1990

The Effects of Crosslinking on the Stability and Autoxidation Rate of Human Hemoglobin a

Thao Yang
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Human hemoglobin has been crosslinked and shown to be stabilized by bis(3,5-dibromosalicyl) fumarate, dimethylpimelimidate, tris(β-chloroethyl) amine and bis(β-chloroethyl) methylamine. Hemoglobin crosslinked by fumarate between the two Lys 82β's (Walder et al. (1980) J. Mol. Biol., 141:195) or Lys 99α's (Chatterjee et al. (1986) J. Biol. Chem., 261:9929) produces markedly higher transition temperature (T_m) of 57°C, while the T_m of Hb A is 41°C. The fumarate crosslinking reaction with deoxyhemoglobin yields a second major product with similar T_m (57°C). This second crosslinked hemoglobin appears to be crosslinked between the β-subunits with one additional modification site.

The Hb A-DMP gave a product with a T_m of 56°C. The oxy β82XLHb A or α99XLHb A was further treated with DMP to produce a double crosslinked hemoglobin, which has a T_m of 60°C. Thus, the second crosslinking only slightly improved the stability by 3°C. The double crosslinked hemoglobins are stabilized by 19°C from Hb A compared to 16°C of single crosslinked hemoglobins.

The hemoglobin crosslinked with tris(β-chloroethyl)
amine (Hiratsuka T., (1988) Biochemistry 27:4110), has a $T_m$ of 49.3°C, and that of hemoglobin treated with BCEA has the same stability as Hb A, 41.3°C.

The autoxidation of $\alpha$99XLHb A is markedly faster than that of $\beta$82XLHb A or Hb A. The first order rate constant of autoxidation of these hemoglobins are $0.022 \text{ h}^{-1}$, Hb A; $0.0269 \text{ h}^{-1}$, $\beta$82XLHb A; and $0.0393 \text{ h}^{-1}$, $\alpha$99XLHb A. The faster rate of autoxidation of oxy $\alpha$99XLHb A compared to oxy $\beta$82XLHb A or Hb A was due to tertiary structure changes by the crosslink on the crosslinked hemoglobins. In $\alpha$99XLHb A the crosslink holds the hemoglobin in the T conformation, and in $\beta$82XLHb A, the R conformation.

Catalase and superoxide dismutase were able to retard the autoxidation of both Hb A and its crosslinked derivatives, $\alpha$99XLHb A and $\beta$82XLHb A by 2 fold. These results proclaim the importance of autoxidation rate and structural stability of crosslinked hemoglobins in the design of blood substitutes.
THE EFFECTS OF CROSSSLINKING ON THE STABILITY AND AUTOXIDATION RATE OF HUMAN HEMOGLOBIN A

by

Thao Yang

A Dissertation submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy

January 1990
DEDICATION

This dissertation is dedicated to the memory of my parents. Even though, they both had passed away, their wisdom, gentle nature and hard-working attitude to support me and my family during my childhood had taught and encouraged me to go forward to learn and acquire a higher degree of education and to push the boundary of knowledge. Their memory will always be with me. It is with great honor, respect, admiration and love that I dedicated this dissertation to my parents, Mr. and Mrs. Nao Ying Yang.
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of their instruments.

Finally, special thanks are extended to his family and relatives especially to his uncle and aunt, Mr. and Mrs. Pao Yang, for their support.
The author of this dissertation, Thao Yang, was born to Nao Ying Yang and Lee (Thao) Yang on June 5, 1959 in a small village, Ban Samkeo, Laos.


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LISTS OF ABBREVIATIONS

AS .................. ammonium sulfate
BBSF ................. bis(3,5-dibromosalicyl) fumarate
BCEA .................. bis(β-chloroethyl) methylamine
BIS-TRIS ............. bis(2-hydroxymethyl)imino tris(hydroxymethyl) methane
CAT .................. catalase
CAT-DMP ............. catalase treated with DMP
CN-Hb A ............. cyanomethemoglobin A
CO-Hb A ............. carbonmonoxyhemoglobin A
CO-Hb S ............. carbonmonoxyhemoglobin S
d ................. density
δ .................. chemical shift
DEAE ................ diethylaminoethyl
DIDS ................ 4,4’-diisothiocyanostilbene-2,2’- disulfonate
DMA .................. dimethyladipimidate
DMP .................. dimethylpimelimidate
DMS .................. dimethylsuberate
DPG .................. 2,3-diphosphoglycerate
DPL .................. 5’-deoxypyridoxal
εoxy .................. extinction coefficient of oxyhemoglobin
εcn .................. extinction coefficient of cyanomethemoglobin
εmM .................. milimolar extinction coefficient
FD .................. fraction denatured
Gdn...............guanidine
Hb A...............normal adult human hemoglobin
Hb A-BCEA.........Hb A treated with BCEA
Hb A-DMP.........Hb A treated with DMP
Hb A-TCEA........Hb A treated with TCEA
Hb A-TFA.........Hb A labeled with $^{19}$F
Hb A$_2$..........normal minor adult human hemoglobin
Hb NY.............hemoglobin New York
Hb S...............sickle hemoglobin
HP................Hewlett-Packard
IHP..............inositol hexaphosphate
MOPS..............4-(N-morpholino) propanesulfonic acid
Mr...............relative molecular weight
NFPLP..........2-nor-2-formyl pyridoxal-5′-phosphate
Ω..................ohm
PBS...............phosphate buffer saline
PFC..............perfluorochemical
PLP..............pyridoxal-5′-phosphate
PLS..............pyridoxal-5′-sulfate
PMP..............pyridoxal-5′-methyl phosphate
PPP..............bis-pyridoxal polyphosphate
RBC...............red blood cell
rpm...............rounds per minute
RT................retention time
SDS...............sodium dodecylsulfate
SDS-PAGE................. sodium dodecylsulfate polyacrylamide gel electrophoresis

SOD....................... superoxide dismutase

TCEA...................... tris(β-chloroethyl) amine

TEMED...................... N,N,N',N'-tetramethyl ethylenediamine

TFAB...................... 3-bromo-1,1,1-trifluoropropanone

Tm......................... melting temperature

Tris...................... tris(hydroxymethyl) aminomethane

U........................... enzyme unit

UV/VIS...................... ultraviolet/visible

XLHb....................... crosslinked hemoglobin

XLHb A..................... second crosslinked hemoglobin from α crosslinking reaction

α99XLHb A.................. fumarate Lys99α1-Lys99α2 crosslinked Hb A

β82XLHb A.................. fumarate Lys82β1-Lys82β2 crosslinked Hb A

α99XLHb A-DMP............. fumarate Lys99α1-Lys99α2 crosslinked Hb A double crosslinked with DMP

β82XLHb A-DMP............. fumarate Lys82β1-Lys82β2 crosslinked Hb A double crosslinked with DMP
This research began with the hope of discovering a method to stabilize unstable hemoglobins and to develop a stabilized crosslinked normal hemoglobin which could be used as a blood substitute. The search for a hemoglobin-based substitute is a logical approach to a complex problem. Crosslinked hemoglobin is practically a natural product and may minimize immunological risks. By crosslinking between the like subunits the hemoglobin molecule is locked in the tetramer form and is prevented from excretion through the glomeruli of the kidneys. Hence, its intravascular retention time in the body is increased. In introducing a crosslink into the hemoglobin molecule, there is a possibility that the crosslinked hemoglobin could be stabilized against heat, be resistant to oxidation, and have unaffected functional properties. However, the opposite possibilities must be considered. A thermally stabilized crosslinked protein could be sterilized against unwanted factors by heating. The resistance to oxidation will unequivocally prolong the storage half-life of the modified protein. While the requirements of a successful blood substitute may go beyond these primary prerequisites, they are indispensable.

The goal of this research is not the immediate creation of a blood substitute but does represent progress
towards its creation. This research proposes two basic
tests. First, a number of crosslinking compounds will be
used to crosslink hemoglobin A. The thermal stability of
the crosslinked hemoglobins will be determined. Secondly,
the autoxidation of these crosslinked hemoglobins will be
evaluated.

The study on thermal stability consists of three
parts. Part A, the stability of fumarate $\beta$-$\beta$ and $\alpha$-$\alpha$
crosslinked hemoglobin A will be compared. Part B, the
stability of the single crosslinking of hemoglobin using
bis(3,5-dibromosalicyl) fumarate, bis($\beta$-chloroethyl) amine,
bis($\beta$-chloroethyl) methylamine, and dimethylpimelimidate
will be measured and compared to that of hemoglobin A.
Part C, the stability of the double crosslinked hemoglobins
with dimethylpimelimidate will be compared. Crosslinking
within a subunit or between two subunits has the potential
to lock the three dimensional or the quaternary structure
of the protein in the conformation which the crosslinking
took place. Hence, hypotheses that the single crosslink
could stabilize the native structure of the protein from
unmodified one, and the double crosslink could also further
stabilize the single crosslinked protein, were developed.

The study on the autoxidation of hemoglobin consists
of two parts. Part A, the rate of autoxidation of fumarate
crosslinked hemoglobin will be compared to hemoglobin A.
Part B, the effect of catalase and superoxide dismutase or
both on the autoxidation of uncrosslinked and crosslinked hemoglobin A will be determined.

A hypothesis in the autoxidation mechanism of hemoglobin in which the T state is a step prior to oxidation is proposed. Such a mechanism is suggested from the autoxidation of $\beta-\beta$ and $\alpha-\alpha$ crosslinked hemoglobin. Since the autoxidation of hemoglobin was proved to generate superoxide radical ($O_2^{-}$) and hydrogen peroxide ($H_2O_2$) (27-29,32,34,37), such anions generated in the reaction would be more damaging to the protein and accelerate the oxidation rate. The prevention of oxidative damage to hemoglobin could be achieved by enzymes and antioxidants (26,36,38,39). The proposal that such a protection by enzymes is realizable in the long term storage of crosslinked hemoglobin is tested by catalase and superoxide dismutase on the crosslinked hemoglobins, since these enzymes were known to scavenge $H_2O_2$ and $O_2^{-}$ (42-47).

Other projects which are of significant importance to this research are the identification of the second unknown crosslinked product from the $\alpha$ crosslinking reaction of Hb A with bis(3,5-dibromosalicyl) fumarate by amino acid analysis, and its thermal denaturation. A preliminary $^{19}$F NMR study in which the Cys 93$\beta$ residue is labeled with 3-bromo-1,1,1-trifluoropropanone in order to probe the conformation of the native and the denatured state is included as an appendix.

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CHAPTER I
INTRODUCTION

The introduction of this dissertation will elaborate briefly on the structural information of hemoglobin, the use of hemoglobin solution as a blood substitute, the modification and crosslinking of hemoglobin using chemical reagents and the changes in hemoglobin properties due to chemical crosslinking. A number of crosslinking compounds which are of interest or related to this research will also be discussed briefly. Finally, the research on which this dissertation is based will be elaborated in detail in the experimental, results and discussion chapters.

HEMOGLOBIN A

Hemoglobin is the oxygen carrying protein in the erythrocyte. Each erythrocyte is packed with 280 million molecules of hemoglobin. The roles of hemoglobin are to bind molecular oxygen to its heme irons in the lungs, deliver it to the various tissues throughout the body and transport carbon dioxide from the tissues back to the lungs to be exhaled (1).

Work to determine the hemoglobin structure began around 1938 by Bernel et al. (2). It was not until 1960 when the three dimensional structure of horse hemoglobin
was revealed by X-ray crystallography by Perutz et al. (3). A molecule of hemoglobin has four polypeptide chains, two \( \alpha \) and two \( \beta \) chains. Each chain contains one heme with a molecular weight of 616 daltons. The \( \alpha \) chain has 141 amino acids with a molecular weight of 15,126 daltons, and the \( \beta \) chain, 146 amino acids and 15,867 daltons. A molecule of hemoglobin, therefore, has a molecular weight of 64,450 daltons. The secondary and tertiary structures of the two types of chains are folded in a similar manner; the \( \alpha \) chain has seven \( \alpha \)-helices whereas the \( \beta \) chain has eight. The helices were named, starting with the N-terminus, A to H consecutively (1).

The heme group is embedded in the globin chain, except for the two propianate groups which project out of the surface. The heme contained an iron atom at the center which plays an important role in ligand binding. The iron ion is coordinated to the four nitrogen atoms of the pyrrole rings of the heme. The fifth coordination site is coordinated to the proximal histidine F8, His 87\( \alpha \) or His 92\( \beta \) (4), and the sixth coordination site is the binding site for ligands (1). Oxygen can only bind to hemoglobin when the iron ion is in the +2 oxidation state. When oxygen is bound to hemoglobin, it is known as oxyhemoglobin, and the quaternary structure is described as a relaxed or R state. The state in which the iron ion remained in the +2 oxidation state, but there is not any
oxygen bound to it, is known as deoxyhemoglobin, and its quaternary structure is described by a tense or T state. When the iron ion is oxidized to Fe$^{+3}$, it is called methemoglobin, and it is ligated to a water molecule. Other anions or ligands can bind to the +2 and +3 oxidation state of hemoglobin as well (1).

The hemoglobin molecule acts in a cooperative manner in binding and releasing molecular oxygen, that is the binding or releasing of four oxygen molecules to or from the four subunits is not independent. The first oxygen attaches weakly to one of the heme; the second, third and fourth have increasing affinity in their order to the hemes. Likewise, the tendency to lose the first oxygen is with difficulty. With the first oxygen lost, the next one comes off easier. The interaction mechanisms during these transitions are well described (1). The cooperativity of oxygenation of hemoglobin was described by the Hill equation (5-7).

Among the numerous small molecules which can bind to hemoglobin with physiological relevance were carbon dioxide, oxygen, 2,3-diphosphoglycerate (DPG), chloride ion and hydrogen ion. These molecules play a very important role in the oxygenation mechanism. A proton has a higher affinity for deoxyhemoglobin, hence at higher pH the oxygen affinity of hemoglobin is higher. This was known as the Bohr Effect (1). Carbon dioxide binds to the amino termini
of the deoxyhemoglobin molecule to form carbamates and is dissociated at the lungs to the less soluble CO₂ gas to be exhaled. DPG binds to deoxyhemoglobin at the amino termini of the two β chains at the central cavity of the four subunits. It is surrounded by eight positive charges, two of each, from the β amino terminus, His β2, Lys β82, and His β143. This binding pushes the two β chains farther apart, which influences the release of oxygen and constrains the hemoglobin molecule in the T state (1). For complete references on the three dimensional structure, conformational changes, and interactions of amino acid residues within the hemoglobin molecule, one is referred to Perutz (5,6,8,9), Perutz et al. (10,11), Kendrew et al. (12), Heidner et al. (13), Ladner et al. (14), Padlan and Love (15,16), and Olsen (17).

HEMOGLOBIN AS BLOOD SUBSTITUTES

The development of hemoglobin solution as a blood substitute has intensified in recent years as better understanding of hemoglobin properties became known. Since hemoglobin in solution has the ability to bind oxygen, in theory it could be used in place of red blood cells (18).

The value of hemoglobin as a blood substitute arose from four major drawbacks engrossed in whole blood: blood for transfusion is limited, blood type must be matched,
whole blood could carry infectious agents, and whole blood has a low storage half-life. A hemoglobin solution would have advantages over whole blood if some of the undesirable functional properties could be overcome. This would suggest the elimination of the need for cross-matching donor and recipient, minimizing any infectious viral agents, improving its half-life and stability (18).

Cell-free hemoglobin in solution does not maintain a T state and has higher oxygen affinity than whole blood due to the absence of DPG. In solution, hemoglobin exists in equilibrium between tetrameric and dimeric forms (19). The dimer are readily excreted by the kidneys within 2-4 hours. Hemoglobin in solution exerts a colloidal oncotic pressure which limits it at lower concentration of 7 g/dL compared to intraerythrocytic concentration of hemoglobin at 30 g/dL (18). Hence, modification of hemoglobin is necessary for improving these disadvantages inherent in cell-free hemoglobin.

The modification and crosslinking of hemoglobin with an external reagent manifests two major principles. The first principle is to achieve a molecular engineering which would produce an active stabilized protein. Recent interest was focussed in crosslinking and modifying the structure of Hb S to produce a modified protein which would be stable and resistant to polymerization when in the deoxy form (20-30). The second principle in modification of
hemoglobin is to develop a suitable hemoglobin derivative for clinical use as a blood substitute (26-32).

The interest in using hemoglobin as blood substitute in recent years was focussed in modified hemoglobin products. The consideration in obtaining suitable hemoglobin solution as blood substitute is currently concentrated in improving its half-life, lowering its oxygen affinity to match that of whole blood, preventing autoxidation, defining the molecular changes chemically, assessing the degree of modification, improving the methods and procedures in modification, feasibility in pharmaceutical consideration, and assessing any adverse effects responded by the immune system to the modified hemoglobin (18).

The prospect for overcoming the limitations inherent in cell-free hemoglobin engrosses two basic approaches: molecular modification of hemoglobin, and environment modification (18). Molecular modification involves the use of a modifying agent or crosslinking agent to react with hemoglobin either intermolecularly or intramolecularly to form dimeric or polymeric species. This approach has the potential to stabilize the hemoglobin molecule in the tetrameric form, possibly increasing the half-life, and preventing autoxidation. The environmental modification requires that hemoglobin be encapsulated within liposomes in the presence of DPG. This approach was investigated by
Chang (33), and Djordjevich and Miller (34). The present investigation lies in the realm of molecular modification of hemoglobin.

PERFLUOROCHEMICALS AS BLOOD REPLACEMENT

Perfluorochemicals (PFC) are fluorinated hydrocarbons, in which most of the hydrogens are replaced by fluorines. Perfluorochemicals received a great deal of attention as a potential blood replacement (35). Perfluorochemicals are not antigenic. The emulsion of perfluorochemicals can dissolve oxygen up to 70% per unit volume (36,37), and their sizes are very small compared to the red blood cells and could pass through occluded blood vessels.

Despite the enthusiasm in perfluorochemicals as blood replacement, the major failure was that when PFC's were given to patients they accumulate in the liver and spleen to the point that these organs were not able to clear other foreign substances (38). The Food and Drug Administration has disapproved PFC's use for these reasons, and they are generally regarded as unsatisfactory blood replacement (39).
REAGENTS RECENTLY USED IN MODIFICATION AND CROSSLINKING OF HEMOGLOBINS

The crosslinking of hemoglobin was shown by Bunn and Jandl in 1968, using bis(N-maleimidomethyl) ether (40). This hemoglobin derivative had high oxygen affinity (P50, 3 mmHg). Subsequent work by Benesh et al. (31), using pyridoxal phosphate, obtained a modified hemoglobin which exhibited lower oxygen affinity (P50, 26-30 mmHg). Greenburg and Maffuid (41) showed that the dialdehyde of ATP (2′-o-[(R)-formyl-(adenine-9-yl) methyl]-3′-triphosphate-3′-deoxy-(S)-glyceraldehyde could react with hemoglobin to yield a derivative with P50 of 11 mmHg (18).

The modification of hemoglobin was investigated by several workers using various reagents to modify the structure of hemoglobin A, and hemoglobin S. Studies, upon which this research is based, were done with aspirin derivatives (20-23), nitrogen mustards (42-44), and imidoesters (45-48). The related aspirins were bis(o-carboxyphenyl) succinate (25), acetyl-3,5-dibromosalicylic acid (24), mono-3,5-dibromosalicyl fumarate (49), bis(3,5-dibromosalicyl) succinate, glutarate, adipate, and fumarate (26-30); and the imidoesters were dimethylpimelimidate (DMP) (46,48,50), dimethylsuberate (DMS) (46), and dimethyl adipate (DMA) (45,47). Others which were of interest were 4,4′-diisothiocyanostilbene-2,2′-disulfonate (DIDS) (19),
and 1,5-difluoro-2,4-dinitro benzene (51). The nitrogen mustards were tris(β-chloroethyl) amine (TCEA) (43), and bis(β-chloroethyl) methylamine (BCEA) (42, 44). BCEA was known to react with hemoglobin (42, 44), and TCEA has been used to crosslink three subunits of adenosine-triphosphatase (ATP-ase) (43).

The crosslinking of hemoglobin with imidoesters (DMA, DMS, DMP) (45-48) resulted in intrasubunits as well as intersubunits crosslinking. It was demonstrated that the imidoesters crosslinked hemoglobin in both the oxy and met forms to give intersubunit crosslinked species of molecular weight equivalent to dimers, trimers, and tetramers (45-48). The dimer species obtained were predominant compared to the trimers and tetramers. Polymeric species were formed as well if the reagent concentration was much higher than hemoglobin (48). Random crosslinking of the hemoglobin molecule by imidoesters occurred, and hence, a heterogeneous species was obtained. Such a heterogeneous species was difficult to purify to homogeneity (45-48). The amidinated hemoglobin had increased oxygen affinity (47).

The crosslinking loci for several reagents which are of interest and directly related to this research are tabulated in Table 1. The use of crosslinking to effect the hemoglobin properties remained of interest, and abundant opportunity to be explored.
Table 1:

The known loci of crosslinking and modification in hemoglobin with various crosslinking compounds related to this research.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>State of Hb in Reaction</th>
<th>Crosslink and/or modified loci</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBSF</td>
<td>Oxy</td>
<td>Lys 82β₁ to Lys 82β₂</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Deoxy</td>
<td>Lys 99α₁ to Lys 99α₂</td>
<td>28</td>
</tr>
<tr>
<td>Aspirins</td>
<td>Oxy</td>
<td>Lys 59β, Lys 144β, Lys 90α</td>
<td>22</td>
</tr>
<tr>
<td>PLP</td>
<td>Deoxy</td>
<td>β N-termini</td>
<td>92</td>
</tr>
<tr>
<td>NFPLP</td>
<td>Deoxy</td>
<td>β₁ N-terminus to Lys 82β₂</td>
<td>32</td>
</tr>
<tr>
<td>PLS</td>
<td>Oxy</td>
<td>α N-termini</td>
<td>92</td>
</tr>
<tr>
<td>DPL</td>
<td>Oxy</td>
<td>α N-termini</td>
<td>92</td>
</tr>
<tr>
<td>PPP</td>
<td>Deoxy</td>
<td>β₁ N-terminus to Lys 82β₂</td>
<td>32</td>
</tr>
<tr>
<td>DMP</td>
<td>Oxy</td>
<td>ε-Amino lysine residues</td>
<td>92</td>
</tr>
<tr>
<td>TCEA</td>
<td>Oxy</td>
<td>Lysine and cysteine residues</td>
<td>43</td>
</tr>
<tr>
<td>BCEA</td>
<td>Oxy</td>
<td>Lysine and cysteine residues</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>suggested: His 2β, His 97β,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>His 143β, His 146β</td>
<td></td>
</tr>
<tr>
<td>DIDS</td>
<td>Oxy</td>
<td>modified at Lys 82β's</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Deoxy</td>
<td>β₁ N-terminus to β₂ N-terminus</td>
<td>19</td>
</tr>
<tr>
<td>DMA</td>
<td>met/oxy</td>
<td>ε-Amino lysine residues</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>known: Lys 7α₁ to Lys 11α₁</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lys 82β₁ to Lys 82β₂</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: BBSF - bis(3,5-dibromosalicyl) fumarate, PLP - pyridoxal-5'-phosphate, NFPLP - 2-nor-2-formyl pyridoxal-5'-phosphate, PLS - pyridoxal-5'-sulfate, DPL-5'-deoxypyridoxal, PPP - bis-pyridoxal polyphosphate, DMP-dimethylpimelimidate, TCEA - tris(β-chloroethyl) amine, BCEA - bis(β-chloroethyl) methylamine, DIDS - 4,4'-dihydrothiocyanostilbene-2,2'-disulfonate, DMA-dimethyladipimidate.
Pyridoxal phosphate and a number of its derivative compounds had been used to crosslink hemoglobin (31,32,92). The reaction of pyridoxal-5'-phosphate (PLP) with hemoglobin under deoxy conditions resulted in modification of the $\beta$ chains at the N-terminal amino groups and in decreased oxygen affinity. A second derivative, 2-nor-2-formyl pyridoxal-5'-phosphate (NFPLP), crosslinked deoxy hemoglobin between the N-terminus of $\beta_1$ and the lysine 82$\beta_2$ (31,92). Other pyridoxal derivatives such as, pyridoxal 5'-sulfate (PLS), pyridoxal-5'-methyl phosphate (PMP), pyridoxal-5'-phosphate monomethyl ether (PME), and 5'-deoxy pyridoxal (DPL) were known to modify the $\alpha$ chains at the N-termini under oxy condition (31,92), and these hemoglobin derivatives had increased oxygen affinity. The negative charge from the phosphate group of the pyridoxal compounds has an electrostatic interaction with the positive charges from the amino acid residues in the DPG cleft, and this effect stabilized the T state which mimics closely the effect of DPG on hemoglobin (92).

Recently, a new class of bis-pyridoxal polyphosphate compounds were developed to crosslink deoxyhemoglobin (32). The crosslinking site was found between the amino terminus of a $\beta$ chain and the lysine 82 of the other $\beta$ chain, as in the case for NFPLP crosslinking (32). The yield of
crosslinked hemoglobin varied with different derivative compounds. These crosslinked hemoglobins had decreased affinity for oxygen and still retained cooperativity (32).

Two pyridoxalated crosslinked hemoglobins which are of interest were the polymeric species double crosslinked by glutaraldehyde (18) and bis(3,5-dibromosalicyl) fumarate (BBSF) (18). In reacting glutaraldehyde to pyridoxalated hemoglobin crosslinked with pyridoxal-5'-phosphate (PLP) produced several polymeric species of molecular weight up to 96,000 daltons (18). The polymerized pyridoxalated hemoglobin still retained a cooperative mechanism in binding oxygen and had lower oxygen affinity compared to unmodified hemoglobin, its p50 value was 32 mmHg while that of whole blood was 29 mmHg. At a given concentration, this polymerized pyridoxalated hemoglobin exerted a lower osmotic pressure compared to unmodified hemoglobin (18). In view of the oxygen binding properties of pyridoxalated crosslinked hemoglobins which exhibited low oxygen binding, the goal of using these crosslinked hemoglobins as blood substitute is realizable.

**ACETYLATION OF HEMOGLOBIN WITH ASPIRIN**

In 1963 Klotz and Tam discovered that aspirin could be used to acetylate Hb S (20). Subsequent reaction with hemoglobin using aspirin was further studied to investigate
the anti-sickling and oxygen binding properties (21). This investigation further proved the acetylation of hemoglobin with aspirin. The acetylation sites were thought to be either at the N-termini of the \( \alpha \) chains as in the case of cyanate (52) and at the \( \epsilon \)-amino group of lysyl residues as those in serum albumin (53).

A more detailed study of acetylation of hemoglobin by aspirin was done by Shamsuddin et al. (22). These investigators discovered that aspirin acetylated hemoglobin randomly, but at least three main sites were the loci where the majority of acetyl groups attached. These were the Lys 59\( \beta \), Lys 144\( \beta \), and Lys 90\( \alpha \). This study led to the suggestion of the development of other acyl salicylate derivatives as potential anti-sickling agents. The investigation by Shamsuddin et al. (22) proved that aspirin slightly prevents gelling of hemoglobin S. The work by Klotz and Tam (20), and Bridges et al. (23) indicated a slight increase in oxygen affinity for the modified hemoglobin. The modification of hemoglobin by aspirin led to a number of acyl compounds which proved to be more potent in the crosslinking of hemoglobin.

ACETYLATION OF HEMOGLOBIN WITH ASPIRIN DERIVATIVES

Walder et al. (24) used acetyl-3,5-dibromosalicylic acid to acetylate Hb S and found essentially the same
effect as the work previously done by Klotz and Tam (20), Shamsuddin et al. (22), and Bridges et al. (23), except the incorporation of acetyl groups into hemoglobin was more effective, that is the percent of modification increased significantly at a given molar ratio of reaction compared to aspirin.

Zaugg et al. (25) synthesized a bifunctional compound from aspirin, using succinyl chloride and salicylic acid. This reagent, succinyl disalicylate, was found to crosslink hemoglobin up to 50% and yielded only dimeric and monomeric species. This indicated that the crosslinking may be intramolecular and selective at a specific site on the hemoglobin molecule.

The interest in diaspirin compounds as crosslinkers intensified. During 1979 and 1980 Walder et al. (29,30) developed a series of diester compounds, namely the derivatives of aspirin: bis(3,5-dibromosalicyl) fumarate, succinate, glutarate and adipate. These reagents were first used to crosslink oxyhemoglobin between the Lys 82β₁ and Lys 82β₂. The structure, oxygen binding properties, and solubility of the β crosslinked hemoglobins A (β82XLHb A) and S (β82XLHb S) were examined (27). In the absence of DPG the β82XLHb A had oxygen affinity slightly higher than or the same as the uncrosslinked Hb A. The presence of DPG did not affect the oxygen affinity of the β82XLHb A. However, in the presence of DPG the oxygen affinity of Hb A
(P50, 12.2 mmHg) was much less than that of the β82XLHb A (P50, 3.3 mmHg) (29).

The examination of the structure of the crosslinked hemoglobin revealed that there was no overall structural changes. However, local perturbation occurred around the crosslink in the DPG binding cavity. This perturbation caused the β F helix, the β EF corner, His 143β and the β amino termini to move slightly toward the center of the cavity (29). This finding became an important consideration in blocking sickling in Hb S.

When reacted with deoxyhemoglobin bis(3,5-dibromosalicyl) fumarate had the ability to crosslink selectively between the α chains between Lys 99α1 and Lys 99α2 (28). The Hb A derivative crosslinked between the α chains (α99XLHb A) had oxygen affinity lower than the uncrosslinked Hb A and similar to that of the whole blood. Under closely matched physiological condition the P50 of α99XLHb A was 29 mmHg compared to 12 mmHg for Hb A (26), and 29 mmHg for whole blood (18). Despite the presence of the crosslink in the hemoglobin, both α and β crosslinked hemoglobins remained highly cooperative in binding oxygen (27-29). The P50 value and the Hill coefficient in 0.05 M Bis-Tris, 0.1 M NaCl, pH 7.0 at 25°C for hemoglobin derivatives were reported as in Table 2.

By crosslinking between the two Lys 82β’s in Hb S the solubility of deoxy Hb S was increased (27). The
solubility of deoxy α99XLHb S apparently arose from the perturbation of the Val-6β acceptor site which constituted the residues Phe-85β and Leu-88β at the start of the F helix. By small displacement of the F helix toward the central cavity these residues were dragged along. Hence, they were less accessible to interact with Val-6 at the surface tetramer. By increasing the length of the crosslink span from four carbons to five and six carbons progressively reduced the magnitude of the perturbation, and consequently, the solubility of deoxy α99XLHb S decreased approaching that of uncrosslinked Hb S (27).

