NUCLEAR MAGNETIC RESONANCE STUDIES OF HEMOGLOBIN AND RED BLOOD CELL FUNCTION

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To Ulrike and James

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Happy Birthday, Jack

NUCLEAR MAGNETIC RESONANCE STUDIES OF HEMOGLOBIN AND RED BLOOD CELL FUNCTION

PART I: NMR Studies of ^{13}CO Binding to Various Heme Globins.

PART II: ^{13}C Magnetic Resonance Studies of the Binding of Carbon Monoxide to Various Hemoglobins.

PART III: ^{13}C Magnetic Resonance Studies of the Binding of Carbon Monoxide to Myoglobins and Hemoglobins Containing Unnatural Hemes.

PART IV: ^{13}C NMR Studies of the Distribution of ^{13}CO Between the Subunits of Partially Liganded Hemoglobins.

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PART IX: Conformation Studies of Various Hemoglobins Using Natural Abundance ^{13}C NMR Spectroscopy.

ABSTRACT

Pulse and Fourier transform nuclear magnetic resonance techniques have been employed to study various aspects of hemoglobin and red blood cell function.

The binding of ^{13}C-enriched carbon monoxide to myoglobins and hemoglobins from a variety of animal species has been studied by ^{13}C NMR. The environments experienced by ^{13}CO bound to α or β subunits, the relative facility with which oxygen displaces ^{13}CO, and the relative thermodynamic affinities of the unliganded subunits for ^{13}CO are found to differ. These results clearly demonstrate the various degrees of nonequivalence that may exist between the subunits of normally occurring hemoglobins which must be accounted for in any physical model of hemoglobin ligation if it is to accurately describe hemoglobin function.

The chemical shifts of ^{13}CO bound to reconstituted myoglobins and hemoglobins containing synthetically modified hemes have been studied and show that the degree of electronic interaction between the heme and the bound ligand may be modulated by the nature of the surrounding protein. T_1 and NOE studies indicate that the bound ^{13}CO ligand interacts strongly with the valine residue (E11) and forms a hydrogen bond with the distal histidine residue (E7) in the ligand binding pocket. These results provide new information about the manner in which the protein environment controls the chemistry and reactivity of the heme.

Observation of the ^{31}P resonances of inorganic phosphate and

2,3-diphosphoglycerate in whole blood has led to the development of a noninvasive technique for the determination of intracellular pH. This technique is generally applicable for the study of intracellular pH in a large number of different cell and vesicle systems.

The reactions of ^{13}C-enriched cyanate with human adult and sickle cell hemoglobins have also been studied by ^{13}C NMR. Results were obtained for the pH dependence of carbamylation for the N-terminal amino groups and lysine residues of these hemoglobins, both in purified hemoglobin solutions and in whole blood. These studies suggest that maximal effects of cyanate in clinical in vitro treatments will be realized when cyanate concentrations are kept below 10-20 mM and reaction pH is in the range 6.5-7.0.

Natural abundance ^{13}C NMR has been used to study the solution conformations of human and rabbit hemoglobins. The results suggest that average hemoglobin quaternary conformations are ligand dependent. Methemoglobin is found to have a solution quaternary conformation roughly intermediate between those of oxyhemoglobin and deoxyhemoglobin at neutral pH.

ABSTRACTS OF THE PROPOSITIONS

PROPOSITION I

A method is proposed for the study of chemotactic receptor sites on polymorphonuclear leukocytes and other phagocytic cells using ^{131}I- and fluorescence-labeled chemotactic factors.

PROPOSITION II

The application of ^{15}N NMR techniques to study the ^{15}N-enriched histidine residue of α-lytic protease is proposed as a means for clarifying the ionization behavior of the serine-histidine-aspartic acid catalytic triad in serine proteases.

PROPOSITION III

The oral administration of the amino acid citrulline to persons suffering from sickle cell anemia is proposed as a possible chemotherapeutic strategy for the inhibition of red cell sickling.

PROPOSITION IV

The application of thallium-203/205 NMR techniques is proposed as a promising new method for the study of specific chemical environments within whole cells.

PROPOSITION V

^{15}N magnetic resonance observation of the ^{15}N-^{13}C spin-spin couplings between ^{13}CO and the nitrogens of ^{13}CO-hemochrome compounds is proposed as a method for dissecting the electronic effects experienced by ^{13}CO into electronic contributions from the porphyrin and contributions from the axial base.

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INTRODUCTION

The most thoroughly studied and best understood class of globular proteins are the hemoglobins. Detailed studies of their physical and chemical properties, their relationships to other heme proteins, and their importance in determining much of the chemistry of the red blood cell, or erythrocyte, have been extensively reviewed over the last twelve years (1-7). The experiments, results and conclusions reported in this thesis represent yet further contributions to the long history of hemoglobin study. In this introduction I describe the problems considered by me to be of greatest importance and the conclusions reached from the experimental work. The body of the thesis, Parts I-IX, contains reprints and preprints of the researches undertaken and the detailed discussions of the results obtained. The reader is referred from the problems and conclusions mentioned in the introduction to the parts of the thesis where these topics are discussed in detail.

The central issues with which I have been concerned are (i) the chemical nature of ligands such as carbon monoxide or, by analogy, oxygen when bound to myoglobins or hemoglobins, (ii) whether or not heme chemistry may be modulated by the surrounding protein and the mechanisms by which this modulation is achieved, (iii) whether or not the α and β subunits of hemoglobins are equivalent in their reactions with ligands and to what extent varying degrees of subunit nonequivalence may effect cooperative hemoglobin ligation, and (iv) to what extent do the solution conformations of hemoglobin depend on the state of the

heme or the nature of the bound ligand. In addition I have been concerned with the relation and significance of hemoglobin studies obtained under restrictive experimental conditions with respect to the realities of hemoglobin function within the erythrocyte, and the extent to which hemoglobin or its intracellular environment may be controlled or beneficially altered so as to relieve the physiological symptoms induced in persons with hemoglobinopathic diseases such as sickle cell anemia.

The Nature of the Ligand

A great deal of controversy has surrounded studies of the geometry and electronic configuration of heme-ligand bonds. Pauling (8) originally proposed a bent iron-oxygen bond involving low spin Fe(II) and molecular oxygen. Weiss (9) argued that a "metsuperoxide" model, involving Fe(III) and superoxide oxygen, provided a better description of heme-oxygen bonding. Gray has argued in favor of an "oxidative addition" model (10) in which peroxide oxygen binds to seven-coordinate low-spin Fe(IV), and Goddard (11) believes that the iron-oxygen bond could be similar to the bonding in ozone; thus he favors a bent, low spin Fe(II)-O-O bond like that originally proposed by Pauling. The bonding of carbon monoxide to the heme is also uncertain. While the overwhelming majority of chemical evidence on metal-carbonyl bonding insists that the iron-CO bond must be linear, X-ray crystallographic studies of erythrocruorin (12) and the monomeric hemoglobin from Glycera dibranchiata (13) indicate that carbon monoxide bonding in these heme proteins assumes a bent configuration.

While these arguments may seem largely semantic in character, they are of importance in that differences in iron-ligand bonding imply differences in the crucial bonding between heme iron and the proximal histidine residue (F8). Perutz (14) has proposed that this heme-histidine bonding constitutes the "trigger" for the allosteric conformation changes observed between deoxy and liganded hemoglobins. Moreover, differences between the bonding interactions of heme-iron with various ligands may be of importance in determining the relative affinities of different myoglobins and hemoglobins for ligands.

In regard to these questions, our detailed studies of the spin-lattice relaxation times (T_1) and nuclear Overhauser effects (NOE) experienced by ^{13}C-enriched carbon monoxide bound to sperm whale myoglobin or human hemoglobin as presented in Part V (15), demonstrate the existence of a strong hydrogen bond between ^{13}CO and the distal histidine residue (E7) of the ligand binding pocket. Thus, depending upon the ability of various bound ligands to form hydrogen bonds with the imidazole ring, the distal histidine is capable of participating in ligand bonding and may influence both bond strength and geometry. We believe participation of the distal histidine in ligand bonding may also explain many of the controversial differences observed between heme model compounds and the native proteins.

Modulation of the Heme

Understanding the nature of the heme-protein interactions which are responsible for the control of ligand affinity in hemoglobin and myoglobin is of considerable importance. Direct effects on the bound ligand, such

as hydrogen-bonding or steric interactions, are probably involved. In addition it is possible that distortion of the heme-proximal histidine bond or electronic effects induced in the porphyrin ring may serve to modulate heme iron reactivity.

To investigate the nature of the electronic interactions between the porphyrin ring and the bound ligand, we studied the ^{13}CO chemical shifts for reconstituted myoglobins and hemoglobins containing hemes modified at the 2 and 4 positions of the porphyrin ring. The results presented in Part III (16) clearly demonstrate that electronic substituent effects are transmitted to the bound ligand, and that the magnitude of these electronic effects is modulated by the nature of the apoprotein. This modulation must either come through heme-proximal histidine interactions or through the direct effects of distal amino acid residues on the bound ligand.

Subunit Nonequivalence

In the early work discussed in Parts I, (17) and II, (18) and more especially in the studies of the distribution of ^{13}CO between the α and β subunits of various hemoglobins under conditions of partial saturation presented in Part IV, (19) we have conclusively demonstrated that the relative affinities of the α and β subunits for ^{13}CO may differ considerably between species. We have also shown that the environments experienced by ^{13}CO bound to α or β subunits are nonequivalent and the facility with which oxygen displaces ^{13}CO differs for different subunits. These results prove that models used to describe hemoglobin cooperativity, such as the allosteric model of Monod et al. (20), do not

accurately describe hemoglobin ligation unless they are specifically modified to account for subunit nonequivalence, such as is the case for the "generalized concerted transition" model of Ogata and McConnell (21-23).

Solution Conformations of Hemoglobins

Part IX (24) describes our early qualitative studies of the solution conformations of hemoglobins using natural abundance ^{13}C NMR. These studies confirmed that lysine residues are immobilized on deoxygenation of hemoglobin, as originally proposed by Perutz (14) and also demonstrated that the solution quaternary conformation of acid (aquo) methemoglobin at neutral pH is roughly intermediate between the conformations of oxy and deoxy hemoglobin. This intermediate configuration, first reported by Huestis and Raftery (25), has since been confirmed in more recent reports (26-29). These results were particularly important because the oxyhemoglobin crystals used in the X-ray diffraction studies of Perutz contained substantial fractions of methemoglobin (30) and the degree to which methemoglobin may have affected the crystallographic data is uncertain.

Intracellular Environment

There is a close association between hemoglobin ligation, intracellular pH, and erythrocyte metabolism in whole blood brought about by the alkaline Bohr effect and the action of 2,3-diphosphoglycerate binding to deoxyhemoglobin (31). In Part VI (32) a very effective ^{31}P NMR method is described for measuring intraerythrocyte pH in whole blood without necessitating the disruption of the cell membrane. This

method should prove to be extremely valuable, not only for the study of red blood cells, but also for the determination of intracellular pH in other cell systems and in synthetic vesicles.

Sickle Cell Anemia

The current understanding of sickle cell anemia suggests that deoxyhemoglobin S tends to form a gel consisting of long tactoid rods which distort the red cells into their characteristic elongated sickle shape. The elongated cells increase the viscosity of the blood and tend to pile up in the capillaries. Reversal of the sickling process has been demonstrated in vitro and to some extent in vivo through the use of various treatments involving urea (33-35), cyanate (36, 37) and carbamyl phosphate (38). All of these treatments were developed in an attempt to block red cell sickling by inhibiting the formation of the hemoglobin S gel.

Our studies on the pH dependence of hemoglobin and whole blood carbamylation using ^{13}C-enriched cyanate are discussed in Parts VII (39) and VIII (40). The results clearly show that α-amino group carbamylation is pH dependent and that the undesirable carbamylation of lysine residues is independent of pH but highly dependent on cyanate concentration. Optimum conditions for in vitro carbamylation of whole blood are obtained at pH ~ 6.5 with cyanate concentrations below 10 mM. No evidence was obtained for carbamylation of cysteine or other residues.

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PART I

NMR Studies of ^{13}CO Binding to Various Heme Globins

Sir:

Whether, and to what extent, the various subunits of hemoglobin interact differently with ligands (such as oxygen or carbon monoxide) remains one of the unanswered questions in the hemoglobin saga. These possible differences may manifest themselves in the kinetic or thermodynamic affinities for ligands or in the environments experienced by ligands when bound to various subunits.

We have studied the binding of ^{13}C enriched carbon monoxide to a variety of heme globins, specifically sperm whale myoblogin,adult human hemoglobin, fetal human hemoglobin, mouse hemoglobin, and rabbit hemoglobin. The technique involved observing, by a Fourier transform method using a Varian Associates XL-100-15 spectrometer, the ^{13}C resonances of carbon monoxide bound to the proteins listed. Representative spectra are shown in Figure 1. All carboxyhemoglobins studied showed two distinct resonances of equal intensity originating from carbon monoxide bound either to the α or β subunits. Data on the chemical shifts for the ^{13}CO resonances of the proteins studied are collected in Table I. (Another report (1) has appeared on ^{13}CO bound to sperm whale myoglobin and human hemoglobin though these earlier data differ considerably from the shifts we have consistently observed.) The results clearly indicate significant differences between the magnetic environments experienced by carbon monoxide bound to α or β subunits. Whether these differences are due to changes in the bond between the iron and carbon monoxide or to other subtler interactions between the ligand and other groups around the heme pocket is presently unknown. Recent work (2) with a spin-labelled analogue of ATP has led to the con-

FIGURE 1

Representative spectra of the carbon monoxide resonances from sperm whale carboxymyoglobin (MbCO), human adult hemoglobin (HbCO-A) and rabbit hemoglobin at 25.15 MHz. Protein concentrations ranged from 2-5 mM in 0.1 M NaCl. Enrichment in ^{13}CO was 90%.

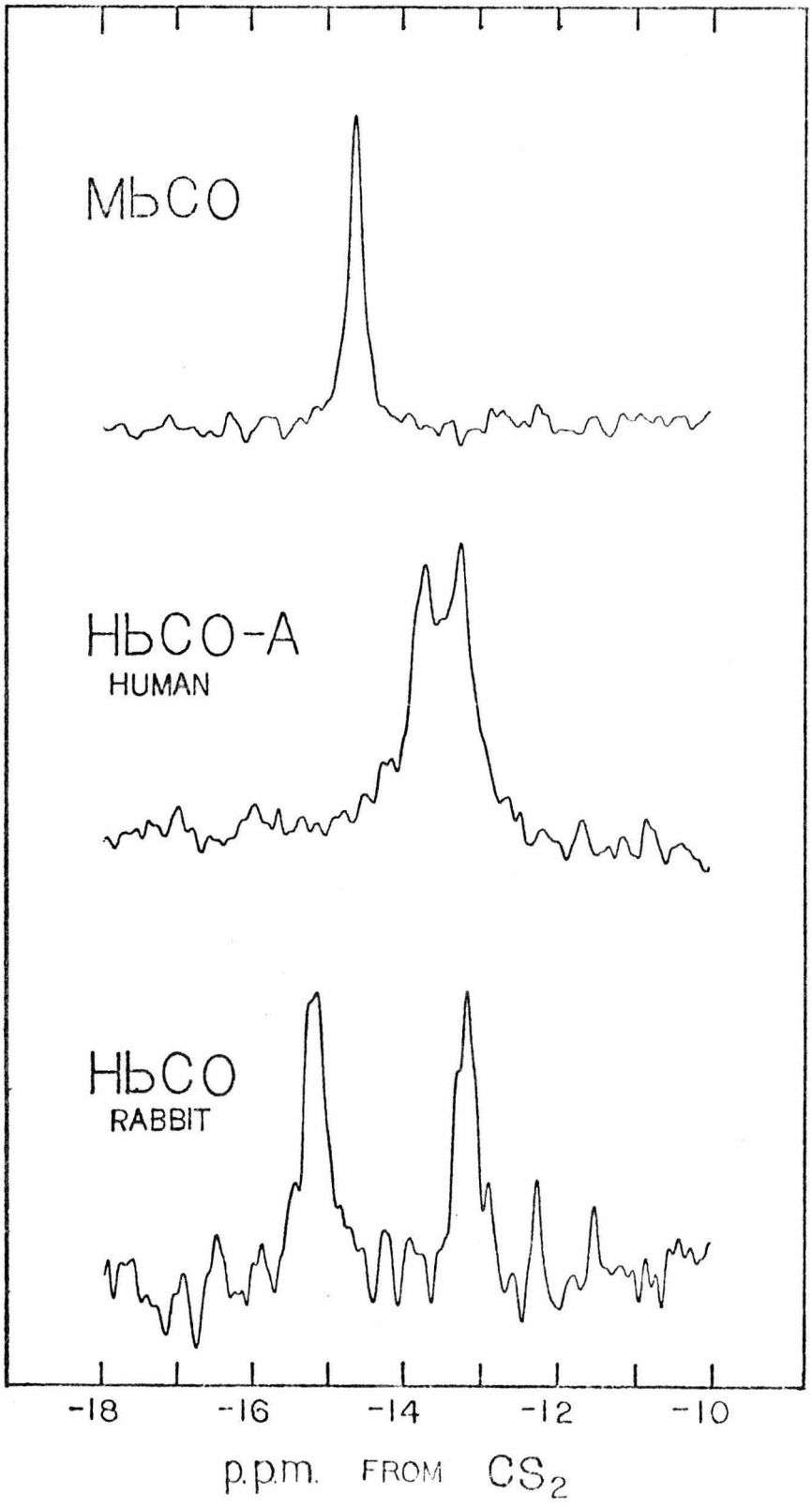
MbCO
HbCO-A
HUMAN
HbCO
RABBIT
-18
-16
-14
-12
-10
p.p.m. FROM CS_2

Table I. Chemical Shifts of Heme-Bound ^{13}CO[a]

MbCO	-14.89		6.79-7.49
(sperm whale)	-14.61		5.42
HbCO-A	-13.74	-13.26	6.35-7.90
(human)			
HbCO-F	-13.74	-13.30	6.80
(human)			
HbCO	-13.58	-13.14	6.21
(mouse)			
HbCO	-15.16	-13.18	6.94-7.38
(rabbit)			

[a] Ppm from CS_2, ± 0.04

conclusion that oxygen binding to hemoglobin may be quantitatively described by a two-state model such as that of Monod, Wyman and Changeux (3), with the additional condition that the α and β subunits are nonequivalent.

The pH dependencies of the ^{13}CO resonances showed that the MbCO resonance was independent over the pH range 6.79 though at pH 5.42 an upfield shift of 0.28 ppm was observed. The resonances of adult human HbCO were independent of pH over the range 6.35 - 7.90 as were the resonances of rabbit HbCO over the range 6.94 - 7.38. These results are somewhat unexpected as Shulman (4) reports that the heme proton chemical shifts of cyanoferrihemoglobin and several related "mixed-state" hemoglobins are markedly sensitive to pH changes in the region near pH 7, although the resonances of these hydrogens, being strongly shifted by unpaired electron density on the carbons to which they are attached, may be much more susceptible to very small changes in protein conformation than the ^{13}C resonances in the carboxy heme globins. Also, the addition of a 2:1 molar excess of 2,3-diphosphoglycerate at pH 7.0 did not affect the chemical shifts of rabbit HbCO.

Interestingly, no significant difference is observed between the shifts of the ^{13}CO resonances when bound to fetal or adult human hemoglobin despite 39 amino acid substitutions (5) in human γ chains with respect to human β chains, i.e., ^{13}CO bound either to a human β or γ subunit apparently experiences essentially the same environment. Conversely, the sequence differences between rabbit and human hemoglobin exert a marked influence on the environment for bound CO.

Studies of the spin-lattice relaxation times (T_1) of the ^{13}CO resonances using the technique of progressive saturation yield values of $T_1 \sim 0.3$ sec for both resonances in rabbit HbCO at pH 7.0 and 3 mM concentration.

Exposure of rabbit HbCO to oxygen results in a significantly faster diminution in the low-field resonance (-15.16 ppm from CS_2) than of the high-field resonance. This indicates a disparity between the relative affinities for carbon monoxide vs. oxygen by the different subunits. Based on kinetic studies using stopped-flow techniques, Gibson (6) has concluded that carbon monoxide replaces oxygen faster in the β than in the α subunits of a variety of hemoglobins. If these kinetic results also reflect the relative thermodynamic affinities (i.e., β subunits prefer carbon monoxide to oxygen as ligands by a larger margin than do α subunits), we should expect carbon monoxide to be replaced by oxygen more readily from α than from β subunits. Accordingly, if Gibson's kinetic results do reflect the differential thermodynamic affinities for oxygen vs. carbon monoxide of α and β subunits, we tentatively conclude that the resonance at lower field (which is the one more readily removed by oxygen) represents ^{13}CO bound to α subunits of rabbit hemoglobin.

In summary, this work indicates that significant differences exist in the nature of the environment experienced by carbon monoxide when bound to α or β subunits of a variety of hemoglobins. The differences do not appear to be significantly affected over the range pH 6.5 - 7.5 and, at least in the case of rabbit HbCO, are not affected by 2,3-diphosphoglycerate. Moreover, the ease of displacement of CO by O_2 differs markedly for CO bound to the α or β subunits of rabbit hemoglobin.

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PART II

^{13}C Magnetic Resonance Studies of the Binding of Carbon Monoxide to Various Hemoglobins

INTRODUCTION

In normal hemoglobin tetramers, the acquisition of a ligand by the iron atom of a particular heme group depends critically on whether or not the other heme groups in the tetrameric molecule are liganded. This dependence of the ligand affinity of one subunit on the existing degree of ligation of other subunits accounts for the positive cooperativity with which most normal hemoglobins bind molecules such as oxygen or carbon monoxide (Antonini and Brunori, 1970). Major conformational differences exist between unliganded deoxyhemoglobin and fully liganded forms of the protein (Muirhead et al., 1967; Perutz et al., 1968; Muirhead and Greer, 1970; Bolton and Perutz, 1970) but exactly how the ligand affinity of a given heme is modulated by events elsewhere in the molecule is not presently known. Some of the unanswered questions are to what extent these effects may be caused by steric factors operating to exclude ligands from a heme in one protein conformation while in another conformation providing for unobstructed ligand approach (Perutz, 1970) or to what extent these effects may reflect electronic factors resulting from changes in the interaction between the iron atom of the heme and the proximal histidine (F8) or by changes in the interaction between substituents about the porphyrin ring (such as the vinyl groups) and the π cloud of the porphyrin. Both families of factors probably contribute and the rigorous dissection into steric or electronic effects undoubtedly oversimplifies the actual situation. For example, the "electronic" effects, though ultimately manifested at the

iron atom, probably have their origin in conformational changes of the polypeptide chain which then influence the proximal histidine. Similarly changes in the orientation of polypeptide chains near the periphery of the porphyrin ring can eventually manifest themselves as "electronic" effects at the iron.

To gain insight into some of these questions provides the focus of this work whose ultimate objective is to resolve the origin of the allosteric cooperativity of hemoglobin. In particular we sought to identify the environmental differences experienced by carbon monoxide when bound to the different subunits of various hemoglobins (Moon and Richards, 1972) with some attention to the relative thermodynamic ease of displacem t of carbon monoxide by oxygen from α or β subunits and the relative affinities of the unliganded subunits for carbon monoxide. We used the ^{13}C nucleus of ^{13}C-enriched carbon monoxide as the probe in this work.

EXPERIMENTAL SECTION

Materials and Methods: Hemoglobin was prepared from freshly drawn, citrated whole blood of human, bovine, mouse (BALB/c), guinea pig (Campbell-Trapani American albino closed colony), rabbit (New Zealand white), chick (white Leghorn, 3 days old), bullfrog, or wild pigeon origin.

The red cells were separated from plasma by centrifugation at 2000 x g and washed several times with 0.1 M sodium chloride. The packed erythrocytes were then lysed with two volumes of distilled water at room temperature for 20-30 minutes. After removal of the stromata by centrifugation at 30,000 x g, the hemolysates were stripped of organic phosphates by extensive dialysis against 0.1 M sodium chloride as described by Bunn et al., (1971). (This method has been shown to reduce the concentration of organic phosphates to a value less than 0.1 mM in a 2 mM hemoglobin solution.) The hemoglobin solutions were then concentrated by ultrafiltration (Amicon, UM-10 membrane) and adjusted spectrophotometrically to a final hemoglobin concentration of 2.0 mM in 0.1 M sodium chloride.

The α and β subunits of normal adult and sickle cell hemoglobin were separated and purified as the p-hydroxymercuribenzoate (HMB) derivatives as described by Geraci et al. (1969). Hemoglobin was allowed to react with HMB overnight at pH 6.15 ± 0.05 and $4^{\circ}C$. The crude reaction product was centrifuged to remove precipitated material and was then applied to a column of Sephadex G-25 equilibrated with

0.01 M phosphate buffer at pH 8.0. The HMB hemoglobin thus eluted was then concentrated by ultrafiltration. The HMB α and HMB β subunits were separated on a single column (2.5 x 30 cm) of DE 52 cellulose (Whatman). The HMB α chains were eluted first with 0.01 M phosphate buffer pH 8.0, followed by residual hemoglobin, eluted with 0.02 M phosphate buffer pH 7.5, and then HMB β subunits eluted with 0.1 M phosphate buffer pH 7.0. The isolated HMB subunits were dialyzed against 0.1 M sodium chloride, and concentrated by ultrafiltration. They were checked for purity by polyacrylamide disc gel electrophoresis. The HMB β subunits contained no detectable impurities while the HMB α subunits typically contained 1-2% (but never more) hemoglobin tetramer.

Extensive treatment with water-saturated carbon monoxide (enriched in ^{13}C by 90-92%) converted the purified, unliganded hemoglobins or separated HMB subunits to their carboxy derivatives. The presence of methemoglobin was measured spectrophotometrically prior to addition of CO by removal of a 10μl aliquot which was diluted to 10 ml in 0.15 M sodium chloride which had been previously saturated with air. The ratios of absorptions at 415, 420 and 430 nm (Benesch et al., 1965) were then immediately determined. In those cases where only the relative chemical shifts of the bound ^{13}CO signals were being monitored, the methemoglobin content was typically 2-3% and never exceeded 10% in any of the various samples studied. No increase in methemoglobin content was detected after determination of any cmr spectrum. In those experiments where the relative degree of ligation

of the two subunits was measured (see below), the amount of methemoglobin never exceeded 3% and did not change detectably during the course of the experiments.

Quantitative treatment of rabbit deoxyhemoglobin with non-saturating amounts of ^{13}CO was accomplished in a specially constructed, closed atmosphere sample tube which has an attached uv cell. This tube was held concentrically in a 12-mm tube containing D_2O which served as a field frequency lock. Before addition of carbon monoxide, the samples were deoxygenated in the cell by extensive flushing with water-saturated nitrogen. Complete deoxygenation was determined by the method of Benesch et al. (1965) using the criterion that the ratio $OD_{670}/OD_{730} \geq 2.3$. Water-saturated ^{13}CO was injected into the sample tube with a gas-tight syringe through a rubber sleeve covering the access port. The extent of hemoglobin saturation with ^{13}CO was followed spectrophotometrically at 650 nm.

Nuclear Magnetic Resonance (nmr): ^{13}C spectra were obtained using the pulse Fourier transform technique on a Varian Associates XL-100-15 spectrometer equipped with a Varian 620i computer (16K memory). Samples were contained in a 12-mm tube with a 5-mm tube containing D_2O inserted concentrically to serve as a field frequency lock. All spectra were obtained under identical conditions at a probe temperature of 34°C using a 90° pulse of 150 μsec duration with a spectrum width of 2500 Hz.

Spin-lattice relaxation time (T_1) measurements were made with proton noise decoupling using the progressive saturation intensity ratio

technique as described by Freeman and Hill (1971). These authors have also investigated the effect of a finite H_1 on progressive saturation experiments of the type used here. They found that, when $\Delta F/H_1$ is less than 0.8, the error in the measured value to T_1 is less than 5%. In our case, H_1 was 1666 Hz (τ_{90° = 150 μsec) and ΔF for the ^{13}CO resonances was about 500 Hz, which, applying the analysis of Freeman and Hill, leads to an error of less than 1% in the measured value of T_1 caused by the finite pulse strength (or long pulse duration). The values reported are based on numerous (7-8) independent measurements of oxygen-free samples. Uncertainties are standard mean deviations of the mean values reported. (The observed T_1 values did not vary detectably as a function of relative methemoglobin concentration over the range 2-60%.) The pulse intervals (0.2 to 2.4 sec) used in these determinations were varied randomly so as to eliminate systematic errors during the long data-acquisition periods (1-10 hrs) necessary to achieve adequate signal-to-noise.

Nuclear Overhauser effects were determined both by direct integration of resonances with and without proton noise decoupling and by comparison of intensity ratios using continuous and gated noise decoupling techniques (Freeman et al., 1972).

RESULTS

Figure 1 displays representative spectra of the ^{13}CO resonances of various carboxyhemoglobins, and Table I collects the chemical shift data for all proteins studied. In general, the carboxyhemoglobins show two distinct resonances of equal intensity which presumably originate from carbon monoxide bound either to the α or β subunits. In contrast, pigeon carboxyhemoglobin gave a single, rather sharp resonance centered at 206.36 ppm from external TMS. Also, the resonances generally occur at very similar absolute chemical shifts with a separation of 0.5 ppm. Pigeon carboxyhemoglobin lacks the resonance at lower field and rabbit carboxyhemoglobin, though having the upfield peak at the usual position, shows a low-field peak shifted considerably further downfield than normal (the separation between the peaks is 2.0 ppm.

The positions of the resonances of carboxyhemoglobin in intact rabbit, human adult, human fetal and sickle cell erythrocytes agree well with those for the corresponding carboxyhemoglobins in solution. The two resonances of human hemoglobins in intact erythrocytes were not resolved, presumably due to magnetic field inhomogeneity and increased viscosity within the cells; the position of the broadened resonances was not, however, shifted. The resonances of carboxyhemoglobin within rabbit erythrocytes were still resolved and occurred at the same position as those in solution. Swelling of rabbit red cells in hypotonic salt solutions led to decreased line widths for the resonances, probably due to decreased intracellular viscosity.

FIGURE 1

Representative spectra of the ^{13}CO resonances from human adult, guinea pig, rabbit and pigeon carboxyhemoglobins at 25.17 MHz.

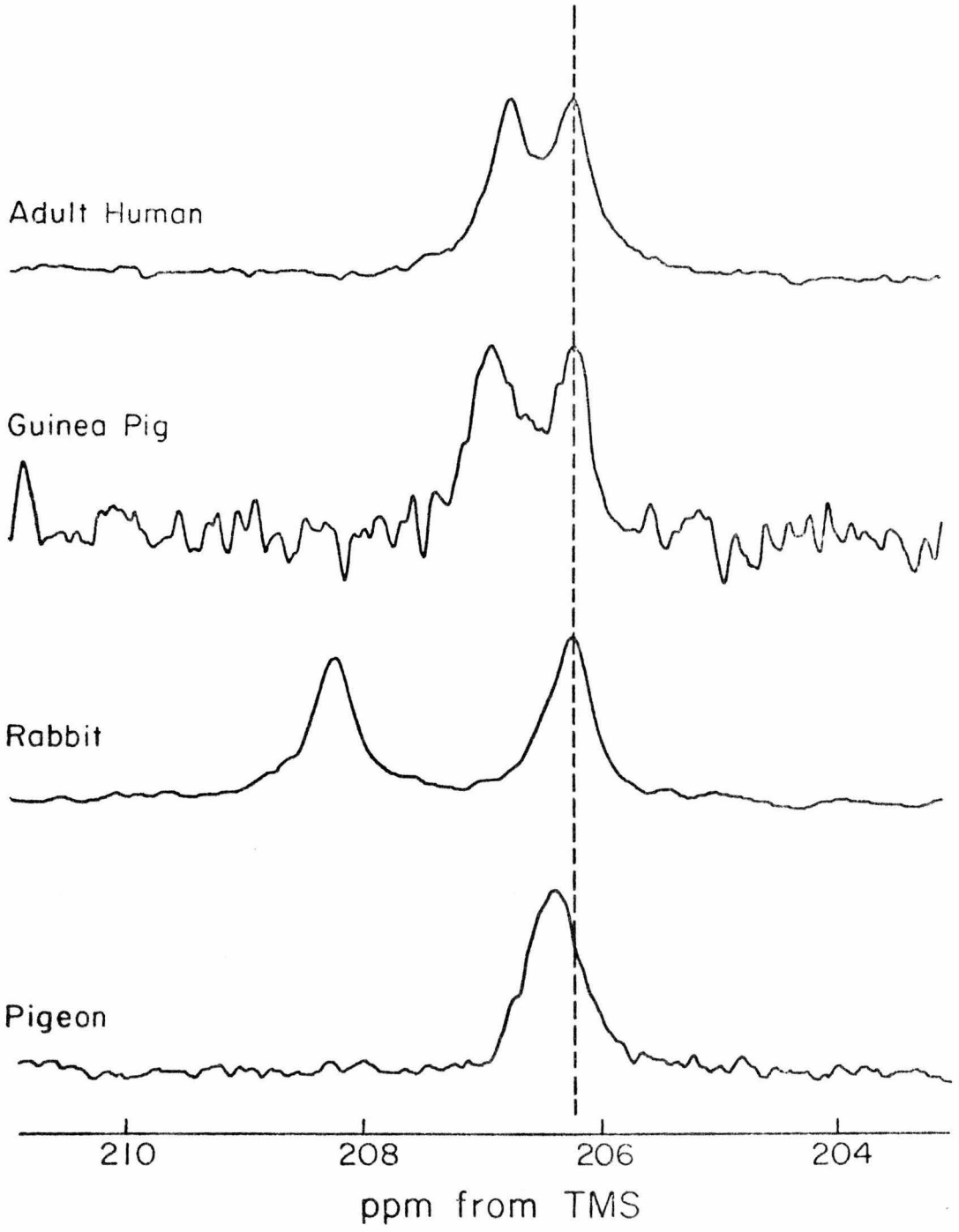
Adult Human
Guinea Pig
Rabbit
Pigeon
210
208
206
204
ppm from TMS

Table I: Hemoglobin ^{13}CO Chemical Shifts[#]

Source	δ (α-subunit)	δ (β-subunit)	pH
Human adult	206.75	206.19	6.35-8.00
Human fetal*	206.75	206.23	6.80
Human sickle cell	206.77	206.22	6.49-7.03
Bovine*	206.69	206.23	7.00
Mouse*	206.67	206.03	6.21
Guinea pig*	206.87	206.13	6.92
Rabbit	208.18	206.18	6.94-7.39
Frog*	206.63	206.19	6.82
Chick	206.73	206.20	7.05-8.70
Pigeon	(single peak: 206.36)		6.68-7.28
PMB-α	206.55	—	7.21-8.78
PMB-β	—	206.17	7.50-9.49
^{13}CO (solution)	184.60		

[#]Chemical shifts in ppm from external TMS ± .04

*Chemical shift based on a single sample of blood.

All the ^{13}CO resonances were pH independent in the range pH 6-8. The addition of a 2:1 or 5:1 molar excess of 2,3-diphosphoglycerate at pH 7.0-7.4 did not affect the chemical shifts of human or rabbit carboxyhemoglobins. Moreover, extensive carbamylation of the ϵ-amino groups of the lysines and of the α-amino groups of the terminal valine residues in human adult, sickle cell, or rabbit hemoglobin had no effect on the chemical shifts for the resulting carbamylated carboxyhemoglobin.

Studies of spin-lattice relaxation times (T_1) for the ^{13}CO resonances, determined by progressive saturation, yield a value of $T_1 = 0.3 \pm 0.1$ sec for both resonances of a single rabbit carboxyhemoglobin sample at pH 7.0 and 3 mM protein concentration. More extensive determinations of T_1 for the ^{13}CO resonances of several carbamylated sickle cell carboxyhemoglobin samples (containing amounts of methemoglobin from 3% to 60%) all give a uniform value of $T_1 = 0.60 \pm 0.16$ sec. As no systematic deviation in T_1 as a function of the relative amount of methemoglobin was observed, we conclude that the presence of this paramagnetic species does not significantly affect the relaxation of carbon monoxide bound to other subunits or molecules. Line widths for individual ^{13}CO resonances were 7 ± 1 Hz.

The observed nuclear Overhauser effects are: for rabbit carboxyhemoglobin, NOE = 1.1 ± 0.1 (for both resonances), and for carbamylated sickle cell carboxyhemoglobin, NOE = 1.00 ± 0.08 (for both resonances).

The subunits were separated as their p-hydroxymercuribenzoate

derivatives and Figure 2 shows the single sharp ^{13}CO resonances of isolated carboxy-HMB α and β subunits. The chemical shift for ^{13}CO bound to HMB β subunits corresponds exactly with that of the higher field resonances of adult and sickle cell carboxyhemoglobin. The chemical shift of the ^{13}CO bound to the HMB α subunits was consistently observed 0.2 ppm upfield of the lower field resonances of adult and sickle cell carboxyhemoglobin*. The positions of the ^{13}CO reso-

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* While this manuscript was in the review process, similar results were reported for normal adult carboxyhemoglobin by Vergamini et al., (1973).

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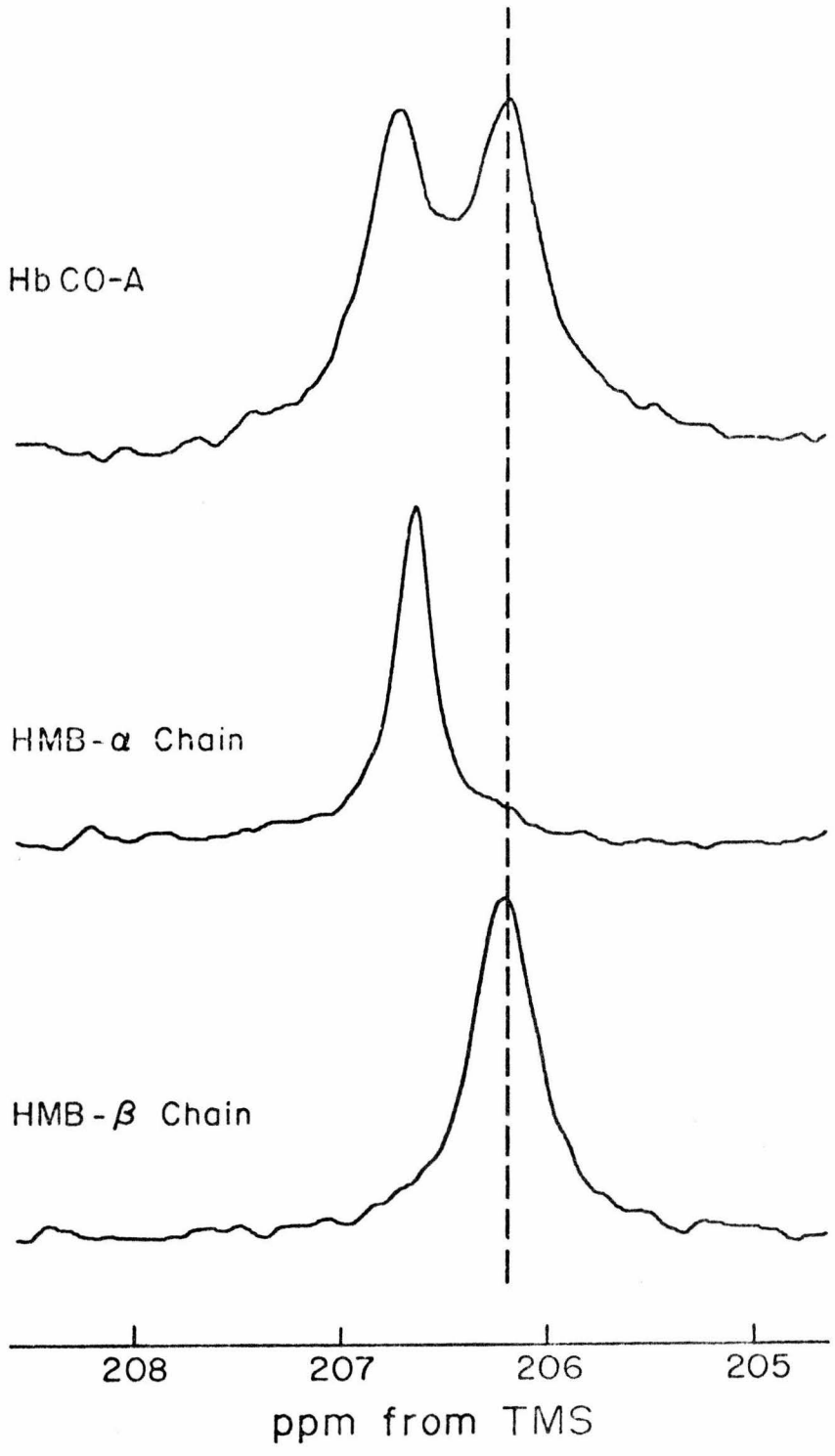
nances did not vary over the pH ranges listed in Table I. Accordingly, we assign the lower field resonance (206.7 ppm) to ^{13}CO bound to the α subunits and the higher field resonance (206.2 ppm) to ^{13}CO bound to the β subunits of adult and sickle cell carboxyhemoglobins.

Antonini et al. (1973) have made similar assignments based on separation of HMB derivatized chains, removal of the HMB, and nmr examination of the resulting mercurial-free ^{13}CO α and ^{13}CO β chains. The mercurial-free ^{13}CO β chains were observed to absorb upfield of the upfield resonance in the tetramer. The mercurial derivatives of both the α and β chains and the mercurial-free α chains exist as monomers whereas the mercurial-free β chains exist largely as tetramers (Yip et al., 1972) which may explain the difference in these results for separated, largely tetrameric β chains compared to ours. Also, the signal-to-noise and resulting resolution of the reported spectra seem

FIGURE 2

Spectra of the ^{13}CO resonances of a sample of intact human adult hemoglobin and the isolated HMB derivatives of its α and β subunits.


Hb CO-A
HMB-α Chain
HMB-β Chain
208
207
206
205
ppm from TMS

to make difficult the precise assignment of chemical shifts.

Addition of small amounts of air to rabbit carboxyhemoglobin causes the lower field resonance to decrease more than the higher field resonance (Figure 3). Similar though much less dramatic effects are observed when solutions of adult and sickle cell carboxyhemoglobins are exposed to limited amounts of oxygen. Reequilibration of such partially oxygenated samples with saturating amounts of ^{13}CO leads to regeneration of equal intensities for the two ^{13}CO resonances (as in spectrum A of Figure 3), thus eliminating the possibility that the preferential decrease in the resonance at lower field is the result of preferential oxidation of the α subunits to the methemoglobin state (Mansouri and Winterhalter, 1973).

Addition of low concentrations of ^{13}CO to solutions of rabbit deoxyhemoglobin at pH 7.0 in the absence of organic phosphates in closed atmosphere sample tubes shows more intense absorption of the higher field than of the lower field resonance; further, addition of saturating concentrations of ^{13}CO to these same samples gives high and low field absorptions of equal intensity (Figure 4).

FIGURE 3

The ^{13}CO resonance spectra of rabbit hemoglobin as a function of increasing exposure to air. The spectra were plotted in the absolute intensity mode to show the rapid decrease in the intensity of the α subunit resonance (low field) relative to the slower decrease in the intensity of the β subunit resonance (high field).

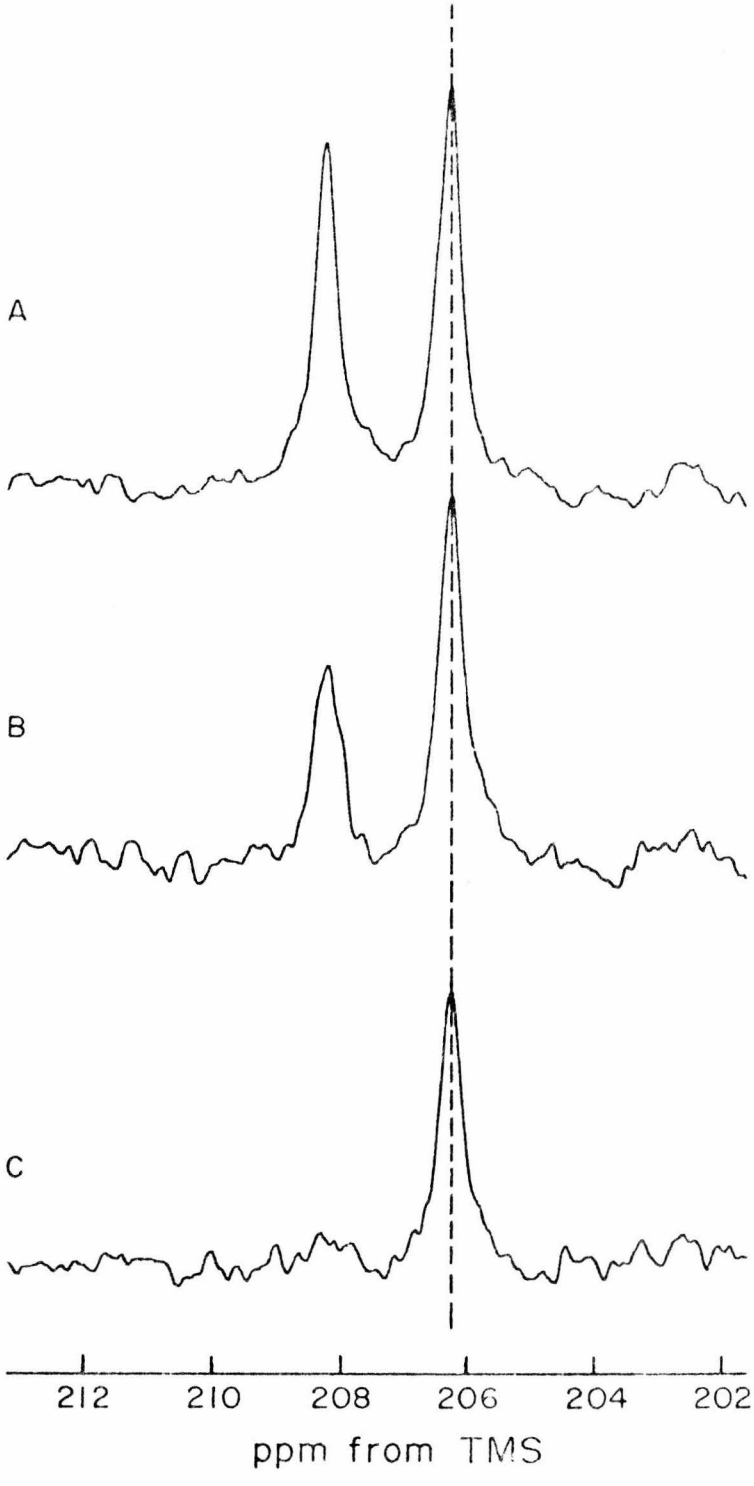
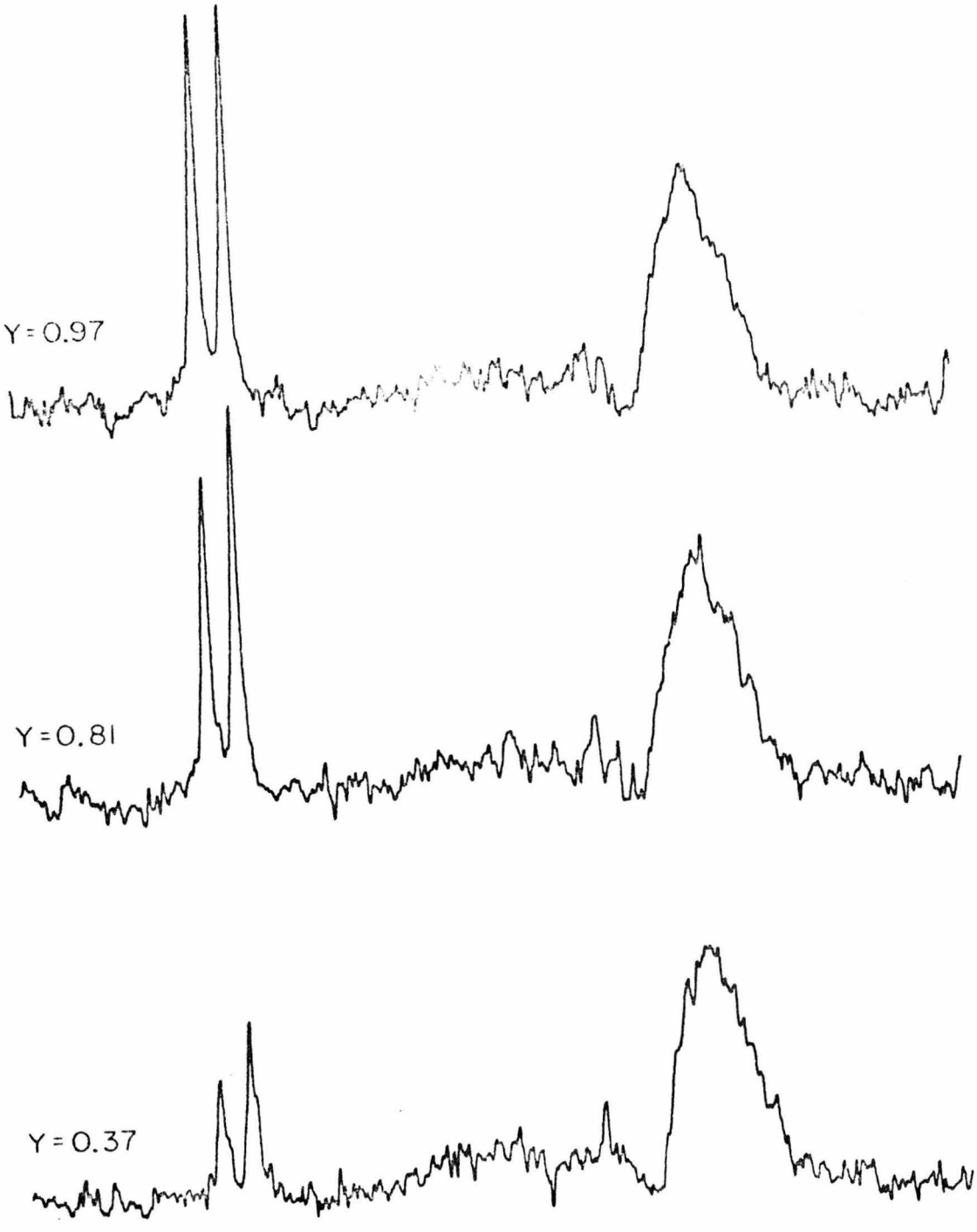

A
B
C
212
210
208
206
204
202
ppm from TMS

FIGURE 4

Spectra of the ^{13}CO and natural abundance peptide carbonyl resonances of rabbit hemoglobin treated with non-saturating amounts of ^{13}CO. The sample contained 5.0 ml of 2.0 mM hemoglobin in 0.15 M sodium chloride/0.05M tris buffer at pH 7.0 in a closed atmosphere sample tube of 76 ml volume. The lower spectrum indicates the distribution of ^{13}CO on the α and β subunits after equilibration against 600μl of ^{13}CO. Integration of the ^{13}CO and carbonyl (656 carbons at natural abundance) resonance regions indicates that the hemoglobin is about 37% saturated; 44% of the ^{13}CO resides on the α hemes and 56% of the ^{13}CO on the β hemes. The center spectrum shows the same sample after a total of 1500 μl of ^{13}CO have been injected into the tube (81% saturated; α 44%, β 56%), and the upper spectrum shows the sample after a total of 2100 μl ^{13}CO have been injected (97% saturated; α 50%, β 50%).


