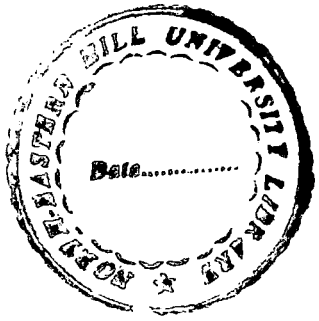
FUNCTIONAL AND CONFORMATIONAL STUDIES OF HEMOGLOBINS OF COW AND WATER BUFFALO



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A Thesis Submitted in Fulfilment of the Degree of

Doctor of Phiolosophy

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Certified that the thesis titled " FUNCTIONAL AND CONFORMATIONAL STUDIES OF HEMOGLOBINS OF COW AND WATER BUFFALO " submitted by Mr. ABANI K. BHUYAN for the award of the degree of DOCTOR OF PHILOSOPHY in Biochemistry of the North-Eastern Hill University, embodies the record of original investigations carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the degree of Ph. D.. This work has not been submitted for any degree of any University.

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PREFACE

This thesis documents a few structural and functional properties of normal adult human hemoglobin and of cow and buffalo hemoglobins. Both cow and buffalo hemoglobins have been chosen with an object of uniquely categorizing the bovine group with regard to conformational and functional allostery of hemoglobins. Human hemoglobin, an extensively studied system and which represents the group of intrinsically high oxygen affinity hemoglobin, has been chosen as a standard to compare and contrast the experimental results on bovine hemoglobins. Primary structural data on buffalo hemoglobin remained unavailable since the writing of this thesis began. Nevertheless, amino acid sequence of cow or ox hemoglobin has been used to discuss the results on the basis of phylogenetic relatedness of cow and buffalo.

The thesis consists of four chapters and two appendices. Chapter 1 concisely describes the mammalian hemoglobin tetramer and warrants the objective of this work. Chapters 2 and 3 deal with structural and conformational analyses of the three hemoglobins. In Chapter 4 detailed functional studies are presented.

Each chapter has been prepared to include an introductory section followed by materials and methods, results and discussion. The introductory section presents a brief review of past work on the subject of that chapter. Materials and methods have been described specifically. Data and figures are briefed in the section 'Results'. Findings, both consistent and contradictory, are presented in detail

under the discussion section. Each chapter carries a list of references cited therein.

A summary of critical roles played by the distal histidine (His E7) in structural and functional properties of hemoglobin is presented in Appendix I. This appendix also highlights the current application of ultrafast spectroscopy and expression of globin genes in E.coli. Finally, Appendix II details the electrophoretic methods.

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A NOTE ON NOMENCLATURE

Three-lettered abbreviations for amino acids have been used throughout, except in Figures 1.4 and 1.5 where amino acid sequences are shown using standard one-lettered form.

The position of an amino acid in the sequence is identified with the segment to which it belongs. For example, HisF8 stands for the histidine at the 8th position of F-helix. Similarly, PheCD1 identifies phenylalanine as the first residue of CD corner.

α or β preceds or follows an amino acid to indicate the subunit which the residue belongs to. For instance, αHisF8 indicates that the histidine belongs to α subunit. Likewise, MetNA2β would identify the methionine residue at the second position of N-terminal nonhelical segment of β subunit. This mode of designation of a residue, however, has not been strictly maintained. In many places words have been used. Thus, MetNA2β could also be written as MetNA2 of β subunit.

Abnormal hemoglobins have been mentioned either through words or by arrows to indicate the amino acid replacements, for example, Hb Hammersmith (βPheC7 $\longrightarrow$ Ser). This means, in Hemoglobin Hammersmith, the PheC7 of β subunits has been replaced by a serine.

Besides, the following short forms have been used in quite a few places:

Short form	stands for
HbO_2	oxyhemoglobin
deoxyHb	deoxyhemoglobin
NOHb	nitrosyl complex of hemoglobin
COHb	carbonmonoxyhemoglobin
CNHb	cyanomet derivative
AqHb	aqueomet form of hemoglobin
HbA	normal adult human hemoglobin
HbC	cow hemoglobin
Hb-A and Hb-B	the two phenotypes of cow hemoglobin
HbB	buffalo hemoglobin

Otherwise indicated, usual convention and notations have been followed throughout.

CHAPTER 1

INTRODUCTION AND OBJECTIVE

1.1. HEMOGLOBIN OF MAMMALS

The mammalian hemoglobin (MW=64,500) is a tetrameric respiratory protein of red blood cells composed of four subunits, namely α_1, α_2, β_1 and β_2. Each subunit consists of a polypeptide chain and a heme group. The heme complex in hemoglobin is iron protoporphyrin IX, illustrated in Figure 1.1. The α and β polypeptide chains normally contain 141 and 146 amino acids, respectively. The sequences are different in the two chains. There are eight helical sections labeled A through H in the β chain. The D-helix is absent in the α chains (see Figure 1.4). Some of the helical stretches are separated by nonhelical regions, while there are no intervening nonhelical links between other helices. Each chain also carries short nonhelical stretches at the N and C termini.

The heme establishes a major contact with the polypeptide chain by means of a covalent bond between the coordination site 5 of the heme iron and the $N_{\epsilon 2}$ nitrogen on the imidazole group of a histidine residue called proximal or heme-linked histidine, which is the eighth residue of the F-helix (HisF8). The heme is also in van der Waals contact with another histidine (HisE7) on the distal side (Figure 1.2). Furthermore, eighteen other amino acid side chains,

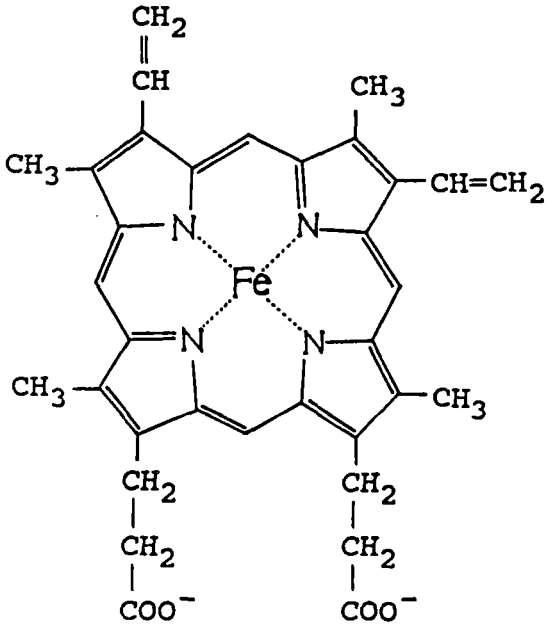


Figure 1.1. Ferrous protoporphyrin IX is the heme chromophore in hemoglobin. This isomer of protoporphyrin has four methyls, two vinyls and two propionates as side chain constituents.

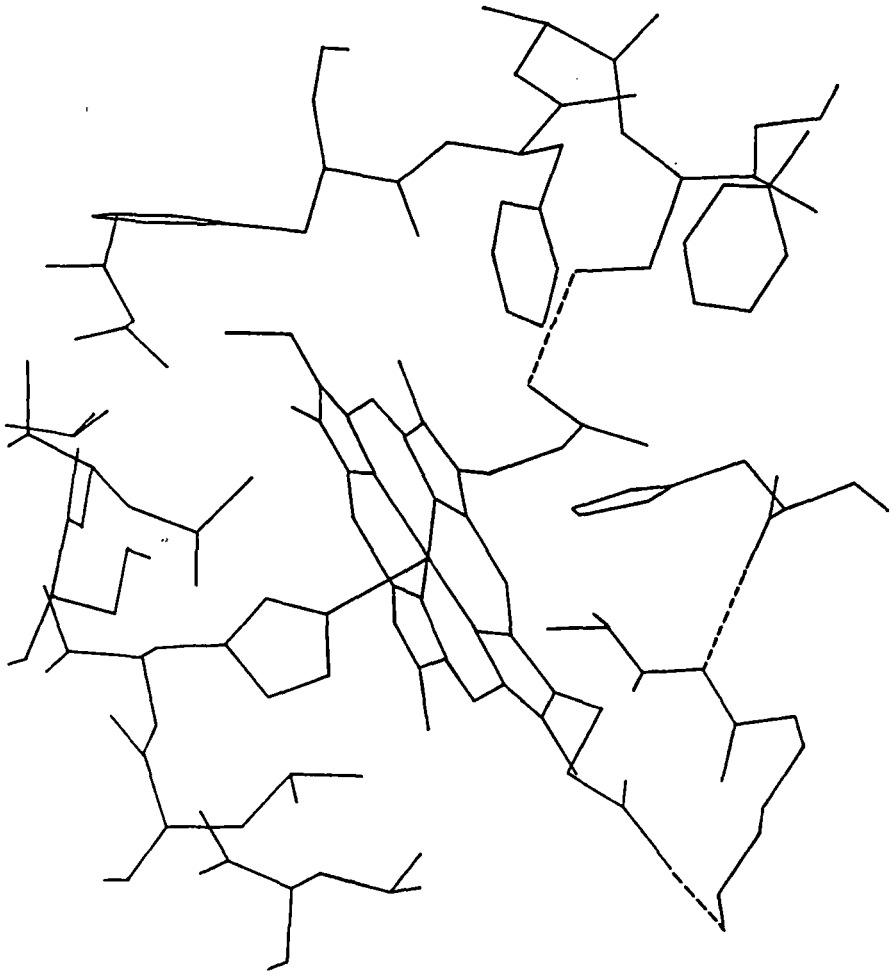


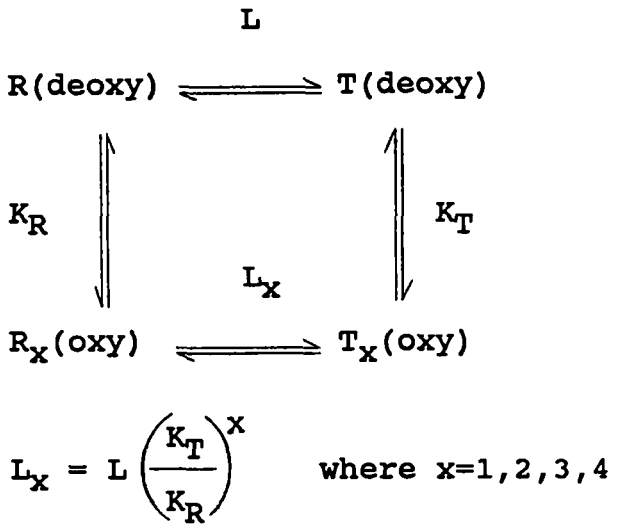
Figure 1.2. The heme group with surrounding residues of β chains of horse aqueomethemoglobin. The residues belong to the CD corner and E- and F helices. This stick diagram, which depicts the compactness of the heme pocket and some of the contacts between the heme and globin, has been reproduced after modification from Dr. Max Perutz's Croonian Lecture of 1968 (see ref. 15). For specific positions of Phe CD1, ValE11, HisE7 and HisF8, see Figure 1 of Appendix I.

most of which are nonpolar, make contacts with the heme. There is a hydrogen bond bridging the propionate side chain of the heme porphyrin with the side chains of HisCD3 of α subunits and LysE10 of β subunits (1-3).

The four subunits of the hemoglobin molecule are packed tetrahedrally by intersubunit nonpolar interactions, salt bridges and hydrogen bonds. The final shape of the tetramer is approximately spherical.

The heme ligands, also called homotropic ligands, bind at the sixth coordination position of the iron which is either in ferric or in ferrous state. Depending upon the ligand field splitting, each valency state may assume high spin or low spin state. Oxygen, carbonmonoxide, nitric oxide, alkylisocyanides, and nitrosoaromatic compounds form ferrous derivatives while H_2O, OH^-, F^-, and N_3^- complexes are ferric derivatives of the heme. Except deoxyhemoglobin (deoxyHb), in which the ferrous heme iron is five-coordinated, all other derivatives of hemoglobin are six-coordinated. Oxygen, the physiological heme ligand, combines reversibly with heme iron to form dioxygen complex. During this process, the high spin ferrous iron goes to low spin configuration with the same valency. The reaction of hemoglobin with oxygen is cooperative with 3.6 kcal/mol per heme as the free energy of cooperativity. The cooperative behavior is manifested in a sigmoid equilibrium curve for the reaction of hemoglobin with oxygen.

At the root of cooperative effect lies a structural concept, namely, an equilibrium between two different structures of the hemoglobin molecule, the R (relaxed) and T (tense) structures. The oxygen affinity of R form is larger than that of T. Their respective association constants with oxygen are K_R and K_T. For $R \rightleftharpoons T$, the equilibrium constant $L=T/R$ (scheme 1.1).



Scheme 1.1

Structural arrangements of the four subunits are different in R and T forms. In traditional nomenclature, the relaxed and tense forms of the individual subunits (i.e., tertiary structure) are labelled by r and t, respectively. The R ⟶ T transition is associated with a rotation of the $\alpha_1\beta_1$ dimer relative to the $\alpha_2\beta_2$ dimer by $12\text{-}15^0$ and a translation of one dimer relative to the other by 0.8 Å (1). During this transition, the contacts $\alpha_1\beta_1$ and $\alpha_2\beta_2$ remain rigid; however, the dimers move relative to each other at $\alpha_1\beta_2$, $\alpha_2\beta_1$, $\alpha_1\alpha_2$ and $\beta_1\beta_2$ contacts.

The allosteric or heterotropic ligands, namely, H^+, CO_2, Cl^-, and organic phosphates lower the oxygen affinity of hemoglobin significantly. More precisely, K_T varies as a function of all of these effectors, while execpt $[H^+]$ the other three ligands do not have much effect on K_R (4,5). These ligands bind to the globin part of the molecule to exert their effects. In deoxyHb, one molecule of organic phosphate salt bridges with an array of positively charged groups in a cavity between the two β chains (6-8). Any replacement or substitution of these amino acids in the β chains results in lesser interaction of organic phosphates

with hemoglobin.

Structural details of liganded and unliganded forms of hemoglobin and changes associated with R $\rightleftharpoons$ T transition have been published in great detail (see 3,4,9-23). Here we discuss briefly the stereochemistry of heme, the role of residues in the heme pocket and the functional role of heterotropic ligands, since they are more relevant to the subject of this thesis.

1.1.A. Stereochemistry of heme:

In deoxyhemoglobin the heme porphyrin is domed (24), so that the iron moves out of the mean porphyrin plane towards His F8. The porphyrin plane flattens up as oxygen binds to iron. This causes the iron to move toward the plane. The inward and outward movements of the iron are controlled by variable Fe-N_{porph} bond lengths and by constraints of the globin chains. As a consequence of toward plane movement of the iron, the distance between His F8 and porphyrin plane shortens by 0.5-0.6 Å in oxy- than in deoxyHb. The bond lengths and distances between planes which are used to describe heme stereochemistry can be found in reference 3. It is gratifying that many of the stereochemical changes in crystal structures of hemoglobin are consistent with those found in solutions probed by spectroscopic methods (see, for example, 25-28). A review of the literature also points out the fact that heme stereochemistry may be significantly different in different porphyrins and hemoglobin types. For example, nickel-porphyrin is planer and the Ni-$N_{\epsilon 2}$ (of HisF8) distance is 3.2 Å, which pushes the R $\rightleftharpoons$ T equilibrium towards right (3,27). In cobalt-porphyrin, the Co-N_{porph} bond is shorter by 0.1 Å than the Fe-N_{porph} bond. As a result, the displacement of Co from porphyrin plane is less than that in iron-porphyrin (29). In Hb Boston, an abnormal human hemoglobin where HisE7 of α subunits is replaced by Tyr

(30), the iron atom is linked to the tyrosine instead of HisF8 (31), and the allosteric equilibrium is strongly favored towards the T structure (32,33).

1.1.B. Stereochemical mechanism and the role of the heme:

The stereochemical mechanism first proposed by Max Perutz in 1970 (9) held that the oxygenation of hemoglobin is accompanied by structural changes in the subunits triggered by shifts of the iron atom relative to the porphyrin and, in the β subunits, also by the steric effects of oxygen itself. The salt bridges which constrain the oxygen-free form are broken by the energy of heme-heme interaction with the release of H^+. There have been much controversy regarding the role of iron displacement in governing R $\rightleftharpoons$ T equilibrium. However, experimental evidences still favor the distances of the iron and the proximal histidine from the porphyrin as the only determinant of allosteric equilibrium in the α subunits (3).

1.1.C. Role of residues on distal side of the heme pocket:

The heme pocket on the distal side is basically formed of PheCD1, HisE7, and ValE11 (Figure 1.2, see also Figure 1 of Appendix I). The role of PheCD1 in regulating the structure and function of hemoglobin is not definitively known. However, this near-heme-aromatic residue changes its orientation substantially with respect to the porphyrin ring in both α and β chains during R $\longrightarrow$ T transition (35). In R structure of α and β chains, the PheCD1 is in van der Waals contact with the carbons of porphyrin. In T form it swings away from the heme. Replacement or substitution of this residue, as in Hb Hammersmith (βPheC7 $\longrightarrow$ Ser) and Hb Bucuresti (βPheC7 $\longrightarrow$ Leu), leads to decrease in oxygen affinity and loss of cooperativity (36-38). It has been suggested that PheCD1 is directly involved in a charge transfer interaction with the heme so as to increase the electron density of the antibonding π^* orbitals of the

porphyrin rings in the R structure (39). Thus, this interaction has been assumed to contribute to the free energy of cooperativity. However, Perutz et al. (3) have argued that the distance between PheCD1 side chain and the porphyrin is 3.8 Å - 4 Å, a distance too large for charge transfer interaction to occur.

Displacement of the distal histidine and the valine is necessary for oxygen binding to the ferrous hemes of β chains (40). Valine imposes steric hindrance at the heme site of β subunits in T structure (9). The heme site of R structure is flexible enough to adjust the relative positions of the heme and ValE11. As a consequence, oxygen can bind favorably. HisE7, on the other hand, forms hydrogen bond with the bound oxygen (41,42). It also produces steric effect and acts as a gate for entry of ligands (43-46). The distal histidine, by swinging out of the way, makes an opening for oxygen to pass through, and this movement of the histidine requires a tertiary structural change (47). The displacements of HisE7 and ValE11 relative to the heme in T structure are initiated through a movement of the E-helix, which is brought about by tertiary structural change.

How HisE7 and ValE11 affect the oxygen equilibrium may be seen from oxygen affinity measurements with engineered hemoglobins (48-51). For example, replacement of the distal histidine by glycine lowers K_R leaving K_T nearly unchanged. On the other hand replacement of HisE7 by Val raises K_T and lowers K_R. When ValE11 is replaced by Ala, K_T rises with no change in K_R. Thus, these residues have individual contribution toward maintenance of the $R \rightleftharpoons T$ equilibrium. It is very important to note that HisE7 is seriously involved in structural and functional properties of hemoglobin. Appendix I at the end of this thesis is devoted to highlight this in finer detail.

1.1.D. Heterotropic ligands:

These *nonheme* ligands preferentially bind to the T structure and lower the oxygen affinity by stabilizing the molecule. The bound ligands are released during T ⟶ R transition. On going from deoxy to oxy form, hemoglobin liberates protons. In fact, the released H^+ come from certain ionizable amino acids, which are actively involved in hydrogen bonding in T structure. The H-bonds break right at the onset of oxygenation, and the protons are discharged (see, for example, 52-56). Conversely, hemoglobin binds H^+ on release of oxygen. The dependence of oxygen affinity of hemoglobin on protons is called Bohr effect. Release of H^+ on ligation at heme above pH 6.0 is called alkaline Bohr effect, and proton uptake below pH 6.0 is referred to as acid Bohr effect.

While hemoglobin binds H^+ ions on deoxygenation, the equilibrium of the reaction of HCO_3^- formation is pushed to the right (see below). Also, as is true with other proteins, CO_2 forms carbamino compounds with hemoglobin. Carbamino adducts are bound to ε-amino group of lysine and NH_2-terminals of α and β chains (see 57).

$$RNH_3^+ \rightleftharpoons RNH_2 + H^+$$
$$RNH_2 + CO_2 \rightleftharpoons RNH\text{-}COO^- + H^+$$
$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$
$$HCO_3^- \rightleftharpoons CO_3^{2-} + H^+$$

Binding of chloride ions to deoxyhemoglobin causes a decrease in oxygen affinity and an increase in Bohr effect (58-60). Four residues, namely, ValNA1 and SerH14 of α chains and LysEF6 and HisH21 of β chains are involved in Cl^- binding (61-71). Chloride also binds to COOH-terminal residues of both α and β subunits (72). In fact, binding of Cl^- to T structure raises the pK's of ValNA1α and LysEF6β, which contribute to Bohr effect (3).

DPG, a major constituent of mammalian red cell, enters a cleft between the two β chains and forms H-bonds with ValNA1, HisNA2, LysEF6 and HisH21 of β chains (8). A schematic sketch of the interaction between DPG and deoxyhemoglobin is shown in Figure 1.3. By stabilizing the structure of deoxyhemoglobin, DPG lowers the oxygen affinity of mammalian hemoglobins. It increases both the alkaline and acid Bohr effect (53). In other forms of vertebrates this function is performed by different organic phosphates. A comparison of the binding sites for Cl^- and DPG shows that these two heterotropic ligands exert their effect competitively. This competitive functional effect is important in regulation of oxygen affinity of mammalian hemoglobins as we shall see later.

1.1.E. Nonphysiological heme ligands:

Ferrous hemoglobin binds two gaseous ligands, nitric oxide and carbonmonoxide, and mainly two nongaseous ligands, namely, isocyanides and nitrosoaromatic compounds at the heme iron (73). Complexes of hemoglobin with these nonphysiological heme ligands have been very useful to elucidate the structure and mechanism of hemoglobin. Studies on these complexes have enabled to test models for the kinetics and structure of HbO_2. Moreover, certain experimental techniques, not easily applicable to HbO_2 can be applied to them. For examnple, nitrosylhemoglobin (NOHb) is the only known paramagnetic derivative of hemoglobin which is amenable to electron spin resonance spectroscopy (74-79). Furthermore, ultrafast laser spectroscopy has been successfully applied to NOHb, COHb, and HbO_2 to observe transient reaction intermediates, their structure and pathway and mechanism of ligand binding (80-85).

1.1.F. Stability of R and T structures and free energy of cooperativity:

In terms of MWC model of allosteric theory (86), the low oxygen affinity of T structure relative to R arises from

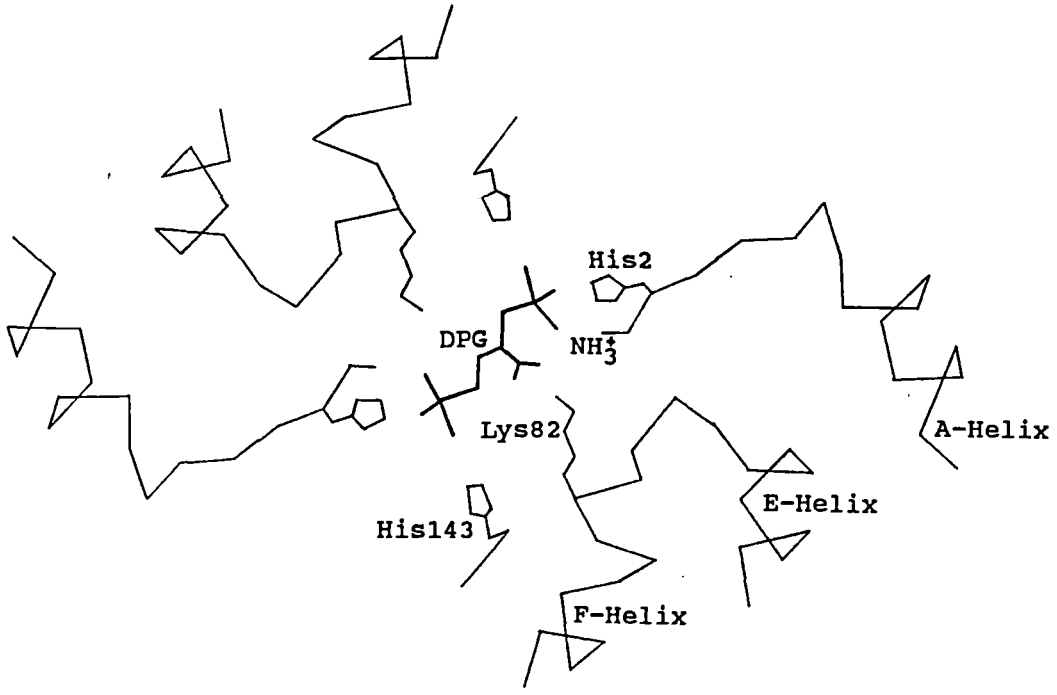


Figure 1.3. A stick diagram to show the cavity formed by the two β chains where DPG is positioned in deoxyhemoglobin. Stereochemical complementarity is notable. DPG forms salt bridges with ValNA1, HisNA2 and HisH21 of both β chains and with LysEF6 of one β chain. This sketch has been reproduced after modification from reference 8.

higher energy of the former. The additional energy comes from extra bonds between subunits in the quaternary T structure compared to those in R. The C terminal residues and DPG form 14 intersubunit salt bridges which are absent in the R structure (3). These additional salt bridges easily account for 14.4 kcal per tetramer, which is the free energy of cooperativity. Thus, it is clear that absence of these salt bridges would be compensated by a lower L and a higher K_T.

Although uncertainty prevails, the free energy of cooperativity is held to be located in both the heme and the globin in the form of strains. Nevertheless, extensive spectroscopic work suggests that the strain takes the form of stretch of the Fe-N bonds (see, for example, 87-95). If the iron atom cannot move to its optimum position on ligand binding, the strain induced is reflected in a reduced affinity for the ligand. It is noteworthy that the location of the strain seems to be different in different hemoglobin derivatives (3,96). Also, the relative strengths of the restraints of the globin and of Fe-N bonds appear to differ amongst different animal species (97-100).

1.2. BOVINE HEMOGLOBINS

Virtually all hemoglobins have the same chromophore, i.e., protoporphyrin IX, to which heme ligands bind. Yet there are diverse groups of hemoglobins which are structurally and functionally different. These differences originate from primary structural variations. In each of the four chains of vertebrate hemoglobins, there are seven invariable sites occupied by the same amino acids. These sites are mainly around the heme and in contacts between the subunits which are essential for allosteric switch between the T and R forms. Otherwise, hemoglobins from different species are not similar with respect to their primary

structure (see 101,102).

Bovine refers to a group of grazing animals which belong to the family Bovidae of the sub-order Ruminants of Artiodactyls. Bovine hemoglobins are functionally distinct and interesting. In fact, of the two group of mammalian hemoglobins, namely, intrinsically high and intrinsically low oxygen affinity hemoglobins, the bovine falls in the second category. Intrinsically low oxygen affinity hemoglobins respond little, or not at all, to the presence of organic phosphates. It was first observed by Bunn (103) that the oxygen affinity of cow hemoglobin is not lowered by 2,3 DPG. He pointed out that the red cells of ruminants have low concentration of DPG and their hemoglobins interact weakly with this allosteric effector. The reason for this weak interaction lies in a unique and common primary structure shared by all ruminant hemoglobins. A comparison of the amino acid sequences of human and ox hemoglobins is presented in Figures 1.4 and 1.5. There is a deletion of the N terminal residue in the β chains. This implies that bovine β chains have 145 amino acids instead of 146. Also, the next residue, which is histidine in the high oxygen affinity mammals, is methionine in bovine and in other ruminants (104). This has been known for quite sometime. Since both these N terminal residues form salt bridges with DPG, the deletion of the ultimate and replacement of the penultimate N- terminal amino acids have been assumed to be the reasons for diminished interaction.

However, even in the absence of organic phosphates bovine hemoglobins have markedly low oxygen affinity. What structural features regulate this behavior is not known. Certainly, it is the globin structure which plays the key role.

