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THE REACTION OF HEMOGLOBIN WITH NITRIC OXIDE

by

CHARLES RUSSELL HILLE

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
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DOCTOR OF PHILOSOPHY

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HOUSTON, TEXAS

MAY, 1979
To my parents, Roy and Ann Hille, who

know where the cholesterol is in eggs.
SUMMARY

The appearance of the three-line hyperfine structure in the EPR spectra of nitrosylhemoglobin samples exhibits a strong correlation with the T quaternary state. Resolution of the contributions of the $\alpha$ and $\beta$ subunits to various EPR spectra has shown that the source of this hyperfine structure is the formation of a modified nitrosylheme configuration within the $\alpha$ chains. These results are in agreement with a large amount of literature on nitrosylhemoglobin in the presence of IP$_6$, the NO hydrates $\alpha_2^NO\beta_2$ and $\alpha_2\beta_2^NO$, and mutant and chemically modified hemoglobins exhibiting abnormal allosteric properties.

The hyperfine structure in the EPR and the corresponding optical absorbance change exhibit a pH dependence that is the result of two independent effects. First, there is an increase in the allosteric constant, $L$, as the pH is lowered which increases the fraction of hemoglobin molecules in the T state at a given level of saturation. Second, as the pH is lowered there is an increase in the fraction of nitrosylheme sites within the T state which exhibit the three-line hyperfine structure. The latter effect is quite dramatic; the fraction of modified $\alpha$ nitrosylheme sites in the T state decreases from 90% at pH 6.5 to 10% at pH 9.0. These results provide strong evidence in favor of the proposal of Kon and Katsoka [Biochem. Biophys. Res. Commun. 8, 4757 (1969)] that the state of protonation of the proximal histidine determines the spectral characteristics of a nitrosylheme site. Accordingly, the protonation of the proximal histidine at a nitrosylheme site is the cause of the formation of the modified nitrosylheme configuration.

The protonation of the proximal histidine and/or the associated configurational change is a relatively slow process and under
normal circumstances takes place only in the α subunits. This process remains slow even when the hemoglobin molecules remain in the T state throughout the reaction, suggesting that the proximal histidine is probably deprotonated when the heme site is unliganded. If it were protonated prior to the binding of NO, the appearance of hyperfine structure might be expected to be immediate.

An optical change which is strikingly similar to that observed on the formation of the protonated nitrosylheme configuration is also found in early stages of the titration of deoxyhemoglobin with oxygen. This result suggests that the proximal histidine of oxyheme sites may also be protonated in the T state and that this modification of the proximal histidine may play a general role in the process of ligand binding to hemoglobin.

Rapid-freeze EPR experiments have been performed to investigate the possibility of differences between the α and β subunits in the rates of NO association, CO association and dissociation, and in the equilibrium of O₂ in the presence and absence of IP₆. In none of the above cases was there any evidence to support chain heterogeneity. Only in the equilibrium of NO was there any data to suggest a difference in ligand affinities, and in view of the extremely long periods of incubation periods required to study the reaction, this difference should be viewed with some degree of reservation. It seems likely that the only ligands of reduced hemoglobin capable of expressing significant amounts of chain heterogeneity are the alkyl isocyanides.
ACKNOWLEDGEMENTS

I would like to thank the following people for their various parts in my development as a scientist and a person:

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# TABLE OF CONTENTS

| I. AN INTRODUCTION TO THE PHYSIOLOGICAL AND STRUCTURAL PROPERTIES OF HEMOGLOBIN | 1 |
| A. Allosteric Effectors of Hemoglobin Function | 1 |
| B. Structural Properties of Hemoglobin | 5 |

| II. MATHEMATICAL AND THERMODYNAMIC FORMULATIONS FOR HEMOGLOBIN | 11 |
| A. The Thermodynamics of Subunit Interactions Within Hemoglobin | 11 |
| B. Model Systems for Hemoglobin | 15 |
| 1. The Concerted Model of Hill | 15 |
| 2. The Adair Scheme | 16 |
| 3. The Sequential Models of Pauling and Koshland | 21 |
| 4. The Allosteric Model of Monod, Wyman, and Changeux | 27 |
| 5. An analysis of the sequential and allosteric models | 30 |

| III. CHAIN HETEROGENEITY | 33 |
| A. Experimental Evidence for Chain Heterogeneity | 33 |
| 1. Methemoglobin | 33 |
| 2. Reduced Hemoglobin | 34 |
| 3. Hybrid Hemoglobins | 37 |
| B. The Effect of Chain Heterogeneity on Model Systems | 40 |

| IV. PRIOR WORK ON THE REACTION OF HEMOGLOBIN WITH NITRIC OXIDE | 48 |

| V. PRELIMINARY WORK ON THE REACTION OF HEMOGLOBIN WITH NITRIC OXIDE | 58 |
| A. Some Spectral Properties of Nitrosoylhemoglobin | 58 |
| 1. Materials and Methods | 58 |
| 2. Results | 66 |
3. Discussion ........................................... 72

B. Experiments with the Nitrosyl Hybrids $\alpha_{2}^{\text{NO}} \beta_{2}$ and $\alpha_{2}^{\text{NO}} \beta_{2}$ .......................... 79
   1. Materials and Methods .............................. 79
   2. Results ........................................... 80
   3. Discussion ....................................... 85

C. Titrations of Nitrosylhemoglobin with IP$_{6}$ and PMB ........................................... 85
   1. Materials and Methods .............................. 85
   2. Results ........................................... 88
   3. Discussion ....................................... 95

VI. THE TITRATION OF HEMOGLOBIN WITH NITRIC OXIDE ........................................... 101
   A. The Titration of Hemoglobin with Nitric Oxide at pH 7.0
      in the Presence and Absence of IP$_{6}$ .......................... 101
         1. Materials and Methods .............................. 101
         2. Results ........................................... 103
         3. Discussion ....................................... 125
   B. The pH Dependence of the Nitric Oxide Titration ........................................... 126
         1. Materials and Methods .............................. 126
         2. Results ........................................... 126
         3. Discussion ....................................... 141
   C. Very Slow Reactions Following the Binding of Nitric
      Oxide to Hemoglobin ................................. 148
         1. Materials and Methods .............................. 148
         2. Results ........................................... 148
         3. Discussion ....................................... 153

VII. THE REACTION OF HEMOGLOBIN WITH CARBON MONOXIDE AND OXYGEN .................. 158
   A. The Reaction of Hemoglobin with Carbon Monoxide ................................. 158
      1. Materials and Methods .............................. 158
ABBREVIATIONS USED

bis-tris — $2,2'$-bis(hydroxymethyl)-2,2',2"-nitrilotriethanol
EPR — electron paramagnetic resonance
Hb — hemoglobin
HbNO — nitrosylhemoglobin
HEPES — 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HPT — 8-hydroxy-1,3,6-pyrene trisulfonate
IP$_6$ — inositol hexaphosphate
nBNC — n-butylisocyanide
NEM — $N$-ethylmaleimide
NMR — nuclear magnetic resonance
PMB — $p$-hydroxymercuribenzoic acid
tris — tris-(hydroxymethyl)-aminomethane
## LIST OF FIGURES AND TABLES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.1. Oxygen saturation curves for hemoglobin and myoglobin</td>
<td>3</td>
</tr>
<tr>
<td>II.1. Thermodynamic cycle for subunit interactions within hemoglobin</td>
<td>13</td>
</tr>
<tr>
<td>II.2. Hill plot of oxygen equilibrium data</td>
<td>18</td>
</tr>
<tr>
<td>II.3. Tetrahedral and square geometries for the sequential model</td>
<td>23</td>
</tr>
<tr>
<td>II.4. The allosteric model</td>
<td>29</td>
</tr>
<tr>
<td>III.1. Saturation equation for a square sequential model incorporating chain heterogeneity</td>
<td>43</td>
</tr>
<tr>
<td>III.2. The Adair scheme expanded to incorporate chain heterogeneity</td>
<td>45</td>
</tr>
<tr>
<td>IV.1. Schematic diagram of a rapid-freeze device</td>
<td>51</td>
</tr>
<tr>
<td>IV.2. Optical difference spectrum of nitrosylhemoglobin ( \pm IP_6 )</td>
<td>54</td>
</tr>
<tr>
<td>V.1. Schematic diagram of a chain preparation</td>
<td>61</td>
</tr>
<tr>
<td>V.2. Schematic diagram of the mixing device used in the optical/EPR experiments</td>
<td>65</td>
</tr>
<tr>
<td>V.3. Optical difference spectra of hemoglobin and isolated chains ( \pm NO )</td>
<td>68</td>
</tr>
<tr>
<td>V.4. The EPR spectra of the nitrosyl derivatives of hemoglobin and isolated chains</td>
<td>71</td>
</tr>
<tr>
<td>V.5. The rapid-freeze titration of hemoglobin with NO</td>
<td>73</td>
</tr>
<tr>
<td>V.6. EPR changes following the mixture of nitrosylhemoglobin with ( IP_6 ) and hemoglobin with subsaturating levels of NO</td>
<td>76</td>
</tr>
<tr>
<td>V.7. Optical changes following the mixture of nitrosylhemoglobin with ( IP_6 ) and hemoglobin with subsaturating levels of NO</td>
<td>78</td>
</tr>
<tr>
<td>V.8. EPR spectra of ( \alpha^{NO}, \beta^{NO}, \alpha_2^{NO} \beta_2 ) and ( \alpha_2^{NO} \beta_2 )</td>
<td>82</td>
</tr>
<tr>
<td>V.9. Kinetic difference spectra for slow changes following the mixture of ( \alpha+\beta, \alpha^+\beta, ) and ( \alpha^+\beta^{NO} )</td>
<td>84</td>
</tr>
<tr>
<td>V.10. Effect of titrating the ( \alpha ) chains with NO in the ( \alpha+\beta ) reaction</td>
<td>87</td>
</tr>
</tbody>
</table>
Figure | Page
--- | ---
V.11. The optical titration of nitrosylhemoglobin with IP₆ | 90
V.12. The optical difference spectrum of IP₆-nitrosylhemoglobin ± PMB | 92
V.13. The optical titration of PMB-nitrosylhemoglobin with IP₆ | 94
V.14. The optical difference of nitrosylhemoglobin ± IP₆ and PMB | 97
V.15. The EPR spectra of nitrosylhemoglobin ± IP₆ and PMB | 99
VI.1. The optical titration of hemoglobin with NO at pH 7.0 | 105
VI.2. The first and last 10% of the titration of hemoglobin with NO | 108
VI.3. The difference between the first and last 10% of the titration of hemoglobin with NO | 110
VI.4. The EPR titration of hemoglobin with NO at pH 7.0 | 112
VI.5. Comparison of the EPR of 10% and fully saturated nitrosylglobin with that of nitrosylhemoglobin + IP₆ | 114
VI.6. Representative fits to optical and EPR spectra | 117
VI.7. Plot of fraction of bound heme sites in the T state versus fraction saturation at pH 7.0 | 119
VI.8. The optical titration of hemoglobin with NO in the presence of IP₆ | 122
VI.9. The EPR spectra of 10% and fully saturated nitrosylhemoglobin in the presence of IP₆ | 124
VI.10. The optical titration of hemoglobin with NO at pH 6.5 | 128
VI.11. The EPR titration of hemoglobin with NO at pH 6.5 | 130
VI.12. The optical titration of hemoglobin with NO at pH 8.0 | 132
VI.13. The EPR titration of hemoglobin with NO at pH 8.0 | 134
VI.14. The optical titration of hemoglobin with NO at pH 9.0 | 136
VI.15. The EPR titration of hemoglobin with NO at pH 9.0 | 138
VI.16. The EPR spectra of 10% nitrosylhemoglobin at pH 6.5, 7.0, 8.0 and 9.0 | 140
VI.17. Plot of fraction of bound heme sites in the T state versus fraction saturation at pH 6.5, 7.0, 8.0, and 9.0 143

VI.18. Very slow changes in the shape of the EPR spectrum of 13% saturated nitrosylhemoglobin 150

VI.19. Very slow changes in the distribution of NO between the subunits in 13% saturated nitrosylhemoglobin 152

VI.20. Very slow changes in the optical spectrum of 15% saturated nitrosylhemoglobin 155

VII.1. The optical titration of hemoglobin with CO 160

VII.2. Comparison of the CO titration with the R→T transition 163

VII.3. The displacement of CO by NO 165

VII.4. The association of CO to hemoglobin 167

VII.5. The optical titration of hemoglobin with O₂ 170

VII.6. The difference between the first and last 10% of the O₂ titration 172

VII.7. The optical titration of hemoglobin with O₂ in the presence of IP₆ 174

VII.8. The difference between the first and last 6% of the O₂ titration in the presence of IP₆ 176

VII.9. The rapid-freeze reaction of hemoglobin with O₂ 178

VII.10. The rapid-freeze reaction of hemoglobin with O₂ in the presence of IP₆ 180

Table Page

VI.1 The dependence of L and the fraction of modified α nitro- sylheme sites in the T state on pH 145
I. AN INTRODUCTION TO THE PHYSIOLOGICAL AND STRUCTURAL PROPERTIES OF HEMOGLOBIN

Hemoglobin has been the object of scientific investigation for over one hundred years, primarily due to the cooperative manner in which it binds molecular oxygen and other heme ligands. The major manifestation of cooperativity is a sigmoidal response of fractional saturation (Y) to ligand concentration, which requires an increase in ligand affinity with an increasing number of bound heme sites. Physiologically, the significance of cooperativity is not that the affinity of hemoglobin for oxygen increases in the lungs, but that it decreases in the tissues. The increase in the ability of the cooperative hemoglobin system to transport oxygen from lungs to tissues over a system exhibiting normal hyperbolic response to ligand concentration is considerable (Fig. I.1).

I.A. Allosteric Effectors of Hemoglobin Function

In addition to the heme ligands, hemoglobin also binds a variety of other molecules and ions. The most important of these are $\text{H}^+$, $\text{CO}_2$, and organic phosphates, all three of which affect the ligand binding properties of hemoglobin by preferentially binding to and stabilizing deoxyhemoglobin over the oxygenated form.

Above pH 6.0 deoxyhemoglobin has a greater affinity for protons than does oxyhemoglobin (1). This situation gives rise to a phenomenon known as the alkaline Bohr effect in which the affinity of hemoglobin for ligand increases with increasing pH. This is advantageous from a physiological standpoint as the pH of the lungs is higher than that of the tissues, enabling hemoglobin to bind relatively more $\text{O}_2$ in the lungs and release more in the tissues than would otherwise be the
Fig. I.1. The effect of oxygen concentration on fraction saturation for myoglobin (dashed line) and hemoglobin (solid line). $\Delta Y_{\text{Mb}}$ and $\Delta Y_{\text{HB}}$ represent the saturation changes in myoglobin and hemoglobin, respectively, on going from the oxygen concentration of the lungs to that of the tissues, i.e., the net amount of oxygen transported by each protein. Data is from White, A., Handler, P., and Smith, E., Principles of Biochemistry, fifth edition (1974), McGraw-Hill Book Company, New York, pp. 837-838.
CO₂ reacts with the N-terminal amino groups of hemoglobin to form carbamino derivatives, and does so to a greater extent with deoxyhemoglobin than oxy (2,3). As is the case with protons, this preferential binding to deoxyhemoglobin causes a decrease in oxygen affinity in the presence of CO₂. This reciprocal relationship between CO₂ and O₂ binding to hemoglobin is also advantageous from a physiological standpoint: the exchange of O₂ for CO₂ being favored in the tissue where CO₂ levels are high, and CO₂ for O₂ in lungs where O₂ is in excess. In addition, the uptake of protons by deoxyhemoglobin in venous blood (due to the Bohr effect) results in a shift of the carbonic acid-bicarbonate equilibrium toward bicarbonate, increasing the total amount of CO₂ capable of being dissolved in the blood. This phenomenon is known as the Haldane effect (4) and effectively doubles the capacity of blood to remove waste CO₂ from the tissues. Thus hemoglobin is responsible not only for oxygen transport from lungs to tissues, but also for some 60% of CO₂ transport from tissues to lungs, either directly as carbamino groups or indirectly as a consequence of the Haldane effect (5).

Organic phosphates, most notably 2,3-diphosphoglycerate (DPG), bind preferentially to deoxyhemoglobin and thus stabilize this form over oxyhemoglobin, again lowering oxygen affinity (6). Since DPG is a major product of glycolysis in the human erythrocyte, the binding of organic phosphates provides a means whereby red cell metabolism is capable of modulating hemoglobin function over the long term. The effect of organic phosphate on hemoglobin function is more pronounced with inositol hexaphosphate (IP₆), a component of avian blood.

The interactions of these physiological effectors of hemo-
globin function, while complex, have in the past decade become well understood in terms of their structural origins within the hemoglobin molecule. On the other hand, the structural basis for the most fundamental property of hemoglobin, cooperativity, has only just begun to be elucidated. Determination of the precise mechanism by which ligand affinity increases with fractional saturation remains a major goal of current hemoglobin research.

I.B. **Structural Properties of Hemoglobin**

Human adult hemoglobin is a tetramer of molecular weight 64,500 (8); hemoglobin A comprises greater than 95% of the hemoglobin in adult erythrocytes (9) and is comprised of two $\alpha$ and two $\beta$ subunits. The primary sequences of these chains and others from minor blood components are known and show striking homologies to the primary sequence of myoglobin from muscle tissues (10).

Perutz and coworkers have undertaken a thorough crystallographic investigation of several forms of hemoglobin A, including a variety of mutant and chemically modified molecules (11-14, 17). They have found that all hemoglobin forms crystallize in one of two readily distinguishable states, differing primarily in quaternary structure. In both states the subunits are tetrahedrally arranged such that like chains occupy equivalent positions. In addition, the subunits in both quaternary structures are arranged in $\alphaβ$ pairs such that the interaction between an $\alpha$ chain with its paired $β(α_1β_1)$ is stronger than its interaction with the other $β(α_1β_2)$.

In the first quaternary structure, characteristic of deoxy-hemoglobin and other forms with low ligand affinity, there are several contacts between subunits not of the same pair. The most important of
there are a set of four salt bridges between the N-terminus of one α subunit and the C-terminus of the other, and a large dovetailing contact at the α₁β₂ interface (15). In this structure the β subunits are relatively far apart and have juxtaposed positive charges in the cleft between them. In this deoxy conformation, the penultimate tyrosine of each chain (tyr 140α and tyr 145β) is tucked into a crevice between two α helices of the same chain near the heme pocket, and the β subunits contain an additional salt bridge between the imidazole of the C-terminal histidine and the carboxyl group of asp 94β. Each heme iron is displaced from the plane of the porphyrin ring some 0.6 Å.

The second quaternary structure is characteristic of the liganded forms of hemoglobin, and several high affinity derivatives and mutants even in the absence of ligand. In this structure the α-α salt bridges are absent and the dovetail at the α₁β₂ interface is less extensive. The two β subunits are some 6 Å closer together than in the deoxy structure. The penultimate tyrosines are out of the crevices rotating freely and the salt bridge between his 146β and asp 94β is absent. The iron atoms have moved squarely into the planes of the porphyrin rings.

Whereas the physical and spectral properties of oxyhemoglobin are adequately described by the sums of the properties of isolated α and β chains, the properties of deoxyhemoglobin are not (15-19). Apparently the more extensive subunit interactions in deoxyhemoglobin constrain the molecule and alter the properties of the subunits. For this reason, the quaternary structure of deoxyhemoglobin has been designated T (or tense) and that for oxyhemoglobin, R (or relaxed). With the structural information on the T and R quaternary structures described
above, the origins of the allosteric effects of $H^+$, $CO_2$, and DPG can be understood.

Several studies have been undertaken in an effort to determine the amino acid residues responsible for the alkaline Bohr effect. The first of these involved reacting the N-terminal amino groups of isolated chains with cyanate to form carbamyl derivatives (2,3). The chains were then recombined to form $\alpha_2^c\beta_2$, $\alpha_2^c\beta_2$, and $\alpha_2^c\beta_2^c$ species, where the superscript c indicates specifically carbamylated subunits. While carbamylation had no effect on the expression of cooperativity, the Bohr effect was reduced by 25% in tetramers having carbamylated $\alpha$ subunits. No such effect was seen on carbamylation of the $\beta$ subunits. Thus the N-terminal amino groups of the $\alpha$ subunits appear to be responsible for a quarter of the alkaline Bohr effect. In the R state these weakly basic groups are freely rotating, but in the T state participate in salt bridges with the C-termini of their partner $\alpha$ subunits (20). Participation in these salt bridges would be expected to raise the pKs, and therefore proton affinities, of these amino groups in going from the R to the T state, and would explain their contribution to the Bohr effect. A second approach in the investigation of Bohr groups involved studies on des-(his 1468) hemoglobin, in which the C-terminal histidine of the $\beta$ subunits was removed by digestion with carboxypeptidase B. It was found that this enzymatically modified protein exhibited a nearly normal amount of cooperativity but only half of the normal Bohr effect (21). A comparison of the nuclear magnetic resonance (NMR) spectra of normal and des-(his 1468) hemoglobins permitted the assignment of a specific peak in the aromatic region to his 1468 (22). The pH dependence of the position of this peak in deoxy- and carboxyhemoglobin suggested pK values
of 8.0 and 7.1, respectively, confirming the role of his 146β in the Bohr effect. In the R state his 146β is freely rotating, but in the T state is involved in an intrasubunit salt bridge with asp 94β. Participation in this salt bridge would be expected to raise the pK of his 146β in the T state. The NMR results are in agreement with work done on hemoglobin modified with N-ethylmaleimide (NEM), which also exhibits only half of the normal Bohr effect (23). NEM reacts with cys 93β and disrupts the formation of the his 146β-asp 94β salt bridge, thereby preventing the increase in pK of his 146β on switching to the T state. Some 25% of the Bohr effect remains unaccounted for, and the search continues for more residues that may be involved in release of protons on binding of ligand to hemoglobin. One particularly likely candidate is his 122β, which undergoes a complex change in environment in going from the R to the T structure (20).