Y = 0.97
Y = 0.81
Y = 0.37

DISCUSSION

The results of this work confirm that carbon monoxide interacts differently with the heme group of α and β subunits in a wide variety of hemoglobins (Moon and Richards, 1972; Matwiyoff et al., 1972; Matwiyoff et al., 1973; Vergamini et al., 1973). Other studies have also revealed differences between the two types of subunits. For example, proton magnetic resonance studies (Lindstrom et al., 1972) show that subtle differences exist in the protein conformation around the α and β heme groups of various human carboxyhemoglobins. Kinetic differences in the reactivities of the α and β subunits have been reported for the binding of nitric oxide (Henry and Cassoly, 1973) and n-butylisocyanide (Olson and Gibson, 1972). Quantitative studies using a spin-labeled analogue of ATP led Ogata and McConnell (1972a,b) to conclude that a quantitative description of hemoglobin ligation requires modification of the simple two-state allosteric model (Monod et al., 1965) by the explicit recognition of the nonequivalence of the α and β subunits.

Chemical Shift - Environment. The studies on separated HMB chains indicate that the lower field resonance arises from ^{13}CO bound to α subunits while the higher field resonance represents ^{13}CO bound to β subunits in adult and sickle cell hemoglobin. By analogy we make similar assignments for the other species of hemoglobin studied including rabbit hemoglobin, the upfield resonance of which falls at the same position as that of other ^{13}CO β subunits. In our view the low field resonance represents ^{13}CO bound to rabbit α subunits which, we

conclude, must be anomalous. These assignments agree with those made previously (Moon and Richards, 1972) on the basis of a rather circuitous analogy to kinetic studies of the relative rates of oxygen displacement by carbon monoxide in α as compared to β subunits (Olson et al., 1971).

The environment of ^{13}CO seems to be essentially the same when bound to the tetramer as when bound to the isolated, monomeric HMB α or β subunit. (The chemical shift for the ^{13}CO β resonance in the tetramer is identical to that for the ^{13}CO HMB β subunit, while that for the ^{13}CO α resonance in the tetramer is 0.2 ppm downfield of that for the ^{13}CO HMB α subunit.) This observation suggests that, in the completely liganded state, interactions between subunits influence little, if at all, the environments of the ligands; such interactions appear to play their dominant role in the unliganded or partially liganded states of the "tight" quaternary conformations of the hemoglobin tetramer.

We observe that the ^{13}CO chemical shifts of carboxyhemoglobins do not depend on pH in the range pH 6-8. Other studies do suggest changes in this pH range. For example, Ogawa and Shulman (1971) reported that the heme-proton chemical shifts of cyanmethemoglobin and several "mixed-state" hemoglobins are markedly sensitive to pH over this range, a sensitivity they attributed to changes in the quaternary conformation of the molecule. Olsen and Gibson (1973) observed a pH and ionic strength dependence of the relative affinities of the α and β subunits for n-butyl isocyanide; the behavior of the two subunits in this regard becomes almost equivalent at high pH. If any similar changes do occur with carboxyhemoglobins they are not manifest in

the environment of the bound carbon monoxide or nature of the bond between the heme iron and the ligand as measured by chemical shifts of the bound ^{13}CO.

What are the origins for the differences between the environments experienced by carbon monoxide bound to α or to β subunits? We interpret the evidence presently at hand to suggest that these chemical shift differences reflect principally effects transmitted from the polypeptide helices through the prophyrin ring or the proximal histidine (F8) to the heme iron and thence to the ligand. These effects may have significant stereochemical contributions arising, for example, from the precise orientation of the heme group within its binding cleft.

More specifically, changes in the region of the C/D helices seem to play an especially dominant role. Three major arguments support this conclusion: (i) absence of a pH dependence of the chemical shifts; (ii) absence of differences in chemical shifts for ^{13}CO bound to α-type or β-type subunits with a wide variety of amino acid substitutions contrasted to a generally consistent difference between ^{13}CO bound to α-type subunits versus ^{13}CO bound to β subunits; (iii) the anomalously low field resonance for ^{13}CO bound to rabbit α subunits.

(i) Though evidence cited earlier suggests that minor differences, particularly in the quaternary structure, may exist as a function of pH and can be reflected within the heme pocket, such differences are not manifest in the observed ^{13}CO chemical shifts. We believe this indicates that small conformational changes may occur within the pocket where the bound carbon monoxide resides without influencing the chemi-

cal shift of this ligand because the bond between it and the heme iron has not been altered.

(ii) In fetal hemoglobin, there are thirty-nine amino acid substitutions in the γ subunits compared to the β subunits of normal adult hemoglobin (Schroeder et al., 1963). Two of these substitutions (E14 and E15) occur within 4Å of the heme (Perutz, 1969) and have been observed to cause changes in the ^{1}H resonances of the methyl protons of the valine residue (E11) to which they are adjacent (Lindstrom et al., 1972). Moreover, valine E11 directly contacts the carbon monoxide ligand in the insect hemoglobin erythrocruorin (Huber et al., 1970) and therefore is presumably at least very near the carbon monoxide in β-type chains of other hemoglobins as well. Yet carbon monoxide has the same chemical shift when bound either to human adult β or to fetal γ subunits. We therefore conclude that the chemical shift does not sensitively reflect such changes in the pocket within which the ligand dwells.

Similar comments apply to α chains as a wide range of α chains of most species exhibit an unchanging resonance for bound ^{13}CO though many amino acid substitutions occur.

However, there is a generally consistent difference between ^{13}CO bound to α compared to β subunits which we believe reflects the major structural difference between α-type and β-type subunits. Subunits of the β-type possess a D helix; subunits of the α-type lack a D helix.

(iii) Many amino acid substitutions have just been shown to be possible without perturbing the chemical shift of ^{13}CO bound to β-type

chains or bound to α-type chains. However, rabbit α chains have markedly anomalous properties not only with respect to the chemical shift of bound carbon monoxide but also with regard to the unusually facile oxidation of the heme iron of rabbit α chains (Matwiyoff et al., 1973). Between rabbit and human α chains there are a total of 25 amino acid substitutions (Ehrenstein et al., 1966). Three of these seem to us of especial significance: Val-29 (B10), Phe-48 (CD7) and Thr-49 (CD8) compared to the human α chain pattern of Leu-29, Leu-48 and Ser-49 (Braunitzer et al., 1961). In two of these three residues (29 and 49) substitutions are unique to rabbits. In the third (48) species variability has been observed but always between homologous amino acids (Val or Leu) (Dayhoff, 1972); only in rabbits is the change to an aromatic residue such as Phe seen. Rabbits also have a unique substitution in Ser-63 (Ehrenstein et al., 1966) but as the side chain of this residue points away from the heme pocket, and as it does not change in the other rabbit hemoglobin allele (see following paragraph) we have not deemed it especially significant compared to the three substitutions on which we should like to focus attention (B10, CD7 and CD8). In the tertiary structure of the α chain residue B10 lies in close proximity to CD7 and CD8 which are in turn directly adjacent to the region occupied by the D helix in β chains. (In fact, because of its uniquely large size, we feel that the substitution of the aromatic residue, Phe 48, at CD8 may well be the dominant change.)

Matwiyoff et al. (1973) recently reported that three resonances are observed for ^{13}CO bound to hemoglobins from some Dutch lop

rabbits, two of which (those at highest and lowest field) occur at the positions we have consistently observed for our rabbit carboxyhemoglobins and a third (at intermediate field) which occurs at a position characteristic for ^{13}CO bound to α subunits of human and most other hemoglobins. The sum of the intensity of the α-^{13}CO resonances at intermediate and lowest fields equals the intensity of the high field β-^{13}CO resonance. This suggests that these rabbits have two types of α subunits and Hunter et al. (1969) have shown that two α chain alleles are, in fact, present in Dutch lop rabbits. One allele has the normal rabbit α chain sequence at 29, 48 and 49, while the other allele has amino acids at these positions found in most other α chains (Leu-29, Leu-48 and Ser-49). Moreover, all other known variations of the normal rabbit α chain (Val-29, Phe-48, Thr-49) involve reversions back to residues common to α chains of hemoglobins from humans and other species (Leu-29, Leu-48, Ser-49).

These observations suggest that changes in the region of the C/D helices can profoundly influence the bound ligand. Deletion of the D helix alters the angle between the E and F helices which in turn should modify interactions between the protein and heme (as well, possibly, as interaction between the iron and proximal histidine F8). Thus, the absence of the D helix in α chains may largely account for the way in which these chains bind ligand differently than β chains. In a similar way, in α chains themselves, changes in the C/D region can affect interaction between the protein and the heme, in turn modifying the interaction between the iron of these hemes and ligands such as carbon

monoxide.

Relative Affinities (Oxygen versus Carbon Monoxide). The results of this work give some indication of the relative thermodynamic affinities of the α and β subunits for oxygen relative to carbon monoxide-particularly for rabbit hemoglobin. In general, hemoglobins have a higher affinity for carbon monoxide relative to oxygen by factors of the order of 230-260: 1 (Joels and Pugh, 1958). We find that admission of small quantities of oxygen to samples of rabbit hemoglobin previously saturated with carbon monoxide and in the absence of organic phosphates leads first to a decrease in the intensity of the low field α chain ^{13}CO resonance indicating displacement of carbon monoxide by oxygen from the α chains. (To ensure that this observation is not caused by preferential oxidation or denaturation of the α chains, we demonstrated that subsequent removal of oxygen and resaturation with ^{13}CO regenerates the original spectrum; monitoring the electronic spectrum at 418 nm also shows no evidence for formation of methemoglobin.) Accordingly we conclude that the α chains are less discriminating than β chains for carbon monoxide as compared to oxygen as a ligand. Though this more facile displacement of carbon monoxide by oxygen from the α chains is especially marked in rabbit hemoglobin, it is also seen to a lesser degree in human and other hemoglobins. Based on the time scale of these experiments (minutes to hours) we identify these differential preferences as thermodynamic and not kinetic effects.

Order of Ligation of Carbon Monoxide. Some disagreement exists on the preferences of the two kinds of subunits for carbon mon-

oxide relative to their unliganded states. Based on studies of the binding of n-butyl isocyanide and carbon monoxide to hemoglobin, Gray and Gibson (1971) identified the β subunit as being more reactive (kinetically) to carbon monoxide. On the other hand, Huestis and Raftery (1972) concluded that the α subunit has the higher equilibrium affinity for carbon monoxide based on their studies of a hemoglobin derivative with a trifluoroacetonyl group attached to cysteine-93β. Our results (Figure 4) demonstrate that for rabbit hemoglobin at moderate saturation with carbon monoxide ($Y \sim 0.37$) more ligand is bound to the β than to the α subunits ($\beta^{CO}/(\alpha^{CO} + \beta^{CO}) \sim 0.56$).

One explanation of this apparent discrepancy may lie in the quaternary conformations of the partially liganded hemoglobins which may conceivably be different in our work and that of Gray and Gibson on the one hand compared to that of Huestis and Raftery on the other. To make the argument, we first note that the oxygen affinity of dissociated β chains has been found to exceed somewhat that of α chains. Indeed, Ogata and McConnell (1972b) incorporated these observations into their generalized concerted transition model where they assume that, in the oxy (relaxed, R) conformation the β chains have a higher affinity for oxygen than do the α chains. Conversely, in the deoxy (tight, T) quaternary state, the α chains seem to have the higher oxygen affinity. (The assumption that the equilibrium ligand affinity of the separated chains reflects that of the chains in the R tetramer finds support in our observation, previously discussed, that the chemical shifts of ^{13}CO are virtually identical whether this ligand is bound

to the intact, completely liganded and, therefore, R tetramer or to the separated HMB subunits.)

Accordingly, if, in the T state, α subunits have a higher affinity while, in the R state, β subunits have the higher affinity, one might well have situations where at low total ligation with the tetramer predominantly in the T state, the α chains preferentially liganded, whereas at moderate total ligation, with the tetramer predominantly in the R state, the β chains have more bound ligand.

Moreover, if the relative ligand affinities of the two types of subunits differ more with carbon monoxide than with oxygen as a ligand as some studies suggest, (Olson and Gibson, 1973; Ogata and McConnell, 1972b), this effect (more ligand on α chains at low saturation, more ligand on β chains at moderate saturation) might be accentuated with carbon monoxide as ligand.

These relative affinities are undoubtedly also strongly influenced by the presence of organic phosphates, and this condition varied within the group of studies cited.

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PART III

^{13}C Magnetic Resonance Studies of the Binding of Carbon Monoxide to Myoglobins and Hemoglobins Containing Unnatural Hemes

INTRODUCTION

The nature of the heme-protein interactions which are responsible for the kinetic and thermodynamic control of heme-ligand reactivity in myoglobin and hemoglobin is not yet clearly understood. The major conformational differences existing between unliganded deoxyhemoglobin and fully liganded forms of the protein have been interpreted to explain much about the mechanism of positive cooperativity in hemoglobin (Perutz, 1970) and suggest the possible role of steric constraints operating to exclude ligands from a heme in one protein conformation while in another conformation providing for unobstructed ligand approach.

Though simple steric effects controlling access to the distal side of the heme may be responsible for the regulation of oxygen affinity, more direct electronic control over heme iron reactivity may also be of importance. Though a general understanding of iron-porphyrin complexes has been developed, there remains considerable confusion about the large variations in heme iron reactivity of the various heme proteins. Early studies of reconstituted hemoglobins containing hemes modified at the 2- and 4- positions of the porphyrin ring clearly demonstrated that such substitutions could have pronounced effects on ligand affinity and the reactivity of the heme (Rossi Fanelli et al., 1959; Antonini et al., 1960). Later studies (Sugita et al., 1971) suggested that the oxygen affinities of reconstituted hemoglobins containing modified hemes reflected the inductive electronic nature of the modifying substituent. More recent studies, however, suggest that these apparent

electronic substituent effects are coincidental and that the alterations in the oxygen affinities of these reconstituted hemoglobins are due to steric perturbations induced in the globin structure by the modifying substituents which result in anomalous allosteric behavior (Yonetani et al., 1974; Yamamoto and Yonetani, 1974).

We have previously reported the application of ^{13}C magnetic resonance to study the chemical environment of ^{13}C-enriched carbon monoxide bound to the heme groups of the α and β subunits of various hemoglobins (Moon and Richards, 1972; Moon and Richards, 1974). These studies clearly demonstrated the inherent differences existing between the environments experienced by the ^{13}C nucleus of ^{13}CO bound to hemes in the α subunits as compared to those in the β subunits. The relative thermodynamic affinities of the α and β subunits for ^{13}CO and the facility with which oxygen displaces ^{13}CO in some hemoglobins was also shown to vary. These differences between the subunits of hemoglobin may be caused by steric factors operating directly on the bound ^{13}CO ligand or electronic effects stemming from changes in the interaction between the iron atom of the heme and the proximal histidine residue (F8) or by changes in the interaction of residues lining the heme pocket with the prophyrin ring.

In this work we report our recent studies of ^{13}CO bound to modified hemes reconstituted with dolphin apomyoglobin and human and rabbit apohemoglobins. The results have been examined with particular reference to the potential role of amino acid residues surrounding the heme pocket in determining the chemical behavior of the heme. We

have studied to what extent substituents at the periphery of the porphyrin ring can affect the electronic nature of bound ligands and the importance of these substituent effects relative to other steric factors which may be dominant in determining the ligand environment. These results are compared with the ^{13}CO chemical shifts obtained for a variety of native myoglobins and hemoglobins from various animal species.

METHODS

Myoglobins

Sperm whale and horse heart myoglobins were obtained from Sigma.

Dolphin myoglobin, used in all reconstitution studies, was prepared from frozen wright's whale dolphin muscle obtained from Marineland of the Pacific in Palos Verdes, California. The isolation procedures used were essentially those of Hugh and Gurd (1970). The dolphin muscle (135 gm) was diced and combined with 300 ml of chilled 65% ammonium sulfate solution in a Waring blender and homogenized for 0.5 hour at 4°C. The crude extract was centrifuged at 15,000 x g for 30 minutes to remove solids. The resulting supernatant was then oxidized with two equivalents of potassium ferricyanide and exhaustively dialyzed against 0.03 M 2-amino-2-methyl-1,3-propanediol buffer at pH 8.7. The crude myoglobin was then applied to a column of DEAE-Sephadex and chromatographed with the same buffer. The fast-moving fraction was collected and concentrated by ultrafiltration.

Hemoglobins

Hemoglobins were prepared from freshly drawn, citrated whole blood obtained from a wide variety of animal sources by the methods described previously (Moon and Richards, 1974). The various animal sources included human, bovine, mouse, guinea pig, rabbit, opossum, chick, pigeon, bullfrog, carp, gar and torpedo ray. The monomeric hemoglobin from the blood worm, Glycera dibranchiata, was prepared from worms obtained through Pacific Biomarine Supply Co. The worms were washed and bled into cold artificial sea water. The red cells were

collected by centrifugation at 500 x g and washed several times with sea water. The packed red cells (about 15 ml) were then lysed hypotonically with three volumes of distilled water at room temperature for 30 minutes. The stromata were removed by centrifugation at 30,000 x g and the resulting hemolysate was dialyzed exhaustively against 0.15 M sodium chloride (three 4 l changes) at 4°C. The crude glycera hemoglobin was then concentrated by ultrafiltration (Amicon UM-10 membrane), centrifuged to remove traces of precipitated material, and applied to a 5 x 100 cm column of G-75 Sephadex. The column was eluted with 0.15 M sodium chloride and the slow moving band of monomeric glycera hemoglobin was collected and concentrated by ultrafiltration.

Modified Hemes

Crystalline protohemin chloride was obtained from Sigma.

Deuterohemin chloride was prepared from protohemin chloride by methods essentially like those of Fischer and Orth (1937a) and similar to those of Caughey et al. (1966) used for the synthesis of deuteroporphyrin IX dimethyl ester. Protohemin chloride (4 g) was mixed with resorcinol (16 g) and heated in an oil bath at 200°C for 15 minutes. The melt was cooled, extracted with ether and filtered. The resulting solid of crude deuterohemin chloride was dissolved in about 10 ml pyridine and mixed with 300 ml chloroform and 300 ml methanol in a 1 l round bottom flask. Hydrogen chloride was bubbled through the mixture for about one hour. The solution was then transferred to a separatory funnel and extracted with 200 ml portions of 10% hydro-

chloric acid (twice), water, 10% ammonium hydroxide (twice), and again with water. The chloroform layer was then dried with anhydrous sodium sulfate, filtered and evaporated to dryness on a rotary evaporator. The crude deuterohemin dimethyl ester was then taken up in 30 ml 1,2-dichloroethane and chromatographed on neutral alumina (activity V) using 1,2-dichloroethane as the eluent. The purified deuterohemin dimethyl ester, eluted in the first large wine-red band off the column, was evaporated to dryness on a rotary evaporator. The yield of the dimethyl ester was 58.2%. A portion of this product (1.2 g) was saved for the synthesis of 2,4-diacetyldeuterohemin chloride. The remainder of the deuterohemin dimethyl ester was saponified in 1 % methanolic potassium hydroxide. The methanolic solution was extracted with chloroform to remove unreacted ester and then acidified with dilute hydrochloric acid to about pH 4. The resulting precipitate of deuterohemin chloride was collected in a sintered glass funnel, washed with water and dried.

2,4-Diacetyldeuterohemin chloride was prepared by a method adapted from that of Fischer and Orth (1937b) and is similar to the method of Brockman et al. (1968). Deuterohemin dimethyl ester (1.17 g, prepared above) was dissolved in 150 ml chloroform. To this mixture was added 150 ml acetic anhydride and 15 ml anhydrous stannic chloride. The reaction mixture was stirred for one hour and then poured into a mixture of ice and ammonium hydroxide. After standing overnight, the methylene chloride layer was washed several times with water, dried over anhydrous sodium sulfate, and concentrated on a rotary

evaporator. The concentrate was then applied to a column of neutral alumina (activity V) and chromatographed with methylene chloride to remove impurities. After all contaminating bands had been eluted, leaving only the green product band, the solvent was changed to chloroform and the 2,4-diacetyldeuterohemin dimethyl ester was immediately eluted. The dimethyl ester was hydrolyzed in 1% methanolic potassium hydroxide and acidified with dilute hydrochloric acid. The precipitate of 2,4-diacetyldeuterohemin chloride was collected in a sintered glass funnel, washed several times with water and dried. The yield was 38.8%.

Mesohemin chloride was prepared by a method adapted from that of Caughey et al. (1966) for the synthesis of mesoporphyrin IX dimethyl ester from protohemin chloride. Wet palladium oxide (3 g dry wt.) was freshly prepared (Stun and Hixana, 1956) and mixed with 1.63 g protohemin chloride and 150 ml 90% formic acid. Hydrogen was passed through this mixture as it was heated at 90 °C for one hour. The reaction was followed spectophotometrically by observing the Soret maxima of small aliquots of the mixture in 80% formic acid. Under these conditions protoporphyrin IX exhibits a Soret band at 407 nm. The reaction mixture was poured into 600 ml 30% ammonium acetate and the precipitate of crude mesoporphyrin IX was collected in a sintered glass funnel. The dry product was converted to the dimethyl ester as in the preparation of deuterohemin dimethyl ester described above. The iron was then reintroduced into the porphyrin ring (Falk, 1964) and the crude mesohemin product was chromatographed on neutral

alumina with 1,2-dichloroethane. The second band (red brown) was collected. The methyl ester was hydrolyzed in 1% methanolic potassium hydroxide and acidified with dilute hydrochloric acid. The mesohemin chloride precipitate was collected in a sintered glass funnel, washed with water and dried. The yield was 34.9%.

Hematoporphyrin IX was obtained from Sigma and converted to hematohemin by introduction of iron into the porphyrin ring (Falk, 1964). The yield of hematohemin was 42%.

Electronic spectral data of the pyridine hemochromes synthesized are collected in Table I. The hemochromes were prepared by dissolving a small amount of the hemin in an aqueous solution of 0.1 M potassium hydroxide and 3 M pyridine. The solutions were then reduced with a small amount of sodium dithionite. Pyridine hemochrome spectra were recorded on a Cary 14 spectrophotometer.

Infrared analysis of the carbonyl absorptions of 2,4-diacetyldeuterohemin dimethyl ester was also carried out. The methyl ester carbonyl stretching frequency was observed at 1750 cm^{-1}. The acetyl carbonyl was observed at 1675 cm^{-1}. The ratio of the two absorption bands, $A_{\text{acetyl CO}}/A_{\text{ester CO}} = 0.75$ is similar to the value obtained by Caughey et al. (1966).

Apomyoglobin

Apomyoglobin was prepared by the method of Hapner et al. (1968). Yields of the lyophilized product ranged between 2.0% and 2.9% apomyoglobin per unit wet weight of muscle.

TABLE I: Electronic Spectra of Pyridine Hemochromes[a]

Heme	α		β		Soret	
	Obs	Lit	Obs	Lit	Obs	Lit
Mesa	546 nm	547 nm	517 nm	518 nm	407 nm	407 nm
Deutero	545 nm	545 nm	516 nm	515 nm	406 nm	406 nm
Hemato	553 nm	549 nm	522 nm	519 nm	415 nm	-
2, 4-Diacetyl-deutero	574 nm	573 nm	538.5 nm	539 nm	439 nm	439 nm

[a] Literature values were taken from J. E. Falk, "Porphyrins and Metalloporphyrins", (Elsevier Publishing Co. 1964) p. 240.

Apohemoglobin

Human and rabbit apohemoglobins were prepared by the method of Winterhalter and Huehns (1964).

Reconstitution of Myoglobins and Hemoglobins

The procedure used for reconstitution of dolphin apomyoglobin with modified hemes was an adaptation of the method described by Atassi and Caruso (1968). Hemin solutions were prepared in 0.1 M sodium phosphate (dibasic) with 1% sodium cyanide. About a two-fold excess of hemin solution was added dropwise to a stirred apomyoglobin solution in distilled water at 0°C. After stirring at 0°-4°C for about two hours the reconstituted myoglobin was applied to a column of G-15 Sephadex and eluted with 0.01 M dibasic phosphate. The samples were then concentrated by ultrafiltration.

Human and rabbit apohemoglobins were reconstituted with modified hemes by a method essentially like that of Winterhalter and Huehns (1964). About 1.0 g of the lyophilized apohemoglobin powder was dissolved in 100-120 ml water and was dialyzed overnight at 4°C against 4 l of phosphate buffer, pH 6.8. The protein was then dialyzed against two 1 l changes of pH 7.5 phosphate buffer for 1.5 hours each. Precipitated material amounting to about 10-15% of the total apohemoglobin was removed by centrifugation at 4°C. About a 1.5-fold excess of hemin was dissolved in a minimal amount of 0.1 N sodium hydroxide (~ 1 ml) and was diluted to 25 ml with 0.01 M sodium cyanide/0.01 M phosphate buffer, pH 7.5. The hemin solution was added dropwise to

the apohemoglobin solution and stirred for several hours at 4°C. The resulting cyanmethemoglobin solutions were dialyzed against cyanide/phosphate, pH 7.5, in an ultrafiltration cell (Amicon PM-10 membrane) to remove excess hemin. The solutions were then dialyzed against 0.15 M sodium chloride and concentrated by ultrafiltration.

NMR Samples

Heme-modified carboxymyoglobin and carboxyhemoglobin samples were prepared by shaking the corresponding cyanmet derivative with 90% ^{13}C-enriched carbon monoxide (Prochem or Analytical Supplies Development Corp.) and a 1.5-fold excess of sodium dithionite in a syringe. The ^{13}CO derivatives were then transferred to an ultrafiltration cell, dialyzed against buffer (0.01 M phosphate for myoglobins; 0.15 M sodium chloride for hemoglobins) and concentrated. Sample concentrations were 1-3 mM myoglobin monomer or hemoglobin tetramer as determined spectrophotometrically from the Soret maxima using an approximate extinction coefficient of 154 mM for the carbon monoxide derivatives. Sample pH was determined on a Radiometer Model 26 pH meter.

NMR Studies

^{13}C magnetic resonance spectra were obtained using the pulse Fourier transform technique on a Varian Associates XL-100-15 spectrometer operating at 25.17 MHz. The spectrometer was interfaced with a Varian 620i computer (16 K memory) for data accumulation and Fourier transformation. A sensitivity enhancement of 0.10 sec was applied to the free induction decay before the Fourier transforma-

tion was carried out. Samples were contained in a 12 mm tube with a 5 mm tube containing D_2O inserted concentrically to serve as a field frequency lock. All spectra were obtained at a probe temperature of 32 °C using a 90° pulse of 150 μsec duration and an acquisition time of 0.8 sec. Proton noise decoupling with a bandwidth of 1.5 KHz centered on water was used throughout.

RESULTS

The observed ^{13}C chemical shifts for the ^{13}CO ligand bound to native myoglobins and hemoglobins obtained from a wide variety of animal sources are collected in Table II. All native myoglobin samples show a single sharp resonance about 207.8 ppm downfield of external tetramethylsilane. All native hemoglobin samples exhibit two distinct resonances except in the case of pigeon hemoglobin and the monomeric hemoglobin from blood worm. The assignment of these hemoglobin resonances to the α and β subunits is based on studies of the individual p-mercuribenzoate (PMB)-derivatized subunits and analogy between species (Moon and Richards, 1974). In general, most β subunit ^{13}CO resonances occur 206.2 ppm downfield of Me_4Si. Within experimental accuracy, there is almost no deviation in the ^{13}CO shift between the β subunits of the species studied. The one major exception is the abnormally high field resonance (205.21 ppm) observed in torpedo ray hemoglobin which we have tentatively assigned to the β subunit. The only other minor differences are observed in mouse and perhaps carp hemoglobins where the β resonances appear slightly upfield of the others. The observed resonances for ^{13}CO bound to α subunits show somewhat more variability. While most species exhibit the α resonance at 206.7 ppm, the ^{13}CO resonances assigned to the α subunits of rabbit (208.18 ppm), opossum (207.42 ppm) and torpedo ray (206.96 ppm) exhibit anomalous shifts to low field. Minor deviations in the α resonances of guinea pig (206.87 ppm) and gar (206.48 ppm) may also be of significance.

TABLE II: ^{13}C Chemical Shifts for ^{13}CO Bound to Native Myoglobins and Hemoglobins[a]

Source	δ (α subunit)	δ (β subunit)
Myoglobins:		
Sperm Whale	207.93	
Dolphin	207.70	
Horse	207.80	
Hemoglobins:		
Human adult	206.75	206.19
Human fetal[b]	206.75	206.23
Human sickle cell	206.77	206.22
Bovine[b]	206.69	206.23
Mouse[b]	206.67	206.03
Guinea pig[b]	206.87	206.13
Rabbit	208.18	206.18
Opossum	207.42	206.13
Chick	206.73	206.20
Pigeon	(unresolved peak: 206.36)	
Frog[b]	206.63	206.19
Carp[b]	206.62	206.07
Gar[b]	206.48	206.19
Torpedo ray[b]	206.96	205.21
Blood worm (monomer)	205.45	
PMB α (adult)	206.55	-
PMB β (adult)	-	206.17
^{13}CO (solution)	184.60	

[a] Chemical shifts in ppm from external Me_4Si ± 0.05.

[b] Chemical shift based on a single sample of blood.

Pigeon hemoglobin shows an unresolved peak at 206.36 ppm suggesting that the α and β subunit resonances are within 0.2 ppm of each other. This may result from a "normal" β resonance at 206.2 ppm and an abnormally high field α resonance at 206.4 ppm. The monomeric glycera hemoglobin from blood worm shows a single resonance, as expected. No change was observed between samples of the high molecular weight oligomeric fraction of glycera hemoglobin and the purified monomer.

The chemical shifts for ^{13}CO bound to the modified hemes of reconstituted dolphin myoglobin, human adult hemoglobin and rabbit hemoglobin are collected in Table III. The apoproteins reconstituted with protoheme exhibited chemical shifts virtually identical to those of the native proteins and had excellent stability. No significant precipitation had occurred in the protoheme-containing samples after several hours of nmr study at 32°C. The effect of sample pH on reconstituted protomyoglobin, as shown in Figure 1, was essentially the same as that observed for native dolphin myoglobin. Samples containing modified hemes all showed reduced stability toward thermal denaturation and precipitation. This was particularly true of hematomyoglobin (not listed in Table III) which precipitated rapidly at 32°C, but gave a consistent shift of 207.71 ppm in three separate samples. No hematohemoglobin samples were prepared. While all reconstituted myoglobin samples were insensitive to pH above 7, myoglobin reconstituted with unnatural hemes showed increased susceptibility to acid denaturation. As shown in Figure 1, the ^{13}CO resonance of protomyoglobin begins

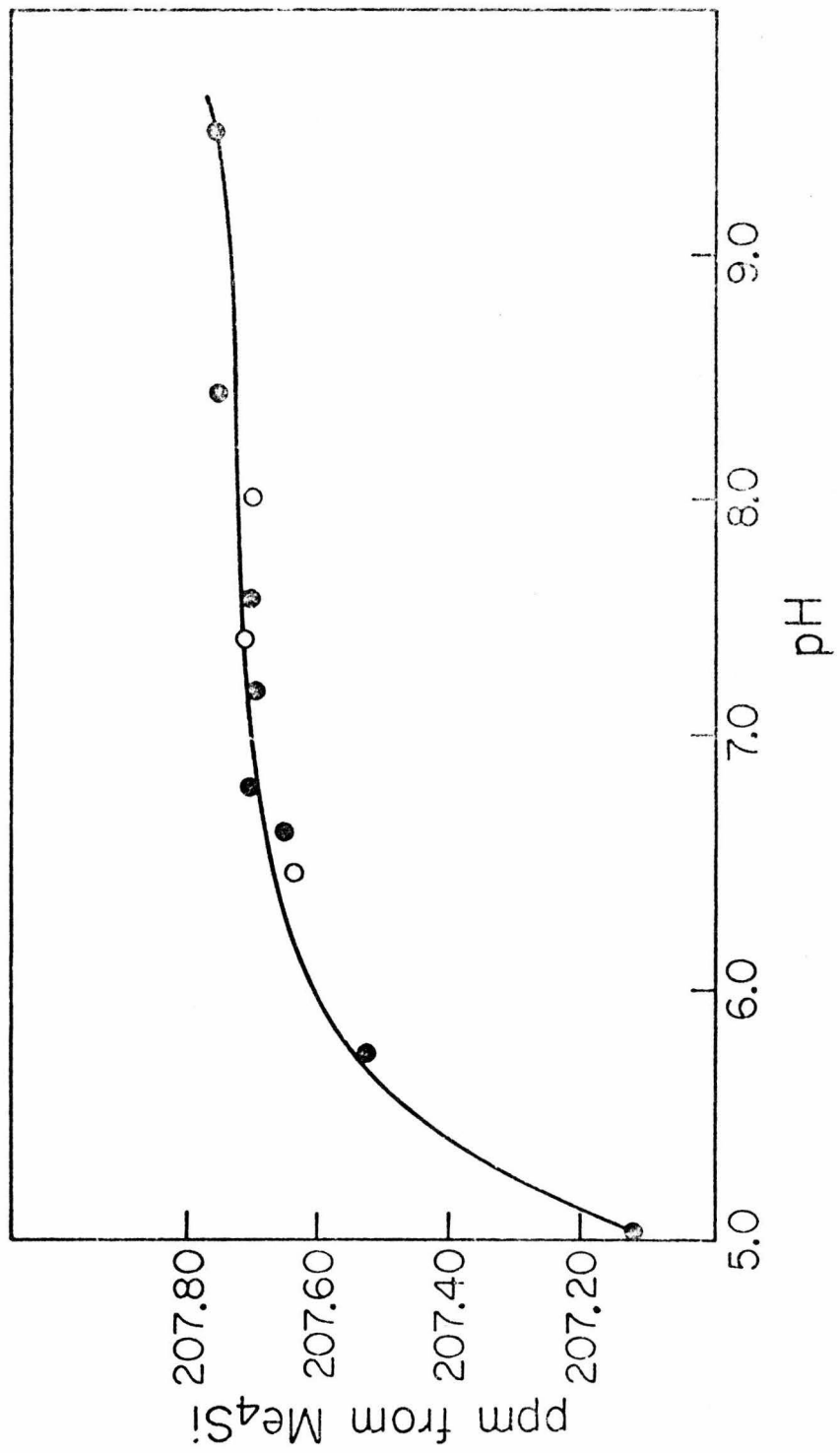
TABLE III: ^{13}CO Chemical Shifts for Dolphin Myoglobin, Adult and Rabbit Hemoglobin Containing Unnatural Hemes[a]

	CH_3CH_2-	H-	CH_2=CH-	CH_3CO-
Myoglobin	207.91	207.71	207.70	207.19
Adult-α	207.04	206.65	206.79	205.85
Adult-β	206.78	206.27	206.16	205.28
Rabbit-α	208.61	208.45	208.21	not observed
Rabbit-β	206.69	206.30	206.15	205.22

[a] Chemical shifts in ppm from external Me_4Si ± 0.08.

FIGURE 1

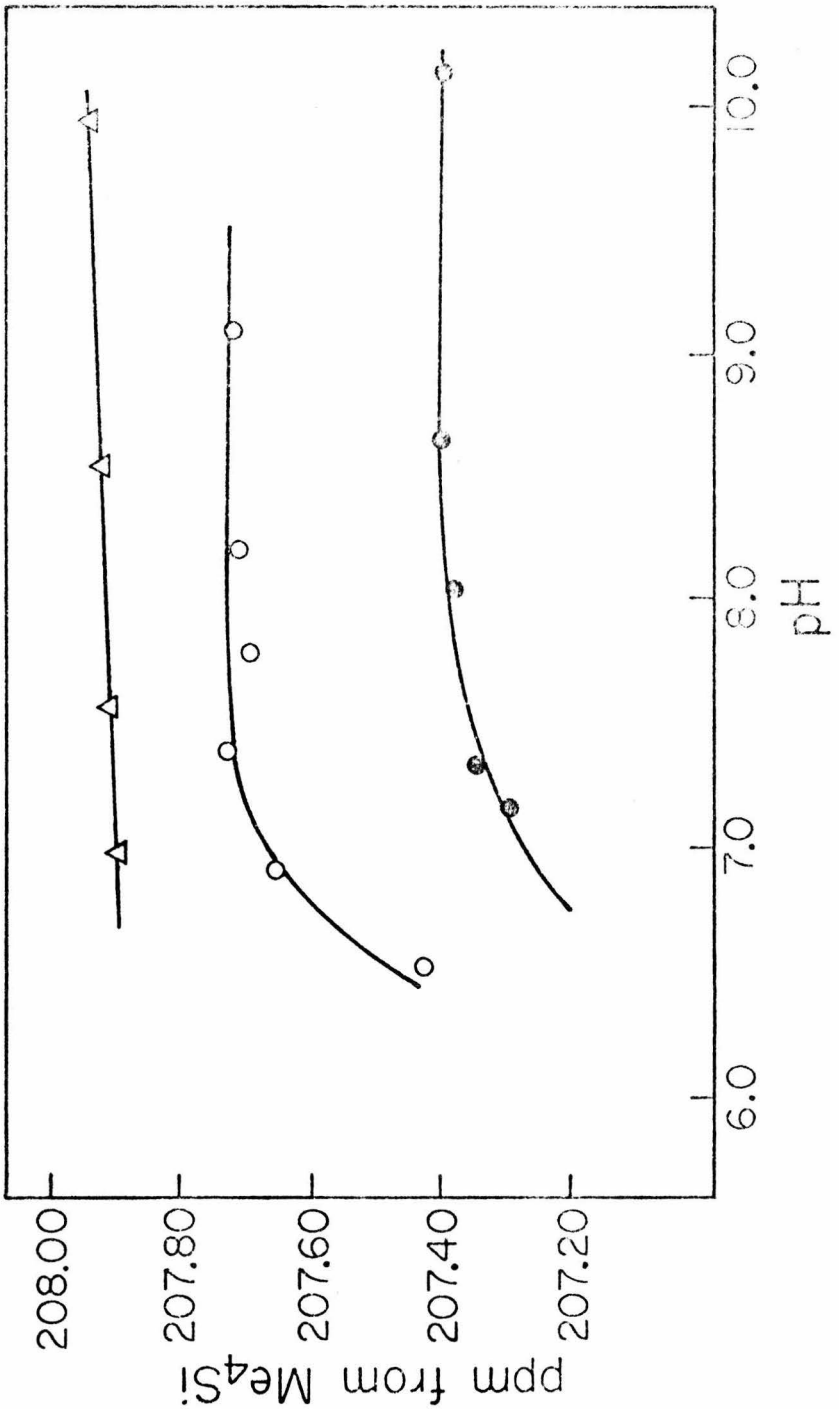
The pH dependence for the ^{13}CO chemical shifts of native (●) and reconstituted (○) protomyoglobin.


ppm from Me4Si
207.80
207.60
207.40
207.20
5.0
6.0
7.0
8.0
9.0
pH

shifting upfield at pH below 6.5 and disappears below pH 5. This shift is accompanied by an increased rate of sample precipitation. This effect is more pronounced in samples containing modified hemes as seen in Figure 2. These samples had good stability at high pH but precipitated rapidly at pH much below 7.2. The hemoglobin samples were independent of pH in the observed pH 6.5-7.5 range. No pH-dependent shifts, even at relatively low pH (pH ~ 5), have been observed for any of the native tetrameric hemoglobins. The hemoglobin samples reconstituted with deuterohemin were somewhat less stable toward precipitation than samples containing mesoheme or 2,4-diacetyldeuteroheme. Only a single ^{13}CO resonance was observed in samples of rabbit 2,4-diacetyldeuterohemoglobin, despite thorough reduction of the hemes and saturation with ^{13}CO. This resonance had virtually the same chemical shift as that of ^{13}CO bound to the β subunit of human 2,4-diacetyldeuterohemoglobin. Since the α subunit of native rabbit hemoglobin has a lower affinity for ^{13}CO than the β subunit (Moon and Richards, 1974) and all samples reconstituted with 2,4-diacetyldeuteroheme were hard to saturate with ^{13}CO and showed persistence of deoxy character in their electronic spectra, it seems likely that the resonance observed for this reconstituted rabbit hemoglobin results from ^{13}CO bound to the β subunit. Presumably the α subunit either does not bind ^{13}CO, or ^{13}CO is in rapid exchange with the solution environment such that the resonance for bound ^{13}CO is not observed. The possibility that the rabbit hemoglobin α subunits simply fail to bind 2,4-diacetyldeuteroheme seems extremely unlikely since these subunits show

FIGURE 2

The pH dependence for the ^{13}CO chemical shifts of reconstituted meso (Δ), deutero (○), and 2,4-diacetyldeutero (◉) myoglobins.


208.00
207.80
207.60
207.40
207.20
ppm from Me4Si
6.0
7.0
8.0
9.0
10.0
pH

normal binding of the other modified hemes and human hemoglobin binds 2,4-diacetyldeuteroheme readily. This possibility also seems unlikely in view of the results of Winterhalter et al. (1971) which suggest that the α subunits have a greater affinity for hemes than do the β subunits.

The data in Table III suggest that the ^{13}CO chemical shifts are influenced by the electronic nature of the substituent groups at the periphery of the porphyrin ring. This is more apparent in Table IV where the observed ^{13}CO chemical shifts for the modified myoglobins and the modified α and β subunits of human adult and rabbit hemoglobins are collected relative to the ^{13}CO shifts observed for the respective native proteins. While the substituent effects of the modifying groups should involve both inductive and resonance contributions, we are unable to predict the relative importance of these contributions in the complex porphyrin ring system. We have therefore chosen the Hammett substituent constants, σ_p, as the simplest measure of the electronic nature of the modifying group. These values are included in Table IV. For reference the ionization constants, pK_3, for neutral 2,4-disubstituted deuteroporphyrins and the carbonyl infrared stretching frequencies for the corresponding pyridine carbonyl hemochromes are also included in the Table. Figure 3 shows a plot of chemical shift, $\delta_{^{13}CO}$, against σ_p for the reconstituted myoglobins. Similar plots of $\delta_{^{13}CO}$ against σ_p for the reconstituted α and β subunits of human adult hemoglobin and the β subunit of rabbit hemoglobin are shown in Figure 4. From these data it is apparent that a good correlation does indeed exist between

TABLE IV: Relative ^{13}CO Chemical Shifts for Globins Containing Modified Hemes

	2,4-Substituent			
	CH_3CH_2-	CH_2=CH-	H-	CH_3CO-
Myoglobin	-5.2 Hz	0	-0.1 Hz	+12.8 Hz
Adult-α	-6.2 Hz	0	+3.5 Hz	+23.7 Hz
Adult-β	-15.6 Hz	0	-3.1 Hz	+22.3 Hz
Rabbit-α	-10.2 Hz	0	-6.0 Hz	not observed
Rabbit-β	-13.6 Hz	0	-4.0 Hz	+23.3 Hz
σ_p[a]	-0.15	-0.02	0	+0.46
pK_3[b]	5.8	4.8	5.5	3.3
ν_{CO}[c]	1973.0 cm^{-1}	1976.6 cm^{-1}	1975.0 cm^{-1}	1983.7 cm^{-1}

[a] σ_p values from McDaniel and Brown (1958) except for vinyl group σ_p which is calculated from the σ_I and σ_R values reported by Taft et al. (1963a, b).

[b] pK_3 values for modified deuteroporphyrins IX from J. E. Falk, "Porphyrins and Metalloporphyrins", (Elsevier Publishing Co., 1964) p. 29.

[c] Infrared carbonyl stretching frequencies for pyridine carbonyl hemochromes from Alben and Caughey (1968).

FIGURE 3

The correlation between the ^{13}CO chemical shifts of heme-modified myoglobins and the substituent constants, σ_p, of the ethyl (Et), vinyl (V), hydrogen (H), and acetyl (Ac) modifying groups. The σ_p values used were taken from McDaniel and Brown (1958) except for the vinyl group for which σ_p was estimated from the values of Taft et al. (1963a, b) in which $\sigma_p = \sigma_I + \sigma_R = 0.01 - 0.03$.