The genetic heterogeneity of bovine hemoglobins is rather divergent (105-108). Many breeds of cattle exhibit

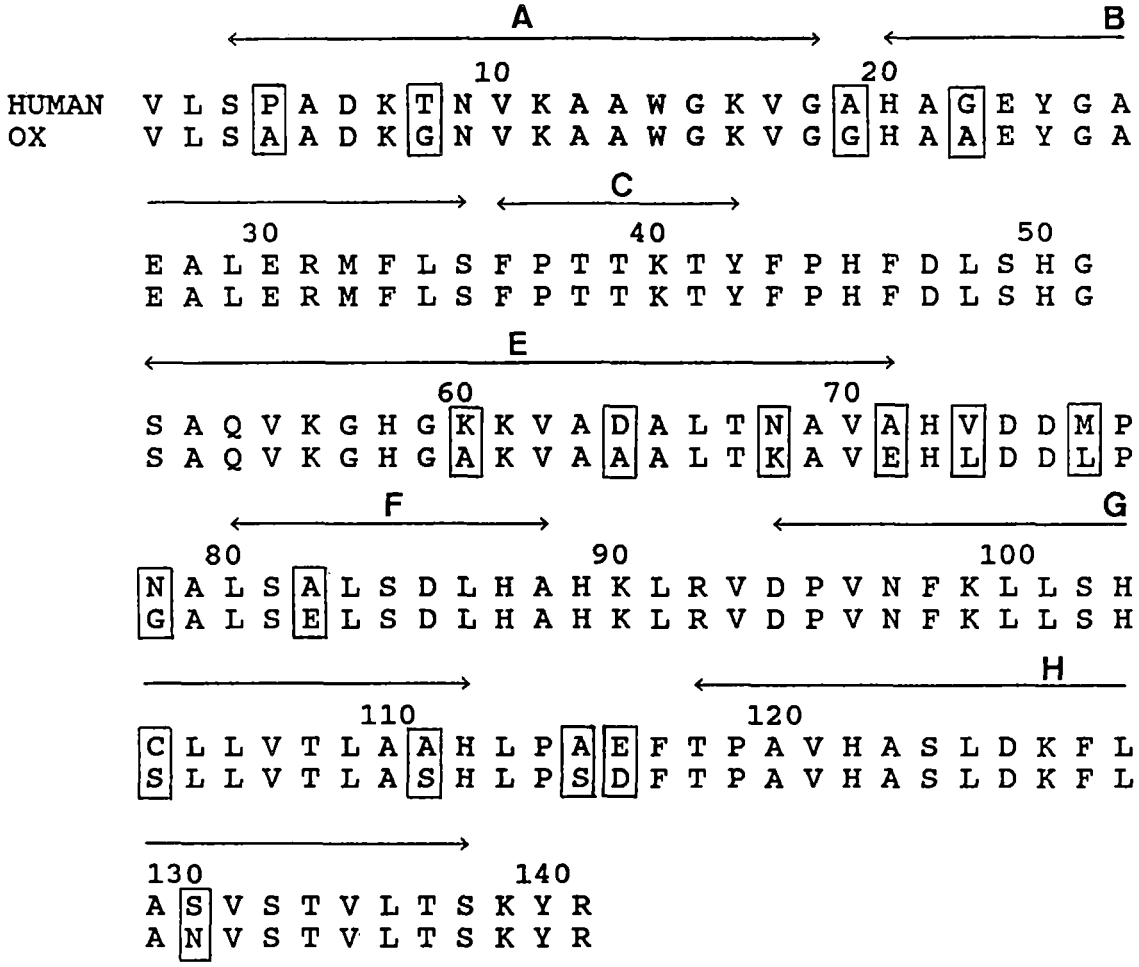


Figure 1.4. Comparison of amino acid sequence of α chains of human and bovine hemoglobins. α chains of both hemoglobins have 141 amino acids. Ox α chain differs from that of human at 17 different positions. Helical segments are marked with arrows above the human sequence. Helices are labelled from A through H. D-helix is absent in α chains.

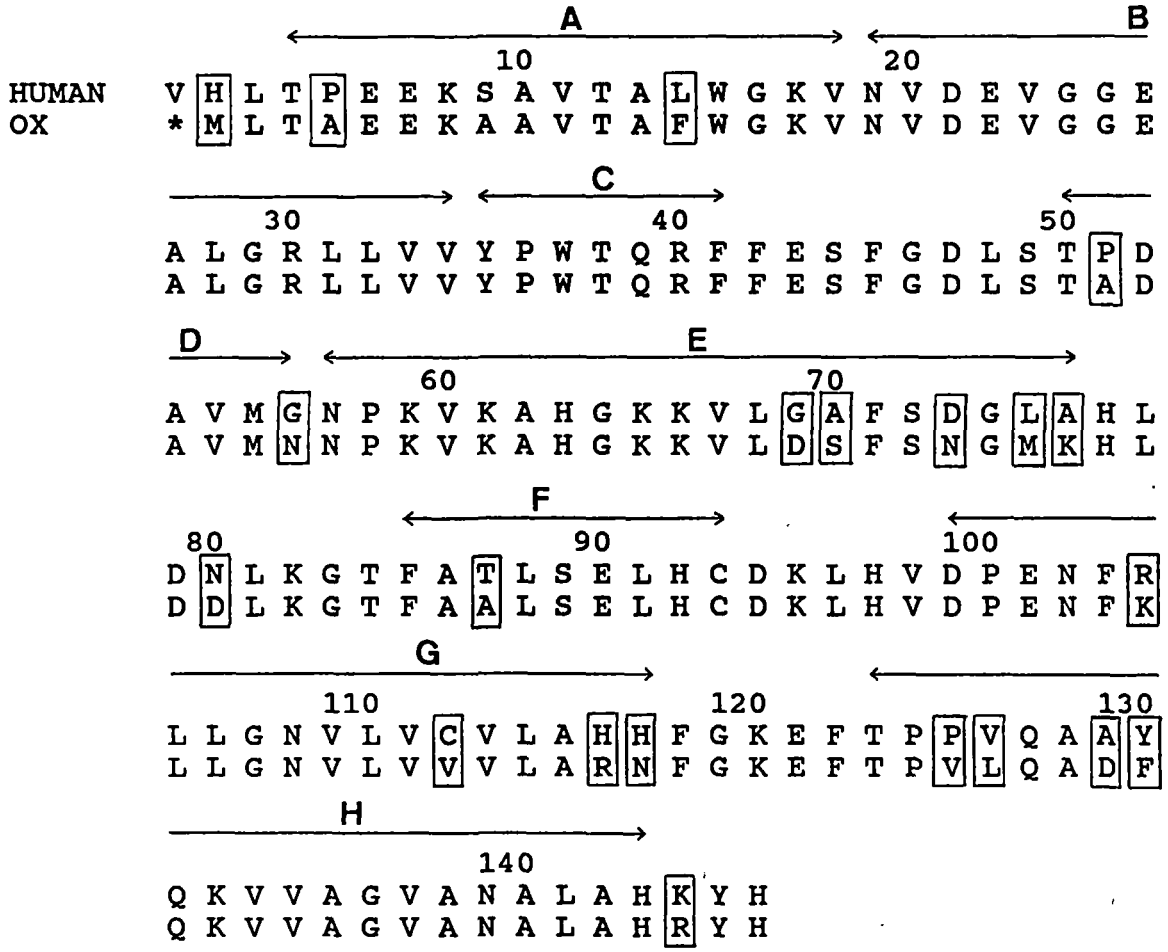


Figure 1.5. Comparison of amino acid sequence of β chains of human and bovine hemoglobins. Human β chain is composed of 146 amino acids. The N-terminal residue is deleted in all ruminants (indicated by *). The helical segments are marked from A through H.

polymorphism involving two hemoglobin types (109). Buffalo hemoglobins, on the other hand, exhibit monomorphism with two component hemoglobins (110-113). The component hemoglobins of cattle share a common α chain, and the genetic differences involve β chain gene locus. In case of buffalo hemoglobins, however, the α chains may be under separate genetic control (112). The nature of genetic control in bovine is unusual. While the human hemoglobin mutants involve single amino acid substitution, the phenotypes of bovine hemoglobins result from multiple random point mutations. It is worth while to mention that sheep and goat hemoglobins also show similar genetic heterogeneity.

It is not known what physiological advantage may be derived from such genetic constitution. The primary structural makeup of bovine hemoglobins might seem to be suited to other regulatory mechanism rather than the effect of DPG.

1.3. OBJECTIVE

Evidently, the underlying mechanism of functional regulation of bovine hemoglobins is puzzling. A number of important questions arise regarding this issue.

1. What is the status of the tertiary and quaternary conformation of the molecule? How stable are the T and the R structures relative to each other? In other words, does a unique $R \rightleftharpoons T$ equilibrium regulate the oxygen affinity?

2. How stable is the bovine tetramer towards acid and alkali, and other denaturation agents? What effects are exerted by the primary structural differences on the chemical nature of the molecule?

3. How do these hemoglobins respond to heterotropic or allosteric ligands? Is there any allosteric effector that is preferably bound to bovine hemoglobins?

4. What is the nature and basis of Bohr effect in bovine? Could an altered Bohr effect regulate the oxygen affinity?

5. Are there differences in functional properties of hemoglobins among different bovine species?

Hemoglobin literature has not repoprted any detailed investigation regarding these problems in proper perspective. An attempt has been made to give answers to some of these questions based on our experimental evidences. We have carried out studies on hemoglobins from two bovine species, namely, cow and buffalo, and from normal adult human. The human hemoglobin was chosen as a control to compare and contrast the results from the two bovine species. Our findings and the conclusions are presented in this thesis.

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CHAPTER 2

CONFORMATION OF BOVINE NITROSYLHEMOGLOBINS: A Test of Quaternary T State Biasness

2.1. INTRODUCTION

In 1971, in a systematic study of the effect of 2,3-Diphosphoglycerate (DPG) on the oxygen affinity of a variety of mammalian hemoglobins, Bunn pointed out that ruminant hemoglobins have intrinsically low oxygen affinity and that DPG does not lower the affinity further (1). He asserted that the reason for less reactivity of DPG with ruminant hemoglobins is related to primary structural differences in the β chains. In the following year X-ray structure of Hb-DPG complex became available (2). The lysines at the 82nd position and the histidines at the 2nd position of β chains were found to hold a DPG molecule by salt bridges in a cleft formed by the two β subunits. Sequence data (3-5) show that the β chains of ruminant hemoglobins (cow, sheep, and goat) have 145 amino acids, i.e., the N-terminal residue is deleted. Furthermore, the next position, which is NA2 is occupied by a histidine in intrinsically high oxygen affinity hemoglobins, has a methionine residue. Thus, it became clear that binding of a molecule of DPG, even if it fits in the proposed cleft of β chains, would be significantly attenuated. Bunn also pointed out a second possible reason that the DPG level in the red cells of ruminants is very low. Thus, it seemed highly probable that

DPG is not a regulator of oxygen affinity in ruminants.

Hemoglobin of water buffalo, another bovine species, also has intrinsically low oxygen affinity. Like other ruminants, buffalo hemoglobins have been known to have very little interaction with DPG. With no primary structure of buffalo hemoglobin as yet available, if we start with a working hypothesis that organic phosphates are not regulators of oxygen affinity of bovine hemoglobins, then what are the determinant factors of functional regulation. It was pointed out by Fronticelli et al.(6) that chloride ions may be the regulators of oxygen affinity since this monovalent anion has larger interaction with cow hemoglobin. However, the cause of low oxygen affinity of bovine hemoglobins even in the absence of allosteric effectors is yet unknown.

It occured to us that it might be the intrinsic conformation of the hemoglobin itself which is somehow constrained in a low affinity form even in the absence of allosteric effectors. In other words, the $R \rightleftharpoons T$ equilibrium might be favored toward T. Here, we also note an early observation of Perutz and Imai (7) that in cow hemoglobin, the allosteric constant L is higher than that in the case of normal adult human hemoglobin (HbA). They also reported a lower value of K_T and the same K_R value as for human; thus indicating stronger constraints on the quaternary T structure.

We tested this contention and our own intuition of a constrained quaternary deoxy structure by employing electron spin resonance (ESR) and visible optical spectroscopy. We also investigated the effect of chloride ions in stabilizing the quaternary T structure to find any possible role of Cl^- in oxygen affinity regulation. Since primary structural data of buffalo hemoglobin is not available preliminary characterization was carried out to

confirm that buffalo β chains also show the deletion and substitution at the amino terminus as common to other ruminants.

2.2. MATERIALS AND METHODS

2.2.A. Separation of chains and determination of N-terminal residues of buffalo hemoglobin:-

Preparation of hemolysate:

Red cells were packed from freshly collected blood samples by washing four times with a four-fold amount of 0.95% saline solution. Hemolysate was prepared according to Drabkin's procedure (8). An equal volume of ice-cold distilled water and 0.4 volume of toluene were added to the washed cells. The supernatant layer of toluene and the pelleted stroma cell debris were separated by centrifugation at 15,000 rpm for 15 minutes. The hemoglobin solution was further cleared by filtering through Whatman 1 paper.

Polyacrylamide disc gel electrophoresis (PAGE):

Component hemoglobins of buffalo were separated by electrophoretic procedure described by Davis and Ornstein (9). The reagents used and the method of gel polymerization are described in Appendix II. Pre-run was carried out at 1 mA per tube for about 20 minutes followed by sample loading and a 2.5 hours of final run at 3 mA per tube. The gels were stained with amido black for about 20 minutes and washed with methanol, acetic acid and water mixed in 5:7:88 ratio.

Triton gel electrophoresis:

The number of polypeptide chains constituting the component hemoglobins and their electrophoretic identities were determined by Triton X-100 polyacrylamide gel electrophoresis method (10,11). Gel constituents and method of polymerization are detailed in Appendix II.

Preparation of hemoglobin components:

Hemoglobin types were fractionated by Whatman CM cellulose chromatography in 0.01 M sodium phosphate buffer, pH 6.2 at 20^0C, containing 1 mM potassium cyanide. For preparative runs a 10x2.6 cm column was employed. The bound hemoglobins were eluted with a linear ionic gradient from 0.01 M NaCl to 0.1 M NaCl, the flow rate being 25 ml per hour.

Preparation of globin:

The heme group was split from globin by using acidified acetone cooled to -20^o C (12,13). Precipitated globin was isolated by centrifugation at 6000 rpm at 0^o C, freeze-dried and stored in cold.

Preparation of globin chains:

The α and β chains were isolated from component globins or whole globin by ion exchange chromatography on Whatman CM 52 cellulose according to Clegg et al. (14). Globin was chromatographed in 0.001 M dibasic sodium phosphate containing 8 M urea and 0.05 M β-mercaptoethanol. pH of the developer was adjusted to 6.7 with dilute orthophosphoric acid. The bound polypeptide chains were eluted from the column using a gradient obtained by mixing equal volume of the starting buffer with 0.035 M dibasic sodium phosphate containing 8 M urea and 0.05 M β-mercaptoethanol, pH 6.7. Preparative runs were carried out using a 2.6x11.5 cm column. The gradient was generated by mixing 475 ml of the starting buffer with 475 ml of 0.035 M dibasic sodium phosphate at the same pH with 8 M urea and 0.05 M β-mercaptoethanol.

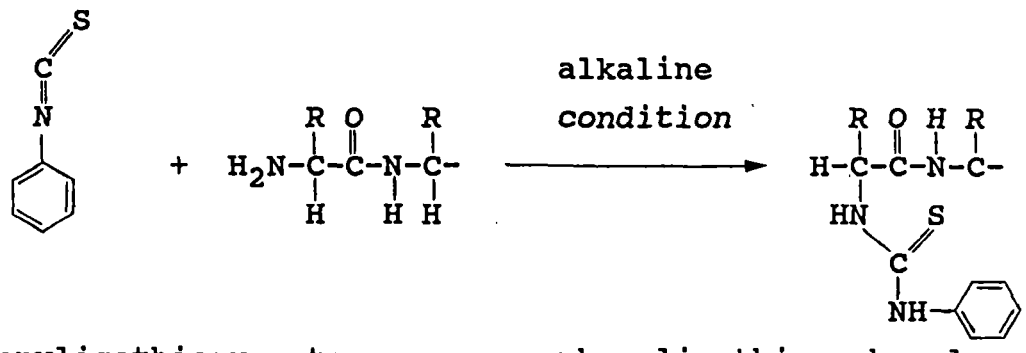
Homogeneity of the isolated chains was checked by Triton gel electrophoresis. Pure chromatographic fractions were desalted by dialyzing against distilled water. Chains were freeze-dried and stored in cold.

N-terminal amino acid determination:

(i) Dansyl chloride method : Amino end groups of polypeptide chains were determined by the method described by Hartley and Massey (15). 0.25 mg of globin was dissolved in 50 µl 0f 1% sodium dodecyl sulfate solution at about 80° C. To this 50 µl of N-ethyl morpholine was added followed by 75 µl of 2.5% dansyl chloride solution in dimethyl formamide. The reaction between dansyl chloride and protein was allowed for 2.5 hours. The dansylated protein was pelleted from the reaction mixture by adding acetone. After a few acetone washes the pellet was dried in vacuum. Hydrolysis was carried out in 100 µl of 5.7 N distilled HCl in a sealed tube at 110°C for 16 hours. For identification of dansylated N-terminal amino acids the hydrolysed sample was chromatographed in two dimensions on polyamide thin layer plates (16,17). For comparison, standard dansylated amino acids purchased from Sigma Chemicals were used. The 5x5 cm polyamide plates were developed in 1.5% formic acid in the first dimension followed by a 9:1 mixture of benzene and acetic acid in the second dimension. Presence of dansylated amino acids in the dried chromatograms was indicated by flourescent spots observed under a 365 nm UV lamp.

(ii) Edman reaction : The N-terminal end groups were also determined by Edman's sequence determination method (18) as follows.

Coupling:- The coupling reaction is:

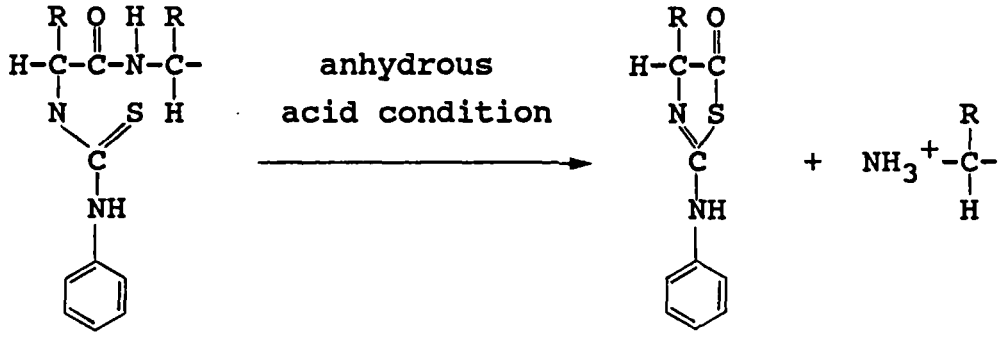


phenylisothiocyanate phenylisothiocarbamyl protein

About 16 mg of globin chain was dissolved in 1 ml of a

pyridine-water mixture in 1:1 ratio to which 7 µl of dimethylallylamine was added. The pH of the solution was lowered to 8.9 with dilute trifluoroacetic acid (TFA). The solution was flushed with nitrogen and 50 µl of phenylisothiocyanate (PTC) was added. This was flushed with nitrogen again. The reaction of PTC with protein took place at 40°C for 90 minutes. At the end of the reaction the PTC coupled protein was repeatedly extracted with benzene. The aqueous phase of the extract was dried in vacuum.

Cleavage:- Under anhydrous acidic conditions, the sulfur of the phenylthiocarbamyl group attacks the carbonyl component of the first peptide bond of the chain, resulting in cleavage of the N-terminal residue as an anilinothiazolinone.

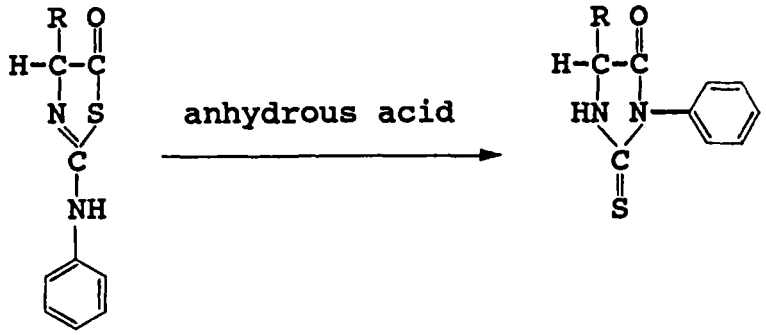


phenylthio carbamyl protein

thiazolinone

The dry phenylthiocarbamyl preparation dissolved in 75 µl of TFA was placed in a water bath at 40°C for 30 minutes. The thiazolinone derivative so formed was repeatedly extracted with ethylene chloride and the extract was dried under a stream of nitrogen.

Conversion:- Since the thiazolinone derivative is less stable, it is converted to the more stable thiohydantoin derivative through the following reaction:



thiazolinone phenylthiohydantoin amino acid

The thiazolinone powder was dissolved in 0.2 ml of 1N HCl, flushed with nitrogen and heated at 80°C for 10 minutes. The solution was cooled and phenylthiohydantoin (PTH) derivative of the N-terminal amino acid was extracted with ethylacetate. The organic phase was evaporated to dryness under a stream of nitrogen. The PTH residue was dissolved in 200 µl of ethylene chloride and was chromatographed on starch paper.

Strips of Whatman 1 paper were coated with 0.5% starch solution in water and air-dried. Before sample loading the paper strips were soaked in a 70:20 mixture of acetone and formamide. 1% solution of standard PTH amino acid was prepared in ethylene chloride. About 10 µl of each of standard as well as test samples was loaded on the paper strips and chromatographed in O-xylene. Strips were dried at room temperature and developed with an aqueous mixture of equal volumes of 0.01 M iodine, 0.5 M potassium iodide and 0.5 M sodium azide.

2.2.B. Preparation of samples for ESR and visible optical measurement :-

Freshly prepared hemolysate was stripped of organic phosphates by passing through a 133x2.6 cm Sephadex G 25

column equilibrated with 0.0075 M Tris-Cl, pH 7.4. Adenosine 5 triphosphate (ATP) and inositol hexaphosphate (IHP) were obtained from Sigma chemicals. All the reagents were analytical grade. Nitric oxide (NO) was prepared by reaction of dilute nitric acid with sodium nitrite. Evolving NO gas was freed from traces of NO_2 by passing through 1 M KOH solution and solid KOH in sequence. No brown color of N_2O_4 was observed in the flask.

Deoxyhemoglobin (deoxyHb) was prepared by first degasing and then by shaking the solution under nitrogen for about 90 minutes at room temperature. DeoxyHb solution was equilibrated with NO gas for 4 minutes. Unreacted or excess NO was removed by evacuation and nitrogen flushing. The nitrosylhemoglobin (NOHb) thus prepared was loaded into quartz ESR tubes. The sample tubes were placed in a methanol bath and frozen in liquid nitrogen. The entire process from NO addition to freezing was carried out within 15 to 20 minutes. ATP and IHP were dissolved in 0.0075 M tris-Cl, pH 7.4 and titrated to the same pH with dilute HCl. These solutions were added to hemoglobin samples prior to deoxygenation. The final concentration of hemoglobin used for ESR was about 0.8 mM with respect to heme. ESR spectra were collected at 77K using a Varian E109 X-band spectrometer of 100 KHz field modulation, with a microwave frequency around 9.15 GHz, microwave power of 10 mW, time constant of 0.5 sec, modulation amplitude of 3.2 gauss, and a sweep time set at 62.5 gauss per minute.

Concentration of protein used for visible optical measurement was 20 mg/ml. Aliquotes of NO-saturated buffer were added to hemoglobin solutions at 20°C. The buffer was evacuated and equilibrated with NO_2-free NO gas by vigorous shaking. Buffer was withdrawn with the help of airtight syringes and added to deoxyHb solutions in an oxygen free atmosphere. Absorbances were measured using a pair of matched cuvettes. For all spectrophotometric measurements, a

UVIDEC 610 instrument of JASCO was employed.

2.3. RESULTS

2.3.A. Chromatographic and electrophoretic identity and N-terminal residues of buffalo hemoglobins:-

A polyacrylamide gel electrophoregram of buffalo hemolysate is shown in Figure 2.1. The thin slow moving band represents the minor component and the fast moving thick band corresponds to the major component. Figure 2.2 shows CM Cellulose ion-exchange fractionation of these two components. The major and the minor components were eluted as the first and the second peak, respectively. Peak homogeneity and the correspondence between electrophoretic and chromatographic identity of the components were checked by polyacrylamide gel electrophoresis (Figure 2.3).

Figure 2.4 shows a Triton X 100 gelelectrophoregram of the hemoglobins. Three chains, one β and two α's constitute the total hemoglobin. The two α chains are resolved into α^{I} and α^{II} constituting the major and the minor components, respectively.

The chromatograms in Figure 2.5 show the separation of the chains by CM Cellulose chromatography in 8 M urea. Chain separation from the whole globin is shown in Figure 2.5.a. The β chain is eluted first, followed by α^{I} and α^{II}. Thus, the order of elution corresponds to their cathodic mobility in triton gel electrophoresis (Figures 2.4 and 2.6). Chromatograms obtained from the individual component globins are presented in Figures 2.5.b and 2.5.c. The peaks are marked according to the content of globin chains. Homogeneity and the identity of the peaks were checked by triton gel electrophoresis (Figure 2.6).

Figure 2.7 schematically shows the polyamide plates indicating the fluorescent spots due to N-terminal DNS amino

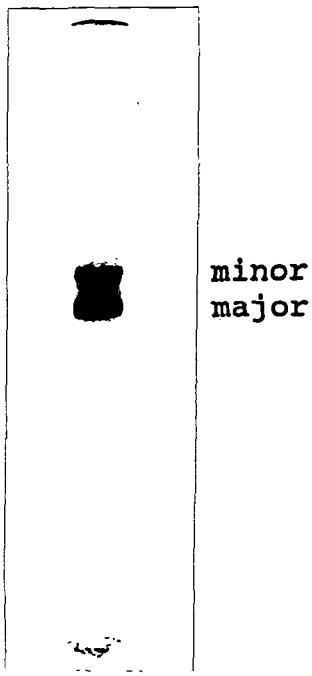


Figure 2.1. Polyacrylamide gel electrophoregram of buffalo hemolysate. Tris-glycine (Tris, 0.005 M and glycine 0.038 M) at pH 8.3 was used as electrode buffer. The upper and the lower band corresponds to minor and major components respectively.

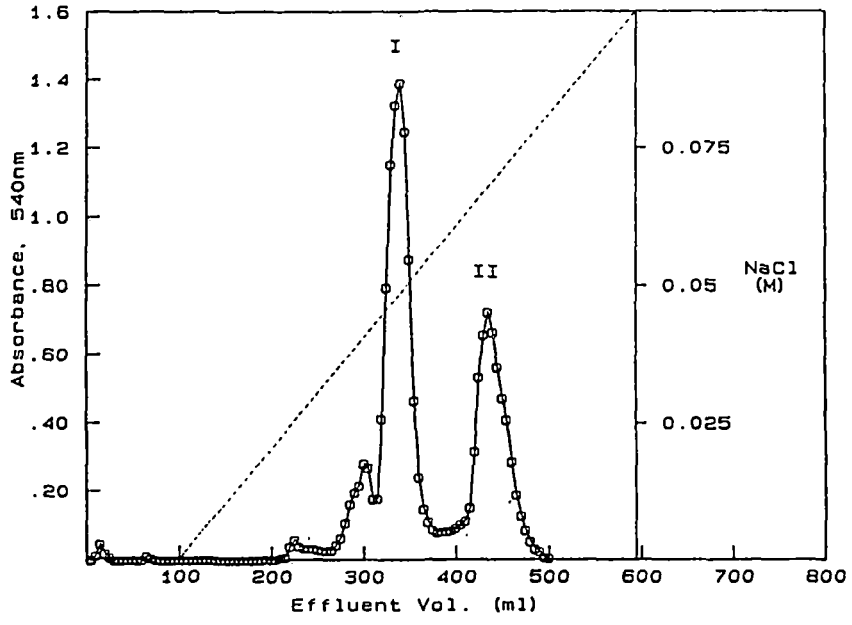


Figure 2.2. Elution pattern of the two component hemoglobins of buffalo during CM cellulose ion exchange chromatography in 0.01 M phosphate buffer, pH 6.2. For elution, a linear ionic gradient was used. Peaks I and II correspond to major and minor components respectively.

