NMR STUDIES OF
COOPERATIVITY IN HEMOGLOBIN

Thesis by
George Adler

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ABSTRACTS

PART I

In this part the binding of ligands, like oxygen and carbonmonoxide to hemoglobin and myoglobin, and the connection between the physiological functions and the structure of these proteins are discussed. The allosteric models, which have been proposed to explain the cooperative binding of oxygen, and the effect of single residue mutations on the structure of hemoglobin are reviewed. Finally, questions which have not yet been satisfactorily answered are raised about the structure and function of hemoglobin.

PART II

The binding of carbon monoxide to rabbit hemoglobin and to trifluoroacetonylated rabbit hemoglobin has been studied by ^{13}C NMR and by ^{19}F NMR. The ^{13}C NMR studies show that CO binds preferentially to the β chains of rabbit hemoglobin in the absence of effectors such as 2,3-diphosphoglycerate (DPG) and inositol hexaphosphate.

The ^{19}F spectrum of trifluoroacetonylated human adult hemoglobin, at low CO or O_2 ligation, indicated preferential α chain ligation (Huestis and Raftery, 1973, Biochemistry 12, 2531). There is a linear

relationship between the fraction of the ligated signal of trifluoroacetonylated rabbit hemoglobin as monitored by ^{19}F NMR and the overall ligation detected by optical density measurements at 650 nm. Therefore, the trifluoroacetonyl label does not, in the case of rabbit hemoglobin, distinguish initial β ligation from random ligation.

Both in the absence of and in the presence of DPG, the ^{19}F spectra of partially ligated trifluoroacetonylated rabbit hemoglobin reveal only two significant resonances. These two resonances, which correspond to the deoxy (tense) and ligated (relaxed) structures of hemoglobin, are joined by two additional resonances when inositol hexaphosphate is added to the solution. The new resonances are presumably due to hemoglobin in intermediate structures between the relaxed and tense states.

The ^{19}F chemical shifts of the deoxy and ligated TF labelled hemoglobin change due to the addition of organic phosphates. This observation implies that the organic phosphates bind to hemoglobin in both the deoxy and the ligated states.

PART III

Titrations of trifluoroacetonyl labelled human deoxy-, oxy-, carbonmonoxy-, nitrosylhemoglobin, aquamethemoglobin and cyanomethemoglobin have been followed by ^{19}F NMR. The titration curve of trifluoroacetonyl human deoxyhemoglobin (Hb^{TF}) in the presence or absence of 2,3-diphosphoglycerate (DPG) is similar to that found by Huestis and Raftery (Proc. Natl. Acad. Sci. USA (1972) 69, 1887). Nitrosyl-, oxy-, and carbonmonoxy-Hb^{TF} in the absence of effectors exhibit pH dependence only below pH 5.5. The addition of inositol hexaphosphate (IHP) resulted in large upfield shifts of the fluorine resonance of $Hb^{TF}(NO)_4$, $Hb^{TF}(O_2)_4$. In addition, the fluorine signal of $Hb^{TF}(CO)_4$ and $Hb^{TF}(O_2)$ exhibited large linewidths which is evidence for the exchange between two structures. The addition of DPG to $Hb^{TF}(NO)_4$ below pH 7.4 results in the appearance of a second resonance which indicates the presence of two different protein structures.

The titration of the labelled aquamethemoglobin in the presence or in the absence of DPG supports the results of Huestis and Raftery (1972). In the presence of IHP trifluoroacetonyl $Hb^{+}(CN^{-})_4$ shows a complicated ^{19}F spectra which suggests that cyanomethemoglobin can have three

different structures. The implication of these results on the mechanism of cooperativity and on the two state model is discussed.

PART IV

The intracellular pH, and the binding of intracellular 2,3-diphosphoglycerate (2,3-DPG) to hemoglobin are studied in AA and SS blood. There is no significant difference in the intracellular pH of AA and SS erythrocytes in the fully oxygenated state. However, in the ^{31}P spectrum of deoxygenated AA blood the resonance due to 2-P of 2,3-DPG is downfield, while in the ^{31}P spectrum of deoxygenated SS blood this resonance is upfield, from the chemical shift of the same phosphorous signal in oxygenated blood. This difference in ^{31}P chemical shifts is likely due to a lower intracellular pH of the SS erythrocytes relative to the AA erythrocytes.

ABSTRACTS OF PROPOSITIONS

Proposition I

A study of the tertiary and quaternary structures of different valency hybrids are proposed.

Proposition II

A study of the effect of the plasma pH on the intracellular pH of AA and SS erythrocytes and on the number of crises which occur in sickle cell patients is proposed.

Proposition III

A study of the mechanism of neoplastic transfromation caused by carcinogens is proposed in mouse fibroblast cells.

Proposition IV

An IR study of the binding of ligands, like N_2, NO,CO, to nitrogenase is proposed. This also involves the evaluation of the number of binding sites in nitrogenase, and the study of competitive inhibition of N_2 reduction by the binding of NO.

Proposition V

The ^{31}P and ^{13}C NMR study of cyanobacteria (blue-green algae) and plant tissues (leaves, stock, sap) are proposed.

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Part III

Part I

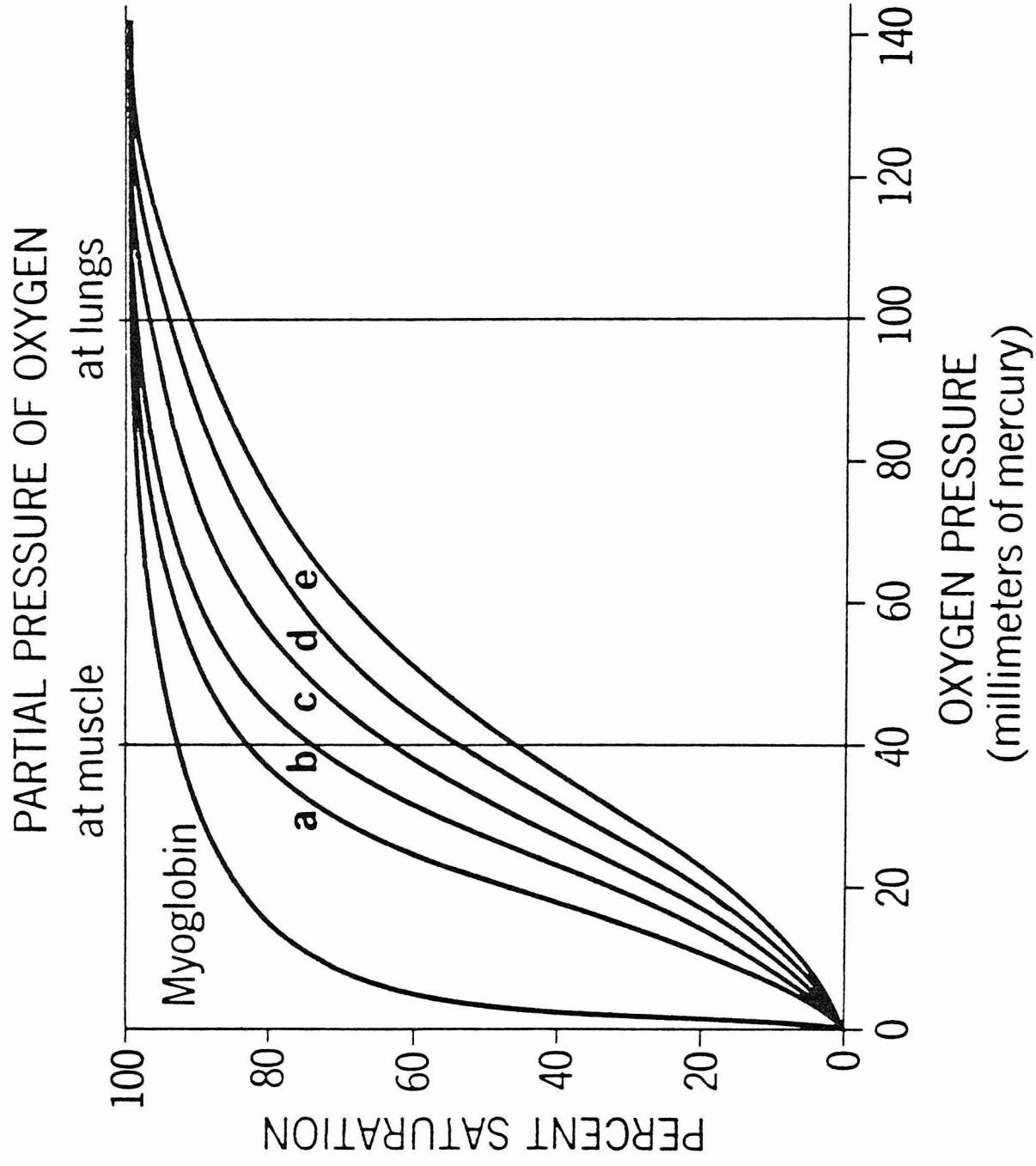
A REVIEW OF THE STRUCTURE AND THE FUNCTION OF HEME PROTEINS

Function of Heme Proteins

Hemoglobin (Hb), the protein which transports oxygen, is one of the most studied proteins in nature (Antonini & Brunori, 1971; Dickerson & Geis, 1969; Bunn, et al., 1977). The reasons for its popularity are its availability in highly pure form and its ability to bind oxygen reversibly with varying affinity. Erythrocytes, the biconcave cells which contain high concentrations of Hb, can be separated from the rest of the blood by simple centrifugation. These cells contain 96 wt. % of Hb. Myoglobin (Mb), the oxygen storage protein of vertebrates, can also bind oxygen reversibly. However, it has a much higher affinity toward oxygen than Hb does. This fact is especially important since Hb has to release the oxygen to myoglobin which is in the muscles. However, Hb not only binds oxygen reversibly and with lowered affinity but it also binds oxygen in a cooperative fashion. The oxygen saturated Hb delivers oxygen to the myoglobin in the muscles. After the release of the first oxygen molecule hemoglobin releases the rest of the oxygen with more and more ease. This happens because there is a larger and larger difference in the affinities between Hb and myoglobin as oxygen is being released, as shown in Figure 1.

FIGURE 1

Oxygen binding curves of myoglobin and hemoglobin with lowering pH (a to e). (The curves were obtained from Dickerson and Geis, 1969).


PARTIAL PRESSURE OF OXYGEN
at muscle
at lungs
Myoglobin
a
b
c
d
e
PERCENT SATURATION
100
80
60
40
20
0
0
20
40
60
80
100
120
140
OXYGEN PRESSURE
(millimeters of mercury)

Hb not only binds oxygen at the lung and releases it to the muscles, but it also helps carry the CO_2 from the tissues to the lung (Parella, 1975; Parella, et al., 1975; Bauer & Kurtz, 1977). Hb binds CO_2 at the N terminals in the following manner (Dickerson & Geis, 1980)

$$-NH_2 + CO_2 \rightleftarrows NH\text{-}COO^- + H^+$$

and by its buffering ability according to the following equation: $Hb(O_2)_4 + H^+ + HCO_3^- \rightleftarrows Hb(H^+) + HCO_3^- + O_2\uparrow$ (Bohr, et al., 1904; Antonini & Brunori, 1971; Dickerson & Geis, 1980).

Hb also responds to heterotropic effectors. In Figure 1 the oxygen binding curves of myoglobin and Hb are shown. The different binding curves of Hb in Figure 1 (a-e) are due to decreasing pH. In the equation above, deoxyhemoglobin binds protons relative to the ligated Hb. This difference in the acidity of Hb is called the Bohr effect. Therefore an increase in protons (decrease in pH) results in a lowering affinity of Hb toward oxygen (Wyman, 1964). Another way to change the binding affinity of Hb is through the presence of the molecule 2,3-diphosphoglycerate (DPG) (Benesch & Benesch, 1967; Benesch, et al., 1968). This molecule which is present in the erythrocytes with about the same concentration as Hb binds

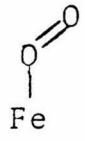
with a much higher affinity to deoxyhemoglobin than to ligated Hb.

$$Hb(O_2)_4 + DPG \rightleftarrows Hb(DPG) + 4O_2\uparrow$$

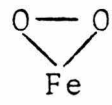
Therefore, people living at high altitudes where oxygen pressure is diminished have up to 50% higher amounts of DPG in their erythrocytes than people living at normal altitude (Lenfant, et al., 1968). This results in a more efficient delivery of oxygen. Thus the available oxygen is lower but the amount of oxygen which is not released by Hb to the tissue is also less. (This change is similar to the change due to the lowering of pH in Figure 1). While deoxy adult hemoglobin binds DPG with high affinity, fetal Hb does not. The difference in affinity between fetal Hb and maternal Hb is mostly due to the heterotropic effect of DPG (Tyuma & Shimizu, 1970; Maurer, et al., 1970). This difference in affinity insures that the maternal Hb delivers oxygen to the fetus.

Ligand Binding to Heme Proteins

The binding of oxygen to iron in Hb and myoglobin is not fully understood. Pauling and Coryell (1936) suggested "end-on" binding (I), while Griffith (1956) suggested that the oxygen binds in a bridging fashion (II).



I



II

Weiss (1964) suggested that actually the iron gets oxidized as it binds oxygen ($Fe(III)O_2^-$). Finally Gray (1971) suggested that the iron in oxyhemoglobin is in the +4 state. The "end-on" geometrical arrangement has been proven by IR and ESR studies (Barlow, et al., 1973; Gupta, et al., 1975). Further, the experimental data, IR, Raman, optical and Mössbauer spectroscopy support the Weiss model (Maxwell, et al., 1974; Barlow, et al., 1973; Yamamoto, et al., 1973; Spiro & Strekas, 1974; Wittenberg, et al., 1970; Lang & Marshall, 1966). More recently, however, Goddard and Olafson (1975) suggested a model similar to the binding in ozone. In this model the unpaired electron on the iron is spin coupled with the unpaired electron on the oxygen (↑Fe-O=O↓). The ozone model can account for the results of Mössbauer spectroscopy without assuming that the iron becomes oxidized. Huynh, et al. (1977) have suggested, in favor of the Pauling model, that Raman spectroscopy can only detect changes in the structure of the heme and not in the oxidation state of the iron. Further, the ozone model and the superoxide

model are consistent with the detected IR stretch of oxygen in $Mb(O_2)_4$ and $Hb(O_2)_4$. Therefore one cannot differentiate between the Weiss and Pauling models using the methods of IR and Raman spectroscopy.

Recent magnetic susceptibility measurements implied that at temperatures, 50-52°K oxyhemoglobin is in thermal equilibrium between a singlet and a triplet state (Cerdonio, et al., 1977). Pauling (1977) disagreed with this interpretation. He suggested that when the Hb solution is frozen, the protein may dehydrate, which can result in the dissociation of the O_2. The calculations of Huynh, et al. (1977) further suggested that the singlet and triplet states are separated by much more in energy than the magnetic susceptibility data suggest.

Carbonmonoxyhemoglobin is diamagnetic and experimental data support the Pauling model in this case, except for Raman spectroscopy which suggests the Weiss model: $Fe(III)CO^-$. Early X-ray diffraction studies on erythrocruorin suggested that the bound CO is bent (Huber, et al., 1970). However, recent X-ray structural studies support that the CO is tilted in $Mb(CO)_4$ and in $Hb(CO)_4$ from the normal of the plane of the heme (Heidner, et al., 1976; Norvell, et al, 1975). One of the reasons which

has been suggested for the tilted CO is that due to the tilt the protein binds CO with lower affinity than the heme models do and therefore excess O_2 can replace the CO (Collman, et al., 1976; Symons & Peterson, 1978). This tilt of the CO is caused by the closeness of histidine E7 and valine E11.

The Structure of Heme Proteins

Myoglobin consists of a single polypeptide chain, 153 amino acids long, which is folded around the heme group (Dickerson & Geis, 1969; Kendrew, et al., 1960; Takano, 1977a,b). The heme, an aromatic porphyrin ring with iron in the +2 or +3 state, is held in a pocket formed by the polypeptide chain by a covalent bond and by hydrophobic interactions. The iron is coordinated by five or six groups depending on its state of ligation; this includes the four pyrrole nitrogens of the porphyrin ring and the nitrogen on the imidazole group of the histidine F8. The iron atom in deoxymyoglobin is out of the plane of the heme by 0.55 Å toward histidine F8 (Takano, 1977b). When it is ligated by carbon monoxide the iron is in the plane of the heme (Norvell, et al., 1975). On the distal side of the heme there is a histidine (E7) which is conserved in the myoglobin and hemoglobin of vertebrates. This

histidine does not bind directly to the heme yet it probably has important functions together with valine E11 in keeping the iron in the +2 state.

The surface amino acids are polar residues in the polypeptide chain while the interior amino acids are mostly hydrophobic residues. Therefore myoglobin is hydrated with a layer of water around it. The water shield around the myoglobin keeps the protein in a monomeric state. The hydrophobic residues in the interior of the myoglobin are necessary for keeping the polypeptide chain in its folded form and for keeping the heme in its pocket. The iron in myoglobin binds O_2, CO, NO and alkyl isocyanates in the +2 state and it binds H_2O, OH^-, N_3^-, CN^- in the +3 state as its sixth ligand. Due to the binding of a ligand the change in structure is localized around the heme pocket in myoglobin.

Hemoglobin, in contrast to myoglobin, has four polypeptide chains, two of a kind ($\alpha_2\beta_2$) arranged in a 2,2,2 symmetry. The α chains have 141 amino acids while the β chains have 146. In the α and β chains the polypeptide is folded around the heme as in myoglobin. Each of these chains can bind one oxygen molecule. The four chains are kept together by hydrophilic and hydrophobic interactions. Some of the polar residues in myoglobin in the C and D helices and in other contact areas have been

replaced by non-polar residues. In deoxyhemoglobin the polar residues like histidines βNA2, βH21, and lysine βEF6, are responsible for the binding of DPG in the cavity formed by the β chains (Arnone, 1972). The oxyhemoglobin also binds DPG probably at the same site as in deoxy-hemoglobin but with less affinity (Garby, et al., 1969; Gupta, et al., 1979).

The Mechanism of Cooperativity in Hemoglobin

A molecular mechanism for the cooperativity of Hb has been proposed based on the crystal structure of deoxy and ligated Hb (Perutz, 1970). In the α_1-β_1 contact changes have not been detected between the deoxy and ligated structures. Most of the difference between the deoxy and ligated structure is in the α_1-β_2 contact. When the Hb becomes ligated the two αβ dimers change their position relative to each other. Upon ligation some hydrogen bonds get broken and others are formed. Two other important parts of the molecule which change are the carboxylic terminals and the heme pocket.

The ligand, when it binds to the iron, changes the configuration of the iron in the heme (Perutz, 1970; 1976). In deoxyhemoglobin, as in deoxymyoglobin, the iron is out of the plane of the heme toward histidine F8 by 0.6 Å (Fermi,

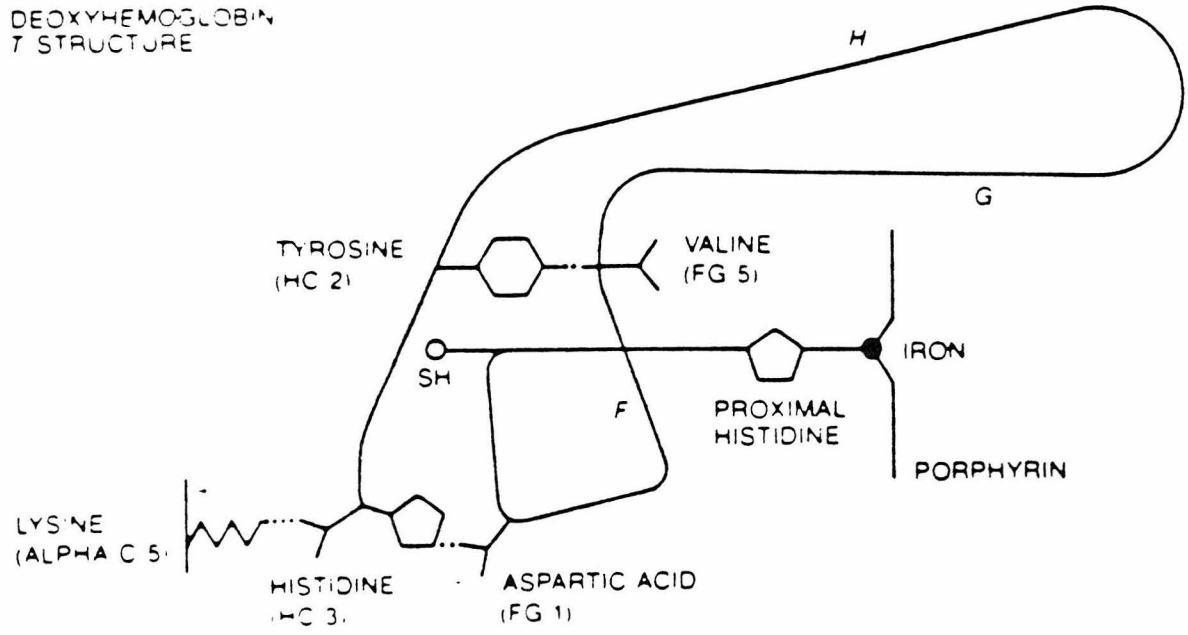
1975). It is coordinated by five groups; four of the ligands are the nitrogens of the pyrrole rings of the porphyrin, and one is the nitrogen of histidine F8. The bond is longer between the proximal histidine and the iron in deoxyhemoglobin than it is in ligated Hb. When the ligand binds the iron moves into the plane of the heme and the bond length decreases between the proximal histidine and the iron. Due to these changes the histidine F8 moves closer to the iron and to the plane of the heme. As the histidine F8 moves the F chain moves with it and changes its structure (Perutz, 1970; Baldwin & Chothia, 1979). The ligand is tilted from the normal of the plane of the heme by the closeness of the histidine E7 and valine E11 (Heidner, et al., 1976).

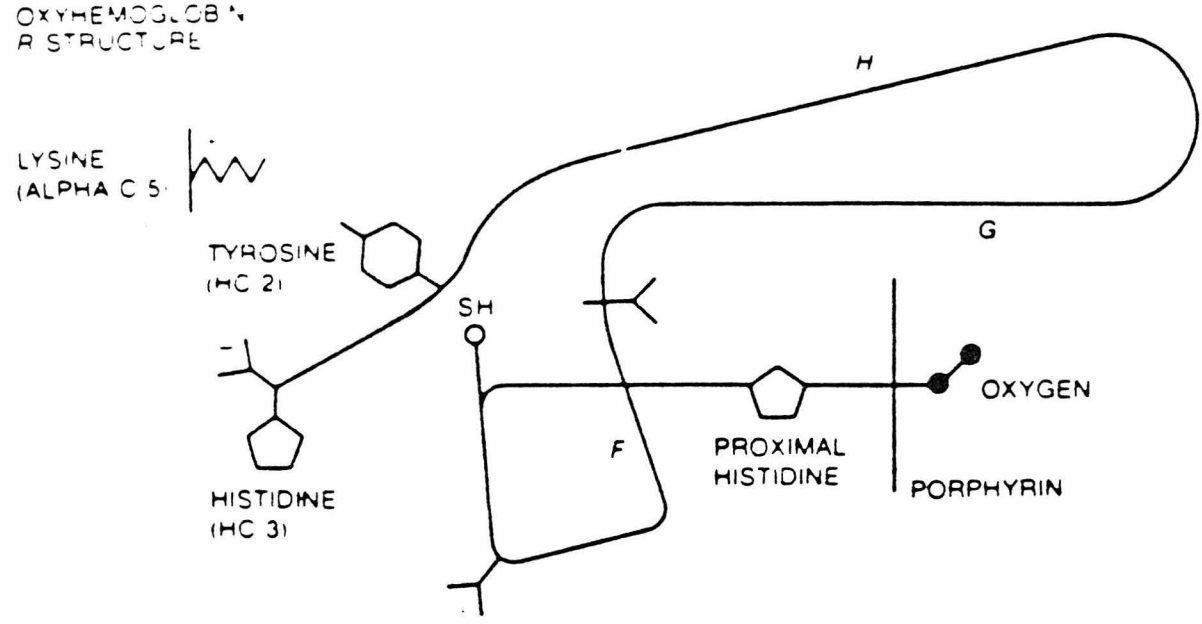
Other changes also occur due to the binding of the ligand. In the α chains the ligand replaces a water molecule which resides in the pocket of the α chains of the deoxyhemoglobin (Fermi, 1975). In the β chain where space is tight and water cannot get in valine E11 has to swing away from the heme so that the ligand can get into the pocket. Recently, it has been suggested that the chain around the heme does not change its structure but that the heme moves and changes its structure relative to its pocket (Baldwin & Chothia, 1979).

The movement of the histidine F8 changes the structure of the F helix. A result of this change is shown in the β chain in Figure 2 where the sulfur of cysteine βF9 moves in conjunction with tyrosine βHC2 (Heidner, et al., 1976; Perutz, 1970). In deoxyhemoglobin the tyrosine βHC2 sits in the pocket between the F and H helices and its hydroxyl group forms a hydrogen bond with the carboxyl group of valine βFG5. The carboxylic group of histidine βHC3 forms a hydrogen bond with the lys αC5 while the histidine group forms a hydrogen bond with aspartate βFG1. When a ligand binds the F and H helices move closer so that tyrosine βHC2 is forced out of its pocket. Valine βFG5 also changes its structure due to the severing of the hydrogen bond between valine βFG5 and tyrosine βHC2. Thus the tyrosine moves out of its pocket and the SH group of cysteine βF9 moves in place of the tyrosine residue. As the tyrosine residue moves out, both of the hydrogen bonds of histidine β146 to aspartate βFG1 and to lysine αC5 are broken. At pH 7.4 a proton is released from the histidine βHC3 which contributes to the Bohr effect (Perutz, et al., 1969; Perutz, 1970; Kilmartin & Wootton, 1970). The hydrogen bond which helped to stabilize the α chains in the deoxy

FIGURE 2

The structure of the histidine F8 side of the heme and the carboxylic end of the β chains. (The drawing was obtained from Perutz, 1978).


DEOXYHEMOGLOBIN
T STRUCTURE
H
G
TYROSINE
(HC 2)
VALINE
(FG 5)
IRON
SH
F
PROXIMAL
HISTIDINE
PORPHYRIN
LYSINE
(ALPHA C 5)
HISTIDINE
(HC 3)
ASPARTIC ACID
(FG 1)


OXYHEMOGLOBIN
R STRUCTURE
H
LYSINE
(ALPHA C 5)
G
TYROSINE
(HC 2)
SH
OXYGEN
F
PROXIMAL
HISTIDINE
PORPHYRIN
HISTIDINE
(HC 3)

state (lys αC5 - his βHC3) is broken so that the α chain is more likely to pick up a ligand when the β chain is ligated.

At a certain point in the ligation the quaternary structure of Hb changes, altering the structure of the groove of valine αFG5 and the orientation of tryptophan βC3. The groove of valine βFG5 in deoxyhemoglobin is occupied by threonine αC6, while in ligated Hb the groove is occupied by threonine αC3 (Perutz, 1976).

When both of the β chains are ligated or when the quaternary structure change occurs, DPG is released. The Bohr protons which thus far have not been released, now are also released. The chains which are not ligated yet have the same high affinity towards ligands as do myoglobins.

The previous description of cooperativity is written with the assumption that the β chains ligate first. However, as Perutz (1970) has pointed out, similar mechanisms can be designed for every sequence of sub-unit ligation. Perutz (1970) in his description of the mechanism has assumed that α chains ligate first.

Two models have been proposed for the triggering of cooperativity and for the storage of the free energy

for cooperativity. Perutz (1970, 1972) has proposed that the triggering occurs from the movement of the iron out of the plane of the heme in deoxyhemoglobin into the plane of the heme in oxyhemoglobin. This movement also moves the histidine F8 which transmits the change through the F and E helices. Further, when the iron is out of the plane of the heme it is under tension and the relief of this tension supplies the energy for the change in the structure of the protein.

The second model, proposed by Hopfield (1973), suggests that the protein responds linearly to changes in the distance between the porphyrin plane and the metal atom. The energy in this model is stored in several electrostatic and hydrophobic interactions. Recently, Gelin and Karplus (1977), Warshel (1977) and Baldwin and Chothia (1979) have revised this model to fit recent results. They proposed that the movement of the heme from its dome shape in deoxyhemoglobin to a planar shape in ligated Hb changes the structure of the protein. The structural change occurs due to the relief of tension, due to van der Waal's interaction, between the heme and the protein contacts.

Allosteric Models

Several models have been proposed to describe the binding behavior of Hb. The first important model has been described by Hill (1910). He proposed that Hb is an oligomer with an unknown number of subunits, and therefore the following equations must be true.

$$Hb_n + nO_2 \rightleftarrows (HbO_2)_n$$

$$K = \frac{[HbO_2)_n]}{[Hb_n][PO_2]^n} \quad \text{or} \quad K(PO_2)^n = \frac{\bar{Y}}{1 - \bar{Y}} \quad \text{when}$$

$$Y = \frac{Hb(O_2)_n}{Hb + Hb(O_2)_n}$$

The number of subunits, n can be determined from a plot of log $\bar{Y}/1-\bar{Y}$ vs log PO_2 and it has been found experimentally that n is 2.8. Since this number is not an integer and does not agree with the later findings that Hb has four binding sites, this model was discarded (Adair, 1923). More recently n has been revived as a measured of co-operativity (Antonini & Brunori, 1971). In a non-cooperative system n = 1, while in a fully cooperative system with four subunits, n = 4.

Adair (1975) suggested a stepwise mechanism in which the cooperativity of hemoglobin can be described by four equilibrium constants.

$$Hb + 4O_2 \overset{K_1}{\rightleftharpoons} Hb(O_2) + 3O_2 \overset{K_2}{\rightleftharpoons} Hb(O_2)_2 + 2O_2$$

$$Hb(O_2)_2 + 2O_2 \overset{K_3}{\rightleftharpoons} Hb(O_2)_3 + O_2 \overset{K_4}{\rightleftharpoons} Hb(O_2)_4$$

This model, of course, is true for the cooperativity of Hb. However, it does not explain why K_1, K_2, K_3, and K_4 have different values.

Two models have been developed which try to explain the differences in the values of the equilibrium constants in the Adair model. These are the Monod, Wyman, Changeux (MWC) and the Koshland, Nemethy, Filmer (KNF) models (Monod, et al., 1965; Koshland, et al., 1966). The MWC model assumes the existence of two quaternary structures and postulates that there are no intermediate structures between these two states. The allosteric protein at intermediate ligation switches its structure from one quaternary state to the other. This model can account for the Bohr effect, DPG binding and has been expanded to account for the effect of the differences between the α and the β chains (Ogata & McConnell, 1971; Edelstein, 1974; Rubin & Changeux, 1966).

The KNF, or induced model, postulates a step by step structural change. Thus when a subunit of Hb is ligated it changes its contacts to the other chains and therefore it is easier for the next subunit to bind a ligand and change its structure.

Both the KNF model and the MWC model can describe the behavior of Hb with well chosen equilibrium constants and interaction values. In order to describe the behavior of Hb the MWC model uses three constants while the KNF model uses four constants. Both models have certain aspects which have been proven to be correct in the molecular mechanism of cooperativity in Hb. However, in Hb the cooperative binding of ligands is due to a single quaternary structural change with many tertiary structural changes (Perutz, 1970; 1976). Recently, a model which fits the behavior of Hb better has been developed but this model is not as simple as the MWC and the KNF models (Szabo & Karplus, 1972).

The Effect of Mutations on Hemoglobin

Replacement of a single amino acid in Hb can have a drastic effect on the function of Hb (Antonini & Brunori, 1971; Perutz & Lehmann, 1968). Some mutated Hbs have

permanently high affinities like Hb Chesapeake (αFG4 arg→leu), Hb Kempsey (βG1 asp→asn) or low affinities like Hb Kansas (βG4 asn→thr) and Hb M Iwate (αF8 his→tyr). The replacement of critical residues in the heme pocket results in the oxidation of the iron, e.g., F8 his→tyr in M Iwate, and E11 val→glu in Hb M Milwaukee. In other cases it results in increased affinity and decreased stability, e.g. β E7 his→arg in Hb Zurich and E11 val→ala in Hb Sydney (Tucker, et al., 1978). Replacement of amino acids in the $\alpha_1\beta_2$ contact results in increased affinity e.g. Hb Chesapeake (αFG4 arg→leu) and J. Capetown (αFG4 arg→gln).

Another important mutation which has a severe effect on the function of Hb is the replacement of βA3 glutamic acid by valine which occurs in sickle cell Hb (Hunt & Ingram, 1959). In sickle cell anemia the mutation occurs in an area which is seemingly unimportant to the structure and function of Hb. Yet this replacement of a glutamic acid with valine results in the polymerization of Hb upon deoxygenation (Finch, et al., 1973). As a result of this polymerization the Hb SS has lower affinity toward oxygen and the erythrocytes deform into the sickle shape in deoxy blood (Gill, et al., 1978; Benesch, et al., 1978;

Bunn, et al., 1977). The result of these deformations is that the sickle cell has a much shorter life span and anemia and severe sickling attacks develop in sickle cell patients. Efforts to inhibit the polymerization have failed due to the ineffectiveness of inhibitors which can get inside the erythrocytes, or due to the poisonous quality of the inhibitors (Brewer & Oelshlegel, 1974; Cerami, 1974; Votano, et al., 1977).

In vitro studies on the polymers and conditions in which polymerization occurs are well documented (Ross et al., 1975; Hofrichter, et al., 1974; Finch, et al., 1973). However, data on in vivo polymerization are scant (Lam, et al., 1979). The mechanism of triggering of the sickling, and the reason for the existence of permanently sickled cells are still unknown.

Even though there has been much work done on Hb, the answers to several questions about the mechanism of cooperativity have eluded scientists. Is there a single set of intermediates or are there several sets of intermediates in the mechanism for the ligation of Hb? Is there a difference in function and in affinity between the α and the β chains? What is the signal which initiates the cooperativity of Hb?

This work deals mainly with the question of what is the proper mechanism for the ligation of Hb. Are there stable intermediates in the ligation of Hb? How do effectors like DPG and IHP affect the structure of Hb? The last part of this thesis tries to increase our understanding of the mechanism of sickling *in vivo*, and suggests a possible new approach to clinical treatment.

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Part II

THE EFFECT OF ORGANIC PHOSPHATES ON THE LIGATION OF RABBIT HEMOGLOBIN

INTRODUCTION

Two simple models have been devised to explain the cooperativity of hemoglobin (Hb): The Koshland, Nemethy, Filmer model which stresses a stepwise mechanism of quaternary structural changes (Koshland, et al., 1966) and the Monod, Wyman, Changeux model which stresses one major quaternary structural change (Monod, et al., 1965). The existence of intermediates would imply that the MWC model for the cooperativity of the Hb is an over-simplification.