The preferential binding of DPG to deoxyhemoglobin is more simply explained than that of protons (20). In the T state the organic phosphate is able to fit into the cleft between the β subunits and balance the charges of the several basic residues of the protein located there. This has the net effect of adding two new salt bridges (one to each β subunit) to the T quaternary structure. In the R state the β subunits have moved closer together and the cleft is simply too small to accommodate the DPG. The enhancement of the Bohr effect by physiological concentrations of DPG can also be explained with structural arguments (24). The strongly acidic DPG would be expected to raise the pKs of the basic residues of the β cleft, and thereby further increase the proton affinity of deoxyhemoglobin over oxy.

The effect of CO₂ on the ligand affinity of hemoglobin has
been studied using the carbamylated derivatives mentioned above (2,3). Blocking the N-terminal amino groups of the protein by reaction with cyanate abolishes the effect of CO₂ on ligand affinity. Since carbamylation has little effect on cooperativity, this is clear evidence that CO₂ produces its allosteric effect by binding to the N-terminal amino groups of hemoglobin. In addition, it has been shown with α₂β₂ and α₂β₂C hybrids that CO₂ binding to β N-termini has a larger effect on ligand affinity than binding to α N-termini (3). The demonstration that CO₂ binds to the β N-termini, which are located in the β-β cleft where DPG binds, accounts for the observation that DPG significantly reduces the effect of CO₂ on ligand affinity. It is known that physiological concentrations of CO₂ inhibit the Bohr effect considerably (25). This is due primarily to the fact that at physiological pH the carbamino groups formed at the N-termini of the protein (predominantly in the T state) dissociate, returning protons to the medium. In this regard it is not surprising that the β N-termini contribute more to the CO₂ effect than do the α as the β N-termini are located in the positively charged β-β cleft. This situation would favor the formation of the negatively charged carbamino group, and its presence in the cleft would help maintain the more open T structure. Formation of the carbamino groups at the α N-termini, on the other hand, would not be expected to stabilize the T structure as much because these groups are already involved in stabilizing salt bridges with the C-termini of their partner α subunits. The fact that CO₂ reaction at the α N-termini does stabilize the T structure (as evidenced by a decrease in ligand affinity on reaction of CO₂ with the α₂β₂C hybrid) indicates that the α carbamino groups must be involved in an interaction even more stabilizing to the T quaternary
conformation than the salt bridge lost on its formation.
II. MATHEMATICAL AND THERMODYNAMIC FORMULATIONS FOR HEMOGLOBIN

Repeated efforts have been made to develop model systems or mathematical formulations to describe the sigmoidal saturation curve of hemoglobin. The primary motivation behind such efforts is the development of a thermodynamic framework within which cooperativity can be understood. This framework is generally couched in terms of subunit interactions: somehow information in the form of free energy must be transferred from one heme site to the others through the intersubunit bonds if ligand affinity is to increase as saturation progresses. What follows is a discussion of (a) a general thermodynamic approach to the subunit interactions of hemoglobin and (b) four model systems that have made significant contributions toward understanding the function of hemoglobin.

II.A. The Thermodynamics of Subunit Interactions Within Hemoglobin

The isolated subunits of hemoglobin exhibit no cooperativity and the response of fractional saturation to ligand concentration is hyperbolic. The equilibrium constant for the ligand binding process ($K^m$) is given by the reciprocal of the ligand concentration at half-saturation and the free energy change ($\Delta G^m$) by $-RT \ln K^m$, where the superscript $m$ denotes the reaction for monomers.

For the tetrameric protein the thermodynamic cycle shown in Fig. II.1 can be constructed (26). Thus the free energy change associated with binding of the $n$th ligand to a heme site within tetrameric hemoglobin ($\Delta G_{n}^c$) can be described in terms of $\Delta G^m$ and the difference between the free energy changes for the association of monomers into tetramers before and after ligand binding:
Fig. II.1. A thermodynamic cycle for successive ligand binding to monomeric and tetrameric hemoglobin. Definitions are as follows: Hb, a free heme site; X, ligand; $\Delta G^m$, the free energy change for ligand binding to a monomer; $\Delta G^t_n$, the free energy for binding of the nth ligand to a tetramer; $\Delta G^{m+t}_n$, the free energy change for association of n ligand-bound monomers and 4-n unbound monomers into a tetramer.
\[(5-n)\text{Hb} + (n-1)\text{HbX} \rightleftharpoons \text{Hb}_4\text{X}_{(n-l)}\]

\[\Delta G^m_{n-l} \rightleftharpoons X \rightleftharpoons \Delta G^m_{n-l} \quad \Delta G^t_{n-l} \rightleftharpoons X \rightleftharpoons \Delta G^t_{n-l} \]

\[(4-n) + n\text{HbX} \rightleftharpoons \text{Hb}_4\text{X}_n\]

Fig. II.1
\[ \Delta G^t_n = \Delta G^m + (\Delta G^m_{n+1} - \Delta G^m_{n-1}) \]

By considering all four ligand binding steps it can be shown that the difference in free energy changes for the binding of four ligand molecules to isolated subunits and the intact tetramer is:

\[ \frac{4}{n=1} \left( \sum \Delta G^t_n \right) - 4 \Delta G^m = \Delta G^m_{4} - \Delta G^m_{1} \]

The term on the left of this equation reflects the amount of ligand-linked subunit interaction, and 1/4 \((\Delta G^m_{4} - \Delta G^m_{1})\) is therefore a measure of the average amount of heme-heme interaction \(\Delta G_I\) associated with cooperativity. This value is different from the more commonly used definition of:

\[ \Delta G_I = -RT \ln \left( \frac{K_4}{K_1} \right) = (\Delta G^m_{4} - \Delta G^m_{3}) - (\Delta G^m_{1} - \Delta G^m_{0}) \]

This second definition describes only the apparent free energy of subunit interaction, since it reflects the differences in subunit aggregation for the binding of the first and last ligand molecules. Equation II.3 has nonetheless become the conventional means of comparing equilibrium data as it can be determined from the upper and lower asymptotes of Hill plots (see section II.B.1), and does reflect the macroscopic mechanism of ligand binding, provided that the \(\alpha\) and \(\beta\) chains have equal affinities.

The implication of Equation II.2 is that subunit aggregation is a necessary but not sufficient condition for cooperativity to occur; there must also be a difference in aggregation in the presence and absence of ligand. The final factor that determines the extent to which a system exhibits cooperativity is the distribution of the ligand-linked
subunit interaction among the individual ligand binding steps. The more symmetric the distribution, the less pronounced will be the expression of cooperativity. In the extreme case where the distribution is even, each ligand binding step will be enhanced to the same extent over ligand binding to monomers, but not at all with respect to one another and cooperativity will not be observed. The primary differences between the various models to be described in the next section reside in the means by which the ligand-linked subunit interaction is distributed among the four steps in ligand binding to hemoglobin and the attending structural implications.

II.B. Model Systems for Hemoglobin

1. The Concerted Model of Hill—The first important model for hemoglobin was put forth by A.V. Hill in 1910 (27). He proposed that n hemoglobin binding sites reacted in a concerted fashion with n molecules of oxygen:

\[
\frac{K}{Hb_n + nO_2} \rightleftharpoons Hb_n(O_2)_n
\]

(11.4)

where \( K \) is the equilibrium constant for a reaction of nth order with respect to \( O_2 \). For this equilibrium the following equation could be derived:

\[
\frac{(Hb_n(O_2)_n)}{(Hb_n)} = \frac{Y}{(1-Y)} = K(O_2)^n
\]

(II.5)

where \( Y \) is the fraction saturation. By plotting \( \log(Y/(1-Y)) \) vs. \( \log(O_2) \) one could obtain \( n \) from the slope and \( \log(K) \) from the intercept at \( \log \left( \frac{Y}{1-Y} \right) = 0 \). The best available data (for \( Y = 0.1-0.9 \)) lent credence to the Hill model, giving a straight line with slope of 2.7
(Fig. II.2). The non-integral value of $n$ appeared to pose no particular problem at the time as proteins were considered to be of indeterminant structure; the value 2.7 was merely taken to reflect the average degree of aggregation of heme groups.

Three pieces of information eventually discounted the Hill theory. The first was the demonstration by Adair that hemoglobin was a discrete molecule of molecular weight 64,500 with four heme sites per molecule (8). A concerted mechanism should have given a value for $n$ of 4, not 3. Second, new data showed that at very high and low levels of saturation the experimental curve approached asymptotes with slopes of unity in a Hill plot (28). A simple concerted mechanism could not account for these new results. Finally, Kinetic work showed that the reaction rate for $O_2$ binding exhibited a low order dependence on oxygen concentration, not a high order one as predicted by the Hill model.

Still, Hill's $n$ provides a convenient empirical index for measuring the amount of cooperativity expressed by a hemoglobin sample and the Hill plot remains the conventional way to present equilibrium data for hemoglobin. An additional advantage in using the Hill plot is that the points where the upper and lower asymptotes intersect $\log(Y/1-Y) = 0$ correspond to the logarithms of the equilibrium constants for the first and last ligand binding steps [$\log(K_1)$ and $\log(K_4)$, respectively. The ratio of these two constants gives an indication of the amount of free energy of heme-heme interaction (c.f. eq. II.3). This value nominally reflects the free energy gain in binding ligand to the $R$ state over the $T$ state, due to the fewer constraints on ligand binding to the $R$ structure.

2. **The Adair Scheme**— After demonstrating the tetrameric nature of
Fig. II.2. A Hill plot of the equilibrium data for hemoglobin shown in Fig. I.1. The asymptotes at high and low saturation intersect the X axis at $K_T = (1/4)K_a$ and $K_R = 4K_a$, respectively.
Fig. II.2
hemoglobin, Adair went on to derive a purely phenomenological equation for the binding of ligand (30). He described the system in its broadest terms with four equilibrium constants corresponding to the sequential binding of four ligand molecules:

$$\text{Hb}_4(X)_{i-1} + X \rightleftharpoons \text{Hb}_4(X)_i \quad \text{II.6}$$

for \(i = 1\) to \(4\). By setting the concentration of \(\text{Hb}_4\) equal to unity, the concentrations of \(\text{Hb}_4(X), \text{Hb}_4(X)_2, \text{Hb}_4(X)_3,\) and \(\text{Hb}_4(X)_4\) could be shown to be equal to \(K_1(X), K_1K_2(X)^2, K_1K_2K_3(X)^3,\) and \(K_1K_2K_3K_4(X)^4\), respectively. The equation for the fraction saturation became:

$$Y = \frac{\text{Hb}_4(X) + 2\text{Hb}_4(X)_2 + 3\text{Hb}_4(X)_3 + 4\text{Hb}_4(X)_4}{4[\text{Hb}_4 + \text{Hb}_4(X) + \text{Hb}_4(X)_2 + \text{Hb}_4(X)_3 + \text{Hb}_4(X)_4]}$$

$$= \frac{K_1(X) + 2K_1K_2(X)^2 + 3K_1K_2K_3(X)^3 + 4K_1K_2K_3K_4(X)^4}{4[1 + K_1(X) + K_1K_2(X)^2 + K_1K_2K_3(X)^3 + K_1K_2K_3K_4(X)^4]} \quad \text{II.7}$$

The equilibrium constants of Adair's equation above were defined for a tetrameric hemoglobin (so-called macroscopic equilibrium constants) and to be compared directly on a per heme basis must be converted to microscopic constants using statistical weighting factors. These weighting factors arise because, for example, the binding of ligand to \(\text{Hb}_4\) is intrinsically four times more likely to occur than binding to \(\text{Hb}_4(X)_3\); similarly, ligand dissociation from \(\text{Hb}_4(X)_4\) is four times more likely than from \(\text{Hb}_4(X)\). Thus the intrinsic equilibrium constants for binding four consecutive ligand molecules are \((1/4)K_1, (2/3)K_2, (3/2)K_3,\) and \(4K_4\).
Equations for the upper and lower asymptotes of the Hill plot can now be considered. At very low levels of saturation, \( Y/1-Y \) becomes equal to \( Y \) and \( K_1(X) \) becomes much less than unity. Because the concentration of \( X \) is so small, the high-ordered terms in \( (X) \) of the Adair equation can be neglected, and:

\[
\frac{Y}{1-Y} = Y = \frac{K_1(X)}{4[1 + K_1(X)]} = (1/4)K_1(X) \quad \text{II.8}
\]

or:

\[
\log(Y/1-Y) = \log(1/4)K_1 + \log(X) \quad \text{II.9}
\]

Thus the lower asymptote of the Hill plot intersects the \( X \) axis at \( 1/4 K_1 \). Turning to the condition at very high levels of saturation, \( Y \) depends only on those terms high-ordered with respect to \( (X) \). In this case Adair's equation (II.7) becomes:

\[
Y = \frac{3 + 4K_4(X)}{4[1 + K_4(X)]} \quad \text{II.10}
\]

and:

\[
1-Y = 1/4[1 + K_4(X)] \quad \text{II.11}
\]

so:

\[
\frac{Y}{1-Y} = 3 + 4K_4(X) \quad \text{II.12}
\]

Since at high ligand concentrations the second term will dominate:

\[
\log(Y/1-Y) = \log(4K_4) + \log(X) \quad \text{II.13}
\]

The \( X \) intercept of the upper asymptote of the Hill plot is therefore \( 4K_4 \). Thus if sufficiently accurate data at the extremes of fraction saturation can be obtained to define the upper and lower asymptotes of the Hill plot, the first and last intrinsic equilibrium constants can
be determined experimentally. Such data does exist (e.g., 28) and it has been determined that \(4K_4\) exceeds \((1/4)K_1\) by a factor of about 300. The ratio of \(4K_4\) to \((1/4)K_1\) reflects the gain in free energy of ligand binding to Hb\(_4\)X\(_3\) state over Hb\(_4\), \(\Delta G_1\) as mentioned, and corresponds to about 3.4 kcal/mol at room temperature. In other words, some 3.4 kcal/mol of energy tied up in the structure of Hb\(_4\) opposes ligand binding and constrains it in the T quaternary structure.

3. **The Sequential Models of Pauling and Koshland** — In 1935 Pauling proposed a model for hemoglobin based on a single fundamental equilibrium constant \(K'\) for ligand binding and an interaction constant \(\alpha\) that reflected the stabilization gained in having adjacent liganded subunits interact (31).

Because subunit interaction is a central feature of the model, the results depend on the protein geometry chosen. With a tetrahedral geometry, for example, there would be no stabilization on binding of the first ligand molecule. Subsequent additions, however, would be stabilized by the formation of as many favorable subunit interactions as there were ligand molecules on the hemoglobin prior to the reaction under consideration, with cooperativity ensuing (Fig. II.3.A). Thus the equilibrium constants from the Adair scheme (again taking into account the statistical weighting factors) become:

\[
\begin{align*}
K_1 &= 4K' \\
K_2 &= (3/2)K'\alpha \\
K_3 &= (2/3)K'\alpha^2 \\
K_4 &= (1/4)K'\alpha^3
\end{align*}
\]

For a square arrangement, the reaction sequence would be that shown in Fig. II.3.B, and the Adair constants become:
Fig. II.3. Two models for the interaction of hemoglobin with ligand according to the scheme of Koshland, Nemethy, and Filmer (refs. 30, 31): A, the tetrahedral geometry, and B, the square geometry.
\[ K_1 = 4K' \]
\[ K_2 = \frac{K' + 2K'\alpha}{2} \]
\[ K_3 = (K'\alpha + K'\alpha^2)/3 \]
\[ K_4 = (1/4)K'\alpha^2 \]

Pauling found that both the tetrahedral and square configurations (but not, for example, a linear one) fit the available equilibrium data adequately, giving \( \alpha = 6.7 \) and 17.3, respectively. These values correspond to a free energy of subunit interaction of between 1 and 1.5 kcal/mol.

The Pauling model assumes a condition of structural promotion, that is, stabilizing interactions between liganded subunits are formed in the sequential addition of ligand molecules. Binding of the first ligand is promoted the least and binding of the last the most. An equally plausible situation would be one of structural constraint, where stabilizing bonds between unliganded subunits are broken on binding successive ligand molecules. In this formulation, three stabilizing bonds would be broken on binding the first ligand, two on the second, one on the third, and none on binding the last for the tetrahedral model. Thus in the context of structural constraint, cooperativity arises because binding of the first ligand molecule is the most constrained and binding of the last the least. In this case the Adair equilibrium constants become:

\[ K_1 = 4K'/\alpha^3 \]
\[ K_2 = (3/2)K'/\alpha^2 \]
\[ K_3 = (2/3)K'/\alpha \]
\[ K_4 = (1/4)K' \]

The difference between structural promotion and constraint is subtle but important. While the two are very similar in mathematical form, structural promotion starts with a molecule having normal ligand
affinity and by means of the favorable interaction between liganded subunits enhances that affinity as ligand binding proceeds. Structural constraint, on the other hand, starts with a molecule whose ligand affinity is considerably reduced by the favorable interaction of unliganded subunits and, as ligand binding progresses, returns that affinity to normal. In the case of structural promotion, the free energy of heme-heme interaction is associated with enhanced ligand affinity in the R state; with structural constraint it is associated with suppressed ligand affinity in the T state. The structural data presented previously in conjunction with the observation that isolated chains exhibit properties similar to the liganded, not the unliganded, form of the intact tetramer indicate that hemoglobin is best described by a system of structural constraint.

Pauling's work has been extended by Koshland and coworkers in recent years (32, 33). Koshland proceeds by assuming two subunit conformations A and B with an equilibrium constant $K_t = (B)/(A)$; ligand binding induces a conformational change from A to B. In this new formulation, Pauling's fundamental $K'$ becomes $K_s K_t$ where $K_s$ is the equilibrium constant for ligand binding to the B tertiary structure. In addition, Koshland defines a new term for the interaction of unlike subunit conformations ($K_{AB}$) as well as like ($K_{BB}$, corresponding to Pauling's $\alpha$). Thus for a tetrahedral geometry the four Adair constants become:

\[
K_1 = 4K_sK_tK_{AB}^3 \quad \quad K_3 = \frac{2K_sK_tK_{BB}}{K_{AB}^2} \quad \quad K_2 = \frac{3K_sK_tK_{BB}}{2K_{AB}^3} \quad \quad K_4 = \frac{K_sK_tK_{BB}^3}{K_{AB}^4}
\]

II.17

With a square subunit arrangement:
\[
K_1 = 4K_s K_{t} K_{AB}^2 \quad K_3 = \frac{K_k K_s K_{BB}^2 + K_s K_k K_{BB}}{3} \\
K_2 = \frac{K_s K_{t} K_{AB}^2 + 2K_s K_{BB}}{2} \quad K_4 = \frac{1}{4} K_s K_{t} K_{BB} K_{AB}
\]

II.18

Since Koshland adopted Pauling's convention of structural promotion, to be directly applicable to the hemoglobin system the \(K_{BB}\) term must be inverted to \(K_{AA}\) so that the Adair constants become:

\[
K_1 = 4K_s K_k K_{AA}^{-3} K_{AB}^3 \quad K_3 = \frac{2}{3} K_s K_{k} K_{AA}^{-1} K_{AB}^{-1} \\
K_2 = \frac{3}{2} K_s K_k K_{AA}^{-2} K_{AB}^{-1} \quad K_4 = \frac{1}{4} K_s K_{k} K_{AA}^{-3}
\]

II.19

for a square geometry:

\[
K_1 = 4K_s K_{t} K_{AA}^{-2} K_{AB}^2 \quad K_3 = \frac{K_s K_{k} K_{AB}^{-2} + K_s K_{k} K_{AA}^{-1} K_{AB}^{-1}}{3} \\
K_2 = \frac{K_s K_{t} K_{AA} K_{AB}^2 + K_s K_{k} K_{AA} K_{AB}^{-1}}{2} \quad K_4 = \frac{K_s K_{t} K_{AB}^{-2}}{4}
\]

II.20

Since A-B bonds are initially formed on ligand binding to \(A_4\), but are lost on saturation to \(B_4(X)\) regardless of geometry, the behavior of the model depends critically on the value of \(K_{AB}\). As \(K_{AB}\) goes to 0 or 1, Koshland's model reduces to that of Hill or Pauling, respectively. As \(K_{AB}\) becomes very large relative to \(K_{BB}\), the sequential binding of ligand can proceed no farther than the second step, as further binding would involve breaking the very stable A-B bonds.