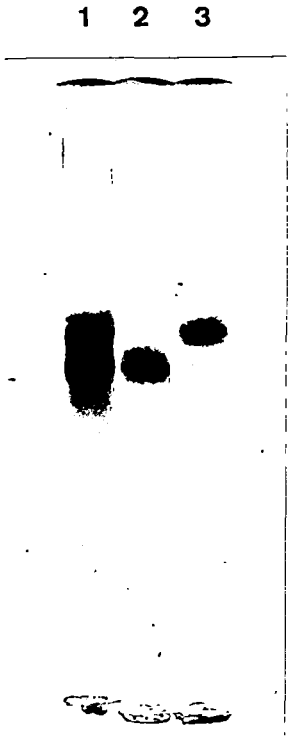


Figure 2.3. Electrophoretic and chromatographic identity of the two hemoglobin components:

1. Total hemolysate,
2. The fast moving band, which is the major component, elutes as peak I in CM cellulose chromatography,
3. Homogeneity of the minor component after ion exchange chromatography.

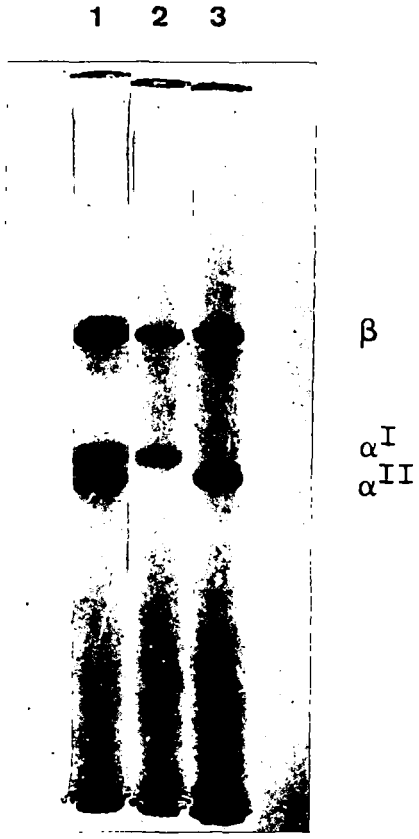
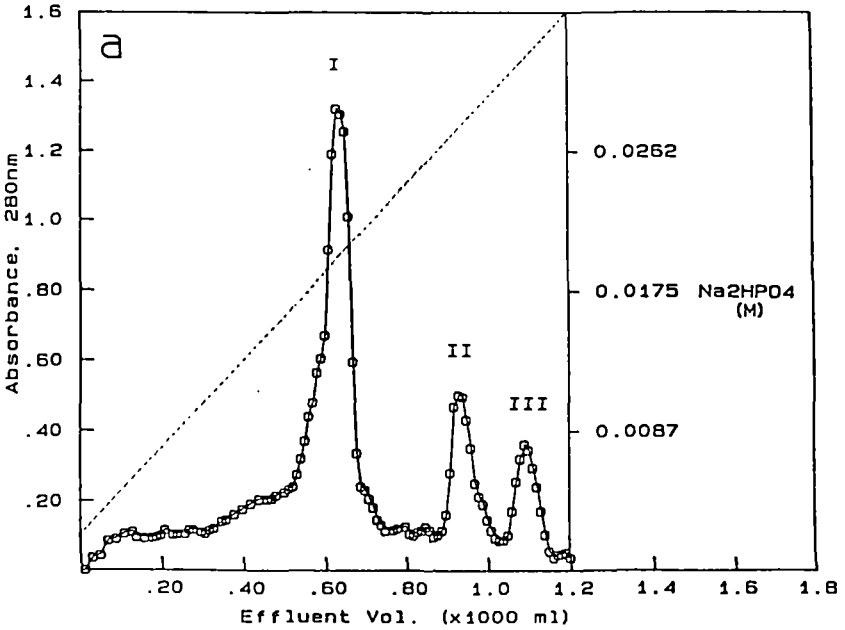
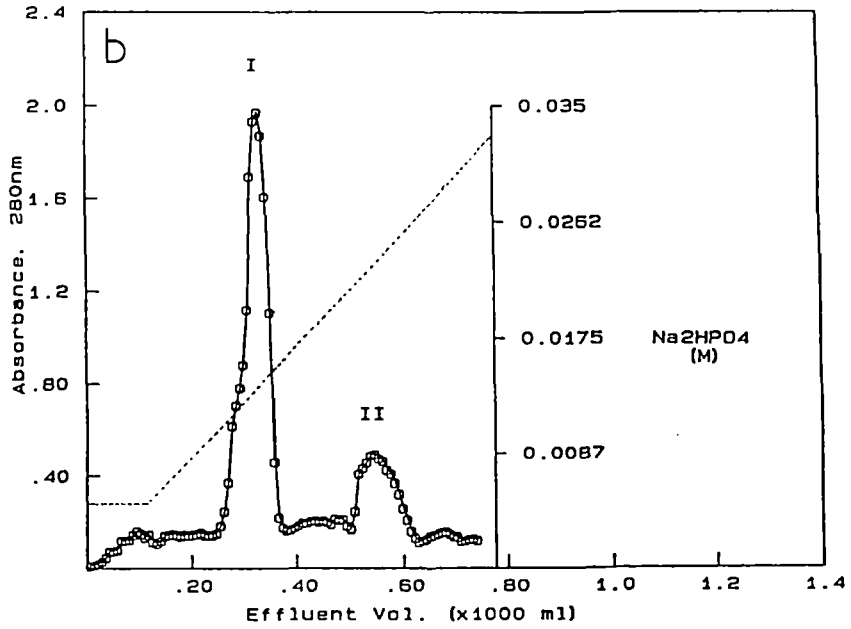


Figure 2.4. Triton X100 electrophoregram of hemoglobins in 5% acetic acid:

1. The three chains of the hemolysate; the fastest moving band corresponds to α^{II} chain followed by α^{I} and β,
2. β and α^{I}, which constitute the major component ($\beta_2\alpha_2^{I}$),
3. Resolution of β and α^{II} of the minor component.


a
I
II
III
0.0262
0.0175 Na2HPO4 (M)
0.0087
Absorbance, 280nm
1.6
1.4
1.2
1.0
.80
.60
.40
.20
.20 .40 .60 .80 1.0 1.2 1.4 1.6 1.8
Effluent Vol. (x1000 ml)


b
I
II
0.035
0.0262
0.0175 Na2HPO4 (M)
0.0087
Absorbance, 280nm
2.4
2.0
1.6
1.2
.80
.40
.20 .40 .60 .80 1.0 1.2 1.4
Effluent Vol. (x1000 ml)

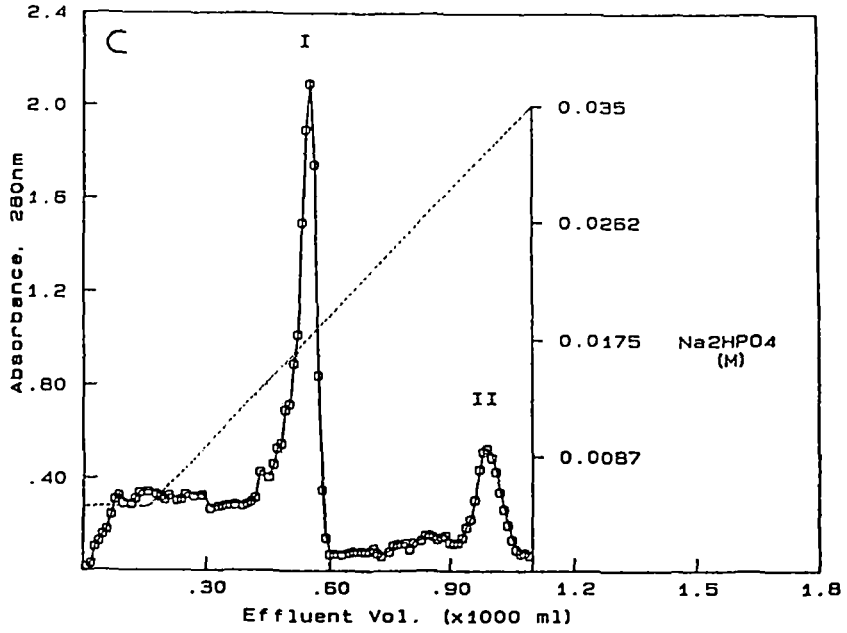


Figure 2.5. Separation of globin chains by CM cellulose chromatography. 0.001 M phosphate buffer containing 8 M urea and 0.05 M β-mercaptoethanol was used at pH 6.7.

a) Separation of all three chains from whole globin; peaks I, II and III correspond to β, α^{I} and α^{II}, respectively,
b) Resolution of β (peak I) and α^{I} (peak II) from major component,
c) Separation of β (peak I) and α^{II} (peak II) from the minor component.

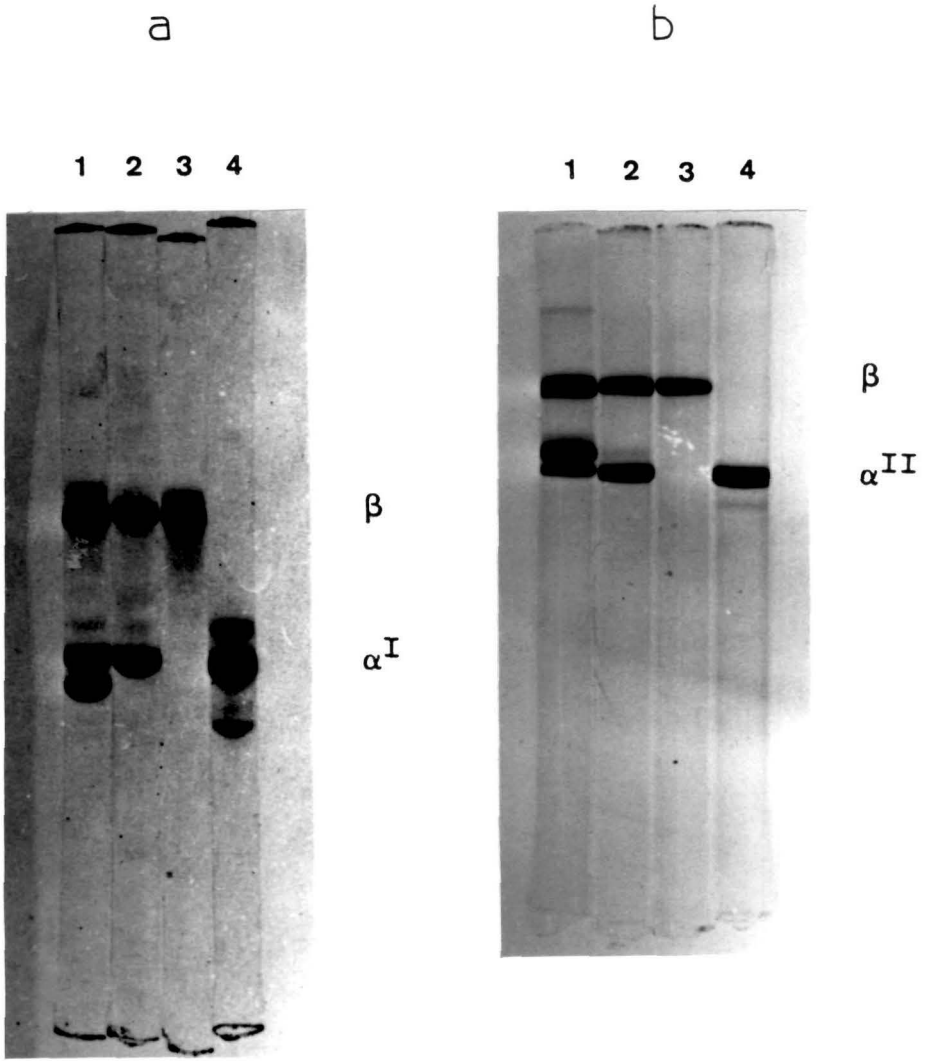


Figure 2.6 . These Triton X100 electrophoregrams show electrophoretic identities of globin chains separated by ion exchange chromatography.

(a) 1. three chains of the whole globin, 2. major component, 3. β and 4. α^{I}, respectively,

(b) In these gels β and α^{II} (third and fourth gels from the left), as obtained from ion exchange chromatography of the globin of minor component, have been shown.

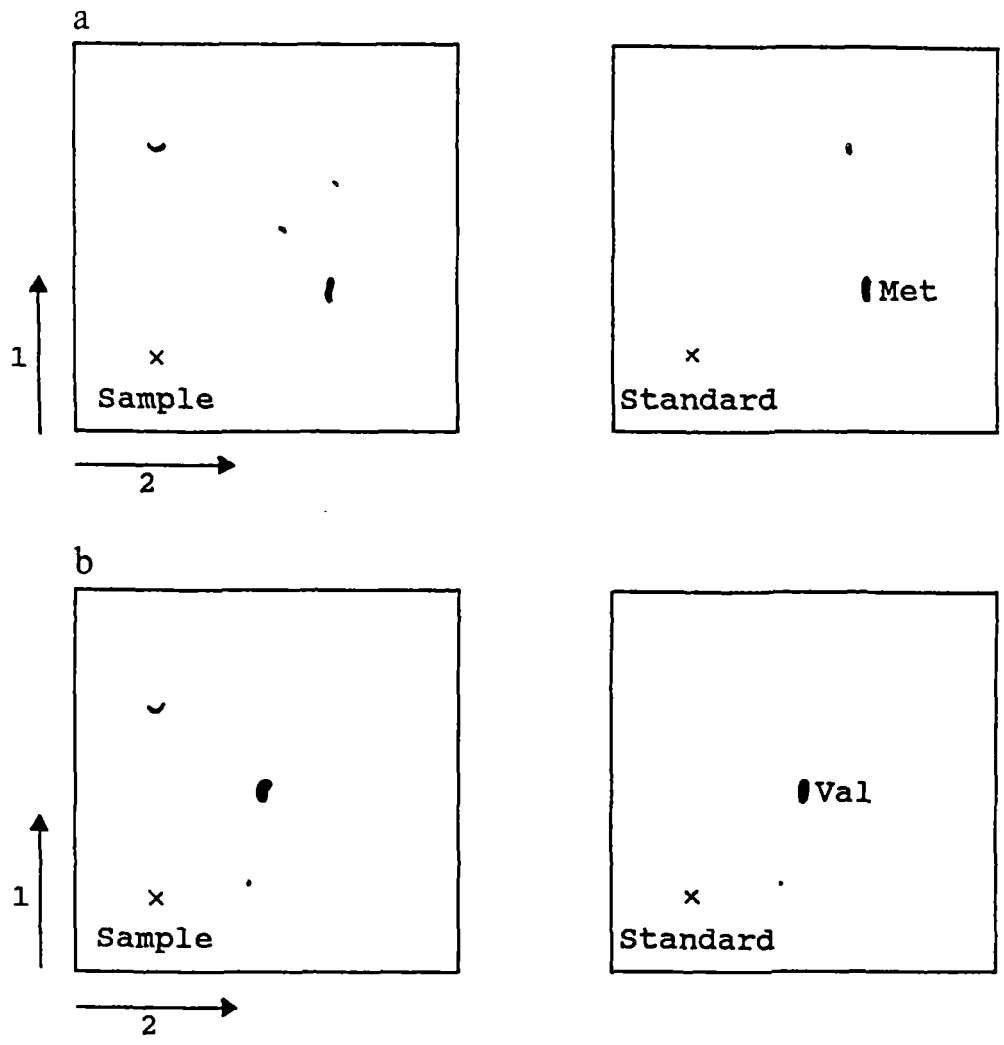


Figure 2.7. Traces of fluorescent spots due to N-terminal DNS amino acids of β, α^{I} and α^{II} chains. (a) β chain; (b) α chains. Comparison with standard DNS amino acids identifies these residues as methionine and valine respectively. Solvent systems are described in the text.

acids of β, α^{I} and α^{II} chains. The N-terminal residues are methionine for β chains and valine for both types of α chains. Identical results were obtained from phenylisothiocyanate procedure of Edman (18).

2.3.B. ESR spectra:-

In general, all the ESR spectra are seen to exhibit g-anisotropy indicating rhombic environment of the free electron site. The effective g-values are shown in the spectra. Figure 2.8 shows the spectra of NOHb obtained from reaction of unstripped hemolysate and nitric oxide. For both the bovine species the hyperfine lines are intense over human. Nitrosyl complex of stripped human hemoglobin at pH 7.4 does not show marked splitting of hyperfine structure; in contrast, the NOHb obtained from stripped bovine hemoglobins present feeble splitting. This is shown in Figure 2.9. In presence of organic phosphates all the three NOHb species exhibit strong splitting of the hyperfine structure. The results obtained in presence of 5 mM ATP are presented in Figure 2.10.

The suggestion of Fronticelli et al. (6) that Cl^- ions may be the regulators, in vivo, of oxygen affinity of cow hemoglobin, induced us to record the ESR spectra of nitrosyl complexes in presence of NaCl. The spectra obtained in presence of 0.6 M NaCl are presented in Figure 2.11. It can be noticed that the resolution of hyperfine lines is more pronounced in case of both the bovine species.

2.3.C. Visible absorption spectra:-

To monitor heme environmental changes, we recorded the visible absorption spectra of the nitrosyl complexes. For human NOHb we obtained similar absorption bands as were reported (19-21). The minor differences in the blue shifts of the Soret line positions in case of the two bovine NOHb species in presence and absence of IHP and NaCl are

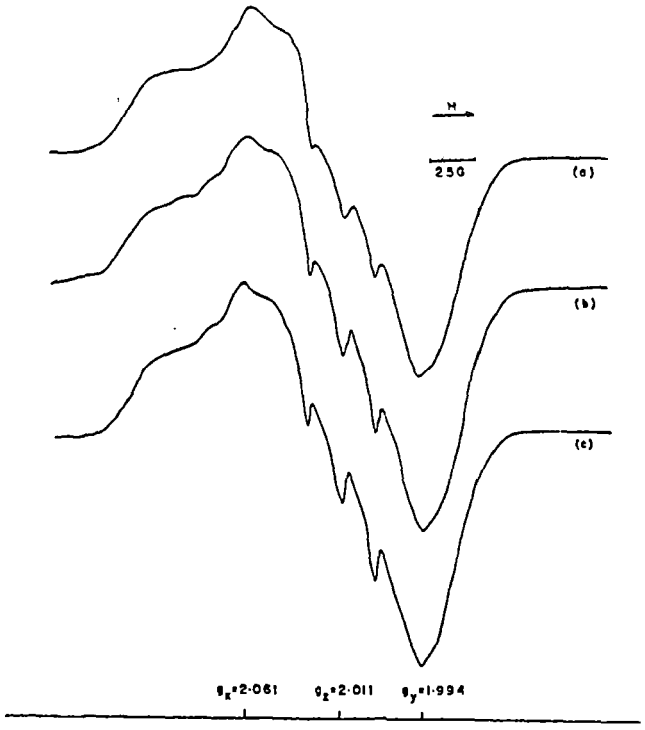


Figure 2.8. ESR spectra of nitrosyl complexes of unstripped hemoglobins. (a) human; (b) cow; (c) buffalo.

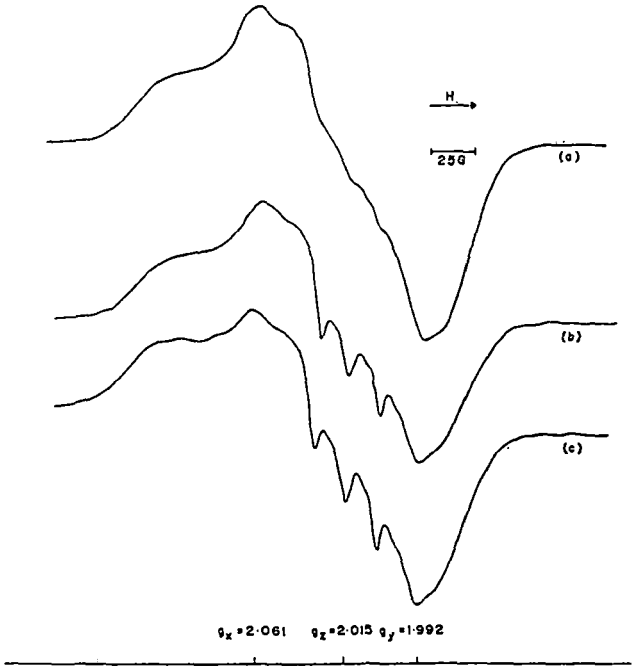


Figure 2.9. ESR spectra of nitrosyl complexes of stripped hemoglobins in 0.0075M Tris-HCl, pH 7.4. (a) human; (b) cow; (c) buffalo.

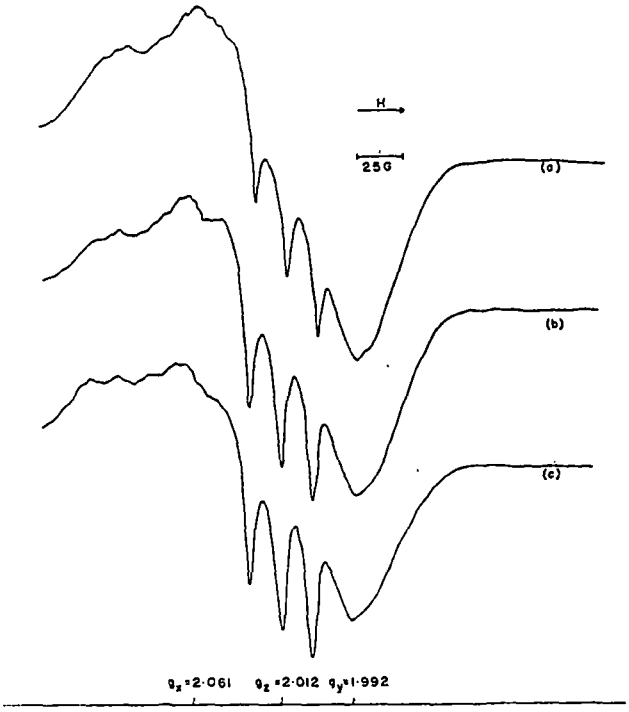


Figure 2.10. ESR spectra of nitrosyl complexes of stripped hemoglobins in presence of 5mM ATP in 0.0075M Tris-HCl, pH 7.4. (a) human; (b) cow; (c) buffalo.

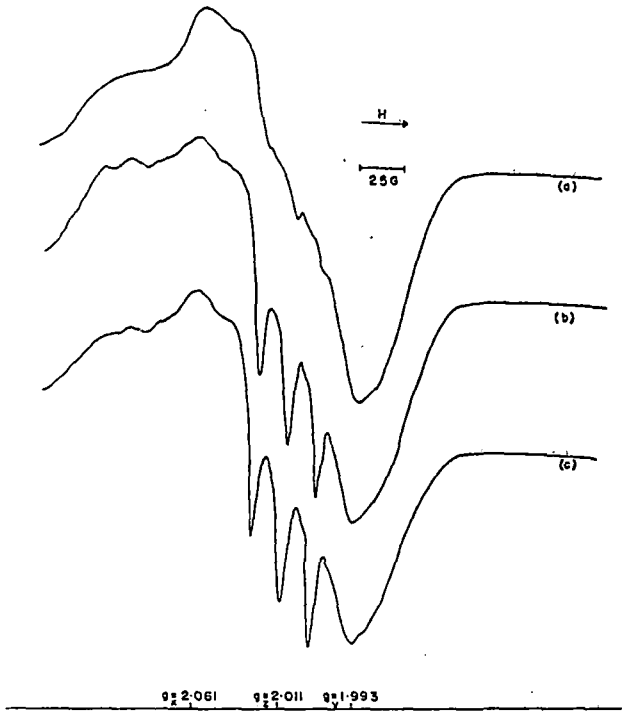


Figure 2.11. ESR spectra of nitrosyl complexes of stripped hemoglobins in presence of 0.6M NaCl in 0.0075M Tris-HCl, pH 7.4. (a) human; (b) cow; (c) buffalo.

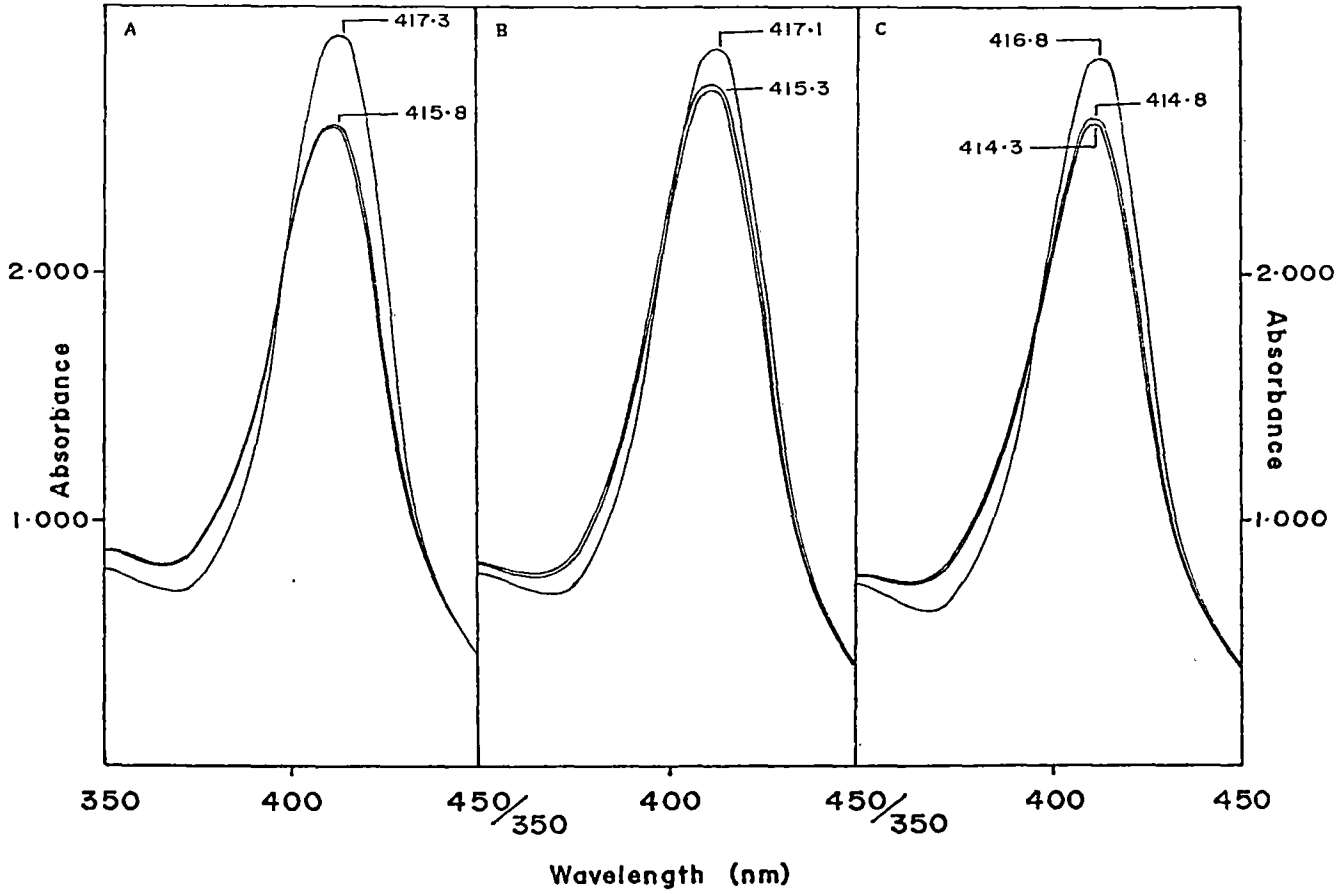


Figure 2.12. Soret spectra of nitrosyl complexes of stripped hemoglobins in presence of ATP and NaCl. (a) human; (b) cow; (c) buffalo.

TABLE 2.1

SORET LINE POSITIONS OF HUMAN, COW AND BUFFALO NITROSYLHEMOGLOBINS IN PRESENCE AND ABSENCE OF ATP AND NaCl AT 20^0C

All wavelengths are in nm

Spectral Component	Human	Cow	Buffalo
NOHb	417.3	417.1	416.8
NOHb in presence of ATP	415.7	415.1	414.3
NOHb in presence of NaCl	415.8	415.3	414.8

presented in Figure 2.12. The wavelengths are also shown in Table 2.1.