Recently, there has been increased interest in the possibility of the two chains of Hb having different affinities towards ligands (Huang & Redfield, 1976; Huestis & Raftery, 1973; 1975; Moon & Richards, 1974; Viggiano & Ho, 1979a,b). Such a difference in the affinities of the two chains would imply that there is a preferential sequence of ligation and that certain intermediates might be observed in the presence of non-saturating amounts of ligand.

There is scant evidence for the existence of intermediate structures which appear during the ligation of Hb. The first suggestions of the possible existence of such intermediates are found in the ESR studies of Ogata and McConnell (1967) and Davies, et al. (1971).

Perutz (1970) also discussed the existence of intermediates, basing his arguments on X-ray structural studies of Hbs. He suggested that such structures formed during the ligation of Hb should, due to their instability, have short lives and be present in small concentrations. Proton NMR studies of artificial hybrids, $\alpha_2{}^{+CN}\beta_2^-$ and $\alpha_2\beta_2{}^{+CN}{}^-$, showed that these hybrids have either a tense (T) or relaxed (R), structure. (Deoxyhemoglobin has a tense structure, while oxyhemoglobin has a relaxed structure.) (Ogawa & Shulman, 1971; 1972; Cassoly, et al., 1971). However, using ^{19}F NMR, Huestis and Raftery (1972b; 1973; 1975) detected intermediates during the ligation with O_2 and CO of trifluoroacetonyl (TF) labelled Hb.

Detection of intermediates in the last case was possible because the TF label attached to cys βF9 is sensitive to changes in the tertiary and quaternary structure of Hb. The location of the sulphur atom of cys βF9, to which the TF label is attached, differs in the deoxy and ligated states (Heidner, et al., 1976). Moreover, the label lies in close proximity to important residues such as βF8 and βFG1. The proximal histidine (βF8), which forms a covalent bound with the heme, is thought to initiate the tertiary and quaternary structural changes following ligation by moving toward the plane of the heme.

In deoxyhemoglobin, an aspartate residue (βFG1) forms a hydrogen bond with the imidazole ring of a histidine (βHC3) while the carboxylate group of histidine (βHC3) forms a hydrogen bond with the amino group of lysine (αC5). Tyrosine βHC2 is located in the binding pocket formed between the F and H helices by the hydrogen bond between asp (βFG1) and his (βHC3). Upon ligation both the lys (αC5)-his (βHC3) and asp (βFG1)-his (βHC3) hydrogen bonds are broken and the tyrosine residue (βHC2) is forced out of its binding pocket. Therefore, the TF label on cys βF9 should be able to detect changes in the tertiary structure of the β chains and the quaternary structure of the entire protein.

The TF label can also detect structural changes due to the binding of effectors such as 2,3-diphosphoglycerate (DPG) (Huestis & Raftery, 1972a, b; 1973; 1975). Addition of DPG to TF labelled human deoxyhemoglobin results in a 12 Hz upfield shift of the ^{19}F resonance (Huestis & Raftery, 1972a). However, addition of DPG to ligated human Hb does not cause a change in the ^{19}F chemical shift.

[DPG binds to deoxyhemoglobin in the cavity between the β chains (Arnone, 1972). It also binds to oxyhemoglobin; however the location of the binding site in this

case is not known (Perutz, 1976). The binding of DPG to deoxyhemoglobin is much stronger than is its binding to oxyhemoglobin (Garby, et al., 1969; Costello, et al., 1976). DPG decreases the affinity of Hb toward O_2 and it enhances the Bohr effect (Benesch, et al., 1968; 1969; Benesch & Benesch, 1969; Riggs, 1971; Tyuma, et al., 1971).]

The effect of IHP on TF labelled Hb has not been previously studied. This effect should be especially interesting since IHP binds to both deoxyhemoglobin and oxyhemoglobin with 10^2 times larger affinity than DPG does (Edalji, et al., 1976; Benesch, et al., 1968; Garby, et al., 1969; Costello, et al., 1976). Both IHP and DPG decrease the affinity of the Hb towards the first three of the oxygen molecules but only IHP has an effect on the binding affinity of the fourth oxygen molecule (Tyuma, et al., 1971). Further, IHP switches the quaternary structure of $Hb(NO)_4$ to the tense state (Perutz, et al., 1976; Briehl & Salhany, 1972; Salhany, et al., 1975) and in the α chains it breaks the proximal histidine to iron bond (Maxwell & Caughey, 1976; Szabo & Perutz, 1976; Taketa, et al., 1978). IHP also alters the structure of $Hb(O_2)_4$ and $Hb(CO)_4$ (Adams & Schuster, 1974; Perutz, et al., 1976).

The trifluoroacetonyl label can also be used to detect preferential α chain ligation in Hb. Since the TF label is on the β chains it should detect β chain ligation. Huestis and Raftery (1972b; 1973) have detected a delay in the appearance of the resonance due to the ligated protein in the ^{19}F spectra of human Hb at low partial pressure of O_2 and CO. This delay occurs whether DPG is present in the solution or not. However, questions have been raised concerning the possibility that, in this study, the TF label may be detecting quaternary structural changes rather than β chain ligation (Edelstein, 1974).

Differences in the chemical environment of the binding pocket of the α and β chains have made it possible to detect differences in affinities between the two chains using ^{1}H and ^{13}C NMR spectroscopy (Davies, et al., 1971; Lindstrom & Ho, 1972; Moon & Richards, 1974).

The ^{1}H NMR data on initial ligation of Hb are inconclusive. Lindstrom and Ho (1972) have found that CO binds randomly to human Hb in the presence or the absence of effectors. The same study reported that, in the presence of a large excess of effectors, oxygen binds preferentially to the α chains. Later, Johnson and Ho (1974) in agreement with the previous study, found that CO ligation is random in the absence of effectors. In

contrast, Johnson and Ho (1974) disagreed with the results of Lindstrom and Ho in the presence of organic phosphates. In this case they found that the α chains bind CO preferentially relative to the β chains. Johnson and Ho (1974) have also concluded that O_2 binds preferentially to the α chains with or without effectors. These same O_2 binding experiments have been redone by Huang and Redfield (1976). They have found, however, that the data obtained by these experiments do not point conclusively towards preferential α chain ligation. More recently, Viggiano and Ho (1979a, b) have repeated the studies of initial O_2 ligation by proton NMR and in the absence of organic phosphates they have agreed with the conclusion of Huang and Redfield (1976). In the presence of organic effectors, however, they support the Johnson and Ho (1974) study which concluded that O_2 ligates preferentially to the α chains. ^{13}C NMR studies of ^{13}CO binding to human Hb revealed no difference in the affinities of α and β chains (Moon & Richards, 1974).

Stop flow studies of CO binding to Hb have shown that CO binds to the β chains faster than to the α chains. The kinetic data, however, cannot give any information about the stability of the structures formed during the ligation (Gray & Gibson, 1971a,b).

^{13}C NMR studies (Moon & Richards, 1974) and IR studies (Satterlee, et al., 1978) have shown that in rabbit Hb there is a perturbation in the α-heme pocket relative to human Hb. This perturbation is probably due to the replacement in rabbit Hb of leu (αCD7) by a phe residue. The perturbation decreases the affinity of the α chains for ligand. Therefore, in the case of rabbit Hb there is a large difference in affinities between the β and the α chains such that the β chains acquire more ligand than the α chains under conditions of partial overall ligation.

Accordingly, in the case of rabbit Hb at partial ligation, forms of Hb should be present in which the β chains are ligated while the α chains are not. In order to learn about the quaternary and tertiary structures of these intermediates we have labelled rabbit Hb with 3-bromo-1,1,1-trifluoroacetone and have studied the ligation with CO of this TF labelled rabbit Hb.

EXPERIMENTAL SECTION

Preparation of Hemoglobin

Samples of blood from New Zealand white rabbits were collected in citrate as anticoagulant. Equal volumes of blood and saline were mixed together and then centrifuged at 630g. The supernatant, containing the blood plasma, was removed, and the entire procedure was repeated twice. The erythrocytes were lysed, by lowering the osmotic pressure of the solvent by the addition of distilled water. The stromata were removed by centrifugation at 30000g. The Hb was pressure dialyzed through an Amicon PM10 membrane for 20 hrs. The pressure dialysis was performed in an Amicon cell fitted with a reservoir containing 0.15 M NaCl solution. After dialysis the Hb was concentrated in the Amicon cell.

The concentration of Hb was measured by the method of Benesch, et al. (1965) at 415, 420, 430 nm. We assumed that the extinction coefficients of rabbit Hb were the same as those of human Hb (Moon, 1975). The concentration of methemoglobin was monitored at each step of the preparation. Samples with higher than 5% Hb+ concentration were discarded.

Labelling of Hemoglobin with the Trifluoroacetonyl Group

The labelling of Hb with the 3-bromo-1,1,1-trifluoroacetone was carried out by the method of Huestis and Raftery (1972a). A solution of Hb was diluted to a concentration of 100 mg/ml. The solution was further diluted to 50 mg/ml of Hb by the addition of 0.4 M phosphate buffer and then adjusted with 1 M NaOH or HCl to pH 7.15. 3-Bromo-1,1,1-trifluoropropanone was obtained from PCR and 50 µl (4.74×10^{-4} mole) was added to 12 ml of this Hb solution (1.75×10^{-5} mole of cys βF9). The pH was maintained as 7.15 for 30 mins by the addition of 1 M NaOH. The precipitate formed by the reaction was removed by centrifugation. The solution was poured on a Biogel-P2 column to remove excess reagent and phosphates. On the column the ionic strength of the solution was adjusted to 15 mM tris (tris(hydroxymethyl)-aminomethane) buffer, 0.1 M NaCl and the pH to be between 6.7 and 7.5. The collected Hb solution was then concentrated and the concentrate was used as a stock solution for preparation of NMR samples.

Preparation of NMR Samples

Samples for the ^{13}C NMR studies were also loaded on a Biogel-P2 column to remove organic phosphates and to adjust the ionic strength of the solution to 15 mM tris,

0.1 M NaCl.

The pentaamonium salt of DPG was obtained from Cal-biochem, and dodecasodium inositol hexaphosphate was obtained from Grand Island Biological Co. Acids were prepared by passing the salts down a Dowex-50-X8 ion exchange column, and stock solutions were prepared from the free acids.

^{19}F NMR samples were prepared by mixing calculated amounts of concentrated Hb in 0.1 M NaCl 0.015 M tris buffer with 1 ml of buffer containing 0.1 M NaCl, 0.175 M tris and when necessary, 0.05 M organic phosphates. This solution was diluted with 0.1 M NaCl 0.015 M tris buffer to a 5 ml sample volume. The method used to prepare ^{19}F NMR samples was also used to prepare the ^{13}C NMR samples. However, the volumes of the solutions were doubled since 10 ml samples were needed for NMR measurements. The samples which resulted from these procedures were 2 mM Hb solutions in 0.1 M NaCl, 50 mM tris, with or without 10 mM organic phosphate. The pH of the samples was adjusted to be between 6.7-7.5. In a few cases 0.1 M NaCl, 0.015 mM tris buffer, prepared in double distilled D_2O, was added in order to allow the use of deuterium lock.

The samples were deoxygenated in a 50 cc syringe by equilibrating them with prepurified nitrogen (99.997% pure). The NMR tubes fitted with a 2 mm cuvette were deoxygenated by flushing with prepurified nitrogen through the access hole for approximately one hr (Huestis, 1972). The NMR tubes were sealed with a rubber sleeve pulled over the access hole. The deoxygenated Hb was injected into these NMR tubes through the rubber sleeve. Deoxygenation was checked using the ratio of the optical density at 670 nm and at 730 nm (Benesch, et al., 1965). If this ratio was less than 2.3 a slight amount of sodium dithionite solution was added to insure complete deoxygenation.

The level of ligation of the Hb was followed by measuring the optical density of the solution at 650 nm. ^{12}CO or ^{13}CO (enriched in ^{13}C to 92%) was injected in measured amounts. The level of ligation in the ^{13}C NMR studies was also followed by a second method in which the level of ligation was calculated from the ratio of the intensity of the $\alpha^{13}CO$ and $\beta^{13}CO$ resonances to the carbonyl resonance.

NMR Conditions

^{19}F and ^{13}C NMR spectra were determined on an XL-100-15 spectrometer equipped with a Varian 620i computer with

16K memory. The ^{19}F spectra were at 94.1 MHz on 12 mm NMR tubes and an internal proton or deuterium lock was used. The deuterium lock was used when the sample contained D_2O. ^{13}C spectra were obtained at 25.1 MHz using 18 mm NMR tubes. 5 mm concentric tubes filled with D_2O for locking were sealed inside each of the NMR tubes. All spectra were observed under identical conditions using 90° pulses. The probe temperature was 25°.

Separation of Heme from Globin

The heme was removed from the rabbit Hb by the method of Winterhalter and Huehns (1964). Hb (1g) in 40 ml of 0.15 M NaCl was added dropwise to 900 ml of acidic acetone (spectroscopic grade acetone containing 0.006 M HCl) at -20°. The precipitated globin was spun down at 760g at -15° and was washed twice with chilled acetone. After the precipitate was dissolved in water it was dialyzed against water, and the globin was lyophilyzed. The heme was obtained by evaporating the acetone on a rotatory evaporator. The heme was dissolved in water in the presence of NaOH and the ^{19}F NMR spectrum of this solution was taken.

Separation of the Chains of Rabbit Globin

The chains of the globin were separated by the method of Wilson and Smith (1959). The globin prepared as described previously was dissolved in 11.7% formic acid. The solution was poured on an amberlite CG50 ion exchange column. The column was washed with pH 1.9 solution of 2 M urea containing HCl. After collecting 250 ml of eluent a nonlinear gradient of 2 M to 8 M urea was used to wash the column. Finally after collecting, in 6 ml fractions, about 2 l of eluent, the column was washed with a solution of 8 M urea. The optical densities of the 6 ml fractions were measured at 280 nm. The eluate was divided into three fractions as shown in Figure 1. These fractions were dialyzed against distilled water and then lyophilized. After each of the three fractions was re-dissolved in 3 ml of water, the ^{19}F NMR spectrum of each sample was taken.

RESULTS

Location of the Trifluoroacetonyl Label

Satterlee (1977) tried to separate the chains of rabbit Hb without success by the usual method of Geracci, et al. (1969). It is possible, however, to separate the globin chains of trifluoroacetonylated

rabbit Hb under denaturing conditions using the method of Wilson and Smith (1959). As a prerequisite for the use of this technique, the globin was separated from the heme by the method of Winterhalter and Huehns (1964). The ^{19}F NMR spectrum of the heme contained no fluorine signal. The column profile of the separation of the chains using this technique is shown in Figure 1. The first fraction contains the α chains while the second and third fractions contain β chain monomers and multimers, respectively (Wilson & Smith, 1959). The ^{19}F NMR spectra of the three fractions indicate that only the second and third fractions contain ^{19}F signal. Thus only the β chains contain the fluorine label.

^{13}CO Ligation of Rabbit Hemoglobin

The ligations of rabbit Hb with ^{13}CO, as followed by ^{13}C NMR observations is shown without effectors in Figure 2, in the presence of DPG in Figure 3 and in the presence of IHP in Figure 4. The chemical shifts of the ^{13}CO bound to the α and β chains of rabbit Hb have been identified by Moon and Richards (1974). Chemical shifts observed in this work are 209.17 $\pm$ 0.02 for the ^{13}CO bound to the rabbit α chains and 207.12 $\pm$ 0.03 for the ^{13}CO bound to the β chains relative to 100% tetramethyl

FIGURE 1

The column profile of the separation of the globin chains. The three collected fractions are labelled as Fr. 1, 2, 3.

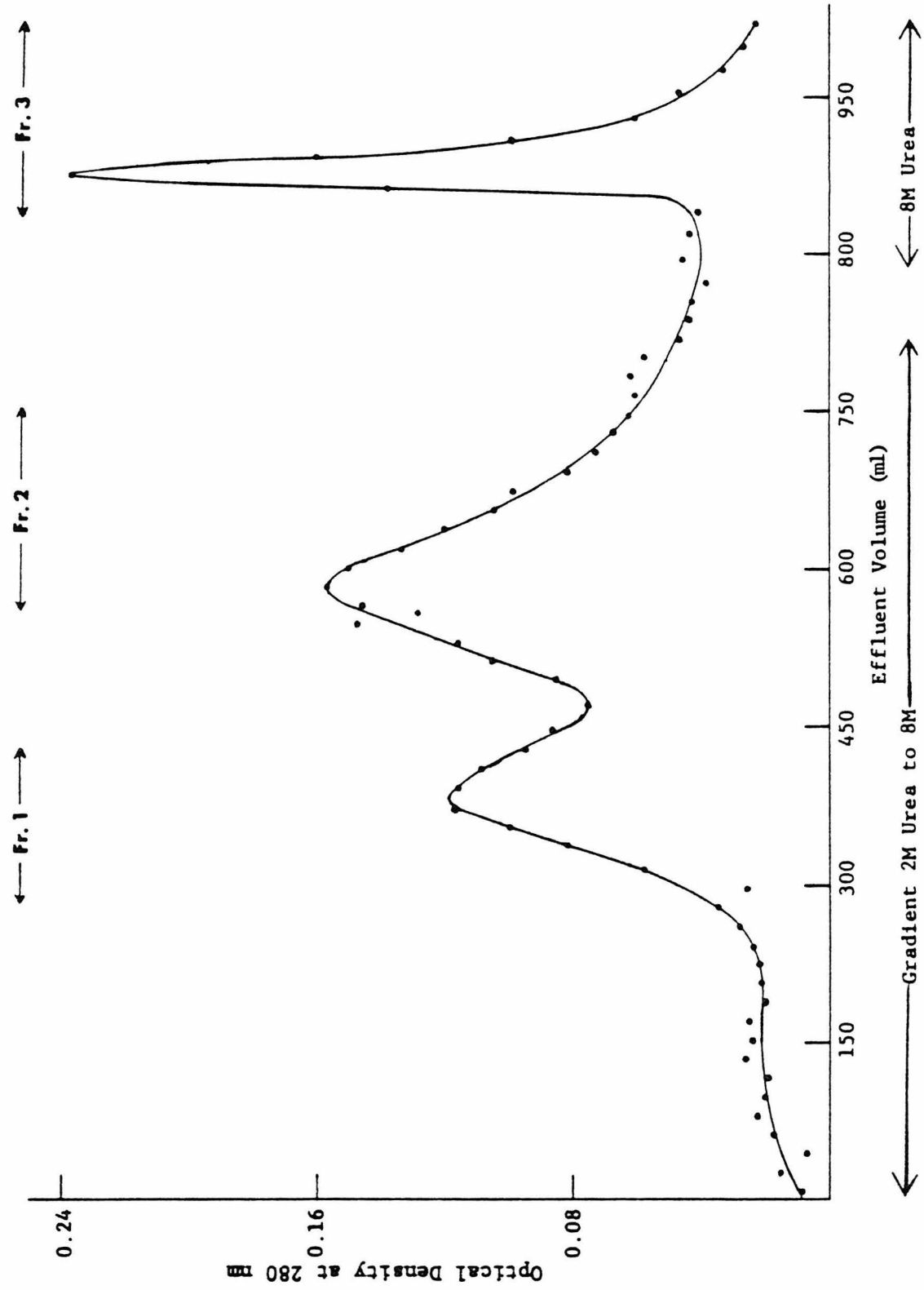

Fr. 1
Fr. 2
Fr. 3
0.24
0.16
0.08
Optical Density at 280 mμ
150
300
450
600
750
800
950
Effluent Volume (ml)
Gradient 2M Urea to 8M
8M Urea

FIGURE 2

The ^{13}C NMR spectra of rabbit carbonmonoxyhemoglobin at different fractions of ligation in the absence of any organic phosphates.

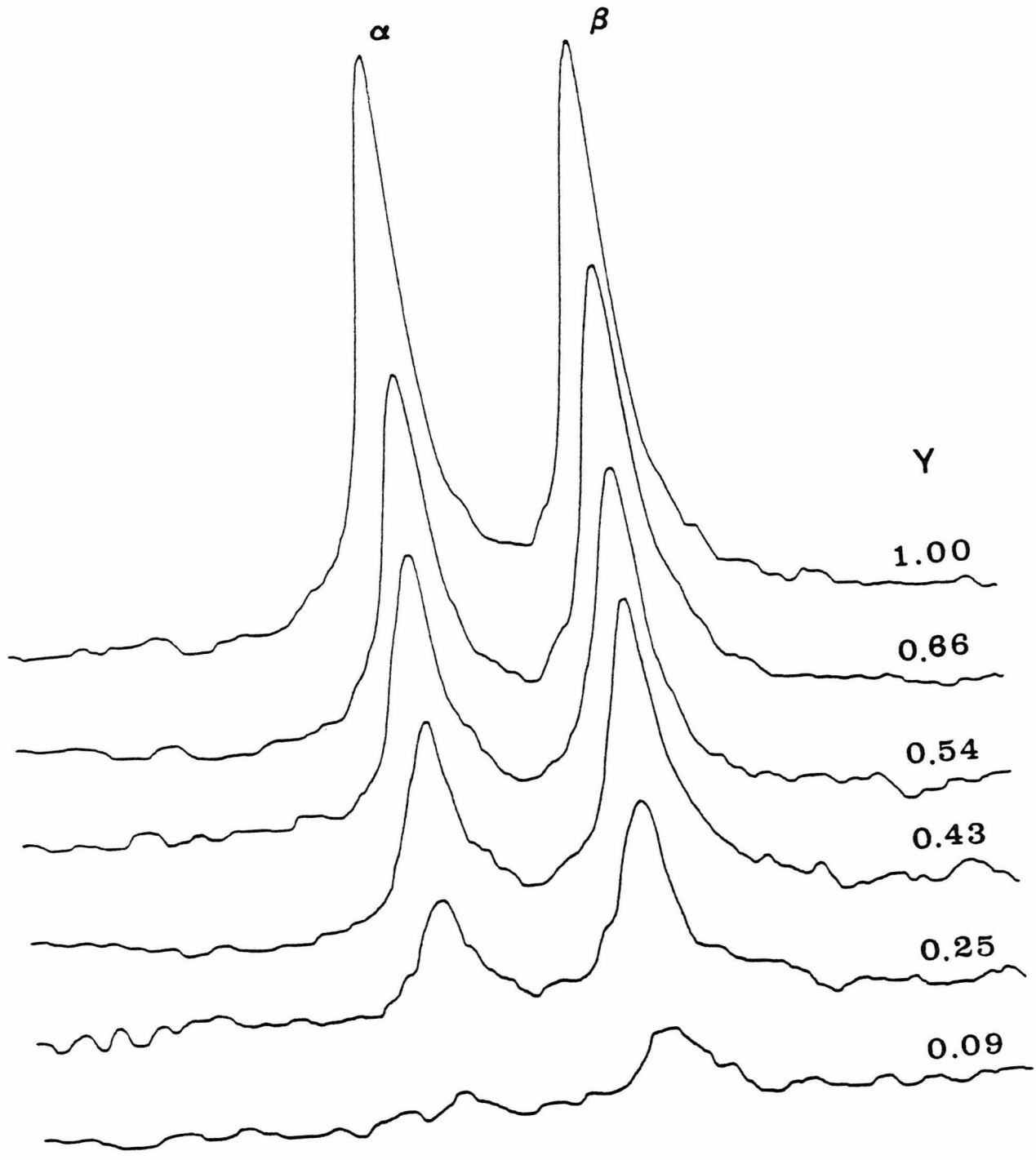

α
β
Y
1.00
0.66
0.54
0.43
0.25
0.09

FIGURE 3

The ^{13}C NMR spectra of rabbit carbonmonoxyhemoglobin at different fractions of ligation in the presence of 2,3-diposphoglycerate.

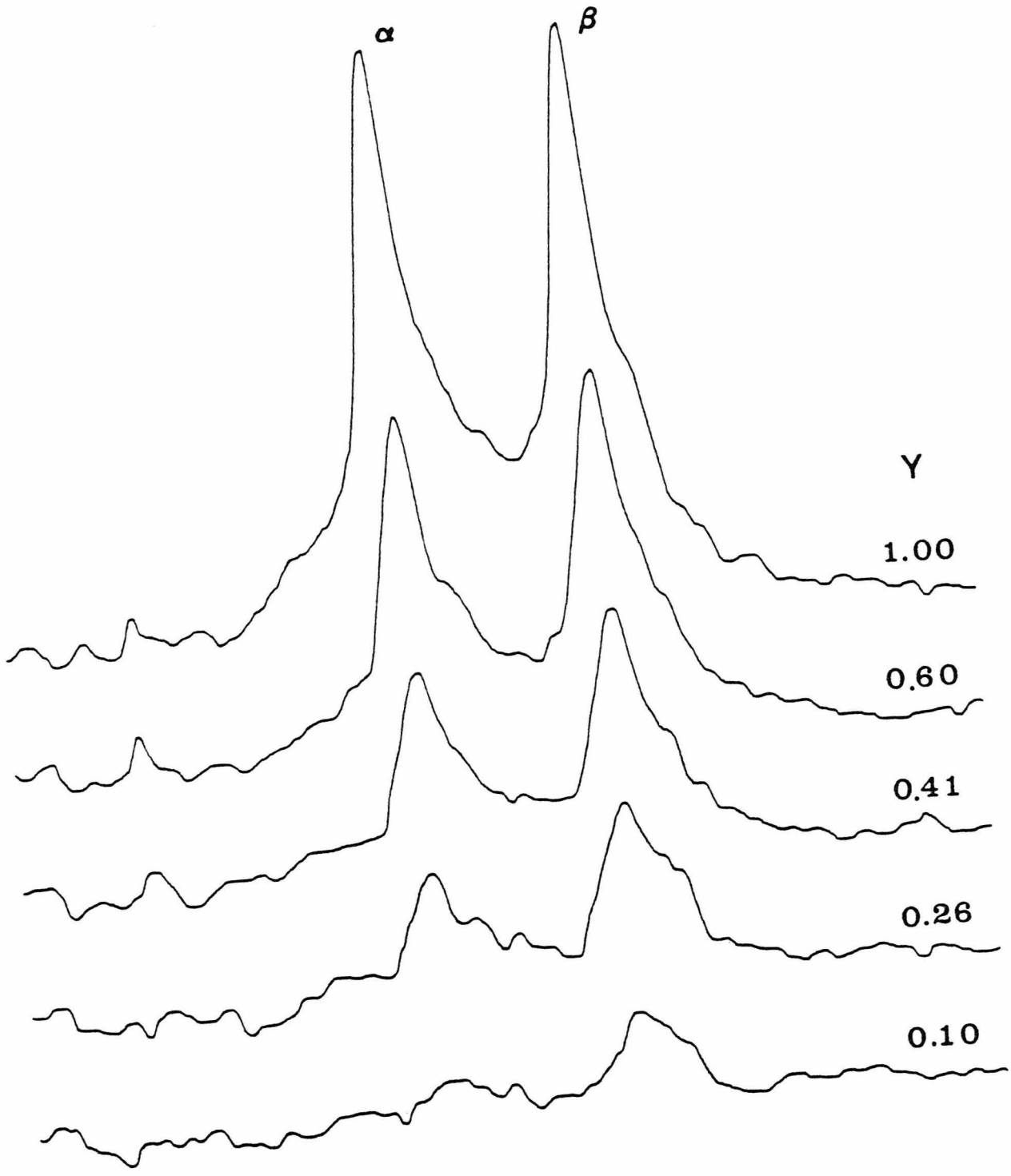

α
β
Y
1.00
0.60
0.41
0.26
0.10

FIGURE 4

The ^{13}C NMR spectra of rabbit carbonmonoxyhemoglobin at different fractions of ligation in the presence of inositol hexaphosphate.

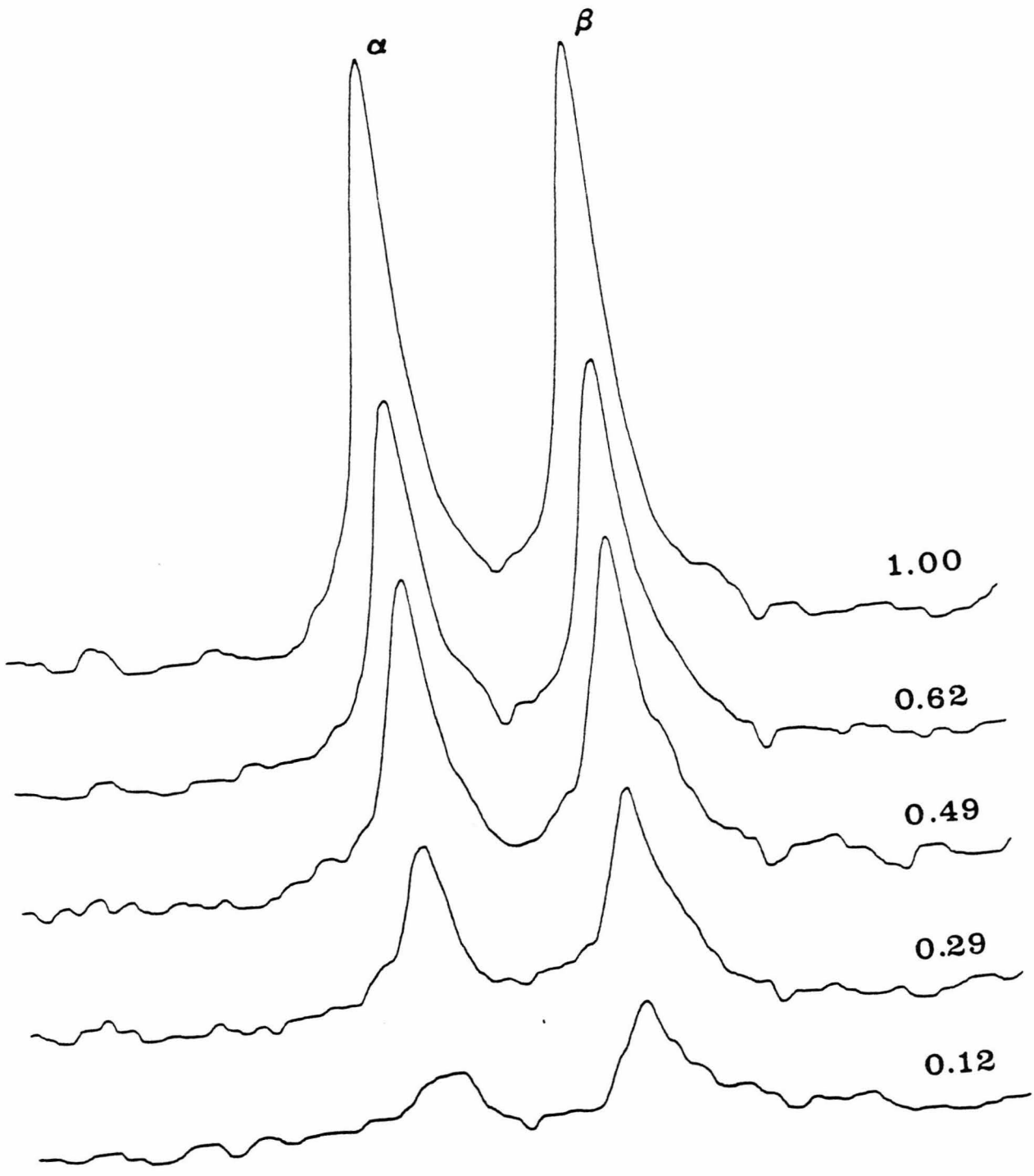

α
β
1.00
0.62
0.49
0.29
0.12

FIGURE 5

The plot of the fractions of the α and β chain ligation by ^{13}CO versus the total fraction of ligation in the absence of any organic phosphates.

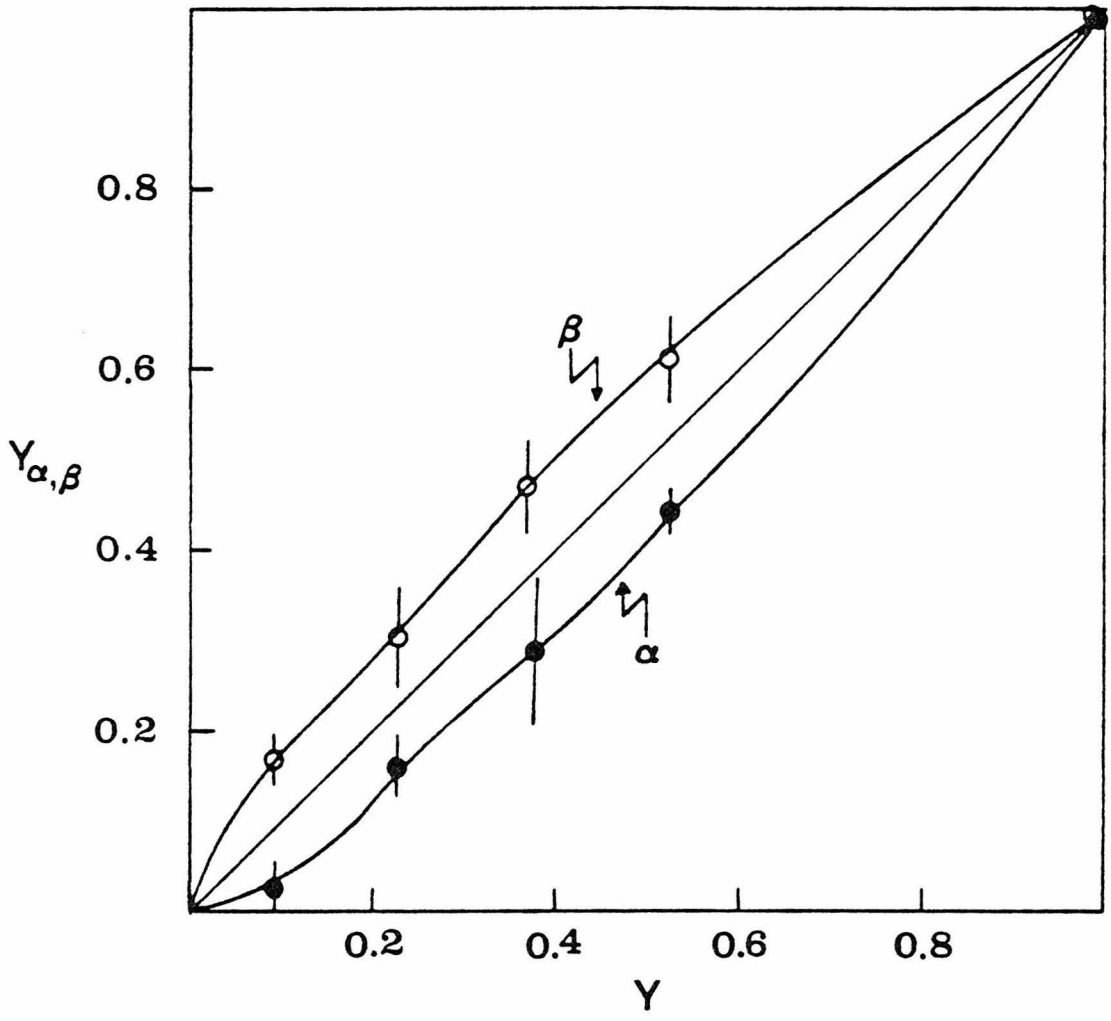

0.8
0.6
0.4
0.2
$Y_{\alpha,\beta}$
β
α
0.2
0.4
0.6
0.8
Y

FIGURE 6

The plot of the fractions of the α and β chain ligation by ^{13}CO versus the total fraction of ligation in the presence of 2,3-diphosphoglycerate.