Kikugawa (54) had used bis(3,5-dibromosalicyl) fumarate to crosslink asymmetrical hybrid hemoglobins between the β chains in hemolysate containing Hb A and S, and Hb S and Hb York (Hb Y) (54). The stability by mechanical shaking of both symmetrical crosslinked Hb A (β82XLHb A) and crosslinked Hb York (β82XLHb Y) improved slightly over the uncrosslinked hemoglobins A and York, and both the asymmetrical hybrid crosslinked Hb AS (82β1A-82β2S XLHb) and crosslinked Hb YS (82β1S-82β2Y XLHb) improved over both the uncrosslinked Hb S and symmetrical crosslinked Hb S (β82XLHb S). However, the symmetrical
Table 2:
The Hill coefficient (n) and P50 (mmHg) values of Hb A and crosslinked Hb A in 0.05 M Bis-Tris, 0.1 M NaCl, pH 7, at 25 °C.

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>P50*</th>
<th>P50, #</th>
<th>n*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>4.0</td>
<td>11.8</td>
<td>2.7a</td>
</tr>
<tr>
<td>b82XLHb A</td>
<td>2.8</td>
<td>2.8</td>
<td>2.3b</td>
</tr>
<tr>
<td>a99XLHb A</td>
<td>13.9</td>
<td>29.0</td>
<td>2.6c</td>
</tr>
<tr>
<td>Whole blood</td>
<td>29.0</td>
<td>29.0</td>
<td>2.6d</td>
</tr>
<tr>
<td>Hb S</td>
<td>4.2</td>
<td>10.0</td>
<td>2.9e</td>
</tr>
<tr>
<td>b82XLHb S</td>
<td>2.5</td>
<td>2.6</td>
<td>2.5f</td>
</tr>
<tr>
<td>Hb A-DMA</td>
<td>0.36</td>
<td>3.7</td>
<td>1.8g</td>
</tr>
</tbody>
</table>

* -No DPG present; # -DPG present in 2 mM; a, b, e and f - from Kikugawa (54); c - from Chatterjee (28); d - from Bolin (18), g - from Pennathur-Das (47).
crosslinked Hb S had the same mechanical stability as the uncrosslinked Hb S (54).

By intramolecular crosslinking between either the $\alpha_1$ and $\alpha_2$ or the $\beta_1$ and $\beta_2$ chains within a molecule of hemoglobin the crosslink prevents the hemoglobin from dissociation into $\alpha\beta$ dimers and hence increased its intravascular life while it was used in transfusion in rats (28).

The use of uncrosslinked hemoglobin as a blood substitute entails two limitations. First, hemoglobin dissociates to $\alpha\beta$ dimers and is filtered through by the glomerulus of the kidneys, and secondly, hemoglobin in solution has inherently higher oxygen affinity due to the absence or very low concentration of DPG. It is reasonable that such limitations could be overcome by crosslinking the hemoglobin molecule in such a way that the hemoglobin molecule could be locked into the tetramer form, and the oxygen affinity is low, as in the $\alpha$ crosslinked hemoglobin (28). In $\beta82XLHb$ A the DPG binding site is blocked and its conformation favors high affinity for oxygen, thus its difficulty in transferring oxygen may limit its use as a blood substitute. On the other hand, in $\alpha99XLHb$ A the oxygen affinity is similar to whole blood (28). Hence the transfer of oxygen would be regulated closely as that of Hb A in the presence of DPG.

Snyder et al. (26) had used $\alpha99XLHb$ A to replace
about 15% of blood volume in rats (Sprague-Dawley rat) and found that the intravascular retention time had increased by about 2 times that of the control experiments using uncrosslinked Hb A solution. Hemoglobin A had a half-life of 90 minutes compared to 3 hours that of α99XLHb A (26). Because of the ability of the crosslink to hold the hemoglobin in the tetramer form, the crosslinked hemoglobin was not readily eliminated by the kidneys. The increasing size of the α99XLHb A molecule crosslinked with bis(sulfosuccinimidyl) suberate to trimers was shown to prevent renal excretion of the modified hemoglobin even further (26). Met α99XLHb A was transfused in rats and found to be reduced in the intravascular system (26). This suggested that either the methemoglobin reductase or another enzyme system in the rats was sufficient to reverse the oxidation of crosslinked hemoglobin.

OTHER REAGENTS WHICH WERE DESIGNED TO REACT WITH HEMOGLOBIN IN THE DPG BINDING SITE

Among other reagents which were designed to react with hemoglobin in the DPG binding site at the lysyl residues or at the amino termini, included 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS) (19), hybrid acylaldehyde derivatives of oxalate, malonate, fumarate, glutarate, 5-formylsalicylacetate (55), and 5-(2-formyl-3-
hydroxyphenoxy) pentanoic acid (BW12C) (56).

Aliphatic carbonyl compounds such as, acetaldehyde, glycoaldehyde, glyoxalic acid, glyceraldehyde, dihydroxyacetone (57), methyl acetyl phosphate (58), and glutaraldehyde (59) also could be used to react with lysine residues on the hemoglobin molecule. The monoaldehyde or bisaldehyde either modifies a lysyl group or crosslinks between two lysines to form a Schiff base or an amide linkage (55). A common properties of these reagents rest upon their randomness in reaction with hemoglobin. Therefore, it was difficult to assess the oxygen binding properties due to difficulty in isolation of a homogeneous species. Nevertheless, hemoglobin treated with dimethyl adipimidate without purification, a reagent which was known to react with hemoglobin to produce heterogeneous species, showed increased oxygen affinity (47).

AUTOXIDATION OF HEMOGLOBINS

Oxyhemoglobin in the body or in solution undergoes autoxidation in which the $\text{Fe}^{2+}$ is oxidized to $\text{Fe}^{3+}$, resulting in methemoglobin. However, in the body the level of methemoglobin remains constant at 1% due to the reduction of methemoglobin to oxyhemoglobin by the methemoglobin reductase, NADH-cytochrome b5 reductase system (60). When oxyhemoglobin is oxidized to
methemoglobin it is no longer able to bind dioxygen. One of the requirement for the successful use of hemoglobin as blood substitute is the prevention of autoxidation. This could be achieved by the presence of enzymes or antioxidants in the hemoglobin solution, which act as superoxide and hydrogen peroxide scavengers since these ions are the products of autoxidation which were known to accelerate the autoxidation rate (61-65).

The autoxidation of isolated $\alpha$ and $\beta$ chains indicates that the $\alpha$ chain autoxidized faster than the $\beta$ chain, and that the rate of autoxidation was affected by oxygen pressure (66) and pH (67). The oxidation rate decreased as the pH or oxygen pressure increased (66). This same relationship holds true for the tetramer hemoglobin as well (68). However, the rate of autoxidation increased at either very low pH or at very high pH (67,69).

In the presence of allosteric anions such as DPG and inositol hexaphosphate (IHP) the individual subunit autoxidized faster than in conditions in which these anions were absent (70). Deoxy hemoglobin A was found to be oxidized at a faster rate than oxy hemoglobin A and carbonmonoxyhemoglobin A (68,70).

Enzymes which were often used to reduce the autoxidation of hemoglobin were catalase and superoxide dismutase (SOD) (61,62,65,71,72). A number of antioxidants were also found to be potent in the protection of
autoxidation of hemoglobin (62-65,71).

In aqueous solution saturated with dioxygen the oxidation potential for Fe$^{+2}$ to Fe$^{+3}$ is 0.46 V (73), whereas the redox potential of hemoglobin is 0.15 V (74). The free heme (Fe$^{+2}$) in solution oxidized immediately to hematin (Fe$^{+3}$) (75). Thus, the tendency for Fe$^{+2}$ to be oxidized to Fe$^{+3}$ is favored over the reverse process. The resistance to oxidation of heme in hemoglobin and myoglobin was due largely to the coordination of the iron to the proximal histidine F8, the steric hindrance from the distal histidine E7 (His 63β, His 58α) (4) of the globin chain, and the hydrophobic environment encapsulating the heme, discriminating water molecule and other ions to react at the iron center (76). These properties were suggested by the picket fence synthetic heme models which were able to bind oxygen reversibly (76).

A discussion of the autoxidation mechanism at the molecular level may help to bring about better understanding of the effect of enzyme such as catalase and superoxide dismutase. The autoxidation of hemoglobin resulted in the production of superoxide radical (O$_2^−$·) and H$_2$O$_2$ which are believed to cause damage to cell membrane and tissues in the body (61,116). Misra and Fridovich (61) proved that O$_2^−$· was produced during the autoxidation of oxyhemoglobin to methemoglobin. Several mechanisms of autoxidation were proposed (61,67-69,71,77). It was
thought that the dioxygen was bound to the Fe$^{+2}$ and one electron from the Fe$^{+2}$ was transferred to the O$_2$ in which the species became a superoxo-ferriheme (Fe$^{+3}$—O$_2^-$) complex (61).

An investigation of the effect of anion-promoted autoxidation of myoglobin (Mb) suggested a $S_N$2 nucleophilic attack by an anion (L$^-$) at the iron center in which an intermediate complex [Mb-Fe$^{+2}$(O$_2$)(L$^-$)] was formed (77). In biological systems the anions accessible for such a reaction were H$_2$O, OH$^-$, and Cl$^-$ (68,77-79). Kinetic experiments using several factors, such as pH, anions, and temperature, to effect the rate of oxidation of either myoglobin or hemoglobin were investigated by several groups of scientists (67-69,71,80). These investigations revealed that the mechanism of autoxidation of these proteins was much more complex than the simple models presented above by Satoh and Shikama (77), and Misra and Fridovich (61). Nonetheless, the autoxidation mechanism proposed by Wallace et al. (68), and Watkins et al. (71) appeared to be reasonable. Such a mechanism is presented below:

\[
\begin{align*}
\text{Hb.Fe(II)O}_2 & \rightarrow \text{Hb.Fe(II) + O}_2 \\
\text{Hb.Fe(II) + H}^+ & \rightarrow \text{Hb.Fe(II)(H}^+) \\
\text{Hb.Fe(II)(H}^+) + \text{O}_2 & \rightarrow \text{Hb.Fe(III)H}_2\text{O} + (\text{H}^+)\text{O}_2^- \\
\text{Hb.Fe(II)O}_2 + 2\text{H}^+ + \text{O}_2^- + \text{H}_2\text{O} & \rightarrow \text{Hb.Fe(III)H}_2\text{O} + \text{O}_2 + \text{H}_2\text{O}_2 \\
\text{Hb.Fe(II) + H}_2\text{O}_2 + \text{H}_2\text{O} & \rightarrow \text{Hb.Fe(III)H}_2\text{O} + \text{OH}^- + \text{HO}^- 
\end{align*}
\]
Hb.Fe(II) + HO· + H₂O → Hb.Fe(III)H₂O + OH⁻

where HbO₂ and Hb represent oxy and deoxy hemoglobin respectively with a single heme center for simplicity.

Based upon this autoxidation mechanism the effect of catalase and superoxide dismutase on the autoxidation of hemoglobin could reasonably be explained since they eliminate the peroxide and superoxide intermediates.

HEMOGLOBIN IN LIPOSOMES

To make a blood substitute in the form of hemoglobin containing liposomes (hemosomes), some investigators have used saturated and unsaturated phospholipids to encapsulated hemoglobin. The rate of oxidation of hemoglobin in the unsaturated liposomes was faster than the one in the saturated liposomes. This oxidation was caused by the lipid peroxidation products (65). Nonetheless, protective agents could be added and the rate of oxidation was reduced (65).

Recently, Jopski et al. (81) had prepared hemosomes containing inositol hexaphosphate, in which the oxygen dissociation (P₅₀, 32.1 mmHg) was found to be nearly the same as that of whole blood, (P₅₀, 27.1 mmHg) (81). The shelf-life of this type of encapsulated hemoglobin was promising. Storage of these hemosomes at 4°C for 300 days
resulted in only 14% methemoglobin (81). In view of these preliminary discoveries of hemoglobin liposomes, they exhibit potential use as a blood substitute. An interest in encapsulated crosslinked hemoglobin has yet to be manifested, and its properties remained to be explored as blood substitute in the future.

THERMAL DENATURATION OF HEMOGLOBIN

The assessment of thermal stability of a protein can be obtained as the melting temperature, the free energy, enthalpy, and entropy of denaturation. Thermal denaturation can lead to important structural information, such as the mechanism of denaturation and the reasons for the stability of the native structure. A denatured state is defined as a major conformational change from the native state of globular proteins without any alteration of the amino acid sequence (82). Thermal denaturation of proteins may be reversible or irreversible depending on the protein in consideration and the conditions used. A reversible denaturation would be one in which the native structure is recovered after denaturation (82).

The thermal denaturation of hemoglobin A and crosslinked hemoglobin A (β82XLHb A) was investigated by White and Olsen (83). Subsequent investigation of the denaturation of carbonmonoxyhemoglobin A (CO-Hb A),
carbonmonoxyhemoglobin S (CO-Hb S), cyanomethemoglobin A (CN-Hb A), hemoglobin New York (Hb NY), and their crosslinked derivatives of the \( \beta 82XLHb \) were examined by Yang and Olsen (84,85). The denaturation of hemoglobins under these conditions was irreversible. The denaturation state of these hemoglobins was thought to be a highly disordered state in which the heme may or may not be intact in the globin chain. The order of thermal stability of methemoglobins A, S, and A2 (86) was S < A < A2 (84,87). The stability of metHb A (metHb A) and its fumarate crosslinked derivatives as characterized by the melting temperature (\( T_m \)) were 41°C for metHb A, 57°C for met \( \beta 82XLHb \) A (83-85).

The thermal transitions of imidoester crosslinked hemoglobins were somewhat broader due to heterogeneous species present during denaturation, and their stability was lower. However, the stability of these hemoglobins increased depending on the conditions during the crosslinking reactions (i.e. at higher pH or higher concentration of reagent). A reaction condition in which the pH was 9.5 and a molar ratio of reagent to hemoglobin was 1000:1, produced crosslinked hemoglobins which have the highest \( T_m \)'s for these hemoglobin derivatives and were comparable to those of fumarate crosslinked hemoglobins. The stability of HbA-DMA was 51.5°C, HbA-DMS 55.4°C, and HbA-DMP 57.0°C (48). A summary of the \( T_m \)'s of crosslinked
hemoglobins determined previously in this laboratory is presented in Table 3.

The thermal denaturation of protein is clearly a valuable method in distinguishing the stability of proteins. This could lead to information such as what crosslinking reagents could stabilize the protein structure the most, and at what temperature the modified protein could be heated without causing denaturation to the protein.

CATALASE AND SUPEROXIDE DISMUTASE: A BRIEF OVERVIEW

In order to understand the prevention of oxidative damage to hemoglobin by catalase and superoxide dismutase, it is necessary to present the known and proposed mechanisms of disproportionation of hydrogen peroxide (H2O2) and superoxide radical by these enzymes. It is of interest and possibly of great value to use enzymes and antioxidants for the protection of hemoglobin in a blood substitute.

Catalase is a heme containing enzyme present in erythrocytes as well as in the tissues of kidney and liver (88). It exists in a tetrameric form, α2β2, and has a molecular weight of 240,000 daltons. Catalase was known to catalyze the reaction (88,89):
Table 3:
The Tm values of crosslinked methemoglobin and mutant hemoglobins determined previously in this laboratory, from the Soret band, 406 nm.

<table>
<thead>
<tr>
<th>met Hb</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A2</td>
<td>43.5 a</td>
</tr>
<tr>
<td>Hb A</td>
<td>41.8 a</td>
</tr>
<tr>
<td>β82XLHb A</td>
<td>56.6 a</td>
</tr>
<tr>
<td>HbA-DMA</td>
<td>51.5 b</td>
</tr>
<tr>
<td>HbA-DMP</td>
<td>57.0 b</td>
</tr>
<tr>
<td>HbA-DMS</td>
<td>55.4 b</td>
</tr>
<tr>
<td>Hb S</td>
<td>39.6 a</td>
</tr>
<tr>
<td>β82XLHb S</td>
<td>56.0 a</td>
</tr>
<tr>
<td>Hb NY</td>
<td>40.9 a</td>
</tr>
<tr>
<td>β82XLHb NY</td>
<td>55.7 a</td>
</tr>
<tr>
<td>Hb Andrew-Minneapolis</td>
<td>40.9 a</td>
</tr>
<tr>
<td>Hb O Indonesia</td>
<td>39.6 a</td>
</tr>
</tbody>
</table>

a Yang, T., (86); M.S. Thesis, Loyola Univ. of Chicago, Chicago.
b Corso, T. D., (48); M. S. Thesis, Loyola Univ. of Chicago, Chicago.
Superoxide dismutases (SOD) are metal containing enzymes distributed throughout the various tissues of aerobic organisms; they exist in the form of either a dimer or tetramer. The metal ions bound to SOD were Cu, Zn, Fe, and Mn. The copper (Cu$^{+2}$) and zinc (Zn$^{+2}$) containing SOD is a dimer containing one of each copper and zinc per subunit and has a molecular weight approximately 32,000 daltons; those containing iron and manganese have molecular weight up to 97,000 daltons (90). The physiological function of SOD is to provide protection for an organism from deleterious effect of the very reactive O$_2$·$^-$ generated in the biological systems (88) by catalyzing the reaction (89):

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$

The role of the zinc ion in SOD is attributed to mostly maintaining stability and organization of the protein and is not directly involved with the catalytic processes. The zinc and the copper centers within a subunit is spanned by a histidine residue (His 61) which separated the two by 6 Å (90). The copper ion center served as the catalytic center for this enzyme. An overall
mechanism involving the copper center was thought to be as (91):

\[
\begin{align*}
Cu^{+2} + O_2^- & \rightarrow CuO_2^+ \rightarrow Cu^{+1} + O_2 \\
Cu^+ + O_2^- + 2H^+ & \rightarrow Cu^{+2} + H_2O_2
\end{align*}
\]

In light of these catalytic reactions by catalase and SOD and the proposed autoxidation mechanisms, it may be possible now to explain why catalase and SOD or antioxidants could provide protection against oxidative damage to hemoglobin. Furthermore, hemoglobin in a blood substitute may need these enzymes to be protected against oxidative damage.
CHAPTER II
MATERIALS AND METHODS

I. MATERIALS

A. CHEMICALS

DEAE-Sephadex A-50 gel was obtained from Pharmacia Fine Chemicals; tris(hydroxymethyl) aminomethane hydrochloride (Tris), 4-morpholinepropane sulfonic acid (MOPS), glycine, acrylamide, N,N’-methylene-bis-acrylamide, bromphenol blue, ammonium persulfate, N,N,N’,N’-tetramethylethlenediamine (TEMED), Amberlite MB-3, Sephadex G-25 gel, SDS protein markers (Dalton mark VII), catalase, and superoxide dismutase, from Sigma Chemical Co.; sodium cyanide, ammonium sulfate, sodium hydroxide, urea, and sodium phosphate, from J. T. Baker Chemical Co.; magnesium chloride, potassium ferricyanide and sodium chloride from Mallinckrodt, Inc.; dimethylpimelimidate, guanidine hydrochloride, trifluoroacetic acid, and sodium dodecylsulfate (SDS), from Pierce Chemical Co.; β-mercaptoethanol, from CalBiochem.; Hydrochloric acid, and glacial acetic acid, from Fisher Scientific Co., N,N-dimethylaniline, N,N-dimethyl formamide, fumaryl chloride, tris(β-chloroethyl) amine, bis(β-chloroethyl) methyl amine, thionyl chloride, 3,5-dibromosalicylic acid, deuterium oxide from Aldrich Chemical Co. Inc.; deuterated dimethyl
sulfoxide from Stohler Isotope Chemicals; carboxymethyl cellulose (CM52), from Whatman Ltd.; and 3-bromo-1,1,1-trifluoropropanone, from PCR Inc.; PHAB buffer, Amido Black 10B, alkaline agarose gels from Corning Medical; 0.2 M sodium citrate pH 2.2, from Beckman; acetone and benzene were distilled from reagent grade.

B. INSTRUMENTS

The instruments used were: a Hewlett-Packard (HP) 8451A UV/VIS diode array spectrophotometer, HP-7470A plotter, HP-9121 disc drive, NESLAB endocal refrigerated circulating bath RTE-9, NESLAB temperature programmer ETP-3, NESLAB digital controller/readout DCR-4, Omega temperature indicator, Orion research digital pH meter, water bath (Blue M Electric Co.), ISCO UA-5 absorbance/fluorescence detector, ISCO gel scanner model 1312, ISCO Tris peristaltic pump, thermometer (Sargent), Varian VXR 300 MHz NMR spectrometer, 5 mm NMR tubes (Wilmad Glass Co. Inc.), and Sorvall RC-5B refrigerated superspeed centrifuge (Dupont), small centrifuge 5414 (Eppendorf), Eppendorf pipettes, Sonicator 1200 (Branson), Vortex-Genie (Fisher Scientific), Columns (Bio-Rad Lab.), conductivity bridge model 31, power supply model PS-4 (Heath Co.), DC power supply model PS 500XT (Hoefer Scientific Instruments), power supply model HV 1000, speed vacuum
Rota-Vapor (Savant Instruments Inc.), SDS slab gel electrophoresis unit SE250 (Hoefer Scientific Instruments), and clinical electrophoresis equipments (Corning Medical), Beckman Microcolumn Amino Acid Analyzer Model 121M (Northwestern Children's Memorial Hospital).

II. CHEMICAL SYNTHESIS

The synthesis of bis(3,5-dibromosalicyl) fumarate was done according to the method of Zaugg et al. (25). In a three-necked 250 mL round bottom flask containing 50 mL of dry benzene was added 3.776 g (0.0128 mole) of 3,5-dibromosalicylic acid, and 3.213 mL (0.0253 mole) of N,N-dimethyl aniline (d, 0.956 g/mL). The solution was stirred slowly to allow homogeneity for 1 minute with a magnetic stirring bar. To this solution 0.675 mL (0.0062 mole) of fumaryl chloride (d, 1.415 g/mL) was added slowly with stirring. Upon completing addition of fumaryl chloride the solution was one phase and turned reddish brown. After three hours of stirring at room temperature 15 mL of water was added, and the solution was acidified with one or two drops of 12 M HCl. Upon increasing mixing speed a large amount of precipitate was readily observed in a two phase system. The stirring was continued for 20 minutes. The product was washed via suction-filtration three times with benzene and was twice recrystallized from minimum 50:50 hot
water-acetone mixture. The crystalline product had a melting point of 226-228°C.

III. PREPARATION OF HEMOGLOBIN

Hemoglobin was prepared from outdated packed Red Blood Cells (RBC) obtained from Life Source according to the method of Dozy et al. (93). The RBC's were washed with phosphate buffer saline (PBS). The PBS was prepared by titrating 5 mM dibasic sodium phosphate (Na$_2$HPO$_4$) to pH 8.0 with 5 mM monobasic sodium phosphate (NaH$_2$PO$_4$), and to this solution was added 8.77 g NaCl per liter (150 mM). To 20 mL of packed cells, 80 mL of (PBS) were added. The mixture was stirred slowly. The blood cells in PBS were placed in centrifuge tubes, balanced to equal weight, and centrifuged at 3,000 rpm (1,075 g) for 10 minutes with a Sorvall RC5B refrigerated superspeed centrifuge, using an SS-34 rotor. The supernatant and buffy coat were removed by aspiration after the centrifugation. The washing of the RBC's with PBS was repeated two more times to ensure that the RBC's were free of the unwanted plasma materials. All these steps were performed at 4°C.

The washed erythrocytes were lysed in 40 mL of ice cold deionized water with gentle stirring for 30 minutes in a cold room at 4°C. To every 80 mL of hemoglobin solution, 20 mL of cold neutral ammonium sulfate (AS) were added.
The neutral AS was prepared by adding 76.7 g of AS per 100 mL of deionized water, and adjusted to pH 7.0 with 6 M sodium hydroxide. The hemoglobin solution in AS was stirred continuously for 30 minutes at 4°C. The mixture was placed in centrifuge tubes, balanced to equal weight, and centrifuged at 12,000 rpm (17,210 g) for 10 minutes. After centrifugation the hemolysate in the supernatant was pooled, and the residues containing the membranes and other precipitates at the bottom of the centrifuge tubes were discarded. The hemolysate was desalted by dialysis in 0.05 M Tris, 1 mM NaCN, pH 8.5. The completion of desalting was checked by adding 1 M BaCl₂ to the dialysis solution in a small beaker to test whether or not BaSO₄ precipitates were still able to form. After desalting was complete, the dialysate was ready for ion-exchange chromatography.

IV. ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange chromatography was performed according to the method of Huisman and Dozy (94), and Dozy et al. (93). DEAE Sephadex A-50 gel was swollen in 0.05 M Tris, 1 mM NaCN, pH 8.5 in the refrigerator for two days, or boiled in deionized water for two hours before use. Column size of 30 x 1 cm or 30 x 1.5 cm was used in these experiments. The Sephadex gels were packed to a height of 25 cm. Prior to chromatography the column was equilibrated with 0.05 M
Tris, 1 mM NaCN, pH 8.5 for approximately 4 hours in the refrigerator with a flow rate of 20 mL per hour. When the pH of the eluent buffer reached 8.5, the equilibration was stopped and the dialyzed hemolysate was applied to the top of the column. Separation of the different hemoglobin was obtained by using a decreasing linear pH gradient from 8.5 to 7.2 with the Tris buffer. The pH gradient system was set up by connecting two aspirator bottles of 250 mL at the base, one containing Tris buffer pH 8.5 and the other 7.2. The bottles were leveled and filled with equal volumes of 250 mL of Tris buffer. During the filling of the bottles the tube connecting the bottles was clamped to prevent mixing of the solutions. The clamp was released and the bottle containing Tris pH 8.5 was stirred to allow formation of the pH gradient. The pH gradient was pumped from the bottle with Tris pH 8.5 to the top of the column. The flow rate was kept constant at 12 mL per hour with an ISCO peristaltic pump. The hemoglobin fractions eluted was monitored by an ISCO UA-5 Absorbance monitor form 280-310 nm. The hemoglobin fractions collected were pooled according to each peak under the chromatogram, CO gassed, and stored at 4°C, or frozen by liquid nitrogen and kept in the freezer (-8°C) for later use.
v. CONCENTRATION OF HEMOGLOBIN BY A PRO-DICON

Concentration of hemoglobin was achieved by a Pro-dicon, or by ammonium sulfate precipitation. The pro-dicon was filled with the desired buffer. The hemoglobin fractions pooled under each peak of the chromatogram were collected, dialyzed in the appropriate buffer, and transferred to the dialysis tube in the pro-dicon. The Micro-prodicon tubing was from Bio-Molecular Dynamics. Vacuum was applied from the water aspirator to the pro-dicon for 1 hour. The prodicon was then set in the refrigerator until the protein was concentrated to the minimum volume allowed in the tubing. If there were any precipitates present in the hemoglobin solution after concentration, the solution was centrifuged at 12,000 rpm (17,210 g). The hemoglobin solution was collected, and the precipitates were discarded.

VI. CONCENTRATION OF HEMOGLOBIN USING AMMONIUM SULFATE

For relatively large volume of hemoglobin solution (100 mL or higher), the protein was concentrated by precipitation with ammonium sulfate (75% or 80% saturation). To 100 mL of hemoglobin solution 56.61 g of ammonium sulfate was added. The solution at ice bath temperature was stirred slowly with a stirring bar until
all the ammonium sulfate dissolved. The hemoglobin precipitates were then centrifuged at 12,000 rpm (17,210 g). The supernatant was discarded and the hemoglobin precipitates were mixed with minimum buffer solution to enable pipetting them into the dialysis tube. Since most of the experiments from this point on were to be carried out in 0.01 M MOPS, pH 7.0, the hemoglobin precipitate was dialyzed in this buffer. The hemoglobin precipitates were dialyzed against a large volume of buffer until the buffer, when tested with 1 M BaCl₂, showed no cloudy precipitates of BaSO₄. Any precipitates due to denatured protein present in the solution were removed by centrifugation (12,000 rpm, 17,210 g).

VII. CROSSLINKING REACTIONS OF HEMOGLOBIN

A. REACTIONS OF HEMOGLOBIN WITH BIS(3,5-DIBROMOSALICYL) FUMARATE

The crosslinking reaction of hemoglobin with bis(3,5-dibromosalicyl) fumarate was carried out in 0.01 M MOPS, 1 mM NaCN, pH 7.0. Other experiments were done at pH 7.2 of the same buffer, but the results were the same. In both the oxy and deoxy crosslinking reactions the molar ratio of hemoglobin tetramers to crosslinking reagent was kept at 1:1.1 for all the experiments, regardless of the concentration of hemoglobin.
For hemoglobin crosslinking in the oxy state, the variation in concentration was from $6 \times 10^{-3} - 0.6$ mM tetramers for different experiments. For hemoglobin crosslinking in the deoxy state, the variation was $2 \times 10^{-3} - 0.5$ mM tetramers; but most experiments were done at 0.2 mM.

The oxy hemoglobin concentration was determined spectrophotometrically by using $\epsilon_{\text{mM}}$ of 13.8 per heme at 540 nm (114), or for higher hemoglobin concentration the $\epsilon_{\text{mM}}$ of 7.12 per heme at 522 nm (95) could be used.

The crosslinking of hemoglobin in the oxy state was done by adding known amount of bis(3,5-dibromosalicyl) fumarate to oxyhemoglobin solution in a flask; the mixture was swirled a few times and incubated at 37°C for 2 hours. The mixture was swirled slowly every 30 minutes within the incubation period. After two hours of incubation, the mixture was submerged in an ice cold bath for 20 minutes to terminate the reaction. The hemoglobin solution was then dialyzed in Tris buffer pH 8.5, or in some experiments the solution was run through a G-25 Sephadex gel equilibrated with 0.01 M MOPS, pH 7.2 followed by dialysis with Tris buffer pH 8.5 before it was applied to a chromatographic column.

For deoxy crosslinking reaction, the oxyhemoglobin solution in a flask with multiple outlets and inlets on top was submerged in a 4°C ice bath and was purged with a slow stream of N₂ gas for 1-4 hours until the solution appeared
purplish and the UV/VIS spectrum of deoxyhemoglobin was confirmed. For carbonmonoxyhemoglobin, the solution was exposed to light at 4°C for 1-2 hours while purging with N₂. After confirmation of deoxy hemoglobin spectrophotometrically, the solution was transferred to a water bath already equilibrated at 37°C. The crosslinking reagent was introduced while purging with N₂; the solution was swirled a few times and incubated for 2 hours. The reaction was terminated in an ice bath for 20 minutes, and the sample was dialyzed against large volumes of 0.05 M Tris, 1mM NaCN, pH 8.5 to remove any excess crosslinking reagent present in the solution. At this point the solution was no longer kept deoxygenated. In some experiments the crosslinking mixture was passed through a Sephadex G-25 column (2 x 50 cm) equilibrated with 0.01 M MOPS, 1 mM NaCN, pH 7.2, followed by dialysis in Tris buffer pH 8.5.