2.4. DISCUSSION

Genetic monomorphism involving two hemoglobin components in buffalo has been well documented (22-25). Detailed survey of blood samples of buffalo population has consistently shown the presence of two component hemoglobins which, on the basis of electrophoretic mobility, have been referred to as buffalo-Hb-fast (fast moving component or higher anodic mobility) and buffalo-Hb-slow (slow moving component) (24). Here, we have called these two hemoglobins as Hb-minor and Hb-major, respectively (Figures 2.1 and 2.2). The component hemoglobins are due to gene duplication leading to non-allelic structural genes in the α chain gene locus (26-30). Thus, the α chains constituting the two hemoglobins of buffalo are different. On the other hand, both the major and the minor components share a common β chain. Two different α chains and a single β chain of adult buffalo hemoglobin were first identified by Balani and Barnabas (24) by analysing the fingerprints of globin chains isolated from the two components. These polypeptide chains are resolvable in triton gel electrophoregram (Figsures 2.4 and 2.6). The two α chians, which we have called α^{I} and α^{II}, show higher cathodic mobility than the single β chain. The two α chains have been shown to differ in three peptide spots in the fingerprint maps of tryptic digest (24). The fingerprints have also indicated that one of the differences between α^{I} and α^{II} involves tryptophan residue (24). Considering the electrophoretic and ion exchange resolutions the two α chains seem to differ in charged amino acids. Such substitutional differences involving charged residues are seen in the case of the two β chains of cow hemoglobin. There are two phenotypes of cow hemoglobin, called Hb-A and Hb-B (31), which differ in their β chains. In Hb-A there are

two lysine residues at 18th and 119th positions of β chain. These two sites are occupied by histidine and arginine, respectively, in the β chain of Hb-B (32). We await the primary structure of buffalo hemoglobin to see the type and nature of substitutional differences between the α chains. However, concerning the N-terminal structural alterations in the β chain, as found in other ruminants, we are sure that the valine at the amino terminus is deleted and the histidine, next to valine in intrinsically high oxygen affinity hemoglobins, is replaced by methionine. With this certainty we are in favorable position to assume that all the bovine hemoglobins employ a common regulatory mechanism. In fact, of the two bovine species examined both share a common tertiary and quaternary conformation.

We have directly looked at the conformation of the hemoglobin molecule with an effort to explain the characteristic low oxygen affinity. ESR spectroscopy is a suitable and powerful technique of choice. Although nonavailability of a paramagnetic center severely limits the use of ESR, spin labelled probes attached to the ferrous iron or elsewhere in the protein chain can be conveniently used to investigate the structure-function relationship (33-41). NO is a strong axial ligand at the sixth coordination position of heme iron and the resultant low spin ferrous complex (i.e., NOHb) is taken to be an analogue of oxyhemoglobin (42,43). NOHb is the only known paramagnetic derivative at the heme site. The unpaired electron density of NO (44) is transferred to the d_{z^2} orbital of the heme iron (19,21,45) and interacts with the quadrupolar nitrogen nucleus of NO. This interaction produces the characteristic three-line hyperfine signal in the ESR spectra of NOHb. Valuable information can be derived from the appearance and intensities of hyperfine lines.

From studies on model compounds (46-50) it has been shown that in NOHb the HisF8-Fe bonds are substantially

weaker than in other hemoglobin derivatives of diatomic ligands (19,51). When the molecule is converted to T form by adding organic phosphates, the already weakened HisF8-Fe bonds are disrupted in about 80% of the α subunits (19,21,52). The reason may be significantly attributed to delocalization of the unpaired electron density from NO to the iron, i.e., the trans effect (53-56), and the greater tension existing in the α hemes compared to β hemes (19). Thus, the T state NOHb contains a mixture of five- and six-coordinate hemes (19,49,57). On the other hand, in R state of NOHb, the HisF8-Fe bond in the β subunit, though weak, are intact. Therefore, both the α and the β hemes are six-coordinate in this case. To summarize, the ESR spectra characteristic of six-coordinate, and a mixture of five- and six-coordinate hemes make possible to distinguish between R and T states of NOHb (57,58).

We shall discuss our results assuming that the T and R states correspond to deoxy and oxy forms respectively. This is, indeed, consistent with chemical and spectroscopic evidences. In Figure 2.8, relatively intense hyperfine lines suggest comparatively weaker αHisF8-Fe bonds in both the bovine species, namely cow and buffalo, than in human. At the physiological pH of 7.4, the ESR spectrum of nitrosyl complex of stripped human hemoglobin indicates six-coordinate heme structure (Figure 2.9). Under identical conditions, the marked splitting of hyperfine lines in case of the two bovine species is to be ascribed to a tendency toward a T state quaternary conformation of these molecules. On the basis of hyperfine splitting centered at g=2.015 in the g_z region of the spectra (Figure 2.9), substantially weaker αHisF8-Fe bonds are implicated. If this is true, then the conformation of the molecule should be such that the Fe-NO bond is strong (smaller Fe-NO bond distance) which would probably lead to a stronger d_π-p_π interaction with the ligand (59). The linkage of distal histidine with the sixth

ligand (21,60,61; also see Appendix I), which has been found to stabilize the R state of NOHb through donor-acceptor interactions between the α distal histidine and NO ligand (21,62,63) is altered when the molecule assumes a T form. Nitrosyl complexes of hemoglobins with substituted distal histidine, in which the donor-acceptor interaction is absent, remain in the T state (62,64,65). But all the bovine α chains sequenced so far (see 32,66-75) show no replacement of distal histidine, and as such, absence of interaction between αHisE7 and NO in the nitrosyl complex of the molecule is hard to conceive when there is no organic phosphate in the system. Then, it could seem possible that multiple site amino acid substitutions in the different α chains of bovine hemoglobins modify the environment around the heme, which in turn could alter the αHisE7-NO interaction even in the absence of organic phosphate. This altered interaction may produce a destabilizing effect on the αHisF8-Fe bond so that a significant portion of the molecules tends to assume a T quaternary state. ESR studies of NOHb with modified heme environment have shown that the g_x region of the spectrum can be identified with changes in the αHisE7 residue or any amino acid substitution which affects the distal histidine, and this has been shown for opossum, rat and rabbit nitrosylhemoglobins (76). However, in Figure 2.10, no significant changes are noticed in the g_x region of the spectra of bovine nitrosylhemoglobins in presence of ATP.

On addition of IHP to NOHb solution, the absorption in the Soret region decreases (52). This is mainly due to spectral changes in the α chains. The already weakened αHisF8-Fe bonds are disrupted and the molecule assumes a T quaternary conformation (19,21,52,77-81) showing three strong hyperfine lines in the g_z region of the spectrum. Bovine hemoglobins are assumed to have very little interaction with organic phosphates. We have found strong

line splitting in the case of both the bovine nitrosylhemoglobins in presence of ATP (Figure 2.10) which is suggestive of substantial interaction of these hemoglobins with organic phosphates. This is consistent with the report of Fronticelli et al. (6) that cow hemoglobin is sensitive to the presence of organic phosphates. The decrease in visible absorbance of bovine NOHb in the Soret region in presence of ATP (Figure 2.12) further illustrates interaction with organic phosphate.

For both the bovine NOHb complexes, the ESR lines in Figure 2.11 split strongly in the g_z region in presence of chloride ions. On the other hand, human NOHb still presents R state spectral features. Thus, agreeing to the earlier report of larger preferential binding of chlorides to cow hemoglobin (6), our result shows that this monovalent anion brings about a constrained quaternary deoxy conformation of bovine hemoglobins.

Quite consistent with earlier reports of visible absorption bands of human nitrosylhemoglobin (19,21) we have recorded a blue shift of 1.6 nm for human NOHb in presence of ATP. Similar small changes in the position and intensity of the Soret line of human NOHb are also noticed when Cl^- ions are present in the system. However, the bovine nitrosyl species show some differences (Figure 2.12 and Table 2.1). Keeping the ESR spectral features in mind the origin of these small changes may be weakly traced out by comparing the structural differences between human and bovine hemoglobins. As Perutz and Imai (7) pointed out, all the amino acid replacements that distinguish the high and low oxygen affinity hemoglobins are conservative and external with a consistent difference at the NA2 position of β chain. However, small heme environmental differences cannot be ruled out. At least in one instance , i.e., in the case of yak hemoglobin , it has been thought that the amino acid substitution ($AlaH13\beta^A \longrightarrow Val\ B^{II}$) changes the heme

environment through hydrophobic interaction (73). Once again we presume heme environmental differences between human and bovine hemoglobins by virtue of polypeptide structural differences which leads to the visible Soret region differences. Such heme environmental diffrences could be electrostatic in origin. More detailed study should help clarify whether this interpretation is correct.

In this chapter we prsented convincing evidence in support of a stable T state structure of bovine hemoglobins which is maintained through stronger constraints in the deoxy quaternary state. We have also qualitatively shown some interaction of these hemoglobins with organic phosphates.

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CHAPTER 3

ASSOCIATION DISSOCIATION PHENOMENA IN BOVINE HEMOGLOBINS

3.1. INTRODUCTION

Ever since the finding of Adair by osmotic measurements that hemoglobin, over a range of pH 6.8 to 8.3, gives a normal osmotic pressure corresponding to a molecular weight of 68,000 (1,2), stability of the hemoglobin molecule over a wide range of solvent conditions became the subject of extensive research. The issue gained further momentum since the announcement of Svedberg and coworkers, in 1927, of the first oil-turbine type of centrifuge (3,4). With an output of centrifugal force 104,000 times that of gravity, Svedberg and Nichols (4) determined the molecular weight, diffusion constant and sedimentation velocity of carbonmonoxyhemoglobin over a pH range 5.4-10.2. In the following years much work was done on the dissociation of tetrameric hemoglobins from different animal species at different pH values and with various denaturants (5-27). A summary of conclusions derived from these investigations is as follows.

1. The tetramer undergoes reversibe dissociation under conditions such as high and low pH, and high salt concentrations.

2. Denaturants, such as formamide, guanidine and

urea also cause dissociation. Guanidine is more effective than other denaturation reagents.

3. Dissociation depends upon ionic strength, nature of the buffer and hemoglobin concentration. Degree of dissociation increases with dilution of the sample. Temperature has little effect on the process.

4. The association-dissociation equilibrium is also affected by the nature of the ligand at the iron atom.

5. Dissociation can be represented by,

$$\alpha_2\beta_2 \overset{K_1}{\rightleftharpoons} 2\alpha\beta \overset{K_2}{\rightleftharpoons} 2\alpha + 2\beta$$

The first step of this reaction shows symmetrical dissociation (see below). The second step predominantly occurs at pH below 4.9 or above 11 (28,29). K_1 is small but measurable at neutral pH and increases at both high and low pH. K_2 is very small at neutral pH and increases at extremes of pH. K_1 is insensitive to presence of NaCl, whereas K_2 assumes a small value in sodium chloride solutions.

6. In certain cases, Hybridized recombination products of two different hemoglobins may be obtained through acid- dissociation followed by neutralization of the mixture. Thus, when acid-dissociated carbonmonoxy derivatives of human and canine hemoglobins were allowed to recombine, two kinds of tetramers were obtained, one with α chains of human hemoglobin and β chains of canine hemoglobin, and the other with α chains of canine and β chains of human hemoglobin (20). Schematically,

$$\alpha_2{}^A\beta_2{}^A + \alpha_2{}^C\beta_2{}^C \rightleftharpoons \alpha_2{}^A\beta_2{}^C + \alpha_2{}^C\beta_2{}^A$$

where A and C refer to human and canine respectively.

7. No evidence for hybridized recombination was obtained between carbonmonoxy complexes of bovine and human, or bovine and canine hemoglobin. This observation led Itano and Robinson (20) to suggest that unlike human and canine, bovine hemoglobin does not dissociate at acid pH. This

thesis gained impetus from the observation that bovine hemoglobin is highly resistant to alkali denaturation (30).

8. Antonini and coworkers (31) subsequently reported that bovine hemoglobin does dissociate at acid and alkaline pH. However, their light scattering measurements gave a molecular weight betwen 45,000 and 50,000 at pH 11.25. This does not indicate complete dissociation of the bovine tetramer, since at this pH hemoglobins from many other species dissociate into monomers.

Hence, the question of dissociation of bovine hemoglobins in acid and alkali remained unanswered (see also 32). We investigated the phenomenon of association-dissociation by hydrodynamic measurements. Since the nature of the heme ligands have been thought to affect the dissociation equilibrium (29), we studied dissociation of aqueomet, cyanomet and carbonmonoxy derivatives of human, cow and buffalo hemoglobins to find to what extent heme ligands exert their effects. Molecular weights were determined solely by using gel filtration methods. Our results definitely show that bovine hemoglobins dissociate at acid and alkaline pH. Further dissociation of bovine dimers to monomers appear to be incomplete in the range of pH employed. Data presented here further show influence of ligands at the heme iron on dissociability of hemoglobins.

3.2. MATERIALS AND METHODS

Preparation of hemoglobin solutions:-

Hemolysates were prepared as already described in Chapter 2, and were passed through a 9.7x1.6 cm column of Sephadex G25 equilibrated with 0.1 M phosphate buffer, pH 7.4.

To prepare carbonmonoxy derivatives the hemoglobin solutions were first deoxygenated by degasing and then by shaking under nitrogen gas. CO was gently bubbled through

the solutions for about 3 minutes. Resultant carbonmonoxyhemoglobins (COHb) were stored in dark at $4^{o}C$. For preparing cyanomet hemoglobins (CNHb), a few drops of a 0.16% solution of an equal mixture of potassium cyanide and potassium ferricyanide were added. CNHb solutions were stored in cold until further use.

Sephadex columns and standard plots:-

Two glass columns of 1.6 cm diameter were packed with Sephadex G100 to a total length of 97 cm and were equilibrated with 0.1 M phosphate buffer, pH 7.4 at room temperature. Blue dextran, bovine serum albumin (BSA), ovalbumin, α chymotrypsinogen, myoglobin, cytochrome c and potassium ferricyanide were used to calibrate the columns in terms of gel filtration parameters. The protein solutions and blue dextran were prepared in the buffer (10 mg ml^{-1}). A few crystals of potassium ferricyanide were used. 0.8 ml of each of the proteins and of blue dextran solutions, and 0.4 ml of potassium ferricyanide solution were applied on the gel surface. Each of these substances were chromatographed individually in duplicate. Thus, each gel filtration run was repeated for reproducibility. Flow rate was 20 ml per hour. Fractions of 4.0 ml were collected. Absorbances were measured at 280 nm or 410 nm. Gel filtration data were used to calculate the void volume (V_o) of the columns, and elution volume (V_e) and distribution coefficient (K_d) for each of the proteins and potassium ferricyanide.

The parameter K_d (33-36) was calculated as,

$$K_d = \frac{V_e - V_o}{V_t - V_g - V_o}$$

where V_t is the total volume of the gel bed and V_g is the volume due to gel grains. V_g was estimated from the following relation,

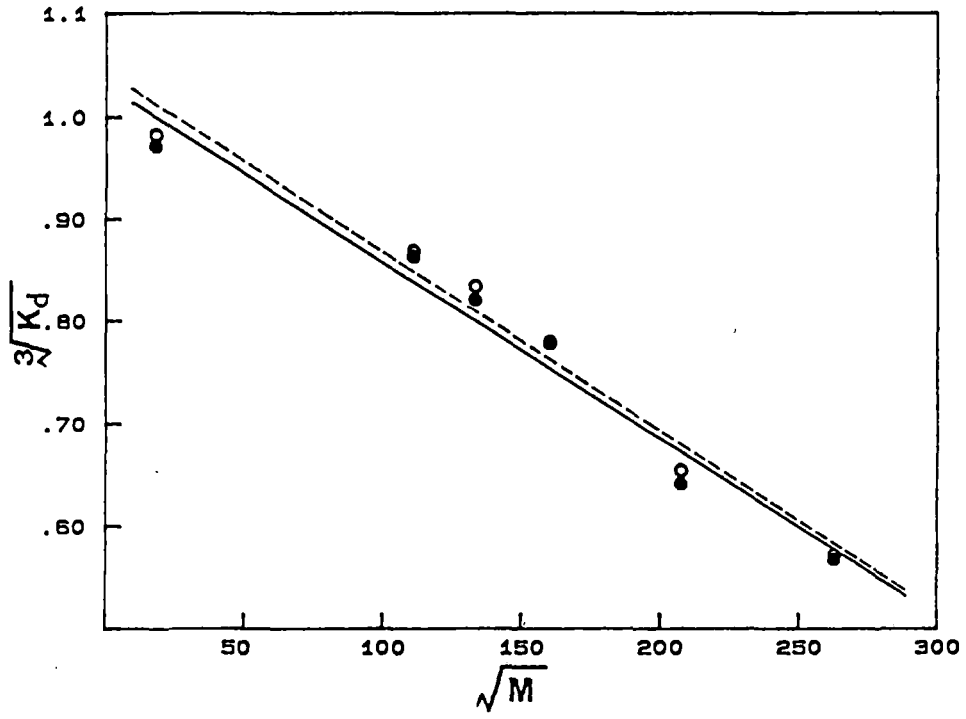


Figure 3.1. Column calibration plot; cube root of K_d, distribution coefficient versus square root of molecular weight of standard proteins and $K_3Fe(CN)_6$ (see Table 3.1). The solid line with filled circles (m=0.0461 , c=2.2829) was drawn from K_d values determined from the first Sephadex G100 column. The second column (broken line and open circles) with m=0.0458 and c=2.2650 gave reproducible result.

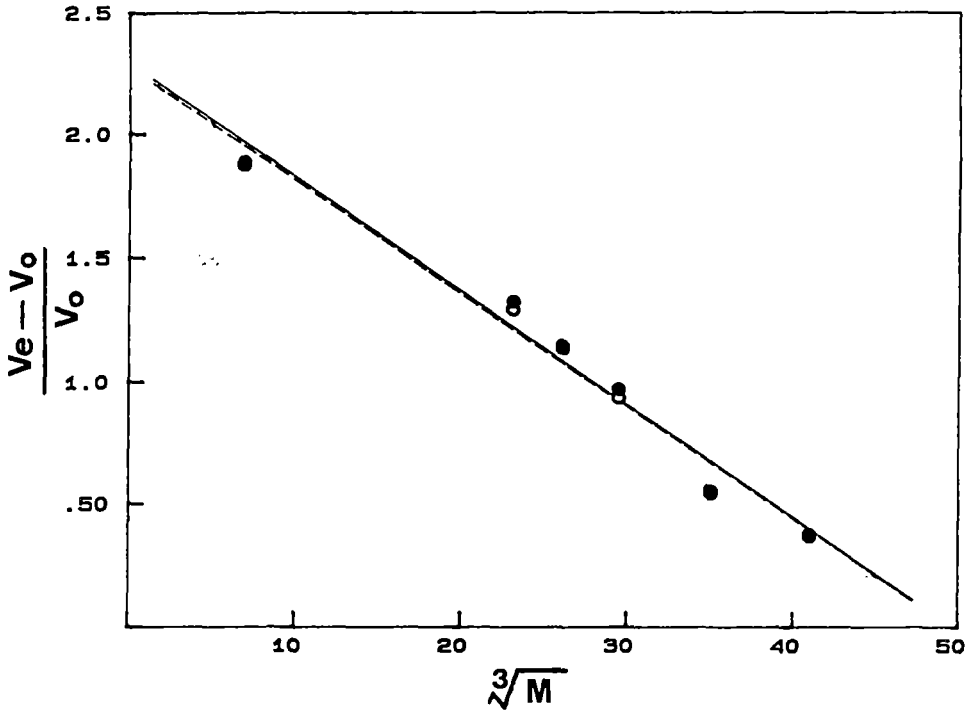


Figure 3.2. Plot of $(V_e - V_o) / V_o$ against cube root of molecular weight of standard proteins and $K_3Fe(CN)_6$. The two lines, solid for filled circles and broken for open circles, were fitted with data obtained from the two Sephadex G100 columns under identical conditions. m and c values for the solid and the broken line are 0.00173 and 1.0318, and 0.00175 and 1.0431 respectively.

$$V_g = \frac{V_t}{B.d}$$

with B =the bed volume per gram of dry Sephadex G100 (approximately 11 ml g^{-1}) and d=density of dry Sephadex G100 (2.9 g ml^{-1} (37)).

Two standard column calibration plots were prepared with the help of the molecular weights (M) of the proteins (Table 3.1) and gel filtration parameters:

(a) The plot of $\sqrt[3]{K_d}$ versus $\sqrt{M}$ is shown in Figure 3.1.
(b) Figure 3.2 shows the plot of $(V_e - V_o)/V_o$ against $\sqrt[3]{M}$

In either case the data were fitted into straight lines using linear least square analysis. These two plots were used to derive the molecular weights of the hemoglobins in the pH range 4-12.

Buffers for gel filtration of hemoglobins:-

0.25 M acetate buffer was employed in the region of pH 4 to 5.5. For pH 5.5 to 7.4, 0.1 M phosphate was used. From pH 8 to 12 glycine-NaOH (0.2 M) seemed to buffer the system adequately.

Each sample of COHb, CNHb and AqHb (aqueomet hemoglobin) was dialyzed briefly against the buffer at a given pH. CO was gently bubbled into the buffer while dialyzing COHb. Gel columns were equilibrated with the same buffer. 1 ml of dialyzed sample (5.5 mg ml^{-1}) was loaded on each gel column and eluted with a flow rate of 20 to 25 ml hr^{-1}. While chromatographing COHb, the columns were covered with aluminium foil to minimize photodissociation. The buffer was degased prior to chromatography. CO was bubbled into the buffer occassionally while elution of COHb proceeded. Since the extent of swelling of the gel matrix differs at different pH, we faced considerable difficulty in maintaining the bed volume. However, every effort was made

to calibrate the columns with due consideration for these circumstances.

Sephadex G100 and the gel filtration set consisting of column, peristaltic pump (P-1) and fraction collector (Radirac) were obtained from Pharmacia. Spectrophotometric measurements were made in a Beckman DU26 spectrophotometer. All experiments were done at room temperature (~25°C).

3.3. RESULTS

Gel filtration parameters and the molecular weights of the standard proteins gave the following staraight line equations:

$$\sqrt[3]{K_d} = 1.0318 - 0.00173\sqrt{M} \qquad 1\ (a)$$

$$\sqrt[3]{K_d} = 1.0432 - 0.00175\sqrt{M} \qquad 1\ (b)$$

$$\frac{V_e - V_o}{V_o} = 2.2829 - 0.04612\sqrt[3]{M} \qquad 2\ (a)$$

$$\frac{V_e - V_o}{V_o} = 2.2650 - 0.0458\sqrt[3]{M} \qquad 2\ (b)$$

(a) and (b) refer to duplicates of the same set of experiment. The two sets of the equations (i.e., 1 and 2) correspond to the two columns used. Plots corresponding to each of these equations are shown in Figures 3.1 and 3.2. Results are seen to be fairly reproducible.

Dissoiation of aqueomet derivatives of human (AqHbA), cow (AqHbC) and of buffalo (AqHbB) hemoglobins induced by acid pH is shown in Figure 3.3. Acid induced dissociation of carbonmonoxy complexes (i.e.,COHbA, COHbC and COHbB) and cyanide derivatives (i.e., CNHbA, CNHbC and CNHbB) are shown in Figures 3.4 and 3.5, respectively. It is clear that each hemoglobin derivative/species undergoes

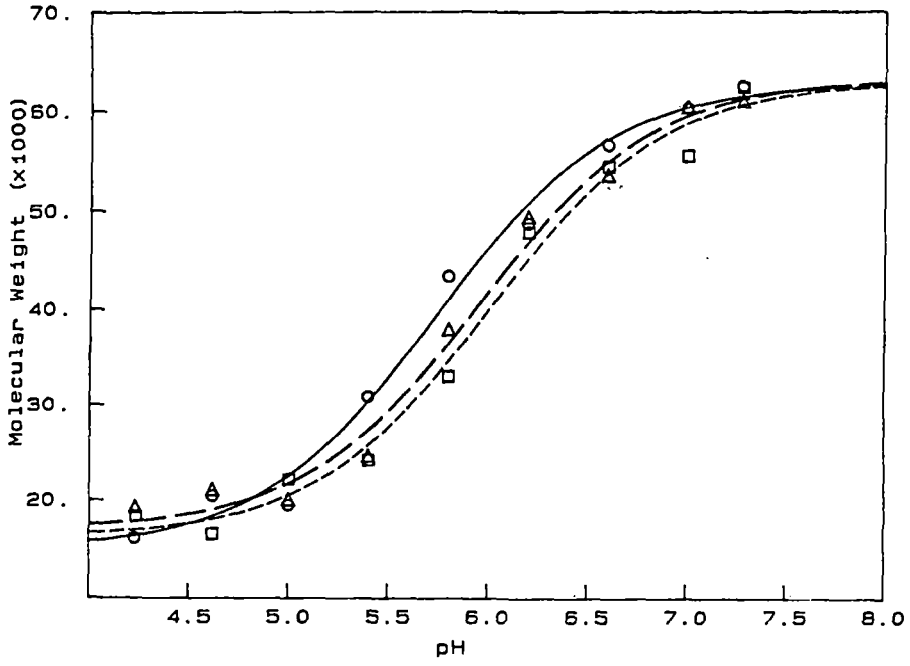


Figure 3.3. Acid dissociation of aqueomethemoglobins;
O AqHbA, □ AqHbC, △ AqHbB

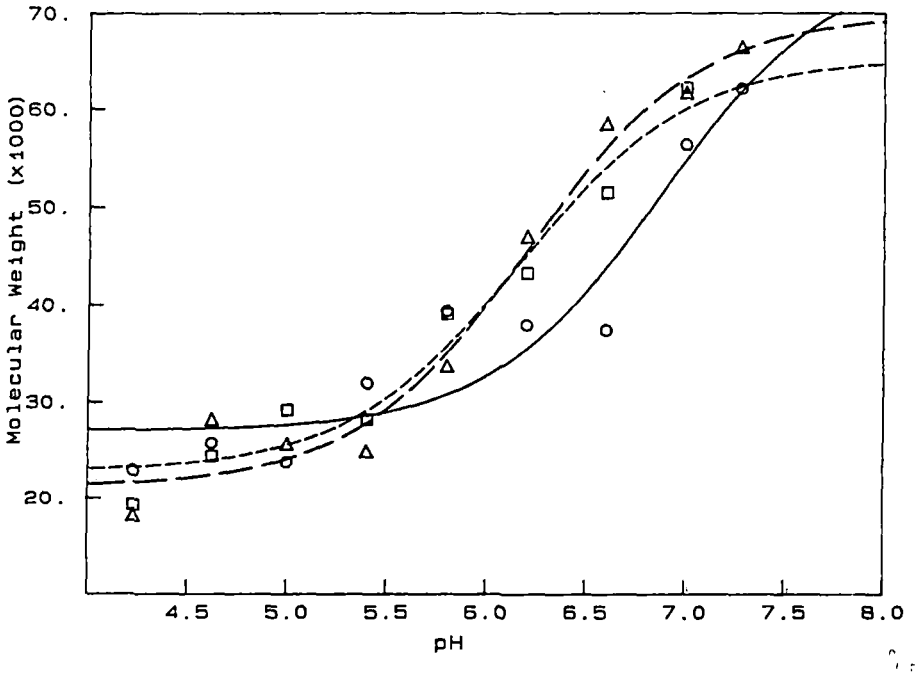


Figure 3.4. Effect of acidic pH on carbonmonoxyhemoglobins;
O COHbA, □ COHbC, △ COHbB

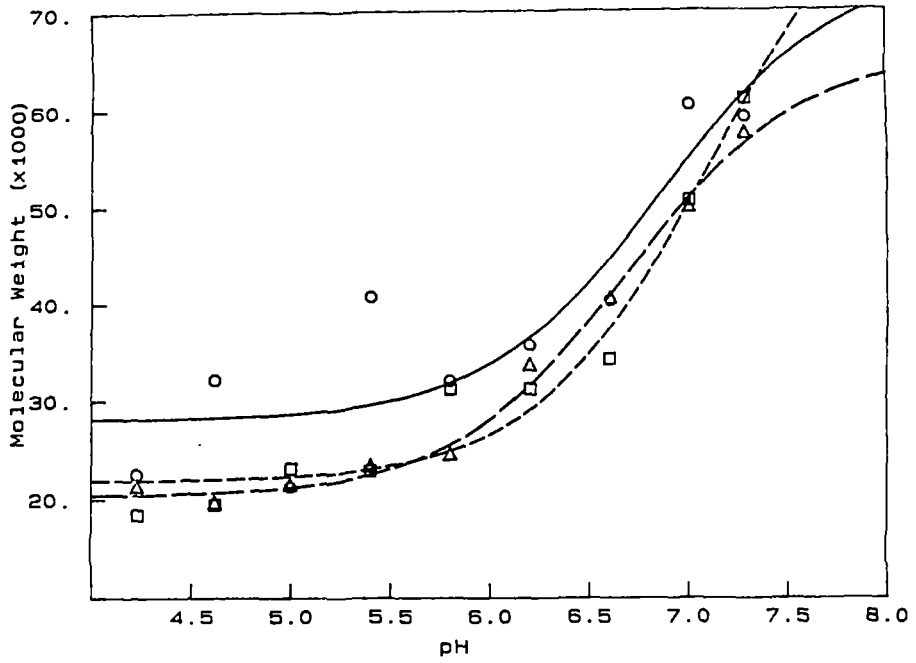


Figure 3.5. Dissociation of cyanomethemoglobins at acid pH
O CNHbA, □ CNHbC, △ CNHbB

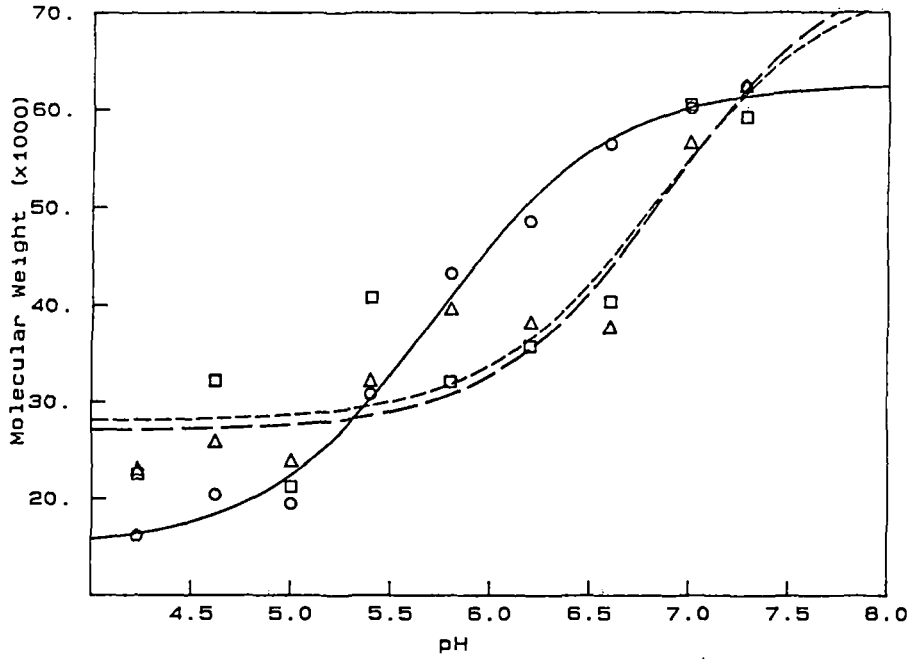


Figure 3.6. Comparison of acid dissociation of the three liganded derivatives of human hemoglobin;
O aqueomet, □ cyanomet, △ carbonmonoxy

dissociation into subunits and the molecular weights of the proteins continue to decrease as the pH is lowered. Although no definite comparison of stability could be made, Figure 3.3 contrasts with Figures 3.4 and 3.5 to indicate the influence of heme ligands on stability of the hemoglobin derivatives. Thus, the aqueomet forms of human and bovine hemoglobin appear to be more stable over the CO and CN derivatives since the transition zones of the latter two hemoglobins are shifted to relatively higher pH values in all three animal species.