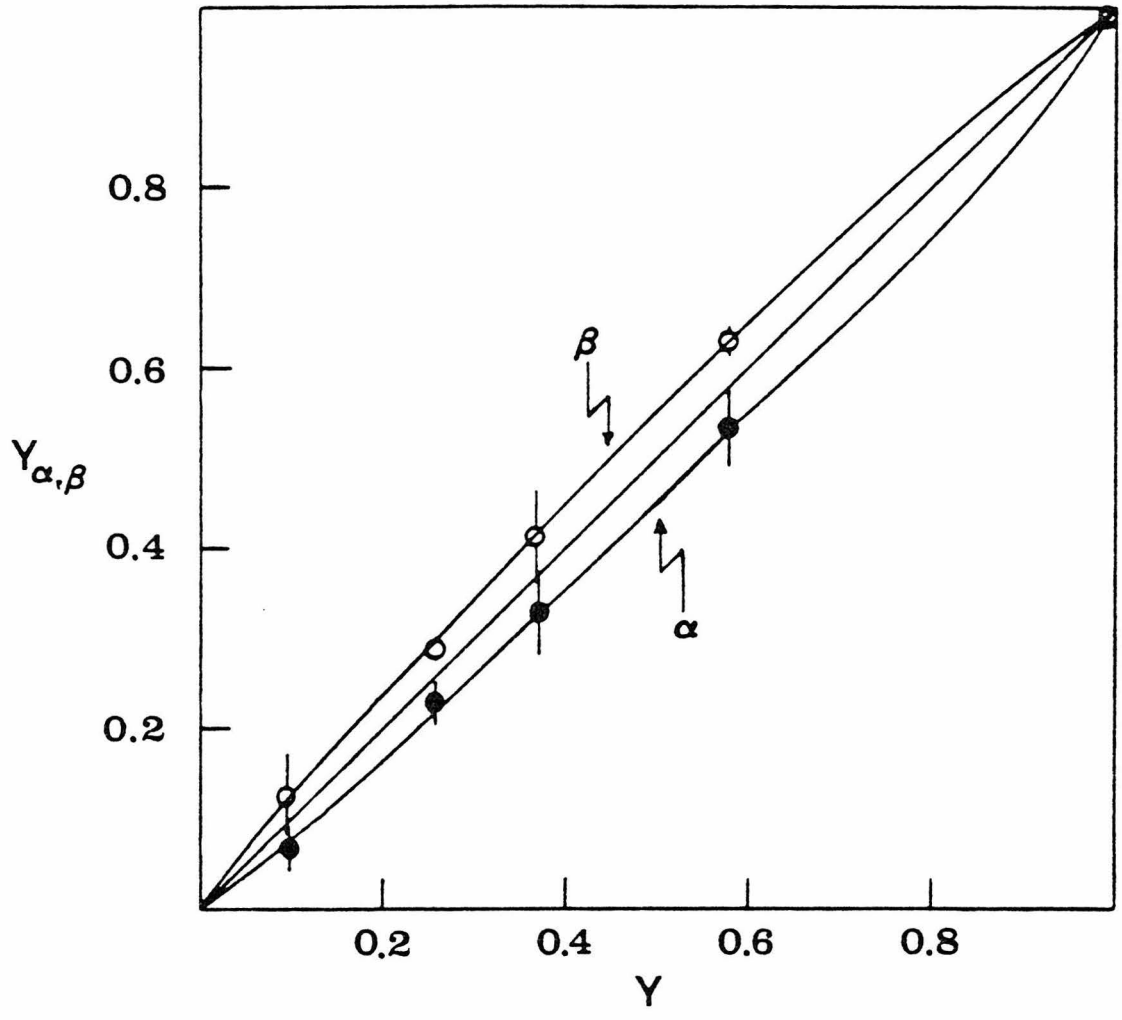
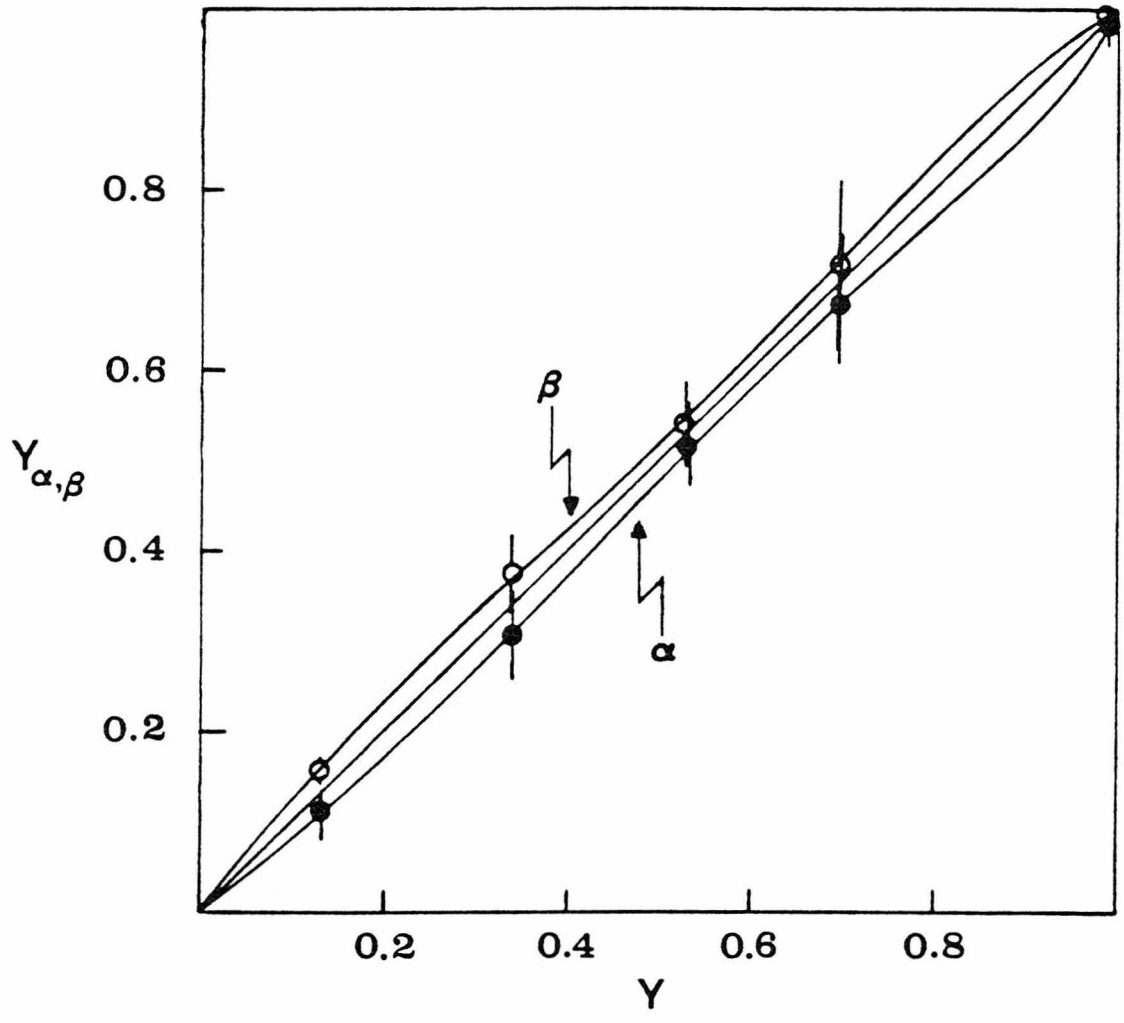

0.8
0.6
0.4
0.2
$Y_{\alpha,\beta}$
β
α
0.2
0.4
0.6
0.8
Y

FIGURE 7

The plot of the fractions of the α and β chain ligation by ^{13}CO versus the total fraction of ligation in the presence of inositol hexaphosphate.


0.8
0.6
0.4
0.2
$Y_{\alpha,\beta}$
β
α
0.2
0.4
0.6
0.8
Y

silane. These observations agree well with earlier results (Moon & Richards, 1974). The chemical shifts of the ^{13}CO bound to the α and β chains do not change when DPG or IHP is added. When effectors are absent the rabbit β chains have a much higher affinity for CO than do the rabbit α chains (Figures 2 and 6). In the presence of DPG and IHP this difference diminishes, as shown in Figures 5, 6, and 7.

^{19}F NMR of TF Labelled Rabbit Hemoglobin

Typical ^{19}F spectra of the CO binding of rabbit Hb are shown in the absence of effectors in Figure 8, in the presence of DPG in Figure 9, and in the presence of IHP in Figure 10. With or without DPG the spectra look quite similar. These two spectra contain only two resonances due to the deoxy (D) and the carbonmonoxy Hb (L). Both of these resonances are 17 Hz wide. However, in the presence of IHP the spectra contain two additional low intensity resonances labelled I (Figure 10).

The chemical shifts of the trifluoracetonylated rabbit Hb under different conditions are shown in Table I. THe difference between the chemical shift of deoxy and carbonmonoxy Hb is greater in rabbit Hb than in human Hb by about 0.24 ppm. Most of this difference occurs be-

FIGURE 8

The ^{19}F NMR spectra of trifluoroacetonylated rabbit hemoglobin at diffraction fractions of total ligation in the absence of any organic phosphates. The deoxy and ligated resonances are labelled as "D" and "L", respectively.

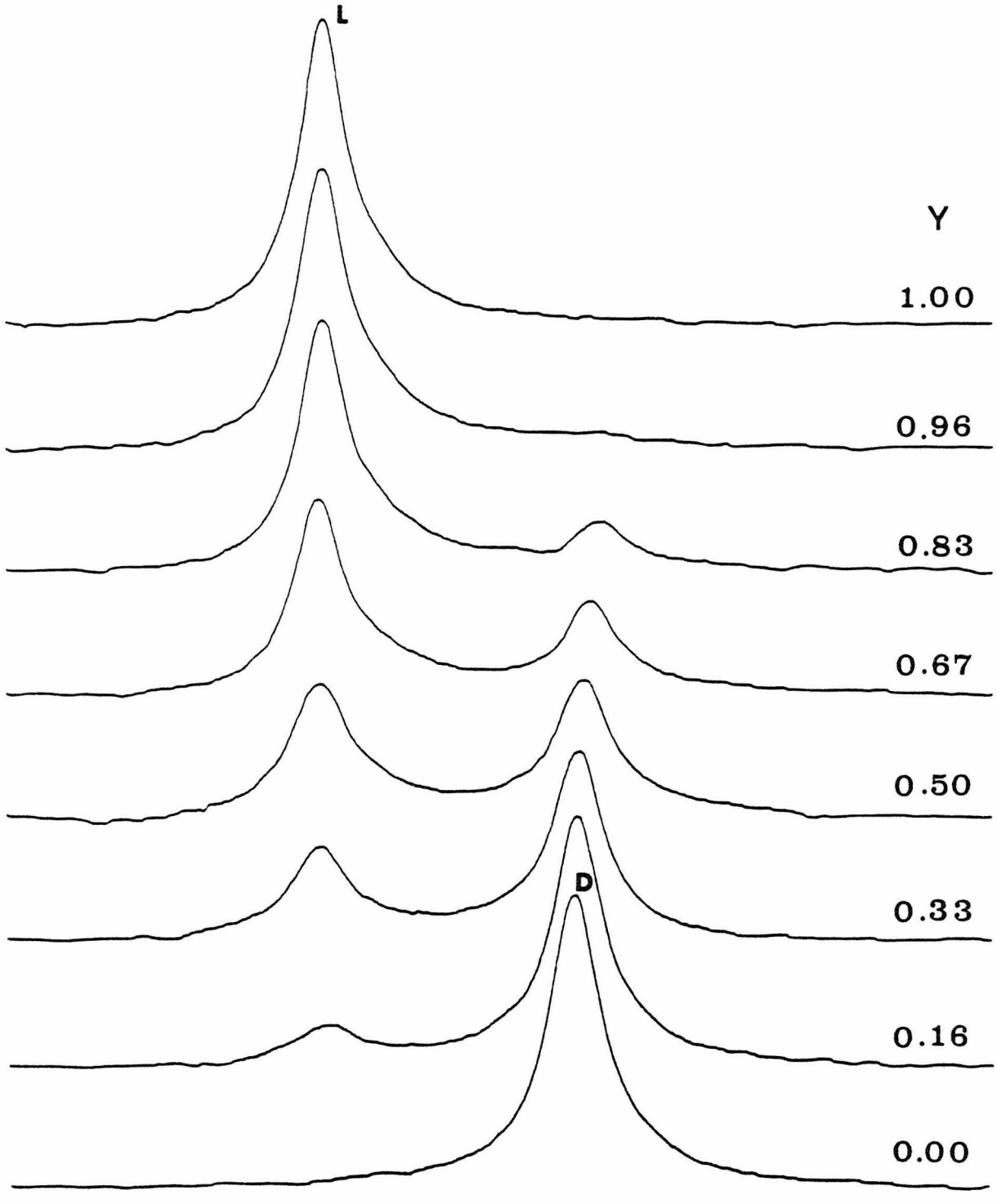

L
Y
1.00
0.96
0.83
0.67
0.50
D
0.33
0.16
0.00

FIGURE 9

The ^{19}F NMR spectra of trifluoroacetonylated rabbit hemoglobin at different fractions of total ligation in the presence of 2,3-diphosphoglycerate. The deoxy and ligated resonances are labelled as "D" and "L", respectively.

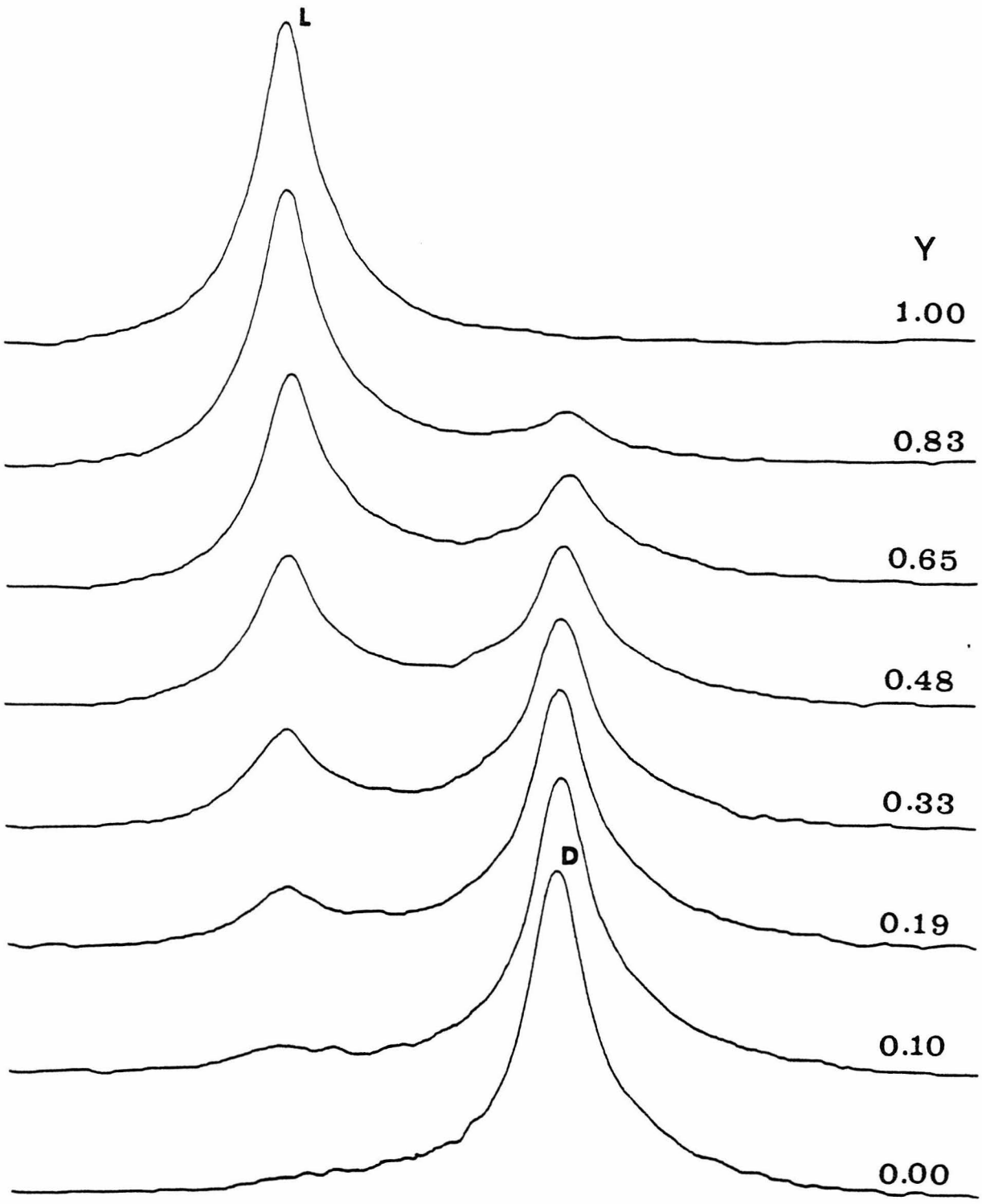

L
Y
1.00
0.83
0.65
0.48
0.33
D
0.19
0.10
0.00

FIGURE 10

The ^{19}F NMR spectra of trifluoroacetonylated rabbit hemoglobin at different fractions of total ligation in the presence of inositol hexaphosphate. The deoxy and ligated resonances are labelled as "D" and "L", respectively. The detected additional resonances are labelled with "I".

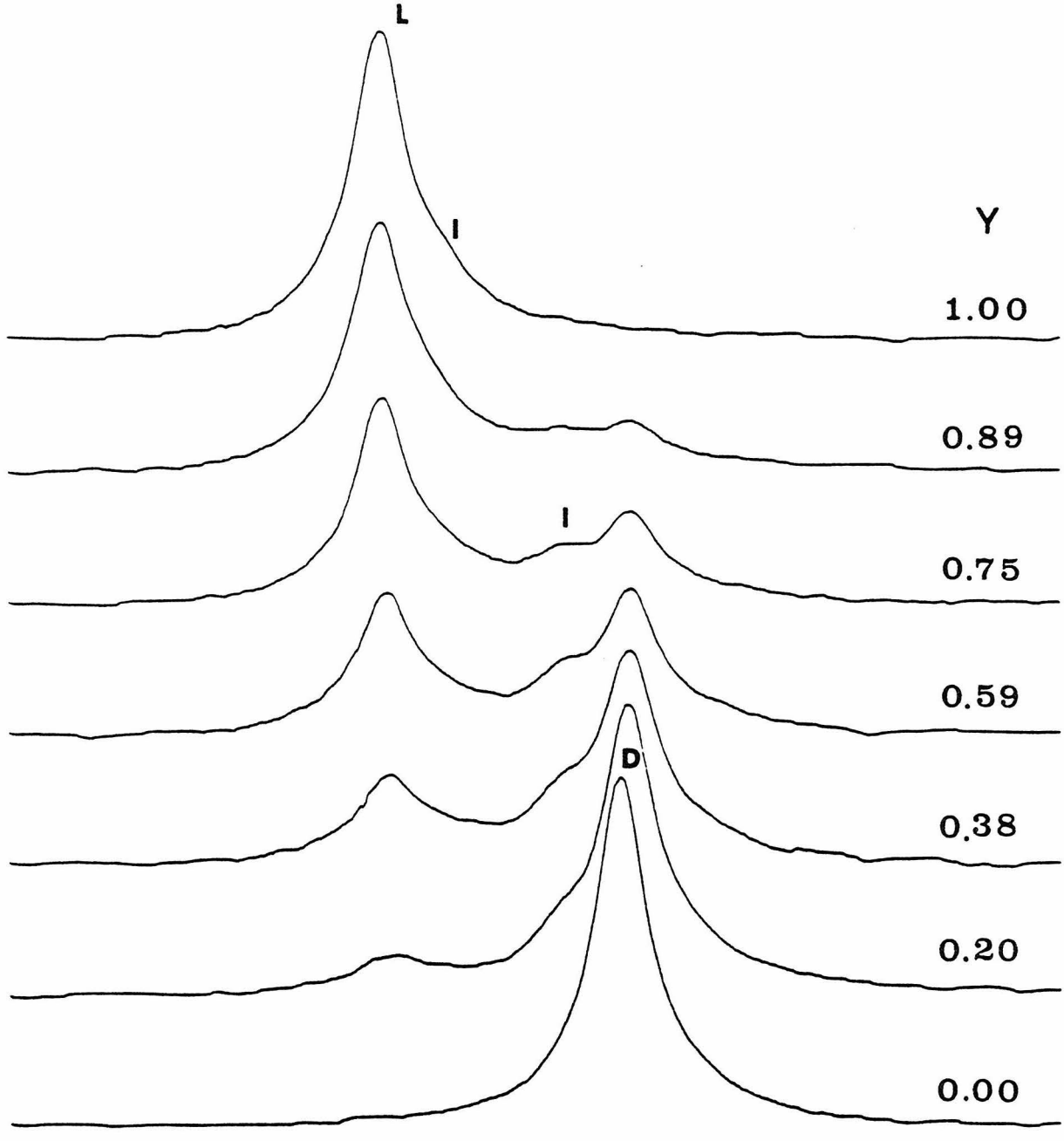

L
I
Y
1.00
0.89
I
0.75
0.59
D
0.38
0.20
0.00

Table I

^{19}F Chemical Shifts of Trifluoroacetonylated Rabbit Hemoglobin*

	No Effectors		DPG		IHP		Additional Signals for IHP	
	$Hb(CO)_4$	Hb	$Hb(CO)_4$	Hb	$Hb(CO)_4$	Hb	$Hb(CO)_4$	Hb
7.5	-8.40	-9.06	-8.49	-9.16	-8.58	-9.16	-8.65	-8.98
7.0	-8.43	-9.16	-8.51	-9.24	-8.56	-9.18	-8.75	-9.00

*All chemical shifts are relative to TFA.

cause the chemical shift of trifluoroacetonyl deoxy rabbit Hb falls at a higher field than does the resonance of the trifluoroacetonyl deoxy human Hb. Organic phosphates also have a different effect on the chemical shifts of human and rabbit Hbs. Huestis and Raftery (1973; 1978) found that DPG moves the chemical shift of human deoxy Hb upfield by about 0.13 ppm without affecting the ligated signal. In rabbit Hb, however, both DPG and IHP shift both the deoxy and the ligated resonances upfield, as shown in Table I. Lowering the pH has a much greater effect on the resonance due to deoxyhemoglobin than it has on the signal due to ligated Hb. This is consistent with the results found in studies on human Hb (Huestis & Raftery, 1972d).

In order to obtain quantitative data about the low intensity resonances a computer program has been written which can generate a theoretical spectrum, assuming Lorentzian line shapes and given experimentally obtained chemical shifts and linewidths (Smallcombe, 1976). This program can minimize the difference between a real and generated spectrum by changing linewidth, area, and chemical shift of the generated resonances. From these computer analyses of the spectra we can get estimates of the sizes of the low intensity resonances. A typical Lorentzian fit is shown in Figure 11. Line E in

FIGURE 11

The Lorentzian fit, calculated by the computer of the NMR, of the spectrum of trifluoroacetonylated rabbit hemoglobin in the presence of inositol hexaphosphate at 0.59 CO saturation. Line A is the calculated spectru, B is the experimentally obtained spectrum. Lines C and D desmonstrate the detected resonances on the shoulder of the large resonances and E shows the difference between the experimental and the calculated spectra (B-A).

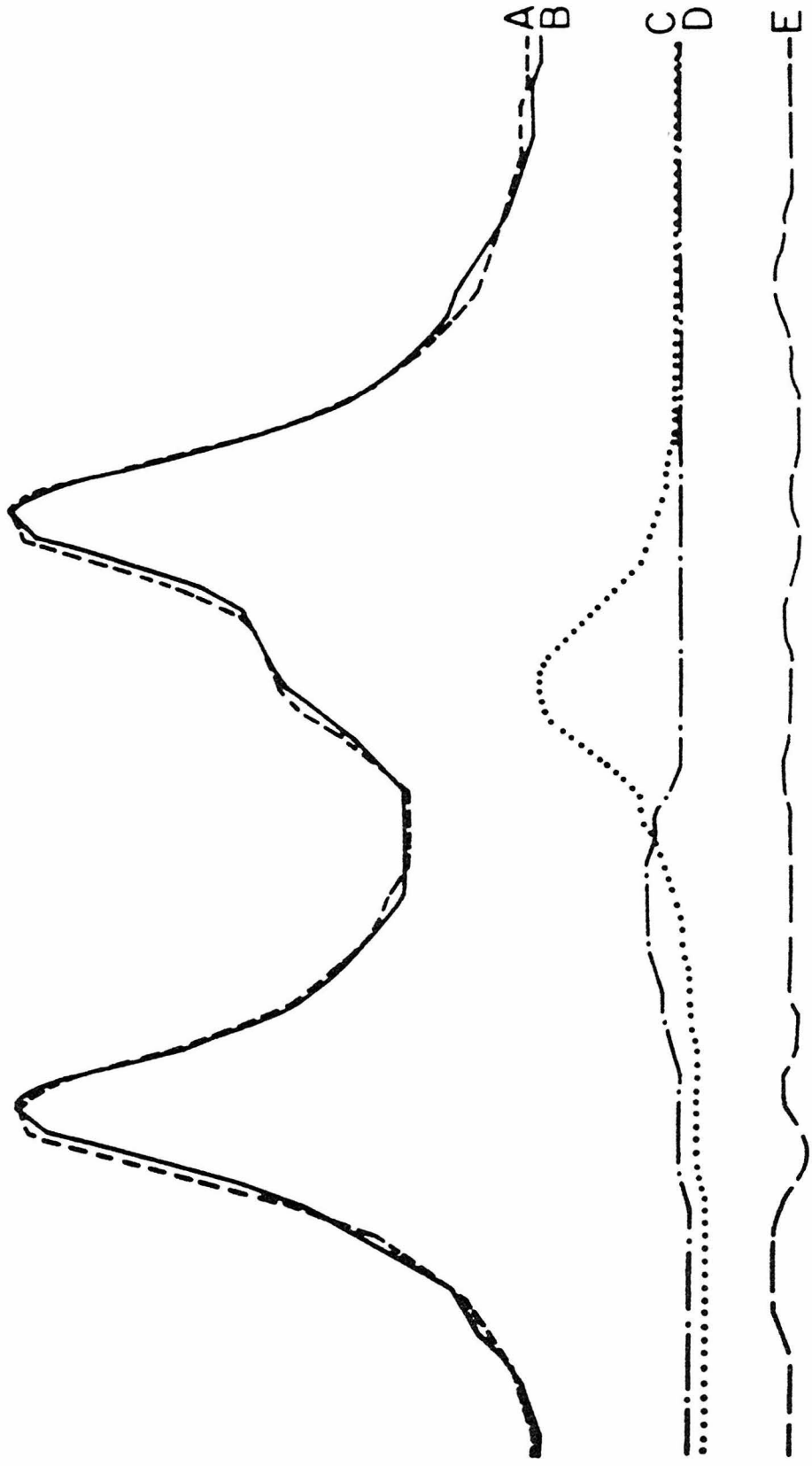

A
B
C
D
E

Figure 11 shows that the difference between the experimental spectrum and the computer generated spectrum is minimal. The integral of the generated resonances plotted against the fraction of ligation is shown in the absence of any effectors in Figure 12, in the presence of DPG in Figure 13, and in the presence of IHP in Figure 14. Significant amounts of intermediates show up only in the presence of IHP. In this case the shoulder on the resonance of deoxy Hb has its maximum size, which is 15% of the total Hb, at about 40% saturation and then decreases as ligation increases. The shoulder on the resonance of ligated Hb becomes significant only at about 75% saturation and it does not go away even at 100% saturation.

In all of these ligation studies no delay has been observed in the appearance of the ligated signal in contrast to human Hb where there is an 18% delay in the appearance of the ligated signal (Huestis & Raftery, 1973). The plots of the fraction of ligated signal versus the fraction of saturation of rabbit Hb determined by optical density measurements at 650 nm in Figure 15 indicate that the intensity of the ^{19}F signal is linear with the ligation of rabbit Hb.

FIGURE 12

The plot of the fraction of the calculated ^{19}F resonances of the deoxy, ligated and the additionally detected resonances versus the total fraction of ligation in the absence of organic phosphates.

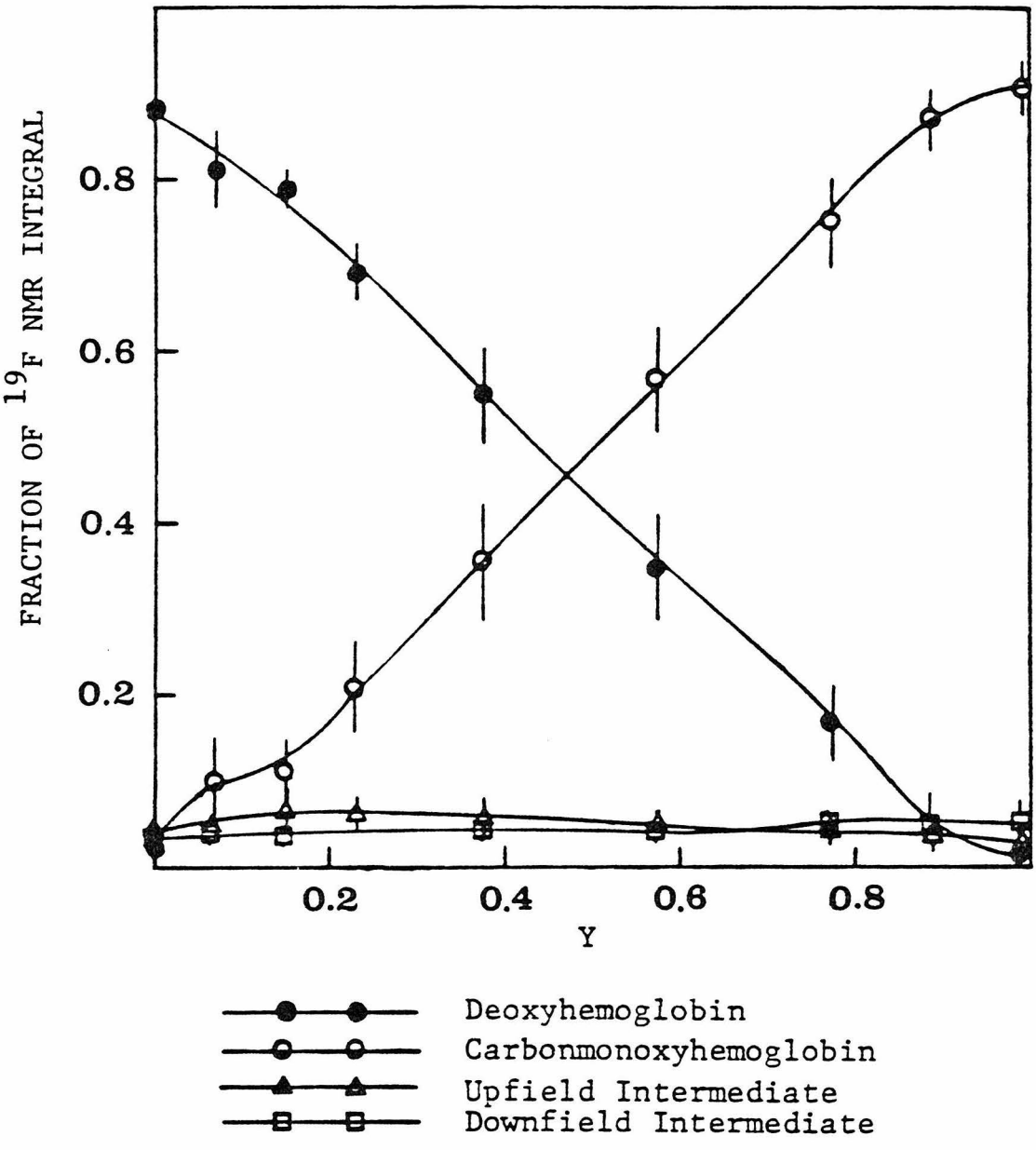

FRACTION OF ^{19}F NMR INTEGRAL
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
Y
Deoxyhemoglobin
Carbonmonoxyhemoglobin
Upfield Intermediate
Downfield Intermediate

FIGURE 13

The plot of the fraction of the calculated ^{19}F resonances of the deoxy, ligated and the additionally detected resonances versus the total fraction of ligation in the presence of 2,3-diphosphoglycerate.