It should be noted that Koshland's model, termed the sequential model because of the step-wise fashion with which it treats ligand binding, does not propose a specific molecular mechanism for hemoglobin. While specific molecular mechanisms based on Koshland's theoretical work can be formulated, in its general form the model only...
describes the four Adair constants in terms of the thermodynamics of various subunit interactions.

4. **The Allosteric Model of Monod, Wyman, and Changeux**

In 1965 Monod, Wyman, and Changeux proposed a model that departed radically from the sequential models discussed above (38). They suggested that a system could exhibit cooperativity provided that it could exist in two different conformational states which are in equilibrium with one another, that the states differ in ligand affinity, and that the state with the lower affinity is preferred in the absence of ligand. Such a system would require structural constraint to maintain the low affinity state in the absence of ligand. Monod et al. referred to the low affinity state as T and the high affinity state as R. This convention was later adopted by Perutz in describing his crystallographic studies.

According to the model, ligand initially is bound to the low affinity T state of hemoglobin. At some point in the course of saturation, sufficient ligand is bound to induce a quaternary conformational change to the R state, thereby raising the affinity of the remaining unfilled heme sites. This formulation, called the two-state or allosteric model, is described by three constants: L, the equilibrium (or allosteric) constant for the R to T conformational change; c, the ratio of the microscopic ligand dissociation constants for the R and T states ($K_R$ and $K_T$, respectively); and $\alpha$, the concentration of free ligand normalized by dividing by $K_R$. The overall scheme is shown in Fig. II.4.A. The ten intermediates ($R_0 - R_4$, $T_0 - T_4$) of this scheme can be described in terms of the three constants given above as shown in Fig. I.4.B. Since:
Fig. II.4. The allostERIC model of Monod, Wyman, and Changeux (ref. 32). A, the R and T state manifolds of the model, where the subscript in the R (R$_n$) or T (T$_n$) state. B, equations for R$_n$ and T$_n$ in terms of the variables of the allostERIC model and R$_0$. L is the allostERIC equilibrium constant between R$_0$ and T$_0$, c the ratio of ligand association constants for the T and R states, and α the free ligand concentration divided by K$_R$. 
\[ R_0 \quad T_0 \]
\[ R_0 + X \equiv R_1 \quad T_0 + X \equiv T_1 \]
\[ R_1 + X \equiv R_2 \quad T_1 + X \equiv T_2 \]
\[ R_2 + X \equiv R_3 \quad T_2 + X \equiv T_3 \]
\[ R_3 + X \equiv R_4 \quad T_3 + X \equiv T_4 \]

A.

\[ T_0 = R_0 L \]
\[ R_1 = 4R_0 a \quad T_1 = 4R_0 Lc a \]
\[ R_2 = (3/2)R_1 a = 6R_0 a^2 \quad T_2 = (3/2)T_1 c a = 6R_0 L(c a)^2 \]
\[ R_3 = (2/3)R_2 a = 4R_0 a^3 \quad T_3 = (2/3)T_2 c a = 4R_0 L(c a)^3 \]
\[ R_4 = (1/4)R_3 a = R_0 a^4 \quad T_4 = (1/4)T_3 c a = R_0 L(c a)^4 \]

B.

Fig. II.4
\[ Y = \frac{(R_1+2R_2+3R_3+4R_4) + (T_1+2T_2+3T_3+4T_4)}{4[(R_0+ R_1+R_2+R_3+R_4) + (T_0+T_1+T_2+T_3+T_4)]} \] II.21

It can be shown after considerable algebra that:

\[ Y = \frac{\alpha(1+\alpha)^3 + L\alpha(1+\alpha)^3}{(1+\alpha)^4 + L(1+\alpha)^4} \] II.22

According to this model the four Adair constants are described by the statistical factor, \(1/K_R\) (inverted because it is defined as a dissociation constant in the allosteric model), and a ratio which describes the fraction of molecules in the R state before and after the addition of the nth ligand molecule (for a given intermediate in the Adair scheme, \(R_n/(R_n+T_n) = 1/(1+Lc^n)\), c.f. ref. 36):

\[ K_1 = 4(1 + Lc)/K_R(1 + L) \]

\[ K_2 = (3/2)(1 + Lc^2)/K_R(1 + Lc) \] II.23

\[ K_3 = (2/3)(1 + Lc^3)/K_R(1 + Lc^2) \]

\[ K_4 = (1/4)(1 + Lc^4)/K_R(1 + Lc^3) \]

Thus the Monod–Wyman–Changeux model attributes the free-energy gain on binding ligand that is associated with cooperativity to an allosteric shift in equilibrium from a low affinity to a high affinity conformation. In contrast to the models of Pauling and Koshland, the allosteric model represents a true molecular mechanism, since two discrete microscopic conformations in equilibrium with one another are proposed.

5. **An Analysis of the Sequential and Allosteric Models**

Of the sequential and allosteric models, the latter is gener-
ally considered to be the more advantageous in describing hemoglobin. In order to construct a specific mechanism from the general model of Koshland, each of the intermediates in the Adair scheme must be assigned a distinct conformation, and a minimum of four microscopic conformational equilibria must be considered. The determination of the corresponding equilibrium constants would pose a serious experimental obstacle. In addition, a simple sequential model has difficulty accounting for such basic experimental observations as the occurrence of only two quaternary conformations and the influence of allosteric effectors on hemoglobin function.

The allosteric model, with its single microscopic equilibrium (T*R), does not suffer from these shortcomings. $K_R$ and $K_T$, and therefore $c$, can often be determined directly from the Hill plot as described previously. The allosteric model is tacitly assumed in describing the crystallographic data of Perutz and coworkers, and the action of allosteric effectors. Still, the allosteric model must be considered only a first approximation in describing hemoglobin function. While the model fits equilibrium data quite well, it falls short when considering kinetic experiments (37, 38). For example, the release of organic phosphates lags behind ligand binding in kinetic experiments, as expected using a model where the only conformational change takes place relatively late in the process of ligand binding. However, proton release does not lag but remains proportional to ligand binding throughout the reaction. Thus important conformational changes at the tertiary level, responsible for the release of Bohr protons on binding of ligand, must be taking place for which the allosteric model cannot account.
Recently, Perutz has proposed a stereochemical model for heme-heme interaction based on his own crystallographic data (20). The central concept of Perutz's model has to do with a change in spin state of the heme iron on ligand binding. In deoxyhemoglobin the iron is high-spin and, having a relatively large atomic radius, is unable to fit into the plane of the porphyrin ring. Perutz proposes that on binding ligand the iron undergoes a shift to lower spin state and, with the concomitant reduction in atomic radius, is now able to move into the plane of the porphyrin ring, pulling the proximal histidine with it. The movement of the histidine triggers further structural changes, eventually breaking the various bonds of structural constraint when enough ligand molecules have bound to the tetramer and thus bringing about the change in quaternary structure. Because of these structural constraints, the proximal histidine prefers the more distant position from the porphyrin ring and it is only the favorable interaction of iron with ligand that induces the histidine to move nearer to the ring. It is this situation that Perutz refers to when he speaks of tension at the hemes (39). Unfortunately, it has been shown that inositol hexaphosphate (IP₆) and the sulfhydryl reagent p-hydroxymercuribenzoate (PMB), two molecules that produce marked and opposite effects on the quaternary structure of hemoglobin (IP₆ stabilizes the T structure, PMB the R) produce very similar changes in the spin state of the iron (40, 41). Thus Perutz's correlation between iron spin state and quaternary structure cannot be totally accurate, and further work is needed.
III. CHAIN HETEROGENEITY

The models discussed in the previous section all make the somewhat arbitrary assumption that the α and β subunits of the hemoglobin tetramer are functionally equivalent in their interactions with one another and with ligands. In the past ten years considerable evidence has been accumulated to suggest that in many, if not most, respects this is not the case: that indeed the chains do not behave identically. Therefore, in their original forms even the relatively sophisticated allosteric and sequential models must be taken as only approximations of hemoglobin behavior. This chapter discusses first the experimental observations implicating chain heterogeneity, and then describes the effects on the various model systems as attempts to accommodate these observations are made.

III.A. Experimental Evidence for Chain Heterogeneity

1. Methemoglobin — The first observations implicating chain nonequivalence came from the laboratory of Q. H. Gibson in stopped-flow experiments with methemoglobin (hemoglobin in which the heme irons have been oxidized to the ferric form with potassium ferricyanide). It was found that under pseudo-first order conditions the reaction of methemoglobin with azide (a ferric heme ligand) was markedly biphasic, with a six-fold difference in the rates of the fast and slow phases (42). Furthermore, there was a distinct wavelength dependence of the signs and amplitudes of both phases, suggesting that the heterogeneous time course was due to the presence of two functionally different heme sites in methemoglobin. Equilibrium and kinetic data for the isolated chains in the met form confirmed the above hypothesis and established that the β subunits were
the faster reacting species, and \( \alpha \) the slower. Biphasic time courses were also observed for nitrite and thiocyanate (42) and imidazole binding (43), but not cyanide or fluoride binding (42). This suggested a correlation between ligand size and the expression of chain differences.

Because methemoglobin was isomorphous to liganded hemoglobin and consequently no conformational change from a low affinity to a high affinity state took place, the chain differences observed with azide and other met ligands did not necessarily pertain to the binding of ferrous ligands such as \( O_2 \) and CO. Chain heterogeneity was first observed in a cooperative system with the reduction of methemoglobin to deoxyhemoglobin by sodium dithionite. It was found that both the reduction potentials \( (E_m^\alpha = +0.052V, E_m^\beta = +0.113V; \text{ ref. 44}) \) and rates of reduction \( (k_\alpha = 6.65^{-1}, k_\beta = 75s^{-1}; \text{ ref. 45}) \) were different for the two chains.

2. Reduced Hemoglobin— With the indication from the methemoglobin studies that larger ligands were more likely to express chain heterogeneity than smaller ones (42), efforts were made to find chain differences in the reaction of reduced hemoglobin with ligands bulkier than \( O_2 \) or CO. The first success was with \( n \)-butylisocyanide (nBNC), where stopped-flow experiments showed that at moderate ionic strength and pH the \( \beta \) chains bound and released ligand approximately ten times faster than \( \alpha \) chains, but that the \( \alpha \) chains had a slightly higher affinity (46,47).

Biphasic time courses were also observed for the displacement of \( O_2 \) from hemoglobin by CO (48) and these results were also interpreted in terms of chain differences. Laser photolysis experiments on hemoglobin solutions in equilibrium with small amounts of \( O_2 \) (\( Y < 0.05 \)) showed that even in the absence of IP, marked chain heterogeneity was
evident for both association and dissociation in the first step of ligand binding (49).

The pronounced chain heterogeneity of the nBNC reaction with hemoglobin provided the basis for investigations into the effects of pH and organic phosphates on the individual chains within the tetramer (38). It was found that both protons and IP$_6$ modified the kinetic properties of the $\alpha$ chains much more than the $\beta$. This was surprising in light of the facts that IP$_6$ binds to the chains exclusively (in the $\beta-\beta$ cleft), and that well over half of the Bohr effect has been attributed to residues on the $\beta$, not the $\alpha$ subunits on the basis of structural studies (c.f. Chapter I.). Further studies were made on the effect of nBNC binding on the affinity of hemoglobin for Bohr protons and organic phosphates (37). It was found that, in agreement with earlier work using CO, the release of a fluorescent analog to IP$_6$ (8-hydroxy-1,3,6-pyrene trisulfonate, HPT) lagged significantly behind ligand binding, indicating that release was associated with the quaternary conformational change from the T state to the R. On the other hand, under conditions where chain heterogeneity was not expressed (high pH or low ionic strength) the release of Bohr protons closely followed the binding of both nBNC and CO. Under conditions favoring chain differences, however, there was a distinct lag in proton release relative to ligand binding. Since the lag was observed only in concurrence with the expression of chain heterogeneity, it was concluded that ligand binding to the more rapidly reacting $\beta$ chains contributed less to the release of Bohr protons than binding to the slower $\alpha$ chains. Analysis showed that the relative contributions of the $\alpha$ and $\beta$ subunits to the release of Bohr protons was 4:1, respectively. This finding was in agreement with the
observation discussed previously that protons had a greater effect on ligand binding to the α chains than to the β (38).

The unique kinetics of the nBNC reaction were also used to determine the contribution of each chain to the absorbance change observed on association of subunits into tetramer (i.e., the conversion from the R to the T state; ref. 50). The difference between the absorption change for nBNC binding to deoxyhemoglobin and the average of the absorption changes for ligand binding to the isolated chains was shown to be equal to the absorption change for chain aggregation (R→T) measured directly (simply by mixing the two deoxy chains in a stopped-flow apparatus). Because the reaction of nBNC with deoxyhemoglobin could be resolved into components for each chain by virtue of its biphasic time course, the relative chain contributions to the R→T absorbance transition at each wavelength could be determined from the following equations:

$$\Delta A_{\alpha}^{R\to T} = (\Delta A_{\text{tot}} - \Delta A_{\text{fast}} f) - \Delta A_{\beta}$$

$$\Delta A_{\beta}^{R\to T} = \frac{\Delta A_{\text{fast}}}{f} - \Delta A_{\beta}$$

where $\Delta A_{\alpha}^{R\to T}$ and $\Delta A_{\beta}^{R\to T}$ are the respective contributions of each chain to the R→T transition, $\Delta A_{\alpha}$ and $\Delta A_{\beta}$ are the absorbance changes for nBNC binding to the isolated chains, $\Delta A_{\text{fast}}$ and $\Delta A_{\text{tot}}$ are the absorbance amplitudes for the fast phase and overall change on binding nBNC, respectively, and $f$ is the fraction of β chains reacting rapidly. It was found that by far the major part of the absorbance change accompanying the R→T transition arose from the α chains, providing further evidence that the two chains were not functionally equivalent.
3. **Hybrid Hemoglobins**—With the clear demonstration of chain heterogeneity under at least certain experimental conditions, efforts were made to prepare artificial intermediates of the Adair scheme and analyze their properties in terms of chain differences. Two types of intermediates were successfully prepared. The first, the so-called symmetrical hybrid, was made by fixing one isolated chain in the liganded form (either by reaction with the very tightly bound NO or by oxidation to the met form followed by reaction with CN−) then recombining it with the other chain to form a tetramer containing only one type of chain capable of reacting with ligands (51,52). The second type of intermediate was the asymmetrical hybrid \((\alpha^{\text{NO}} \beta^{\text{NO}})\alpha\beta\) and was prepared by allowing a mixture of nitrosylhemoglobin and oxyhemoglobin to dissociate into dimers and reassociate randomly, then removing the oxygen with dithionite (53). Investigations of these two types of intermediates generated a considerable amount of information which, although somewhat confusing, must be reconciled in any working hypothesis of hemoglobin function.

Initial studies with the cyanomet hybrids \(\alpha^{\text{II}}_2 \beta^{\text{III-CN−}}_2\) and \(\alpha^{\text{III-CN−}}_2 \beta^{\text{II}}_2\) showed that only the former showed any Bohr effect in binding \(O_2\) (51). This agrees with the nBNC studies suggesting that the Bohr effect was primarily associated with ligand binding to the \(\alpha\) chains (57). It was found in further studies (54) that the reactions of both hybrids with CO were very similar, being strongly biphasic and exhibiting wavelength heterogeneity. In both cases the addition of \(\text{IP}_6\) resulted in the disappearance of the faster phase. Since in each experiment only one type of chain was reacting with CO, the biphasic time courses were taken to reflect a situation where the hybrids were in equilibrium between the R and T states under the experimental conditions. The addi-
tion of IP_{6} shifted that equilibrium over to the T state. Other experiments, however, were not so easily explained in terms of the allosteric model. While IP_{6} changed both hybrids from predominately rapidly reacting species (approximately 70%) to slowly reacting forms, the optical absorbance change characteristic of the R→T transition was observed only with α_{2} ^{II} β_{2} ^{III-CN}. Also, even in the absence of IP_{6} both hybrids exhibited the extremely small equilibrium constants for dissociation into dimers (K_{4,2} = 10^{-12} M) characteristic of the T, not the R, state.

The symmetrical nitrosyl hybrids α_{2} ^{NO} β_{2} and α_{2} ^{NO} β_{2} were first prepared and studied by Antonini and coworkers (50), who showed that both hybrids reacted with CO at rates intermediate between those characteristic of the R and T states, with α_{2} ^{NO} β_{2} reacting somewhat faster than α_{2} ^{NO} β_{2}. It was later found that, unlike α_{2} ^{NO} β_{2} or either of the cyanomet hybrids, α_{2} ^{NO} β_{2} exhibited the unsuppressed Soret optical spectrum characteristic of the T state (55). In addition, the binding of CO to α_{2} ^{NO} β_{2}, but not α_{2} ^{NO} β_{2}, was shown to be followed by a slow first order spectral change. The implication of all these results was that α_{2} ^{NO} β_{2} and α_{2} ^{NO} β_{2} existed in different conformations.

Further work with the nitrosyl hybrids was stimulated by the characterization of the electron paramagnetic resonance (EPR) spectra of nitrosyl complexes of hemoproteins (56,57). It was shown that the spectra of several hybrids with NO on the β chains (α_{2} ^{NO} β_{2}, α_{2} ^{NO} β_{2}, α_{2} ^{NO} β_{2}, α_{2} ^{NO} β_{2}, α_{2} ^{NO} β_{2}) were all identical to the spectrum of isolated β chains. The spectra of the corresponding α hybrids, however, were not all identical but could be divided into two groups. The first (α_{2} ^{NO} CO, α_{2} ^{NO} O_{2}, α_{2} ^{NO} IIIECN−, α_{2} ^{NO} IIIN−) contained β chains whose heme irons were low-spin, and had EPR spectra identical to isolated α chains. The second
group \((\alpha_2^{\text{NO}} \beta_2, \alpha_2^{\text{NO}} \beta_2 \text{IEF}^-, \alpha_2^{\text{NO}} \beta_2 \text{IEF}^2\text{O})\) contained high-spin \(\beta\) heme irons and gave very similar EPR spectra, characterized by a pronounced three-line hyperfine structure. These results indicated that the \(\alpha\) subunits were sensitive to subtle differences in the structure of the \(\beta\) subunits (but that the converse was not true) and that the spin-state of the \(\beta\) heme irons was an important contributing factor to this effect.

In contrast to the results with the symmetrical hybrids, it was found that the asymmetrical \((\alpha^{\text{NO}} \beta^{\text{NO}})\alpha\beta\) reacted with CO at a slow rate very close to that of the T state (53). While these results are only preliminary, the implication is that, at least in the special case of NO, the properties of the Adair intermediate \(\text{Hb}_4\text{X}_2\) depend on the arrangement of the two ligands within the tetramer. For NO, the intermediates increased in R-like properties in the order \((\alpha^{\text{NO}} \beta^{\text{NO}})\alpha\beta < \alpha_2^{\text{NO}} \beta_2 < \alpha_2^{\beta} \beta_2\).

Sugita and coworkers took a different approach to the investigation of chain differences in hemoglobin function (58–60). By substituting a modified heme (mesoheme) for the naturally occurring protoheme in one chain then recombinating it with the native form of the other chain, it was possible to make a tetramer whose \(\alpha\) and \(\beta\) subunits exhibited easily distinguishable spectral properties. It was found that the aggregation of \(\alpha^\text{meso}\) and \(\beta^\text{proto}\) chains showed the same optical change associated with the R\(\rightarrow\)T transition as \(\alpha^\text{meso} + \beta^\text{meso}\); similarly \(\alpha^\text{proto}\) and \(\beta^\text{meso}\) recombined to give a spectral change very similar to \(\alpha^\text{proto} + \beta^\text{proto}\) (60). Thus it was established independent of the previously discussed nBNC studies (50) that the R\(\rightarrow\)T optical change arises predominantly from the \(\alpha\) chains. Because of the significantly different spectral characteristics of meso- and protohemoglobin, a
given chain could be observed spectrally for any reaction with the heme hybrids simply by observing the reaction at an isosbestic point for the heme in the other chain. The dissociation of $O_2$ from the hybrids was studied in this manner and it was found in agreement with the laser photolysis and CO displacement work described above (49,61) that $O_2$ dissociated faster from the $\beta$ chains than the $\alpha$ in both $\alpha^\text{meso}_2\_\beta^\text{proto}_2$ and $\alpha^\text{proto}_2\_\beta^\text{meso}_2$ (59).