The crosslinked hemoglobin was purified by ion-exchange chromatography by the same method above. The unused purified crosslinked hemoglobin was CO gassed and kept in the refrigerator at 4°C or was frozen and kept in the freezer at -80°C until the next use.
B. MODIFICATION REACTIONS OF HEMOGLOBIN WITH TRIS(\(\beta\)-CHLOROETHYL) AMINE AND BIS(\(\beta\)-CHLOROETHYL) METHYLAMINE

The reaction of hemoglobin with tris(\(\beta\)-chloroethyl) amine was carried out by the methods of Hiratsuka (43). The first reaction was carried out by mixing 9 mL of oxy Hb A at 0.446 mM tetramers in 0.01 M MOPS, 0.05 M NaCl, pH 7.88 directly with 0.011 g solid (5.07 mM) of tris(\(\beta\)-chloroethyl) amine (TCEA). The final molar ratio of TCEA to hemoglobin tetramers was 11:1.

The second reaction of hemoglobin with tris(\(\beta\)-chloroethyl) amine was done by adding 5 mL of 0.0319 M tris(\(\beta\)-chloroethyl) amine (TCEA) in 0.01 M MOPS, 0.05 M NaCl, pH 7.88, to 9 mL of 0.446 mM tetramers of oxy hemoglobin A in MOPS buffer. The final molar ratio of TCEA to hemoglobin tetramers was 40:1.

The reaction of hemoglobin with bis(\(\beta\)-chloroethyl) methylamine (BCEA) was done according to Roth et al. (42), and Fung et al. (44). To 4 mL of 0.627 mM tetramers of oxy Hb A was added 4.8x10^{-3} g of BCEA (6.23 mM). The final molar ratio of BCEA to hemoglobin concentration tetramers was 10:1.

All three reactions were carried out at room temperature (21°C) for one hour with slow stirring. The reactions were terminated by adding 2.3 mL of 1 M Glycine
to the reaction flasks. The hemoglobin solutions were passed through a G-25 Sephadex gel equilibrated with 0.01 M MOPS, pH 7.0 and dialyzed two times against 2 L of 0.01 M MOPS, pH 7.0 at 4°C overnight. Any precipitates formed in the solutions were centrifuged before storage.

C. MODIFICATION REACTIONS OF HEMOGLOBIN WITH DIMETHYLPIMELIMIDATE AND DOUBLE CROSSLINKING

The reaction of hemoglobin with dimethylpimelimidate (DMP) was done in 0.1 M borate, pH 9.5 at room temperature for one hour. The reactions of oxy Hb A, oxy β82XLHb A, and oxy α99XLHb A were performed in the same condition and concentration. Two reaction conditions were used in which the molar ratio was 500:1 and 50:1 DMP to hemoglobin tetramers. For the lower molar ratio reaction the DMP was simply added in lesser amount. All the other conditions remained unchanged.

A sample reaction of hemoglobin with DMP at 500:1 molar ratio was as follows: To 24.418 mL of buffer containing 0.051 g of DMP was added 410 µL of 1 M NaOH to adjust pH to 9.5, and then 172 µL of oxy Hb A (2.275 mM tetramers, 145.6 mg/mL) was added. The pH was periodically monitored. The reaction was carried out at room temperature (22°C). The final concentrations of reagents were 7.871 mM DMP, and 0.01564 mM hemoglobin tetramers or 1
mg/mL. Hence, the molar ratio of reagent to hemoglobin tetramers was 500:1. The pH was checked periodically during the reaction and maintained at 9.4-9.6. After one hour the reaction was terminated by adding 5 mL of 0.5 M glycine in borate buffer. The reaction mixture was dialyzed in 0.01 M MOPS, pH 7.0 to get rid of the excess crosslinking reagent and glycine.

A double crosslinking reaction was performed by reacting a purified α99XLHb A or β82XLHb A with DMP. These reactions were performed at 500:1 molar ratio of DMP to hemoglobin. All other conditions were the same as in the reactions of DMP with uncrosslinked Hb A.

VIII. CROSSLINKING OF HEMOGLOBIN TO CATALASE WITH DMP

The crosslinking of hemoglobin (Hb A) to catalase was performed by using DMP to bridge the two molecules. The reaction conditions and concentration of protein were the same as the DMP reaction with hemoglobins. The final molar ratio of DMP to hemoglobin and to catalase was 2000:4:1.

A sample reaction of DMP crosslinking between hemoglobin and catalase was as follows: A 0.051 g of DMP was dissolved in 24.14 mL of 0.1 M borate buffer adjusted to pH 9.5 with 410 µL of 1 M NaOH. To this solution was added 172 µL of Hb A (145.6 mg/mL, 2.275 mM) and 278 µL of catalase (90 mg/mL). The final concentration of reagents
were 7.871 mM DMP, 0.0156 mM tetramers Hb A or 1 mg/mL, and 0.0042 mM tetramers catalase or 1 mg/mL. The reaction was allowed to proceed for 1 hour at room temperature (22°C). The reaction was terminated by adding 5 mL of 0.5 M glycine in borate buffer. The reaction mixture was dialyzed in 0.01 M MOPS, pH 7.0 to get rid of the excess crosslinking reagent and glycine.

Two reaction conditions were used in which the molar ratio of DMP to hemoglobin and catalase were 2000:4:1 and 1000:4:1. All other experimental conditions remained unchanged.

A control experiment in which DMP was reacted with catalase was also performed at molar ratios of 2000:1 and 1000:1 DMP to catalase. A protocol of this experiment at 2000:1 molar ratio was as follows: A 0.051 g of DMP was added to 24.31 mL of 0.1 M borate buffer, adjusted to pH 9.5 with 410 µL of 1 M NaOH. To this solution 278 µL of catalase (90 mg/mL) was added. The final concentration of reagents were 7.87 mM DMP and 0.0042 mM or 1 mg/mL catalase. The rest of the experimental conditions were the same as that in the DMP and hemoglobin reactions.

IX. PREPARATION OF GLOBIN CHAINS

AND AMINO ACID HYDROLYSIS

The purification of globin chains was by the method
of Ascoli et al. (96). The hemoglobin was dialyzed in excess deionized water to remove all the salt present in the solution. The heme was removed by adding the deionized hemoglobin solution dropwise to rapidly stirred cold acetone (-20°) containing 2% concentrated HCl. A white precipitate was formed, which was the globin chains, and the acetone solution became brownish in the presence of hemins. The sample was centrifuged at 5,000 rpm (2,987 g, 4°C) to remove the precipitates. The precipitates were washed three times with ether followed by centrifugation to remove any remaining reddish particles on the precipitates and dried in air while the centrifuge tubes were submerged in ice.

The separation of the subunits was carried out using CM-Cellulose (CM52), from Whatman Ltd., with a linear increasing ion gradient. The gradient system consisted of two buffer chambers, designated buffer I and buffer II (100 mL each). Buffer I contained 0.005 M Na₂HPo₄, 0.05 M 2-mercaptoethanol, 8 M urea, pH 6.7. Buffer II contained 0.05 M Na₂HPo₄, 0.05 M 2-mercaptoethanol, 8 M urea, pH 6.7. The conductivity of the buffers I and II, by a Conductivity Bridge Model 31, were 1,020 \( \mu \Omega^{-1} \) and 6,800 \( \mu \Omega^{-1} \), respectively. The column packed with CM-52 gel (25cm x 1 cm) was equilibrated with buffer I for at least 2 hours or until the conductivity of the eluent matched that of the buffer I. When the conductivity of the equilibrated eluent
and that of the buffer I matched within 1-2%, then the globin chains sample was loaded on to the top of the column and was washed with buffer I for 15 minutes, and then the gradient was started.

The globin chains obtained through the ion exchange chromatography were freed from urea, salt, and \( \beta \)-mercaptoethanol by dialysis against a large volume of deionized water. The globin solution was placed in acid cleaned test tubes and the water was evacuated by using a Speed Vacuum Rota-Vapor. After the water was completely evacuated the required amount of solid globin chains were mixed with 1 mL of 6 M predistilled HCl. The middle of the test tube above the solution was made smaller by heating and pulling the test tube while it was melting. The test tube was allowed to cool down. The bottom of the test tube was immersed in dry ice cold acetone solution, and air was evacuated from the test tube. After it was evacuated the small middle portion of the test tube was sealed by flame, and the test tube was placed in 110°C oil bath to allow hydrolysis of the polypeptide chains. The hydrolyzate was filtered, mixed with 0.2 M sodium citrate, pH 2.2 buffer (from Beckman), and analyzed by a microcolumn amino acid analyzer (model 121M, Beckman) at Northwestern University of Chicago Children's Hospital in Dr. Mir Shamsuddin's laboratory. The number of amino acids was estimated by multiplying the average residues (142) to the
area ratio of individual amino acid to the total area of all residues from the $\alpha$ and $\beta$ chains. The $\alpha$-chain has 141 residues and the $\beta$-chain has 146 residues. The average residue is calculated as shown below:

$$\text{Average Residue} = \frac{(141 + 146 - 3)}{2} = 142$$

Thus, the average residue is 142. The subtraction of 3 was due to the three tryptophan residues per $\alpha\beta$ dimer.

X. NMR SAMPLES PREPARATIONS AND EXPERIMENTS

The $^1$H and $^{13}$C spectra were taken in deuterated DMSO in 5 mm tubes. For the proton spectrum the nucleus was set at 1.250 with a spectral width of 4000 Hz. The frequency was 300 MHz. The acquisition time was 3.752 sec.; the pulse width was 20 $\mu$sec. and no delay time was used. The number of scans collected was 4-16.

The $^{13}$C spectrum was run at 75 MHz with the nucleus set at 13.250 and no delay time was used. The spectral width was 16501.7 Hz. The acquisition time and pulse width were set at 1.639 sec. and 8.7 $\mu$sec., respectively. The total number of transients was 7,248.
XI. CLINICAL ALKALINE AGAROSE GEL ELECTROPHORESIS

The alkaline agarose gel electrophoresis was performed, using commercially available Corning Medical films. The agarose film consists of 1% (w/v) agarose, 5% (w/v) sucrose, 0.002 M Na₂EDTA, 0.0059 M boric acid, and 0.053 M Tris buffer, pH 8.8.

The electrode buffer, also commercially prepared by Corning Medical, contained 17.7 g sodium barbital, 2.6 g barbital, 1.0 g sodium chloride, 0.7 g disodium EDTA, sucrose octaacetate. This buffer was dissolved in 2 L of deionized water to produce 0.05 M buffer with 0.035% EDTA, pH 8.6. The buffer was stored at 4°C in the refrigerator for later use.

Prior to electrophoresis the power supply was warmed up for about 10 minutes. The alkaline agarose film was peeled off from the protective plastic, and the wells were each filled with 1 µL of the sample. The two chambers in the electrophoresis cell were each filled with 75 mL of the electrode buffer. The loaded agarose film was inserted face down in the electrophoresis cell cover with the matching of the electrode marks (anode to anode and cathode to cathode), and was fitted on to it. The electrophoresis was carried out for 20 minutes at 240 volts. The film was removed and stained in Amido Black 10B for 10 minutes. Then it was transferred to a 5% acetic acid solution for 30
seconds with slow stirring to wash off the excess stain. The film was washed with deionized water and dried in an oven at 55-65°C for 1 hour. After the film was dried, it was taken out from the oven and cooled to room temperature for 10 minutes. Then it was destained in a 5% acetic acid with agitation for 1 minute. This process was repeated in a new acetic acid solution one more time, and the film was immersed in water to remove the excess acid and dried at 55-65°C. After drying at this step the film was ready for visual inspection and gel scanning.

XII. SDS GEL ELECTROPHORESIS

The sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in slab gel according to Laemmli (97), with minor modifications. Two set of gels were used; one (Savant) with dimension of 22 x 16 x 0.15 cm, and the other (Hoefer mighty small) with 7.5 x 8.0 x 0.05 cm.

For the larger gel after the glass plates system was set up, the gel plug was polymerized by using a fourth of the volumes of the solutions of the separating gel at the bottom of the glass plates. The 11% acrylamide separating gel was polymerized by mixing the following reagents: 12 mL of 1.875 M TRIS, pH 8.8; 22 mL of 30% acrylamide (15 g acrylamide, 0.41 g N,N'-methylene-bis-acrylamide in 50 mL
of deionized water); 0.6 mL of 10% SDS in deionized water; and 26.96 mL of deionized water. The mixture was degassed for 15 minutes before adding 400 µL of 10% freshly made ammonium persulfate, and 80 µL of N,N,N’N’-tetramethylenediamine (TEMED). The final concentration of the separating gel was: 0.375 M TRIS, 0.1% SDS, 11% acrylamide, pH 8.8.

The stacking gel was polymerized by mixing the following reagents: 7.52 mL of 1.0 M TRIS, pH 6.8; 3.0 mL of 30% acrylamide; 300 µL of 10% SDS; and 18.96 mL of deionized water. The mixture was again degassed to prevent air bubbles before adding 100 µL of 10% ammonium persulfate, and 20 µL of TEMED. The final concentration of the stacking gel was: 0.273 M TRIS, 0.1% SDS, 3.2% acrylamide, pH 6.8.

The electrode buffer contained 0.025 M TRIS, 0.192 M glycine, 0.1% SDS, pH 8.8. The digestion mixture solution contained 0.229 M TRIS, pH 6.8, 3.7% SDS, 18.3% glycerol (v/v), 8.3% β-mercaptoethanol (v/v), and 0.02% bromphenyl blue.

The protein markers used were bovine serum albumin (Mr 66,000), egg albumin (Mr 45,000), glyceraldehyde-3-phosphate dehydrogenase (Mr 36,000), carbonic anhydrase (Mr 29,000), trypsinogen (Mr 24,000), trypsin inhibitor (Mr 20,100), and α-lactalbumin (Mr 14,200).

The electrophoresis was carried out by a power
supply model PS-4 (Heath Co.) at constant current at 35 mA until the dye reached the bottom of the gel. This process took approximately 8 hours. After the electrophoresis, the proteins were fixed in the gel with 40% methanol and 7% acetic acid solution for 4 hours and stained with coomassie brilliant blue for 4 hours. The fixing step was not necessary. The gel was destained by repeated washing with a solution of 40% methanol and 7% acetic acid. After destaining, they were stored in 5% acetic acid. The molecular weights of the unknown proteins were estimated from the graph of the log of molecular weight versus relative mobility of the marker proteins, and the percentage of crosslinking can be estimated by gel scanning.

XIII. DENATURATION EXPERIMENTS

The thermal denaturation of uncrosslinked and crosslinked hemoglobins were carried out according to White and Olsen (83), and Yang and Olsen (84). Thermal denaturation was done in 0.01 M MOPS, pH 7.0, in the presence of 0.9 M guanidine. The guanidine solution was prepared fresh prior to the experiments. The optical density of the Soret band of each experiment was kept at 1.0; this corresponded to a hemoglobin concentration of approximately 7 µM per heme. The hemoglobins were
thermally denatured as methemoglobins. Prior to denaturation the oxy hemoglobin was oxidized to methemoglobin by 9.7 µM potassium ferricyanide for 15 minutes.

A movable seven cuvette holder in the spectrophotometer was used. Seven cuvettes were used in each experiment; six were hemoglobin samples and one was the reference. The program for this denaturation experiment was written to take a measurement of each sample in 15 second intervals. The time interval between the same sample was 1.75 minutes. For each round of measurement the reference which contained 9.7 µM potassium ferricyanide was taken prior to measurement of the samples. The wavelengths used to monitor the denaturation were 280 nm, 406 nm, 418 nm, 522 nm, 540 nm, 576 nm, and 630 nm. Seven entire spectra were taken from 200-650 nm in every 7°C interval for observation of base line shift. The method of analysis was based on a two-state model, in which the protein was thought to be in equilibrium between either the native or the denatured state (82,89). The fraction denatured was calculated from the following equation:

\[ F_D = \frac{(A - A_N)}{(A_D - A_N)} \]

where \( A \) was the absorbance at a specified temperature. \( A_N \) and \( A_D \) were the absorbance effect of the native state and
denatured state due to temperature, respectively. From the value of the $F_D$ the apparent thermodynamic parameters of $K_D$, $\Delta G_D$, $\Delta H_D$, and $\Delta S_D$ could be estimated.

From the plot of $F_D$ versus temperature for each sample, the $T_m$ was the temperature at which the $F_D$ equaled 0.5. A second method to obtain the $T_m$ was to calculate the first derivative of the absorbance with respect to temperature. The temperature corresponding to the minimum or maximum was the $T_m$.

XIV. AUTOXIDATION EXPERIMENTS

The autoxidation of hemoglobins was monitored by the formation of methemoglobin according to the method of Tomita et al. (99). In a sample mixture of oxy and met hemoglobins all the methemoglobin could be converted to cyanomethemoglobin. The percent methemoglobin could be determined indirectly from oxy and cyanomet hemoglobins by using the ratio of extinction coefficient of oxyhemoglobin to cyanomethemoglobin, the resulting absorbance of oxy and cyanomethemoglobin mixture and that of pure cyanomet hemoglobin. Figure 1 shows the theoretical derivation of this method. At 100% oxyhemoglobin and cyanomethemoglobin the absorbancy at 576 nm are indicated by $A_3$ and $A_2$, respectively. In a sample mixture of oxy and met hemoglobins after converting all the methemoglobin to
Figure 1:

The visible spectra of hemoglobin used for the calculation of percent met hemoglobin in autoxidation. The absorbance of 576 nm at A₁ is 100% oxyhemoglobin at zero time, at A₂ after the hemoglobin is partially autoxidized, and at A₂ after conversion to 100% cyanomet hemoglobin.
cyanomethemoglobin with cyanide, the resulting absorbancy is $A_1$. The value of $k$, the ratio of molar extinction coefficient of oxy to cyanomet form, could be determined experimentally by dividing the absorbance of 100% oxyhemoglobin to the absorbance of cyanomethemoglobin of the same sample after conversion to 100% cyanomet hemoglobin at 576 nm. Multiplying $k$ by the absorbancy $A_2$ is equal to $A_3$, corresponding to the absorbancy of 100% oxyhemoglobin.

$$k = \frac{\epsilon_{\text{oxy}}}{\epsilon_{\text{cn}}} = \frac{A_3}{A_2} \quad \text{(at 100% oxy or cyanomet hemoglobin)}$$  

$$kA_2 = A_3$$  

The percent of methemoglobin is calculated by:

$$\% \text{ methemoglobin} = \left[ \frac{(A_3 - A_1)}{(A_3 - A_2)} \right] \times 100\%$$  

$$= \left[ \frac{(kA_2 - A_1)}{(kA_2 - A_2)} \right] \times 100\%$$  

$$= \left[ \frac{(k/k-1) - (1/k-1) \times (A_1/A_2)}{} \right] \times 100\%$$  

The experimental value of $k$ determined by Tomita et al. (68) was 2.285, and the percent of methemoglobin is then calculated by the following equation or by substituting the experimental $k$ value for equation 5 above:

$$\% \text{ methemoglobin} = \left[ 1.778 - 0.778 \times \frac{A_1}{A_2} \right] \times 100\%$$  

$A_1$ is the absorbance at 576 nm of oxy and cyanomet
hemoglobin mixture at the first measurement, and $A_2$ is the absorbance at 576 nm after conversion of any oxy to all cyanomet form at the second measurement.

For the determination of $k$ value the spectra of oxyhemoglobin at 2 mM heme, which produced an optical density (O.D.) equaled to 1.4 at 540 nm, were recorded. A crystal of potassium ferricyanide was added to the oxyhemoglobin solution in the cuvette; the cuvette was capped and tilted upside down three to four times, then the solution in the cuvette was allowed to equilibrate for 5 minutes at room temperature. The oxyhemoglobin was all converted to methemoglobin at this point. Then a crystal of sodium cyanide was added to the methemoglobin solution to convert all of it to the cyanomet form. The spectrum of the cyanomethemoglobin was recorded. The value of $k$ is then calculated experimentally by dividing the absorbance of 100% oxyhemoglobin ($A_3$) to that of 100% cyanomet hemoglobin ($A_2$). The $k$ values determined in this laboratory for oxy Hb A, oxy α99XLHb A, and oxy β82XLHb A were 2.163 ± 0.009, 1.953 ± 0.048 and 2.047 ± 0.008, respectively. The percent methemoglobin in this research was calculated based on these $k$ values.

The experiments were done at 37°C at room temperature (22°C). Fresh hemoglobin was used for these experiments. The hemoglobin samples were incubated in sterilized test tubes from Nutrex. The variation in
hemoglobin concentration in the test tubes between experiments was 1.9 mM, 2.2 mM, and 3.2 mM per heme. The incubation of hemoglobins was carried out in 0.01 M MOPS, pH 7.0. Prior to this incubation the hemoglobin samples were dialyzed and concentrated by a pro-dicon in 0.01 M MOPS, pH 7.0 to get rid of any free cyanide from previous preparation steps. During the incubation the test tubes containing the hemoglobin samples were covered with polystyrene test tube caps and sealed with parafilm to prevent evaporation of water. At the specified times, an aliquot of hemoglobin solution predetermined to yield an absorbance of 1.4 at 540 nm was pipetted and mixed with appropriate amount of the reference buffer. The sample cuvette was capped and tilted up side down several times to obtain a homogeneous mixture prior to measurement of the absorbance. For the first measurement, the hemoglobin solution was mixed with 1 µL of 0.1 M NaCN solution buffered at pH 7.0 or in some experiments, a crystal of sodium cyanide was added directly in the cuvette. The hemoglobin solution was allowed to equilibrate at room temperature for 5 minutes before measurement. After 5 minutes any met hemoglobin present in the solution would have been converted to cyanomethemoglobin, and the remaining was oxy hemoglobin. The spectrum from 500-700 nm of the hemoglobin sample was then recorded.

For the second measurement, the hemoglobin solution
from the first measurement was mixed with 1 µL of 0.1 M potassium ferricyanide buffered at pH 7.0 or a crystal of potassium ferricyanide, and the solution was allowed to equilibrate at 37°C for 20 minutes before measurement. After this incubation period the hemoglobin sample were all converted to cyanomet hemoglobin. The spectra were recorded from 500-700 nm simultaneously by the Hewlett-Packard diode array spectrophotometer 8451A.

XV. AUTOXIDATION OF HEMOGLOBIN IN THE PRESENCE OF CATALASE AND SUPEROXIDE DISMUTASE

The experiments on the autoxidation of hemoglobin in the presence of catalase and superoxide dismutase were carried out as described above for the autoxidation of hemoglobin (99), except catalase and superoxide dismutase or both were added to the hemoglobin solution under investigation. For catalase, a 9.1 µL solution of catalase (90 mg protein/mL, 44,000 U/mg protein) was added to 500 µL of oxyhemoglobin (2.22 mM heme) prior to the start of incubation. The total enzyme units added were 36,036 U, corresponding to 70,784 U/mL, or 16,530 U/mM heme. A unit of activity of catalase is defined by the supplier (Sigma) as the amount of enzyme which will decompose 1 µmole of H₂O₂ per minute at pH 7.0 at 25°C while the H₂O₂ concentration falls from 10.3 to 9.2 µmoles per mL of
reaction mixture.

For superoxide dismutase, a 2 mg solid sample of SOD (Cu/Zn SOD, 4,300 U/mg solid) was dissolved in 200 µL of buffer, and 40 µL of this solution was added to 500 µL of oxyhemoglobin (2.22 mM heme) prior to incubation. The total enzyme units added were 16,000 U, 3,185 U/mL or 835 U/mM heme. In one experiment, the SOD (2,990 U/mg solid) was added directly at 8.8 mg to 500 µL of oxyhemoglobin (2.20 mM heme). The total units were 26,400 U, 52,600 U/mL or 12,000 U/mM heme.

In the experiments in which both catalase and SOD were present the above described conditions and amount of enzymes were added to 500 µL of oxyhemoglobin (2.22 mM heme). The autoxidation was followed by the 576 nm wavelength as described above. The SOD and catalase were used without further purification and the enzymes activity was assumed to be as stated by the supplier.

The first order rate constant was obtained by plotting the \(-\log[A_0 - A]/[A_0 - A_\infty]\) versus time, where \(A_0\) and \(A_\infty\) represented the absorbance at 576 nm of 100% oxyhemoglobin and 100% cyanomethemoglobin, respectively, and \(A\) was the absorbance of oxyhemoglobin at 576 nm at the end of each time interval. The apparent rate constant \(k_{app}\) (hr\(^{-1}\)) was obtained by the slope of this plot. The percent inhibition of autoxidation by the enzymes was estimated by the percent difference of the slopes.
CHAPTER III

RESULTS

I. SYNTHESIS OF BIS(3,5-DIBROMOSALICYL) FUMARATE

The bis(3,5-dibromosalicyl) fumarate was a white crystalline powder with a melting point of 226-228°C. A small amount of the crystal was dissolved in a 1 M ferric chloride solution and the color remained yellowish, indicating the absence of a phenolic proton. Figures 2(A) and (B) show the $^1$H NMR spectra of bis(3,5-dibromosalicyl) fumarate and its presumed structure. There were four resonances, matching the four H's on half of the molecule. The singlet resonance at 7.34 ppm was from the fumarate H's. The doublet resonance at 8.33 ppm and 8.34 ppm arose from the benzyl proton of position 4, and the one at 8.08 ppm and 8.09 ppm was due to the benzyl proton at position 6. The broad resonance at 3.35 ppm was from the acidic proton of the carboxylic acid group. The multiplets (pentets) centered at 2.49 ppm was due to the H from the dimethyl sulfoxide (DMSO). The integration of the resonance at 7.34 ppm, 8.08 ppm, and 8.33 ppm indicated a ratio of 2:1:1, respectively.

Chemical shifts of substituted benzene and substituted ethylene from the literature were used to predict the NMR spectra. The observed chemical shifts and
Figure 2:

(A): The $^1$H NMR spectrum of bis(3,5-dibromosalicyl) fumarate (BBSF) ran at 300 MHz in deuterated DMSO and its presumed structure. The resonance at 2.49 ppm is from the proton of DMSO.

(B):

The expanded $^1$H NMR spectrum of BBSF. The resonances are: fumarate $^1$H, 7.34 ppm; H(4), 8.33 and 8.34 ppm; and H(6), 8.08 and 8.09 ppm.
predicted ones matched within 0.2 ppm for the $^1H$ resonances. The following equations were used to calculate the predicted chemical shifts (100,101).

For $^1H$ in substituted benzene:

$$\delta(\text{ppm}) = 7.27 + \sum S(\delta)$$

For $^1H$ in substituted ethylene:

$$\delta(\text{ppm}) = 5.28 + \sum S(\delta)$$

For $^{13}C$ in substituted benzene:

$$\delta(\text{ppm}) = 128.5 + \sum S(\delta)$$

The $S(\delta)$ is the incremental shift of each individual $^1H$ or $^{13}C$ in relation to either the $^1H$ or $^{13}C$ of nonsubstituted ethylene or benzene. The $^1H$ incremental shifts for substituted benzene and ethylene were taken from Gunther (Table 4.6 and 4.7, p. 98-99) (100). The predicted and observed chemical shifts for $^1H$ were tabulated in Table 4, and those of $^{13}C$, in Tables 5 and 6. Figures 3(A) and (B), the $^{13}C$ spectra show 9 resonances which agree with the nine identical carbons of bis(3,5-dibromosalicyl) fumarate. The chemical shift of each carbon on the benzene due to the substituted groups were calculated by using the
Table 4:
The predicted and observed $^1$H chemical shifts of bis(3,5-dibromosalicyl) fumarate from TMS.

<table>
<thead>
<tr>
<th>Substituents</th>
<th>$^1$H(4) $^a$</th>
<th>$^1$H(6) $^a$</th>
<th>H(fumarate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{CO}_2\text{H}$</td>
<td>0.20</td>
<td>0.80</td>
<td>___</td>
</tr>
<tr>
<td>$\text{OCOR}$</td>
<td>-0.02</td>
<td>-0.02</td>
<td>___</td>
</tr>
<tr>
<td>$\text{Br(3)}$</td>
<td>0.22</td>
<td>-0.03</td>
<td>___</td>
</tr>
<tr>
<td>$\text{Br(5)}$</td>
<td>0.22</td>
<td>0.22</td>
<td>___</td>
</tr>
<tr>
<td>cis, $\text{OCOR}$</td>
<td>___</td>
<td>___</td>
<td>1.15</td>
</tr>
<tr>
<td>gem, $\text{OCOR}$</td>
<td>___</td>
<td>___</td>
<td>0.84</td>
</tr>
<tr>
<td>Predicted</td>
<td>7.89</td>
<td>8.24</td>
<td>7.27</td>
</tr>
<tr>
<td>Observed</td>
<td>8.08</td>
<td>8.33</td>
<td>7.34</td>
</tr>
<tr>
<td></td>
<td>8.09</td>
<td>8.34</td>
<td></td>
</tr>
</tbody>
</table>

* H(4) and H(6) are protons on the benzene ring at positions 4 and 6 respectively.

$^a$ Values for the incremental shift were taken from Gunther (100) Tables 4.6 and 4.7, p. 98-99.
Solvent for the observed values was deuterated DMSO.
Table 5:
The predicted $^{13}$C chemical shifts using the incremental $^{13}$C shifts\(^a\) in ppm of the aromatic carbon atoms of monosubstituted benzene from 128.5 ppm of benzene carbons referencing from TMS.

<table>
<thead>
<tr>
<th>C(^b)</th>
<th>COOH</th>
<th>OCOCH(_3)</th>
<th>Br(3)(^c)</th>
<th>Br(5)(^c)</th>
<th>$^{13}$Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>2.1*</td>
<td>-6.4</td>
<td>1.7</td>
<td>1.7</td>
<td>127.6</td>
</tr>
<tr>
<td>C2</td>
<td>1.5</td>
<td>23.0*</td>
<td>3.4</td>
<td>-1.6</td>
<td>151.8</td>
</tr>
<tr>
<td>C3</td>
<td>0.0</td>
<td>-6.4</td>
<td>-5.5*</td>
<td>1.7</td>
<td>118.3</td>
</tr>
<tr>
<td>C4</td>
<td>5.1</td>
<td>1.3</td>
<td>3.4</td>
<td>3.4</td>
<td>141.7</td>
</tr>
<tr>
<td>C5</td>
<td>0.0</td>
<td>-2.3</td>
<td>1.7</td>
<td>-5.5*</td>
<td>122.4</td>
</tr>
<tr>
<td>C6</td>
<td>1.5</td>
<td>1.3</td>
<td>-1.6</td>
<td>3.4</td>
<td>133.1</td>
</tr>
</tbody>
</table>

\(\text{a} \) Values were taken from Silverstein (101), Table VII, p. 265.