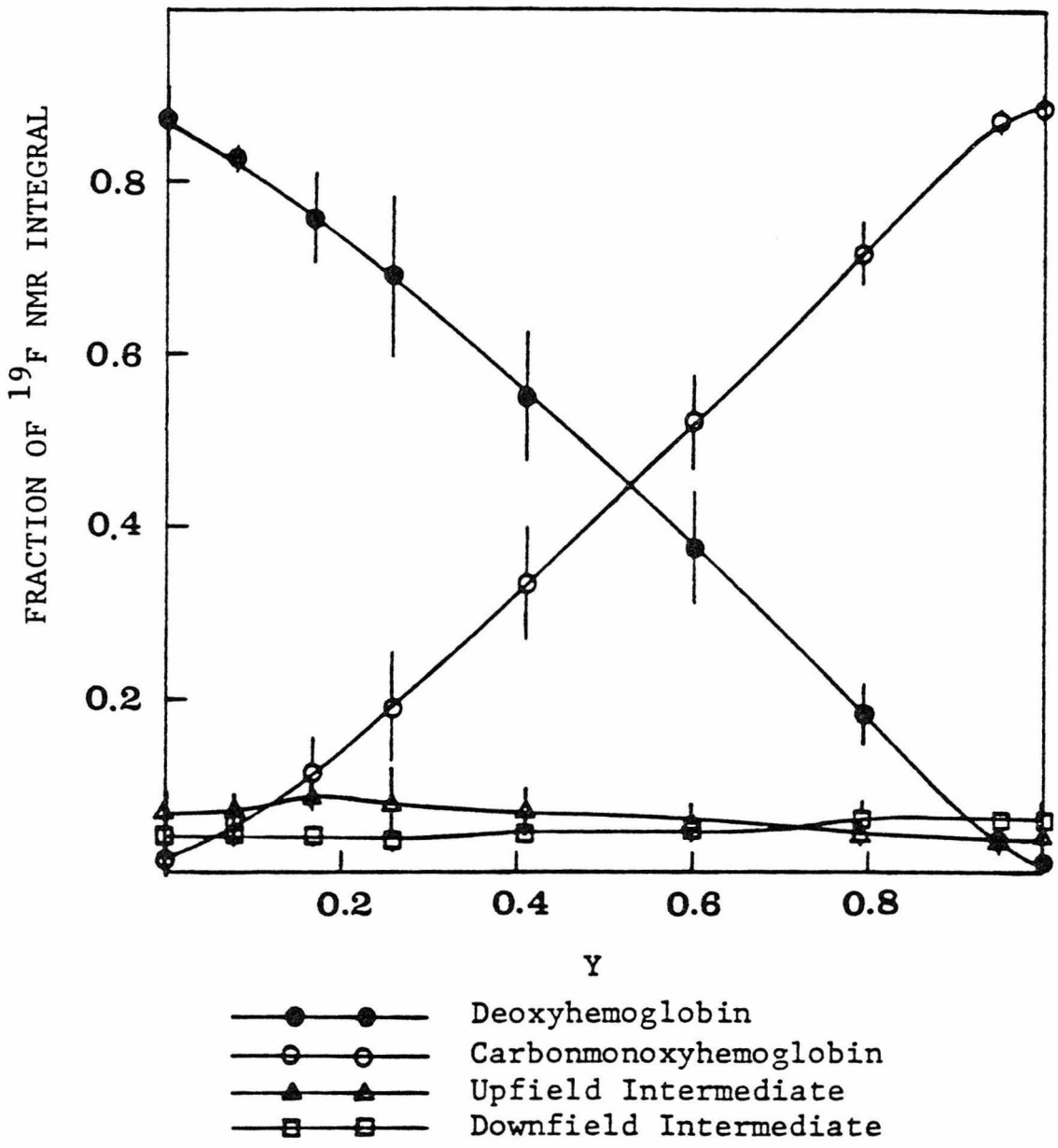

FRACTION OF ^{19}F NMR INTEGRAL
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
Y
Deoxyhemoglobin
Carbonmonoxyhemoglobin
Upfield Intermediate
Downfield Intermediate

FIGURE 14

The plot of the fraction of the calculated ^{19}F resonances of the deoxy, ligated and the additionally detected resonances versus the total fraction of ligation in the presence of inositol hexaphosphate.

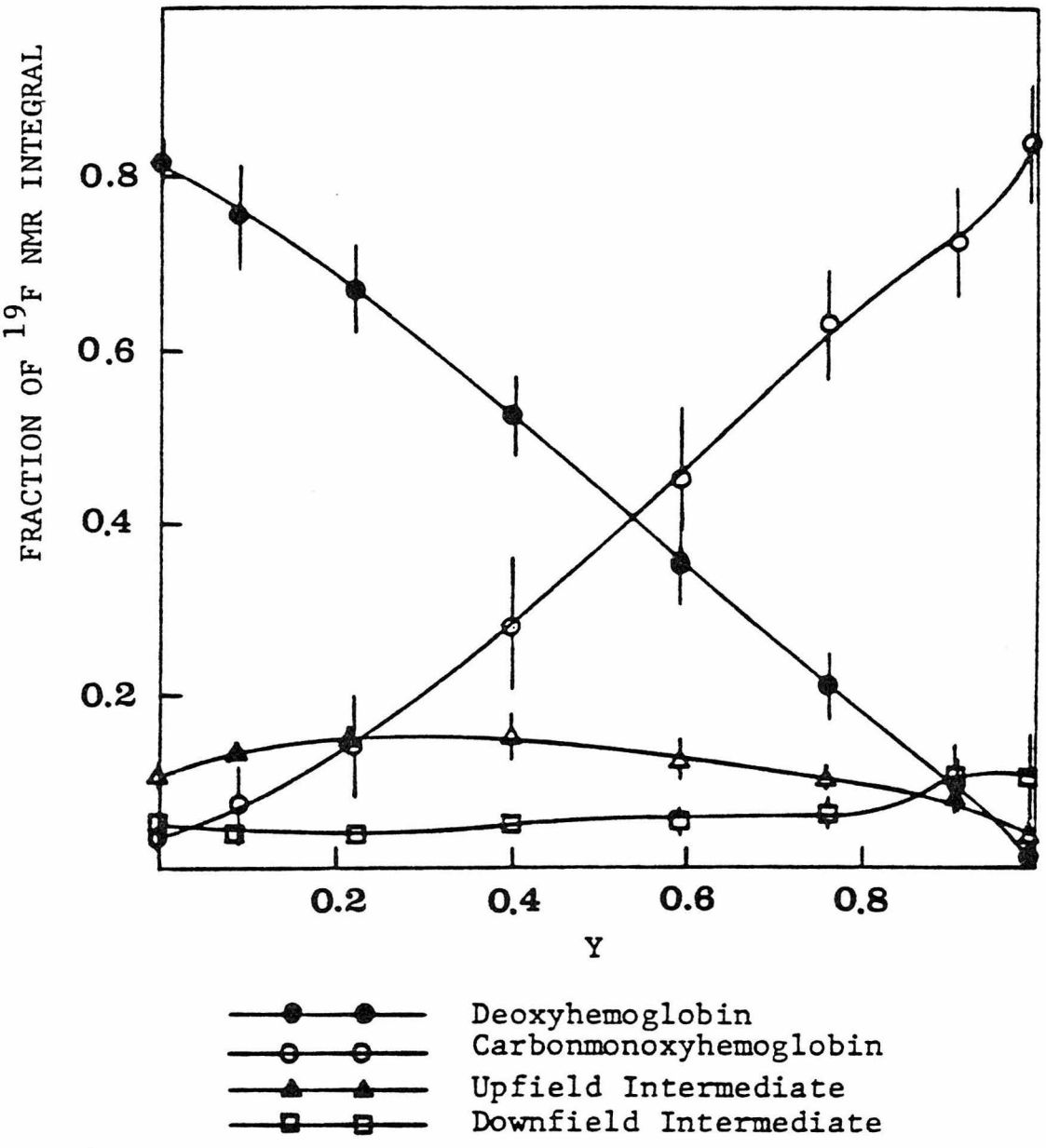
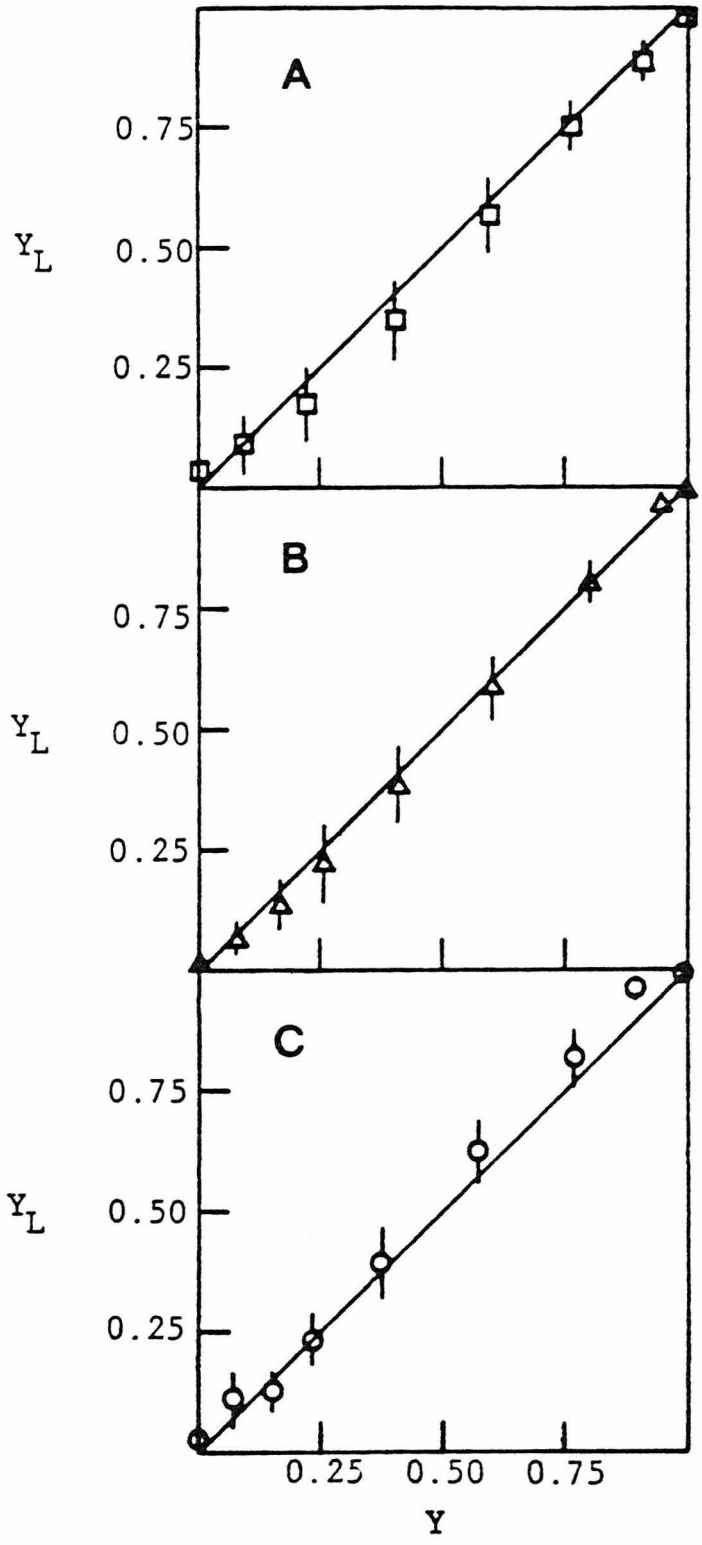

FRACTION OF ^{19}F NMR INTEGRAL
0.8
0.6
0.4
0.2
0.2
0.4
0.6
0.8
Y
Deoxyhemoglobin
Carbonmonoxyhemoglobin
Upfield Intermediate
Downfield Intermediate

FIGURE 15

The plots of the fraction of the calculated ^{19}F resonance of the ligated signal versus the total fraction of saturation in the presence of inositol hexaphosphate (A); in the presence of 2,3-diphosphoglycerate (B); and in the absence of any organic phosphates (C).


A
B
C
0.75
0.50
0.25
Y_L
0.25
0.50
0.75
Y

DISCUSSION

The Effect of Organic Phosphates on Chain Heterogeneity

In order to find intermediate structures in the ligation of Hb it is worthwhile to study Hb in which one chain ligates preferentially. Studying TF labeled human Hb, which ligates preferentially at the α chains, Huestis and Raftery (1973; 1975) detected intermediates in which the α chains were ligated while the β chains were not. As the difference between the affinities of α and β chains increases, more intermediates can in principle be detected. Therefore, it is worthwhile to look for intermediates in rabbit Hb, in which the β chains have a much higher affinity towards CO than do the α chains as shown in Figure 2 (Moon & Richards, 1974).

We have studied the effect of organic phosphates on the binding of ^{13}CO to rabbit Hb. CO binds with a much higher affinity to β chains than to α chains when effectors are not present (Figures 2, 5). The addition of DPG (Figures 3, 6) and IHP (Figures 4, 7) reduces the inequality of the affinities between the β and α chains. In the presence of IHP the ligation is close to random (Figures 4, 7).

The reduced affinity of the α chains of rabbit Hb relative to the α chains of human Hb is probably due to

the perturbation caused by the replacement of leucine at position αCD7 in human Hb by a phenylaniline residue in rabbit Hb (Moon & Richards, 1974; Satterlee, et al., 1978). The probable result of this mutation is that the histidine E7 is pushed closer to the bound CO, thus destabilizing the iron CO bond in the α chains and stabilizing intermediates like $\beta^{CO}\beta\alpha\alpha$ or $\beta^{CO}\beta^{CO}\alpha\alpha$.

There is a disagreement about which chain is effected more by the binding of organic phosphates. Since organic phosphates bind between the β chain one would expect a larger change in the affinity of the β chains than in the α chains. The results of Figures 2-7 support this statement. Further, in their study of the binding of ethyl isocyanide to human Hb, Dill, et al. (1978) concluded that IHP has a greater effect on the affinity of the β chains than on the affinity of the α chains.

In contrast, the studies of Gray and Gibson (1971a, b) indicate that, in the presence of IHP, β chains react faster than do α chains. This result is supported by Olson and Gibson (1973) who found that a time delay occurs in the proton release from Hb during ligation, when IHP is present. Since proton release is largely due to α chain ligation they concluded that the β chains ligate faster than α chains.

A possible structural explanation for the effect of IHP on the α chains may be that the heme pockets of deoxy β chains do not change their structure due to the binding of IHP. However, the heme pocket of the α chain may decrease in size due to the binding of IHP. This conclusion is also supported by the effect of IHP on $Hb(NO)_4$. In this case the histidine F8 iron bond of the α chains are severed without much change in the β chains (Perutz, et al., 1976; Cassoly, 1975; Taketa, et al., 1978).

The discrepancy between the thermodynamic and kinetic studies of initial ligation can be explained by the fact that though the β chains react kinetically faster than the α chains, ligated β chains are less stable thermodynamically than are the α chains, in the presence of IHP.

Another explanation for this discrepancy has been suggested by Huestis and Raftery (1975). They proposed that the fast component, in the kinetic study, may be due to the $\alpha^{L}\alpha^{L}\beta\beta$ species. Therefore, the kinetic study may be measuring the rate of the ligation of this species.

The Environment Around the Ligand in the Heme Pocket

Chemical shifts of the ^{13}CO bound to the α and β chains of rabbit Hb do not change when organic phosphates are added. Changes in the heme pocket due to the presence of DPG have been detected by proton NMR (Ho, et al., 1973). Therefore, we can conclude that the ^{13}CO chemical shifts of the α-^{13}CO and β-^{13}CO are not sensitive to small changes in the structure of the heme pocket. In contrast, the chemical shift of the ^{13}CO bound to the α chains of rabbit Hb is downfield by 1.51 ppm from that of human Hb, due to the perturbation caused by the phe at αCD7 in rabbit Hb (Moon & Richards, 1974). This change in chemical shift indicates that the replacement of the leu by phe results in an alteration of the structure of the heme pocket.

Detection of Intermediate Structures in the Ligation of Hemoglobin

Though ^{13}C NMR studies have revealed information concerning the ligation of the α and β chains, they have not supplied any information about structural changes occurring within the protein as a result of ligation. Only gross structural changes due to ligation can be detected in Hb by natural abundance ^{13}C NMR (Moon &

Richards, 1973). To detect detailed changes in the structure of Hb the cys βF9 can be labelled with a spin label (Ogawa & McConnell, 1967; McConnell, et al., 1969; Colemen, 1977; Johnson, et al., 1978), or an NMR label (Huestis & Raftery, 1972a, b, c; 1973; 1975). Spin labels, however, are too large and can perturb the local protein structure extensively (Moffat, 1971) as is seen by the decrease in the Hill coefficient to 2.3 from the normal 2.8-3.0 when spin labels are attached to Hb (Ogawa & McConnell, 1967). A smaller NMR label, like the TF group, affects the local structure less, and changes the Hill coefficient only slightly from 3.0 to 2.8 (Lee, et al., 1973).

The Location and Effect of the TF Label

Questions have been raised concerning the specificity of the trifluoroacetonyl label and its effect on the structure of Hb and on cooperativity (Knowles, 1975; Edelstein, 1974). Huestis and Raftery (1978) have shown that the label attaches exclusively to the cys βF9 in human Hb. Lee, et al (1973) have further shown that the label has minimal effect on the affinity of Hb and on cooperativity.

In rabbit Hb, which has one less cysteine in the β chains (Dayhoff, 1972) than does human Hb, the separation of chains is not possible using the usual method of Geraci, et al. (1969)(Satterlee, 1977). In our studies we separated the chains of TF labelled rabbit Hb under denaturing conditions using the method developed by Wilson and Smith (1959). The heme was removed and tested for ^{19}F content to insure that the TF label did not attach to it. ^{19}F signal has been found only in the rabbit β chains and not in the rabbit α chains or in the heme fraction, contrary to Knowles' (1975) conclusions. These results agree fully with the results of Huestis and Raftery (1978) who concluded that the label attaches exclusively to the cysteine βF9 in human Hb.

The TF label increases the affinity of the human Hb slightly (Lee, et al., 1973; Huestis & Raftery, 1973) by weakening the asp βFG1-his βHC3 hydrogen bond, or by destabilizing the βHC2 tyrosine in its binding pocket in deoxyhemoglobin. Our ^{13}C NMR studies have not detected any variation in the relative affinities of the chains in TF labeled rabbit Hb. The presence of the label does not alter the chemical shifts of ^{13}CO bound to the α and β chains. Infrared studies of CO binding to unlabelled and TF labelled rabbit Hb have revealed no changes in

stretching frequencies and intensities (Satterlee, et al., 1978b).

The Effect of Chain Heterogeneity and Organic Phosphates on the Structural Changes Occurring Upon Ligation.

Studies of the ligation of TF labelled rabbit Hb with CO in the presence or absence of DPG revealed only two structures (Figures 9, 10, 13, 14). The dissimilarity of these two states results from differences in quaternary and tertiary structures. The resonances revealed by computer simulation of the observed spectra are small and do not change significantly with ligation; they are more likely due to line shapes of the NMR signals being not ideally Lorentzian (as was assumed in the computer simulations) than they are due to discrete intermediates. Therefore, intermediates in which more β than α chains are ligated ($\beta^{CO}{}_{3\alpha\alpha}$, $\beta^{CO}{}_{\beta}{}^{CO}{}_{\alpha\alpha}$, $\beta^{CO}{}_{\beta}{}^{CO}{}_{\alpha}{}^{CO}{}_{\alpha}$) have the same quaternary structures as do either deoxy (T) or ligated (R) Hb. This conclusion is supported by studies of artificial hybrids (Ogawa & Shulman, 1971, 1972; Cassoly, et al., 1971; Cassoly & Gibson, 1972) which have shown that $\beta^{+CN^-}{}_{\beta}{}^{+CN^-}{}_{\alpha\alpha}$ is largely in the R state.

A second possibility is that each of the above mentioned intermediates has a unique structure which does not reach sufficiently high concentration to be detected by ^{19}F NMR. If such is the case, we would interpret the formerly mentioned small resonances in Figures 13 and 14 as evidence of low concentration intermediates in partially ligated Hb. However, this is unlikely since the concentration of the intermediates does not decrease to zero in deoxy or fully ligated Hb as is expected when low concentration intermediates with different quaternary structures are present.

Using ^{19}F NMR Huestis and Raftery (1973) found evidence for the presence of intermediates in TF labelled human Hb. These intermediates were identified as the $_{\alpha}CO_{\alpha}CO_{\beta\beta}$ and $_{\alpha}CO_{\alpha}CO_{\beta}CO_{\beta}$ structures. Such intermediates should not be detected in rabbit Hb because the β chains of rabbit Hb ligate preferentially. Therefore, the fact that we have not found intermediates in rabbit Hb is consistent with the results obtained in studies of human Hb (Huestis & Raftery, 1973, 1975).

When IHP is present, two additional resonances, which appear as shoulders on the sides of the major resonances, are seen during the ligation of rabbit Hb (Figures 11 and 15). Since there is no preferential

ligation under these conditions (Figure 5 and 8) the intermediates cannot be identified as to their levels and types. However, studies of artificial hybrids suggest that both $\alpha^{+CN^-}{}_\alpha{}^{+CN^-}{}_{\beta\beta}$ and $\alpha\alpha\beta^{+CN^-}{}_\beta{}^{+CN^-}$ convert to the T state in the presence of IHP (Ogawa & Shulman, 1972; Cassoly, et al., 1971; Cassoly & Gibson, 1972). These structures, however, may have different tertiary structure from deoxyhemoglobin. In the case of TF labelled $\alpha^{+CN^-}{}_\alpha{}^{+CN^-}{}_{\beta\beta}$ Huestis and Raftery (1973) have shown this to be the case. Thus structures, similar to the artificial hybrids, may be detected by the ^{19}F NMR as intermediates. The large size of the shoulder on the resonance due to deoxyhemoglobin can be explained by the existence of many intermediates with similar quaternary structures in partially ligated Hb.

The shoulder on the resonance due to $Hb(CO)_4$ appears only at very high ligation, which suggests that the shoulder is probably due to a 3 or 4 liganded structure. On the basis of the oxygen exchange studies of carbonmonoxy rabbit Hb it is probable that if a three liganded structure does exist it is in the $\beta^{CO}{}_\beta{}^{CO}{}_\alpha{}^{CO}{}_\alpha$ form (Moon & Richards, 1974). This however, is unlikely due to the fact that Hb is saturated under these conditions.

The shoulder on the resonance due to $Hb(CO)_4$ may also be caused by a four liganded structure which is not in an altered R state. As the fraction of ligation of Hb increases, the shoulder on the resonance due to $Hb(CO)_4$ also increases. This signal would be attributed to a slow exchange on the NMR time scale between the quaternary structure, which causes the shoulder, and the R structure.

IHP has an extensive effect on the structure of Hb. IHP binds to deoxy Hb with a binding constant of 10^7, while DPG binds to deoxyhemoglobin with a binding constant of 2×10^5 (Edalji, et al., 1976; Benesch, et al., 1968). The free energy released by the binding of a mole of CO to Hb is about -9.6 kcal (Antonini & Brunori, 1971). Even more interesting is that the free energy of cooperative interaction is estimated to be 2.4 kcal/mole of Hb (Wyman, 1967). In contrast, the binding of IHP to deoxyhemoglobin results in the release of about -10.9 kcal/m. These values of free energy suggest that IHP can probably modify the quaternary structure of Hb in solution in order to find a structure with lower energy than the R structure. Therefore, the high field low intensity resonance in the spectra of $Hb^{TF}(CO)_4$ in the presence of IHP, can be due to a ligated species with an altered R structure.

Evidence for the drastic effect of IHP on the structure of Hb has been found in the NO binding studies of Hb (Perutz, et al., 1974a; 1976; Maxwell & Caughey, 1976). These studies have shown that the bond between the proximial histidine and the iron of the α chains of $Hb(NO)_4$ is broken when IHP is present. The severing of this bond is due to the tension created by the binding of IHP to $Hb(NO)_4$ and by the large trans effect on the NO. Under these conditions $Hb(NO)_4$ is completely in the T state (Perutz, et al., 1976; Briehl & Salhany, 1975; Cassoly, 1975; Maxwell & Caughey, 1976). IHP binding has also been shown to have a great effect on artificial hybrids as well as on methemoglobin (Ogawa & Shulman, 1972; Cassoly, et al., 1971; Cassoly & Gibson, 1972; Perutz, et al., 1974b, c; Cassoly, 1976).

An alternative explanation for the presence of intermediates is based on the theory that only α ligated intermediates can be detected by the TF label. β chain ligated intermediates cannot be detected because the TF label lies in the β chains and changes due to the ligation of the β chains are much larger than the quaternary changes due to the α chain ligation. When α chains ligate first, the quaternary change can be detected since there is no other large change in the β chains. Therefore, inter-

mediates such as $\alpha^{CO}\alpha^{CO}\beta\beta$ and $\alpha^{CO}\alpha^{CO}\beta^{CO}\beta$, which are found in human Hb, can only be detected in the presence of IHP in rabbit Hb. However, based on this argument, large amounts of intermediates would suggest preferential α ligation, which the ^{13}C NMR results have shown in Figure 5d is not the case.

^{19}F chemical shifts of TF rabbit Hb change when DPG and IHP are present in the deoxy and the ligated states of Hb (Table I). In contrast, DPG has no effect on the chemical shift of ligated TF labeled human Hb (Huestis & Raftery, 1972; 1973; 1975). The chemical shift of the ^{31}P resonance of DPG in the rabbit Hb solution changes upon ligation. This indicates that the interaction between DPG and rabbit Hb differs in the deoxy and the ligated states (Pinnell & Walter, 1975).

Initial Ligation of TF Labelled Hemoglobin

Huestis and Raftery (1973) found that human Hb binds CO preferentially at the α chains. They based their conclusions on the ^{19}F NMR studies of human Hb which revealed a delay in the appearance of a ligated signal relative to the total ligation. When we tried to detect preferential β chain ligation of TF labelled rabbit Hb we found, however, that there is a linear relationship

between the appearance of the resonance due to ligated Hb and the total ligation of rabbit Hb (Figure 15).

Edelstein (1974) suggested that the TF label detects the state function $\bar{R}$ which is the fraction of Hb with the R quaternary structure. Our result does not agree with this interpretation since, based on the Edelstein argument, there should have been a lag in the appearance of the resonance due to ligated Hb as there is in human Hb. Since this is not the case we interpret these results to imply that the TF label detects a combination of tertiary and quaternary structural changes. When α chains ligate preferentially the only effect which is possible to detect with the TF label is quaternary structural change. Therefore, a lag in the appearance of the ligated signal suggests α chain ligation. The label, however, cannot differentiate β chain ligation from random ligation.

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Part III

THE EFFECT OF pH AND ORGANIC PHOSPHATES ON THE STRUCTURE OF HEMOGLOBINS IN DIFFERENT STATES OF OXIDATION AND LIGATION

INTRODUCTION

Hemoglobin (Hb) is known to exist in two quaternary structures (Haurowitz, 1938; Muirhead & Perutz, 1963; Perutz, 1970). Monod, Wyman, Changeux (1965) (MWC) knowing this fact proposed a mechanism for the cooperativity of Hb in which they suggested that both deoxy and fully ligated Hb can exist in the T and R states. In support of the MWC model, Ogawa and Shulman (1971) have shown that the artificial intermediates, $\beta^{+CN^-}\beta^{+CN^-}\alpha\alpha$ and $\alpha^{+CN^-}\alpha^{+CN^-}\beta\beta$, can exist in both the T and R forms depending on the conditions. Further, mutants, like Hb Kempsey and carboxypeptidase A treated Hb, are in the R state even when they are deoxygenated while other mutants, such as Hb Kansas, stay in the T state even when they are ligated in the presence of inositol hexaphosphate (IHP) (Ogawa, et al., 1972; Ho, et al., 1973; Lindstrom, et al., 1973; Perutz, et al., 1974a).

Perutz (1970) has suggested in his theory for the mechanism of cooperativity in hemoglobin that there is a link between the spin state of the iron and the quaternary structure of the protein. Later he has proposed that the low affinity of Hb is due to a tension created by the protein on the heme which opposes the movement of the iron into the plane of the heme (Perutz,

1972). The tension is maximum when a ligand binds to the iron and the iron moves into the plane of the heme yet the protein is still in the T quaternary state (Perutz, et al., 1978). In fact the IR stretching frequencies of the azide methemoglobin in the presence and in the absence of IHP differ by 1 kcal which is 30% of the difference in free energy between the R and T structures of Hb (Perutz, et al., 1978; Messana, et al., 1978). This tension, together with the trans effect of NO, was cited as being responsible for the breakage of the histidine αF8 iron bond in $Hb(NO)_4$ in the presence of IHP (Salhany, 1974; Salhany, et al., 1974; Perutz, et al., 1976; Maxwell & Caughey, 1976).

Evidence against this theory has been collected by X-ray absorption which detected no difference between the iron-pyrrole nitrogen distance in deoxy- and oxyhemoglobin (Eisenberger, et al., 1976), by Raman spectroscopy which detected no difference between heme models and heme proteins (Spiro & Burke, 1976), and by X-ray analysis of deoxyhemoglobin which indicated that the iron is out of the plane of the heme in deoxyhemoglobin just as in the five coordinated model compounds (Fermi, 1975).

Hopfield (1973) suggested an alternate model for the cooperativity of Hb. In this model the energy for the cooperativity of Hb is distributed in the protein and only a very small fraction of this energy is stored at the hemes. In support of this model Rakshit and Spiro (1974) suggested from their studies of heme proteins by resonance Raman spectroscopy that the spin state may be a function of heme protein structure.

Gelin and Karplus (1977) and Warshel (1977) have revised the Hopfield model. It has been shown that the porphyrin is domed in deoxyhemoglobin (Hoard & Scheidt, 1972; Gelin & Karplus, 1977). Since there is no change in the iron-pyrrole nitrogen bond length upon ligation Gelin and Karplus (1977) suggested that instead of the iron moving into the plane of the heme, the heme becomes planar (Eisenberger, et al., 1976). This change in the structure of the heme results in pressure on the protein by the periphery of the heme due to van der Waal's interactions, to change its structure to the R state. This tension is mainly on valine FG5 and on the histidine F8 (Perutz, 1970; Gelin & Karplus, 1977; Baldwin & Chothia, 1979). However, there is no significant amount of energy stored in the domed structure which would get released upon ligation as Perutz (1970) has suggested (Warshel,

1977; Little & Ibers, 1974). The energy for the cooperativity of the chains must be stored in many interactions in the protein (Hopfield, 1973).

The effector which maximally increases the tension in the ligated forms of Hb is IHP. In the presence of IHP mutant Hbs like Hb Kempsey, and altered Hbs like NES-des-Arg Hb (N-ethylsuccinimide derivative of Hb which has its terminal residue on the α chains removed by carboxypeptidase B), which are in the R form even in the deoxy state switch their quaternary structure to the T form. In these mutated Hbs the spin state does not change (Perutz, 1974a) . In addition, IHP can change the structure of carboxy Hb Kansas from the R to the T state (Ogawa, et al., 1972).

IHP has a small effect on the structure of oxy and carbonmonoxy Hb. In the visible spectra of oxyhemoglobin in the presence of IHP Adams and Schuster (1974) have detected changes relative to Hb in the absence of IHP suggesting that IHP alters the structure of oxyhemoglobin. They have interpreted these changes as evidence for an R to T switch in quaternary structure. Perutz, et al. (1976), however disagreed with this conclusion. They suggested that the key markers of quaternary structures like tryptophan βC3, which absorbs in the UV region in

the T state indicate that oxy and carbonmonoxyhemoglobin in the presence of IHP stay in the R quaternary structure. The results of proton NMR supports this conclusion (Perutz, et al., 1974b). Upon addition of IHP to carbonmonoxyhemoglobin no change is detected in the H^1 chemical shifts of the resonances which are markers for the R structure. Some of these resonances however become broader due to the addition of IHP.

The effect of IHP is the largest on $Hb(NO)_4$. In this case it has been shown by UV, visible and NMR spectroscopy that the protein below pH 7.5 is in the T state (Salhany, 1974; Salhany, et al., 1974; 1975; Perutz, et al., 1976). Further, Briehl and Salhany (1975) have shown that nitric oxide sickle cell Hb gels in the presence of IHP. Kon (1975) and Szabo and Perutz (1976) suggested that the change in the ESR spectra due to the addition of IHP to $Hb(NO)_4$, first detected by Rein, et al. (1972), is due to the severing of the iron histidine F8 bond. Maxwell and Caughey (1976) confirmed this conclusion by IR spectroscopy. They also found that in the presence of IHP only 50% of the iron histidine F8 bond is broken in $Hb(NO)_4$. The results of Cassoly (1975) and Taketa, et al. (1978) suggest that the severing of the iron histidine F8 bond occurs only in the α chains.

In the case of oxidized Hb the effect of IHP is also quite large. Aqua- and fluoromethemoglobin are normally in the R state but when IHP is added they switch to the T state (Fermi & Perutz, 1977; Perutz, et al., 1978). Although azide and cyanomethemoglobin bind IHP, they do not change their quaternary structure in the presence of IHP from the R to the T state (Perutz, et al., 1974b,c; Perutz, et al., 1978). However, evidence exists for unknown alterations of the structure of azide and cyanomethemoglobin due to the binding of IHP (Perutz, et al., 1974c; 1978).

As it has been shown in Part I of this work, and in the work of Huestis and Raftery (1972a,b; 1973; 1975) the trifluoroacetonyl (TF) label has been useful for following tertiary and quaternary structural changes in Hb. Studies of the TF labelled Hb, between pH 6 and 8, and in the presence or absence of DPG, resulted in useful information about the structure of Hb. The titration of TF labelled deoxyhemoglobin with an apparent pK of 7.4 has been interpreted as due to the cooperative ionization of his βHC3 and two or three other residues (Huestis & Raftery, 1972b). Removal of the his βHC3 or his βHC3 and tyr βHC2 resulted in the disappearance of the titration curve detected by ^{19}F NMR. Titration

of TF labelled methemoglobin resulted in a titration curve with an apparent pKa of 7.8. DPG increased the slope of the titration in the TF labelled deoxyhemoglobin. Further, addition of DPG shifts the ^{19}F resonance of TF labelled deoxyhemoglobin upfield by 0.13 ppm without changing the ^{19}F resonances of ligated TF labelled Hbs.

In order to study the effect of organic phosphates on the tertiary and quaternary structures of Hb we have studied the ^{19}F, ^{13}C and ^{31}P NMR of TF labelled human and rabbit Hb in their deoxy-, oxy-, carboxy-, nitrosyl- aquamet- and cyanomet- forms in the presence and in the absence of organic effectors.

MATERIALS AND METHODS

Preparation of Hemoglobin

Fresh blood for these studies was obtained from humans and from New Zealand rabbits. The blood was prepared as described in Material and Methods of Part I. Trifluoroacetonylated Hb was prepared using the method of Huestis and Raftery (1972a).

Trifluoroacetonylated methemoglobin was prepared by adding a calculated amount of $K_3Fe(CN)_6$ solution to the reaction mixture after the reaction with 3-bromo-1,1,1-trifluoropropane (10% excess).

The reaction mixture in the oxy- or met- form, was loaded on a biogel P2 column equilibrated with 0.1 M NaCl and 0.015 M 2,2-bis-(hydroxymethyl)-2,2',2''-nitrilothmethanol (bis-tris) which was obtained from Aldrich. The collected Hb solution was then concentrated and was later used as a stock solution for the preparation of NMR samples.

Preparation of NMR Samples

Samples for the ^{13}C NMR studies were prepared as described in Material and Methods of Part I with the exception of the substitution of bis-tris buffer for tris. For the ^{19}F NMR studies 0.5 mM Hb samples were prepared so that the final concentration of the salts present were 0.1 M NaCl, 0.05M bis-tris and 2.5 mM effector, if any. For the ^{31}P NMR studies samples were prepared as for ^{19}F NMR with the exception of the ratio of the concentration of the effector to the Hb. This ratio was 1:1 in the phosphorous studies as distinct from an effector: hemoglobin ratio of 5:1 in the ^{19}F studies.

The pH of the solution was adjusted by measuring out appropriate amounts of buffer (bis-tris buffer containing DPG or IHP, if any) and titrating the buffer with 1M NaOH or HCl to the appropriate pH. After this was done

the measured amount of Hb stock solution was added to the buffer. Fine correction of pH was done on the total sample. Any precipitate that formed was removed by centrifugation at 600g. For each pH a separate sample was prepared in order to avoid irreversible changes and denaturation due to the addition of acid or base.