III.B. The Effect of Chain Heterogeneity on Model Systems

With the unequivocal demonstration that the $\alpha$ and $\beta$ subunits of hemoglobin are not functionally equivalent, attempts have been made to incorporate chain heterogeneity into the various models used to describe hemoglobin function. In the case of the allosteric model, the following saturation equations for each chain can be derived (36):

$$Y_\alpha = \frac{a(1+a)(1+b) + L c_a(1+c_a)(1+c_b)}{(1+a)^2(1+b)^2 + L(1+c_a)^2(1+c_b)^2}$$  \hspace{1cm} \text{III.3.}

$$Y_\beta = \frac{b(1+b)(1+a) + L c_b(1+c_a)(1+c_b)}{(1+a)^2(1+b)^2 + L(1+c_a)^2(1+c_b)^2}$$  \hspace{1cm} \text{III.4.}

where $a = (X)/K^\alpha_R$, $b = (X)/K^\beta_T$, $c_a = K^\alpha_R/K^\alpha_T$, $c_b = K^\beta_R/K^\beta_T$ and $L = (T)/(R)$ as before. The overall saturation equation (Eq. II. A) becomes the average of $Y_\alpha$ and $Y_\beta$:

$$Y_{tot} = \frac{Y_\alpha + Y_\beta}{2} = \frac{(1+a)(1+b)[a(1+b) + b(1+a)] + L(1+c_a)(1+c_b)[c_a(1+c_b) + c_b(1+c_a)]}{2(1+a)^2(1+b)^2 + 2L(1+c_a)^2(1+c_b)^2}$$  \hspace{1cm} \text{III.5}

The sequential model has even more difficulty accommodating chain differences, due to its emphasis on intersubunit interactions. Not only must different chain affinities be taken into account, but possible sub-
unit interactions for like and unlike chains in both A and B conformational arrangements must be considered. Even by assuming that $K_{AB}$ is equal to unity for all subunit interactions between unlike conformations, the overall saturation equation becomes that shown in Figure III.1 for a square geometry. It should be noted that the $K_s$ and $K_t$ terms of equations II.18 and II.20 always appear together. For this reason it is impossible to distinguish between chain heterogeneity arising from different ligand affinities for the B conformations of the two chains and that arising from different equilibrium constants for the $A \leftrightarrow B$ conformational change within each chain. The equation in Figure III.1 has been derived arbitrarily assuming that chain differences are expressed in the ligand affinities ($K_s^\alpha$ and $K_s^\beta$) only. The Adair scheme has also been modified to include chain differences (47), the result being an array of ten intermediates whose relative concentrations are determined by 16 equilibrium constants (Fig. III.2). The saturation equation for this expanded scheme remains in the general form of equation II.7:

$$Y = \frac{A_1(X) + 2A_2(X)^2 + 3A_3(X)^3 + 4A_4(X)^4}{4[1+A_1(X) + A_2(X)^2 + A_3(X)^3 + A_4(X)^4]} \quad \text{III.6}$$

where now $A_1 = K_{1,2} + K_{1,3}$; $A_2 = K_{1,2}(K_{2,4} + K_{2,5}) + K_{1,3}(K_{3,6} + K_{3,7})$; $A_3 = K_{1,2}K_{2,4}K_{4,8} + K_{1,3}K_{3,7}K_{7,9}$; and $A_4 = K_{1,2}K_{2,4}K_{4,8}K_{8,10}$; and $K_{x,y}$ is the equilibrium constant between intermediates $x$ and $y$.

Clearly, the expression of chain heterogeneity complicates the interpretation of equilibrium data. The problem is even more severe when considering kinetic data, where chain differences may be manifested in either the association or dissociation rate constants for a given step, or both, with the possibility of cancelling one another
Fig. III.1. The saturation equation for a square Koshland model taking into account chain heterogeneity. The equation was derived assuming $K_{AB}$ for all subunit interactions was equal to unity and that chain heterogeneity was expressed only in the $K_s$ term of the product $K_s \cdot K_t$. All terms are used as defined in the text with the notations $\alpha$ and $\beta$ referring to constants for the individual subunits.
\[ y = \frac{0.5(k_6^0k_e + k_6^0k_e)(S) + 0.5(k_6^0k_e)^2 + (k_6^0k_e)^2 + 4(k_{ba}k_{ba} + k_{bb}k_{ba} + 1)(k_6^0k_e)(k_6^0k_e)(S)^2 + 1.5(k_{ba}k_{ba}k_{bb}k_{ba})(k_6^0k_e)(k_6^0k_e)(k_6^0k_e)(k_6^0k_e)(S)^3 + (k_6^0k_e)^2 (k_6^0k_e)^2 k_{ba}k_{ba}k_{bb}k_{ba}(S)^4}{(1 + 2(k_6^0k_e + k_6^0k_e)(S) + (k_6^0k_e)^2 + (k_6^0k_e)^2 + 4(k_{ba}k_{ba} + k_{bb}k_{ba} + 1)(k_6^0k_e)(k_6^0k_e)(S)^2 + 2(k_{ba}k_{ba}k_{bb}k_{ba})(k_6^0k_e)(k_6^0k_e)(k_6^0k_e)(k_6^0k_e)(S)^3 + (k_6^0k_e)^2 (k_6^0k_e)^2 k_{ba}k_{ba}k_{bb}k_{ba}(S)^4} \]

Fig. III.1
Fig. III.2. The Adair scheme expanded to include chain heterogeneity.
Fig. III.2
out. A phenomenological description of the kinetic time course of lig-
and binding now requires the determination of 32 rate constants instead
of the 8 needed in the absence of chain differences. While only 25 of
these rate constants are independent due to detail balance (62), each
of the rate equations for the ten Adair intermediates must be numerical-
ly integrated to simulate data using this scheme. This is tedious at
best to both man and computer. Nonetheless, the need to incorporate
chain heterogeneity into computer simulations of both equilibrium and
kinetic data is unavoidable.

Chain differences also complicate the determination of the
average free energy of heme-heme interaction, \(\Delta G_I\), discussed in section
II.A (26). At low levels of saturation, ligand molecules will preferen-
tially bind to the chain with the higher affinity, and at high levels
of saturation the chain with the lower affinity will be less saturated
and more available for ligand binding. Thus the lower asymptote in a
Hill plot of the equilibrium data will be biased toward the high affini-
ty chain and the upper asymptote toward the low affinity chain. This
moves the asymptotes closer together and results in apparent decreases
of both \(\Delta G_I\) and the Hill coefficient \(n\). It should be noted that the
decrease in \(\Delta G_I\) is only apparent, since in the presence of chain hetero-
geneity this term is given by the average of the heme-heme interactions
for the individual chains:

\[
\Delta G_I = -1/2 \ RT [\ln(K_4^\alpha / K_1^\alpha) + \ln(K_4^\beta / K_1^\beta)]
\]  

III.7

and not merely by the horizontal distance between the asymptotes [i.e.,
\(-RT\ln(K_4 / K_1)\)]. Thus it is possible for two ligands to have similar mech-
anisms for binding to hemoglobin (as evidenced by similar values for \(\Delta G_I\))
even though the indices of cooperativity, n, may differ markedly due to chain heterogeneity.

Cooperativity and chain heterogeneity have opposing effects on the kinetics of ligand binding to hemoglobin. Cooperativity normally causes an acceleration of the reaction (an increase in the observed rate constant with time) while chain heterogeneity results in a deceleration (decreasing rate constant with time) as the faster reacting species is depleted earlier in the reaction, leaving only the slower to bind ligand later in the reaction. In the absence of cooperativity, chain differences would be relatively easy to observe as a biphasic time course. Cooperativity, however, is capable of masking even a three- to fivefold difference in ligand binding rates by increasing the rate of binding to the intrinsically slower chain late in the reaction (62). Equilibrium data are even less sensitive to chain differences (apart from the effects on $\Delta G^\circ$ discussed above) and a tenfold difference in chain affinities causes no obvious qualitative change in the shape of a Hill plot to indicate chain heterogeneity. Thus significant chain differences can be masked by the inherent cooperativity of the hemoglobin system and it is only through the careful examination of kinetic and equilibrium data that these chain differences can be discerned.
IV. PRIOR WORK ON THE REACTION OF HEMOGLOBIN WITH NITRIC OXIDE

The reaction of nitric oxide was discovered in 1865 by L. Hermann (63) who showed, using gasometric methods, that NO had at least a fivefold greater affinity for hemoglobin than CO, and was able to replace CO bound to hemoglobin on a 1:1 basis. It was not until much later, however, that the first quantitative work with NO appeared. Taking advantage of the higher affinity of hemoglobin for NO than CO, the replacement reaction was used to determine $k_4$, the rate constant for the dissociation of the fourth CO molecule from sheep hemoglobin (64). It had been shown previously (65) for the replacement of O$_2$ by CO that the observed rate of displacement was given by

$$\frac{r}{4} = \frac{k_4}{k_4(0_2) + \frac{4}{1^i_4}(CO)}$$

where $k_4^i$ and $k_4$ are the rate constants for the association and dissociation of the fourth O$_2$ molecule and $1^i_4$ is the rate constant for the association of the fourth CO molecule. By plotting $1/r$ versus (O$_2$) at constant (CO), a straight line could be obtained with Y intercept $= 1/k_4^i$ and slope $= 4k_4^i/1^i_4k_4(CO)$, permitting the easy determination of both $k_4$ and $k_4^i/1^i_4$. Similarly, $1_4$ and $1_4^i/j_4^i$ ($j_4^i$ being the association rate constant for the fourth NO molecule) were obtained from the replacement of NO for CO, giving values of 0.03 s$^{-1}$ and 0.25, respectively, at pH 9.0 and 20°C (64).

Experiments with the then new stopped-flow spectrometer on the reaction of NO with sheep hemoglobin showed that the microscopic rate of binding the fourth NO molecule was 3.3 times faster than binding of the
first \(j'_4 = 25 \times 10^6 \text{M}^{-1}\text{s}^{-1}\) as opposed to \(j'_1 = 7.5 \times 10^6 \text{M}^{-1}\text{s}^{-1}\) at pH 9.0 and 0.5°C; refs. 66,67). Later work with human hemoglobin (68) indicated that the microscopic association constant remained unchanged at \(j'_1 = 2.5 \times 10^7 \text{M}^{-1}\text{s}^{-1}\) throughout the ligand binding process at pH 7.0 and 20°C. The release of the fluorescent IP₆ analog HPT, however, lagged significantly behind ligand binding, implying that a quaternary conformational change from the T to the R state was indeed taking place in the reaction.

The dissociation of NO from human hemoglobin was examined by treating nitrosylhemoglobin with an excess of dithionite (to consume the NO once it dissociated) in the presence and absence of excess CO (69). It was found that the fourth NO molecule dissociated some 100 times more slowly than the first \(j'_4 = 10^{-5} \text{ s}^{-1}\) as opposed to \(j'_1 = 10^{-3} \text{ s}^{-1}\). Thus in the reaction of NO with hemoglobin, cooperativity appears to be expressed as a 100-fold decrease in the rate of ligand dissociation on switching from the low affinity to the high affinity state.

The EPR spectrum of nitrosylhemoglobin was first reported by Kon (56). Later work by Shiga and coworkers (70) showed that the isolated \(\alpha\) and \(\beta\) subunits exhibit markedly different EPR spectra and that the spectrum of nitrosylhemoglobin was given by the sum of the spectra for the isolated chains. This demonstration that NO can be used as a chain-specific spin label led to rapid-freeze experiments in which the more rapidly reacting chain in the nBNC reaction with hemoglobin was unequivocally shown to be \(\beta\) (71). In these experiments, an apparatus like the one shown schematically in Fig. IV.1 was used. Hemoglobin was first mixed with a saturating amount of nBNC and then, after a period
Fig. IV.1. A schematic diagram of a rapid-freeze apparatus.

Hemoglobin is mixed with a solution of ligand in the first mixer and allowed to react for a period of time proportional to the length of tubing between mixers. This mixture is then mixed with a solution of NO to rapidly fill all empty heme sites. Finally, the total reaction mix passed out through a nozzle into cold (-140°C) isopentane and is quenched.
of incubation proportional to the distance between the two mixers, was mixed a second time with a nitric oxide solution to rapidly fill all empty heme sites. Finally, the reaction mix was frozen rapidly in cold (-140°C) isopentane and packed into EPR tubes. The spectra of samples collected after short periods of incubation showed a clear enhancement of the α contribution relative to the β, indicating that nBNC had preferentially bound to the β chains, thereby rendering them unavailable to react with NO in the second mixing.

The reaction of NO itself was also examined by means of EPR spectroscopy (72). Since NO reacts extremely rapidly and in effect irreversibly with hemoglobin, the distribution of NO between α and β subunits after mixing hemoglobin with a subsaturating amount of NO in solution reflects the relative rates of NO binding to the subunits. With this in mind, nitrosylhemoglobin samples at low levels of saturation (20%) were prepared and their EPR spectra analyzed in terms of the spectra of isolated \( \alpha^\text{NO} \) and \( \beta^\text{NO} \). The results suggested that the α chains bound NO approximately twice as fast as β. This result is inconsistent with the optical kinetics work which indicates no increase in \( j' \) with time (68), and suggests that chain heterogeneity and cooperativity cancel one another out in the association reaction. As will be discussed in the next chapter, the original EPR interpretation (69) is probably in error due to the failure to take into account a change in the shape of the \( \alpha^\text{NO} \) spectrum at low levels of saturation and re-equilibration of the NO in favor of the α chains during long periods of incubation.

Cassoly was the first to observe that IP6 induces a large spectral change in nitrosylhemoglobin (Fig. IV.2; ref. 55). The spec-
Fig. IV.2. The optical difference spectrum of nitrosylhemoglobin ± IP₆. The spectrum was obtained using the mixing device described in section V.A.1. Hemoglobin concentration was 0.16 mM before mixing; solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
tral change is slow \( (k = 2s^{-1} \text{ at pH 7.0 and 20°C}) \) and apparently takes place subsequent to, not concurrent with, IP6 binding. IP6 binding to \( \alpha_{NO. CO}^{NO} \beta_{2}^{\beta} \), but not \( \alpha_{2}^{CO. NO} \beta_{2}^{\beta} \), causes the same spectral change as for \( \alpha_{2}^{NO. NO} \beta_{2}^{\beta} \), suggesting that only the \( \alpha \) chain spectrum is altered. This idea is supported by the observation that the slow change observed following CO binding to \( \alpha_{2}^{NO} \beta_{2}^{\beta} \) is equal to but opposite in sign to the spectral change seen on addition of IP6 to nitrosylhemoglobin. The addition of CO to \( \alpha_{2}^{NO} \beta_{2}^{\beta} \) caused no such extra spectral change. Later work with the heme hybrids of Sugita (73) also implicated the \( \alpha \) chains as the sole source of the slow change following the binding of IP6 to nitrosylhemoglobin.

Shulman and coworkers (74,75) compared the nuclear magnetic resonance (NMR) and circular dichroism (CD) spectra of nitrosylhemoglobin with the spectra of modified and mutant hemoglobins whose quaternary structures were well established. They concluded that the addition of IP6 caused nitrosylhemoglobin to switch from the R to the T state. Furthermore, these workers showed that the slow spectral change was due to a perturbation within the T state and not to the R-T transition, per se.

It has been proposed on the basis of infrared studies that the addition of IP6 to nitrosylhemoglobin causes a transition from a six- to a five-coordinate geometry in approximately half of the heme sites (presumably in the \( \alpha \) chains; 76,77). This conclusion was arrived at after comparing the N-O stretching frequencies of nitrosylhemoglobin \( \pm \text{ IP6} \) to the frequencies of inorganic nitrosylheme model compounds whose geometries had been established by x-ray crystallography. However, if this were the case, nitrosylhemoglobin \( \pm \text{ IP6} \), being pentacoordinate, would be expected to be high-spin with \( S = 3/2 \) (\( S = 2 \) from the high-spin heme iron antiferromagnetically coupled to \( S = 1/2 \) of the unpaired NO
electron) and give an EPR signal centered at \( g = 4 \). Such is not the case; and the only detectable EPR signal being centered at \( g = 2 \), typical of low-spin \((S = \frac{1}{2})\), six-coordinate nitrosylheme complexes. In addition, the position and intensity of the EPR hyperfine in samples used in the infrared study indicate that the protein may have been de-natured (78).

An alternative explanation for the effects of IP$_6$ on nitrosyl-hemoglobin has recently been put forth, again on the basis of work with inorganic model compounds (79). In a series of nitrosylheme compounds with \( p \)-substituted pyridine derivatives in the sixth ligand position, a nine-line hyperfine structure was observed when the \( p \)-substituted group of the pyridine was electron-donating and a three-line hyperfine structure when that group was electron-withdrawing. Since the \( g \) anisotropy of the EPR signal was found not to vary significantly in the two cases, it was concluded that the iron remains hexacoordinate at all times. It was suggested that in the case of hemoglobin, changes in the state of protonation of the proximal histidine could bring about the same effect, a deprotonated histidine (at \( N_1 \) of the imidazole ring) being electron-donating and a protonated one being electron-withdrawing. According to this hypothesis, the proximal histidine of the \( \alpha \) chains has a lower \( pK \) in the T than in the R state, and the IP$_6$-induced appearance of the three-line hyperfine is due to the conversion to the T state and subsequent slow protonation of the proximal histidine. Such a correlation between quaternary conformation and state of protonation of the proximal histidine (as evidenced by the appearance of the three-line hyperfine structure) has been observed (78). Other conditions favoring the T state, in particular low levels of saturation, would be expected to
have a similar effect on the shape of the EPR signal.

With this material as background, a study was begun in an effort to describe quantitatively the interaction of nitric oxide with human hemoglobin. By taking advantage of both the optical and EPR spectral properties of nitrosylhemoglobin, it was hoped to establish clearly the role of chain heterogeneity in the binding of nitric oxide to hemoglobin and the nature of the slow spectral change which appears to reflect the protonation of the proximal histidine within the T state.
V. PRELIMINARY WORK ON THE REACTION OF HEMOGLOBIN WITH NITRIC OXIDE

Before an analysis of the components in the optical and EPR titrations of hemoglobin with nitric oxide can be undertaken, certain background work must be done. This work involves: 1) the characterization of the components of the optical difference spectrum of hemoglobin $\pm$ NO and the EPR spectrum of nitrosylhemoglobin in order to establish the endpoints of both titrations; 2) an investigation of the relative rates of NO binding to the $\alpha$ and $\beta$ subunits in order to determine any necessary weighting factors for the calculation of the relative amounts of NO bound to the two chains; and 3) an investigation of the optical and EPR properties of the NO hybrids $\alpha_2^N\beta_2$ and $\alpha_2^B\beta_2^N$ in order to characterize as fully as possible the optical and EPR properties of the nitrosylhemoglobin geometry induced by IP$_6$.

V.A. Some Spectral Properties of Nitrosylhemoglobin

1. Materials and Methods—Human hemoglobin for all experiments was prepared in the following manner: Units of packed red blood cells, generally less than three weeks since the date of drawing, were obtained from the Institute of Hemotherapy in Houston, Texas, and washed three times. This was done by centrifuging at 3,000 rpm for ten minutes, removing the supernatant and leukocytes by suction from an aspirator, and resuspending the red cells in isotonic saline solution. The washed red cells were lysed by suspension into an approximately equal volume of glass-distilled water and allowed to stand at room temperature for 45–60 minutes. After this time the suspension was made 3% (w/v) with NaCl and centrifuged for thirty minutes at 10,000 rpm to remove cell debris. The red cell lysate was decanted off, treated with a small amount of diatomaceous earth to help coagulate any remaining cell debris, and
centrifuged a second time at 10,000 rpm for thirty minutes. The resulting lysate was dialyzed overnight against 0.01 M tris-HCl, 0.1 M NaCl, pH 8.0, then run through a Sephadex G-25 column equilibrated with the same buffer to remove organic phosphates and other small molecules. The hemoglobin was then concentrated in an Amicon Diaflo membrane ultrafiltration cell to a final concentration of approximately 10 mM heme and placed in a tonometer equipped with a septum-fitted injection port. The solution was deoxygenated by repeatedly evacuating the tonometer and flushing with nitrogen gas that had been scrubbed of oxygen by passage over a hot copper coil. Samples could be withdrawn as needed through the injection port by means of a Hamilton syringe. Hemoglobin stock solutions prepared in this manner and stored in a refrigerator were stable indefinitely.