\(\text{b} \) The carbon positions on the benzene ring.

\(\text{c} \) Br(3) and Br(5) are the bromine from position 3 and 5 on the benzene ring.

\(\text{d} \) $^{13}$C chemical shifts were calculated by adding the sum of the incremental $^{13}$C chemical shifts due to each substituted group to 128.5 ppm.

\* The substituent group is attached to the carbon corresponding to this row number.
Table 6:
The predicted and observed $^{13}$C chemical shifts of bis(3,5-dibromosalicyl) fumarate from TMS.

<table>
<thead>
<tr>
<th>$^{a}C$</th>
<th>Predicted (ppm)</th>
<th>Observed (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl</td>
<td>127.6</td>
<td>127.39</td>
</tr>
<tr>
<td>C2</td>
<td>151.8</td>
<td>146.07</td>
</tr>
<tr>
<td>C3</td>
<td>118.3</td>
<td>119.53</td>
</tr>
<tr>
<td>C4</td>
<td>141.7</td>
<td>133.87</td>
</tr>
<tr>
<td>C5</td>
<td>122.4</td>
<td>118.97</td>
</tr>
<tr>
<td>C6</td>
<td>133.1</td>
<td>133.37</td>
</tr>
<tr>
<td>C7</td>
<td>165-185$^{b}$</td>
<td>163.32</td>
</tr>
<tr>
<td>C8</td>
<td>140-190$^{c}$</td>
<td>161.39</td>
</tr>
<tr>
<td>C9</td>
<td>136.3$^{d}$</td>
<td>138.94</td>
</tr>
</tbody>
</table>

$^{a}$ This column represents the carbon position in the benzene ring.

$^{b}$ Value for the carboxylate carbon of carboxylic acid from Silverstein et al. (101), Appendix C, p. 289.

$^{c}$ Value for the ester carbon with two R groups, one attached to the carbon and the other to the oxygen, from Gunther (1973), p. 364.

$^{d}$ Value from Wehrli and Wirthlin (115), Table 2.10, p. 41-42.

Solvent for the observed values was deuterated DMSO.
Figure 3:

(A): The decoupled $^{13}$C NMR spectrum of BBSF ran at 75.4 MHz in deuterated DMSO. Inset is the DMSO peak.

(B):

The expanded $^{13}$C spectrum of BBSF, showing 9 resonance peaks.
values in Table 5 (p. 265) from Silverstein et al. (101). The ester group was assumed to be an acetate group. The $^{13}$C chemical shifts were assigned in accordance with the agreement between the predicted values and observed values as shown in Table 6. The septet centered at 39.50 ppm was from the carbon of DMSO. These data were consistent with the presumed structure of bis(3,5-dibromosalicyl) fumarate.

II. PURIFICATION AND CROSSLINKING OF HEMOGLOBINS

A. PURIFICATION OF NORMAL AND CROSSLINKED HEMOGLOBINS

Hemoglobin A was isolated from the minor hemoglobins and other red cell components by ion exchange chromatography, using Sephadex A-50 gel. The resolution of each component was achieved by a linear gradient from pH 8.5 to 7.2. Figure 4 shows the chromatogram for the purification of Hb A from a hemolysate obtained from packed red blood cells. The resultant chromatogram shows four peaks. After purification the protein fractions under each peak were pooled and clinical gel electrophoresis was performed to identify the proteins and check for contaminants. The protein under peak II was analyzed to contain Hb A$_2$; peak III, Hb A; and peak IV, Hb A$_{IC}$ or glycosylated Hb A. Since the net charge on the Hb A$_2$ molecule is more positive (+4) than that on the Hb A molecule, it is expected to elute before Hb A. In Hb A$_{IC}$
Figure 4:

The chromatogram showing the elution of Hb A from Sephadex A-50 gel. Peak II is Hb A2; peak III, Hb A; and peak IV, Hb A1c. The pH gradient profile is plotted on top of the chromatogram.
the amino termini of the $\beta$ chains reacted with glucose in the red cell, and as a consequent two positive charges at the amino termini were deleted from the hemoglobin molecule. Therefore, the Hb $A_{1c}$ would be more negatively charged and retained on the A-50 gel longer and eluted later than Hb A. The break on peak IV was due to upsetting the pH gradient upon addition of buffer after the original gradient buffer ran out. A small peak (peak I) prior to the elution of Hb $A_2$ was probably due to other red cell proteins, most likely glyceraldehyde-3-phosphate dehydrogenase.

The purified Hb A represented in peak III of Figure 4 was used to crosslink with bis(3,5-dibromosalicyl) fumarate in either the oxy or deoxy state and was resolved on Sephadex A-50 gel. The purification of crosslinked hemoglobins was carried out by the same chromatographic method described above. Figure 5 shows a chromatogram for the elution of the $\beta 82XL\text{Hb A}$ sample. The crosslinking was not quantitative and a small percentage of Hb A remained uncrosslinked. Also, a small percent of side reactions may have occurred. The resultant elution shows two major peaks. After crosslinking between two lysines the positive charge on the hemoglobin molecule would have been lowered by two, and hence, it was expected to elute after uncrosslinked Hb A. The protein under peak I was analyzed by clinical gel electrophoresis to be Hb A and peak II,
Figure 5:

The chromatogram for the elution of $\beta$82XLHb A from sephadex A-50 gel. Peak I is uncrosslinked Hb A and peak II, $\beta$82XLHb A.
The small amount of the unknown crosslinked product obtained was apparent on the shoulder of peak II. No further characterization of this modified protein was carried out.

Figure 6 shows the chromatogram for the elution profile of an α99XLHb A sample. This chromatogram shows three clear peaks. Again, the crosslinking between two lysyl residues would have deleted two positive charges, and thus, the α99XLHb A was expected to elute after Hb A under these conditions. The protein in peak I was Hb A; peak II, α99XLHb A; and peak III, an unknown crosslinked hemoglobin, denoted XLHb A. The yield of this second crosslinked product was higher than in the β crosslinking reaction, and it may contain a mixture of the β or α crosslinked hemoglobin with additional reacted sites. Amino acid analysis of the globin chains of this second unknown product (Fig. 6, peak III) suggested that at least one product was a β-β crosslinked hemoglobin.

Tables 7 and 8 show the percent of crosslinking of hemoglobin with bis(3,5-dibromosalicyl) fumarate in the oxy and deoxy conditions respectively. The percent of β crosslinked hemoglobin obtained at 1:1:1 molar ratio of reagent to hemoglobin was higher than the α crosslinked hemoglobin. In the oxy state the crosslinking ranged from 70-90%; in the deoxy state it was also in this range, but the yield of α99XLHb A was lower due to the formation of
Figure 6:

The chromatogram for the elution of α99XLHb A from Sephadex A-50 gel. Peak I is uncrosslinked Hb A; peak II, α99XLHb A; and peak III, an unknown crosslinked hemoglobin, denoted XLHb A.
Table 7:

Percent yield of β82XLHb A and other products in the crosslinking reaction of oxy hemoglobin with bis(3,5-dibromosalicyl) fumarate.

<table>
<thead>
<tr>
<th>No.</th>
<th>[Hb]$^a$</th>
<th>[BBSF]$^a$</th>
<th>% β82XLHb A</th>
<th>% Hb A</th>
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<td>1.</td>
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$^a$ [Hb] and [BBSF] are in mM.
Table 8:

Percent yield of \( \alpha 99XLHb \) A and other products in the crosslinking reaction of deoxy hemoglobin with bis(3,5-dibromosalicyl) fumarate.

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<th>% ( \alpha 99XLHb )</th>
<th>% XLHb</th>
<th>% Hb A</th>
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\( ^{a} \) [Hb] and [BBSF] are in mM.
the unknown crosslinked species in this reaction. The \(\alpha_99\text{XLHb A}\) obtained ranged from 30-50\% plus the additional unknown crosslinked protein which ranged from 20-50\%.

B. **PURIFICATION OF \(\alpha\) AND \(\beta\) GLOBIN CHAINS AND AMINO ACID ANALYSIS**

The globin chains of the second crosslinked hemoglobin from the \(\alpha\) crosslinking reaction shown in Figure 6, peak III, were abstracted free of hemes, purified by carboxymethyl cellulose (CM52) chromatography in urea and hydrolyzed to amino acids. The number of amino acids were compared to those known ones in the \(\alpha\) and \(\beta\) chains to identify whether the crosslink span was between the \(\alpha\) or \(\beta\) chains. Figure 7 shows the elution profile for the non-crosslinked \(\alpha\) and \(\beta\) globins by CM52 gel. The elution resulted in two peaks of relatively equal amount (peak A 56\%, peak B 44\%) as expected for the \(\alpha\) and \(\beta\) chains. Under these chromatographic conditions, the more negatively charged protein would be eluted first, and thus, peak A would be the \(\beta\) chain (Fig. 23, lane 3), and peak B the \(\alpha\) chain (Fig. 23, lane 8).

Figure 8(A), (B) and (C) show the CM52 chromatograms for the elution of globin chains from \(\alpha_99\text{XLHb A}, \beta_82\text{XLHb A}\) and \(\text{XLHb A}\), respectively. In Figure 8(A), the protein under peak C and D were analyzed by SDS (Fig. 22, lanes 1,
Figure 7:

The chromatogram for the elution of \( \alpha \) and \( \beta \) globins from CM52 gel. Peak A is the \( \beta \) globin and peak B, \( \alpha \) globin.
Figure 8:

(A): The chromatograms for the purification of globin chains from α99XLHb A. Peak C is α-α crosslinked globins and peak D, β globin.

(B): The chromatogram for the purification of globin chains from β82XLHb A. Peak E is β-β crosslinked globins and peak F, α globin.

(C): The chromatogram for the purification of globin chains from the second unknown crosslinked hemoglobin from peak III of figure 6. Peak G represents crosslinked globins and peak H, uncrosslinked one.
2 and 3) and shown to contain dimers and monomers respectively. Hence, the globin chains in peak C would be the \( \alpha - \alpha \) crosslinked globins, and those under peak D, \( \beta' \)'s. In Figure 8(B), the globin in peak E was analyzed to be dimers which would be the crosslinked \( \beta - \beta \) chains (Fig. 22, lane 4) and thus, peak F, the \( \alpha \) chains (Fig. 22, lane 6). In Figure 8(C), the protein under peak G contained dimers (Fig. 23, lanes 4 and 5) and peak H, monomers (Fig. 23, lanes 6 and 7). These conclusions were based on the SDS gel electrophoresis results of the globin chains eluted under each peak of the chromatogram. The elution time of the different globins in these chromatograms could not be compared since the columns were repacked and buffers were remade. These changes may cause differences in the column bed volume and slight deviation in the salt gradient. After the globin chains were hydrolyzed to amino acids, their amount were quantitated. Figures 9(A), (B), and (C) show the chromatograms for the elution of the individual amino acids. Figure 9(A) was a standard run containing 17 amino acids, excluding tryptophan, asparagine, and glutamine. Figures 9(B) and (C) show the elution profile for the amino acids obtained from the purified \( \alpha \) and \( \beta \) chains, respectively (Fig. 7, peak B and A). These chromatograms show the presence of 15 amino acids in the hydrolysate. The four amino acids missing were Ile, Trp, Gln, and Asn. Neither of the \( \alpha \) nor \( \beta \) globin contains Ile.
Figure 9:

(A): The chromatogram showing the elution of standard amino acids at 0.25 µM each.

(B): The chromatogram showing the elution of amino acids from the α globin.

(B): The chromatogram showing the elution of amino acids from the β globin.
The Trp was destroyed in the acid hydrolysis and would not be present in the hydrolysate. The Gln and Asn residues were oxidized to glutamate and aspartate, respectively and would be eluted under the known glutamate and aspartate peaks. Figures 10(A) and (B) show the chromatograms for the elution of the amino acid hydrolysate obtained from peaks G and H, respectively. The number of amino acids were consistent with those in Figure 9. Table 9 shows the printout results of the retention time, the relative area and the total amount of the amino acids corresponding to the chromatograms on Figures 9 and 10. The number of amino acid residues found experimentally were compared to the theoretical number. Table 10 shows the data obtained for the amino acid residues from the α and β chains compared to the amino acid residues obtained from the globins of the unknown crosslinked Hb A. The columns for peaks H and G correspond to the number of amino acid residues found from the globin chains eluted in the chromatogram of figure 8(C), peaks H and G. The number of amino acid residues obtained under peak G (Fig. 8(C) and Fig. 10(A)) matched closely with the experimental and theoretical residues of the β chain, and those obtained under peak H (Fig. 8(C) and Fig. 10(B)) matched with the number of residues from the experimental and theoretical values of the α chain. Based on these findings the globins under peak G must be β-β crosslinked.
Figure 10:

(A): The chromatogram showing the elution of amino acids from the globins under peak G of figure 8(C).

(B): The chromatogram showing the elution of amino acids from the globins under peak H of figure 8(C).
Table 9:

Amino acid composition as determined by the Beckman Microcolumn Amino Acid Analyzer Model 121M. The amount is in µM. Runs 35 and 36 correspond to α and β chains respectively; runs 73 and 74 correspond to peak G and H of figure 8(C).

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Comparison of the number of amino acid residues found from peaks G and H of figure 8(C) to the theoretical and experimental residues of the $\alpha$ and $\beta$ chains. Large differences in the number of amino acids between the $\alpha$ and $\beta$ chains are underlined.

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<tr>
<td>Lys</td>
<td>11</td>
<td>11</td>
<td>11.0</td>
<td>11.1</td>
<td>10.8</td>
<td>14.7</td>
<td>14.4</td>
</tr>
<tr>
<td>Arg</td>
<td>3</td>
<td>3</td>
<td>3.0</td>
<td>3.1</td>
<td>3.0</td>
<td>2.9</td>
<td>3.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} From theoretical values.
\textsuperscript{*} From experimental values.
\textsuperscript{a} Underline indicates large differences in amino acid between $\alpha$ and $\beta$ chains.
C. CROSSLINKING OF HEMOGLOBIN A WITH 
DIMETHYLPIIMELIMIDATE, TRIS(β-CHLOROETHYL) 
AMINE AND BIS(β-CHLOROETHYL) METHYLAMINE

The hemoglobins crosslinked with dimethylpimelimidate, tris(β-chloroethyl) amine, and bis(β-chloroethyl) methylamine were not isolated by ion exchange chromatography due to the heterogeneous nature of the products as indicated by the alkaline clinical gel electrophoresis. Such a separation would not have yielded a single crosslinked species. Nevertheless, the SDS gel electrophoresis indicated several species of molecular weight ranging from monomers to polymers.

D. CLINICAL ALKALINE AGAROSE GEL 
ELECTROPHORESIS RESULTS

The clinical alkaline agarose gel electrophoresis was used to identify the different proteins and check for homogeneity of the proteins. In this gel system the proteins were native, and those proteins which possessed higher negative charge would be moving faster toward the anode. Since the net surface charge on the Hb A molecule is more negative than that of Hb A₂, Hb A would be expected to move faster anodically. Figure 11 shows the clinical gel of Hb A and Hb A₂. The pools of hemoglobin samples
Figure 11:

The clinical alkaline agarose gel electrophoresis of Hb A and Hb A₂. Lane 1 is Hb A and lane 2, Hb A₂ from previous preparation. Lanes 3 is Hb A (Fig. 4, peak III), and lane 4, Hb A₂ (Fig. 4, peak II).
under each peak (Fig. 4, peaks II and III) corresponding to Hb A2 and Hb A were shown to be homogeneous based on this condition.

Figure 12(A) shows the clinical gel of uncrosslinked and crosslinked Hb A prior to purification. Figure 12(B) shows the clinical gel electrophoresis scans for the α crosslinked hemoglobin sample. The α crosslinked hemoglobin samples on the clinical gel showed at least three major bands which were well separated from each other; one corresponded to the uncrosslinked hemoglobin, and the other two were modified proteins which had higher electrophoretic mobility than uncrosslinked Hb A. This property demonstrates that the crosslinked proteins could be purified free of contamination from each other. Of the two modified hemoglobins, one had slightly higher electrophoretic mobility than the other, and were less intense as judged by this gel. The gel scans (Fig. 12(B), scans 1 and 2) show a broad shoulder indicating the presence of this second modified protein.

Figure 13 shows the clinical gel for the purified α99XLHb A sample from Figure 6. After purification by ion exchange chromatography the crosslinked hemoglobin could be resolved to a single band. Both the α99XLHb A and β82XLHb A have the same electrophoretic mobility (Fig. 13, lanes 2, 3 and 5). Figures 14(A) and (B) show the clinical gel for the β82XLHb A sample and its gel scans, respectively. The
Clinical gel electrophoresis of fumarate crosslinked hemoglobins prior to purification. Lane 1 is α99XLHb A sample at higher hemoglobin concentration and at pH 7.0 during reaction (Fig. 6); lane 2, α99XLHb A sample at lower concentration and at pH 8.4 during reaction; lane 3, control Hb A; lanes 4 and 5 are repeats of lanes 1 and 2; lane 6, purified β82XLHb A; lane 7, control Hb A₂.

Gel scans of the clinical gel of fumarate crosslinked hemoglobins in figure 12(A). The scan numbers correspond to the lane numbers in figure 12(A). The crosslinked hemoglobins have higher electrophoretic mobility toward the cathode since two positive charges from two lysines were deleted due to covalent crosslinking to the fumarate group.
Fig. 12(A)
Fig. 12(8)

Distance (cm)

1

2

3

4

5

6

α99XLHb A sample

Hb A

82XLHb A
Figure 13:

Clinical gel electrophoresis of purified fumarate crosslinked hemoglobins. Lane 1 is Hb A (Fig. 6, peak I); lane 2, purified α99XLHb A (Fig. 6, peak II); lane 3, purified β82XLHb A; lane 4, the unknown crosslinked hemoglobin (XLHb A) (Fig. 6, peak III); lane 5, purified α99XLHb A; lane 6, Hb A; lane 7, control Hb A; and lane 8, control Hb A2.
Figure 14:

(A): Clinical gel electrophoresis of $\beta$82XLHb A sample. Lanes 1 and 2 are unpurified $\beta$82XLHb A; lane 3, purified $\beta$82XLHb A; lane 4 and 5, control Hb A and Hb A2, respectively; lane 6, co-mixture of Hb A and unpurified $\beta$82XLHb A; and lane 7, co-mixture of Hb A and purified $\beta$82XLHb A.

(B): Gel scans of the clinical gel of figure 14(A) showing the relative position of the $\beta$82XLHb A to Hb A. The scan numbers correspond to the lane numbers in figure 14(A). The $\beta$82XLHb A moves faster toward the cathode than Hb A.
Fig. 14(B)

β82XLHb A sample

β82XLHb A sample

β82XLHb A

Distance (cm)
The crosslinked hemoglobin samples show two major bands which corresponded to the uncrosslinked Hb A and the \( \beta_{\text{82XLHb A}} \) as shown on the clinical gel of Figure 14(A) lanes 1, 2, 6 and 7, and scans 1 and 2 of Figure 14(B). The \( \beta_{\text{82XLHb A}} \) could be purified from contamination of Hb A to homogeneity by ion exchange chromatography as shown in figure 5 (peak II) and lane 3 of figure 14(A).

Figures 15(A) and (B) show the clinical gel for hemoglobin treated with dimethylpimelimidate (DMP) and its gel scans, respectively. Figures 16(A) and (B) show the clinical gel and its scans, respectively, for \( \alpha_{\text{99XLHb A}} \) treated with DMP. Figures 17(A) and (B) show the clinical gel and its scans for the nitrogen mustards treated hemoglobins.

The crosslinking of hemoglobins using the imidoester and the nitrogen mustards caused diffused bands for the crosslinked hemoglobin on the clinical gel. The amount of modification is dependent on the crosslink concentration. When the same imidoester crosslink between two lysines the net charge remains the same, but if one end is hydrolyzed then a positive charge would have been added since the imidate nitrogen is positively charged under these conditions. Thus, the microheterogeneity was caused by the reaction being nonselective and reacted at only one functional end of the reagent. Hence, depending upon the extent of modification on the hemoglobin molecule, those
Clinical gel electrophoresis of Hb A-DMP. Lane 1, control Hb A; lane 2, control β82XLHb A; lane 3, Hb A-DMP; and lane 4, β82XLHb A-DMP.

Gel scans of the clinical gel in figure 15(A). The scan numbers correspond to the lane numbers of figure 15(A). The DMP treated hemoglobins indicate heterogeneous modified species in both Hb A-DMP and β82XLHb A-DMP. In the β82XLHb A-DMP more negatively charged species were obtained. The broaden shoulders on both sides of scans 3 and 4 indicate both negatively and positively charged species were formed. A small amount of modified species move cathodically.
Fig. 15(A)
(A): Clinical gel electrophoresis of DMP treated $\alpha_{99XLHb}$ A sample. Lane 1 is control Hb A; lane 2 and 4, $\alpha_{99XLHb}$ A-DMP; and lane 3, control $\beta_{82XLHb}$ A.

(B): Gel scans of the clinical gel in figure 16(A). The scan numbers correspond to the lane numbers of figure 16(A). The DMP treated $\alpha_{99XLHb}$ A indicates heterogeneous modified species with different electrophoretic mobility were formed. The broaden shoulder on both sides of the peak (scans 2 and 4) indicates that both negatively and positively charged species were obtained.
Fig. 16(A)
Fig. 16(B)
**Figure 17:**

(A) Clinical gel electrophoresis of TCEA and BCEA treated hemoglobins. Lanes 1 and 2 are Hb A-TCEA; lane 3, Hb A-BCEA; lane 4, control Hb A; lane 5, control α82XLHb A; and lane 6, control α99XLHb A. The Hb A-TCEA and Hb A-BCEA appear as diffused bands on the gel due to heterogeneous modified species. However, the Hb A-BCEA is less diffused.

(B) Gel scans of the nitrogen mustards treated hemoglobins from figure 17(A). The scan numbers correspond to the lane numbers of figure 17(A). The amount of positively charged species obtained is predominated in Hb A-TCEA.
Fig. 17(B)

Distance (cm)
derivatives that are highly modified due to higher reagent concentration will have lower electrophoretic mobility and move slower than others which have fewer modifications. This agrees with the results from side reaction of the imidoester with ammonia, glycine and trizma buffer (48). In some reactions, a small amount of the highly DMP modified species moved cathodically instead. Based on the net charge of the modified protein DMP reacted with Hb A, α99XLHb A and β82XLHb A, to give heterogeneous species (Fig. 15 and 16). In the α99XLHb A-DMP and β82XLHb A-DMP at least one modified protein which had the same net charge as Hb A was predominant (Fig. 15(B), scan 4; Fig. 16(B), scans 2 and 4).

The nitrogen mustard derivatives also caused diffuse bands for the crosslinked hemoglobins on the clinical gel. This was due to the same reasons as for the imidoester crosslinked hemoglobins. The nitrogen mustards reacted with hemoglobin randomly to yield heterogeneous species. Some of these modified species having large number of positively charge groups on them due to the positive charge of the nitrogen, moved cathodically. The hemoglobin crosslinked with BCEA diffused less than the hemoglobin crosslinked with TCEA (Fig. 17(A) lanes 1, 2, and 3). The pK\textsubscript{a}'s of diethylamine and triethylamine are 10.99 and 10.76, respectively (117). Under the conditions of the clinical gel used (pH 8.6) both of these reagents would
bear positive charges at the nitrogen. By analogy to the derivatives of these compounds, BCEA and TCEA which are tertiary amine, would argue strongly in favor of a positive charge at the nitrogen of these reagents. The TCEA appears to be more reactive than BCEA, and hence the BCEA reaction yielded less crosslinked species.

In the Hb A-TCEA reaction at least three predominant modified hemoglobin species were obtained based on the net charge of the proteins. Some crosslinked species yielded in this reaction were more positively charged than Hb A, and a small fraction was more negatively charged (Fig. 17(B), scans 1 and 2). The SDS results indicated clearly two crosslinked species having molecular weight corresponding to a dimer (Mr 32,000) and a trimer (Mr 48,000) species in addition to some tetrameric and polymeric species (Fig. 19(A) and (B), lanes or scans 2, 3, and 4).

In the Hb A-BCEA reaction only dimeric crosslinked species were obtained as judged by SDS results (Fig. 19(A), lane 2). The gel scan shows one predominant peak corresponding to that of Hb A, and a broaden shoulder at the base of the peak indicating the diffused species shown on the clinical gel (Fig. 17(A), lane 3). This demonstrated that the crosslinked species were present in less amount compared to uncrosslinked species.
Figure 18 shows the SDS gel results of purified crosslinked hemoglobins. From these results all the crosslinked proteins show two bands of equal intensity corresponding to molecular weight of a monomer ($M_r 16,000$) and a dimer ($M_r 32,000$). This demonstrated that the crosslinking span was between two subunits, and that the crosslinking was selective at specific sites. The Hb A shows only one band corresponding to a monomer.

Figures 19(A), (B), (C) and (D) show the SDS results for the hemoglobin crosslinked with DMP and nitrogen mustard derivatives. The DMP reactions with both uncrosslinked and crosslinked hemoglobins at 500:1 molar ratio of reagent to hemoglobin yielded crosslinked species which have molecular weight corresponding to dimers ($M_r 32,000$), trimers ($M_r 48,000$), tetramers ($M_r 64,000$) and polymers ($M_r > 64,000$) as shown on lane 8 (Fig. 19(A)) or scan 8 (Fig. 19(B)) and lanes 1 and 7 (Fig. 21). The reaction of DMP with hemoglobin resulted in crosslinked species which did not concentrate as sharp bands on the SDS gel but were diffused compared to other known crosslinked species ran on the same gel. The crosslinked species were predominant compared to the monomers present. The low amount of monomers, as shown by the SDS gels, would be in agreement with the large percent of crosslinked species.
Figure 18:
SDS gel electrophoresis of fumarate crosslinked hemoglobins. Lanes 1, 2, 9 and 10 are repeats of α99XLHb A; lane 3, unknown crosslinked hemoglobin (XLHb A) (Fig. 6, peak III); lanes 4 and 8, control Hb A; lanes 5 and 6, β82XLHb A; and lane 7, marker proteins. The marker proteins used are: bovine serum albumin (Mr 66K), egg albumin (Mr 45K), glyceraldehyde-3-phosphate dehydrogenase (Mr 36K), carbonic anhydrase (Mr 29K), trypsinogen (Mr 24K), trypsin inhibitor (Mr 20.1K) and α-lactalbumin (Mr 14.2K). The direction of movement is from top to bottom.
SDS gel electrophoresis of DMP and nitrogen mustards treated hemoglobins. Lane 1 is Hb A; lane 2, Hb A-BCEA; lane 3, Hb A-TCEA from reaction I; lane 4, Hb A-TCEA from reaction II; lanes 5 and 10, marker proteins; lane 6, catalase; lane 7, mixture of CAT-DMP and Hb A-DMP (200:4:1); lane 8, α99XLHb A-DMP (500:1); and lane 9, Hb A-DMP (50:1). The direction of movement is from top to bottom.

Estimation of the molecular weight of crosslinked species in Hb A-TCEA by the plot of Log(MW) versus relative mobility. This estimation was from the large SDS gel. T represents trimer; D, dimer; and M, monomer.

Estimation of the crosslinked species in DMP and nitrogen mustards treated hemoglobins. The majority of crosslinked species are dimeric, trimeric and tetrameric as indicated by X's.
Fig. 19(A)
Fig. 19(C)

\[ Y = -0.897973903442 X + 5.03452869203 \]

- I: 46.847 g/mol
- D: 30.162 g/mol
- M: 16.400 g/mol
- HbA: 16.130 g/mol

RELATIVE MOBILITY
obtained. The gel scan resolution showed that the crosslinked species in relative percent were 4.3% monomers, 19.7% dimers, 30.3% trimers, and 45.7% both tetramers and polymers for α99XLHb A-DMP. The percent of each species obtained in β82XLHb A-DMP are 5.2%, monomers; 14.7%, dimers; 27.2%, trimers; and 52.9%, tetramers.

The percent of trimeric and tetrameric species obtained in the Hb A-DMP (500:1) compared to those of α99XLHb A or β82XLHb A (500:1) were lower, but the dimer was higher. The crosslinked species in the Hb A-DMP (500:1) had the same molecular weights as those in the α99XLHb A-DMP and β82XLHb A-DMP (500:1). The percent of species in Hb A-DMP obtained from SDS gels were 6.7% monomers, 40.9% dimers, 27.0% trimers and 25.4% tetramers.

The DMP reaction with Hb A at 50:1 yielded predominantly dimers, some trimers, and a small fraction of tetramers. The corresponding percent of each species as judged by SDS results were 22.4% monomers, 55.1% dimers, 18.4% trimers and 4.1% tetramers.

In Figure 19(C), the estimation of the molecular weight of the Hb A-TCEA crosslinked species was done on a larger SDS slab gel. The plot of log(MW) versus relative mobility showed that the crosslinked species obtained in this crosslinking reaction were largely dimers and trimers; a small percent of polymers were obtained as well. The corresponding percentage of these species as determined by
the SDS gel scan were 25.6% monomers, 46.5% dimers, 18.5% trimers and 9.3% tetramers (Fig. 19(A) lanes 3 and 4; Fig. 19(B) scans 3 and 4). For the Hb A treated with BCEA the SDS gel scan showed 43.9% dimers and 54.8% monomers (Fig. 19(A) lane 2; Fig. 19(B) scan 2).

In Figure 19(D), the estimation of the molecular weight of species crosslinked by DMP, TCEA and BCEA show that the crosslinked species fall in three regions: dimer, trimer and tetramer. But there are more scattering points around the region of tetramer due to polymeric species. Table 11 shows the percent of uncrosslinked and crosslinked hemoglobins treated with DMP, TCEA, and BCEA as obtained from the SDS gel scan results. Table 12 shows the difference in $T_m$'s with respect to the number of crosslinks per tetramer or species. Figure 20 shows the average minimum number of crosslinks per tetramer or species with respect to $\Delta T_m$. The number of crosslinks per species is reflected in the increase in $\Delta T_m$.