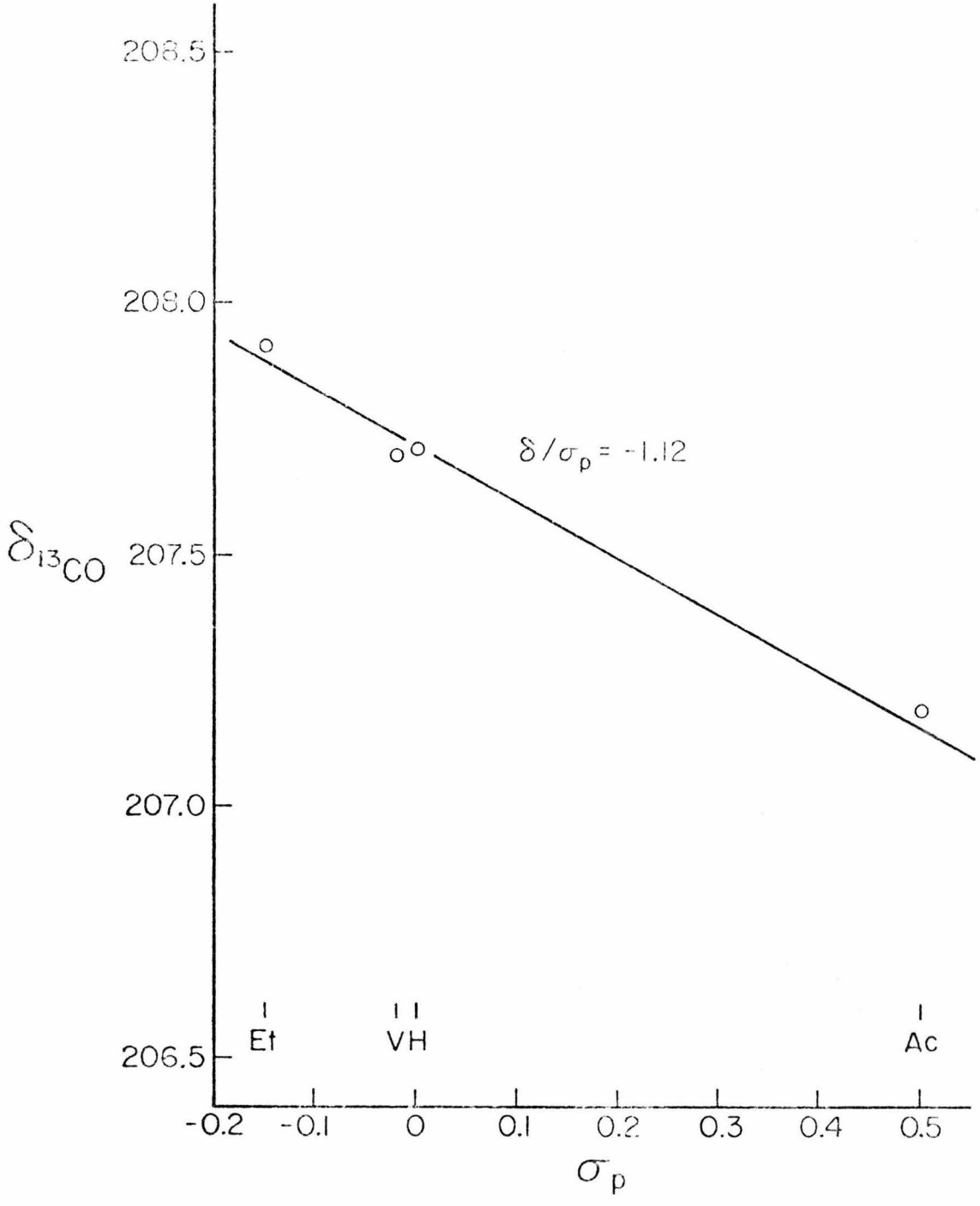
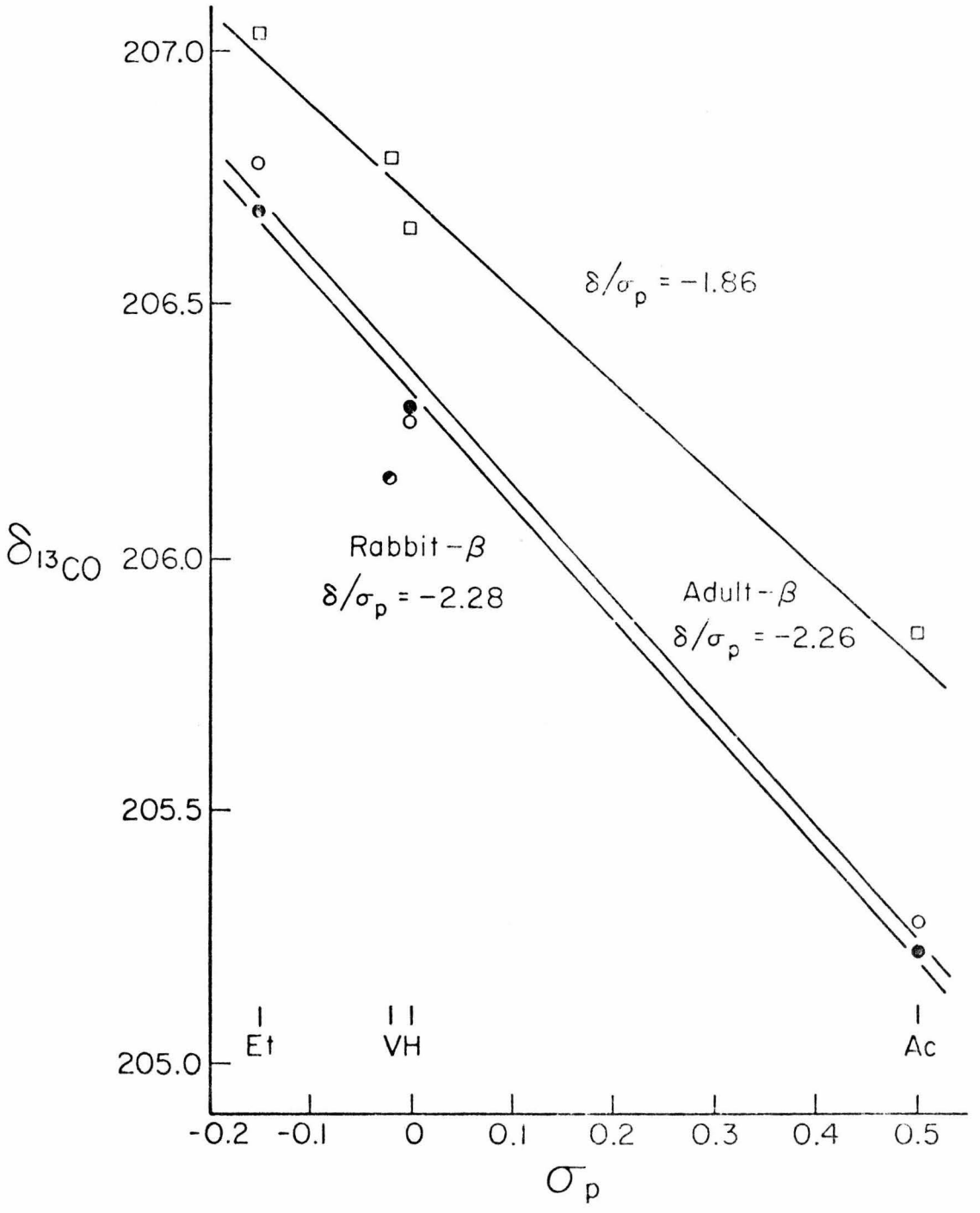

208.5
208.0
207.5
207.0
206.5
$\delta_{^{13}CO}$
$\delta/\sigma_p = -1.12$
Et
VH
Ac
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
σ_p

FIGURE 4

The correlation between the ^{13}CO chemical shifts of heme-modified hemoglobins and the σ_p values of the modifying groups. The $\delta_{^{13}CO}$ data for the α (□) and β (○) subunits of human adult hemoglobin and the β subunit of rabbit hemoglobin (●) are shown.


207.0
206.5
206.0
205.5
205.0
δ_{13CO}
$\delta/\sigma_p = -1.86$
Rabbit-β
$\delta/\sigma_p = -2.28$
Adult-β
$\delta/\sigma_p = -2.26$
Et
VH
Ac
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
σ_p

the chemical environment of the ligand and the electronic nature of the substituent group. The slopes of the plots in Figures 3 and 4 are also of interest. The slope, $\delta_{^{13}CO}/\sigma_p$, is an indication of the magnitude of the substituent effect experienced by the bound ^{13}CO ligand. An excellent correlation is seen between the slopes of adult and rabbit β subunits (-2.26 and -2.28, respectively). A slight decrease in slope is observed for adult α subunits, but this difference is probably within the experimental limits of the data. A very definite decrease, however, is observed for the slope of myoglobin (-1.12), indicating that the magnitude to the substituent effect experienced by the bound ligand in myoglobin is about half as great as it is in hemoglobin. This is in contrast to results from studies of the electronic spectra of heme-modified myoglobins and hemoglobins in which the Soret band shifts induced by a particular heme are nearly equal for both proteins (Antonini et al., 1964). The electronic effects experienced by ^{13}CO in myoglobin and hemoglobin are further demonstrated by comparing $\delta_{^{13}CO}$ with the chemical properties of simple porphyrins removed from the protein environment. Figure 5 shows the correlation between $\delta_{^{13}CO}$ and the pK_3 values for the ionization of the analogous neutral porphyrins. Similarly Figure 6 shows the agreement between $\delta_{^{13}CO}$ and the infrared CO stretching frequencies of the pyridine carbonyl hemochromes. These data offer strong evidence that the substituent effects observed for simple porphyrins are present and clearly measurable in the intact reconstituted myoglobin and hemoglobin samples. Again the magnitude of the substituent effect in hemoglobin subunits appears greater than in myoglobin.

FIGURE 5

A plot of the relation between the ^{13}CO chemical shifts of heme-modified myoglobins and hemoglobins and the pK_3 values for the ionization of the analogous 2, 4-disubstituted deuteroporphyrins. The $\delta_{^{13}CO}$ data for dolphin myolgobin (Δ), the α(□) and β(○) subunits of adult hemoglobin and the α(▣) and β(◐) subunits of rabbit hemoglobin are shown. The pK_3 data were obtained from J. E. Falk, "Porphyrins and Metalloporphyrins", (Elsevier Publishing Co., 1964) p. 29.

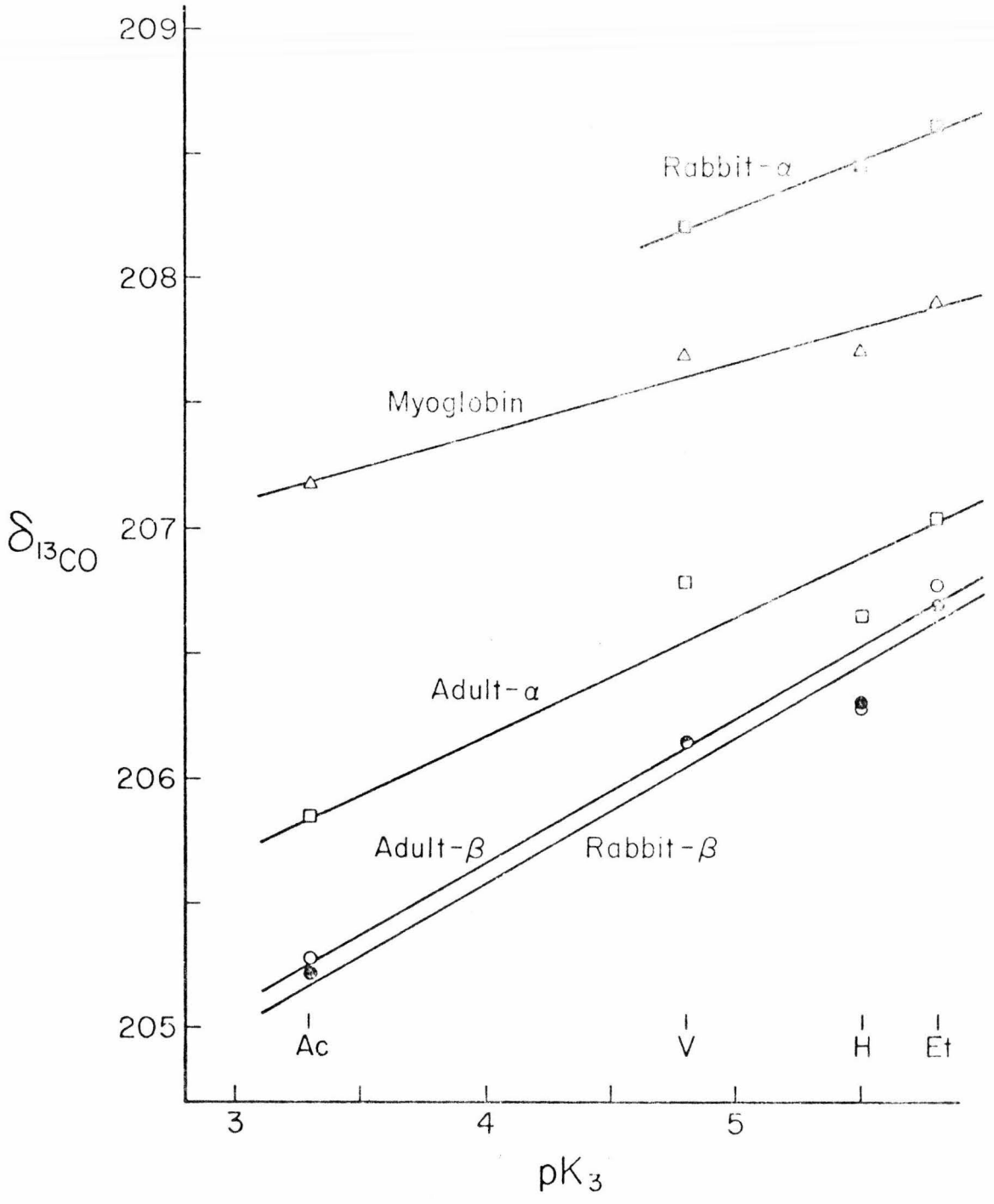
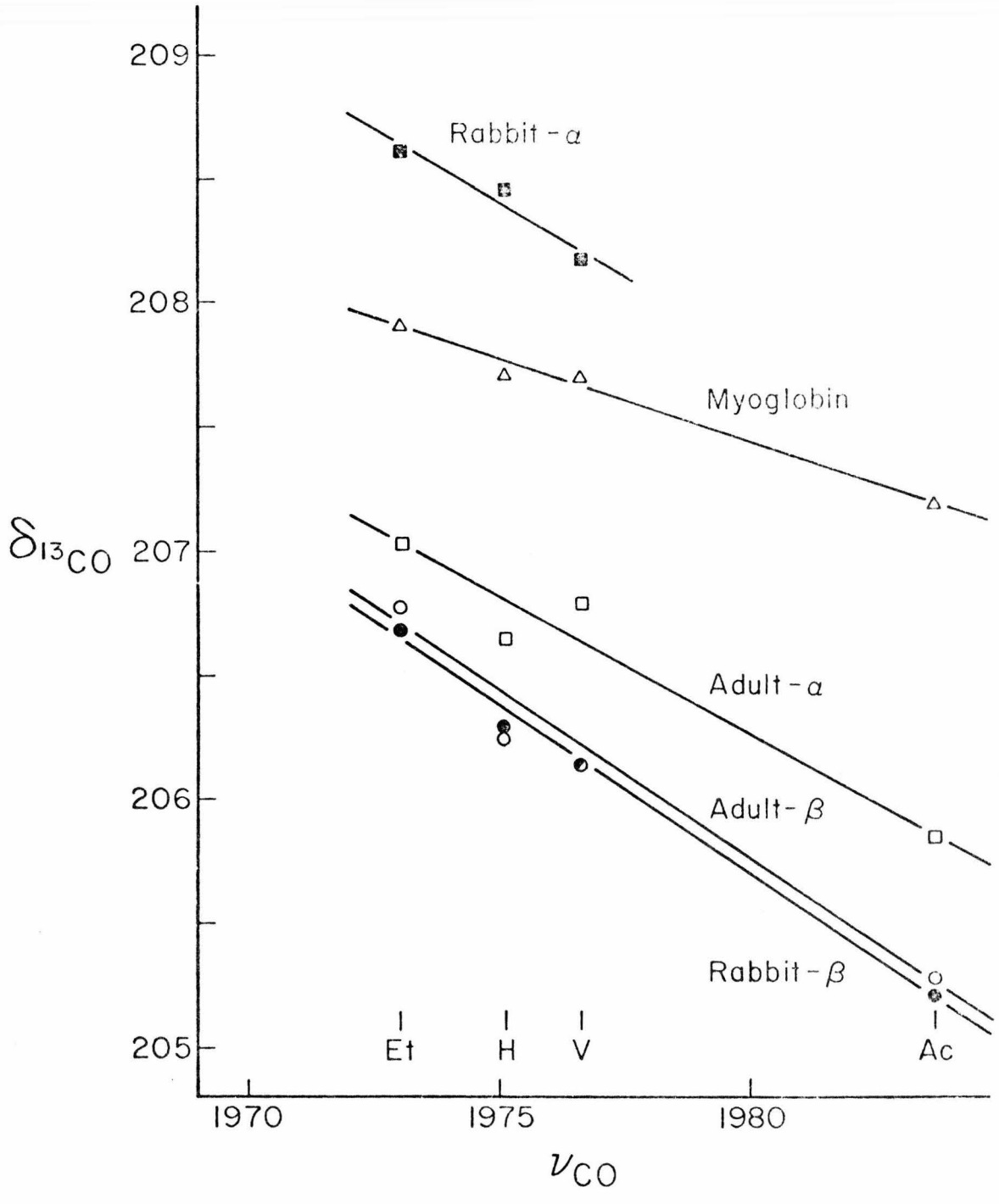

209
208
207
206
205
$\delta_{^{13}CO}$
Rabbit-α
Myoglobin
Adult-α
Adult-β
Rabbit-β
Ac
V
H
Et
3
4
5
pK_3

FIGURE 6

A plot of the relation between the $\delta_{^{13}CO}$ data and the infrared carbonyl stretching frequencies (ν_{CO}) of 2,4-disubstituted pyridine carbonyl hemochromes. The $\delta_{^{13}CO}$ data for myoglobin (Δ), α (□) and β (○) adult hemoglobin, and α (■) and β (●) rabbit hemoglobin are shown. Infrared data were obtained from Alben and Caughey (1968).


209
208
207
206
205
δ_{13CO}
Rabbit - α
Myoglobin
Adult - α
Adult - β
Rabbit - β
Et
H
V
Ac
1970
1975
1980
ν_{CO}

DISCUSSION

It has been well established that the interaction of carbon monoxide with iron (II) porphyrins is sensitive to the electron withdrawing or donating ability of the substituents at the periphery of the porphyrin ring and to the relative basicity of the trans ligand (Caughey, 1967). The results of various studies on modified porphyrins and hemes (Phillips, 1960; Alben and Caughey, 1962; Falk, 1966; Alben and Caughey, 1968) all suggest that electron withdrawal at the 2, 4-positions of the porphyrin ring reduces the strength of the iron-ligand bond for ligands such as carbon monoxide and oxygen. The possibility that electronic substituent effects could be of importance in hemoglobin was explored by Sugita et al. (1971). These workers reported that reconstituted hemoglobins containing modified hemes were relatively stable and exhibited a reasonable degree of allosteric cooperativity. The oxygen affinities of these hemoglobins demonstrated an apparent substituent effect where the 2, 4-substituent order, ethyl > hydrogen > α-hydroxyethyl > vinyl, reflects the relative oxygen affinities of 5:2:1.3:1. These data were in agreement with earlier kinetic studies of the binding of oxygen to hemoglobins containing modified hemes (Antonini and Gibson, 1960) and implied that the oxygen affinity was proportional to the negative inductive character of the substituent. More recent studies, however, suggest that these apparent substituent effects are coincidental and argue against the importance of the electronic substituent effects. It has been proposed that the size of the substituent group is of impor-

tance (Yamamoto et al., 1974) and that the substituent group effects observed in myoglobins and hemoglobins are stereochemical, rather than electronic in nature (Yonetani et al., 1974). It has been further suggested that these stereochemical effects induced in the heme pocket by the heme substituents may perturb the intersubunit contacts in hemoglobin in such a way as to directly influence hemoglobin cooperativity (Yamamoto and Yonetani, 1974).

A problem may exist, however, in that measurements of the kinetic and thermodynamic parameters for ligand binding to modified heme myoglobins and hemoglobins may be difficult to interpret. The well established nonequivalence of the α and β subunits of hemoglobin (Ogata and McConnell, 1972; Moon and Richards, 1974, Johnson and Ho, 1974), coupled with the effects of hemoglobin allostery, make comparison between myoglobins and hemoglobins very difficult. These sorts of measurements are also incapable of discriminating between electronic substituent effects experienced at the heme iron and the various sorts of steric interactions which could be induced by the modified hemes into the protein so as to affect ligand access to the heme and the stability of the heme-ligand bond. Since the ^{13}CO chemical shifts of the α and β subunits are easily distinguished and relatively independent of hemoglobin quaternary structure (Vergamini et al., 1973; Moon and Richards, 1974), a comparison between the ^{13}CO shifts of the various subunits and the expected electron withdrawing or donating properties of the substituent groups should indicate the degree to which electronic effects are experienced by the bound ligand and the relative magnitude of these

effects compared to the differences existing between various native hemoproteins.

The results of the ^{13}CO chemical shift data for heme-modified myoglobins and hemoglobins indicate the following conclusions: (i) modified hemes reconstitute with apomyoglobin and apohemoglobin in a sufficiently regular manner as to provide for the continued significance of electronic substituent effects within the protein, while at the same time maintaining the inherent nonequivalence of the various subunits, (ii) the degree of electronic coupling between the porphyrin ring and the bound ligand may vary between proteins, and (iii) electronic effects which might be induced in the porphyrin ring by steric interactions with peripheral residues of the heme pocket are not responsible for the differences (as experienced by ^{13}CO) existing between native myoglobins and the α and β subunits of modified hemoglobins.

Heme Reconstitution

Any report of the properties of reconstituted myoglobins or hemoglobins containing unnatural hemes may be greeted with the healthy suspicion that reconstitution induces gross stereochemical changes in the protein resulting in anomalous ligand binding characteristics. This is especially true of heme-modified hemoglobins in which the Hill constants, n, are invariably lower than in native hemoglobin (Sugita and Yoneyama, 1971; Yonetani et al., 1974; Yamamoto and Yonetani, 1974). Thus, a simple explanation for the apparent absence of electronic effects in reconstituted myoglobins and hemoglobins containing both iron and cobalt hemes (Yonetani et al. 1974; Yamamoto et

al., 1974) could be that the modified hemes reconstitute with the apoprotein in such an abnormal fashion that the steric differences existing around the various modified hemes are great and tend to wash out the electronic effects normally observed in simple porphyrin model compounds. While results such as the EPR studies of reconstituted proto-, meso- and deuteromyoglobins (Hori, 1972) show no change in heme plane orientation, it may be argued that these studies are not sufficiently sensitive to detect major stereochemical changes around the ligand binding site.

Our results obtained from studies of the ^{13}CO chemical shifts offer further evidence that protoheme reconstitutes with apoprotein in a manner wholly indistinguishable from the native protein. This is especially evident in that the inherent differences existing between native myoglobin and the subunits of human and rabbit hemoglobins are fully retained in the reconstituted proteins. Thus the precise heme-protein interactions responsible for the observed differences in the native proteins are uninterrupted in the reconstituted proteins. This property of the reconstituted proteins is further dramatized by the fact that the modified hemes reconstitute with apoprotein in such a way as to maintain the differences between the subunits while at the same time demonstrating a clearly linear relationship with the electronic nature of the substituent groups. The possibility that the ^{13}CO chemical shifts could be related to the steric size of the 2,4-substituent groups is highly unlikely since the ethyl and acetyl substituent effects shift the ^{13}CO resonances in opposite directions from the resonances

observed for the deuteroheme-containing proteins. If such steric factors dominated the ^{13}CO shifts, one would expect little or no correlation with σ_p for the substituent groups.

Further evidence that electronic effects dominate the ^{13}CO chemical shifts of the reconstituted proteins is obtained from the nature of the chemical shifts. In general, ^{13}C chemical shifts are dominated by paramagnetic contributions to nuclear shielding (Karplus and Pople, 1963), where distortion of the carbon p orbitals results in a downfield chemical shift. The 2,4-substituents of the heme have been shown to vary the basicity of the pyrrole nitrogens and should therefore vary the electron density of the iron. An increase in electron density on the iron will cause increased bonding between the heme and the ^{13}CO ligand due to increased d-π overlap between the orbitals of the carbon and the iron (Alben and Caughey, 1968). Since this overlap distorts the p orbitals of the carbon, increased electron density on the iron is expected to result in a downfield paramagnetic shift of the ^{13}CO resonance. It is thus reasonable that in mesoheme-containing proteins, where the ethyl groups are electron donating, the ^{13}CO binds tighter to the iron and the resonance is shifted downfield, while in 2,4-diacetyldeuteroheme proteins the ^{13}CO binds less strongly and the resonance shifts upfield. This analysis also agrees with studies of other metal carbonyl compounds in which increased electropositive character on the metal results in an upfield chemical shift (Levy and Nelson, 1972).

By demonstrating the importance of electronic effects in determining the ^{13}CO chemical shifts of heme-modified myoglobins and

hemoglobins, we do not imply that steric effects are of no importance to the ligand binding properties of these proteins. Both factors probably contribute. Very precise steric interactions at the heme periphery may induce changes in the protein conformation which affect the kinetic and thermodynamic properties of the proteins (Yonetani et al., 1974; Yamamoto et al., 1974) without affecting the bound ligand. Since the σ_p values for hydrogen and vinyl substituents are quite close, the differences observed between ligand affinities of deutero- and protoheme proteins may well be dominated by the action of steric effects well removed from the factors dominating the ^{13}CO chemical shift.

Magnitude of the Substituent Effects

Previous reports of the oxygen affinities of heme-modified hemoglobins containing iron (Sugita and Yoneyama, 1971) and cobalt (Yonetani et al., 1974) show that the nucleophilicity of the 2,4-substituent groups could be of importance in determining affinities of these hemoglobins for ligands. However, studies of the corresponding reconstituted iron and cobalt myoglobins exhibit differences in the order of the relative oxygen affinities from the order observed in the hemoglobins, leading Yonetani et al. (1974) to conclude that the order of the oxygen affinities is dependent on the nature of the apoprotein and that the correlation between the nucleophilicity of the porphyrin substituents and the oxygen affinities of reconstituted myoglobins and hemoglobins was "a fortuitous coincidence observed under limited experimental conditions". These authors argued that the differences between myoglobins and hemoglobins could not be reconciled strictly in terms of

substituent group electronic effects acting on the metal-ligand bond and considered that the effect of the substituents must therefore be stereochemical rather than electronic. We believe that both factors are of importance. The data in Figures 3 and 4 clearly show that the substituent effects are more pronounced in the hemoglobin subunits than in myoglobin. Thus we agree with Yonetani et al. (1974) that the nature of the apoprotein is of importance in determining the relative substituent effects observed. However, our results suggest that the differences in the oxygen affinities observed between myoglobins and hemoglobins may be easily reconciled. Steric and electronic forces are of importance in both proteins but the relative importance of electronic effects is sufficiently reduced in the myoglobins that the steric effects dominate. In the hemoglobins the electronic interaction between the heme and the bound ligand is sufficiently great that the electronic substituent group effects are still of importance and correlate much more closely with results obtained from porphyrin and hemochrome model compounds. Thus the apoprotein is capable of modulating the extent to which the bound ligand experiences electronic changes in the porphyrin ring.

It seems likely that the interaction between the proximal histidine residue (F8) and the heme may contribute to the modulation of the degree of electronic coupling between the porphyrin and the ligand by effecting the electronegativity of the iron. It is also possible that hydrogen bonding or steric interactions acting directly on the ligand may perturb the iron-carbon bond in such a way as to reduce the

magnitude of the substituent effects experienced by the ^{13}CO ligand. In this regard it is interesting to note that x-ray diffraction studies of the monomeric oxygen carrying heme proteins from chironomus (Huber et al., 1971) and glycera (Padlan and Love, 1974) indicate that the Fe-C-O bond is bent, not perpendicular to the heme plane as would normally be expected.

Subunit Nonequivalence

The forces responsible for the different ^{13}CO chemical shifts observed for native myoglobins and the α and β subunits of native hemoglobins must have their origin in some combination of steric and electronic effects acting directly on the heme and the bound ligand. The possibility exists that the observed differences between the various heme proteins (Table II) could be due to minor interactions between the residues of the heme pocket and the porphyrin ring. Though the electronic spectra of the α and β subunits of hemoglobin are identical, the ^{13}CO chemical shifts might be sufficiently sensitive to changes in the electronic nature of the porphyrin ring that the α and β subunit ^{13}CO resonances could differ due to electronic changes in the porphyrin ring too small to be detected in the electronic spectra. Our results with modified hemes, however, show that substituent modifications, which produce very noticeable changes in the Soret spectra of the hemes, produce changes in the ^{13}CO shifts only comparable to, but not significantly greater than, the differences seen between the α and β subunits of the various hemoglobins. Thus the electronic effects influencing the ^{13}CO shifts do not manifest themselves in the Soret

spectra and therefore do not originate in the porphyrin ring.

The infrared spectrum of rabbit carboxyhemoglobin exhibits two carbon monoxide carbonyl stretching bands at 1951 cm^{-1} and 1928 cm^{-1} and these bands have been shown to correlate respectively with the high field (206.18 ppm) and low field (208.18 ppm) ^{13}CO resonances in the NMR spectrum of rabbit hemoglobin (Matwiyoff et al., 1973). It is therefore possible that a major change in the Fe-CO bond is responsible for the difference between the ^{13}CO chemical shifts of the rabbit α and β subunits. These changes could originate either in the proximal histidine-heme interaction, which may effect the ligand without strongly influencing the Soret spectrum of the porphyrin ring, or the differences in ^{13}CO chemical shift could be due to specific interactions between the ligand and the distal histidine residue (E7) in the ligand binding pocket. In this regard it is especially interesting to note that heme proteins which do not possess a distal histidine residue such as glycera hemoglobin (Padlan and Love, 1974) and the α subunits of opossum hemoglobin (Waterman and Stenzel, 1974), show anomalous shifts of their ^{13}CO resonances relative to the ^{13}CO shifts observed in most hemoglobins. These results suggest that the ^{13}CO ligand is sensitive to a carefully constructed ligand environment on the distal side of the heme. More precise discussions of the role of the distal histidine in ligand bonding (Moon et al., 1975) and the nature of the amino acids responsible for maintaining the ^{13}CO environment (Moon and Richards, 1975) will follow.

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PART IV

^{13}C NMR Studies of the Distribution of ^{13}CO Between the Subunits of Partially Liganded Hemoglobins

INTRODUCTION

The allosteric properties of hemoglobin ligation have been largely accounted for in the classic stereochemical mechanism for hemoglobin subunit cooperativity proposed by Perutz (1, 2). In this mechanism the hemoglobin tetramer is proposed to exist in either of two dominant quaternary conformations corresponding to the fully deoxygenated (T) or oxygenated (R) forms of the protein. Ligand binding proceeds first at the unliganded subunits in a sequential manner resulting in the destruction of structural constraints responsible for maintaining the T quaternary conformation. Once these constraints are removed the tetramer shifts into the R conformation and allows further ligation of subunits to proceed more readily. Thus this mechanism contains features of the two-state concerted MWC allosteric model (3) with the exception that the tertiary structures of the individual subunits are determined more directly by the presence of bound ligand, as predicted in the KNF sequential model (4), than by the nature of the quaternary conformation. Studies of the binding of a spin-labeled analog of ATP to hemoglobin at various stages of partial oxygenation led Ogata and McConnell (5) to conclude that the oxygen affinities of the α and β subunits must differ. They proposed that a "generalized concerted transition" model (GCT), based on the simple two-state MWC model, could account for these differences by the explicit recognition of the nonequivalence of the α and β subunits. This model has recently

been discussed in more detail (8) and specific functions for the ligation of the individual α and β subunits, $\bar{Y}_{\alpha}$ and $\bar{Y}_{\beta}$, have been derived. Thus the application of techniques capable of directly determining the equilibrium ligation of the individual α and β subunits should be valuable in further refining the model systems so that a more precise understanding of hemoglobin function may be achieved.

Several previous reports have clearly demonstrated that ^{13}C-enriched carbon monoxide ligands bound to the α or β subunits of hemoglobin are readily distinguished by ^{13}C magnetic resonance (9-13). We recently reported that the relative thermodynamic ease with which oxygen displaces ^{13}CO in human hemoglobin differs for the α and β subunits (14). This difference was shown to be much greater in rabbit hemoglobin. Further we observed that the relative affinities of the unliganded α and β subunits of rabbit hemoglobin for ^{13}CO differ such that the α subunit has a lower equilibrium affinity for ^{13}CO than the β subunit.

To gain a more quantitative understanding of the relative affinities of the α and β subunits for carbon monoxide we have determined the distribution of ^{13}CO between the α and β subunits at various stages of partial hemoglobin ligation. These studies have been conducted with human (Hb-A), rabbit (Hb-R), and opossum (Hb-O) hemoglobins to test the possibility that the relative affinities of the α and β subunits for ^{13}CO may vary between species. We have chosen rabbit and opossum hemoglobins because they contain amino acid substitutions near the ligand binding sites of their α subunits and seem most likely

to exhibit differences in the relative affinities of their subunits for ligands as compared to human hemoglobin.

EXPERIMENTAL

Hemoglobin was prepared from freshly drawn, citrated whole blood of human adult, rabbit (New Zealand white), or opossum (Didelphius marsupialis) origin.

The red cells were separated from plasma by centrifugation at 2000 x g and washed several times with 0.15 M sodium chloride. The packed erythrocytes were then lysed hypotonically with three volumes of distilled water at ambient temperature for 20 minutes. After removal of the stromata by centrifugation at 30,000 x g, the hemolysates were dialyzed overnight against a large volume of 0.15 M sodium chloride at 4°C. The crude hemoglobin solutions were then concentrated by ultrafiltration (Amicon UM-10 membrane) and further dialyzed against distilled water under nitrogen in the ultrafiltration apparatus. The concentrated hemoglobin solution (~ 2 mM in tetramer) was then applied to a column of Sephadex G-50 and eluted with water. The void volume, containing high molecular weight contaminants, was discarded. The salt-free hemoglobin was concentrated by ultrafiltration, dialyzed against 0.15 M sodium chloride under nitrogen, and concentrated to a final concentration of about 3 mM in hemoglobin tetramer.

Samples for NMR study were prepared from the hemoglobin stock solutions, 1 M Tris buffer in 0.15 M sodium chloride, pH 7.0, and 0.15 M sodium chloride. The final sample volume was 5.0 ml of 2.0±0.1 mM hemoglobin tetramer in 50 mM Tris and 0.15 M sodium chloride, pH 7.0±0.1. Samples containing 2,3-diphosphoglycerate

were prepared by the addition of 0.1 M 2,3-diphosphoglycerate in 0.15 M sodium chloride, pH 7.0, prior to final dilution with 0.15 M sodium chloride. Samples containing inositol hexaphosphate were prepared by dissolving the solid phosphate in a minimum amount of 0.15 M hydrochloric acid so as to obtain a pH of about 6.5. This solution was added to the hemoglobin solution prior to addition of 0.15 M sodium chloride. Final pH adjustment was made with 0.15 M hydrochloride added and the sample was then diluted to 5.0 ml with 0.15 M sodium chloride. Final hemoglobin concentrations were determined spectrophotometrically using the ratios of absorptions at 415, 420, and 430 nm (15). The amount of methemoglobin never exceeded 3% and did not change detectably during the course of the experiments.

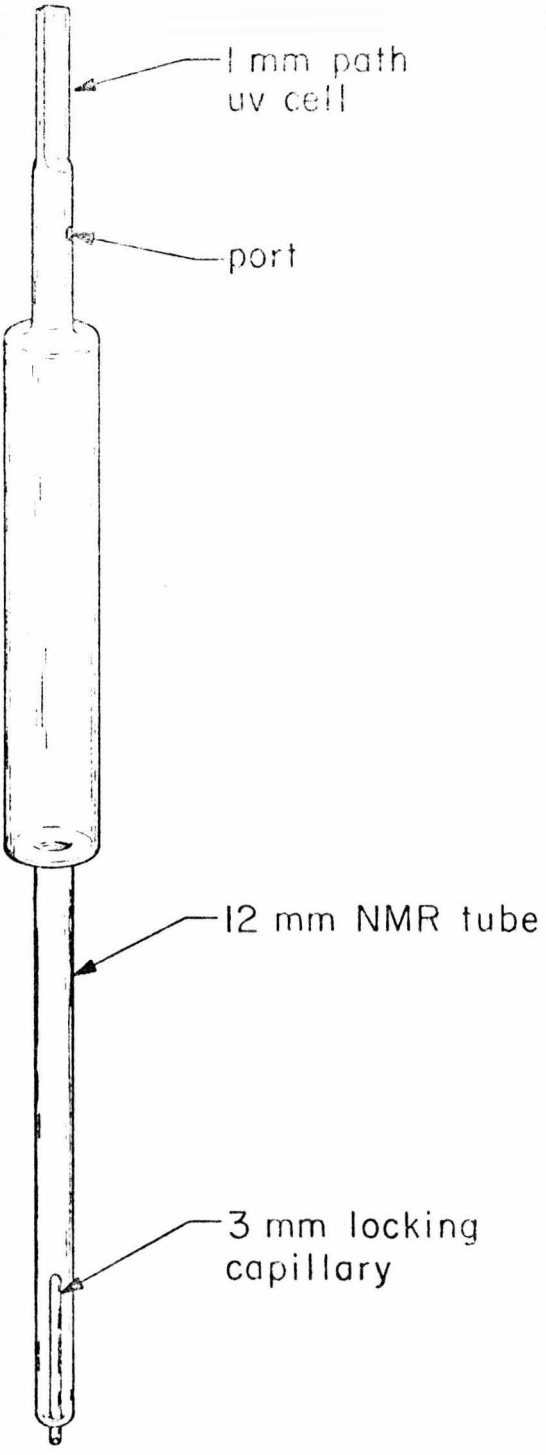
Quantitative treatment of deoxyhemoglobins with nonsaturating amounts of ^{13}CO was accomplished in a specially constructed, closed atmosphere sample tube which has an attached UV cell (Figure 1). The 5.0 ml hemoglobin samples were deoxygenated in the cell by extensive flushing with water-saturated nitrogen. Complete deoxygenation was determined spectrophotometrically (15) using the criterion that the ratio $OD_{670}/OD_{730} \geq 2.3$. Water-saturated ^{13}CO was injected into the sample tube with a gas-tight syringe through a rubber sleeve covering the access port. The extent of hemoglobin saturation with ^{13}CO was determined spectrophotometrically at 650 nm both before and after NMR study.

^{13}C magnetic resonance spectra were obtained using the pulse Fourier transform technique on a Varian Associates XL-100-15

spectrometer operating at 25.17 MHz. The spectrometer was interfaced with a Varian 620i computer (16 K memory) for data accumulation and Fourier transformation. A sensitivity enhancement of 0.10 sec was applied to the free induction decay before the Fourier transformation was carried out. Samples were contained in the specially constructed sample tube shown in Figure 1. A 3 x 50 mm capillary containing D_2O was mounted concentrically in the bottom of the sample tube to serve as a field frequency lock. All spectra were obtained under identical conditions at a probe temperature of 32 °C without proton noise decoupling. A 90° pulse of 55 μsec duration was used with an acquisition time of 0.8 sec. Since both the α and β subunit resonances have virtually identical spin-lattice relaxation times (14,16), any errors in quantitation due to partial saturation of the resonances are minimized.

FIGURE 1

Closed-atmosphere sample tube. The tube has an attached 1 mm path length uv cell at the top and a 12 mm NMR tube at the bottom. A rubber sleeve covering the port served as an injection septum for addition of ^{13}CO. The 3 mm capillary mounted concentrically at the bottom of the NMR tube was filled with D_2O for locking. Total cell volume was 118 ml. The spinning rate for this sample tube was typically 8-10 Hz.


1 mm path
uv cell
port
12 mm NMR tube
3 mm locking
capillary

RESULTS

Representative spectra of the ^{13}CO resonances for human adult, rabbit, and opossum carboxyhemoglobins are collected in Figure 2. Assignment of these resonances to ^{13}CO bound to the α or β subunits of the respective hemoglobins is based on studies of the isolated PMB-derivatized α and β subunits of adult hemoglobin (13, 14) and comparative studies of the carboxyhemoglobins from a wide variety of animal species (14, 17). The ^{13}CO carbons are not significantly spin-coupled with neighboring protons making it possible to observe these resonances clearly in the absence of proton noise decoupling and thereby eliminate any contributions to the signal intensity caused by nuclear Overhauser effects. Since the ^{13}CO relaxation times for the α and β subunits do not differ detectably in any of these hemoglobins (14, 16), the relative ^{13}CO saturation of the α and β subunits, S_α and S_β, may be determined directly from the signal intensities. The extensive overlap of the resonances of Hb-A necessitates the estimation of S_α and S_β by simple measurement of the relative peak heights. This method is somewhat inaccurate, especially in that small differences between the subunits become more difficult to detect. The ^{13}CO resonances of Hb-R and Hb-O, however, are sufficiently well resolved that they may be integrated with considerable accuracy.

Figures 3 and 4 contain representative spectra of rabbit and opossum hemoglobins at various stages of partial ^{13}CO ligation. From these spectra it is apparent that the α subunits of Hb-R exhibit a reduced

FIGURE 2

Representative spectra of the ^{13}CO resonances from the α and β subunits of human, rabbit and opposum hemoglobins at 25.17 MHz. Sample concentrations were 2.0 mM (tetramer) in 0.15 M sodium chloride. Enrichment in ^{13}CO was 90-92%.

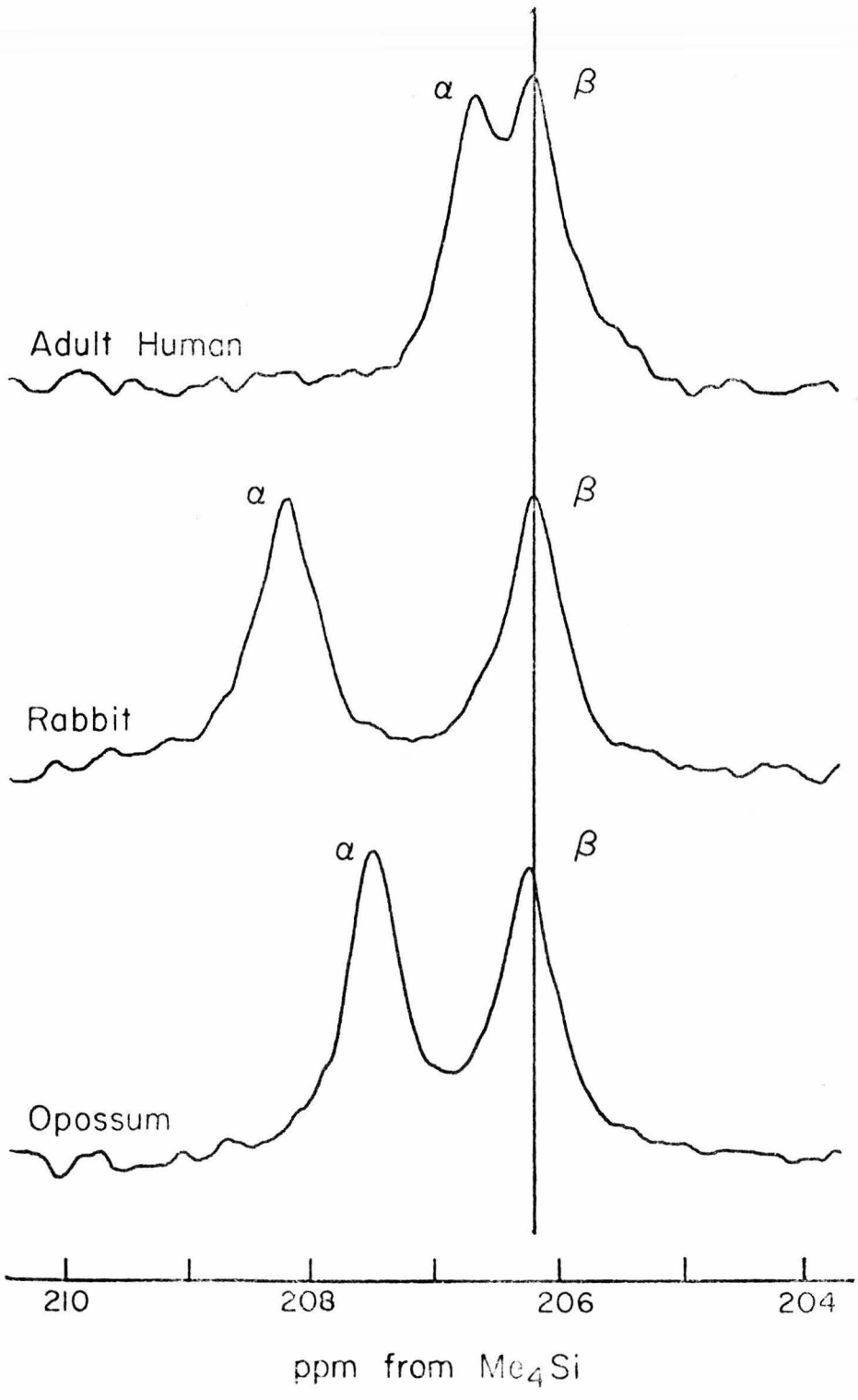

α
β
Adult Human
α
β
Rabbit
α
β
Opossum
210
208
206
204
ppm from Me_4Si

FIGURE 3

Typical ^{13}CO spectra of rabbit hemoglobin at various stages of partial ^{13}CO ligation. Sample concentrations were 2.0 mM Hb-R in 0.05 M tris/0.15 M sodium chloride, pH 7.0. The spectra are plotted in the absolute intensity mode so that the relative amount of ^{13}CO bound to each subunit is easily visible as the level of total ^{13}CO saturation increases.