Hemoglobin chains were prepared according to the scheme shown in Fig. V.1 (88). Roughly 50 ml of 5 mM hemoglobin in 0.025 M K2HPO4 were flushed with CO2, then made 0.025 M with respect to KH2PO4 and 0.1 M with respect to NaCl. The hemoglobin was then treated with 50 mg of p-hydroxymercuribenzoate (PMB) per gram of protein. The pH was adjusted to pH 5.9 with 2.0 M acetic acid and the solution stirred overnight in a cold box. The next day the precipitate was centrifuged out and the solution of PMB-hemoglobin split into two equal portions. The solutions were checked for complete separation into PMB-chains by cellulose acetate strip electrophoresis in a tris-EDTA-glycine buffer system purchased from Gelman Instrument Company, Ann Arbor, Michigan. In this system, the α chains were nearly stationary and the β chains ran toward the anode. The first portion of PMB-hemoglobin was passed through a 5 x 40 cm Sephadex G-25 column equilibrated with 0.005 M inorganic phos-
Fig. V.1. A schematic diagram of the preparation of isolated
\( \alpha \) and \( \beta \) subunits from hemoglobin.
HEMOGLOBIN

\[ \text{p-MB, pH 5.9, 24 hr.} \]

\[ \text{spin down precipitate} \]

\[ \text{pMB-CHAINS} \]

G-25

0.005 M \( \text{NaPi}, \text{pH 8.0} \)

↓

elute with same buffer

↑

DE-52

0.005 M \( \text{NaPi}, \text{pH 8.0} \)

↓

elute with same buffer

↓

make 15 mM with ME

↑

CM-23

0.1 M \( \text{NaPi}, \text{pH 6.7} \)

↓

wash with same buffer,

↓

elute with 0.1 M Tris-HCl, pH 8.5

α

G-25

0.005 M \( \text{NaPi}, \text{pH 5.9} \)

↓

elute with same buffer

CM-52

0.005 M \( \text{NaPi}, \text{pH 5.9} \)

↓

elute with a gradient of

0.01 M \( \text{NaPi}, \text{pH 6.7} \)

and 0.03 M \( \text{Na}_2\text{HPO}_4 \)

G-25

0.1 M \( \text{NaPi}, 0.1 M \text{ ME}, \text{pH 6.8} \)

↓

elute with same buffer

G-25

0.005 M \( \text{NaPi}, \text{pH 5.9} \)

↓

elute with same buffer

CM-52 SLURRY

0.005 M \( \text{NaPi}, \text{pH 5.9} \)

↓

pack column,

↓

elute with 0.1 M \( \text{NaPi}, \text{pH 7.0} \)

β

Fig. V.1
phate (P$_i$), pH 8.0, and eluted with the same buffer. The pH of the eluent was checked and raised to pH 8.0 with 2.0 N NaOH if necessary. This solution was then applied to a 3 x 15 cm column of DE-52 equilibrated with 0.005 M P$_i$, pH 8.0, and the PMB-α chains eluted with the same buffer (the PMB-β chains remained tightly bound to the column). The eluent was made 15 mM with respect to mercaptoethanol and the pH adjusted to 6.7 with dilute H$_3$PO$_4$. The solution was applied to a 3 x 5 cm column of CM-23 equilibrated with 0.01 M P$_i$, pH 6.7, and washed with 300-500 ml of the same buffer to remove mercaptoethanol. The PMB-free α chains were then eluted as a tight band with 0.1 M tris-HCl, pH 8.5, and collected in a small Erlenmeyer flask.

The second portion of the PMB-hemoglobin solution was passed through a 5 x 40 cm Sephadex G-25 column equilibrated with 0.005 M P$_i$, pH 5.9, and eluted with the same buffer. The pH was adjusted to 5.9 with 2.0 M acetic acid, if necessary, and the eluent applied to a 3 x 15 cm column of CM-52 equilibrated with 0.005 M P$_i$, pH 5.9. The PMB-β chains were eluted with a linear gradient of 500 ml 0.01 M P$_i$, pH 6.7, and 500 ml 0.03 M Na$_2$HPO$_4$. The eluent was made 0.1 M with respect to mercaptoethanol and passed through a 5 x 30 Sephadex G-25 column equilibrated with 0.1 M P$_i$, 0.1 M mercaptoethanol, pH 6.8. The PMB-free β chains were eluted with the same buffer, then applied to another G-25 column equilibrated with 0.005 M P$_i$, pH 5.9 and eluted with the same buffer to remove mercaptoethanol. Finally, the dilute solution of β chains was treated with a small amount of a CM-52 slurry equilibrated with 0.005 M P$_i$, pH 5.9. A column was poured and the concentrated β chains eluted as a tight bound with 0.1 M P$_i$, pH 7.0. Solutions of both chains were titrated with PMB while observing at 250 nm
to ensure that all PMB had been removed (α chains have one free sulfonyldryl group per heme, β have two). Care was taken throughout the procedure to bubble all buffers with CO to prevent oxidation of the chain solutions by atmospheric oxygen.

Optical spectra were recorded on a Cary 118 spectrophotometer. Because of the sensitivity of nitrosylhemoglobin to air, flow cells connected to a dual-syringe mixing device (shown schematically in Fig. V.2) were used instead of cuvettes to record the difference spectra. Deoxygenated buffers were prepared by bubbling solutions with O₂-free nitrogen for thirty minutes. Deoxyhemoglobin samples were prepared by injecting an appropriate volume of the stock hemoglobin into the desired nitrogen-bubbled buffer, and then adding a few grains of sodium dithionite. Nitric oxide solutions were prepared by injecting deoxygenated buffer (without dithionite) into a NO-flushed tonometer, followed by equilibration at 1 atm. Deoxyhemoglobin and deoxygenated buffer were mixed 1:1 with the mixing device connected first to the reference flow cell, then to the sample, and the baseline for the difference spectrum was recorded. A saturated NO solution was then substituted for the deoxygenated buffer, mixed with deoxyhemoglobin through the sample flow cell, and the difference spectrum recorded. Optical kinetic data were obtained with a Gibson-Durrum stopped-flow apparatus.

EPR spectra were recorded on a Varian E-6 (X-band) spectrometer and stored digitally in an Interdata 7/16 minicomputer. The following instrument settings were used: microwave frequency, 9.24 GHz; field modulation, 2.5 G; microwave power, 10 milliwatts; scan width, 400 G; centerfield, 3280 G; time constant, 0.3 sec; sweeptime, 100 G/min;
Fig. V.2. A schematic diagram of the mixing device used in the optical/EPR experiments. Two syringes (A and B) are mounted in a thermostated water bath and driven by a pneumatic driving block that forces the solutions to be mixed through an eight-jet tangential mixer (m), then on to a flow cell in the spectrophotometer. The drive syringes are filled from storage syringes (S₁ and S₂) via two Hamilton valves (V₁ and V₂).
temperature, 110 K. Rapid-freeze samples were prepared by mixing deoxyhemoglobin with solutions of varying NO concentration in a device similar to that shown in Fig. IV.2. The solutions were allowed to react for approximately 20 ms, then mixed either with N₂- or CO-equilibrated buffer and quenched by squirting through a nozzle into cold (-140°C) isopentane and the samples packed into EPR tubes. The time of reaction with NO could be varied by changing the length of the tubing between the two mixers.

IP₆ and PMB were purchased as the sodium salts from Sigma Chemical Company, St. Louis, Missouri. Stock solutions of 0.1 M IP₆ were prepared by dissolving 1.58 g of IP₆ into a few ml of water, neutralized with HCl, and diluted to 10.0 ml. Stock solutions of 0.05 M PMB were prepared by dissolving 180 mg PMB in a small amount of 0.1 N KOH, titrated to pH 7.0 with 2.0 N HCl, and diluted to 10.0 ml. Such stock solutions were kept frozen until needed. Sodium dithionite was purchased from Aldrich Chemical Company, Atlanta, Georgia, as was tris-(hydroxymethyl)-aminomethane (tris). Unless otherwise stated, experiments were performed in 0.1 M 2,2'-bis-(hydroxymethyl)-2,2',2''-nitrilotriethanol (bis-tris, purchased from Aldrich in the Gold Label form), 0.1 M NaCl, pH 7.0 at 20°C. Nitric oxide gas was purchased from Matheson Gas Co., LaPorte, Texas, in lecture bottles and used within two months of purchase to minimize artifacts due to decomposition of the gas. All column materials were purchased from Pharmacia, Inc., Piscataway, New Jersey.

2. Results— The optical difference spectra of α chains, β chains, and hemoglobin ± NO are shown in Fig. V.3. In addition, the absorbance change observed kinetically on mixing the unliganded subunits in the
Fig. V.3. The optical difference spectra of hemoglobin (solid line), α chains (dashed line), and β chains (dotted line). In addition, the kinetic difference spectrum obtained from mixing unliganded chains in the stopped-flow apparatus is shown (R→T transition, dotted-dashed line). The average of the difference spectra for the chains minus the R→T transition is given by the solid circles at various wavelengths. The conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
stopped-flow apparatus (i.e., the R+T conversion) is shown. This spectral change must be taken into account because in the difference spectrum of hemoglobin ± NO the reference solution (deoxyhemoglobin) is in the T state and the sample solution (nitrosylhemoglobin) is in the R state, whereas the isolated chains are in the R state even when unliganded. The closed circles in the figure represent the average of the subunit difference spectra minus the R+T spectral change. As can be seen, the difference spectrum of hemoglobin ± NO is adequately described by these three components.

The EPR spectra of nitrosylhemoglobin and nitrosyl chains are shown in Fig. V.4. In agreement with the work of Shiga et al. (70), the two subunits exhibit distinct signals, the average of which (closed circles) describes the spectrum of nitrosylhemoglobin. With the EPR spectrum of nitrosylhemoglobin thus characterized, a rapid-freeze titration of hemoglobin with NO was performed to determine any variation in the contributions of the two subunits to the EPR spectra as a function of fraction saturation. Because the binding of NO to hemoglobin is essentially irreversible under the experimental conditions (69), the distribution of NO between the subunits should reflect the relative rates of binding NO. Any variation in the contribution of the chains to the total signal in the course of the titration would therefore indicate that the subunits bound NO at different rates. The results of the titration are shown in Fig. V.5. The titration exhibits a well-defined inflection point and all spectra are described well by equal amounts of α and β signals. These findings indicate that NO binds to the α and β subunits of hemoglobin at comparable rates, and are in disagreement with the work of Henry and Cassoly (72), who performed a
Fig. V.4. The EPR spectra of nitrosylhemoglobin (solid line), nitrosyl α (dashed line), and nitrosyl β (dotted line) using the instrument settings given in the text. The heme concentration of the chains were one-half that of hemoglobin; the closed circles represent the sum of the spectra of the chains. The solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0.
Fig. V.5. The rapid-freeze titration of deoxyhemoglobin with nitric oxide. 0.80 mM hemoglobin was reacted with 0.08, 0.20, 0.40, 0.60 and 0.80 mM NO. The samples were prepared in the rapid-freeze apparatus discussed in the text using N₂-equilibrated buffer in the second mix. Identical results were obtained when a saturated CO solution was used in place of the N₂-equilibrated buffer. Solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
similar titration and reported a relatively greater contribution of $\alpha^\text{NO}$ at low levels of saturation. The major difference in methodology between the present and the previous work is that in the latter, nitrosyl-hemoglobin samples were allowed to incubate some 30 minutes before freezing. As shown in the next chapter, during that 30 minutes a re-equilibration of NO takes place, resulting in a shift in NO from the $\beta$ to the $\alpha$ chains.

To further investigate the effect of reaction time on the results of the rapid-freeze titration, the EPR spectrum of subsaturated nitrosylhemoglobin was examined as a function of time elapsed between mixing and freezing. As can be seen in Fig. V.6A, there is a marked change in the shape of the EPR spectrum with time. No such change was observed with fully saturated nitrosylhemoglobin. Fig. V.6B shows a similar experiment for the reaction of nitrosylhemoglobin mixed with IP$_6$. In both cases the major qualitative change is the appearance of a three-line hyperfine structure centered at $g = 2.01$. This feature is characteristic of the protonated histidine configuration discussed in the previous chapter.

The binding of subsaturating amounts of NO to hemoglobin in the stopped-flow apparatus is also followed by a slow spectral change in the visible absorption spectrum (Fig. V.7, closed circles); again, the change is qualitatively similar to that observed following the binding of IP$_6$ to fully saturated nitrosylhemoglobin (Fig. V.7, open circles). In addition, the rates of the EPR and optical changes for both experiments are very similar (Fig. V.7, insat), with a rate constant of approximately 1.6 s$^{-1}$.

3. Discussion—The results of the rapid-freeze titration shown in
Fig. V.6. The EPR time courses following: A, the reaction of deoxyhemoglobin with 32% saturating NO; and B, the reaction of fully saturated nitrosylhemoglobin with inositol hexaphosphate. The time between mixing and freezing is given beside each spectrum. Conditions were as in Fig. V.4.
Fig. V.7. The kinetic difference spectra of slow changes following the binding of inositol hexaphosphate to nitrosylhemoglobin (open circles) and 32% saturating NO to deoxyhemoglobin (closed circles). The data were obtained at each wavelength from the total absorbance change of a kinetic trace in the stopped-flow apparatus. Inset: time courses for the slow spectral changes shown in the main body of the figure. The open and closed triangles are timepoints obtained from the EPR data of Fig. V.5A and V.5B, respectively, by normalizing the area of the hyperfine structure. Conditions were as in Fig. V.4.
Fig. V.5 clearly indicate that, within the limits of the EPR analysis, NO reacts with the \( \alpha \) and \( \beta \) subunits of hemoglobin at identical rates. This is in agreement with the kinetic results of Cassoly and Gibson (68), which showed that the reaction of NO with hemoglobin in the stopped-flow exhibits neither chain heterogeneity nor cooperativity. On the basis of comparisons with the optical and EPR spectral changes following the binding of \( I{P}_6 \) to nitrosylhemoglobin, the slow changes following the binding of subsaturating levels of NO to hemoglobin are taken to reflect the formation of the same modified nitrosylheme configuration at low levels of saturation as induced by \( I{P}_6 \) in fully saturated nitrosylhemoglobin. The occurrence of such extra spectral changes precludes the analysis of the EPR titration of hemoglobin with nitric oxide in terms of the spectra of isolated \( \alpha^{\text{NO}} \) and \( \beta^{\text{NO}} \), exclusively. Before such an analysis can be undertaken, the extra spectral change due to the formation of the hyperfine species must be characterized. For this reason, the NO hybrids \( \alpha_2^{\text{NO}} \beta_2 \) and \( \alpha_2 \beta_2^{\text{NO}} \) have been prepared and their spectral and kinetic properties investigated.

V.B. Experiments with the Nitrosyl Hybrids \( \alpha_2^{\text{NO}} \beta_2 \) and \( \alpha_2 \beta_2^{\text{NO}} \)

1. Materials and Methods— Isolated chains were prepared as discussed previously. The nitrosyl hybrids, in turn, were prepared as follows. Approximately 10 ml of each chain in the oxygenated form were prepared at the same concentration (approximately 50 \( \mu \)M) and loaded into 10 ml syringes. The solutions were then treated with a few grains of dithionite to remove oxygen. 2.0 ml of each solution were placed by means of a needle into two separate septum-fitted nitrogen-flushed vials and a stoichiometric amount of dissolved NO added. Finally, 2.0 ml of the other chain solution was added to each vial. All work
was carried out in a coldroom to minimize protein dematuration, and all samples were used within 30 minutes of preparation. Samples were checked for recombination into tetramers by cellulose acetate strip electrophoresis after reaction with CO to fill all empty heme sites.

EPR, optical and kinetic experiments were performed as described above.

2. Results—The EPR spectra of $\alpha^\text{NO}, \beta^\text{NO}, \alpha_2^\text{NO} \beta_2$ and $\alpha_2^\text{NO} \beta_2$ are shown in Fig. V.8. In agreement with the work of Henry and Banerjee on a wide variety of nitrosyl hybrids (57), the spectrum of $\alpha_2^\text{NO} \beta_2$ is seen to be identical to that of isolated $\beta^\text{NO}$, but $\alpha_2^\text{NO} \beta_2$ is different from $\alpha^\text{NO}$, having a pronounced three-line hyperfine structure at $g = 2.01$. In addition, $\text{IP}_6^\text{NO}$ affected the shape of neither spectrum, suggesting that $\alpha_2^\text{NO} \beta_2$ does not form the modified nitrosylheme configuration, and $\alpha_2^\text{NO} \beta_2$ is fully modified even in the absence of $\text{IP}_6^\text{NO}$.

The kinetics of reassociation of the $\alpha$ and $\beta$ chains was also examined with one or the other chain bound with NO. In the case of $\alpha + \beta^\text{NO}$, only an extremely small spectral change could be observed. This spectral change appeared to be equivalent to the $R\leftrightarrow T$ transition (Fig. V.9, dotted line) but was much smaller in extinction, as though most of the newly formed $\alpha_2^\text{NO} \beta_2$ tetramers remained in the $R$ state (Fig. V.9, dotted line). The reassociation of $\alpha^\text{NO} + \beta$, on the other hand, showed a relatively large slow spectral change, very similar in shape, amplitude, and rate to that observed following the binding of $\text{IP}_6^\text{NO}$ to nitro-

sylhemoglobin (Fig. V.9, solid line).

As a means of corroborating the above results, an experiment was performed in which $\alpha$ and $\beta$ chains were mixed in the stopped-flow while progressively titrating the $\alpha$ chains with NO. Observing the
Fig. V.8. The EPR spectra of $\alpha^{\text{NO}}(A)$, $\beta^{\text{NO}}(B)$, $\alpha_2^{\text{NO}}\beta_2(C)$ and $\alpha_2\beta_2^{\text{NO}}(D)$ in 0.1 M bis-tris, 0.1 M NaCl, pH 7.0. The hybrids were prepared as described in the text.
Fig. V.9. The kinetic difference spectra of α+β (closed squares), 
α^{NO}+β (closed circles), and α+β^{NO} (open circles). Each data point 
was obtained from the total absorbance change of a given wavelength 
in the stopped-flow apparatus. Each chain solution was 50 μM and 
the solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 
20°C.
reaction at 590 nm, a maximum for the α+β reaction and an isosbestic point for the NO$_2$+β reaction, the results shown in Fig. V.10 were obtained. As can be seen, the magnitude of the absorbance change decreases to zero as the α chains become saturated with NO. This indicates that the optical change which generally accompanies the R→T transition of unliganded subunits occurs only in the α subunits. The change cannot take place in the NO$_2$+β reaction because the α subunits are bound with NO.

3. Discussion—The EPR results of Fig. V.8 suggest that only the heme sites of the α subunits form the modified configuration. This is supported by the observation that the extinction of the slow spectral change following the recombination of NO$_2$+β is very close to that of the IP$_6$-induced spectral change with nitrosylhemoglobin. It seems reasonable, therefore, to conclude that this slow spectral change occurs only in the α chains. This conclusion is in agreement with a considerable amount of literature (57, 73-75). Furthermore, since IP$_6$ induces no further increase in the amount of hyperfine present in the EPR spectrum of NO$_2$+β, and since that spectrum exhibits no intensity in a region (g = 2.03 to 2.04) where the spectra of both NO$_2$ and NO do, it is reasonable to assume that all the nitrosylheme sites of NO$_2$+β are in the modified configuration. This EPR spectrum (Fig. V.8.C) can therefore be used in analyzing the EPR titration of hemoglobin with NO to determine the relative contribution of the modified species as a function of fraction saturation.

V.C. Titrations of Nitrosylhemoglobin with IP$_6$ and PMB

1. Materials and Methods—Titrations of nitrosylhemoglobin with IP$_6$ and PMB were performed by mixing titrant solutions of increasing concen-
Fig. V. 10. The effect of titrating the $\alpha$ chains with NO in the reaction $\alpha + \beta$. A, 0% NO; B, 25% NO; C, 50% NO; D, 75% NO; E, excess NO. The wavelength of observation was 590 nm, an isosbestic point for the $IP_6$-induced spectral change and a maximum for the $\alpha + \beta$ reaction. Conditions were as in Fig. V.9.
tration with nitrosylhemoglobin in the mixing device described previously. In the case of PMB, extreme care had to be taken to avoid contamination of the system with oxygen since dithionite reacts with PMB and could therefore not be used.

2. Results—The optical titration of nitrosylhemoglobin with IP₆ at pH 7.0 is shown in Fig. V.11. The spectral change is monotonic and exhibits the expected hyperbolic response of fraction saturation to IP₆ concentration (Fig. V.11, inset).