Dissociation of aqueomet, carbonmonoxy and cyanomet forms of human hemoglobin are compared in Figure 3.6. Similarly, individual comparisons amongst the three derivatives of cow and buffalo hemoglobin are shown in Figures 3.7 and 3.8, respectively. These three figures very clearly show least stability of the cyanide derivatives of human as well as of bovine hemoglobins. In human, carbonmonoxyhemoglobin is seen to have dissociation transition similar with cyanomet forms (Figure 3.6). For both the bovine species, carbonmonoxyhemoglobins are as stable as the aqueomet forms.

The alkali dissociation curves of AqHbA, AqHbC and AqHbB are shown in Figure 3.9. Similar transition curves for carbonmonoxy and cyanide derivatives of human and bovine hemoglobins are presented in Figures 3.10 and 3.11, respectively. As in the case of acid dissociation, here also no clear comparison can be made regarding stability of human and bovine hemoglobins except in Figure 3.10 where the dispersed transition curves indicate higher stability of COHbA over COHbC and COHbB. The discernible general trend in each case is decreasing molecular weight as the pH is raised from 8 to 11.8. Around pH 10 the molecular weight is about half that of the full size molecule. This is consistent with earlier report of Hasserodt and Vinograd (18) that

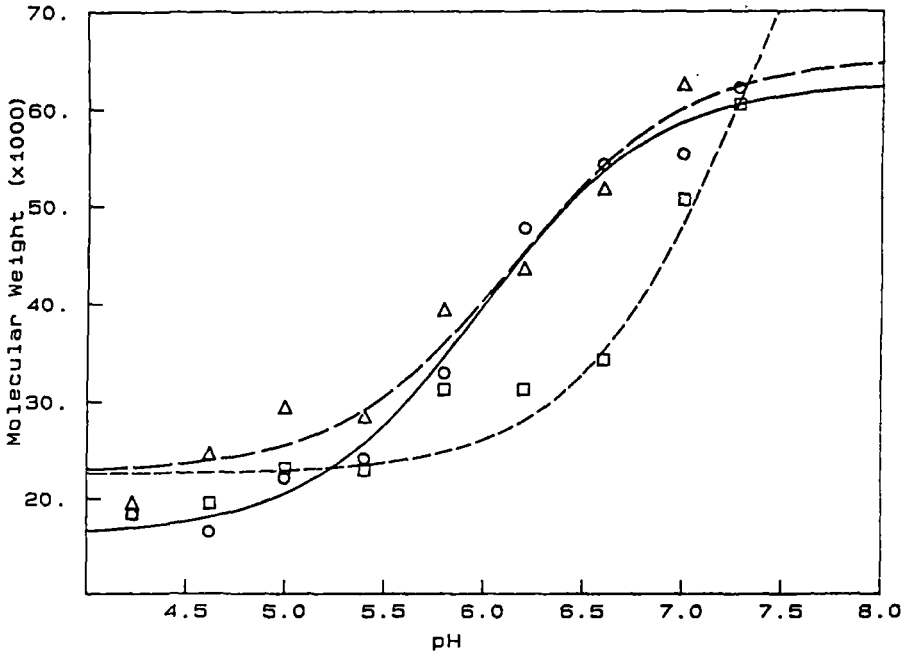


Figure 3.7. Comparison of acid dissociation of the three liganded derivatives of cow hemoglobin;
O aqueomet, □ cyanomet, Δ carbonmonoxy

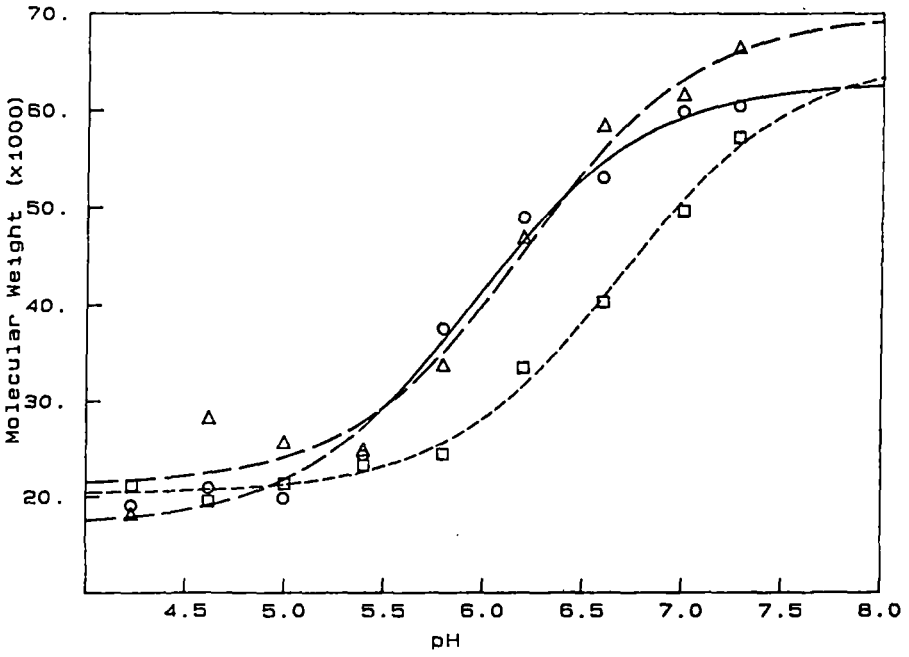


Figure 3.8. Comparison of acid dissociation of the three liganded derivatives of buffalo hemoglobin;
O aqueomet, □ cyanomet, Δ carbonmonoxy

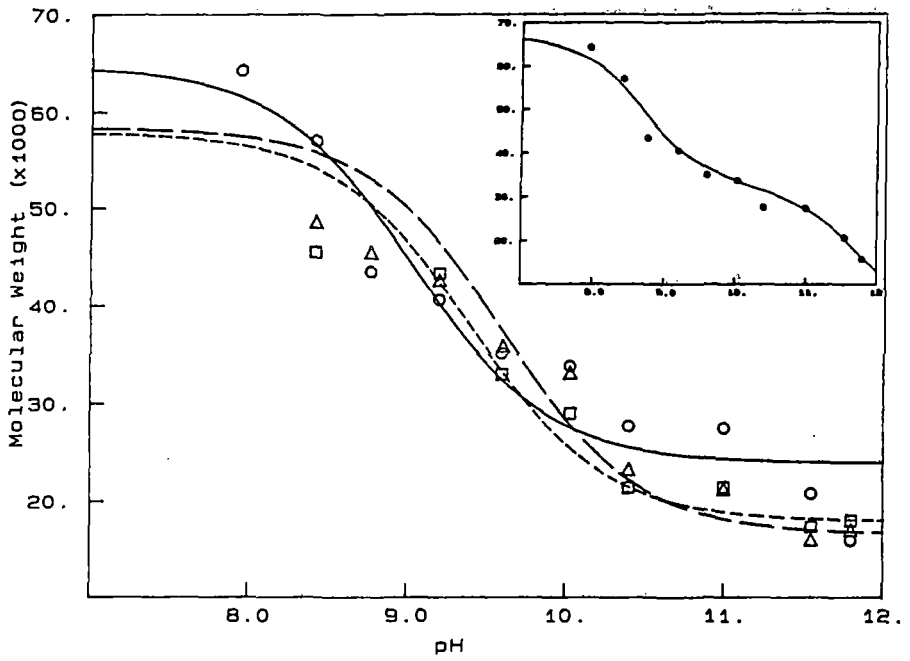


Figure 3.9. Alkali dissociation of aqueomethemoglobins; O AqHbA, □ AqHbC, Δ AqHbB. The curve in the inset has been drawn to indicate the possibility of a stability zone between pH 9.5 and 11 for human aqueomethemoglobin

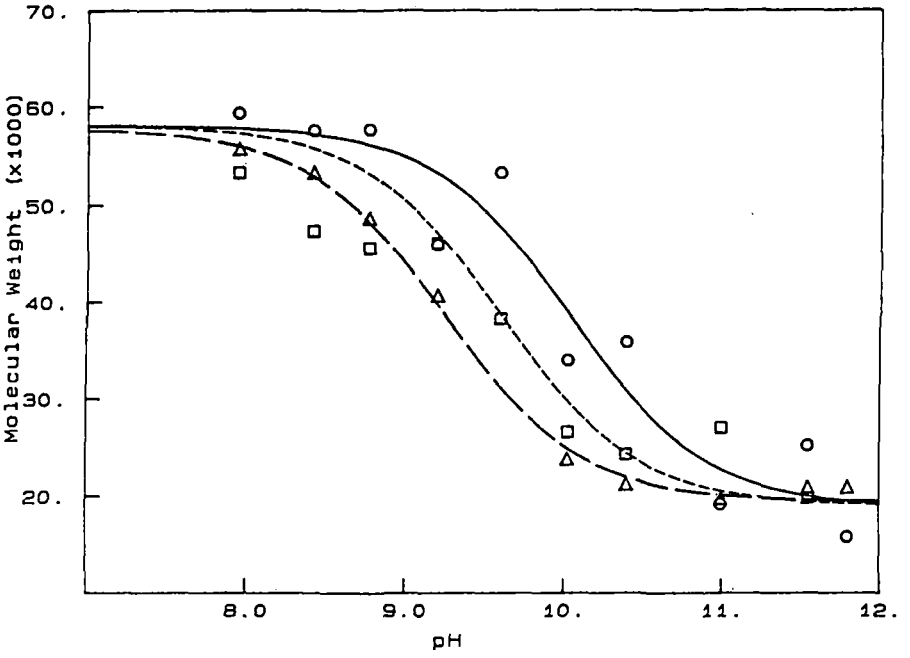


Figure 3.10. Dissociation of carbonmonoxyhemoglobins at alkaline pH; O COHbA, □ COHbC, Δ COHbB

dissociation into dimer takes place between pH 9 and 11. Above pH 11, the hemoglobins continue to dissociate to monomers. According to Kurihara and Shibata (23) the alkaline dissociation of horse hemoglobin could be described by a flat zone between pH 9.5 and 10.5. Dissociation into dimer takes place below about pH 9.5, and the phase above pH 10.5 represents dissociaton of dimer into monomer. Our alkali dissociation curves for AqHbA and CNHbA may also be described with a somewhat flat zone between pH 9.5 and 11. These curves are shown in insets in Figures 3.9, 3.11 and 3.12.

To compare ligand effect on dissociation of a given hemoglobin, molecular weights of human aqueomet-, carbonmonoxy- and cyanomethemoglobins in the alkaline pH zone are plotted in Figure 3.12. Similar comparisons of ligand effect for cow and buffalo hemoglobins are shown in Figures 3.13 and 3.14. For the bovines, within a given species, influence of heme ligands on alkali dissociation could not be discerned clearly. In case of human hemoglobin, the dissociation curves for the three liganded derivatives in Figure 3.12 are fairly dispersed indicating ligand effect on alkali dissociation.

3.4. DISCUSSION

3.4.A. Mechanism of dissociation:-

It is well established that at high concentrations of neutral electrolyte, or of urea, and under the influence of acid and alkaline pH the hemoglobin tetramer dissociates into dimer symmetrically, i.e., $\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta$ (29,38). At extremes of pH the dimer further splits into monomers unsymmetrically. The terms symmetrical and unsymmetrical are related to the modes of rupture of intersubunit contacts which hold the four monomers into a tetramer. Figure 3.15 explains the meaning of symmetrical and unsymmetrical splitting.

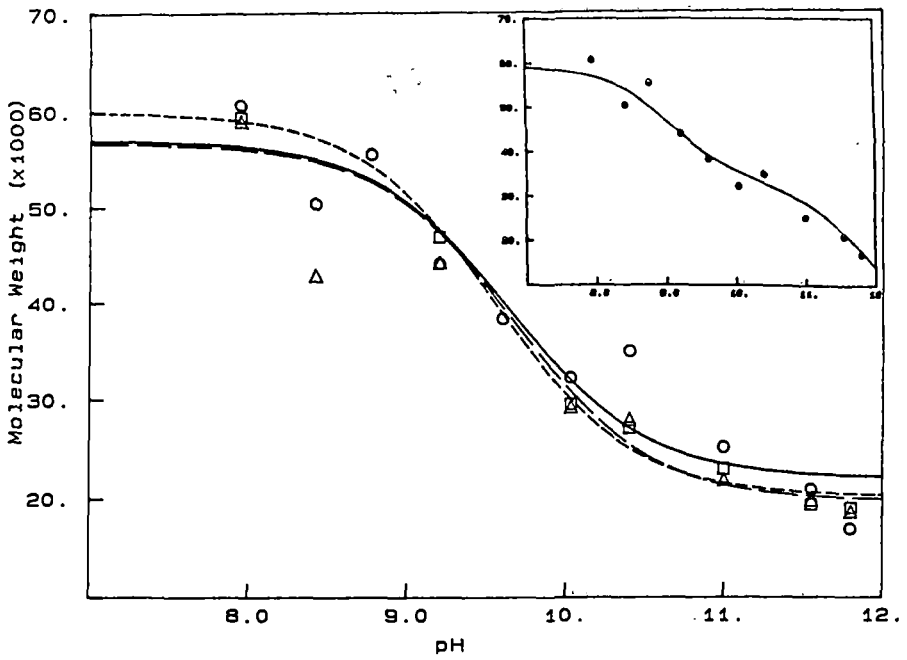


Figure 3.11. Dissociation of cyanomethemoglobins in alkali solutions; O CNHbA, □ CNHbC, Δ CNHbB. Shown in the inset is the dissociation curve of CNHbA with the flat pH zone (see text)

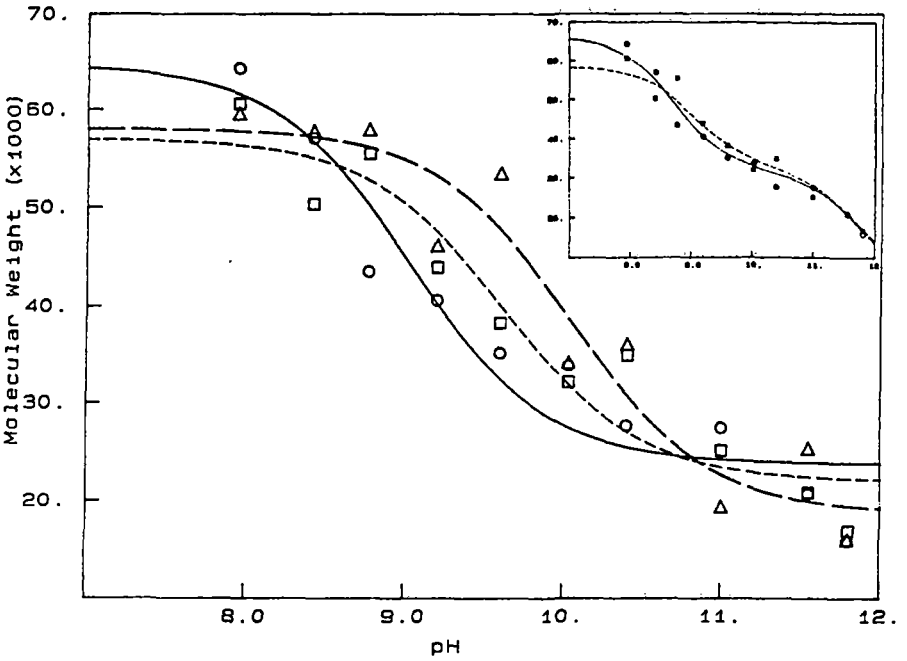


Figure 3.12. Comparison of alkali dissociation of the three liganded derivatives of human hemoglobin; O aqueomet, □ cyanomet, Δ carbonmonoxy. The curves in inset show flat pH zones for aqueomet (O) and cyanomethemoglobins (□)

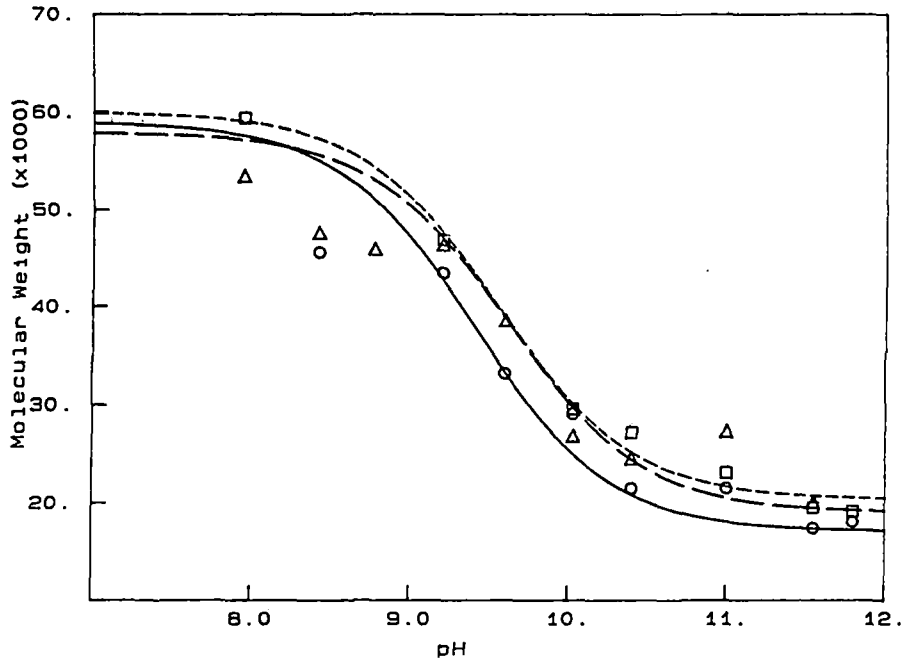


Figure 3.13. Comparison of alkali dissociation of the three liganded derivatives of cow hemoglobin; O aqueomet, □ cyanomet, △ carbonmonoxy

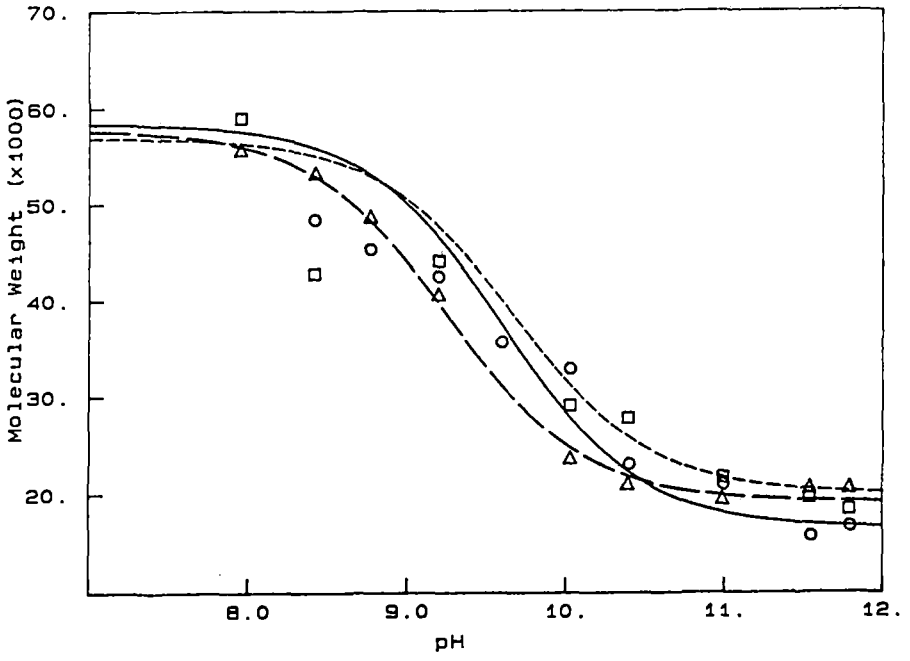
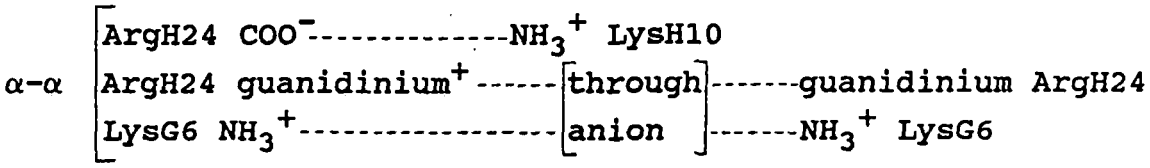
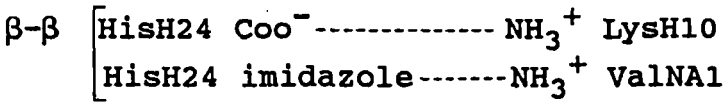


Figure 3.14. Comparison of alkali dissociation of the three liganded derivatives of buffalo hemoglobin; O aqueomet, □ cyanomet, △ carbonmonoxy

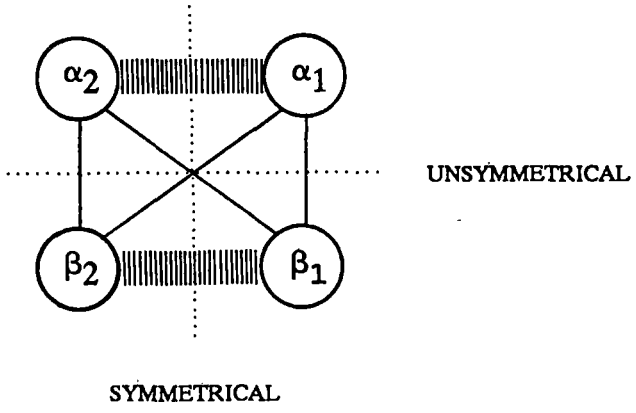
The reason why the tetramer dissociates into dimer symmetrically lies in the nature of intersubunit contacts. The like subunits interact by polar contacts in contrast with nonpolar interactions between unlike subunits (29). As shown below, there exist three salt bridges between the two α subunits and two salt bridges between the β subunits.



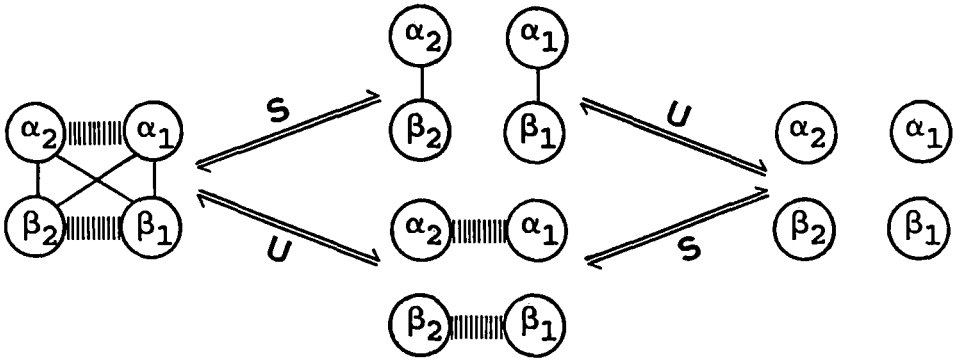


At acidic and alkaline pH these salt bridges become weak. Amongst the nonpolar subunit interactions, the contacts $\alpha_1\beta_2$ and $\beta_1\alpha_2$ are established by fewer number of residues than the contacts $\alpha_1\beta_1$ and $\alpha_2\beta_2$ (29,39). Thus, for tetramer to dimer dissociation, symmetrical splitting is favored over the unsymmetrical one. At the extremes of pH, contacts $\alpha_1\beta_1$ and $\alpha_2\beta_2$ cease to occur and the dimers dissociate into monomers.

The dissociation reaction is influenced by primary structure of hemoglobins. The extent of influence is determined by the variation of binding energy brought about by amino acid replacements. In Hb Kansas, where AsnG4 in one β chain is replaced by a threonine residue, fails to make the native hydrogen bond with AspG1 of an α chain. Instead, a nonnative hydrogen bond is formed between the hydroxyl group of the threonine residue and the main chain carbonyl of AspG1 (29). This structural alteration, which, otherwise, stabilizes the $\alpha_1\beta_2$ dimer, causes the binding energy of these subunits to diminish. Consequently, the tetramer-dimer dissociation constant of oxy form of Hb Kansas is doubled



a



b

Figure 3.15. (a) Illustrative sketch of unsymmerical and symmetrical modes of splitting of the hemoglobin tetramer; polar contacts ||||||||||| , nonpolar contacts ——— ; (b) Tetramer to monomer splitting to show order of rupture of intersubunit contacts associated with given mode of splitting; S symmetrical, U unsymmetrical

relative to normal human hemoglobin (29).