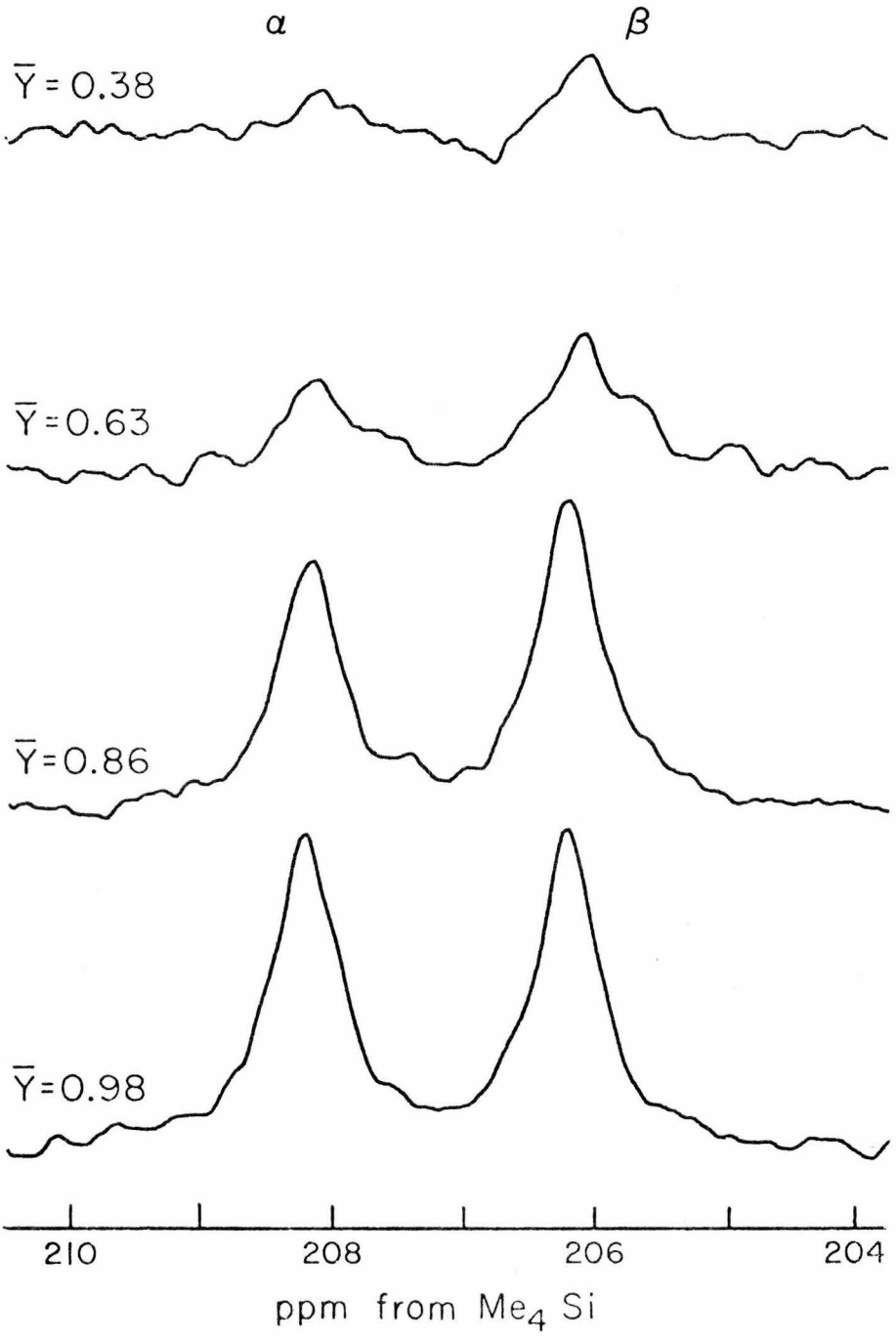
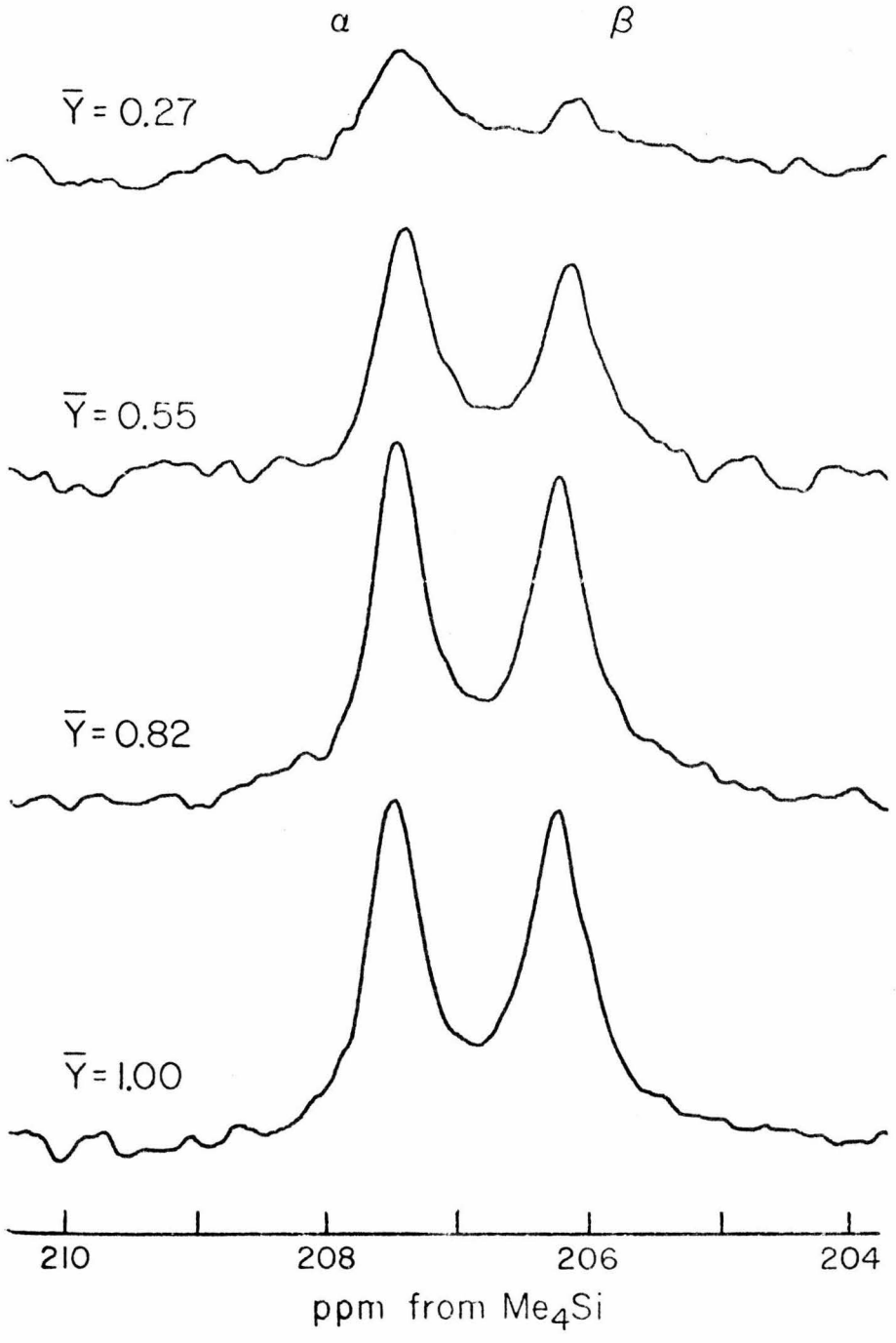

α
β
$\bar{Y}$ = 0.38
$\bar{Y}$ = 0.63
$\bar{Y}$ = 0.86
$\bar{Y}$ = 0.98
210
208
206
204
ppm from Me_4 Si

FIGURE 4

The ^{13}CO resonances of opossum hemoglobin at various stages of partial ^{13}CO ligation. The spectra are plotted in the absolute intensity mode. Sample conditions are the same as in Figure 3.


α
β
$\bar{Y}$ = 0.27
$\bar{Y}$ = 0.55
$\bar{Y}$ = 0.82
$\bar{Y}$ = 1.00
210
208
206
204
ppm from Me_4Si

affinity for ^{13}CO relative to the β subunits, while the α subunits of Hb-O have a higher affinity for ^{13}CO than the β subunits.

The fractional saturation of the individual α and β subunits is obtained from the relation

$$\bar{Y}_{\alpha} = 2\bar{Y} \cdot \frac{S_{\alpha}}{S_{\alpha} + S_{\beta}}$$

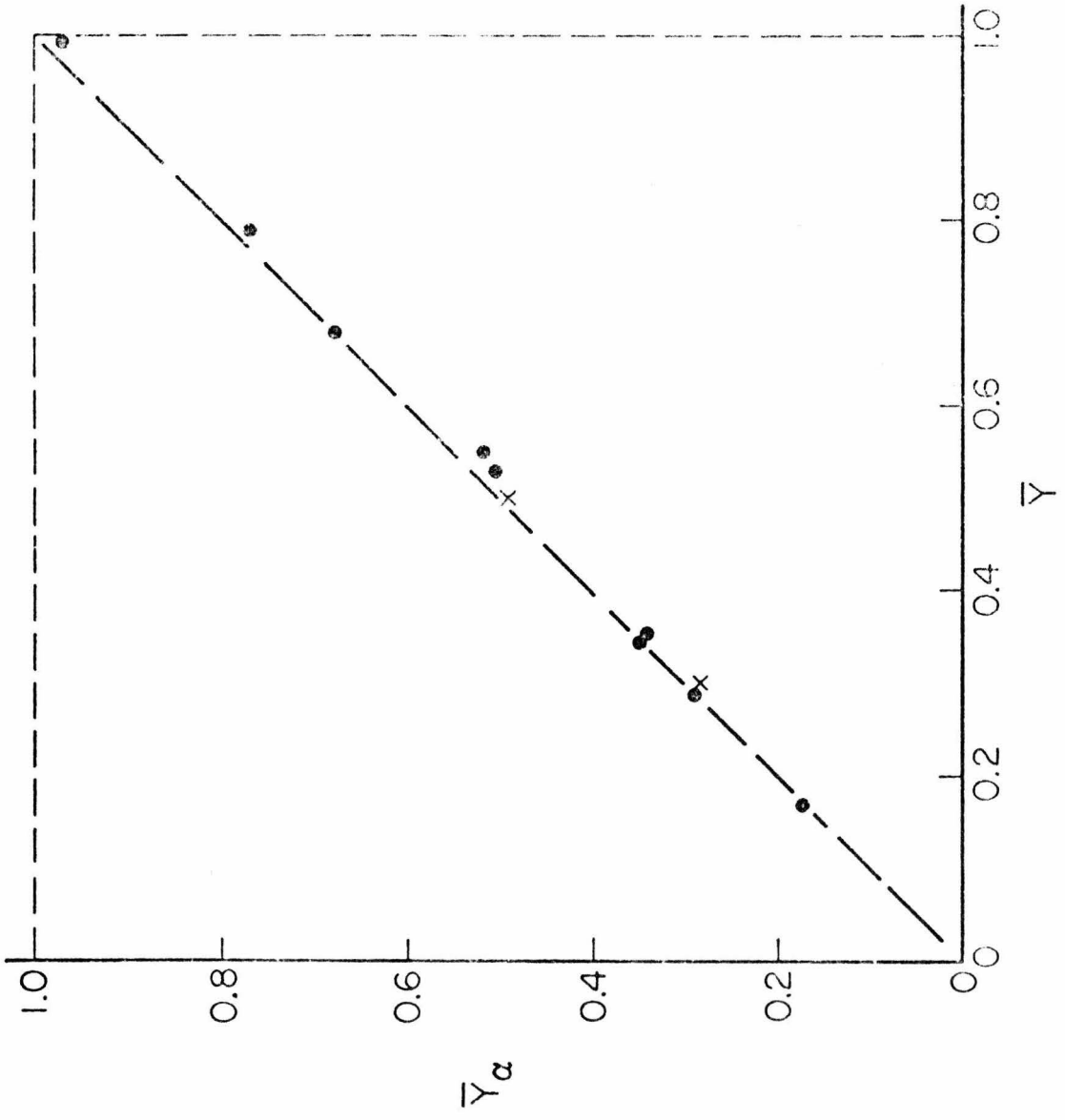
$$\bar{Y}_{\beta} = 2\bar{Y} \cdot \frac{S_{\beta}}{S_{\alpha} + S_{\beta}}$$

where $\bar{Y}_{\alpha}$ and $\bar{Y}_{\beta}$ represent the fraction of liganded α and β subunits, and $\bar{Y}$ is the fractional saturation of hemoglobin with ^{13}CO as determined spectrophotometrically at 650 nm. Figure 5 shows a plot of $\bar{Y}_{\alpha}$ vs. $\bar{Y}$ for Hb-A. It is apparent from these data that the fractional saturation of the α and β subunits closely follows $\bar{Y}$ and that the α and β subunits have nearly equal affinities for ^{13}CO. The intensity of the α resonance appears, on the average, somewhat less intense than the β resonance, suggesting that the β subunits may have a slightly higher equilibrium affinity for ^{13}CO than the α subunits. However, this difference is quite small (1-2%) and could easily be accounted for if the majority of the methemoglobin present (2-3%) was represented by oxidized α subunits (18).

Addition of organic phosphates had no observable effect on the distribution of ^{13}CO between the subunits of Hb-A. Even in the presence of 50 mM inositol hexaphosphate, as shown in Figure 5, we were unable to detect any significant change in the relative affinities of the α and β subunits for ^{13}CO.

FIGURE 5

A plot of the fractional saturation of the α subunits of Hb-A as a function of the overall fractional saturation of Hb as determined spectrophotometrically at 650 nm. Both the phosphate-free (●) and inositol hexaphosphate containing (X) samples show little, if any, deviation between $\overline{Y}_{\alpha}$ and $\overline{Y}$.


1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
$\bar{Y}_\alpha$
$\bar{Y}$

A plot of $\bar{Y}_\alpha$ and $\bar{Y}_\beta$ against $\bar{Y}$ for rabbit hemoglobin is shown in Figure 6. It is quite evident from these data that the α subunits have a lower affinity for ^{13}CO than do the β subunits at all stages of ligation in the range $0.2 < \bar{Y} < 1.0$. The slight discrepancy between the intensities of the α and β resonances near total saturation ($\bar{Y} = 0.98$) is probably due to the majority of the methemoglobin contained in the sample (~2%) being present as oxidized α hemes.

The effect of 10 mM inositol hexaphosphate on the distribution of ^{13}CO between the α and β subunits of Hb-R is to reduce the amount of ^{13}CO bound to β subunits somewhat more than the reduction of ^{13}CO bound to the α subunits. Thus inositol hexaphosphate reduces the difference between the α and β subunits by primarily affacting the binding of ^{13}CO at the β subunits.

Figure 7 shows the relative affinities of the subunits of Hb-O for ^{13}CO. The α subunits of Hb-O exhibit a significantly higher affinity for ^{13}CO than the β subunits at all stages of partial ligation in the range $0.2 < \bar{Y} < 1.0$. At total ^{13}CO saturation the ligation of Hb-O subunits is equal as expected.

Figure 8 presents the data of Figures 6 and 7 replotted to show the dependence of the ratio $\bar{Y}_\alpha/\bar{Y}_\beta$ on $\bar{Y}$ in the absence of organic phosphate.

FIGURE 6

A plot of $\overline{Y}_{\alpha}$ (●) and $\overline{Y}_{\beta}$ (○) against $\overline{Y}$ for rabbit hemoglobin.

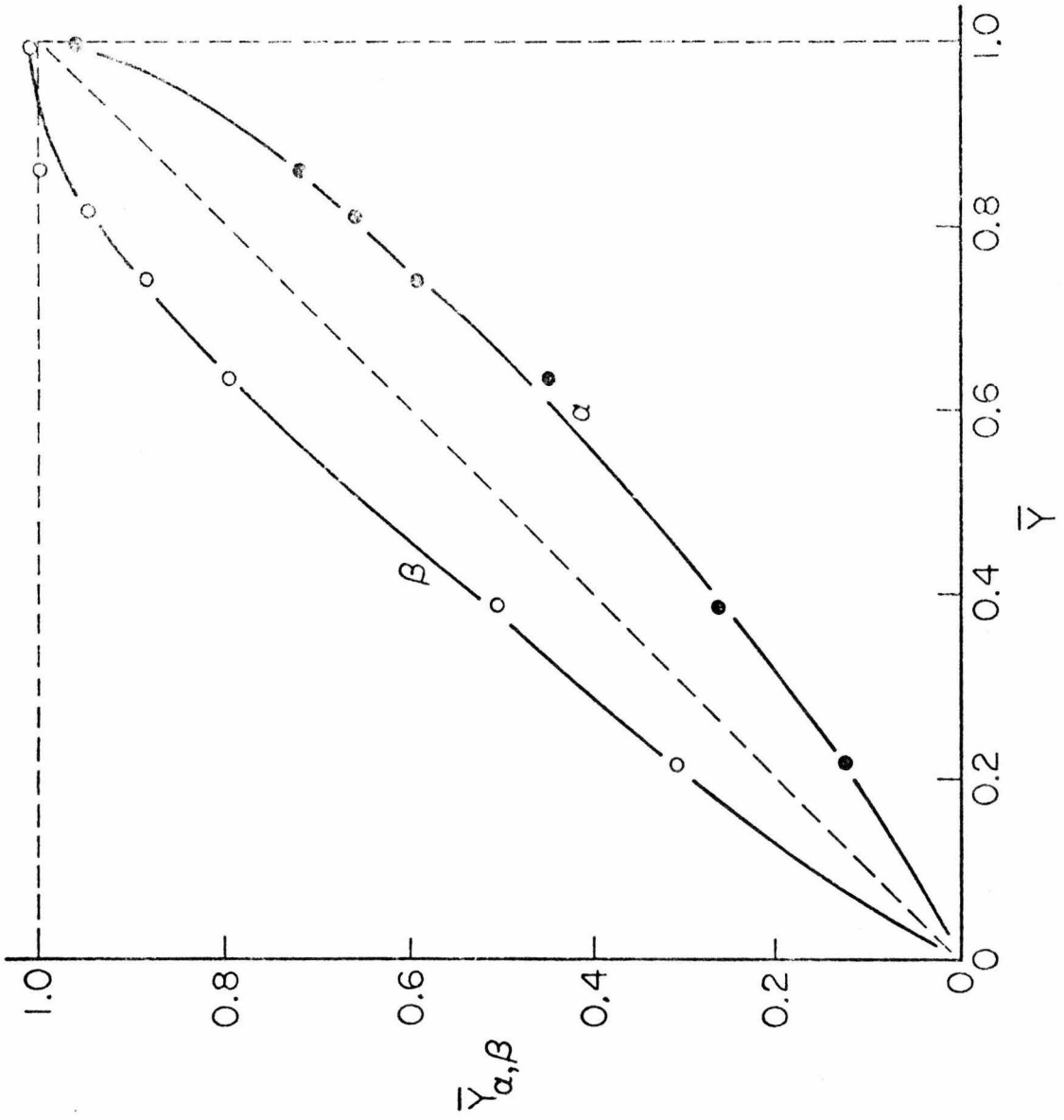

1.0
0.8
0.6
0.4
0.2
0
0.2
0.4
0.6
0.8
1.0
$\bar{Y}_{\alpha,\beta}$
$\bar{Y}$
α
β

FIGURE 7

A plot of $\overline{Y}_{\alpha}$ (●) and $\overline{Y}_{\beta}$ (○) against $\overline{Y}$ for opossum hemoglobin.

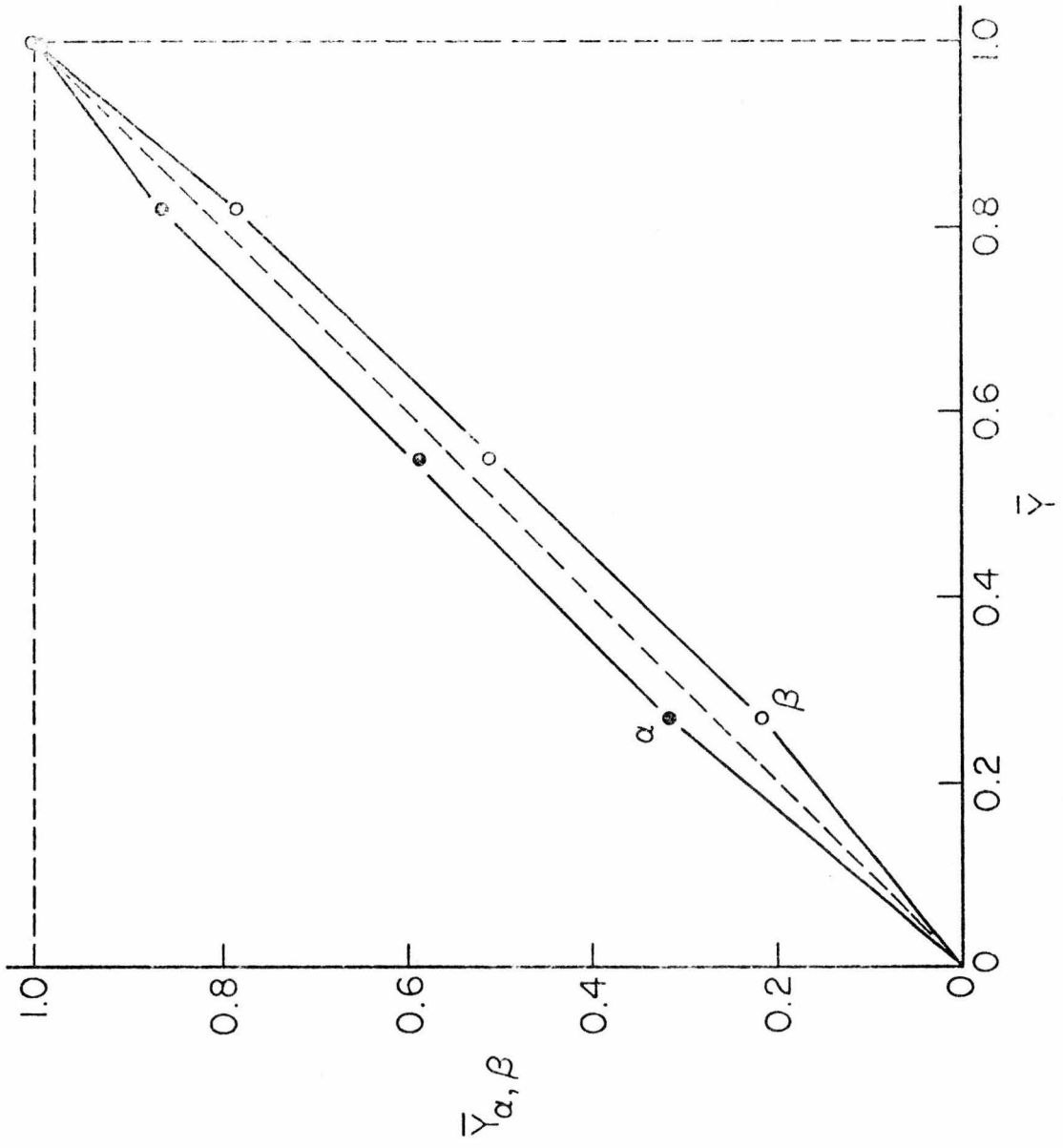
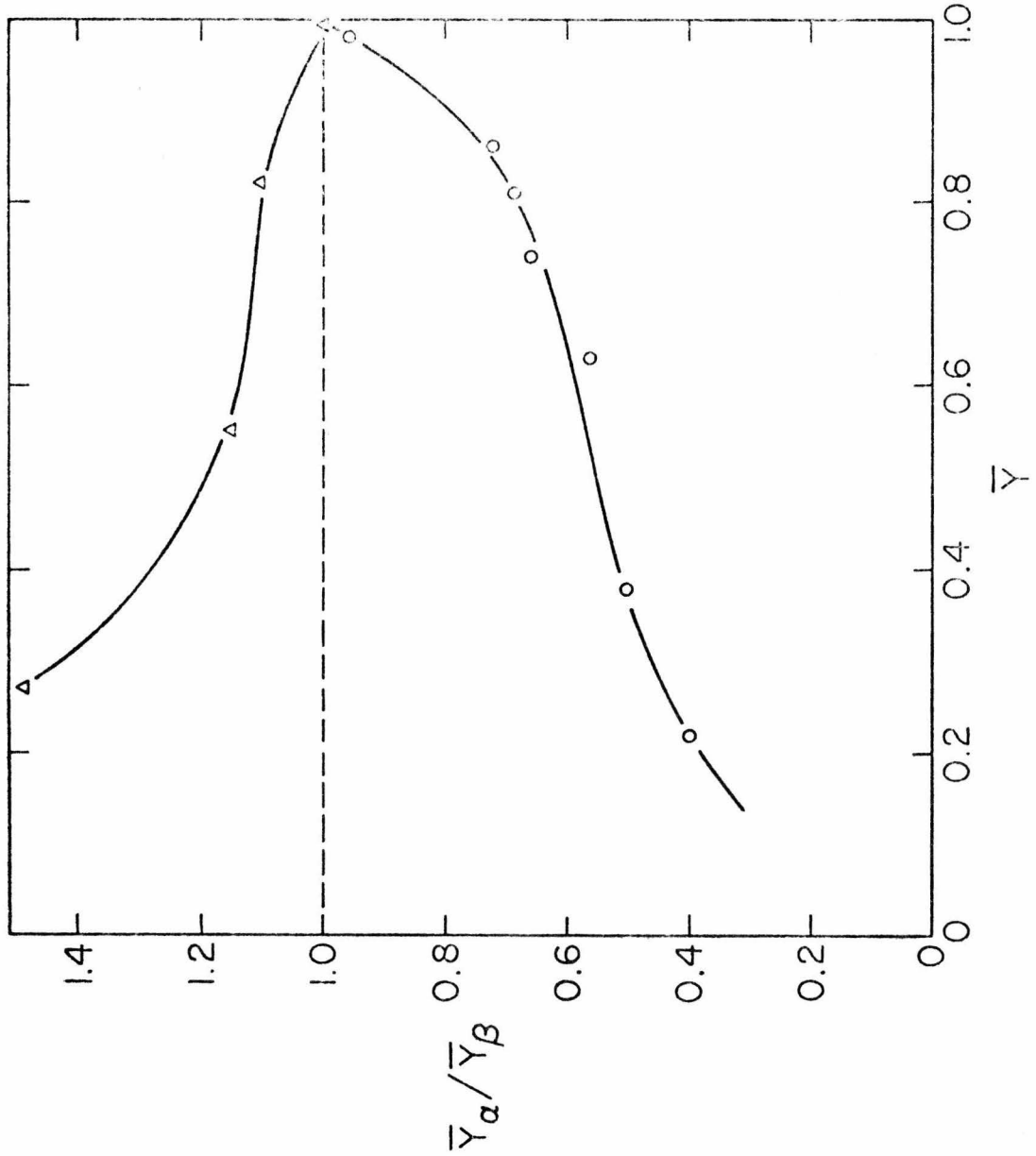

1.0
0.8
0.6
0.4
0.2
0
$\bar{Y}_{\alpha,\beta}$
α
β
$\bar{Y}$

FIGURE 8

A plot of the ratio of partial subunit saturation, $\overline{Y}_\alpha/\overline{Y}_\beta$, against overall fractional saturation, $\overline{Y}$, for rabbit (○) and opossum (Δ) hemoglobins. All values were obtained in the absence of organic phosphates.


1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
$\overline{Y}_\alpha / \overline{Y}_\beta$
0
0.2
0.4
0.6
0.8
1.0
$\overline{Y}$

DISCUSSION

We previously reported preliminary studies of the equilibrium binding of ^{13}CO to the subunits of Hb-R and demonstrated that the α subunits have lower affinity for ^{13}CO than do the β subunits (14). Proton magnetic resonance studies of the differential binding of oxygen and carbon monoxide to the subunits of Hb-A provide a somewhat different result (19). These studies employed intensity measurements of contact shifted heme proton resonances peculiar to the unliganded α and β subunits. The extent of individual subunit ligation was estimated from the relative intensity decrease in these α and β heme resonances. The results suggested that CO binds randomly to the α and β hemes of Hb-A in the absence of organic phosphates. It was further reported that the α subunits may have a slight preference for CO in the presence of 2,3-diphosphoglycerate and that these subunits have a small but definite preference for CO in the presence of inositol hexaphosphate. Our results reported here are in close agreement with the ^{1}H nmr results for CO binding to Hb-A in the absence of organic phosphates. We should like to point out, however, that while both the ^{1}H and ^{13}CO experiments reach similar conclusions, these methods are of fundamentally different character. The ^{1}H nmr studies monitor the absence of ligand, are most sensitive at low $\bar{Y}$, and observe hemoglobin molecules primarily in the T quaternary conformation. Our studies monitor the presence of bound ^{13}CO, are most sensitive at higher $\bar{Y}$, and observe hemoglobin which is expected to be primarily in the R quaternary conformation. Thus the agreement between these respective techniques

suggests very strongly that the affinities of the α and β subunits, of Hb-A for CO are indeed virtually equal at all stages of partial saturation.

A small but perhaps significant difference exists between the ^{1}H and ^{13}CO studies of carbon monoxide binding to Hb-A in the presence of organic phosphates. While the ^{1}H nmr results suggest that CO binds with a slight preference for α hemes in the presence of organic phosphates, we have been unable to detect any changes in the differential affinities of the subunits for ^{13}CO, either in the presence of 2-5 mM 2, 3-diphosphoglycerate or 50 mM inositol hexaphosphate. There are several possible explanations for this small discrepancy. The easiest is simple criticism of the techniques employed. The heme proton resonances lie on the "tail" of the large resonance from residual water and overlap several aromatic proton resonances introducing the possibility of systematic errors in the data (19). On the other hand, the ^{13}CO resonances, while well removed from all other carbon resonances, overlap each other making the detection of differential binding more difficult. Since the ^{1}H nmr results indicated that the maximum difference in CO saturation for the α and β subunits in the presence of inositol hexaphosphate was only 6%, it may well be that the differences between the ^{1}H and ^{13}C results are insignificant. We tend to believe, however, that we could detect differences in Hb-A subunit affinities as small as 2-3%. Another possibility is that the differences observed result from the natures of the two types of experiments. The chemical shifts and line widths of the ^{13}CO resonances are independent of organic phosphate concentration, quaternary conformation (14) and applied magnetic field strength (16) and therefore reflect only the relative

abundance of ^{13}CO liganded subunits ($\bar{Y}_\alpha$ and $\bar{Y}_\beta$). However, the line widths of the α and β heme proton resonances in fully deoxygenated hemoglobin samples are field dependent and increase from ~200 Hz at 90 MHz to ~270 Hz at 250 MNz (19). This suggests the possibility of fast or intermediate spin exchange between two or more unliganded heme species. For example, it is possible that the absolute chemical shift of these heme protons in a particular unliganded subunit in the totally unliganded T quaternary conformation differs from the shift of these same protons in the totally unliganded R conformation. Likewise, these shifts may differ from those of unliganded subunits in various partially liganded hemoglobin intermediates. Since high concentrations of inositol hexaphosphate are known to reduce Hb-A cooperativity, as reflected by the Hill coefficient (20), it is likely that increased concentrations of partially CO liganded hemoglobin species are present in inositol hexaphosphate-containing samples. Thus an apparent preferential binding of CO to α subunits could be observed if the heme proton resonances of unliganded α subunits in partially liganded Hb-A intermediates were sufficiently broadened or shifted away from their normal positions so as not to be observed. In any case, the observed absence or relative insignificance of differential CO binding in Hb-A suggests that the equilibrium constants for CO binding to the α and β subunits of Hb-A, either in the T or R quaternary conformations, must not differ appreciably (i.e., $K_T^\alpha \sim K_T^\beta$ and $K_R^\alpha \sim K_R^\beta$ for CO binding to Hb-A).

The situation is much different for rabbit and opossum hemo-

globins. Here the affinities of the α and β hemes for ^{13}CO are unmistakably different. The data of Figures 6-8 clearly demonstrate the existence of substantial amounts of partially liganded hemoglobin species under equilibrium conditions. For Hb-R in the region Y ~.5 the data in Figure 8 suggest that the dominant partially liganded hemoglobin species is probably $\alpha^{O}\alpha^{L}\beta^{L}\beta^{L}$.

As mentioned before, the presence of 10 mM inositol hexaphosphate in Hb-R samples reduces the ^{13}CO affinity of the β subunit more than it does the α subunit. This fact could be easily rationalized by the usual argument that organic phosphates should be expected to have their greatest effect on the β hemes because organic phosphates bind between the β subunits (21). The effects observed for Hb-R, however, may also be explained if we assume that the $\alpha^{O}\alpha^{L}\beta^{L}\beta^{L}$ species has the R quaternary conformation. The addition of large quantities of organic phosphate into partially liganded Hb-R samples would then be expected to reduce the concentration of $\alpha^{O}\alpha^{L}\beta^{L}\beta^{L}$ by shifting the R—T equilibrium toward T. This effect would result in a larger observed decrease in $\bar{Y}_{\beta}$ than would be observed for $\bar{Y}_{\alpha}$. In general, one would expect organic phosphate-induced shifts in the R—T equilibrium to principally effect $\bar{Y}_{\beta}$ if, in the R quaternary conformation, the β subunits have the higher affinity for ligand.

It is particularly interesting that all of these hemoglobins (Hb-A, R, O) exhibit virtually the same high degree of allosteric cooperativity. We have determined that the Hill coefficient for oxygen binding to Hb-R under conditions similar to those reported here is $n = 3.0 \pm 0.1$ (22). The Hill coefficient for O_2 binding to Hb-O is

$n = 2.89 \pm 0.18$ (23). In general, mammalian hemoglobins all have values of $n = 2.8\text{-}3.0$ and the shape of the curves for CO binding are similar to those for O_2 binding (24). Thus these results show that substantial amounts of partially liganded hemoglobin species may exist under equilibrium conditions without altering the experimentally determined Hill coefficient. It follows from this that any physical model used to describe hemoglobin ligation must, as a minimal requirement, include terms which account for the nonequivalence of the subunits. The GCT model (6-8) meets this requirement. It would seem useful to apply conventional methods and further test this and other models in their ability to describe Hb-R and Hb-O ligation where subunit nonequivalence is greater than in Hb-A.

The peculiar ^{13}CO affinities observed for the α subunits of Hb-R and Hb-O are probably caused by the substitution of residues which affect the ligand binding site at the distal side of the heme. We previously identified the presence of a phenylalanine residue at position CD7 of the rabbit α subunit as being responsible for the abnormal ^{13}CO chemical shift (14). We believe this residue interacts with the distal histidine residue (E7) and is also responsible for the abnormal ligand affinity of this subunit (17). In this regard it is of particular interest that we have recently obtained experimental evidence for the existence of a hydrogen bond between the distal histidine and the ^{13}CO ligand (16). In the case of Hb-O, the distal histidine residue of the α subunit has been replaced by glutamine (25) and should be the primary cause of the abnormal ^{13}CO chemical shift and affinity of this subunit.

This provides further evidence that the absolute ligand affinities of hemoglobin subunits may be controlled by the nature of the distal heme environment without pathologically affecting the stability of the heme toward oxidation.

In their ^1H magnetic resonance studies, Johnson and Ho (19) demonstrated that the relative affinities of the α and β subunits of Hb-A vary depending on the nature of the ligand. They observed that while CO binds to the subunits in a virtually random manner, O_2 binds with a slight preference for the α subunits. This preference was enhanced in the presence of organic phosphates. Further, the binding of n-butyl isocyanide to Hb-A has been reported to occur preferentially at the β subunits (26). It is likely, therefore, that the relative affinities of the subunits of Hb-R and Hb-O will also vary as a function of the type of ligand. Again we believe that these differences result from the nature of precisely controlled environments at the distal side of the heme. From model building studies it has been suggested (2) that steric effects due to the long butyl side chain of n-butyl isocyanide are responsible for decreased reactivity of this ligand with the α subunits. However, as Johnson and Ho pointed out (19), it is much more difficult to explain the differences between the binding properties of O_2 and CO since these ligands are of about the same size and shape. In this regard we believe that the potential for hydrogen bond formation between the distal histidine residue and various bound ligands may be of considerable importance in determining the relative and absolute affinities of various hemoglobin subunits for ligands. We will discuss this possibility and the general nature of the ligand environment as sensed by

^{13}CO in considerable detail elsewhere (17).

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PART V

Determination of Spin-Lattice Relaxation Times and Nuclear Overhauser Effects for ^{13}CO Bound to Myoglobin and Hemoglobin

INTRODUCTION

The useful application of ^{13}C magnetic resonance to discriminate between the heme environments experienced by ^{13}C-enriched carbon monoxide bound to myoglobin and the α and β subunits of hemoglobins has been demonstrated in a number of recent reports (Moon and Richards, 1972; Matwiyoff and Needham, 1972; Matwiyoff et al., 1973; Vergamini et al., 1973; Moon and Richards, 1974). These studies are particularly valuable in that they provide precise information about the character of the bound ligand which may be compared with other studies of hemoprotein function, such as ^{1}H magnetic resonance studies of the heme environment (Shulman et al., 1972; Johnson and Ho, 1974) or the nature of various probes of hemoglobin quaternary conformation (Ogata and McConnell, 1972; Huestis and Raftery, 1973), to yield a more detailed picture of the chemical processes responsible for oxygen transport and storage in mammals and other animals.

Previous ^{13}CO studies have shown conclusively that the chemical environments experienced by the bound ^{13}CO ligand differ between myoglobin and the α and β subunits of hemoglobin. More recently we were able to show that the relative thermodynamic affinities of hemoglobin subunits for ^{13}CO may be different for some hemoglobins (Moon and Richards, 1974). In our most recent work we have employed quantitative measurements of the intensities of the α and β subunit ^{13}CO resonances to gain information about the relative equilibrium affinities of the subunits for carbon monoxide at various stages of partial ^{13}CO saturation (Moon and Richards, 1975). We have also studied the ^{13}CO

chemical shifts of myoglobins and hemoglobins containing hemes modified at the 2- and 4-positions of the porphyrin ring to obtain information about the electronic substituent effects transmitted through the heme to the bound ligand and to better understand the nature of the heme-protein interactions responsible for determining the ligand environment and modulating heme-ligand reactivity (Moon et al., 1975).

One of the principle objectives in our work has been to answer several fundamental questions about the nature of the ligand binding sites in myoglobins and hemoglobins. To what extent do residues surrounding the heme participate in ligand bonding? How important are steric factors operating around the bound ligand in determining the stability of the heme-ligand bond? What are the exact functions of the highly conserved distal histidine (E7), valine (E11) and phenylalanine (CD1) residues which line the ligand binding sites of most myoglobins and hemoglobins? It is the objective of the present work to provide some insight into these questions through the study of the spin-lattice relaxation times (T_1) and nuclear Overhauser effects (NOE), exhibited by the ^{13}CO ligand when bound to sperm whale myoglobin or human adult hemoglobin. From the results of these studies we wish to draw particular attention to a methyl group of valine (E11) and the imidazole of histidine (E7) as being of especial importance in determining the environment of the ^{13}CO ligand.

EXPERIMENTAL

Sperm whale myoglobin was obtained from Sigma. The impure commercial product was dissolved in 0.03 M phosphate buffer, pH 6.5, and applied to a column of SP Sephadex. The column was eluted with the same buffer and the fourth myoglobin fraction, containing the major oxidized component, was collected and concentrated by ultrafiltration (Amicon, PM-10 membrane). This product gave a single band on polyacrylamide gel electrophoresis. The purified metmyoglobin was then shaken with a 1.5-fold excess of sodium metabisulfite in a syringe containing ^{13}C-enriched carbon monoxide (90-92% enriched). The resulting carboxymyoglobin was dialyzed against 0.10 M phosphate, pH 7.0, and concentrated by ultrafiltration for NMR study.

Human adult hemoglobin was prepared from the freshly drawn citrated whole blood of a normal donor. The red cells were separated from plasma by centrifugation at 2000 x g, washed several times with 0.15 M sodium chloride, and lysed hypotonically with three volumes of distilled water at ambient temperature for 20 minutes. The stromata were removed by centrifugation at 30,000 x g and the resulting hemolysates were dialyzed overnight against two 4 l changes of 0.15 M sodium chloride at 4°C. The hemoglobin solutions were concentrated by ultrafiltration (Amicon, UM-10 membrane) and further dialyzed against water in the ultrafiltration apparatus under nitrogen. The concentrated hemoglobin solutions (~ 3 mM in tetramer) were then applied to a column of Sephadex G-50 and eluted with water. The first fraction immediately following the void volume, which contained high mo cular

weight contaminants, was discarded. The purified hemoglobin was concentrated by ultrafiltration, dialyzed against 0.15 M sodium chloride under nitrogen, and again concentrated for NMR study.

Carboxymyoglobin concentrations were determined spectrophotometrically at 423 nm using an extinction coefficient of ϵ = 187 mM. Final hemoglobin concentrations were determined using the ratios of absorptions at 415, 420, and 430 nm (Benesch et al., 1965). Methemoglobin concentrations never exceeded 3%. The sample pH was in the range pH 6.9 - 7.0. The hemoglobin samples were then shaken in a syringe with ^{13}CO and sealed in the NMR tube with excess ^{13}CO over the sample.

^{13}C magnetic resonance spectra were obtained using the pulse Fourier transform technique on a Varian Associates XL-100-15 spectrometer equipped with a Varian 620 i computer (16 K memory) for data accumulation and Fourier transformation. Samples were contained in a 12 mm tube with a 5 mm tube containing D_2O inserted concentrically to serve as a field frequency lock. All spectra were obtained under identical conditions at a probe temperature of 34°C using a 90° pulse of 55μ sec duration with a spectrum width of 2500 Hz.

Spin-lattice relaxation time (T_1) measurements were made with proton noise decoupling using the progressive saturation intensity ratio technique as described by Freeman and Hill (1971). At least four independently determined T_1 values were obtained for each of the oxygen-free myoglobin and hemoglobin samples. The pulse intervals used in these determinations were varied randomly so as to eliminate systematic

errors which might develop during the long data-acquisition periods (1-10 hrs) necessary to achieve adequate signal-to-noise.

Nuclear Overhauser effects (NOE) were determined both by direct integration of the ^{13}CO resonances with and without proton noise decoupling and by comparison of intensity ratios using continuous and gated noise decoupling techniques (Freeman et al., 1972). In either case, the pulse intervals used for NOE determinations were about $3T_1$ for the corresponding ^{13}CO resonances. Myoglobin samples used for NOE studies were concentrated to 3-4 mM. All hemoglobin samples used for NOE determinations were 2.0 mM in tetramer.

Solution viscosities for carboxymyoglobin samples in 0.1 M phosphate and carboxyhemoglobin samples in 0.15 M sodium chloride were measured with an Ostwald capillary viscometer at 34°C. Solution densities were determined in a picnometer (1.00 ml) equilibrated at 34°C. The viscosity and density measurements were carried out over the entire range of sample concentrations used in the T_1 and NOE studies.

RESULTS

T_1 Studies

The dependence of T_1 on protein concentration for the ^{13}CO resonances of carboxymyoglobin (MbCO) and carboxyhemoglobin (HbCO) is shown in Figures 1 and 2. The T_1's obtained for HbCO at 14.1 kG (Figure 2) were obtained on a Varian DFS-60 spectrometer equipped with a 10 mm probe and Fourier transform capability (Armitage et al. 1974). The error bars denote standard mean deviations of the individual T_1 values obtained at each concentration. Since the ^{13}CO resonances for the α and β subunits of hemoglobin overlap considerably, the intensities of both these resonances were taken together in all T_1 and NOE studies. No systematic differences between the peak heights of these resonances were detected in any of the samples, suggesting that the T_1's of these resonances are equal within the experimental limits of the instrumentation.

The data in Figures 1 and 2 show that the T_1's increase steadily with increasing protein concentration. While this effect is only barely visible in the MbCO samples, it becomes very apparent at higher HbCO concentrations where longer rotational correlation times, τ_c, would be expected. Furthermore, the T_1's obtained for HbCO in a 14.1 kG magnetic field are shorter than those obtained at 23.5 kG. These results would be expected if the rotational correlation time of ^{13}CO bound to MbCO or HbCO is on the order of 10^{-8} sec and ^{13}CO relaxation is dominated by a $^{13}C-^{1}H$ dipolar mechanism (Doddrell et al., 1972; Farrar and Becker, 1971). Thus, it is likely that the bound ^{13}CO ligand

FIGURE 1

The dependence of T_1 for ^{13}CO on myoglobin concentration. The values were obtained for MbCO samples in 0.10 M phosphate, pH 7.0. Error bars represent standard mean deviations for at least four independent measurements. Other points represent single determinations.

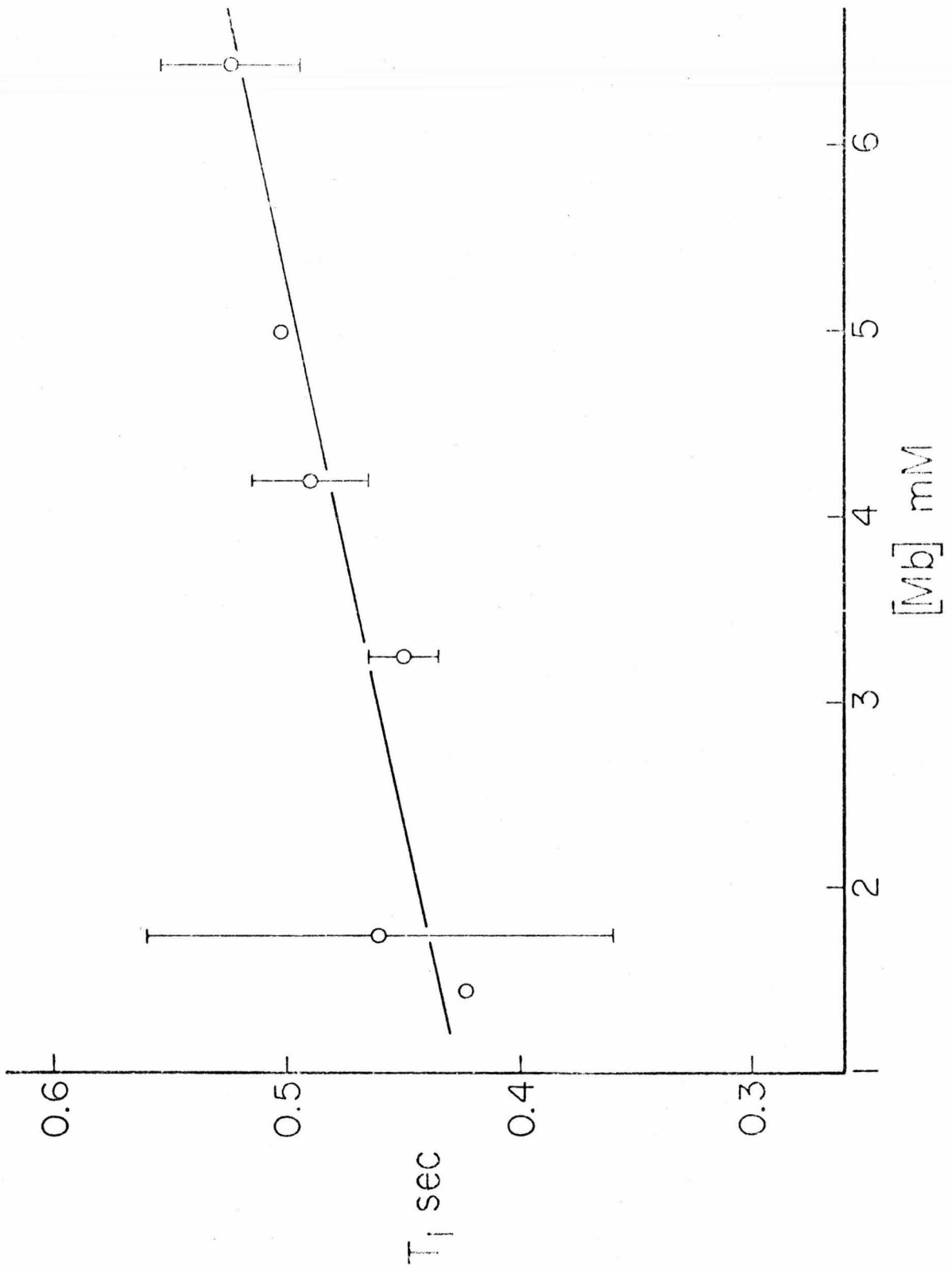
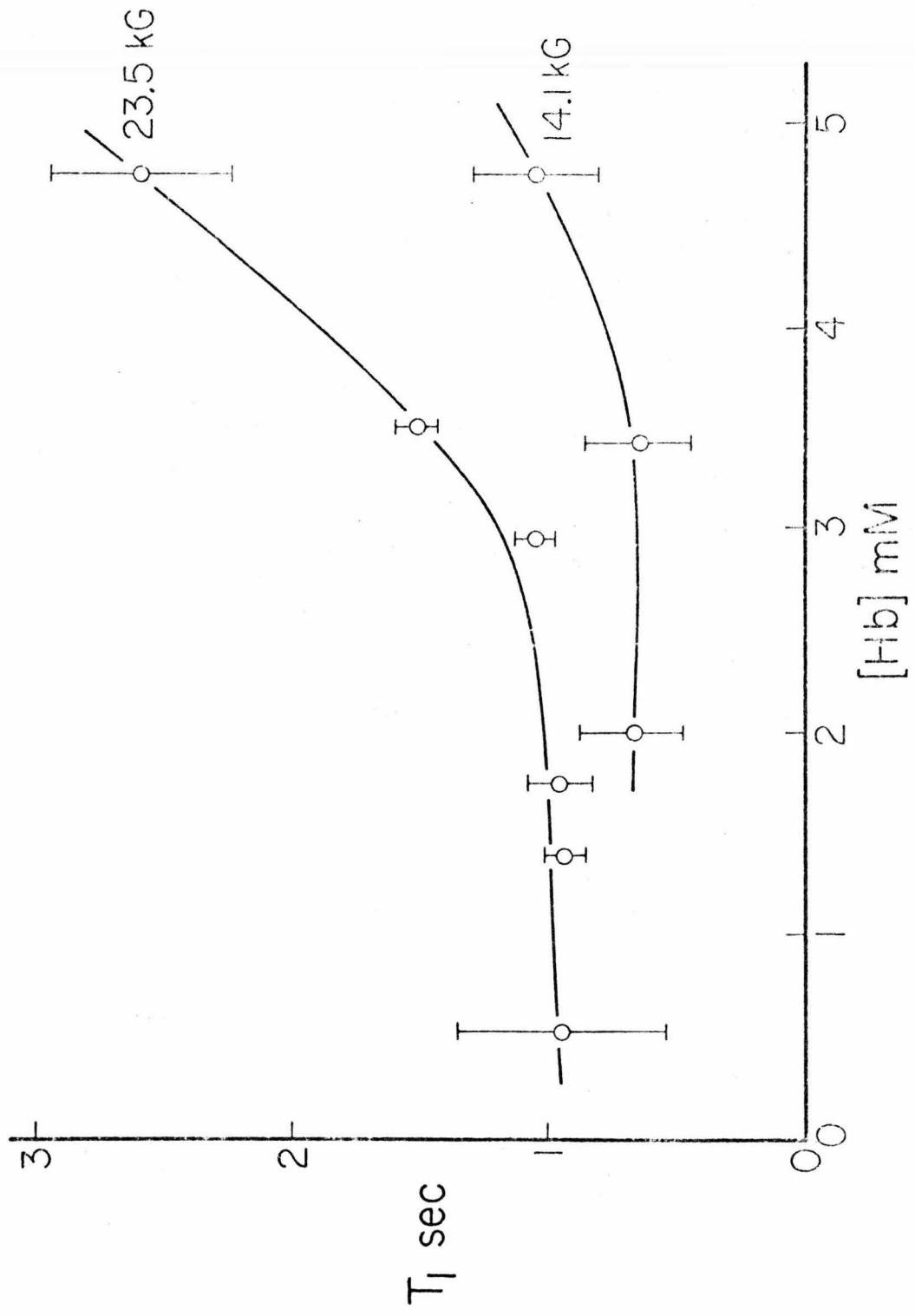

0.6
0.5
0.4
0.3
T_i sec
1
2
3
4
5
6
[Mb] mM

FIGURE 2

The dependence of T_1 for ^{13}CO on hemoglobin concentration. The values were obtained for HbCO samples in 0.15 M sodium chloride, pH 6.9-7.0. Error bars represent standard mean deviations for at least four independent T_1 determinations.