Spectra of oxyhemoglobin were obtained from the samples prepared in 12 mm NMR tubes. These samples were then deoxygenated in a syringe and injected into the closed NMR tube containing nitrogen, as described previously (Part I, Materials and Methods), their spectra were obtained once again. Spectra of $Hb(CO)_4$ and $Hb(NO)_4$ were obtained after adding saturating amounts of CO or NO to the deoxygenated samples and equilibrating the gas with Hb for five minutes. Spectra of cyanomethemoglobin were obtained in normal 12 mm NMR tubes. Then calculated amounts of KCN were added to obtain $Hb^+(CN^-)_4$ samples (at least eight times more than necessary to saturate the Hb^+ solution). The pH of these samples was measured again since the addition of KCN changed the pH slightly.

NMR Conditions

^{19}F, ^{13}C, ^{31}P spectra were obtained at 94.1 MHz, 25.1 Mhz, and 40.5 MHz, respectively, on an XL100-15

spectrometer using the Varian 620i computer. ^{13}C spectra were obtained using 18 mm NMR tubes, while 12 mm NMR tubes were used to obtain ^{19}F and ^{31}P spectra. In obtaining fluorine spectra internal proton lock was used. In order to obtain ^{13}C and ^{31}P NMR spectra, specially made tubes, which have a 5 mm or 2 mm concentric tube containing D_2O fused into them for locking, were used. All spectra were obtained under identical conditions at 24.5°C. The samples were pre-equilibrated for 20 minutes to insure complete temperature equilibration.

RESULTS

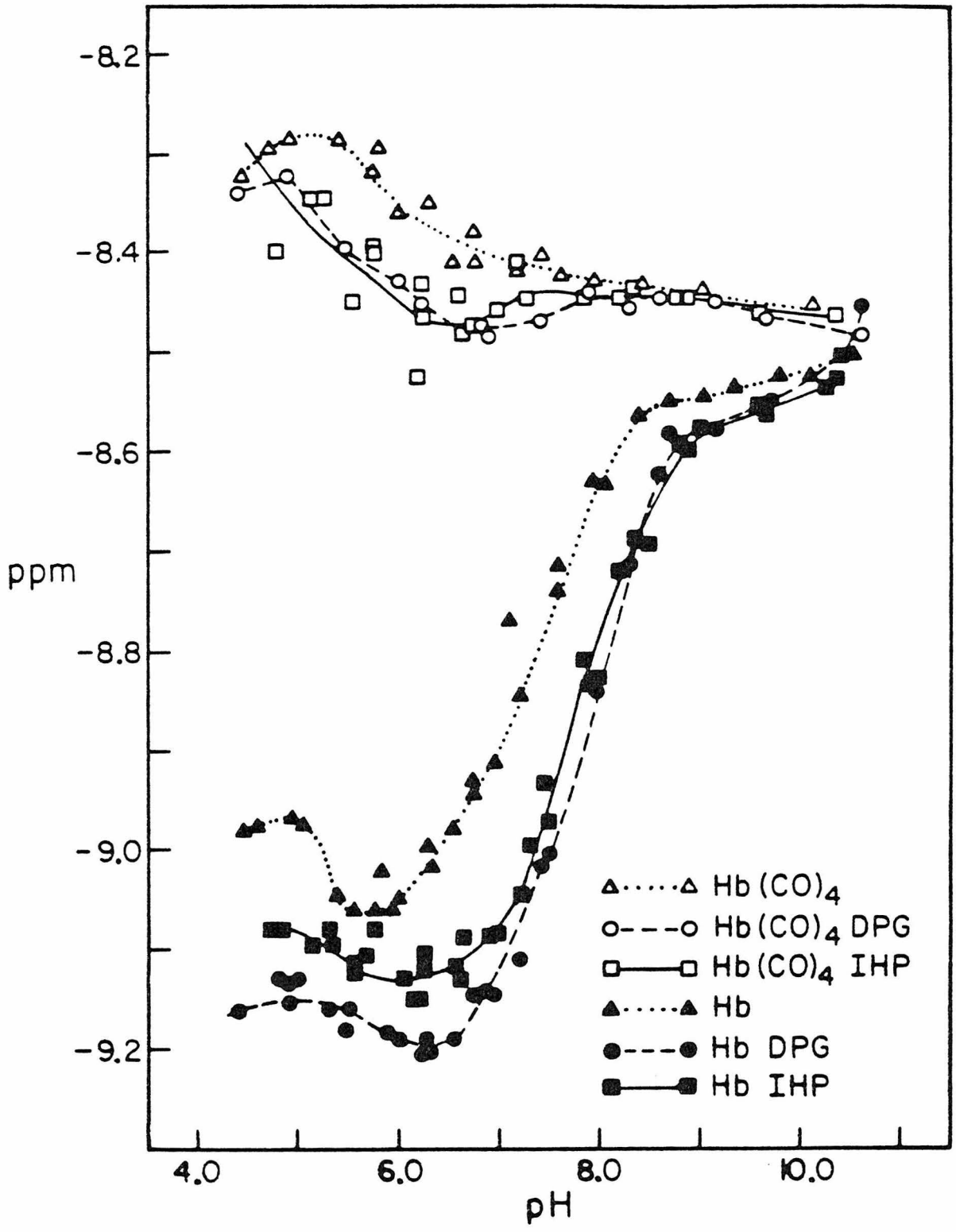
The Effect of Organic Phosphates on the Titration of Hemoglobin in the +2 Oxidation State

The Titrations of TF Labelled Deoxy and Carbonmonoxyhemoglobin

In Figure 1 the titrations of TF labelled human Hb (Hb^{TF}) are shown in the deoxy and carboxy state in the absence of any effectors, in the presence of DPG and in the presence of IHP. The titrations of deoxyhemoglobin in the presence and in the absence of DPG are similar to those reported by Huestis and Raftery (1972b). DPG shifts the resonance due to deoxyhemoglobin more than

FIGURE 1

The plots of the ^{19}F chemical shifts of trifluoroacetonylated human deoxy- and carbonmonoxyhemoglobin in the absence of any effectors, in the presence of 2,3-diphosphoglycerate and in the presence of inositol hexaphosphate versus pH.


-8.2
-8.4
-8.6
-8.8
-9.0
-9.2
ppm
4.0
6.0
8.0
10.0
pH
Hb (CO)4
Hb (CO)4 DPG
Hb (CO)4 IHP
Hb
Hb DPG
Hb IHP

IHP does. Addition of DPG and IHP to carbonmonoxyhemglobin results in a small upfield chemical shift below pH 8.0. Both deoxy and carbonmonoxy TF labelled Hb have a small titration curve below pH 6 which is observed both in the presence of or in the absence of effectors.

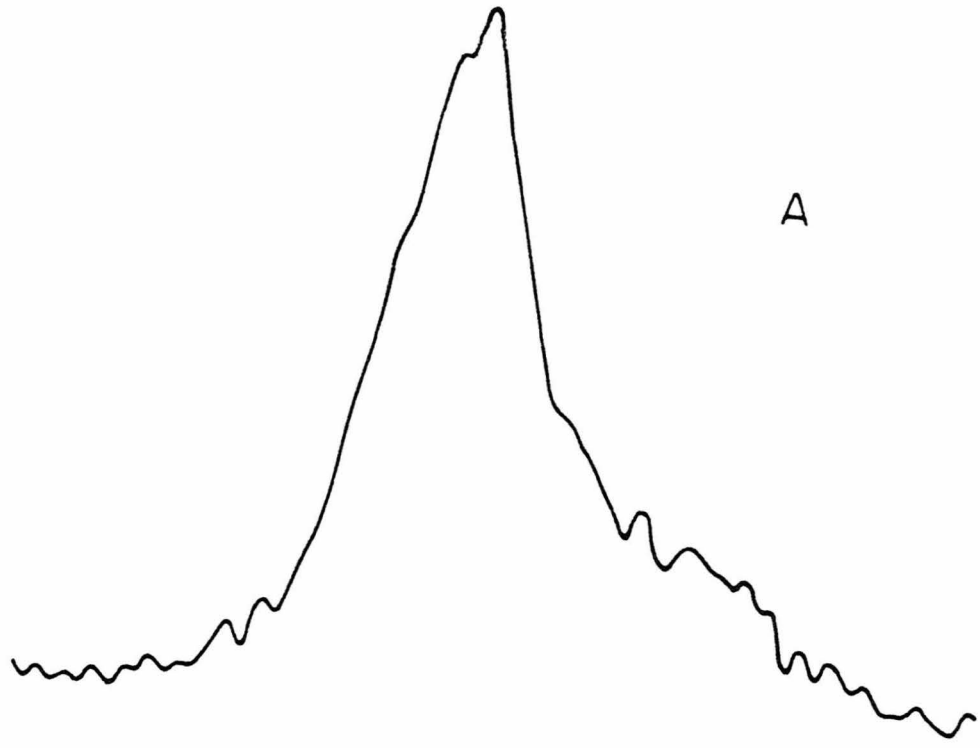
The resonance due to $Hb^{TF}(CO)_4$ has a large linewidth below pH 7 in the presence of IHP. Figure 2 shows the spectra of TF labelled $Hb(CO)_4$ in the presence of IHP and in the absence of organic phosphates. The half linewidth in the absence of IHP is 18 Hz while in the presence of IHP it reaches about 60 Hz at pH 6.2 Further lowering of pH lowers the linewidth.

The Titrations of Different Forms of Hemoglobin

The titrations of TF labelled $HB(CO)_4$, $Hb(O_2)_4$, $Hb(NO)_4$ and Hb are shown in Figure 3 in the absence of organic phosphates. Above pH 8.5 the chemical shift of Hb^{TF} is more downfield than $Hb^{TF}(O_2)_4$ and $Hb^{TF}(NO)_4$. These results suggest that above pH 8.5 there is little difference between deoxy and ligated Hb in the environment of the TF label. The lowering of the pH below pH 6 does not shift the resonances of $Hb^{TF}(O_2)_4$ and $Hb^{TF}(NO)_4$ as much as it does the ^{19}F resonance of $Hb^{TF}(CO)_4$.

FIGURE 2

The spectra of trifluoroacetonylated human carbonmonxy-hemoglobin at pH 6.2 in the presence of inositol hexa-phosphate (A) and in the absence of organic phosphates (B).


A

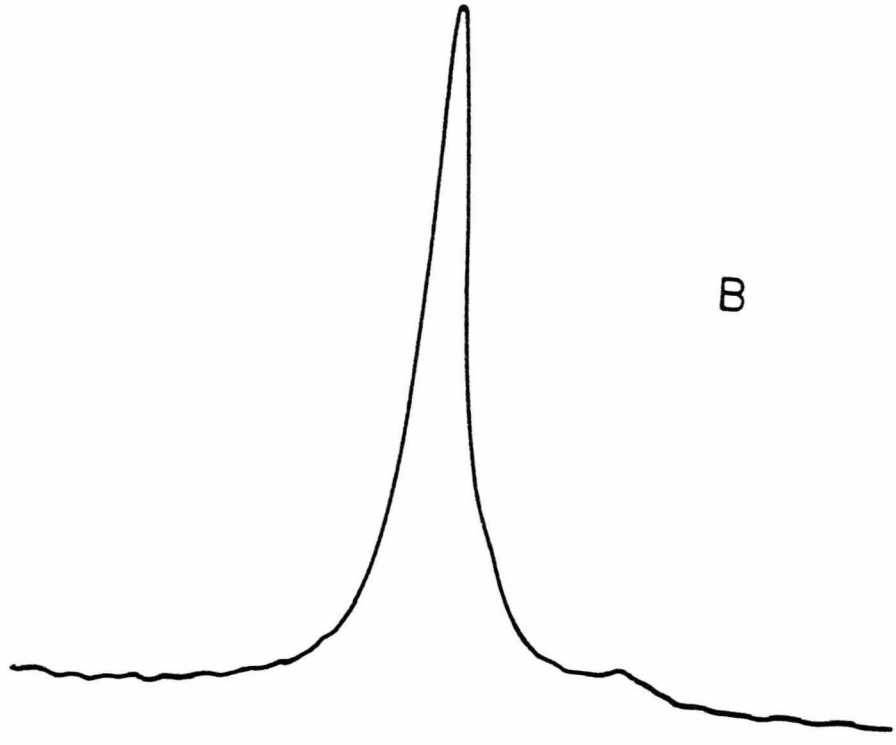
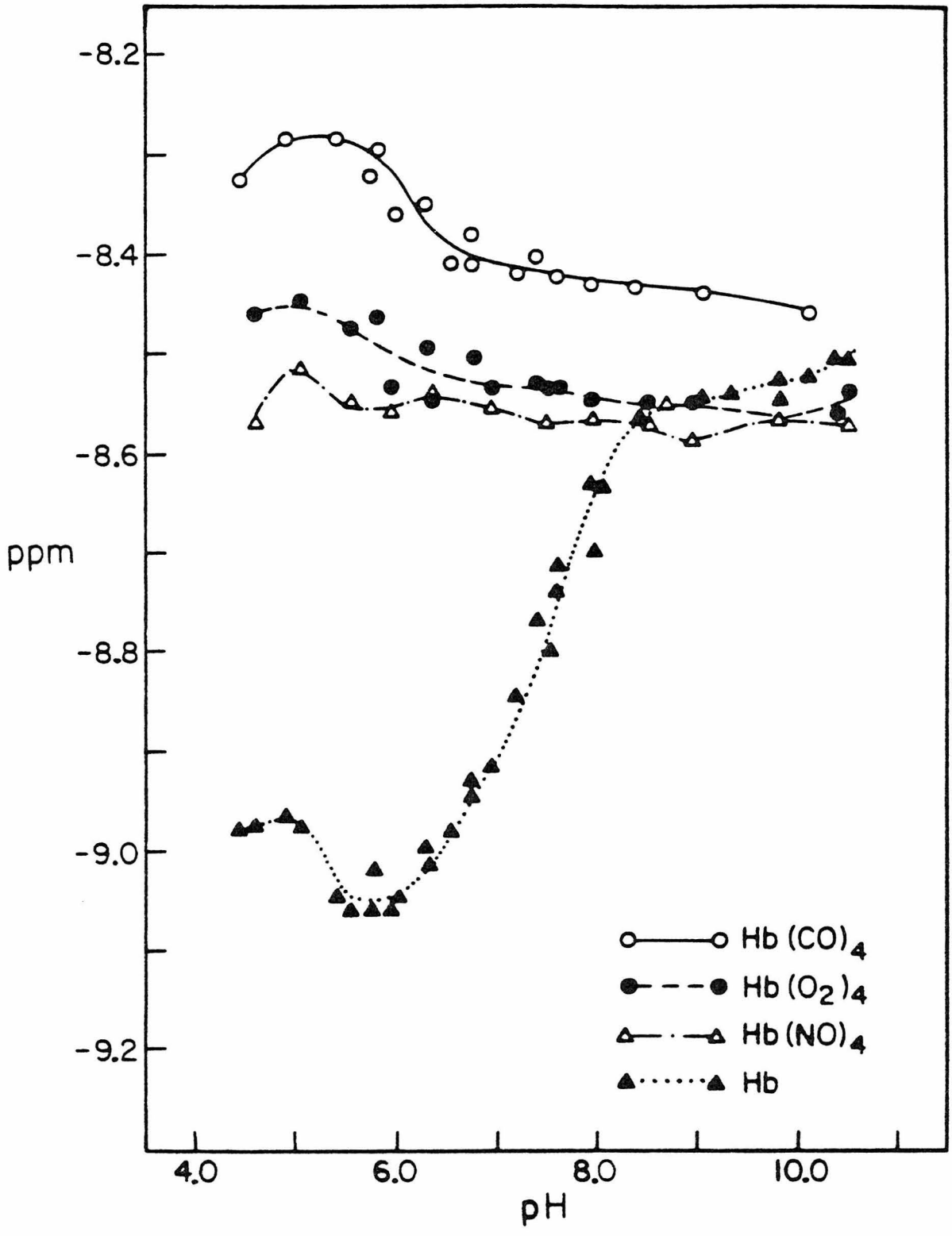

B

FIGURE 3

The plots of the ^{19}F chemical shifts of trifluoroacetonylated human carbonmonoxy-, oxy-, nitrosyl-, and deoxyhemoglobin in the absence of organic phosphates versus pH.


-8.2
-8.4
-8.6
-8.8
-9.0
-9.2
ppm
4.0
6.0
8.0
10.0
pH
Hb (CO)4
Hb (O2)4
Hb (NO)4
Hb

The Titrations of Different Forms of Hemoglobin in the Presence of DPG

Addition of DPG results in an upfield shift of the resonance due to $Hb(CO)_4$ as shown in Figure 1. The resonance due to $Hb^{TF}(O_2)_4$ and $Hb^{TF}(CO)_4$ shifts very little due to the addition of DPG (Figure 4). Thus the difference between the resonances of $Hb^{TF}(NO)_4$, $Hb^{TF}(O_2)_4$ and $Hb^{TF}(CO)_4$ is smaller in the presence of DPG, as shown in Figure 4. However, a second resonance upfield from the normal ligated resonance appears in the spectra of $Hb^{TF}(NO)_4$ in the presence of DPG below pH 7. This resonance has a chemical shift similar to deoxy Hb^{TF} and it reaches 55% of the total signal in the spectrum at pH 6.3. Figure 5 shows the dependence of the spectrum of $Hb(NO)_4$ on pH in the presence of DPG.

The Titrations of Different Forms of Hemoglobin in the Presence of IHP

Addition of IHP to different forms of Hb results in dramatic changes, as shown in Figure 6. Besides the changes mentioned earlier in $Hb^{TF}(CO)_4$ and Hb^{TF} the titration of $Hb^{TF}(O_2)_4$ and $Hb^{TF}(NO)_4$ detected by ^{19}F NMR also changes due to the addition of IHP. In $Hb^{TF}(NO)_4$ in the presence of IHP there is a slow exchange on the

FIGURE 4

The plots of the ^{19}F chemical shifts of trifluoroacetonylated human carbonmonoxy-, oxy-, nitrosyl-, and deoxyhemoglobin in the presence of 2,3-diphosphoglycerate versus pH.

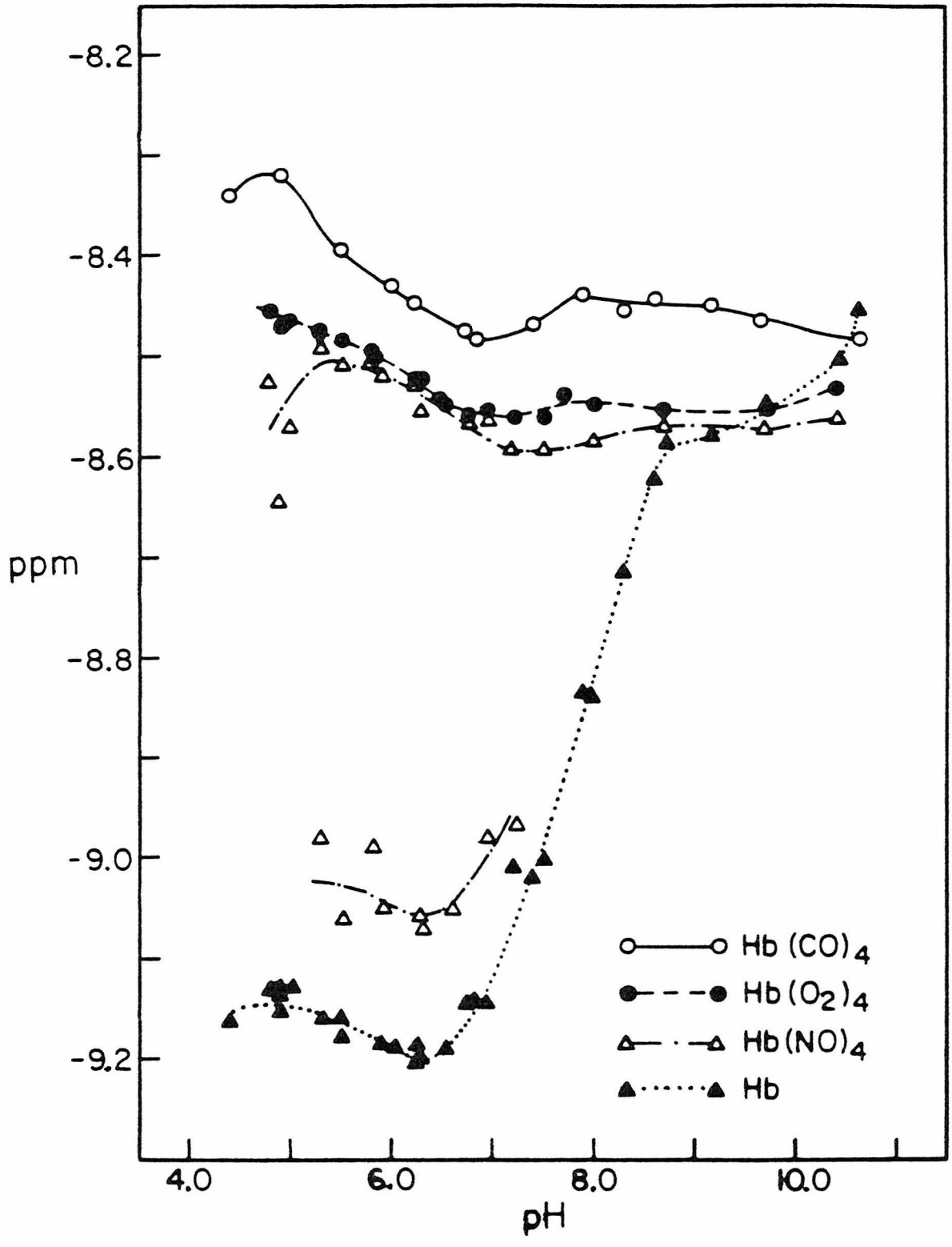

-8.2
-8.4
-8.6
-8.8
-9.0
-9.2
ppm
4.0
6.0
8.0
10.0
pH
Hb (CO)4
Hb (O2)4
Hb (NO)4
Hb

FIGURE 5

The ^{19}F NMR spectra of trifluoroacetonylated human nitrosyl hemoglobins at different pHs, in the presence of 2,3-diphosphoglycerate.

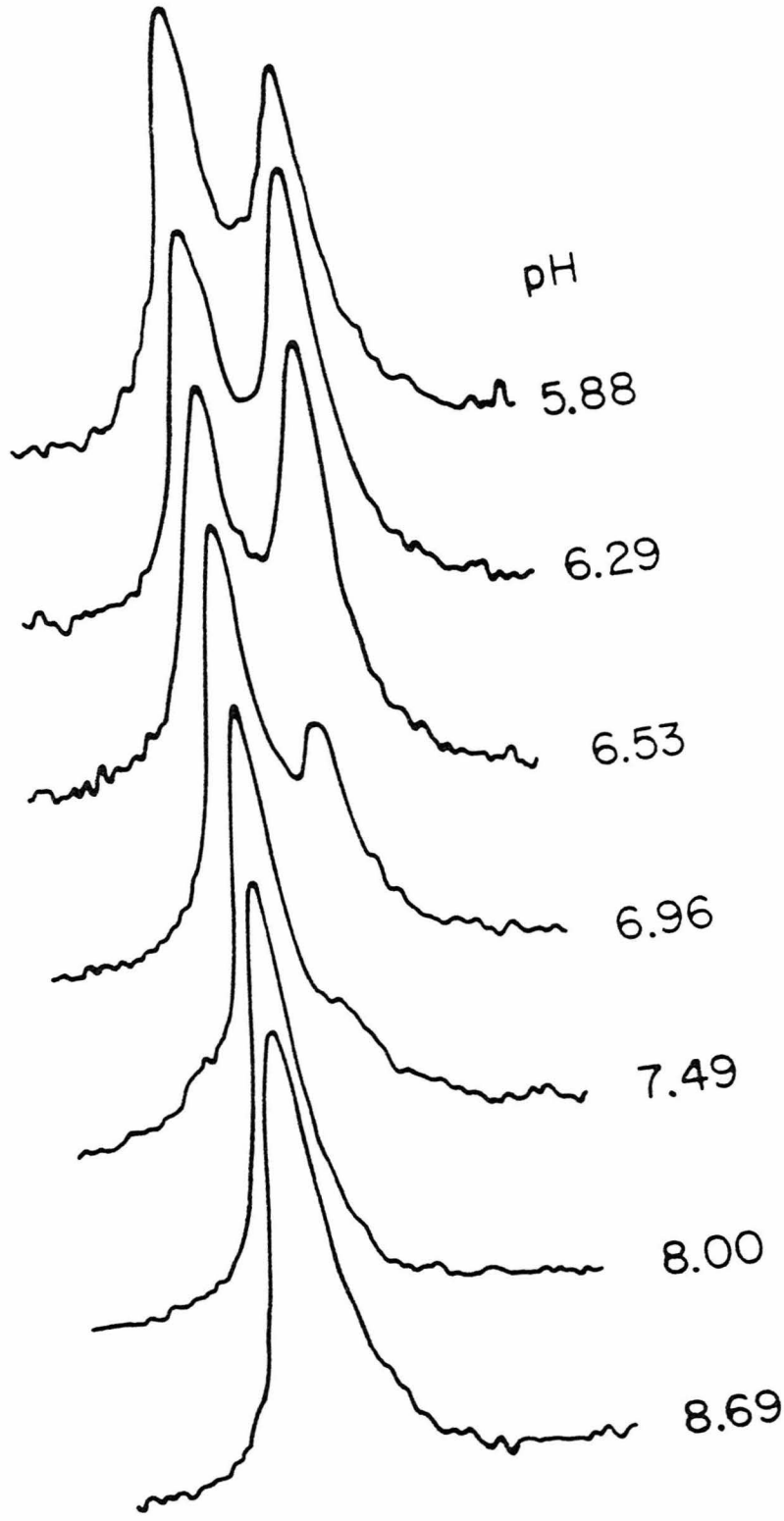
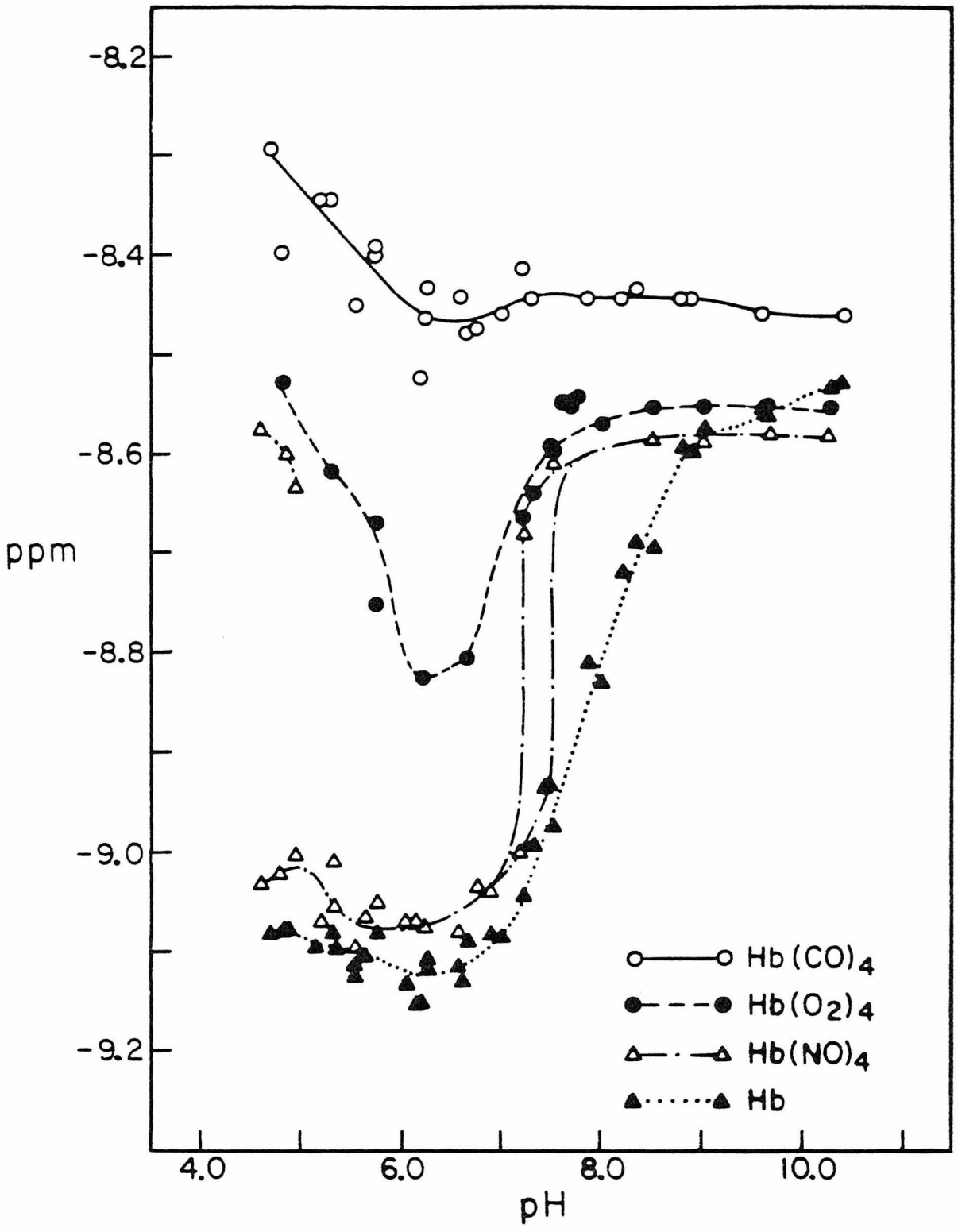

pH
5.88
6.29
6.53
6.96
7.49
8.00
8.69

FIGURE 6

The plots of the ^{19}F chemical shifts of the trifluoroacetonylated human carbonmonoxy-, oxy-, nitrosyl-, and deoxyhemoglobin in the presence of inositol hexaphosphate versus pH.


-8.2
-8.4
-8.6
-8.8
-9.0
-9.2
ppm
4.0
6.0
8.0
10.0
pH
Hb (CO)4
Hb(O2)4
Hb(NO)4
Hb

NMR timescale between the two resonances which is due to the existence of two different states of $Hb^{TF}(NO)_4$ between pH 7.2 and 7.5. Below pH 7.2 only the upfield resonance appears with a chemical shift similar to that of deoxy Hb^{TF}. The NMR spectra of $Hb^{TF}(NO)_4$ in the presence of IHP are shown in Figure 7. The resonance due to $Hb^{TF}(O_2)_4$ has a large linewidth below pH 7 just as $Hb^{TF}(CO)_4$, and its chemical shift is intermediate between the deoxy resonance and the resonance due to $Hb(O_2)_4$ in the absence of any effectors (Figure 8).

In order to insure 100% ligation in case of $Hb^{TF}(O_2)_4$ and $Hb^{TF}(CO)_4$ at low pH and in the presence of IHP, the difference visible spectra were recorded between the samples with identical pH and concentrations in the presence of IHP and in the absence of organic effectors. In the difference visible spectra of $Hb^{TF}(CO)_4$ and $Hb^{TF}(O_2)_4$ we have recorded there was no significant difference in the Soret region between samples which contained IHP and samples which did not contain any organic effectors.

The Effect of IHP on the Heme Pocket

In order to learn about the effect of pH and IHP on the heme pocket rabbit $Hb(^{13}CO)_4$ was studied by ^{13}C NMR.

FIGURE 7

The ^{19}F NMR spectra of trifluoroacetonylated human nitrosylhemoglobin, at different pHs, in the presence of inositol hexaphosphate.