3.4.B. Dissociation of bovine hemoglobins:-

The basic result from the data presented here is that bovine hemoglobins do dissociate at acid and alkaline pH unlike the earlier suggestion of Itano and Robinson (20). The evidence for undissociability of bovine hemoglobin was solely based on failure of cattle hemoglobin to hybridize with human hemoglobin when both were mixed and subjected to action of acid. As the rate of hybridization between two different hemoglobins is a function of the rate of monomer formation and since cow hemoglobin was not found to hybridize with the human counterpart, K_2 of the reaction

$$\alpha_2\beta_2 \overset{K_1}{\rightleftharpoons} 2\alpha\beta \overset{K_2}{\rightleftharpoons} 2\alpha + 2\beta$$

must be significantly different for the two hemoglobins. Magnitude of K_2 is governed extrinsically by pH, ionic strength, nature of the buffer and concentration of hemoglobin, and intrinsically by the interactions which constitute subunit contacts. Under identical pH and solvent conditions, any difference in dissociability must originate from primary structure of the molecule. Rosemeyer and Huehns (38) showed a correlation between p-chloromercuribenzoate (PCMB)-induced monomer formation and sulphydril content in a number of animal hemoglobins. According to them reactivity of CysF9 and CysG14 of β chains and CysG11 of α chains determine the extent of monomer formation. The role of cysteine reactivity on dissociation has also been assumed in the case of Hb Philly where all the six cysteines are reactive (29). In bovine, the hemoglobin tetramer has only two cysteine residues as against six in human. Only CysF9 of β chain is present in bovine. This residue is not involved in αβ contact. Both CysG14 of β and CysG11 of α chains contribute to $\alpha_1\beta_1$ contact. In addition HisG18 of human β chain, which also forms $\alpha_1\beta_1$ contact, is substituted by an

arginine residue in bovine. We do not know whether these replacements exist in buffalo hemoglobin also. These substitutions in case of cow hemoglobin which affect $\alpha_1\beta_1$ contact are summarized in Table 3.2. In light of these replacements, the binding energy between the subunits in bovine tetramer is expected to be different from human hemoglobin. More specifically, on the basis of reduced sulphydril reactivity, the binding energy between unlike subunits of bovine hemoglobin may be higher. This could result in no monomer formation in bovine. This view is also consistent with the report that bovine hemoglobin is highly resistant to the action of alkali (30). Although a clear tilt of the dimer $\rightleftharpoons$ monomer equilibrium to the left is not discernible from the data prsented here, the alkali dissociation curves of aqueomet and cyanomet derivatives of human hemoglobin shown in the inset of Figures 3.9, 3.11 and 3.12 indicate complete monomer formation.

In case of human carbonmonoxyhemoglobin (COHbA), the dissociation to monomer could not be seen clearly. Nevertheless, the present data show incomplete monomer formation in case of both the bovine hemoglobins. A correlation between no monomer formation and presence of only one cysteine in the β chain of human fetal hemoglobin and horse, rabbit and pig hemoglobin has been reported (38). It is likely that two out of four cysteines (at least in cow hemoglobin) are unreactive in bovine. A detailed investigation on the chemistry of cysteines in bovine hemoglobins should be helpful to confirm this.

3.4.C. Effect of heme ligands on dissociation:-

In solution, the balance of the dissociation equilibrium also depends upon the nature of the heme ligand (29). Early studies indicated that salt-induced dissociation curves of deoxy-, oxy- and carbonmonoxyhemoglobins of human and of ferrihemoglobin of cow are similar (24,32,40). On the

TABLE 3.1

MOLECULAR WEIGHTS OF THE PROTEINS USED FOR COLUMN CALIBRATION

Potassium ferricyanide (MW=329.26) was also included to prepare the standard curves shown in Figures 3.1 and 3.2.

Protein	Molecular Weight (M)
Bovine Serum Albumin	69,000
Ovalbumin	43,000
α-chymotrypsinogen	25,700
Myoglobin	17,800
Cytochrome c	12,400

TABLE 3.2

SUBSTITUTIONS OF RESIDUES INVOLVED IN $\alpha_1\beta_1$ CONTACT

Residue #	Segmental position	Human	Bovine
α104	G11	Cys	Ser
β111	G14	Cys	Val
β115	G18	His	Arg

other hand, cyanomet-, oxy- and carbonmonoxyhemoglobins were found to differ greatly in their stability when denatured in acid (41,42). Present data suggest dispersed behavior amongst the three liganded hemoglobins studied. In acidic pH, the dissociation curve of human aqueomet hemoglobin appears to be very different from those of cyanomet and carbonmonoxy forms (Figure 3.6). In case of both the bovine species, in acid pH, respective aqueomet and cyanomet forms seem to have nearly identical dissociation curves (Figures 3.7 and 3.8). Thus, aqueomet hemoglobins of all the three mammals seem to be stable relative to carbonmonoxy and cyanomet hemoglobins. Even in alkaline pH the aqueomet forms appear to be stable over the other two liganded derivatives of human and cow hemoglobin (Figures 3.12 and 3.13).This trend is not seen in case of buffalo, where stability increases in the order, cyanomet-, aqueomet- and carbonmonoxyhemoglobin. (Figure 3.14).

Inconsistent dissociation behavior of different liganded hemoglobins, both within a given species and amongst different animal species, observed here as well as in previous reports (see, for example, 27,43,) is hard to explain. Yet, influence of the heme ligand on dissociation is clear. Properties of heme ligand-linked dissociation must have the basis of electrostatic interaction as the major driving force. Specificity and the number of contacts between the heme and globin and between α and β subunits are quite different among different liganded derivatives (see, for example, 39). Furthermore, there are small differences in the tertiary structure of the subunits in the region of the heme groups and at the C-termini of the subunits (39). Therefore, influence of heme ligands on dissociation properties is not hard to conceive. Even the oxidation status of the iron atom can affect the total stability of heme proteins. For example, our recent NMR and stopped-flow kinetic experiments have definitely established the

stability of reduced cytochrome c over the oxidized one by about 6.5 kcal mol^{-1} (44). We believe that the effects of the heme and the iron atom on the stability and the structural properties of heme proteins originate from differences in electrostatic interaction. In case of hemoglobin, stability and variability in dissociation behavior amongst different liganded forms might arise from different interactions between heme and globin and between the subunits.

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CHAPTER 4

REGULATION OF OXYGEN AFFINITY OF BOVINE HEMOGLOBINS

4.1. INTRODUCTION

Oxygen affinity of vertebrate hemoglobins is known to be modulated by pH, partial pressure of CO_2 and a variety of organic phosphates. Although the effect of protons on oxygen equilibrium has been known for nearly a century (1), the knowledge of the influence of organic phosphates on hemoglobin function is relatively recent. In 1967, it was discovered that 2,3 Diphosphoglycerate (DPG), the principal organic phosphate constituent inside the human erythrocyte, lowers the oxygen affinity of hemoglobin (2,3). Subsequent research demonstrated that all organic phosphates alter the oxygen affinity of hemoglobin. The particular organic phosphate present in the red cells of vertebrates vary in a taxonomic pattern (4). Thus, large amount of inositol pentaphosphate is contained in avian red cells. On the other hand, concentration of adenosine triphosphate (ATP) is higher in the red cells of fish and reptiles (except in alligator). Mammalian red cells, in general, are fairly rich in DPG. On the basis of content of red cell DPG, mammals have been divided into three groups: (1) very high (about 10 mM), as in rat, mouse, pig and rabbit; (2) high (about 5 mM), exemplified by human, horse, dog, porpoise and guneapig; and (3) low (less than 2 mM), as found in the

erythrocytes of cat and ruminants.

Various studies were carried out in seventies in which many species of mammals were tested for red cell DPG, oxygen affinity and the effect of DPG on stripped hemoglobin (5-7). In the erythrocytes of deer, cow, goat and sheep, DPG concentration was found to be less than 0.1 mM. Hemoglobin of these animals have intrisically low oxygen affinity. Bunn and coworkers (5-7) also showed that the oxygen affinity of stripped hemoglobin from these ruminants do not alter much when DPG is added. It, therefore, appeared that the species with low or negligible concentration of organic phosphate have hemoglobins that are insensitive to nucleotide phosphates. Then the obvious questions are : (1) what regulates the oxygen affinity of hemoglobin from animals with low DPG; (2) why organic phosphates do not bind to these hemoglobins; and (3) why oxygen affinity should be intrinsically low.

Less attention has been paid to these questions. Some reports concerning the oxygen affinity of cow hemoglobin have appeared (8,9). In the present study, we have examined the organic phosphate sensitivity of cow and buffalo hemoglobin in more detail. In addition, we have examined the possible role of various allosteric effectors on the function of these two hemoglobins. Of all, chloride ions have been found to have maximum effect on oxygen affinity. Also, an appreciable sensitivity to organic phosphates has been detected. We have also evaluated the effect of urea on oxygen affinity. These results have been compared and contrasted with those of human hemoglobin. The mechanism of functional regulation in bovine appears to be distinct from that in human. In particular, electrostatic effects seem to play a major role in bovine hemoglobins.

4.2. MATERIALS AND METHODS

Human blood was obtained from a normal, healthy adult individual. Cow and buffalo blood samples were collected from animal slaughter house. All samples were anticoagulated with sodium heparin solution containing 0.09 M NaCl. Hemolysate was prepared as already described in Chapter 2. After removing cellular debris by centrifugation at 10,000 rpm the supernatant was filtered through Whatman 1 filter paper followed by passage through a 133x2.6 cm Sephadex G25 column equilibrated with 0.0075 M Tris-HCl, pH 7.4. Prior to gel filtration, a portion of unstripped hemoglobin was set apart for oxygen affinity measurements. Whole blood measurements were done before processing the blood samples.

Oxygen binding measurements were carried out at 37^{o}C with a Hem-O-Scan instrument of SLM Aminco. Hemoglobin concentration was about 95 mg ml^{-1}. Organic phosphates (ATP, GTP, CTP and DPG) were added in the form of solid or solutions. All the experiments were performed in about 48 hours from collection of blood samples. About 5% ferric hemoglobin was formed during measurements.

Reagents were of analytical grade. Tris buffer was prepared at room temperature by titrating the reagent with dilute distilled HCl before adjusting the pH. NaCl and ExcelaR grade urea were obtained from Glaxo. Urea solutions were deionized with mixed bed resin (Amberlite MB3 from Sigma) and buffered at the desired pH before use. Organic phosphates were purchased from Sigma.

In denaturation experiment, hemoglobin solutions were mixed with urea solutions buffered in 0.05 M Tris, pH 7.4 so as to give a final protein concentration 8 micromolar heme ml^{-1}. Complete denaturation was achieved in buffered 6

M guanidinium hydrochloride. The solutions were incubated at room temperature for 2 hours and absorbances were measured at 415 nm. Data were treated as (60),

$$n_\mu = \frac{A_\mu - A_g}{A_0 - A_g} \times 100 \qquad (1)$$

where n_μ is the percentage of native structure retained by the hemoglobin molecule in an urea solution of concentration μ. A_μ is absorbance after incubation in the solution of μ concentration, A_g is the absorbance of hemoglobins in 6 M guanidinium hydrochloride and A_0 is absorbance without urea or guanidine. Urea concentrations used in the experiment varied from 0.2 to 9 M.

Preparation of nitrosyl complexes of hemoglobin has been described in Chapter 2. 0.5 M deionized and buffered urea was added before nitrosylation. ESR spectra were measured as described in Chapter 2.

pH of solutions was measured using a Control Dynamics pH meter (No. 301). Spectrophotometric measurements were done in a UVIDEC 610 spectrophotometer of JASCO.

4.3. RESULTS

Oxygen affinity of whole blood in both the bovine species is markedly lower than that of human (Figure 4.1). Similar observation was made in case of stripped hemoglobins in 0.0075 M Tris-Cl, pH 7.4 at 37°C. As shown in Figure 4.2, human hemoglobin has a higher intrinsic oxygen affinity than that of cow and buffalo. The P_{50} values noted for whole blood and stripped hemoglobins are shown in Table 4.1. Of the two bovine species, buffalo hemoglobin appears to have lower oxygen affinity than that of cow. The n values of human, cow and buffalo hemoglobins were found to be 2.7, 3.09 and 3.13, respectively.

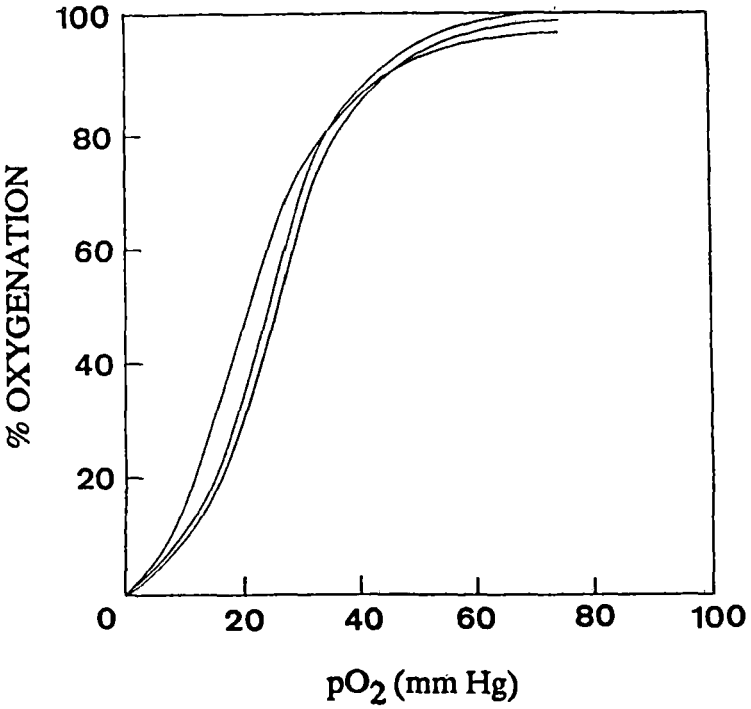


Figure 4.1. Oxygen equilibrium curves of whole blood at 37°C. The curves from left to right are of human, cow and buffalo.

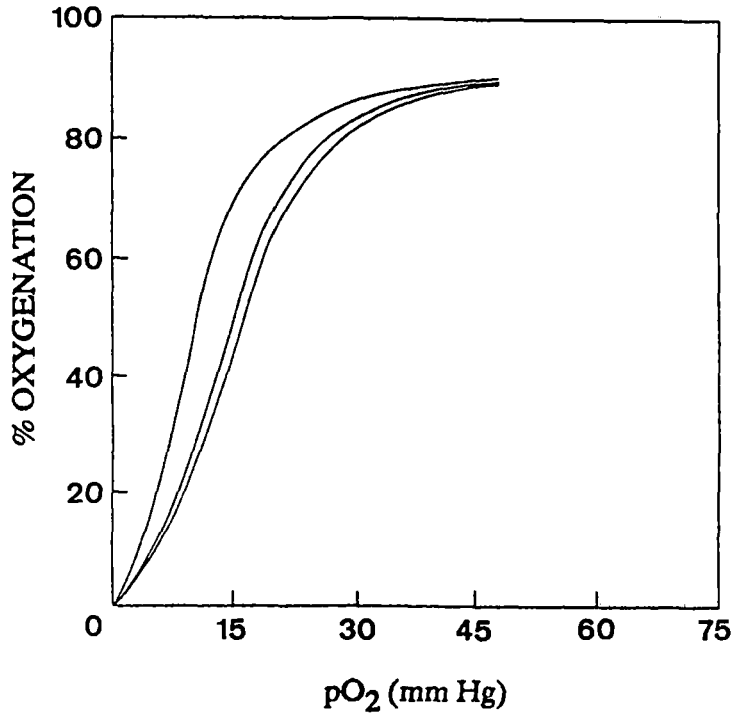


Figure 4.2. Oxygen equilibrium curves of stripped hemoglobins at 37°C, 0.0075 M Tris-Cl, pH 7.4. The curves from left to right are of human, cow and buffalo.

TABLE 4.1

P_{50} VALUES OF HUMAN, COW AND BUFFALO HEMOGLOBIN
(in mm Hg)

Experimental condition	Human	Cow	Buffalo
Whole blood at 37°C	20.5	24.5	26.9
Stripped hemoglobin in 0.0075 M Tris-Cl, pH 7.4, 37°C	9.75	14	15.38

The effect of NaCl on oxygen affinity is shown in Figures 4.3 and 4.4. Both bovine hemoglobins are relatively more sensitive to the presence of chloride ions (Figure 4.3). At lower concentration of Cl^- the three curves tend to converge. At higher salt concentrations steepness of the curves result from dissociation of the tetramer into dimer, which is less pronounced in case of human hemoglobin. In low salt, sligltly low oxygen affinity of buffalo hemoglobin compared to that of cow becomes apparent. Greater effectiveness of chlorides in case of the two bovine species is also seen in Figure 4.4. In this figure, Bohr effect of the three hemoglobins in presence of 0.075 M and 0.15 M NaCl is shown. As a first approximation, steepness of the two bovine curves indicate larger Bohr effect than that in human. All the three hemoglobins have minimum oxygen affinity near pH 6. As expected, oxygen affinity increases above and below this pH zone.

Figure 4.5 shows effect of organic phosphates on oxygen affinity of stripped hemoglobins at pH 7.4. Human hemoglobin was tested only for DPG effect (Figure 4.5.c). In all cases of organic phosphates tested, full saturation of the effect on oxygen affinity is observed for concentrations greater than 4 mM (effect of GTP was tested only upto 3 mM). For the bovine hemoglobins, saturation is noticed even at lower concentration of DPG (Figure 4.5.c). These results indicate binding of organic phosphates to stripped hemoglobins of cow and buffalo.

Effect of urea on oxygen affinity of human, cow and buffalo hemoglobins is shown Figures 4.6 and 4.7. Urea causes an increase in oxygen affinity of all the three hemoglobins. This effect is relatively greater in the case of human hemoglobin. At higher concentration of urea (>3 M) a saturation effect is observed. Effect of urea in

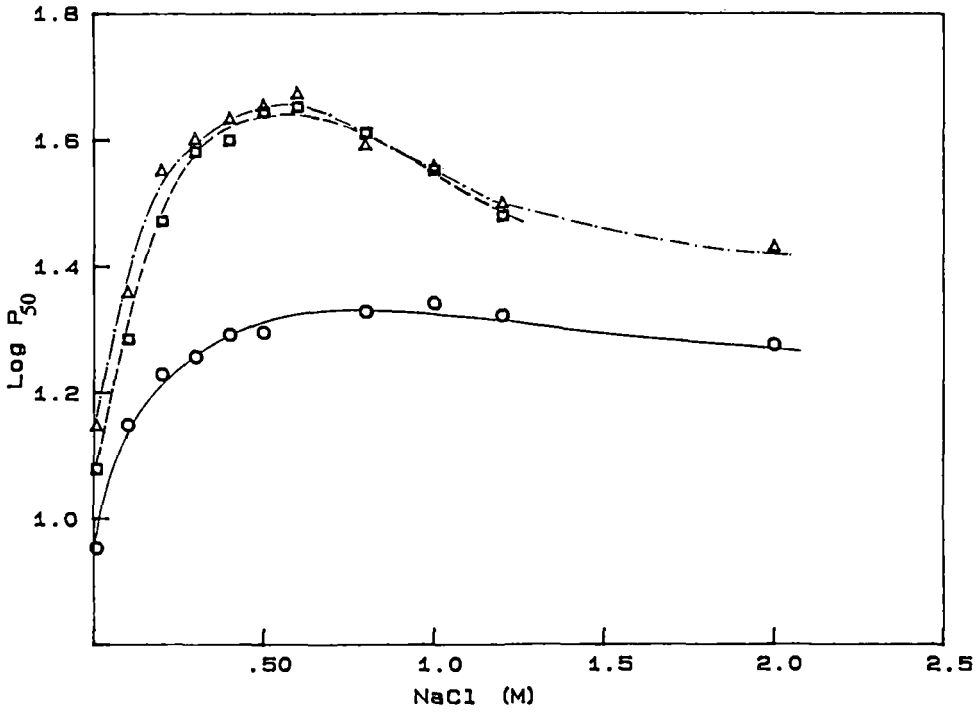


Figure 4.3. Effect of NaCl concentration on the oxygen affinity at 37°C in Tris buffer, pH 7.4.
○ Human, □ Cow, △ Buffalo.

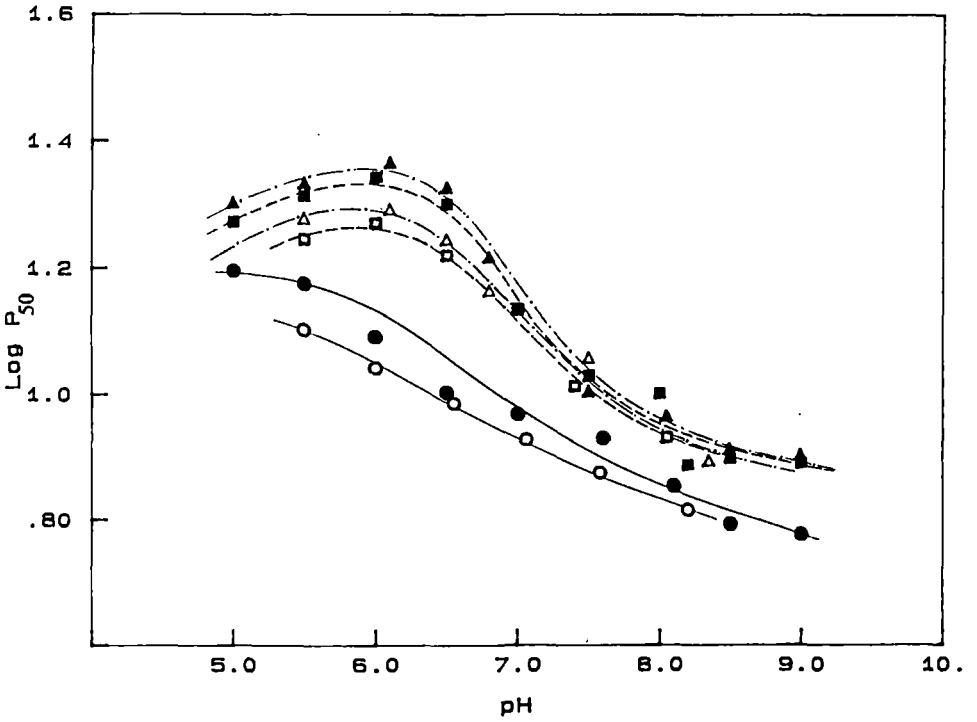
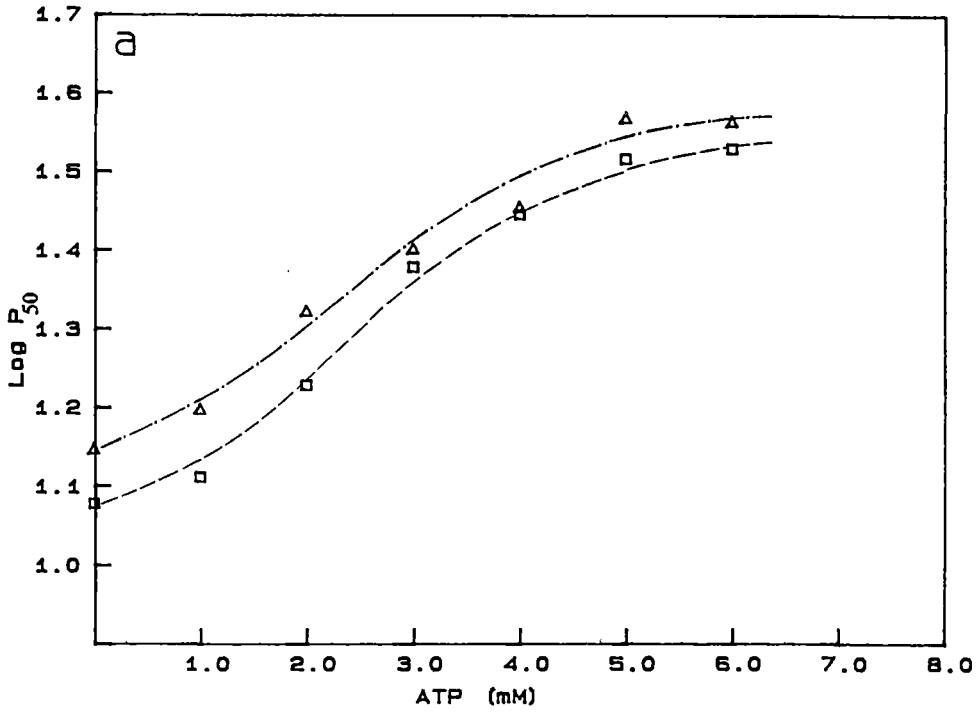
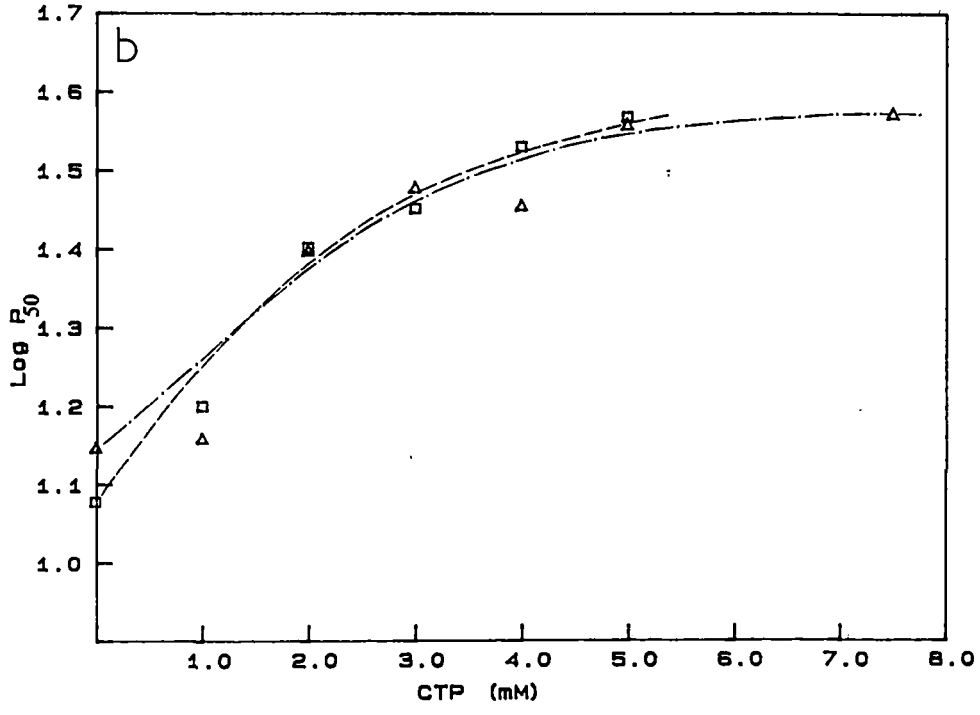


Figure 4.4. Bohr effect of human and bovine hemoglobins;
● Human, ■ Cow, ▲ Buffalo in presence of 0.15 M Cl^-,
○ Human, □ Cow, △ Buffalo in presence of 0.0075 M Cl^-


a
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
Log P_{50}
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
ATP (mM)


b
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
Log P_{50}
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
CTP (mM)