Figure 21 shows the SDS results for the reaction of DMP with Hb A and CAT. This was an attempt to try to crosslink the Hb A to CAT with DMP, to form hemoglobin-catalase complex (CAT-Hb A-DMP). The DMP was able to crosslink both the CAT and Hb A separately, but did not bridge the hemoglobin molecule to the catalase molecule. The reaction with a molar ratio of 2000:4:1 of reagent to Hb A to CAT yielded species with molecular weight similar
### Table 11:

Percent of uncroslinked and crosslinked hemoglobins reacted with DMP, TCEA, and BCEA as determined by SDS gel scans.

<table>
<thead>
<tr>
<th>Hb</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Trimer</th>
<th>Tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A-BCEA</td>
<td>54.8</td>
<td>45.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb A-TCEA I</td>
<td>25.6</td>
<td>46.5</td>
<td>18.5</td>
<td>9.3</td>
</tr>
<tr>
<td>Hb A-TCEA II</td>
<td>31.0</td>
<td>48.3</td>
<td>17.2</td>
<td>3.4</td>
</tr>
<tr>
<td>Hb A-DMP (50:1)</td>
<td>22.4</td>
<td>55.1</td>
<td>18.4</td>
<td>4.1</td>
</tr>
<tr>
<td>Hb A-DMP (500:1)</td>
<td>6.7</td>
<td>40.9</td>
<td>27.0</td>
<td>25.4</td>
</tr>
<tr>
<td>α99XLHb A</td>
<td>45.5</td>
<td>54.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β82XLHb A</td>
<td>35.4</td>
<td>64.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XLHb A</td>
<td>49.1</td>
<td>50.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α99XLHb A-DMP (500:1)</td>
<td>8.3</td>
<td>18.8</td>
<td>29.2</td>
<td>43.7</td>
</tr>
<tr>
<td>β82XLHb A-DMP (500:1)</td>
<td>5.2</td>
<td>14.7</td>
<td>27.2</td>
<td>52.9</td>
</tr>
<tr>
<td>Minimum Number of Crosslinks per Species</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Minimum Number of Crosslinks per Tetramer</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 12:
The minimum average number of crosslinks per species or tetramer for crosslinked hemoglobins.

<table>
<thead>
<tr>
<th>Hemoglobins</th>
<th>$\Delta T_m$</th>
<th>Crosslinks per Species</th>
<th>Crosslinks per Tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A-BCEA</td>
<td>-0.1</td>
<td>0.45</td>
<td>0.90</td>
</tr>
<tr>
<td>Hb A-TCEA</td>
<td>8.2</td>
<td>0.93</td>
<td>1.41</td>
</tr>
<tr>
<td>Hb A-DMP (500:1)</td>
<td>15.1</td>
<td>1.71</td>
<td>2.12</td>
</tr>
<tr>
<td>$\alpha_{99XLHb}$ A-DMP (500:1)</td>
<td>17.0</td>
<td>2.08</td>
<td>2.27</td>
</tr>
<tr>
<td>$\beta_{82XLHb}$ A-DMP (500:1)</td>
<td>19.4</td>
<td>2.28</td>
<td>2.42</td>
</tr>
</tbody>
</table>
Figure 20:

The average number of crosslinks per species (O) and per tetramer (x) with respect to $\Delta T_m$. From lower to higher $\Delta T_m$ the points represent consecutively Hb A-BCEA, Hb A-TCEA, Hb A-DMP (500:1), $\alpha_{99\text{XL}}$Hb A-DMP (500:1) and $\beta_{82\text{XL}}$Hb A-DMP (500:1).
\[ Y = 10.0132461296X + 2.9997367331 \]
Figure 21:

SDS gel electrophoresis of DMP treated hemoglobin and CAT-Hb A-DMP complex. Lane 1 is Hb A-DMP (500:1); lane 2, CAT-Hb A-DMP complex (2000:4:1); lane 3, CAT-DMP (2000:1); lane 4, Hb A-DMP (50:1); lane 5, CAT-Hb A-DMP (200:4:1); lane 6, CAT-DMP (200:1); lane 7, α99XLHb A-DMP (500:1); lane 8 and 10, marker proteins; and lane 9, Hb A.
to that in the Hb A-DMP 500:1, except there was one additional band at high molecular weight (Mr 116,000). This high molecular weight species was due to the crosslinked dimer of CAT, and the catalase monomer band was buried with the tetramer band of the Hb A-DMP (Fig. 21, lanes 1 and 2). The reaction with the molar ratio of 200:4:1 yielded four bands (lane 5), but two of the bands with molecular weight of 58,000 daltons and 116,000 daltons corresponded to the monomer and dimer of CAT (Fig. 21, lanes 3 and 6); and the other two bands at molecular weight of 32,000 daltons and 48,000 daltons corresponded to the dimer and trimer of Hb A (Fig. 21, lane 4). If Hb A had been crosslinked to CAT at least a species of molecular weight 74,000 daltons would have been observed. Thus, the DMP was unable to bridge the Hb A and the CAT to form a hemoglobin-catalase complex at both of these conditions.

Figures 22 shows the SDS results of globin chains obtained by cation exchange chromatography from Figures 8(A) and (B). The globin chains in lanes 1, and 2 indicated that only dimer species were present under peak C (Fig. 8(A)). Since these globins were from α99XLHb A, the globin chains under peak C were the α-α crosslinked globins, and those under peak D (Fig. 8(A)) were the uncrosslinked β chains as indicated by the monomer species. The globin chains on lanes 4 and 6 were from the β82XLHb A. On lane 4 the globins present were dimers, and lane 6 were
**Figure 22:**

SDS gel electrophoresis of globin chains purified from CM52 gel. Lane 1 and 2 are repeat of globin chains under peak C of figure 8(A) (α-α crosslinked globins); lane 3, globin chains under peak D of figure 8(A) (β globin); lane 4, globin chains from peak E of figure 8(B) (β-β crosslinked globins); lane 5, marker proteins; and lane 6, globin chains from peak F of figure 8(B) (α globin).
monomers. This demonstrated that the globins in peak E (Fig. 8(B)) were the β-β crosslinked globins, and lane 6 (Fig. 8(B), peak F) contained the uncrosslinked α globins.

Figures 23 shows the SDS results of globin chains from Hb A (Fig. 7) and the unknown crosslinked hemoglobin (Fig. 6, peak III). Lanes 1, 3, and 8 all indicated that monomers were present, which should be the case since these lanes contained uncrosslinked globin chains. Lanes 4 and 5 contained only dimers (Fig. 8(C), peak G) and lanes 6 and 7 contained only monomers (Fig. 8(C), peak H). The globins in peak G were identified to be β-β's and peak H, α's.

III. THERMAL STABILITY OF HEMOGLOBINS

A. THERMAL STABILITY OF α99XLHb A VERSUS β82XLHb A AND NORMAL Hb A

The thermal stability of hemoglobins crosslinked between the α chains was determined and compared with that of the β crosslinked and uncrosslinked hemoglobins. Figure 24 shows the plot of absorbance versus wavelength as temperature was raised for a sample of Hb A. The temperature effects a decrease on the Soret absorbance of the hemoglobin as the temperature was raised. However, the chromophore at the near ultraviolet region was increased as the temperature was raised. The denaturation of hemoglobin is not reversible. After the denaturation experiment the
Figure 23:

SDS gel electrophoresis of globin chains from Hb A and the unknown crosslinked hemoglobin (Fig. 6, peak III). Lane 1 is Hb A (α and β chains); lane 2, marker proteins; lane 3, globin chains under peak A (β globin) (Fig. 7); lanes 4 and 5, repeat of globin chains under peak G of figure 8(C) (crosslinked globins); lanes 6 and 7, repeat of globins under peak H of figure 8(C) (uncrosslinked globins); and lane 8, globin chain under peak B (α globin) (Fig. 7).
Figure 24:

The effect of temperature on the absorbance of hemoglobin plotted as absorbance with respect to wavelength. From top to bottom at 410 nm, the solid line (_____ ) represents the absorbance at 25°C, 31°C, 39°C, 48°C, 56°C, 65°C and 74°C. The (— • —) is the return spectrum after cooling to room temperature (22°C).
The sample was cooled back to room temperature and the original spectrum was not recovered fully, however, the return spectrum was recovered nearly 50% as shown by the dash-dot line. Figure 25 shows the denaturation transition monitored by using the absorbance at 406 nm with respect to temperature. The temperature transition on the Soret band is broad and begins at approximately 30-50°C until it reaches a plateau.

Figure 26 shows the first derivative spectra of the absorbance at 406 nm with respect to temperature for Hb A, α99XLHb A, β82XLHb A, and the unknown XLHb A. The single crosslink on the hemoglobin molecule either between the Lys 82β₁ and Lys 82β₂ or Lys 99α₁ and Lys 99α₂ causes a higher denatured transition in the diaspirin cross linked hemoglobins than in the uncrosslinked Hb A. The temperature transition spans from 46-66°C. The minimum of the α99XLHb A is at 56.7°C, those of the β82XLHb A and XLHb A are 56.6°C and 57.6°C respectively, compared to 41°C of Hb A; within the limit of error all of these crosslinked proteins have the same thermal stability.

B. THERMAL STABILITY OF SINGLE CROSSLINKED HEMOGLOBINS WITH DIMETHYLPIMELIMIDATE, TRIS(β-CHLOROETHYL) AMINE AND BIS(β-CHLOROETHYL) METHYLAMINE.

The imidoester and nitrogen mustard derivatives
Figure 25:

The plot of absorbance versus temperature showing the denaturation transition at 406 nm of hemoglobin.
Figure 26:
The first derivative of absorbance with respect to temperature at 406 nm for Hb A (---), α99XLHb A (....), β82XLHb A (--- · --- · ---), and XLHb A (--- ---). The $T_m$ is the temperature at the minimum. The α99XLHb A, β82XLHb A and XLHb A have the same stability.
reacted with hemoglobins randomly and did not produce a single crosslinked protein as judged by clinical and SDS gels. The thermal stability of these modified proteins thus represents an average value of the crosslinked species in the mixture. At the high end temperature the hemoglobin treated with DMP formed turbidity in the solution during thermal denaturation and thus caused light scattering. This problem was corrected by subtracting the absorbance of a wavelength which did not change over the entire denaturation time span of a sample in which turbidity was not observed. During the denaturation span turbidity was not observed in the Hb A sample, and the optical density at 522 nm did not change. Thus, the absorbance of 522 nm was subtracted from the absorbance of a wavelength under examination at each temperature of the same sample. Figure 27 shows the effect of temperature on the absorbance at 406 nm, 522 nm and the application of the subtraction method. There was very little or essentially no effect on the absorbance at 522 nm as the temperature was raised (Fig. 27, dotted line). The solid line is the non-subtractive plot; the dash-dot-dash line, closely tracing the non-subtractive curve, is a demonstration of the subtraction from the absorbance at 522 nm, and the dotted line is the absorbance tracing at 522 nm without any subtraction. In this case there was not any precipitation in the sample, so using the subtractive and non-subtractive method yielded
The effect of subtraction in a non-precipitated sample at 406 nm (___) and 522 nm (....) of Hb A. The subtracted absorbance of a non-precipitated Hb A sample at 406 nm (— • — • —). For a non-precipitated sample the non-subtracted or subtracted curve has the same transition.
the same temperature transition.

Figure 28 shows the application of the subtraction method for an α99XLHb A-DMP sample at 406 nm and 522 nm. The solid line is the non-subtractive plot; the dash-dot-dash line is the subtractive plot, and the dotted line is the absorbance tracing at 522 nm. It was assumed that the temperature effect on the optical density at 522 nm of a crosslinked sample did not change as in the case of Hb A, and that the changes observed in the absorbance at 522 nm of the DMP treated samples were caused by light scattering due to precipitation of the samples. This assumption means that the absorbance at 522 nm with respect to temperature must be a straight line; and it is so if any changes in absorbance are subtracted from themselves. The effect of light scattering on the absorbance at 522 nm and the absorbance of a wavelength under examination is assumed to be the same. Since the temperature at the initial increase in absorbance at 522 nm corresponds to the same temperature as the break due to precipitation of the non-subtractive plot, any increase in absorbance beyond this point was due to precipitation. All the non-precipitating samples did not show any increase in absorbance at 522 nm. This subtraction corrects the base line of all spectra to be the same. The extrapolation to correct the base line by plotting absorbance versus $\lambda^4$ and the subtraction using 630 nm yielded the same Tm's. Therefore, this assumption
Comparison of the subtracted and non-subtracted transition curve at 406 nm for α99XLHb A-DMP sample. The absorbance at 522 nm (....) is subtracted from that at 406 nm (____). The (____ . ____ . ____) is the result of the subtracted curve at 406 nm. All the DMP treated hemoglobin samples are analyzed by this method.
should be correct. All the hemoglobins modified with DMP were analyzed by the subtractive method.

Figure 29 shows the effect of stabilization by single crosslinking on Hb A plotted as the fraction denatured \( f_D \) with respect to temperature at 406 nm. The transition for hemoglobin treated with BCEA, TCEA and DMP is much broader than the ones observed for Hb A and the diaspirin crosslinked Hb A. The transition of Hb A-BCEA covered from 32-56°C, that of Hb A-TCEA, 32-60°C, and 38-66°C for Hb A-DMP. All the modified hemoglobins have a temperature transition higher than that of Hb A, except for the hemoglobin crosslinked with BCEA. The Hb A-BCEA (10:1) has a transition equal to that of Hb A. The hemoglobin crosslinked with diaspirin produced the most stable single crosslink proteins. These are the \( \alpha 99XLHb \) A and \( \beta 82XLHb \) A; their \( T_m \)'s are 57°C. The next most stable hemoglobin derivative is Hb A-DMP at 500:1 molar ratio of reagent to hemoglobin; its \( T_m \) is 56.1°C. The Hb A-DMP at 1000:1 molar ratio has comparable \( T_m \) (57°C) to those of the diaspirin crosslinked hemoglobins (48). The Hb A-TCEA at 10:1 molar ratio yielded an intermediate temperature transition; its \( T_m \) is 49.3°C (48).

C. THERMAL STABILITY OF DOUBLE CROSSLINKED HEMOGLOBINS.

The \( \alpha 99XLHb \) A and \( \beta 82XLHb \) A were double crosslinked
The effect of stabilization on the singly crosslinked hemoglobins plotted as $f_D$ with respect to temperature for Hb A (---), Hb A-BCEA (....), Hb A-TCEA (---), Hb A-DMP (500:1) (---), $\alpha_{99XL}$Hb A (---), and $\beta_{82XL}$Hb A (---).
with DMP at a molar ratio of 500:1 pH 9.5 in order to produce crosslinked proteins with higher molecular weight. Figure 30 shows the effect of double crosslinking on the thermal stability of the α and β crosslinked hemoglobins analyzed by the first derivative of the absorbance with respect to temperature. The diaspirin double crosslinked hemoglobins have slightly higher Tm's than the singly crosslinked proteins. The increase in Tm of the double crosslinked hemoglobins is approximately 3°C compared to that of the singly crosslinked hemoglobins. The β82XLHb A-DMP has slightly higher stability than the α99XLHb A-DMP, but within the limit of error they both have the same stability. In comparison to Hb A the Tm of the double crosslinked proteins has increased by 18°C.

Table 13 shows the values of Tm's for all the crosslinked hemoglobins determined from the Soret band at 406 nm, and 418 nm; and the aromatic chromophore at 280 nm. Table 14 shows the change in Tm's of crosslinked hemoglobins relative to uncrosslinked Hb A. The average Tm's of Hb A was taken to be 43°C for the data determined at 280 nm, and 41°C for those determined from 406 nm and 418 nm.
The effect of stabilization on the double crosslinked hemoglobins plotted as the first derivative with respect to temperature for Hb A (___), α99XLHb A (___ __ ___ ___), α99XLHb A-DMP (___ ___), β82XLHb A (___ __ ___), β82XLHb A-DMP (___ . ___ . ___).
Table 13:
The $T_m$ values of modified hemoglobins at 280 nm, 406 nm, and 418 nm as determined by the fraction denatured with respect to temperature.

<table>
<thead>
<tr>
<th>Hb</th>
<th>280 nm</th>
<th>406 nm</th>
<th>418 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>42.5 ± 1.9</td>
<td>40.9 ± 0.75</td>
<td>40.8 ± 1.5</td>
</tr>
<tr>
<td>Hb A-BCEA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41.7 ± 2.0</td>
<td>41.3 ± 1.1</td>
<td>40.8 ± 1.8</td>
</tr>
<tr>
<td>Hb A-TCEA I&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.5 ± 0.82</td>
<td>49.3 ± 1.2</td>
<td>50.8 ± 0.96</td>
</tr>
<tr>
<td>Hb A-TCEA II&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.1 ± 1.3</td>
<td>45.2 ± 1.6</td>
<td>46.5 ± 1.3</td>
</tr>
<tr>
<td>Hb A-DMP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>58.5 ± 0.71</td>
<td>56.1 ± 1.5</td>
<td>56.2 ± 1.9</td>
</tr>
<tr>
<td>α99XLHb A</td>
<td>57.9 ± 1.1</td>
<td>56.7 ± 1.1</td>
<td>56.5 ± 1.2</td>
</tr>
<tr>
<td>β82XLHb A</td>
<td>57.9 ± 1.8</td>
<td>56.6 ± 0.37</td>
<td>56.2 ± 0.24</td>
</tr>
<tr>
<td>XLHb A</td>
<td>59.0 ± 0.21</td>
<td>57.6 ± 0.32</td>
<td>57.3 ± 0.33</td>
</tr>
<tr>
<td>α99XLHb A-DMP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.5 ± 1.4</td>
<td>58.7 ± 0.36</td>
<td>59.4 ± 0.57</td>
</tr>
<tr>
<td>β82XLHb A-DMP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>61.1 ± 3.9</td>
<td>60.4 ± 1.7</td>
<td>61.4 ± 1.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> The molar ratio of reagent to hemoglobin used in the reactions were 10:1 for BCEA, 11:1 for TCEA I, and 40:1 for TCEA II. For the TCEA II the reagent was dissolved in the buffer before mixing with hemoglobin.

<sup>b</sup> The DMP molar ratio to hemoglobin was 500:1.
Table 14:

The $\Delta T_m$ values showing the effect of stabilization due to single and double crosslinking relative to the $T_m$ of Hb A. For Hb A, at 280 nm the average $T_m$ is 43°C and at 406 nm, 41°C.

<table>
<thead>
<tr>
<th>Hb</th>
<th>280 nm</th>
<th>406 nm</th>
<th>418 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A-BCEA</td>
<td>-2.0 ± 1.5</td>
<td>-0.1 ± 1.6</td>
<td>0.3 ± 0.88</td>
</tr>
<tr>
<td>Hb A-TCEA I</td>
<td>7.5 ± 0.82</td>
<td>8.2 ± 1.2</td>
<td>11.0 ± 1.4</td>
</tr>
<tr>
<td>Hb A-TCEA II</td>
<td>5.8 ± 1.5</td>
<td>4.2 ± 1.6</td>
<td>6.4 ± 1.4</td>
</tr>
<tr>
<td>Hb A-DMP</td>
<td>15.5 ± 0.71</td>
<td>15.1 ± 1.5</td>
<td>15.2 ± 1.9</td>
</tr>
<tr>
<td>$\alpha 99X$Hb A</td>
<td>14.9 ± 1.2</td>
<td>15.4 ± 0.78</td>
<td>15.5 ± 1.3</td>
</tr>
<tr>
<td>$\beta 82X$Hb A</td>
<td>14.9 ± 1.8</td>
<td>15.6 ± 0.37</td>
<td>14.8 ± 0.21</td>
</tr>
<tr>
<td>XHb A</td>
<td>16.0 ± 0.25</td>
<td>16.6 ± 0.37</td>
<td>16.3 ± 0.40</td>
</tr>
<tr>
<td>$\alpha 99X$Hb A-DMP</td>
<td>11.5 ± 1.4</td>
<td>17.0 ± 1.7</td>
<td>17.9 ± 1.3</td>
</tr>
<tr>
<td>$\beta 82X$Hb A-DMP</td>
<td>18.1 ± 3.9</td>
<td>19.4 ± 1.7</td>
<td>20.4 ± 1.5</td>
</tr>
</tbody>
</table>
IV. AUTOXIDATION OF HEMOGLOBINS

A. THE AUTOXIDATION OF Hb A, α99XLHb A AND β82XLHb A

The hemoglobin used in the autoxidation experiments was freshly purified and used within four days. The purification of hemoglobins by the method described elsewhere in this dissertation should have removed all the DPG and other red cell proteins. Figure 31 shows the change of the Hb A spectra between 500-700 nm as a result of autoxidation at 37°C between zero and 107 hours. At 107 hours the absorbance at 540 nm and 576 nm decreased, and that at 630 nm increased (dash-dot-dash curve); this demonstrated that met hemoglobin was formed in the hemoglobin solution, and the oxy hemoglobin concentration was diminished. After addition of sodium cyanide to the solution the result due to oxy and cyanomet hemoglobins present in the mixture is shown by the dash-double-dash curve. The decrease in absorbance at 576 nm compared to the original spectrum at zero hour (solid curve) indicated that little oxy hemoglobin was present and a large amount of it was converted to cyanomethemoglobin. After complete conversion to 100% cyanomet hemoglobin the spectrum was shown by the dash curve.

Figure 32 shows the linearity of the absorbance versus oxy hemoglobin concentration with an optical density below 2.0 for the wavelengths at 540 nm (solid line), 576
Figure 31:
The visible spectral change of hemoglobin during autoxidation between zero and 107 h at 37°C; at zero hour, 100% oxy hemoglobin (______); at 107 h, mixture of met and oxy hemoglobin (____ . ___ . ___); after addition of NaCN (____ - - ____ - - ____); and after complete conversion to 100% cyanomet hemoglobin (___ ___ ___).
Figure 32:

Linearity of absorbance versus hemoglobin concentration at 540 nm (___), 576 nm (____), and 630 nm (___ . ____ . ____). The absorbance has a linear relationship with concentration up to 2.0 unit of absorbance.
nm (dotted line), and 630 nm (dash-dot-dash line). All three wavelengths still have linear relationship with respect to hemoglobin concentration at an optical density below 2.0. The deviation of absorbance from linearity with concentration begins at 2.6 for this spectrophotometer. This result agrees with that obtained by Corso (48), and by using a sample of ferricyanide as well (data not shown).

Figure 33(A) shows the development of met hemoglobin with respect to time for Hb A and diaspisin crosslinked hemoglobins. At 37°C the percent of met hemoglobin in α99XLHb A (open circle curve) at any given time is higher than what was in the β82XLHb A (open triangle curve), or Hb A (solid circle curve). The met hemoglobin developed in β82XLHb A was slightly higher than what was in Hb A. The autoxidation of Hb A at room temperature (22°C, solid triangle curve) is slower than at 37°C (solid circle curve) and at 4°C, in the refrigerator, Hb A essentially had not autooxidized during the period of autoxidation experiment (open square curve). After 130 h the met hemoglobin remains at 5%. Figure 33(B) shows the formation of met hemoglobin during the first 20 hours of autoxidation. The percent of met hemoglobin developed under these conditions is directly proportional to time. Table 15 shows the percent of met hemoglobin with respect to time for Hb A, α99XLHb A and β82XLHb A.

Figure 34(A) and (B) show the rate of autoxidation
Figure 33:

(A): The development of met hemoglobin as a function of time for Hb A at 4°C (□□□□), Hb A at 22°C (▲▲▲), Hb A at 37°C (●●●●), β82XLHb A at 37°C (△△△), and α99XLHb A at 37°C (○○○○).

(B): The autoxidation of hemoglobin within 20 h at 37°C for Hb A (●●●●), β82XLHb A (△△△), and α99XLHb A (○○○○), showing the linear relationship of met hemoglobin with respect to time.
Table 15:

Autoxidation of oxy Hb A and α99XLHb A in 0.01 M MOPS, pH 7.0 at 37°C. The concentration of hemoglobins were 1.95 mM Hb A, 1.90 mM α99XLHb A, and 1.90 mM β82XLHb A per heme.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% met Hb A</th>
<th>% met α99XLHb</th>
<th>% met β82XLHb</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>1.52 ± 0.63</td>
<td>4.50 ± 0.13</td>
<td>1.8 ± 1.05</td>
</tr>
<tr>
<td>1.0</td>
<td>__________</td>
<td>__________</td>
<td>1.56 ± 0.00</td>
</tr>
<tr>
<td>2.5</td>
<td>4.11 ± 3.55</td>
<td>13.55 ± 2.44</td>
<td>4.63 ± 1.20</td>
</tr>
<tr>
<td>5.0</td>
<td>8.68 ± 3.92</td>
<td>21.27 ± 0.88</td>
<td>10.76 ± 1.69</td>
</tr>
<tr>
<td>7.5</td>
<td>12.00 ± 7.23</td>
<td>32.95 ± 2.63</td>
<td>17.78 ± 2.55</td>
</tr>
<tr>
<td>10.0</td>
<td>19.35 ± 5.95</td>
<td>39.07 ± 2.09</td>
<td>24.51 ± 3.03</td>
</tr>
<tr>
<td>15.0</td>
<td>28.73 ± 5.04</td>
<td>47.31 ± 0.06</td>
<td>35.39 ± 2.03</td>
</tr>
<tr>
<td>20.0</td>
<td>37.53 ± 5.23</td>
<td>58.38 ± 2.45</td>
<td>43.37 ± 1.95</td>
</tr>
<tr>
<td>30.0</td>
<td>__________</td>
<td>__________</td>
<td>56.62 ± 2.25</td>
</tr>
<tr>
<td>54.0</td>
<td>__________</td>
<td>__________</td>
<td>71.39 ± 1.77</td>
</tr>
<tr>
<td>59.0</td>
<td>66.47 ± 2.16</td>
<td>86.30 ± 3.01</td>
<td>__________</td>
</tr>
<tr>
<td>63.0</td>
<td>__________</td>
<td>__________</td>
<td>78.20 ± 1.58</td>
</tr>
<tr>
<td>67.5</td>
<td>71.47 ± 2.11</td>
<td>87.81 ± 0.71</td>
<td>__________</td>
</tr>
<tr>
<td>83.0</td>
<td>75.47 ± 3.05</td>
<td>90.96 ± 0.23</td>
<td>__________</td>
</tr>
<tr>
<td>91.5</td>
<td>77.81 ± 1.04</td>
<td>90.38 ± 0.88</td>
<td>__________</td>
</tr>
<tr>
<td>93.5</td>
<td>__________</td>
<td>__________</td>
<td>85.01 ± 1.44</td>
</tr>
<tr>
<td>116.5</td>
<td>__________</td>
<td>__________</td>
<td>85.98 ± 1.39</td>
</tr>
<tr>
<td>127.0</td>
<td>80.28 ± 3.23</td>
<td>92.98 ± 0.01</td>
<td>__________</td>
</tr>
<tr>
<td>132.0</td>
<td>83.44 ± 1.32</td>
<td>94.12 ± 0.12</td>
<td>__________</td>
</tr>
<tr>
<td>141.5</td>
<td>__________</td>
<td>__________</td>
<td>85.98 ± 1.39</td>
</tr>
</tbody>
</table>
Figure 34:

(A): The rate of autoxidation of hemoglobin plotted as $-\log \left( \frac{(A_0 - A)}{(A_0 - A_w)} \right)$ with respect to 130 h at 37°C for Hb A (--), β82XLHb A (--), and α99XLHb A (--).

(B): The autoxidation of hemoglobin in the first phase (20 h) at 37°C for Hb A (--), β82XLHb A (--), and α99XLHb A (--).
within 130 and 20 hours respectively for Hb A and diaspirin crosslinked hemoglobins plotted as the \(-\log[(A_0-A)/(A_0-A_\infty)]\) with respect to time. The autoxidation of hemoglobin is complex and does not have a simple linear relationship with respect to time; it appears to be bi-phasic, indicating a multistep autoxidation mechanism. Nevertheless, the first phase below 20 hours appeared to have a linear relationship with respect to time. Thus, an apparent rate constant \(k_{app}\) could be estimated for the autoxidation of hemoglobin within this period. Since the autoxidation of hemoglobin is slow, the apparent rate constant based on a linear relationship within 20 hours would be reasonable despite the multiple mechanistic steps. As expected from the measurement of the percent of met hemoglobin with respect to time, the rate of autoxidation of \(\alpha99\text{XLHb A}\) (open circle curve) is faster than either one of the \(\beta82\text{XLHb A}\) (open triangle curve) or Hb A (solid circle curve). Table 16 shows the \(k_{app}\) values and the percent inhibition due to the addition of SOD and catalase. The \(k_{app}\) of Hb A is \(2.20\times10^{-2}\) hr\(^{-1}\); and those of \(\alpha99\text{XLHb A}\) and \(\beta82\text{XLHb A}\) are \(3.93\times10^{-2}\) hr\(^{-1}\) and \(2.69\times10^{-2}\) hr\(^{-1}\), respectively (Table 16). The \(\alpha99\text{XLHb A}\) autoxidizes faster than Hb A by a factor of 1.8. The \(\beta82\text{XLHb A}\) has an intermediate rate between Hb A and \(\alpha99\text{XLHb A}\). Of the two crosslinked hemoglobins, the \(\alpha99\text{XLHb A}\) autoxidizes 1.5 fold faster than \(\beta82\text{XLHb A}\).
Table 16:

Autioxidation of hemoglobins in the presence or absence of CAT and SOD. The concentrations of CAT and SOD were 5.77 μM tetramers and 23.1 μM dimers, respectively.