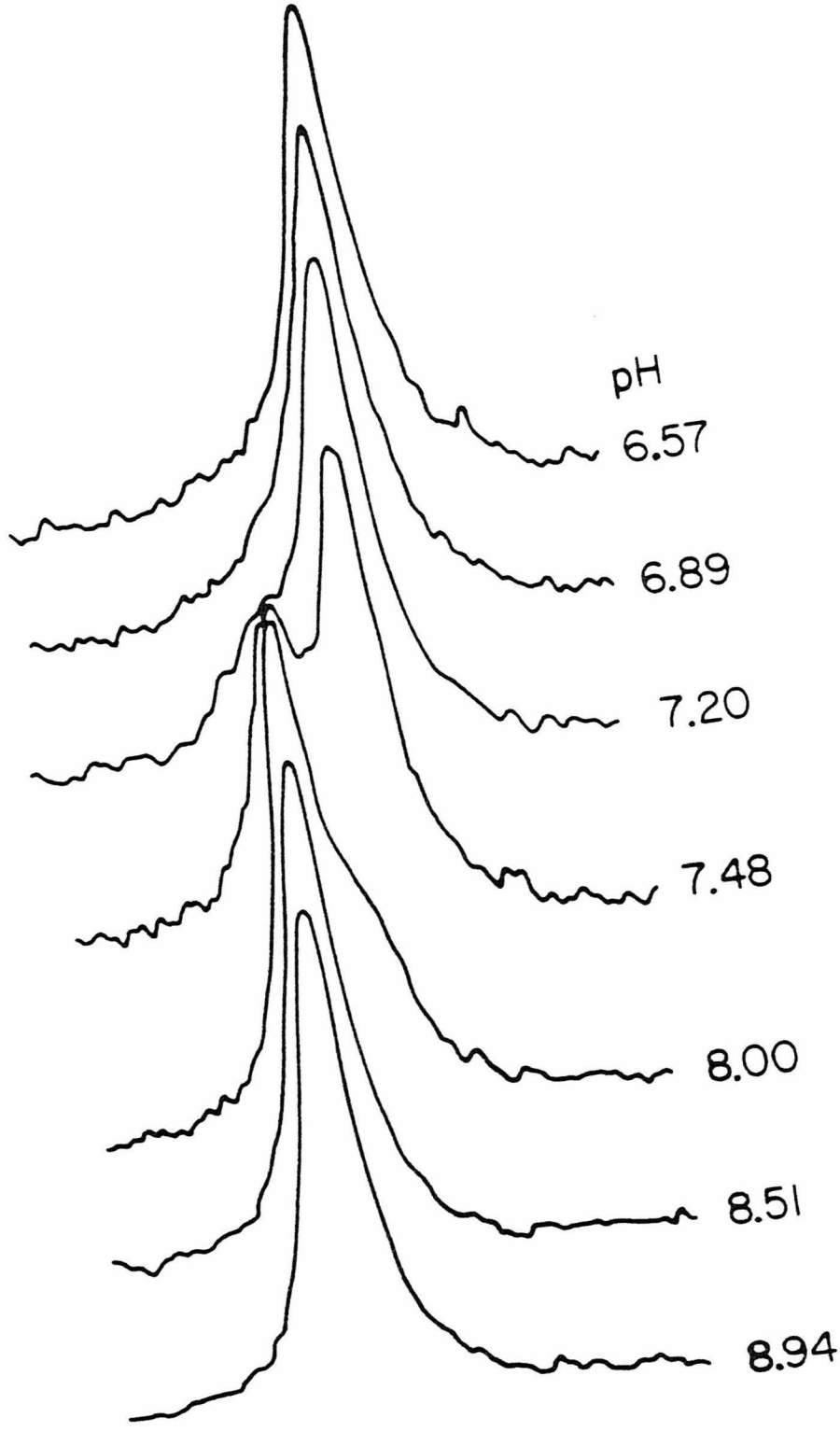
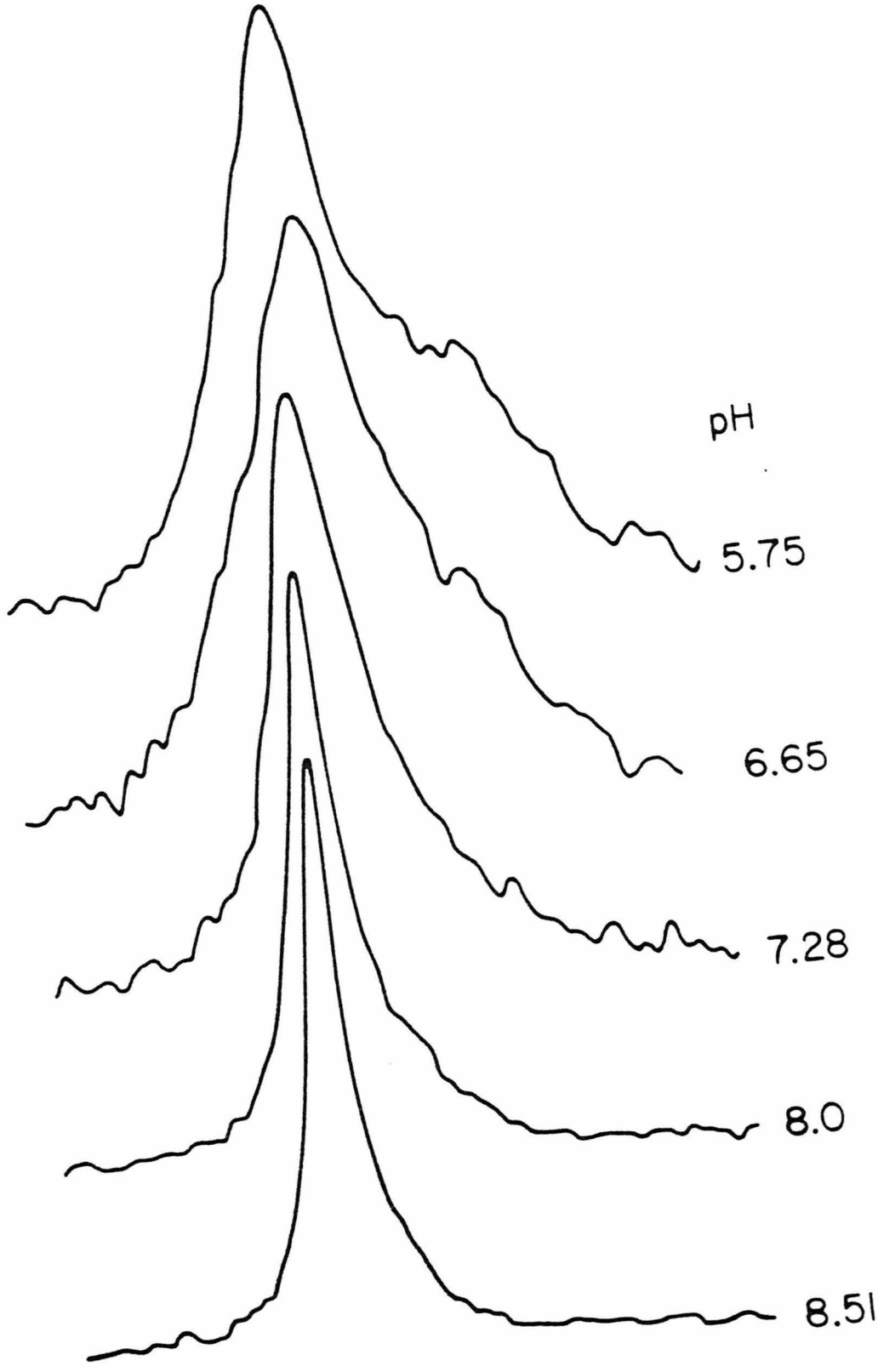

pH
6.57
6.89
7.20
7.48
8.00
8.51
8.94

FIGURE 8

The ^{19}F NMR spectra of trifluoroacetonylated human oxyhemoglobin, at different pHs, in the presence of inositol hexaphosphate.


pH
5.75
6.65
7.28
8.0
8.51

The results are shown in Figure 9. The changes in chemical shift with pH are identical to those of Satterlee, et al. (1980) who studied the ^{13}C NMR of carbonmonoxy rabbit Hb without IHP. Thus no change occurs in the heme pocket due to the addition of IHP which can be detected by ^{13}C NMR.

31P NMR Study of the Binding of IHP

The binding of IHP to deoxy and ligated rabbit Hb was studied by ^{31}P NMR. However, due to the complexity of the spectra, the analysis of the results was impossible. The two titrations are shown in Figures 10 and 11. Approximate relative intensities of the detected resonances are indicated on the graphs. Differences between the spectra of ^{31}P NMR of the IHP bound to deoxy and ligated Hb samples can be seen through the whole pH range. The titration of free IHP by ^{31}P NMR shows different titrations patterns from those shown in Figure 10 or 11 (Zuiderweg, et al., 1979; Pinnell & Walter, 1972).

The Effect of Organic Phosphates on the Titration of Hemoglobin in the +3 Oxidation State

Figure 12 shows the titration of $Hb^{TF+}(CN^-)_4$ and $Hb^{TF+}(H_2O, OH^-)_4$ by ^{19}F NMR in the presence and in the

FIGURE 9

The plot of the ^{13}C chemical shifts of the α and β chains of rabbit carbonmonoxyhemoglobin, at different pHs, in the presence of inositol hexaphosphate versus pH.

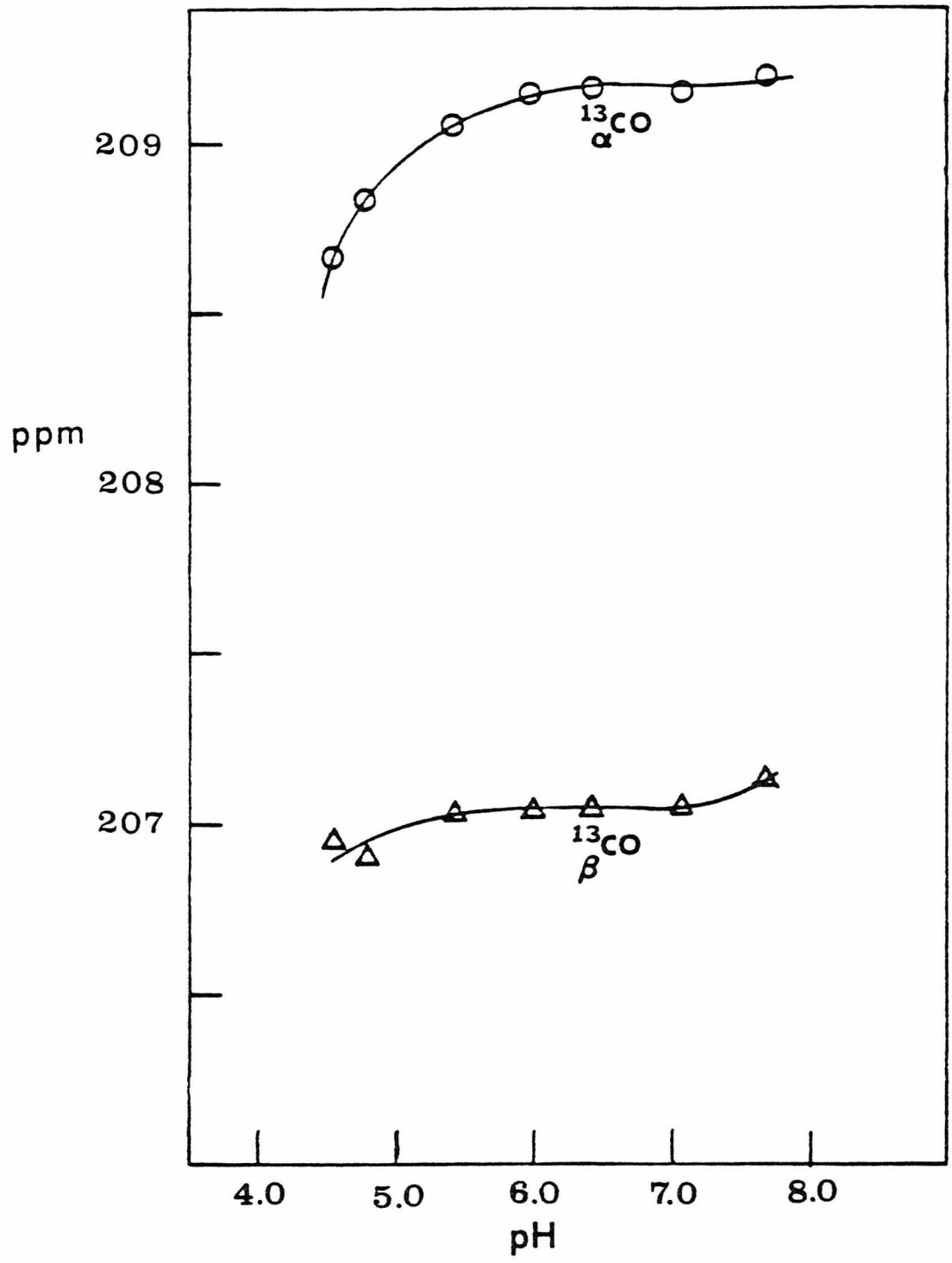

209
208
207
ppm
^{13}CO α
^{13}CO β
4.0
5.0
6.0
7.0
8.0
pH

FIGURE 10

The plot of the ^{31}P chemical shifts of inositol hexaphosphate bound to deoxygenated rabbit hemoglobin versus pH. The relative intensities of the detected resonances are indicated on the plot.

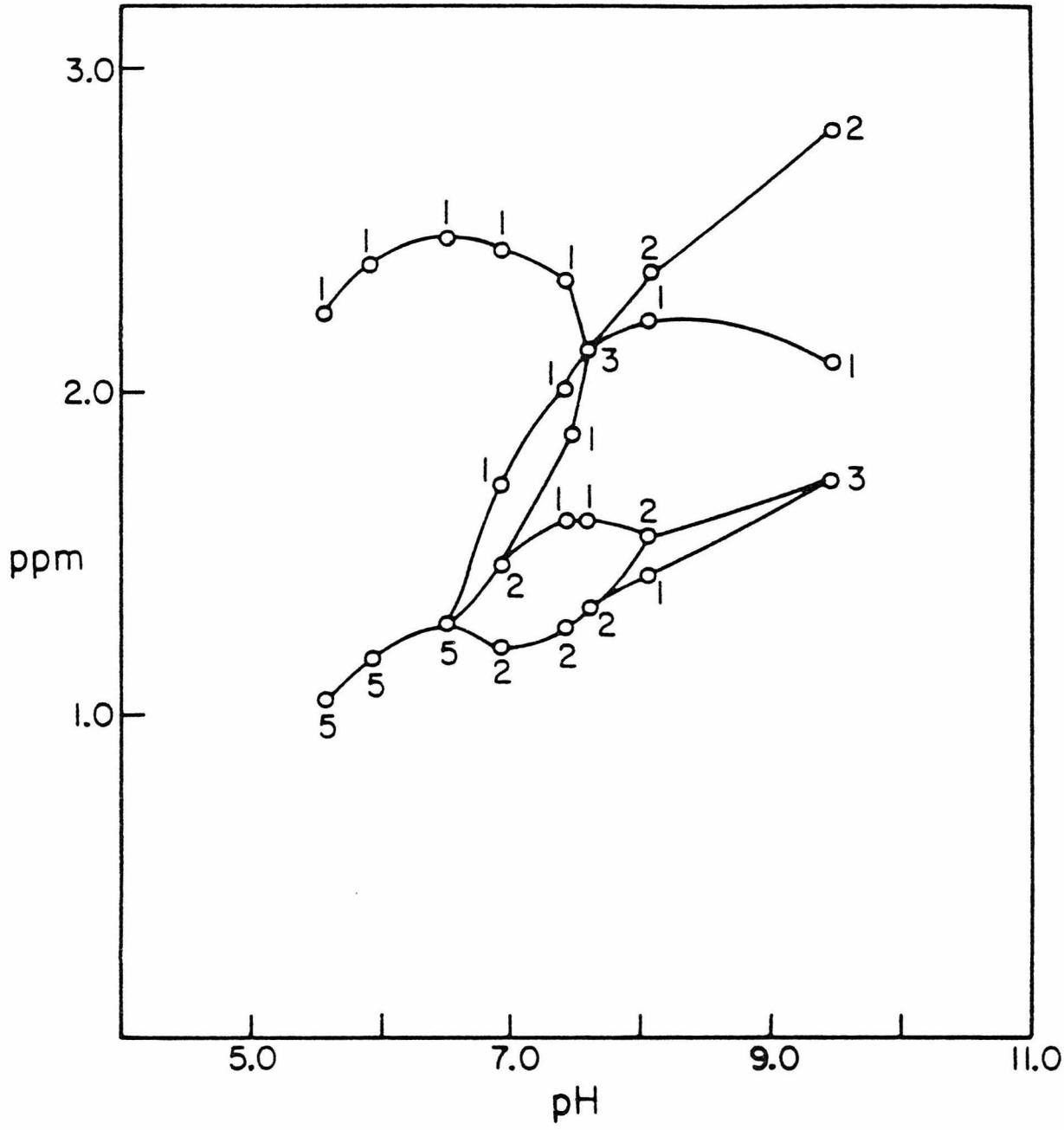
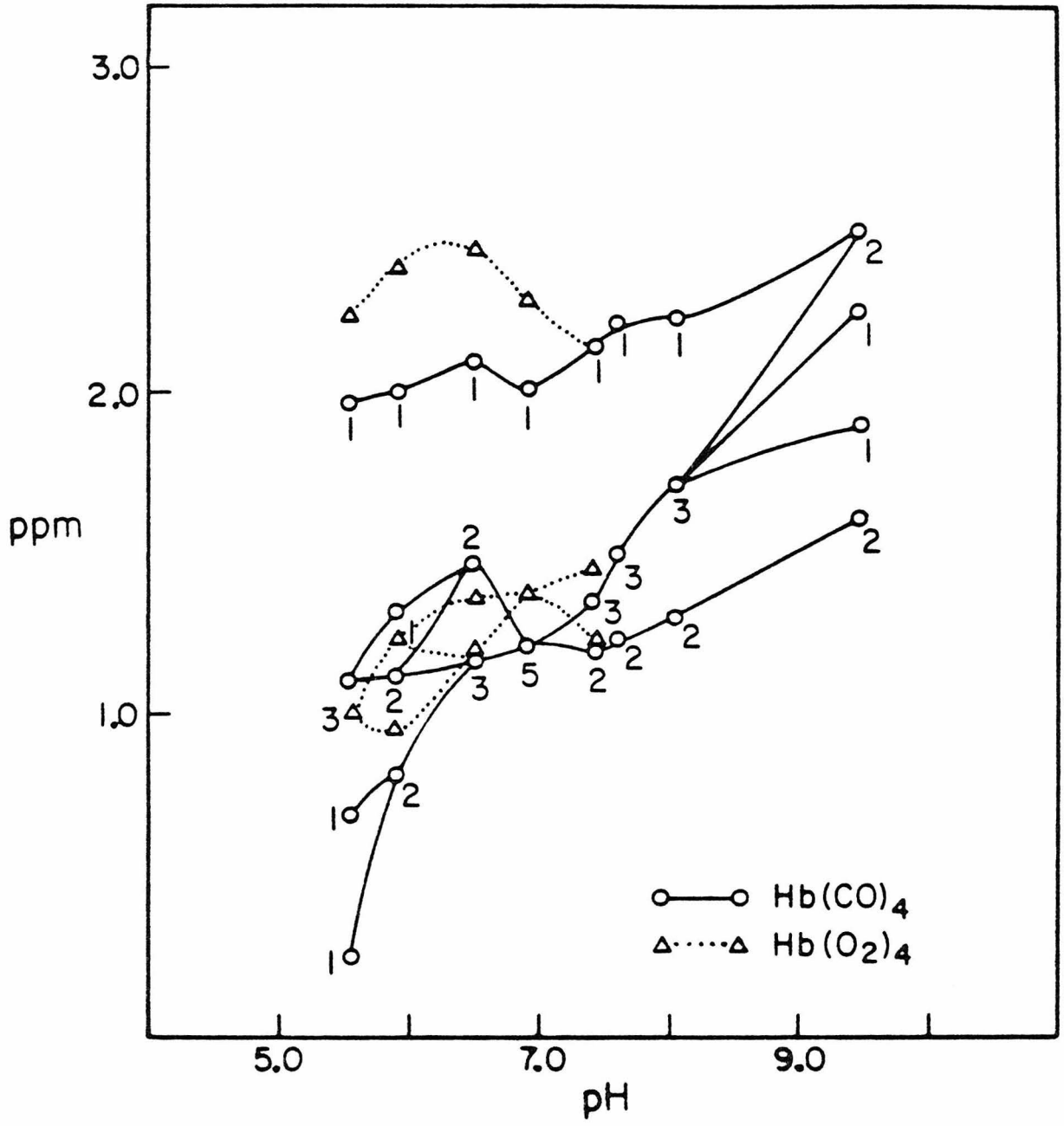

3.0
2.0
1.0
ppm
5.0
7.0
9.0
11.0
pH

FIGURE 11

The plot of the ^{31}P chemical shifts of inositol hexaphosphate bound to ligated rabbit hemoglobin versus pH. The relative intensities of the detected resonances of IHP bound to carbonmonoxyhemoglobin are indicated on the plot.


3.0
2.0
1.0
ppm
5.0
7.0
9.0
pH
Hb(CO)4
Hb(O2)4

absence of DPG. The titrations of $Hb^{TF+}(CN^-)_4$ show a very similar behavior to $Hb^{TF}(CO)_4$. Aquamethemoglobin however, has a titration curve which is similar to that of deoxyhemoglobin except the titration curve of $Hb^{TF+}(H_2O)_4$ has a smaller slope. DPG has practically no effect on the structures of $Hb^{TF+}(H_2O)_4$ and $Hb^{TF+}(CN^-)_4$.

IHP has a large effect on methemoglobin as shown in Figure 13. Between pH 8.2 and 7.0 aquamethemoglobin is present in two forms. In the presence of IHP the titration of $Hb^+(H_2O,OH^-)_4$ has a larger slope than that of deoxyhemoglobin. Below pH 7.4 $Hb^{TF+}(CN^-)_4$ is present in at least three different forms. The spectra are broad, probably due to exchange, and all three resonances overlap. Addition of KCN doesn't change the spectra suggesting that Hb^+ is completely saturated with CN^-.

DISCUSSION

Effect of Organic Phosphates on Hb^{TF} and $Hb^{TF}(CO)_4$

Huestis and Raftery (1972b) have found using ^{19}F NMR that deoxy Hb^{TF} in the absence of effectors has a large titration curve with a pKa $\approx$ 7.4. They interpret this titration to be due to the cooperative ionization of several groups including possibly his βHC3 and βH21

FIGURE 12

The plots of the ^{19}F chemical shifts of trifluoroacetonylated human aquamet and cyanomethemoglobin versus pH in the presence and in the absence of 2,3-diphosphoglycerate.

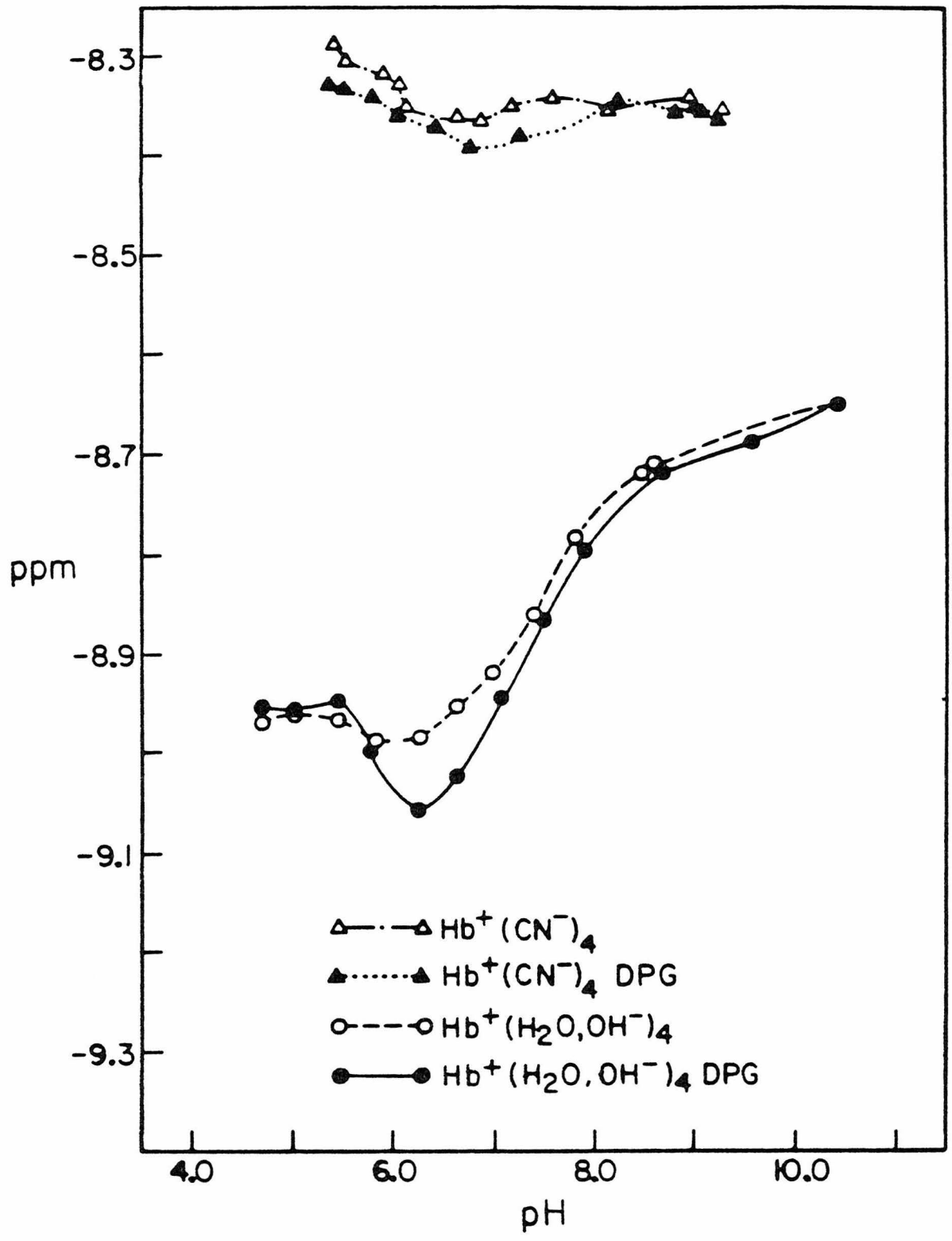
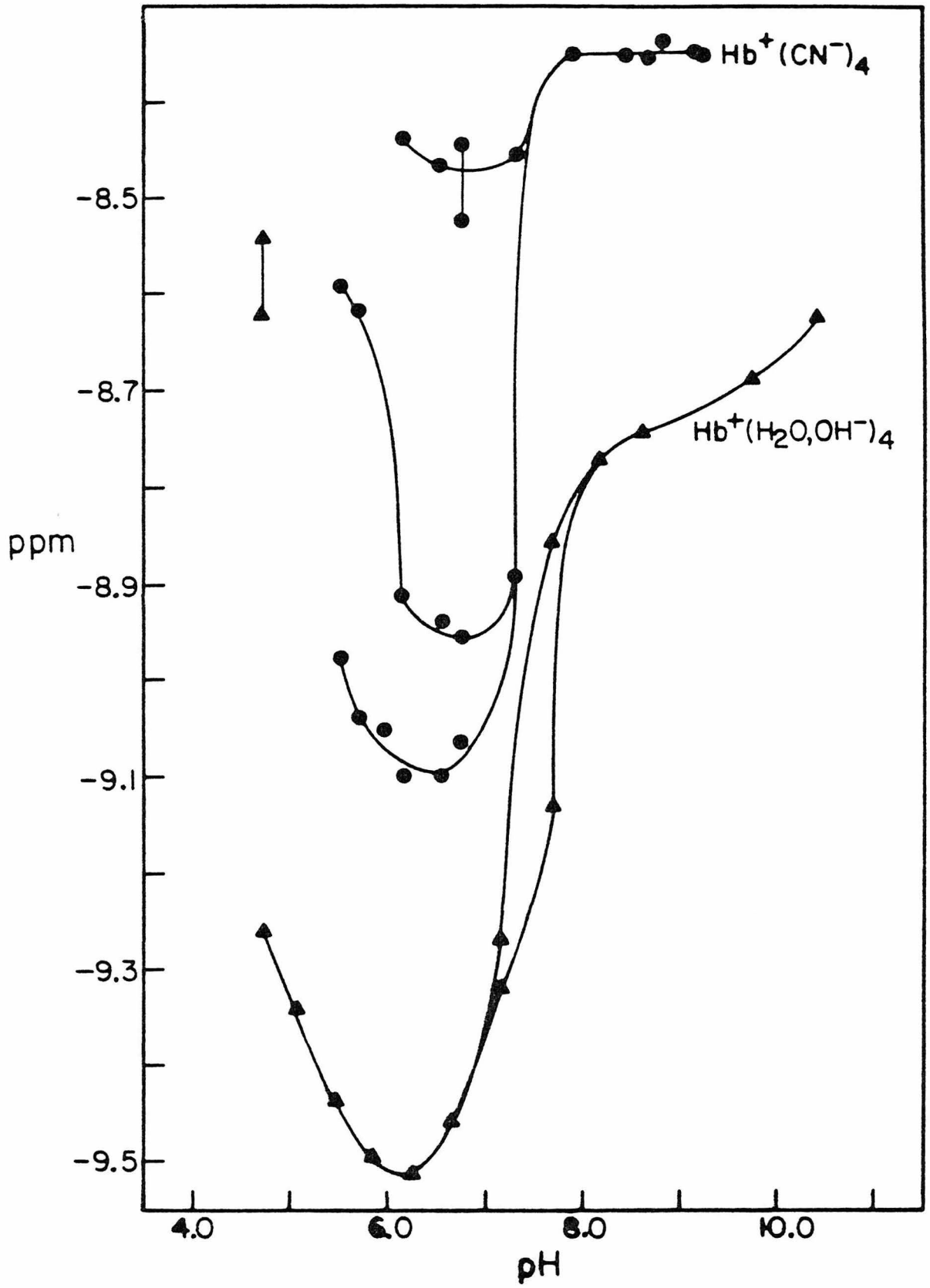

-8.3
-8.5
-8.7
-8.9
-9.1
-9.3
ppm
4.0
6.0
8.0
10.0
pH
Hb+ (CN−)4
Hb+(CN−)4 DPG
Hb+(H2O,OH−)4
Hb+(H2O,OH−)4 DPG

FIGURE 13

The plots of the ^{19}F chemical shifts of trifluoroacetonylated human aquamet- and cyanomethemoglobin versus pH in the presence of inositol hexaphosphate.


Hb+(CN−)4
Hb+(H2O,OH−)4
-8.5
-8.7
-8.9
-9.1
-9.3
-9.5
ppm
4.0
6.0
8.0
10.0
pH

with which organic phosphates interact. The results in Figure 1 agree well with the results of Huestis and Raftery (1972b). The effect of IHP on the titration of Hb^{TF} is slightly smaller than that of DPG even though the binding constant of IHP, binding to Hb^{TF}, is 1.6×10^7 while that of DPG is 2×10^5 (Edalji, et al., 1976; Benesch, et al., 1968). However, the small difference in chemical shifts is indicative of the similarity of the way the two organic phosphates bind to Hb^{TF} (Arnone, 1974; Arnone & Perutz, 1974). Both DPG and IHP bind to the β chains by electrostatic interactions and with hydrogen bonds.

Recently evidence has been mounting for the proposal that ligated Hb also binds organic effectors (Garby, et al., 1969; Costello, et al., 1976). Our results suggest that this theory is true below pH 8. Above pH 8 the protons which keep histidines βH21 and βNA2 positively charged in the binding pocket between the β chains, are removed. As a result no electrostatic interaction can develop between the negatively charged organic phosphate and the neutral residues in the binding pocket. Recently the results of Gupta, et al. (1979) suggest that organic phosphates bind between the β chains of the ligated Hb just as in deoxyhemoglobin.

Both in the presence of DPG and IHP the fluorine chemical shift of $Hb^{TF}(CO)_4$ moves slightly upfield. In addition below pH 7 the linewidth of $Hb^{TF}(CO)_4$ in the presence of IHP increases as shown in Figure 2. The increased linewidth suggests that spin-spin (T_2) relaxation has increased. An increase in T_2 is either due to change in structure or due to chemical exchange. It is unlikely that a change in the structure of Hb would increase spin-spin relaxation as drastically as has been detected. Such a drastic change is expected in gelled Hb (Allison, 1957; Pople, et al., 1959). A change of the spin state of Hb from low spin to high spin could also cause the linebroadening due to paramagnetic relaxation. However, $Hb(CO)_4$ has been shown to be diamagnetic (Cerdonio, 1977; Perutz, et al., 1974a). The most probably cause for the change in T_2 is due to chemical exchange.

Chemical exchange can appear in three different ways in the NMR spectrum. In the limit of slow exchange there are two resonances in the spectrum due to the slowness of the reaction relative to the NMR scale. An example of slow exchange is shown in Figures 5 and 7. In the case of intermediate exchange, as in Figures 2 and 8 there is a single resonance between the two states with broadened

linewidths. Since in the amount of time the NMR signal is collected the detected group moves one detects an average signal between the two extreme positions of the detected group. This signal is broad due to the change in the environment of the detected group while collecting the data. In the fast exchange limit one detects an average of the two environments with normal signal width since the NMR cannot detect the movement of the reported group. Fast exchange happens in the binding of DPG to Hb as detected by ^{31}P NMR (Huestis & Raftery, 1972c).

In the case of $Hb^{TF}(CO)_4$ in the presence of IHP the chemical exchange can be due to change in the quaternary or tertiary structure of Hb, change in the binding of IHP and change in the binding of CO. The $Hb^{TF}(CO)_4$ in the presence of IHP does not release CO since the visible spectra of $Hb(CO)_4$ in the presence and in the absence of IHP is the same. Further, the titration detected by ^{13}C NMR in rabbit $Hb(CO)_4$ in the presence of IHP in Figure 9 is identical to that that was found by Satterlee, et al. (1980) without IHP. Therefore, no significant structural change occurs in the heme pocket due to the binding of IHP. The ^{31}P NMR spectra of the IHP binding to deoxy and carboxy Hb^{TF} are different (Figures 10 and 11). However, both of these spectra are different from the ^{31}P NMR spectra

of the titration of free IHP (Pinnell & Walter, 1977, Zuiderweg, et al., 1979). In addition the ^{31}P chemical shifts of solutions with IHP concentration five times the concentration of Hb is virtually the same as free IHP chemical shifts at the same pH. Therefore, the IHP is not likely to be in a fast exchange between a bound and unbound state.

These results imply that the intermediate chemical exchange detected in the ^{19}F NMR is due to the change in the environment of the TF label due to quaternary or tertiary structural changes. The TF label detects a difference in the titration between of the T and R structures (Huestis & Raftery, 1972b). Thus deoxyhemoglobin has an upfield chemical shift relative to ligated Hb due to the protonation of residues around the TF label. So intermediate exchange is likely to be caused by the protonation and deprotonation of these residues. The change in protonation, of course, reflects quaternary structural change in Hb. The exchange can also be due to a change in the orientation of cys βF9 and the label. In this case the exchange detected in the ^{19}F NMR of TF labelled $Hb(CO)_4$ is due to an equilibrium between the two tertiary structures of the β chains (Heidner, et al., 1976).

Differences in the Structure of the Different Forms of Human Hemoglobin in the Absence of Effectors

The chemical shifts of $Hb^{TF}(O_2)_4$ and $Hb^{TF}(NO)_4$ are upfield from that of $Hb^{TF}(CO)_4$ as shown in Figure 3. Higher than pH 8.5 the structure of deoxyhemoglobin is similar to $Hb(O_2)_4$ and $Hb(NO)_4$. This result agrees completely with that of Huestis and Raftery (1972b). At pH 8.5 the hydrogen bond between his βHC3-asp βFG1 is already broken, due to the neutral histidine. The tyrosine βHC2 moves out of its pocket or the label detects it in its "R" state structure.

At low pH a small downfield shift occurs in both the deoxy and ligated structure below pH 6. This titration is different from that detected by Satterlee, et al. (1980), and it is also detected in Figure 9. This titration has a pKa which is less than 4.7 and was interpreted to be due to the histidine E7. Another possible group which may be responsible for the titration detected below pH 6 is the propionic acid group of the heme (Shaw & Hartzell, 1975; Fuchsman & Appleby, 1979). The fact that this change in chemical shifts is mostly independent of quaternary structure supports this conclusion.

Effect of DPG on Hemoglobin Structure

Addition of DPG results in the appearance of a second resonance in the spectrum of $Hb(NO)_4$ below pH 7 as shown in Figure 4. In the case of $Hb(NO)_4$ it has been suggested that DPG can switch the structure of the protein partially to the T state (Perutz, et al., 1976). In fact in Figure 5, it can be seen that the amount of "T" state protein reaches more than 50% of the total $Hb(NO)_4$.

Another interesting observation is that DPG changes very little the ^{19}F chemical shifts of $Hb^{TF}(O_2)_4$ and the downfield chemical shifts of $Hb^{TF}(NO)_4$. However, DPG shifts upfield the chemical shift of $Hb^{TF}(CO)_4$ as shown in Figure 1. These results suggest that DPG tightens up the structure of $Hb(CO)_4$ but does not change the structure of $Hb^{TF}(O_2)_4$. Huestis and Raftery (1973) have not detected the effect of DPG on $Hb^{TF}(CO)_4$ because the effect is small and they have only obtained ^{19}F spectra of $Hb^{TF}(CO)_4$ in the presence of DPG around pH 7.4.