The change in the optical spectrum of nitrosylhemoglobin on addition of IP₆ is brought about, presumably, by virtue of the fact that organic phosphates function as negative allosteric effectors (i.e., that they shift the allosteric equilibrium toward the T state, thereby inducing the modified nitrosylheme configuration). To see what effect a positive allosteric effector has on the spectral properties of nitrosylhemoglobin, titrations using PMB were undertaken. PMB reacts rapidly only with cys 893, disrupts the salt bridge between his 146 and asp 94, and thereby destabilizes the T state relative to the R. The difference spectrum of nitrosylhemoglobin plus IP₆ in the presence and absence of PMB is shown in Fig. V.12. The spectral change is very similar in shape to the IP₆-induced change but is opposite in sign and smaller in magnitude, implying that PMB is capable of reversing some, but not all, of the IP₆ effect. The addition of PMB to nitrosylhemo-
globin in the absence of IP₆ caused no appreciable spectral change in the optical or EPR, indicating that the only effect of PMB is to reverse a portion of the IP₆-induced spectral change. This effect could also be observed in the titration of PMB-nitrosylhemoglobin with IP₆ as shown in Fig. V.13. The spectral change is exactly the same in shape as
Fig. V.11. The optical titration of nitrosylhemoglobin with inositol hexaphosphate. The spectra were obtained by mixing nitrosylhemo-
globin with solutions of varying IP$_6$ concentration in the apparatus shown in Fig. V.1: A, 0.01 mM; B, 0.02 mM IP$_6$; C, 0.03 mM;
D, 0.04 mM; E, 0.05 mM; F, 0.20 mM. Hemoglobin concentration was
0.14 mM before mixing. Inset: the absorbance change as a function
of IP$_6$ concentration. Solution conditions were 0.1 M bis-tris,
0.1 M NaCl, pH 7.0, 20°C.
Fig. V.12. The difference spectrum of IP₆-nitrosylhemoglobin ± PMB. Hemoglobin concentration was 0.10 mM and the PMB concentration 0.05 mM before mixing. Solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
Fig. V.13. The titration of PMB-nitrosylhemoglobin with IP$_6$. The titration was obtained as in Fig. V.10. A, 0.01 mM IP$_6$; B, 0.02 mM; C, 0.03 mM; D, 0.04 mM; E, 0.08 mM; F, 0.40 mM. Hemoglobin concentration was 0.130 mM before mixing. The solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
in the titration in the absence of PMB, but some 25% smaller. The end-
points of all three titrations are shown in Fig. V.14, where it can be
more readily seen that the sum of the spectral changes for nitrosyl-
hemoglobin ± IP₆ and IP₆-nitrosylhemoglobin ± PMB (closed circles)
closely resembles the spectral change for PMB-nitrosylhemoglobin ± IP₆.
The effects of IP₆ and PMB on the EPR spectrum of nitrosylhemoglobin
are shown in Fig. V.15. Again, while not perturbing the spectrum of
nitrosylhemoglobin alone, PMB decreases the effect of IP₆ by about 25%.
3. Discussion— The effects of PMB and IP₆ on the optical and EPR
spectral properties of nitrosylhemoglobin can be described by the fol-
lowing thermodynamic cycle:

\[
\begin{align*}
\text{Hb•NO} & \xrightarrow{\varepsilon_1} \text{[Hb•NO]}^{\text{IP}_6} \\
\varepsilon_3 & \uparrow \downarrow \quad \uparrow \downarrow \quad \varepsilon_2 \\
\text{[Hb•NO]}^{\text{PMB}} & \xleftarrow{\varepsilon_4} \text{[Hb•NO]}^{\text{IP}_6}_{\text{PMB}}
\end{align*}
\]

and:

\[
\varepsilon_1 + \varepsilon_2 = \varepsilon_3 + \varepsilon_4
\]

where \(\varepsilon_n\) represents the extinction change in the optical or EPR spec-
trum for a given binding step. Since in both cases \(\varepsilon_3\), the extinction
change taking place on binding of PMB to nitrosylhemoglobin, is equal
to zero, equation V.2 reduces to:

\[
\varepsilon_1 + \varepsilon_2 = \varepsilon_3
\]

The spectra shown in Figs. V.14 and V.15 are in agreement with equation
V.3.

It should be pointed out that both IP₆ and PMB interact ex-
clusively with the \(\beta\) chains, but that the observed changes in optical
Fig. V.14. The endpoints of the IP₆ titrations in the absence (A) and presence (B) of PMB from Figs. V.10 and V.12. Also shown is the difference spectrum of IP₆-nitrosylhemoglobin ± PMB from Fig. V.11 (C). The closed circles represent the sum of spectral changes A and C.
Fig. V.15. The EPR spectra of nitrosylhemoglobin (A), nitrosylhemoglobin + PMB (B), nitrosylhemoglobin + IP₆ (C), and PMB-nitrosylhemoglobin + IP₆ (D). Samples were prepared by adding PMB, IP₆, or both to 1.0 mM nitrosylhemoglobin solutions in stoppered vials. Aliquots were withdrawn with a Hamilton syringe, placed in septum-fitted, N₂-flushed EPR tubes and frozen by slow immersion into liquid nitrogen. Solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
and EPR spectra are the result of conformational changes only in the α subunits. As mentioned previously in section III.A.3 (54,55) in regard to the dependence of the $^{\text{NO}}\alpha$ signal shape on the spin state of the β heme irons in nitrosyl hybrids, it appears that the α subunits are extremely sensitive to small conformational changes taking place in the β subunits.
VI. THE TITRATION OF HEMOGLOBIN WITH NITRIC OXIDE

In the previous chapter it was shown that at low levels of saturation extra spectral changes were observed in both the optical and EPR spectra of nitrosylhemoglobin. These changes were found to be due to the formation of a modified nitrosylheme configuration in the α subunits. As there was no contribution of this modified species to the spectra at high levels of saturation, it was evident that there was a dependence of the formation of the altered species on fraction saturation. With the establishment of the optical and EPR spectral properties of the modified species, it was possible to perform optical and EPR titrations of hemoglobin with nitric oxide and analyze each spectrum for the relative contribution of this modified nitrosylheme configuration.

VI.A. The Titration of Hemoglobin with Nitric Oxide at pH 7.0

in the Presence and Absence of Inositol Hexaphosphate

1. Materials and Methods—Optical/EPR titrations were performed in the mixing device described in section V.A.1. The only modification was the addition of a Hamilton valve/needle assembly between the mixer and flow cell. This assembly allowed sample flow to be directed either through a 1.0 mm flow cell in the Cary 118 spectrophotometer or through the needle into septum-fitted EPR tubes which had been previously flushed with N₂. In this manner, optical and EPR measurements could be made on samples of identical composition. EPR samples were frozen in liquid nitrogen 20 ± 5 seconds after collection to allow for the completion of the slow spectral change. EPR spectra were recorded as discussed previously. Absolute optical spectra were recorded digitally in a Data General Nova 2/10 minicomputer equipped with an OLIS A/D con-
verter interface and a Ball dual drive floppy disk system. The spectrum of deoxyhemoglobin was subsequently subtracted from each of the other spectra in the titration and the resulting difference spectra plotted on an X-Y recorder in order to follow the course of the titration. Hemoglobin concentrations in such experiments were approximately 2.0 mM before mixing.

The spectra from both optical and EPR titrations were fit to two (optical data) or three (EPR data) standard spectra using a least squares procedure based on the statistical method of maximum likelihood (30). In this procedure, it is assumed that the fit most closely describing the theoretical parent distribution from which a given set of data has been obtained is the one that maximizes the probability of making the observed set of measurements. For a Gaussian distribution with standard deviation \( \sigma \) about the parent distribution, the probability of making a single observation \( y_i \) at \( x_i \) is given by:

\[
P_i = \frac{1}{\sigma_i \sqrt{2\pi}} \exp \left[ -\frac{1}{2} \left( \frac{y_i - y(x_i)}{\sigma_i} \right)^2 \right]
\]

where \( y(x_i) \) reflects the "real" value of \( y \) at \( x_i \) as determined by the parent distribution. The probability of making the entire set of observed measurements is given by the product of the probabilities for all the points:

\[
P = \prod \left\{ \frac{1}{\sigma_i \sqrt{2\pi}} \exp \left[ -\frac{1}{2} \sum_{i=1}^{n} \left( \frac{y_i - y(x_i)}{\sigma_i} \right)^2 \right] \right\}
\]

This probability is maximized when the summation in the exponent of equation VI.2 is minimized. When \( \sigma \) is constant over \( x \), this summation,
defined as \( \chi^2 \), simplifies to:

\[
\chi^2 = \sum_{i=1}^{n} [y_i - y(x_i)]^2
\]

For the fitting procedures used here, \( y(x_i) \) is given by the sum of the amplitudes of the standard spectra, each multiplied by a weighting factor:

\[
y(x_i) = w_1 A_1^i + w_2 A_2^i + w_3 A_3^i
\]

Thus \( \chi^2 \) is a function of the weighting factors sought in the fitting procedure, and the following partial differential equations can be derived:

\[
\frac{\delta \chi^2}{\delta w_1} = -2 \sum_{i=1}^{n} (A_1^i) [y_i - (w_1 A_1^i + w_2 A_2^i + w_3 A_3^i)] = 0
\]

\[
\frac{\delta \chi^2}{\delta w_2} = -2 \sum_{i=1}^{n} (A_2^i) [y_i - (w_1 A_1^i + w_2 A_2^i + w_3 A_3^i)] = 0
\]

\[
\frac{\delta \chi^2}{\delta w_3} = -2 \sum_{i=1}^{n} (A_3^i) [y_i - (w_1 A_1^i + w_2 A_2^i + w_3 A_3^i)] = 0
\]

To obtain a fit, these three simultaneous equations are solved for \( w_1 \), \( w_2 \), and \( w_3 \) with a BASIC computer program using the method of determinants (c.f. Appendix).

2. **Results**—The optical difference titration of hemoglobin with NO between 700 and 450 nm is shown in Fig. VI.1. Although isosbestic points are observed, the titration exhibits a lack of uniformity which implies the occurrence of more than one spectral change on binding of ligand. The effect is particularly pronounced in the 525 to 500 nm region where the absorbance increase with each incremental addition of NO gradually decreases to zero and becomes negative in the final 10-15%
Fig. VI.1. The optical titration of deoxyhemoglobin with nitric oxide at pH 7.0. Spectra were recorded after mixing 0.20 mM deoxyhemoglobin with solutions of varying NO concentration in the mixer shown in Fig. V.I. The lack of uniformity in the titration is particularly evident in the 500–520 nm region. Solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
of the titration. Fig. VI.2 shows the spectral changes for the first (dotted line) and last (solid line) 10% of the titration, and it can be seen that the two spectra are markedly different. Fig. VI.3 compares the difference between the two spectra in Fig. VI.2 (A) with the spectral change observed on addition of IP\_6 to nitrosylhemoglobin (B). The similarity in the spectra indicates that an extra spectral change very much like the one induced by IP\_6 with nitrosylhemoglobin is present in the difference spectrum of the first 10% of the titration but not the last 10%. This similarity suggests that the modified heme configuration formed by the addition of IP\_6 to nitrosylhemoglobin is also formed at low levels of saturation in the absence of IP\_6. It is the appearance of this modified species that destroys the uniformity of the NO titration.

An EPR titration of hemoglobin with NO is shown in Fig. VI.4, where again the lack of uniformity is readily apparent. Fig. VI.5 shows the spectra of fully saturated nitrosylhemoglobin (A), 10% saturated nitrosylhemoglobin from Fig. VI.4 times 10 (B), and nitrosylhemoglobin +IP\_6 (C) for comparison. As with the optical work, the similarity between the EPR spectra of subsaturating nitrosylhemoglobin and nitrosylhemoglobin +IP\_6 is striking. The three-line hyperfine structure in both spectra is though to be due to the appearance of the modified nitrosylheme configuration.

In order to determine the contribution of the modified species as a function of fraction saturation, each optical and EPR spectrum was fit to the sum of two or three components as discussed in the Materials and Methods section. In the case of the optical titration, the absorbance data were fit using as standards the fully
Fig. VI.2. Spectral changes for the first (dashed line) and last (solid line) 10% of the titration shown in Fig. VI.1. As can be seen, the spectra are quite different.
Fig. VI.3. The difference between the two spectra in Fig. VI.2. (A) and the IP₆-induced difference spectrum (B) under the same conditions. The similarity of the two spectra indicates the formation of the modified nitrosylheme configuration at low levels of saturation but not at high. Hemoglobin concentration in B was 0.10 mM.
Fig. VI.4. The EPR titration of deoxyhemoglobin with NO at pH 7.0. Samples were prepared as described in the text. Note the appearance of hyperfine structure at lower levels of saturation. The solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C. Identical results were obtained in 0.1 M HEPES, 0.1 M NaCl, pH 7.0, 20°C, indicating no detectable effects of possible pH changes on freezing the samples.
Fig. VI.5. A comparison of the EPR spectra of fully (A) and 10% saturated (B, x10) nitrosylhemoglobin from Fig. VI.4. The spectrum of nitrosylhemoglobin + IP₆ is shown (C) for comparison, and the similarity to the 10% nitrosylhemoglobin spectrum is striking. As with the data shown in Fig. VI.3, the results indicate the formation of the modified nitrosylheme species at low levels of saturation but not at high.
saturated difference spectrum from the titration and the difference spectrum obtained by the addition of IP₆ to nitrosylhemoglobin. The first spectrum was taken to represent binding of NO, per se, and the second to indicate the relative number of α nitrosylheme sites in the modified configuration. For the EPR titration, the fits were made using the spectra of $\alpha^{\text{NO}}$, $\beta^{\text{NO}}$ and $\alpha^{\text{NO}}_2 \beta_2$ as the standard spectra. The first two spectra were taken to represent the normal nitrosylheme configuration in each subunit and the third to represent the modified α nitrosylheme species.

The results of typical fits for both optical and EPR spectra are shown in Fig. VI.6, A and B, respectively, where it can be seen that the fitting procedure gives quite satisfactory results. The actual data for the optical and EPR fits are shown with the fitting programs in the appendix. It should be pointed out that in the case of the EPR fits, the sum of the contributions of the $\alpha^{\text{NO}}$ and $\alpha^{\text{NO}}_2 \beta_2$ spectra was consistently close to that of $\beta^{\text{NO}}$ even though no precautions were taken in preparing the computer program to ensure this situation. Since it is known from the rapid-freeze data shown in Fig. V.3 that the NO is evenly distributed between the α and β subunits, these results provide further evidence that the modified nitrosylheme configuration occurs only in the α subunits.

The results of three independent optical/EPR titrations are shown in Fig. VI.7 as a plot of the fraction of α heme sites in the T state configuration vs. fraction saturation. For the optical data, the relative number of modified α nitrosylheme sites was determined by dividing the contribution of the standard spectrum for the modified nitrosylheme configuration to the fit by the fraction saturation. The
Fig. VI.6. Representative fits of optical (A) and EPR (B) spectra using the method described in the text. The solid lines in each case represent the data, and the broken lines the components as described by the fitting procedure. For the optical spectrum, these components were the difference spectrum of deoxyhemoglobin $\pm$ NO (dashed line) and the LP$_6$-induced spectral change (dotted line). For the EPR data, the components were the spectra of $\alpha^{\text{NO}}$ (dashed line), $\beta^{\text{NO}}$ (dotted line), and $\alpha^{\text{NO}}_2 \beta_2$ (dotted dashed line). The sums of the components in each case are given by the closed circles. Hemoglobin concentration was 2.0 mM and the fraction saturation was 41%.
Fig. VI.7. A plot of the fraction of bound heme sites in the T state as determined from the modified nitrosylheme contribution vs. fraction saturation for one optical and two optical/EPR titrations performed as described in the text using 0.1 M bistris, 0.1 M NaCl, pH 7.0, 20°C. Closed symbols are for optical data, open symbols for EPR. The solid line gives the fraction of hemoglobin molecules in the T state as a function of the fraction saturation as calculated from the allosteric model using \( L = 1 \times 10^7 \) and \( c = 0.008 \).
fraction saturation in turn was determined either from the contribution of the fully saturated difference spectrum to the fit or from the relative absorbance change at 460 nm, an isosbestic for the IP₆-induced spectrum. The two methods of determining the fraction saturation generally agreed within 2%. For the EPR data, the fraction of modified α nitrosylheme sites was determined from the ratio of the contribution of the modified nitrosylheme spectrum to the total α subunit contribution (both normal and modified spectra). The fraction saturation was determined from the integrated signal intensity relative to that of fully saturated nitrosylhemoglobin and was in good agreement with the results from the optical part of the titration. As can be seen in Fig. VI.7, the results of the three titrations are in reasonable agreement, giving a smooth curve over the entire range of fraction saturation. Since some question has arisen in the literature as to the pH effects of freezing samples when using certain buffers (81), a titration was performed in 0.1 M HEPES, 0.1 M NaCl, pH 7.0 (the pKₐ of this buffer was judged to have only a small pH effect on freezing). The results were identical to those obtained using bis-tris.

An optical titration of hemoglobin with nitric oxide in the presence of IP₆ is shown in Fig. VI.8. Unlike the titration in the absence of IP₆, this set of curves exhibits a monotonic increase in spectrum amplitude with increasing NO concentration and well-defined isosbestic points. The EPR spectra of fully saturated and 10% saturated nitrosylhemoglobin (times 10) in the presence of IP₆ are shown in Fig. VI.9. As with the optical titration, there is no apparent change in spectrum shape with fraction saturation. Both spectra in Fig. VI.9 can be adequately described by equal amounts of α₂NO β₂ and βNO signal.
Fig. VI.8. The optical titration of deoxyhemoglobin + IP₆ with NO at pH 7.0. Note the uniformity of the titration in the 500-520 nm region. Hemoglobin concentration was 0.10 mM before mixing; there was a four-fold excess of IP₆. Solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
Fig. VI.9. The EPR spectra of 10% (A, x10) and fully saturated (B) nitrosylhemoglobin + IP$_6$. Both spectra can be adequately fit by equal amounts of the spectra of $\beta^{\text{NO}}$ and $\alpha_2^{\text{NO}} \beta_2$ indicating that there is no change in spectral shape in the course of the NO titration in the presence of IP$_6$. Hemoglobin concentration was 1.80 mM before mixing; solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
Apparently IP₆ induces the maximum amount of modified nitrosylheme species even at high levels of saturation and all the α nitrosylheme sites remain in the modified configuration throughout the titration.

3. Discussion — From previous work, the appearance of the modified configuration has been associated with the T state (75). If this relationship is valid then the contribution of modified species to a spectrum at a given level of saturation in a titration should reflect the number of molecules in the T state at that level of saturation. Using the allosteric model to determine the dependence of the fraction of hemoglobin molecules in the T state on the fraction saturation, the data in Fig. VI.7 have been simulated by varying the allosteric parameters L and c. The solid line in Fig. VI.7 represents such a simulation using values of L = 10⁷ and c = 0.008, and as can be seen, the simulation adequately describes the experimental results. The values of L and c used in the simulation are in good agreement with independently determined values for the NO reaction reported in the literature (L = 1.4 x 10⁷, ref. 44; and c = 0.01, ref. 69). It should be pointed out that to some extent changes in L and c are capable of cancelling one another out in plots of the type in Fig. VI.7. Choices for L between 10⁶ and 10⁸ can be made to approximate the data by varying c from 0.1 to 0.005. Nonetheless, it remains clear that reasonable values of L and c provide a good approximation of the data.

In order to further investigate the correlation between the modified nitrosylheme configuration and T state conformation, the titration of hemoglobin with nitric oxide has been studied as a function of pH. Since the value of L is known to be dependent on pH (as a result of the Bohr effect), the pH dependence of the contribution of
the modified configuration should prove to be a critical test of this correlation. The results of this study are presented and discussed in the next section.

VI.B. The pH Dependence of the Nitric Oxide Titration

1. Materials and Methods—Optical/EPR titrations were carried out and analyzed as discussed in the previous section. The following buffers were used: 0.1 M glycine, 0.1 M NaCl, pH 9.0; 0.1 M tris, 0.1 M NaCl, pH 8.0; 0.1 M bis-tris, 0.1 M NaCl, pH 6.5. Glycine and tris buffers were purchased from Sigma.