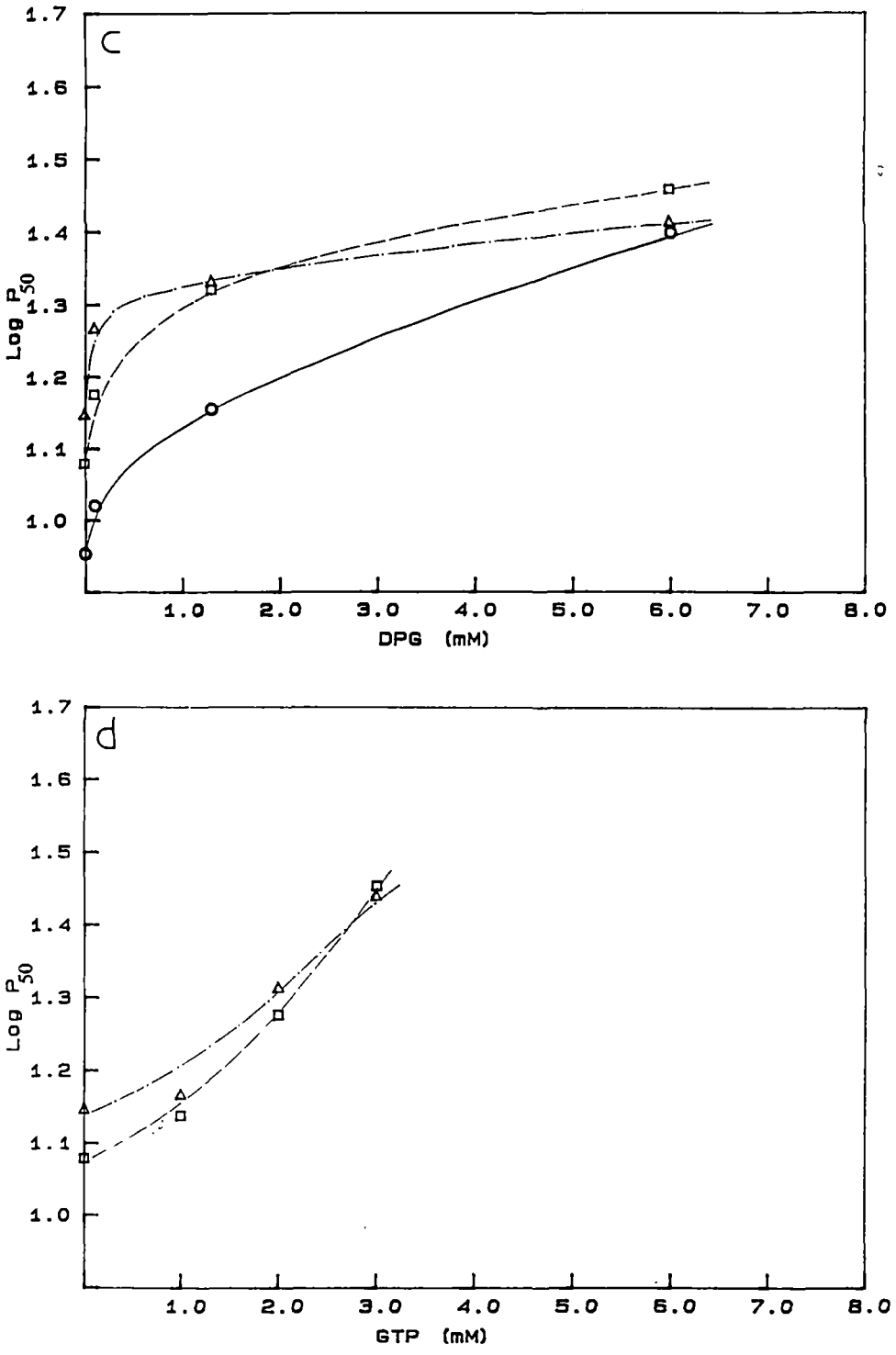


Figure 4.5. Effect of organic phosphates on the oxygen affinity at 37°C in 0.0075 M Tris-Cl, pH 7.4.
(a) ATP effect, □ Cow, △ Buffalo;
(b) CTP effect, □ Cow, △ Buffalo;
(c) DPG effect, O Human, □ Cow, △ Buffalo;
(d) GTP effect, □ Cow, △ Buffalo

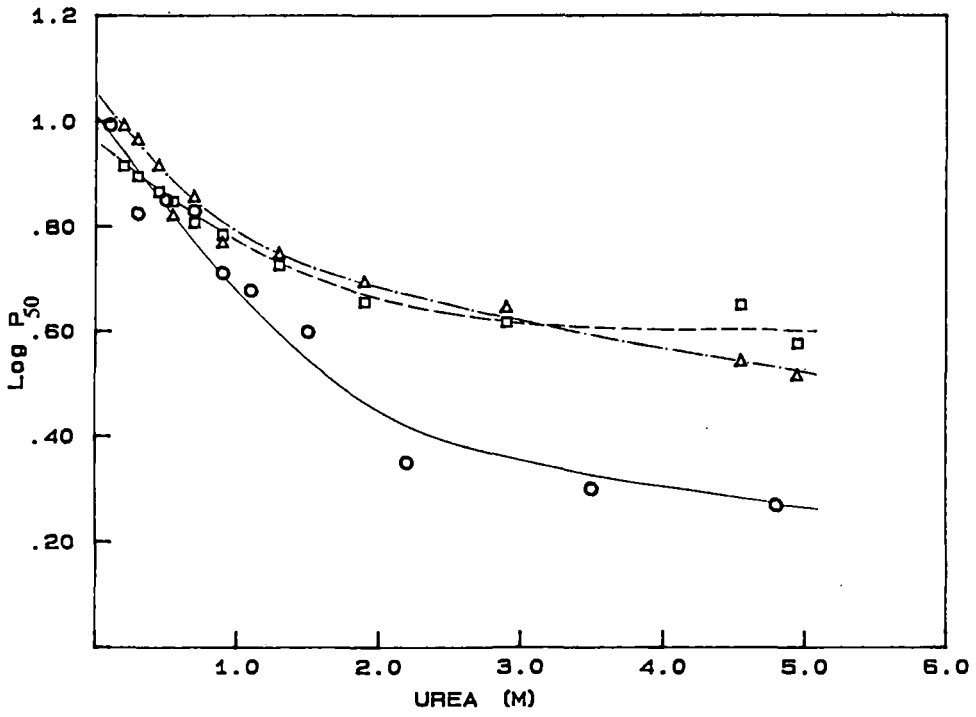


Figure 4.6. Effect of urea on the oxygen affinity; O human, □ cow and Δ buffalo hemoglobin in 0.0075 M Tris-Cl, pH 7.4 at 37°C

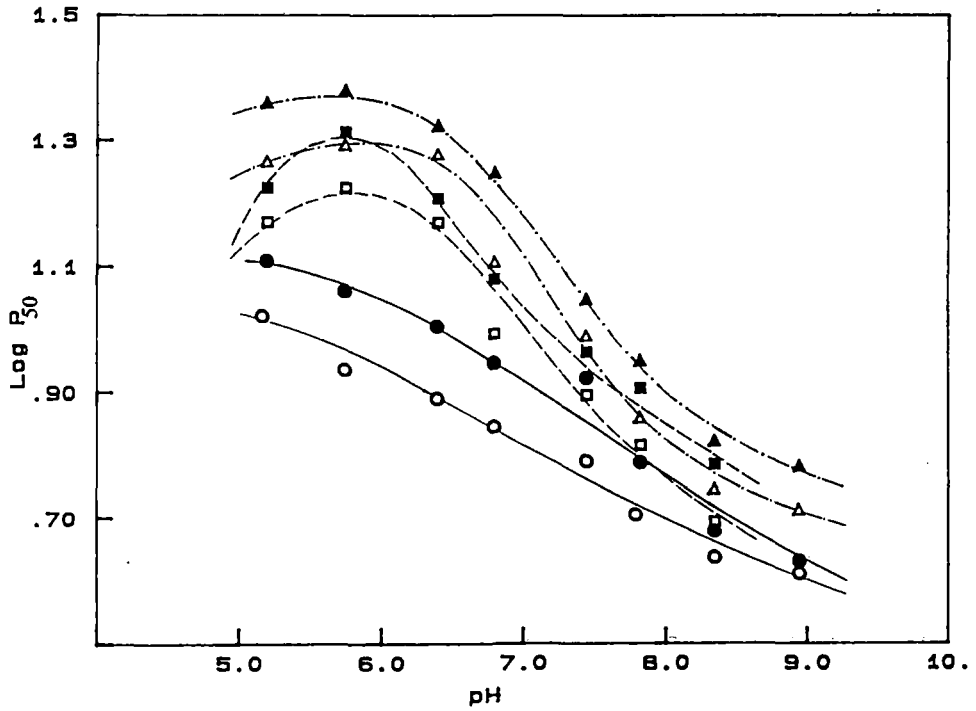


Figure 4.7. Bohr effect of human and bovine hemoglobins in presence of urea; ● human, ■ cow, ▲ buffalo in presence of 0.05 M urea, O human, □ cow, Δ buffalo in presence of 0.35 M urea

increasing the oxygen affinity is also apparent from Figure 4.7. This figure shows Bohr effect of hemoglobins in presence of 0.05 and 0.35 M urea. The curves seem to converge at higher pH. As was seen in Figure 4.4, here also Bohr effect is more pronounced in case of bovine.

Stability of human and bovine hemoglobins in presence of different concentrations of urea, as measured by Soret intensity, is shown in Figure 4.8. Normalized values of percent native structure have been plotted as a function of urea concentration. Figure 4.9 shows the ESR spectra of nitrosyl complexes of all three hemoglobins in presence of 0.5 M urea.

4.4. DISCUSSION

4.4.A. Intrinsically low oxygen affinity of bovine hemoglobins:-

In connection with oxygen affinity, intrinsically low refers to low sensitivity of hemoglobins to DPG (8). Given the experimental conditions of pH 7.4, 20 to 25^{o}C, and in presence of 0.1 M NaCl, the low affinity hemoglobins (stripped) have P_{50} values of between 10 and 20 mm Hg (8). P_{50} values of cow and buffalo hemoglobins (stripped) in the present study have been found to be 14 and 15.38 , respectively (Figure 4.2 and Table 4.1), and since the effect of organic phosphates on oxygen affinity is relatively less (Figure 4.5), the two bovine species may be considered to represent the group of animals with intrinsically low oxygen affinity. In the following discussion, we would assume structural and functional nearness of the two bovine hemoglobins.

Characteristically low intrinsic affinity for oxygen, as in the case of the two bovine species, can be described in terms of the two conformational states, R and

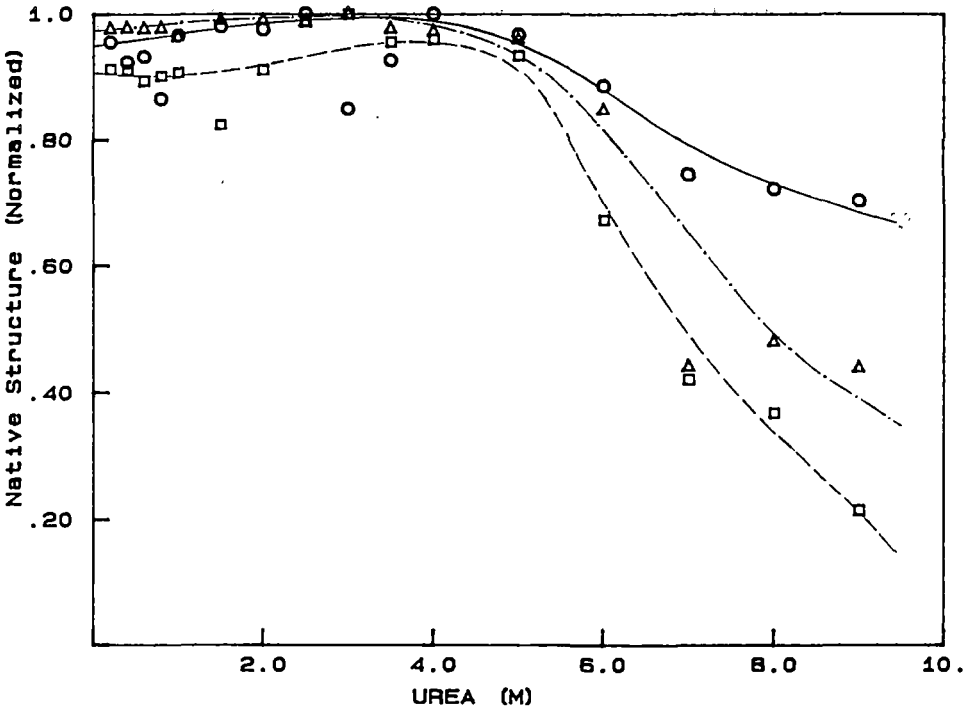


Figure 4.8. Hemoglobin native structure (n) remaining after 2 hour incubation at 37°C in 0.0075 M Tris-Cl, pH 7.4 containing different concentrations of urea;
O human, □ cow, Δ buffalo

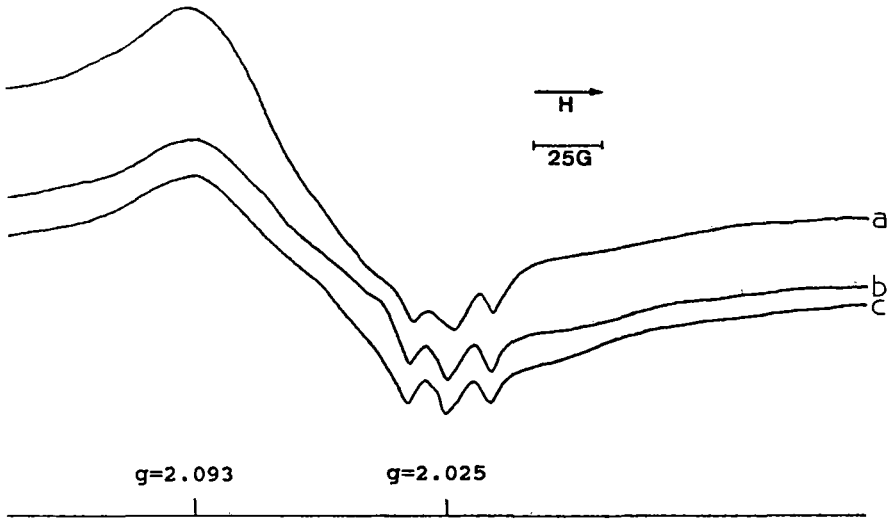


Figure 4.9. ESR spectra of nitrosyl complexes of stripped hemoglobins in presence of 0.5 M urea in 0.0075 M Tris-Cl, pH 7.4. Spectra were recorded at 77 K; (a) human, (b) cow, (c) buffalo. See text in Chapter 2 for ESR conditions.

T, within the framework of MWC model (10). For the allosteric equilibrium

$$R \rightleftharpoons T$$

the allosteric constant, L = T/R. Both R and T states have individual affinity for ligands, which can be described through the magnitudes of their respective microscopic dissociation constants, K_R and K_T.

$$RF \rightleftharpoons R + F \quad \text{where F is ligand}$$

$$K_R = \frac{[R][F]}{[RF]} \tag{2a}$$

Similarly, for

$$TF \rightleftharpoons T + F$$

$$K_T = \frac{[T][F]}{[TF]} \tag{2b}$$

For hemoglobins with intrinsically high oxygen affinity, K_R is smaller than K_T, which indicates larger affinity of the R state for ligands. According to allosteric theory, cooperativity is more marked when the ratio of K_R to K_T is small ($c = K_R/K_T$). It was actually shown by Perutz and Imai (8) that K_R is same in stripped hemoglobins of cow and human, but K_T in case of cow is only about a half of that in human. This indicates that c is larger in cow, which in turn implies low cooperativity in this species of ruminants. Even the magnitude of L is higher in case of bovine hemoglobins. In 0.1 M NaCl, the value of L in cow is about 60 fold higher than that in human (8). A qualitative picture of largeness of L is provided by our ESR experiments on nitrosyl complexes of both cow and buffalo hemoglobins (see Chapter 2). This is indicative of a greater concentration of deoxy quaternary structure in bovine. Smallness of K_T, on the other hand, points to the stability of the T structure. Thus, a strong tilt of the allosteric equilibrium towards a constrained and stable T conformation explains intrinsically low oxygen affinity of bovine hemoglobins. The origin of this allosteric tip and stability of the T conformation must lie in the structure of the molecule as well as in the mode

of its interaction with nonheme ligands.

4.4.B. Binding of Cl^- ions to bovine hemoglobin:-

Cl^- ions are known to influence the oxygen affinity of hemoglobins (see, for example, 11). In quite a few instances, including certain abnormal human hemoglobins, the observed oxygen affinity has been explained on the basis of varying chloride binding properties of hemoglobins (9,29,58,59). A larger chloride binding, in case of cow hemoglobin, was experimentally observed by Fronticelli and coworkers (9). Our ESR experiments hinted at the role of Cl^- in controlling the allosteric equilibrium (see Chapter 2). So, we performed oxygen equilibrium measurements of cow and buffalo hemoglobins in presence of varying concentrations of chloride. More effectiveness of Cl^- in lowering the oxygen affinity of cow and buffalo hemoglobins is clearly seen in Figures 4.3 and 4.4. Both cow and buffalo hemoglobins are seen to have nearly identical sensitivity to increasing concentration of NaCl. The number of chloride ions released (or bound) by the hemoglobin tetramer upon oxygenation (or deoxygenation), at a given pH, can be determined according to the Cl^- linkage equation (12,13),

$$\Delta Cl = \left(\frac{\partial \log P_{50}}{\partial \log [Cl^-]} \right) \qquad (3)$$

where $[Cl^-]$ indicates chloride ion activity in solution. ΔCl, the number of chloride ions exchanged per heme can be estimated from the slopes of the curves in Figure 4.3. At 0.2 M NaCl, pH 7.4, oxyhemoglobin of human, cow and buffalo release approximately 1, 2.1 and 1.8 chloride ions, respectively. This result indicates about two fold larger interaction between bovine deoxyhemoglobin and Cl^-.

For specific binding of any allosterically-linked ligand there must be certain distinct binding sites present

in the protein. The number of Cl^- ions bound to the hemoglobin molecule depends on the number of binding sites available as well as the affinity constant of each of these binding sites for chloride in deoxy and oxy structure, i.e.,

$$\Delta Cl = \sum_i \left[\frac{K_i'[Cl^-]}{1+K_i'[Cl^-]} - \frac{K_i[Cl^-]}{1+K_i[Cl^-]} \right] \quad (4)$$

where K_i' and K_i are affinities of the i^{th} site for Cl^- ions in deoxy and oxy hemoglobin respectively. Fronticelli et al. (9) computed the values of K_i' and K_i for two values of i, 1 and 3, so as to simulate the experimental curves of P_{50} vs. Cl^- ion concentration which they obtained for cow hemoglobin. Their simulation indicated that the ratio K'/K is higher for cow hemoglobin than for human hemoglobin. This means that in cow the affinity of deoxyhemoglobin for Cl^- ions is higher than in human. Considering the functional alikeness and structural homology between cow and buffalo hemoglobins a similar behavior may be expected in the case of buffalo hemoglobin as well.

But what about the nature and the number of chloride binding sites? This question is best answered by chemical modification, and spectrophotometric and NMR titration of susceptible groups. NMR data indicates that chloride binds at or near HisHC3β and ValNA1α (14-16). X-ray crystallography also indicated that ValNA1 is associated with chloride binding (17). Several chemical modification experiments showed that a number of other positively charged residues including LysEF6, HisH21 and HisHC3 of β subunits and ValNA1 of α subunits are involved in chloride binding (18-23). Since the effects of Cl^- and 2,3-DPG have been shown to be competitive (24,25), these anions might share the same binding sites. Two chloride binding sites, one located between the α amino group of ValNA1 and the β hudroxyl group of SerH14 on the same α chain, and another positioned between α amino group of ValNA1 and the

guanidinium group of ArgHC3 on the opposite α chain, have been determined from X-ray diffraction (17). Also, chloride binds to the COOH terminal residues of both α and β chains (16,26). An inspection of the amino acid sequence of cow hwmoglobin (Figures 1.4 and 1.5 in Chapter 1 and ref. 27,28) would show that all these amino acids are present. In addition to these, there might be other chloride binding sites in bovine which are absent in human hemoglobin. It would be possible to say something more conclusive once the primary structure of buffalo hemoglobin becomes available.

Assuming that the amino acid sequences of ruminant hemoglobins do not directly contribute to extra Cl^- binding sites, it should be possible to account for the difference in ΔCl between human and bovine hemoglobins in terms of molecular solvation and electrostatic energy. A preliminary and short version of difference in protein solvent interaction between human and cow hemoglobin has already appeared (8,9). The suggestion of Perutz and Imai (8) that deletion and substitution in the primary structure at the N-terminal end of cow β chain distorts the initial segment of the β subunit, was used by Fronticelli and coworkers (9) to explain additional chloride binding. According to them this distortion might produce larger interaction of the protein with the solvent and an increased polarity of the environment. In addition, a new steric situation may be produced with consequent repulsion for Cl^- in the oxy structure. Thus, this model is partially based on the structural changes at the amino terminus of β chains. It is worth while to note that the distortion mentioned here might not always cause increased Cl^- binding. For example, Hb Leiden, in which the 4th or the 5th residue of the A-helix at the N-termini of β chains is deleted, shows a decreased response to chlorides both in equilibria and kinetics of ligand binding (29).

Instead of focusing at the N-terminus alone, it would perhaps be more promising to consider the whole molecule electrostatically. The electrostatic energy, ΔG^{o} of a molecule is composed of contributions from Coulombic interaction between pairs of charged atoms on the molecule ΔG^{o}_{c}, interaction of the charges with the solvent ΔG^{o}_{p}, and the interaction of the charges with the ion atmosphere in the solvent ΔG^{o}_{a} (30,31),

$$\Delta G^{o} = \Delta G^{o}_{c} + \Delta G^{o}_{p} + \Delta G^{o}_{a} \qquad (5)$$

For n number of charges,

$$\Delta G^{o} = \sum_{i=1}^{n} (\Delta G^{o}_{c,i} + \Delta G^{o}_{p,i} + \Delta G^{o}_{a,i}) \qquad (6)$$

Magnitude of each of these terms will vary with composition and replacement of amino acids. For two conformations of a macromolecule the main difference would be charge-solvent interaction. It is possible that hemoglobins showing variable interactions with anions mostly differ in interaction of their cationic charges with the ion atmosphere in the solvent. This is a speculation only. It is, however, clear that application of electrostatic treatment would assume conformational heterogeneity amongst hemoglobin types. New methods and improvement upon old procedures of calculation of electrostatic energy are now available (30-35). Application of these methods to hemoglobin should be useful to understand the mechanism of interaction of allosteric ligands in greater detail.

4.4.C. Binding of organic phosphates to bovine hemoglobins:-

In 0.1 M NaCl, pH 7.2, 20°C, and in presence of 0.2 mM DPG, Bunn (36) found very less rise in P_{50} of stripped hemolysate of cow. For his experiments Bunn used 0.05 mM hemoglobin (tetramer). However, we find that oxygen affinity

of hemoglobins of both cow and buffalo are affected by each of the four organic phosphates used in this study. DPG appears to be most effective (Figure 4.5). Actually, phosphate effects are sometimes unnoticed when low concentrations of organic phosphates are tested. At the same time it is desirable to use a moderately concentrated hemoglobin solution (see ref. 37 for a discussion on DPG/hemoglobin molar ratio and dilution of the solution). These two facts could form part of the reasons why effects of organic phosphates were not noticed appreciably in earlier studies. Since chloride ions are highly effective in lowering the oxygen affinity of bovine hemoglobins, for appreciable detection of effect of organic phosphates it is desirable to use low concentration of NaCl in the solution. Effect of DPG on the oxygen affinity of stripped hemolysate of cow was also observed by Fronticelli et al. (9).

The curves in Figure 4.5, in principle, should obey the equation (38,39),

$$\log P_{50} = \text{constant} + \frac{1}{n} \log \frac{1 + \frac{c}{K_D}}{1 + \frac{c}{K'}} \qquad (7)$$

where n is Hill coefficient, c is effector concentration, K_D is approximate dissociation constant of deoxyhemoglobin-effector complex and K' represents an average value for binding of effector to intermediate oxygenation states such as HbO_2 and HbO_4. At higher concentration of effectors, when $c >> K >> K_D$, the above equation reduces to

$$\log P_{50} = \text{constant} + \frac{1}{n} \log \frac{K'}{K_D} \qquad (8)$$

This indicates that the ratio of K' to K_D will primarily determine the oxygen affinity. K_D varies largely with the type of polyanion. K_D for DPG is far lower compared to that

for ATP. On the other hand, K′ values for a variety of polyanions are close to each other (39). It is, thus, clear that the magnitude of K_D will eventually take the leading role in determining oxygen afiinity. On the basis of gathered evidences in favor of weaker interaction of bovine hemoglobins with organic phoshates it can be assumed that K_D in bovine, with respect to a given polyanion, is larger compared to that in human. This explains why oxygen affinity of bovine hemoglobins is not much lowered by organic phosphates. Notice that the influence of n is not great, since variation in the ratio of K′ to K_D in two different hemoglobins can more than compensate for the effect of n.

To account for larger K_D for a given polyanion (e.g., DPG) in bovine, a structural description of the binding site is essential. In normal adult human hemoglobin the binding site of DPG is constituted of four residues, namely, ValNA1 , HisNA2, LysEF6 and HisH21 of β chains (41,42). The deletion of ValNA1 and substitution of HisNA2 by a methionine reidue in the β subunit of cow and sheep hemoglobins have been considered (5,8). Does this structural alteration fully abolish binding of organic phosphates? In HbA as well as in other intrinsically high oxygen affinity hemoglobins, the amino group of ValNA1 and the side chain of HisNA2 of β chains are external and capable to form salt bridges with organic phosphates. In ruminant hemoglobins, the side chain of MetNA2β will point to the interior of the protein. Amino group of this residue can, however, interact with organic phosphates, the binding potential of which would be determined by the extent to which the amino group is free. The probable conformation of N-terminal segment of the β chains of cow and sheep hemoglobin has been published by Perutz and Imai (8). There is no indication of involvement of the amino group in any interaction. Furthermore, the N-terminus will certainly be influenced by the positive end of A-helix dipole. Consequently, the amino

group of MetNA2 should have a potential to bind phosphates. In many proteins phosphates bind at a distance of 3-5 Å from the amino terminus of a helix (43). The other two DPG binding sites, namely, LysEF6 and HisH21 are present in those ruminant hemoglobins the primary structure of which have been determined. These two groups should retain some interaction with DPG unless the conformation of intrinsically low affinity hemoglobins is very different from the high affinity group. Thus, interaction of organic phosphates with bovine hemoglobins cannot be fully eliminated. The N-terminal conformation of β subunits, due to apolar character of MetNA2 side chain, is certainly unfavorable for phosphate binding. This, however, diminishes interaction but does not abolish fully. The diminished interaction is reflected in a higher dissociation constant, K_D of deoxyhemoglobin-organic phosphate complex in bovine. If our contention is correct one should be able to obtain a reasonable correlation between experimentally observed P_{50} with calculated P_{50} values by employing the equation above. To do so one needs to determine K_D and K' experimentally. This can be done by monitoring changes in the proton concentration of unbuffered solutions as polyanions bind to hemoglobin (44-46). A few preliminary experiments on interaction of IHP and ATP with cow and buffalo hemoglobins have qualitatively indicated higher K_D values. More carefully controlled experiments will be needed to obtain quantitative data and to confirm this result.

4.4.D. Effect of urea on oxygen affinity of bovine hemonglobins:-

It has been known for many years that in presence of urea hemoglobin has an increased oxygen affinity. It was first demonstrated by Taylor and Hastings (47) that horse hemoglobin, in presence of 4 M urea, has an increased oxygen affinity. Since then urea effect has been shown in several mammalian hemoglobins (48-50). No report, however, exists on

oxygen equilibrium of bovine hemoglobins in presence of urea. Our results furnish data for increased oxygen affinity of cow and buffalo hemoglobins in a wide range of urea concentrations (Figures 4.6 and 4.7). The changes in oxygen equilibrium are not the same for human and the bovine. In low concentration (<1 M), urea effect is nearly same for all the three hemoglobins. In concentrations greater than about 1 M, rise in oxygen affinity is far greater for human than for the two bovine species. In still higher concentrations (>3 M), a saturation effect is observed for all the three hemoglobins.