Effect of IHP on Hemoglobin Structure

The addition of IHP to different Hbs results in drastic changes in structure (Ogawa & Shulman, 1972; Perutz, et al., 1976; 1978). In the case of $Hb(NO)_4$ IHP not only changes the structure of the protein but it also breaks

the α-iron histidine F8 bond (Maxwell & Caughey, 1976; Szabo & Perutz, 1976; Taketa, et al., 1977). In the ^{19}F NMR titration it is obvious that the change occurs from the R to the T state between pH 7.2 and 7.5 (Figure 6). The two forms are in slow exchange on the NMR timescale in this pH range as shown in Figure 7.

Adams and Schuster (1974) have presented difference UV and visible spectrum data which suggest that in the presence of IHP the $Hb(O_2)_4$ changes its structure to the T state. The ^{19}F NMR results in Figure 6 suggest that this switch of structure does not completely happen. Perutz, et al. (1976) have concluded that $Hb(O_2)_4$ changes its tertiary structure but its quaternary structure does not change. According to this interpretation the ^{19}F NMR detects an exchange between the T and R tertiary structures in the β chains but $Hb(O_2)_4$ stays in the R structure.

Another interpretation is that the ^{19}F NMR detects both tertiary and quaternary structural changes because the TF label on the β chains is sensitive to α chain ligation (Part I of this work, Huestis & Raftery, 1975). Thus the chemical exchange in $Hb(O_2)_4$ in the presence of IHP can be between the T and R quaternary structure. If such an equilibrium exists, then the UV and visible

spectrum of $Hb(O_2)_4$ in the presence of IHP should reflect the sum of the spectra due to the T structure and the R structure. As a result one would expect an intermediate spectrum between the T state and R state of Hb. Adams and Schuster (1974), in fact, have detected such a UV spectrum. Further, the absorption due to trp βC3 at 294 nm is only a shoulder on the side of the 288 nm absorption in the difference UV spectrum between the samples in the presence of IHP and the samples in the absence of organic phosphates. Thus, for example, a 50% decrease in intensity of the 294 nm band due to a 1:1 ratio between $Hb(O_2)_4$ in the T and R state would result in a shoulder that is too small to be detected.

Adams and Schuster (1974) have also detected changes in the UV spectra due to the addition of IHP to $Hb(CO)_4$ but these changes are much smaller. Perutz, et al. (1976; 1978) have also detected these differences and they have interpreted them as due to tertiary structural changes. However, from the previous paragraph one can also conclude that $Hb^{TF}(CO)_4$, in the presence of IHP, is also in a chemical exchange between the T and R quaternary state. Only in this case the R structure is predominant. Therefore the changes detected in the difference UV and visible spectra are very small (Adams

& Schuster, 1974).

The Structure of $Hb(H_2O, OH^-)_4$ and $Hb^+(CN^-)_4$

Perutz (1970) has based his allosteric mechanism on the assumption that the structure of oxyhemoglobin is quite similar to that of $Hb^+(H_2O)_4$. Huestis and Raftery (1972b) have disagreed with this idea because the ^{19}F NMR chemical shift of $Hb^{TF+}(H_2O)_4$ was intermediate between the deoxy and ligated state. Heidner, et al. (1976) have proposed that the difference between $Hb(CO)_4$ and $Hb^+(H_2O)_4$ is localized around the heme and around the cysteine βF9 area. They have proposed that cysteine can take two different positions. In the R state the cysteine βF9 replaces tyrosine βHC3 in the pocket formed between the F and H helices, while in the T state the tyrosine βHC2 is in the pocket and the cysteine βF9 is rotated and buried. Heidner, et al. (1976) suggested that in $Hb^+(H_2O)_4$ the cysteine oscillates between its two possible orientations. Johnson, et al. (1978) found from their study of spin labelled Hb that in fact not two but three different environments exist in all forms of Hb for the spin label which was attached to cys βF9. A further complication is that at pH 8.2 $Hb^+(H_2O)_4$ is in equilibrium with $Hb^+(OH^-)_4$. $Hb^+(H_2O)_4$ prefers the

high spin state while $Hb(OH^-)_4$ is mostly in the low spin state.

The titration curve which has been detected, reflects structural change and has a pKa of 7.5. The detected pKa is lower than that detected by Huestis and Raftery which is 7.8 (1972b). The results of Chevion, et al. (1979), from proton relaxation studies, also agree with the fact that the change in spin state happens at a different pH than the change in structure.

The iron of $Hb^+(CN^-)_4$ is in the low spin state and the protein structure of $Hb^+(CN^-)_4$ is in the R state (Perutz, et al., 1974b,c; 1978). The difference between the X-ray crystal structure of $Hb^+(CN^-)_4$ and $Hb^+(H_2O)_4$ has been studied and the results indicate small localized differences between $Hb^+(CN^-)_4$ and $Hb^+(H_2O)_4$ (Deatherage, et al., 1976). They propose however that the structure of $Hb^+(CN^-)_4$ is closer to that of $Hb(O_2)_4$ than $Hb^+(H_2O)_4$. This conclusion agrees well with the ^{19}F NMR titration results in Figure 12. The chemical shifts of $Tb^{TF+}(CN^-)_4$ are quite similar to that of $Hb(CO)_4$. The ^{19}F resonance of $Hb^{+TF}(CN^-)_4$ changes very little with pH.

The Effect of Organic Phosphates on $Hb^{+}(CN^{-})_4$ and $Hb^{+}(H_2O,OH^{-})_4$

The addition of DPG to TF labelled $Hb^{+}(H_2O)_4$ or $Hb^{+}(CN^{-})_4$ changes the chemical environment of the label very little (Figure 12). The addition of IHP, however, drastically changes the structure of $Hb^{+}(H_2O)_4$ and $Hb^{+}(CN^{-})_4$ as shown in Figure 13. The titration curve of $Hb^{+}(H_2O)_4$ in the presence of IHP has a larger slope. Therefore, more groups are involved in the cooperative ionization. Between pH 7 and 8.2 there are two resonances in the spectra probably due to the slow exchange between the two states of $Hb^{+}(H_2O)_4$. This observation is similar to the slow exchange of $Hb(NO)_4$ in the presence of IHP. The titration of $Hb^{+TF}(CN^{-})_4$ indicates the presence of three different structures below pH 8. The largest of the three is the one with the most upfield chemical shift. The resonances overlap and they have large linewidths. Since all of these resonances are downfield from the resonance due to $Hb^{+TF}(H_2O)_4$ in the presence of IHP, the $Hb^{+TF}(CN^{-})_4$ does not change its quaternary structure (Figure 13). From Figure 13 one can also conclude that even though $Hb^{+}(CN^{-})_4$ does not change its quaternary structure as compared to $Hb^{+}(H_2O)_4$, its tertiary and the TF label

structure definitely changes. The cys βF9 oscillates between three different orientations as has been suggested by Johnson, et al. (1979).

These results agree well with the conclusions of Perutz, et al. (1974a,b,c; 1976) that the addition of IHP can change the structure of methemoglobin. Hensley, et al. (1975a,b) and Edelstein and Gibson (1975) have argued that the spin state is independent of the quaternary structure. They have found that cooperativity of the oxidation of deoxyhemoglobin diminishes in the presence of IHP. They have argued that IHP instead of stabilizing the T state decreases the cooperativity by increasing the difference between the affinities of the α and the β chains for electrons. Further, they argued that since $Hb^{+}SS\ (H_2O)_4$ in the presence of IHP gels at much higher concentrations than deoxyhemoglobins SS in the presence of IHP the $Hb^{+}(H_2O)_4$ is not actually in the T state. However, since then $Hb(NO)_4$ has been shown to have a T structure in the presence of IHP, and $HbSS(NO)_4$ gels at about the same concentrations as $Hb^{+}(H_2O)SS$ (Briehl & Ewert, 1974; Briehl & Salhany, 1975); the argument made by Hensley and Edelstein and Gibson is not valid. Edelstein and Gibson (1975) pointed out that none of the methemoglobins has been proven to change its structure

due to the addition of IHP. This latest point has been disproven by Fermi and Perutz (1977) who have shown that $Hb^{+}(F)_4$ is in the T state in the presence of IHP.

CONCLUSIONS

Our results suggest that quaternary (tertiary) structure depends on the structure of the heme. This conclusion is due to the fact that the amount of structural change detected by ^{19}F NMR follows this sequence: $Hb(NO)_4 > Hb(O_2)_4 > Hb(CO)_4$ and $Hb^{+}(H_2O, OH^{-})_4 > Hb^{+}(CN^{-})_4$. One would expect this sequence to be the opposite of the spectrochemical sequence ($NO > CO > O_2$ and $CN^{-} > H_2O$, OH^{-} if the crystal field splitting of the ligand would contribute to the stability of the structure of Hb. This is true in the case of O_2 and CO and in the case of CN^{-} and H_2O. The ligand NO does not follow the sequence because in this case it has such a large trans effect that it severs the iron-histidine F8 bond in the α chains. As a result the histidine F8 moves into its stable configuration in the T structure; IHP binds with higher affinity to the T structure than to the R structure.

The position of the iron also follows this sequence. Thus in $Hb(NO)_4$ in the presence of IHP the iron is out of the plane at the heme toward the NO while in $Hb(O_2)_4$, $Hb(CO)_4$, in the presence or in the absence of IHP, and in $Hb(NO)_4$ in the absence of IHP it is in the plane of the heme (Perutz, et al., 1976; Scheidt & Frisee, 1974; Heidner, et al., 1976). In $Hb^+(CN^-)_4$ in the presence or absence of organic effectors the iron is in the plane of the heme, while the iron is out of the plane toward histidine F8 in $Hb^+(H_2O)_4$ in the presence or absence of IHP (Deatherage, et al., 1976; Perutz, et al., 1974c; Messana, et al., 1978).

Warshel (1977) and Gelin and Karplus (1977) argued that the iron is equally stable in the plane of the heme or outside of the plane of the heme. The position of the iron relative to the plane of the heme depends on the ligand and the quaternary structure of the protein. Warshel (1977) has argued that the severing of the bond between the heme and the protein in $Hb(NO)_4$ in the presence of IHP is a special case. Our results suggest that ligands which keep the iron in the plane of the heme (in the low spin state) when they bind to Hb do not allow the switch in the structure of Hb from the R to the T state completely. Therefore the spin state of the

iron contributes to the selection of the stable structure of the protein. This is not true in the case of $Hb(NO)_4$ where the combined effect of the trans effect of NO and the effect of IHP break the heme protein bond. This conclusion is also not true in the case of $Hb^+(H_2O)_4$ in the absence of IHP. $Hb^+(H_2O)_4$, in the absence of effectors, is in the R structure even though the iron in $Hb^+(H_2O)_4$ is mostly in the high spin state. The structure of $Hb^+(H_2O)_4$ is not a true "R" structure, however, as has been shown by the studies of the ^{19}F NMR and of $Hb^{TF+}(H_2O)_4$ and by the studies of the X-ray crystal structure of $Hb^+(H_2O)_4$ (Huestis & Raftery, 1972b; Deatherage, et al., 1976).

Our conclusions are consistent with the theory of Perutz (1970) in that the heme structure does contribute to the structure of the protein. However the spin state does not determine the quaternary structure of the protein. In contrast these conclusions disagree with the suggestion of Rakeshit and Spiro (1974) that the spin state of the heme is a function of the protein structure. It is more likely that the spin state of the heme is a function of the protein and the ligand as has been proposed by Warshel (1977).

The ^{19}F results have been criticized on the grounds that the changes occurring are due to localized tertiary changes (Heidner, et al., 1976). Heidner, et al. (1976), however, have ignored the importance of the hydrogen bond between lys αC5 and his βHC3 in the T state. In Part I it has been shown that initial β chain ligation is not detected by the TF label. This result is inconsistent with the fact that the TF label detects only tertiary structural changes. Huestis and Raftery (1973; 1975) have also shown that the TF label can detect the ligation of the α chains.

The conclusion that IHP can switch the R state ligated Hb to the T state is also consistent with the difference UV and visible spectra of $Hb(O_2)_4$ and $Hb(CO)_4$ in the presence and in the absence of IHP. Due to the simultaneous presence of R and T state $Hb(O_2)_4$ the difference spectra show changes due to IHP but not large enough to indicate 100% T structure (Perutz, et al., 1976; 1978; Adams & Schuster, 1974).

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Part IV

CHANGES IN THE INTRACELLULAR pH OF AA AND SS ERYTHROCYTES DUE TO OXYGEN BINDING

INTRODUCTION

It has been known for a long time that in the blood of individuals suffering from sickle cell anemia the erythrocytes are distorted in the deoxy state (Herrick, 1910). Pauling, et al. (1949) have proven that this abnormality is due to a molecular disease. This was done by showing that normal and sickle cell Hbs (Hb and HbSS) have different electrophoretic mobilities. Later, Ingram (1957) identified the single mutation which causes this abnormality. The negatively charged glutamate β6,A3 residue in normal Hb is replaced by a hydrophobic valine residue in sickle cell Hb.

The gel formation of deoxyhemoglobin has been studied in vitro by optical calorimetric methods (Ross, et al., 1975; Hofrichter, et al., 1973) and by ultracentrifugation (Bartles, et al., 1970; Briehl & Ewert, 1973; Williams, 1975). The ultracentrifugation studies have shown that HbSS is present in two forms: the solution as monomer Hb and in the sediment as gelled Hb. The gel contains six long Hb polymer rods wound around each other so that the cross section of the rods always contains six Hb molecules in a hexagonal form (Finch, et al., 1973; Edelstein, et al., 1973).

Wishner, et al. (1975) have been able to crystallize sickle cell deoxyhemoglobin solution containing polyethylene glycol. They have studied the X-ray diffraction of these crystals and they found that each crystal contains four Hb molecules in a double stranded form. This configuration agrees well with the model of Finch, et al. (1973) and Edelstein, et al. (1973) for the gel as the broken up pieces of the polymers of HbSS. Further, specific residues on the surface of one Hb have been found to interact with complementary parts of the other Hb molecules. These include valine β6,A3 aspartate β73,E17 glutamates β121,GH4, β22,B4, β21,B3, and histidines β116,G18 and α20B1, plus other hydrophobic residues. Each Hb molecule interacts with four other Hb molecules. The fact that the α chains are also involved in the polymerization have been shown by the fact that βSS_4, which is in the T state, does not polymerize (Benesch, et al., 1973).

The large effect of the mutation of a single residue at β6,A3 is due to a change in the secondary structure. In HbAA the first three residues of the β chains form a random coil and the following three residues, including glutamate β6,A3, are the initial residues of the A α helix (Chou, 1974). In HbSS the first six residues are more stable in a β sheet structure due to the replacement

of the glutamate at β6,A3 in HbAA by a valine residue. The β sheet structure is stable because valine strongly prefers a β sheet conformation while glutamate destabilizes a β sheet conformation (Chou & Fasman, 1974a,b).

As a result of the sickling of the erythrocytes the affinity of HbSS toward oxygen decreases (Gill, et al., 1978; Benesch, et al., 1978). Since less oxygen is delivered to the tissue the erythrocytes try to readjust, as they do at high altitudes in individuals with AA blood, by increasing the DPG concentration inside the erythrocytes (Briehl & Ewert, 1973). Of course, the increase in the concentration of DPG results, inside the erythrocytes, in more sickling. However, the increased concentration of DPG does not fully account for the increase in the partial pressure of oxygen at 50% oxygen saturation in cells (Seakins, et al., 1973). A decrease of pH in the erythrocytes could account for the disparity between the affinity of normal and SS Hb.

The results of experiments which try to evaluate the effect of intracellular pH on the sickling of SS erythrocytes are confusing. Intracellular pH can be measured with a microelectrode after freeze-thaw lysis, or by addition of weak organic acids, which after passing through the membrane equilibrate between their protonated

and anionic forms according to intracellular pH (Waddell & Bates, 1969). The former technique may detect incorrect pH values because due to the lysis buried molecules become accessible to water and these molecules may bind or release, H^+ ions (Kong & Alleyne, 1969; Barreras & Diggs, 1964; Ringelhann & Konotey-Ahulu, 1971; Warth, et al., 1977). The latter method is inadequate due to the large scatter in the data probably caused by the interaction of the organic acid with other molecules and ions inside the cell. As a result the behavior of acid may not follow the internal pH, and the acid may also perturb the cell (Irvine, et al., 1960).

Ueda, et al. (1979) studied the interaction of pH, pO_2 and gelation of HbSS in the erythrocytes. They measured P_{50} as a function of intracellular pH, using the freeze-thaw method, and they found that HbSS in the erythrocytes has a lower affinity (higher P_{50}) with lowering pH than HbAA does.

Two other approaches have been tried to measure changes in the intraerythrocyte pH. The first has been an attempt to calibrate the measured saturation curves with changing pH (Winslow, 1977). This approach, however, could only suggest that the pH gradient through the membrane is larger in HbSS blood than in AA blood. Further,

this change in pH can account for the difference in the affinity between the two cells detected by Seakins, et al. (1973). Dash and Brewer (1978) have used another method to measure intracellular pH. They have measured the amount of lactate in the cell and calculated the contribution of the differences in concentrations of lactate to pH. This method suggests that the intracellular pH is 0.1 unit lower in deoxy SS blood than in deoxy AA blood. However, this method cannot detect absolute pH values. Further, it cannot detect differences, if any, in the intracellular pH of oxy AA blood and oxy SS blood.

Recently, a non-destructive method has been developed using ^{31}P NMR for the measurement of intracellular pH (Moon & Richards, 1973). This method has now been applied to oxy SS erythrocytes and it has been found that the intracellular pH of SS erythrocytes is 0.15 pH unit lower than AA erythrocytes even though the external pH was the same (Lam, et al., 1979).

In order to follow the effect of ligation on intracellular pH the ^{31}P NMR spectra of SS and AA erythrocytes have been obtained at different oxygen saturations.

EXPERIMENTAL SECTION

Blood was collected from donors with AA and SS blood by venous puncture into heparinized vacutainers or syringes. The SS blood was obtained from donors at the USC Sickle Cell Center. The blood, kept on ice, was used as soon as possible to avoid metabolic degradation of DPG. Each blood sample was injected, through a small access hole, into a 12 mm NMR tube; fused with a 2 mm cuvette containing a small concentric capillary of D_2O for field frequency lock. Then the blood sample in the NMR tube was equilibrated at 4°C, through the access hole, with a gas mixture containing 95% oxygen and 5% CO_2 to obtain oxygenated Hb, or with a gas mixture containing 95% nitrogen and 5% CO_2 to obtain partially or totally deoxygenated Hb inside the erythrocytes. The NMR tube was sealed by pulling a gas tight rubber sleeve on the access hole. After equilibrating the sample with the gas mixture, the NMR spectrum of the sample was taken. Then the visible spectrum of the blood sample was recorded between 850 and 700 nm. Finally, a sample was removed from the NMR tube through the access hole for measurement of the plasma pH. Deoxygenation or oxygenation of the sample was assumed to be complete when

the visible spectrum of the blood did not change further after repeated equilibration of the sample with the appropriate gas mixture.

The ^{31}P NMR spectra were recorded at 24°C on a Varian XL100-15 spectrometer operated at 40.5 MHz. About 2000 transients were collected with 0.3 sec acquisition time.

The fraction of saturation (Y) was calculated from the absorbance (A) of fully oxy and deoxy samples at 750 nm according to equation 1.

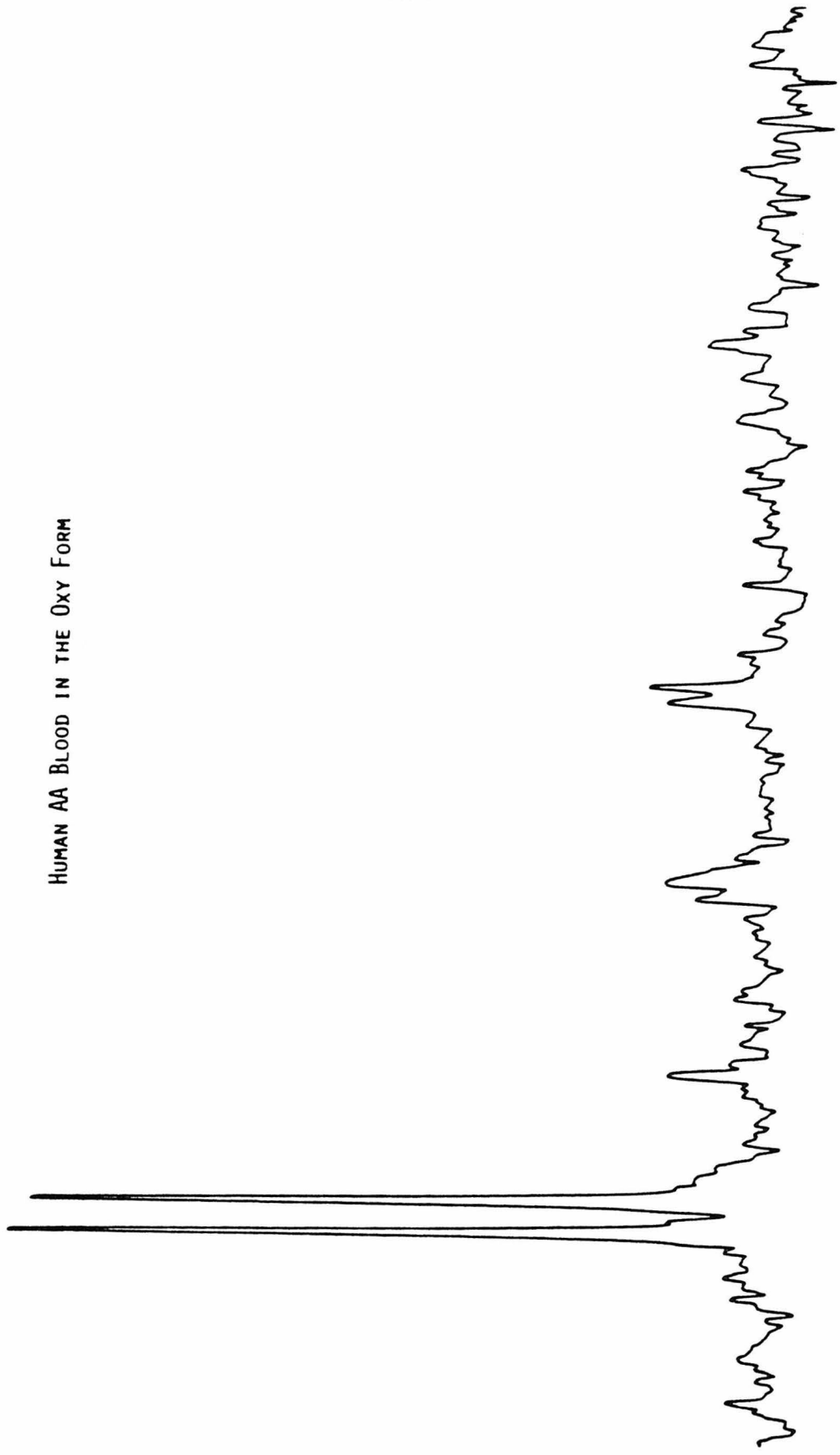
$$Y = \frac{A_{\text{unknown sample}} - A_{\text{deoxy sample}}}{A_{\text{oxy sample}} - A_{\text{deoxy sample}}} \qquad (1)$$

RESULTS

Figure 1 shows a typical ^{31}P NMR spectrum of whole AA blood in the fully oxygenated state. The two large resonances are due to the phosphorous in the 3 and 2 positions of DPG. Upfield from the DPG ^{31}P resonances there are a couple of smaller resonances which are due to the ^{31}P resonances of the plasma phospholipids, and the γ and α phosphorous of ATP. Inorganic phosphates, intracellular or external, appear upfield of the 2-P resonance of DPG, if there are any present.

FIGURE 1

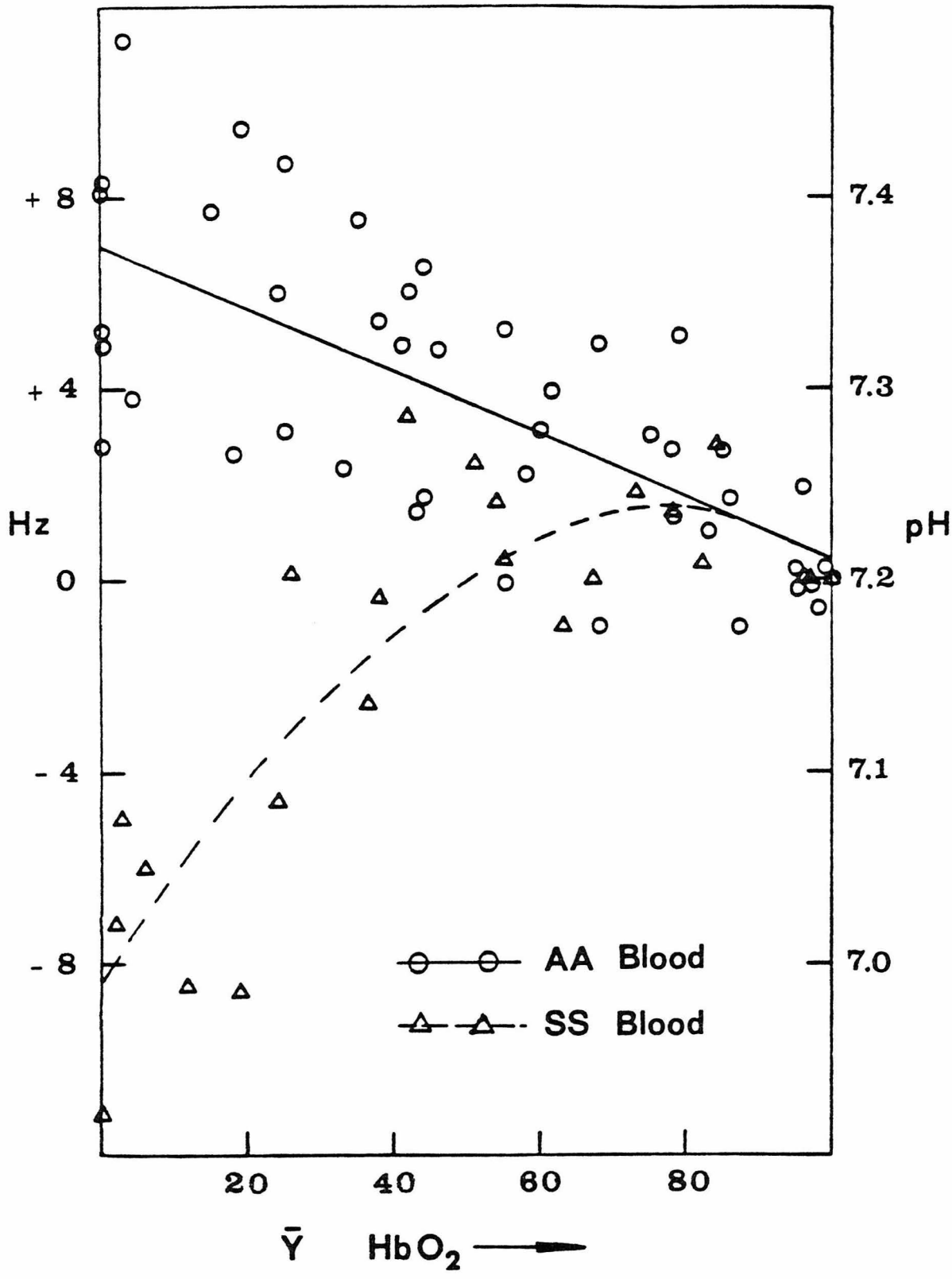
A ^{31}P NMR spectrum of totally oxygenated human whole AA blood.



HUMAN AA BLOOD IN THE OXY FORM

FIGURE 2

The plot of ^{31}P chemical shifts and pH of whole human AA and SS blood versus the percent of oxygen saturation of the hemoglobin inside the cells.


+ 8
+ 4
0
- 4
- 8
Hz
7.4
7.3
7.2
7.1
7.0
pH
AA Blood
SS Blood
20
40
60
80
$\bar{Y}$ HbO_2

The chemical shift versus pH correlation has been calculated by linear least square analysis from the data of Moon and Richards (1973). Between pH 6.4 and 7.6 the correlation coefficient is 0.985 for the data of Moon and Richards (1973).

Just as Lam, et al. (1979), we have also detected some difference between the intracellular pH of AA and SS erythrocytes in whole blood. In AA blood the erythrocytes have an intracellular pH of 7.26 $\pm$ 0.08 while in SS blood the erythrocytes have a pH of 7.13 $\pm$ 0.1. Our standard deviations are the same in AA blood and slightly larger in SS blood than those obtained by Lam, et al. (1979). However, in this study the two intracellular pH values for AA and SS blood, are not significantly different statistically according to the student's "t" test.

Figure 2 shows the change in the ^{31}P chemical shifts of the 2-P of 2,3-DPG and the change in the intracellular pH as a function of the percent of oxygen saturation in both AA and SS erythrocytes, relative to the 2-P chemical shift of 2,3-DPG in fully oxygenated blood (AA or SS). The ^{31}P chemical shifts of the 3P resonance of 2,3-DPG have shown very small changes with the percent of oxygen saturation, and the changes seemed to be random. The percent of saturation was calculated from the collected optical absorbance data of the samples at 750 nm. The optical

density had to be collected at 750 nm because of the high absorbance of the sample below 700 nm. Due to the variation between the blood samples collected from different individuals one could not obtain extinction coefficients. Therefore, for each sample the percent of saturation has been calculated from the spectra of fully oxy and deoxy samples according to equation 1. The correlation between the ^{31}P chemical shifts of 2-P of 2,3-DPG in AA blood and the percent of oxygen saturations was analyzed by linear least square fitting. The correlation line was Y = 0.0657 x -6.97 with a correlation coefficient of 0.712.

The plot of the ^{31}P chemical shifts or pH versus the percent of saturation of AA blood indicates that upon deoxygenation the chemical shifts of 2-P of 2,3-DPG and the intracellular pH increases by about 7 Hz or 0.18 pH units. As the sickle cell blood samples are deoxygenated the ^{31}P chemical shift of 2-P of 2,3-DPG and the intracellular pH decreases by 8 Hz or 0.2 pH units. Therefore, there is a difference of about 15 Hz on the chemical shift of 2-P of 2,3-DPG, and 0.4 pH units between the deoxygenated AA and SS erythrocytes in whole blood.

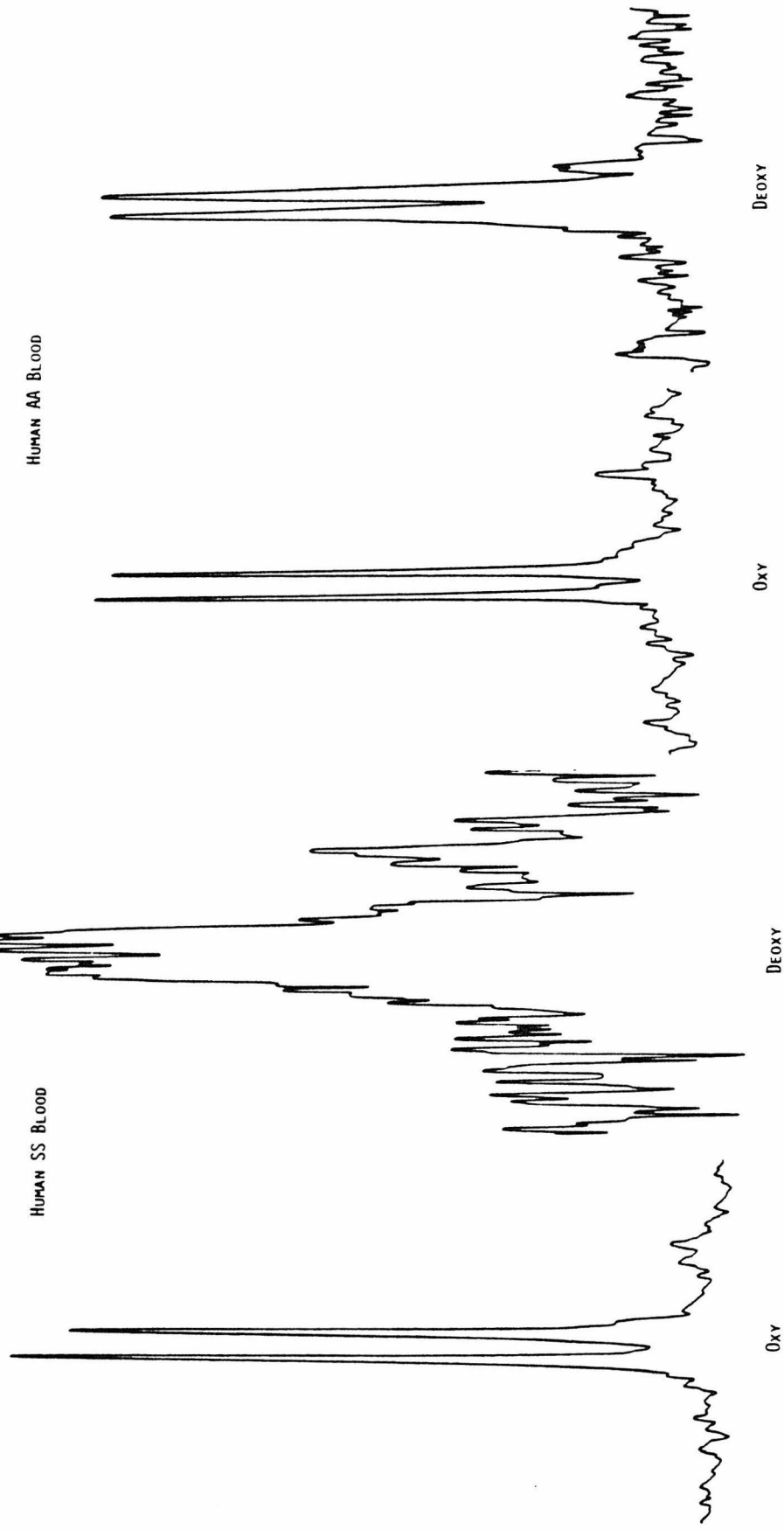
Due to the deoxygenation of AA blood the DPG resonances have a small, ∿ 8 Hz increase in linewidths. However, the linewidths of DPG increase up to 90 Hz in SS blood below 30% oxygen saturation, as shown in Figure 3.

DISCUSSION

The effects of deoxygenation on the ^{31}P chemical shift of 2-P or 2,3-DPG and on intracellular pH of AA and SS erythrocytes are shown in Figure 2. The detection of intracellular pH has been possible due to the sensitivity of the ^{31}P NMR to pH changes (Gorenstein, et al., 1976; Evans and Kaplan, 1977). Recently, Costello, et al. (1976) and Marshall, et al. (1977) have suggested that the ^{31}P chemical shifts of 2,3-DPG in the presence of Hb can also follow changes due to Hb ligation. However, they found no difference between the ^{31}P chemical shifts of 2-P of 2,3-DPG deoxy and ligated Hb at pH 7.38. However, they found that below pH 7.38 the ^{31}P signal moves downfield upon deoxygenation (Costello, et al., 1976). HbSS monomers and HbAA monomers should bind DPG the same way, therefore the effect of binding on the chemical shift should cancel out as long as there is no significant amount of gel present in the SS cell.