2. Results—The optical and EPR titrations at pH 6.5, 8.0, and 9.0 are shown in Figs. VI.10 through VI.15. It can be seen from the EPR titrations that the absolute contribution of the modified species, at a given level of saturation, decreases markedly as the pH increases. At pH 6.5 the three-line hyperfine structure is conspicuous in each spectrum of the titration, but at pH 9.0 it is barely observable, even at low levels of saturation. This situation is more readily observed in Fig. VI.16, which shows the EPR spectra of 10% saturated nitrosylhemo-
globin from each of the four pH titrations (including that at 7.0). According to the allosteric model (34), at this low a level of saturation most of the hemoglobin molecules having NO bound remain in the T state, even assuming the smallest values of L (10^5-10^6) that might be expected at the higher pHs. The decrease in hyperfine structure with increasing pH must be due to a decrease in the fraction of α nitrosyl-
heme sites in the T state which take on the modified configuration and cannot be the result of allosteric effects. This fraction of modified T state α nitrosylheme sites decreases from approximately 90% at pH 6.5 to 10% at pH 9.0 (Table VI.1).
Fig. VI.10. The optical titration of deoxyhemoglobin with NO at pH 6.5. Spectra were recorded as discussed in the text. Hemoglobin concentration was 1.92 mM before mixing; solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 6.5, 20°C.
Fig. VI.11. The EPR titration of deoxyhemoglobin with NO at pH 6.5. Conditions were as in Fig. VI.10.
Fig. VI.12. The optical titration of deoxyhemoglobin with NO at pH 8.0. Hemoglobin concentration was 1.97 mM before mixing; solution conditions were 0.1 M tris, 0.1 M NaCl, pH 8.0, 20°C.
Fig. VI.13. The EPR titration of deoxyhemoglobin with NO at pH 8.0. Conditions were as in Fig. VI.12.
Fig. VI.14. The optical titration of deoxyhemoglobin with NO at pH 9.0. Hemoglobin concentration was 1.96 mM before mixing; solution conditions were 0.1 M glycine, 0.1 M NaCl, pH 9.0, 20°C.
Fig. VI.15. The EPR titration of deoxyhemoglobin with NO at pH 9.0. Conditions were as in Fig. VI.14.
Fig. VI.16. The EPR spectra of 10% saturated nitrosylhemoglobin at pH 6.5 (A), 7.0 (B), 8.0 (C), and 9.0 (D). Spectra were taken from Figs. VI.11, VI.4, VI.13, and VI.15, respectively. Note the marked decrease in hyperfine structure in going from pH 6.5 to 9.0.
The pH titration data can be normalized and compared directly by dividing the observed fraction of modified nitrosylhemes by the maximum possible in the T state. The results of such an analysis are shown in Fig. VI.17. Assuming a pH-independent value of 0.008 for c, the values for L obtained are: $10^8$ at pH 6.5; $3 \times 10^6$ at pH 8.0; and $10^6$ at pH 9.0 (Table VI.1). It was found that $0.008 \pm 0.002$ was the only value of c that gave reasonable fits at all four pHs. Thus, these graphically determined values for L can be taken to be accurate despite the multiplicity of possible combinations of L and c giving reasonable fits at a single pH.

3. Discussion—The effect of protons on the contribution of the modified configuration to the optical and EPR spectral properties of nitrosylhemoglobin appears to be twofold. First, there is a 100-fold increase in L as the pH drops from 9.0 to 6.5, consistent with the interpretation of the Bohr effect in terms of the allosteric model (34, 36). As the proton concentration increases, the allosteric equilibrium between the R and T state is shifted toward the T, permitting the formation of more of the modified species. Second, protons appear to enhance the stability of the modified configuration within the T state. As the pH is lowered, the contribution of the hyperfine signal at 10% saturation increases, as shown in Fig. VI.16. At this low level of saturation, 75% of the nitric oxide bound is associated with $\text{Hb}_4\text{NO}$ species, and most of the remaining 25% with $\text{Hb}_4(\text{NO})_2$. From the allosteric parameters used in the simulations, both of these molecules must be predominantly in the T state. At pH 9.0, where conditions most favor the R state the T/R ratios for $\text{Hb}_4\text{NO}$ and $\text{Hb}_4(\text{NO})_2$ are 8,000 and 64, respectively. Therefore the increase in the hyperfine signal at
Fig. VI.17. Plot of fraction of molecules in T state vs. fraction saturation for pH 6.5 (squares), 7.0 (triangles), 8.0 (circles), and 9.0 (diamonds). Two titrations, one optical and one optical/EPR, were done at each pH (data shown is for EPR only, for sake of clarity). Solid lines represent fits according to the allosteric model using $c = 0.008$ and $L = 10^8$, $10^7$, $3 \times 10^6$, and $10^6$ at pH 6.5, 7.0, 8.0, and 9.0, respectively.
Fraction of Bound

Freeze States in T State

Fig. VI.17
TABLE VI.1. The dependence of the value of $L$ and the fraction of
modified $\alpha$ nitrosylheme sites in the $T$ state as a function of $pH$.
Data was taken from the EPR results of Figs. VI.11, VI.4, VI.13,
and VI.15.
<table>
<thead>
<tr>
<th>pH</th>
<th>L</th>
<th>Fraction of modified α nitrosylheme sites in T state</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>$10^8$</td>
<td>0.90</td>
</tr>
<tr>
<td>7.0</td>
<td>$10^7$</td>
<td>0.80</td>
</tr>
<tr>
<td>8.0</td>
<td>$3 \times 10^6$</td>
<td>0.40</td>
</tr>
<tr>
<td>9.0</td>
<td>$10^6$</td>
<td>0.10</td>
</tr>
</tbody>
</table>
10% saturation as pH is lowered cannot be due to a further shift in the allosteric equilibrium toward the T state but, as mentioned, must reflect an increase in the stability of the modified nitrosylheme configuration.

The pH dependence of the fraction of α nitrosylheme sites in the T state exhibiting modified spectral properties lends credence to the proposal of Kon and Kataoka (79) that the modified nitrosylheme configuration is the result of the protonation of the proximal histidine of NO-heme sites in the T state. This hypothesis has been taken up and extended by Peisach and coworkers (78, 82, 83) who have shown that, like the NMR and CD probes to quaternary structure, the presence or absence of the hyperfine EPR structure can be related to changes in the ligand affinity of hemoglobin. On the basis of molecular orbital considerations these workers further suggest that on protonation of the proximal histidine, the NO switches from a bent to a linear binding geometry with respect to the iron-N-O bond (78). Thus in the R state the nitrosylheme sites of both subunits contain deprotonated histidine residues and bent Fe-N-O bonds, but in the T state the histidines of the α subunits become protonated and the Fe-N-O bond becomes linear. Single-crystal EPR studies on a low affinity mutant hemoglobin (84) have found that the Fe-N-O bond angle is 105° in the β subunits and 167° in the α subunits. This increase in bond angle is associated with a decrease in the unpaired electron spin density at the NO nitrogen from 63% to 37%. In addition, the proximal histidine interacts with the unpaired spin density in the α subunits, giving rise to a hyperfine splitting of 7-8 gauss, but not in the β subunits to any significant extent. These results support the proposal of Chevion et al. (78) re-
garding the change in Fe–N–O bond angle on protonation of the proximal histidine in the α subunits. In their infrared studies, Maxwell and Caughey assumed that the Fe–N–O bond angle was always in the bent configuration. Thus it is possible that the increase in N–O stretching frequency they observed on addition of IP₆ to nitrosylhemoglobin was due to a change in Fe–N–O bond angle and not to the formation of a penta-coordinate nitrosylheme geometry in the α subunits. The decrease in unpaired electron spin density at the NO nitrogen (associated with the increase in Fe–N–O bond angle) would be expected to raise the order of the N–O bond, since the unpaired electron occupies a π* orbital in NO (85). This in turn could give rise to the increase in the N–O stretching frequency observed by Maxwell and Caughey.

The protonation of the proximal histidines of α nitrosylheme sites and the concomitant change in Fe–N–O bond angle is a slow process (Fig. V.7, inset; also ref. 75), and takes several seconds for completion. This is true even in samples that remain in the T state throughout the reaction (e.g., deoxyhemoglobin + subsaturating NO or IP₆–deoxyhemoglobin + saturating NO). Thus it is likely that, in the absence of NO, the proximal histidine is not protonated in the T state. Consequently, the pK effects of NO binding to α heme sites in the T state that give rise to the protonation of the proximal histidine probably do not contribute to the Bohr effect except in an indirect way. In fact, Chevion et al. maintain that the proton associated with the proximal histidine in the T state is not in equilibrium with the solvent and is therefore not titratable at all. It is clear, however, from the pH dependence of the hyperfine contribution to the EPR spectra in Fig. VI.16 that this is definitely not the case and that there are solvent effects
on the environment of the proximal histidine.

VI.C. Very Slow Reactions Following the Binding of Nitric Oxide to Hemoglobin

1. Materials and Methods— Solutions of 2.0 mM hemoglobin and 2.0 mM NO were mixed in the apparatus used in previous experiments. This device was fitted with a piece that allowed a 2.0 ml syringe to be screwed into place to accept the reaction mix. The solutions were mixed and, after a period of incubation between 30 seconds and one hour, this syringe was detached from the mixer, fit with a needle, and portions of the sample were squirted into septum-fitted EPR tubes which had been flushed with either N₂ or CO. The nitrogen samples were frozen immediately in a dry-ice/acetone bath. The CO samples were shaken to ensure full reaction of the empty heme sites with CO and after a period of time roughly equal to half the first incubation were frozen. The experimental conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0.

2. Results— The results of the experiments described above are shown in Figs. VI.18 (N₂-flushed tubes) and VI.19 (CO-flushed tubes). As can be seen in Fig. VI.18, the spectrum after a 30-second incubation is composed of about equal amounts of the $\alpha_2 N O_2$ and $\beta N O$ signals, as observed before. However, within 11 minutes the contribution of $\beta N O$ disappears and most of the nitrosylheme sites of the sample exhibit a hyperfine signal. The loss of $\beta N O$ signal could be due to one of two events, or both. First, a re-equilibration of NO during the incubation period from the $\beta$ chains to the $\alpha$ could be taking place. The initial distribution of NO between the subunits is determined by the relative rates of NO binding and is therefore 1:1. However, if the $\alpha$ subunits had a higher affinity for NO than the $\beta$, as the system approached
Fig. VI.18. Very slow changes in the shape of the EPR signal of 13% saturated nitrosylhemoglobin. Samples were prepared as described in the text. Note the nearly complete disappearance of NO signal as evidenced by the very low signal intensity in the 3220-3260 G region. Time elapsed between mixing and freezing is: A) 0.5 min; B) 1.5 min; C) 5.0 min; D) 11 min. Hemoglobin concentration was 0.50 mM and the fraction saturation was 0.13. Solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20° C.
Fig. VI.19. Very slow changes in the distribution of NO between
the subunits within 13% nitrosylhemoglobin. Sample A was reacted
with CO to convert the protein to the R state immediately after
mixing and frozen as discussed in the text conditions were as in
Fig. VI.18. Sample B was reacted with CO after an 11 min. incuba-
tion, and frozen five minutes later.
equilibrium, the distribution of NO would favor the \( \alpha \) subunits by a factor equal to the ratio of the subunit affinities. The dissociation of NO from the T state is known to be relatively fast compared to the R state (approximately \( 10^{-3} \text{s}^{-1} \) vs. \( 10^{-5} \text{s}^{-1} \)) and such a re-equilibration might indeed be taking place. The loss of \( \beta^{\text{NO}} \) signal could also be due to the very slow formation of a protonated configuration within the \( \beta \) subunits which exhibited spectral properties indistinguishable from that in the \( \alpha \) subunits. The results from the CO experiment help distinguish between these two possibilities. Since on reaction of the empty heme sites of the sample with CO the protein switches to the R state, the spectra of Fig. VI.19 contain no hyperfine contribution and can be described completely in terms of the spectra of \( \alpha^{\text{NO}} \) and \( \beta^{\text{NO}} \). As can be seen in Fig. VI.19, even after 11 minutes there is still a significant contribution of \( \beta^{\text{NO}} \) signal, although it has decreased by some 10% relative to the \( \alpha^{\text{NO}} \) signal. Thus it is clear that while some re-equilibration does take place during prolonged incubation, the \( \beta \) subunits do appear to be able to form a modified nitrosylheme configuration, albeit very slowly.

Very slow changes in the absorbance spectrum of nitrosylhemoglobin at low levels of saturation are also observed, as can be seen in Fig. VI.20. The spectral change after the initial difference spectrum was recorded (about one minute) has a rate of appearance on the same order as that for the hyperfine structure in Fig. VI.18 (i.e., several minutes). As in the case of the EPR study, the very slow spectral change is presumably taking place in the \( \beta \) subunits and is similar to the change which had already occurred in the \( \alpha \) subunits.

3. **Discussion**— The re-equilibration of NO toward the \( \alpha \) chains helps to
Fig. VI.20. Very slow changes in the optical spectrum of 15% saturated nitrosylhemoglobin. Each spectrum was obtained by subtracting 15% of the absorbance change of deoxyhemoglobin ± NO from the absorbance change on addition of 15% saturating NO recorded at various times after mixing: A, 2 min; B, 4 min; C, 9 min; D, 14 min. Hemoglobin concentration was 0.20 mM before mixing; solution conditions were 0.1 M bis-tris, 0.1 M NaCl pH 7.0, 20°C.
explain the conflicting results of Henry and Cassoly (64). These workers reported that at low levels of saturation the α heme sites were preferentially filled under conditions where the distribution of NO was determined kinetically. In their experiments, the samples sat for 30 minutes at 0°C before being saturated with CO and frozen, ample time to allow rearrangement to take place even at low temperature. Also, the reconversion to the normal nitrosylheme configuration on addition of CO to samples of low saturation was found to be slow, and if adequate time were not allowed between the addition of CO and freezing, this too could affect the results. The modified nitrosylheme species has a relatively intense signal in the 3120–3200 G region, where only α^{NO} makes any significant contribution to the signal of nitrosylhemoglobin. If the hyperfine structure was not resolved in the sample spectrum, it would give the appearance of a much larger α^{NO} contribution than was actually the case.

It was surprising to find that the β subunits appear to be capable of forming a modified nitrosylheme configuration. The extremely slow formation and disappearance of this species in the β subunits makes its study extremely difficult. Nitrosylhemoglobin is relatively unstable and denatures on prolonged incubation, particularly at low levels of saturation. The protein is especially sensitive to oxidation under conditions favoring the formation of the modified nitrosylheme configuration (77). Under these conditions dithionite cannot be used to keep the protein reduced as it reacts with NO, removing it from the protein. The results shown in Figs. VI.18 and VI.19 should therefore be viewed with some degree of reservation. In addition, even if nitro- sylhemoglobin was stable indefinitely, the slow re-equilibration toward
the \( \alpha \) subunits would have to be accurately determined and taken into account in the various titrations. This would greatly complicate the interpretation of the data. For these reasons, the formation of the modified nitrosylheme configuration in the \( \beta \) subunits was not pursued further.

The appearance of the modified \( \beta \) species does not interfere with the previous results to any appreciable extent. In the EPR titrations, the samples were frozen 20 seconds after mixing and the \( \beta \) nitrosylheme reaction could not have proceeded far. This is borne out by the fact that in the EPR fitting procedures that the sum of the contributions of the \( \alpha_{\text{NO}} \) and \( \alpha_{\text{NO}} \beta_{\beta} \) signals was equivalent to that of \( \beta_{\text{NO}} \).

Had the \( \beta \) subunits been contributing significantly to the hyperfine \( \alpha_{\text{NO}} \beta_{\beta} \) signal this would not have been the case. In the optical titrations, it took longer to record the spectra (approximately one minute) and a \( \beta \) contribution to the observed spectral change due to the appearance of the modified nitrosylheme configuration may have been appreciable (10-20%). However, the optical data was not analyzed in terms of the contributions of the two subunits, but only in terms of the dependence of the spectral change on the formation of the T state. Since this dependence must hold for the \( \beta \) subunits as well as the \( \alpha \) (as judged by the disappearance of the hyperfine structure in the EPR spectra of Fig. VI.19 on addition of CO) the results of the optical titrations remain valid. In neither optical nor EPR titrations has the re-equilibration had time to become important since only a 10% change in the distribution of NO had taken place in 11 minutes.
VII. THE REACTION OF HEMOGLOBIN WITH CARBON MONOXIDE AND OXYGEN

With the quantitative description of the titration of hemoglobin with nitric oxide, similar, preliminary studies with carbon monoxide and oxygen were undertaken. Because of the slow rate of CO dissociation from hemoglobin, the distribution of CO between α and β sub-units at low levels of saturation could be taken to reflect the relative rates of CO binding to the chains, as in the case of the NO titration. Because of the extremely fast dissociation rate of oxygen, such experiments with O₂ were not feasible even at low temperatures. Instead, the oxygen experiments were performed under equilibrium conditions.

VII.A. The Reaction of Hemoglobin with Carbon Monoxide

1. Materials and Methods—Optical titrations were performed as described with NO, using 1.0 cm flow cells. Rapid-freeze experiments were performed using the apparatus shown in Fig. IV.1. Deoxyhemoglobin was reacted with CO solutions of varying concentration, quenched with NO to fill all empty heme sites, and frozen. Since the dissociation of CO is extremely slow, the distribution of CO between the two chains was determined by the relative on constants, as was the case with NO. Measurements on the rate of CO dissociation were made by mixing a solution of carboxyhemoglobin with an excess of dissolved NO, withdrawing aliquots after various times into EPR tubes, and freezing manually. EPR spectra were recorded as described in section V.A.1.

2. Results and Discussion—The optical titration of hemoglobin with CO is shown in Fig. VII.1, where it can be seen that the reaction appears to exhibit well-defined isosbestic points. In theory this
Fig. VII.1. The optical titration of deoxyhemoglobin with CO. Spectra were recorded after mixing deoxyhemoglobin with solutions of varying CO concentration in the mixing apparatus shown in Fig. V.1. Hemoglobin concentration was 0.10 mM before mixing; solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
should not be the case, since the R↔T spectral change of unliganded sites must also be taken into account at higher levels of saturation. However, as can be seen in Fig. VII.2, the spectral changes for hemoglobin ± CO and this R↔T transition are quite similar in shape but opposite in sign. As a result, an analysis similar to that used with the NO reaction is impossible since the contribution of the latter transition (which would in any case be small) is lost in the inherent uncertainty of determining the fraction saturation. This situation persists even in the presence of IP₆, and the data was not further analyzed.

In an effort to detect any chain heterogeneity in the dissociation of CO from hemoglobin, the displacement of CO by NO was observed by EPR. The results, shown in Fig. VII.3, agreed well with the literature (64) giving a half-time of approximately 1.3 min. The shape of the spectrum did not change during the course of the reaction, indicating that CO dissociated from the α and β subunits at equal rates.

The association of CO was also examined by EPR using the rapid-freeze technique. The results are given in Fig. VII.4. Again, the spectrum shape does not change in the course of the reaction and thus the subunits appear to bind CO at equal rates. This result is in agreement with previous work (48) which has interpreted heterogeneous time courses for the CO binding reaction in terms of differential binding between the R and T states and not chain differences.

VII.B. The Reaction of Hemoglobin with Oxygen
1. Materials and Methods— Optical titrations were performed as described with NO, using 1.0 cm flow cells. The rapid-freeze experiment was performed by mixing hemoglobin solutions in equilibrium with various amounts of O₂ with NO using only the lower two syringes of the
Fig. VII.2. The endpoint of the CO titration shown in Fig. VII.1 (A) and the R-T transition (B). The spectra exhibit similar wavelength maxima and isosbestic points to the extent that resolution between the two is impossible in the analysis discussed in the text.
Fig. VII.2
Fig. VII.3. The displacement of CO by NO. A solution of 0.10 mM carboxyhemoglobin was mixed 1:1 with a solution of 2.0 mM NO into a tonometer flushed with $N_2$. Samples were withdrawn at various times afterwards (from 0.5 to ten minutes) with a Hamilton syringe, injected into EPR tubes and frozen in liquid nitrogen solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
Fig. VII.3
Fig. VII.4. The association of carbon monoxide to deoxyhemoglobin. 1.0 mM deoxyhemoglobin was mixed with solutions of varying CO concentration, then reacted with NO to fill all empty heme sites and quenched by squirting the reaction mix into cold (-140°C) isopentane using the rapid-freeze apparatus shown in Fig. IV.1. Solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 20°C.
apparatus shown in Fig. IV.1. The apparatus was packed in ice to slow the dissociation of O₂ from hemoglobin in the time between mixing with NO and freezing. All experiments were carried out in 0.1 M bis-tris, 0.1 M NaCl, pH 7.0.

2. Results—The optical titration of deoxyhemoglobin with O₂ is shown in Fig. VII.5. Careful examination reveals that the relative peak intensities in the difference spectra of 460 and 575 nm change in the course of the titration and that the spectral change on addition of O₂, like NO, is not isosbestic. The difference between the first and last 10% of the O₂ titration is given in Fig. VII.6, and is strikingly similar to the corresponding difference in the NO titration both in shape and in amplitude (c.f. Fig. VI.3). The optical titration of deoxyhemoglobin ±IP₆ with O₂ is shown in Fig. VII.7, and the difference between the first and last 10% of that titration in Fig. VII.8. Again, a spectral change reminiscent of the IP₆-induced spectral change with nitrosylhemoglobin is observed.

Rapid-freeze titrations of hemoglobin with O₂ were carried out in the presence and absence of IP₆ to determine any chain heterogeneity in the O₂ equilibrium. The results are shown in Figs. VII.9 and VII.10. In both cases the shape of the EPR signal due to nitrosylheme sites remained the same throughout, suggesting that at equilibrium O₂ is evenly distributed between the chains.