<table>
<thead>
<tr>
<th>Hemoglobins plus enzymes</th>
<th>k_100 (hr⁻¹)</th>
<th>k_100 (hr⁻¹)</th>
<th>% In</th>
<th>% In per μM enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb A</td>
<td>2.20±0.300</td>
<td>-1.55±2.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hb A+CAT</td>
<td>0.785±0.500</td>
<td>-2.60±3.33</td>
<td>64</td>
<td>11.1</td>
</tr>
<tr>
<td>Hb A+SOD</td>
<td>1.16±0.400</td>
<td>0.122±0.257</td>
<td>47</td>
<td>2.0</td>
</tr>
<tr>
<td>Hb A+CAT+SOD</td>
<td>0.399±0.160</td>
<td>0.924±1.70</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>β82XLHb A</td>
<td>2.69±0.150</td>
<td>-2.92±2.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β82XLHb A+CAT</td>
<td>1.38±0.240</td>
<td>0.498±3.57</td>
<td>49</td>
<td>8.5</td>
</tr>
<tr>
<td>β82XLHb A+SOD</td>
<td>1.52±0.140</td>
<td>0.420±2.08</td>
<td>43</td>
<td>1.9</td>
</tr>
<tr>
<td>β82XLHb A+CAT+SOD</td>
<td>0.670±0.040</td>
<td>-3.20±1.43</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>α99XLHb A</td>
<td>3.93±0.680</td>
<td>11.35±21.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α99XLHb A+CAT</td>
<td>1.72±1.44</td>
<td>18.57±13.82</td>
<td>56</td>
<td>9.7</td>
</tr>
<tr>
<td>α99XLHb A+SOD</td>
<td>2.44±0.266</td>
<td>11.10±15.06</td>
<td>38</td>
<td>1.6</td>
</tr>
<tr>
<td>α99XLHb A+CAT+SOD</td>
<td>1.29±0.799</td>
<td>6.68±11.45</td>
<td>67</td>
<td></td>
</tr>
</tbody>
</table>

% In - total percent of enzyme inhibition.
% In per μM enzyme - total percent of enzyme inhibition per μM of each enzyme.
B. THE EFFECT OF CATALASE AND SUPEROXIDE DISMUTASE ON THE RATE OF AUTOXIDATION OF Hb A, α99XLHb A AND β82XLHb A

The autoxidation of hemoglobin was known to generate superoxide radical and subsequently reducing it to hydrogen peroxide (29). The $\cdot O_2^-$ and $O_2^{-2}$ are reactive and can cause severe damage to tissues by oxidizing the lipids on the membrane (116). The presence of $\cdot O_2^-$ and $O_2^{-2}$ would accelerate the rate of autoxidation of hemoglobin by oxidizing the Fe(II). Two of the enzymes which are able to catalyze the redox reactions of $\cdot O_2^-$ and $O_2^{-2}$ are superoxide dismutase and catalase, respectively; thus, reducing the damage caused by these species. These enzymes were used to reduce the autoxidation of hemoglobin. Figure 35 shows the effect of catalase, superoxide dismutase and both on the autoxidation rate of Hb A. Both enzymes are able to reduce the rate of autoxidation by 11.1%/µM of enzyme by CAT and 2.0%/µM by SOD (Table 16).

The effect of inhibition on autoxidation by both enzymes together is stronger than just one acting independently. The two enzymes together are capable of inhibiting the autoxidation of Hb A up to 82% (Table 16). Tables 17, 18 and 19 show the autoxidation of Hb A in the presence of SOD, CAT and both. The results of these experiments were normalized to 2% met hemoglobin initially.
Figure 35:
The effect of CAT and SOD or both on the autoxidation of Hb A at 37°C: control Hb A (● ●), Hb A plus SOD (△ △), Hb A plus CAT (○ ○), and Hb A plus SOD and CAT (▲ ▲). The concentrations are: Hemoglobin, 0.48 mM tetramers; CAT, 5.77 µM tetramers; and SOD, 23.1 µM dimers.
Table 17:
The autoxidation of Hb A in the presence of CAT, SOD and both, measured as percent of methemoglobin. The concentration of Hb A was 2.22 mM heme, of CAT, 5.77 µM tetramers, and of SOD, 23.1 µM dimers. Experiment was done on 3-14-89.

<table>
<thead>
<tr>
<th>Time</th>
<th>None</th>
<th>CAT</th>
<th>SOD</th>
<th>SOD+CAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>1.0</td>
<td>5.92</td>
<td>0.00</td>
<td>3.55</td>
<td>3.33</td>
</tr>
<tr>
<td>2.5</td>
<td>8.47</td>
<td>7.18</td>
<td>5.62</td>
<td>7.19</td>
</tr>
<tr>
<td>5.0</td>
<td>16.09</td>
<td>15.30</td>
<td>9.91</td>
<td>8.39</td>
</tr>
<tr>
<td>15.0</td>
<td>38.77</td>
<td>26.19</td>
<td>23.70</td>
<td>11.40</td>
</tr>
<tr>
<td>25.0</td>
<td>48.65</td>
<td>34.36</td>
<td>36.91</td>
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</tr>
<tr>
<td>38.5</td>
<td>61.90</td>
<td>32.65</td>
<td>48.90</td>
<td>33.45</td>
</tr>
<tr>
<td>48.5</td>
<td>70.17</td>
<td>46.13</td>
<td>68.77</td>
<td>39.25</td>
</tr>
<tr>
<td>62.0</td>
<td>77.60</td>
<td>54.35</td>
<td>80.44</td>
<td>49.43</td>
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Table 18:

The autoxidation of Hb A in the presence of CAT, SOD and both, measured as percent methemoglobin. The concentration of Hb A was 2.22 mM heme, of CAT, 5.77 µM tetramers, and of SOD, 23.1 µM dimers. Experiment was done on 3-23-89.

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Table 19:
The autoxidation of Hb A in the presence of SOD, CAT and both, measured as percent methemoglobin. The concentration of Hb A was 2.20 mM heme, of CAT, 5.77 µM tetramers, and of SOD, 23.1 µM dimers. Experiment was carried out on 5-19-89.

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* The experiment with SOD or both with SOD and CAT were not carried out.
Under the conditions used, SOD and CAT were also able to slow the rate of autoxidation for the crosslinked hemoglobins as well. The reduction in the rate of autoxidation by SOD, CAT and both appear to be lower than that for uncrosslinked Hb A. However, the pattern of inhibition by SOD, CAT and both on the crosslinked hemoglobins appear to be the same as that in the uncrosslinked Hb A. Figure 36 shows the inhibition of SOD, CAT and both on the autoxidation of β82XLHb A. The inhibition are 1.9%/µM by SOD, and 8.5%/µM by CAT; both enzymes together inhibited the autoxidation up to 75% (Table 16). Tables 20 and 21 show the autoxidation of β82XLHb A in the presence of SOD, CAT and both, measured as percent met hemoglobin. Figure 37 shows the inhibition of SOD, CAT and both on the autoxidation of α99XLHb A. The inhibition on the autoxidation of α99XLHb A by SOD and CAT are 1.6%/µM, and 9.7%/µM, respectively (Table 16). The inhibition by both enzymes is 67% (Table 16). Tables 22, 23 and 24 show the percent of met hemoglobin developed during the autoxidation of α99XLHb A in the presence of SOD, CAT and both. Table 25 shows the relative rate constants of autoxidation of Hb A either in the absence or presence of SOD and CAT or both to the corresponding crosslinked hemoglobin samples in the absence or presence of the enzymes.

By assuming a first order rate for autoxidation, a
The effect of CAT and SOD or both on the autoxidation of β82XLHb A at 37°C; control β82XLHb A (●●), β82XLHb A plus SOD (ΔΔ), β82XLHb A plus CAT (OO), and β82XLHb A plus SOD and CAT (▲▲). The concentration are: Hemoglobin, 0.48 mM tetramers; CAT, 5.77 µM tetramers; and SOD, 23.1 µM dimers.
The autoxidation of β82XLHb A in the presence of SOD, CAT and both, measured as percent methemoglobin. The concentration of β82XLHb A was 2.22 mM heme, of CAT, 5.77 µM tetramers, and of SOD, 23.1 µM dimers. Experiment was carried out on 3-14-89.

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Table 21:

The autoxidation of β82XLHb A in the presence of SOD, CAT and both, measured as percent methemoglobin. The concentration of β82XLHb A was 2.20 mM heme, of CAT, 5.77 µM tetramers, and of SOD, 23.1 µM dimers. Experiment was carried out on 6-3-89.

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Figure 37:
The effect of CAT and SOD or both on the autoxidation of α99XLHb A at 37°C; control α99XLHb A (●●), α99XLHb A plus SOD (△△), α99XLHb A plus CAT (○○), and α99XLHb A plus SOD and CAT (▲▲). The concentration are: Hemoglobin, 0.48 mM tetramers; CAT, 5.77 μM tetramers; and SOD, 23.1 μM dimers.
Table 22:

The autoxidation of α99XLHb A in the presence of SOD, CAT and both, measured as percent methemoglobin. The concentration of α99XLHb A was 2.22 mM heme, of CAT, 5.77 µM tetramers, and of SOD, 23.1 µM dimers. Experiment was carried out on 3-23-89.

<table>
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* The experiment with SOD and both SOD and CAT were not carried out.
Table 23:

The autoxidation of α99XLHb A in the presence of SOD, CAT and both, measured as percent methemoglobin. The concentration of α99XLHb A was 2.20 mM heme, of CAT, 5.77 mM tetramers, and of SOD, 23.1 μM dimers. Experiment was carried out on 5-19-89.

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</table>

* The experiment with SOD or both SOD and CAT were not carried out.
Table 24:
The autoxidation of α99XLHb A in the presence of SOD, CAT and both, measured as percent of methemoglobin. The concentration of α99XLHb A was 2.20 mM heme, of CAT, 5.77 µM tetramers, and of SOD, 23.1 µM dimers. Experiment was done on 6-3-89.

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### Table 25:

Comparison of relative rate\(^a\) in the presence or absence of SOD, CAT or both of Hb A to α99XLHb A and β82XLHb A assuming first order rate of autoxidation.

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<th>CAT+SOD</th>
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<td>1.0</td>
<td>1.0</td>
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<td>1.8±0.3</td>
<td>1.3±0.1</td>
<td>1.7±0.1</td>
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<tr>
<td>α99XLHb A</td>
<td>1.8±0.3</td>
<td>2.2±2.1</td>
<td>2.1±0.2</td>
<td>3.2±2.0</td>
</tr>
</tbody>
</table>

\(^a\) The relative rate is calculated by taking the ratio of \(k_{\text{app}}\) of crosslinked hemoglobins to \(k_{\text{app}}\) of Hb A.
reasonable estimate of the half-life ($t_{1/2}$) of oxyhemoglobin could be determined. Table 26 shows the $t_{1/2}$ of oxyhemoglobins based on the assumption that autoxidation follows first order kinetics. The $t_{1/2}$ of α99XLHb A (17.6 h) was shorter than either one of Hb A (31.5 h) or β82XLHb A (25.8 h). The effects on the $t_{1/2}$ of Hb A by CAT, SOD and both are 2.2, 2.0 and 6.0 (Table 27) fold increases, respectively. The increase in $t_{1/2}$ of β82XLHb A in the presence of SOD and CAT or both are 2.0, 1.8 and 4.0 fold, respectively (Table 27). For α99XLHb A the $t_{1/2}$ increase by 1.9, 1.6 and 4.5 (Table 27) fold due to the presence of SOD, CAT, and both enzymes, respectively. Table 27 shows the fold increase due to the presence of enzymes relative to hemoglobin samples without any enzymes. This indicated that CAT is slightly more effective in inhibiting the autoxidation of hemoglobin than SOD. Both enzymes, CAT and SOD, can inhibit the autoxidation by approximately 4 fold.
Table 26:
Comparison of average $t_{1/2}$ (h) values for autoxidation of hemoglobins in the presence of CAT and SOD, assuming first order rate of autoxidation.

<table>
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<th>CAT+SOD</th>
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</thead>
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<td>88.3</td>
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<td>173.7</td>
</tr>
<tr>
<td>$\beta 82X$Hb A</td>
<td>25.8</td>
<td>50.2</td>
<td>45.5</td>
<td>103.4</td>
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<tr>
<td>$\alpha 99X$Hb A</td>
<td>17.6</td>
<td>40.3</td>
<td>28.4</td>
<td>53.7</td>
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</tbody>
</table>
Table 27:

Comparison of the average fold changes due to the presence of SOD, CAT and both to those samples without any enzymes.

<table>
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</thead>
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<td>-6.0±2.4*</td>
</tr>
<tr>
<td>β82XLHb A</td>
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<tr>
<td>α99XLHb A</td>
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</table>

* The negative sign indicates the rate is decreasing.
CHAPTER IV

DISCUSSION

The following discussion will explain the significance of this research and provide an explanation as to what these discoveries mean. The goal is to present an interpretation which will explain the data. The results from crosslinking and thermal stability of hemoglobins using four reagents will be discussed. The effect of single and double crosslinking on the thermal stability of hemoglobin will be addressed.

The findings on the autoxidation of uncrosslinked and crosslinked hemoglobins will be examined with mechanisms and their relationship to the three-dimensional structure. The effect of slowing down the autoxidation rate of hemoglobin by superoxide dismutase and catalase will be examined based on the proposed autoxidation mechanisms. Any implications or problems relevant to the making of blood substitute will be suggested. Finally, conclusions will be drawn from these findings and a summary of the discoveries will be attached to emphasize the importance of these findings.

I. SYNTHESIS OF BIS(3,5-DIBROMOSALICYL) FUMARATE

The bis(3,5-dibromosalicyl) fumarate (BBSF) was
synthesized from 3,5-dibromosalicylic acid and fumaryl chloride by a base catalyzed reaction using N,N-dimethyl aniline. Figure 38 shows the reaction mechanism for this reaction.

The bis(3,5-dibromosalicyl) fumarate has a m.p. of 226-228°C which agrees well with what was reported by Walder et al. (30). When the compound was tested with ferric chloride for the presence of phenolic proton the result was negative (yellowish color), indicating that the ester linkage between the fumaryl and the phenol groups has occurred. A color for a positive test of the phenolic proton of 3,5-dibromosalicylic acid is purplish. The singlet resonance at 7.34 ppm is due to the two fumaryl protons. These two protons are trans to each other and are chemically equivalent, and there are no adjacent protons. Hence, the signal is a singlet. The two doublets at 8.08 ppm and 8.09 ppm, and 8.33 ppm and 8.34 ppm are the benzyl protons at position 4 and 6, respectively. These benzyl protons are different in their chemical environment and are not equivalent. Although there are no adjacent protons next to H4 and H6, in high magnetic field the proton on the benzene ring can couple with each other through four bonds. Therefore, a doublet resonance arises for each of these protons. The integration ratio of the resonance at 7.34 ppm to the ones at 8.08 ppm and 8.33 ppm was 2:1:1. This agrees with the ratio of the fumaryl protons to the two
Figure 38:

The reaction mechanism for the synthesis of bis(3,5-dibromosalicyl) fumarate.
benzyl protons of the presumed structure of BBSF. The assignment of the resonance position to each proton was by prediction using the incremental shifts from the literature (100) and the reasoning from the inductive effect of the substituent groups on the benzene ring.

In the presumed structure of bis(3,5-dibromosalicyl) fumarate there are nine carbons. The $^{13}C$ NMR spectrum demonstrates that indeed the compound synthesized contained nine $^{13}C$ signals. The calculated and observed $^{13}C$ chemical shifts of the benzene ring agreed within 3\% of error, except for the carbon at position 4 which had an error of 5.8\%. These errors may have been arisen from the fact that a different solvent was used in measuring the $^{13}C$ spectrum. The $^{13}C$ chemical shifts used for the prediction were taken in CDCl$_3$, and the solvent used in this research was deuterated DMSO. At the C2 position on the benzene ring the OCOR group was assumed to be OCOCH$_3$. This assumption may contribute to the difference in the predicted $^{13}C$ chemical shift for the C2 and the rest as well. The $^{13}C$ of the ester carbon (C8), carboxylate carbon (C7) and fumaryl carbon (C9) are sensitive to change depending on the exact substituent group attached to these carbons and their chemical environment, thus, a prediction of an exact value is not possible. Nevertheless, the $^{13}C$ shift of these carbons fell within the normal range observed. With the structural information from the NMR data coupled with the
physical data, it is without doubt that bis(3,5-dibromosalicyl) fumarate has the presumed structure.

II. PURIFICATION AND CROSSLINKING OF HEMOGLOBINS
A. PURIFICATION OF UNCROSSLINKED HEMOGLOBINS

The hemoglobin used for crosslinking reactions and subsequent experiments was purified to homogeneity as judged by the clinical and SDS gel electrophoresis results. All the hemoglobins eluted with the Sephadex diethylaminoethyl (DEAE) A-50 gel were consistent with their known properties, such as the net surface charge on the protein. In the anion exchange chromatographic system used, the A-50 gel surface is permeated with positive charges, and the proteins were separated as a function of net surface charge. A protein with higher negative charge would be retained on the column longer than one which is less negatively charged. All three hemoglobins, Hb A, Hb A\text{ic}, and Hb A\text{2} have different net charge from each other; consequently, they can be separated by the A-50 gel. The Hb A\text{ic} is a derivative of Hb A in which the amino termini of the $\beta$ subunits had reacted with glucose forming a Schiff base. When the glucose attached to the $\beta$ subunits are oxidized to fructose, the hemoglobin derivative becomes stable (52). This reaction would have deleted two positive charges on the Hb A molecule. As a consequence, the Hb
AIC has different electrophoretic mobility from Hb A. Since two positive charges were deleted, the Hb AIC would have higher net negative charge and be retained on the A-50 gel longer, thus it would be eluted later than Hb A (Fig. 4). Of the three hemoglobins, Hb A has an intermediate charge between Hb A2 and Hb AIC; therefore, it eluted in the middle (Fig. 4). In Hb A2 the δ chain has 10 amino acids different from the β chain of Hb A. As a result of these differences, the net charge on the Hb A2 molecule is more positive than Hb A by +4 (4). Hence Hb A2 would elute from the column before Hb A and Hb AIC.

B. PURIFICATION OF α AND β CROSSLINKED HEMOGLOBINS

The bis(3,5-dibromosalicyl) fumarate was used to crosslink Hb A in the oxy state between Lys 82β1 and Lys 82β2 (29,30) and between Lys 99α1 and Lys 99α2 in the deoxy state (28). The crosslinking reaction could occur in three ways:

1. \[ \text{Lys-NH}_2 + \text{β}_{1(82)} - \text{Lys-NH} - \text{CCH=HCC-HN-Lys-(82)β}_2 \]
2. \[ \text{Lys-NH}_2 + \text{α}_{1(99)} - \text{Lys-NH} - \text{CCH=HCC-HN-Lys-(99)α}_2 \]
3. \[ -\text{Lys-NH} - \text{CCH=HCC-O}^- \]
The reaction pathways 1 and 2 were well established by Walder et al. (29,30). If one end of the reagent reacted with an amino group and the other was hydrolyzed before it had a chance to react, then reaction 3 would have occurred. If only this reaction occurred the hemoglobin molecule when analyzed by the SDS gel would have resulted only in monomer species and with different electrophoretic mobility from Hb A on the clinical electrophoresis gel. This sort of modified species was not observed to occur alone but as extra modification on the already crosslinked hemoglobin.

The crosslinking is highly selective and specific at these lysine residues mentioned; theoretically it should be quantitative. However, in practice a small amount of hemoglobin did not get crosslinked due to hydrolysis of the reagent before it had a chance to react with it. The molar ratio in the crosslinking reaction was 1:1:1 hemoglobin to reagent, regardless of what the concentration of hemoglobin may be (Tables 7 and 8). The efficiency of the β crosslinking was near 90%, but the α crosslinking yielded much lower crosslinked species, 50% (Table 8).

The β crosslinking reaction yielded predominantly one crosslinked species (Fig. 5, peak II). A small percent of side reactions may have occurred as shown by the shoulder on the right side of the chromatogram in Figure 5 peak II. The clinical gel electrophoresis results of the β
crosslinked sample showed that there were three bands; one corresponding to uncrosslinked Hb A, and the other two, crosslinked hemoglobins (Fig. 14(A)). Of the two crosslinked hemoglobins, one band corresponded to the \( \beta 82XLHb \) A, and the other, to an unknown crosslinked hemoglobin. This second crosslinked product may have arisen from extra modification on the \( \beta 82XLHb \) A, since its electrophoretic mobility was slightly higher than that of the \( \beta 82XLHb \) A. The second crosslinked protein was very small compared to what was obtained in the \( \alpha \) crosslinking reaction, and could not be resolved well by ion exchange chromatography. Therefore, this product was not further investigated.

In the \( \alpha \) crosslinking reaction, besides the \( \alpha 99XLHb \) A product, the second unknown crosslinked hemoglobin was much higher than in the \( \beta \) crosslinking reaction. The yield of this product was up to 50% (Table 8). The unknown crosslinked hemoglobin either in the \( \beta \) or \( \alpha \) crosslinking reaction always eluted after the \( \beta 82XLHb \) A or \( \alpha 99XLHb \) A. Since it eluted from the column after the \( \alpha 99XLHb \) A or \( \beta 82XLHb \) A, its net charge must be more negative than that of the known crosslinked protein indicating extra modification on the lysine residues. The extra modification would have deleted the positive charge and made the protein more negatively charged. Hence it would have had a stronger interaction with the positively charged
A-50 gel and eluted after the $\beta$82XLHb A or the $\alpha$99XLHb A. Both $\beta$82XLHb A and $\alpha$99XLHb A eluted from the A-50 gel after Hb A. Thus, it was possible to purify the crosslinked hemoglobins from contamination.

The clinical gel electrophoresis showed that both the $\alpha$99XLHb A and $\beta$82XLHb A have the same electrophoretic mobility, but the unknown crosslinked hemoglobin moved slightly faster than either the $\beta$ or $\alpha$ crosslinked hemoglobins (Fig. 13, lane 4). This agrees with the fact that in either the $\alpha$ or $\beta$ crosslinking reaction two positive charges were deleted by the crosslink, and in the unknown crosslinked hemoglobin at least one more positive charge is deleted by the extra modification. The SDS gel electrophoresis demonstrated that $\beta$82XLHb A, $\alpha$99XLHb A and the unknown crosslinked protein consist of dimers and monomers only in relative amount of 50% each (Fig. 18 and Table 11). If an extra lysine was modified in the unknown crosslinked hemoglobin, the charge on the protein would have decreased by -2 due to the hydrolysis at the ester linkage of the reagent. Based on these results, it is most likely that only one extra modification occurred in the unknown crosslinked hemoglobin, as depicted in the fumarate crosslinking reaction pathway 3 above or a crosslink within a subunit had occurred. The modification could be on the $\alpha$ or $\beta$ subunits.
C. CROSSLINKING OF HEMOGLOBIN WITH DIMETHYLPIIMELIMIDATE

The hemoglobin crosslinked with DMP by the condition described elsewhere in this dissertation demonstrates that hemoglobin crosslinked with these reagents resulted in heterogeneous crosslinked species (Fig. 15(A), lanes 3 and 4). The condition used to analyze the proteins on the clinical gel was non-denaturing, and the proteins were in their native state. They did not move on the gel in discrete bands but were smeared on it. The cause of diffusion of the derivative proteins on the clinical gel may have arisen from two possibilities. Firstly, the DMP may have caused a conformational change in the hemoglobin, thus exposing negatively charged residues. Secondly, the hemoglobin may have been highly and randomly modified, producing derivative hemoglobins which bear different surface charges from that on Hb A. The first possibility would not validate the fact that the visible spectral characteristics of the oxy Hb A-DMP was still the same as the unmodified oxy Hb A. Therefore, if any conformational changes occur at all it is at minimum level. The hemoglobin is not expected to be denatured, since the thermal denaturation still displays a temperature transition which indicates the unfolding of the modified protein. Furthermore, several samples of already denatured protein after re-equilibration and one already denatured in
1 mM SDS were subjected to denaturation again and found to display no temperature transition, except for a slight decrease in absorbance of the denatured protein due to the effect of temperature. Figure 39 demonstrates this observation. The second possibility agrees with the usual observation of the electrophoretic results based on the charge property of the protein.

The imidoester compounds exhibit high specificity for reacting with the $\epsilon$-amino group of lysine. The reaction between an imidate and an amine results in an amidine, which has a $pK_a$ value of approximately 11. Thus, in a protein solution of pH less than 9 the amidine maintains the charge characteristic of a lysine, and hence a charge-induced conformational change of the protein is not expected (45). An analysis to determine the number of reacted lysines per mole of hemoglobin at 1000:1 molar ratio was found to be 11 lysines per one mole of hemoglobin (48).

It is conceivable that at high pH the nitrogen on the DMP could act as a nucleophile, and making the two DMP centers available for reaction with electrophilic carbons. However, the condition used in this research has a pH of 9.5, and therefore, the DMP should exist in the protonated form. Since other nucleophiles besides lysine could have conceivably reacted with the electrophilic carbon of DMP, the reaction is complex and could result in crosslinking
Figure 39:

Demonstration for the absence of denaturation transition in an already denatured Hb A; transition from an undenatured Hb A (___), from a denatured Hb A (.....).
the protein at other amino acid residues in addition to the lysyl residues. Figure 40 shows the general reaction in which any nucleophilic amino acid residues may react with DMP. Therefore, this mechanism does not preclude the reaction of sulfhydryl of cysteine or the carboxyl groups of aspartic and glutamic acid with the DMP. In reaction pathway 1 both ends of the DMP reacted to crosslink the protein, whereas in pathway 2 one end is hydrolyzed before reacting with the nucleophile.

Ferris and Smith (45) had used DMA, a similar imidoester with four carbons and a crosslink span of 8.6 Å, to crosslink hemoglobin and found two crosslinks between Lys 7α1 and Lys 11α2 (9 Å), and Lys 82β1 and Lys 82β2 (6 Å). Also their SDS results showed that there were four species, monomers, dimers, trimers and tetramers, which agree with the present DMP-treated hemoglobin result. Obviously the DMA was able to crosslink the hemoglobin between several subunits to form the above described species. The DMP span is 9.2 Å, and it should have similar ability to crosslink protein the same way as DMA, except for steric reasons due to the length of DMP being longer. Examination of the crystal structure of hemoglobin reveals six possible α-β crosslink candidates with interresidue distance of 11, 15, 18, 20, 21 and 21 Å; seven β-β crosslink candidates with interresidue distance of 6, 9, 12, 15, 18, 18, and 19 Å, and ten α-α possibilities with
Figure 40:

A general reaction for a nucleophile to react with DMP. Pathway 1 demonstrates a crosslinking reaction; pathway 2, a modification reaction in which one ends of the DMP is hydrolyzed. AH₂ represents a nucleophile.
DMP reaction with two nucleophiles:

\[ \text{CH}_3\text{O} - \text{C} - (\text{CH}_2)_5\text{C} - \ddot{\text{O}} - \text{CH}_3 \rightarrow \text{CH}_3\text{O} - \text{C} - (\text{CH}_2)_5\text{C} - \ddot{\text{O}} - \text{CH}_3 \]

\[ \text{NH}_2^+ \quad \text{NH}_2^+ \]

\[ \text{H} - \dddot{\text{A}} - \text{H} \quad \text{H} - \dddot{\text{A}} - \text{H} \]

DMP reaction with one nucleophile:

\[ \text{CH}_3\text{O} - \text{C} - (\text{CH}_2)_5\text{C} - \ddot{\text{O}} - \text{CH}_3 \rightarrow \text{CH}_3\text{O} - \text{C} - (\text{CH}_2)_5\text{C} - \ddot{\text{O}} - \text{CH}_3 \]

\[ \text{NH}_2^+ \quad \text{NH}_2^+ \]

\[ \text{H} - \dddot{\text{A}} - \text{H} \quad \text{H} - \dddot{\text{A}} - \text{H} \]

\[ \text{H} - \dddot{\text{A}} - \text{H} \quad \text{H} - \dddot{\text{A}} - \text{H} \]

\[ \text{2 CH}_3\text{OH} \]

\[ \text{2 CH}_3\text{OH} \]
interresidue distance of 5, 10, 11, 12, 13, 15, 16, 17, 17, and 19 Å (45). Although the interresidue distances of these ε-amino groups are much wider than the DMP span of 9.2 Å, in solution the mobility of the lysines may come close enough for the crosslink to occur.

The SDS gel electrophoresis of the Hb A-DMP indicates that the derivative hemoglobin was crosslinked between subunits to form four bands at the positions expected for monomer, dimer, trimer and tetramer (Fig. 20, lane 1). In addition a small percent of polymeric species were formed as well. Under this condition the hemoglobin concentration was 1 mg/ml (62 µM heme), and intermolecular crosslinking may be minimized. Nevertheless, the observation of polymeric species is a proof that some of the crosslinks are on the surface of the subunits. Lockhart and Smith (46) had used DMA to crosslink hemoglobin to haptoglobin and suggested that the hemoglobin-haptoglobin-DMA complex was formed by the crosslink being on the surface of the hemoglobin molecule.

The tetramer on the SDS gel was visibly composed of two bands. The dimer and trimer regions were diffused, perhaps indicating a variety of crosslinks randomly on the hemoglobin molecule causing a slight difference in molecular weight of the derivative hemoglobins. Thus, a variety of crosslinks could be introduced into the subunits in addition to the intersubunit crosslinks, preventing
complete unfolding of the protein into individual \( \alpha \)- and \( \beta \)-subunits by SDS. The crosslinking between subunits is observable on the SDS gel, however it does not disprove intrasubunit crosslinking. Based on the molecular weight of the marker proteins used the crosslinked species have the following molecular weight: dimer, 27,200 daltons; trimer, 45,400 daltons and tetramers, 62,400 daltons (Fig. 19(D)). The species with molecular weight higher than 64,000 daltons did not form well defined bands on the SDS gel (Fig. 19(A), lane 9; Fig. 21, lane 1) due to the fact that these species are highly crosslinked and have different molecular weights.

In this reaction the amount of crosslinked species is dependent upon the concentration of DMP. In comparison between the crosslinking condition at 50:1 and 500:1 molar ratio, the amount of trimers and tetramers in the 50:1 molar ratio are less than those in the 500:1 (Fig. 19(A), lane 9; Fig. 21, lane 1). The second additional band in the tetramers is not apparent in the 50:1 molar ratio reaction.

Looking at the pairs of lysines suggested by Ferris and Smith (45) as potential candidates for intersubunit crosslinking, it is certainly possible to yield dimeric, trimeric and tetrameric species. Besides, if other nucleophilic residues reacted with the DMP as suggested, then the possibilities of intersubunit crosslinking
increases even higher. At least one can conclude that the reaction of hemoglobin with DMP is random, and heterogeneous crosslinked species could be obtained in the form of dimer, trimer and tetramer.

D. CROSSLINKING REACTION OF HEMOGLOBIN WITH NITROGEN MUSTARDS

The nitrogen mustard derivatives, tris(β-chloroethyl) amine (TCEA) and bis(β-chloroethyl) methylamine (BCEA) were compared in crosslinking hemoglobin. Nitrogen mustard is a good alkylating agent. It was known to react with Hb S, producing modified Hb S with increased solubility (42). Its derivatives, TCEA and BCEA, have been used to crosslink ATPase (43) and hemoglobin (44). The TCEA is a potential tri-functional alkylating agent and the BCEA, bi-functional. The crosslink span of these agents is 5 Å. It is hypothesized that the TCEA can crosslink between three subunits and the BCEA, two subunits. Nitrogen mustard was known to react with anionic sites on proteins (42,75). The typical reactions of nitrogen mustards with nucleophilic residues are shown in Figure 41.