The measurements were made at two different magnetic field strengths: H_0 = 23.5 kG and 14.1 kG.


23.5 kG
14.1 kG
T_1 sec
[Hb] mM
0
1
2
3
4
5

has a correlation time equal to that of the entire protein molecule and undergoes ^{13}C spin relaxation by dipole-dipole interactions with one or more protons bound to the residues lining the ligand binding site on the distal side of the heme plane. Since the ^{13}CO T_1's observed for a totally dipolar $^{13}C-^{1}H$ relaxation mechanism depend upon both τ_c and the distance, r, between the dipoles, we may test the likelihood of dipolar relaxation between ^{13}CO and neighboring protons by first estimating τ_c at various protein concentrations by an independent means and then calculating the values of r using the experimental T_1's obtained at 14.1 kG and 23.5 kG for each of the various samples. If the dipolar mechanism dominates, a consistent value of r should be obtained.

If we make the reasonable assumption that the bound ^{13}CO has the same correlation time as the entire protein molecule, we may obtain good estimates of τ_c using the microviscosity theory of Gierer and Wurtz (1953). Here the rotational correlation time of a spherical molecule is given by the equation

$$\tau_c = \eta V_m f_r / kT \qquad (1)$$

where η is the solution viscosity, V_m is the volume of the sphere, f_r is the microviscosity correction term which accounts for solvent molecules of finite size, k is the Boltzman constant, and T is the absolute temperature. The microviscosity term is a function of the difference in size between the solvent and solute particles and is given by

$$f_r = [6\, r_s/r_o + (1 + r_s/r_o)^{-3}]^{-1} \qquad (2)$$

Hence the microviscosity factor for pure liquids ($r_o = r_s$) becomes

$f_r = 1/6.125 \approx 0.163$ (Noggle and Schirmer, 1971). In the limit $r_o \gg r_s$, which is more nearly the case for macromolecules in water, $f_r \sim 1$.

The concentration dependence of solution viscosity under our experimental conditions was determined experimentally from the solution densities, ρ (Figure 3), and values for η/ρ obtained by Ostwald viscometry. The concentration dependence of η is shown in Figure 4. Using these values of η and appropriate values for the effective radii of the HbCO, MbCO and water particles, we may calculate the τ_c's for HbCO and MbCO in our samples (Figures 5 and 6). In the HbCO calculations we used an average value for the radius of gyration of oxyhemoglobin (Hb: $r_o = 24.2$Å) obtained from neutron and x-ray diffraction studies (Schneider et al., 1969; Conrad et al., 1969). The radius of gyration for MbCO (Mb: $r_o = 16.2$Å) was estimated from the intrinsic viscosities of myoglobin (3.41 cm^3/g, Banaszak et al., 1963) and hemoglobin (3.1 cm^3/g, Marcy and Wyman, 1941) and the known radius of hemoglobin (24.2Å), using the relation (Tanford, 1961)

$$R^3 = \frac{3M}{10\pi N_o} \cdot [\eta] \qquad (3)$$

where $[\eta]$ is the intrinsic viscosity, M is the molecular weight, and N_o is Avogadro's number. Using the same relation we estimated the radius of water to have a maximum value of about $r_s = 1.5$Å. Using these radii the microviscosity terms become $f_r = 0.76$ for MbCO and $f_r = 0.83$ for HbCO.

The general equation for the actual dependence of T_1 for a nuclear

FIGURE 3

The dependence of solution density, ρ, on MbCO and HbCO concentration under our experimental conditions at 34° C.

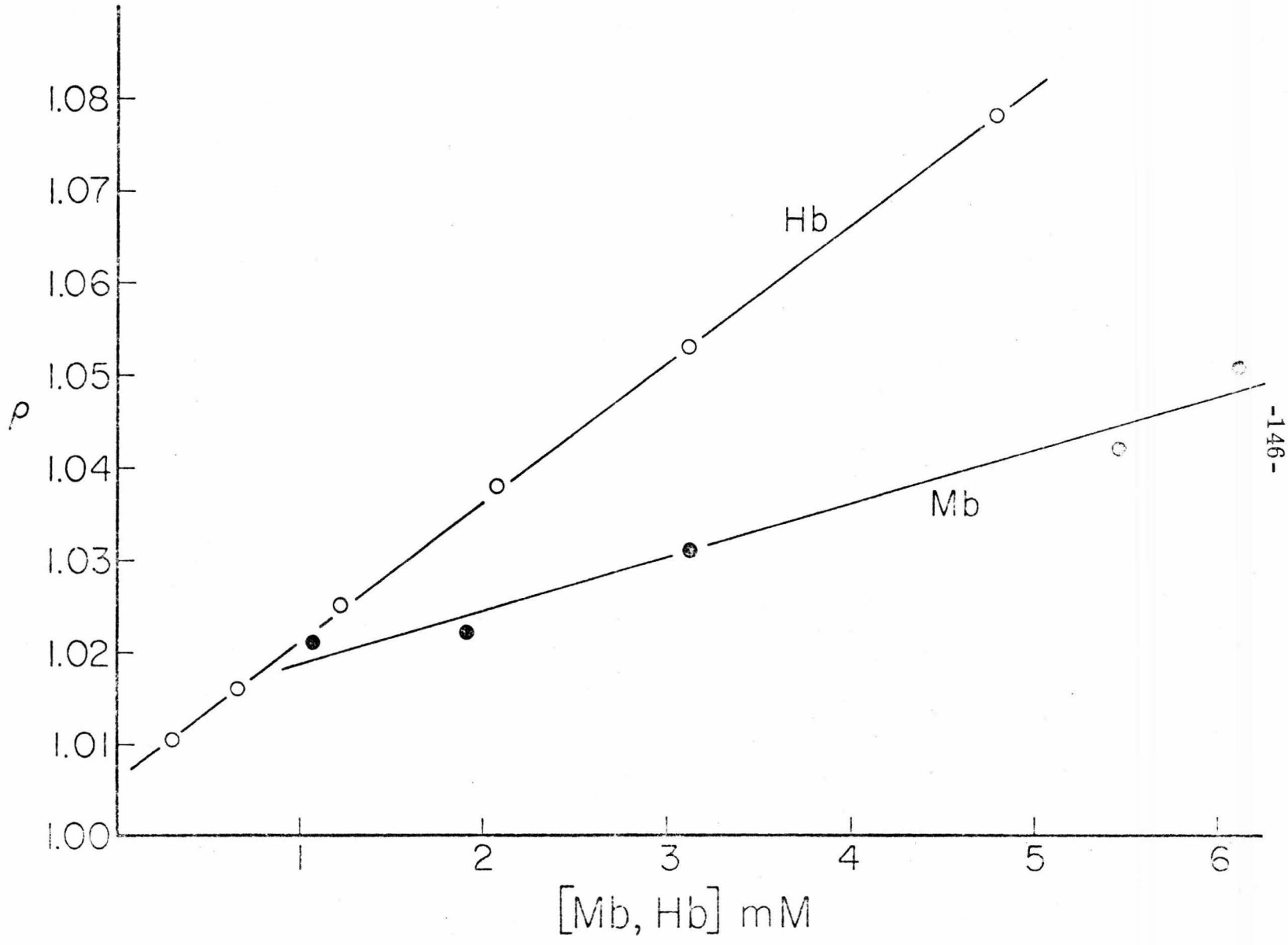

1.08
1.07
1.06
1.05
1.04
1.03
1.02
1.01
1.00
ρ
Hb
Mb
1
2
3
4
5
6
[Mb, Hb] mM

FIGURE 4

The dependence of solution viscosity, η, on sample concentration under our experimental conditions.

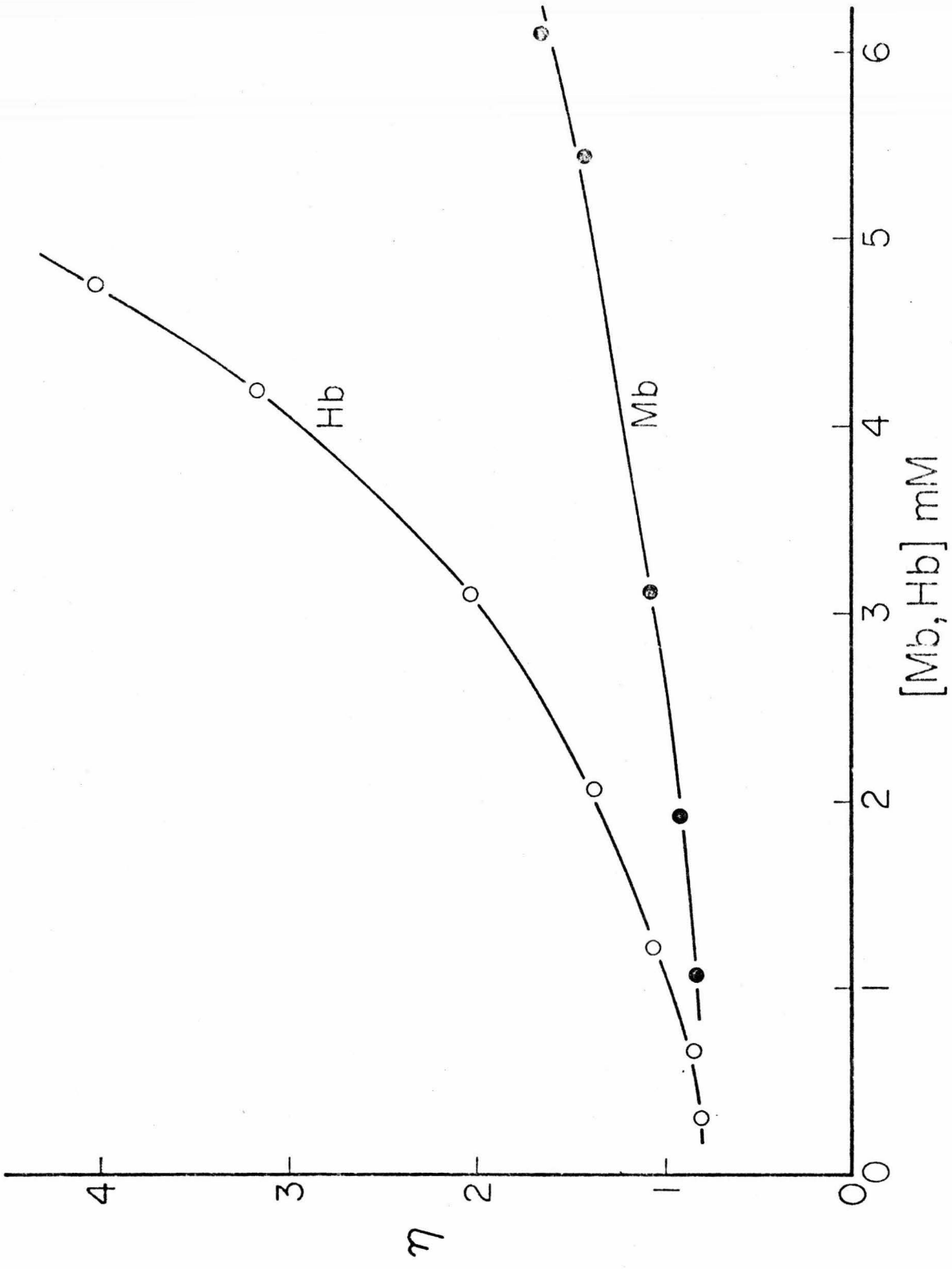

Hb
Mb
η
[Mb, Hb] mM
0
1
2
3
4
5
6

FIGURE 5

The dependence of rotational correlation time, τ_c, on MbCO concentration. The values of τ_c were calculated using the correction for microviscosity according to equations (1, 2). The τ_c* values are uncorrected for microviscosity, $f_r = 1$, and are included for comparison.

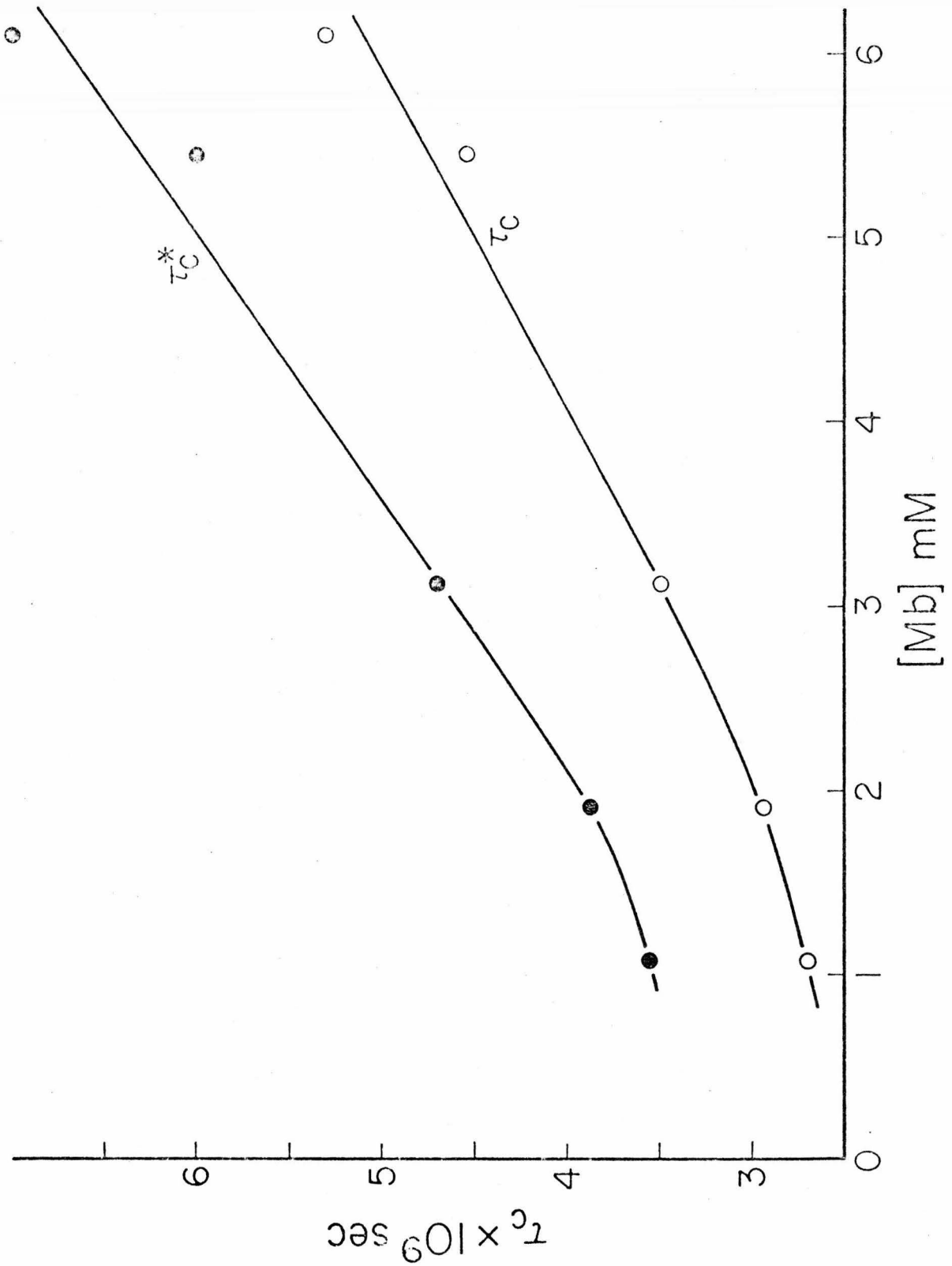
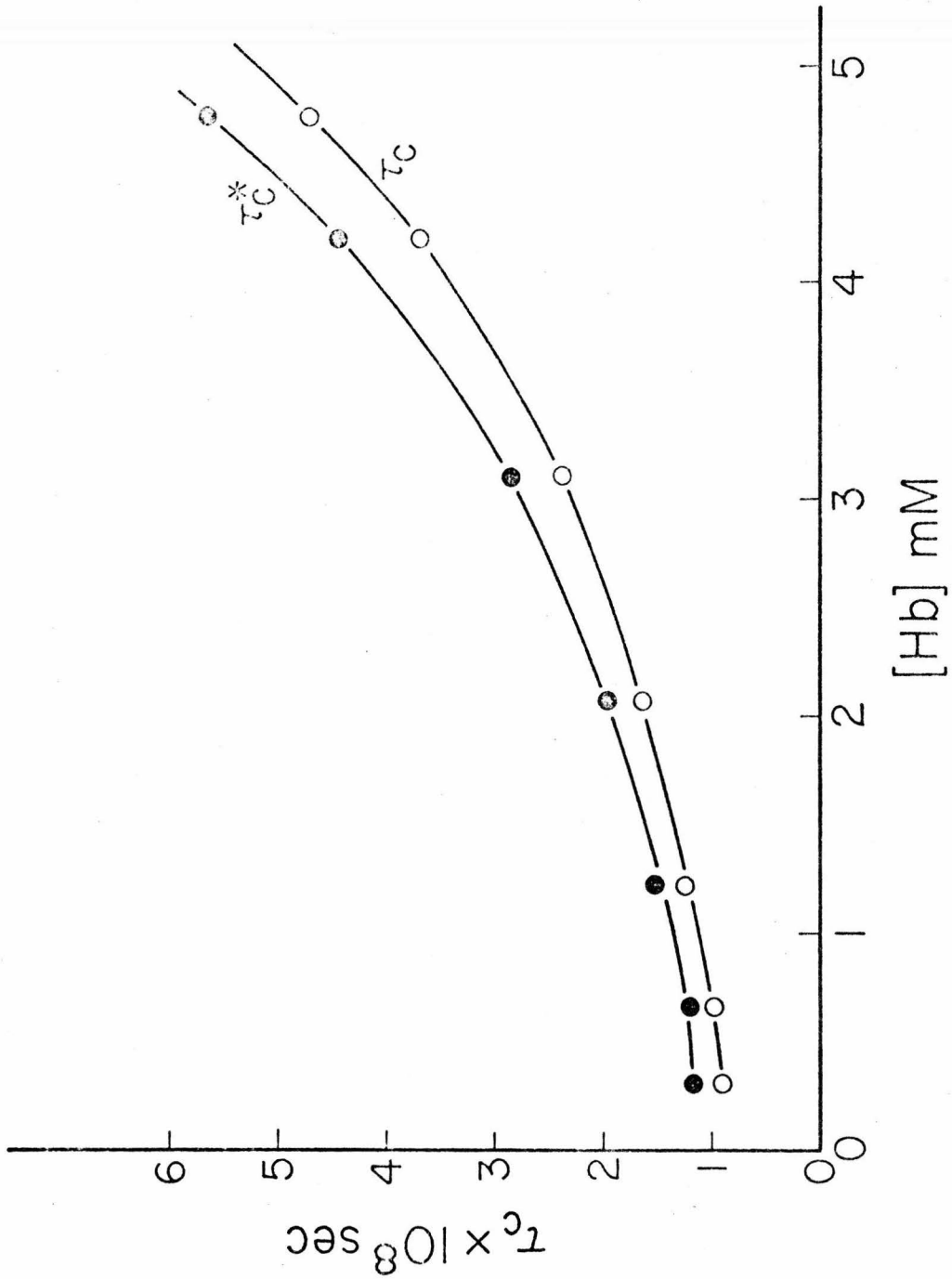

$\tau_c \times 10^9$ sec
3
4
5
6
0
1
2
3
4
5
6
[Mb] mM
τ_{c_2}
$\tau^*_{c_2}$

FIGURE 6

The dependence of τ_c on HbCO concentration. The microviscosity corrected, τ_c, and uncorrected, τ_c*, were calculated as in Figure 5.


$\tau_c \times 10^8$ sec
0
1
2
3
4
5
6
[Hb] mM
0
1
2
3
4
5
τ_{c_2}
$\tau^*_{c_2}$

spin, I, relaxing via a dipolar mechanism to a neighboring spin, S, is given by (Farrar and Becker, 1971)

$$R_1^I = T_1^{-1} = \gamma_I^2 \gamma_S^2 \hbar^2 S(S+1)\{\frac{1}{12} J_0(\omega_I - \omega_S) + \frac{3}{2} J_1(\omega_I) + \frac{3}{4} J_2(\omega_I + \omega_S)\}$$

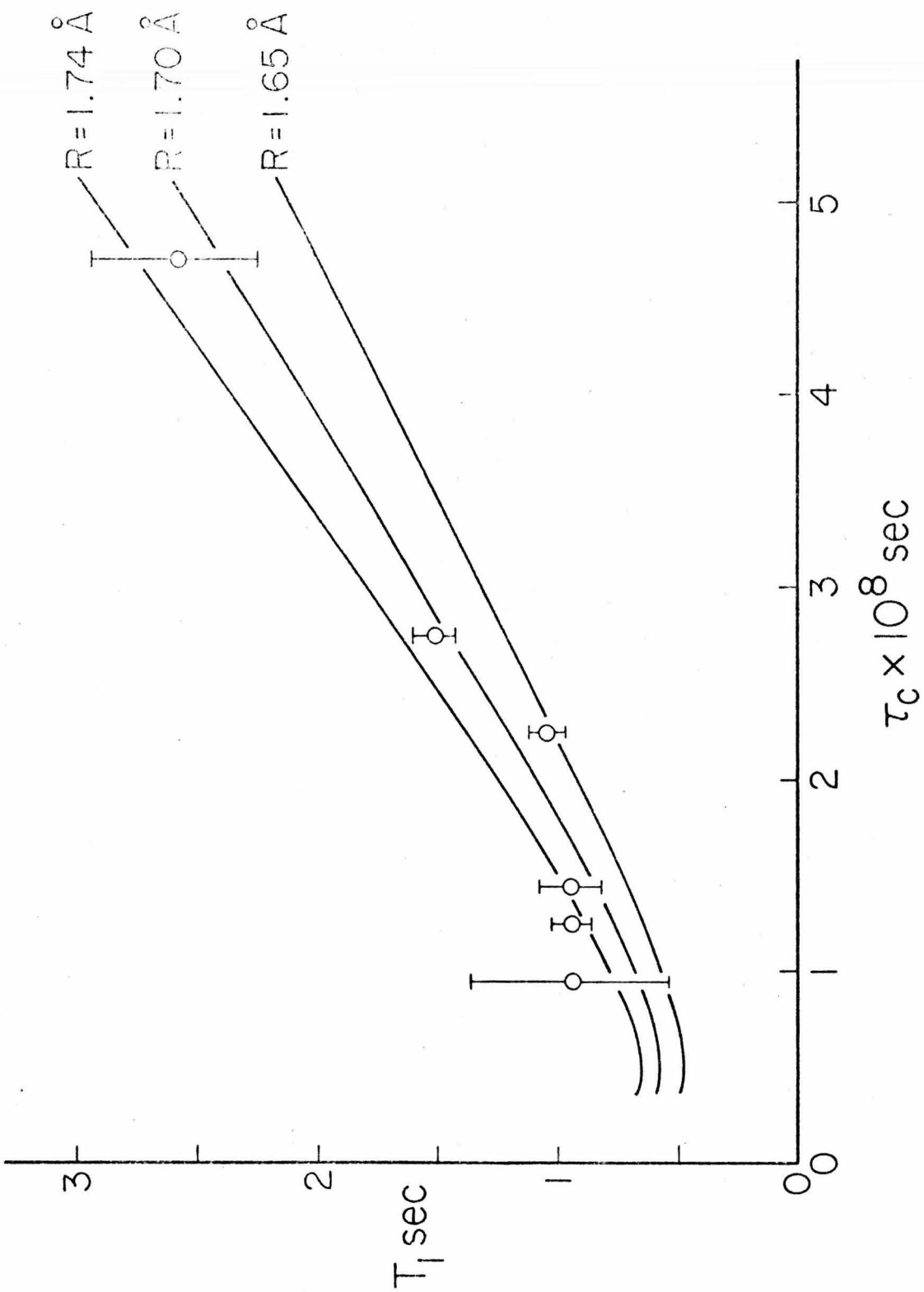
where
$$J_0(\omega) = (24/15r^6)[\tau_c/(1 + \omega^2\tau_c^2)]$$
$$J_1(\omega) = (4/15r^6)[\tau_c/(1 + \omega^2\tau_c^2)] \qquad (4)$$
$$J_2(\omega) = (15/15r^6)[\tau_c/(1 + \omega^2\tau_c^2)]$$

For our purposes γ_I and γ_S are the magnetogyric ratios of ^{13}C and ^{1}H nuclei, ω_I and ω_S are the Lamor frequencies of ^{13}C and ^{1}H at 23.5 kG or 14.1 kG, and S = 1/2. Using this equation we may generate curves for the dependence of T_1 on τ_c for various assumed values of r, and compare these calculated curves with the experimentally determined T_1's. Figure 7 shows the T_1 values obtained for HbCO at 23.5 kG plotted as a function of the correlation time and compared with several theoretical curves based on selected values of r. The MbCO T_1's of Figure 1 lie near the minimum of the r = 1.65Å curve. Using our values of T_1 and τ_c, and solving equation (4) for r, we obtain r = 1.77 ± .05 Å for HbCO at 23.5 kG, r = 1.77 ± .07Å for HbCO at 14.1 kG, and r = 1.65 ± .02Å for MbCO at 23.5 kG.

The only assumptions made in these calculations are that (i) ^{13}CO relaxes by a totally dipolar mechanism and (ii) only one proton is involved. These assumptions may be tested by studying the nuclear Overhauser effects experienced by ^{13}CO.

FIGURE 7

The dependence of T_1 on correlation time. The curves represent the theoretical dependence of T_1 on τ_c for ^{13}CO relaxing by a dipolar mechanism to a single proton at various distances calculated according to equation (4). The experimental T_1 values for HbCO at 23.5 kG (Figure 2) are included for comparison.


R=1.74 Å
R=1.70 Å
R=1.65 Å
T_1 sec
$\tau_c \times 10^8$ sec
0
1
2
3
4
5

NOE Studies

If a ^{13}CO nucleus relaxes by a dipolar mechanism to a neighboring proton the integrated intensity of the ^{13}C resonance will increase if the resonance absorption of the proton is saturated (Noggle and Schirmer, 1971). These nuclear Overhauser effects for ^{13}C interacting with protons may vary depending on correlation time and the extent to which a dipolar mechanism dominates relaxation. Doddrell et al. (1972) have presented a detailed theoretical discussion of the NOE's expected for proton-decoupled ^{13}C nuclei in macromolecules. For isotropic reorientation, in which a single correlation time, τ, describes molecular reorientation, the increase in ^{13}C signal intensity is expressed by

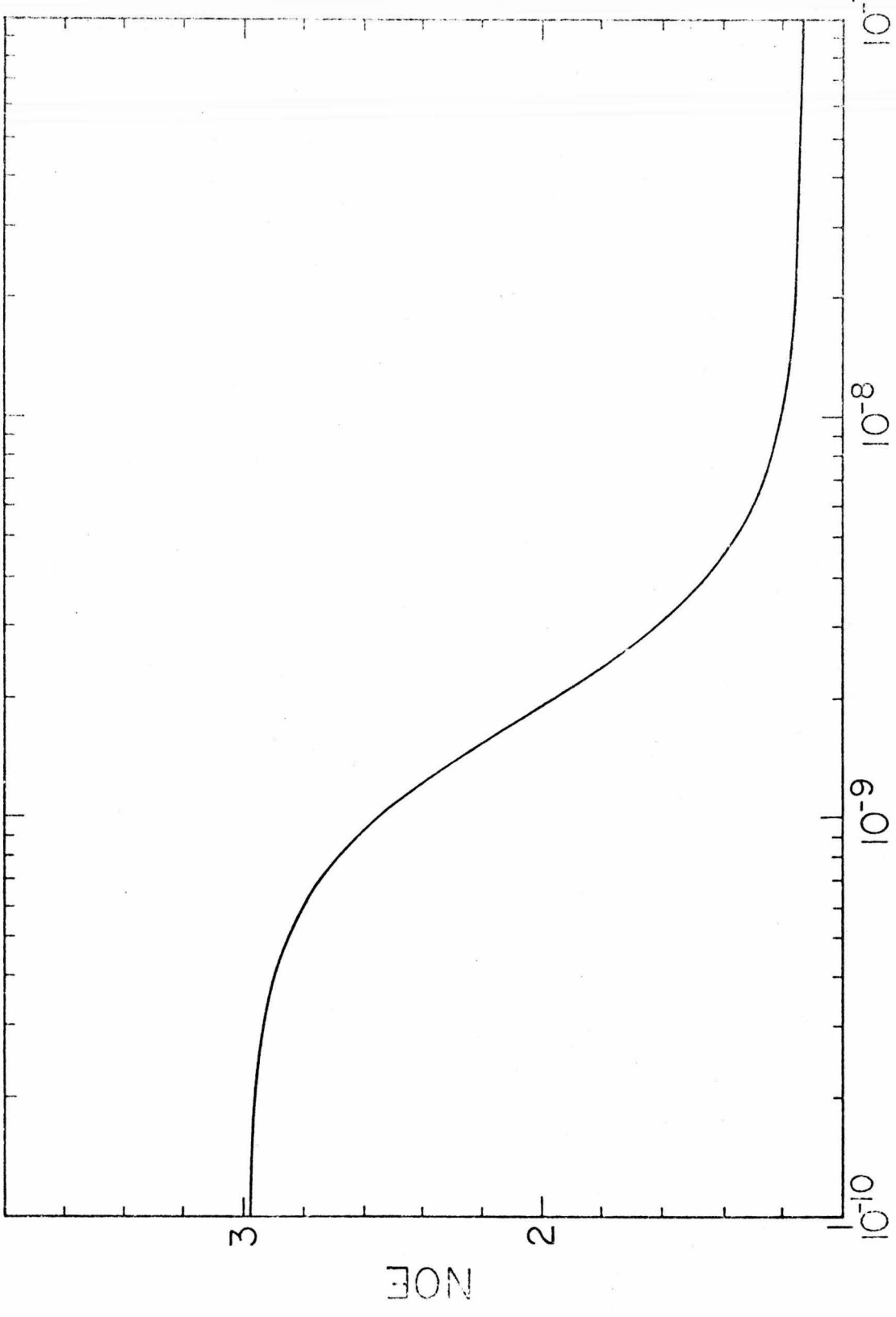
$$NOE = 1 + \frac{\gamma_H}{\gamma_C} \cdot \frac{\dfrac{6\tau}{1+(\omega_H+\omega_C)^2\tau^2} - \dfrac{\tau}{1+(\omega_H-\omega_C)^2\tau^2}}{\dfrac{\tau}{1+(\omega_H-\omega_C)^2\tau^2} + \dfrac{3\tau}{1+\omega_C^2\tau^2} + \dfrac{6\tau}{1+(\omega_H+\omega_C)^2\tau^2}} \quad (5)$$

For 100% dipolar relaxation the NOE will vary between 2.99 and 1.17 depending on the correlation time, as shown in Figure 8.

In a previous report (Moon and Richards, 1974), using broad band ^1H noise decoupling, we were unable to confirm the importance of dipolar relaxation for ^{13}CO bound to hemoglobin since we obtained a value of NOE = 1.1 ± 0.1. However, it is apparent from Figures 6 and 8 that the maximum NOE obtainable for HbCO is about NOE = 1.2. Since the T_1 results reported here suggest that dipolar relaxation is indeed important, we sought to repeat our NOE studies in greater detail to see if an NOE for MbCO and HbCO could be detected. More importantly,

FIGURE 8

The dependence of nuclear Overhauser effects on τ_c. The curve was calculated for a ^{13}C nucleus relaxing by a dipolar mechanism to protons according to equation (5).


NOE
3
2
1
10^{-10}
10^{-9}
10^{-8}
10^{-7}
τ_c(sec)

we wished to employ narrow band gyrocode decoupling in various regions of the ^{1}H NMR spectrum in an attempt to determine, roughly, the ^{1}H chemical shifts of the protons involved in ^{13}CO relaxation.

The ^{1}H frequency dependent NOE data obtained for MbCO and HbCO are presented in Figures 9 and 10. The horizontal bars in the figures indicate the gyrocode noise bandwidths used in the experiments. In Figure 9 it is clearly evident that ^{13}CO bound to myoglobin experiences an NOE due to its interaction with protons in two distinctly different chemical environments. The apparent chemical shifts of these two types of protons are about 1 ppm and 25 ppm downfield of external Me_4Si. The experimental uncertainty in these shifts is about ± 2 ppm. A similar pattern is observed for HbCO (Figure 10), though the nonequivalence of the subunits and longer T_1's for HbCO result in decreased signal-to-noise and increased scatter in the NOE data. Nevertheless, it is still quite evident that the bound ^{13}CO interacts with protons in two widely differing environments. Here the apparent chemical shifts are about 5 ppm upfield and 25 ppm downfield of Me_4Si with an uncertainty of about ± 3 ppm.

While the presence of at least two protons affecting ^{13}CO relaxation does not affect the NOE (Kuhlmann et al., 1970), the T_1 expression will be affected. In this case the r^6 terms in equation (4) must be replaced by $1/\sigma$, such that

$$\sigma = \sum_j r_j^{-6} \qquad (6)$$

where r_j is the distance between the carbon and the jth hydrogen

FIGURE 9

The dependence of NOE on ^{1}H decoupler frequency for MbCO. ^{1}H decoupler offsets are shown in ppm from external Me_4Si. The horizontal bars indicate a gyrocode noise bandwidth of 200 Hz.

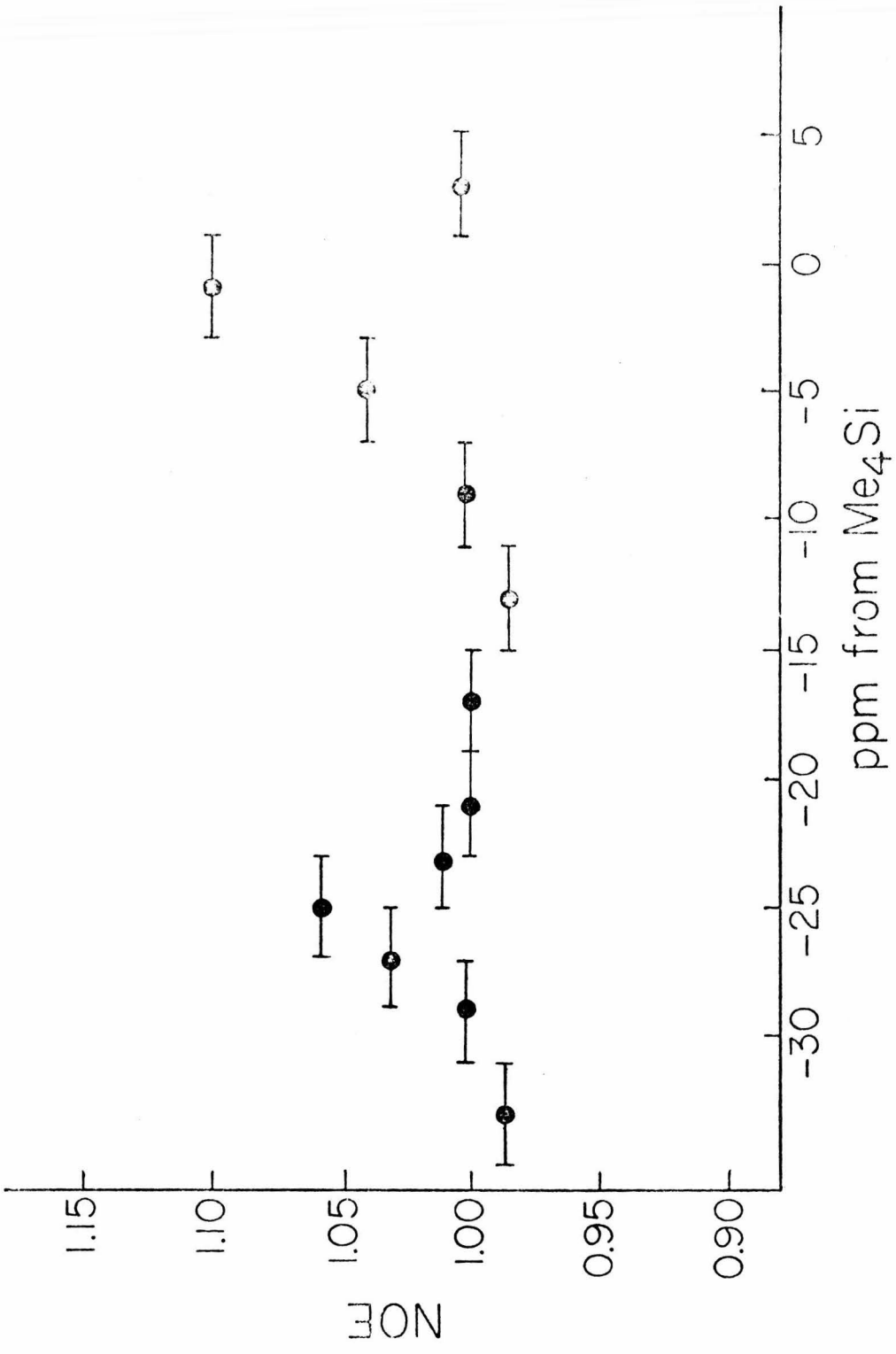
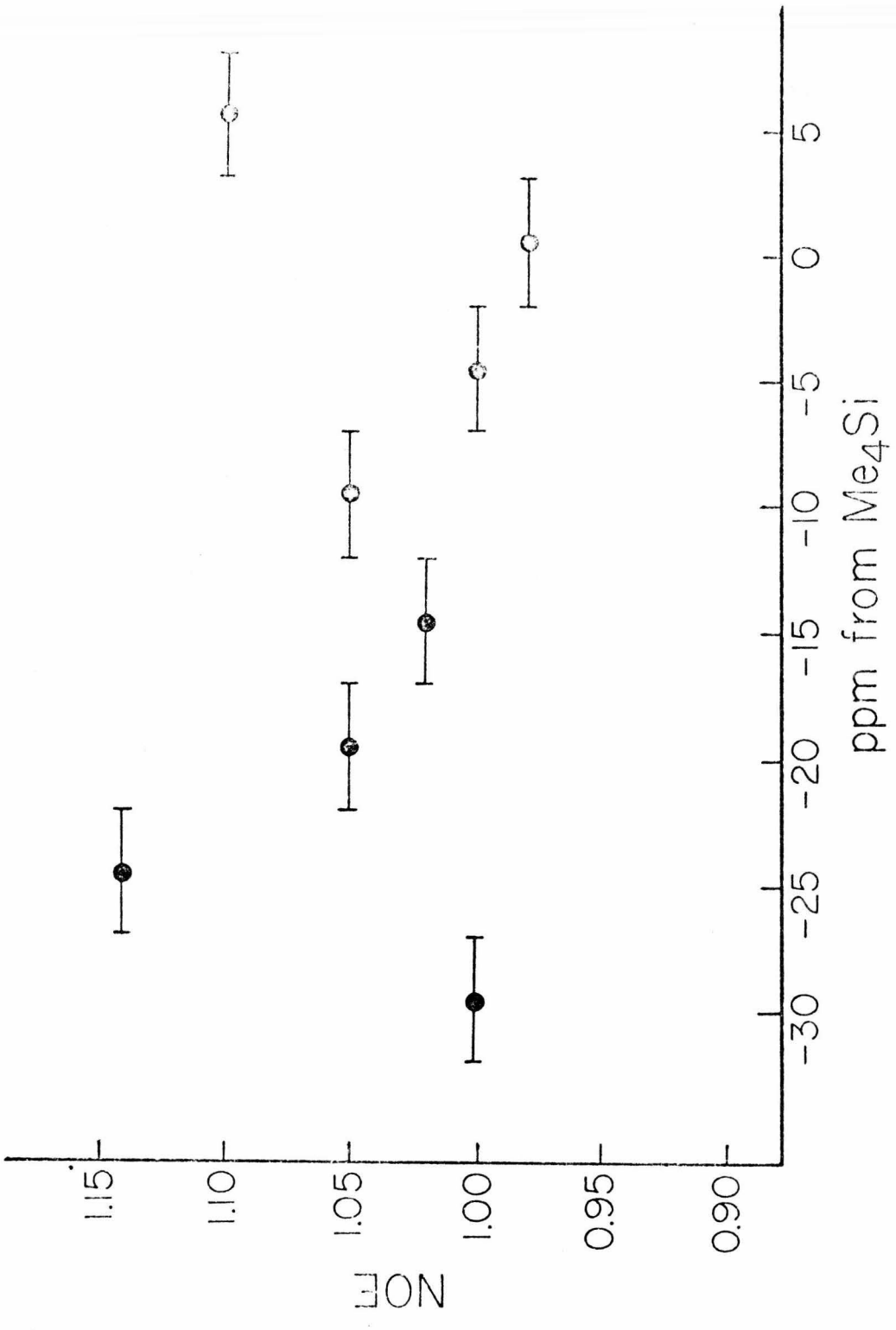

NOE
1.15
1.10
1.05
1.00
0.95
0.90
5
0
-5
-10
-15
-20
-25
-30
ppm from Me4Si

FIGURE 10

The dependence of NOE on ^1H decoupler frequency for HbCO. A gyrocode noise bandwidth of 250 Hz was used at various offsets indicated in ppm from Me_4Si.


NOE
1.15
1.10
1.05
1.00
0.95
0.90
5
0
-5
-10
-15
-20
-25
-30
ppm from Me4Si

(Doddrell et al., 1972). Thus the values of r calculated from the experimental T_1 and τ_c data represent only the effective distances of a single proton from ^{13}CO which would account for the observed relaxation behavior. In fact the bound ^{13}CO must be interacting with at least two protons. However, since the interaction between dipoles varies with the inverse sixth power of r_j it is only possible for a few protons to contribute significantly to the sum in equation (6), even in the case of the nonprotonated ^{13}CO carbon (Doddrell et al., 1972).

DISCUSSION

Nobbs and Watson (1968) have conducted detailed X-ray crystallographic studies of the ligand environment in oxymyoglobin. Though considerable difficulty was experienced in obtaining suitable diffraction data for the precise geometry of the $Fe-O_{\alpha}-O_{\beta}$ bond, these authors found that oxygen interacts primarily with valine E11/68 and the distal histidine E7/64. They concluded that the $C_{\gamma 1}$ methyl carbon of valine and the $N_{\epsilon 2}$ of histidine were, respectively, 3.0 Å and ~2.5 Å distant from O_{α} of oxygen. They further suggested a probable hydrogen-bond existing between oxygen and $N_{\epsilon 2}$ of the distal histidine. Our results are consistent with these findings and provide direct experimental evidence for hydrogen-bonding. The ^1H chemical shift of the E11 valine methyl group nearest the center of the heme in horse carboxymyoglobin has tentatively been assigned (Wuthrich et al. 1972) to a resonance of three protons at 2.6 ppm upfield of internal sodium-2, 2-dimethyl-2-silapentane-5-sulfonate (DSS). The γ_1 methyl resonances of the valine E11 residues in the α and β subunits of carboxyhemoglobin have been reported at 6.48 ppm and 6.58 ppm from HDO, respectively, where HDO is -4.84 ppm downfield of DSS (Lindstrom et al. 1972; Lindstrom and Ho, 1973). These results are in good agreement with the approximate ^1H chemical shifts we obtain

for the high field component of the ^{13}CO NOE (Figures 9 and 10).

While it is difficult to imagine a proton resonance -25 ppm downfield of Me_4Si, and the presence of such a resonance in MbCO or HbCO has not previously been reported, we believe that the data in Figures 9 and 10 for the low field NOE component are sufficiently good that such a proton must exist. We tentatively assign this proton resonance to the $N_{\epsilon 2}$ proton of the distal histidine. Owing to its large downfield shift we suggest that this proton must be involved in a strong hydrogen-bond. The N—H proton resonance of histidine is normally expected about -13 ppm from Me_4Si in the absence of hydrogen bonding. However, in the presence of H-bonding interactions, these N—H protons have been observed as broad resonances with shifts as great as -18 ppm. (Robillard and Shulman, 1972). Since intermolecular hydrogen-bonding has been observed to shift proton resonances downfield by as much as 10 ppm (Lynden-Bell and Harris, 1969), we believe that our result of 25 ± 2 ppm is quite reasonable if the histidine is indeed involved in a strong hydrogen bond. Further, because this proton must be very close to the bound ^{13}CO, and ^{13}CO is the only possible H-bond acceptor in the ligand binding site, the proposed hydrogen bond between the ligand and the distal histidine seems to be the only reasonable explanation for the observed shift.

If we assume that contributions to the ^{13}CO T_1 from valine and

histidine are about equal, we may reexamine the T_1 data by equation (4) using appropriate values of σ (equation 6). For ^{13}CO dipolar relaxation to a single valine proton and the N—H proton we have $\sigma = r_{his}^{-6} + r_{val}^{-6}$ where $r_{his} \approx r_{val}$. Calculation of r by equation (4) gives $r \sim 1.9$ Å. If, on the other hand, all three valine protons are equidistant from ^{13}CO, we have $\sigma = r_{his}^{-6} + 3r_{val}^{-6}$. Using this relation we obtain $r_{his} \sim 1.9$ Å and $r_{val} \sim 2.4$ Å.

The existence of a hydrogen-bond between carbon monoxide and the distal histidine may serve to explain the disparity between the infrared results obtained for oxygen bound to heme model compounds and the native heme proteins. The $^{16}O—^{16}O$ stretching frequencies observed in oxyhemoglobin and oxymyoglobin have been reported to be 1107 cm^{-1} and 1103 cm^{-1}, respectively (Barlow et al. 1973; Maxwell et al. 1974). However, the $^{16}O—^{16}O$ stretching frequencies observed in dioxygen complexes of "picket fence" porphyrins at low temperature are observed at 1385 cm^{-1} (Collman et al. 1974). Since hydrogen-bonding between histidine and oxygen should be at least as effective as that for ^{13}CO, it is quite possible that the histidine-oxygen interaction results in a reduction of O—O bond order and thereby decreases ν_{O_2} in MbO_2 and HbO_2. In the absence of this hydrogen-bond, the O_2 bond order would increase to that observed in the model compound. If this is true, we suspect that ν_{O_2} for monomeric Glycera dibranchiata

hemoglobin, in which the distal histidine has been replaced by isoleucine (Padlan and Love, 1974), will be greater than ν_{O_2} observed in other hemoglobins and may more nearly approximate ν_{O_2} in the model.

Johnson and Ho (1974) have reported that the differential ligation of the α and β hemoglobin subunits depends upon the nature of the ligand. While substantial differences are observed between the O_2 affinities of the subunits, the CO affinities of the subunits of human adult hemoglobin are virtually equal. It is possible, in fact likely, that the histidine is largely responsible for these differences. The degree of hydrogen-bonding to oxygen or carbon monoxide should differ because the geometry of the Fe—O—O bond should be bent, while that of Fe—C—O should be more nearly linear. Furthermore, ligands such as alkyl isocyanides are expected to be relatively poor H-bond acceptors and their bonding may deviate considerably from that of O_2. It is also quite conceivable that the differences between the environments experienced by ^{13}CO bound to α or β subunits are due to differences between histidine-ligand interactions in different subunits. In this regard it is interesting that substitution of the distal histidine for any other residue results in a significant shift in the ^{13}CO resonance (Moon et al. 1975).

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PART VI

Determination of Intracellular pH by ^{31}P Magnetic Resonance

INTRODUCTION

Eventually studies of the hemoglobin molecule in solution must be related to its function within the intact red cell. Organic phosphates, in particular 2,3-diphosphoglycerate, bind preferentially to unliganded deoxyhemoglobin, thereby reducing the oxygen affinity of hemoglobin (1,2). The degree of hemoglobin oxygenation significantly affects pH through the alkaline Bohr effect and the intracellular pH in turn exerts a strong influence on the level of 2,3-diphosphoglycerate within the erythrocyte (3). Moreover, 2,3-diphosphoglycerate serves as a potent inhibitor of several red cell enzymes (4). A close association thus exists within the red cell between hemoglobin oxygenation, pH, and metabolism which is mediated in large measure by 2,3-diphosphoglycerate. In order to gain a better understanding of these interrelationships within the functioning erythrocyte we have undertaken ^{31}P magnetic resonance studies of hemolysates, red cell suspensions, and whole blood.

