoglobins of bovine and sheep, 'C' haemoglobins of goat and sheep, bovine Hb-B, and sheep Hb-A and Hb-B.
This is, however, an incomplete phylogeny since the sequences of β chains of many other animals species are now known.

In the line leading to 'C' haemoglobins of goat and sheep, there has been a deletion of a portion of DNA strand in the C-locus, since 'C' haemoglobins of goat and sheep, apparently have 5 amino acids missing from the N-terminal end, when they are compared to adult haemoglobins of goat and sheep.

In the line leading to bovine, there is allelism of β genes. These two alleles, however, differ by multiple but random point mutations. Likewise in the line leading to sheep, there is also allelism of β genes. βA and βB genes of sheep also have multiple but random point mutations.

In the line leading to goat, in addition to the normal, there is also another β gene product, Hb-3. This haemoglobin is prevalent in Indian goats. From the fingerprint maps it looks as though the difference between β-3 and normal β of goat is restricted to a small number of amino acid residues. Thus, in Indian goats, there are at least five structural genes for αI, αII, β, βIII and γ chains.

Avians

In the avians, the multiple haemoglobins of each species appear to differ polytopically involving both the α and β subunits, the differences being more in the β chains. Muller (177) from the study of the nature of the
polypeptide chains in chick haemoglobins has suggested that these chains of the chick minor and major haemoglobins are probably non-allelic. Our work on chick haemoglobins is in conformity with Muller's work and we propose that the \( \alpha \) and \( \beta \) subunits in chick are products of non-allelic genes. Duck haemoglobins also appear to follow the same pattern as that of chick haemoglobins. Thus, from these studies it appears that perhaps all the haemoglobins are different from one another.

4. **Evolution of Haemoglobins (in general)**

Several proposals have been made to describe the evolutionary relationship among the genes that control the structure of polypeptide chains in the normal human and infra-human haemoglobins. The best mean for evaluating these proposals is by analysis of the sequence of the amino acids in polypeptide chains of haemoglobins. With this information homologies of chain structures as shown by correspondence between the sequence of amino acids in entire chains or portion of the chain held to establish the trend of evolution of the haemoglobin gene. In our present work we have chosen three different groups of animals two of which are mammals and one is of avians. The interspecies comparison of the haematological studies, fragility of erythrocytes, paper electrophoretic mobilities of haemoglobin, the elution pattern on CM-cellulose column,
resistance to denaturation by alkali, mobility of the 
polypeptide chains on paper electrophoresis and finally 
the tryptic peptide maps of the haemoglobins of human 
and infra-human species provides us with the valuable 
information about the evolution of haemoglobin. The 
two monkey haemoglobins, namely langur and bonnet, 
closely resemble human haemoglobins in all the above 
properties. As we go away from the primates to ruminants 
to avians to fishes and eels the differences in the 
structure and other properties become wider and wider. 
Thus, we find that the fingerprint map of buffalo 
haemoglobin and that of human involve at least 7 to 8 
different tryptic peptides. The fingerprints of avian 
haemoglobins are much more different as compared to that 
of human. And finally, the fingerprinting results (obtained 
by Dr. P. Ramakrishnan in our laboratory) of fishes and 
eels resemble least to that of human.