On the clinical electrophoresis gel the nitrogen mustard hemoglobin derivatives, Hb A-TCEA and Hb A-BCEA, appear diffused (Fig. 17(A), lanes 1, 2 and 3). These
Figure 41:

The TCEA and BCEA reactions with nucleophiles. The AH$_2$ represents a nucleophile from the amino acid side chain.
BCEA reaction with nucleophiles:

\[
\begin{align*}
\text{Cl-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-Cl} + \text{H-A-H} & \rightarrow \text{Cl}\text{-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-Cl} + 2 \text{HCl} \\
\text{ClCH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{Cl} + \text{H-A-H} & \rightarrow \text{Cl}\text{-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-Cl} + 3 \text{HCl}
\end{align*}
\]

TCEA reaction with nucleophiles:

\[
\begin{align*}
\text{Cl-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-Cl} + \text{H-A-H} & \rightarrow \text{Cl}\text{-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-Cl} + 2 \text{HCl} \\
\text{ClCH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{Cl} + \text{H-A-H} & \rightarrow \text{Cl}\text{-CH}_2\text{CH}_2\text{-N-CH}_2\text{CH}_2\text{-Cl} + 3 \text{HCl}
\end{align*}
\]
results show that the TCEA reaction produced mostly slower moving species than those obtained in the BCEA reaction. In the TCEA reaction the hemoglobin appeared to be highly modified compared to the BCEA reaction (Fig. 17(A), lanes 1 and 2; Fig. 17(B), scans 1 and 2). It was believed that the nitrogen mustards alkylated the negatively charged carboxyl group of the C-terminal, aspartic acid, glutamic acid and the ring nitrogens of histidines (102) and produced electrophoretic species which would migrate slower toward the anode. Roth et al. (42) had suggested that conformational changes due to alkylation may expose new negative charges at the protein surface and accelerate migration of protein toward the anode. The results with TCEA and BCEA appear to diffuse in both directions. Thus, if conformational changes were responsible for this behavior, then the changes resulted in heterogeneous species. However, the modified hemoglobins still retain the same spectral characteristics. Moreover, and the thermal denaturation of these nitrogen mustard hemoglobin derivatives still exhibits temperature transition; therefore, the proteins were not denatured by these agents (Fig. 39). If the protein had already been unfolded by the presence of the nitrogen mustards, then such a transition would not have been present in the course of denaturation of the proteins. The clinical gel of hemoglobin modified with TCEA and BCEA in this research confirms the
heterogeneous nature of the modified hemoglobins as reported by Roth et al. (42). Nitrogen mustard is also known to undergo strong electrophilic chemical reactions with lysine (42) and cysteine residues (43). It is conceivable that various nucleophilic substances could react with these agents. If the TCEA and BCEA reacted at only one functional group at a lysyl residue, then the crosslinked species would be unaffected by the charge at neutral pH. However, if it is modified at a carboxyl group, then the net positive charge on the crosslinked species would be +1 relative to the unmodified hemoglobin at physiological pH. On the other hand, if the BCEA and TCEA reacted with all their functional groups at the lysyl residues, then the net charge on the protein would be decreased by -1 for BCEA and -2 for TCEA.

The hemoglobin modified with TCEA is more diffused on the clinical gel than the product obtained in the reaction with BCEA at the same molar ratio. Roth et al. (42) observed that at increasing molar ratio both fast and slow moving species were observed and at low molar ratio only the fast moving species predominated.

The clinical gel results demonstrate that the TCEA and BCEA reacted with hemoglobin in a random manner. This created some modified species which are more positively charged than Hb A, as seen by the diffusion of the derivative proteins on the clinical gels toward the cathode
It is difficult to pinpoint and relate the charge property to that of Hb A because the species are highly heterogeneous and discrete bands were not obtained. Therefore, the charge could not be related in magnitude to that of Hb A. This remains to be determined.

The BCEA was used to modify Hb S in order to test for its antisickling effect. It was discovered that at 20:1 molar ratio of BCEA to hemoglobin the resonance at -3.68 ppm vanished completely. The resonance at -3.68 ppm arises from the proton of C2 of \( \beta 2 \) histidine. This was interpreted that the BCEA reacted with \( \beta 2 \) histidines (44). The \( \beta 97, \beta 143 \) and \( \beta 146 \) histidine residues between -3.2 and -3.6 ppm were broaden. These residues are located near the \( \alpha 1/\beta 2 \) subunit contact. The NMR spectrum in this region is used to probe conformational changes of the hemoglobin molecule. Therefore, the NMR results suggested that BCEA either reacted with one of these histidines or all of them, or the changes may come from the induction of conformational changes due to the binding at \( \beta 2 \) histidine. From this investigation the BCEA treatment of Hb S does not alter the heme environment or the subunit interfaces (44), and the oxygen affinity remains the same (42). This conclusion most likely is also true in Hb A.

The crosslinked species obtained from reacting hemoglobin with BCEA were predominately dimers as
determined by SDS gel electrophoresis (Fig. 19(A), lane 2), whereas the TCEA reaction produced some trimers, tetramers and polymers in addition to the dimers (Fig. 19(A), lanes 3 and 4). This can only mean that the protein is crosslinked between two subunits. Therefore, the BCEA must have reacted with other nucleophilic amino acid residues to crosslink the protein besides the histidines. With the SDS results, the modification loci could not be determined, but at least the inference of intersubunit crosslinking could be concluded. The dimeric, trimeric, tetrameric and polymeric species indicated that intersubunit crosslink had occurred. Intermolecular crosslinking is not likely, since the hemoglobin concentration is dilute (0.45 mM tetramer). The crosslinked species observed on the SDS gel were slightly diffused. This diffusion would indicate that within a crosslinked species, either a dimer, trimer or tetramer, several modifications also occurred thereby creating a slight difference in the apparent molecular weight of the crosslinked species (Fig. 19(A) lanes 2, 3 and 4). Based on the molecular weight of the marker proteins used, the dimer has a molecular weight of 30,162 daltons and trimer, 46,847 daltons (Fig. 19(C)). The species with molecular weight matching that of the tetramers and polymers are weak and could not be estimated, but such species in the TCEA reactions are present (Fig. 19(A), lanes 3 and 4; Fig. 19(B), scans 3 and 4). These
higher molecular weight species did not form well defined bands on the SDS gel due to the fact that they are highly crosslinked and have different molecular weights.

In general, the possibilities of crosslinked species formed by reacting hemoglobin with BCEA would yield the same results as the DMP reactions since it is a bi-functional reagent. Since the TCEA is a tri-functional reagent and the BCEA, bi-functional, the TCEA would be more reactive than the BCEA and thus more crosslinked species would be generated with this reagent.

E. DOUBLE CROSSLINKING OF α99XLHb A AND β82XLHb A WITH DIMETHYLPIIMELIMIDATE

The fumarate crosslinked hemoglobins, α99XLHb A and β82XLHb A, were further reacted with DMP to produce what is called "double crosslinked hemoglobin". In the first reaction of hemoglobin with bis(3,5-dibromosalicyl) fumarate, either in the deoxy or oxy state, resulted in crosslinking between the α or β subunits. The reaction of DMP with α99XLHb A and β82XLHb A produced additional crosslinked species, trimers, tetramers, and polymers, which are also observed in the DMP reaction with uncrosslinked hemoglobin (Fig. 19(A) lane 8; Fig. 19(B) scan 8; Fig. 21 lane 7). At the same molar ratio of reagent to hemoglobin (500:1) the amount of dimer in the
double crosslinked hemoglobin, $\alpha_{99XLHb} \text{ A-DMP}$ or $\beta_{82XLHb} \text{ A-DMP}$, is depleted and the amount of trimer and tetramer are increased compared to those in Hb A-DMP (Table 11). This suggested that when a crosslinked hemoglobin was double crosslinked the dimers already present were easily crosslinked within a molecule by the second crosslinking reagent (DMP) to form the higher crosslinked species.

The chance for the intersubunit crosslinking between two molecules is feasible also, but the probability is low since the hemoglobin concentration in the reaction mixture is low (0.015 mM tetramer). The loci of modification have not been determined, and one can only make qualitative assessment whether or not the crosslink is bridged between the subunits. Obviously in both the $\alpha_{99XLHb} \text{ A-DMP}$ and $\beta_{82XLHb} \text{ A-DMP}$, a small amount of monomer (8%) is still present as judged by the SDS gels. Therefore, a small amount of the uncrosslinked subunits, either the $\beta$ in $\alpha_{99XLHb} \text{ A-DMP}$ or $\alpha$ in $\beta_{82XLHb} \text{ A-DMP}$, remained uncrosslinked.

F. PURIFICATION OF $\alpha$ AND $\beta$ GLOBINS
AND AMINO ACID ANALYSIS

The purification of $\alpha$ and $\beta$ globins was carried out with the cation exchange chromatography using the carboxymethyl cellulose (CM52) gel in the presence of urea
(Fig. 7). In the α chain the number of positively charged amino acids are higher than in the β's by +4, so in this chromatographic system the β chain would elute from the column first.

In the β82XLHb A the β-β crosslinked globins were expected to elute from the column before the uncrosslinked α globin. The purified globin under each peak (Fig. 8(B)) was analyzed by the SDS to determine which was the crosslinked globin and which one was uncrosslinked (Fig. 22, lanes 4 and 6). As expected the first peak (peak F) was the crosslinked β-β globins. The globin in the second peak (peak F) contained only monomers, the α globin. Since the content of the amino acids in the β globin already resulted in more negative charge than those in the α globin, the two crosslinked β globins together would be even more negative than the native β globin by -2 and would move even faster than the native β globin through the CM52 gel.

In the α-α crosslinked hemoglobin (Fig. 8(A)), the globin under peak C was determined by SDS to be dimer (Fig. 22, lanes 1 and 2), and the one under peak D, monomer (Fig. 22, lane 3). The α-α crosslinked globins move faster through the CM52 gel than the uncrosslinked β globin. This elution is not consistent with the net charge property of the α-α globins and the uncrosslinked β globin. The native β globin has a net charge of -4 compared to the native α
globin. When the \( \alpha \) globins are crosslinked, a -2 charge was added. The overall net charge of the crosslinked \( \alpha-\alpha \) globins together is -2 compared to -4 of the native \( \beta \) globin. A difference of -2 charge still remains for the native \( \beta \) globin over the crosslinked \( \alpha-\alpha \) globins. Due to other properties not yet understood, the \( \alpha-\alpha \) crosslinked globins elute from the column before the uncrosslinked native \( \beta \) globin.

In the unknown crosslinked hemoglobin (Fig. 8(C)), the globins under peak G were of dimers (Fig. 23, lanes 4 and 5), and those under peak H, monomers as determined by SDS (Fig. 23, lanes 6 and 7). At this point it was not clear whether the crosslinked globins were the \( \alpha' \)s or \( \beta' \)s. The globins under both peaks were hydrolyzed to amino acids, and their amino acids were compared to those of the \( \alpha \) and \( \beta \) globins (Table 10). The amino acid residues were corrected for errors by using the average area from the concentration.

The results of amino acid from the globin under peak H matches those of the \( \alpha \) chain, and those from peak G matches those of the \( \beta \) chain. Therefore, the globins under peak G must be \( \beta-\beta \) crosslinked ones. Chatterjee et al. (28) had suggested that the unknown crosslinked protein under the third peak of the \( \alpha \) crosslinking reaction contained a mixture of crosslinked proteins, and that at least one product was the \( \beta-\beta \) crosslinked hemoglobin.
Based on the electrophoretic mobility of this crosslinked hemoglobin compared to the α or β crosslinked one, it must have an additional modification site on the hemoglobin molecule since it moves faster than both the α and β crosslinked hemoglobins. The locus of modification on the subunit has not been determined. The modification could be on the β-β crosslinked or α uncrosslinked subunit. It is most likely that only one additional modification had occurred.

III. THERMAL STABILITY OF CROSSLINKED HEMOGLOBINS

A. THE EFFECT OF α CROSSLINKING ON

THE THERMAL STABILITY OF Hb A

The thermal stability of met β82XLHb A was investigated by White and Olsen (83). Its Tm was determined to be 57°C. Subsequent denaturation of met β82XLHb S (84) and β82XLHb New York (85) showed that their Tm's are 55°C and 54°C, respectively. The crosslinked hemoglobin, α99XLHb A, has the same thermal stability as the β82XLHb A. Its Tm is 56.7°C (Fig. 26). Crosslinking between two subunits of the same type resulted in the same thermal stability.

The hemoglobin in solution, particularly in dilute solutions, exists in equilibrium between the tetramer and dimer, such as α2β2 = 2αβ. The equilibrium constant for
deoxyhemoglobin is $2.4 \times 10^{-11} \text{ M}^{-1}$ (74). The crosslinking of hemoglobin between the $\beta$ subunits was believed to stabilize the hemoglobin in the tetramer form according to LeChatelier's principle (83, 84). The crosslink prevents the hemoglobin from dissociating to $\alpha\beta$ dimers. The same principle should apply to crosslinking between the two $\alpha$ subunits. Therefore, crosslinking between either the two $\beta$'s or $\alpha$'s prevent dissociation of the hemoglobin to $\alpha\beta$ dimers. This shift in equilibrium favoring the tetramer is very important in thermal stability as well as in clinical aspects.

It was believed that before thermal unfolding of the subunits, they must be dissociated from the tetramer to dimer (84). During the denaturation processes, in the $\beta 82\text{XLHb A}$, when the subunits dissociate to $\alpha\beta$ dimer it is believed that the $\beta$ chains are still hook up together by the crosslink (84). Such a rationale would still be true in the $\alpha 99\text{XLHb A}$. Thus, when the $\alpha 99\text{XLHb A}$ dissociates to $\alpha\beta$ dimer, the $\alpha$ chains are intact by the crosslink. The resistance to dissociation provides an additional stability barrier in the denaturation besides what comes from the crosslink on the globin chains. The most important factor in resisting denaturation, however, is from the crosslink itself holding the subunits together, thus preventing them from unfolding.

The thermal stability of the second crosslinked
hemoglobin (XLHb A) from the α crosslinking reaction, which was determined to be β-β crosslinked with an additional modification site also has the same thermal stability as the α99XLHb A and β82XLHb A. Its $T_m$ is 57.6°C (Fig. 26). Within the limit of errors, the three crosslinked hemoglobins β82XLHb A, α99XLHb A and XLHb A have the same stabilities. The additional modification on the XLHb A did not significantly stabilize or destabilize the crosslinked protein. Since the crosslink is between the β chains, the effect on the equilibrium and thermal denaturation would be practically the same as the β82XLHb A.

In view of the clinical aspect of a blood substitute, the ability of a crosslink to stabilize the hemoglobin in the tetrameric form would eliminate the problems of kidney filtration in which the αβ dimer could easily pass through the glomeruli. When the hemoglobin is in the tetrameric form, its size is large and cannot pass through the glomeruli of the kidneys. Therefore, the stability of the tetramer form would enhance the intravascular life time of the hemoglobin in vivo.
THE EFFECT OF SINGLE CROSSLINKING BY
DIMETHYLPIMELIMIDATE, TRIS(β-CHLOROETHYL) AMINE
AND BIS(β-CHLOROETHYL) METHYLAMINE ON THE THERMAL
STABILITY OF Hb A

The thermal denaturations of hemoglobins crosslinked with DMP, TCEA and BCEA were done according to the same method described elsewhere in this dissertation. In these reactions the crosslinking and modification are non-specific and dependent on the concentration of the reagent and pH. Thus, heterogenous species were produced as judged by the clinical and SDS gels electrophoresis. From these results at least the heterogenous species are dimers, trimers, tetramers, and some polymers (Fig. 19(A), lanes 2, 3, 4, 8 and 9; Fig. 21, lanes 1, 4, and 7).

Since these crosslinked species are heterogeneous, it is difficult to separate them into unique species. So, the thermal transition of these samples represents an average transition of all the modified species present in a sample. In this interpretation the less modified protein would have a low temperature transition similar to that of unmodified Hb A, and the highly modified protein would have an increased transition temperature. The $\Delta T_m$ increases linearly with respect to the number of crosslinks per species (Table 12, Fig. 20). A transition temperature representing an overall average value would appear to be
broad, and this was observed to be so. The hemoglobin sample crosslinked with DMP at 500:1 molar ratio has a T_m of 56.1°C (Fig. 29). The broadness of the transition indicates that some modified species, which are less modified have lower temperature transitions, and some which are highly crosslinked have high temperature transitions. This result indicates that in the Hb A-DMP at least one crosslinked species has nearly the same stability as the fumarate crosslinked hemoglobins.

The hemoglobin crosslinked with TCEA and BCEA at 10:1 molar ratio exhibit lower temperature transitions than the Hb A-DMP. The T_m of Hb A-TCEA and Hb A-BCEA are 49.3°C and 41.3°C, respectively (Fig. 29). The transition temperature of the Hb A-TCEA shifts higher by 8°C than that of Hb A, but the stability of the Hb A-BCEA remains the same as that of Hb A. The nitrogen mustards crosslinked hemoglobins analyzed by SDS showed that there are dimeric, trimeric, tetrameric and polymeric species in Hb A-TCEA, but only the dimeric species predominate in the Hb A-BCEA sample (Fig. 19(A), lanes 2, 3 and 4; Fig. 19(B), scans 2, 3 and 4). The higher T_m in Hb A-TCEA is also reflected in the higher numbers of crosslinks per species compared to Hb A-BCEA (Fig. 20, Table 12).

All the single crosslinked hemoglobins, Hb A-DMP, Hb A-TCEA, α99XLHb A and β82XLHb A are more stable than Hb A, except for Hb A-BCEA which has the same stability as Hb A.
Even though, there are dimeric, trimeric, tetrameric and polymeric crosslinked species present in Hb A-DMP, Hb A-TCEA and Hb A-BCEA, the thermal transition of these crosslinked hemoglobins are not higher than the single fumarate crosslink hemoglobins, α99XLHb A and β82XLHb A. One would expect that the presence of trimers and tetramers due to multiple crosslinks would stabilize the protein more, but such an expectation was not observed. The explanation for this incongruity would mean that not all crosslinks stabilize proteins. Putting several crosslinks on the protein may destabilize its secondary and tertiary structure, leading to a less stable protein even though three or four subunits are linked together. In the hemoglobins crosslinked with DMP, TCEA and BCEA such an interpretation would be reasonable since the temperature transition did not effect a higher T_m for the dimers, trimers and tetramers present compared to the fumarate crosslinked hemoglobins.

The specific single crosslink either between the two α- or β-subunits produced the most stable single crosslinked hemoglobin derivatives. The hemoglobin treated with DMP has a transition temperature comparable to the diaspirin crosslinked hemoglobins depending on the degree of modification. In the reaction at 1000:1 molar ratio pH 9.5 the sample has a T_m (57°C) equal to that of the fumarate crosslinked hemoglobins (48). The BCEA hemoglobin
derivative is less stable than all the crosslinked hemoglobins investigated.

C. THE EFFECT OF DOUBLE CROSSLINKING ON THE THERMAL STABILITY OF HEMOGLOBIN

The thermal denaturations of double crosslinked hemoglobins, α99XLHb A-DMP and β82XLHb A-DMP, were done according to the same method described above. As mentioned before the second DMP reaction generated heterogeneous species which are difficult to purify, thus, the thermal transition of these double cross linked hemoglobins represent an overall average value for the α99XLHb A-DMP and β82XLHb A-DMP. The Tm of α99XLHb A-DMP and β82XLHb A-DMP at 500:1 molar ratio are 58.7°C and 60.4°C respectively, compared to 56.1°C of Hb A-DMP (Table 13; Fig. 30). The β82XLHb A-DMP has slightly higher stability than α99XLHb A-DMP. This may be due to the R state being more favorable for DMP crosslinking than the T state. The number of crosslinks per species (Table 12) is higher in β82XLHb A-DMP, hence, its ΔTm is higher. When Hb A is crosslinked with DMP the first time, the stability is increased by 15°C, but in the α99XLHb A-DMP and β82XLHb A-DMP it increases by only 3-4°C. Therefore, the effect due to DMP crosslinking (ΔTm) is not additive.
IV. AUTOXIDATION OF HEMOGLOBINS

A. THE AUTOXIDATION OF HB A

The autoxidation of HB A was monitored spectrophotometrically in 0.01 M MOPS, pH 7, according to the method of Tomita et al. (99). The autoxidation of hemoglobin appears to be bi-phasic. In the first phase the methemoglobin rises linearly with respect to time until it reaches 20 h (Fig. 33(B)). Beyond 20 h the autoxidation reaches a plateau (Fig. 33(A)). This curve shape indicates that the autoxidation involves multisteps in the autoxidation mechanism. The autoxidation of HB A at room temperature (22°C) and at 4°C was compared to that at 37°C and found to be much slower. At 4°C the autoxidation within 130 h essentially has not changed; the percent met hemoglobin remains at 5%. At room temperature the autoxidation is slightly faster than at 4°C but still is much slower than at 37°C. The autoxidation still maintains a linear curve within 160 h for the experiments done at room temperature and 4°C (Fig. 33(A)).

The plot of the rate of disappearance of oxy hemoglobin with respect to the whole time (130 h) indicates that the rate of autoxidation is non-linear (Fig. 34(A)). Since the first phase of autoxidation is linear, it can be interpreted as pseudo-first order rate of autoxidation dependent upon the concentration of hemoglobin. For this
reaction the rate is given by:

\[-\frac{d[HbO_2]}{dt} = k_{\text{app}} \times [\text{HbO}_2]\]

From this assumption the apparent pseudo-first order rate constant could be determined by plotting \(-\log(A_0-A)/(A_0-A_\infty)\) with respect to time, where the numerator term indicates the concentration of oxyhemoglobin at time \(t\) and the denominator represents the total oxyhemoglobin concentration at the beginning. The \(k_{\text{app}}\) determined for Hb A based on the above equation is 0.022 h\(^{-1}\). This value agrees well with 0.032 h\(^{-1}\) determined by Mansouri and Winterhalter (66) and 0.042 h\(^{-1}\) by Watkins et al. (71).

When adding a crystal of sodium cyanide to the hemoglobin solution the pH may rise a little, but the cyanomethemoglobin spectrum is independent of pH, and such a change in pH was not observed when 1 µl of 0.1 M buffered sodium cyanide was added to the solution. The buffer was not expected to cause the autoxidation of hemoglobin.

The temperature effects an increase in the rate of autoxidation due to the shift in equilibrium toward the release of \(O_2\). Therefore, at 37°C the autoxidation would be faster than at lower temperature. The use of temperature at 37°C is only to facilitate the monitoring of the autoxidation of hemoglobin and to match that of the body temperature (37°C).
The autoxidation of Hb A was investigated by Misra and Fridovich (61) and found to be caused by the self generation of \( O_2^- \) and \( H_2O_2 \). The autoxidation transition in which \( O_2 \) is ligated to the iron atom was believed to be caused by the migration of one electron toward the \( O_2 \), and such species has the superoxo-ferriheme structure, \([Fe^{+3-}_\cdot O_2^-]\). Misra and Fridovich (61) used epinephrine to prove that \( O_2^- \) and \( H_2O_2 \) are generated in the autoxidation of hemoglobin. The \( H_2O_2 \) causes peroxidation of epinephrine, and \( O_2^- \) oxidizes epinephrine to adrenochrome. The oxidation of epinephrine to adrenochrome was inhibited by either SOD or catalase, or both. This proved that the autoxidation of hemoglobin generates superoxide anion and hydrogen peroxide (61). Catalase and SOD do not inhibit epinephrine oxidation completely. The inhibition by catalase is about 70%, and that by SOD is 60%. This further proves the multistep mechanism in which catalase only inhibits the oxidation caused by \( H_2O_2 \), and SOD inhibits the oxidation caused by \( O_2^- \). The oxidation of epinephrine is almost completely inhibited by both enzymes (61).

Satoh and Shikama (77) concluded that the autoxidation of oxy myoglobin was induced by anion, and the mechanism is by a nucleophilic displacement. Such a mechanism is a \( SN_2 \) mechanism, and may be written as:
\[
\text{MbFe(II).O}_2 + \text{L}^- \rightarrow [\text{MbFe(III)}_{\text{L}^- \text{O}_2^-}] \rightarrow \text{MbFe(III).L}^- + \text{O}_2^-
\]

where L\(^-\) represents an anion. The \(\text{H}_2\text{O}\) and \(\text{OH}^-\) can act as anions and react with the iron as described \textit{in vivo} (77). Sugawara and Shikama (67) proposed a similar mechanism in which \(\text{H}_2\text{O}\) reacts directly at the iron, and the distal histidine is involved in hydrogen bonding to the ligated dioxygen.

The effect of pH on the autoxidation of myoglobin and hemoglobin was investigated by a number of investigators (66-70). These investigators found that autoxidation is dependent upon pH, at higher pH the rate of autoxidation decreases and at lower pH it increases. The allosteric anions, 2,3-diphosphoglycerate (DPG) and inositol hexaphosphate (IHP), cause the autoxidation rate to increase (66,70). The increase in oxygen pressure causes a decrease in the autoxidation rate (66,70). Deoxy hemoglobin was found to autoxidize two order of magnitude faster than oxy hemoglobin (68,70).

The mechanisms proposed so far do not explain satisfactorily the fact that SOD and catalase have partial inhibition on the autoxidation despite its simplicity and attractiveness. Obviously, the presence of \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\) accelerate the autoxidation rate, and the catalytic breakdown of these species slows down the autoxidation rate. So, the autoxidation is caused in part by \(\text{O}_2^-\) and \(\text{H}_2\text{O}_2\).
Site-directed mutagenesis to replace the highly conserved distal histidine (His E7) in sperm whale myoglobin demonstrates that the autoxidation rate increases by 40-350 fold (80). Springer et al. (80) suggested that the increase in autoxidation rate appears to be due to a decrease in oxygen affinity and an increase in solvent anion accessibility to the distal pocket (80). The idea that iron autoxidation is at least regulated by the distal His is supported by three evidences. First, X-ray crystallographic analysis of model heme-CO complexes (103) have shown that the distal histidine sterically prevents the formation of linear Fe=C=O bonding. Second, neutron diffraction studies of sperm whale myoglobin have revealed that the distal histidine is hydrogen bonded to the bound O$_2$ at the iron atom (104,105). Third, Traylor et al. (106) had suggested that local polarity of the ligand also contribute to the iron autoxidation.

According to the theories above, the distal histidine would certainly play a role in regulation of the channel for the ligand to come in and react with the iron. Other residues in the ligand binding vicinity may also have a role in steric regulation of ligand binding as well. It was well known that the quaternary and tertiary structures of the oxy and deoxy hemoglobins are different (11). During the transition from R to T state, at the heme environment, the F helix moves clockwise with respect to
the heme axis; the CD corner moves away from the heme, but the E helix moves closer to it. These displacements cause the Phe CD1, Leu F7 and His F8 to move away from the heme, but His E7, Val E11 and Leu FG3 move closer to it (17). In the T state it would seem that His E7 and Val E11 would block the ligand pathway to the iron, but despite this seemingly paradox about the heme environment being more closed, it is possible that the ligand could enter easily from the Phe CD1 channel since it moves away from the heme. Ligands such as, H2O, HO-, O2- and H2O2, are small enough that this slight closing at the iron center may not matter, and the pathway is still large enough for these ligands to diffuse in and react with the iron. This argument is not in contrary to the fact that deoxy hemoglobin autoxidizes faster than oxy hemoglobin. Another contributing factor to the slower autoxidation rate of oxy hemoglobin compared to deoxy hemoglobin is that in oxy hemoglobin O2 is bound to the Fe(II), so any ligands which will cause the oxidation of the Fe(II) must interact with this complex. Hence, the O2 may simply act in protecting the Fe(II) from oxidation, whereas in deoxy hemoglobin the Fe(II) is free to react with any ligands which come in contact with it. These arguments suggest the reasons that deoxyhemoglobin would autoxidize faster than oxy hemoglobin.

For these many reasons the multistep mechanism in the autoxidation of hemoglobin which appears to
satisfactorily explain the bi-phasic nature of the autoxidation curve was proposed by Wallace et al. (68) and modified by Watkins et al. (71). In addition to the proposed reactions a number of reactions could still be postulated where the superoxide radical, hydrogen peroxide and hydroxyl radical are thought to react with Fe(II) in hemoglobin:

\[
(1) \quad \text{Hb.Fe(II)}O_2 \quad = \quad \text{Hb.Fe(II)} + O_2 \\
(2) \quad \text{Hb.Fe(II)} + H^+ \quad = \quad \text{Hb.Fe(II)}(H^+) \\
(3) \quad \text{Hb.Fe(II)}(H^+) + O_2 + H_2O \rightarrow \text{Hb.Fe(III)}H_2O + H^+O_2^- 
\]

In these reactions the initial required step is the dissociation of \(O_2\) from the Fe(II), and the formation of \(O_2^-\). Once \(O_2^-\) is formed it can spontaneously dismutate to hydrogen peroxide (107) or react directly with deoxyhemoglobin at the iron.

\[
(4) \quad 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \\
(5) \quad \text{Hb.Fe(II)}(H^+) + H^+O_2^- + H_2O \rightarrow \text{Hb.Fe(III)}H_2O + H_2O_2 
\]

The \(H_2O_2\) formed from the dismutation reaction and from \(O_2^-\) reacting with Fe(II) can react with deoxyhemoglobin to produce \(OH^-\) and the very reactive hydroxyl radical, \(HO^-\). This is the well known Fenton reaction (91).
When \( \cdot \text{HO} \) is formed it can react with \( \text{H}_2\text{O}_2 \) producing \( \cdot \text{HO}_2 \) and water. It can further react with itself to give \( \text{H}_2\text{O}_2 \) and dioxygen (91).

\[
\begin{align*}
\text{(8)} & \quad \cdot \text{HO}_2 + \cdot \text{HO}_2 \rightarrow \text{H}_2\text{O}_2 + \text{O}_2
\end{align*}
\]

The \( \cdot \text{HO}_2 \) can reduce the Fe(III) in met hemoglobin to the Fe(II) (91).

\[
\begin{align*}
\text{(9)} & \quad \text{Hb.Fe(III)H}_2\text{O} + \cdot \text{HO}_2 \rightarrow \text{Hb.Fe(II)} + \text{O}_2 + \text{H}^+ + \text{H}_2\text{O}
\end{align*}
\]

The \( \cdot \text{HO} \) can oxidize the iron in oxy and deoxy hemoglobin to methemoglobin and \( \text{OH}^- \). This reaction is part of the Haber-Weiss reaction (91).

\[
\begin{align*}
\text{(10)} & \quad \text{Hb.Fe(II)} + \cdot \text{HO} + \text{H}_2\text{O} \rightarrow \text{Hb.Fe(III)H}_2\text{O} + \text{OH}^-
\end{align*}
\]

Since there are many possible reactions at the iron center, these reactions are different in reactivity and perhaps mechanism as well. Thus, their rates of reaction are different, and the superimposed rates of all these reactions happening at the same time would appear to be a higher order rate of reaction.
The autoxidation of the isolated α and β chains appear to be linear for 100 hours (66, 70). The α chain autoxidizes faster than the β chain. The first order rate constant for the α chain is 0.025 h⁻¹ and 0.009 h⁻¹ for the β chain at pH 7.2. The two phases of autoxidation may be contributed from the fast and slow components of the α and β chains respectively.