We have found ^{31}P NMR to provide a useful and direct means for determining the intraerythrocyte pH and the levels of several important red cell metabolites. Since ^{31}P is present at 100% natural abundance, exhibits a wide spectrum of chemical shifts, and is contained in many metabolites, it holds considerable promise as a probe in many biological systems.

EXPERIMENTAL PROCEDURE

Fig. 1 shows the pH dependencies of the chemical shifts for several organic phosphates and emphasizes the large variation in this parameter as a function of the state of ionization. All spectra were obtained by pulsed Fourier transform techniques on a Varian Associates XL-100-15 spectrometer operating at 40.5 Hz. Fig. 2 shows a more detailed representation of the titration behavior of the 2- and 3-phosphate groups of 2,3-diphosphoglycerate. Assignment of the resonance at lower field to the 3-phosphate (which titrates with $pK_a = 6.3$) depends on the ^1H-coupled spectrum in which this ^{31}P resonance appears as a triplet with J = 5 Hz which is due to coupling to a CH_2 group. The resonance at higher field (which titrates with pK = 7.0) was assigned to the 2-phosphate because its ^1H-coupled spectrum gives a doublet with J = 10 Hz which is expected for attachment to a CH group. The relative chemical shift separation between the 2- and 3-phosphate ^{31}P nuclei depends on pH as shown in Fig. 2, and this separation may be used to measure pH directly in cases where diamagnetic or other bulk effects may complicate determinations based on absolute chemical shifts. Except in the region 6.4 to 6.6, the absolute chemical shift of the nuclei will distinguish between the 2-fold degeneracy inherent in the use of the difference parameter alone.

Typical physiological concentrations for 2,3-diphosphoglycerate in human red cells of 5 mM and in rabbit red cells of 7 mM make the ^{31}P resonances from 2,3-diphosphoglycerate easily observable by pulsed

FIGURE 1

The pH-dependent chemical shift behavior of a variety of biological organic phosphates. Chemical shifts are reported relative to external 1.0 M phosphoric acid. F6P, fructose 6-phosphate; DPG, 2,3-diphosphoglycerate; CP, carbamyl phosphate.

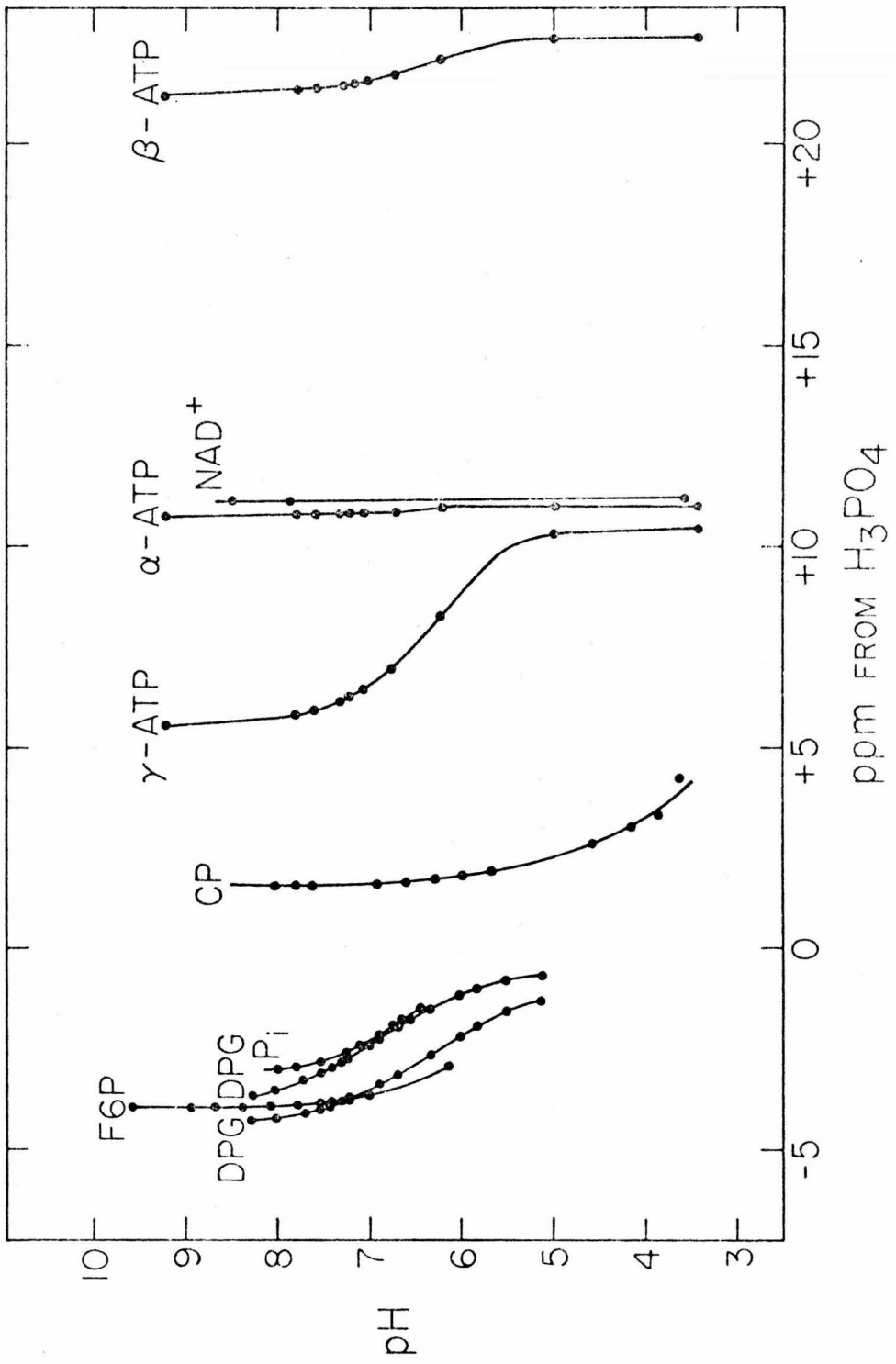
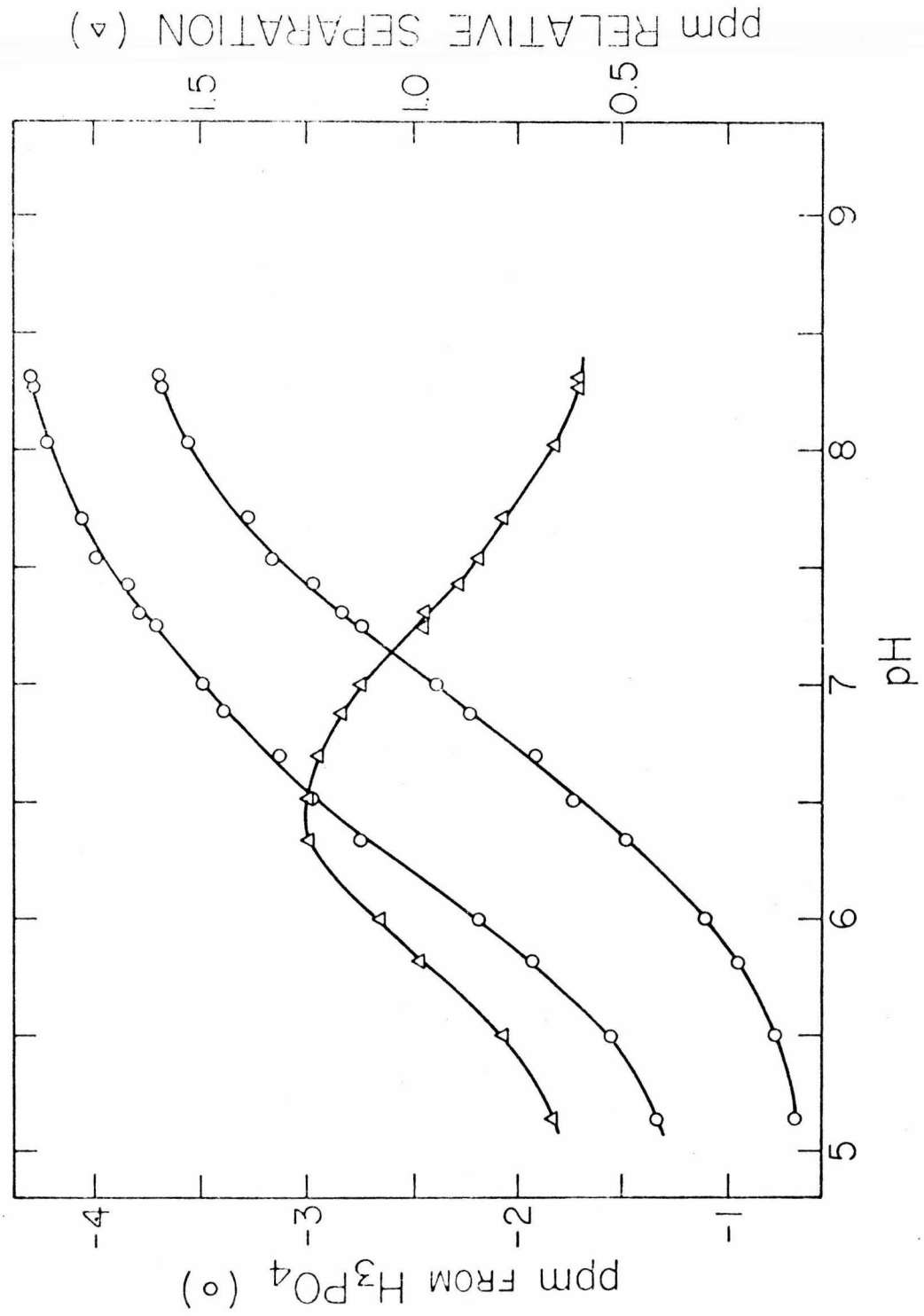

β- ATP
α- ATP
NAD+
γ- ATP
CP
F6P
DPG
DPG
Pi
+20
+15
+10
+5
0
-5
ppm FROM H3PO4
10
9
8
7
6
5
4
3
pH

FIGURE 2

The detailed pH-dependent chemical shift behavior of 2,3-diphosphoglycerate. Absolute shifts from external H_3PO_4 are indicated on the left ordinate while the relative separation of the ^{31}P resonances are shown on the right. The upper and lower titration curves correspond to phosphates at positions 3 and 2 of 2,3-diphosphoglycerate, respectively.


ppm RELATIVE SEPARATION (▵)
1.5
1.0
0.5
ppm FROM H_3PO_4 (○)
-4
-3
-2
-1
5
6
7
8
9
pH

Fourier transform NMR in hemolysates, packed red cells, or whole blood. Fig. 3 shows a typical spectrum of carbon monoxide-treated whole rabbit blood. The sample was contained in a 10-mm tube which was not spun as this leads to packing of the erythrocytes around the sides of the tube which increases the magnetic field inhomogeneity within the sample and thereby artificially broadens the resonances.

Rabbit red cell hemolysates which were first treated with carbon monoxide so as to reduce interactions between 2,3-diphosphoglycerate and hemoglobin as much as possible by completely saturating the hemoglobin with ligand gave titration results similar to those of Fig. 2. Fig. 4 shows a typical spectrum of such a hemolysate and Fig. 5 summarizes the titration behavior of 2,3-diphosphoglycerate and inorganic phosphate in such a hemolysate.

These data, together with comparable titration data for the ^{31}P resonance of inorganic phosphate in the hemolysate, were used to estimate the intracellular pH of erythrocytes in whole blood which had been treated with carbon monoxide such as the sample shown in Fig. 3. The pH estimates indicated are based on both the differences in chemical shift for the two ^{31}P nuclei of the intracellular 2,3-diphosphoglycerate and for the ^{31}P nuclei of P_i inside and outside the cell.

FIGURE 3

Typical ^{31}P spectrum of carbon monoxide-treated whole rabbit blood obtained at 40.5 MHz by pulsed Fourier transform NMR. The anticoagulant used was acid-citrate-dextrose (ACD, Formula B). The whole blood pH was 6.70 ± 0.02 as determined on a Radiometer model 26 pH meter. The estimated intracellular pH values indicated are based on both the absolute chemical shifts and the relative separation of the 2,3-diphosphoglycerate (DPG) resonances, and the relative separation of the intra- and extracellular P_i resonances. The phospholipid resonances of the red cell membrane are also indicated.

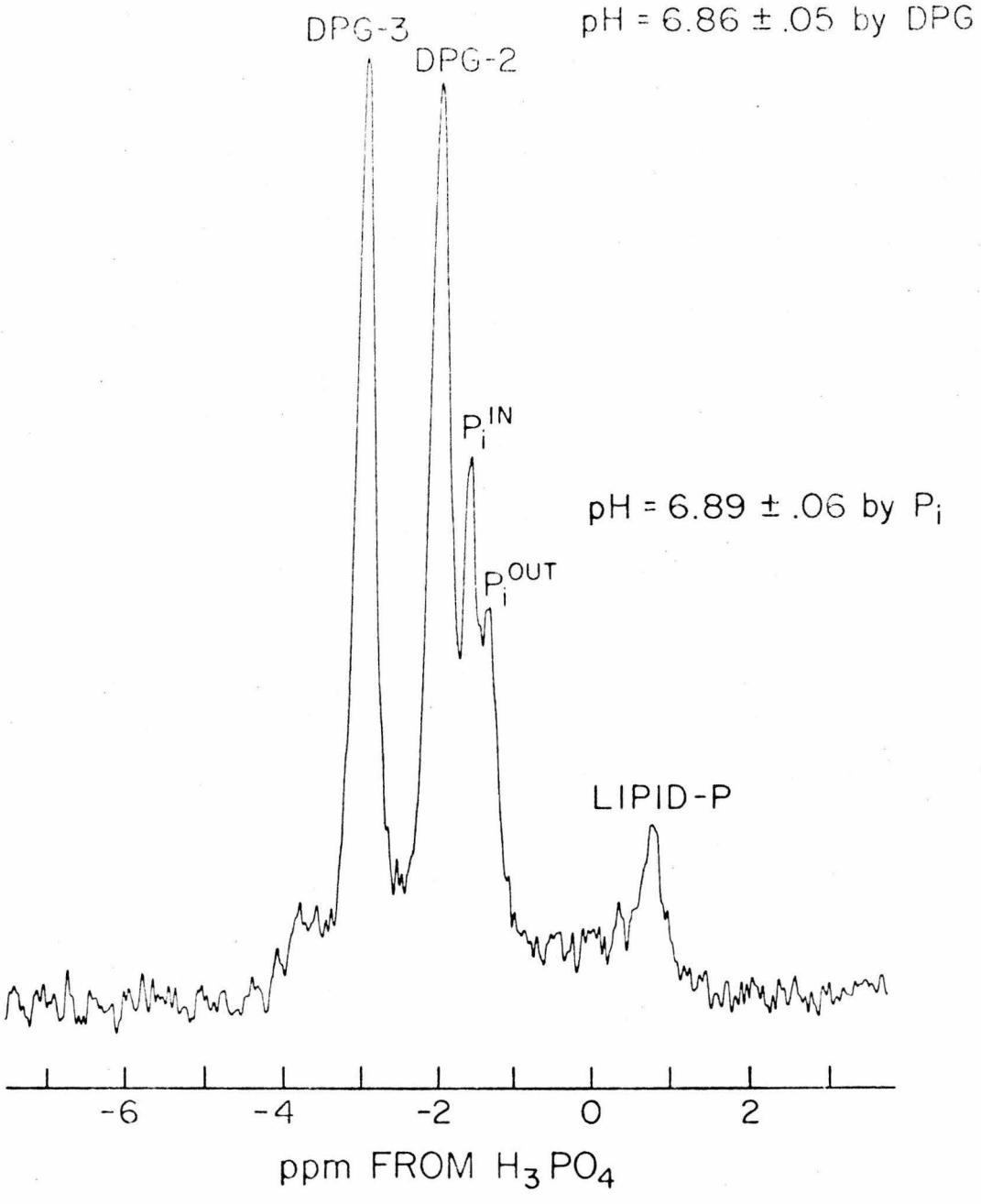

DPG-3
DPG-2
pH = 6.86 ± .05 by DPG
P_i^{IN}
pH = 6.89 ± .06 by P_i
P_i^{OUT}
LIPID-P
-6
-4
-2
0
2
ppm FROM H_3PO_4

FIGURE 4

A typical ^{31}P spectrum of a rabbit hemolysate obtained from sonicated cells. Sample pH = 7.52 ± 0.02. Data accumulation time was 5 1/2 hours for this sample. Sufficient signal-to-noise ratios for determining 2,3-diphosphoglycerate (DPG) and P_i chemical shifts are obtained after several minutes, however.

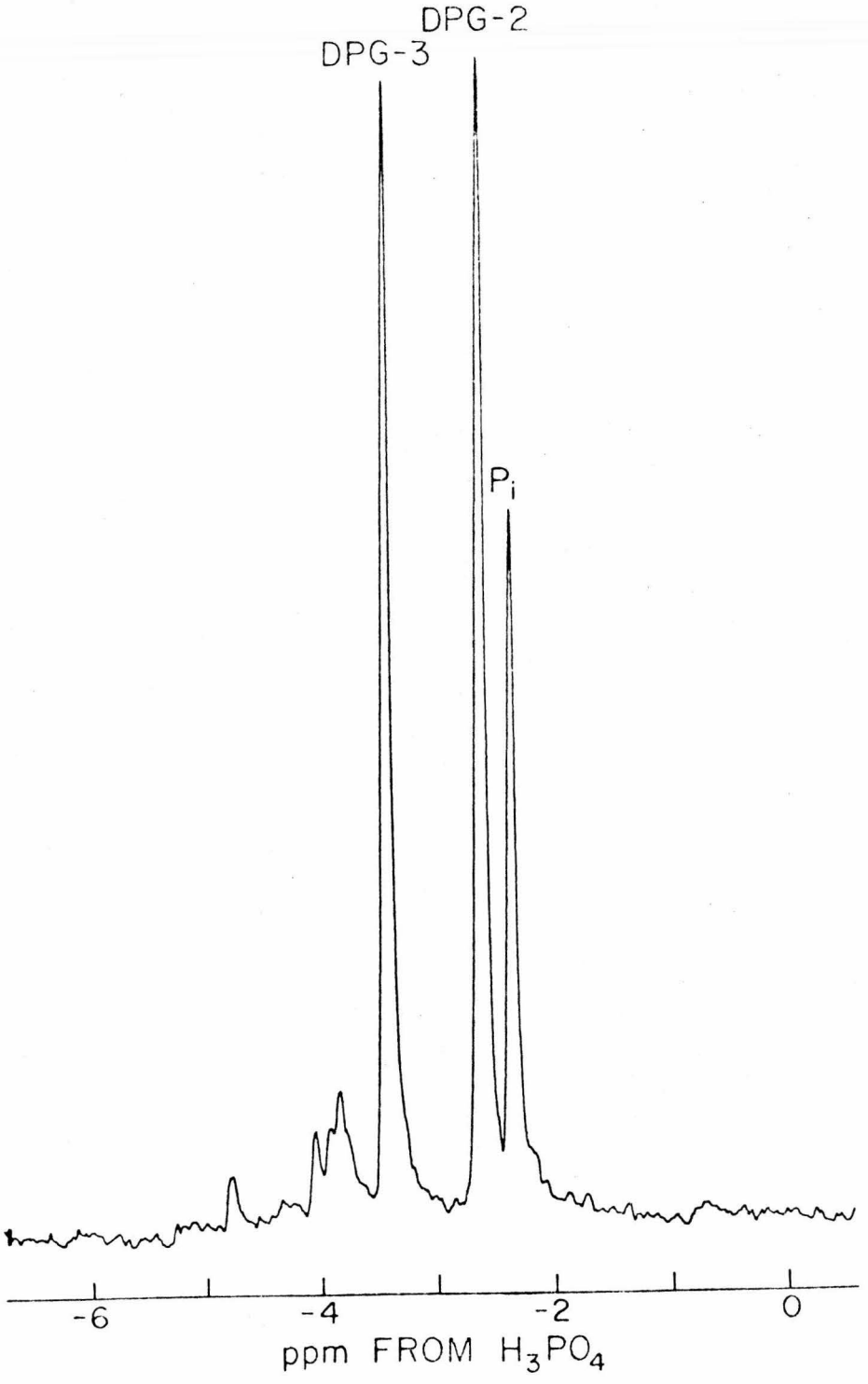
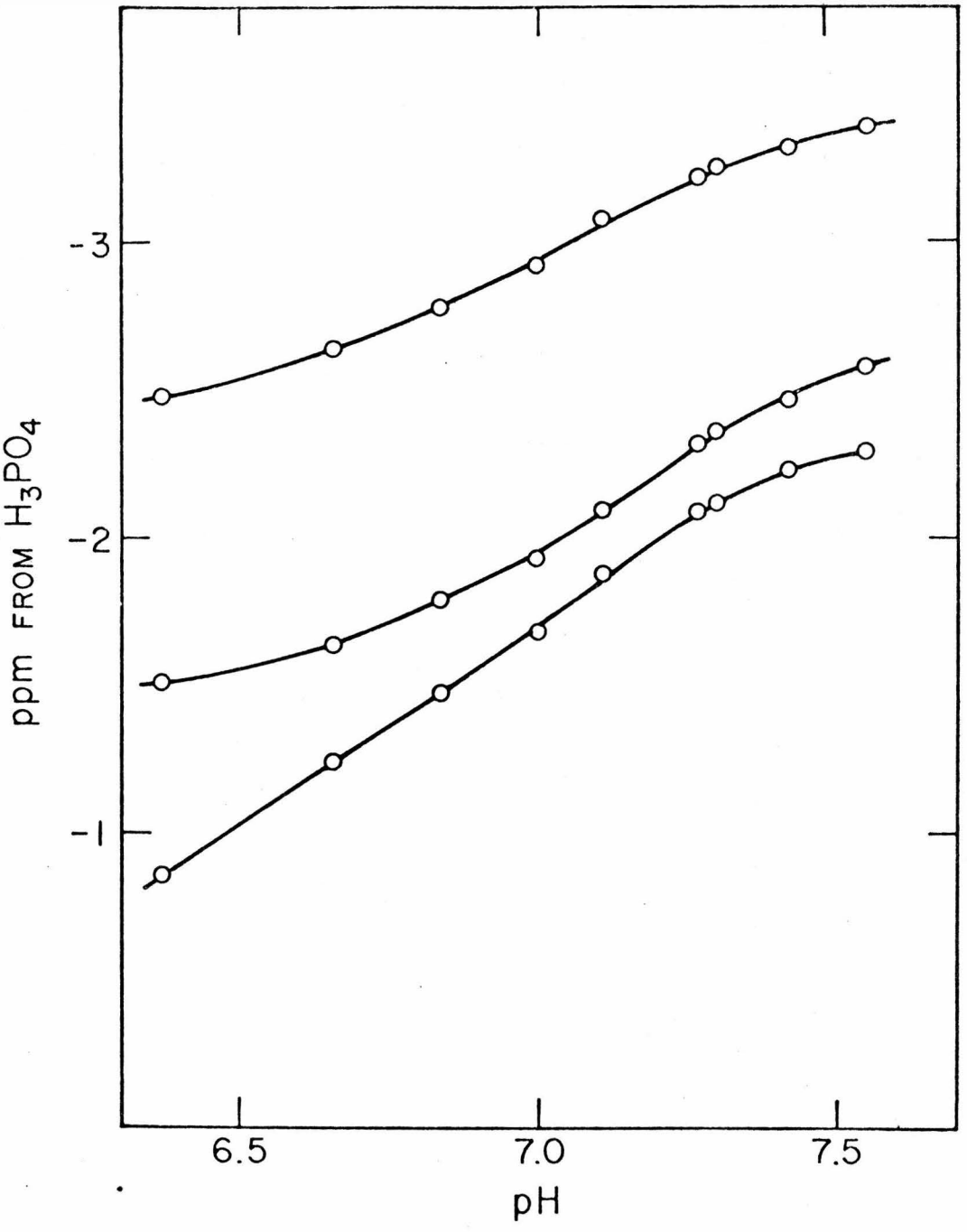

DPG-2
DPG-3
P_i
-6
-4
-2
0
ppm FROM H_3PO_4

FIGURE 5

A plot of chemical shift versus pH for carbon monoxide-treated rabbit hemolysates. The upper and middle titration curves correspond to phosphates at positions 3 and 2 of 2, 3-diphosphoglycerate, respectively, and the lower curve corresponds to the titration behavior of P_i.


-3
-2
-1
ppm FROM H_3PO_4
6.5
7.0
7.5
pH

DISCUSSION

Although providing a nondestructive and reasonably accurate and rapid way of measuring intraerythrocytic pH, this method must be used with caution as the interpretation of the observed resonances can be complicated by the interaction between 2, 3-diphosphoglycerate and deoxyhemoglobin. On binding to deoxyhemoglobin, the 2, 3-diphosphoglycerate resonances experience a large downfield shift which probably results largely from an increase in degree of ionization for the bound 2, 3-diphosphoglycerate, as the magnitude of the observed downfield shift closely parallels that observed on ionization of 2, 3-diphosphoglycerate in solution. Other factors, such as aromatic ring currents or bond anisotropies, for example, may also influence chemical shift. (The 2, 3-diphosphoglycerate exchanges rapidly relative to $\sqrt{2}/2\pi\Delta$ so that the observed signal represents the weighted average for the 2, 3-diphosphoglycerate in its two environments-solution and bound to hemoglobin.[1]) Accordingly, one must calibrate the experiment by first titrating a hemolysate which has the same relative oxygen tension as the blood sample of interest if one is to obtain accurate data. Nevertheless this chemical shift data may be acquired in a relatively short time (only several minutes per point are required) and the technique is therefore comparable in ease to other methods of measuring intraerythrocytic pH, i.e., electrode determination of the pH in hemolysates obtained from packed red cells which have been lysed by repeated freezing and

[1] R. B. Moon and J. H. Richards, unpublished observations.

thawing of the cells (5) or by sonication. In this regard we have found no change in intraerythrocytic pH in packed red cells or their hemolysates with respect to whole blood samples. This technique offers a further advantage in that no disruption of the cell membrane is necessary; measurements can be made of intact, functioning cells. Thus one can directly study the response of intracellular pH to various external stimuli. Further, the total concentrations of 2,3-diphosphoglycerate and P_i can be determined by integration of their respective ^{31}P resonances in spectra requiring data accumulation times of an hour or less.

While most other cellular systems do not possess significant concentrations of 2,3-diphosphoglycerate, they often do contain other phosphates such as P_i, ATP, and inositol hexaphosphate with concentrations approaching 1 mM. Use of analogous ^{31}P techniques may therefore provide a basis for intracellular pH measurements in a variety of biological systems. Once again appropriate controls in solution must be carried out so that one can unambiguously dissect the influence on chemical shift of pH changes from other effects such as interaction with intracellular macromolecules or cations, for example. Moreover, phosphates incorporated into synthetically prepared vesicles may provide a probe for measurements of internal pH for the study of Donnan equilibrium or other properties of model membranes.

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PART VII

^{13}C Magnetic Resonance Studies of Hemoglobin Carbamylation

INTRODUCTION

The in vitro effects of cyanate on the red blood cells of individuals suffering from sickle cell anemia have been shown to be of benefit in inhibiting the sickling process and prolonging the mean survival times of reinfused cells (1-5). The majority of cyanate carbamylation is known to occur at the N-terminal valine α-amino groups of hemoglobin with carbamylation of lysine ϵ-amino groups occurring to a lesser extent (6). However, the extent of amino group carbamylation is strongly dependent on pH at values near the pK_a of the reacting amino groups (7). Since the α-amino groups of the terminal valines in hemoglobin have values of pK_a near 7 (8) the carbamylation of hemoglobin should be strongly pH dependent within the physiological pH range.

To better understand the reactions of cyanate with hemoglobin we have employed carbon-13 magnetic resonance techniques to study the in vitro effects of ^{13}C-enriched cyanate at various concentrations on hemoglobin and whole blood over the pH range 6.7 - 8.2.

MATERIALS AND METHODS

Sodium cyanate was prepared from ^{13}C-enriched sodium cyanide (Prochem) by the method of Smith and Yates (9). Enrichment in ^{13}C was 91%. Potassium cyanate (90.8% ^{13}C-enriched) was purchased from Thompson-Packard. Citrulline was prepared from L-ornithine hydrochloride (Sigma) and 90% ^{13}C-enriched urea (Prochem) by the method of Kurtz (10). All other carbamylated amino acids and peptides were prepared with ^{13}C-enriched sodium or potassium cyanate by methods essentially those described by Stark (11, 12).

Human adult hemoglobin was prepared from freshly drawn citrated whole blood as described elsewhere (13) and treated with carbon monoxide (Thompson-Packard, 90.8% ^{13}C-enriched). Carbamylation of 10 ml samples of carboxyhemoglobin-A (1-2 mM) with potassium cyanate (10-40 mM) was conducted for 2.5 hours at 37°C in 0.15 M sodium chloride. Sample pH was adjusted with 0.1 M HCl or 0.1 M NaOH on a Radiometer model 26 pH meter. The pH was either measured before and after reaction or kept constant during the reaction by addition of 1.0 M HCl on a Radiometer model TTT 1c pH-stat. The reaction was terminated by addition of 200 ml ice cold 0.15 M sodium chloride followed by dialysis against 3 l. 0.15 M NaCl at 4°C.

Heparinized whole sickle cell blood was obtained with informed consent from adolescent male and female patients with electrophoretically proven sickle cell anemia who at the time of venipuncture were

not acutely ill. Carbamylation of oxygenated sickle erythrocyte suspensions (10 ml samples, PCV = 30-50%) with sodium cyanate (1-50 mM) was completed within six hours of venipuncture. Reactions were conducted for 2.5 hours at 37°C. Sample pH was determined before and after reaction. The reaction was terminated by washing the red cells three times with 30 ml ice cold 0.15 M NaCl followed by lysis with 30 ml distilled water and dialysis of the hemolysates against 3 l. 0.15 M NaCl at 4°C.

All carbamylated hemoglobin samples were concentrated by ultrafiltration (Amicon UM-10 membrane) and adjusted spectrophotometrically to 2.0 mM (tetramer) in 0.15 M NaCl for nmr study.

^{13}C nmr spectra were obtained using the pulse Fourier transform technique on a Varian Associates XL-100-15 spectrometer equipped with a Varian 620i computer (16K). Samples were contained in a 12 mm tube with a 5 mm tube containing deuterium oxide inserted concentrically to serve as a field frequency lock. All spectra used for quantitation of the carbamyl resonances were obtained under identical conditions at 34°C without proton noise decoupling. A 90° pulse of 150μsec duration was used with a spectrum width of 2500 Hz. Spin-lattice relaxation time (T_1) measurements were made with proton noise decoupling using the progressive saturation technique (14). Nuclear Overhauser effect (NOE) determinations were made both by direct integration of the resonances with and without proton noise decoupling and by comparison of intensity ratios using continuous and gated noise decoupling techniques.

RESULTS AND DISCUSSION

Representative spectra of carbamylated carboxyhemoglobin-S are collected in Figure I. Chemical shift, T_1, and NOE data for carbamylated hemoglobin and the ^{13}C-enriched carbamyl group of citrulline are summarized in Table I.

Assignment of the heme-bound carbon monoxide resonances to the individual α or β subunits of hemoglobin is based on nmr studies of ^{13}CO bound to isolated α or β subunits (13). Assignment of the resonances of carbamyl groups bound either to the α-amino groups of the N-terminal valines or the ϵ-amino groups of lysine residues is based on the following data. Both resonances are independent of pH within the physiological range (pH 5-9) and do not converge in acid denatured, heme-free $\alpha\beta$ globin solutions indicating that the carbamyls are bound to chemically different functional groups rather than to the same functional group in different environments of the native protein. The chemical shifts of carbamyl groups bound to various amino acids and peptides are shown in Table II. These data show that carbamyls attached to the α-amino groups of L-valyl-L-valine and poly-L-valine are shifted upfield of the carbamyl group of N_α-acetyl-N_ϵ-carbamyl-lysine or citrulline while the carbamyl group of S-carbamylcysteine is shifted far downfield of all carbamyls attached to amino groups.

The low field carbamyl resonance of carbamylated hemoglobin is only slightly shifted from the resonances of N_α-acetyl-N_ϵ-carbamyllysine and citrulline and the high field carbamyl resonance of hemoglobin

FIGURE I

Typical spectra of carbamylated carboxyhemoglobin-S showing the ^{13}CO resonances at low field (206 ppm), the broad carbonyl resonance region (275-280 ppm), and the ϵ- and α-amino carbamyl resonances at higher field (161 ppm). Reactions of cyanate with oxygenated red cell suspensions were conducted under various conditions. (A) = 10 mM cyanate for 2.5 hrs at 37°C, pH 7.8, PCV = 30% in plasma; (B) = 20 mM cyanate for 2.0 hrs at 40°C, pH 8.0, PCV = 35% in 0.1 M NaCl; (C) = 50 mM cyanate for 3.0 hrs at 40°C, pH 8.2, PCV = 50% in 0.1 M NaCl.

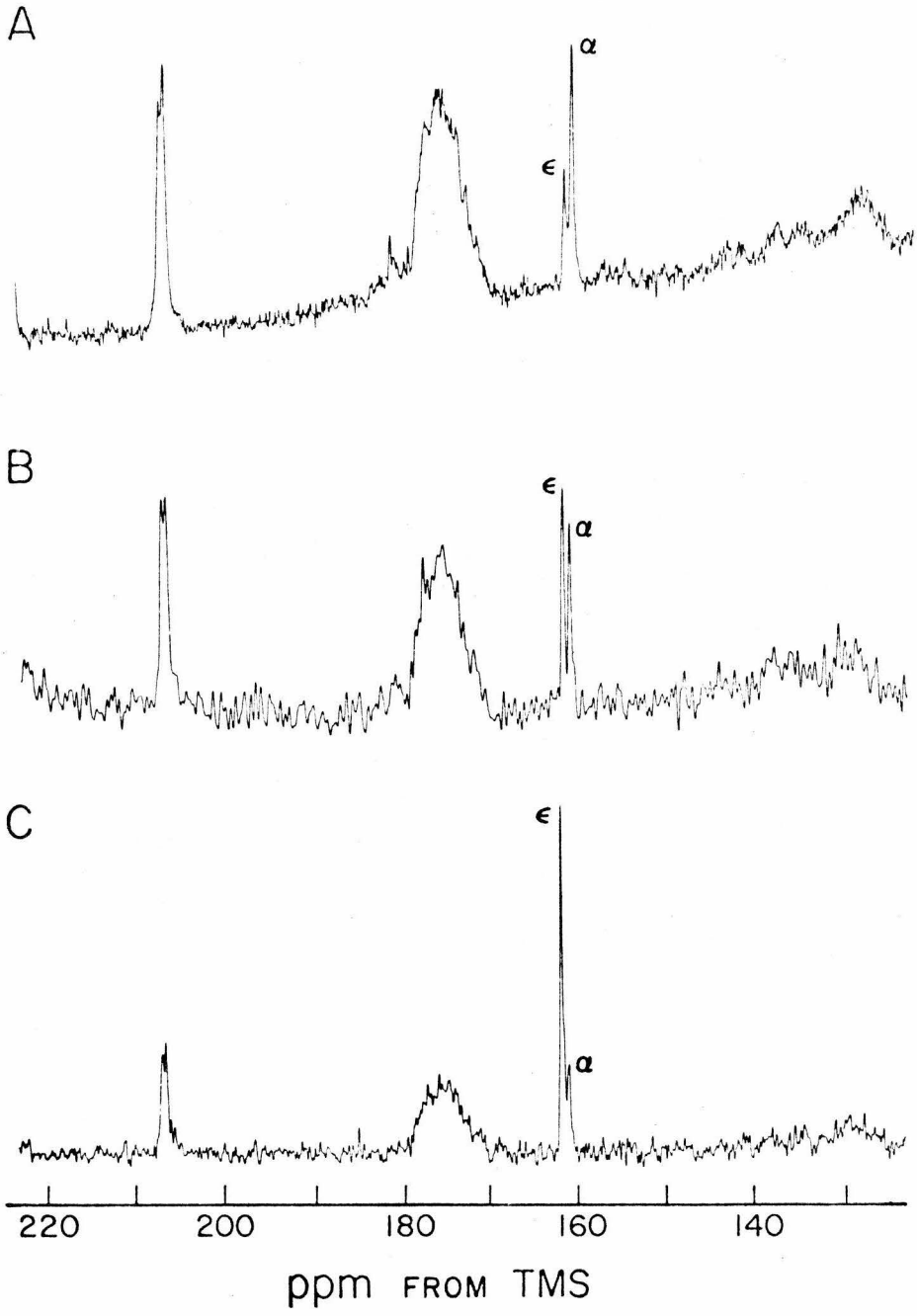

A
α
ϵ
B
ϵ
α
C
ϵ
α
220
200
180
160
140
ppm FROM TMS

Table I. Carbamylated Hemoglobin-S

Group	δ	T_1	NOE
α-Carbamyls	160.99	0.49 ± .07	1.45 ± .09
ε-Carbamyls	161.82	0.45 ± .13	1.57 ± .12
^{13}CO (α-Subunit)	206.67	0.60 ± .16	1.00 ± .08
^{13}CO (β-Subunit)	206.13	"	"
Citrulline (Carbamyl)	162.08	14.64 ± .76	2.09 ± .11

(δ in ppm from external TMS ± .04)

Table II. Chemical Shifts of Various Carbamyl Groups

Species	δ
N_{α}-Carbamyl-L-valyl-L-valine	161.49
N_{α}-Carbamyl-poly-L-valine	160.77
N_{α}-Acetyl-N_{ϵ}-carbamyllysine	162.14
N_{δ}-Carbamylornithine (Citrulline)	162.08
N_{α}-Carbamyl-L-lysylglycine	161.32
N_{α}-Carbamyl-N_{ϵ}-carbamyl-L-lysylglycine (α)	161.39
(ϵ)	162.11
S-Carbamyl-L-Cysteine	171.45
Cyanate (pH 8.5; Triplet, J_{C-N} = 11 Hz)	129.17

(δ in ppm from external TMS ± .04)

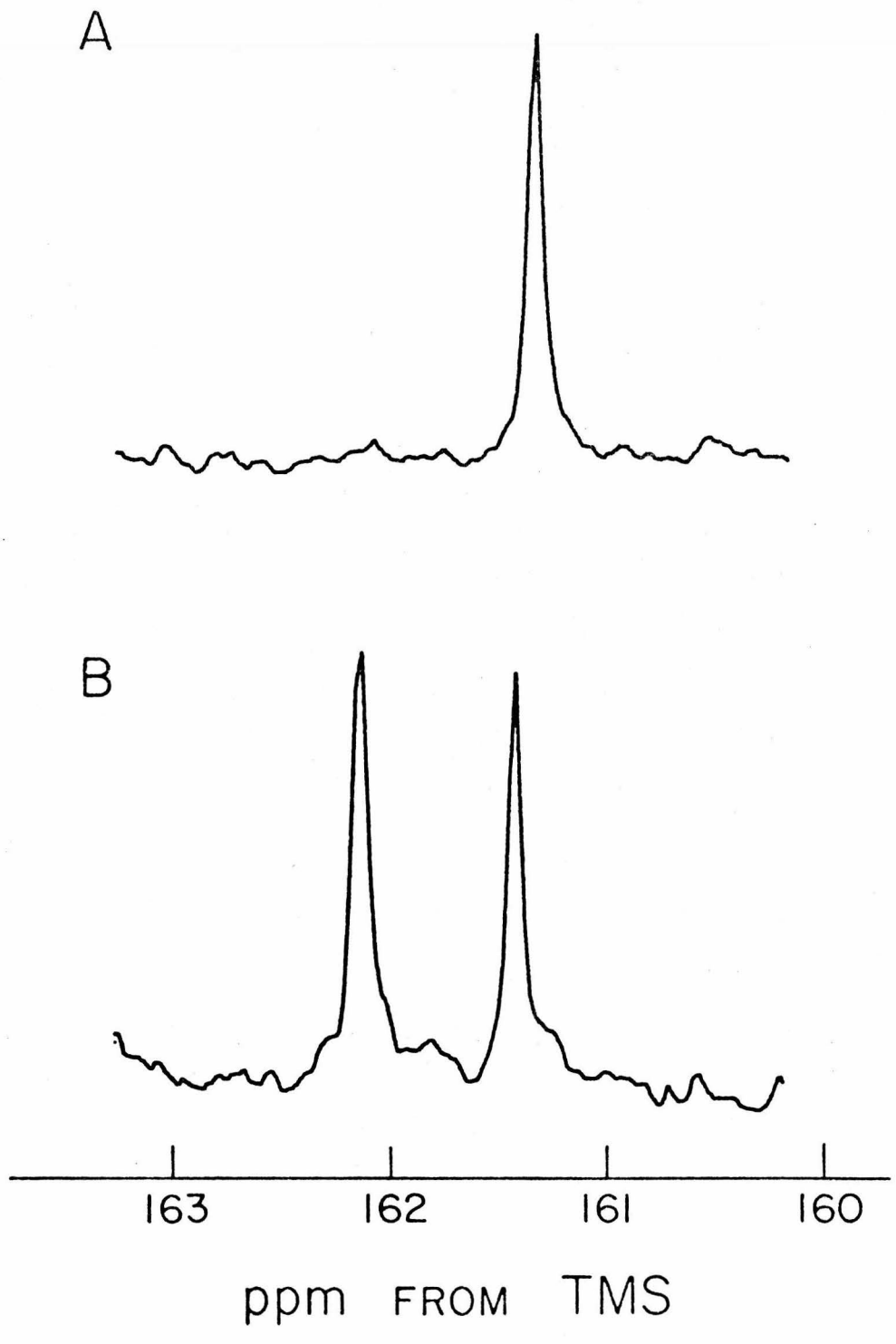
falls between those of N_{α}-carbamyl-L-valyl-L-valine and N_{α}-carbamyl-poly-L-valine. Accordingly, the downfield resonance of carbamylated hemoglobin is assigned to carbamyl groups attached to the ϵ-carbon of lysines and the higher field resonance to carbamyl groups attached to the α-amino group of the N-terminal valines of the α and/or β chains. These assignments are borne out by studies on L-lysylglycine. Mild treatment of L-lysylglycine with one equivalent of cyanate leads only to carbamylation of a single amino group (161.32 ppm) as shown in Figure IIA. After extensive carbamylation of L-lysylglycine with excess cyanate, however, a second carbamyl resonance (162.11 ppm) is observed to lower field as shown in Figure IIB. Since cyanate reacts with α-amino groups about 100 times faster than with the ϵ-amino groups of lysine residues at or below neutral pH (11) we assign the high and low field carbamyl resonances of carbamylated L-lysylglycine to carbamyls attached to the α-amino and ϵ-amino groups, respectively. The chemical shift difference between these resonances is almost identical to that between the two carbamyl group resonances of carbamylated hemoglobin.

The T_1 values listed in Table I are based on numerous independent measurements of undegassed samples. All uncertainties in T_1 are standard mean deviations of the mean values reported.

Since carbamyl carbons are not significantly spin-coupled with neighboring protons it is possible to observe these resonances clearly in the absence of proton noise decoupling, thereby eliminating the NOE. Thus, using a suitably long pulse interval ($\geq 4T_1$) the extent of hemo-

FIGURE II

Carbamylated L-lysylglycine treated with cyanate under mild and vigorous conditions. (A) = 0.9 equivalents ^{13}C potassium cyanate at 25 °C for 4.0 hrs, pH 5.8; (B) = 4.5 equivalents of ^{13}C potassium cyanate at 100 °C for 3.8 hrs. pH 10.0.


A
B
163
162
161
160
ppm FROM TMS

globin carbamylation may be determined by integrating the carbamyl resonances (at 91% ^{13}C) and comparing with integrals of either the carbonyl resonance region (656 carbons at 1.1% ^{13}C) or the bound ^{13}C resonances (4 carbons at 91% ^{13}C). Since the carbonyl resonances have $T_1 \sim 0.416$ sec (15), which is very similar to that of the carbamyls, any errors in the spectrum due to partial saturation of the resonances are minimized.

Spectra of carbamylated hemoglobin-A obtained without proton noise decoupling are shown in Figure III. These samples were obtained by reaction of hemoglobin (1.6 mM) with cyanate (40 mM) at various pH. Quantitation of these spectra leads to the pH dependent carbamylation pattern shown in Figure IV. Reaction of hemoglobin-A (2.0 mM) with somewhat less cyanate (20 mM) results in a more dramatic pH dependent carbamylation of the α-amino groups with essentially pH independent carbamylation of lysine residues as shown in Figure V.

This pH effect on the extent of hemoglobin carbamylation is also evident in studies of whole blood carbamylation. Figure VI shows the extent of hemoglobin-S carbamylation at various cyanate concentrations in whole sickle blood at pH = 7.51± 0.06. Increasing the whole blood pH to 8.0± 0.2 results in a considerable decrease in the extent of hemoglobin-S carbamylation as shown in Figure VII. Of special interest is the observation that the extent of carbamylation of the α-amino groups of valine has decreased much more than the extent of carbamylation of the ϵ-amino groups of lysine. These data therefore show that relatively small differences in sample pH result in significant changes in both the total amount of carbamylation as well as the

FIGURE III

Spectra of carbamylated carboxyhemoglobin-A. Carbamylation reactions were conducted with 1.6 mM carboxyhemoglobin-A and 40 mM cyanate for 2.5 hrs at 37°C. Sample pH was measured at the beginning and end of the reaction. Average pH values are indicated in the figure. The small resonance at 186 ppm is due to free ^{13}CO in solution.

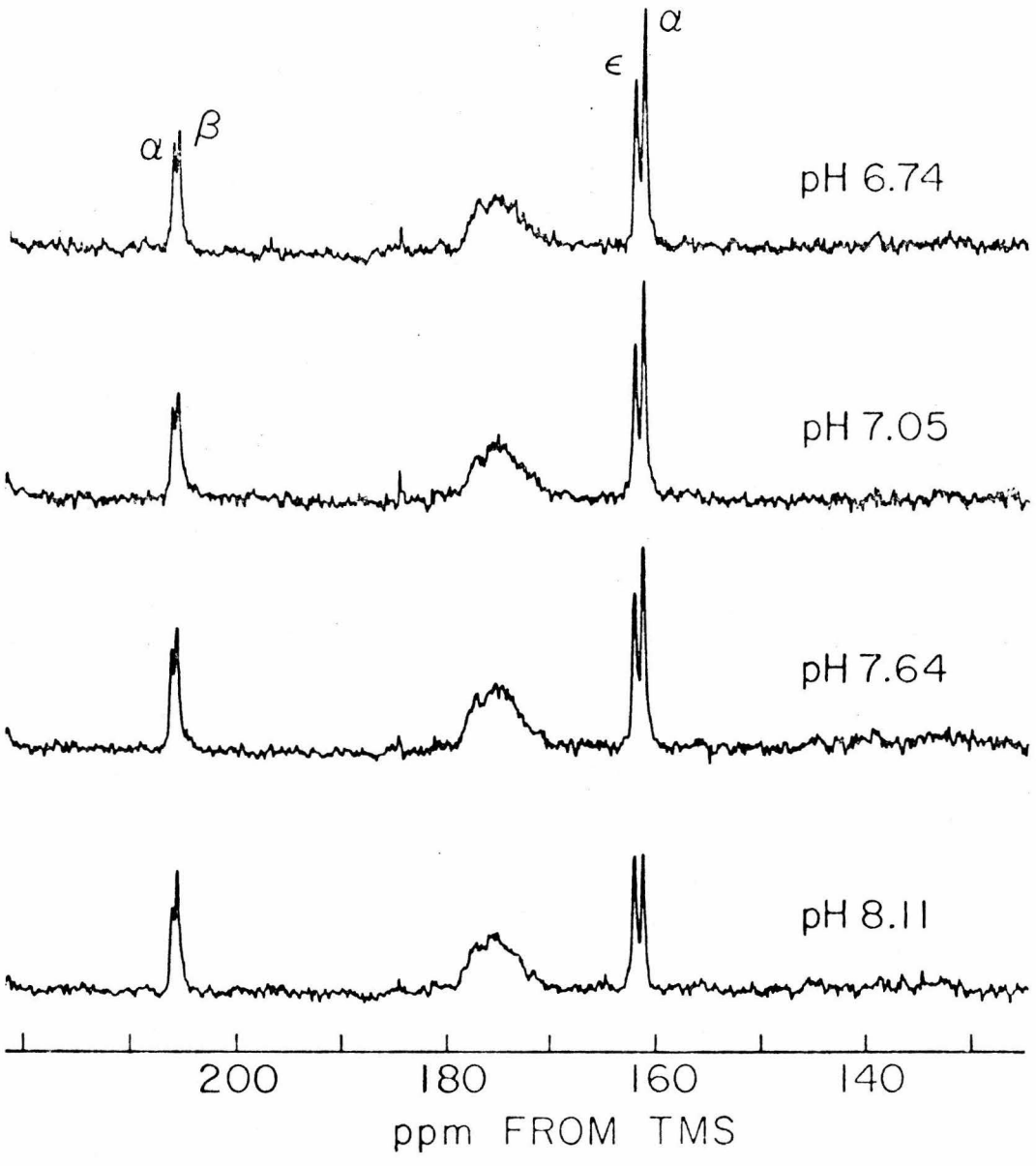

α
β
ε
α
pH 6.74
pH 7.05
pH 7.64
pH 8.11
200
180
160
140
ppm FROM TMS

FIGURE IV

A plot of the extent of α-amino ($\clubsuit$) and ϵ-amino (Φ) group carbamylation as a function of reaction pH for carboxyhemoglobin-A using the data of Figure III. The horizontal error bars indicate the increase in pH between the beginning (left limit) and end (right limit) of the reaction. Vertical error bars indicate the uncertainty of carbamyl group quantitation based on spectrum signal/noise ratio, resonance overlap and the experimentally determined accuracy of the integrator (4%).