3. Discussion—The results of the optical titrations shown in Figs. VII.6 and VII.8 suggest that the state of protonation of the proximal histidine may play a role in the interaction of oxygen with hemoglobin. The differences in the electronic structures of NO and O₂ and the absorbance spectra of nitrosyl- and oxyhemoglobin are pronounced. It is
Fig. VII.5. The optical titration of deoxyhemoglobin with O₂.
Spectra were recorded after mixing deoxyhemoglobin with solutions
of varying O₂ concentration in the mixing device shown in
Fig. V.1. Hemoglobin concentration was 0.11 mM before mixing;
solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0,
20°C.
Fig. VII.6. The difference between the first and last 10% of the titration shown in Fig. VII.5. Note the similarity between this difference spectrum for $O_2$ and the ones in Fig. VI.3 for NO.
Fig. VII.7. The titration of deoxyhemoglobin + IP₆ with O₂. The procedure was identical to that in Fig. VII.5 except that the deoxyhemoglobin was treated with a two-fold excess of IP₆ (0.05 M).
Fig. VII.8. The difference between the first and last 6% of the titration shown in Fig. VII.7. Note the similarity to the spectra in Figs. VII.5 and VI.3.
Fig. VII.9. The rapid freeze reaction of oxyhemoglobin at 20% (A) and 80% (B, x4) saturation with NO. The spectrum of fully saturated nitrosylhemoglobin is shown for comparison (C). Note the lack of change in signal shape with O₂ saturation. Hemoglobin and NO concentrations were 1.0 mM before mixing. Solution conditions were 0.1 M bis-tris, 0.1 M NaCl, pH 7.0, 4°C.
Fig. VII.10. The rapid freeze reaction of oxyhemoglobin at 20% (A) and 80% (B x 4) with NO in the presence of a two-fold excess of IP₆. Conditions were as in Fig. VII.9. Again, note the lack of any change in signal shape with O₂ saturation.
therefore surprising that the difference spectra shown in Figs. VII.6 and VII.8 for $O_2$ bear such a close resemblance to the one in Fig. VI.3 for NO.

The difference spectra in Figs. VII.6 and VII.8 for the $O_2$ titration bear no resemblance to the spectral change produced by the addition of IP₆ to oxyhemoglobin (86). This difference spectrum closely resembles the R+T spectral change following the recombination of unliganded isolated chains. If the protonation of the proximal histidine was playing a part in the $O_2$ reaction, the addition of IP₆ would be expected to give rise to a spectral change similar to the difference between the first and last 10% of the oxygen titration and not the R+T spectral change. The discrepancy between these results and those of the oxygen titrations are unresolved and further work is re-
quired.

The results of the rapid-freeze experiments appear to dis-
agree with the work of Sawicki and Gibson (49). These workers reported, on the basis of computer fits to laser flash photolysis experiments with oxyhemoglobin, that the $\alpha$ subunits had a two-fold greater affinity for $O_2$ than the $\beta$ subunits in both the R and T states. However, the rapid-freeze experiments were probably unable to detect such small dif-
ferences in affinities. A further complication exists since the oxy-
hemoglobin solutions were at equilibrium in these experiments and the distribution of oxygen among the hemoglobin molecules was therefore not necessarily statistical. As a result of cooperativity, much of the oxygen would be expected to be bound to $Hb_4(O_2)_4$. Such a situation would necessarily mean that the $\alpha$ and $\beta$ subunits were equally populated with oxygen and would tend to mask any difference in $O_2$ affinities be-
tween the subunits.
VIII. CONCLUSION

The appearance of the three-line hyperfine structure in the EPR spectra of nitrosylhemoglobin samples exhibits a strong correlation with the T quaternary state (e.g., Fig. VI.17). Resolution of the contributions of the α and β subunits to the various EPR spectra has shown that the source of this hyperfine structure is the formation of a modified nitrosylheme configuration within the α chains. These results are in agreement with a large amount of literature on nitrosylhemoglobin in the presence of IP₆ (55, 74, 75), the NO hybrids $\alpha_{2}^{\text{NO}}\beta_{2}$ and $\alpha_{2}^{\text{NO}}\beta_{2}$ (56,57), and mutant and chemically modified hemoglobins exhibiting abnormal allosteric properties (75, 78).

The hyperfine structure in the EPR and the corresponding optical absorbance change (Fig. IV.2) exhibit a pH dependence that is the result of two independent effects. First, there is an increase in the allosteric constant, L, as the pH is lowered which increases the fraction of hemoglobin molecules in the T state at a given level of saturation (Fig. VI.17). Second, as the pH is lowered there is an increase in the fraction of nitrosylhemoglobin sites within the T state which exhibit the three-line hyperfine structure. The latter effect is quite dramatic; the fraction of modified α nitrosylheme sites in the T state decreases from 90% at pH 6.5 to 10% at pH 9.0. These results provide strong evidence in favor of the proposal of Kon and Kataoka (79) that the state of protonation of the proximal histidine determines the spectral characteristics of a nitrosylheme site. According to these authors, a deprotonated histidine would give rise to the normal spectral properties observed with isolated chains and a protonated histidine to the hyperfine structure in the EPR and the associated change in
the optical spectrum. Accordingly, the protonation of the proximal
histidine at a nitrosylheme site is the cause of the formation of the
modified nitrosylheme configuration.

The protonation of the proximal histidine and/or the asso-
ciated configurational change is a relatively slow process (Fig. V.7,
inset) and under normal circumstances takes place only in the α sub-
units. This process remains slow even when the hemoglobin molecules
remain in the T state throughout the reaction (75), suggesting that the
proximal histidine is probably deprotonated when the heme site is un-
liganded. If it were protonated prior to the binding of NO, the
appearance of hyperfine structure would be expected to be immediate.

An optical change which is strikingly similar to that observed
on the formation of the protonated nitrosylheme configuration is also
found in early stages of the titration of deoxyhemoglobin with oxygen
(Figs. VII.6 and VII.8). This result suggests that the proximal histi-
dine of oxyheme sites may also be protonated in the T state and that
this modification of the proximal histidine may play a general role in
the process of ligand binding to hemoglobin.

Rapid-freeze EPR experiments have been performed to investi-
gate the possibility of differences between the α and β subunits in the
rates of NO association, CO association and dissociation, and in the
equilibrium of O₂ in the presence and absence of IP₆. In none of the
above cases was there any evidence to support chain heterogeneity. Only
in the equilibrium of NO was there any data to suggest a difference in
ligand affinities, and in view of the extremely long periods of incuba-
tion periods required to study the reaction, this difference should be
viewed with some degree of reservation. It seems likely that the only
ligands of reduced hemoglobin capable of expressing significant amounts of chain heterogeneity are the alkyl isocyanides (46, 47, 87).
APPENDIX: COMPUTER PROGRAMS FOR FITTING PROCEDURES

A.1. PROGRAM FOR FITS TO EPR DATA

10 DIM X[50,1], Y[50,1], A[50,1], B[50,1], F1[50,1], F2[50,1]
20 DIM S[50,1]
30 DIM X$[50]
40 DIM D[3,3], M1[3,3], M2[3,3], M3[3,3], P[3,3], Q[3,3], R[3,3]
50 DIM C$[50], F3[50,1], G[50,1]
60 DIM U[3,3]
70 DIM A$[50], B$[50]
80 DIM Y$[50]
90 PRINT "WHAT IS THE NUMBER OF POINTS PER SPECTRUM(<=50)?"
100 INPUT N
110 PRINT
120 PRINT "INPUT X COORDINATES"
130 INPUT X$
140 FILE #1;X$
150 FOR L=1 TO N
160 READ #1;X[L,1]
170 NEXT L
180 PRINT
190 PRINT "WHAT IS THE NAME OF THE SPECTRUM TO BE FIT?"
200 INPUT Y$
210 FILE #2;Y$
220 FOR L=1 TO N+2
230 READ #2;Y[L,1]
240 NEXT L
250 PRINT
260 PRINT "WHAT IS THE NAME OF THE FIRST PRIMARY SPECTRUM?"
270 INPUT A$
280 FILE #3;A$
290 FOR L=1 TO N+2
300 READ #3;A[L,1]
310 NEXT L
320 PRINT
330 PRINT "WHAT IS THE NAME OF THE SECOND PRIMARY SPECTRUM?"
340 INPUT B$
350 FILE #4;B$
360 FOR L=1 TO N+2
370 READ #4;B[L,1]
380 NEXT L
390 PRINT
400 PRINT "WHAT IS THE NAME OF THE THIRD PRIMARY SPECTRUM?"
410 INPUT C$
420 FILE #5;C$
430 FOR L=1 TO N+2
440 READ #5;C[L,1]
450 NEXT L
460 IF Y[N+1,1]<1000 THEN 1370
470 IF Y[N+2,1]<1000 THEN 1440
480 IF A[N+1,1]<1000 THEN 1510
490 IF A[N+2,1]<1000 THEN 1580
500 IF B[N+1,1]<1000 THEN 1650
510 IF B[N+2,1]<1000 THEN 1720
520 IF C[N+1,1]<1000 THEN 1780
530 IF C[N+2,1]<1000 THEN 1850
540 LET D[1,1]=0
550 LET D[1,2]=0
560 LET D[1,3]=0
570 LET D[2,1]=0
580 LET D[2,3]=0
590 LET D[3,3]=0
600 LET T1=0
610 LET T2=0
620 LET T3=0
630 FOR L=1 TO N
650 LET D[1,2]=D[1,2]+A[L,1]*B[L,1]
700 LET T1=T1+A[L,1]*Y[L,1]
710 LET T2=T2+B[L,1]*Y[L,1]
720 LET T3=T3+C[L,1]*Y[L,1]
730 NEXT L
740 LET D[2,1]=D[2,1]
750 LET D[3,1]=D[3,1]
760 LET D[3,3]=D[3,3]
770 LET P=INV(D)
780 LET D=DET(D)
790 LET M1=D
800 LET M1[L,1]=T1
810 LET M1[L,2]=T2
820 LET M1[L,3]=T3
830 LET Q=INV(M1)
840 LET M1=DET(M1)
850 LET M2=D
860 LET M2[L,1]=T1
870 LET M2[L,2]=T2
880 LET M2[L,3]=T3
890 LET R=INV(M2)
900 LET M2=DET(M2)
910 LET M3=D
920 PRINT
930 PRINT "IT WORKED BY GOLLY!!!"
940 PRINT
950 PRINT "X",A$,B$,C$
960 LET M3[1,3]=T1
970 LET M3[2,3]=T2
980 LET M3[3,3]=T3
990 MAT U=INV(M3)
1000 LET M3=DET(M3)
1010 LET W1=M1/D
1020 LET W2=M2/D
1030 LET W3=M3/D
1040 FOR L=1 TO N
1050 LET F1[L,1]=A[L,1]*W1
1060 LET F2[L,1]=B[L,1]*W2
1070 LET F3[L,1]=C[L,1]*W3
1090 PRINT X[L,1],A[L,1],B[L,1],C[L,1]
1100 NEXT L
1110 PRINT
1120 PRINT "X","FIT FOR","FIT FOR","FIT FOR"
1130 PRINT "A$,B$,C$
1140 FOR L=1 TO N
1150 PRINT X[L,1],F1[L,1],F2[L,1],F3[L,1]
1160 NEXT L
1170 PRINT
1180 PRINT "X",Y$,"TOTAL FIT"
1190 FOR L=1 TO N
1200 PRINT X[L,1],Y[L,1],S[L,1]
1210 NEXT L
1220 PRINT
1230 PRINT "WEIGHTING FACTOR FOR ";A$" = ";W1
1240 PRINT
1250 PRINT "WEIGHTING FACTOR FOR ";B$" = ";W2
1260 PRINT
1270 PRINT "WEIGHTING FACTOR FOR ";C$" = ";W3
1280 PRINT
1290 LET R=0
1300 FOR L=1 TO N
1310 LET R=R+ABS(S[L,1]-Y[L,1])
1320 NEXT L
1330 LET Z=R/N
1340 PRINT
1350 PRINT "MEAN RESIDUAL = ";Z
1360 END
1370 LET G=1000/Y[N+1,1]
1380 FOR L=1 TO N
1390 LET Y[L,1]=Y[L,1]*G
1400 NEXT L
1410 LET Y1=1000
1420 GOTO 470
1430 END
1440 LET C=1000/Y[N+2,1]
1450 FOR L=1 TO N
1460 LET Y[L,1]=Y[L,1]*C
1470 NEXT L
1480 LET Y2=1000
1490 GOTO 480
1500 END
1510 LET G=1000/A[N+1,1]
1520 FOR L=1 TO N
1540 NEXT L
1550 LET A1=1000
1560 GOTO 490
1570 END
1580 LET C=1000/A[N+2,1]
1590 FOR L=1 TO N
1610 NEXT L
1620 LET A2=1000
1630 GOTO 500
1640 END
1650 LET G=1000/B[N+1,1]
1660 FOR L=1 TO N
1670 LET B[L,1]=B[L,1]*G
1680 NEXT L
1690 LET B1=1000
1700 GOTO 510
1710 END
1720 LET C=1000/B[N+2,1]
1730 FOR L=1 TO N
1740 LET B[L,1]=B[L,1]*C
1750 NEXT L
1760 LET B2=1000
1770 GOTO 520
1780 LET G=1000/C[N+1,1]
1790 FOR L=1 TO N
1800 LET C[L,1]=C[L,1]*G
1810 NEXT L
1820 LET C1=1000
1830 GOTO 530
1840 END
1850 LET C=1000/C[N+2,1]
1860 FOR L=1 TO N
1870 LET C[L,1]=C[L,1]*C
1880 NEXT L
1890 LET C2=1000
1900 GOTO 540
1910 END
A.2. AN EXAMPLE OF AN EPR FIT (c.f. FIG. VI.6A)

WHAT IS THE NUMBER OF POINTS PER SPECTRUM(<=50)?
?41
INPUT X COORDINATES
?XEPFR
WHAT IS THE NAME OF THE SPECTRUM TO BE FIT?
?41D
WHAT IS THE NAME OF THE FIRST PRIMARY SPECTRUM?
?2ANO
WHAT IS THE NAME OF THE SECOND PRIMARY SPECTRUM?
?2BNO
WHAT IS THE NAME OF THE THIRD PRIMARY SPECTRUM?
?3ANOB
IT WORKED BY GOLLY!!!

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MEAN RESIDUAL = 0.532549
A.3. PROGRAM FOR FITS TO OPTICAL DATA
THIS PROGRAM WAS WRITTEN BY PAUL REISBERG

10 REM ****** MULTIPLE LINEAR REGRESSION *******
20 REM ****** EQANAL W/ CARY DECODE *******
30 REM N=#PTS N1=#TERMS N2=#CURVES X=SPECIES Y=DATA
40 REM FILENAME0=DEOX Y,FILENAME1=LIGANDED,FILENAME2=DATA
50 REM J,IR,AR9,Y5,X2,S0,R1,Y1,X5,S3,R,A,S2,A2,A3,D,N3,K,A0,F
60 REM X1=WVLNGTHS,F$=FILENAME,D$,S$
70 REM F2=ATTENUATION FACTOR
80 REM N4,N5,S1$,S2$,H=[HB],C1=[RNC]1UL/ML,CO=[STOCK],U=UL/ML
90 REM U1=UL/ML SLTN
100 DIM X[401,10],F$[16],S1[401],Y1[401],A[10]
110 DIM S2[10],R[10]
120 DIM S3[10],A2[10,10],A3[10,10]
130 DIM S$[15],D$[50],X1[401],Y[401]
140 DIM S1$[10],S2$[10]
150 REM  INITIALIZATION
210 INPUT "#OF CURVES? ",N2
220 INPUT "PRINT EVERY ",N5
230 INPUT "FILENAME? ",F$
240 LET N1=2
250 LET N4=N1-1
260 FOR L=0 TO N4
270 GOSUB 1380
280 FOR I=1 TO N
290 LET X[I,L+1]=Y[I]
300 NEXT I
310 NEXT L
320 FOR L=2 TO N2
330 LET A9=0
340 LET Y5=0
350 LET X2=0
360 LET S0=0
370 LET R1=0
380 FOR I=1 TO N1
390 LET Y1[I]=0
400 NEXT I
410 FOR J=1 TO N1
420 LET X5[J]=0
430 LET S3[J]=0
440 LET R[J]=0
450 LET A[J]=0
460 LET S2[J]=0
470 NEXT J
480 MAT A2=ZERO[N1,N1]
490 MAT A3=ZERO[N1,N1]
500 REM  ACCUMULATION OF SUMS
510 GOSUB 1380
FOR I=1 TO N
   LET Y5=Y5+Y[I,J]
FOR J=1 TO N1
   LET X5[J,J]=X5[J,J]+X[I,J]
NEXT J
NEXT I
LET Y5=Y5/N
FOR J=1 TO N1
   LET X5[J,J]=X5[J,J]/N1
NEXT J
FOR I=1 TO N
   LET S0=S0+(Y[I,J]-Y5)^2
FOR J=1 TO N1
   LET D=X[I,J]-X5[J,J]
   LET S3[J,J]=S3[J,J]+D^2
   LET R[J,J]=R[J,J]+(Y[I,J]-Y5)
   FOR K=1 TO J
   NEXT K
NEXT J
NEXT I
LET N3=N-1
LET S2=SQR(S2/N3)
FOR J=1 TO N1
   LET S3[J,J]=SQR(S3[J,J]/N3)
   LET R[J,J]=R[J,J]/(N3*S3[J,J]*S0)
   FOR K=1 TO J
      LET A2[J,K]=A2[J,K]/(N3*S3[J,J]*S3[K,J])
   NEXT K
NEXT K
NEXT J
MAT A3=INV(A2)
LET A0=Y5
FOR J=1 TO N1
   FOR K=1 TO N1
   NEXT K
NEXT J
PRINT "A";J;"=";A[J]
LET A0=A0-A[J]*X5[J]
FOR I=1 TO N
NEXT I
NEXT J
PRINT "A 0 =";A0
FOR I=1 TO N
   LET Y1[I,J]=Y1[I,J]+A0
   LET X2=X2+(Y[I,J]-Y1[I,J])^2
NEXT I
LET X2=X2/(N-(N1+1))
FOR J=1 TO N1
1030 LET S2[J]=SQR(A3[J]*J)*X2/(N3*S3[J]^2)
1040 PRINT "SIGMA A";J;" = ";S2[J]
1050 LET A9=A9+A[J]
1060 LET R1=R1+A[J]*R[J]*S3[J]/S0
1070 NEXT J
1080 LET F=(R1/N1)/(N-R1)/(N-(N1+1))
1090 LET R1=SQR(R1)
1100 LET S=X2/N
1110 FOR J=1 TO N1
1120 FOR K=1 TO N1
1130 LET S=S+X2*X5[J]*X5[K]*A3[J,K]/(N3*S3[J]*S3[K])
1140 NEXT K
1150 NEXT J
1160 LET S=SQR(S)
1170 PRINT " SIGMA A0 = ";S
1180 PRINT " ONE MINUS SUM OF COMPONENTS",1-A9
1190 PRINT " R(1) = ";R[1]
1200 PRINT " R(2) = ";R[2]
1210 PRINT " F TEST",F
1220 PRINT "MULTIPLE LINEAR CORRELATION COEF = ";R1
1230 PRINT "WAVELENGTH","OD","ODFIT","RESIDUALS"
1240 FOR I=1 TO N STEP N5
1250 PRINT X1[I],Y[I],Y1[I],Y[I]-Y1[I]
1260 NEXT I
1270 LET Y3=A[2]/A9
1280 PRINT
1290 PRINT
1300 PRINT
1310 PRINT
1320 PRINT
1330 PRINT
1340 PRINT
1350 PRINT
1360 NEXT L
1370 GOTO 1680
1380 REM
1390 LET D$=F$+STR(L)
1400 FILE #1;D$
1410 LET N=1
1420 REWIND #1
1430 READ #1;D$
1440 READ #1;D$
1450 READ #1;D$
1460 IF EOF(1)=6 THEN 1540
1470 IF D$[2,2]<>"0" THEN 1450
1480 LET S$=D$[8,18]
1490 LET X1[N]=VAL(S$)
1500 LET S$=D$[20,34]
1510 LET Y[N]=VAL(S$)
1520 LET N=N+1
1530 GOTO 1450
1540 LET N=N-1
1550 RETURN
1680 END
A.4. AN EXAMPLE OF AN OPTICAL FIT (C.F. FIG. VI.6B)

$\#$OF CURVES? 3
PRINT EVERY 1
FILENAME?AZ

A 1 = .126353
A 2 = .438687
A 0 = -2.85599E-4

SIGMA A 1 = 1.25502E-2
SIGMA A 2 = 5.35161E-3
SIGMA A0 = 4.6774E-4
ONE MINUS SUM OF COMPONENTS = .43496

R(1) = .425726
R(2) = 1.18239
F TEST = -39.0995

MULTIPLE LINEAR CORRELATION COEF = 1.19024

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