**B. THE EFFECT OF CROSSLINKING ON THE AUTOXIDATION OF HEMOGLOBIN**

The crosslinking of hemoglobin by bis(3,5-dibromosalicyl) fumarate in the oxy state puts the crosslink between Lys 82β₁ and Lys 82β₂ (30), and in the deoxy state, Lys 99α₁ and Lys 99α₂ (28). This is due to the fact that in the oxy state the two Lys 82β's are closer to each other (6 Å) than the two Lys 99α's (9 Å), whereas in the deoxy state the Lys 82β's are 8.1 Å apart and the Lys 99α's, 5 Å apart. Also in the oxy state the Lys 82β's are stereochemically favorable for the crosslink to occur and not the Lys 99α's, whereas the reverse is true in the deoxy state. This is the reason why all four lysines are not crosslinked in attempting to double crosslink the hemoglobin with BBSF.

The hemoglobins crosslinked between either the α- or β-subunits display markedly different autoxidation rates.
The autoxidation of α99XLHb A is faster than that of β82XLHb A or Hb A (Fig. 33(A)). The β82XLHb A autoxidizes slightly faster than Hb A, but the limit of error is close. The rate of autoxidation of α99XLHb A is 0.0393 h⁻¹ compared to 0.0269 h⁻¹ and 0.022 h⁻¹ of β82XLHb A and Hb A, respectively (Table 16). Since the autoxidation of α99XLHb A is faster than Hb A or β82XLHb A, its half-life in the oxy state is also shorter. The half-life is 17.6 h for α99XLHb A, compared to 25.8 h for β82XLHb A and 31.5 h for Hb A, as determined by the first-order rate approximation.

The faster rate of autoxidation of α99XLHb A than that of β82XLHb A results from the different quaternary structures due to crosslinking in the two proteins. The crosslinking in the α99XLHb A took place in the deoxy state and in β82XLHb A, in the oxy state. The crosslink in the α99XLHb A holds it in the T state and in β82XLHb A, in the R state. After reoxygenation of the deoxy α99XLHb A, a conformation closely related to the T state (a pseudo T state) is still retained. The facts that α99XLHb A binds oxygen less tightly than the β82XLHb A (28,29) and that deoxyhemoglobin autoxidizes faster than oxy hemoglobin (68,70) supports this proposal.

Another possible explanation is that the crosslink causes tertiary structural changes at the heme environment enabling water molecules and other intermediates generated during the autoxidation, such as superoxide radical,
hydrogen peroxide, and hydroxy radical to enter and react with the iron. The study of mutant sperm whale myoglobins in which the distal histidine was replaced demonstrates that these mutant myoglobins autoxidize faster than normal myoglobin, and that the heme environment was more accessible to ligands to get into the heme pocket (80). As stated above, in the deoxyhemoglobin the F helix moves clockwise with respect to the heme axis, the CD corner moves away from the heme, but the E helix moves closer to it. These displacements cause the Phe CD1, Leu F7 and His F8 to move farther away from the heme, but His E7, Val E11 and Leu FG3 move closer to it. During the crosslinking reaction of α99XLHb A the hemoglobin was in the deoxy state, thus, these changes must have occurred. After reoxygenation of the deoxy α99XLHb A, these displacements may not go back to their original positions in the oxy state due to structural constraints of the crosslink. Therefore, it is expected that the crosslink will hold these displacements even after reoxygenation and may cause additional tertiary or secondary structural changes around the crosslink itself.

The X-ray crystallographic structure of β82XLHb A showed that the F helix and EF corner were displaced toward the bisphosphoglycerate binding cavity, but the overall tertiary structure of the hemoglobin does not change (29). The Lys 82β is located on the EF corner (EF6) and Lys 99α,
on the G helix (G6). The heme is covalently coordinated to His F8. In oxy hemoglobin the two Lys 82β's are 6 Å apart, and in deoxy hemoglobin the two Lys 99α's are separated by 5.2 Å. The fumarate crosslink has a span of 5 Å. Thus the crosslink span is shorter than the inter-residue distance between the pairs of lysines. By crosslinking the two lysines are pulled toward each other. So, it is expected to cause local displacement of the F helix in β82XLHb A and in α99XLHb A, of the G helix. Thus, these displacements by crosslinking may cause the heme to be displaced and the heme pocket to be opened up allowing ions to enter easily and react with the iron. In the T state it would seem that the His E7 and Val Ell would block the ligand pathway, but since the Phe CD1 moves away from the heme it is possible that the ligand could enter from this channel. If the tertiary structure of oxy α99XLHb A resembles that of the deoxyhemoglobin then it would be autoxidized faster than Hb A or β82XLHb A. A pseudo T conformation would most likely be oxidized to methemoglobin faster than one at the R state because during the oxidation process the ligand must be kicked off prior to oxidation.

The evidence that α99XLHb A is held in the T state or pseudo T state and β82XLHb A, in the R state, agrees with the finding that deoxy hemoglobin autoxidizes faster than oxy hemoglobin (68,70), and that the heme environments opened up more to allow ligands to come in and react at the
iron as suggested by Springer et al. (80).

In the $\beta 82\text{XLHb A}$ the crosslink took place when the hemoglobin was in the oxy state. Thus, it is believed that the crosslink holds the $\beta 82\text{XLHb A}$ in the R state. Since the R state of oxy $\beta 82\text{XLHb A}$ is essentially the same as oxy Hb A, except for the local perturbation around the crosslink, it should autoxidize at the same rate as oxy Hb A. Such a similarity in the autoxidation rate is confirmed for $\beta 82\text{XLHb A}$ and Hb A (Table 16). Therefore, overall the faster rate of autoxidation of the crosslinked hemoglobins compared to uncrosslinked hemoglobin seems to be principally due to structural differences in the R and T states and local structural changes at the crosslink in the two crosslinked hemoglobins.

C. THE EFFECT OF CATALASE AND SUPEROXIDE DISMUTASE ON THE AUTOXIDATION OF Hb A, $\alpha 99\text{XLHb A}$ AND $\beta 82\text{XLHb A}$

The autoxidation of hemoglobin can be partially inhibited by either catalase or superoxide dismutase (Fig. 35, 36 and 37). This is because $O_2^{-\cdot}$, HO$^{-\cdot}$ and H$_2$O$_2$ are generated during the autoxidation. As indicated above in the autoxidation reactions of hemoglobin, the $O_2^{-\cdot}$, HO$^{-\cdot}$ and H$_2$O$_2$ are known to react with the Fe(II) at the heme center.

Superoxide dismutase catalyzes the break down of $O_2^{-\cdot}$ to H$_2$O$_2$. The catalytic break down of $O_2^{-\cdot}$ to H$_2$O$_2$ by
SOD appears to be as follows (89):

$$\text{SOD-Cu(II)} + \text{O}_2^- \rightarrow \text{SOD-Cu(I)} + \text{O}_2$$

$$\text{SOD-Cu(I)} + \text{O}_2^- \rightarrow \text{SOD-Cu(II)} + \text{H}_2\text{O}_2$$

Thus, in the autoxidation reactions above SOD would inhibit the progress of the oxidation of Fe(II) $\rightarrow$ Fe(III) in reaction 5. Reaction 4 is a spontaneous dismutation reaction of $\text{O}_2^-$ to form $\text{H}_2\text{O}_2$ and may occur independently of the presence of SOD.

Catalase is present in the red cell and the cytoplasm of other cells. Its role is to catalyze the destruction of $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and $\text{O}_2$. The decomposition of hydrogen peroxide by catalase is by means of two reactions. The first reaction involves the formation of a primary complex in which a molecule of $\text{H}_2\text{O}_2$ reacts with the Fe(III) in catalase (108):

$$\text{CAT-Fe(III)-OH} + \text{H}_2\text{O}_2 = \text{CAT-Fe(III)-OOH} + \text{H}_2\text{O}$$

The second reaction involves the primary complex reacting with a second molecule of $\text{H}_2\text{O}_2$ (108).

$$\text{CAT-Fe(III)-OOH} + \text{H}_2\text{O}_2 \rightarrow \text{CAT-Fe(III)-OH} + \text{H}_2\text{O} + \text{O}_2$$

By destroying $\text{H}_2\text{O}_2$, catalase would prevent $\text{H}_2\text{O}_2$ from
reacting with the Fe(II) of the hemoglobin, thus, protecting the hemoglobin from oxidation. Therefore, the catalase would have acted to protect directly the progress of reactions 6 and 7, and indirectly reaction 10 since the HO· is one of the products of H₂O₂ reacting with the Fe(II) of hemoglobin.

In both the crosslinked and uncrosslinked hemoglobins SOD and catalase inhibit the autoxidation partially. But when both are applied together the inhibition is increased (Fig. 35, 36 and 37). The catalase inhibits 11.1%/µM enzyme in the Hb A sample, 8.5%/µM in β82XLHb A and 9.7%/µM in α99XLHb A. The SOD inhibits 2%/µM enzyme in Hb A, 1.9%/µM in β82XLHb A and 1.6%/µM in α99XLHb A (Table 16).

The inhibition by catalase observed in both crosslinked and uncrosslinked hemoglobins was 49-64% and 38-47% by SOD. Thus, the SOD inhibits autoxidation slightly less than catalase. This seems to suggest that either the amount of H₂O₂ generated in the autoxidation was higher than O₂·⁻ or that certain dismutation reactions were responsible for the break down of O₂·⁻ and thus, they are not intercepted by SOD. When both enzymes were applied the inhibition was 67-82%. Hence, not all the O₂·⁻ and H₂O₂ were intercepted by the enzymes. The inhibition of autoxidation by SOD and catalase is without doubt a proof of the generation of O₂·⁻ and H₂O₂ in the reaction. The effect of inhibition observed by using SOD and catalase
should provide a clue to the protection of hemoglobin autoxidation.

D. CROSSLINKED HEMOGLOBIN AS POTENTIAL BLOOD SUBSTITUTE

The interest in using crosslinked hemoglobin as a blood substitute is being investigated quite extensively by several groups of scientists (18, 27-30, 33, 34, 40, 49). Of the several required properties of a crosslinked hemoglobin as a potential blood substitute, the obvious ones are oxygen binding, structural stability and non-lability to oxidation. For a crosslinked hemoglobin to be considered as a potential blood substitute the oxygen affinity should not deviate significantly from that of whole blood, it must be crosslinked between the like-subunits to prevent dissociation to αβ dimer, and it should be less labile to oxidation.

Of all the crosslinked hemoglobins investigated so far, the most promising one is the α99XLHb A. The α99XLHb A has lower oxygen affinity, similar to that of whole blood (28), but the β82XLHb A has higher oxygen affinity (29). The α99XLHb A, β82XLHb A, and their double crosslinked derivatives have the highest thermal stability (Table 13), which would imply the highest structural stability for these crosslinked proteins. The α99XLHb A autoxidizes faster than β82XLHb A or Hb A, and β82XLHb A autoxidizes
slightly faster than Hb A (1.2 fold). Two important factors in making a blood substitute from modified hemoglobin lie in the autoxidation and stability. The stability of crosslinked hemoglobin may increase the intravascular retention time of the hemoglobin in the body during circulation because the equilibrium is shifted toward the tetrameric form. Thereby, preventing renal elimination. With stable crosslinked protein, the solution could be sterilized at high temperature without causing denaturation. Also stabilized crosslinked hemoglobin could increase storage shelf-life and allow storage at room temperature. From this point of view, the crosslinking in both α and β crosslinked hemoglobins would satisfy these requirements. Since the autoxidation rate of α99XLHb A is faster than that of normal Hb A and β82XLHb A, it is not known if this rate would be acceptable. Snyder et al. (68) had proved that in Sprague-Dawley rats the methemoglobin reductase system was able to reduce the met α99XLHb A progressively from nearly 100% methemoglobin to 27% met hemoglobin within 5 hours. Based on this experiment the methemoglobin reductase system should be able to slow down the rate of autoxidation of the α99XLHb A in vivo. So, in vivo the rate of autoxidation at 0.0396 h⁻¹ may not matter, but for storage purposes such hemoglobin would need protection from autoxidation by either antioxidants or enzyme systems. Such an effect was demonstrated by the
reduction of autoxidation of hemoglobins by SOD and catalase or both.

During emergency in which a massive amount of blood substitute is needed to carry oxygen to the tissues in a short time the patient would need a fully oxygenated blood substitute. Any methemoglobin present in the blood substitute given to a patient may take a while for it to be reduced. Thus, the high autoxidation rate of $\alpha_{99XLHb A}$ may present a problem in using it as a blood substitute.

The thermal stability of Hb A-DMP, Hb A-TCEA, Hb A-BCEA, $\alpha_{99XLHb A-DMP}$ and $\beta_{82XLHb A-DMP}$ is dependent on the amount of crosslink on them. The double crosslinked hemoglobins, $\alpha_{99XLHb A-DMP}$ and $\beta_{82XLHb A-DMP}$, are the most stable ones, followed by Hb A-DMP (at 500:1 molar ratio) and Hb A-TCEA. The Hb A-BCEA has the same stability as Hb A. In regard to stability, some of these crosslinked hemoglobins (Hb A-DMP, $\alpha_{99XLHb A-DMP}$ and $\beta_{82XLHb A-DMP}$) should also satisfy this requirement. However, this is questionable, since all the DMP treated hemoglobins exhibit low solubility in aqueous solution. Pennathur-Das et al. (47) have shown that the oxygen affinity of DMA treated hemoglobin is increased. Thus, it is likely that DMP treated hemoglobins are also affected the same way.

The nitrogen mustards are highly toxic compounds, so Hb A-TCEA and Hb A-BCEA are ruled out as potential blood substitutes. Furthermore their stability is low, but the
model of tri-functional crosslinking should provide a further search for such reagent with less toxicity. Roth et al. (42) have shown that the oxygen affinity of nitrogen mustard treated hemoglobin remains the same as Hb A.
APPENDIX

19F LABELING ON HEMOGLOBIN

PROCEDURES

A. MODIFICATION REACTIONS OF HEMOGLOBIN WITH 3-BROMO-1,1,1-TRIFLUOROPROPANONE

The reaction of hemoglobin with 3-bromo-1,1,1-trifluoropropanone (TFAB) was done according to the method of Huestis and Raftery (109). The labeling of oxyhemoglobin with 19F was done in three different solvents, 0.2 M phosphate, 0.1 M NaCl, pH 7.2, 0.1 M MOPS, 0.1 M NaCl, pH 7.2, and 0.01 M MOPS, pH 7.2. The mole ratio of TFAB to Cys β93 was 2.5:1.0. TFAB was dissolved in the corresponding buffer, which the hemoglobin was in, or added neat directly to the hemoglobin solution. The pH of the solution tended to drop because of the acidity of the TFAB solution, hence, 5-20 µL of 0.2 M NaOH was added at a time to maintain the pH at 7.2.

A 2 mL of 10.3% oxyhemoglobin A (103.4 mg/mL, 6.47 mM heme, 1.29 x 10^-5 mole heme, 6.45 x 10^-6 mole Cys β93) in 0.01 M MOPS, 0.1 M NaCl, pH 7.2, was reacted with 1.8 µL of TFAB (d 1.72 g/mL, 1.61 x 10^-5 mole) at room temperature (22°C) for 30 minutes. The reaction was carried out in a small test tube with slow stirring by a mini stirring bar at the bottom and the pH was constantly monitored. If the
pH dropped below 7.0, the solution was adjusted with 0.2 M NaOH, 5-20 µL at a time. For reactions carried out in MOPS buffer the pH changed by 0.1-0.2 unit only, and therefore, pH adjustment was not needed. For different reactions the hemoglobin concentration was varied from 15-105 mg/mL, however most of the reactions had 50 mg/mL or 100 mg/mL. The molar ratio of TFAB to Cys β93 was kept at 2.5:1 for most of the experiments, except for some of the initial experiments, which were 25:1. After the reaction was completed the hemoglobin solution was put in an ice cold bath for 10 minutes, centrifuged to remove any precipitates formed and passed through a Sephadex G-25 column equilibrated with 0.01 M MOPS, pH 7.0. The labeled hemoglobin (Hb-TFA) was concentrated by the pro-dicon in 0.01 M MOPS, pH 7.0. The reactions of hemoglobin with TFAB were also done with CO deoxy and cyanomet hemoglobins in the same manner. The extent of modification at the cysteine residues was characterized by Ellman’s reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (110,111).

B. NMR SAMPLE PREPARATION

The hemoglobin solution for NMR studies consisted of 20-100 mg/mL hemoglobin in 0.01 M MOPS, pH 7.0 and 10% D2O. In the absence of guanidine three different buffers (0.01 M MOPS, pH 7.0; 0.1 M NaCl, pH 7.0; and 0.2 M phosphate, 0.1
M NaCl, pH 7.0) were used, but most of the experiments established in the presence of guanidine were done in 0.01 M MOPS, 10% D2O, pH 7.0 with various guanidine concentration (0.9-4.6 M). A 5 mm diameter NMR tube (from Wilmad Glass), fitted to a 0.7 mm diameter coaxial external reference capillary tube (from Wilmad Glass) was used. The external reference in the capillary tube contained 3 x 10^-3 M of trifluoroacetic acid (TFA) in 0.01 M MOPS, pH 7.0.

A protocol of an NMR sample preparation of 10% hemoglobin, 10% D2O, and 1 M guanidine in 0.01 M MOPS, pH 7.0 would be as follows:

A 233 µL of oxy HbA-TFA (257.5 mg/mL) in 0.01 M MOPS, pH 7.0 was mixed with 60 µL of D2O, 60 µL of 0.01 M MOPS, pH 7.0, and 247 µL of 2.5 M guanidine in 0.01 M MOPS, pH 7.0.

The 19F NMR spectra were recorded using a Varian VXR 300 MHz NMR spectrometer tuned to a frequency of 282.2 MHz (7.047 T). The probe was at room temperature (21± 0.5°C). The nucleus was set at 19.0. The acquisition time was 0.296 second. The pulse width was 15 µsec. The spectral width was 8,000 Hz. The number of scans were between 5,000-10,000. The 19F spectra were run with the decoupler off.
RESULTS

In the hope of studying the hemoglobin conformation by NMR, the hemoglobin was labeled at Cys 93β with $^{19}$F. The reagent used for labeling was 3-bromo-1,1,1-trifluoropropanone (TFA, F$_3$CCOCH$_2$Br). The labeling method was according to Huestis and Raftery (109,112,113). When a protein goes from one state to another it is likely that the environment at Cys 93β may change. This change might expose the $^{19}$F on the Cys 93β to different environment and may cause a change in magnetic properties, such as chemical shift or relaxation time. It was hoped that the signal from $^{19}$F could be used to probe the different states of the hemoglobin, such as the native or denatured state. Different chemical shifts have been observed for different ligated state of the hemoglobin (109,112,113). The denaturation state of the hemoglobin could be studied as a function of temperature or guanidine concentration.

The spectra of different ligated TFA treated hemoglobin (Hb A-TFA) appear as broad singlets ranging from -8.4 to -8.9 ppm, with the met state being at higher field and cyanomet at lower field. Table 28 shows the chemical shifts observed by Huestis and Raftery compared to those from this research. Figure 42 shows the $^{19}$F NMR signal from the Hb A-TFA. The chemical shifts observed by Huestis and Raftery (109,112,113) are at lower field than those
Table 28:
The $^{19}$F chemical shifts observed with 94.1 MHz at various ligand states in Hb A-TFA at pH 7.2 and 22°C relative to trifluoroacetic acid. The NMR samples contained 2.5-10% hemoglobin.

<table>
<thead>
<tr>
<th>Ligated state</th>
<th>$\delta$(ppm)$^a$</th>
<th>$\delta$(ppm)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanomet</td>
<td>-4.89</td>
<td>-8.42 ± 0.04</td>
</tr>
<tr>
<td>CO</td>
<td>-4.99</td>
<td>-8.56 ± 0.07</td>
</tr>
<tr>
<td>Oxy</td>
<td>-5.13</td>
<td>-8.62 ± 0.04</td>
</tr>
<tr>
<td>Met</td>
<td>-5.58</td>
<td>-8.94 ± 0.05</td>
</tr>
<tr>
<td>Deoxy</td>
<td>-5.68</td>
<td>-8.86 ± 0.06</td>
</tr>
</tbody>
</table>

$^a$ Chemical shifts observed by Huestis and Raftery at 94.1 MHz (109,112,113).

$^b$ Chemical shifts observed in this research at 282.2 MHz.
Figure 42:

The \(^{19}\text{F}\) chemical shifts observed for different ligated states of hemoglobins in 10\% D\(_2\)O, 0.01 M MOPS, pH 7.2, at room temperature (21°C). The hemoglobin concentration was 10\%. 
CN-Hb

CO-Hb

Oxy-Hb

Deoxy-Hb

Met-Hb

-10 PPW
obtained in this research. This may be due to difference in the reference used. The reference, trifluoroacetic acid, used in this research was diluted in water and used externally; so it may have been dissociated to the anion form, since trifluoroacetic acid is a strong acid. Huestis and Raftery did not comment whether they diluted the trifluoroacetic acid in water or they used it neat. Nevertheless, the difference in chemical shift is the same in both results.

It is possible to observe the hemoglobin in different ligated state in a sample. Figure 43 shows an oxy hemoglobin sample which a fraction of it has been oxidized to met hemoglobin.

Probing the denatured state of hemoglobin using increasing guanidine concentration could not be realized because the Cys 93β in the native state is on the surface of the hemoglobin molecule and in the denatured state it is also on the surface. So, the environment around the $^{19}$F probe is the same in both states, thus, no significant chemical shift was observed. Table 28 compares the $^{19}$F chemical shifts in hemoglobin in the presence or absence of guanidine. The $\text{F}_3\text{COCH}_2\text{Br}$ in aqueous solution appears at $-8.19 \pm 0.03$ ppm from trifluoroacetic acid. The $^{19}$F in carbonmonoxyhemoglobin appears at $-8.56$ ppm, and that of met hemoglobin is at $-8.94$ ppm. This means that the $^{19}$F in carbonmonoxyhemoglobin is more exposed to the polar
**Figure 43:**

Observation of different ligated states of hemoglobins in the same sample. The peak at -8.6 ppm is due to the oxy form, and at -9.1 ppm, the met form.
Table 29:

The $^{19}$F chemical shifts in CO and met Hb A-TFA in the presence or absence of guanidine at room temperature ($21^\circ$C).

<table>
<thead>
<tr>
<th>[Gdn]</th>
<th>$\delta$ of CO-Hb</th>
<th>$\delta$ of Met-Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>-8.56 $\pm$ 0.07</td>
<td>-8.94 $\pm$ 0.05</td>
</tr>
<tr>
<td>0.9</td>
<td>-8.70</td>
<td>-8.55</td>
</tr>
<tr>
<td>1.0</td>
<td>-8.65 $\pm$ 0.11</td>
<td>-8.61</td>
</tr>
<tr>
<td>2.0</td>
<td>-8.75 $\pm$ 0.05</td>
<td>-8.68</td>
</tr>
<tr>
<td>3.0</td>
<td>-8.71</td>
<td>-8.67</td>
</tr>
<tr>
<td>3.6</td>
<td>-8.71</td>
<td>-8.70</td>
</tr>
<tr>
<td>4.0</td>
<td>-8.71</td>
<td></td>
</tr>
</tbody>
</table>
solvent, water; therefore, it is in a more hydrophilic environment than in the met state. When Hb A-TFA is denatured in 3 M Gdn, the $^{19}$F resonance appears at -8.70 ppm. According to the results obtained, the Cys 93β in the denatured state of the protein would be in an intermediate environment between the met and CO hemoglobin states since its chemical shift is at -8.70 ppm. At 3 M guanidine hemoglobin is already denatured.

In the denatured state if the protein is in a random coil state, one would have expected the $^{19}$F probe to have, if not close to, the same chemical shift as the reagent itself (-8.2 ppm). The chemical shift would go from higher to lower field. In the met hemoglobin it appears to be so, but in the CO hemoglobin it is shifting upfield. Unless, at this concentration the CO state is changing to the met state, and its chemical shift is still shifting upfield and has not reached the state where it would go downfield. This could only be so if the CO and met hemoglobin have not denatured completely at 3 M Gdn.

Three experiments in which hemoglobin was mixed with 0.9 M Gdn accompanied by raising the temperature to 65°C showed the $^{19}$F shifted to -8.1 ppm, which would agree with the interpretation that the $^{19}$F probe would be in the same environment in the denatured state of the protein as the free reagent. But when the temperature was raised to 65°C the hemoglobin sample had already aggregated. Thus, it is
not certain whether this chemical shift represents the denatured state. It is also possible that temperature could effect such a chemical shift in the denatured protein. Aggregation is one of the reasons why the use of temperature to induce denaturation of hemoglobin was not very useful in NMR due to the ease of formation of aggregates at high hemoglobin concentration. Figure 44 shows the effect of temperature on the $^{19}$F chemical shift of hemoglobins.
Figure 44:

The effect of temperature on the $^{19}$F chemical shift of Hb A-TFA; curves 1 and 2 are oxy Hb A-TFA; curve 3, oxy $\beta$82XLHb A-TFA; and curve 4, met Hb A-TFA.
SUMMARY

Hemoglobin has been crosslinked using four different reagents: bis(3,5-dibromosalicyl) fumarate, dimethylpimelimidate (DMP), tris(β-chloroethyl) amine (TCEA), and bis(β-chloroethyl) methylamine (BCEA). The hemoglobins crosslinked with imidoester and nitrogen mustard derivatives all produced heterogeneous species which were not resolved well enough to a single homogeneous crosslinked species. Increasing the molar ratio of nonspecific crosslinking reagents, TCEA, BCEA and DMP, to hemoglobin during reaction produced higher crosslinked species. Nonspecific crosslinking reagents can crosslink hemoglobin to form dimeric, trimeric, tetrameric, and polymeric species. The tri-functional reagent, TCEA, crosslinks hemoglobin to produce higher percentages of dimers and trimers compared to the bi-functional reagent, BCEA, whereby only dimers were predominant. Since the hemoglobin solution of the DMP, TCEA and BCEA treated hemoglobins were of heterogeneous nature, their thermal transition represents an average value of the crosslinked sample.

All the crosslinked proteins have structural transitions at higher temperature than Hb A, except for Hb A-BCEA which is equal to Hb A. The specific crosslinker, bis(3,5-dibromosalicyl) fumarate, produces the highest
stability in the singly crosslinked protein. These are the $\beta 82X$LHb A and $\alpha 99X$LHb A. The nonspecific crosslink reagents DMP, TCEA and BCEA stabilize hemoglobin by a lesser extent compared to the specific crosslinker. However, the stability is still higher than normal Hb A by 8-15°C. The $T_m$ of these crosslinked hemoglobins from the Soret data are $41.3^\circ C$, Hb A-BCEA; $49.3^\circ C$, Hb A-TCEA; $56.1^\circ C$, Hb A-DMP (500:1); $56.7^\circ C$, $\alpha 99X$LHb A; $56.6^\circ C$, $\beta 82X$LHb A; $57.6^\circ C$, XLHb A; $58.7^\circ C$, $\alpha 99X$LHb A-DMP (500:1); and $60.4^\circ C$, $\beta 82X$LHb A-DMP (500:1).

Since higher molecular weight crosslinked species are present in DMP, TCEA and BCEA treated hemoglobins, but their thermal transition is lower or only comparable to the $\alpha 99X$LHb A and $\beta 82X$LHb A at very high molar ratio of reagent to hemoglobin, the crosslinks in Hb A-DMP, Hb A-TCEA and Hb A-BCEA must have destabilized the proteins. So, not all crosslink will result in stabilizing the protein structure.

The data from 280 nm and the Soret region indicate that the crosslinks stabilize some parts of the protein more than others. Double crosslinking of $\beta 82X$LHb A or $\alpha 99X$LHb A with DMP produces the highest change in stability, $(60^\circ C)$. The increase in stability due to the second reaction of DMP with crosslinked hemoglobin is much less than the stability due to the first reaction, $3^\circ C$ compared to $16^\circ C$. Thus, the increase in $T_m$ is not additive.
The autoxidation rate of $\alpha_{99}$XLHb A is faster than those of the $\beta_{82}$XLHb A and Hb A by 1.5 and 1.8 fold, respectively. The $\beta_{82}$XLHb A and normal Hb A have nearly the same autoxidation rates within the limit of error. The apparent first order rate constant of autoxidation of hemoglobins are $2.20 \times 10^{-2} \text{ h}^{-1}$, Hb A; $2.69 \times 10^{-2} \text{ h}^{-1}$, $\beta_{82}$XLHb A; and $3.93 \times 10^{-2} \text{ h}^{-1}$, $\alpha_{99}$XLHb A. The faster rate of autoxidation of the crosslinked hemoglobins is believed to be due to the tertiary structural change by the fumarate crosslink holding the oxy $\alpha_{99}$XLHb A in the T conformation and the $\beta_{82}$XLHb A, in the R conformation.

Superoxide dismutase and catalase are able to slow down the rate of autoxidation independently or together. The ability of SOD and CAT to protect hemoglobins from autoxidation is a further proof of the generation of $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ during autoxidation. This research emphasizes two important factors for the design of a blood substitute, stability and iron autoxidation. For a blood substitute, at least the modified hemoglobin is required to be stabilized by a crosslink and resistant to oxidation.
REFERENCES:


86. Yang, T., (1986); The Effect of Crosslinking by Bis(3,5-dibromosalicyl) Fumarate on the Stability of Abnormal Hemoglobins and Hemoglobin Derivatives. M.S. Thesis, Loyola Univ. of Chicago, Chicago.


100. Gunther, H., (1973); NMR Spectroscopy; John Wiley & Sons, Chichester, New York, Brisbane, Toronto.


115. Wehrli, F. W., and Wirthlin, T., (1976); Interpretation of Carbon-13 NMR Spectra; Heyden & Son Ltd.


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The dissertation is therefore accepted in partial fulfillment for the requirements for the degree of Doctor of Philosophy.

12/12/84
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