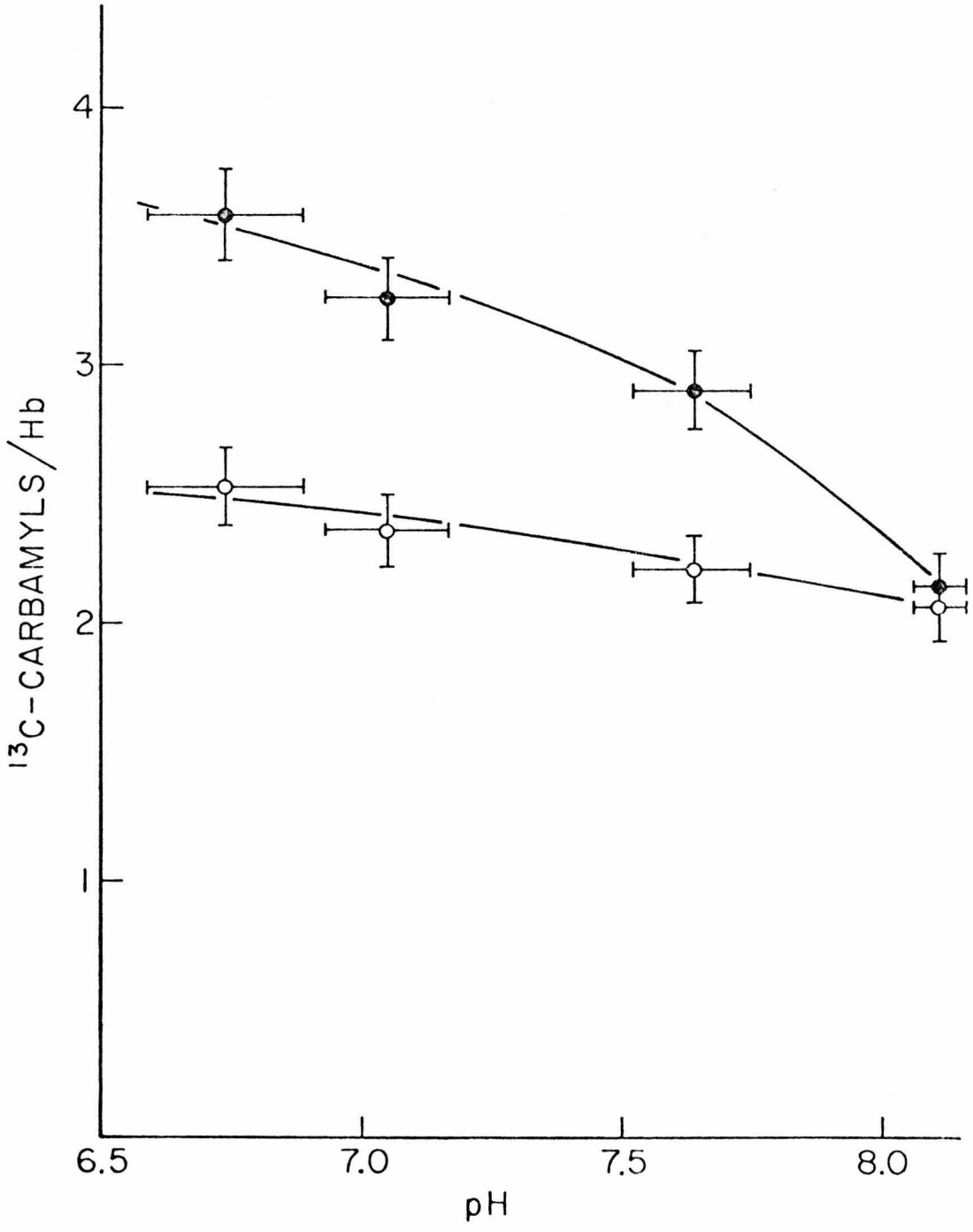

4
3
2
1
^{13}C-CARBAMYLS/Hb
6.5
7.0
7.5
8.0
pH

FIGURE V

A plot of α-amino (Φ) and ϵ-amino (Φ) group carbamylation vs. reaction pH. Carbamylation reactions were conducted with 2.0 mM carboxyhemoglobin-A and 20 mM cyanate for 2.5 hours at 37°C. Sample pH was kept constant during the reaction with a pH-stat. Estimated errors in carbamylquantitation were determined as in Figure IV.

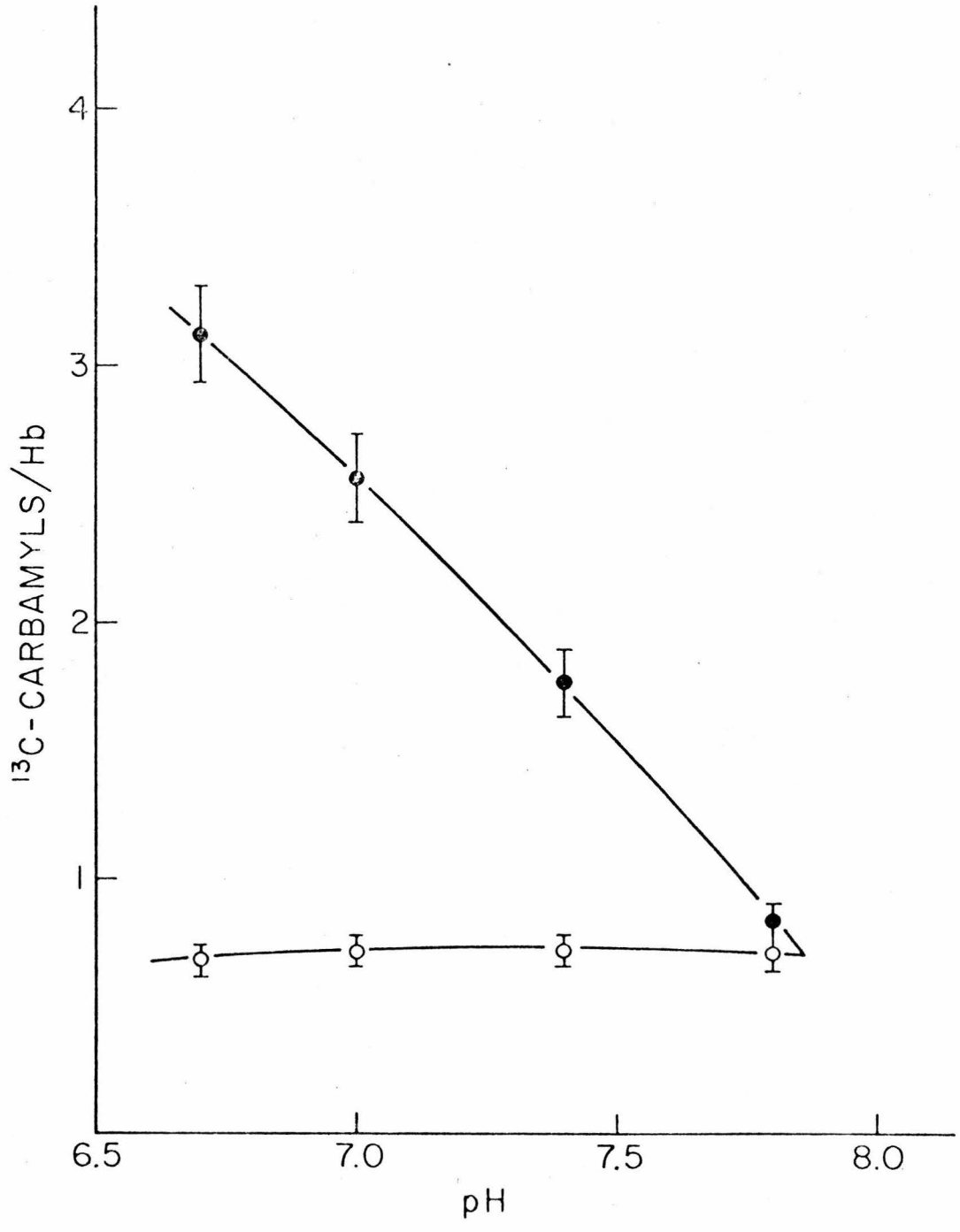

4
3
2
1
^{13}C-CARBAMYLS/Hb
6.5
7.0
7.5
8.0
pH

FIGURE VI

A plot of α-amino (Φ) and ϵ-amino (Φ) group carbamylation vs. cyanate concentration for hemoglobin-S in oxygenated whole sickle blood. The reactions were conducted for 2.5 hours at 37°C. Average whole blood pH = 7.51 ± 0.06 as determined at the beginning and end of the reactions. The PCV was adjusted to 30% for all samples.

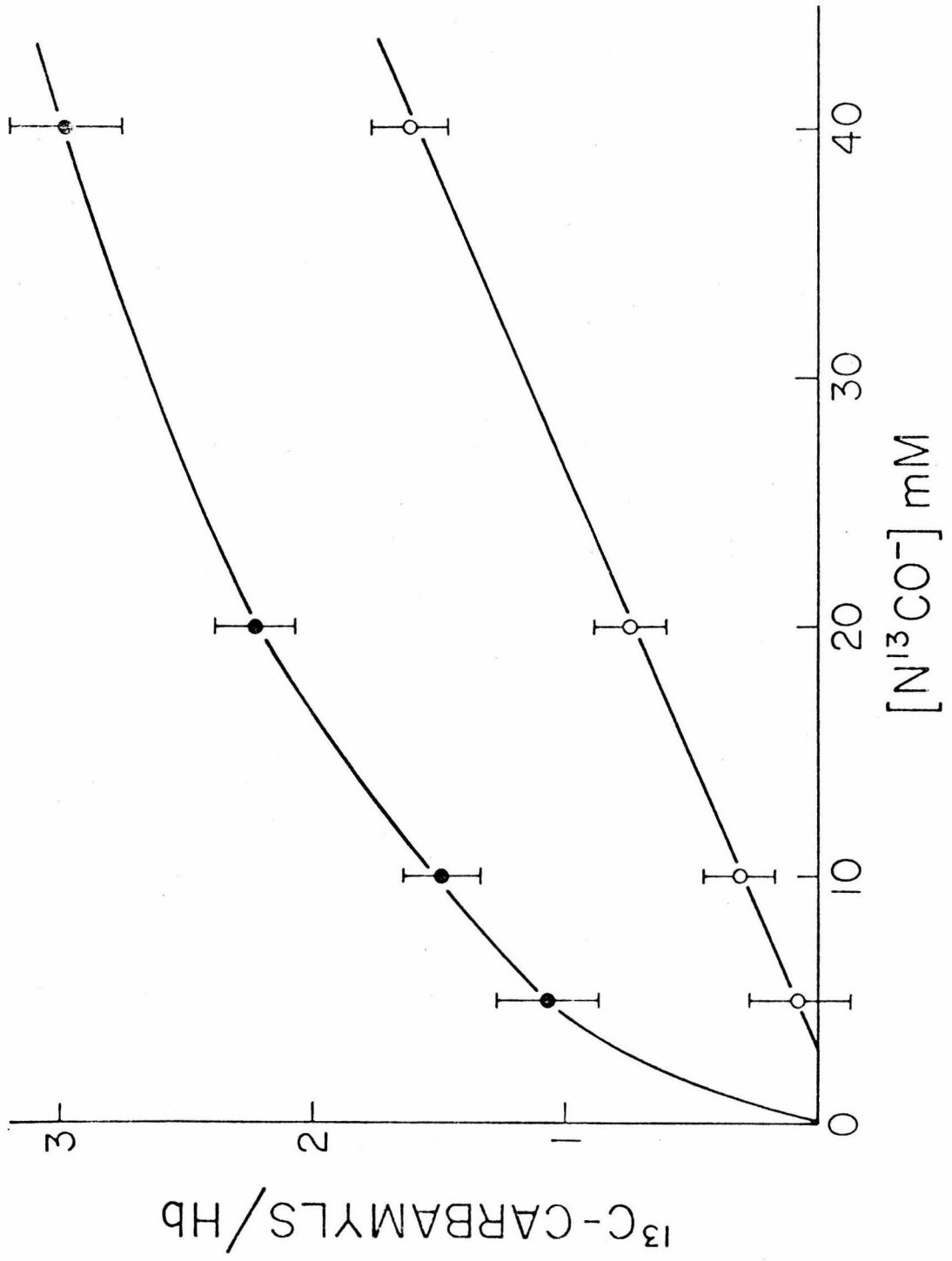
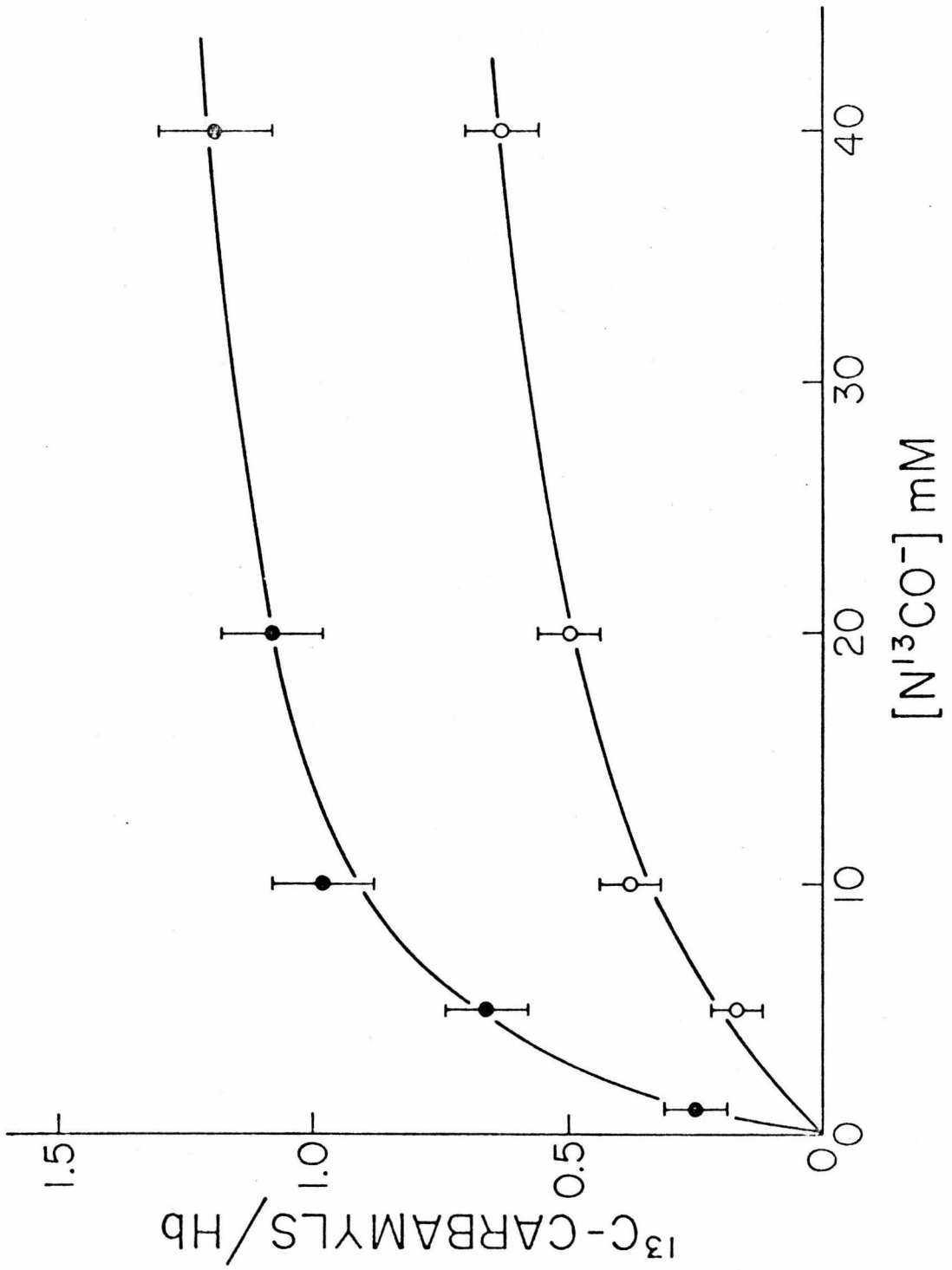

^{13}C-CARBAMYLS/Hb
3
2
1
0
10
20
30
40
[N^{13}CO$^-$] mM

FIGURE VII

A plot of α-amino ($\clubsuit$) and ϵ-amino (Φ) group carbamylation vs. cyanate concentration for hemoglobin-S in oxygenated whole sickle blood. Average whole blood pH = 8.0 ± 0.2 as determined at the beginning and end of the reaction. All other conditions were identical to those in Figure VI.


^{13}C-CARBAMYLS/Hb
1.5
1.0
0.5
0
0
10
20
30
40
[N^{13}CO$^-$] mM

relative degrees of carbamylation of the α-amino groups of the N-terminal valines as contrasted to the ϵ-amino groups of lysine for hemoglobin-S, both in solution and within the red blood cell. Moreover, if the pK_a's of the α-amino groups differ in the α and β subunits, the distribution of carbamyl groups between the subunits should also show considerable pH dependence. Accordingly, careful control of whole blood as well as intracellular pH is of critical importance when evaluating cyanate as a potential therapy in sickle cell anemia. ^{13}C nmr is also shown to be a rapid, convenient method for study of hemoglobin carbamylation.

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PART VIII

^{13}C Magnetic Resonance Studies of Whole Blood Carbamylation

INTRODUCTION

The effects of cyanate for the in vitro carbamylation of red blood cells from persons suffering from sickle cell anemia have been demonstrated to be of value for inhibiting the sickling process and prolonging the mean survival times of reinfused sickle cells (1-7). Though carbamylation of hemoglobin occurs principally at the N-terminal valine α-amino groups of the α and β subunits, carbamylation of the ϵ-amino groups of the lysine residues also occurs (8, 9). While it has been reported that the reactivities of the N-terminal α-amino groups are about two orders of magnitude more reactive than are the lysine ϵ-amino groups (8), the presence of eleven lysine residues per α-amino group results in a significant fraction of the total carbamylation occurring on lysines (9). Owing to the considerable pH dependence for carbamylation of α-amino groups under physiological and typical in vitro conditions (9, 10), the relative α/ϵ amino groups carbamylation ratio must vary considerably with pH. No control of sample pH was mentioned for the actual carbamylation of red cells following the addition of cyanate in several accounts of hemoglobin (11) and whole cell (1, 2, 7, 12) carbamylation. Further, some of the reported procedures involved the direct addition of unbuffered cyanate solutions (2, 7, 11) or solid sodium cyanate (12) to the samples, which is expected to result in increased sample pH.

To better understand the reactions of cyanate with normal and sickle erythrocytes we have employed carbon-13 nuclear magnetic resonance techniques to study the in vitro effects of ^{13}C-enriched cyanate on whole cell suspensions over the pH range 6.6-8.0. ^{13}C magnetic resonance is particularly useful for these studies because the extent of lysine carbamy-

lation is easily distinguished from valine α-amino group carbamylation (9) and the need for radioactive labels or extensive amino acid analysis techniques is eliminated.

MATERIALS AND METHODS

Potassium cyanate (90.8% ^{13}C-enriched) and carbon monoxide (92% ^{13}C-enriched) were purchased from Analytic Supplies Development Corporation.

All samples of normal adult blood were drawn from the same donor (MN). Whole sickle blood was obtained with informed consent from adolescent male and female patients (PF, AF, DC, PP, CH, TJ, GG,KM) with electrophoretically proven homozygous sickle cell anemia who, at the time of venipuncture, were not acutely ill. Heparin was used as anti-coagulent in all cases.

The packed cell volume (PCV) was adjusted to 50% by removal of plasma or addition (for MN) of 0.15 M sodium chloride solution. After the blood was saturated with ^{13}C-enriched carbon monoxide, the whole blood pH was adjusted slowly with a small amount of either 1.0 N hydrochloric acid or 0.5 N sodium hydroxide using a Radiometer model 26 pH meter. No cell lysis was observed. Appropriate amounts of 0.1 M ^{13}C-enriched potassium cyanate/0.15 M sodium chloride solution, which had been adjusted to the proper pH, and 0.15 M sodium chloride were mixed with whole cells to yield samples of 10 ml volume with PCV = 30% and 20 mM potassium cyanate. Each reaction series consisted of five normal and five sickle cell samples contained in tightly capped tubes. The samples were incubated in a water bath at 37.0° ± 0.1 °C with occasional mixing for 150 minutes. Sample pH was measured at the beginning and end of the

reaction. Reactions were completed within six hours of venipuncture.

The reactions were terminated by washing the cells three times with 30 ml volumes of ice-cold 0.15 M sodium chloride, followed by hypotonic lysis in 30 ml of distilled water. The stromata were removed by centrifugation (30,000 x g) and the hemolysates were dialyzed overnight against two - four liter changes of 0.15 M sodium chloride at 4 °C.

The samples were concentrated by ultrafiltration in an Amicon Diaflow apparatus (UM-10 membrane) and adjusted spectrophotometrically (13) to 2.0 mM hemoglobin tetramer in 0.15 M sodium chloride for NMR study.

^{13}C NMR spectra were obtained using the pulse Fourier transform technique on a Varian Associates XL-100-15 spectrometer equipped with a Varian 620i computer (16K). Samples were contained in 12 mm sample tubes. Since the carbamyl carbons are not significantly proton-coupled, and because the presence of nuclear Overhauser effects (NOE) complicates quantitation of the resonances, spectra were obtained without proton noise decoupling. The ^{1}H resonance of the water in the sample was used as a field frequency lock. A 90° pulse of 55 μsec duration was used with a spectrum width of 2500 Hz. The pulse interval was 1.64 sec corresponding to about 3 T_1 for all resonances being quantitated (9). Typical data accumulation time was 4-5 hours for each spectrum.

Quantitation of the levels of α- and ϵ-amino group carbamylation was accomplished by comparing the integrals of the carbamyl resonances with the integral of the peptide carbonyl region, containing 656 carbonyl carbons at natural abundance (1.1% ^{13}C).

RESULTS AND DISCUSSION

Representative ^{13}C NMR spectra of carboxyhemoglobin-S obtained from the whole cell carbamylation reactions described above are collected in Figure 1. Chemical shift, T_1 and NOE data for carbamylated hemoglobin, and the assignment of the ^{13}C resonances from carbamyl groups attached to the N-terminal valine α-amino groups or lysine ϵ-amino groups has previously been reported (9). Assignment of the heme-bound carbon monoxide resonances to the individual α or β subunits of hemoglobin A and S is based on NMR studies of ^{13}CO bound to isolated subunits (14).

Since the carbamyl carbons are not significantly spin-coupled with neighboring protons it is possible to observe these resonances clearly in the absence of proton noise decoupling, thereby eliminating the NOE. Thus, by using a suitably long pulse interval ($\geq 3T_1$), the extent of hemoglobin carbamylation may be determined by integrating the carbamyl resonances (at 91% ^{13}C) and comparing these integrals with the integral of the carbonyl resonance region (656 carbons at 1.1% ^{13}C). Since the carbonyl resonances have $T_1 \sim 0.416$ sec (15), which is very similar to the T_1's of the α and ϵ carbamyls ($T_1 \sim 0.49$ and 0.45 sec, respectively) (9), any errors in the integrals due to partial saturation of the resonances are minimized.

The results of the whole cell carbamylation studies are presented in Figure 2. These data clearly show the dependence of α-amino group carbamylation on extracellular pH. The carbamylation of lysine residues, however, is relatively independent of pH within the 6.6-8.0 pH range studied. These results are in general agreement with results obtained from studies on purified carboxyhemoglobin A solutions (9). Further, the overall shape of the curve for α-amino group carbamylation is essentially the same as that obtained for deoxyhemoglobin A (10). It is also evident

FIGURE 1: Spectra of carbamylated carboxyhemoglobin-S. Carbamylation reactions were conducted with carbon monoxide-saturated whole cell suspensions (PCV = 30%) and 20 mM cyanate for 2.5 hours at 37°C. Sample pH was measured at the beginning and end of the reaction. Average pH values are indicated in the figure. The ^{13}CO resonances of the α and β subunits (206 ppm) and the resonances of the ^{13}C-enriched carbamyl carbons (161 ppm) attached to α and ϵ groups are also indicated. The natural abundance carbonyl carbon resonances are in the 170-180 ppm region of the spectrum.

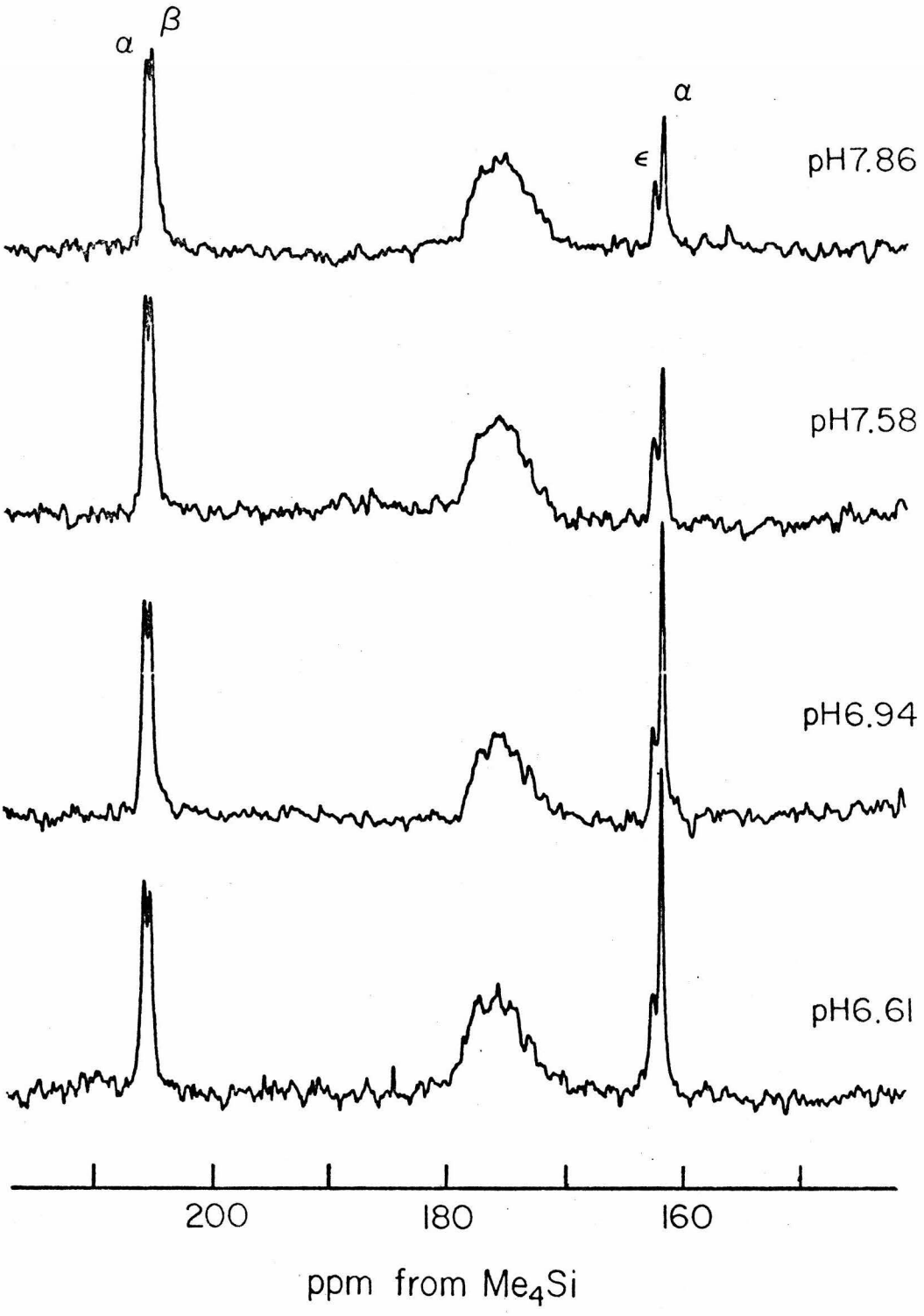
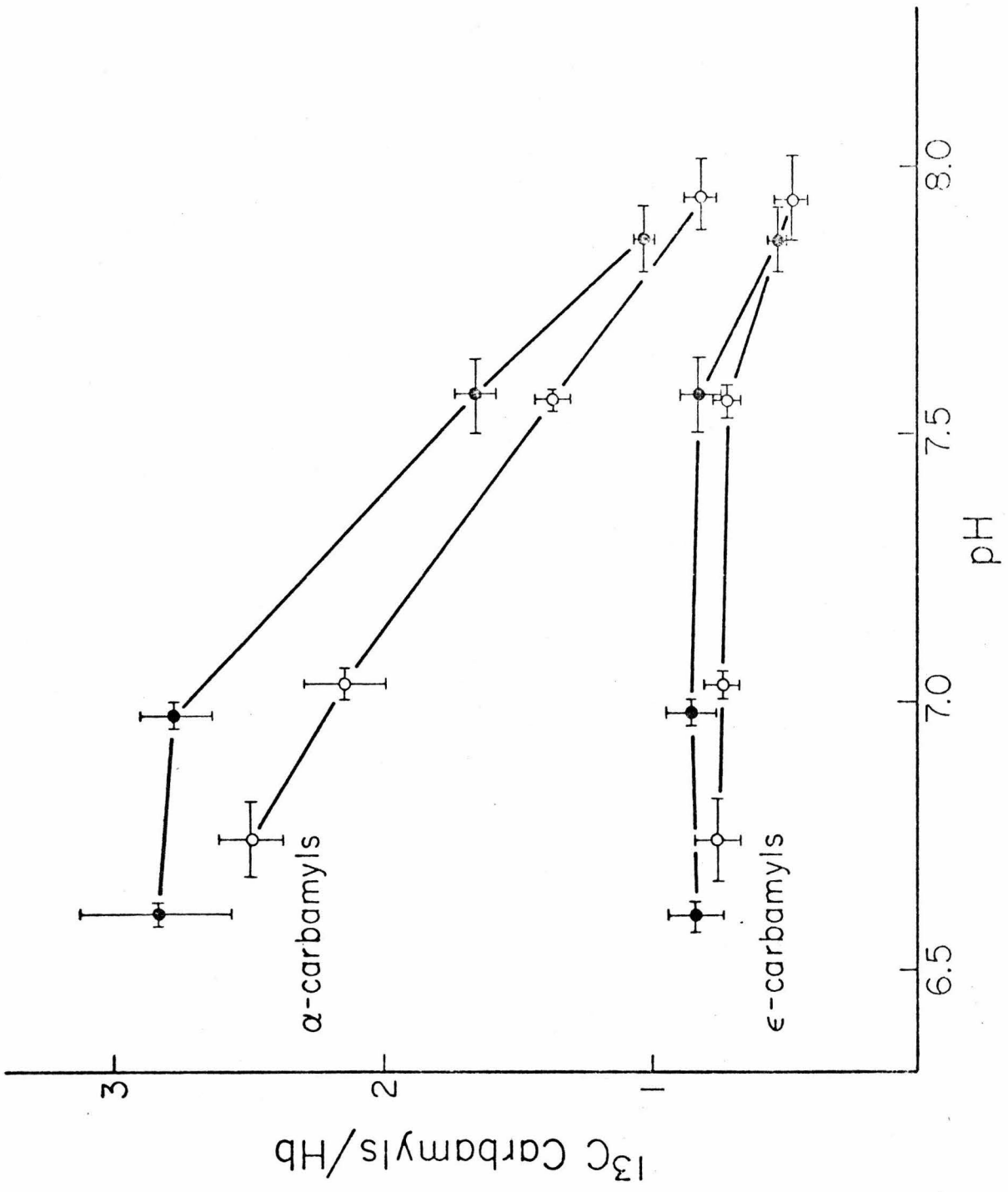

α
β
α
ϵ
pH7.86
pH7.58
pH6.94
pH6.61
200
180
160
ppm from Me4Si

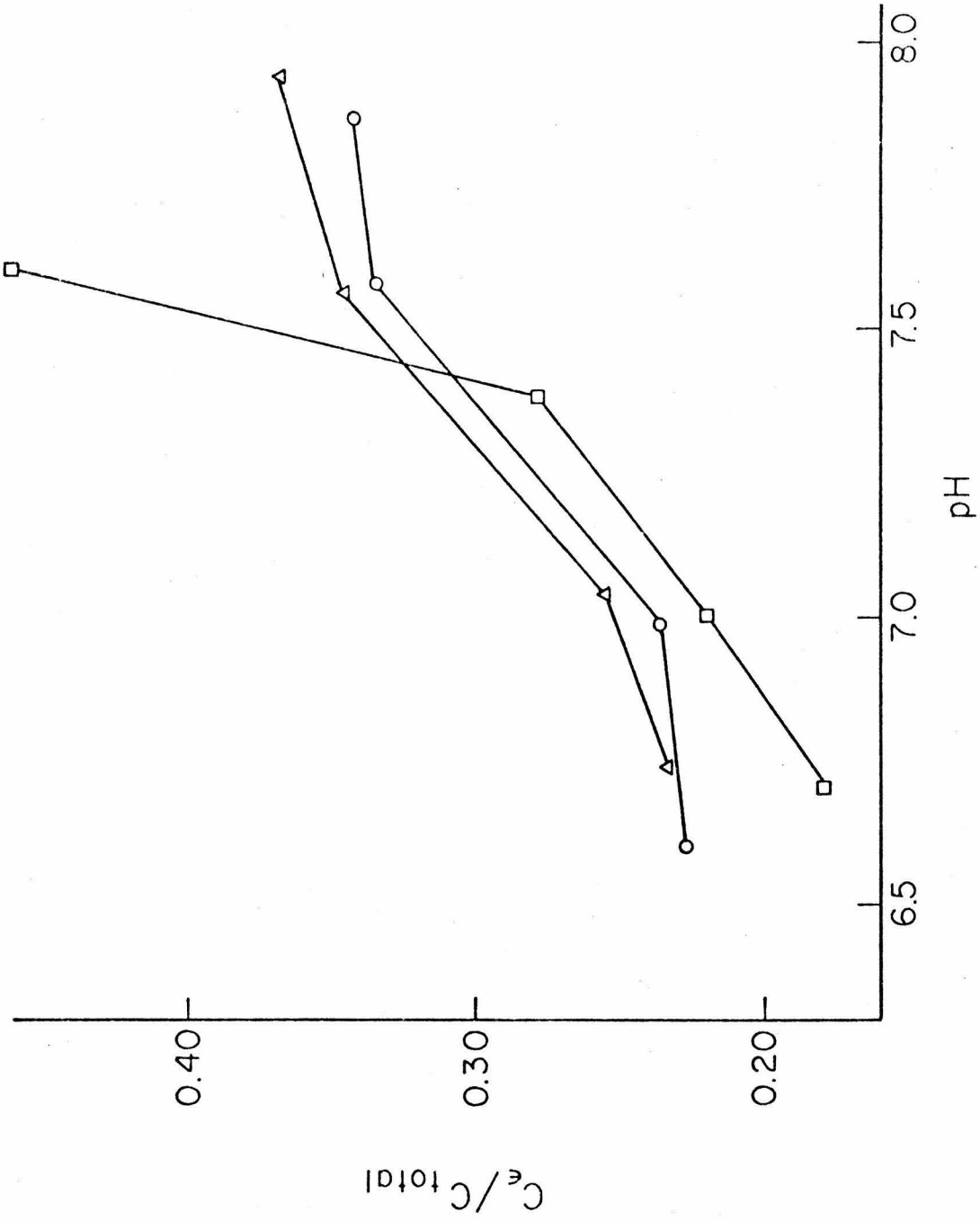
FIGURE 2: A plot of the extent of hemoglobin α and ϵ amino group carbamylation for sickle cell (●) and normal adult (○) erythrocytes as a function of mean whole blood pH. The horizontal error bars indicate the standard mean deviations in sample pH for five identical samples at the beginning and end of the reaction. Vertical error bars indicate the standard mean deviations in the carbamyl group quantitation based on ^{13}C NMR spectra obtained for each of the five samples. Resonance overlap and the experimentally determined accuracy of the integrator (4%) have also been accounted for.


^{13}C Carbamyls/Hb
3
2
1
α-carbamyls
ε-carbamyls
6.5
7.0
7.5
8.0
pH

from Figure 2 that, in the region below pH 8, hemoglobin in whole sickle blood carbamylates to a significantly greater extent than does hemoglobin in the normal adult red cells of the control. Both α-amino group and ϵ-amino group carbamylation show this effect. Lee and Manning (8) have shown that, in stripped hemoglobin solutions at pH 7.4, carboxyhemoglobin-S and carboxyhemoglobin-A carbamylate with the same apparent first order rate constant. This suggests that no difference exists between the pK_a values for the α-amino groups of the two liganded hemoglobins. It has also been established that deoxyhemoglobins A and S are about twice as reactive as the corresponding oxyhemoglobins (8, 10) and that the effect of 2, 3-diphosphoglycerate (P_2-Glyc) on hemoglobin carbamylation is to inhibit reaction at the β chain α-amino groups without significantly affecting carbamylation of the α chain α-amino groups. No data exists, however, to suggest any dependence of lysine carbamylation on either the state of hemoglobin ligation or the level of P_2-Glyc present. Since we have observed increases in the molar carbamylation levels for both the α- and ϵ-amino groups of hemoglobin S in sickle cell blood relative to hemoglobin A in the control, it seems unlikely that differences in degree of hemoglobin ligation or P_2-Glyc concentration are directly responsible. Rather, the increased level of lysine carbamylation in sickle cells seems to be due to increased permeability of the sickle cell membrane toward diffusion of cyanate anion into the cell. This explanation would also fit the observations of others that the sickle erythrocyte shows radically altered permeability to small ions (16).

Figure 3 shows a plot of the pH dependence of the fraction of total carbamylation accounted for by lysine residues. Again a slight difference is observed between the fraction of lysine carbamylation in sickle erythocytes

FIGURE 3: A plot of the fraction of total carbamylation occurring on lysine residues of carboxyhemoglobin-A (□) and carboxyhemoglobin in normal (○) and sickle cell (△) erythrocytes. Carboxyhemoglobin-A reactions were conducted with 2.0 mM hemoglobin and 20 mM cyanate for 2.5 hours at 37°C.

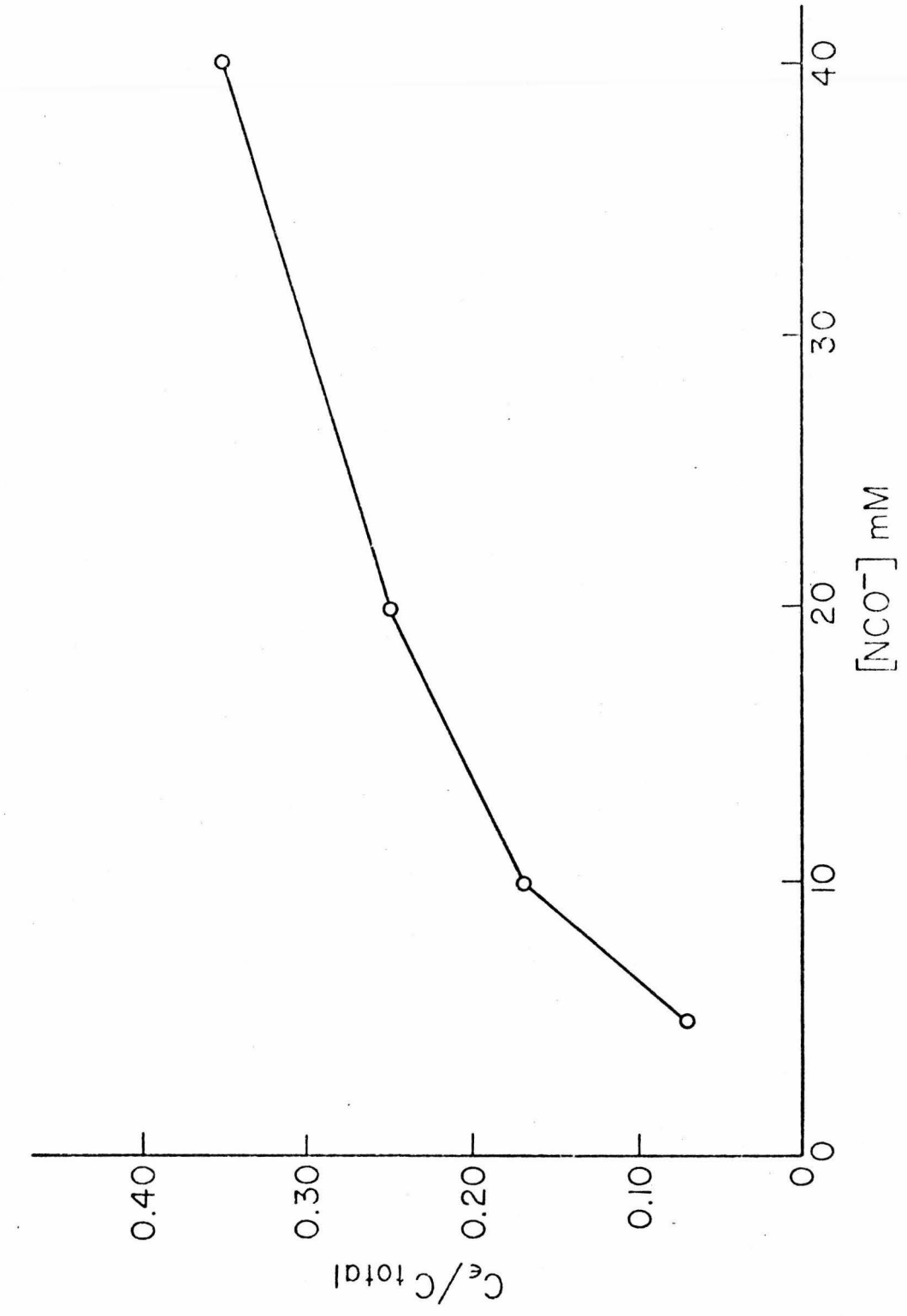

0.40
0.30
0.20
C_e/C_{total}
6.5
7.0
7.5
8.0
pH

with respect to the control. A larger difference is observed in the fraction of lysine carbamylation observed in phosphate-free hemoglobin solutions carbamylated under similar conditions (9). Since the fraction of lysine carbamylation under otherwise constant conditions is expected to be constant, intracellular pH seems most likely to be influencing this ratio. Thus, the intracellular pH appears to be buffered relative to the whole blood pH and the pH within the sickle cells appears to be slightly lower than that of the control. The data suggest that the sickle erythroctye pH is about 0.05-0.1 pH unit lower than that of the control cells. The fact that intracellular pH is buffered relative to extracellular pH has been well demonstrated (17-19). Further, increased P_2-Glyc concentration should lead to a reduction of intracellular pH (20). Since elevated P_2-Glyc concentrations have been reported in sickle erythrocytes (21), this is a plausible explanation for the apparent difference in the fraction of lysine carbamylation observed between sickle and normal red cells.

Figure 4 shows the dependence of the fraction of lysine carbamylation on cyanate concentration at pH 7.5 ± 0.06. These data are in agreement with the results of others (10, 11) and confirm that high cyanate concentrations lead to more extensive lysine carbamylation.

The absence of a carbamyl resonance in the 171-172 ppm region of the spectrum suggests that no detectable carbamylation of cysteine residues has occurred. While the carbamyl group resonance of S-carbamyl-L-cysteine occurs at 171.45 ppm (9), the absence of this resonance in all of our hemoglobin samples, including those reacted at pH ~6.5 and kept below pH 7 during workup and spectral analysis, suggests that S-carbamylation is not a problem in samples carbamylated in the pH 6-7 range. Thus, concern over carbamylation of cys 93 in the β chain (11, 22, 23) does not appear to

FIGURE 4: A plot of the fraction of total carbamylation occurring on lysine residues of hemoglobin-S as a function of cyanate concentration. The reactions were conducted on oxygenated whole sickle blood for 2.5 hours at 37°C. Average sample pH = 7.51 ± 0.06 as determined at the beginning and end of the reactions. The PCV was adjusted to 30% for all samples.


0.40
0.30
0.20
0.10
0
0
10
20
30
40
C_e/C_{total}
$[NCO^-]$ mM

be a problem under our conditions. Further, any S-carbamylation is expected to be reversible above pH 7 (24).

These results suggest that optimal carbamylation of the α-amino groups of hemoglobin-S within sickle erythrocytes is achieved in the pH 6.5-7.0 range and that cyanate concentrations are best kept below 10-20 mM if minimal carbamylation of lysine residues is to be realized. Thus in vitro carbamylation of whole blood might best be accomplished in ACD anticoagulant solutions containing buffered 10-20 mM cyanate at pH 6.5.

These results also show that ^{13}C magnetic resonance techniques are capable of monitoring carbamylation levels as low as 0.1 mole per mole of hemoglobin without the use of clinically undesirable radioactive isotopes or extensive protein manipulation. ^{13}C NMR shows the further advantage of being able to directly distinguish carbamylation of the N-terminal valine residues from other potentially reactive sites such as lysine and cysteine residues.

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PART IX

Conformational Studies of Various Hemoglobins Using Natural Abundance ^{13}C NMR Spectroscopy

INTRODUCTION

An enormous amount of research has been devoted to hemoglobin (1-5), the oxygen carrying protein of erythrocytes. However, many of the molecular details of its action remain unknown, for example, the nature and geometry of the iron-oxygen bond (6-8), the exact relationship between the conformations (9,10), inter alia, of deoxyhemoglobin (Hb), oxyhemoglobin (HbO_2), carboxyhemoglobin (HbCO), acid (or aquo) methemoglobin (Hi) and cyanmethemoglobin (HiCN), the relative importance of the α and β subunits to the allosteric cooperativity of the hemoglobin molecule (11-14), (for example, the possible existence of quaternary--and tertiary--conformations intermediate between the conformations of Hb and HbO_2) the nature of the salt bridges between subunits in various quaternary conformations, and the exact mechanisms by which organic phosphates such as 2,3-diphosphoglycerate (2,3-DPG) influence hemoglobin oxygen affinity and allostery.

The present paper presents our results with natural abundance ^{13}CO nuclear magnetic resonance studies of normal human and rabbit hemoglobin. The main molecular differences observed by these techniques depend on the relative mobility of various amino acid side chains that reflect, in turn, the conformational differences between the various forms of hemoglobin.

A point of special importance in analysis of the spectra we obtained is that particular attention has been paid to changes observed for differently liganded hemoglobins from a given biological source, rather than detailed interpretation of each of the absorptions of individual

spectra; accordingly, differences between the various spectra are the major source of the information we consider. Moreover, by using native proteins under physiological conditions and concentrations, one can monitor changes which are unperturbed by introduction of labels or other alterations of the natural protein. Natural abundance studies have a further potential advantage in that observation of the entire ^{13}C range should lead to more information about changes in the protein than would be obtained by observation of a specific label which, though sensitive to changes in its immediate environment, may fail to reflect important changes elsewhere in the protein.

Previous studies of proteins using ^{13}C pulsed Fourier transform spectroscopy have been reported for ribonuclease A (15), for lysozyme (16) and for carboxymyoglobin and carboxyhemoglobin (17), though the last work was carried out under conditions of such a low signal-to-noise ratio that little molecular information was available.