A novel molecular basis for the effect of urea on function of hemoglobin is not certainly known. Urea does perturb the secondary and tertiary structure of hemoglobin, as is also true for other proteins (see, for example, ref 51,52). Existing data on various hemoglobin systems, however do not suggest dissociation of the tetramer by urea at a concentration below 4 M. Although there exists discrepancies regarding stability of various vertebrate hemoglobins in urea solutions, many hemoglobins appear to be fairly stable upto about 4 M urea (50,60). This is also seen from our urea-induced denaturation experiments. Figure 4.8 indicates no loss of native structure of human and bovine hemoglobins upto about 5 M urea. Since the rapid rise in oxygen affinity takes place in presence of urea concentrations lower than about 3 M (figure 4.6), it would appear that neither conformational instability nor dissociation of the hemoglobin molecule is the basis for the observed properties. Another fact is that the effect of organic phosphates can be observed even in presence of urea (see, for example, ref 53). Phosphates can influence oxygen binding only when the hemoglobin is a tetramer (41,54).

Difference electron density maps calculated from three dimensional X-ray intensity measurement on crystals of

α-chymotrypsin in 3 M urea (55) have provided evidence that urea binding may not cause any structural change to the protein. Also, low concentration of urea can massively penetrate into the protein interior. It is likely that even at low concentrations, urea penetrates into the heme pocket and exerts local effect. It is hard to explain the modes of these effects. It is, however, worth noting at this point that the ESR spectra of nitrosyl complexes of human and bovine hemoglobins, in presence of 0.5 M urea, show changes in heme electronic structure leading to loss of native heme environment (Figure 4.9). We shall make no effort to explain these spectra here, since more careful measurements are warranted. But it is possible that urea-induced oxygenation is caused via heme structural alterations. It is also likely that cyanate present in urea solutions reacts irreversibly with ϵ amino group of lysine and α amino groups of other residues in the hemoglobin tetramer. Reversible reaction of cyanate with functional groups of proteins such as -SH, phenolic -OH and -COOH is well known (56). Cerami et al. (57) have discussed the effect of carbamylation of N-terminal valines with cyanate.

There is another contention that urea in its cationic form binds to carboxyl termini of α and β chains of hemoglobin (ref. 14 quoted in 53). Weber et al. (53) have pointed out that the greater effect of urea on oxygen affinity at high than at low pH supports the possibility of electrostatic interaction of urea with negatively charged carboxyl termini of the protein. However, such an explanation should take Bohr effect into account. Figure 4.7 shows the oxygen affinity of human, cow and buffalo hemoglobin in presence of 0.5 and 0.35 M urea as a function of pH. The Bohr effect is faithfully represented. A comparison of these curves with those in Figure 4.4 reveals that in presence of urea, the curves are steeper, which might be indicative of greater magnitude of Bohr effect. Yet

in Figure 4.7, for a given hemoglobin, the slopes of the two curves corresponding to the two different concentrations of urea are roughly the same. This implies that urea does not directly contribute to Bohr effect. It would rather indicate stronger electrotatic interaction of urea with terminal COO^- groups at higher pH. A rigorous analysis of the urea effect and the underlying mechanism do not seem to be easy. It is as if a number of specific and nonspecific events produced by urea leads to a cumulative energetic significance which is manifested in high oxygen affinity.

Effect of urea, at low concentrations, on the oxygen affinity of human and bovine hemoglobins seems to be similar. Above about 0.5 M urea, oxygen affinity of human hemoglobin abruptly increases whereas for bovine the increase is smooth. Without a full knowledge of molecular basis of urea effect we cannot offer an explanation at this stage. It is worth noting that the extent to which urea affects oxygen affinity of a hemoglobin is related to the blood and erythrocyte concentration of urea in that species (see, for example, 60).

4.4.E. Bohr effect in bovine hemoglobins:-

Bohr effect is essentially a heterotropic linkage between proton and oxygen. In other words, this is the pH dependence of oxygen affinity in hemoglobin. The protonic linkage equation (12) is

$$\left(\frac{\partial \log P}{\partial pH}\right)_y = \left(\frac{\partial H^+}{\partial y}\right)_{pH} \qquad (9)$$

or

$$-\frac{d\log P_{50}}{dpH} = \Delta H^+ \qquad (10)$$

where P is partial pressure of oxygen, y, the fractional saturation of hemoglobin with oxygen, and H^+ is the average

number of protons per heme bound by hemoglobin.

When oxygen binds at a given pH the number of protons dissociated, $\triangle H^+$ can be estimated according to the proton linkage equation by plotting log P_{50} as a function of pH. The slopes of the curves yield values of $\triangle H^+$. It is estimated from Figure 4.4 that on oxygenation in presence of 0.075 M Cl^-, human, cow and buffalo hemoglobins liberate about 1.2, 1.8 and 2 protons respectively. This is an indication of larger Bohr effect in bovine, and is consistent with earlier report (9, also see 61).

Bohr effect originates from interaction between positively charged weak bases called Bohr groups and negatively charged groups in deoxyhemoglobin (62). It is these salt bridges which contribute to increased structural stability and low oxygen affinity of deoxyhemoglobin. Formation and maintenance of these salt bridges are strongly pH dependent. At higher pH, the weak bases involved in salt bridging become uncharged. Consequently, the salt bridges break and pK's of the Bohr groups decrease. The resultant quaternary structure has high oxygen affinity. Actually, oxygenation of deoxyhemoglobin is accompanied by the pK shift of Bohr groups mentioned here. As pK falls hydrogen ions are liberated. This relationship is described by

$$\triangle H^+ = \sum_i \left[\frac{K_i'}{[H]+K_i'} - \frac{K_i}{[H]+K_i} \right] \qquad (11)$$

where K_i and K_i' are ionization constants of the i^{th} group in deoxy and oxy structure, respectively. [H] is activity coefficients of hydrogen ion.

The amino acid residues responsible for alkaline Bohr effect are ValNA1 and HisH5 of α chains and HisHC3 and LysEF6 of β chains (22,62-64). All these Bohr groups are

present in cow hemoglobin, and our present knowledge of primary structure of buffalo hemoglobin indicates presence of αValNA1. Thus, we can assume i to be same for both human and bovine. To account for large H^+ in bovine we can consider magnitudes of K′ and K. For K′/K to be large, which should be the case for larger ΔH^+, the pK′s of Bohr groups in bovine deoxy tetramer must be higher than those in human. This, in turn, should yield a stable deoxy structure which we have qualitatively shown in Chapter 2.

The same value of i in human and bovine, and yet larger ΔH^+ in the latter might arise from the net effect of interplay of a series of localized electrostatic interactions which are dependent on ionic strength and dielectric constant of the solvent, ion pairs in the protein and electrostriction effect of the protein on water dipoles. None of these effects can be interpreted so simply (see, for example, 65).

We conclude that the paricularly interesting features of oxygen equilibrium properties of bovine blood can be accounted for by the intrinsic properties of their hemoglobins. Low oxygen affinity of bovine hemoglobins can be correlated with their stable deoxy structure. Chloride ions strongly reduce oxygen afinity of bovine. Urea, on the other hand, increases oxygen affinity. Hemoglobins of both cow and buffalo are sensitive to organic phosphates. They are also endowed with a larger Bohr effect which might seem to originate from protein-protein and protein-solvent electrostatic interactions.

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SUMMARY

The following is a consummate summary of results of the present investigation.

The phenotypes and subunit composition of buffalo hemoglobin:

The total hemoglobin of adult water buffalo is composed of two component hemoglobins. They share a common β subunit, the α chains constituting the two components are different. By labelling the two α chains as α^{I} and α^{II}, subunit composition of the two phenotypes can be written as $\alpha_2^{I}\beta_2$ (major component) and $\alpha_2^{II}\beta_2$ (minor component). In polyacrylamide gel electrophoresis anodic mobility of $\alpha_2^{I}\beta_2$ is higher than that of $\alpha_2^{II}\beta_2$. In presence of Triton X100 cathodic mobilities of the globin chains follow the order $\alpha^{II} > \alpha^{I} > \beta$. Chromatographically, the two component hemoglobins are separable on a CM cellulose column at pH 6.2 employing a linear ionic gradient from 0.01M to 0.1M NaCl. The three globin chains are readily separable by the method of Clegg et al. (ref. 14 of Chapter 2) at pH 6.7. For elution of chains, concentration of dibasic sodium phosphate may be raised from 0.001M to 0.035M linearly.

The N-terminal residues of α^{I}, α^{II} and β chains of buffalo hemoglobin:

In either of the α chains valine is the N-terminal amino acid. Methionine occupies the N-terminus of the β

chain, a characteristic feature of ruminant β chains.

Quaternary structure of cow and buffalo hemoglobins in terms of allostery:

The central theme conveyed by this work is a stable quaternary deoxy structure of bovine hemoglobins. On the basis of quantitative oxygen equilibrium analysis and model building it was suggested a decade ago that quaternary T structure of cow hemoglobin is strongly constrained. This work provides a qualitative picture of the constraints on deoxy conformation of hemoglobins of both cow and buffalo. Electron spin resonance (ESR) measurements on nitrosylated hemoglobins have shown that bovine hemoglobins, even under physiological conditions, have a stable quaternary deoxy structure. This is crucial to understanding low oxygen affinity of ruminant hemoglobins in vivo. The constraints on quaternary T structure increase when allosteric effectors are added. This fact is qualitatively demonstrated by the ESR spectra recorded in presence of organic phosphates and sodium chloride. More intense hyperfine structures in presence of organic phosphates and Cl^- ions suggest interaction of these effectors with bovine hemoglobins. Chloride-induced hyperfine structure is more intense than induced by organic phosphate. Notably, chloride fails to produce hyperfine lines in the ESR spectra of human nitrosylhemoglobin. These results are suggestive of binding of allosteric effectors to bovine deoxy tetramer.

Pronounced hyperfine structure (i.e., T state quaternary feature) seen in the ESR spectra of bovine hemoglobins under physiological conditions is a certain indication of a tilt of the allosteric equilibrium $R \rightleftharpoons T$ to the right. In terms of allosteric theory, L of bovine hemoglobin is higher than the L of normal adult human hemoglobin, where L is the allosteric constant (i.e., T/R).

Visible soret band positions of nitrosyl complexes of both cow and buffalo hemoglobins are somewhat different from the soret position of human nitrosylhemoglobin. Although not conclusive, the band position differences are likely to originate from heme environmental differences.

Dissociation of bovine hemoglobins in acid and alkali:

The question of dissociation of bovine hemoglobins in acid and alkali, a hitherto unresolved issue, has been effectively answered. The bovine tetramer has been found to exhibit the reaction $\alpha_2\beta_2 \rightleftharpoons 2\alpha\beta \rightleftharpoons 2\alpha + 2\beta$. There are minor differences in dissociation behavior between human and bovine hemoglobins. The most likely reason for these differences could be less sulphydril reactivity and altered cysteine content of bovine hemoglobins. It is also speculated that subunit binding energy, especially between unlike subunits, is higher in case of the bovine tetramer.

Heme ligands have been found to affect the dissociation behavior, although relative effects produced by different ligands could not be placed in a specific order.

Intrinsically low oxygen affinity:

Under physiological conditions both the bovine hemoglobins have low oxygen affinity. P_{50} of cow hemoglobin is slightly higher than the P_{50} of buffalo hemoglobin. P_{50} of stripped adult human hemoglobin is about 0.7 fold and about 0.64 fold less than the stripped hemoglobin of cow and buffalo, respectively.

Organic phosphate binding to bovine hemoglobins:

It is found that in presence of low Cl^- ion concentration, organic phosphates lower the oxygen afffinity of bovine hemoglobins. This result is in conformity with the

ESR measurements which also showed interaction of organic phosphates with bovine deoxyhemoglobin. Since ValNA1β is deleted and HisNA2 $\longrightarrow$ MetNA2 substitution occurs in bovine β subunits, organic phosphates are likely to bind weakly. It has been argued that K_D, the dissociation constant of deoxyhemoglobin-effector complex is higher in bovine than in human.

Influence of chloride ion on oxygen affinity:

Both cow and buffalo hemoglobins show appreciable sensitivity to the presence of Cl^- ions. The effect of chlorides on oxygen affinity of hemoglobins is much larger in bovine than in human indicating strong binding to the former. The ability of chlorides to constrain the deoxy T state of bovine tetramer is also seen from the sharp hyperfine lines of ESR spectra. Both the bovine species show nearly similar sensitivity to Cl^-.

Effect of urea on oxygen affinity:

The present work furnishes data, for the first time, on oxygen affinity of bovine hemoglobins in a wide range of urea concentration. Urea increases the oxygen affinity . In concentrations greater than about 1M, the rise in oxygen affinity is far greater for human than for the two bovine species. Above about 3M urea a saturation effect is observed for all the three hemoglobins. Neither conformational stability nor dissociation of the hemoglobin molecule appears to give rise to the observed functional properties in presence of urea. It is rather likely that electrostatic interaction of urea with COO^- termini of subunits and heme structural changes are responsible for rise in oxygen affinity.

Bohr effect:

Bovine hemoglobins are endowed with a large Bohr

effect. It is very highly probable that the pK's of the Bohr groups in bovine deoxy tetramer are higher than those in human. Localized electrostatic interactions, which are dependent on solvent properties and protein-solvent interactions, seem to make significant contributions to Bohr effect in bovine.

APPENDIX I

PRINCIPAL STRUCTURAL AND FUNCTIONAL ROLE OF HisE7 IN HEMOGLOBIN

The 58th amino acid of α and β chains of hemoglobin which forms the E helix, and is located in the distal side of the heme pocket is known to be critical for normal functioning of the molecule. This distal histidine residue is largely conserved. Replacement of HisE7 leads to functional impairment. Following is a brief summary of our current understanding of the structural and functional role of this residue.

In 1963, an X-ray crystallographic study of azido and aquometmyoglobins done by Stryer et al. (1) suggested that the distal histidine is involved in hydrogen bonding with the heme ligands. Since then the importance of this residue has been shown by electrochemical (2), infra red and NMR (3-5), ESR (6-9), X-ray crystallography (10-16), neutron diffraction (17), ligand binding kinetics (18), time-resolved optical spectroscopy (19,20), site-directed mutagenesis and protein engineering followed by X-ray crystallography and resonance Raman scattering (21), ligand binding kinetics (22) and flash photolysis and low temperature time resolved Fourier transform IR (23) studies. The great volume of data published thus far has conclusively shown two pivotal roles played by HisE7. Nonbonded

interaction with the ligand and the steric effect produced by this residue are crucial for fine-tuning the ligand affinities of hemoglobin and myoglobin.

Differential effect of geometry and orientation of iron-oxygen bond on the interaction of His E7 with oxygen :-

In oxyhemoglobin a hydrogen bond between the N_ϵ of distal histidine imidazole and the second oxygen atom of the bound oxygen molecule seems to be necessary to stabilize the ligated structure (Figure 1). Extent of this bonding is related to the geometry of iron-oxygen bond (24) as well as to the orientation of residues in the distal heme pocket. In oxyhemoglobin the Fe-O-O bond angle is 156^o which places the terminal oxygen near the N_ϵ of HisE7 to promote a hydrogen bond. In α subunits, where the distance between the terminal oxygen and N_ϵ of HisE7 is 2.7Å, a hydrogen bond can form favorably; in contrast, the N_ϵ of the β chain-distal histidine is almost equidistant from the two oxygens. These distances are 3.4Å and 3.2Å for the first and the second oxygen atom respectively (24). With this geometry of an isosceles triangle both the oxygen atoms can form hydrogen bonds with N_ϵ of HisE7. Since these distances are large compared with 2.7Å in the case of α subunits, H-bonds in β subunits are substantially weaker or may be absent also. Additional support in favor of a stronger hydrogen bond in α subunit comes from enhanced rate of autooxidation of α chains after low intensity flash photolysis (25-28). As Shaanan has pointed out (24), a stronger H-bond in the α subunit may polarize the oxygen molecule more, thus allowing more rapid autooxidation.

Absence of a strong hydrogen bond in β subunits results in sterically favorable orientation of the oxygen molecule. Also, since HisE7 is not in close proximity, there is an open space in the heme pocket rendering the oxygen

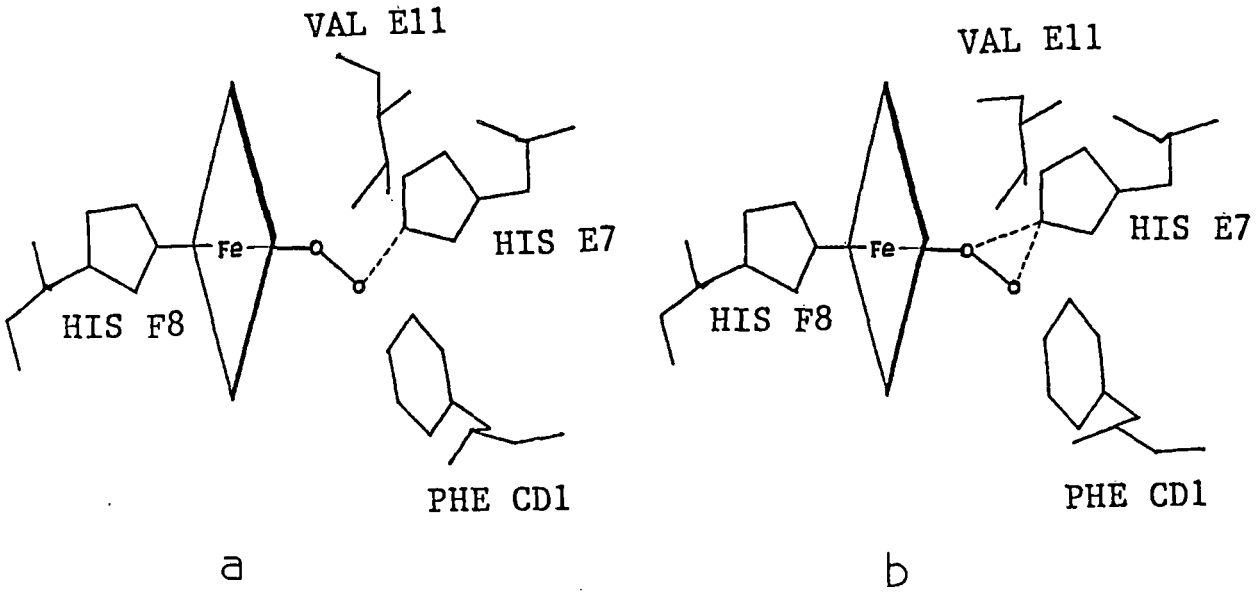

Figure 1. Schematic representation of heme environment in *oxyhemoglobin; (a) α subunit, (b) β subunit. Broken lines* indicate probable hydrogen bond.

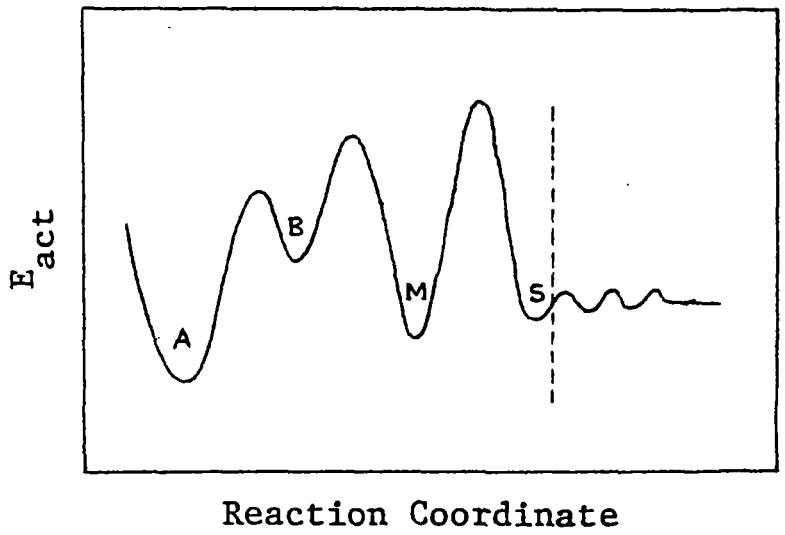

Figure 2. Sequential barrier model of ligand binding; S,M,B and A stand for solvent, protein matrix, heme pocket and heme iron respectively.

molecule enormous freedom of rotation about its equilibrium position (15). Besides hydrogen bonding and steric hindrance, proximity and orientation of HisE7 may influence the ligand molecule through dipole-dipole force, donor-acceptor interactions and electromagnetic interactions. The latter two factors have been demonstrated to be operative in the case of carbonmonoxyhemoglobin (5,29).

Oxygen affinity of hemoglobins with substituted distal histidine :-

Hemoglobin Zürich, where HisE7 of the β chains is replaced by Arg (30), has an abnormally high oxygen affinity (31). By contrast, opossum hemoglobin with HisE7 substituted by glutamine in the α chains (32,33) has low oxygen affinity (34). Furthermore, in Hemoglobin M Boston (αHisE7 ⟶ Tyr) and Hemoglobin M Saskatoon (βHisE7 ⟶ Tyr) the affected subunits stay in nonfunctional ferric form (35). Thus, in all cases of distal histidine substitution the normal function of oxygen binding is altered.

Role of distal histidine in stabilization of quaternary structure :-

The high oxygen affinity of Hemoglobin Zürich can be explained on the basis of influence of HisE7 in maintaining an unconstrained β heme. In normal hemoglobins, the N_ϵ and the C_ϵ of the distal histidine make contact with the lower part of the heme and orients it in the correct position (36). Absence of these contacts in Hb Zürich leaves the β heme unconstrained in deoxy state (quaternary T structure). ESR studies of nitrosyl complexes of opossum hemoglobin have shown that in this molecule the R quaternary state is unstable (8). It has been argued that absence of His E7 may be the genesis of a switch of quaternary structure from R to T state. Hemoglobin M Boston also

assumes a T quaternary state upon NO ligation (37,38). Thus, the distal histidine has a definite role in maintaining the quaternary structure of the molecule.

Contribution of HisE7 toward energetics of ligand binding :-

Studies on geminate recombination following photodissociation of liganded hemoglobins have been fairly useful to trace the pathway of the ligand as it approaches the heme iron (see ref. 39 for a review). The ligand starts from solvent , migrates through the protein matrix to the heme pocket and eventually binds to the heme iron:

$$\underset{\text{(solvent)}}{S} \rightleftharpoons \underset{\text{(globin matrix)}}{M} \rightleftharpoons \underset{\text{(heme pocket)}}{B} \longrightarrow \underset{\text{(heme iron)}}{A}$$

The process involves at least three barriers as shown in Figure 2. No matter from which point the ligand starts it has to overcome the final barrier 'BA' between the heme pocket (B) and the iron atom (A) (19,20,40,41). It has been shown that HisE7 contributes significantly to the barrier height, E(ba) (18). In hemoglobin Zürich the average barrier between B and A is about 2 KJ per mole which is about half of the corresponding barrier height in normal β chains (19,20).

Effect of substitution of distal histidine studied by site-directed mutagenesis :-

Recent work on high level expression of myoglobin and hemoglobin genes in E.coli (42-45) has made it possible to produce mutant hemoglobins. Nagai et al. (21) have studied the equilibrium and kinetics of ligand binding in mutant hemoglobins where HisE7 of the β subunits was replaced by Gln, Val or Gly. Substitution by Gly was found to produce a large space in the oxygen binding site such that ligands can enter the heme pocket unhindered. The Gly and the Val mutants were found to be unstable. Unlike these,

the Gln substitution appeared to be tolerable. Probably Gln, owing to its flexible side chain, occupies the same position as that of HisE7 and can form a hydrogen bond with the oxygen molecule. Substitution of HisE7 of α subunits by glycine in R state hemoglobin has been found to cause marked decrease in oxygen affinity. In contrast, the same replacement in β subunits of R state hemoglobin produces no change in ligand binding properties (22). These observations suggest the following two points. Firstly, the bulkiness of HisE7 seems to be a controlling factor. Volume of the glycine residue is small compared with histidine. Valine assumes a rather rigid conformation when attached to an α helix (46). Besides being flexible the bulk of the glutamine R group is comparable with that of histidine. This may be the probable reason why glutamine substitution is tolerated. Secondly, the space in the heme pocket is critical. R state β subunits have an open space in the heme pocket, there is no hydrogen bonding, and substitution of histidine by a residue having smaller volume might be tolerated. On the other hand, the hydrogen bonds in α subunits seem to be essential for controlling oxygen affinity.

The enormity of X-ray data together with physical methods have shown the principal role of distal histidine in the hemoglobin molecule. It is to be noted that HisE7 contributes only to the structure of the heme pocket. Substitution of this residue by other groups does not alter globin conformation. Bulkiness of histidine side chain and its capability to interact with ligand are critical but not sufficient for normal functioning of the molecule. As has been discussed above Gln can form a hydrogen bond and this substitution seems to be tolerable, yet opossum hemoglobin exhibits intrinsically low oxygen affinity. Not much is known about the dynamic role of HisE7 in the course of ligand combination. Data on flash photolysis and geminate recombination has established the gating role of HisE7

(19,20). Extension of the recent approach of molecular dynamics simulations (47-49) are expected to add more to our current understanding of the problem. A mutant might show normal functional property if the substituting amino acid fully mimics the dynamic behavior of HisE7. X-ray data has shown steady state heme-histidine interactions which have been suggested to maintain the T state quaternary conformation (36). It would be worth investigating this aspect in subnanosecond regime which might elucidate the dynamics of heme orientation and functional cooperativity of hemoglobin.

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APPENDIX II

A. POLYACRYLAMIDE GEL ELECTROPHORESIS FOR SEPARATION OF COMPONENT HEMOGLOBINS

Solutions and reagents :-

The 7.3% arylamide gels were prepared as follows:

Solution A:	Acrylamide	1.46 g
	N,N -methylenebisacrylamide	0.06 g

Dissolved the two upto 10 ml in water

Solution B:	TEMED (N,N,N ,N -tetramethylene diamine)	5.50 µl
	1N HCl	1.20 ml
	Tris	0.915 g

Dissolved these in water to 5 ml

Solution C:	Ammonium persulphate	0.014 g

Dissolved in 5 ml water (0.28 % soln.)

Solutions A, B and C were mixed as 2:1:1. The mixture was poured into gel tubes (~9cm long with ~0.55 cm internal diameter). The disc gel tops were immediately overlayed with distilled water to make the surfaces even and left at room temperature for about 90 minutes for polymerization to occur.

Electrode buffer :-

0.005 M Tris and 0.038 M glycine buffer, pH 8.3 (0.6 g Tris and 2.88 g glycine contained in a litre).

Anodic preelectrophoresis :-

1 mA per tube for 20 minutes.

Final run :-

3 mA per tube for 2.5 hours.

Stain :-

0.1% amido black in 5% acetic acid/ 25% methanol for about 15 minutes.

Destaining :-

Overnight destaining with a mixture of methanol, acetic acid and water mixed as 5:7:88.

B. TRITON GEL ELECTROPHORESIS FOR SEPARATION OF GLOBIN CHAINS

Solutions and reagents :-

Solution A:	Urea	6.075 g
	Ammonium persulfate	33 mg

Dissolved the two in 12.6 ml water. The urea concentration in this solution is 3.75 M

Solution B:	Acrylamide	2.025 g
	N,N -methylenebisacrylamide	27 mg

Dissolved in 4.95 ml water.

Solution C:	Acetic acid	1.5 ml
	TEMED	150 µl

These two were mixed in with 2.1 ml water

To the mixture of A, B and C, 135 µl Triton X-100 was added and mixed thoroughly. After casting gels, the gel tops were overlayed with water and left at room temperature overnight.

Preelectrophoresis running conditions :-

Reverse polarity

The first preelectrophoresis was carried out in 5% acetic acid at 2.5 mA per tube until the voltage increased to about 200 V (~3 hours).

For second preelectrophoresis the anodal compartment was filled with fresh 5% acetic acid. The gels were overlayed with 1 M cysteamine hydrochloride. Run was continued at the same setting as in first preelectrophoresis until the voltage dropped to about 85 V (~1 hour).

At the end of second preelectrophoresis cysteamine.HCl was removed from gel tops and both anodal and cathodal compartments were filled with fresh 5% acetic acid.

Preparation of sample :-

To prepare sample, a mixture of 1.25 ml 8M urea aquous solution, 1.25 µl of acetic acid and 25 µl β-mercaptoethanol was used. To about 50 µl of this mixture 10 to 15 µl sample was added, mixed and left at room temperature for about 2 hours.

Final run :-

Reverse polarity

Electrophoresis was carried out at 1 mA per tube for 15 minutes followed by 2 mA per tube for about 3.5 hours.

Gel staining and destaining :-

Same as in the procedure for component separation.