FIGURE 3

^{31}P NMR spectra of human AA and SS blood in the oxy and deoxy state.


Human AA Blood
Deoxy
Oxy
Human SS Blood
Deoxy
Oxy

However, when HbSS gels the extra DPG which is free should be detected preferentially as compared to the DPG which is bound to HbSS, because of the increased linewidth of the bound signal relative to the free signal due to the slow tumbling of the HbSS polymer. The signal due to DPG which is not bound also becomes broader (but not as much as the bound DPG signal) due to the increased viscosity of the solution inside the SS erythrocytes (Figure 3). So the change in the intracellular pH of SS erythrocytes upon deoxygenation may be more accurate as opposed to the change in intracellular pH of AA erythrocytes upon deoxygenation which may be smaller than detected due to the effect of the binding of DPG by deoxyhemoglobin on the ^{31}P chemical shifts. Therefore, the pH values calculated from the results of Moon and Richards (1973) and indicated in Figure 2, should be used only as a guide.

Lam, et al (1978) have found a difference of 0.15 pH units between the intraerythrocytes pH of AA and SS blood in the oxy state. This difference in this study was found to be 0.13 pH units which was not found statistically significant using the student's "t" test. Thus in this study at 100% oxygen saturation the intracellular pH of AA and SS blood does not differ significantly.

Upon deoxygenation the ^{31}P resonance of the 2-P of 2,3-DPG in AA blood moves downfield. This shift is an indication of the binding of DPG or increase in the pH by $\leq$ 0.2 pH units. Since the plasma pH is 7.5 in AA blood, the gradient between the intracellular and external pH in the deoxy state is smaller than in the oxy state. Upon deoxygenation the intracellular pH should increase, as one would expect from the Bohr effect, due to the uptake of protons by Hb.

The gradient between external and internal pH in oxygenated SS blood is the same as in AA blood. However, as SS blood is deoxygenated, the ^{31}P chemical shift and therefore the intracellular pH decreases by $\geq$ 0.2 pH units. Therefore, if one ignores the effect of the binding of DPG to deoxy HbAA and to deoxy HbSS on the ^{31}P chemical shift the decrease in intracellular pH in SS blood is approximately the same as the increase in the intracellular pH due to the Bohr effect in AA blood. If we assume that the Bohr effect in HbSS is normal then there are twice as many protons released due to the gellation of HbSS as there are protons taken up due to the normal Bohr effect. A decrease in the intraerythrocyte pH can be due to increased proton transportation from outside through the membrane. This, however, is unlikely since Dash and

Brewer (1978) found by measuring the amount of lactate in the cell, that the change due to deoxygenation in pH inside the erythrocytes is similar to that found in cell lysates. Another possible explanation for the decrease in the intracellular pH of SS erythrocytes upon deoxygenation is that the protons are released from the surface of Hb molecules which form the gel due to the interaction between neighboring Hb molecules. There are four histidines in each Hb molecule which are involved in the interaction between Hb molecules (Wishner, et al. 1976). These histidines are probably protonated around pH 7.2. Upon gelation, due to the hydrophobic environment found by the Hb-Hb contact, the histidines release their protons. A neutral histidine is more stable in a hydrophobic environment than a positively charged one. Other residues like glutamate and aspartate do not contribute to this proton release since they are not protonated at this pH.

Winslow (1977) has suggested, from his studies of the oxygen saturation curves of SS blood, that there is a larger pH gradient across the membranes of SS erythrocytes than across the membranes of AA erythrocytes. The results in Figure 2 support his suggestion, but only

in the deoxygenated state. In ligated blood the pH gradient between external and internal pH is the same in both SS and AA blood. Upon deoxygenation, however, the pH gradient across the membrane slightly decreases in AA blood while it increases by 0.2 pH units in SS blood. A large gradient between the external and internal pH in the SS erythrocytes would have a large effect on the affinity of HbSS as suggested by Winslow (1977). Since the internal pH of SS erythrocytes decreases the affinity of HbSS also decreases.

Ueda, et al. (1979) have tried to detect differences in the partial pressure of oxygen at 50% saturation (P_{50}) at different intracellular pH values (Bohr effect) in SS and AA erythrocytes. They have found that P_{50} increases more due to the lowering of pH, in SS blood than in AA blood. This difference in P_{50}, between AA and SS blood was small. (As the P_{50} increases the affinity of Hb for oxygen decreases). Since the largest difference in the affinity between AA and SS blood, is due to the difference in intracellular pH below 30% oxygen saturation, therefore Ueda, et al. (1979) could only detect small differences in the affinity between AA and SS blood at 50% oxygen saturation.

The results of Dash and Brewer (1978) are similar to those shown in Figure 2. However, they have not been able to detect the full change in intracellular pH due to the fact that they have not been able to deoxygenate the SS blood further than 20% saturation. Further, the differences in the lactate concentrations inside the cells may not reflect only changes in pH. Their assumption, the pH changes linearly with percent of oxygen saturation, is also incorrect. In Figure 2, it is shown that the largest change in pH occurs in SS blood below 30% saturation.

These results show that gel formation of HbSS results in the lowering of intracellular pH. A decrease in the intracellular pH lowers the affinity of HbSS which results in an increase in the concentration of deoxy HbSS inside the cells. The increase in the concentration of HbSS results in more gel formation.

There is a controversy in the literature concerning whether or not there is any change in the intracellular pH of normal and sickle red blood cell (Rhodes, et al., 1974; Ringelhann & Konotey-Ahula, 1971; Greenberg & Kass, 1958; Barreras & Diggs, 1964). Studies of treatment of sickle cell patients in crisis with bicarbonate and lactate show that these substances have little effect in relieving pain However, in vitro studies have shown that lowering the

pH of HbSS solution increases the amount of gel and the rate of gel formation (Briehl, 1978; Shibata, et al., 1977). NH_4Cl treatment has been shown to initiate sickle cell crisis in patients, probably due to lowering the pH of the blood (Greenberg & Kass, 1958; Barreras & Diggs, 1964).

Two explanations may be possible for the discrepancy between the effect of pH in vivo and in vitro. First, it is possible that a small increase in external pH may not be enough to affect the cells which are sickled and occlude a vein. Further, since the blood flow is stopped, due to the occlusion, the blood with higher pH cannot get to the sickled cells. Second, the oxygen saturation of gelled cells returns to 100% only after a long time (Winslow, 1977). Thus, change in pH in the blood may affect the gelled cells very slowly. It might be more fruitful to obtain data on the number of occurrences of crises in a given time in a given patient, with blood pH. Further, it would be a useful approach to try to prevent sickle cell crises by administration of bicarbonate or lactate on a regular schedule to keep the pH of the blood at the high end of the normal pH scale.

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PROPOSITIONS

PROPOSITION I

In order to find out what is the mechanism of co-operativity in ligand binding of hemoglobin one has to learn all the structural changes which occur in hemoglobin (Hb) upon ligation. The structure of deoxy and fully ligated Hb has been solved by crystal structure of Hb (Perutz, 1976, Introduction of this work). However, one cannot study the intermediate states in ligation by crystallographic methods due to the fact that intermediate structures are present only in low concentrations during the ligation.

The existence of intermediates has been established indirectly by ESR of spin labelled Hb studies (Ogawa, et al., 1968), and directly by ^{19}F NMR of TF labelled Hb (Huestis & Raftery, 1973; 1975, Part I of this work). Recently, from the highly accurately determined oxygen binding curves of Hb it has been possible to calculate all four Adair constants by least square analysis (Mills, et al., 1976; Imai & Adair, 1977). From the binding constants it was possible to calculate the amount of singly, doubly, and triply ligated intermediates (Mill, et al., 1976; Imai & Adair, 1977).

Since the intermediates are present in low concentrations it is difficult to obtain data about their

quaternay and tertiary structures. In order to obtain data about the structures of the intermediates Ogawa and Shulman (1971, 1972) have prepared Hb in which either the α or the β subunits are in the cyanomet-state. They called these structures valency hybrids. In the deoxy state these structures behave as structures which have two chains ligated. The structures of the artificial intermediates were studied by ^{1}H NNR, and the initial ligation kinetics of these intermediates were also studied by visible spectroscopy (Ogawa & Shulman, 1971, 1972; Cassoly & Gibson, 1971, 1972). It has been found that the artificial intermediates can exist in two states deoxy (T) and ligated (R) state. Banerjee, et al. (1973) and later Nagai (1976) have shown differences in their Hill plots between $\alpha_2^{+L}\beta_2$ and $\beta_2^{+L}\alpha_2$, and in different valency hybrids with L being CN^-, N_3^-, H_2O, F^-. A Hill coefficient of greater than 1 is expected for the artificial hybrid which has a quaternary structural change. Further a decrease in the affinity of the artificial hybrids is an indication of the presence of T state Hb. (This is true for L = F^-, H_2O).

Artificial intermediates can be useful, in distinguishing between the Monod Wyman and Changeux (1965) model (MWC) and the Koshland Nemethy and Filmer (1966) model (KNF). The former model proposes that intermediates should be present in either the T or R state while the

latter model proposes that intermediates can have quaternary structures which are different from the T and R states.

In order to determine which allosteric model can explain the behavior of artificial intermediates two series of experiments are proposed.

A series of artificial intermediates, such as $\beta_2^{+CN^-}\alpha_2$, $\beta_2^{+N_3^-}\alpha_2$, $\beta_2^{+H_2O}\alpha_2$, $\beta_2^{+F^-}\alpha_2$, $\alpha_2^{+CN^-}\beta_2$, $\alpha_2^{+N_3^-}\beta_2$, $\alpha_2^{+H_2O}\beta_2$, and $\alpha_2^{+F^-}\beta_2$, should be prepared as done be Banerjee, et al (1973). The Bohr effect and Hill plots of these intermediates in the presence and absence of organic phosphates should be determined. Following these experiments the intermediates can be labelled by the trifluoroacetonyl label and from their NMR spectra their tertiary and quaternary structures can be determined as has been done for Hb in Part 3 of this work. Further the quaternary structures of these Hbs, as defined by Perutz (1970), can be determined from their UV spectra (Perutz, et al., 1974b, 1978).

A second set of artificial intermediates like $\alpha^{+L}\alpha\beta\beta$, $\beta^{+L}\beta\alpha\alpha$, $\alpha^{+L}\alpha^{+L}\beta^{+L}\beta$ and $\beta^{+L}\beta^{+L}\alpha^{+L}\alpha$, should be prepared. Intermediates like $\alpha^{+CN^-}\alpha\beta\beta$ or $\alpha^{+CN^-}\alpha^{+CN^-}\beta^{+CN^-}\beta$ can possibly be prepared from a 1:1 mixture of $Hb(O_2)_4$ and $\alpha^{+CN^-}\alpha^{+CN^-}\beta O_2\beta O_2$, or $Hb^+(CN^-)_4$ and $\alpha^{+CN^-}\alpha^{+CN^-}\beta O_2\beta O_2$ respectively. The intermediates form according to the following equations:

$$_{\alpha}O_{2\alpha}O_{2\beta}O_{2\beta}O_{2} \overset{K_A}{\rightleftharpoons} 2\ _{\alpha}O_{2\beta}O_{2}$$

$$_{\alpha}{}^{+CN^-}\ _{\alpha}{}^{+CN^-}\ _{\beta}O_{2\beta}O_{2} \overset{K_B}{\rightleftharpoons} {}_{\alpha}{}^{+CN^-}\ _{\beta}O_{2}$$

$$2\alpha O_{2\beta}O_{2} + 2\alpha{}^{+CN^-}{}_{\beta}O_{2} \overset{1/K_{AB}}{\rightleftharpoons} 2\ \alpha{}^{+CN^-}{}_{\alpha}O_{2\beta}O_{2\beta}O_{2}$$

then

$$Y\left[{}_{\alpha}{}^{+CN^-}\ _{\alpha}O_{2\beta}O_{2\beta}O_{2}\right] = \frac{1}{1 + 2\dfrac{K_{AB}}{\sqrt{K_A K_B}}}$$

This equation implies that if $K_{AB} = \sqrt{K_A K_B}$ than 1/3 of the Hb tetramers would be in the mixed tetramer state. In fact the dissociations constants of different ligated Hbs are similar: at 20° the dissociation constants of $Hb(O_2)_4$ and $Hb^+(CN)_4$ are 1.0×10^{-6} and 2.4×10^{-5} respectively (Ip & Ackers, 1977; Hensley, et al., 1975).

An evidence for the feasibility of this experiment is the study of Moffat (1974). He found that hybridization of HbS and $Hb^+(CN^-)_4$ decreases the minimum gelling concentrations (MGC) of a solution containing these two Hbs. If the mixing of the two Hb solutions is carried out in oxy state, the MGC is lower than when the solutions are

mixed in the deoxy state.

If trifluoroacetonylated Hbs are mixed then the ^{19}F NMR of the mixture can detect the differences between the structures, if any. Huestis and Raftery (1972,1973) did show that the ^{19}F chemical shifts of $(\alpha^{+CN^-}{}_\alpha{}^{+CN^-}{}_{\beta\beta})^{TF}$, Hb^{TF} and $Hb^{TF}(CO)_4$ are different. However, Husetis and Raftery (1973) did not detect the intermediate $(\alpha^{CO}{}_{\alpha\beta\beta})^{TF}$ in the ligation of TF labelled human Hb even though the results of Mills, et al (1976) imply that in the ligation of Hb the one liganded intermediate should be present in the largest concentration during the early states of ligation. The $(\alpha^{CO}{}_\alpha{}^{CO}{}_\beta{}^{CO}{}_\beta)^{TF}$ intermediate was detected in the ^{19}F NMR of partially ligated TF labelled Hb and therefore the detection of the hybrid, $\alpha^{+CN^-}{}_\alpha{}^{+CN^-}{}_\beta{}^{+CN^-}{}_\beta$ maybe used as a test for the hybridization.

The quaternary structure of the hybrid intermediates can also be tested by obtaining the UV spectra of the solution of the mixture and subtraction of the known UV spectra of Hb and $\alpha^{+CN^-}{}_\alpha{}^{+CN^-}{}_{\beta\beta}$ or $\alpha^{+CN^-}{}_\alpha{}^{+CN^-}{}_{\beta\beta}$ and $Hb^+(CN^-)_4$, respectively.

The structural studies of these artificial hybrids would help in distinguishing between the two allosteric models. If there are only two structures which are detected in these hybrids then the MWC model should dominate while if there are many structures then that would support

the KNF model. However, if one detects a variation in the structures of liganded intermediates (as in the structures of $\alpha^{+CN^-}\alpha^{+CN^-}\beta\beta$, $\alpha^{+F}\alpha^{+F}\beta\beta$, $\alpha^{+H_2O}\alpha^{+H_2O}\beta\beta$ etc.) then that implies that the ^{19}F NMR is more sensitive toward localized tertiary structural changes than to quaternary structural changes.

APPENDIX

$$T_A \overset{K_A}{\rightleftharpoons} 2D_A \qquad K_A = \frac{D_A^2}{T_A} : \quad D_A^2 = K_A T_A$$

$$T_B \overset{K_B}{\rightleftharpoons} 2D_B \qquad K_B = \frac{D_B^2}{T_B} : \quad D_B^2 = K_B T_B$$

$$2T_{AB} \overset{K_{AB}}{\rightleftharpoons} 2D_A + 2D_B \qquad K_{AB}^2 = \frac{(D_A)^2 (D_B)^2}{(T_{AB})^2}$$

where T stands for tetramer while D stands for dimers

$$K_{AB}^2 = \frac{(K_A T_A)(K_B T_B)}{(T_{AB})^2}$$

or

$$\frac{K_{AB}^2}{K_A K_B} = \frac{T_A T_B}{T_{AB}^2}$$

$$\frac{K_{AB}}{\sqrt{K_A K_B}} = \frac{\sqrt{T_A T_B}}{T_{AB}}$$

if the concentrations of T_A and T_B mixed are equal and equal to "a" while "x" is the amount of hybrid tetramer formed then

$$\frac{K_{AB}}{\sqrt{K_A K_B}} = \frac{\sqrt{(a - x/2)^2}}{x} = \frac{a - x/2}{x}$$

$$x \frac{K_{AB}}{\sqrt{K_A K_B}} = a - x/2$$

$$x\left(\frac{1}{2} + \frac{K_{AB}}{\sqrt{K_A K_B}}\right) = a$$

$$\frac{x}{a} = \frac{1}{\frac{1}{2} + \frac{K_{AB}}{\sqrt{T_A K_B}}}$$

$$Y = \frac{x}{2a} = \frac{1}{1 + 2\,\frac{K_{AB}}{\sqrt{K_A K_B}}}$$

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PROPOSITION II

The evidence for the lower intracellular pH in SS blood than in AA blood is mounting. Evidence for the lower pH in SS blood first was obtained by Winslow (1977). He found that the oxygen binding curves of SS erythrocytes have a higher P_{50} than one would expect based on the external pH, and on the intracellular DPG concentrations. Therefore, he argued in favor of a larger pH gradient between the external and internal pH in SS erythrocytes than in AA erythrocytes. Dash and Brewer (1978) have analyzed the amount of lactate inside the erythrocytes and found that upon deoxygenation SS blood has a lower intracellular pH than AA blood by about 0.1 pH units. The ^{31}P NMR study of AA and SS blood in Part 4 support these studies. Further the detected change in intracellular pH upon deoxygenation is larger than Dash and Brewer (1978) have found.

The effect of the lower intracellular pH in SS blood than in AA blood is quite dramatic (Bookchin, et al., 1976; Ueda, et al., 1979). The lower intracellular pH in SS blood than in AA blood decreases the affinity of HbSS toward oxygen, increases the amount of gel present in the cell, increases the number of new sickled cells and increases the pseudo first order rate constant for gelation (Briehl, 1978; Shibata, et al., 1977; Gill, et

al. 1978). The more gel forms the lower the intracellular pH should be depressed. Thus the lowering of pH upon gelation enhances the viscious cycle.

Another problem is as Winslow (1977) has pointed out that the cells after complete deoxygenation do not return to 100% saturation. It takes a long time to saturate SS erythrocytes to 100% oxygen saturation because the monomerization of the gell upon oxygenation is a very slow process.

In vivo studies of the effect of pH on patients in sickling crisis have been confusing. Greenberg and Kass (1958) have shown that NH_4Cl treatment of sickle cell patients can initiate sickle cell crisis. The results of this experiment have been interpreted as a result of lowering blood pH by the NH_4Cl. Treatment of patients in sickle cell crisis by increasing the blood pH has been shown to be effective in this study. Further, Barreras and Diggs (1964) proposed that due to the delivery of insufficient amount of oxygen to the tissues lactic acid builds up in patients suffering from sickle cell attack. Thus the effect is again a lowering of blood pH. Injections of bicarbonate has been found again to be effective in relieving the pain is sickle cell patients.

This result is expected based on the result of Winslow (1977). He found that after deoxygenation of the SS cells they do not return to 100% oxygen saturation after exposing them to oxygen for a long time.

There may be another explanation for the ineffectiveness of the bicarbonate or lactate treatment of SS patients to relieve pain. The higher blood pH may have no effect on sickled cells which are occluding a vein. The reason for this is that there is no blood flow through an occluded vein and therefore the sickled cells cannot equilibrate with the blood. Thus, one would expect that bicarbonate or lactate would have very little or no effect on patients suffering from a sickle cell crisis. In order to evaluate this possiblity it is proposed that data should be collected if there is any correlation between the blood pH of patients and the occurance of crises from which they suffer. Further, in order to prevent sickle cell crisis, a study of the blood pH of SS blood is proposed in which patients should be kept on the higher end of the normal scale by the administration of lactate or bicarbonate on a routine basis. The blood *pH* of these patients should be correlated with their occurance of crises. A decrease in the number of crises in these patients would indicate the efficacy of the increased blood pH.

In contrast Kong and Alleyne (1969) and Ringelhann and Konotey-Ahulu (1971) and Rhodes et al (1974) have found no evidence for the efficiency of increasing the blood pH by injection of bicarbonates or lactate on the sickle cell crises of patients. Further, aspirin has also been shown to help in relieving the pain of sickle cell patients even though aspirin is acidic (Hadlock, 1971).

In order to evaluate the possible effect of external pH on the internal pH a study of the ^{31}P NMR of SS and AA blood is proposed, at different percents of ligation, at different plasma pHs, in the range of pH 6.5-8.0. The plasma pH can be adjusted by lactic acid or by lactate. The results of this study would reveal if the problem in the treatment of patients is due to the possibility that the external pH has very little effect on the intracellular pH. Another important study would be to study the effect of external pH on the ^{31}P NMR of SS cells at about 20% oxygen saturation and at 100% saturation. If at 100%, and 20% oxygen saturation the internal pH increases as the external pH increases, but the linewidth of the ^{31}P signal at 20% oxygen saturation does not decrease then the change in pH is not very effective in decreasing the gelation due to the slowness of the monomerization process.

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PROPOSITION III

It has been shown that neoplastic transformation *in vivo* is a complicated process involving many intermediate stages (Barrett & Ts'o, 1978). Recently, *in vitro*, it has been shown that neoplastic transformation in hamster embryo cells involves several stages (Barrett, et al., 1977; 1978). After two weeks of incubation of hamster embryo cells with a carcinogen benzo [a] pyrine (BP), some morphological changes are detected and there is enhanced fibrinolytic activity. After about 10 weeks more than 90% of the cultures which survive have increased fibrinolytic activity and go through morphologic changes. 18 percent of these cultures acquire the ability to grow in soft agar; they become anchorage independent.

Evidence exists for the interdependence of neoplastic transformation and anchorage independency (Sponditos & Simmovitch, 1977). Sponditos and Simmovitch (1977) have also shown, that the anchorage independence can be transferred by metaphase chromosomes. BP treated cells which have not yet acquired anchorage independence can grow in soft agar after a transfer of chromosomes of cells which are anchorage independent. The implication of this fact is that there is a genetic mechanism or mutation which causes anchorage independence.

Results, which show that mutations caused by carcinogens are not the reasons for transformations, have been collected. It has been shown that for every mutated cell there is at least 10 cells which are transformed (Chan & Little, 1978). However, there have been studies which have estimated the ratio of transformation to mutation by a carcinogen, as high as 500:1 (Parodi & Brombilla, 1977; Barrett, et al., 1978). These studies, of course, imply that a mechanism exists for transformation and it is not caused by somatic mutations.

A recent study of mouse fibroblast cells has shown that both chemically and virally transformed cells can be classified into different populations depending on their ability to start tumors in syngeneic mice (Patek, et al., 1978). Patek, et al. (1978) found that some of the transformed cells called "O" cells have the ability to grow in agarose yet they do not start tumors in normal or in immunodeficient (T cell depleted) mice. They found that other cells, labelled I, start tumors in immunodeficient mice but not in normal mice. In this case Patek, et al (1978) suggest that this difference in tumogenecity is due to the fact that normal mice have more natural killer cells than the immunodeficient mice. Finally, another cell population was found from which cells (labelled C) injected into both normal and immunodeficient mice start tumors. They propose that although

they do not have any evidence for the possibility that all these cell populations are steps in the same mechanism of transformation (O→I→C) they do show that there is an additional necessary event in the I cells, which causes cancer only in immunodeficient cells, toward complete transformation (I→C).

However, there may be another explanation for the previous experiments. Recently, Bittner & Ruddon (1976) and Gehly, et al. (1979), have shown that there is a time dependency of the effectiveness of chemical carcinogen to cause transformation in 3T3 and 10T 1/2 mouse cells. They have shown that cells which are in their growing stages have a higher rate of hydrocarbon hydroxylase activity. This activity drops as the cells reach confluence. However, once confluence is reached the hydrocarbon hydroxylase activity is restored. Further, confluent cultures in used up media have a very low level of hydrocarbon hydroxylase activity. Addition of a fresh media results in rapid increase in the hydrocarbon hydroxylase activity (Bittner & Ruddon, 1976). A dependence of transformation on the concentration of chemical carcinogen (BP) has also been shown in embryonic hamster cells (Huberman & Sacks, 1966).

In order to prove that there is a single transformation in mouse fibroblast cells it is proposed that a study of the time of incubation by the carcinogen and concentration dependence of the different cell populations should be carried out on the described system of Patek, et al. (1978). The study found that 70% of the chemically treated cells are in the "last" detected state of transformation while only 20% of the cells are in the I state. If the distribution of the cells between the O, I and C states is dependent on the time of incubation with the carcinogen than the O→I→C mechanism is plausible. If the decrease in the time of incubation results in lower number of transformed cells with the same distribution, than the O→I mutation is independent of the O→C mutation. Similar studies of the transformation with varying concentrations of carcinogen is proposed.

Further it is proposed that the I cells should be cloned without the presence of carcinogen. In this case the hydrocarbon hydroxylase activity decreases. Then these cultures should be tested for the number of spontaneous transformation by injecting them into normal and immunodeficient mice. The number of cultures which causes tumors would show how stable the I state is. Following these experiments, the carcinogen again should be added to the culture. If significantly higher than 70% the I

cells transform under these conditions to the C state than that means that I is an intermediate stage in the transformation. If only 70% of the I cells transform that implies that there are two independent transformations in this sytem.

It would be also interesting to obtain data on the distribution of W, I, and C cells if the carcinogen is added before confluence, at confluence or after confluence. These studies are currently being done in Dr. Melvin Cohn's laboratory (private communication). The dependence of the populations on the concentration of the carcinogen should also be studied. Further it is proposed that the I cells should be cloned without the presence of carcinogen than they should be tested for their effect on normal and immunodeficient mice. If their effect have not changed again carcinogen should be added in different states of of the culture cycle and see if the I→C transformation occurs. If the I→C transformation is independent of the O→I transformation one would expect that $\approx$ 70% of the I cells would transform to the C state. If on the other hand I is an intermediate in an O→I→C mechanism then one would expect all the I cells to transform.

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PROPOSITION IV

Nitrogenase is a large protein complex. This complex is responsible for N_2 fixation and reduction in plants (Ljones, 1979). Besides reducing N_2 it is capable of reducing N_2O, N_3^-, C_2H_2, HCN, CH_3NC, H^+. The reduction of N_2 happens by the following equation:

$$N_2 + 6e^- + 12Mg\ ATP + 8H^+ \rightarrow 2NH_4^+ + 12Mg\ ADP + 12Pi$$

It also hydrolyses at least 2ATP/e^- however this ratio varies. In some cases the ATP to e^- ratios can reach up to 10:1 (Ljones & Burris, 1972). The reduction of N_2 to NH_3 uses a lot of ATP even though this reduction is an energetically favored process. The ATP is used to overcome a large activation energy.

There is a lot known about the structure of nitrogenase (Ljones, 1979). It is known that it is comprised of two parts: the Fe protein which contains only irons and "MoFe" protein which contains both molybdenums and irons. The Fe protein binds ATP and ADP. The ESR spectrum of Mo Fe protein does not change in the presence of ATP (Bui & Mortenson, 1968; Ome-Johnson, et al., 1972).

The Fe protein has a molecular weight of 55000-65000 daltons and has two identical subunits each of which binds one ATP. After the second ATP is bound, a structural

change occurs (Ljones & Burris, 1972; Ljones, 1979). The Fe protein is only functional when it is in its reduced form (Kelly & Lang, 1970; Yates, 1972). Reduction of the protein is accomplished by $Na_2S_2O_4$ *in vitro* and by NADPH, or by chloroplast activity *in vivo* (Yates, 1972). Due to its similarity to ferrodoxin it is proposed that a Fe_4S_4 group is its functional unit (Gillan, et al., 1977; Ljones, 1979). This structure is like a cage which is held by the protein by four cysteine residues. When the Fe protein is reduced and 2ATP binds to it, the cage becomes exposed to water. This fact is supported by the fact that the cage is more accessible to chelating agents (Walker & Mortenson, 1974) and the Fe protein has an ESR spectra which is similar to the one obtained under denaturing conditions, as in the presence of urea (Zumft, et al., 1973).

The transferred electron is then used by the MoFe protein to reduce its substrate. After every electron transfer the MoFe-Fe protein complex falls apart (Hageman & Burris, 1979). Although it is not known where substrates bind to this protein it is, however, believed that the Molybdenums are involved (Chatt, et al., 1969; Ljones, 1979). There are two molybdenum and 16 irons in the MoFe protein (Shah & Briehl, 1977). It has also been shown that the molybdenums and irons are part of a cofactor

which can be separated from the rest of the protein. Further, this cofactor can carry out 80% of the activity of the full protein (Shah & Briehl, 1977).

It is not known how the nitrogen binds to the MoFe protein. Inorganic complexes bind N_2 in two ways: in one case it is bridged between two metals, in the other it is bound in end-on fashion to the metal, (Winfield, 1955; Chatt, et al., 1975; Shilov, et al., 1971; Manniquez & Bercaw, 1974). An interesting change has been detected by Chatt et al. (1969) in the IR frequency of this complex: $[Re\ Cl(N_2)(P\ Me_2\ Ph)_4]$ when $MoCl_2 \cdot 2\ Et_2O$ was added. Free N_2 has a stretching frequency of 2371 cm^{-1} which changes to 1922 cm^{-1} when it is bound by the complex. Addition of $MoCl_2 \cdot 2\ Et_2O$ however changes its infrared absorption to 1680 cm^{-1}. This change represents an overall change of 100 kcal in the binding energy. Thus the IR stretching frequency of N_2 is quite sensitive to its binding mode.

The results of Ljones (1973) suggest that CN^- inhibits the Fe protein even though the MoFe protein can reduce it to CH_4 and NH_4^+. Bui and Mortenson (1968) found however, that CN^- does not bind to the Fe protein. CO and NO are also potent inhibitors of the protein even though both get reduced by nitrogenase. CO is a non-

competitive inhibitor while NO is a competitive inhibitor of nitrogen reduction (Burris, 1976).

IR spectroscopy has been used to study the binding of oxygen, carbonmonoxide and nitric oxide to hemoglobin and the binding of azide to methemoglobin (Caughey, 1978; Satterlee, et al., 1978; Maxwell & Caughey, 1976; Perutz, et al., 1978). With the development of Fourier transform IR it has even been possible to detect the weaker infrared absorption of sulfhydryls in hemoglobin (Bates, 1976; Alben , et al., 1974; Bates, et al., 1975). Therefore an IR study of the binding of CO, NO, N_2 by nitrogenase is proposed. The absorptions can be detected in a D_2O solution as has been shown by Maxwell and Caughey (1976) since in the region in which all these ligands absorb, between 2200-1500 cm^{-1}, D_2O absorbs weakly. The way N_2 binds can also be determined from its absorption as has been indicated by Chatt, et al., 1969. The stretching frequency of N_2 is quite sensitive to the way in which it is bound. Competitive studies can also be carried out, e.g., in the case of NO and N_2. The different binding sites of CO from N_2 should also be detected by IR. The effect of reduced Fe protein in the presence of ATP on the MoFe protein can also be studied. All these studies should contribute to what is known to the structure and function of nitrogenase.

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PROPOSITION V

There is a need for the development of new methods to follow metabolic changes in plants. Methods which have been developed use expensive and highly sophisticated equipment or they are inconvenient to collect routine data (Wolk, et al. 1976; Meeks, et al., 1978; Bauer, et al. 1977; Schaefer, et al., 1979). The best available data, about the use of nitrogen in the metabolic pathways of plants, follow ^{13}N labelling of plant tissues after incubation of plants with $^{13}N_2$. ^{13}N decays with a half life of 10 minutes, therefore the experiment has to be finished in less than two hours (Wolk, et al., 1976). Another experiment uses ^{15}N labelling and it is detected by spectrometric analysis after a laborious extraction and separation of the labelled material (Meeks, et al., 1978; Bauer, et al., 1977). This method suffers from the complicated method for the separation of metabolites and from the destruction of the plant tissue which may cause irreversible changes in the sample. Therefore, the results of this study are not very reliable.

Another method has been tried used solid state ^{15}N NMR (Schaefer, et al., 1979). This method uses highly expensive instrumentation and although it detects the ^{15}N labelling it does not differentiate between amino acids

very well. The only conclusion this method has produced is that the plant can use both NH_4^+ and NO_3^- as nitrogen source.

Recently useful information has been obtained from the ^{31}P and ^{13}C NMR of intact cells about their metabolism. These include red blood cells (Moon & Richards, 1973; Henderson, et al., 1974), muscle cells (Hoult, et al., 1974; Burt, et al., 1976) and yeast cells (Burt, et al., 1976). Further studies of rat live mitochondria gained information about oxidative phosphorylation (Ogawa, et al., 1978). These results show that ATP to ADP conversion results in OH^- uptake from the cytoplasm and $H_2PO_4^-$ release inside the mitochondria. All of these results support Mitchell's (1961, 1966) theory of chemiosmotic coupling.

The detection of ^{31}P NMR signals has also been possible in whole rabbit hearts (Hollis, et al., 1978; Nunnally & Hollis, 1979). These studies have shown that reduction in coronary flow (ischemia) results in lowering of the intracellular pH and increase in the orthophosphate concentration with a drastic decrease in the concentration of creatine orthophosphate.

Further results have been obtained by the specific ^{13}C labelling of glycerol in rat liver cells and following its uptake and usage by ^{13}C NMR. The results show the production of glutamate, aspartate lactate, etc (Cohen, et al., 1979). A study of Friend leukemic cells has also

been carried out by ^{15}N NMR (Lapidot, et al., 1975). However, a general ^{15}N source, glycine, was used to label the cells so useful structural information has not been gained.

In order to learn more about the metabolic behavior of plant cells a study of the metabolism of cyanobacteria (blue-green algae) is proposed. The possibility that ^{13}C NMR can be obtained on algae is suggested by the study of the ^{13}C NMR of red algae (Bhattacharjee, et al., 1979). The effect of light, and nitrogen source like, N_2 or NH_4NO_3 should be studied. Although ^{15}N NMR spectra of algae would be the most interesting spectra to study of these cells, its information would be limited to long term effects due to the low signal sensitivity and it is not very sensitive to differences in amino acids as shown by Lapidot, et al., (1975). However, nitrogen fixation should result in increased amino acid formation and increased usage of ATP. The detection of the depletion of ATP due to the formation of amino acids, and the amino acids themselves, should be possible using ^{31}P and ^{13}C NMR. The effect of too little and too much light, lack of nutrients, etc. should be studied in algae. These studies then can be used as a control for the studies of the plant tissues. The most obvious plant tissue which can be studied by this method is the sap. However,

the detection of ^{31}P by NMR in leaves, pods, or stocks of plants should also be explored. Later, data can be obtained by whole plant Zeugmatography. This method can map the distribution of metabolites or phosphorous nucleotides throughout the plant (Lauterbur, 1977). This information would be useful in the study of transportation of metabolites in plants.

Since no physical technique is available for evaluating the metabolic state of plants it would be useful to explore the possibility of using NMR techniques in this area.

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