EXPERIMENTAL

Hemoglobin was prepared from whole blood freshly drawn from humans or New Zealand white rabbits. The erythrocytes were separated from plasma, washed several times with 0.1 M NaCl, and hemolyzed by treatment with water. After removal of the stromata by centrifugation, the hemolysates were extensively dialyzed against 0.1 M NaCl.

Methemoglobin was prepared by direct oxidation of oxyhemoglobin with ferricyanide, followed by extensive dialysis against 0.1 M NaCl.

Cyanmethemoglobin was prepared from methemoglobin by treatment with sodium cyanide, followed by dialysis against 0.1 M NaCl.

Oxy-, carboxy-, and deoxyhemoglobins were prepared by extensive treatment of hemoglobin with water-saturated oxygen, carbon monoxide, or nitrogen.

Protein was concentrated either using an Amicon ultrafiltration apparatus or by ultracentrifugation. Final concentrations were adjusted spectrophotometrically to 3.0 mM; a molecular weight of 68,000 was assumed. The pH of all samples was adjusted, where necessary, by dialysis against 0.1 M NaCl in 0.01 M Tris buffer of appropriate pH. Sample pH was varied within the range 6.8 - 7.5.

^{13}C NMR Spectra were obtained by use of the pulsed Fourier transform technique on a Varian Associates XL-100-15 spectrometer equipped with a Varian 620i computer. Samples were contained in a 10-mm diameter tube, concentrically held within a 12-mm tube containing deuterium oxide, which was used as a field-frequency lock. All spectra were proton noise decoupled and were obtained under identical conditions at 34°C.

A 50° pulse of 60μsec duration was used, with an acquisition time of 0.2 sec. A total of 130,000 - 175,000 transients were obtained per spectrum, requiring data accumulation times of 8-10 hours.

All spectra used in subsequent discussion obtained under any given set of conditions were highly reproducible. Moreover, no significant change in relative intensities was observed when longer acquisition times were used for rabbit hemoglobin.

RESULTS

Representative spectra of the many observed are collected in Figures 1-3. Figure 1 shows acid-denatured $\alpha\beta$ globin from rabbit, Figure 2 collects typical spectra of various human hemoglobins (HbO_2, Hi and Hb and a sample of Hb in the presence of 2,3-DPG), and Figure 3 shows results with rabbit hemoglobins (HbO_2, HbCO, HiCN, Hi and Hb).

FIGURE 1

Acid-denatured $\alpha\beta$-globin from rabbit, pH 0.75.

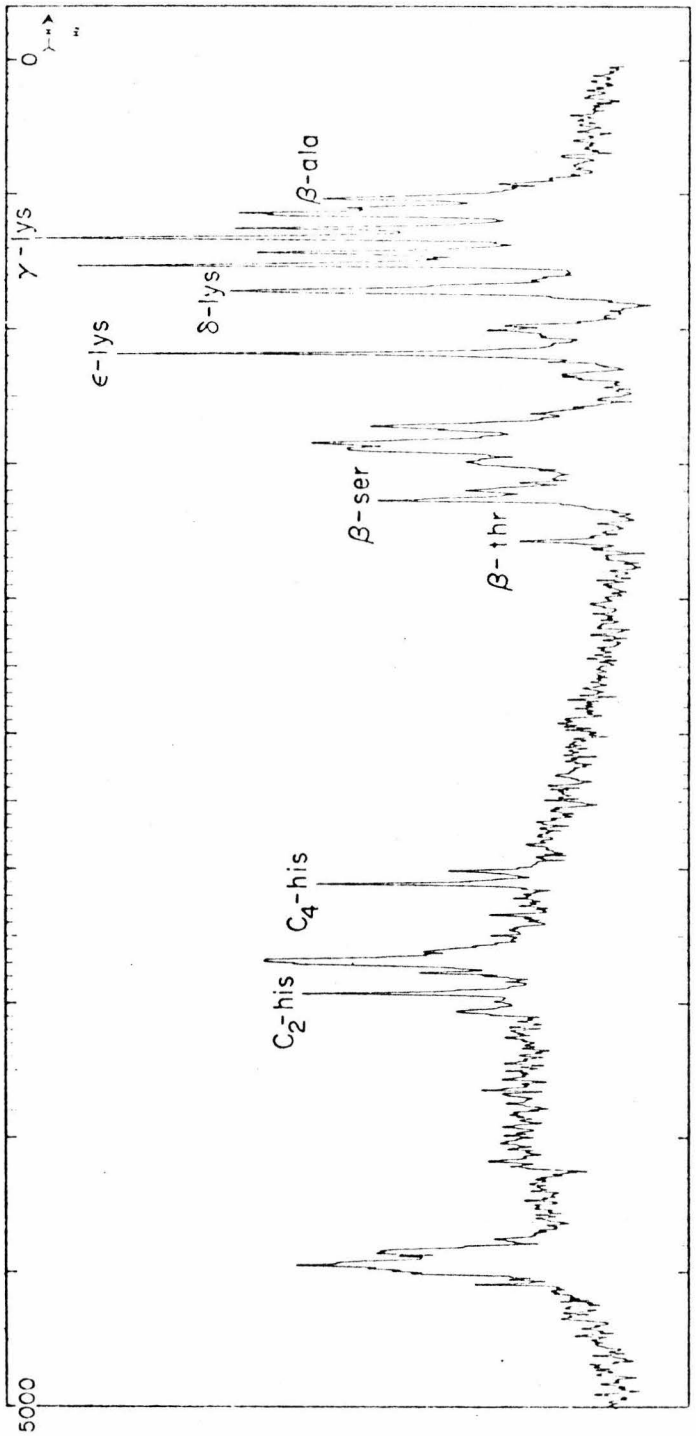

0
5000
γ-lys
β-ala
δ-lys
ε-lys
β-ser
β-thr
C4-his
C2-his

FIGURE 2

Typical spectra of human hemoglobins: (A) = HbO_2, pH 7.25; (B) = Hi, pH 7.12; (C) = Hb, pH 7.41; (D) = Hb + 2,3-DPG (5mM), pH 7.38. The resonances of ϵ-lysine and β-alanine carbons are indicated (1 and 2, respectively).

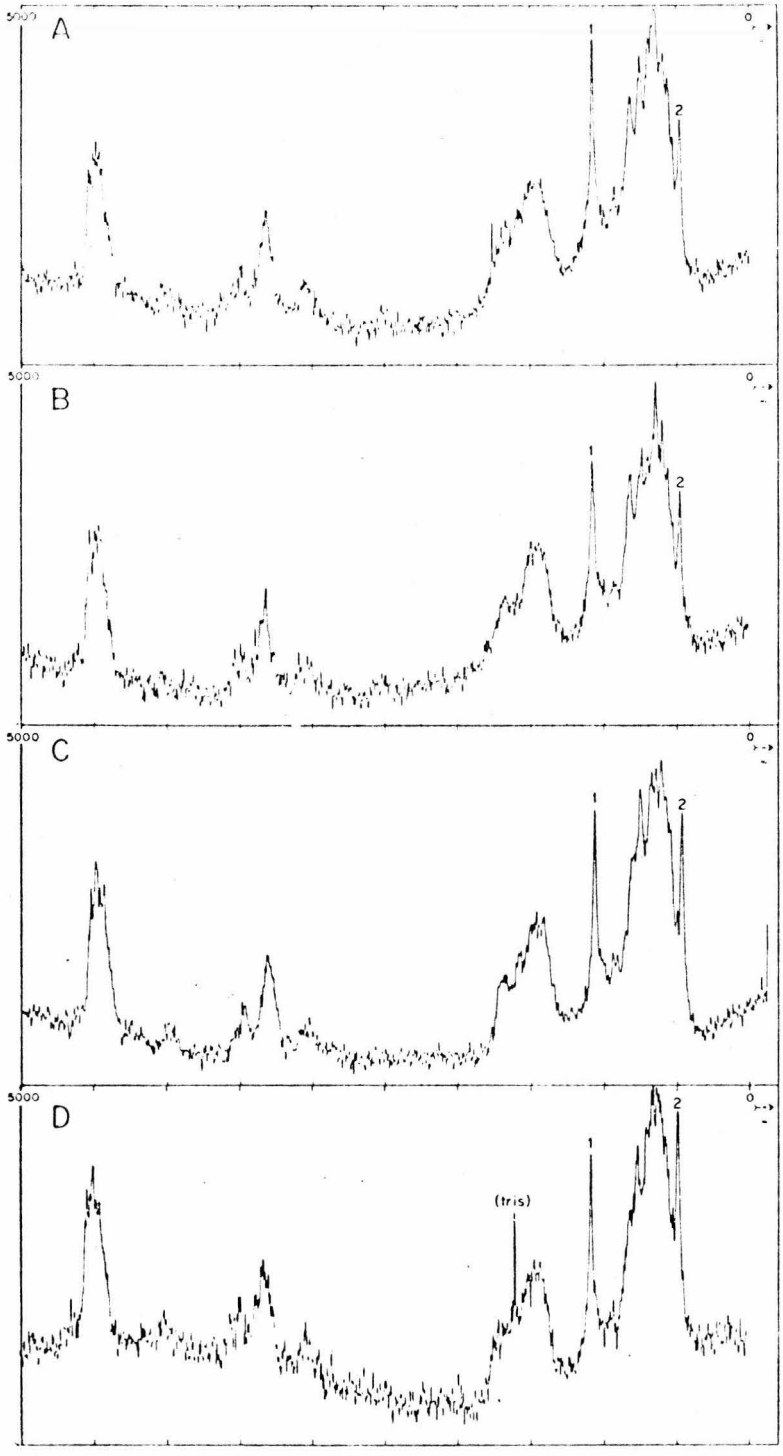
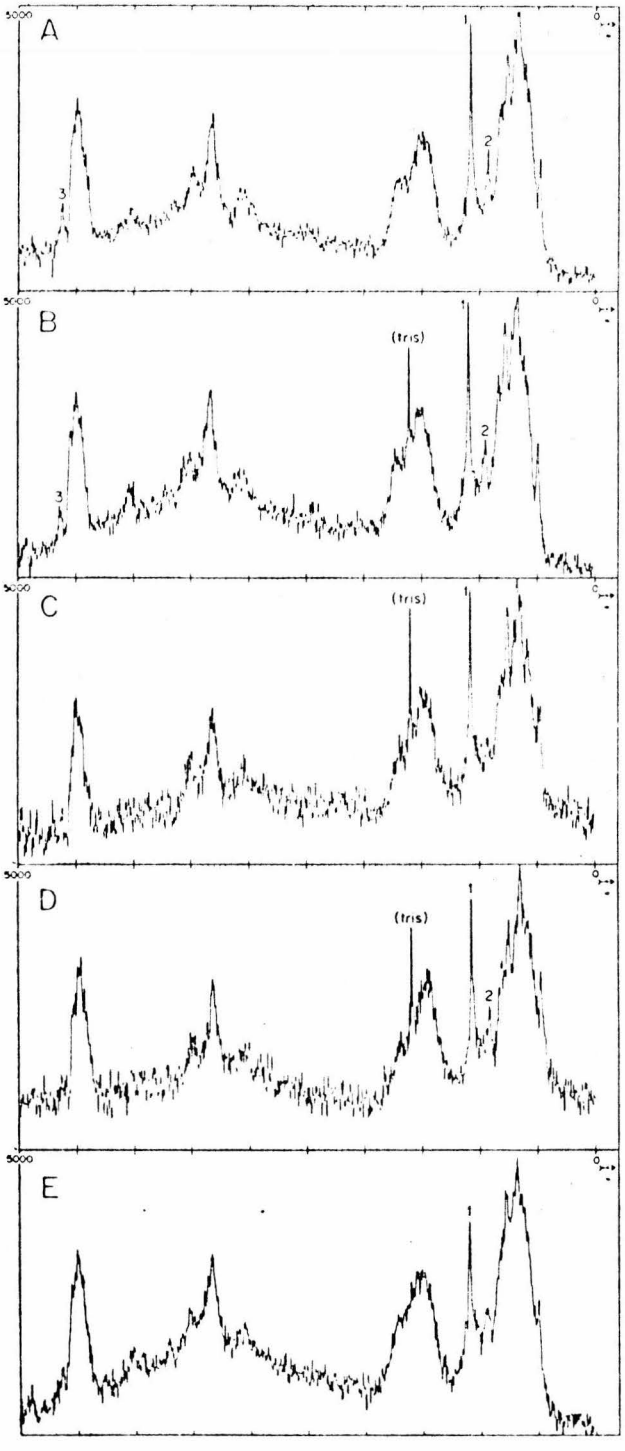

5000
A
0
1
2
5000
B
0
1
2
5000
C
0
1
2
5000
D
0
1
2
(tris)

FIGURE 3

Typical spectra of rabbit hemoglobins: (A) = HbO_2, pH 6.90; (B) = HbCO, pH 7.29; (C) = HiCN, pH 7.45; (D) = Hi, pH 7.41; (E) = Hb, pH 7.02. The resonances of ϵ-lysine, γ-glutamic acid and δ-glutamic acid carbons are indicated (1, 2, and 3, respectively).


A
1
2
3
B
(tris)
1
2
3
C
(tris)
1
D
(tris)
1
2
E
1

DISCUSSION

Analysis of Spectra

The spectrum of denatured rabbit hemoglobin of Figure 1 shows the resonances anticipated for a polypeptide of appropriate amino acid composition in a random coil conformation with extensive segmental motion which results in the observed, relatively sharp absorptions. The general features of this and other spectra used in this work, including the detailed justifications of assignments of particular resonances to particular amino acids, will be discussed more extensively elsewhere.

In the various forms of human hemoglobins shown in Figure 2, the main changes of note among the series HbO_2, Hi, Hb and Hb in the presence of 2, 3-DPG are (i) a steady decrease in the resonance due to the ϵ-carbon of lysine residues, (ii) a somewhat less obvious decrease in the resonances due to the δ and γ carbons of lysine and (iii) an increase (two-fold over the entire series) in intensities of resonances due to the methyl groups (β carbons) of the alanine residues.

The decrease in intensities of the resonances of the side-chain carbons of lysine residues arises, in our view, because of immobilization of these side chains as the protein conformation becomes tighter, and lysines that are free in solution in oxyhemoglobin become involved in salt bridges as the structure changes to that of deoxyhemoglobin. As these side chains become immobilized, their relaxation times decrease (18) and their absorptions broaden and are removed from the sharp resonances, characteristic of mobile side chains, and are added to the broad, unresolved absorptions.

This interpretation is in accord with studies of the conformational and segmental motion of ribonuclease A (19), and of the resulting relaxation times, that led to the general conclusion that backbone carbons and carbons in side chains that experience little if any motion independent of their stretch of polypeptide chain have spin-lattice relaxation times less than 0.1 sec. Two major exceptions to this generalization are the peptide carbonyl carbons, which have T_1 about 0.416 sec, and the ϵ-carbons of lysine side chains which have T_1 about 0.330 sec, due to their relative freedom in ribonuclease A. Using the technique of progressive saturation, we have independently determined the relaxation times characteristic of the ϵ-carbons of "mobile" lysine side chains has an average $T_1 = 0.4 \pm 0.1$ sec.

A similar explanation can account for the increase in intensity of the methyl groups, though in this case, the increase in intensity depends on the saturation behavior of these absorptions. These methyl groups, which are likely to experience free-spin rotation, will have $T_1 > 0.5$ sec. The spectra were observed with acquisition times of 0.2 sec, so that under our conditions these resonances will be largely saturated. They do not, therefore, appear with their full intensity in the observed spectra of oxyhemoglobin. As the protein becomes progressively more rigid, as in conversion to Hi, Hb or Hb in the presence of 2, 3-DPG, these groups will become increasing immobilized, their relaxation times will decrease, and their apparent intensity will increase because the absorptions have become less saturated under conditions of observation.

Figure 3 shows the natural abundance spectra of the various rabbit hemoglobins (HbO_2, HbCO, HiCH, Hi and Hb). In general, the ob-

served variations between the differently liganded species agree well with those observed for human hemoglobins. Again one of the prominent differences focuses on the signal from the ϵ-carbons of lysine residues. Between HbO_2 and HbCO, little change is observed. There is a slight decrease in HiCN relative to HbO_2 or HbCO and a very significant decrease for Hi. Deoxyhemoglobin shows the most marked decrease. About half the total decrease observed between HbO_2 and Hb has occurred in acid (or aquo) methemoglobin (Hi).

The spectra of similarly liganded hemoglobins from rabbits and humans show further interesting differences, which seem to indicate a generally looser structure for rabbit than for human hemoglobins. Thus the spectra of rabbit HbO_2 and HbCO show more detail than that of human HbO_2. In rabbit HbO_2 and HbCO, the γ and δ carbons of some glutamic acid residues are visible, whereas these are only barely, if at all, detectable in the spectrum of human HbO_2. The intensity of these resonances due to the γ and δ carbons of glutamic acid correspond to their intensity in denatured rabbit hemoglobin. There are four more glutamic acid residues in rabbit than in human hemoglobin, but this fact alone is insufficient to account for the marked spectral differences in this regard between rabbit and human hemoglobin, which must arise from the considerably greater mobility of the γ and δ carbons of glutamic acid residues in rabbit compared to human HbO_2.

Whereas the intensity of the resonance due to the ϵ-carbon of lysine residues shows little change between rabbit HbO_2 and HiCN, the glutamic acid resonances are appreciably reduced in rabbit HiCN relative to HbO_2.

The greater general flexibility of rabbit compared to human hemoglobins seems to be further confirmed by the absence of an increase in the intensity of the alanine methyl groups in the conversion of rabbit HbO_2 to Hb; presumably in both forms these methyl carbons have sufficiently long relaxation times that they are saturated in our experiments using acquisition times of 0.2 sec. Moreover, the intensity of this resonance in the native proteins corresponds to its relative intensity in either acid or base denatured rabbit $\alpha\beta$ globin.

Another general feature of all the spectra is the steady decrease in the overall aliphatic region of the spectra, measured relative to the carbonyl and α-carbon resonances (which show very little change with changes in ligands) as one moves from HbO_2 to Hb either in the rabbit or human series. These general changes correlate with the more discrete differences already discussed (for example, resonances due to the ϵ-carbon of lysines).

Conformational Differences

HbO_2 Compared to Hb. The most striking spectral change on deoxygenation of either rabbit or human hemoglobin is the decrease in intensity of the resonance from the ϵ-carbons of lysine. This difference is enhanced in the presence of 2,3-DPG and results from immobilization of lysine side chains. Though we cannot, in the absence of a better quantitative knowledge of the T_1 and T_2 values for the resonances in question, decide unequivocally how many lysine residues are immobilized in the conversion of HbO_2 to Hb, we can estimate that about 25% of those residues that are free in human HbO_2 are immobilized on deoxy-

genation, while 33% of the free lysine residues are immobillzed on deoxygenation of rabbit hemoglobin.

From Perutz's x-ray results (5), two lysine (40α and 127α) seem to be strongly immobilized on conversion of human HbO_2 to Hb. Immobilization of an additional lysine (82β) should occur in the presence of 2,3-DPG. On this basis, we would estimate that about half of the total lysines (11 residues) are always immobilized in intact hemoglobin, whatever the nature of its ligands.

As the intensities of the ϵ-carbons of lysine are about the same in rabbit and human hemoglobin, we guess that both hemoglobins have about the same number of bound lysines. Deoxygenation of rabbit HbO_2 shows a greater decrease in the intensity of the ϵ-carbon of lysine than for deoxygenation of human HbO_2, suggesting that deoxygenation of rabbit HbO_2 causes immobilization of one or two more lysine residues than in the case of deoxygenation of human HbO_2.

Rabbit Compared to Human Hemoglobin. As noted above, the variously liganded rabbit hemoglobins seem generally to have more flexibility and mobility than their human counterparts. Comparison of the amino acid compositions of these two types of hemoglobins provides a possible rationalization of these results; rabbit hemoglobin contains (i) more amino acids with larger side chains than does human hemoglobin and (ii) fewer proline residues.

Specifically, of the 25 amino acid substitutions between rabbit and human α-chains, 18 of these involve sterically larger residues and only 6 involve sterically smaller residues in rabbit, as compared to human,

hemoglobin. There are 14 amino acid substitutions between rabbit and human β subunits. Eight of these are sterically larger and two are sterically smaller in rabbit, compared to human, β-chains. Moreover, rabbit β-subunits lack three proline residues present in normal human hemoglobin.

These substitutions could cause rabbit hemoglobin to have a significantly less compact, looser structure than human hemoglobin. Additionally, the larger number of proline residues in human hemoglobin should increase its rigidity as compared to rabbit hemoglobin.

Charge and polarity differences between the two kinds of hemoglobin may contribute to the differences in segmental motion and flexibility between them, but, in our view, the steric factors discussed above best explain the observed resluts.

Rabbit HbCO and HiCN Compared to HbO_2, Hi and Hb. Though previously assumed to have conformations equivalent to that of human HbO_2, human HbCO and HiCN seem to differ subtly from HbO_2, as monitored by a fluorine label attached to Cys 93β(20). Our data on rabbit hemoglobins suggest that the conformational differences between HbO_2, HbCO, and HiCN are small compared to the larger differences between these liganded species and the weakly- or nonliganded forms Hi and Hb (which, also of course, show significant differences from each other). Our data also show that significant difference between HbO_2 and HbCO, if any, is unobservable, suggesting that bonding of either oxygen or of carbon monoxide causes very similar changes in the tertiary structures of the subunits, and leads to essentially equiva-

lent quaternary structures for these hemoglobins. Moreover, the conformation of HiCN differs significantly from that of HbO_2 or HbCO. This evidence argues against the "metsuperoxide" bonding description (21, 6) (as a metsuperoxide should have the same structure as HiCN), and favors either the original suggestion of Pauling and Coryell (22) or that of Gray (23), in which oxygen forms two bonds to iron and adopts a geometry similar to that, for example, of ethylene in many metal-organic complexes. In these model cases, the bonding of both ethylene and carbon monoxide, though having different geometries, cause very similar spectral changes.

The differences between HbO_2 and HiCN appear largely manifest in greater mobility of the glutamic acid residues in HbO_2; they seem to be much more restricted in HiCN (as well as in Hi and Hb, though in these two forms the lysine residues are also immobilized; they are not in HiCN). Thus, only the glutamic acid side chains show marked differences between HbO_2 and HiCN. Though the molecular origin of these changes is uncertain, an intriguing possibility is that the side chains of glutamic acid are chiefly involved in intra-subunit H-bonding interactions and are, therefore, particularly sensitive to changes in subunit tertiary structure. Lysine residues may, on the other hand, be principally involved in intersubunit interactions, and changes in lysine resonances may largely reflect changes in quaternary structure of the hemoglobin.

HbO_2 Compared to Hi. From x-ray diffraction studies of structures of single crystals, Perutz has suggested (24) that HbO_2 and Hi have similar structures, whereas this work would suggest that in solution the conformations of these hemoglobins differ appreciably, and that at physio-

logical pH both rabbit and human Hi have structures roughly intermediate between those of HbO_2 and Hb. These conclusions agree with those obtained by use of a fluorine label on Cys 93β which show, at least in the vicinity of the label, strong similarities between human Hb and Hi (20). This similarity is further supported by the observation that Hi binds ATP (25); such binding is normally a property of Hb, but not of HbO_2.

In this regard, the effect of ATP on the electron paramagnetic resonance spectrum of Hi deserves comment. In the absence of ATP, two resonances are observed (26), one attributed to high-spin and the other to low-spin Fe III; these forms are in equilibrium. Addition of ATP causes the virtual elimination of the signal due to low-spin Fe III, and a concomitant increase in the signal due to high-spin Fe III. Though no explanation of these results was offered, one possibility is that the forms in equilibrium are acid methemoglobin (Hi) with high-spin Fe III and alkaline methemoglobin (HiOH) with low-spin Fe III. The HiOH structure should approximate that of HiCN, for which separation between the β-subunits should be small. Accordingly, addition of ATP should shift the equilibrium toward the high-spin, acid methemoglobin form. On this basis, one would anticipate that elevation of this pH should cause the ^{13}C spectrum of Hi to appear increasingly similar to that of HiCN and, in fact, this is a result we have observed.

General Comments. Because the actual crystals used in determining the structure of "oxyhemoglobin", though crystallized as HbO_2, are generally understood to have undergone considerable oxidation to

methemoglobin, and because the solution conformation of Hi appears to differ appreciably from that of HbO_2, some question has been raised as to whether oxyhemoglobin actually, in solution, has the structure assigned to it (27).

This may, however, not be so serious an objection. One should distinguish clearly between differences in the energies of various species, on the one hand, and differences in their conformations on the other. There is no requirement that these two properties be simply related. Thus, some conformational changes may represent relatively large energy differences, while other conformation changes, which appear to be as extensive, may occur with relatively much smaller changes in the energy of the system. Lattice effects in a crystal that was formed as HbO_2 and in which considerable oxidation of HbO_2 to Hi may have occurred may well cause the Hi to retain a conformation very similar to that of HbO_2, in which case the reported structure of oxyhemoglobin may be essentially correct.

CONCLUSION

The use of natural abundance ^{13}C NMR spectroscopy, which focuses particularly on differences between the spectral characteristics of closely related proteins (as in the sequence HbO_2, HbCO, HiCN, Hi and Hb) has been used to study the conformation of protein molecules in solution.

The similarity between rabbit HbO_2 and HbCO suggests that both these forms have nearly identical quaternary conformations for the $\alpha_2\beta_2$ tetramer, as well as for the individual α and β subunits. The differences between these forms (HbO_2 and HbCO) and rabbit HiCN suggest to us that, though HiCN has the quaternary structure of HbO_2 or HbCO, there have been subtle conformational changes in the tertiary structures of the α and β subunits in rabbit HiCN.

The quaternary structure of Hi seems to be between that of the strongly liganded hemoglobin and deoxyhemoglobin. We do not suggest an intermediate quaternary structure for Hi, but interpret our results in terms of an equilibration between two limiting quaternary structures (that for strongly liganded hemoglobins on the one hand and deoxyhemoglobin on the other).

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PROPOSITION I

An extremely important response of the immune system involves the migration of leukocytes, monocytes and other phagocytic cells into areas of local infection or inflammation. This migration of phagocytic cells may result from either the direct effect of chemotactic agents which are recognized by the cells and stimulate their accumulation in the infected area or by the indirect effect of chemotactic inhibitors which block the normal movement of the cells once they are localized in the infected area.

With the development of semi-quantitative techniques for the study of cell chemotaxis through micro-pore filters came the discovery that the complement enzyme system of plasma played some role in the generation of chemotactic substances (1).

The complement enzyme system, as described in relation to the immune hemolysis reaction, involves at least nine immunologically distinct serum proteins. When antibody-sensitized sheep red blood cells are treated with whole serum, the complement components in the serum react in a sequential manner with the sensitized red cells resulting in cell lysis (2). During the course of this reaction a protein fragment, $C5_a$, is now known to be released which possesses anaphylatoxic activity (3) and is chemotactic (4) for polymorphonuclear leukocytes (PMN's). This fragment has a molecular weight of about

15,000 (5) and has been shown to be chemotactic for neutrophils (6), eosinophils (7), and monocytes (8) as well as PMN's.

The action of the proteolytic enzyme plasmin on the third component of complement, C3, has also been shown to result in the generation of a protein fragment with chemotactic and anaphylatoxic activity (9). This factor, $C3_a$, has a molecular weight of about 7,000 and may also be generated by the action of trypsin, thrombin, the C3 convertase enzyme complex of complement or the C3 inactivator complex of plasma (10), though the resulting $C3_a$ molecules from each of these preparations show differences in their chemical and physiological properties.

More recently, the "dialyzable transfer factor" from human leukocytes, which has a molecular weight of 5000 or less, has been shown to be strongly chemotactic for granulocytes and weakly chemotactic for monocytes in vitro (11). This result is particularly interesting because "transfer factor" is a highly variable molecule and has been shown to be capable of transferring antigen specific delayed hypersensitivity from sensitized individuals to previously unresponsive recipients (12).

Though the in vitro activity of these chemotactic molecules has been well demonstrated, one may ask if their importance carries over to in vivo immune responses of clinical significance. It is now reasonably clear that, in at least some circumstances, complement

derived chemotactic factors are of principal importance for the accumulation of phagocytic cells in areas of infection or inflammation. Inflammatory rheumatoid synovial fluids have been shown to contain $C5_a$ chemotactic activity while inflammatory non-rheumatoid synovial fluids contain $C3_a$ (13). Neither of these components could be detected in normal synovial fluids. The accumulation of PMN's in the peritoneal cavities of mice following injection of endotoxin has been shown to be complement dependent with $C5_a$ exhibiting the majority of chemotactic activity (14), while leukotactic activity around myocardial infarcts in rats has been shown to be $C3_a$ dependent (15). The importance of complement-derived chemotactic factors to the migration of macrophages toward Mycobacterium tuberculosis and other organisms has also been demonstrated (16).

The mechanism of action for these chemotactic factors is not yet understood. $C5_a$ has been shown to increase aerobic glycolysis and hexose monophosphate shunt activity in neutrophils (17) but this alone is not sufficient for chemotaxis. $C5_a$ has also been shown to provoke the secretion of lysozomal hydrolase enzymes from phagocytes leading to the hypothesis that a plasma membrane receptor exists which is linked to a system for controlling intracellular cGMP levels (18). Evidence has also been presented for more than one chemotactic receptor on eosinophils which could lead to a variety of responses depending upon the nature of the chemotactic factor

involved (19).

Working on the reasonable assumption that discrete receptor sites of some kind exist for chemotactic agents on or within phagocytic and immuno-competent cells, we wish to propose a relatively simple and direct technique for the study of specific binding interactions between chemotactic substances, particularly $C3_a$ and $C5_a$, and a variety of cell types. Using this technique we further propose to answer or gain insight into the following questions:

1) Do all cells within a given morphological classification interact equally with a given chemotactic agent or do some cells interact more strongly with one agent while other cells within the same morphological class interact with a different agent?
2) Do different chemotactic agents interact with the same loci on a given cell or do specific loci exist for each type of chemotactic substance?
3) Do inhibitors of chemotaxis inhibit binding of chemotactic factors or do they inhibit other processes of the chemotactic response?
4) What are the relative binding affinities of various cells for various chemotactic substances?
5) Where on a given cell are bound chemotactic substances located; cytoplasmic membrane, nuclear membrane,

mitochondria, cytoplasm, etc.?

Experimentally, we first propose to study the interactions of $C3_a$ and $C5_a$ with phagocytic cells obtained from several different sources and to determine the effect of the chemotactic inhibitor cytochalasin B on the normal binding interactions of $C3_a$ and $C5_a$.

Chemically pure C3 and C5 will be prepared by the usual methods (20, 21). $C3_a$ is then prepared from C3 by each of the methods described by Bokisch et al. (10) and these preparations will be radiolabelled with ^{131}I using the chloramine T method (22). $C5_a$ will be prepared from C5 using both the trypsin digest method (23) and the normal complement $EAC1_a42_a3$ enzyme complex method (5). The purified $C5_a$ preparations will be fluorescence-labelled with fluorescein isothiocyanate by the usual technique (24). A mixture of ^{131}I- and fluorescein-labelled chemotactic factors (e.g., trypsin digest preparations of ^{131}I-$C3_a$ and fluorescein-$C5_a$) may then be mixed with a heterogenous cell preparation from buffy-coat or peritoneal exudates. The cells are then washed gently to remove excess chemotactic factor and are plated out on a microscope slide and fixed by the usual hematologic methods (25). The slide is then examined with a fluorescence microscope and the locations of the fluorescence dots within several suitable fields are recorded photographically. Next, the slide is treated with photographic emulsion and is exposed for about one week. The slide is then developed and stained (26) with a standard Romanowski

stain (e.g., Giemsa's, Wright's, or Leishman's). On optical microscopic examination the cells will appear like normal Romanowski-stained cells so that their morphologies may easily be determined. Moreover, the presence of ^{131}I-$C3_a$ is detected directly by counting photographic emulsion grains over the cells that contain the labelled $C3_a$. Since the cytology of the cells may be observed directly, the location of $C3_a$ molecules (i.e., nucleus, cytoplasmic membrane, etc.) in the cells which bind $C3_a$ may be determined. Further, a comparison of the fluorescence data with the photographic data will indicate which cells bind each of these chemotactic factors and whether or not certain cells of a particular morphological type are specific for one chemotactic factor or the other.

If some cells are observed to bind both $C3_a$ and $C5_a$, a second experiment will determine if the two chemotactic factors are binding to common or independent binding sites. After counting a statistical sample of the cells observed to bind both $C3_a$ and $C5_a$, a second cell preparation is treated with a large excess of $C5_a$ but the same amount of $C3_a$. A slide is prepared as before. If a large reduction in the number of cells binding both $C3_a$ and $C5_a$ is observed, or if a sizeable decrease in the photographic intensity of $C3_a$ with respect to $C5_a$ is observed for cells binding both molecules, it may be assumed that binding of $C3_a$ and $C5_a$ is competitive for chemotactically active sites on the cell. If, however, the photographic intensity of $C3_a$

does not measureably decrease, it may be concluded that separate binding sites exist for each of the two chemotactic molecules.

In a similar experiment the effect of cytochalasin B may be studied. Cytochalasin B is known to inhibit $C5_a$ stimulated chemotaxis of PMN's (27). By statistically comparing the binding of labelled $C3_a$ and $C5_a$ molecules to cell preparations in the presence or absence of cytochalasin B, it may be determined if cytochalasin B inhibits the binding efficacy of the chemotactic molecules or plays some role in blocking chemotaxis. Similar techniques may be used to study the relative binding of $C3_a$ molecules prepared by different methods. Of course a number of control experiments will be necessary, such as switching labels (i.e., ^{131}I-$C5_a$ and fluorescein-$C3_a$) and varying relative concentrations of the chemotactic factors, but no serious difficulties are otherwise anticipated.

In addition to providing information about phagocytic cell chemotaxis, these studies should also provide valuable information about membrane characteristics and membrane-small protein binding interactions. The techniques described here should also find wide application in a variety of cell-protein binding studies.

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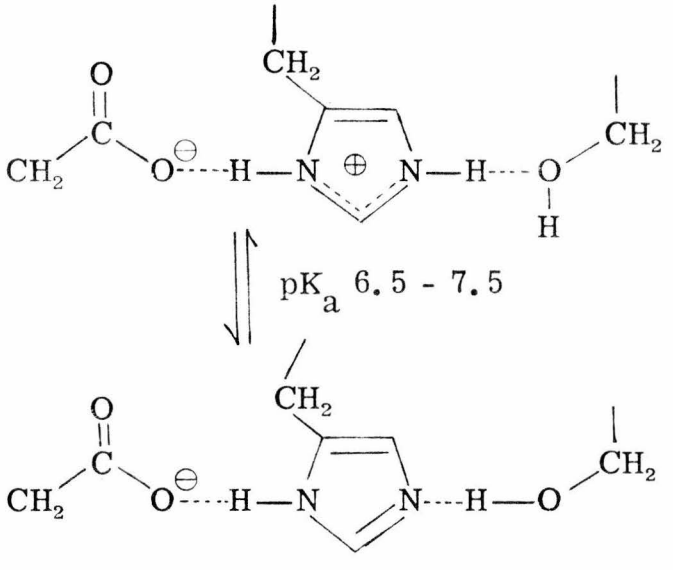
PROPOSITION II

Serine proteases, such as trypsin, chymotrypsin, elastase, subtilisin and α-lytic protease, form an important class of enzymes containing invariant serine, histidine and aspartic acid residues at their catalytic centers. A characteristic ionization involving these residues occurs with an apparent pK of about 6.5 - 7.5 and corresponds to the acquisition of catalytic activity by these enzymes. Based on the X-ray diffraction data for α-chymotrypsin (1), these residues were proposed to form a catalytic triad in which the buried aspartate is hydrogen-bonded to the histidine. Thus the catalytically inactive and active forms of this "charge relay" system were thought to have the forms



These two different views of the ionization behavior in serine proteases have profound implications concerning the mechanism of catalysis.

Since the assignment and interpretation of the N—H proton chemical shift is questionable (3), and because the alleged reversal of ionization behavior is novel, it is necessary to provide further experimental evidence capable of rigorously distinguishing between the alternative possibilities. We propose here a straightforward ^{15}N magnetic resonance experiment to positively assign the chemical shift of the proton shared between histidine and aspartic acid and to determine the precise distance of that proton from the imidazole nitrogen.

L-[3—^{15}N] Histidine will be prepared from L-2, 5-diamino-4-ketovaleric acid and ^{15}N-enriched potassium thiocyanate by methods analogous to those previously reported (3-5). The ^{15}N-enriched histidine will then be biosynthetically incorporated into α-lytic protease (3) and studied by ^{15}N NMR on the new Bruker WH-180 spectrometer.

The N—H proton chemical shift will be determined by a method similar to the application of INDOR for determining polypeptide configuration (6). The ^{15}N spectrum will be observed using narrow band noise decoupling at various parts of the proton spectrum. When the correct ^{1}H frequency is obtained the N—H proton resonance will

experience an NOE (7). The analogous ^{13}C NMR technique has been used in the study of hydrogen-bonding between histidine and the ^{13}CO ligand bound to hemoglobin with considerable success (8). Since we expect that the relaxation of the ^{15}N nucleus will be dominated by dipolar interaction with the N—H proton, the distance of the proton from the imidazole nitrogen may be determined by a careful study of T_1. By repeating these studies at several different pH we will obtain exact knowledge of the pH behavior of the proton chemical shift and its absolute distance from the nitrogen, thus clearing up questions as to where the proton resides and whether or not the imidazole or the carboxylic acid is formally charged.

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trations of cyanate as low as 0.01 M could reverse sickling to the same extent. It was demonstrated that cyanate treatment resulted in the irreversible carbamylation of α-amino groups in Hb-S. This increases the oxygen affinities of the erythrocytes and decreases their tendency to sickle (6). Labeling of erythrocytes with ^{51}Cr showed that the in vivo mean 50% survival time of sickle cell erythrocytes increased from 9.9 to 20.7 days after prior in vitro treatment of the cells with sodium cyanate (7). Comparative studies (8) of the effects of urea and cyanate have concluded that the clinical usefulness of urea in reversing sickling is negligible while cyanate was shown to be extremely potent in its ability to block sickling.

Though the molecular mechanism for sickling is now believed to involve the formation of intermolecular β-sheet structures (9), cyanate is still considered to be an effective drug for blocking sickling. The principal reservation about the administration of cyanate to humans is the potential carbamylation of other proteins throughout the body (10). In fact, there is now increasing evidence that long term administration of cyanate to humans may result in significant neurological damage (11).

Carbamyl phosphate (CP) has also been shown to be highly effective in reversing sickling (12). While CP is a naturally occurring metabolite in most parts of the body, it is only found in low concentrations in blood plasma as part of the urea cycle. Elevation of blood CP levels in vitro has been shown to result in the diffusion of CP into the red cells where it dissociates into cyanate and phosphate; the intracellular cyanate is then free to carbamylate the hemoglobin (13). Since

CP is readily converted to ammonia, carbon dioxide and phosphate in most body tissues without the formation of cyanate, it was reasoned that CP represents a much safer treatment for sickle cell anemia than cyanate, but is as effective as cyanate for treatment of red cell sickling.

The one obvious drawback to CP is that it decomposes immediately at acid or alkaline pH and is stable for only short times at neutral pH. This necessitates that the clinical administration of CP be by frequent, low dose, intravenous injection of CP solutions, as oral administration of CP would result in its immediate conversion to cyanate and phosphate in the gastro-intestinal tract, and large single injections of CP solutions would result in significant decomposition of CP to cyanate in the plasma before the CP had time to diffuse into the red cells or be metabolized by normal enzymatic processes. Thus CP action would occur chiefly through the generation of cyanate and would offer no significant advantage of this drug.

The potency of cyanate in reversing sickling, coupled with the known disadvantages of the current methods of clinical treatment, leads us to propose the use of the amino acid citrulline as a safe, convenient and hopefully effective means of treating patients with sickle cell anemia. Citrulline is a naturally occurring intermediary metabolite of the urea cycle. It is ordinarily produced by the reversible action of the liver enzyme ornithine carbamyl transferase on ornithine and carbamyl phosphate. Citrulline levels in blood are comparable to those of ornithine (6 and 8 μg/ml, respectively), and carbamyl phosphate levels in ureotelic animals have been shown to be strongly dependent upon orni-

thine and citrulline concentration (14). We believe that by elevating blood citrulline levels it may be possible to elevate the CP concentration to a level therapeutically effective for the control of red cell sickling. Furthermore, production of CP by enzymatic degradation of citrulline could not result in a CP overdose which might lead to extensive extracellular cyanate formation.

We propose to study the in vitro and in vivo efficacy of citrulline as an indirect carbamylating agent of Hb-S in whole blood. Citrulline will be incubated with freshly drawn whole blood samples containing added ornithine carbamyl transferase at 37°C for varying lengths of time and the degree of carbamylation will be determined by the newly developed column chromatographic techniques of Williams (15). If the results of these studies appear promising, in vivo studies involving oral and intravenous administration of citrulline to animals will be conducted. Since citrulline is quite stable at moderately acid and alkaline pH, it should be quite stable in the digestive tract and should be readily transported into the circulatory system.

If the effect of citrulline on Hb-S is as we suppose, one might wonder why normal dietary ingestion of foods rich in citrulline does not result in detectable hemoglobin carbamylation. We suspect that it does. Interestingly, the only known food source containing significant quantities of citrulline is watermelon (16). Normal citrulline concentrations in watermelon range around 40 mg per pound of juice. Thus one might expect that routine ingestion of watermelon could result in sufficient carbamylation of Hb-S (around 5%) to be therapeutically effective in

reducing the long term debilitating effects of sickle cell anemia.

Though considerable study will be necessary to determine the long term toxicity of citrulline in human subjects, we are encouraged that citrulline will be proven relatively safe by our inability to find any recorded evidence of fatality or chronic illness attributable to watermelon overdose.

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PROPOSITION IV

Some of the most interesting chemistry in nature is carried out within the ordered chemical environments of intact cells. To facilitate the study of these biochemical reactions the classical enzymologist has sought to isolate and purify specific cellular components and then to characterize their chemical properties under a variety of conditions. Unfortunately this method requires the disruption of normal cell function and the physiological conditions most important to the component of interest. It is therefore necessary that biochemical behavior observed under restricted conditions be related back to the behavior of the functioning cell. To this end we wish to propose a method for the study of intracellular environments in normally functioning cells.

In principle the application of magnetic resonance techniques to study intracellular function holds promise owing to the ability of NMR and ESR methods to probe specific chemical environments in otherwise complicated systems. In practice, however, the problems of spin-sensitivity, resolution, and the physiological consequences of introducing specific labels into cells complicate the actual experiments.

While ESR spin-labeling techniques offer excellent sensitivity and have been useful in studying the properties of membranes (1), it is difficult to incorporate spin-labels into cells without causing adverse effects. Another disadvantage of ESR is that the chemical shift parameter, so useful in NMR for resolving effects due to differing environments, does not exist in the ESR spectrum. On the other hand, NMR

techniques have limited sensitivity. In the ^{1}H NMR spectrum, where sensitivity is best, whole cell samples exhibit so many resonances that interpretation of the spectrum is often impossible. The resonance from H_2O is easily observed but this resonance rarely shifts and is therefore only useful for relaxation time measurements, as in the estimation of bulk intracellular viscosity (2, 3). Fluorine nuclei have reasonable sensitivity, a good chemical shift range, and low natural abundance in biological samples, suggesting that the incorporation of fluorine labels into cells might be useful (4). Unfortunately F^- ion is not tolerated by cells and most other labels either do not cross the cell membrane or adversly affect cell metabolism. ^{31}P NMR studies of the naturally occurring inorganic and organic phosphates in cells provide a means for determining intracellular pH (5), but the phosphate chemical shift range is relatively small and sensitivity is marginal for detailed studies of intracellular function in whole cells. Most other nuclei are not sufficiently sensitive to serve as probes of intracellular environments due to the necessarily low concentrations involved. NMR studies of thallium-203/205 nuclei, however, may be ideally suited to the study of whole cells.

$^{203/205}Tl^+$ has good sensitivity and an enormous chemical shift range (6). The Tl^+ ion exhibits solvent shifts of ~ 2000 ppm and, marvelously, Tl^+ mimics the behavior of K^+ in whole cells (7). Since Tl^+ is not normally present in biological systems but, like K^+ is concentrated into cells when present in the media, this probe offers great potential for studying cell function.

The Tl^+ chemical shift is very sensitive to OH^- concentration suggesting that this probe should provide another method for determining intracellular pH. Further, if the mean lifetime τ of Tl^+ in a specific environment is greater than $\sqrt{2}/2\pi\,\Delta$, the nucleus will be in slow exchange and will provide a separate signal for that environment. Since the chemical shifts, Δ, for Tl^+ are large, this condition should be met whenever $\tau \geq 5 \times 10^{-5}$ sec. This should be the case when Tl^+ resides in various cell compartments separated by membranes, or when dissociation of Tl^+ from specific cell components is slow. For each of these slowly exchanging environments it will be possible, within the sensitivity limits of the instrument, to determine relaxation parameters and nuclear Overhauser effects so as to provide information about the mobility of Tl^+ and the nature of its surroundings. Thus we envision the productive application of $^{203}/^{205}Tl$ magnetic resonance to study such problems as Tl^+ transport across membranes in the Na^+/K^+-dependent ATPase reaction, intracellular pH and viscosity, the effects of compartmentalized cell structures on Tl^+ distribution, and the effects experienced by Tl^+ in different environments induced by the addition of other chemical agents. These studies should be possible with only minor modification of existing NMR instrumentation (8).

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PROPOSITION V

One of the major aims of our research has been to obtain direct and unequivocal evidence concerning the nature of the electronic and steric interactions between the heme and surrounding protein which are responsible for the control of heme-ligand reactivity. We have shown the importance of direct steric and hydrogen-bonding effects on the bound ligands which we believe are of considerable importance (1). We have also shown that, while electronic effects which could be induced in the porphyrin rings of native hemoproteins are not likely to significantly affect iron-ligand bonding, the magnitude of artificially induced electronic effects on the ligand are modulated by the nature of the protein surrounding the heme (2). Though this modulation may result from the interaction of the ligand with distal amino acid residues, it is also possible that perturbations induced in the proximal histidine-heme bond could be of importance. In this light it is interesting that substitution of pyridine for imidazole as the axial base in heme model compounds results in large changes in the differential affinities of the compounds for oxygen or carbon monoxide (3, 4). Studies of p-substituted pyridine hemochromes also show that ligand binding is markedly sensitive to the basicity of the axial base (5). Thus perturbing the histidine-iron bond in α subunits in a slightly different manner from that in the β subunits could be responsible for the differential subunit affinities observed in Hb-A for the binding of O_2 vs. CO (6, 7). In order that we may better understand the relative importance of electronic

effects transmitted through the axial base and tetrapyrole nitrogens to the iron and thence to the ligand, we propose to study the ^{13}C-^{15}N spin-spin coupling interactions between ^{13}C-enriched carbon monoxide or alkyl isocyanide ligands with the nitrogens of a series of modified hemochromes.

While data on two bond ^{13}C-X-^{15}N coupling is lacking, studies on hydrazines, nitrosamines and amides (X = C or N) indicate that an intervening π - system is necessary for transmission of spin-spin interactions (8). Typical couplings for these compounds are $0 < J_{CN} < 10$ Hz. It therefore seems likely that couplings of about 10 Hz or more will be observed in the extensively π-delocalized hemochromes. Changes in the ^{13}C-Fe-^{15}N couplings in hemochromes modified with various substituent groups on the axial base or the porphyrin ring should indicate the relative effects of the substituents on the electronic couplings through the iron to the ligand. Thus it should be possible to dissect the relative importance of porphyrin- and axial base-derived electronic interactions in determining the nature of the iron-ligand bond in various ligands. Further, studies of the ^{15}N-Fe-^{15}N coupling in [^{15}N]-pyridine hemochromes will show the relative symmetry of electronically induced changes in the iron-base and iron-ligand bonds caused by various substituents on the porphyrin ring.

In addition to providing significant information about porphyrin and axial base effects transmitted to the ligand, these studies will also provide badly needed experience for determining other areas of biochemical research that might be best studied by ^{15}N magnetic resonance.

Of course, all of this research presupposes the availability of instrumentation sufficiently sensitive to observe natural abundance ^{15}N at concentrations of about 0.08 M.

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